P2Y receptor regulation of mitogenesis in vascular smooth muscle cells

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by

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Abstract

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Intimal proliferation of vascular smooth muscle (VSM) plays a central role in the pathology of cardiovascular disease (Ross 1993). Platelet derived growth factor (PDGF) is implicated as a mediator of neointimal formation following angioplasty (Yang *et al.*, 1998). Extracellular ATP and UTP acting via G-protein coupled P2Y receptors have been shown to stimulate DNA synthesis in rat VSMCs (Boarder & Hourani 1998; Erlinge 1998). This study investigates the effects of nucleotides on the regulation of mitogenesis in explant cultures of human saphenous vein (SV) VSM both in the presence and absence of PDGF at several levels in the mitogenic pathway.

[^{3}H]-thymidine incorporation was used as an index of DNA synthesis and proliferation. PDGF induced increases in proliferation of SV VSMCs. Increases in ERK and JNK activation, but not p38 MAPK, were also observed using phospho-specific antibodies in Western blotting. MEK inhibitors, PD98059 and U0126, attenuated both PDGF-induced ERK activation and DNA synthesis. The activation of JNK was also found to be dependent upon ERK activation when these inhibitors were used. SB203580, LY294002 and Y27632, which are inhibitors of p38 MAPK, PI3K and ROCK respectively, attenuated the PDGF-induced increase in DNA synthesis but did not block ERK or JNK activation.

Nucleotides such as ATP, ADP, UTP, UDP, 2MeSATP, 2MeSADP and ATPγS *per se* did not induce increases in [^{3}H]-thymidine incorporation in SV cells. However, ATP in the presence of PDGF synergistically enhanced DNA synthesis in SV VSMCs, whereas UTP and UDP inhibited the PDGF-mediated DNA synthesis response. Other nucleotides had no effect. This suggested an antipropliferative role for UTP and UDP and a proliferative role for ATP. Neither ATP or UTP alone or with PDGF stimulated a detectable accumulation of inositol 1,4,5 trisphosphate (Ins(1,4,5)P_3) although ATP, UTP and PDGF elevated intracellular calcium levels.

ATP was found to increase the activation of ERK to the same extent as PDGF when incubated on cells for longer than 10 minutes. In the presence of PDGF, ATP synergistically enhanced the PDGF-induced ERK response. UTP and UDP alone did not increase the activation of ERK. Nucleotides did not increase the activation of JNK or p38 MAPK. However in the presence of PDGF, UTP attenuated the PDGF-induced activation of JNK.

In contrast to the human studies, both ATP and UTP increased DNA synthesis, ERK activation and PLC activity in VSMCs derived from spontaneously hypertensive rats (SHR) compared to normotensive rats (WKY). RT-PCR and pharmacological receptor characterisation studies suggested that mitogenic signalling in SHR cells were mediated through $P2Y_2$, whereas in WKY cells both $P2Y_2$ and $P2Y_4$ receptors were mediating nucleotide signalling.

In summary the ATP-mediated proliferative response is dependent upon ERK activation and the UTP-mediated anti-proliferative effect is dependent upon JNK attenuation most likely via $P2Y_4$ receptor. In rat VSMCs the enhanced mitogenic signalling observed in cells from hypertensive rats may be due to the absence of $P2Y_4$ receptor expression, which may attenuate proliferation in normotensive rats.

Publications and abstracts

Papers

White, P.J., **Kumari, R.**, Porter, K.E., London, N.J.M., Ng, L.L., Boarder, M.R.
Antiproliferative effect of UTP on human internal mammary artery and saphenous vein vascular smooth muscle cells.
American Journal of Physiology, 279, H2735-2742, 2000 (see Appendix).

Kumari, R., White, P.J., London, N.J.M., Ng, L.L., Boarder, M.R.
UTP acting at P2Y receptors inhibits both PDGF-stimulated c-Jun amino-terminal kinase and proliferation in human vascular smooth muscle cells: role of extracellular signal-related kinase.
In preparation

Kumari, R., Webb, T.E., Boarder, M.R.
Increased mitogenic responses in hypertensive rat aortic smooth muscle cells regulated by P2Y receptors.
In preparation

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Abbreviations

α,β-MeATP	α,β–methylene adenosine triphosphate
β,γ-MeATP	β,γ–methylene adenosine triphosphate
2MeSATP	2-Methylthio-adenosine triphosphate
2MeSADP	2-Methylthio-adenosine diphosphate
ADP	adenosine diphosphate
AII	angiotensin II
ANOVA	analysis of variance
ANP	anti-naturetic protein
ARL67156	6-N,N-diethyl β,γ-dibromomethylene-D-ATP
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
ATPγS	adenosine 5'-0-(2-thiodiphosphate)
BAECs	bovine aortic endothelial cells
BSA	bovine serum albumin
BSS	balanced salt solution
$[Ca^{2+}]i$	intracellular calcium ion concentration
CaBP	calcium sensor proteins
cAMP	cyclic adenosine monophoshate
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CHO	chinese hamster ovary
CHOP	CREB homologous protein
CNS	central nervous system
COX	cycloxygenase
CREB	cAMP responsive element binding protein
DAG	sn-1,2-diacylglycerol
DEPC	dimethylpyrocarbonate
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
d.p.m.	disintegrations per minute

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(beta-aminoethylether)-N,N'- tetraacetic acid
ERK	extracellular regulated kinase
ET-1	endothelin-1
et al.	*et alia*
FAK	focal adhesion kinase
FCS	foetal calf serum
FPL 66096	2-propylthio-D-β,γ-difluromethylene ATP
FPL67085	2-propylthio-D-β,γ-dichloromethylene ATP
G Protein	guanine nucleotide-binding protein
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
Go 6850	bisindolylmaleimide I; GF 109203X; 2-[1-(3-dimethylamino propyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide
Grb2	growth factor receptor binding protein 2
GRK	GPCR kinase
GSK	glycogen synthase kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEK	human embryonic kidney
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	horseradish peroxidase
IB-1	islet brain-1
IGF	insulin-like growth factor
$InsP_3$	inositol (1,4,5) trisphosphate
$InsP_X$	inositol (poly)phosphates
Ins $(1,3)P_2$	inositol (1,3) bisphosphate
Ins $(1,4)P_2$	inositol (1,4) bisphosphate
Ins $(1,4,5)P_3$	inositol (1,4,5) trisphosphate
Ins $(1,3,4,5)P_4$	inositol (1,3,4,5) tetraphosphate
ITP	inosine triphosphate

JIP-1	JNK interacting protein-1
JNK	c-Jun N-terminal kinase
JSAP	JNK/SAPK-associated protein
K^+	potassium ions
Log_{10}	logarith to base 10
LPA	lysophosphatidic acid
MAPK	mitogen activated protein kinase
MAPKAP-K	MAPK-activate protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MBP	myelin basic protein
MEK	mitogen and extracellular regulated kinase
MEKK	MEK kinase
min	minutes
MLC	myosin light chain
MLCK	MLC kinase
mRNA	messenger RNA
MSK-1	mitogen and stress-activated protein kinase 1
Na^+	sodium ions
NA	noradrenaline
NANC	non-adrenergic non-cholinergic
NC-IUPHAR	International Union of Pharmacology Committee on receptor Nomenculature and Drug Classification
NFAT4	nuclear factor of activated T cells 4
NHE-1	Na^+/H^+ exchange protein 1
NPY	neuropeptide Y
OD	optical density
ORCC	outward rectifying chloride channels
PA	phosphatidic acid
PAK	p21 activated protein kinase
PC12	phaeochromocytoma 12 cells
PD 98059	2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor

PGE_2	prostaglandin E_2
PGF_2	prostaglandin F_2
PGI_2	prostaglandin I_2
PH	pleckstrin homology
PI	phosphoinositide
PIP2	phosphotidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKN	protein kinase N
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethyl sulphonylfluoride
PPADS	pyridoxal-phosphate-6-azophenyl-2'-4'-disulphonic acid
PRK	PKC-related protein kinase
PTB	protein tyrosine binding
$PtdIns(4,5)P_2$	phosphatidylinositol (4,5) bisphosphate
PTX	pertussis toxin
RAFTK	related adhesion focal tyrosine kinase
RBECs	rat brain microvascular endothelial cells
RNA	ribonucleic acid
RNAse	ribonuclease
ROCK	Rho-operated coiled-coil kinase
RKK	MAPKAP-K2 reactivating kinase kinase
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
RTK	receptor tyrosine kinase
Sap-1	serum response factor accessory protein-1
SAPK	stress-activated protein kinase
SDS PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEK	stress-activated and extracellular related kinase
Ser/Thr	serine/threonine
SH2/3	Src homology 2/3

Chapter 1

Introduction

1.1 History of P2 receptors

The effects of ATP in the vasculature were first reported in 1929 by Drury and Szent-Gyorgi who showed slowing of heart (bradycardia), drop in blood pressure and vasodilatation following intravenous injection of adenosine and ATP into anaesthetised guinea-pig. ATP is synthesised during the process of cellular respiration, in which the metabolic breakdown of glucose yields energy for the synthesis of ATP from ADP and inorganic phosphate and was first recognised as an intracellular source of energy for many important biochemical reactions by Lipmann in 1941. Later it was classed as a neurotransmitter when Holton (1959) demonstrated its release from sensory nerves. In 1972 Burnstock formed the 'purinergic nerve hypothesis', which described nerves using ATP for non-adrenergic and non-cholinergic (NANC) transmission, or as a co-transmitter with noradrenalin, acetylcholine or other neurotransmitters (Burnstock 1976). The receptors for adenine compounds were termed purinoceptors and were divided after further pharmacological characterisations into P1 and P2 receptors (Burnstock 1978). P1 receptors had an agonist potency order of adenosine>>ATP and were selectively antagonised by methylxanthines. P2 receptors had an agonist potency order of ATP>>adenosine and were not antagonised by methylxanthines and are discussed further in this thesis. Burnstock and Kennedy (1985) further subdivided P2 receptors based on rank order of agonist potencies and the nature of responses to ATP. The first subtype was P_{2X}, which was selectively stimulated by the ATP analogue α,β-methylene ATP. These receptors mediated vasoconstriction and contraction in the vas deferens and bladder smooth muscle. The second subtype was P_{2Y}, which mediated vasodilatation and relaxation in guinea-pig taenia coli and longitudinal muscle of rabbit portal vein and was potently stimulated by 2-methylthio-ATP (2MeSATP). Later studies lead to the re-definition of these receptor subtypes, using upper-case X and Y. P2X receptors were defined as ligand gated ion channels and P2Y receptors as G protein-coupled, seven transmembrane receptors. In 1986, Gordon proposed further subdivisions of P2 receptors with the addition of two more classes. These were P_{2T}, stimulated by ADP leading to platelet aggregation, but antagonised by ATP and P_{2Z}, which was activated by ATP^{4-} (tetrabasic form of ATP) found on macrophages, mast cells and lymphocytes. It soon became clear that the uridine nucleotides were also active in a wide variety of tissue, which generated the term pyrimidinoceptor (reviewed by Seifert & Schultz, 1989, Von Kugelgen *et al.*, 1987). The

addition of more subclasses and the use of the alphabetical nomenclature continued. Where UTP and ATP acted equipotently receptors were termed P_{2U} (von Kugelen & Starke, 1991, O'Connor *et al.*, 1991, 1992, Keppens *et al.*, 1992) or P_{2N} receptor (Dubyak & El-Moatassim, 1993). The putative P_{2D} receptor was stimulated by diadenosine polyphosphates, which is stored and released from synaptic vesicles (Floggaard & Klenow 1982, Pintor-Torres *et al* 1991, Pintor *et al.*, 1993, Pintor & Miras-Portugal, 1993, Castro *et al.*, 1992).

The meeting of the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) in 1992 established that the alphabetical naming of P2 receptor nomenclature was unhelpful and making the addition of new subtypes difficult and inconsistent with NC-IUPHAR guidelines (Abbrachio *et al.*, 1994, Fredholm *et al.*, 1994, Kenakin *et al.*, 1992). In 1994 Abbracchio and Burnstock proposed a nomenclature system based on numbering receptor subtypes, which was accepted by IUPHAR after further additions (Fredholm *et al.*, 1997). This numbered nomenclature system is discussed in Section 1.6 and used throughout this thesis.

1.2 Sources of extracellular nucleotide

ATP is present in the cytosol at high concentrations (~5 mM) predominantly in the Mg^{2+}-complexed form (MgATP). Extracellular ATP levels remain low, maintained by rapid breakdown of ATP to adenosine by ectonucleotidases. Transient release of ATP in response to specific physiological and/or pathological conditions occurs due to regulated release in three ways, vesicular release, non-vesicular release and release from ruptured cells.

1.2.1 Vesicular release

Holton first demonstrated the neuronal release of ATP from sensory nerves following antidromic stimulation of sensory nerve fibres in the skin of rabbit ear in 1959. Since then the release of ATP from intracellular stores via vesicular release (exocytosis) has been shown to occur in both neuronal and non-neuronal cells.

There is substantial evidence to demonstrate that ATP and ADP are co-stored and co-released with other neurotransmitters. ATP and noradrenaline (NA) were found to be

co-stored in the synaptic vesicles of adrenergic nerves in both the peripheral and central nervous system (CNS) (Thureson-Klein *et al.*, 1979, Whittaker 1982). Several reports show it to be co-released with acetylcholine neurotransmitter (Burnstock, 1976; Sneddon & Burnstock, 1984; Richardson & Brown, 1987). Adrenomedullary chromaffin granules were found to store ATP at a concentration of approximately 100 mM (Hillarp & Thieme, 1959) and stimulation of these cells resulted in exocytosis (Rojas *et al.*, 1985). Large amounts (100 mM – 1 M) of ATP and ADP are stored in platelets in dense granules (Carty *et al.*, 1981, Gordon 1986) and are known to release these stores when activated (Born & Kratzer, 1984). UTP was found also to occur in platelet dense granules but at approximately one tenth the concentration of ATP (Goetz *et al.*, 1971)

1.2.2 Transmembrane transport

Nucleotides are also released from most intact cells that have no exocytotic release pathway or are nonexcitable. Burnstock in 1989 described the release of ATP from vascular endothelial cells during hypoxia or ischaemia that caused initial vasodilation of the vasculature (also see Ralevic *et al.*, 1992). Vascular endothelial cells were also shown to release UTP, UDP, UMP and uridine due to increased fluid sheer stress (Saiag *et al.*, 1994) by an undetermined process. Lazarowski and collegues (1995) showed release of ATP from 1321N1 astrocytoma cells (P2Y receptor null cell line), which was sufficient to stimulate the ATP-sensitive P2Y receptor that was transfected. The same group later showed that mechanical movement of extracellular cell medium caused a large release of UTP from 1321N1 cells (Lazarowski *et al.*, 1997b). Mechanical stretching of vascular smooth muscle cells caused a time and strength-dependant rise in ATP levels in the extracellular media (Hamada *et al.*, 1998). One proposal for the release of nucleotides is that they are pumped out via the ABC group of transmembrane transporter proteins, such as the multidrug resistance (MDR) protein or cystic fibrosis transmembrane conductance regulator (CFTR) (Sedaa *et al.*, 1990, Abraham *et al.*, 1993, Reisin *et al.*, 1994, Brake & Julius 1996). A defect in this mechanism has been implicated in cystic fibrosis and is discussed further in Section 1.11.5.1.

1.2.3 Cell lysis

An obvious source of ATP would be via the lysis of cells, which stores ATP at high mM concentrations. Such an event would result in high, localised concentrations of ATP in the extracellular space. An example of this would arise during blood vessel injury, where endothelial cell lysis would release ATP. By acting on P2 receptors expressed by most of the surrounding cell types this event may aid the wound healing process. This is further discussed in section 1.10.

1.3 Nucleotide Metabolism

Extracellular nucleotides are rapidly hydrolysed by ectonucleotidases on the surface of all cells or phosphorylated by ectonucleotide diphosphokinases. This interconversion of nucleotides has a large impact on the study of P2 receptors.

1.3.1 Ectonucleotidases

A transient increase in extracellular ATP occurs in response to specific physiological and/or pathological conditions resulting in receptor activation. ATP is sequentially degraded to ADP then AMP and then to adenosine by the family of ectonucleotidases, which includes ecto-ATPase, ecto-ADPase and 5'-ectonucleotidase. ATP, ADP and adenosine act at their extracellular receptors to exert their influences, whereas AMP has little effect at either P1 or P2 receptors. UTP and UDP act at different P2Y receptors and conversion to uridine poses less of a problem than adenosine conversion from ATP, as no known uridine receptor exists.

1.3.1.1 EctoATPase

EctoATPase is found in a variety of cell types including endothelial (Pearson & Gordon, 1979), smooth muscle (Pearson & Gordon, 1985), cochlear hair cells (Mockett *et al.,* 1994), myocytes (Meghji *et al.*, 1992) and cholinergic nerve terminals (Richardson & Brown 1987). This ectonucleotidase is a 519 amino acid glycoprotein (Najjar *et al.,* 1993) containing an extracellular catalytic domain, transmembrane anchor region and a

cytoplasmic region containing two phosphorylation sites (Rees-Jones & Taylor, 1985). These sites are phosphorylated by insulin or growth factor mediated events possibly as a mechanism of regulation. Both Ca^{2+}/Mg^{2+}-dependent and independent forms of ectoATPases exist with an optimum activity at pH 7.5. They act by removing the terminal or γ phosphate group off a broad range of nucleoside triphosphates, although ATP is the preferred substrate (Ziganshin *et al.*, 1994a). Purine and pyrimidine nucleoside triphosphates (ATP, GTP and UTP, CTP respectively) are sequentially dephosphorylated at approximately the same rate (Welford *et al.*, 1986 & 1987). The phosphorothioate analogues ATPγS and ATPβS, but not ATPαS, are dephosphorylated more slowly than ATP. Homo-ATP is degraded at the same rate as ATP, whereas α,β-MeATP and β,γ-MeATP are very stable.

1.3.1.2 EctoADPase

EctoADPase removes the β phosphate group from ADP to produce AMP. ATP and its non-hydrolysable analogues β,γ-imidoATP selectively inhibit it, thereby regulating the output of AMP levels (Pearson & Gordon, 1985, Meghji 1993). In ventricular myocytes ADPase preferentially delivers AMP to 5' ectonucleotidase rather than into the bulk phase. Also ADP supplied from ATPase was more efficiently processed than ADP added suggesting preferential supply between ectoenzymes. This allows efficient production of adenosine and preventing the accumulation of AMP. This can vary with cell-type, as can the efficiencies of all three ectoenzymes.

1.3.1.3 5'-ectonucleotidase

5' ectonucleotidase (ecto-monophosphatase) is linked to the plasma membrane via a phosphatidylinositol-glycan anchor and catalyses the production of adenosine from AMP. Immunocytochemical localisation shows broad distribution of this ectonucleotidase on the surface of neurons (Maienchein & Zimmermann, 1996). In endothelial and aortic smooth muscle cells a difference in the rate of adenosine production was observed (Gordon *et al.*, 1986; 1989). On endothelial cells high levels of ADP and ATP selectively inhibited 5' ectonucleotidase, therefore decreasing the efficiency of adenosine production. On aortic smooth muscle cells, however, the hydrolysis of ADP to AMP then to adenosine occurred more efficiently than predicted. The feed forward inhibition of 5' ectonucleotidase hinders

the conversion of AMP to adenosine in the presence of ADP or ATP, whereas preferential delivery of AMP from ectoADPase increases the hydrolysis of AMP.

1.3.1.4 The fate of adenosine

This metabolism of ATP to adenosine not only allows the removal of unwanted ATP, but also allows the accumulation of adenosine. In cholinergic nerve terminals from the striatum, adenosine prevents further release of acetylcholine. This was not due to direct release of adenosine into the synapse, but rather due to the production by degradation of released ATP (Richardson *et al.,* 1987). Excess adenosine can be deaminated by an extracellular deaminase, an enzyme which is restrictively distributed (Meghji 1993). Uptake by a specific, bi-directional uptake carrier is more common and can be inhibited by dipyriamole and p-nirtobenzyl-6-thioguanosin (Jacobson 1990). Once in the cell adenosine is inactivated either by deamination to inosine by adenosine deaminase or by phosphorylation to AMP by adenosine kinase.

1.3.2 Nucleoside diphosphokinase

The conversion of nucleoside diphosphates to triphosphates is just as important as the degradation pathway. Pearson *et al.* (1980) reported that the conversion of [^{3}H]ADP led to production of [^{3}H]ATP on pig aortic endothelial cells, showing the presence of an ecto-enzyme with nucleoside diphosphokinase activity. This enzyme transfers the terminal γ-phosphate group from a nucleotide triphosphate to a nucleotide diphosphate. More recently, Nicholas and colleagues (1996b) reported nucleoside diphosphokinase activity on 1321N1, which exhibits a higher affinity than any 1321N1 cell-associated nucleotidase present (Lazarowksi *et al.*, 1997a). Thus the addition of UDP in the presence of ATP does not lead to production of UMP but the conversion to UTP and ADP (Harden *et al.*, 1997, Lazarowksi *et al.*, 1997a). Using [^{3}H]UDP and then quantifying the conversion to [^{3}H]UTP using HPLC, Lazarowski *et al.* (1997b) showed that after 5 minutes of incubation there was approximately 3% conversion of UDP to UTP in the presence of endogenously released ATP (induced by medium change) and approximately 20% in the presence of 10 μM ATP. This activity has masked the true rank order of potencies for several cloned P2 receptors.

1.3.3 The effects of nucleotide metabolism on P2Y receptors

The interconversion of nucleotides obscures not only their true potency, but the metabolic product produced also misinterprets the effects of the agonist. A single tissue may express multiple P1 and P2 receptors, therefore a response to ATP may be masked by ADP acting at other P2 receptors and adenosine acting at P1 receptors. The problem of nucleotide interconversion persists and has been tackled by using regeneration systems, hydrolysis resistant nucleotides and ectonucleotidase inhibitors. High-pressure liquid chromotography (HPLC) can also be used to generate pure solutions of nucleotides from commercially available nucleotides that are often contaminated with other nucleotides.

1.3.3.1 Nucleotide regeneration systems

Triphosphate regeneration systems using creatine phosphokinase and phospho-creatine as the phospho donor enable solutions of ATP to remain ADP free. Similarly, preventing the accumulation of nucleoside triphosphates could also be achieved using hexokinase and glucose as the phospho recipient. This method has proved useful in studies of P2Y receptor pharmacology. UDP had previously been profiled as a full agonist at $P2Y_2$ receptors but after analysis using purified UDP, hexokinase and glucose (to prevent the accumulation of UTP) it was discovered to be inactive at $P2Y_2$ (Nicholas *et al.*, 1996b, Lazarowski *et al.*, 1997a). The $P2Y_6$ receptor was initially thought to be UTP selective (Chang *et al.*, 1995), however further analysis using hexokinase showed it to be more selective for UDP over UTP (Nicholas *et al.*, 1996b). The $P2Y_4$ receptor also was reported to have a different profile than initially thought (Communi *et al.*, 1995, Nguyen *et al.*, 1995). UDP was thought to act at $P2Y_4$, however it was activated most potently by UTP, less potently by ATP, but not at all by nucleotide diphosphates (Nicholas *et al.*, 1996b).

Pre-treatment of cells with apyrase has been useful in removing endogenously released nucleotides from the cell medium (ATP or ADP→ AMP) thereby reducing any associated receptor desensitisation. It must, however, be washed out from cells if nucleotides are to be used as agonists, although hydrolysis resistant compounds such as ATPγS and UTPγS are not affected. Apyrase may therefore be used to remove contamination from these resistant nucleotides.

1.3.3.2 Hydrolysis resistant nucleotides

Several resistant nucleotides have been widely used to distinguish between the various P2 subtypes. α,β–methyleneATP and β,γ–methyleneATP are both very stable and selective for P2X receptors (Welford *et al.*, 1986). ATPγS has been widely used, as it is equipotent to ATP in most tissues when ectonucleotidase activity is low. A more recent addition is UTPγS (Lazarowski *et al.*, 1996), which can be produced by the enzyme nucleoside diphosphokinase using UDP and either GTPγS or ATPγS as the γS donor. UTPγS was shown to be resistant to hydrolysis by apyrase, alkaline phosphatase, acid phosphatase and incubation on epithelium cells (Lazarowski *et al.*, 1996). UTPγS has been used on 1321N1 cells transfected with $P2Y_2$ receptors and observed to be equipotent with UTP and ATP.

1.3.3.3 Ectonucleotidase inhibitors

EndoATPases are inhibited by ouabain, sodium azide and oligomycin. These however are not effective at ectoATPases (Ziganshin *et al.*, 1995). Only one useful inhibitor of ectonucleotidases has been found. ARL 67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP, formly FPL67156) is a synthesised analogue of ATP. It was found to inhibit ectoATPase in a human blood cell assay (Crack *et al.*, 1995), but also exhibits weak agonist effects at $P2Y_2$ ($p[A_{50}] \approx 3.5$) receptors and weak antagonist action at P2X and P2T receptors ($pA_2 \approx 3.3$ and 3.5 respectively). ARL 67156 has been shown to enhance sympathetic purinergic neurotransmission in isolated vas deferens of guinea pig consistent with the inhibition of ATP hydrolysis (Westfall *et al.*, 1996).

Cyclopiazonic acid (CPA) has been described as a selective inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum of skeletal and smooth muscles (Kurebayashi & Ogawa, 1991, Uyama *et al.*, 1992). However, the potentiation of the ATP response produced by CPA (16%) in guinea-pig urinary bladder was found to be non specific as the contractile responses to KCl were also increased (Ziganshin *et al.*, 1994b).

A further complication is that many P2 antagonists used also inhibit nucleotide degradation. The ATP binding domains of P2 receptors and ATPases are very similar and are also recognised by antagonists resulting in antagonism of receptor and inhibition of ATPases. Therefore, small antagonist effects on concentration-response curves may be due to inhibition of nucleotide degradation. Suramin has many biological actions including P2 antagonism (Leff *et al.*, 1990). Its inhibition of ectonucleotidases has also

been widely shown. For example suramin acts as a non-competitive inhibitor of ATP breakdown in the guinea-pig urinary bladder (Hourani & Chown, 1989), as a competitive inhibitor of a Ca^{2+}/Mg^{2+}-dependant ectonucleotidase in *Xenopus* oocytes (Ziganshin *et al.*, 1995, 1996) and inhibits ectonucleotidase activity in a blood cell assay (Crack *et al.*, 1994) and on endothelial cells (Meghji & Burnstock 1995). Other P2 antagonists like NF023, a structural analogue of suramin, have also been observed to decrease ATP breakdown on human blood cells to 51% of control values (Beukers *et al.*, 1995). Reactive red 2, a structural analogue of reactive blue-2, reduced ATP degradation on vas deferens and taenia coli by up to 95% despite being a strong antagonist of P2X receptors in rat vas deferens and P2Y receptors in guinea-pig taenia coli (Bultmann & Starke, 1995). Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is also a P2 antagonist, which inhibited ectonucleotidase activity by approximately 81% in the rat vas deferens (Khakh *et al.*, 1994) and in other tissues such as bovine pulmonary artery endothelium (Chen *et al.*, 1996a).

1.4 Methods for P2 receptor classification

The potency of an agonist depends upon its intrinsic efficacy and affinity. This agonist-specific property is not constant, but varies across different cell and tissue type due to the heterogeneity of transducer mechanisms, receptor density and the efficiency of receptor coupling to effectors. It is therefore simpler to classify receptors according to their sensitivity to antagonists, as they possess affinity for the receptor but no efficacy, therefore eliminating the tissue specific variation. (Kenakin *et al.*, 1992). However, the limited availability of selective antagonists hinders studies of P2 subtype differentiation. Classification has therefore depended heavily on agonist potencies.

1.4.1 Agonists

Functional studies using only agonists give information on potency, but not affinity. Changes in receptor number and efficacy can alter the observed agonist potency, which may result in different agonist potency values for the same receptor in different tissues. Endogenous agonists and synthetic agonists that are used to distinguish between P2 receptor subtypes are shown in Figure 1.1. The endogenous agonists include ATP,

UTP, ADP, UDP, AMP, and adenosine. The synthetic compounds are produced by modifying ATP in several ways, including the addition to the second carbon of the adenine ring to generate 2-methlthioadenosine (2MeSATP), replacement of the interphosphate oxygen with methylene to generate 5'-(α,β-methylene) triphosphate (α,β-MeATP) and substitution of an oxygen in the phosphate chain with sulphur to generate adenosine 5'-O-(3-thiotriphsophate) (ATPγS). The rank order of agonist potency of P2 receptor is characterised using such agonists, therefore allowing differentiation and classification of receptor subtypes. Agonist potencies at P2 receptors are discussed further in Section 1.5 and characterisation of the receptors is summarized later in Table 1.1.

1.4.2 Antagonist

Antagonists rather than agonists are the preferred tool for classification since agonist potency depends not only on its binding but also on the entire signal transduction pathway (Kenakin *et al.,* 1992). However, specific high affinity antagonists for P2 receptors are not available. Despite this some compounds have been nominated as selective antagonists and used in the classification of P2 receptors. The structures of some of these are shown in Figure 1.2.

1.4.2.1 Suramin

Suramin is a highly negatively charged sulphonic acid derivative of naphthalene used as a trypanocidal drug. Dunn and Blakeley (1988) first reported suramin as a P2X receptor antagonist in mouse vas deferens. Since then it has been widely reported as a P2X antagonist in many tissues including visceral smooth muscle cells, blood vessels, platelets and cultured coelic ganglion neurons (Dunn & Blakley, 1988, von Klugelgen *et al.,* 1989, Hoyle *et al.*, 1990, Leff *et al.*, 1992, Evans *et al.*, 1992a, Hourani *et al.*, 1992, Brake *et al.*, 1994) and as a P2Y antagonist in tissues such as taenia caeci (den Hertog *et al.*, 1989, Voogd *et al.*, 1993). This was further investigated by studying the effects of suramin on $P2Y_1$ and $P2Y_2$ receptors transfected into 1321N1 cells (Charlton *et al.*, 1996). The pA_2 value for suramin at $P2Y_1$ was found to be 5.77 ± 0.11, whereas it had a lower affinity at $P2Y_2$ (pA_2 = 4.32 ± 0.13). It is therefore very useful for distinguishing between $P2Y_1$ and $P2Y_2$ responses where both receptors are expressed simultaneously, such as bovine aortic artery (Wilkinson *et al.*, 1994a). Suramin was also shown not to antagonise the cloned

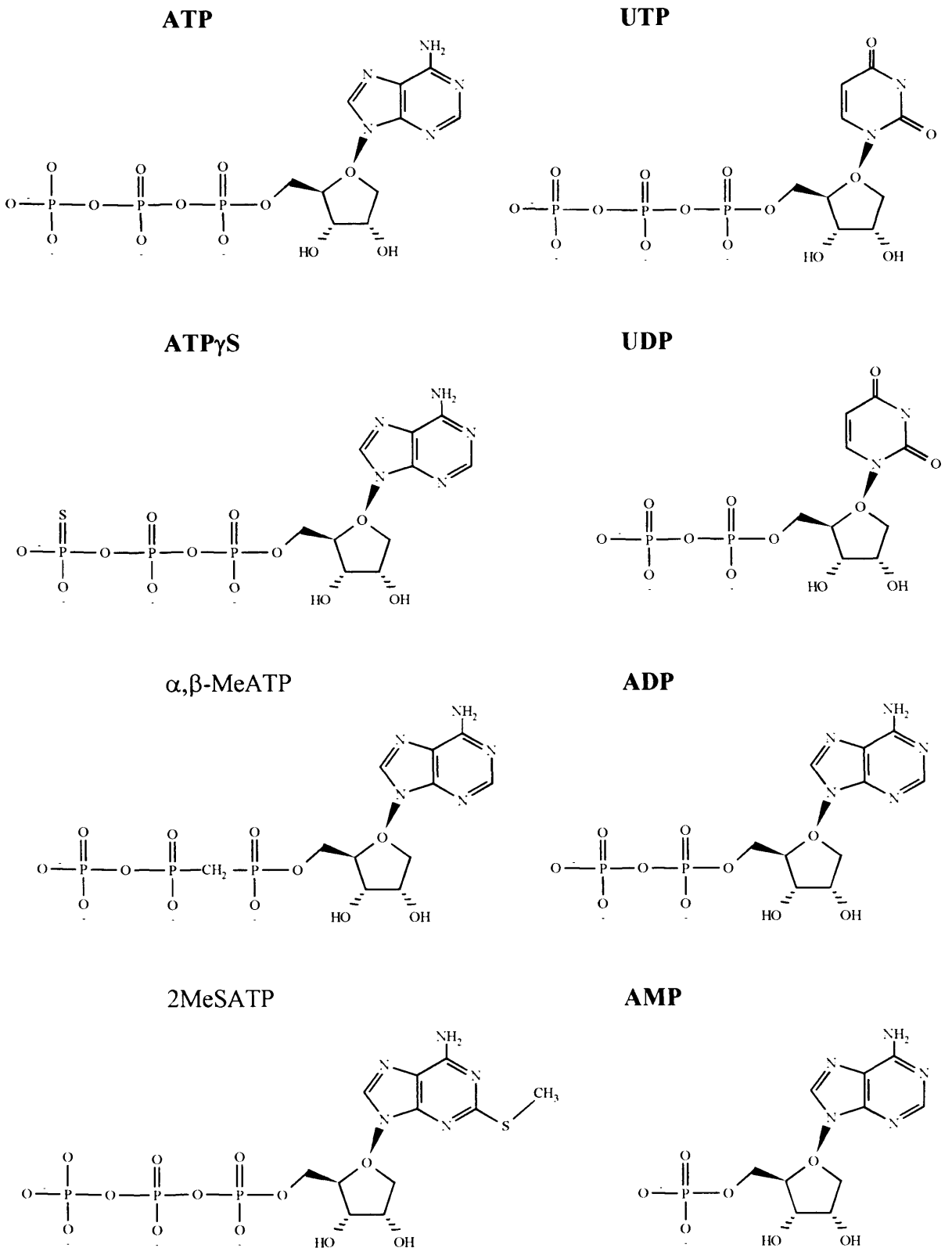


Figure 1.1 Chemical structures of P2 agonists

$P2Y_4$ receptor (Charlton *et al.*, 1996b). Communi *et al.* (1999) showed that suramin was not effective at $P2Y_6$ but was at $P2Y_{11}$ with a pA_2 value of 6.09 ± 0.52 when transfected in CHO-K1 cells. Suramin acts by inhibiting the formation of the ternary complex of agonist/receptor/G protein by binding to the Gα subunit, thus preventing spontaneous GDP exchange and Gα binding to the receptor (Beindl *et al.*, 1996).

However, suramin has been reported to be a non-selective antagonist for P2 receptors and has many biological effects (Voogd *et al.*, 1993). Boyer *et al.* (1994) demonstrated that suramin decreased the maximal PLC response to isoprenaline at β-adrenoceptors in turkey erythrocytes in a non-competitive manner. P2 receptor antagonism is also complicated by suramins ability to inhibit ectonucleotidases as discussed in Section 1.3.3.3. It also has anti-reverse transcriptase and anti-proliferative activities through the inhibition of the binding of various growth factors to their cell surface receptors (Nakajima *et al.*, 1991). The selectivity of suramin is therefore questionable, but it still remains the most commonly used P2 receptor antagonist.

NF023, a novel suramin analogue, is the symmetrical 3'-urea of 8 (benzamido) naphthalene-1,3,5-trisuphonic acid. This specific competitive antagonist is selective for P2X receptors over $P2Y_1$ and $P2Y_2$, displaying a pA_2 value of 5.96 ± 0.04 at P2X (Ziyal *et al.*, 1997, Lambrecht *et al*, 1996). Like suramin it has many non-specific biological actions such as inhibition of ecto-nucleotidases (Beukers *et al.*, 1995) and uncouples Gα subunits from their effectors (Freissmuth *et al.*, 1996, Biendl *et al.*, 1996). Whereas suramin is selective for recombinant $G\alpha_s$ subunits, NF023 is selective for recombinant $G\alpha_i$ subunits.

1.4.2.2 PPADS

Pyridoxal-phosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS) was initially sythesised and used as a P2X antagonist with a pA_2 value of 6.4 in rabbit vas deferens (Ziyal *et al.*, 1997). It was shown not to antagonise α_1-adrenoceptors, muscarinic m2 and m3 receptors, histamine H1 and adenosine A1 receptors (Lambrecht *et al*, 1992). It was also shown to antagonise native $P2Y_1$ receptors expressed on bovine aortic endothelial cells (Brown *et al.*, 1995) and native turkey erythrocyte $P2Y_1$ receptors with a pA_2 value of approximately 6 (Boyer *et al.*, 1994). PPADS is a useful tool in distinguishing responses in tissues expressing multiple P2 receptors as it is ineffective at $P2Y_2$, $P2Y_4$, $P2Y_6$ or $P2Y_{11}$ (Brown *et al.*, 1995, Charlton *et al.*, 1996, Charlton *et al.*, 1996b, Communi *et al.*,

1999). Like suramin and NF023, PPADS inhibits ectonucleotidases (Chen *et al.*, 1996a) and is non selective (Shenaz *et al.*, 2000), which complicates interpretations.

1.4.2.3 RB-2

The anthraquinone dye reactive blue 2 (RB-2) was first shown to competitively antagonise the relaxant effect of ATP in gastrointestinal tract (Manzini *et al.*, 1986). It showed no effect on P2X–mediated vasoconstriction but was effective on P2Y-mediated vasodilatation in dog coronary artery (Houston *et al.*, 1987) and rat mesenteric artery (Burnstock & Waland 1987). It was later shown to be a potent antagonist selective for $P2Y_1$ receptors over P2X receptors (Bultmann & Starke, 1995) in guinea-pig taenia coli.

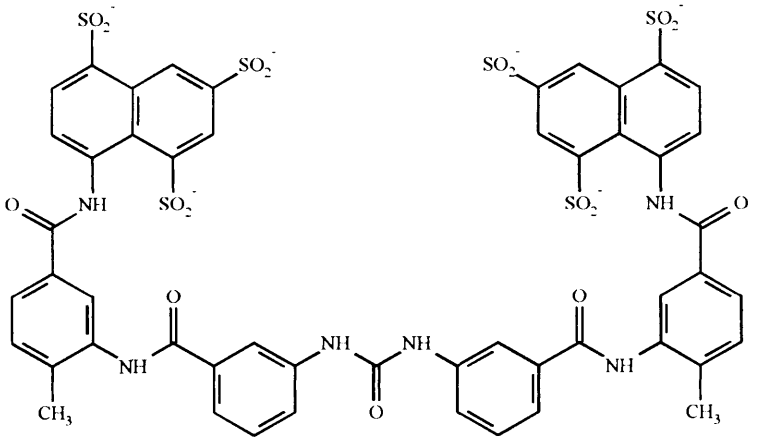
1.4.2.4 A3P5PS

Adenosine-3'-phosphate-5'-phosphosulphonate (A3P5PS) was reported to be a turkey $P2Y_1$ receptor antagonist with a pA_2 value of 6.46 ± 0.17 (Boyer *et al.*, 1996a). Conversely it was shown to stimulate agonist activity at the turkey $P2Y_1$ receptor to a small degree, but not at the human $P2Y_1$ receptor. It also appears to be specific to PLC-coupled $P2Y_1$ receptors and is ineffective at the C6 glioma $P2Y_1$-like adenylate cyclase coupled receptor and also 1321N1 cells transfected with $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors. This makes it the most selective $P2Y_1$ antagonist to date. A more recent addition to $P2Y_1$ antagonists is an A3P5PS derivative N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (N6MABP) (Boyer *et al.*, 1998).

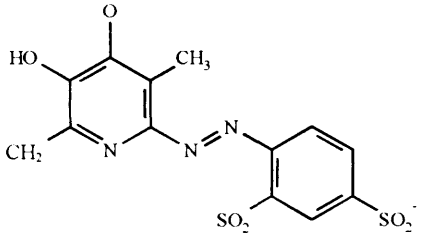
1.4.2.5 FPL66096 & FPL67085

2-propylthio-D-β,γ-difluoromethylene ATP (FPL66096) and 2-propylthio-D-β,γ-dichloromethylene ATP (FPL67085) were developed and found to be selective and potent P_{2T} antagonists with a pA_2 value of 8.7 and 8.9 respectively (Humphries *et al.*, 1994, 1995a, 1995b). Antagonism though potent is not purely competitive and also shows weak antagonism at $P2Y_1$ and P2X receptors. It is hoped that FPL67085 will be useful as an effective antithrombotic agent and is undergoing Phase 1 clinical studies.

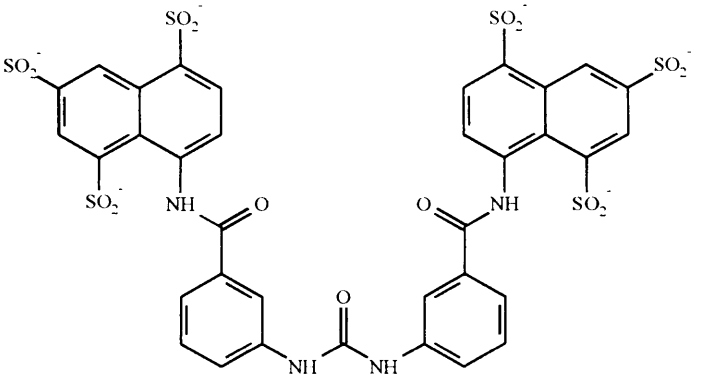
Suramin



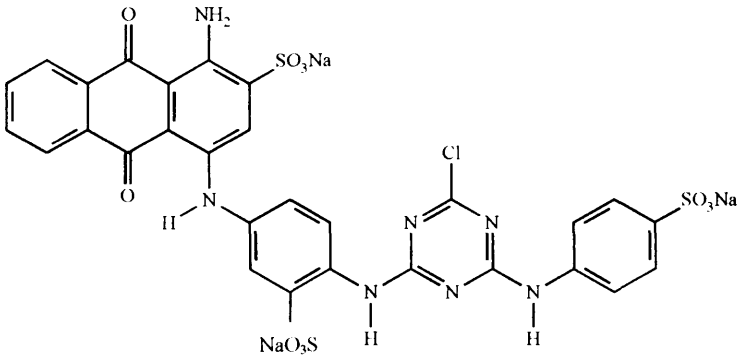
PPADS



NF023



Reactive Blue 2



A3P5P

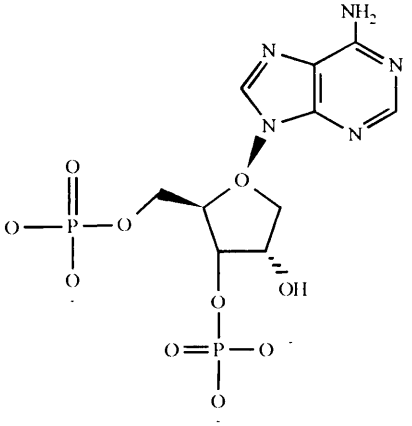


Figure 1.2 Chemical structures of P2 anatgonists

1.4.3 Radioligand binding studies

Successful radioligand binding studies require selective, high affinity, stable ligands for the receptor of interest. Preferably these are neutral antagonists, but agonists can be used although their affinity for the receptor may be affected by the action of binding. The antagonists for P2 receptors are non selective, competitive antagonists and interact with many other proteins such as suramin. Using agonists of P2 receptors causes problems as their binding affinities are only slightly higher than their affinities for other ATP binding proteins and are subject to hydrolysis by ectonucleotidases. Despite this, several groups have attempted binding assays. Cooper *et al* (1989) developed a radioligand binding assay using [^{35}S]ADPβS, and reported specific labelling of P2Y receptors stimulating PLC responses in turkey erythrocyte membranes. The same compound was found to label intact cultured bovine endothelial cells specifically (Wilkinson & Boarder 1995), but was disputed as being reliable as it also bound to non-$P2Y_1$ possessing adrenal medullary endothelial cells.

The photoaffinity ATP analogue 3'-0-(4-Benzoyl)benzoyl ATP (BzATP) has been used to competitively label $P2Y_1$ receptors on turkey erythrocytes plasma membranes (Boyer & Harden 1989). Upon exposure to UV light BzATP becomes covalently associated with $P2Y_1$ receptors. [^{32}P]BzATP was also shown to label $P2Y_2$ receptors transfected into K565 human leukaemia cells (Erb *et al.*, 1993), but was found not to bind to the agonist-binding site, as UTP binding was not inhibited.

The most popular compound for radiolabelling is [^{35}S] deoxyadenosine 5'(α-thio)triphosphate or [^{35}S]ATPαS in P2 receptor studies. This has also been used to attempt to identify new P2Y receptor subtypes such as p2y5 and p2y7 (Webb *et al.*, 1996c, Akbar *et al.*, 1996). However these studies have been disputed on the basis that [^{35}S]ATPαS is not a specific ligand at P2Y receptors as binding occurred on membranes from 1321N1 or COS-7 cells either $P2Y_1$ transfected, sham transfected with an empty vector or in wildtype state (Schachter & Harden 1997). These cells were described to possess a large number of high affinity binding sites for [^{35}S]ATPαS, which are not related to $P2Y_1$. This method has also been used to study $P2Y_1$ distribution in chick and rat brain and revealed surprisingly high levels of expression in both species (B_{max} = 37-39 pmol/mg protein, Simon *et al.*, 1995, Webb *et al.*, 1998a). High affinity agonist binding to GPCR is thought to require a stoichiometry of 1:1 between the receptor and the G protein. However the density of

receptor expressed in the rat and chick brain reported is far in excess of the level of G protein expected in the brain (Harden *et al.*, 1995). Schäfer and Reiser (1997) used [^{35}S]ATPαS to label preparations of rat brain cortical synaptosomes with high affinity (K_d = 22 nM, B_{max} = 14 pmol/mg protein). Displacement of [^{35}S]ATPαS by agonists and antagonists of P2 purinoceptors revealed an affinity profile characteristic of $P2Y_1$ receptor. Binding also found to be regulated by GTP, which is an indication of GPCRs. Further developments of specific high affinity ligands are required if reliable radioligand binding assays are to be performed. However, optimal radioligands for binding studies are antagonists, rather than the agonists described above. Selective high affinity antagonists for $P2Y_1$ receptors have now been developed (Boyer *et al.*, 1998, 1996a) though attempts at binding studies are still uncertain.

1.5 Cloning of P2 receptor subtypes

Characterising P2 receptors using purely pharmacological means is difficult due to the instability and low affinity of P2 receptor agonists and non-selective properties of antagonists. Molecular biology has provided structural data, which has aided receptor characterisation. Cloning and functional expression of P2Y receptors has also been used to characterize receptors according to their agonist potencies. Current P2 receptor cloning has expanded P2X and P2Y nomenclature from $P2X_{1-4}$ and $P2Y_{1-7}$ (Burnstock & King 1996) to $P2X_{1-7}$ and $P2Y_{1-13}$, although the P2Y cloned and sequenced subtypes are not all pharmacologically characterised as yet. The uncharacterised receptors may be pseudo-receptors that have a sequence in the genome but no function, while others may be closely related receptors that are unresponsive to nucleotides.

P2Y receptors belong to the family of seven transmembrane G protein-coupled receptor (GPCR) superfamily. The cloning of the first P2Y receptor was achieved by polymerase chain reaction (PCR) using degenerate primers based on the sequences in transmembrane domains II and VI, which are conserved in many G protein-coupled receptors. Further P2Y receptors were cloned using degenerate primers based on transmembranes III and VII of available P2Y receptor sequences. It was found that P2Y receptors were closely related to interleukin-8, thrombin, vasoactive intestinal peptide, platelet activating factor and angiotensin II receptors, whereas P1 and P2 receptors showed little homology in their amino acid sequence.

1.5.1 $P2Y_1$

The first P2Y receptor was cloned by Webb *et al.* (1993) from chick brain and was designated as $P2Y_1$ in accordance with NC-IUPHAR guidelines. The 362 amino acid receptor is highly expressed in chick brain at the time of hatching. Hydropathicity analysis revealed the classical seven hydrophobic transmembrane domains typical of a GPCR. Later the turkey $P2Y_1$ homologue was identified by Filtz *et al.* (1994). This was cloned and transfected into the null cell line 1321N1 and reported to respond to 2-MeSATP stimulations resulting in activation of PLC, which was pertussis toxin insensitive. This indicated a possible role of G_q protein in signal transduction.

Since then cDNA has been located in rat heart, brain, lung, liver, muscle, kidney, in small amounts in the spleen but absent from the testis (Tokuyama *et al.*, 1995). $P2Y_1$ receptor was also cloned from bovine aortic endothelium (Henderson *et al.*, 1995). The human $P2Y_1$ receptor was cloned from a human genomic library (Schachter *et al.*, 1996) and several other tissues including placenta (Leon *et al.*, 1997), erythro-leukemia cell line (Ayyanthan *et al.*, 1996), brain (Schachter *et al.*, 1996), prostate and ovary (Janssens *et al.*, 1996) and platelets (Jin *et al.*, 1998). Human tissue distribution studies showed expression in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The amino acid sequence is 83% identical to the chick $P2Y_1$ receptor and is approximately 95% homologous to the rat mouse and bovine receptors. Chromosomal localisation experiments identified the human $P2Y_1$ receptor gene to be present on chromosome 3, position q25 (Ayyanathan *et al.*, 1996). The mammalian homologues compared to the avian homologue possess the addition of 11 amino acids near the N-terminus and substitution of different amino acids, mainly in transmembranes IV and V.

Originally, functional analysis of the $P2Y_1$ receptor gave a rank order of potency of 2MeSADP > 2MeSATP > ADP > ATP (Webb *et al.*, 1993, Filtz *et al.*, 1994, Henderson *et al.*, 1995). However, recent characterisation studies of the $P2Y_1$ receptor have disputed this. Transfection of the human $P2Y_1$ receptor in Jurkat cells showed increases in cytosolic calcium when stimulated with HPLC purified agonists. The rank order of agonist potency found was 2MeSADP and ADP to be agonists and ATP and its derivatives to be weak competitive antagonists (Leon *et al.*, 1997), which was similar to the profile of the P_{2T} receptor expressed on platelets. Hechler and collegues (1998) also showed this to be the case for rat $P2Y_1$ and human $P2Y_1$ receptors using purified agonists. The speculation that

the $P2Y_1$ receptor was the same as the P_{2T} receptor was further investigated by Fagura *et al.*, (1998). They used the P_{2T}–selective antagonist ARL 66096 (Humphries *et al.*, 1994) and the $P2Y_1$-selective antagonist A3P5PS (Boyer *et al.*, 1996a) to show that platelets co-expressed both receptor subtypes therefore ADP is capable of activating P_{2T} and $P2Y_1$ receptors present.

The activity of ATP was verified by Palmer and colleagues (1998). When purified agonists were used to stimulate human $P2Y_1$ transfected into 1321N1 cells and native $P2Y_1$ receptors in HEK 293 cells, the rank order of potency of 2MeSADP > ADP > 2MeSATP > ATP was found. ATP did not act as a weak antagonist and its activity was not due to conversion to ADP. They suggested that difference between these results and those observed for in Jurkat transfected receptors was due to differences in receptor reserve in the two types of cells. If ATP were an agonist with low efficacy then in high receptor expressing system it would act as a full agonist, whereas in a low receptor expressing system it would act as a partial agonist or a silent antagonist. Down-regulation of human $P2Y_1$ receptors in 1321N1 cells was achieved with adenosine-5'-0-(2-thiodiphosphate) ($ATP\gamma S$) for 24 hours. After receptor down-regulation the concentration-effect curve for ADP was rightward-shifted by 10-fold and ATP agonist activity was abolished. This therefore demonstrated a lower receptor reserve, as may occur in Jurkat cells. 2MeSATP potency, which was thought to be greater than ADP was due to commercial contamination of 2MeSATP with 2MeSADP, which was eliminated by HPLC purification.

The majority of studies have shown that $P2Y_1$ receptors couple to inositol phosphoinositide hydrolysis and Ca^{2+} mobilisation. However, a $P2Y_1$-like receptor was described linked to the inhibition of adenylate cyclase in rat C6 glioma cells (Boyer *et al.*, 1993). This had a similar agonist profile to the $P2Y_1$ receptor, activating PLC in turkey erythrocytes membranes, which is blocked by pre-treatment with pertussis toxin. However, PPADS was found not to act as an antagonist at the C6 glioma $P2Y_1$-like receptor despite antagonising turkey erythrocyte $P2Y_1$ receptor (Boyer *et al.*, 1994). This was strong evidence to suggest that the rat C6 glioma $P2Y_1$-like adenylate cyclase linked receptor and the turkey $P2Y_1$ PLC linked receptor may be different $P2Y_1$ subtypes, which may differentially link to different secondary messenger systems. PPADS was therefore able to discriminate between these two receptors. Supporting this was the activation of PLC by transfected human $P2Y_1$ receptors in the C6 glioma cell line, which was PPADS sensitive (Schachter *et al.*, 1996). The PLC activity was not enhanced by activation of the endogenous C6 glioma $P2Y_1$ receptor suggesting two types of P2Y receptors.

The rat C6 glioma cell and the B10 rat brain capillary endothelial cell line P2Y receptors, which couple to adenylate cyclase, was reported to be rat $P2Y_1$ (Webb *et al.*, 1996). However, when the rat $P2Y_1$ receptor, which links to adenylate cyclase proposed by Webb and colleagues (1996) was transfected into rat C6 glioma cells, it was reported to activate PLC and not adenylate cyclase (Schachter *et al.*, 1997). Therefore the rat $P2Y_1$ receptor, like the human $P2Y_1$, is distinct from the native rat C6 glioma P2Y-like receptor.

1.5.2 $P2Y_2$

Lustig and colleagues (1993) first described the cloning of a 373 amino acid $P2Y_2$ receptor from a mouse neuroblastoma cell-line (NG108-15), previously known as P_{2U}, which shares only 40% identity with chick $P2Y_1$. It was equally sensitive to ATP and UTP when transfected into *Xenopus* oocytes. The pharmacological order of agonist potency of the mouse $P2Y_2$ was found to be ATP = UTP > ATPγS >> 2MeSATP (Lustig *et al.*, 1993). In 1994, Parr and co workers cloned the human $P2Y_2$ receptor from human airway and colonic epithelial cells. Further $P2Y_2$ receptors were cloned from human bone (Bowler *et al.*, 1995), and rat lung, pituitary gland and endothelium (Rice *et al.*, 1995, Chen *et al.*, 1996b, Godecke *et al.*, 1996). The rat and human $P2Y_2$ share over 95% and 89% homology with the mouse $P2Y_2$ and couples to PLC activation and increases in intracellular Ca^{2+}. $P2Y_2$ receptors are thought to be the most widely distributed P2Y subtype in the body, being found in the heart, liver, lung, kidney, placenta, skeletal muscle, brain, spleen and testis (Parr *et al.*, 1994, Lustig *et al.*, 1996).

The agonist profile of the human $P2Y_2$ receptor transfected into 1321N1 cells was found to be similar to that of the mouse $P2Y_2$ (Lazarowski *et al.*, 1995). Both ATP and UTP were full potent agonists, with little or no effect with 2MeSATP, 2MeSADP and α,β-MeATP. ATPγS was also a full agonist, but approximately 10 times less potent. This was also consistent with Lustig *et al.* (1993) who reported ATPγS to be between 7 and 11 times less potent than UTP or ATP. Initial reports placed ADP and UDP as full agonists at $P2Y_2$ receptors (Parr *et al.*, 1994), but Nicholas and colleagues (1996b) showed that this was due to contamination of di-phosphates with tri-phosphates or conversion to tri-phosphates by nucleoside diphosphokinase.

$P2Y_2$ subtypes have also been suggested to exist based on suramin sensitivity observations in native tissue. Stimulation of $P2Y_2$ receptors was competitively inhibited

by suramin in PC12 cells and rat aortic rings (Murrin & Boarder, 1992, Dainty *et al.*, 1994), whereas stimulations of $P2Y_2$ receptor are insensitive to suramin in bovine aortic endothelial cells and canine tracheal epithelium (Wilkinson *et al.*, 1993; Dainty *et al.*, 1994). $P2Y_4$ receptors from the rat have a similar agonist profile as $P2Y_2$ receptors (Webb *et al.*, 1998b, Bogdanov *et al.*, 1998), but are insensitive to suramin (Charlton *et al.*, 1996b). It is possible, therefore, that the latter responses reported as suramin insensitive, may be due to the presence of $P2Y_4$ receptors.

1.5.3 $P2Y_3$

Webb and colleagues (1996b) also cloned a protein from chick brain with a similar structural motif to $P2Y_1$, which was activated by nucleoside diphosphates. This receptor shared only 39% amino acid sequence identity with $P2Y_1$ and was termed $P2Y_3$. It is expressed in brain, spinal cord, kidney, lung and in the spleen. Initially this receptor was expressed in Xenopus oocytes where ADP gave the highest activity, but the data was misleading as $P2Y_3$ mRNA displaced cytotoxic effects on the cells. This receptor was then expressed in Jurkat cells and assayed with various nucleotides for calcium responses. UDP was found to be the most potent, being 10-fold potent than UTP and ADP. The rank order of potency was UDP > UTP > ADP > ATP with ATP being only a partial agonist. Suramin and RB-2 both were able to antagonise the calcium responses of $P2Y_3$ receptors. The mammalian $P2Y_6$ shares 65% identity with the chick $P2Y_3$ receptor making it the closest P2Y receptor relative and it has been suggested that the chick $P2Y_3$ is the avian homolog of the mammalian $P2Y_6$ receptor (Li *et al.*, 1998a). The turkey $P2Y_3$ receptor was isolated and expressed in 1321N1 cells and was functionally compared to 1321N1 cells transfected with rat $P2Y_6$. UDP was the most potent agonist at both receptors, whereas UTP had a lower potency. However, ATP and its derivatives were slightly more active at the turkey $P2Y_3$ receptor than at the rat $P2Y_6$.

1.5.4 $P2Y_4$

Communi and colleagues (1995) isolated an intronless coding sequence that was 1059 base pairs long from the human placenta cDNA library. This was stably expressed in 1321N1 cells and found to stimulate PLC activity when treated with UTP and UDP and

was termed human $P2Y_4$ receptor. This 365 amino acid protein is most closely related to the $P2Y_2$ receptor exhibiting 51% sequence identity and 35% with the chick $P2Y_1$ receptor. Nguyen *et al.* (1995) also isolated the sequence encoding the $P2Y_4$ receptor from the human genomic DNA and located the gene encoding it on the region q13 of the X chromosome using fluorescence in situ hybridisation. They also found that when transfected into 1321N1 cells UTP and UDP were full agonists. However, Nicholas and co workers (1996b) showed the action of UDP on $P2Y_4$ receptors was due to cell surface conversion of UDP to UTP and also due to impurities in UDP stocks with UTP. The treatment of stock solutions with hexokinase and glucose or HPLC purification to produce pure UDP resulted in the conclusion that UDP was ineffective at $P2Y_4$.

The rat brain $P2Y_4$ receptor was cloned by Webb *et al.* (1998b) and Bogdanov *et al.* (1998), and shows 83% sequence identity with the human $P2Y_4$ receptor. 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 ATP and UTP are full agonists at the $P2Y_4$ receptor. However, 1321N1 cells expressing the human $P2Y_4$ showed UTP to stimulate the formation of inositol phosphates with equal potency above ATP a partial agonist and ADP relatively inactive (Communi *et al.*, 1995, Nguyen *et al.*, 1995. To resolve this problem, human and rat $P2Y_4$ receptors were transfected and compared in the 1321N1 cell line by Kennedy *et al.* (2000). Human $P2Y_4$ receptors responded maximally to UTP for both Ca^{2+} and inositol phosphate levels detected, whereas ATP was ineffective at increasing Ca^{2+} levels, acting as a weak antagonist. Therefore ATP was found to be ineffective at human $P2Y_4$. The rat $P2Y_4$ receptor displayed UTP to be more potent than ATP but both being full agonists. The newly cloned rat $P2Y_4$ receptor was reversibly antagonized by RB-2 but not by suramin or PPADS. In comparison, $hP2Y_4$ is moderately blocked by PPADS (Charlton *et al.*, 1996b) and to a lesser extent by RB-2 and is also suramin insensitive (Communi *et al.*, 1996a, Charlton *et al.*, 1996). The agonist profile of rat $P2Y_4$ therefore, is more like the P2U receptor subtype, such as $rP2Y_2$ or $hP2Y_2$, rather than human $P2Y_4$.

1.5.5 $P2Y_5$

The orphan GPCR 6H1 was cloned from activated chicken T cells by Kaplan *et al.* (1996) and was proposed as being a P2Y receptor (Webb *et al.*, 1996c) as it displayed approximately 30% amino acid sequence identity with $P2Y_1$ and $P2Y_2$. It was therefore designated as $P2Y_5$. Displacement studies of bound [^{35}S]dATPαS gave a rank order of potency for this receptor as ATP > ADP > 2MeSATP >> UTP. However, this has been disputed (Schachter & Harden 1997) as discussed earlier (Section 1.4.3). Li *et al.*, (1997) reported that no nucleotide-induced responses were observed in 1321N1 cells stabling expressing the cloned turkey homolog of $P2Y_5$. No functional data has been acquired for the so-called $P2Y_5$ receptor at present. It remains as either a pseudo-receptor or a receptor for which a natural ligand has yet to be found, but is not classed as a P2Y receptor.

1.5.6 $P2Y_6$

The $P2Y_6$ receptor was first isolated from a rat aortic smooth muscle cell cDNA library by Chang and co-workers (1995) and found to display 44% and 38% identity with rat $P2Y_2$ and chick $P2Y_1$ receptors respectively. The tissue distribution of rat $P2Y_6$ included lung, stomach, intestine, spleen, mesentery, heart and aorta. When transfected into C6 glioma cells, rat $P2Y_6$ was found to couple to PLC activation but not adenylate cyclase, and displayed a rank order of potency as shown by mobilisation of intracellular Ca^{2+} as UTP > ADP = 2MeSATP > ADPβS > ATP = ATPγS. Preliminary investigation showed that both suramin and RB-2 inhibited these responses. The human $P2Y_6$ sequence was published by Communi *et al.* (1996b) and was also cloned from human T cells (Southey *et al.*, 1996) and placenta where it is produced in different lengths possibly due to alternate splicing (Maier *et al.*, 1997). 1321N1 expression of human $P2Y_6$ led to PLC activation agonist potency order of UDP > 5-bromo-UTP > UTP > ADP > 2MeSATP >> ATP (Communi *et al.*, 1996b). Using stable purified nucleotides Nicholas *et al.* (1996b) found that UTP weakly activated $P2Y_6$ and any activity instigated by UTP was due to contamination or breakdown to UDP. UDP, therefore, is the most potent activator of $P2Y_6$ with UTP, ATP and ADP only weakly activating it. The $P2Y_3$ subtype has very similar agonist potency profile to that of $P2Y_6$ receptor. It is possible that these receptors are species homologues of the same receptor.

1.5.7 $P2Y_7$

The $P2Y_7$ receptor was first cloned by Akbar and co-workers (1996) from human erythro-leukemic cells. This was transfected into COS-7 cells and the apparent rank order of potency was found to be ATP = ATPγS > 2MeSATP > βγ-MeATP > ADP = UTP. However, when Herold *et al.* (1997) transfected the receptor into 1321N1 cells they were unable to stimulate the $P2Y_7$ receptor with nucleotides. This was explained when cloning and functional analysis of a leukotriene B_4 receptor was published by Yokomizo and colleagues (1997). It was found to have the same sequence as the $P2Y_7$ receptor. The apparent nucleotide signalling observed by Akbar *et al.*, (1996) was thought to have occurred due to the presence of native purinoceptors on COS-7 cells, therefore responded to ATP. This receptor is therefore termed as a leukotriene B_4 receptor and not a P2Y receptor.

1.5.8 $P2Y_8$

Bogdanov and co-workers (1997) reported the cloning of a novel nucleotide receptor isolated from the neural plate of *Xenopus* embryos. It was found to be highly expressed in embryos and functionally linked to Ca^{2+} mobilization with a rank order of agonist potency of ATP = UTP = ITP = CTP = GTP. However, no other species homolog has been cloned.

1.5.9 $p2y_9$

Bohm *et al.* (1997a) and Janssens *et al.* (1997) cloned a putative P2Y receptor from human genomic DNA from a sequenced-tagged site that was similar to the receptor $P2Y_5$ and when Janssens and co-workers carried out transfections of this receptor into COS-7, 1321N1 and CHO-K1 cells no functional responses were found despite using a wide range of nucleotides. This receptor was termed $p2y_9$ receptor and may not be a nucleotide receptor or may be activated by a rare nucleotide or 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 (1997b) but no functional studies have yet established if $P2Y_{10}$ is in fact a P2Y receptor.

1.5.11 $P2Y_{11}$

The human $P2Y_{11}$ was cloned from placental cDNA and genomic library by Communi and colleagues (1997) and shares 33% amino acid identity with the human $P2Y_1$ receptor. This receptor is unusual in that its gene structure contains an intron (not seen in other P2Y receptors) and is also only expressed in the spleen and in HL60 cells. When transfected into 1321N1 cells the human $P2Y_{11}$ shows a rank order of potency of ATP > 2MeSATP >>> ADP with no activity observed with UTP and UDP. Another atypical observation seen was that when expressed in 1321N1 cells and CHO-K1 cells the $P2Y_{11}$ receptor coupled to PLC and adenylate cyclase. This promiscuous coupling is a novelty in the P2Y family and was further investigated by Communi and colleagues (1999). The $P2Y_{11}$ receptor was transfected into 1321N1 cells to characterise the PLC-mediated response rather than the adenylate-mediated response as these cells already possess an adenosine receptor coupled to adenylate cyclase. The CHO-K1 cells were also transfected with $P2Y_{11}$, and these cells were then used to assess the adenylate cyclase activity as these cells express $P2Y_2$ receptors which couple to PLC. No difference in agonist or antagonist sensitivity between the two signalling pathways was observed and the rank order of potency was concluded as ATPγS = BzATP > ATP > ADPβ > 2MeSATP with no activity stimulated with UTP. The adenosine receptor antagonist 8-SPT had no effect on nucleotide responses, which verified that the responses were not mediated via ATP breakdown to adenosine. Surprisingly, FPL67085, a P_{2T} receptor antagonist, was found to be a potent agonist at $P2Y_{11}$. A2P5PS and A3P5PS, both $P2Y_1$ antagonists, had no effect. Suramin was found to be the most effective antagonist (pA_2 = 6.09), whereas RB2 was less effective and PPADs had no effect.

More recently Zambon and colleagues (2001) cloned the canine $P2Y_{11}$ receptor which displayed approximately 70% amino acid sequence identity with the human $P2Y_{11}$ receptor. The same group then went on to transfect both the canine and human $P2Y_{11}$ receptors in CHO-K1 and 1321N1 cells and characterised their nucleotide selectivity and

second messenger coupling (Qi *et al.*, 2001b). Differences between the two homologues were observed. The canine $P2Y_{11}$ receptor was an ADP-preferring receptor, whereas the human $P2Y_{11}$ receptor was an ATP-preferring receptor. The canine $P2Y_{11}$ receptor was also reported have higher efficiency in coupling to cAMP formation and more selective for 2MeSATP and 2MeSADP. The canine $P2Y_{11}$ receptor was argued not to be a distinct member from human $P2Y_{11}$ receptor as it too simultaneously coupled to inositol lipid hydrolysis and cAMP synthesis. The difference in ATP and ADP agonist selectivity was suggested to be due to a difference in residue at position 265 located in the juxtaposition of transmembrane domain 6 and the third extracellular loop. In human $P2Y_{11}$ receptor this residue is an arginine whereas in the canine homolgue this residue is a glutamine.

1.5.12 $P2Y_{12}$

ADP mediates platelet aggregation by stimulating two different P2 GPCRs. The first is the $P2Y_1$ receptor coupled to G_q, thereby mobilizing intracellular Ca^{2+}. The second is P_{2T} receptor coupled to the inhibition of adenylyl cyclase via G_i. The molecular characterization of this P_{2T} receptor remained elusive until recently when Hollopeter and collegues (2001) cloned this receptor from the cDNA library of rat platelets and designated it as $P2Y_{12}$. The human homologue was also isolated from a human platelet library. When expressed in Xenopus oocytes, both were sensitive to pertussiss toxin and 2MeSADP was found to be more potent agonist than ADP. No other nucleotides were found to be effective. Surprisingly this receptor only showed 19% sequence homology to $P2Y_1$ receptor, but displayed 44% sequence homology with the UDP-glucose receptor, both of which are localised on the same chromosome (3q24-25). They also showed that patients with bleeding disorders had a defect in the $P2Y_{12}$ gene, preventing adenylyl cyclase inhibition. Antithrombotic drugs such as clopidogrel are proposed to inactivate G_i coupled receptors by modifying cysteine residues in the receptor. The $P2Y_{12}$ receptor contains four extracellular cysteine residues, which may be the target sites of these antithrombotic agents.

Around the same time another group also cloned a receptor which was stimulated by ADP and designated it as SP1999 (Zhang *et al.*, 2001). The same group later reported evidence for this receptor to be $P2Y_{12}$ by generating $P2Y_{12}$ null mice (Foster *et al.*, 2001). These mice were normal except they exhibited prolonged bleeding times, poor aggregation

of platelets in response to ADP and failed to inhibit adenylate cyclase. Clopidogrel was also reported not to inhibit aggregation induced by ADP. Previous studies using $P2Y_1$ null mice reported ADP-induced $P2Y_1$ receptor activation to mediate shape change through Ca^{2+} mobilization and that these mice displayed partial platelet aggregation (Fabre *et al.*, 1999, Leon *et al.*, 1999), whereas Foster *et al.* (2001) show $P2Y_{12}$ not to participate in Ca^{2+} mobilization. Therefore these receptors signal independently but work synergistically to produce aggregation in response to ADP.

1.5.13 $P2Y_{13}$

Recently Communi and colleagues (2001) cloned the $P2Y_{13}$ receptor from human spleen, which was previously described as a orphan receptor called GPR86 (Wittenburg *et al.*, 2001). It was found to have 43% amino acid identity with the human $P2Y_{12}$ receptor. This receptor is predominantly expressed in the spleen and the brain as well as lymph nodes and bone marrow. Similar to the $P2Y_{12}$ receptor, $P2Y_{13}$ is also stimulated by ADP when transfected into 1321N1 cells. However in contrast to $P2Y_{12}$, 2MeSADP stimulated PLC activation with equal potency to that of ADP, whereas it is more poent than ADP at the $P2Y_{12}$ receptor (Zhang *et al.*, 2001, Hollopeter *et al.*, 2001). Also, ATP and 2MeSATP had no effect. Coexpression of $G\alpha_{16}$ protein was necessary to couple the receptor to PLC. The responses were pertussis toxin sensitive suggesting a synergy between $G\alpha_{16}$ and $G\alpha_i$.

$P2Y_{13}$ receptor was also expressed in CHO-K1 cells and displayed biphasic properties. Low concentrations (< 30 nM) of ADP inhibited forskolin-stimulated increases in cAMP levels, whereas higher concentrations stimulated increases in cAMP levels. In the presence of pertussis toxin the inhibition of adenylate cyclase was abolished and stimulation of adenylate cyclase was potentiated. It was suggested that $P2Y_{13}$ simultaneously coupled to two G proteins with opposing effects.

1.5.14 P2X receptors

The fast neurotransmitter properties of ATP are mediated via ligand-gated P2X ion channels. The first P2X receptor was cloned from rat vas deferens by Valera *et al.* in 1994 and later in rat urinary bladder by the same group (Valera *et al.*, 1995). Structural predictions of this 399 amino acid protein suggest it contains two membrane-spanning

regions with a pore-forming motif. This was designated as $P2X_1$ and when transfected into Xenopus oocytes or human embryonic kidney (HEK) cells, ATP activated cation-selective ion channels with a relatively high permeability to calcium to generate an inward current. This current was desensitised with repeated application of agonist and displayed suramin and PPADS sensitivity. 2MeSATP was found to be the most potent agonist, along with α,β–MeATP, which was less potent.

Brake and colleagues (1994) cloned the $P2X_2$ receptor from rat phaeochromocytoma PC12 cells. This receptor shares 33% sequence homology with $P2X_1$ and is suramin and PPADS sensitive (IC_{50} values between 1-5 μM, Evans *et al.*, 1995). But unlike $P2X_1$, $P2X_2$ did not desensitise with repeated application of ATP and was also not stimulated by α,β-MeATP (Brake *et al.*, 1994). $P2X_2$ expression was found in the brain, spinal cord, pituitary gland, intestine, bladder and vas deferens. The genomic encoding of this receptor is 2839 base pairs long, whereas its cDNA only consists of 1481 base pairs. The presence of ten introns in the genomic DNA accounts for this difference, which also allows for potential splice variations. So far reverse transcriptase polymerase chain reaction (RT-PCR) analysis of rat tissues has revealed just two splice variants (Brandle *et al.*, 1997).

$P2X_3$ was cloned from the rat dorsal root ganglia cDNA by Chen *et al.* in 1995 and was shown to be selectively expressed in the sensory neurones. The rank order of agonist potency was shown as 2MeATP > ATP > α,β–MeATP with rapid desensitisation upon repeated application. Again this receptor was also found to be suramin and PPADS sensitive with an IC_{50} value of approximately 1 μM. $P2X_2$ and $P2X_3$ were shown to form a heteromultimer together, which resulted in different characteristics from the individual receptors (Lewis *et al.*, 1995). Although this is the most frequently reported form of heteromultimerisation, most of the cloned P2X receptors are capable of forming such association with other P2X receptors (Le *et al.*, 1999, Torres et al., 1998, 1999), except for $P2X_7$. $P2X_6$ was also found to be the exception to homomer formation (Torres *et al.*, 1999). $P2X_1$ through to $P2X_6$ therefore can generate many forms of multimers, which may account for the diversity of signalling seen with ATP in native sytems.

The $P2X_4$ receptor was cloned from rat cDNA by Bo *et al.* in 1995, sharing 49%, 45%, and 45% sequence homology with $P2X_1$, $P2X_2$ and $P2X_3$ respectively (Buell *et al.*, 1996). ATP stimulated this receptor when transfected into HEK cells, whereas α,β–MeATP was ineffective. Suramin and PPADS were not effective antagonists, which

is useful when differentiating between $P2X_4$ and other P2X receptors. $P2X_4$ is widely distributed in the central and peripheral nervous system (Collo *et al.*, 1996).

Both $P2X_5$ and $P2X_6$ were cloned from rat hippocampus (Collo *et al.*, 1996). $P2X_5$, like $P2X_2$, is not responsive to α,β–MeATP and slow to desensitise. $P2X_6$, like $P2X_4$ receptor is insensitive to α,β–MeATP, suramin and PPADS.

The $P2X_7$ receptor was cloned from rat brain (Suprenant *et al.*, 1996) and proposed to be the ATP stimulated P_{2Z} receptor responsible for macophage cell lysis. It is homologous to other P2 receptors, but has a unique, longer carboxy-terminal domain that is required for ATP-induced cell lysis in $P2X_7$-transfected HEK cells.

The P2X receptor family are ligand-gated ion channels, which are permeable to Na^+, K^+ and Ca^{2+} expressed mainly in excitable or secretory cells (Bean 1992). The binding of ligand produces a conformational change allowing influx of Ca^{2+} and Na^+ primarily, leading to depolarisation of the cell. As a result voltage sensitive Ca^{2+} channels open and/or calcium-induced calcium release causes increases in intracellular calcium levels.

1.5.15 P2 agonist & antagonists

Of the P2Y receptors discussed above, only seven have been cloned and characterised, whereas all the P2X receptors cloned have been characterised. A summary of the rank order of agonist potencies and effective antagonist are summarized in Table 1.1.

	Rank order of agonist potency	**Antagonist activity**
$P2Y_1$	2MeSADP>ADP>2MeSATP>ATP not UTP,UDP	Suramin pA_2=6.0 PPADS pA_2=6.0
$P2Y_2$	UTP=ATP Not ADP, 2MeSADP, 2MeSATP	Suramin pA_2=4.3 PPADS inactive
$hP2Y_4$	UTP>ATP Not ADP, ATP, 2MeSADP	Suramin inactive PPADS inactive
$rP2Y_4$	UTP=ATP Not ADP, ATP, 2MeSADP	Suramin inactive PPADS inactive
$P2Y_6$	UDP>> UTP>ADP>2MeSATP Not ADP, 2MeSADP	Suramin inactive PPADS inactive
$P2Y_{11}$	ATPγS>ATP>2MeSATP>>ADP=2MeSADP Not UTP, UDP	Suramin pA_2=6.09 PPADS inactive
$P2Y_{12}$	2MeSADP>ADP Not ATP, UDP	Full analysis not available
$P2Y_{13}$	ADP=2MeADP Not ATP, 2MeSATP	Full analysis not available
$P2X_1$	2MeSATP=ATP>α,β-meATP>>ADP,UTP	SuraminIC_{50}=1μM PPADS IC_{50}=1μM
$P2X_2$	ATP>>2MeSATP>>ADP>>UTP Not α,β-MeATP	SuraminIC_{50}=1μM PPADS IC_{50}=1μM
$P2X_3$	2MeSATP>ATP>α,β-MeATP>>ADP>>UTP	SuraminIC_{50}=1μM PPADS IC_{50}=1μM
$P2X_4$	ATP>ADP>2MeSATP>>α,β-MeATP Not UTP	SuraminIC_{50}=100μM PPADS IC_{50}=100μM
$P2X_5$	ATP=2MeSATP>ADP Not UTP, α,β-MeATP	SuraminIC_{50}=5μM PPADS IC_{50}=5μM
$P2X_6$	ATP=2MeSATP>>ADP not α,β-MeATP	SuraminIC_{50}=100μM PPADS IC_{50}=100μM
$P2X_7$	ATP>2MeSATP>>ADP Not UTP, α,β-MeATP	SuraminIC_{50}=100μM PPADS IC_{50}=50μM

Table 1.1 Characterisation of functional P2 receptors.

1.6 Signal Transduction of P2Y receptors

P2Y receptors are seven transmembrane G protein-coupled receptors activating phospholipase C, which results in the production of inositol polyphosphates and raised intracellular Ca^{2+} levels. Further studies of P2Y receptors show coupling to other signalling molecules, which include PLD, PLA_2, PKC, adenylate cyclase, and mitogen activated protein kinases (discussed in detail in Section 1.8). The structure of P2Y receptors and their utilisation of these molecules in signal transduction mechanism are discussed below.

1.6.1 Structure of P2Y receptors

Hydropathicity analysis shows that P2Y receptors consist of seven α-helical transmembrane 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 receptor sequences are approximately 350 amino acids in length and are amongst the smallest of G protein-linked receptors. 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). There are conserved cysteine residues in the first and second extracellular loops that may represent a site for a disulphide bridge, which stabilizes the protein structure (Jiang *et al.*, 1997a, Moro *et al.*, 1998, 1999). The human $P2Y_1$ receptor contains two disulphide bridges rather than one (Moro *et al.*, 1999). Site-directed mutagenesis in the mouse $P2Y_2$ receptor showed that amino acids with a positive charge in transmembrane domains III, VI and VII were the most likely targets for ligand interaction as the fully ionised forms of the nucleotides are the most active (Erb *et al.*, 1995). Mutations in H262L, R265L and R292L all caused a 100-850 fold decrease in the potency of ATP and UTP and rendered ADP and UDP ineffective. The mutation K289R led to an increase in the activity of ADP and UDP 100 fold above ATP and UTP. It was concluded that positively charged His262, Arg265 and Arg292 all have interactions with the phosphate moiety of nucleotides.

Similarly mutational analysis at the human $P2Y_1$ receptor by Jiang *et al.*, (1997a) uncovered several different areas of ligand interactions. Mutation S317A did not alter 2MeSATP potency, whereas F131, H132, Y136, F226 or H277 residues replaced with

alanine all resulted in 7-18 fold decrease in potency. Mutations K280A and Q307A both greatly reduced ligand binding with a large reduction in potency. Mutations R128A, R310A and S314A however showed no activity of 2MeSATP. These mutations suggest that TM3 and TM7 are critical for the ligand-binding pocket.

Recently Qi and colleagues (2001) performed mutational studies in canine and human $P2Y_{11}$ receptors. The canine homologue was found to be more selective for ADP whereas the human $P2Y_{11}$ receptor was more selective for ATP. Mutational analysis showed that the change of the Arg-265 residue in human $P2Y_{11}$ receptor (similar to the His-277 residue in $P2Y_1$ receptor as described in Jiang *et al.,* 1997a), which is located in the juxtaposition of transmembrane domain 6 and the third extracellular loop, to glutamine increased the potency and efficacy relative to ATP. The reverse mutation, where Gln-268 was mutated to arginine, increases the potency of ATP potency in the canine $P2Y_{11}$ receptor. This data suggested that the Arg-265 in the $P2Y_{11}$ receptor is involved in nucleotide biding and functions to discriminate between ATP and ADP. Unfortunately due to the lack of reliable radioligand binding assay, the involvement of this residue cannot be confirmed. However mutational analysis has proved a useful tool in identifying important residues and will help future development of more specific and potent agonists and antagonists.

1.6.2 G Proteins

The superfamily of guanine nucleotide-binding proteins share considerable sequence homology at their guanine nucleotide-binding site. G proteins are just one of the sub-families. These are membrane bound heterotrimeric guanine-nucleotide binding proteins, composed of α, β, and γ proteins, each of which has different isoforms. Rodbell and co-workers first showed that certain receptors required GTP for adenylate cyclase activation in rat liver membranes (Lin *et al.*, 1977). Since then a large body of work has developed showing the tremendous complexity of the GPCR and G protein signalling mechanisms.

1.6.2.1 Mechanism of action

G proteins cycle between a GTP-bound active form and a GDP-bound inactive form (Figure 1.3). In its resting state the G protein consists of an αβγ-trimer with GDP bound to the α-subunit. In this state it is able to bind to an inactive receptor. Agonist binding activates the receptor causing a conformational change, which is transmitted to the G protein. The affinity for GDP is reduced causing dissociation resulting in a ternary complex consisting of the agonist bound receptor and the nucleotide free G protein. This transient state is unstable and short-lived. GTP, present in higher concentration in the cell than GDP, binds to the G protein causing disassociation into the active GTP bound G_α subunit and the βγ subunit, which are free to diffuse in the membrane and associate with effectors (reviewed in Clapman & Neer 1993). The βγ subunit does not dissociate unless denatured. Dissociation from the receptor reduces its affinity for its ligand resulting it receptor ligand dissociation. Binding of the G_α subunit increases its GTPases activity and catalyses the conversion of GTP to GDP. This terminates the interaction with the effector and enables the inactive GDP-bound G_α subunit to re-associate with the βγ subunit. This complex can then associate with inactive receptor to begin the cycle again (for review see Fields & Casey 1997).

1.6.2.2 G protein subtypes

Sequence homology and bacterial toxin sensitivity shows four subfamilies of G protein α subunits (G_s, G_i, G_q, and $G_{12/13}$) ranging between 39 to 52 kDa in weight (Hepler & Gilman, 1992). G protein α_s subunits are sensitive to the cholera toxin, which causes ADP-ribosylation of a conserved arginine residue in the α_s subunit preventing GTPase activity and binding to subunit βγ. The α_s subunits ability to stimulate adenylate cyclase was recognised by Gilman, (1987).

Conversely the α_i subunit was found to inhibit adenylate cyclase activity (Neer & Clapham, 1988) and includes α_{i-1}, α_{i-2}, α_{i-3}, α_o (predominantly neural), α_t, α_{t2} (retinal subunits), and α_z. These G proteins are sensitive to pertussis toxin, which has been shown to ADP-ribosylate a conserved cysteine residue preventing activity except α_z, which lacks the consensus site for ADP-ribosylation (Fong *et al.*, 1988).

The α_q class couple to the activation of phospholipase C with a rank order of PLCβ1 > PLCβ2 > PLCβ3 (Nakamura *et al.*, 1995) and includes members α_q, α_{11}, and α_{16}.

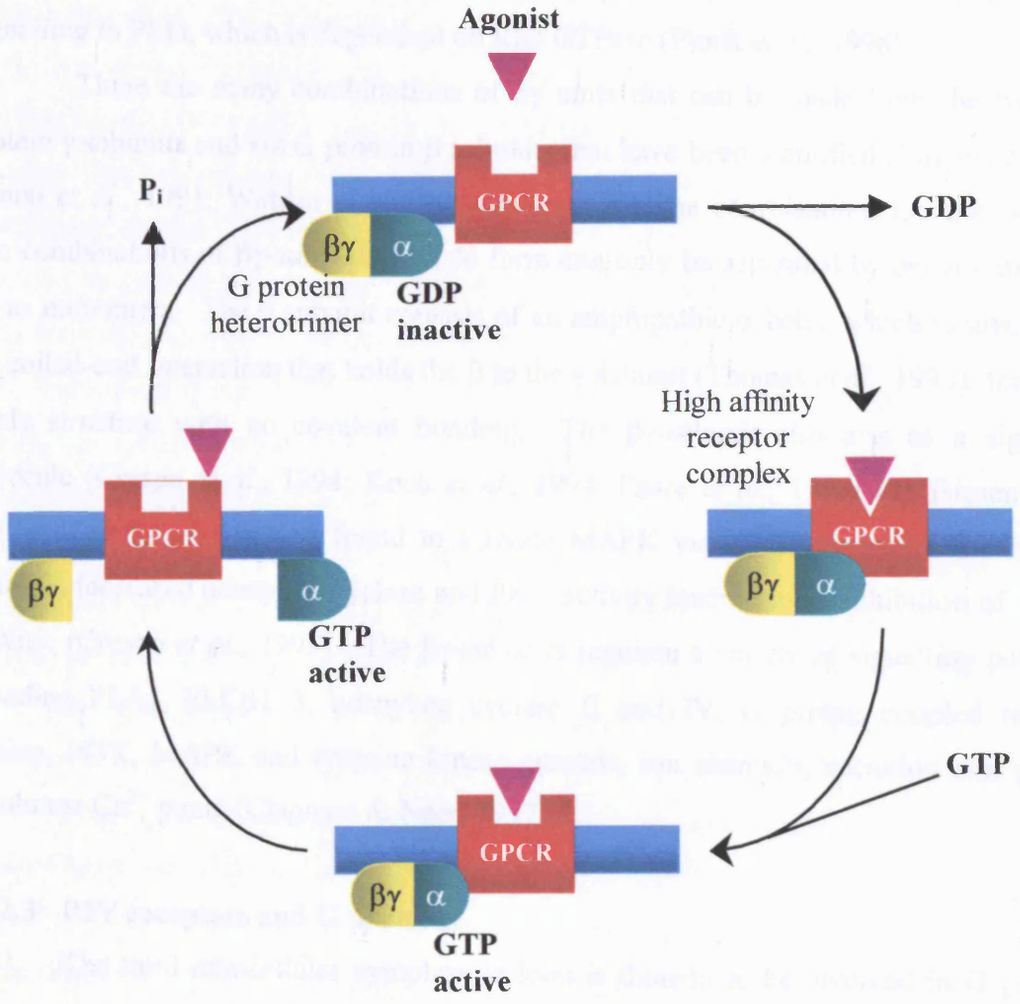


Figure 1.3 G protein ternary complex cycling between activation and inactivation

Receptor activation was found to induce the phosphorylation of tyrosine residue (Tyr356) of $G_{q/11}$,which was essential for activation (Umemori *et al.*, 1997).

The α_{12} and α_{13} class, which are pertussis toxin insensitive, have no well defined targets of action (see Offermanns & Schultz, 1994), but may be involved in mitogenesis (Aragay *et al.*, 1995; Collins *et al.*, 1996), apoptosis (Berestetskaya *et al.*, 1998) and signalling to PLD, which is dependant on Rho GTPase (Plonk *et al.*, 1998).

There are many combinations of βγ units that can be made from the 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), however some combinations are not possible. The combinations of βγ-subunits that do form can only be separated by denaturation and act as monomers. The β subunit consists of an amphipathic α helix, which is involved in the coiled-coil interaction that holds the β to the γ subunit (Thomas *et al.*, 1993), forming a stable structure with no covalent bonding. The βγ-subunit also acts as a signalling molecule (Crespo *et al.*, 1994; Koch *et al.*, 1994; Faure *et al.*, 1994; van Biesen *et al.*, 1995). The βγ-subunit was found to activate MAPK via a Ras-dependant mechanism, while G_s increased adenylate cyclase and PKA activity leading to an inhibition of MAPK activity, (Crespo *et al.*, 1995). The βγ-subunits regulate a variety of signalling pathways including PLA_2, PLCβ1–3, adenylate cyclase II and IV, G protein-coupled receptor kinases, PI3K, MAPK and tyrosine kinase cascade, ion channels, secretion and plasma membrane Ca^{2+} pump (Clapham & Neer, 1997).

1.6.2.3 P2Y receptors and G proteins

The third intracellular cytoplasmic loop is thought to be involved in G protein-receptor interaction (Strader *et al.*, 1995). In P2Y receptors a histidine residue near transmembrane domain three is conserved and is believed to be essential in coupling the receptor to G protein activation (Fraser *et al.*, 1988).

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, will link to a defined set of G protein subunits regardless of cell type. However, different tissues may express receptors at different levels, causing differential linkage to signalling pathways. Receptors may also 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 βγ subunit.

Chlorea toxin and pertussis toxin are useful tools in distinguishing which G proteins couple to P2Y receptors. The $P2Y_1$ receptor was reported as pertussis toxin insensitive and activating PLC in bovine aortic endothelial cells (BAECs) (Purkiss *et al.*, 1994; Motte *et al.*, 1993; Pirotton *et al.*, 1996), which suggests coupling to G_q protein. The $P2Y_2$ receptor on the other hand was shown to be pertussis toxin sensitive in BAEC's (Purkiss *et al.*, 1994; Erb *et al.*, 1993; Pirotton *et al.*, 1996) most likely coupling to $G_{i/o}$ protein. This has also been shown to be the case in other native cells such as human skin fibroblast cells and CF/T43 human airway endothelial cells, where $P2Y_2$ responses were found to be pertussis toxin sensitive and in porcine coronary artery and rat liver where $P2Y_1$ receptor responses were insensitive (Fine 1989, Brown *et al.*, 1991, Flavaham *et al.*, 1989, Siddiqui & Exton 1992). In rat aortic smooth muscle cells, Pediani and co-workers (1999) observed the activation of Ca^{2+} signalling by UTP to be both pertussis toxin sensitive and insensitive. This was suggested to be due to the co-expression of $P2Y_2$ and $P2Y_4$ receptors and simultaneous coupling of different G proteins. In *Xenopus* oocytes $P2Y_2$ couples to two different ion channels, Ca^{2+}-dependant chloride channel and inward-rectifier potassium channels, both of which were suramin sensitive (Mosbacher *et al.*, 1998). However only the latter was found to be pertusiss toxin sensitive suggesting that this receptor couples to two different G proteins.

The $P2Y_4$ receptor induced inositol phosphate accumulation was shown to be pertussis toxin sensitive at a single stimulation time point of 30 seconds when transfected into 1321N1 cells (Communi *et al.*, 1996a). It was suggested that $P2Y_4$ couples to multiple G proteins, including the G_i protein, in transfected 1321N1 cells. In a lung submucosal derived cell line the same group showed that $P2Y_4$ receptor activation was inhibited by pertussis toxin, suggesting $G_{i/o}$ involvement (Communi *et al.*, 1999b). However, another group reported that human $P2Y_4$ activation in 1321N1 cells leads to a time or dose dependant PLC activation which is pertussis toxin insensitive (Roberts *et al.*, 1999).

The $P2Y_6$ receptor is thought to link to G_q proteins as PLC activation was shown to be pertussis toxin insensitive (Chang *et al.*, 1995, Robaye *et al.*, 1997). However, when cRNA for $P2Y_6$ was injected into neurons Filippov and co-workers (1999) observed the activation of the $P2Y_6$ receptor, which led to the inhibition of N-type Ca^{2+} currents that were pertussis toxin sensitive and M-type K^+ currents that were insensitive respectively. This was evidence for multiple coupling by one receptor.

The $P2Y_{11}$ receptor, when expressed in CHO-K1 and 1321N1 cells, activated adenylyl cyclase and PLC (Communi *et al.*, 1997, Qi *et al.*, 2001a). The $P2Y_{11}$ receptor is probably linked directly to G_s coupled to the activation of adenylyl cyclase as well as another G protein linked to PLC. On the other hand activation of adenylyl cyclase could be independent of G_s and may involve activation via intracellular Ca^{2+} increases or PKC (see 1.6.5).

1.6.3 Phospholipases

Membrane phosphospolipids are hydrolysed by phospholipases to generate lipid signalling molecules which act as second messengers in the signal transduction process initiated by activated receptors. The most studied phospholipases include PLC, PLA_2 and PLD, are discussed below. They are named by the position at which they attack the phospholipid backbone (see Figure1.4A)

1.6.3.1 Phospholipase C (PLC)

PLC catalyses the hydrolysis of the phosphodiester bond on the third (SN-3) position of the glycerol (Figure 1.4) in the membrane phospholipid phosphotidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2 or PIP_2] to produce two intracellular messengers, inositol triphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) (Figure 1.5). DAG is lipophilic and remains bound to the membrane where it activates protein kinase C (Rhee & Choi, 1992). Ins(1,4,5)P_3 is soluble and diffuses through the cytosol to stimulate the release of Ca^{2+} from intracellular stores by binding to specific Ins(1,4,5)P_3 receptors (Berridge, 1993), which are tetrameric Ca^{2+} channels. Ins(1,4,5)P_3 signalling is terminated by either phosphorylation to produce inositol 1,3,4,5-tetrakiphosphate (Ins(1,3,4,5)P_4) by 3-kinase or dephosphorylation to inositol which is recycled to the membrane phospholipid pool. Ins(1,3,4,5)P_4 may also stimulate the influx of calcium into cells (Luckhoff & Clapham, 1992, Cullen *et al.*, 1990).

Three PLC isoforms, β, γ and δ, have been purified and cloned (Rhee & Choi 1992). A fourth member PLCε was cloned more recently (Song *et al.*, 2001, Lopez *et al.*, 2001, Jin *et al.*, 2001). All isoforms have a pleckstrin homology (PH) domain near the N-terminus (see Figure 1.4B) and two catalytic domain regions known as X and Y (Lee & Rhee 1995). The PH domain is required for membrane and PIP_2 binding but the enzyme

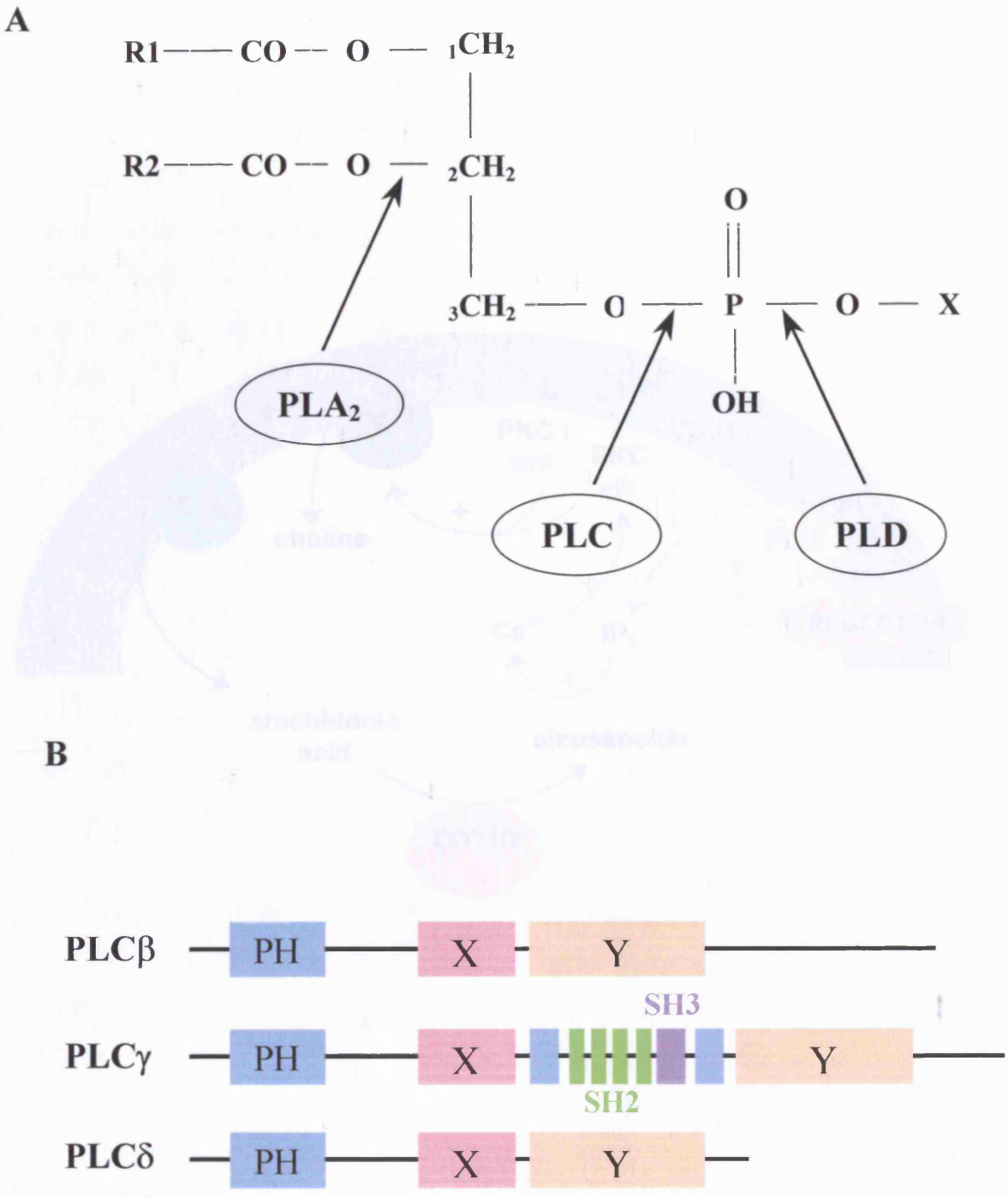


Figure 1.4
A) Cleavage sites of membrane phospholipids by phospholipases
B) Structure of PLC isoforms

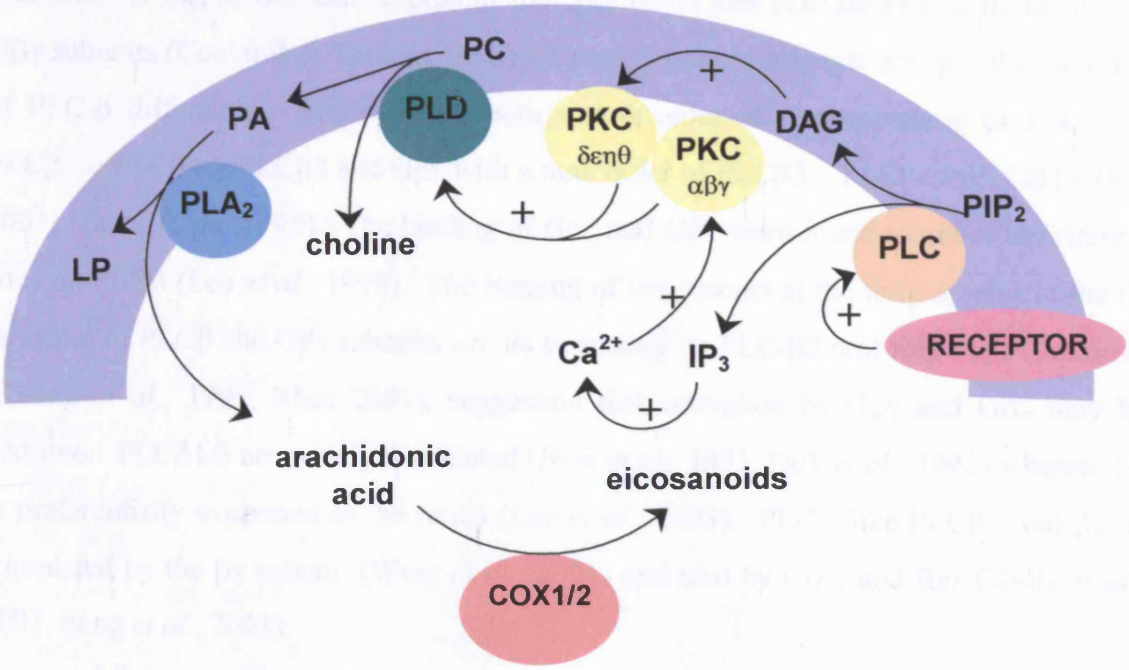


Figure 1.5 Summary of phospholipid metabolism involving PLC, PKC, PLD, PLA and Cox

remains active in its absence (Cifuentes et al., 1993). The region between X and Y domains is short in PLC-β and PLC-δ but longer in the PLC-γ isoform where it contain the SH2 and SH3 domains, enabling interaction with tyrosine phosphorylated proteins.

The PLC-β isoform has four different subtypes, referred to as PLC-β1-4. Activation of PLC-β can be via GTP bound $G\alpha_q$ proteins, which are pertussis toxin insensitive and by Gβγ subunits, although PLCβ-4 is not activated by Gβγ subunits (Lee *et al.*, 1994, Jiang *et al* 1994). Some responses however were found to be pertussis toxin sensitive. It was shown that G_i protein subtypes could also activate PLC-β by release of Gβγ subunits (Cockroft & Thomas 1992). These G protein subunits activate the isoforms of PLC-β differently. $G\alpha_q$ activates with a rank order of responsiveness of PLCβ1 > PLCβ2 > PLCβ4 > PLCβ3 and Gβγ with a rank order of PLCβ3 > PLCβ2 > PLCβ1 (Rhee 2001, Lee & Rhee, 1995). The binding of $G\alpha_q$ and Gβγ were found to occur at different sites on PLCβ (Lee *et al.*, 1993). The binding of $G\alpha_q$ occurs at the third a helix in the C-terminus of PLCβ and Gβγ subunits occurs separately on PLC-β2 probably via PH domain (Kuang *et al.*, 1996, Rhee 2001), suggesting that activation by Gβγ and $G\alpha_q$ may be additive. PLCβ1-3 are widely distributed (Jhon *et al.*, 1993, Park *et al.*, 1992) whereas β4 is preferentially expressed in the retina (Lee *et al.*, 1993). PLCε, like PLCβ2 and β3, is stimulated by the βγ subunit (Wing *et al.*, 2001) and also by $G\alpha_{12}$ and Ras (Kelly *et al.*, 2001, Song *et al.*, 2001).

Like many G protein coupled receptors, the cloned mammalian P2Y receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ also couple to PLC-β activation via $G_{q/11}$ when transfected into 1321N1 cells (Palmer *et al.*, 1998, Nguyen *et al.*, 1995, Nicholas *et al.*, 1996, Schachter *et al.*, 1996, Parr *et al.*, 1994, Communi *et al.*, 1995a, Chang *et al.*, 1995, Communi *et al.*, 1997).

PLC-γ has two isoforms, PLC-γ1 and γ2. These are activated by growth factor tyrosine kinase receptors including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and nerve growth factor (NGF) (Rhee & Choi, 1992). Growth factor receptors dimerize upon activation, which increases the catalytic activity of its tyrosine kinase (discussed in Section 1.7). $PLC\gamma_1$ associates with the tyrosine residues on the activated receptor and undergoes tyrosine phosphorylation at Tyr771, Tyr783 and Tyr1254 (Kim *et al.*, 1991). This increases its activity causing the hydrolysis of PIP_2 to $Ins(1,4,5)P_3$ and DAG.

1.6.3.2 Phospholipase A2 (PLA_2)

PLA_2 cleaves membrane phospholipids to generate a 20 carbon, polyunsaturated fatty acid called arachidonic acid and lysophospholipids (Figure 1.4A). These are precursors for other lipid mediators such as prostaglandins, leukotrienes and platelet activating factor (Dennis 1987). PLA_2 exist as two isoforms, which are secreted PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$). These phospholipases are regulated by intracellular Ca^{2+} levels, phosphorylation by PKC, cAMP-dependent kinases and receptor tyrosine kinase (Glaser *et al.* 1993, Wightman *et al.*, 1982, Goldberg *et al.*, 1990). However, Ca^{2+}-independent forms of PLA_2 and PKC-independent mechanism of PLA_2 activation have been observed in some cells (Miyake & Gross, 1992, Qui & Leslie, 1994). Secreted PLA_2s are small, 13-18 kDa water-soluble proteins found in snake and bee venoms, and in the mammalian pancreas (Davidson & Dennis 1990). After secretion $sPLA_2$ are thought to bind to the exterior of cells and carry out hydrolysis of phospholipid substrates. The $cPLA_2$ shows preference to arachidonic acid containing phospholipids and its translocation to the membrane is dependant on calcium (Clark *et al.*, 1991; Sharp *et al.*, 1991). Activation of PLA_2 can either be direct from G proteins and protein tyrosine kinases or involve second messengers. For example, the $cPLA_2$ is regulated by phosphorylation at ser505 by the MAP kinase cascade (Lin *et al.*, 1993). Arachidonic acid is metabolised to produce prostaglandins (PGE_2, PGI_2 and PGD_2) by cycloxygenase 1 or 2 COX1/2 (Scott *et al.*, 1999) (Figure 1.5). PLA_2-induced production of PGI_2 is also regulated by tyrosine kinases and MAPK (Patel *et al.*, 1996). Prostaglandins stimulate increases in cAMP levels in VSMCs (Graves *et al.*, 1993, Vegesna & Diamond 1986) via their receptors, EP_1 receptor for PGE_2, IP receptor for PGI_2 and DP receptors for PGD_2. The production of prostaglandins thus is downstream of the ERK pathway. The addition of COX inhibitor, indomethacin, resulted in a decrease in PDGF-induced accumulation of cAMP in human arterial SMCs (Graves *et al.*, 1996). This was consistent with PDGF inducing prostaglandin production which results in cAMP production.

P2Y receptor regulation of PLA_2 has been demonstrated in many cell types including astrocytes (Bolego *et al.*, 1997), BAEC's (Patel *et al.*, 1996), RAW 264.7 macrophage cells (Lin & Lee, 1996), and HL60 cells (Xing *et al.*, 1994).

1.6.3.3 Phospholipase D (PLD)

PLD cleaves membrane phospholipids containing phosphatidylcholine at their terminal phosphodiester bond (Figure 1.4A) to generate phosphatidic acid (PA) and free polar head groups e.g. free choline (Figure 1.5). PA can be metabolised by PA phosphohydrolase to form DAG that leads to the activation of PKC, therefore PLD has the potential to activate PKC. DAG can also be converted to PA by the enzyme DAG kinase. At present only one isoform of PLD has been cloned (Hammond *et al.*, 1995) though evidence exists for further subtypes (Exton *et al.*, 1994). Receptor activation of PLD can occur via PLC dependent or independent methods. PDGF receptor-induced activation of PLD was shown to be dependant on PLCγ, tyrosine phosphorylation and PKC activation (Pfeilschifter & Merriweather, 1993, Lee *et al.*, 1994; Yeo *et al.*, 1994). PLC independent pathways involve direct coupling to low-molecular-mass G proteins (Malcolm *et al.*, 1995, Bowman *et al.*, 1993, Brown *et al.*, 1993; Cockcroft *et al.*, 1994). Both phosphorylation-dependent and independent activation by PKC have been suggested (Conricode *et al.*, 1992, Balboa *et al.*, 1995).

$P2Y_2$ receptor activation of PLD has been demonstrated in bovine adrenal medullary endothelial cells that correlated with PLC activation (Purkiss *et al.*, 1993). In BAECs Purkiss *et al.*, (1992), also demonstrated that $P2Y_2$ leads to PLD activation probably downstream of PLC. However the production of PA was found not only produced from PLD-induced conversion of phosphatidylcholine, but also from the action of DAG kinase on DAG. Therefore the detection of increased PA levels does not always correlate with increased PLD activity. Malcolm *et al.* (1995) observed P2 receptor activation of PLD in rat liver plasma membrane, which was PLC independent.

1.6.4 Protein Kinase C (PKC)

Signal transduction from the plasma membrane requires diffusible second messengers to interact with proteins to alter their activity and induce a cellular response. The second messenger DAG, produced by PIP_2 hydrolysis by PLC, activates a serine/threonine family of protein kinases called PKC (Figure 1.5). This family consists presently of a family of 11 isotypes (α, β_I, β_{II}, γ, δ, ε, ζ, θ, ι, λ and η) requiring threonine phosphorylation for activation. However, PKC isoforms are subdivided into Ca^{2+}-dependent and independent groups and further divided on the basis of conserved structural

features and their activators. The Ca^{2+}-dependent PKC isoforms (sub group C) consists of PKC isoforms α, β_I, β_{II} and γ subspecies and are activated by Ca^{2+}. The Ca^{2+}-independent PKC isoforms (sub group N) consists of δ, ε, η, and θ subspecies activated by phosphotidyl serine, phorbol esters and DAG. The final group (sub group A) consists of atypical PKC isoforms includes ζ, λ, and ι, activated by phosphotidyl serine but not Ca^{2+}, phorbol esters or DAG (Andrea & Walsh 1992). All PKC isoforms are constructed with a kinase catalytic site and variable regulatory domains called the pseudosubstrate site (House & Kemp 1987). Inhibtion of the kinase activity is relieved when DAG binds to the pseudosubstrate site.

Tumour promoting phorbol esters, such as 12-0-tetradecanoyl phorbol 13-acetate (TPA) and PMA, strongly activate PKC (Nishizuki 1992) Ro 31-8220 was developed as a competitive PKC inhibitor based on bisindolylmaleimide (Davis *et al.,* 1992), which is selective for protein kinase C (IC_{50}=10nM) over CaM kinase II (IC_{50} = 17 μM) and PKA (IC_{50} = 900 nM). Ro 31-8220 however also has non-specific effects and has been reported to inhibit MAP kinase phosphatase-1 expression and activate the stress activated protein kinase, JNK-1 (Beltman *et al.,* 1996). The indolocarbazole Go 6976 is a novel inhibitor of Ca^{2+} sensitive PKC isoforms (Martiny-Baron *et al.*, 1993), inhibiting PKC α (IC_{50}=2.3nM) and β1 (IC_{50} = 6.2 nM) whereas micromolar concentrations do not inhibit PKC δ, ε and ζ isoforms, which are Ca^{2+} insensitive. Go 6850, also known as GF 109203X, is based on bisindolylmaleimide and is a general competitive PKC inhibitor acting at the ATP binding site of PKC. It inhibits PKC isoforms α, β_I, β_{II}, γ, δ and ε with a rank order of subtype inhibition of $\alpha > \beta 1 > \varepsilon > \delta > \zeta$.

Modulation of signalling from P2Y receptors via PKC has been shown to be important in many signalling pathways. The use of phorbol esters has shown that PKC can modulate P2Y receptor activated PLC activity. Purkiss *et al.*, (1994) showed that $P2Y_1$ receptor activation lead to the production of $InsP_3$ that was inhibited by TPA, but potentiated by Ro 31-8220, whereas $P2Y_2$ receptor activation of PLC was unaffected in BAECs. This suggested that P2Y receptor PLC responses were differentially regulated by PKC. The short-term stimulation of PKC with TPA resulted in attenuation of the agonist response, whereas long-term stimulation of PKC with TPA resulted in potentiation of the response due to the down-regulation of PKC (Hepler *et al.*, 1988, Purkiss *et al.*, 1994). Variation of the time of TPA stimulation affects PKC isoforms in a different manner (Olivier & 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.*, 1996). An 8 hour exposure in rat mesangial cell decreased levels of PLCα and δ isoforms, while 24 hour exposure decreased ε isoforms (Pfeilshifter & Merriweather 1993).

PKC has also been shown to be involved in P2Y mediated mitogenesis, prostaglandin and nitric oxide (NO) release. In porcine aortic VSMCs ATP-induced increases in mitogenesis were partially inhibited by PKC downregulation (Wang *et al.*, 1992). In rat aortic VSMC proliferation, P2Y receptor signalling to the p42/p44 MAPK cascade involved PKC-α and/or PKC-δ acting upstream of Raf kinase (Yu *et al.*, 1996). In BAECs Patel *et al.*, (1996) demonstrated that $P2Y_1$ and $P2Y_2$ receptor activation of p42/p44 MAP kinase was required for endothelial cell production of PGI_2, which previously had been shown to be PKC dependant. Extracellular ATP was linked to p42/p44 MAP kinase and PKC-ε and PKC-δ in cardiac cells (Vassort & Puceat, 1997). Recently Soltoff and colleagues (1998a, 1998b) reported that $P2Y_2$ receptors link to p42/p44 MAP kinase in a PKC dependent manner that is proposed to be via PKC-δ in PC12 cells. The precise mechanism of MAPK activation by PKC is still unclear.

1.6.5 Adenylate cyclase

Adenylate cyclases are a family of intracellular enzymes containing two transmembrane regions, each containing six transmembrane helices, and two intracellular regions, which catalyse the production of 3',5'-cyclic adenosine monophosphate (cAMP), stimulated by hormones such as epinepherine, glucagons and vasopressin. This is an important second messenger, which regulates cAMP-dependent protein kinases such as protein kinase A (PKA). PKA has many substrates, which include phosphorylase kinase, glycogen synthase, myosin light chain kinase, hormone-sensitive lipase, pyruvate kinase, phosphatase inhibitor 1, phosphodiesterases (which hydrolyse cAMP), ion channels and many more. PKA has been shown to inhibit ERK by inhibiting Raf-1 (Wu *et al.*, 1993b, Cook & McCormick 1993). However, activation of ERK has also been demonstrated and shown to be mediated via a small G protein Rap-1, which activates B-Raf (Vossler *et al.*, 1997). Modulation of both adenylate cyclase and phophodiesterases by drugs is important in the treatment of cardiovascular diseases and asthma.

There are currently nine cloned mammalian isoforms (I-IX) all having a similar structure (Hurley, 1999). The isoforms show diversity in their regulatory characteristics

and can be divided into three groups. The first group consists of isoforms I, II, and VIII and are positively modulated by Ca^{2+}/calmodulin in response to $G\alpha_s$, whereas in the absence of Ca^{2+}/calmodulin $G\alpha_s$ is ineffective, while $G\alpha_i$ and $\beta\gamma$ subunits are inhibitory. The second group consists of isoforms II and IV, which are effectively stimulated by $G\alpha_s$ and PKC with Ca^{2+} and $G\alpha_i$ having little influence. The third group consists of type V and VI, which are stimulated by $G\alpha_s$ and inhibited by $G\alpha_i$ by binding to the catalytic site of adenylate cyclase (Dessauer *et al.,* 1998). Forskolin positively regulates all isoforms except IX and is a useful tool in studying agents that have a inhibitory influence on adenylate cyclase. Protein phosphorylation of adenylate cyclase can stimulate or inhibit cAMP production. PKC activates type II adenylate cyclase by serine and threonine phosphorylation (Bol *et al.,* 1997) whereas CaM kinase II inhibits type III by serine phosphorylation (Wei *et al.*, 1996). Adenylate cyclase expression shows variations between different isoforms. Types V and VI are widely expressed, whereas type I is abundant in the brain. Differential expression of isoforms therefore allows differing influences on cAMP production

Extracellular nucleotides have been shown to inhibit adenylate cyclase production of cAMP in 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 & Mills, 1993). Inhibition of adenylate cyclase was found to be pertussis toxin sensitive implicating $G\alpha_i$ sub-unit rather than accumulation of Ca^{2+}.

Nucleotide stimulation of adenylate cyclase production of cAMP was observed in HL-60 cells (Choi *et al.,* 1997; Jiang *et al.*, 1997b), BAECs (Tada *et al.*, 1992), and NG108-15 cells (Matsuoka *et al.*, 1995). Recently, $P2Y_{11}$ receptor was cloned (Communi *et al.,* 1997) and reported to couple to adenylate cyclase in CHO-K1 cells together with the activation of PLC showing multiple signalling pathways activated by a single receptor. When expressed in 1321N1 cell-line, none of the P2Y 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).

1.6.6 Phosphoinositide 3-kinase (PI3K)

PI3K phosphorylates the 3'-hydroxy group on the inositol ring of Ptd Ins, Ptd Ins 4-phosphate or Ptd Ins 4,5-phosphate (see Fig 1.5). P13K was purified and cloned from bovine brain and found to consist of two subunits, the regulatory p85 and the catalytic p110 subunit (Otsu *et al.*, 1991, Hiles *et al.*, 1992). The p85 subunit contains two SH2 and one SH3 domains, which can interact with protein and growth factor receptors. Binding of the p85 subunit to receptors brings the catalytic subunit closer to its membrane substrates. There are many members of the PI3K family. Three members of the p110 subunit include p110α, β and γ and two members of the p85 subunit are α and β (Hu *et al.*, 1993, Stoyanov *et al.*, 1995). However, p110γ does not associate with any regulatory p85 species and is activated by both α and βγ subunits of G proteins. PI3K activity is found in most cell types and has been implicated in many biological responses including cell proliferation (Valius & Kazlauskas 1993, Fantl *et al.*, 1992), cell survival (Yao & Cooper 1995), membrane ruffling (Wennstrom *et al.*, 1994), and chemotaxis (Kundra et al., 1994).

Small GTP-binding proteins such as the Ras-related Cdc42, which regulates cytoskeletal reorganization, also bind the p85α subunit (Zheng *et al.*, 1994, Chen 1994). Activated GTP-bound Ras can bind directly to the catalytic p110α subunit, which may suggest a mechanism for P13Ks involvement in mitogensis (Rodriguez-Viciana *et al.*, 1994). Another small GTP-binding protein Rho has also been implicated as an upstream regulator of PI3K (Kumagi *et al* 1993, Ridley *et al.*, 1992). PI3K activity can be inhibited using inhibitors such as LY294002 and wortmannin. These have proved useful tools in determining the actions of PI3K (Vlahos *et al.*, 1994, Davies *et al.*, 2000). In human promonocytic U937 cells, UTP induced ERK activation was inhibited by LY294002 and wortmannin and also src inhibitors radicicol and PP2 (Santiago-Perez *et al.*, 2001). It was suggested that $P2Y_2$ receptor coupling to ERK pathway was mediated via PI3K and src. However in rat aortic SMC, PI3K inhibitors did not inhibit ATP-induced ERK activation (Wilden *et al.*, 1998) suggesting that coupling to PI3K by P2Y receptors varies between cell types.

1.7 Growth factor receptors

Understanding the mechanism of growth factor receptor signal transduction is important as the components of this pathway have been implicated not only in long term events such as proliferation and differentiation, but also in P2Y G protein linked receptor signalling (Bowden *et al.*, 1995; Graham *et al.*, 1996; Soltoff *et al.*, 1998a, b). Growth factor receptors are members of receptor tyrosine-kinase (RTK) family that consist of a transmembrane polypeptide with a protein tyrosine-kinase domain in their intracellular portion. Growth factors bind to their receptor causing dimerization and RTK activation (Heldin 1995). This tyrosine kinase domain is a highly conserved amino acid sequence, although in some RTKs this sequence is interrupted by an inserted sequence, e.g. PDGF receptor (PDGFR). This kinase activity allows the dimerized receptors to phosphorylate themselves on tyrosine residues, which promotes further kinase activity. In the mouse PDGFR the Tyr825 residue in the tyrosine kinase domain is phosphorylated upon receptor activation, inducing a conformational change, which increases autophosphorylation and phosphorylation of substrates (Fantl et al., 1993). The phosphorylated tyrosines 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 & Williams, 1994; Macias *et al.*, 1994) (Figure 1.6). Many cellular enzymes contain these binding motifs including PLCγ (Ronnstrand *et al.*, 1992), Ras GTPase activating protein (Ras-GAP), and the tyrosine kinase, Src. Other proteins containing these domains, which have no apparent activity, function as adapters and form complexes with proteins. These include growth factor receptor binding protein 2 (Grb2) (Arvidsson *et al.*, 1994), Shc (Kavanaugh *et al.*, 1994), Ras activator Sos and the p85 subunit of PI3K (Coughlin *et al.*, 1989). They are usually subjected to phosphorylation by the tyrosine kinase and contain more SH2, SH3, PTB and PH domains therefore bringing together more adapters and also other tyrosine phosphorylated enzymes. The formation of complexes containing RTK, adapters and enzymes, recruits cytoplasmic enzymes to the plasma membrane into close proximity with their substrates. Grb2 associated with the RTK and Shc through its SH2 domains. Shc also associates with the receptor and with non-receptor tyrosine kinases such as Src. Grb2 also binds to the proline-rich sequence in Sos through its SH3 domain. This complex of protein-protein interaction allows activation of Ras, which has a pivotal role in RTK-mediated proliferation (Pronk & Bos 1994). This small

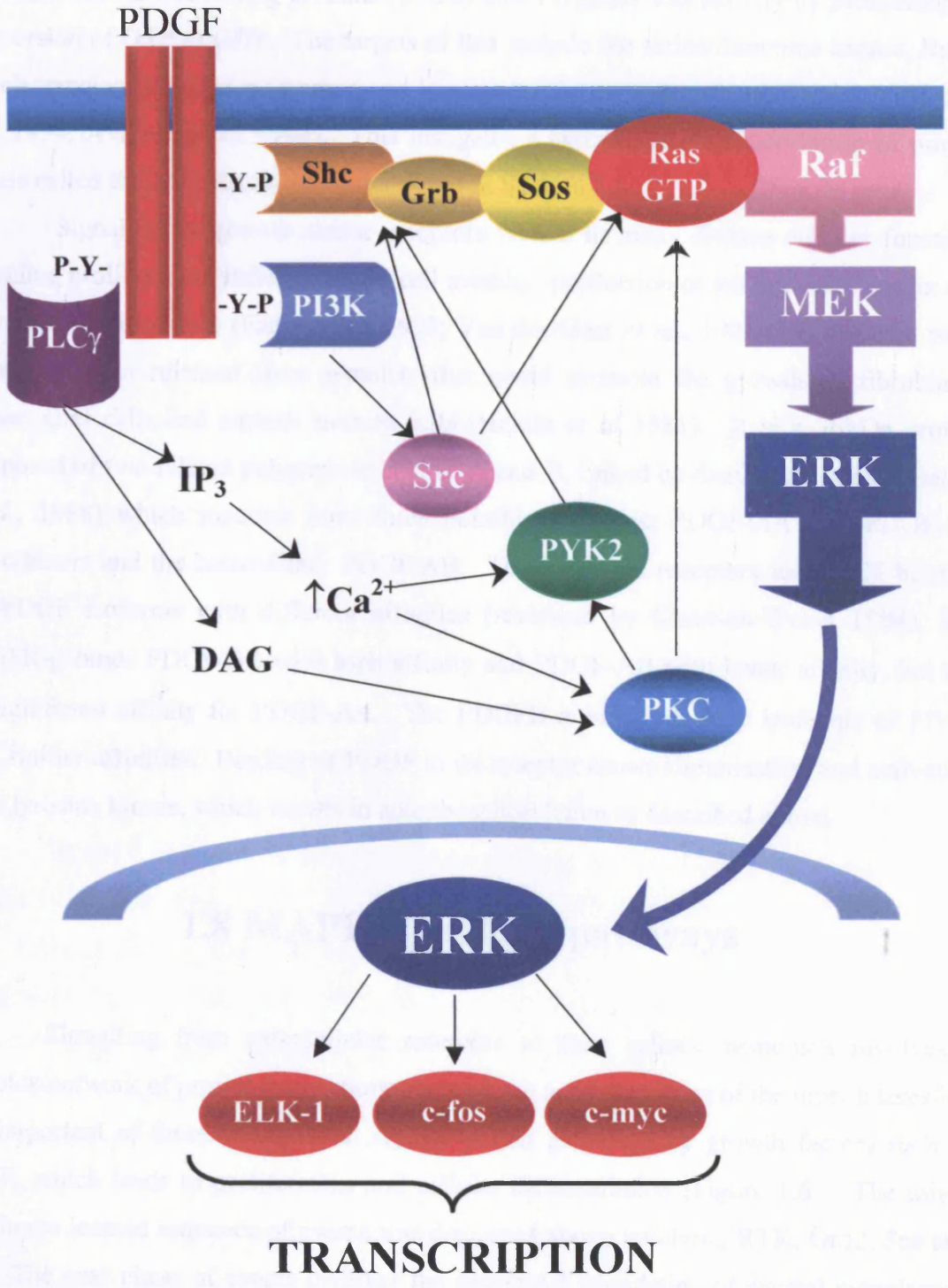


Figure 1.6 PDGF mitogenic signalling pathway

GTPase cycles between inactive GDP-bound form and active GTP-bound form. Guanine nucleotide exchange factors (GEFs) activate Ras by increasing the exchange of GTP for GDP and GTPase-activating proteins (GAPs) down regulate Ras activity by promoting the conversion of GTP to GDP. The targets of Ras include the serine/threonine kinase, Raf-1, which translocate to the membrane and is activated by an unknown mechanism (Prank & Bos 1994, Avruch *et al.*, 1994). This instigates a cascade of phosphorylation of protein kinase called the MAPKs, which is discussed in Section 1.8.

Signalling of growth factor receptors results in many diverse cellular functions including proliferation, differentiation, cell motility, production of extracellular matrix and altered gene expression (Fantl *et al.*, 1992; Van der Geer *et al.*, 1994). PDGF is a well-known mitogen released from platelets that could promote the growth of fibroblasts, human glial cells and smooth muscle cells (Heldin *et al* 1981). It is a 30kDa protein composed of two related polypeptide chains, A and B, linked by disulphide bonds (Heldin *et al.*, 1988) which together form three possible isoforms: PDGF-AA and PDGF-BB homodimers and the heterodimer PDGF-AB. There are two receptors for PDGF binding the PDGF isoforms with different affinities (reviewed by Claesson-Welsh 1994). The PDGFR-β binds PDGF-BB with high affinity and PDGF-AB with lower affinity, but has no significant affinity for PDGF-AA. The PDGFR-α binds all three isoforms of PDGF with similar affinities. Binding of PDGF to its receptor causes dimerisation and activation of its tyrosine kinase, which results in autophosphorylation as described above.

1.8 MAPK signalling pathways

Signalling from extracellular receptors to their cellular responses involves a complex network of protein interactions and enzyme activities. One of the most interesting and important of these extracellular signals is that generated by growth factors such as PDGF, which leads to proliferation and cellular differentiation (Figure 1.6). The initial membrane located sequence of events was discussed above involving RTK, Grb2, Sos and Ras. The next phase of events involves the sequential stimulation of several cytoplasmic protein kinases known as the mitogen-activated protein kinases (MAPKs). This was originally identified as a 42-kDa tyrosine phosphorylated protein (Cooper & Hunter, 1981 & 1983). It was later identified as a serine/ threonine kinase based on its ability to phosphorylate microtubule associated protein 2 after insulin stimulation (Ray & Sturgill,

1987). It required both tyrosine and threonine phosphorylaton for activation and was designated as p42MAPK (Rossomando *et al.*, 1989, Ray & Sturgill, 1988). Molecular cloning of p42MAPK and the highly related p44MAPK revealed that these MAPKs belonged to a family of ser/thr kinases related to Fus3 and Kss1 kinases from yeast (Boulton *et al.*, 1990). Since then mammalian p42MAPK and p44MAPK are also known as extracellular regulated kinase 2 and 1 respectively (ERK2 and ERK1). However, Seger and Krebs (1995) suggested the use of the term ERK for p42 and p44 MAPK isoforms as the MAPK family is comprised of several different parallel protein kinase cascades, which have now been identified (see Figure 1.7). These cascades involve sequential activation of distinct isoforms of kinases with little or no cross-reactivity between them. The kinases at the same level in the separate cascades are referred to by a general name. For example, RTK activation of Ras stimulates MAP kinase kinase kinase (MAPKKK) activation (known as Raf-1 in the ERK signalling cascade), which phosphorylates MAPKK (MEK1), which in turn phosphorylates MAPK or ERK. Parallel pathways include the c-jun N-terminal kinase (JNK) pathway and the p38 kinase pathway, each phosphorylated and activated by their own MAPKK and MAPKKKs. These three pathways are discussed in more detail below.

1.8.1 ERK signalling pathway

ERK is activated by dual phosphorylation of a Tyr-Glu-Thr 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 & Gotoh, 1993) and threonine 202 and tyrosine 204 for human ERK1 (Sturgill *et al.*, 1988; Payne, 1991). Phosphorylation on both residues is essential for enzyme activity. Point mutation of either threonine or tyrosine causes inactivity (Robbins *et al.*, 1993). The upstream activator of ERKs was purified and shown to be a kinase that catalysed ser/thr and tyrosine phosphorylation, belonging to a family of dual specificity kinases (Matsuda *et al.*, 1992, Posada & Cooper 1992, Seger *et al.*, 1992a). Cloning of this kinase showed that it contained all the conserved residues of protein kinases and was referred to as MAPK/ERK kinase 1 & 2 (MKK1/2 or MEK1/2) (Crews & Erikson 1992, Seger *et al.*, 1992b, Ahn *et al.*, 1991, Wu *et al.*, 1993). MEK itself is activated by phosphorylation on two serine residues in its catalytic domain (Gomez & Cohen, 1991, Ahn *et al.*, 1993, Huang *et al.*,

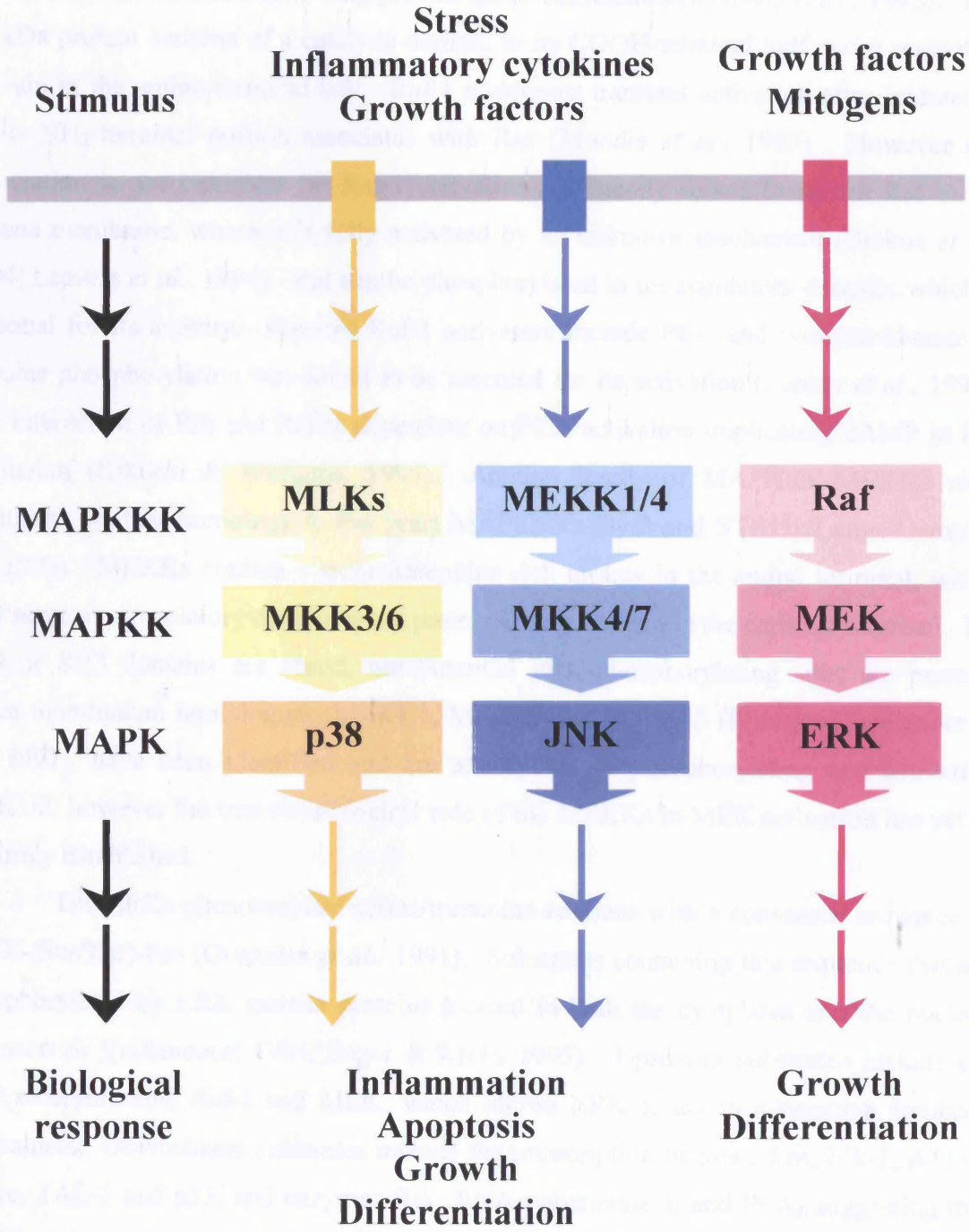


Figure 1.7 Parallel MAPK signalling

1993; Zheng & Guan, 1994). Unlike ERK, several upstream regulators can activate MEK, known as MAPKKKs or MEK kinases (MEKKs). Raf-1, a serine/threonine kinase, was shown to stimulate MEK following growth factor stimulation (Kyriakis *et al.,* 1992). This 75-kDa protein consists of a catalytic domain in its COOH-terminal half and a regulatory domain in the amino-terminal half. Raf-1 undergoes transient activation after interaction of its NH_2-terminal portion associates with Ras (Moodie *et al.*, 1993). However this association is not sufficient for Raf-1 activation. It merely serves to recruit Raf to the plasma membrane, where it is fully activated by an unknown mechanism (Stokoe *et al.*, 1994, Leevers *et al.*, 1994). Raf can be phosphorylated in the regulatory domain, which is essential for its activity. Possible Raf-1 activators include PKC and tyrosine kinases as tyrosine phosphorylation was found to be essential for its activation (Fabian *et al.*, 1993). The interaction of Ras and Raf is dependent on PKA activation implicating cAMP in Raf regulation (Kikuchi & Williams, 1996). Another family of MAPKKK/MEKKs were identified by their homology to the yeast MAPKKKs Byr2 and STE11 (Lange-Carter *et al.*, 1993). MEKKs contain a serine/threonine rich moiety in the amino terminal, which may serve as a regulatory domain and a protein kinase domain in the carboxy-terminal. No SH2 or SH3 domains are found, but potential PKC-phosphorylating sites are present. Three mammalian homologues, MEKK1, MEKK2 and MEKK3 (Ellinger-Ziegelbauer *et al.,* 1997), have been identified and are all capable of phosphorylating and activating MEK1/2, however the true physiological role of the MEKKs in MEK activation has yet to be firmly established.

The ERKs phosphorylate serine/threonine residues with a consensus sequence of Pro-X-(Ser/Thr)-Pro (Gonzales *et al.*, 1991). Substrates containing this sequence that are phosphorylated by ERK include proteins located in both the cytoplasm and the nucleus (Johnson & Vaillancourt 1994, Seger & Krebs 1995). Upstream substrates include the EGF receptor, Sos, Raf-1 and MEK, which allows ERK to act in a negative feedback mechanism. Downstream substrates include the transcription factors c-Fos, Elk-1, ATF-2, c-myc, TAL-1 and p53, and enzymes Rsk, RNA polymerase II and PLA_2 suggesting that ERK is an important regulator of cytoplasmic and nuclear cell physiology.

The direct involvement of ERK in proliferation has been demonstrated using dominant negative and antisense techniques. Antisense Raf-1 and inhibitory Raf-1 were shown to limit NIH3T3 cell proliferation whereas constitutively active Raf-1 accelerated it (Miltenberg *et al.*, 1993). Overexpression of nonactivatable forms of MEK1 also reduced the rate of cell proliferation and ERK activity, whereas a constitutively active form

accelerated proliferation and ERK activity. Dominant negative forms of ERK and antisense cDNA for ERK also reduced cell proliferation (Pagés *et al.*, 1993). Active Ras, which is a protooncogene, has been shown to be important in cell growth and transformation (Trahey & McCormick, 1987), whereas mutations in Ras block growth factor mediated activation of both Raf and MAPK (Troppmair *et al.*, 1992; de Vries-Smits *et al.*, 1992). A summary of this pathway can be seen in Figure 1.6.

An approximately 80 kDa MAPK distantly related to ERK has been characterized and termed big mitogen activated protein kinase (BMK1) or ERK5. It contains a Thr-Glu-Tyr motif in its activation loop (Lee *et al.*, 1995) and is selectively activated by MEK5 (Kato *et al.*, 1997) or MEKK3 (Chao *et al.*, 1999). It can be stimulated by oxidative stress and phosphorylate transcription factors MEF2C and c-myc (English *et al.*, 1998). Inactive ERK5 constructs block EGF-stimulated cell proliferation suggesting it to play a key role in EGF-stimulated cell growth (Kato *et al.*, 1998). It was recently shown that GPCR via $G\alpha_q$ and $G\alpha_{13}$ could potently activate ERK5 although the mechanism is still not fully understood (Marinissen *et al.*, 1999, Gutkind 2000).

1.8.1.1 P2Y receptor activation of ERK

G-protein coupled receptors are coupled to mitogenesis through tyrosine kinase and MAPK cascade (Gutkind, 1998; Bourne, 1990, Bourne *et al*, 1995). Although originally thought to be as a distinct separate pathway from growth factor signalling, it is now known to link or overlap the growth factor signalling pathway. G_i and G_q-coupled receptors can activate MAPK cascade by their free $\beta\gamma$ subunits. This was first demonstrated when overexpression of the $\beta\gamma$ subunit was found to sufficiently stimulate ERK (Faure *et al.*, 1994, Crespo *et al.*, 1994) and was found not to require PKC but Ras activation (Crespo *et al.*, 1994, Koch *et al.*, 1994). This also revealed that GPCR and RTK mitogenic signalling converged at the level of Ras (See figure 1.8). The PI3K inhibitor wortmannin inhibited ERK activity induced by LPA-stimulated GPCR via $G\alpha_i$ and $G\beta\gamma$ subunits suggesting a role for PI3K in GPCR to ERK signalling (Hawes *et al.*, 1996). The PI3Kγ isoform, which has an absent regulatory domain, was the most likely candidate to be activated by $G\beta\gamma$ complexes (Stoyanov *et al.*, 1995). This was also shown to result in the recruitment of Src-like kinases, Shc, Grb2, Sos and Ras (Lopez-Ilasaca *et al.*, 1997).

Tyrosine kinases apart from RTK have also been suggested to participate in GPCR signalling to ERK. These nonreceptor tyrosine kinases such as Src or Src-like

tyrosine kinase were found to mediate the phosphorylation of Shc and the subsequent association of Grb2 and mSos between G protein βγ-subunits and the Ras-MAPK cascade (Lutterell *et al.,* 1996). However the precise mechanism of GPCR activation of Src is not well understood. It has been suggested that upon GPCR activation the subsequent phosphorylation by GPCR kinase (GRK) results in the recruitment of arrestin. This acts as a adapter molecule and recruits Src to the GPCR which may lead to its activation (Luttrell *et al.*, 1999). The internalisation of the GPCR-arrestin–Src complex is then required for MAPK activation.

Another non-receptor tyrosine kinase that has been implicated in GPCR signalling to ERK is Pyk2, which is a Ca^{2+} and PKC-dependent kinase. $G\alpha_q$ increases PLC activation, which results in increased Ca^{2+}, PKC activation and thus Pyk2 activation (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 leads to the complex formation that includes Src and Grb2 resulting in MAPK activation (Giancotti & Ruoslahti 1999, Clark *et al.*, 1995, Gutkind & Robbins, 1992; Rankin *et al.,* 1994). PKCs are also reported to activate Raf-1 in a Ras independent manner (Pace *et al.*, 1995, Kolch *et al* 1993, Heidecker *et al.*, 1992). G_q coupled receptor activation of MAPK may be fully PKC-dependent (Hawes *et al.*, 1995), partially PKC-dependent (Crespo *et al.*, 1994) or completely PKC-independent (Charlesworth & Rozengurt, 1997) although this is not fully understood as yet (Burgering & Bos 1995).

RTK can also play a role in GPCR signalling to ERK. For example EGF and PGDF receptors become active upon GPCR activation (Luttrell *et al.*, 1997, Linseman *et al.,* 1995, Daub *et al.*, 1996 & 1997, Cunnick *et al.,* 1998) resulting in activation of the Ras/MAPK signalling cascade (van Biesen *et al*, 1995, Chen *et al.,* 1996c). How these RTK are activated is unclear. A possibility is that βγ-activated Src may phosphorylate the EGF receptor (Luttrell *et al.*, 1997). This type of transactivation of the EGF receptor has also been observed by angiotensin II in rat liver epithelial cells (Li *et al.*, 1998b) and also for the PDGF receptor (Linseman *et al.,* 1995; Daub *et al.,* 1996). GPCRs are therefore able to utilise the non-receptor and receptor tyrosine kinase to create the protein complexes required for MAPK signalling.

In 1994 P2Y receptors were first shown to activate ERK (referred to as MAPK in most of the following examples) in primary cultures of rat cerebral cortical astrocytes (Neary & Zhu, 1994) and rat renal mesangial cells (Huwiler & Pfeilschifter, 1994).

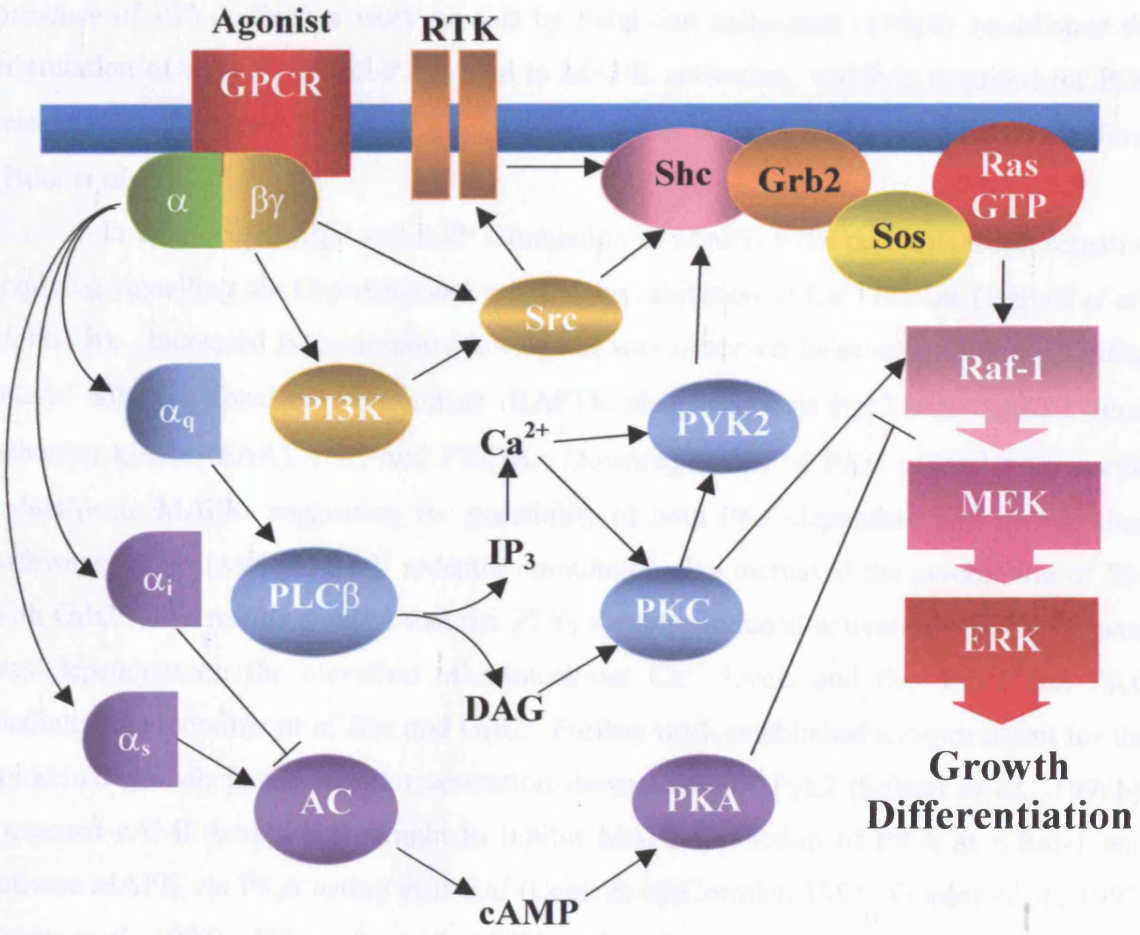


Figure 1.8 GPCR signalling to ERK

Endothelial cells have also been studied for P2Y activation of MAPK. In EAhy 926 human endothelial cell line MAPK activation by UTP and ATP was reported, but not by 2MeSATP, indicating $P2Y_2$ receptor stimulation (Graham *et al.*, 1996). This was also found to be dependant on PKC and extracellular Ca^{2+} and pertussis toxin insensitive. In bovine aortic endothelial cells 2MeSATP stimulated MAPK activation indicating the presence of $P2Y_1$. Further work on this by Patel and colleagues (1996a) established the stimulation of both $P2Y_1$ and $P2Y_2$ lead to MAPK activation, which is required for PGI_2 release. This was later shown to require PKC, which is most likely to be PKC-ε isoform (Patel *et al.,* 1996).

In PC12 cells, UTP and ATP stimulation of MAPK were pertussis toxin sensitive implying signalling via G_i protein and sensitive to inhibition of Ca^{2+} release (Soltoff *et al.,* 1998a, b). Increased tyrosine phosphorylation was observed in other proteins including 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, suggesting the possibility of both PKC-dependent and independent pathways being involved. P2Y receptor stimulation also increased the association of Shc with Grb2. The results suggest that the $P2Y_2$ receptor-induced activation of MAP kinase was dependent on the elevation of intracellular Ca^{2+} levels and that Pyk2 and PKC mediated the recruitment of Shc and Grb2. Further work established a requirement for the epidermal growth factor receptor activation downstream of Pyk2 (Soltoff *et al.,* 1998b). Increased cAMP levels are thought to inhibit MAPK by action of PKA at c-Raf-1 and activate MAPK via PKA acting at B-Raf (Cook & McCormick 1993, Vossler *et al.*, 1997, Daaka *et al.*, 1997). This suggests that P2Y mediated MAPK activation may be modulated by both PKC and PKA. Though some theoretical pathways have been established for GPCR stimulation of ERK, further work still needs to be carried out for role of the different P2Y subtypes in the activation of ERK.

Vascular smooth muscle proliferation is important in diseases such as atherosclerosis and hypertension and ATP has been shown to cause VSM proliferation that involves MAPK activation (Erlinge 1998). This is discussed in more detail in Section 1.10.

1.8.1.2 Inhibitors of ERK pathway

Selective inhibitors of the ERK pathway were first sought after by Dudley and colleagues (1995). This was achieved by screening a compound library with an assay that measured myelin basic protein (MBP) phosphorylation in the presence of GST-MEK and GST-ERK fusion protein. A synthetic compound 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one or PD98059 was found to reversibly inhibit MBP phosphorylation. They also showed that PD98059 blocked the activity of MEK and not ERK with an IC_{50} of approximately 10 μM. The same group later showed that PD98059 prevented the activation of MEK by Raf by binding to the inactive form of MEK (Alessi *et al.*, 1995). PDGF-induced ERK phosphorylation and DNA synthesis in 3T3 cells was inhibited with an IC_{50} of approximately 10 μM and 7 μM respectively, indicating that PD98059 could cross the cell membrane. Other kinases such as Raf, PKC, PKA, EGF kinsae, PDGF kinase, v-src, insulin receptor kinase, PI3K, JNK and p38 MAPK were not inhibited showing that PD98059 is a selective inhibitor of MEK and depending on the cell type and stimulation technique in intact cells the IC_{50} ranges between 2 – 10 μM (Dudley *et al.*, 1995, Pang *et al,* 1995).

U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) was also identified as an inhibitor of MEK by Favata and colleagues (1998). Like PD98059, U0126 inhibits the activation of MEK rather than blocks its activity (Favata *et al.*, 1998, Davies et al., 2000) and is highly selective non competitive inhibitor. Both inhibitors were suggested to share a common or overlapping binding site for MEK. These inhibitors are valuable tools in elucidating the role of ERK in a many tissue types.

It was also reported that both inhibitors affected the activation of ERK5. PD98059 was shown to prevent the activation of MKK5 rather than prevent its activity and thus prevent the phosphorylation of ERK5 (Kamakura *et al.*, 1999). PD98059 is also reported to inhibit ($IC_{50} \approx 1$ μM) cyclo-oxygenase 1 and 2 (COX 1 and 2), which catalyses the production of prostaglandins and leukotrienes and platelet aggregation (Börsch-Haubold *et al.*, 1998). U0126 however has no influence.

1.8.2 JNK signalling pathway

Investigations to identify the kinase responsible for phosphorylation of serines 63 and 73 on the amino terminus of the c-Jun transcription factor were performed by separate

groups. The first group found two kinase of 46 kDa and 55 kDa, which when activated by UV irradiation led to c-jun gene activation (Pulverer *et al.*, 1991). These kinases were termed c-Jun NH2-terminal kinases (JNK) (Hibi *et al.*, 1993, Derijard *et al.*, 1994). A second group cloned a cDNA encoding an enzyme structurally related to the MAPKs, by using degenerate PCR primers based on ERK1/2 (Smeal *et al.*, 1991). The cDNA cloned displayed the catalytic properties of JNK1 when expressed in mammalian cells. A third group also cloned a kinase from a rat-brain library which was activated by stimuli such as heat shock, translational inhibitors and tumour necrosis factor and were therefore termed as stress-activated protein kinases (SAPK) (Kyriakis *et al.*, 1994). The genes for SAPKα and SAPKβ encode 55 kDa kinases similar in size to JNK2 whereas SAPKγ encodes a 45 kDa kinase similar to 46 kDa JNK1 (Kyriakis *et al.*, 1994, Derijard *et al.*, 1994). The major isoforms of JNK are p46 JNK1 (SAPKγ), p54 JNK2 (SAPKα), and p49 JNK3 (SAPKβ), (Kyriakis *et al.*, 1994), although their can be up to 10 splice variants (Gupta *et al.*, 1996).

The substrates of JNK include transcription factors c-Jun, Elk-1/2, Sap-1 (serum response factor accessory protein-1), NFAT4 (nuclear factor of activated T cells 4) and ATF2 (activating transcription factor 2 (Widman *et al.*, 1999, Kyriakis *et al.*, 1994, Derijard *et al.*, 1994, Gupta *et al.*, 1995, Whitmarsh *et al.*, 1995). The JNK pathway is activated by oxidative stress, heat shock, ultraviolet light, cytokines (TNF family and interleukins), osmotic shock agents (such as sorbitol), protein synthesis inhibitors (such as cyclohexamide and ansiomycin), mechanical sheer stress and growth factors (reviewed in Kyriakis & Avruch 2001) (Figure 1.9). Their main role appears to be involved in inhibiting cell growth and regulating apoptosis (Kyriakis *et al.*, 1996)

JNKs undergo phosphorylation on the threonine 183 and tyrosine 185 residues in the sequence Thr-Pro-Tyr, which is required for activation (Derijad *et al.*, 1994), whereas ERKs have phosphorylation sequence of Thr-Gly-Tyr, therefore JNK is not recognised by ERKs upstream kinase MEK (Kyriakis *et al.*, 1994). JNK is phosphorylated by dual serine/threoine specific MAPKK known as SEK1/MKK4/JNKK (Sanchez *et al.*, 1994, Yan *et al.*, 1994). MKK4 was shown to phosphorylate JNK1 (Figure 1.9) and have homology with MKK1/2 (MEK1/2) and SEK1, the murine homologue of MKK4, phosphorylates all isoforms of JNK (Derijard *et al.*, 1995, Sanchez *et al.*, 1994). MKK7 (MEK7 or JNKK2) also phosphorylates all isoforms of JNK and is strongly activated by chemokines. SEK1/MKK4 cannot stimulate ERK (Lin *et al.*, 1995) and Raf-1 and

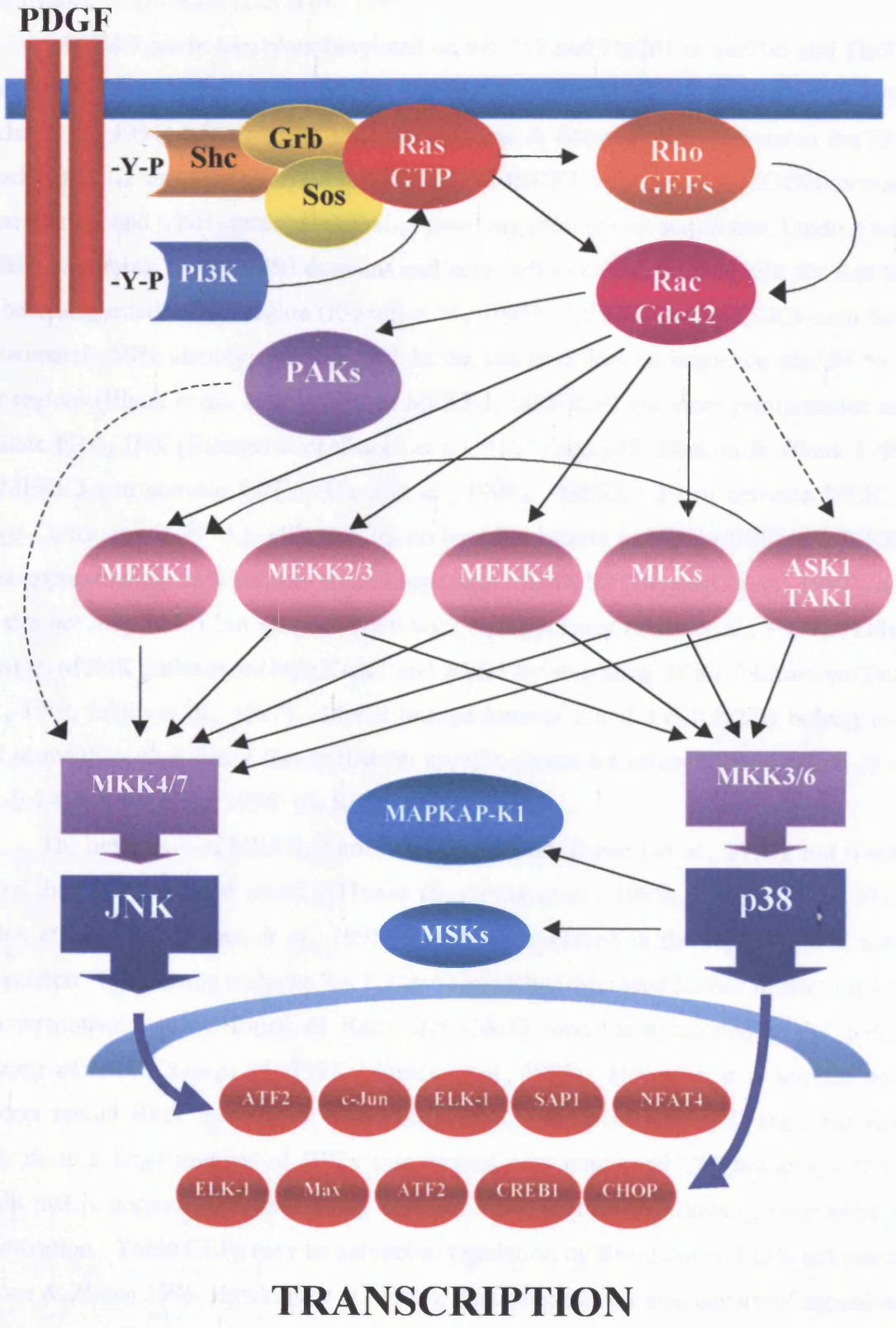


Figure 1.9 PDGF signalling to JNK and p38 MAPK

MEK1/2 do not to activate JNK (Minden *et al.*, 1994, Sanchez *et al.*, 1994) and Raf-1 does not activate SEK1/MKK4 (Lin *et al.*, 1995).

MKK4/7 are in turn phosphorylated on Ser 257 and Thr261 or Ser206 and Thr210 respectively (Derijard *et al.*, 1995,) by MAPKKK called MEKK1 (Yan *et al.*, 1994, Minden *et al.*, 1994), MEKK2 and MEKK3 (Deacon & Blank 1999), whereas in the ERK cascade MEK1/2 are phosphorylated by Raf-1. MEKK1 consists of a COOH-terminal kinase domain and a NH_2-terminal domain containing proline rich sequences, binding sites for SH3-containing proteins, PH domains and acid rich motif. A binding site for Ras has also been suggested in this region (Russell *et al.*, 1995). MEKK2 and MEKK3 each have approximately 50% identity with MEKK1 in the catalytic domain sequence and 94 % in other regions (Blank *et al.*, 1996). Unlike MEKK1, MEKK2/3 are more promiscuous and stimulate ERK, JNK (Ellinger-Ziegelbauer *et al.*, 1997) and p38 (Deacon & Blank 1999) and MEKK3 can activate ERK5 (Chao *et al.*, 1999). MEKK1/2 can activate MEK1/2 (Lange-Carter *et al.*, 1993,), although its preferred substrate is MKK4/SEK1. MEKK4 shares approximately 55% sequence homology with MEKK1/2 (Gerwins *et al.*, 1997)) and activates not only SEK1 but also MKK3/6 with equal potency (Sells *et al.*, 1997). Other activators of JNK pathway include TAK1 and ASK1 by activating SEK1 (Ninomiya-Tsuji *et al.*, 1999, Ichijo *et al.*, 1997). Mixed lineage kinases 2 and 3 (MLK2/3) belong to a small serine/threonine kinase family that are specific potent activators of JNK (Hirai *et al*, 1997 & 1998, Rana *et al.*, 1996) via SEK1 and MKK7.

The activation of MEKK is not fully understood (Russell *et al.*, 1995), but it may involve the Rho family of small GTPases (Bagrodia *et al.*, 1995a, Coso *et al.*, 1995a, Minden *et al.*, 1995, Olson *et al.*, 1995), which are involved in the regulation of actin cytoskeleton. This family includes Rac1, Cdc42 and Rho (discussed further in Section 1.9) and constitutively active forms of Rac1 and Cdc42 were demonstrated to be potent activators of JNK (Coso *et al.*, 1995, Minden *et al.*, 1995). However, it is unclear how receptors recruit Rac1 and Cdc42 to stimulate JNK. As with other GTPases, the Rho family share a large number of GEFs that consist of a conserved Dbl homology (DH) domain that is necessary for promoting GDP dissociation thereby allowing GTP binding and activation. These GEFs may be subject to regulation by Ras-induced PI3K activation (Cerione & Zheng 1996, Hawkins *et al.*, 1995), thus suggesting a mechanism of signalling from receptor to Rac1/Cdc42. Ras has also been shown to recruit Rac to mediate mitogen-induced membrane ruffling, suggesting Rac to be an effector of Ras (Ridley *et al.*, 1992). Downstream mechanisms from Rac1/Cdc42 to JNK are also under investigation. PAKs

(PAK1, PAK2, β-PAK/PAK3 and γ-PAK) are 60-70 kDa serine/threonine kinase proteins with C-terminal catalytic domain and a Rac1/Cdc42 interaction and binding domain (CRIB) in the N-terminal, which interacts directly with the effector loops of Rac1/Cdc42 (Leberer *et al.*, 1992, Manser *et al.*, 1994, Martin *et al.*, 1995, Bagrodia *et al.*, 1995a, Teo *et al.*, 1995). The binding of GTP-bound form of Rac1/Cdc42 stimulates PAK activation, thus regulating the actin cytoskeleton (Sells 1997 & 1999). However the PAK substrate that couples it to the JNK pathway is still unknown, but suggested to be via MKK4/7 activation (Tournier *et al.*, 1997, Whitmarsh *et al.*, 1997, Fanger *et al.*, 1997, Widmann *et al.*, 1999).

JNK is not only activated by inflammatory cytokines (TNF-α and IL-1β), but increasing evidence shows JNK to be regulated by growth factors such as EGF, VEGF and PDGF (Hashimoto *et al.*, 1999, Minden *et al.*, 1994, Pedrum *et al.*, 1998, Yu *et al.*, 2000, Assefa *et al.*, 1999, Lallemand *et al.*, 1998), which resulted, in some cases, in proliferation (Pedrum *et al.*, 1998, Izumi *et al.*, 2001, Li *et al.*, 1999). The implication of JNK in proliferation in discussed further in Section 1.10.

1.9.2.1 GPCR regulation of JNK

GPCR can regulate the stress-activated pathways through the Ras-related small GTP- binding proteins Rac 1 and Cdc42. Coso and colleagues (1996) demonstrated JNK activation by muscarinic m1 and m2 receptors transfected into COS-7 cells. However this was not mimicked when cells were expressed with α_q, α_{i1}, α_s, or α_{13} G protein subunits, but was when the free βγ subunits were overexpressed. Other groups on the other hand demonstrated JNK activation coupled to activated $G\alpha_{12}$ and $G\alpha_{13}$ subunits (Prasad *et al.*, 1995, Voyno-Yasenetskaya *et al.*, 1996, Collins *et al.*, 1996). These reports also demonstrated a role for Rac1 and Cdc42, using dominant-negative mutants of these GTPases, resulting in attenuated JNK activation. Other proteins such as GEFs have been suggested to play a role in transmitting signals from GPCR to Rac 1 and Cdc42 (Kiyono *et al.*, 1999, Fan *et al.*, 1998, Nishida *et al.*, 1999). The βγ subunit recently was shown to activate JNK through MKK4 through Cdc42 and Rho and to a lesser extend MKK7 through Rac1 (Yamauchi *et al.*, 1999). $G\alpha_{q/11}$ was shown to use Src family kinases to stimulate JNK (Nagao *et al.*, 1998). Non receptor tyrosine kinases Pyk2 and FAK have also been demonstrated to signal to JNK through the recruitment of an adaptor protein Grb2 and Crk (Oktay *et al.*, 1999, Blauket *et al.*, 1999) or paxiliin (Igishi *et al.*, 1999)

again by activating GEFs for Rho family of GTPases (Kiyokawa *et al.*, 1998, Dolfi *et al.*, 1998). Rac and Cdc42-independent activation of JNK pathway was also demonstrated by Berestetskaya and colleagues (1998). The $G\alpha_{12}$ and $G\alpha_{12}$ subunits activated the JNK pathway in COS-7 cells in a MEKK1 and ASK1 (a putative MEKK)-dependent manner resulting in apoptosis. A summary is shown in Figure 1.10.

More recent studies have shown P2Y receptor stimulation of the JNK pathways. In rat glomerular mesangial cells the stimulation of JNK with UTP and ATP was shown to be mediated through $P2Y_2$ receptors (Huwiler *et al.*, 1997). Similarly, JNK was stimulated by UTP in 1321N1 human astrocytoma cells stably expressing recombinant human $P2Y_2$ or $P2Y_4$ receptors (Paul *et al.*, 2000). In cells expressing human $P2Y_1$ receptors JNK was stimulated by 2MeSADP (Sellers *et al.*, 2001). Increases in JNK activation was found to be mediated by an autocrine ATP stimulation of P2Y receptors in response to stretch in rat aortic SMCs (Hamada *et al.*, 1998). Conversely, in Eah926 endothelial cells UTP was found to have a negative influence on JNK and inhibited TNFα-induced JNK activation (Paul *et al.*, 2000), which also demonstrates a novel mechanism of P2Y and TNF receptor cross-talk regulation. Other MAPK pathways may play a role in JNK signal transduction. An example of cross-talk between the ERK and JNK pathways was observed in bovine aortic endothelial cells (Pedrum *et al.*, 1998). VEGF-induced JNK activation was demonstrated to be ERK-dependent, possibly via SEK1 modulation by ERK. ERK can also have an inhibitory role in the regulation of JNK. The inhibition of glucose-induced JNK activation in SHSY5Y cells by insulin like growth factor-1 (IGF-1) was found to be mediated via ERK (Cheng *et al.*, 1998). There is now increasing evidence to support a model of crosstalk between MAPKs (Frost *et al.*, 1997), adding to the complexity of MAPK signalling pathways.

1.9.2.2 Inhibitor of JNK

The islet brain 1 (IB-1) and the closely related isoform JNK interacting protein 1 (JIP-1) are natural regulators of JNK and highly expressed in pancreatic β-cells (Bonny *et al.*, 1998, Negri *et al.*, 2000). They interact with JNK through their JNK binding domain (JBD) (Negri et al., 2000, Yasuda et al., 1999) and competitively block the interaction between JNK and c-jun (Dickens *et al.*, 1997, Bonny *et al.*, 2001). A peptide of 20 amino acids derived from IB-1 (18 amino acids from IB-2) was reported to sufficiently block activation of c-jun by JNK (Bonny *et al.*, 2001). A 10 amino acid HIV-TAT sequence,

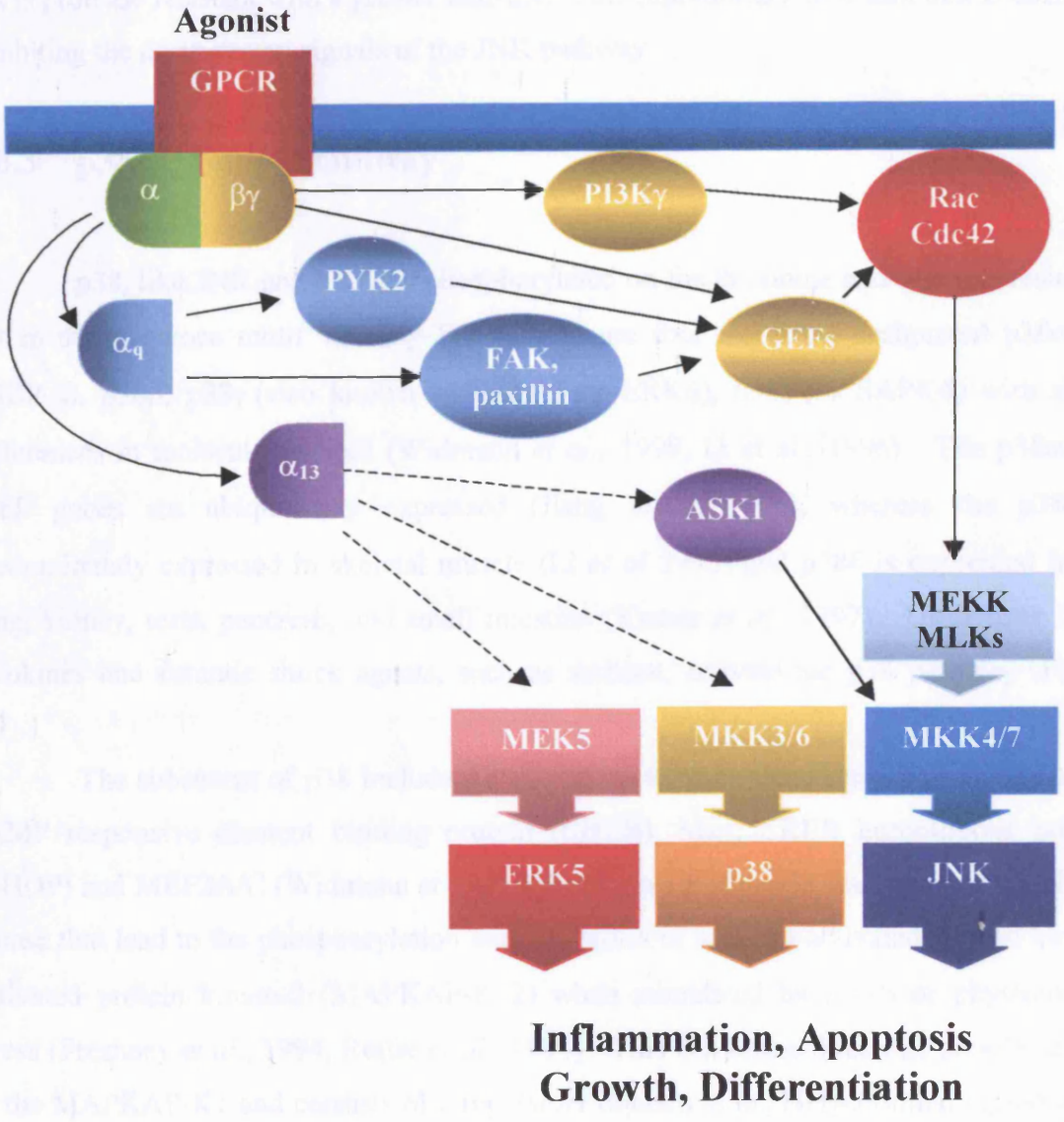


Figure 1.10 Summary of GPCR signalling to JNK, p38 MAPK and ERK5

which directs cellular imports was covalently linked to the 20 amino acid peptide inhibitor of JNK to produce a cell permeable JNK inhibitor. This compound termed JNK inhibitor JNKI) was shown to inhibit IL-1β-induced apoptosis of β-cells. They also synthesised the all-D retro-inverso (D-JNKI) that had the same properties of the L-enatiomer (L-JNKI), but is protease resistant with a greater half-life. This inhibitor is a new tool that is useful in inhibiting the downstream signals of the JNK pathway

1.8.3 p38 signalling pathway

p38, like JNK and ERK, is phosphorylated on the threonine and tyrosine residues, but in the sequence motif Thr-Gly-Tyr. There are four isoforms, designated p38α (or CSBP-1), p38β, p38γ (also known as SAPK3 or ERK6), p38δ (or SAPK4) with slight differences in molecular weight (Widmann *et al.*, 1999, Li et al., 1996). The p38α and p38β genes are ubiquitously expressed (Jiang *et al*, 1996), whereas the p38γ is predominantly expressed in skeletal muscle (Li *et al* 1996) and p38δ is expressed in the lung, kidney, tests, pancreas, and small intestine (Kumar *et al.*, 1997). Ultraviolet light, cytokines and osmotic shock agents, such as sorbitol, activate the p38 pathway (Figure 1.9).

The substrates of p38 include the activating transcription factor-2 (ATF2), ElK-1, cAMP responsive element binding protein (CREB), Max, CREB homologous protein (CHOP) and MEF2A/C (Widmann *et al.*, 1999) (Figure 1.10). p38 was also identified as a kinase that lead to the phosphorylation and activation of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K 2) when stimulated by IL-1β or physiological stress (Freshney *et al.*, 1994, Rouse *et al.*, 1994). This is a serine/threonine closely related to the MAPKAP-K1 and consists of a regulatory domain in the NH_2-terminal region and a kinase in the COOH-domain and can phosphorylate the small heat shock protein HSP27, which is involved in F-actin reorganization into stress fibres, thereby affecting motility (Hout *et al.*, 1997, Lambert *et al.*, 1999, Stokoe *et al.*, 1992). Mitogen and stress-activated protein kinases (MSKs) are also serine/threonine kinases similar to MAPKAP-K1 and was found to be a substrate for p38 (and ERK) (Deak *et al.*, 1998). MSK-1 was shown to be a potent activator of CREB.

The upstream activators are not yet fully elucidated but p38 is phosphorylated by MAPKKs known as MKK3/6 (MEK3/6) (Enslen *et al.*, 1998). MKK3/6 are highly

selective for p38 and do not activate JNKs or ERKs. MKK3 preferentially activates p38α and p38β and like SEK1 is strongly activated by physical and chemical stresses, whereas MKK6 activates all isoforms MKK4 has also been shown to stimulate p38α (Cuenda *et al.*, 1995 & 1996). MKK3 is phosphorylated on Ser 189 and Thr 193 and MKK6 is phosphorylated on Ser207 and Thr 211 (Cuenda *et al.*, 1995, English *et al.*, 1995, Rainguard *et al.*, 1996). MAPKAP kinase-2 reactivating kinase kinase (RKK), a partially purified IL-1β and stress-activated MAPKK, phosphorylates and activates p38 but not other MAPKs (Freshney *et al.*, 1994, Rouse *et al.*, 1994). MAPKKKs include ASK1 and TAK1 (Widmann *et al.*, 1999), MEKK3, which phosphorylates MKK6 (Deacon & Blank 1999) and MEKK4, which phosphorylates MKK3/6 (Takekawa *et al.*, 1997). The small GTP-binding proteins Rac1 and Cdc42 are implicated in activation of the p38 pathway, similar to the activation of the JNK pathway (Bagrodia *et al.*, 1995a, Zhang *et al.*, 1995). The coupling of Rac1 and Cdc42 to p38 is believed to be via PAK, which phosphorylates and activates the MAPKKKs.

GPCR were soon found to be able to regulate p38 kinase although the full mechanism is not fully understood. The βγ subunit and $G\alpha_{q/11}$ have been shown to activate p38 (Yamauchi *et al* 1997) and $G\alpha_q$ coupled receptors have been shown to stimulate p38α and p38γ (Marinissen *et al.*, 1999). $G\alpha_{12}$ and $G\alpha_{13}$ can activate the putative MAPKKK ASK1, which activates both JNK and p38 (Berestetskaya *et al.*, 1998). Two non-receptor tyrosine kinases Btk and Src have also been implicated (Bence *et al.*, 1997, Nagao *et al.*, 1998).

More recent studies have shown P2Y receptor stimulation of the p38 pathways. In rat glomerular mesangial cells the stimulation of p38 with UTP and ATP was shown to be mediated through $P2Y_2$ receptors (Huwiler *et al.*, 2000). Similarly, p38 were stimulated by UTP in 1321N1 human astrocytoma cells stably expressing recombinant human $P2Y_2$ or $P2Y_4$ receptors (Paul *et al.*, 2000). In human platelets, p38 was activated by ADP acting at the $P2Y_1$ receptor (Dangelmaier *et al.*, 2000).

The main role of p38 appears to be involved in inhibiting cell growth and regulating the cell cycle and apoptosis (Kyriakis *et al.*, 1996). However it also plays an important role in inflammation stimulating the production of proinflammatory cytokines (IL-1b, TNK-a and IL-6), the induction of COX-2 and the induction of adhesion proteins such as VCAM-1 (for review see Ono & Han 2000). The p38 pathway also regulates the proliferation and differentiation of cells of the immune system. GPCR stimulated by

thrombin and angiotensin II stimulated p38 in platelets and rat VSMCs respectively suggesting a role for p38 in platelet aggregation and hypertrophy (Kramer *et al.*, 1995, Ushio-Fukae *et al.*, 1998). Interestingly both p38α and p38β activity has been shown to increase during cardiomyocyte hypertrophy, where p38β was more potent at inducing hypertrophy and p38α appeared to be more important in cardiomyocyte apoptosis (Wang *et al.*, 1998a).

1.8.3.1 Inhibitor of p38 MAPK

A highly selective inhibitor of p38α and p38β MAPK was found by Cuenda and collegues (1995). The pyridinyl imidazole SB203580 has an impressive high degree of specificity demonstrated by its inability to inhibit other kinases including closely related JNK and ERK and surprisingly p38γ and p38δ (Davies *et al.*, 2000). An IC_{50} of 50-500 nM was observed whereas other kinases, such as protein kinase B (PKB) and glycogen synthase kinase (GSK), were inhibited at IC_{50} that was 100-500 fold higher (Davies *et al.*, 2000). SB203580 has also been shown inhibit COX 1 and 2 activity with similar potency as PD98059 (IC_{50} = 2 and 1 μM respectively) (Börsch-Haubold *et al.*, 1998).

1.9 Rho-operated coiled coil kinase (ROCK)

There are more than 50 Ras-related small GTPases identified in mammals and yeast. Rho was the first to be identified in 1985 and was termed Ras homologue (Madaule & Axel 1995). Purification of several other small GTPases lead to the identification of proteins closely related to Rho and collectively these were known as the Rho family of small GTP-binding proteins and include 3 isoforms of Rho (RhoA, B and C), two isoforms of Rac (Rac 1 and 2) and Cdc42 (Hall 1994, Foster *et al* 1996). This family is primarily involved in regulating the organization of the actin cytoskeleton. Both Rac and Cdc42 stimulate JNK and p38 MAPKs (see Kyriakis & Avruch 2001 for review). RhoA on the other hand does not regulate JNK or p38. RhoA is ubiquitously and abundantly expressed and therefore is the most studied and is reported to control the assembly of actin stress fibres, cell adhesion through focal adhesion complexes, cytokinesis, smooth muscle contraction and cell proliferation (Hall 1994, Harata *et al.*, 1992, Noda *et al.*, 1994, Narumiya 1996). Roles for Rho B and C are still unclear. RhoA, like Rac and Cdc42 is

active in the GTP bound form and requires an extracellular signal to activate it, such as serum, lysophosphatidic acid (LPA), thrombin and bombesin (Ridley & Hall, 1992, Jalink *et al.*, 1994, Rankin et al., 1994). Therefore GPCRs appear to be the major activators of Rho. G_{12} and G_i have been shown to couple LPA to Ras activation and cell growth, which was pertussis toxin sensitive (Moolenaar 1995). $G\alpha_{12}$ and $G\alpha_{13}$, but not $\beta\gamma$ subunits, were shown to induce the development of stress fibres, which was inhibited by the Rho inhibitor C3 exoenzyme (Buhl *et al.*, 1995). This was assumed to be mediated via Rho guanine nucleotide exchange factors (Rho GEFs). However the downstream effectors from the G proteins mediating signals to Rho remain to be identified.

Downstream events of Rho have been elucidated using C3 exoenzyme. During the same time as Rho identification an ADP-ribosyltransferase in *Clostridium botulinum* was found to ADP-ribosylate proteins in mammalian cells (Ohashi & Narumiya 1987, Aktories *et al.*, 1987). Its substrate botulinum C3 was found to be Rho protein (Narumiya *et al.*, 1988, Kikuchi *et al.*, 1988) and ADP-ribosylated Asn residue in the effector binding domain thereby blocking Rho signalling (Sekine *et al.*, 1989). This revealed that Rho played a role in stimulus-evoked reorganization of the actin cytoskelton. LPA induced stimulation of protein kinases, FAK and paxillin, were inhibited by C3 exoenzyme indicating that these lay downstream of Rho (Kumagai *et al.*, 1993, Ridley & Hall 1994). There is evidence to suggest that activation of Rho down regulates $p27^{kip}$ and increases DNA synthesis, therefore implicating Rho in the regulation of cell growth (Lamarche *et al.*, 1996, Narumiya *et al.*, 1997, Olson *et al.*, 1995, Seasholtz *et al.*, 1999).

Using yeast two hybrid screening combined with ligand overlay assay with [^{35}S]GTPγS-Rho direct targets of Rho were identified (Watanabe *et al.*, 1996). These included serine/threonine protein kinases PKN and rhophilin, both of which contained Rho-binding sequences (Narumiya *et al.*, 1997). Another downstream effectors of Rho identified by ligand overlay assay and purification was a 160 kDa Ser/Thr kinase (Ishizaki *et al.*, 1996). This was termed Rho-associated coiled-coil kinase (ROCK), also known as ROKβ (Figure 1.10A). An isoenzyme of p160ROCK was also identified as Rho kinase/ROKα/ROCK-II and displays 90% sequence identity in the kinase domain (Leung *et al.*, 1995, Matsui *et al*, 1996, Nakugawa *et al.*, 1996). ROCK is highly expressed in heart, lung, skeletal, muscle, kidney and pancreas, whereas Rho-kinase is highly expressed in the brain (Leung *et al.*, 1995, Ishisaki *et al.*, 1996). The ser/thr kinase domain in located in the NH_2 terminal domain region followed by approximately 600 amino-acid long α-

helix, which is a coiled-coil structure and cysteine rich Zinc finger like motif and PH region in carboxy terminus (Figure 1.11A). The GTP-bound Rho binding region is located between the carboxyl end of the coiled coil region and the PH domain (Leung *et al.*, 1995, Fujisawa *et al.*, 1996). It is likely that upon binding the N-terminal coiled coil region the C-terminal lipid-binding region become exposed allowing ROCK to interact with other proteins and attach to the membrane. This aids subsequent phosphorylation of substrates by translocating ROCK in close proximity with its substrates and allowing binding. Indeed detectable amounts of ROCK were found to translocate to the cytoskeletal when platelets were stimulated with thrombin (Fujita *et al.*, 1997). ROCK was reported to increase the formation of stress fibres, recruitment of integrins to focal adhesions (Leung *et al.*, 1996, Amano *et al.*, 1997, Ishizaki *et al.*, 1997) and also enhances myosin light chain (MLC) to increase the contractility of actinomyosin, therefore increasing smooth muscle contraction (Amano *et al.*, 1996, Kureshi *et al.*, 1997) (Figure 1.11B). The substrates for ROCK include LIM-kinase, which phosphorylates the actin-binding and depolymerising protein cofilin (Maekawa *et al.*, 1999, Ohashi *et al.*, 2000) and Na-H exchanger-1 (NHE-1) (Kaneko *et al.*, 2000).

A highly specific inhibitor of ROCK, Y-27632, was found by a Japanese group and reported not only to inhibit agonist induced contraction in VSMC, but also reduced the blood pressure in spontaneously hypertensive rats (Uehata *et al.*, 1997). Y-27362 has proved to be a useful tool in determining the functions and involvement of ROCK. However, recently Y27632 was shown to inhibit PKC-related protein kinase 2 (PRK2) with similar potency to that of ROCK (Davies *et al.*, 2000). Inhibition of other kinases such as mitogen- and stress-activated protein kinase 1 (MSK1) and MAPK-activated protein kinase 1β (MAPKAP-K1β) were inhibited with a potency 10 and 24 (respectively) fold less than that of ROCK. In rat aortic, SMCs thrombin-induced DNA synthesis and cell migration was blocked using Y27632 (Seasholtz *et al.*, 1999) as well as endothelin-1 (ET-1) induced hypertrophy in neonatal rat cardiac myocytes (Kuwahara *et al.*, 1999). Interestingly, the stretch-activated stimulation of ERK, which resulted in DNA synthesis, was also inhibited by Y27632 (Numaguchi *et al.*, 1999), suggests that Rho/ROCK plays an important role in mediating the mechanical stretch signal to ERK activation. The PDGF and LPA-stimulated DNA synthesis in human aortic SMCs and downregulation of $p27^{kip}$ in rat VSMCs were also reduced (Sawada *et al.*, 2000) suggesting that ROCK plays a pivotal role in G_1 cell cycle progression, gene transcription and cell proliferation. Recently $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors were shown to couple to Rho and Rho kinase in vascular

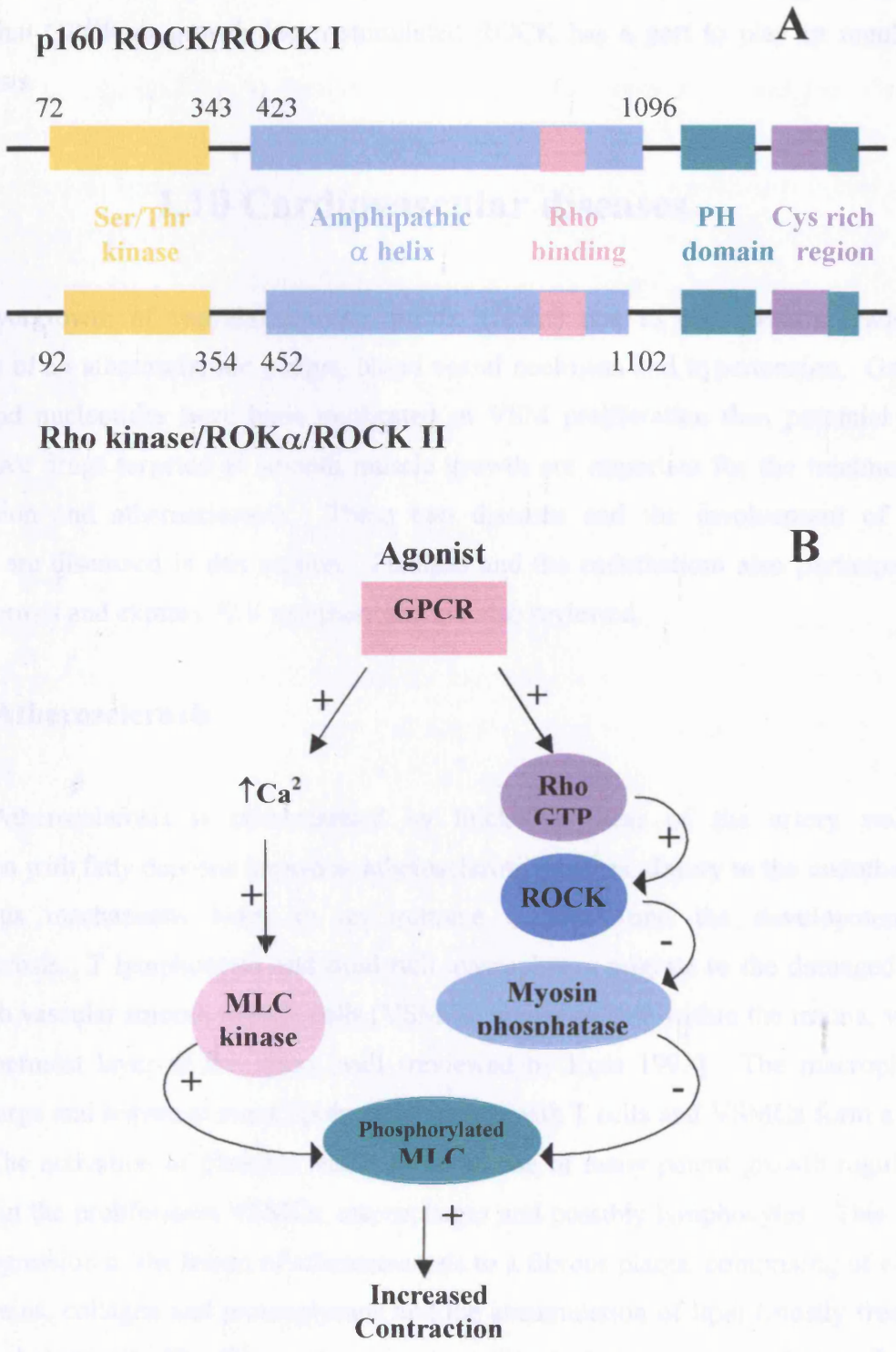


Figure 1.11
A) Structure of ROCK and Rho kinase
B) ROCK activation

myocytes using Y27632 (Sauseau *et al.*, 2000). There is therefore increasing evidence to suggest that GPCR or growth factor-stimulated ROCK has a part to play in regulating mitogenesis.

1.10 Cardiovascular diseases

Overgrowth of vascular smooth muscle (VSM) due to proliferation leads the formation of an atherosclerotic plaque, blood vessel occlusion and hypertension. Growth factors and nucleotides have been implicated in VSM proliferation thus potential anti-proliferative drugs targeted at smooth muscle growth are important for the treatment of hypertension and atherosclerosis. These two diseases and the involvement of P2Y receptors are discussed in this section. Platelets and the endothelium also participate in atherosclerosis and express P2Y receptors and are also reviewed.

1.10.1 Atherosclerosis

Atherosclerosis is characterised by thickened areas of the artery wall in association with fatty deposits known as atherosclerotic plaques. Injury to the endothelium by various mechanisms leads to an immune response and the development of atherosclerosis. T lymphocytes and lipid rich macrophages migrate to the damaged area along with vascular smooth muscle cells (VSMCs) and aggregate within the intima, which is the innermost layer of the artery wall (reviewed by Ross 1993). The macrophages become large and scavenge more lipids and together with T cells and VSMCs form a fatty streak. The activation of platelets leads to the release of many potent growth regulators resulting in the proliferation VSMCs, macrophages and possibly lymphocytes. This leads to the progression of the lesion of atherosclerosis to a fibrous plaque comprising of elastic fibre proteins, collagen and proteoglycans and the accumulation of lipid (mostly free and esterified cholesterol). The fibrous plaque projects into the lumen and impedes the flow of blood. The core of the plaque contains lipids and necrotic debris and is surrounded by dense aggregation of connective tissue and VSMCs.

The treatment for patients with atherosclerosis is either by bypass grafting performed with autologous saphenous vein (SV) or internal mammary artery (IMA) or percutaneous transluminal coronary angioplasty (dilation of occluded vessels by inserting a

balloon catheter). However, the failure rate is extremely high due to restenosis, which is caused by further inflammatory responses, smooth muscle proliferation and thrombosis. The patient's saphenous vein is used most often in aortocoronary bypass surgery (Cameron *et al.,* 1996), despite venous graft occlusion occurring within 10 years of implantation in 50% of patients. IMA grafts, on the other hand, have a higher patency and lower patient mortality than venous grafts (Loop *et al.,* 1986, Cameron *et al.,* 1996) and have been shown at post-mortem examination to have little or no evidence of atherosclerosis (Kay *et al.,* 1976). Bypass grafting using autologous long SV is also used for critical lower-limb ischaemia. More than 30% of leg vein grafts develop restenosis in the first year following the operation (Varty *et al.,* 1993) and the vein graft develops a thickened intima due to intimal hyperplasia. Intimal hyperplasia (IH) is a fibro-proliferative response to vascular injury caused by surgical intervention or angioplasty. It is characterised by excessive smooth muscle cell migration and subsequent proliferation and secretion of glycosaminoglycans, elastin and collagen into the matrix leading eventually to restenosis. Why IMA grafting is more successful than SV is still unclear.

VSMCs display two different phenotypes based on the distribution of myosin filaments and the formation of large amounts of secretory protein (Nagai *et al.,* 1989). The majority of cells in the media of adult vessels are in the contractile state containing thick and thin myofilaments. In this contractile phenotype, they respond to agents that induce either vasoconstriction or vasodilation and do not respond to proliferative stimulants (Chamley-Campbell *et al.,* 1981). VSMCs in the synthetic phenotype can express genes for a number of growth regulatory molecules and cytokines, respond to growth factors and synthesise extracellular matrix and is necessary for wound repair. The synthetic phenotype cells respond to PDGF in an autocrine way and other growth stimulators and also stimulate neighbouring SMCs (Libby *et al.,* 1988, Sjolund *et al.,* 1988). Various observations suggest that smooth muscle cells in lesions switch from the contractile phenotype to the synthetic phenotype (Dilley & Schwartz, 1989; Majack *et al.,* 1996). Freshly isolated VSMC were reported to change their phenotype from the contractile to the synthetic phenotype after passaging (Chamley-Campbell *et al.,* 1981; Majack *et al.,* 1996). VSMCs rapidly lost the ability to contract and increased their synthesis activity allowing cells to proliferate. Thus VSMC used in culture may represent the synthetic phenotype since the method of explant culture selects for cells that can migrate and proliferate. Cells in culture may reflect VSMCs involved in atherosclerosis as both display synthetic characteristics and allows studies of VSMCs from diseased vasculature to be performed in culture.

1.10.2 Hypertension

Hypertension is defined by an increase in blood pressure and if left untreated, increases the probability of secondary cardiovascular diseases such as arteriosclerosis, congestive heart disease, artherothrombic brain infarction and nephrosclerosis. There are two types of hypertension - essential hypertension and secondary hypertension. Essential hypertension refers to the sustained elevation in systemic arterial blood pressure for which there is no discernible origin. Several mechanisms are believed to be involved including renal Na^+ handling, Na^+ induced Ca^{2+} changes in VSM, increased catecholamine release from sympathetic nervous system and the actions of AII and anti-naturetic protein (ANP). These pathophysiological changes lead to the expansion of the blood volume and increased peripheral resistance through vasoconstriction thereby increasing blood pressure. High dietary salt intake, obesity and genetic predisposition are also implicated. Secondary hypertension causes are believed to be caused by physiological conditions such as renal artery atherosclerosis, which leads to renal hypertension and ischaemia resulting in excessive release of renin and AII production. Another example is the release of mineral corticoids and corticosteroids following endocrine pathology or drug treatment. Hypertension may be benign, which is asymptomatic developing over many years or malignant, developing rapidly in a brief period of time (Friedman 1984).

AII has been shown to stimulate hypertrophy and hyperplasia in the spontaneously hypertension rat (SHR) leading to the thickening of vascular walls and suggesting that it may play a part in initiating hypertension (Bunkenburg *et al.,* 1992, Wilkie *et al.,* 1996, Paquet *et al.,* 1989). The SHRs are the most commonly used experimental model of the human hypertension disease state. The SHR strain was developed at Kyoto University in Japan in 1963 by inbreeding Wistar rats, which showed persistent high blood pressure (b.p.>150mmHg) (Okamoto & Aoki 1963). By the third filial generation all offspring were genetically predisposed to develop hypertension spontaneously and displayed high blood pressure (>150mmHg) seven weeks after birth. The original Wistar strain from Kyoto was used as a normotensive model and therefore called Wistar Kyoto rat (WKY). Vascular smooth muscle cells from aortas of SHR and WKY rats are then cultured. SHR VMCs have been shown to differ from WKY in many ways although receptor number and affinity have been shown not to differ (Bolger *et al.,* 1991). SHR VSMCs display enhanced mitogenic responses compared to WKY VSMC to

several G protein-couple receptors (Morton *et al.,* 1995, Paquet *et al.,* 1990, Bunkenburg *et al.,* 1992). PIP_2 hydrolysis, $[Ca^{2+}]_i$ elevation and mitogenesis occur at higher levels in SHR cells compared to WKY cells (Osani & Dunn 1992, Morton *et al.,* 1995, Turla & Webb 1990). Elevated levels of tyrosine kinase activity in SHR cells have been reported, as well as tyrosine phosphorylation and PLC and PLD activation (Wilkes *et al.,* 1993). ERK activity stimulated by AII and PMA (PKC activator) was also found to be increased in SHR compared to WKY (Wilkie *et al.,* 1997).

1.10.3 Mediators of proliferation of VSMCs

PDGF and other growth factors such as basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-1) can all induce smooth muscle proliferation. Following venous grafting or angioplasty they are released from SMCs, macrophages and activated platelets and thus play an important role in intimal thickening in atherosclerosis and restenosis (Varty *et al.,* 1993, Ross *et al.,* 1990, Schwarz *et al.,* 1995). In vascular smooth muscle cells, PDGF-AA and PDGF-BB both equally induce early response genes but only PDGF-BB stimulates proliferation (Sachinidis *et al.,* 1993). PDGF gene expression is also increased in macrophages in lesions of atherosclerosis (Ross *et al.,* 1990) and increased numbers of PDGF-β receptors on adjacent intimal smooth muscle cells is also seen (Rubin *et al.,* 1988). PDGF was shown to enhance proliferation and increase ERK activities in SV VSMCs with little increases seen in IMA VSMCs despite abundant expression of PDGF β-receptor mRNA in both cell types (Yang *et al.,* 1998). Other evidence to show that PDGF is a mediator of VSMC proliferation in atherosclerosis was shown in rat carotid artery (Sirois *et al.,* 1997). Myointimal proliferation was found to be dependent on both PDGFR-β overexpression and its activation by PDGF-BB and that using antisense oligonucleotides to PDGFR–β suppressed neointimal formation by up to 80%.

Little is known about the role of JNK and p38 in VSMCs. The inflammatory cytokines (IL-1β and TNF-α) activate both JNK and p38. These act upon macrophages and VSMCs within the atherosclerotic plaque and are believed to be critical regulators of the progression of the plaque and rupturing (Force *et al.,* 1996). Hypertension generates large pressures and mechanical sheer stress in cardiomyocytes and VSMCs and the release of vasoactive peptides such as endothelin-1 and angiotensin II. These vasoactive compounds strongly activate JNK in cardiomyocytes (Choukroun *et al.,* 1998, Shapiro *et*

al., 1996) suggesting a major role in cardiovascular regulation. Mechanical stress in rat VSMCs was found to increase JNK, p38 and ERK activities (Li *et al.*, 1999a). The activity of p38 was found to increase during the progression of cardiomyocyte hypertrophy and constitutively activated MKK3/6 also elicited hypertrophy (Wang *et al.*, 1998a,b). The results also showed that p38γ was more important in promoting hypertrophy whereas p38α and p38β were critical for eliciting cardiomyocyte apoptosis. Ischeamic infarcts are characterised by the apoptosis and necrosis of cardiomyocytes and hypertrophy of the surrounding vessels. Bogeoyevitch *et al.* (1996) demonstrated increases in p38 activity in ischaemia of the heart. Izumi *et al.* (2001) showed rapid activation of JNK and ERK in balloon injured rat arteries and when a dominant negative JNK was transfected into these cells, neointimal formation was prevented. The three MAPK pathways of ERK, JNK and p38 may therefore mediate growth factor signalling and regulate proliferation, growth and apoptosis of VSMCs in atherosclerosis.

1.10.4 Role of P2Y receptors in the endothelium

Studies in bovine aortic endothelial cells report $P2Y_1$ and $P2Y_2$ receptors to couple to PLC activation and Ca^{2+} increases through G_q and $G_{i/o}$ respectively (Wilkinson *et al*, 1995, Motte *et al.*, 1993). Action of nucleotides on the endothelial cells causes production and release of vasodilators such as nitric oxide (NO) and PGI_2. (Boeynaems & Pearson, 1990, Wilkinson *et al.*, 1994a). The release of ATP during increased stress such as increased blood flow or pressure (Bodin *et al.*, 1992) therefore instigates vasodilatation. In EAh926 endothelial cells UTP was found to have a negative influence on JNK and inhibited TNFα-induced JNK activation (Paul *et al.*, 2000). In bovine aortic endothelial cells VEGF-induced ERK activation was found to be necessary for JNK activation (Pedrum *et al.*, 1998). Both ERK and JNK were found to be necessary for endothelial cell proliferation in response to growth factors, which therefore suggests that JNK suppression by P2Y receptors may prevent endothelial cell growth.

1.10.5 Role of P2Y receptors in the platelets

Platelets contain a large source of ATP and other inflammatory mediators and express the $P2Y_1$ and P_{2T} receptors (Gordon *et al.*, 1986, Fagura *et al.*, 1998) or $P2Y_{12}$, as

it is now known (Hollopeter *et al.,* 2001, Foster *et al.,* 2001) (see Section 1.5.12). ATP and ADP may be stored in platelets in concentrations as high as 1 M. In normal healthy vasculature platelet aggregation releases nucleotides (Hourani & Hall, 1994). The serum concentration of ATP and ADP may rise to approximately 20 μM (Gordon 1986). These nucleotides act on the endothelial layer P2Y receptors, which mediate the release of NO and PGI_2, which facilitate smooth muscle relaxation and antiproliferation. These also act on platelets and prevent further platelet aggregation and nucleotide release and inflammatory mediators. Nucleotides are rapidly hydrolysed to adenosine, which acts on P1 receptors to prevent further platelet aggregation and stimulates smooth muscle relaxation. So in healthy individuals, nucleotides overall will cause vasodilatation, decreased platelet activation and prevent smooth muscle proliferation, (Figure 1.12A). However if the endothelial layer is damaged (Figure 1.12B) due to vascular disease or trauma, releases of nucleotides from these ruptured cells leads to amplification of platelet aggregation. Nucleotides and other mediators act directly at smooth muscle cells rather than the endothelial layer causing constriction, proliferation and hypertrophy via P2X and P2Y receptors (Rongen *et al* 1997, Hourani and Hall, 1996; Boarder and Hourani, 1998).

1.10.5.1 Atherothrombosis

Platelet activation at sites of injury leads to an accumulation of platelets, release of fibrinogen, ADP and thromboxane and the formation of a clot. Inappropriate or over activation of platelets leads to thrombosis. Therapeutic agents have therefore been directed at platelet P_{2T}/$P2Y_{12}$ receptors. The thienopyridine compounds clopidogrel and ticlopidine effectively inhibit ADP-induced platelet activation (Dormandy 1998). It is believed they are converted to unknown metabolites, which are non-competitive antagonists at P_{2T} receptors, although the full mechanism is not clear (Quinn & Fitzgerald 1999). Clopidigrel and ticlopidine are undergoing phase III trials and evidence is emerging that these antagonists reduce the risks of recurrent strokes and heart attacks, especially when combined with aspirin (CAPRIE 1996, Yusuf *et al.*, 2001). The competitive antagonist action of compounds FPL67085 and FPL66096 at P_{2T} receptors also acts as a potent anti-thrombotic agent (Humphries *et al.*, 1995) and are currently undergoing phase I trials.

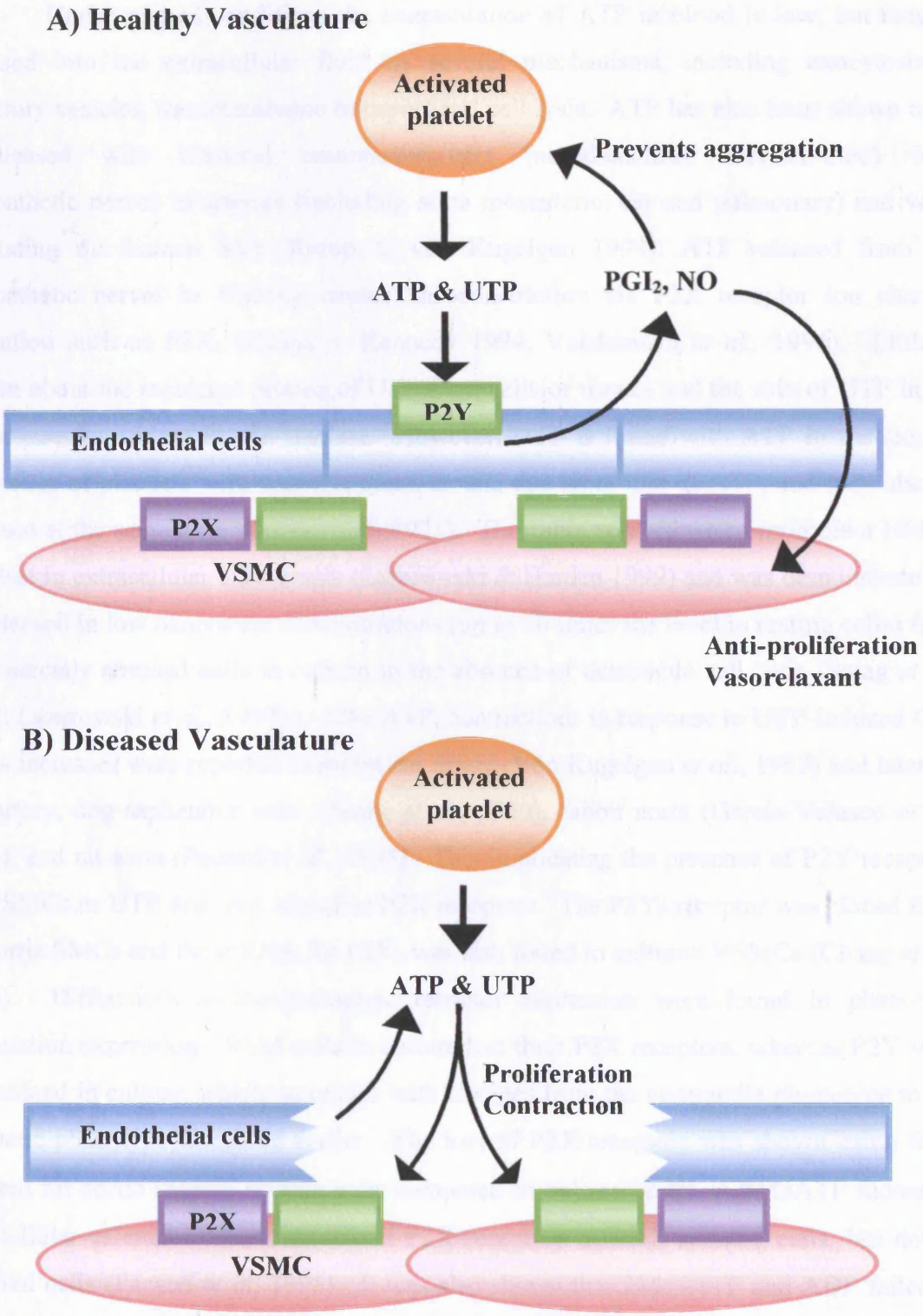


Figure 1.12 The role of nucleotides in A) healthy and B) diseased vasculature

1.10.6 Role of P2Y receptors in the vasculature

Under normal conditions the concentration of ATP in blood is low, but may be released into the extracellular fluid by several mechanisms, including exocytosis of secretory vesicles, transmembrane transport and cell lysis. ATP has also been shown to be co-released with classical neurotransmitters (noradrenaline, acetylcholine) from sympathetic nerves in arteries (including aorta mesenteric, ear and pulmonary) and veins (including the human SV) (Rump & von Kugelgen 1994). ATP released from the sympathetic nerves in VSMCs causes vasoconstriction via P2X receptor ion channel activation such as $P2X_1$ (Evans & Kennedy 1994, Vulchanova *et al.,* 1996). Little is known about the regulated release of UTP from cells or tissues and the role of UTP in the cardiovascular system is still unclear. However, UTP is found with ATP in the storage organelles of platelets with concentrations around one tenth that of ATP, and may also be released at the same time (Goetz *et al.,* 1971). Thrombin was shown to promote a 10-fold increase in extracellular UTP levels (Lazarowski & Harden 1999) and was demonstrated to be released in low nanomolar concentrations (up to 20 times the level in resting cells) from mechanically stressed cells in culture in the absence of detectable cell lysis (Saiag *et al.,* 1994; Lazarowski *et al.,* 1997b). Like ATP, contractions in response to UTP-induced Ca^{2+} levels increases were reported in rabbit ear artery (Von Kugelgen *et al.*, 1987) and later rat tail artery, dog saphenous vein (Saiag *et al.*, 1990), rabbit aorta (Garcia-Velasco *et al.*, 1995), and rat aorta (Pacaud *et al.*, 1995). This implicating the presence of P2Y receptors on VSMCs as UTP does not stimulate P2X receptors. The $P2Y_6$ receptor was cloned from rat aortic SMCs and the mRNA for $P2Y_2$ was also found in cultured VSMCs (Chang *et al.,* 1995). Differences in the purinergic receptor expression were found in phenotype modulation expression. VSM cells in culture lost their P2X receptors, whereas P2Y were maintained in culture, which correlates with changes from the contractile phenotype to the synthetic phenotype discussed earlier. The loss of P2X receptors was shown using fresh isolated rat aortic smooth muscle cells compared to cultured cells. α,βMeATP increased intracellular calcium concentrations via P2X receptors in fresh isolated cells, but not in cultured cells (Pacaud *et al*, 1995). It was also shown that 2MeSATP and ADP failed to increase calcium levels via P2Y receptors in isolated cells, but did in cultured cells. Later Erlinge and colleagues (1998) reported a down-regulation of the contractile $P2X_1$ receptor in culture whereas in intact rat arterial tissue it was found to play a role in the contractile

response. Also, the up-regulation of $P2Y_1$ and $P2Y_2$ mRNA (subtypes implicated in mitogenic signalling mediated by nucleotides) in synthetic phenotype cells was also seen. Furthermore, upregulation of the $P2Y_2$ receptor has been demonstrated in the neointima *in vivo* following experimental balloon angioplasty (Seye *et al.,* 1997*)* and the auto-upregulation of expression of P2Y receptors by ATP itself and other growth factors has been demonstrated (Hou *et al.,* 1999). PDGF may therefore enhance proliferation by differentially upregulating the number of P2Y receptors expressed and the addition of ATP acting as a competence factor (Miyagi *et al.,* 1996) may enhance the response further in a synergistic manner to above those levels stimulated by PDGF.

Increasing evidence shows the involvement of P2Y receptors in regulating mitogenesis (reviewed in Erlinge 1998). Wang *et al.* (1992) reported increases in DNA synthesis in porcine SMCs induced by ATP, which was synergistically enhanced by PDGF, IGF and EGF. The mitogenic responses were also shown to be mediated via increases in cAMP, arachidonic acid and prostanglandin E_2 production and pertussis toxin sensitive. However, Yu *et al.* (1996) demonstrated that P2Y receptor activation was coupled to a pertussis toxin insensitive G protein, which triggered Ptd Ins production and subsequent activation of PKC, Raf-1, and ERK in rat aortic VSM. ATP also caused accumulation of c-fos and c-myc mRNAs, increased DNA synthesis and cell proliferation implicating P2Y receptors in VSMC proliferation. Harper and co-workers (1998) showed increases in PLC activity in response to nucleotides in SHR VSMCs. ATP responses were shown to be partial compared to UTP in SHR VSMC, which was the only full agonist found. UDP, ADP, 2MeSATP and αβ methylene ATP were all ineffective. This shows that responses were not elicited through $P2Y_1$ or $P2Y_6$ receptors, but most likely through $P2Y_4$ and $P2Y_2$. UTP also increased phosphorylation of p42/p44 MAPK and induced cell proliferation, suggesting the involvement of $P2Y_4$ and $P2Y_2$ in mitogenesis. RT-PCR analysis of SHR VSMC also showed high levels of mRNA expressing $P2Y_2$, $P2Y_4$ and $P2Y_6$, but not $P2Y_1$. However $P2Y_4$ involvement alone in causing mitogenesis was questionable due UTP and ATP both acting as full agonists at rat $P2Y_4$ ($rP2Y_4$) similar to $rP2Y_2$ (Bogdanov *et al* 1998). Both $rP2Y_2$ and $rP2Y_4$ may therefore regulate mitogenesis in SHR VSMCs. The sheer stress caused by hypertension results in damage to the endothelium, therefore nucleotides released from activated platelets stimulate proliferation of VSMCs, which is a pivotal process in the development of atherosclerosis and maintaining hypertension.

Wilden and colleagues (1998) showed that UTP-stimulated P2Y receptor led to the activation of ERK that was dependent on MEK activation but PI3K-independent. However, both PI3K and ERK were required for cell proliferation implying separate pathways were required for proliferation in these cells. Increases in DNA synthesis stimulated by ATP and UTP were not only reported in rat aortic SMC but also in vena cava SMCs (Erlinge *et al.*, 1993). Later the same group attempted to characterize the P2Y receptor or receptors involved (Erlinge *et al.*, 1995) and reported the potency of agonists as UTP = ATP ≥ ADP >> AMP = adenosine and suggested the P2U ($P2Y_4$ or $P2Y_2$) to be engaged in mitogensis.

UTP has also been shown to be involved in the regulation of the cell cycle in rat aortic smooth muscle cells, either alone or with PDGF (Miyagi *et al*, 1996). PDGF stimulated quiescent cells to move from G_0 phase to G_1, but no further, whereas ATP and UTP could not achieve this. But UTP, ATP and ATPγS stimulated cell cycle progression of PDGF induced G_1 phase to completion of the cell cycle. Growth factors such as PDGF are therefore thought to be essential for nucleotides to exert their mitogenic effects on smooth muscle cells. Alone however, nucleotides have been shown to induce limited cell cycle progression in rat arterial SMCs (Malam-Souley *et al.*, 1993 & 1996). Progression from G1 to S phase was not observed, and therefore DNA synthesis does not occur which is characteristic of S phase. In passaged human coronary VSMCs, P2Y receptor were shown to generate Ca^{2+} responses in response to ATP, UTP and 2MeSATP (Strobaek *et al.*, 1996) and ATP was found to induce proliferation in human SMCs alone and synergistically with neuropeptide Y (Erlinge *et al.*, 1994).

Increasing evidence shows P2Y receptor stimulation of the p38 and JNK pathways. A recent study of rat aortic SMCs by Hamada and colleagues (1998) showed that increases in JNK activation was found to be mediated by an autocrine ATP stimulation of P2Y receptors in response to stretch. This is further evidence to show the involvement of JNK in P2Y signalling and also implies that JNK may be activated in VSMCs under stressful conditions which may be as a result of hypertension or atherosclerosis.

Investigating nucleotide signalling in VSMCs and the mechanism of inducing proliferation is therefore important. This would be of great clinical importance to generate better drugs in dealing with such conditions as atherosclerosis and hypertension

1.11 P2Y receptor physiology in other tissues & clinical relevance

The wide spread expression of P2Y receptor and physiological responses to nucleotides observed in many tissues shows the importance of these receptors. Any defects in P2Y receptor signalling could potentially be involved in pathological disease states and this would implicate P2Y receptors as potential drug targets. However, since most tissues express more than one subtype, it would be advantageous that therapeutic drugs be highly selective for their receptors to avoid possible unwanted effects. Here the various roles that P2Y receptors have in selected tissues apart from the vasculature is discussed and the clinical relevance of these receptors in certain diseases states is also approached later.

1.11.1 Nervous system

P2X and P2Y receptors are both present in the brain and may be co-expressed in certain regions. ATP has unambiguously been shown to act as a fast neurotransmitter in the central nervous system (CNS) (Bean *et al.*, 1992; Evans *et al.*, 1992a; Edwards *et al.*, 1992). These effects were mediated primarily by P2X receptors, which are ATP-gated cation channels, allowing sodium and potassium ions to flow across the membrane causing rapid depolarisation and subsequent action potential firing.

ATP is known to modulate release of neurotransmitters such as noradrenaline (Allgaier *et al.*, 1995) and acetylcholine (Cunha *et al.*, 1994). In rat sympathetic neurones Boehm *et al.* (1995) reported that ATP and UTP triggered [^{3}H]noradrenaline which was suramin-sensitive. This was response was suggested to be mediated by the $P2Y_4$ receptor. Similar studies in chick sympathetic neurones showed P2Y receptors to increase electrically evoked noradrenaline release (Allgaier *et al.*, 1993) and in rat striatum P2Y receptors mediated an increase in dopamine release (Zhang *et al.*, 1996a).

1.11.2 Urogenital

Both P1 and P2 receptors exist in the bladder where ATP mediates contraction via P2X receptors and adenosine acting at P1 receptors causes relaxation (King *et al.*, 1997).

ATP and adenosine can therefore modulate detrusor muscle responses. McMurray *et al.*, (1998) have also shown that P2Y receptors regulate relaxation in marmoset urinary bladder smooth muscle. Relaxation was found to be inhibited by suramin and cibacron blue (RB-2) but not 8-PT (P1 antagonist). Inhibition by tos-phechloromethylketone (PKA inhibitor) was also observed suggesting a role for cyclic AMP-dependent PKA in the relaxation response. Similarly, mouse vas deferens displayed contraction via ATP stimulating P2X receptors followed by relaxation via P2Y receptor activation (Bland *et al.*, 1992). In rat bladder UTP was reported to instigate a rapid and concentration dependant contraction that was not desensitised by α,β-meATP, suggesting that this response was not linked to P2X receptors (Bolego *et al.*, 1995a). In contrast, Hashimoto and Kokubun, (1995) did find α,β-meATP to desensitise UTP induced contraction in rat bladder smooth muscle.

1.11.3 Gastrointestinal

The gastrointestinal tract is innervated by sympathetic and parasympathetic and enteric nervous system, controlling relaxation and contraction of multiple muscle layers. ATP was first recognised to act as a neorotransmitter in the gut in 1970 by Burnstock. In the rat duodenum adenosine or ATP induced relaxation while UTP and ATPγS caused contraction (Johnson *et al.*, 1996), which seemed contradictory at first. Separation of the duodenum into longitudinal muscle and the muscularis mucosae showed that a $P2Y_1$ receptor on longitudinal muscle mediated relaxation only and the muscularis mucosae expressing P2X and P2U receptors both mediated contraction only. Hourani *et al.*, (1993) also demonstrated contraction after application of ATP, UTP or adenosine acting via a P2Y receptor or an A1 adenosine receptor in the rat colon muscularis mucosae. In pig small intestine ATP was found to inhibit peristalsis via $P2Y_1$ (Heinemann *et al.*, 1999). The different layers of the gut expressed different receptors therefore allowing differential gut modulation

1.11.4 The immune system

P2 receptors are found on a variety of cells of the immune system including mast cells, macrophages and lyphocytes (Wiley *et al.*, 1994, Zambon *et al.*, 1994, DiVirgilio 1995). ATP is co-released during mast cell degranulation with histamine. ATP acts in a

paracrine fashion to activate P2 receptors on adjacent mast cells, potentiating further degranulation (Osipchuk & Cahalan 1992). ATP stimulation of Ins (1,4,5)P_3 formation and increases in Ca^{2+} levels in inflammatory cell types, including neutrophils, monocytes and macrophages, is believed to be mediated via P2Y receptors. Increases in PLC and PLA_2 activity and the production of inflammatory mediators is also linked to P2Y receptor stimulation (Cockroft & Stutchfield 1989, Balazovich & Boxer 1990). P2Y receptors may also facilitate the migration of inflammatory cells by upregulating the expression of adhesion molecules on monocyte and granulocyte plasma membranes (Freyer *et al.*, 1988).

1.11.5 Therapeutic role of P2Y agonists / antagonists

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

1.11.5.1 Cystic Fibrosis

Cystic fibrosis is a result of a mutation in cystic fibrosis transmembrane conductance regulator (CFTR) causing defective epithelial cell chloride transport. As an outcome, disease characteristics include dehydration and thick mucal secretions in the lung, gall bladder and small intestine (Boucher *et al.*, 1986; Noone and Knowles, 1993). Since ATP and UTP have been shown to effectively enhance mucociliary clearance, potential therapeutic benefits for patients with this disease has been proposed (Sabater *et al.*, 1999). UTP or ATP activation of the $P2Y_2$ receptor increases the release of chloride ions through activation of calcium dependant outward rectifying chloride channels (ORCC) thereby relieving the symptoms of the disease (Mason *et al.*, 1991). In normal conditions the CFTR provides a mechanism for ATP release, which stimulates $P2Y_2$ coupling to ORCC, thereby increasing Cl^- release (Schwiebert *et al.*, 1995). A defective CFTR is unable to supply ATP leading to decreased chloride transport. Most studies have implicated $P2Y_2$ as the main receptor involved but some reports have also shown UDP (Lazarowski *et al.*, 1997c) and adenosine (O' Reilly *et al.*, 1998; Stutts *et al.*, 1995) to having modulatory effects. $P2Y_2$-null mice were generated using homologous recombinant techniques to investigate this further (Cressmann *et al.*, 1999). It was found

that 85-95% of the nucleotide-stimulated Cl^- release was due to $P2Y_2$ in the excised airway epithelium. Current therapy involves aerosol application of UTP that directly activates ORCC chloride release. However potent agonists that are specifically selective for $P2Y_2$ receptors in the airways would be advantageous. UTPγS would also be a good candidate as it would be resistant to any ectonucleotidases present (Lazarowski *et al.*, 1996).

1.11.5.2 Diabetes

Activation of the P2Y receptors in insulin-secreting pancreatic β-cells leads to $Ca2^+$ increases and subsequent insulin secretion and improved glucose tolerance (Li *et al.*, 1991, Hillaire-Buys *et al.*, 1992,1993). While P2Y receptors are involved in insulin secretion, A1 adenosine receptors are implicated in antilipolytic action on adipocytes (Petit *et al.*, 1996). Ectonucleotidase resistant ADPβS has been shown to stimulate insulin production in diabetic rats remaining effective after oral administration (Hillaire-Buys *et al.*, 1992,1993). There is therefore a potential for P2Y and A1 agonist as therapeutic agents in the control of diabetes.

1.11.5.3 P2X receptors

P2X and P2Y receptors have been found in vas deferens (Bland *et al.*, 1992). A $P2X_1$-null mouse was generated by deletion of the $P2X_1$ gene to investigate its role in ejaculation (Mulryan *et al.*, 2000). It was reported that male fertility was reduced by 90% in these mice despite normal copulation and sperm production. The reduced fertility was suggested to be due to the reduced contractile response of the vas deferens, which propels sperm into the ejaculate. Therefore $P2X_1$-selective antagonists may possibly be developed and used as a male non-hormonal contraceptive pill.

1.12 Thesis Objectives

Intimal smooth muscle proliferation is regulated by cell surface receptors such as RTK and GPCRs. Understanding the signalling mechanisms of these receptors is important as overgrowth leads to artherosclerosis, narrowing of blood vesels and hypertension (Ross 1993). PDGF has been shown to act as a mitogen in human VSMCs (Yang *et al.,* 1998) and rat VSMCs (Sirois *et al.*, 1997). Several animal studies (reviewed in Erlinge 1998) have shown that nucleotides are able to mediate a mitogenic response both alone and in the presence of other growth factors (Wang *et al.*, 1992). Similarly in human SMCs, ATP was found to stimulate proliferation alone and synergistically with neuropeptide Y (Erlinge *et al,* 1994). The primary objective of this study was to investigate the ability of nucleotides *per se* or in the presence of PDGF to stimulate proliferation in human VSMC. This was initially assessed by [^{3}H]-thymidine incorporation to identify mitogenic nucleotides and attempt to characterise P2Y receptors on these cells that instigate mitogenesis. Changes in intracellular calcium levels and $InsP_3$ production were also investigated in order to characterise P2Y receptors.

The second aim was to elucidate the mechanism of PDGF and nucleotide signalling. Protein kinases play an important role in intracellular signalling. The MAPKs, for example, are important in cellular processes such as growth, differentiation, inflammation and apoptosis. The involvement of the MAPKs ERK, JNK and p38 in PDGF and nucleotide signalling were studied by quantifying phosphorylation using phospho-specific antibodies. Another protein kinase (ROCK) was similarly investigated. Protein kinase inhibitors were used to investigate whether these protein kinases were necessary for PDGF-induced DNA synthesis.

Alongside studies on human cells, nucleotide influences on rat VSMCs were also investigated. There have been many reported differences in VSMC signalling between the rat hypertensive model compared to the normotensive rat. Earlier work by Harper *et al.* (1998) showed nucleotide mitogenic influences in SHR cells, but, in the light of subsequent publications, has left unanswered questions. The final aim of this study was to pursue this further and determine any differences in P2Y receptor signalling and expression between SHR and WKY VSMCs.

Chapter 2

Methods and Materials

2.1 Cell culture

2.1.1 Human vascular smooth muscle cells

Human saphenous vein (hSV) and internal mammary artery (hIMA) primary explants were prepared by Dr. Karen Porter (Dept. of Medicine, Leicester Royal Infirmary) with approval of the local Ethical Committee. Briefly, excised tissue from SV or IMA recovered during coronary artery bypass operations was stripped of endothelium and connective tissue and chopped using scissors. Small sections were then placed in 25 cm^2 flask in 7 ml whole growth medium (RPMI 1640, supplemented with 10% foetal calf serum (FCS) 50 I.U./ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine). After 2 weeks incubation, cells grown out from the tissue were passaged and cultured, as described below, into 80 cm^2 into growth medium (Dulbecco's modified Eagles medium (DMEM) with 1000 mg/l glucose with pyridoxine, supplemented with 10% foetal calf serum (FCS) 50 I.U./ml penicillin, 50 μg/ml streptomycin). 60-70% of cells stained positive for smooth muscle actin immunostaining with monoclonal mouse anti-human smooth muscle actin (White *et al.*, 2000). Cells from different patients were referred to as cell lines. Cells were used between passages 2 and 7 as they reached confluency. Most experiments were performed on SV cells although a few earlier experiments (Chapter 3) were also performed on IMA cells (as stated in text).

2.1.2 Rat vascular smooth muscle cells

SHR and WKY cell lines were prepared by Dr. Leong L. Ng (Dept. of Medicine and Therapeutics, University of Leicester), using the method described in Davies *et al* (1991). Briefly, arterial blood pressure of 12 week old SHR and WKY rats was determined using tail cuff measurements. The rats were decapitated and the thoracic aorta was removed and the adventitia was stripped. The media was cut into 1 mm pieces and enzymatically digested. Cells were centrifuged and resuspended in growth medium. Clonal cultures were established and those colonies with smooth muscle cell morphology were combined after five days incubation. The resulting colonies showed 100% positive immunoreactivity for smooth muscle actin.

SHR and WKY VSMCs were maintained in complete medium consisting of Dulbecco's modified Eagles medium (DMEM) with 4.5 g/l of glucose supplemented with 10% Foetal Calf Serum (FCS), 50 I.U./ml penicillin, 50 μg/ml streptomycin and 2 mM L-

glutamine in 175 cm^2 tissue culture flasks and kept in a sterile humidified atmosphere of an incubator at 37^0C with 5% CO_2.

2.1.3 1321N1 transfected cell line

Human 1321N1 astrocytoma cell line is a P2 receptor null cell line exhibiting no P2 agonist-stimulated PLC and Ca^{2+} responses. 1321N1 cells transfected with human $P2Y_2$ ($hP2Y_2$) and $hP2Y_4$ receptors were donated by Dr. G.A. Weisman, University of Missouri-Columbia, U.S.A and Dr. T.K. Harden, University of North Carolina, Chapel Hill, U.S.A respectively. 1321N1 cells were maintained and cultured in DMEM (4.5 g/L of glucose supplemented with 10% FCS, 50 I.U./ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine) and stored in a sterile incubator at 37^0C with 5% CO_2.

2.1.4 Passaging of cells

All cell culture work was performed in Class II microbiological cell culture cabinet. Confluent cells grown in 175 cm^2 flasks were split by washing three times with 10 ml of Earle's balanced salt solution (EBSS). The adherence of the cells to the surface of the flask, via the actions of the anchor proteins, was inhibited due to the absence of calcium and magnesium. This allowed the removal of the cells from the flask when 5 ml of trypsin/EDTA (ethylenediamine tetra-acetic acid) solution was added. The flask was also swirled and incubated at 37^0C for 5 minutes and agitated to ensure clumps of cells were broken up. 10 ml of complete medium was then added to inhibit any further trypsin digestion. The detached cells were transferred to 30 ml sterile tube and centrifuged at 200 X g for 5 minutes. The supernatent was decanted and the cell pellet was resuspended in 5 ml of complete medium and passed through an 18-gauge needle 5 times. The cell suspension was then diluted out as required in complete medium. Typically, the cells were split with a ratio of 1:4 depending upon surface area. Each 175cm^2 flask received total volume of 50 ml of cell suspension, a 6 well plate received 3 ml/well of cells, a 24 well plate received 1 ml/well and 96-well plate received 200 μl/well of cells. Human VSMC, however, were not centrifuged nor passed through a needle as cells dispersed into a fine suspension efficiently and further agitation damaged the cells. They were, therefore, diluted as required and seeded with a ratio of no more then 1:3. Human VSMCs were larger in comparison to rat VSMC and grew at a slower rate and therefore needed to be seeded at a higher density.

Prior to experimentation, rat VSMCs were serum-starved for 24 hours after reaching 80% confluency, normally within 2 days of seeding cells. This allowed cell to reach quiescence after which they were ready to use. 1321N1 cells were also serum-starved for 24 hours, but at 40% confluency within 1 day of seeding. Human VSMCs reached 80% confluency after 7 days and required 48 hours serum starvation.

2.1.5 Storage of cells

When not in use, cells were frozen down and stored under liquid nitrogen. The cells were grown to confluence in a 175 cm^2 flask washed 3 times with 10 ml EBSS (without Mg^{2+}/Ca^{2+}) and 5 ml trypsin/EDTA solution added to detach the cells from the flask. Cells were then pelleted in a centrifuge and resuspended in 1 ml of freezing medium [90% FCS and 10% dimethylsulphoxide (DMSO)] through an 18-gauge needle, transferred to a cryo-vial and frozen at –80°C and then in liquid nitrogen. Cells were grown from liquid nitrogen storage by adding one vial to 50 ml warmed complete medium in a 175 cm^2 flask.

2.2 Total [^{3}H] inositol polyphosphates

Lithium is an uncompetitive inhibitor of inositol monophosphate recycling to inositol and the subsequent incorporation into the membrane phospholipid pool (Nahorski *et al.*, 1992, Hallcher & Sherman, 1980). Therefore the recycling of Ins(1,3,5)P_3 (produced by PLC) is impaired, causing an accumulation of inositol monophophates. At higher concentrations (above 5 mM) the Ins(1,4)P_2 and Ins(1,3,4)P_3 1-phosphatase enzyme is also inhibited resulting in the accumulation of larger inositol phosphates. Lithium has therefore been used as a tool for the study of PLC activity (Berridge *et al.*, 1993, Nahorski *et al.*, 1991).

Cells were cultured to 80% confluence in 24 well multi-well plates. The medium was then replaced with 500 µl radio-labelled serum free M199 medium supplemented with 50 I.U./ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine and 1 µCi/ml (0.074 MBq/ml) D-myo-[2-^{3}H] inositol). Cells were incubated in this medium for 24 hours. The plates were then placed in a 37^{0}C water bath and each well was incubated with 10 mM LiCl for 10 minutes. In some experiments suramin or hexokinase was also used and 50 µl was added at 11 times the final concentration with 110 mM LiCl in BSS at this stage.

Cells were then stimulated for 20 minutes by the addition of 50 µl of agonist solutions at 12 times the final concentration in BSS plus 10 mM LiCl. The reaction was stopped by aspiration of the stimulating media and addition of 0.5 ml of cold 0.5 M trichloroacetic acid (TCA). The plates were then placed on ice for 1 hour for [^{3}H]-inositol phosphate extraction. 400 µl of sample from each well was then transferred into polypropylene vials and 100 µl of 10 mM EDTA was added to collate calcium. This was followed by the addition of 500 µl 1:1 mixture of tri-n-octyl-amine and 1,1,2 tri-chloro-tri-fluroethanol. This part of the procedure was carried out in the fume cupboard. The samples were vortexed and left to settle for 5 minutes and then 400 µl of the aqueous phase was removed and 50 µl of 250 mM $NaHCO_3$ added to neutralise the samples. Samples were incubated at 4^0C for 90 minutes. Dowex-1 Cl^- ion exchange resin columns were charged with formate ions by washing with 1 M formic acid. Samples were added to the columns and then washed with 10 ml water to remove excess [^{3}H]-inositol and 10 ml 60 mM ammonium formate to elute contaminating glycerophosphoinositides. 5 ml 2 M ammonium formate was then added to elute labelled inositol phosphates into large scintillation vials. 15ml of FLO-SCINT^{TM} IV scintillation cocktail was added to each vial as it has an aqueous capacity of 1:3. Vials were vortexed to ensure mixing of the two phases and counted for [^{3}H].

2.3 Measurement of intracellular calcium

Increases in cytosolic calcium concentrations are achieved by stimulation of cell surface receptors, which eventually released calcium from internal stores, by influx through plasma membrane, or via both mechanisms (McFadzeon & Gibson, 2002). Calcium signalling is important in smooth muscle contraction and relaxation. Intracellular calcium concentrations can be measured using fluorescent calcium indicators and single cell imaging techniques in response to various agonists as a function of time. The fluorescent indicator for calcium used was fura-2, which binds to calcium with a 1:1 ratio. It is most useful because it has different excitation wavelengths in its free and bound form. In its free form fura-2 has high excitation efficiency at 380nm and low excitation efficiency at 340nm. However, in its calcium bound form it has a high excitation efficiency at 340nm and a low excitation efficiency at 380nm. Therefore with increasing calcium concentrations the excitation efficiency at 380nm decreases and 340nm

simultaneously increases. By measuring the fluorescence emitted at 500nm by fura-2 when separately illuminated by both 340 and 380nm wavelengths, the amount of fura-2 bound can be measured. Ratio of fluorescence obtained at 340nm to 380nm can be used as an index of changes in free cytosolic calcium levels. Fura-2 is unable to cross the cell membrane, therefore it is applied to the cells as the acetoxymethylester (fura-2AM), which can cross the plasma membrane. A 30 minutes incubation period is sufficient to allow fura-2AM to diffuse across. As it enters the cell it is hydrolyzed by intracellular esterases thus trapping it inside the cell.

Cells were cultured on coverslips and made serum free for 24 hours. Cells were plated on three coverslips and each was taken individually and stimulated. The only alteration in protocol for each coverslip was the order in which the agonists were added. Each coverslip was washed with physiological saline solution and incubated for 30 minutes with 4 μM fura-2AM in 0.1% pluronic acid. Excess dye was washed off and 1 ml of saline was placed in a coverslip dish and placed on the microscope stage in the perfusion apparatus. Cells were continuously perfused with saline and various drugs were added a different time intervals for one minute using a multibarrelled perfusion system which was aligned so that flow was in one direction across the cells on the coverslip. Using software program (ImproVision microspectrofluorimetry system) cells were illuminated to visualize them. An appropriate field of view of healthy, clear cells was selected. The intensifier gain of the CCD camera was then set to a level that shows cells clearly and so that the widest range of 256 grey levels were available. The camera was set to record and captured images with alternating 340 and 380nm excitation wavelength with a constant emission wavelength of 500nM. The image sequence was then subjected to background subtraction by the software program. Image analysis software also ratios background corrected image pairs (340:380) pixel by pixel. The cell of interest was outlined and the mean ratio of that cell was calculated for each field of view. The same was done for the other cells in the field. Therefore the mean ratio of cells of interest were collected at each frame and analyzed as a function of time.

2.4 Cell stimulation and extraction

The following stimulation method was used for preparing lysates from either rat VSMCs, human VSMCs, or 1321N1 astrocytoma cell lines for Western blotting or kinase

assay. Cells were cultured in 6 well multi-well plates or in some cases, in 6 cm round dishes until they reached confluence. 1 ml of serum-free media was added per well of a 6 well plate or 2 ml per 6 cm dish for 24 hours prior to stimulation. Human VSMC were serum starved for 48 hours. Cells were stimulated for the required time by the addition of 100 µl of 11 times final concentration of agonist (or 22 times for 6 cm dishes) in a 37^{0}C water bath. For stimulation times of more than 10 min the cells were maintained in CO_2 incubators at 37^{0}C. The stimulation was stopped by quickly inverting plates onto tissue and adding liquid nitrogen to the cells. Cells were lysed and scraped using a 1 ml syringe plunger into 100 µl of ice-cold lysis buffer (20 mM Tris-HCl pH 8, 0.5% Triton X-100, 250 mM NaCl, 3 mM EDTA, 3 mM ethylene glycerol bis-aminoethylether tetra-acetic acid (EGTA), 1 mM phenylmethyl sulphonylfluoride (PMSF), 2 mM sodium orthovanadate, 5 µg ml^{-1} leupeptin, 20 µg ml^{-1} aprotinin) or a similar buffer as stated in text. After scraping the wells, the lysates were transferred to eppendorf tubes, vortexed and then centrifuged for 10 minutes at 14,000 r.p.m. at 4°C. 10 µl of the supernatant was used in Bradford assay for protein concentration determination. At this stage samples were either prepared for immunoprecipitation or equal volume of a double concentration of Laemmli's sample buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 4% 2-mercaptoethanol, and 0.01% bromophenol blue) was added to the remainder of the supernatant. The samples were boiled and either stored at -20^{0}C or used immediately.

2.5 Bradford protein assay

The Bradford protein estimation technique (Bradford M.M. 1976) was used to quantify the amount of protein in a cell lysate. The mechanism behind the Bradford assay is the absorbance maximum of the Bradford reagent (Sigma) shifts from 465nm to 595nm due to the protein-dye complex stabilisation. A standard curve was generated by creating a dilution series made up of BSA, from 0 to 25 µg/ml, and addition of 1 ml 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 polystyrene cuvettes on a spectrophotometer. Unknowns were diluted to 1 ml and following addition of 1 ml of Bradford reagent, read on the spectrophotometer. The standard absorbances produced a linear plot from which unknowns were calculated.

2.6 Western blotting

Following cell stimulation, protein extraction and protein determination as described in Section 2.4-5 and 2.9 for ROCK detection, extracts were analysed by Western blotting.

2.6.1 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel setting and electrophoresis (SDS-PAGE) was carried out using Biorad minigel apparatus. Each gel consisted of two components, the running (or resolving) gel and the stacking gel. A 10% running gel was mixed using 10% acrylamide: bisacrylamide (29:1%), 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, 200 µl of isopropanol was laid on top to aid polymerisation and provide a horizontal set gel. The role of the running gel was to separate the proteins in the in the cell extract according to their relative molecular weights. The smaller the protein the further it would migrate towards the cathode when a current is passed through. The migratory distance was also dependent on the percentage of acrylamide in the gel. The higher the percentage of acrylamide in the running gel the slower the proteins run through the gel. Most of the experiments carried out involved the use of 10% running gels, except those, which required the observation of smaller proteins such as JNK or p38, where 12% gels were used. After 25 minutes the running gel had set and the ethanol was removed and excess washed away with a 0.1% 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 a 10 well comb inserted 0.5 cm from the running gel. The purpose of the stacking gel was to ensure all the proteins in the cell extract in each well were focused into a tight band by the time they reached the running gel. This was allowed to set for 15 minutes and the comb removed. The wells were then washed with running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS) through a needle and syringe. The final dimensions of a mini-gel were 8.5 cm by 5.5 cm by 0.75 cm.

The gels were assembled in the apparatus and upper and lower reservoirs filled with running buffer ready for loading. Samples were boiled at 100^0C for 5 minutes prior to loading. Prestained protein molecular weight standards (250 kDa, 150 kDa, 100 kDa, 70

kDa, 50 kDa 37 kDa, 27 kDa and 15 kDa) were boiled for 1 minute at 100^0C. Eight out of the 10 lanes were routinely used with outer lanes used for prestained markers. Between 5 and 20 μg protein was loaded per lane for each extract (10-25 μl volume). Electrophoresis was carried out at constant 150V for 90 minutes or until the samples reached the bottom of the running the gel. The power supply was switched off and the apparatus was disassembled and the gels were removed.

2.6.2 Blotting

The blotting procedure was carried out using Biorad blotting apparatus. The gels were washed for 15 minutes in blotting buffer (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol) before assembling a sandwich. This consisted of the gel in direct contact with a sheet of nitrocellulose with 4 pieces of 3 mm blotting paper and a final fibre pad on each side, which was held in place by a plastic grid blotting cassette. The cassette was placed in the transfer tank filled with blotting buffer with the gel positioned towards the cathode plate in the tank and the nitrocellulose towards the anode. This, therefore, allowed the proteins to transfer from the gel (positive side) to the nitrocellulose membrane (negative side) when the power supply was switched on to 100V for 1 hour. A cooling coil was also placed in the tank. The proteins were transferred to the surface of the nitrocellulose and were now more accessible for antibody detection

To check sufficient transfer, the nitrocellulose was removed from the cassette and stained with Ponceau S to visualise the proteins. The protein standards were also marked using a biro and the nitrocellulose was trimmed. The blots were washed with 10 ml/blot TBS-T (pH 7.5, 20 mM Tris-HCl, 137 mM sodium chloride and 0.05% Tween-20) for 5 minutes, three times. Non specific binding sites were blocked by incubating the nitrocellulose overnight at 4°C (or 2 hours at room temperature) in 10% dried milk (10 ml) or 1% BSA depending on antibody to be used in the next stage.

2.6.3 Antibody incubation

Excess blocking agent was removed by washing 5 times for 5 minutes in 15 ml TBS-T before antibody was added. The first antibody or the primary antibody was diluted as required in 5% marvel or 0.1% BSA according to the manufacturer's recommendations. The nitrocellulose was placed in a small container and 5 mls of diluted primary antibody was added and incubated at 4°C overnight. The nitrocellulose was then washed 6 times in

15 mls TBS-T for 5 minutes. The horseradish peroxidase (HRP) conjugated secondary antibody was diluted as required in 5% dried milk or 0.1% BSA for 90 minutes at room temperature. After this incubation the nitrocellulose was again washed six times with 15 ml TBS-T. The secondary antibody recognises the primary antibody, which was raised against and specifically detects the protein of interest. The target protein can then be visualised due to the peroxidase tail using Amersham enhanced chemiluminesence plus reagents (ECL plus). ECL is a light emitting method detection. Immobilised specific antigens are conjugated directly or indirectly with HRP conjugated antibodies, which catalyses the oxidation of the Lumigen PS-3 acridan substrate to generate thousands of acridinum intermediates per minute (Akhaven-Tafti *et al.*, 1994 & 1995). These react with hydrogen peroxide in slight alkaline conditions to produce a sustained, high intensity chemiluminesence. This light-emitting reaction can be captured by autoradiography. Alternatively to quantify chemiluminesence emissions a gel documentation system was used (Chemi-Genius). ECL-incubated blots were placed in a dark room containing a CCD camera. The blots were focused and iris fully opened. A series of images of the blots was captured using Gene Snap acquisition software with exposure times of 1, 5, 10, 15, 20 and 30 minutes. Gene Tools Match analysis software was used to select bands at required molecular weight, allow background correction and quantify each band by calculating the mean pixel density. Saturation is difficult to achieve due to the broad range of grey scale (0-65536) for every pixel, which makes this system more advantageous than autoradiography. Quantification is also performed on the original raw data collected even after image manipulations are performed such as altering the contrast or brightness, which are used to improve the appearance of the blot. The original data remains uncorrupted despite these functions. Quantification data was analysed using Graphpad Prism software

2.7 JNK & p38 assays

2.7.1 Plasmid

These assays were performed with the kind help of Dr. J.L. Blank, Dr. K. Deacon and Mr. D. Burdon. To assess JNK activity an assay was carried to measure the phosphorylation of a recombinant protein fragment of c-Jun by JNK. Constructs for bacterial expression of c-Jun as translational fusions with glutathione *S*-transferase (GST)

were kind gifts from Roger J. Davis (as described in Derijard *et al.*, 1995). The N terminus of the c-Jun transcription factor (codons 1 to 79) was expressed as a recombinant fusion protein of glutathione-S-transferase from the plasmid vector pGEXcJUN. 10 ml of 2 X YT media (10 g L^{-1} NaCl, 10 g L^{-1} yeast extract, 16 g L^{-1} bacto-tryptone), with ampicillin at 100 μg ml^{-1}, was inoculated with a single colony of *E.coli* (strain JM109) containing pGEXcJUN and incubated overnight at 37°C with shaking at 200rpm. 1L of 2 X YT, with ampicillin at 100 μg ml^{-1} was inoculated with the overnight 10ml culture and shaken at 200 rpm at 37°C until the OD A_{600nm} reached 0.5. Fusion protein expression was then induced with iso-propyl-thiogalacto-pyranoside (IPTG) at 0.4 mM for 3 to 6 hours at 30°C with shaking at 200 rpm. The bacterial culture was separated from the culture media by centrifugation at 4000 X g for 10 minutes at 4°C. Bacterial pellets were resuspended in 50 ml of GST lysis buffer (20 mM Tris-HCL, pH 7.5, 1 M NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.1% Triton X-100, 0.5mM PMSF, 2 μgml^{-1} leupeptin, 0.2 mg ml^{-1} aprotinin) and frozen at -20°C for 3 hours. Frozen resuspended pellets were rapidly defrosted in a water bath at 50°C and subjected to sonication with an immersed probed at maximum for 3 x10 seconds. Liquid and solid phases were then separated by centrifugation at 10 000 X g for 15 minutes at 4°C. GST-c-JUN protein was affinity purified from the cleared lysate by incubation with 5ml of 70% glutathione-sepharose (Amersham -pharmacia) for 1 hour at 4°C. GST-cJUN beads were washed three times with 50ml of lysis buffer and then once protein kinase assay lysis buffer before resuspension as a 25% v/v slurry in assay lysis buffer. Samples of prepared GST-cJUN bead were resolved by SDS PAGE and stained with Coomasie-brilliant blue to assess fusion protein quantity and quality using bovine serum albumin (BSA) to construct a standard curve.

2.7.2 Immune complex kinase assay

Cells were grown and stimulated similar to that described in Section 2.4. Briefly, cells were grown to confluence, serum starved for 48 hours and stimulated for 30 minutes with agonists. The stimulation was stopped and cells lysed with liquid nitrogen and the addition of lysis buffer. Extracts were cleared by centrifugation at 14 000 X g for 10 minutes at 4^0C and then supernatent was protein equalised. Extracts were incubated with 20 μl of a 25% (v/v) slurry of glutathione-Sepharose coupled to GST-c-Jun (5 μg protein) for 1 hour at 4^0C. Extracts were centrifuged 14 000 X g for 25 seconds at 4^0C to collect the beads and washed twice in 200 μl lysis wash buffer (20 mM Tris-HCl pH 8, 1% Triton

X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg ml^{-1} leupeptin) and twice in 200 μl kinase buffer (20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol and 50 μM Na_3VO_4). After the final wash the reaction was initiated with the addition of 40 μl kinase buffer containing 250 μM ATP and 5 μCi [γ^{32}P]ATP and incubated at 30^{0}C for 20 minutes. Samples were votexed once during this incubation. The reaction was stopped by the addition of 40 μl 2 X Laemmli sample buffer, vortexed and then boiled. After brief centrifugation the phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

2.7.3 Immunoprecipitation of JNK & p38

An alternative method to that described above involved immunoprecipitating JNK using JNK polyclonal antibody and then measuring its activity using c-Jun. This methods was also used for assessing p38 activity. Cell were grown and stimulated similar to that described in Section 2.4. Briefly, cells were grown to confluence, serum starved for 48 hours and stimulated for 30 minutes with agonists. The stimulation was stopped and cells lysed with liquid nitrogen and the addition of lysis buffer. Extracts were cleared by centrifugation at 14 000 X g for 10 minutes at 4^{0}C and then supernatent was protein equalised. Extracts were incubated with 1μg of rabbit anti-JNK1 antisera (Santa Cruz), for 1 hour at 4^{0}C. For p38 MAPK assays lysates were incubated with 10μg of goat anti-p38 antisera (Santa Cruz [sc-535-G]). Immune complexes were incubated with either protein-A (for rabbit antisea) or protein-G sepharose (for goat antisera) (Amersham) for a further 1 hour at 4^{0}C. Samples were centrifuged 14 000 X g for 25 seconds at 4^{0}C and the precipitates were then washed twice with lysis buffer and twice with protein kinase buffer (25mM HEPES, pH 7.2, 25mM β-glycerophosphate, 25mM MgCl2, 2mM dithiothreitol and 100μM Na_3VO_4). Protein kinase assays were initiated with the addition of 40μl of protein kinase buffer with 250μM ATP, 5μCi γ [^{32}P]-ATP and either 2ug of GST-cJUN for JNK-1 assays or 5μg of GST-ATF2 for p38 MAPK to each sample (both GST-c-Jun and GST-ATF2 were kindly purified and supplied by Dr. Karl Deacon by affinity chromatography). These were incubated at 30°C for 20 minutes. Reactions were stopped with an equal volume of 2 X Lamelli sample buffer, vortexed, boiled and resolved by SDS-PAGE and visualized by autoradiography.

2.8 Methods used to investigate ROCK activity

2.8.1 Immunoprecipitation

Cells were grown to confluence in 6 well plates and stimulated as previously described for western blotting (Section 2.4) except for the following alterations. Following stimulation, reaction was stopped with liquid nitrogen and then cells were lysed into 200 µl/well of ROCK extraction buffer (140 mM NaCl, 20 mM EPPS, 5mM Na_2EDTA, 100 mM NaF, 100 mM $Na_4P_7O_2$, 5 mM Na_3VO_4, 1 mM Na_2MOO_4, 0.1% NP-40, 1 µM PMSF, 5 µg/ml leupeptin, 20 µg/ml aprotinin), dishes were scraped and lysates pipetted into eppendorfs. Lysed cells were vortexed and cleared be centrifugation for 10 minutes at 14000 rpm, 4°C. 180 µl of the supernatant was removed for the immuno-precipitation procedure and 10 µl for protein determination. Approximately 2 µl (1 µg antibody/50 µg protein in sample) of rabbit anti-ROCK polyclonal antibody (kindly provided by Dr. L.L. Ng) was added to the cell lysate and the samples were vortexed, briefly spun down and incubated for 90 minutes at 4^0C. Immune complexes were further incubated in 70 µl of 15% Protein A sepharose at 4^0C for 90 minutes on rollers. The immunoprecipitation lysate was then spun for 30 secs at 14,000 X g and precipitates were washed four times in ROCK wash buffer (25 mM HEPES, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM dithiothreitol (DTT), 10% Glycerol, 0.1% NP-40). After final spin the buffer was carefully removed off the sepharose beads and the immunoprecipitated protein was now ready to use either separated by electrophoresis as described in Section 2.6 or used in kinase assay as described below (Section 2.8.3)

2.8.2 Autophosphorylation

For Western blotting 30 µl of Lamelli buffer was added to immunoprecipitated pellets, samples were boiled followed by a short spin and samples loaded on 10% acrylamide gels as described in Section 2.6. Nitro-cellulose membrane probed using anti-phospho tyrosine/serine/threonine antibodies to measure autophosphorylation of ROCK as an indication of its activation.

2.8.3 Kinase Assay

To measure the kinase activity of ROCK, an assay was developed based on the myelin basic protein (MBP) being phosphorylated by ROCK. An alternative substrate was

also used. ROCK is able to phosphorylate a Ser648 on Na^+H^+-exchange protein-1 (NHE-1). An oligopeptide substrate representing amino acid sequences 632-656 of NHE-1 sequence (RKILRNNLQKTRQRLRSYNRHTLVA called NHE-1 peptide) was kindly provided by Dr. L. L. Ng and used in this assay. After immunoprecipitating ROCK (Section 2.8.1) precipitates were resuspended in 40 µl ROCK assay buffer (25 mM HEPES, 10 mM $MGCl_2$, 10 mM ATP, 3 mM $MnCl_2$, 1 mM DTT, 1 mM Na_3VO_4, either 5 mM MBP or 0.2 mM NHE-1 peptide) was added to the samples. The reaction was started with the addition of 1 µl of assay buffer with 1 µCi/µl ^{32}P-ATP to the assay mixture and incubated for 30 min at 30°C. The reaction was stopped by the addition 20 µl of ice cold 20% TCA. The samples were spotted onto P81 phospho-cellulose squares and washed with 75 mM phosphoric acid 3 times for 10 minutes. The P81 squares were then added to scintillation vials with emulsifier safe scintillant and counted for [^{32}P].

2.8.4 Translocation

Translocation of ROCK to the membrane was also investigated. Stimulated cells were lysed in 100 µl ROCK hypotonic buffer (20 mM EPPS, 100 mM NaF, 100 mM $Na_4P_7O_2$, 5 mM Na_3VO_4, 1 mM Na_2MOO_4, 1 µM PMSF, 5 µg/ml leupeptin, 20 µg/ml aprotinin)and cell extracts were centrifuged at 8000g for 10minutes at 4°C. The nuclear fraction pellet produced was washed in 500 µl hypotonic buffer, twice and then resuspended in 25 µl lysis buffer. 5 µl was used in protein determination and 20 µl of 2 X Lamelli buffer was added to the remainder. The supernatant from the first spin was centrifuged at 100,000g for 30 mins at 4°C. The plasma membrane fraction pellet produced was washed twice in 500 µl hypotonic buffer and resuspended directly into Lamelli buffer as resuspension into any other buffer proved difficult. The supernatant from the final spin represented the soluble cytosolic fraction and equal volume of 2 times Lamelli buffer was added. All the samples were boiled, briefly spun and then separated on 10% acrylamide gels and nitro-cellulose probed with anti-ROCK antibody to determine if any translocation from different fractions was evident as an indication of ROCK activation upon stimulation.

2.9 Incorporation of [^{3}H] thymidine

Measuring the incorporation of [^{3}H] thymidine into newly formed DNA in S-phase of the cell cycle was used as an index of the rate of DNA synthesis and therefore the rate of mitogenesis.

Rat VSMCs were plated into 24 well muti-well plates and grown to 80% confluence. Media was aspirated from the wells and replaced with 0.5 ml serum free medium (DMEM containing 1 g/L glucose supplemented with 50 I.U./ml penicillin, 50 μg/ml streptomycin) for 24 hours at 37^{0}C. The cells were stimulated for 1 hour by addition of 100 μl of 6 times final concentration of required agonists at 37^{0}C. The stimulating media was then replaced with 0.5 ml serum free medium and left for a further 19 hours. Following this incubation, [^{3}H]-thymidine (1 μCi/ml final concentration) was then added to each of the wells and incubated for a further 4 hours at 37^{0}C. Plates were placed on ice and the medium was aspirated and cells washed sequentially for 5 minutes with two 1 ml washes with BSS, two 500 μl washes with 5% TCA and two 500 μl washes with ethanol. After the final ethanol wash the wells were aspirated and the plates were left to air dry. 500 μl of 0.5 M NaOH was added and the plates frozen and thawed. Samples were transferred to scintillation vials and 5 mls of emulsifier safe scintillation cocktail was added, vortexed and counted for ^{3}H.

For human VSMC, cells were plated in 96 well multi-well plates and 100 μl serum free medium was added for 48 hours when cells reached 80% confluence. 100 μl of 2 times final concentration of required agonists was used to stimulate the cells and after an hour the stimulating media was then replaced with 100 μl serum free medium for 19 hours. As above [^{3}H]-thymidine (1 μCi/ml final concentration) was then added and incubated for 4 hours. Plates were placed on ice and cells were washed sequentially for 5 minutes with two 200 μl washes with BSS, two 100 μl washes with 5% TCA and two 100 μl washes with ethanol. 100 μl of 0.5 M NaOH was added after drying the ethanol off and the procedure continued as described above.

2.10 Polymerase chain reaction (PCR)

RT-PCR was designed by Mullis & Fallona in 1987. This method isolates messenger RNA (mRNA) to determine which genes are transcribed from genomic DNA in a particular tissue or cell type. During transcription double-stranded DNA dissociates and mRNA is synthesised along one strand of the DNA, catalysed by RNA polymerase. The resulting single stranded mRNA then leaves the nucleus and its sequence is translated into a polypeptide chain by ribosomes in the cytosol. In RT-PCR, the mRNA that has been isolated from the cytosol is reverse transcribed into DNA using an enzyme called reverse transcriptase. This DNA is termed complimentary DNA (cDNA), as it is complimentary to the mRNA strand from which it was synthesised. Specific pairs of primers are designed using known sequences in the gene of interest and the cDNA between these primers is amplified using DNA polymerase (Taq polymerase). By repeating this process many times, the cDNA is amplified exponentially. The amplified cDNA can be visualized when subjected to electrophoresis on an agarose gel containing ethidium bromide. Ethidium bromide intercalates into the structure of DNA and fluoresences under ultra-violet illumination. Base pair markers are electrophoresed alongside cDNA on the gel to ensure the correct product is visualised.

2.10.1 Preparation

Cultured SHR and WKY VSMCs were grown to confluence in three large petri dishes (approximately $6x10^6$ cells with a total area of 190 cm^2) and washed with PBS three times. TRIzol reagent (a monophasic solution of phenol and guanidine isothiocyanate) was used to disrupt the cells and was an effective reagent for dissolving the cellular components whilst maintaining RNA integrity in the samples. An initial 2 mls was added to the first dish, the cells dislodged by scraping and samples pipetted up and down several times and then transferred to the next dish, scraped and then transferred to the last dish before pipetting into a sterilin tube. A second volume of 2 ml of Trizol was added to each plate as before to ensure all cells had been removed. Finally, the samples were passed through an 18 gauge needle to disrupt all the cells in preparation for RNA extraction.

2.10.2 RNA extraction

The solutions used for RNA extraction were diethylpyrocarbonate (DEPC)-treated water (0.01% w/v) to inhibit RNA degrading enzymes, known as ribonucleases (RNAases). The RNA was extracted using TRIzol reagent, following the supplier's instructions. Briefly, samples were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes and then isopropanol was added (0.2 ml per ml of TRIzol), tubes were shaken vigorously and then the samples were incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000 X g for 15 minutes at 4°C. The samples separated into three phases; the lower phenol/chloroform phase containing cellular debris, an interface phase of DNA and an upper aqueous phase containing RNA. The aqueous phase was therefore carefully removed and transferred to a fresh tube. RNA was then precipitated using isopropyl alcohol. 0.5 ml per ml of TRIzol originally used was added to samples, vortexed and then centrifuged at 12,000 X g for 5 minutes at 4°C. The supernatent was removed and the resulting RNA pellet was washed with 1 ml per ml of TRIzol of 70% ice-cold ethanol, vortexed and centrifuged at 7,500 X g for 5 minutes at 4°C. The ethanol was removed and the samples were air dried until the pellet turned transparent around the edges. The pellet was then resuspended in 100µl DEPC-treated water. The pellet was not allowed to dry out completely as this would decrease its solubility in this last step. Remaining traces of cellular DNA was removed by incubating the samples with 200 units DNase 1 for 15 min at 37°C. RNA was re-extracted with addition of 500 µl TRIzol, the procedure described above was repeated and the pellet was finally resuspended in 50 µl DEPC-treated water. RNA concentration was determined spectrophotometrically. Ideally the $OD_{260/280}$ of dissolved RNA is greater than 1.8. Contamination with protein or DNA would reduce this value. An RNA solution with an $OD_{260} = 1$ has an approximate concentration of 40 µg/ml RNA.

2.10.3 First-strand cDNA synthesis

First strand cDNA was synthesised using 5 ug RNA with 25 µg/ml oligo-$(dT)_{17}$ primer, 500 µM dNTPs (dATP, dCTP, dGTP and dTTP) and distilled water in a reaction volume of 26 µl. This was incubated for 5 minutes at 65°C, snap cooled on ice and then spun down. The following components were added to make a final volume of 40 µl; 10 µM dithiothreitol (DTT), 10 units/µl Superscript II RT, and 8 µl 5 x 1st strand buffer (25 mM Tris-Cl, 50 mM $MgCl_2$, 100 mM β- mercaptoethanol and 250 mM KCl, pH 8.3 at

Receptor	Primer	Sequence	Product length
Rat $P2Y_1$	Forward	5′-TGGTGGCCATCTCCCCTATTCTCTT-3′	595bp
	Reverse	5′-ATCTCGTGCCTTCACAAACTC-3′	
Rat $P2Y_2$	Forward	5′-TTCCACGTCACCCGCACCCTCTATTACT-3′	539bp
	Reverse	5′-CGATTCCCCAACTCACACATACAAATGATTG-3′	
Rat $P2Y_4$	Forward	5′-CTTCTCTGCCTGGGTGTTTGGTTGGTAGTA-3′	474bp
	Reverse	5′-TCCCCCGTGAAGAGATAGAGCACTGGA-3′	
Rat $P2Y_6$	Forward	5′-GCCAGTTATGGAGCGGGACAATGG-3′	352bp
	Reverse	5′-AGGAACAGGATGCTGCCGTGTAGGTTG-3′	
Rat $P2Y_{12}$	Forward	5′-ATCTGGGCCTTCATGTTCCTGCTGTC-3′	532bp
	Reverse	5′-GTTTCGTTGCCAAAGCCCTCGGTGCTCTC-3′	

Table 2.1 P2Y receptor primer sequences

42°C). Reaction tubes were then incubated at 42°C for 1 hour, 70°C for 15 minutes and finally 5°C for 5 minutes. Reactions containing no RT were also included in 1st strand synthesis step and run parallel with sample containing RT. This acted as a control for the detection of contaminating genomic DNA in the RNA samples.

2.10.4 PCR reaction

Forward and reverse primers designed specifically for rat P2Y receptors were used to amplify cDNA. These are shown in Table 2.2. Primers for a house keeping gene GAP, which is expressed in both cell types were also used. 1-3 µl of RT reaction mixture was used in a PCR reaction volume of 50 µl, which included forward and reverse primers (each at 0.3 pmol/µl), 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.05 units/µl Bio-Taq, 5 µl 10 x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% w/v gelatine) and water. Regions of each receptor were amplified using the following profile: 5 minutes at 94°C to allow template to denature, followed by 35 cycles of 45 seconds at 94°C, 30 seconds at annealing temperature of 65°C, 45 seconds at 72°C and a final extension step of 7 minutes at 72°C. PCR reaction with no cDNA was also carried out to detect for any contaminating DNA in the PCR reaction. Products were separated by electrophoresis through a 2% (w/v)

agarose gel containing ethidium bromide (0.5 mg/l) in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) and bands were visualised and captured under UV light.

2.11 Materials

All tissue culture medium and plastic-ware were supplied by Gibco. Nucleotides were supplied by Sigma and RBI-Sigma for 2MeSATP. PD98059, U0126, SB203580 and LY294002 were supplied by Tocris. Y27632 was obtained from Yoshitomi Pharmaceutical Industries, Osaka, Japan. Radioactive isotopes [^{3}H] thymidine, [^{3}H] inositol and γ [^{32}P]-ATP were supplied from Amersham Life Sciences. Glutathione sepharose was obtained from Amersham-Pharmacia. Equipment for SDS PAGE and blotting were supplied by Biorad, Hert., U.K. Equipment for gel documentation system was supplied Syngene, Synoptics Ltd., Cambridge. Polyacrylamide (ratio 37.5:1) was supplied by Anachem. Anti active-p44/p42 MAPK and anti-active JNK primary polyclonal antibodies were supplied from Promega, USA. Phospho-p38 antibody was supplied by New England Biolabs. JNK1 and p38 MAPK antisera were obtained from Santa Cruz, U.S.A. Anti-mouse and anti-rabbit IgG HRP secondary antibody were supplied by Sigma. Autoradiography film and ECL reagent was supplied by Amersham Life Sciences. TRIzol reagent was obtained from Gibco BRL. Most other chemicals and general reagents were supplied by Sigma or Fisher Scientific.

2.12 Statistical Analysis of Data

Results are expressed, as means $\pm$ standard error of the mean (S.E.M.) using Graph Pad Prism 3.0. Generation of sigmoidal concentration-response curves was also produced by this software using the following equation which assumes a standard slope factor (or Hill slope) of 1.0;

$$Y = \text{bottom} + \frac{(\text{top-bottom})}{1+10^{\log EC50-X}}$$

The log EC_{50} was also calculated and is stated as the negative log EC_{50} (pEC_{50}). Statistical analysis was performed using the appropriate statistical tests such as Students t test, one way or two way ANOVA with Dunnet's or Bonferroni's post-test on the original raw data including those figures plotted as normalised data. P values of less than 0.05 were considered to be significant.

Chapter 3

PDGF & P2Y receptor mitogenic signalling in human VSMCs

3.1 Introduction

The saphenous vein (SV) is used to replace diseased coronary arteries in coronary bypass. However, a common drawback to this procedure following surgery is restenosis. This involves the proliferation of SV smooth muscle, resulting in occlusion of the grafted vessel. Healthy vasculature retains an intact endothelium, which provides protection and anti-proliferative influences on VSMCs and attenuates platelet activity. However, this is compromised in vascular diseases or damage as a result of surgery and as a result the activation of platelets is enhanced. PDGF is released from platelets and acts on both the endothelium and VSMCs. PDGF, a potent mitogen, increases proliferation in VSMCs derived from SV (Bornfeldt *et al.*, 1997, Yang *et al.*, 1998) and thus may play an important role in intimal thickening in atherosclerosis and restenosis. Extracellular nucleotides are also implicated in this process. They may come from a variety of sources including local nerve terminals and platelets. ATP is a co-transmitter released with noradrenaline from sympathetic nerves causing vasoconstriction, primarily by increasing intracellular calcium concentrations through P2X intrinsic ion channels in VSMC (Burnstock 1990, Brizzolora & Burnstock 1991). However when the extracellular concentrations of ATP increase, due to release by platelets, endothelial cells or damaged cells (Gordon 1986, Goetz *et al* 1971), vasodilatation, proliferation and hypertrophy may be induced through P2Y receptors (reviewed in Boarder & Hourani, 1998 and Kunapuli & Daniel, 1998). Nucleotides have been shown to stimulate the proliferation of human VSMCS (reviewed in Erlinge 1998) as well as animal VSMCs (Erlinge *et al.*, 1993, Harper *et al.*, 1998, Malam- Souley *et al.*, 1993, Wang *et al.*, 1992). Therefore nucleotide signalling, through P2Y receptors, is important in the regulation of proliferation of VSMCs. It would be of great clinical importance to understand the role of nucleotides in atherosclerosis and restenosis. This may lead to improved pharmacological intervention. The aim of this chapter was to further investigate the role of P2Y receptors on human SV VSMCs in the control of proliferation.

3.2 PDGF induced DNA synthesis

Human SV VSMCs were grown to near confluence (80-90%) at early passage. Cells were serum starved for 24 hours prior to stimulation with a range of PDGF-BB concentrations (100 pM – 30 nM) for 1 hour. Stimulating agonists were then removed.

Following incubation for a further 19 hours in serum free medium, [^{3}H]-thymidine was added for 4 hours and incorporation of [^{3}H]-thymidine was then measured as an index of mitogenesis (Section 2.9). The results are shown in Figure 3.1 and were collected from three separate experiments, each performed in quadruplicate and the concentration response curve was constructed with data plotted as percentage of basal stimulation. DNA synthesis increased dose dependently with increasing PDGF concentration with an EC_{50} of 3.5 ± 2.95 nM ($P<0.0001$, $n = 3$, one way ANOVA). 1 nM PDGF significantly induced a response which was 267.8 ± 8.34% of basal, ($P<0.05$) and 3 nM PDGF produced a response which was 578 ± 90.6% of basal ($P<0.01$). Concentration of 1 nM was used for further experiments (unless otherwise stated), as this was cost effective.

3.3 The effects of nucleotides on DNA synthesis

ATP, UTP and UDP were initially used to see if they influenced DNA synthesis. Cells were grown and serum starved as above and stimulated for 1 hour with 300 μM nucleotides either alone or in the presence of 1 nM PDGF. The results are shown in Figure 3.2. Alone, ATP, UTP and UDP at 300 μM did not significantly influence DNA synthesis. However, in the presence of PDGF, ATP significantly potentiated the PDGF-induced response (260.34 ± 73.4% of the PDGF response, $P<0.01$, one way ANOVA). Unexpectedly, UTP and UDP both attenuated the PDGF-induced DNA synthesis (43.8 ± 7.0 and 36.3 ± 3.28 % of PDGF response respectively, both $P<0.01$). Approximately 90% of all the cell lines from different patients displayed inhibitory responses to UTP and approximately 50% of cell lines displayed proliferative properties to ATP. To establish if these nucleotides were acting directly or whether their break down products were stimulating these response similar experiments were performed by Pam White, using HPLC purified nucleotides and nucleotide regeneration systems (see White *et al.*, 2000). Creatine phosphokinase was used to regenerate tri-phosphates such as ATP and UTP and prevent the accumulation of di-phosphates such as ADP and UDP. Hexokinase was used to regenerate UDP and prevent the accumulation of UTP. The results produced were found to be similar to those shown in Figure 3.2. This suggests that the responses produced by ATP, UTP and UDP were not as a result of their metabolised products, such as ADP, UDP or UTP respectively. This was consistent with cell number studies, where UTP was found to inhibit PDGF-induced increases in cell number (White *et al.*, 2000).

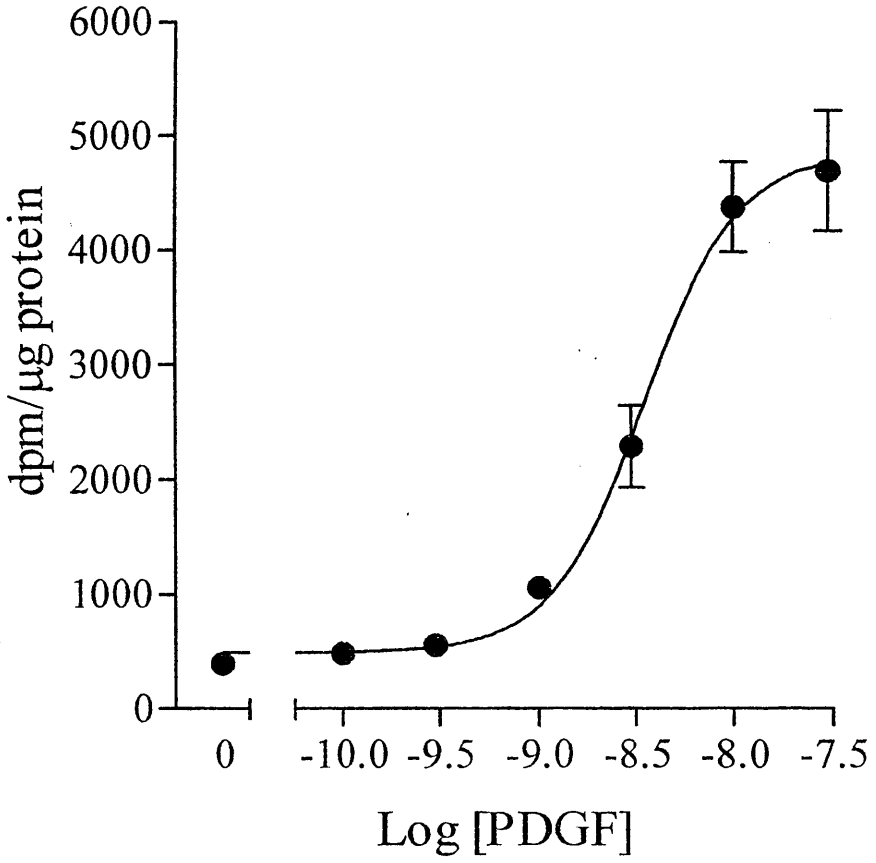


Figure 3.1 DNA synthesis PDGF concentration response curve

[^{3}H] Thymidine incorporation as a measure of DNA synthesis stimulated for one hour with a PDGF concentration range of 100 pM to 30 nM. Data presented as dpm per µg protein ± S.E.M. from three experiments each performed in quadruplicate. Data collected in collaboration with P.White.

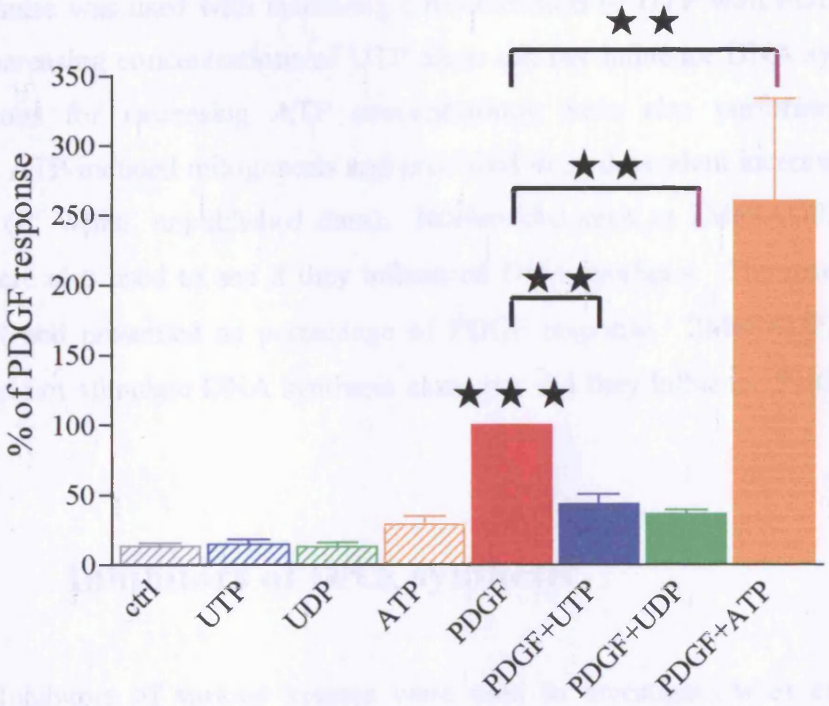


Figure 3.2 DNA synthesis in human VSMCs

[^{3}H] thymidine incorporation into DNA in human SV VSMCs stimulated with 1nM PDGF (red) with and without 300μM ATP (orange), UTP (blue) or UDP (green). Data presented as percentage of the PDGF stimulated response ± S.E.M. from 4 separate experiments (3 experiments for ATP ± PDGF) each carried out in triplicates. Statistics were performed using one way ANOVA followed by Bonferroni's multiple comparison test on raw data (★★★P<0.001, ★★P<0.01).

Next increasing concentrations of UTP (1 – 300 μM) were used to investigate the dose dependent relationship between UTP inhibition and PDGF-induced DNA synthesis response on cell lines showing UTP-induced inhibition. The results are shown in Figure 3.3. UTP significantly inhibited PDGF-induced DNA synthesis (pIC_{50} = 4.70 ± 0.38 [IC_{50} = 20.0 μM] $P<0.001$, one way ANOVA). Similar results were produced when creatine phosphokinase was used with increasing concentrations of UTP with PDGF (White *et al.*, 2000). Increasing concentrations of UTP alone did not influence DNA synthesis. Further investigations for increasing ATP concentrations were also performed on cell lines displaying ATP-induced mitogenesis and produced dose dependent increase in the presence of PDGF (P. White, unpublished data). Nucleotides such as 2MeSADP, 2MeSATP and ATPγS were also used to see if they influenced DNA synthesis. The results are shown in Figure 3.4 and presented as percentage of PDGF response. 2MeSADP, 2MeSATP and ATPγS did not stimulate DNA synthesis alone nor did they influence PDGF-induced DNA synthesis.

3.4 Inhibitors of DNA synthesis

Inhibitors of various kinases were used to investigate what effects on PDGF-induced DNA synthesis would occur if they were blocked. The MAPKs are important components of the proliferative pathway. The MAPKs include ERK, JNK and p38. ERK is phosphorylated by its upstream kinase MEK, which can be inhibited using PD98059 and U0126 (Dudley *et al.*, 1995, Favata *et al.*, 1998, Davies *et al.*, 2000). SB203580 specifically inhibits p38 MAPK (Cuenda *et al.*, 1995, Davies *et al.*, 2000). PI3-kinase, which is activated by PDGF receptor (Valius & Kazlauskas, 1993) is able to activate Ras, the primary stimulant for the MAPK pathways and can be blocked with LY294002 (Vlahos *et al.*, 1994). ROCK is activated by Rho (Uehata *et al.*, 1997), which is also a substrate of Ras. Y27632 is a specific inhibitor of ROCK (Uehata *et al.*, 1997) and was also used.

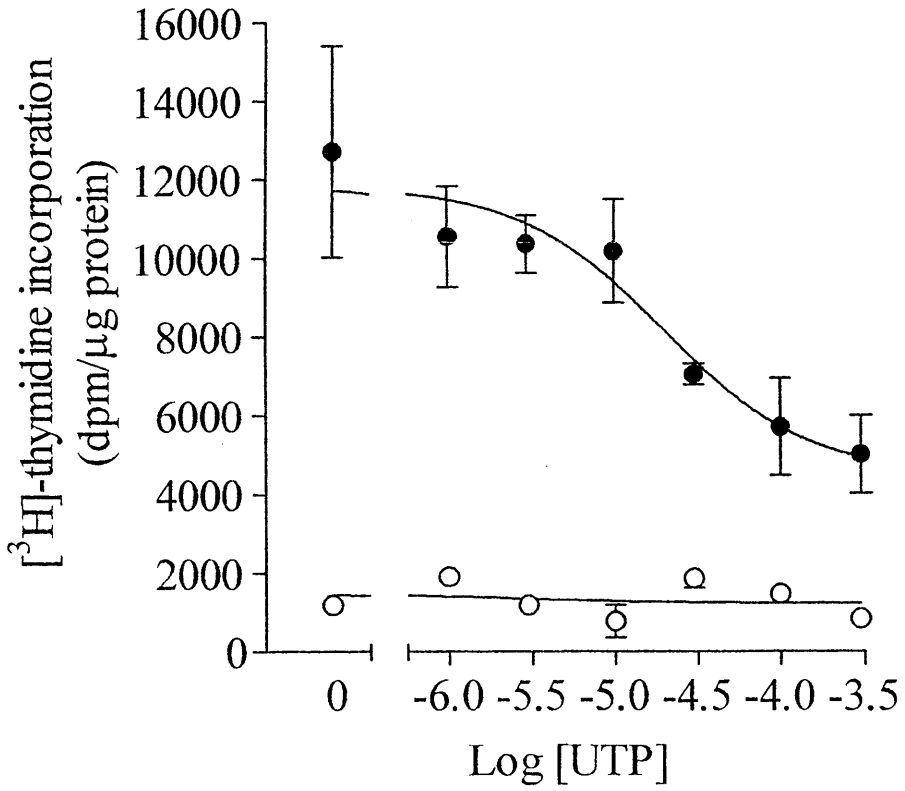


Figure 3.3 Effects of increasing concentration of UTP on DNA synthesis

The effects of UTP concentration response curve (1-300 μM) on [^{3}H]-thymidine incorporation stimulated with (•) and without (○) 1 nM PDGF. Data presented as dpm/μg protein ± S.E.M. from 3 separate experiments each carried out in triplicates. The effects of UTP were significant $P<0.001$ (one way ANOVA).

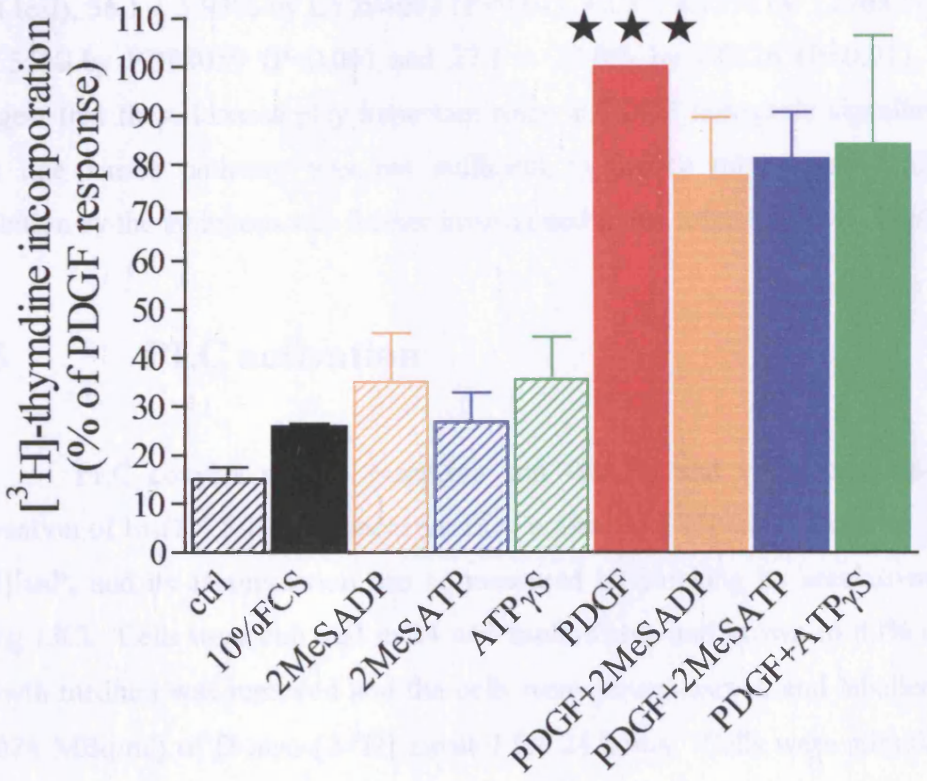


Figure 3.4 DNA synthesis in human VSMCs

[^{3}H] thymidine incorporation into DNA in human SV VSMCs stimulated with 300 μM 2MeSADP (orange), 2MeSATP (blue), ATPγS (green) and 1 nM PDGF (red). Block colours represent agonists in the presence of 1 nM PDGF and slashed bars represent agonists in the absence of PDGF. Data presented as percentage of the PDGF stimulated response ± S.E.M. from 3 separate experiments each carried out in triplicates. Statistics were performed using one way ANOVA followed by Dunnett's multiple comparison test on raw data (★★★$P<0.001$).

Figure 3.5 shows the effects of 1 μM SB203580, 1 μM LY294002, 30 μM PD98059, 10 μM U0126 and 30 μM Y27632 in the presence and absence of 1 nM PDGF and normalised to maximal PDGF response. These inhibitors had no significant influence on basal levels of ERK phosphorylation. PDGF significantly stimulated DNA synthesis as described before ($P<0.001$). All the inhibitors used significantly inhibited this PDGF-induced DNA synthesis. The responses were reduced to 23.9 ± 8.32 % of the PDGF response by SB203580 ($P<0.01$ One-way ANOVA with Dunnett's multiple comparison post test), 36.1 ± 5.93% by LY294002 ($P<0.01$), 42.5 ± 4.93% by Y27632 ($P<0.01$), 44.43 ± 7.35% by PD98059 ($P<0.05$) and 27.1 ± 12.0% by U0126 ($P<0.01$). These results suggest that these kinases play important roles in PDGF mitogenic signalling pathway and that one kinase pathway was not sufficient to induce mitogenesis. Dose dependent inhibition by the inhibitors was further investigated in the following two chapters.

3.5 PLC activation

PLC couples to TK receptors and GPCRs and when activated catalyses the formation of $Ins(1,4,5)P_3$. To measure PLC activation [^{3}H] inositol can be used to produce [^{3}H]$InsP_x$ and its accumulation can be measured by blocking its breakdown and recycling using LiCl. Cells were cultured in 24 well multi-dishes and grown to 80% confluence. The growth medium was removed and the cells were serum-starved and labelled with 1 μCi/ml (0.074 MBq/ml) of D-myo-[2-^{3}H] inositol for 24 hours. Cells were stimulated with 1 nM PDGF, 300 μM UTP or ATP or co-stimulation with PDGF plus UTP or ATP for 20 minutes in the presence of 10 mM LiCl. The reaction was stopped using 1 M TCA and total cellular inositol phosphates were extracted using tri-*n*-octylamine/1.1.2-trichloro-trifluoroethane and separated as described in Section 2.2. The results are shown in Figure 3.6 and presented as the percentage of the basal response. These results were collected in cooperation with Pam White. PDGF significantly increased PLC activity (266.9 ± 51.5% of basal, $P<0.01$, one way ANOVA), however nucleotides alone or in the presence of PDGF generated no response. In parallel studies the formation of mass $Ins(1,4,5)P_3$ was also found not to be stimulated with nucleotides (White *et al.*, 2000)

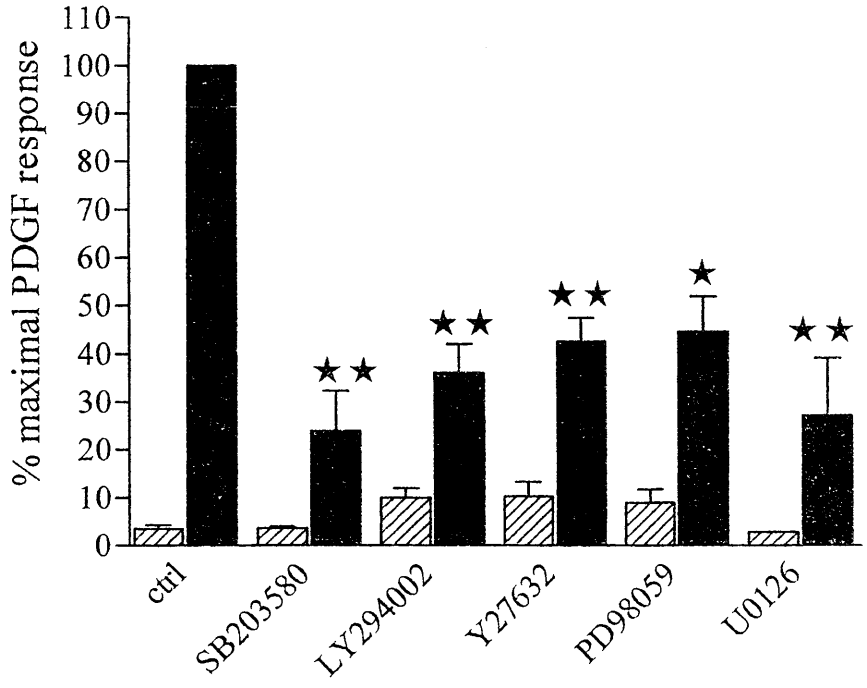


Figure 3.5 The effects of various inhibitors on DNA synthesis

Basal (hatched) and 1 nM PDGF (solid bars) stimulated [^{3}H] thymidine incorporation with 1 μM SB2035880, 1 μM LY294002, 30 μM Y27632, 30 μM PD98059 and 10 μM U0126. Data presented as percentage of the PDGF stimulated response ± S.E.M. from 3 separate experiments each carried out in triplicates. Statistics were performed using one way ANOVA followed by Dunnett's multiple comparison test on raw data (★P<0.05, ★★P<0.01).

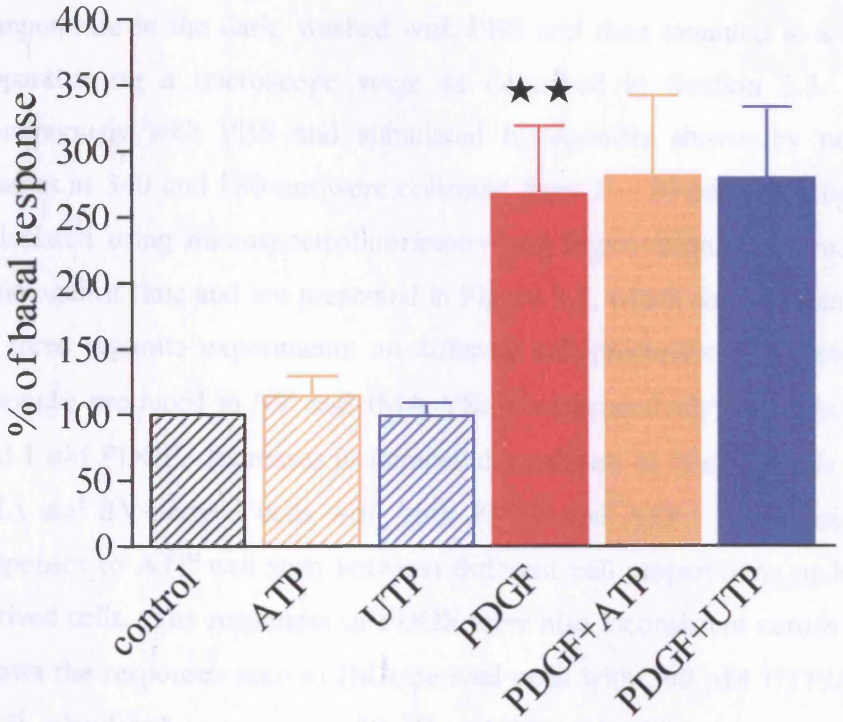


Figure 3.6 PLC activation by PDGF in human VSMCs

The effects of 20 minutes stimulation with 300 μM ATP (orange) or UTP (blue) in the absence (hatched) and presence (solid bars) of 1 nM PDGF (red) on PLC activity. Data presented as percentage of the basal stimulated response ± S.E.M. from 3 separate experiments each carried out in quadruplicates. Statistics were performed using one way ANOVA followed by Dunnet's multiple comparison test on raw data (★★$P<0.01$). Data collected in collaboration with P. White.

3.6 Intracellular calcium

Cells were cultured on 22mm coverslips and serum starved before they reached confluence for 24 hours. They were loaded with Fura 2-AM for 30 minutes at room temperature in the dark, washed with PBS and then mounted in a multi-barallel perfusion apparatus on a microscope stage as described in Section 2.3. Cells were perfused continuously with PBS and stimulated by agonists shown by perfusate for 1 minute. Images at 340 and 380 nm were collected from 8 – 20 cells and then 340/380 ratios were calculated using microspectrofluorimetry and Improvision software. Data was plotted as ratio against time and are presented in Figure 3.7, which show traces that are representative of three separate experiments on different cell preparations. Traces A and B show the response produced in SV and IMA VSMCs (respectively), stimulated with 300 μM ATP and 1 nM PDGF. Increases in intracellular calcium or $[Ca^{2+}]_i$ levels were observed in both IMA and SV-derived cells with both PDGF and ATP. No consistent difference in the responses to ATP was seen between different cell preparations or between IMA and SV-derived cells. The responses to PDGF were also inconsistent across experiments. Trace C shows the responses seen in IMA-derived cells with 300 μM UTP/ATP and 1 nM PDGF. UTP stimulated increases in $[Ca^{2+}]_i$. UTP and ATP responses showed no consistent difference across experiments. No differences in the UTP response between IMA and SV-derived cells was seen (P. White, unpublished data) and no differences between ATP and UTP dose dependent $[Ca^{2+}]_i$ increase was observed in SV VSMCs. ATP and UTP stimulation was sustained in the absence of extracellular Ca^{2+}, which was removed using EGTA (White *et al*, 2000). The P2X agonist α,β-methylene-ATP was also found not to raise $[Ca^{2+}]_i$.

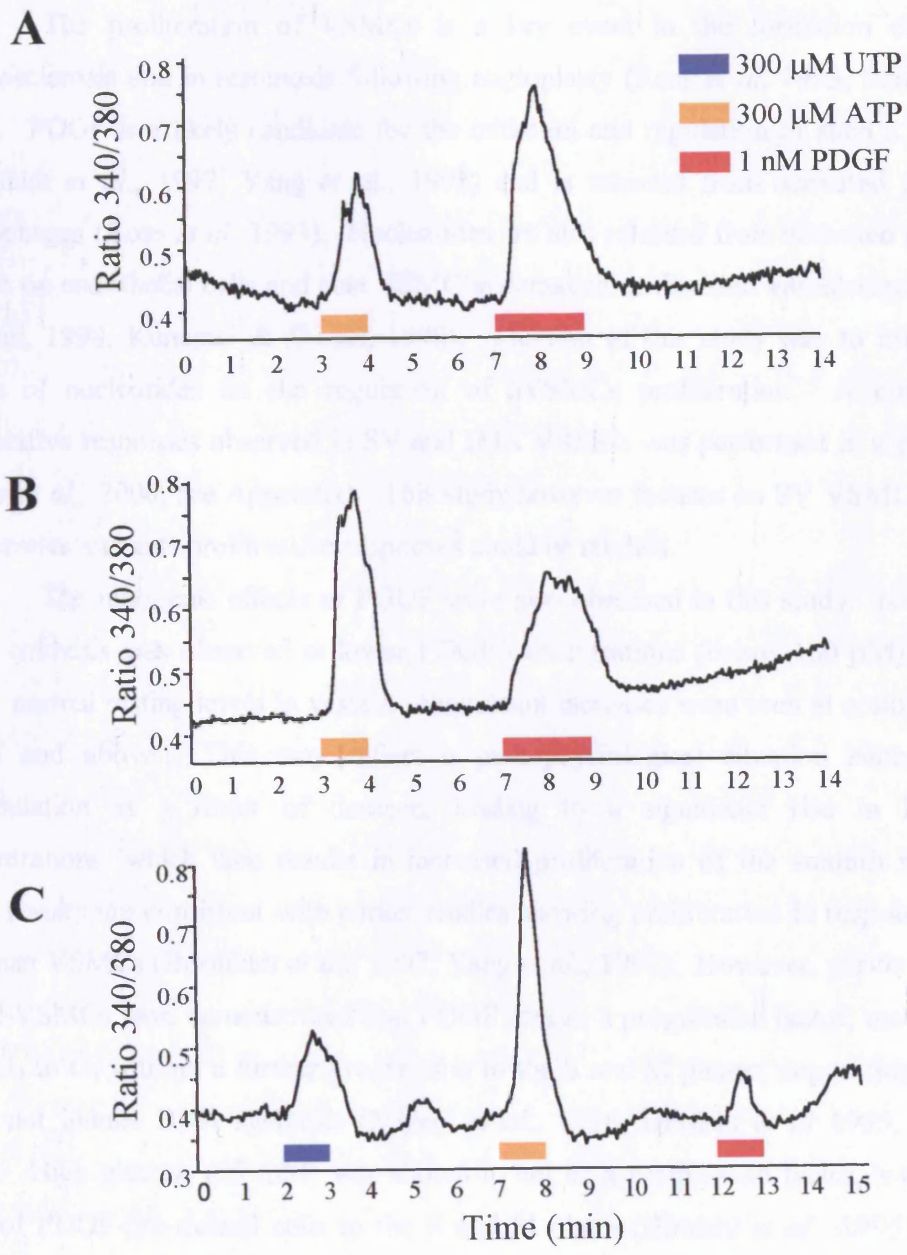


Figure 3.7 Intracellular calcium responses

$[Ca^{2+}]_i$ responses to 1 nM PDGF (red), 300 μM ATP (orange) and 300 μM UTP (blue) in SV (**A**) and IMA (**B** & **C**) VSMCs. Data presented as the averaged 340/380 ratio from 8 - 20 serum starved human VSMCs and is representative of 3 separate experiments carried out at least 3 times on different cell preparations.

3.7 Discussion

The proliferation of VSMCs is a key event in the formation of lesions of artherosclerosis and in restenosis following angioplasty (Ross *et al*, 1993, Schwartz *et al.*, 1995). PDGF is a likely candidate for the initiation and regulation of such a proliferation (Bornfeldt *et al.*, 1997, Yang *et al.*, 1998) and is released from activated platelets and macrophages (Ross *et al*, 1993). Nucleotides are also released from activated platelets and can act on endothelial cells and also VSMC in damaged or diseased vasculature (Boarder & Hourani, 1998, Kunapuli & Daniel, 1998). The aim of this study was to investigate the effects of nucleotides on the regulation of hVSMCs proliferation. A comparison of proliferative responses observed in SV and IMA VSMCs was performed in a parallel study (White *et al.*, 2000, see Appendix). This study however focuses on SV VSMCs since both proliferative and anti-proliferative responses could be studied.

The mitogenic effects of PGDF were also observed in this study. No increase in DNA synthesis was observed at lower PDGF concentrations (below 100 pM), which may reflect normal resting levels in vessels. Significant increases were seen at concentrations of 1 nM and above. This may reflect a pathophysiological situation such as platelet degranulation as a result of damage, leading to a significant rise in local PDGF concentrations, which then results in increased proliferation of the smooth muscle cells. These results are consistent with earlier studies showing proliferation in response to PDGF in human VSMCs (Bornfeldt *et al.*, 1997, Yang *et al.*, 1998). However, previous studies in animal VSMCs have demonstrated that PDGF acts as a progression factor, mediating entry from G_0 to G_1 without a further progression to the S and M phases, suggesting that PDGF could not induce DNA synthesis (Miyagi *et al.*, 1996, Hiroishi *et al* 1995, Jahan *et al* 1996). High glucose (25 mM) was shown to act as a progression factor, which allowed entry of PDGF-pre-treated cells to the S and M phase (Hiroishi *et al.*, 1995). Thus the ability of PDGF to induce progression of the cell cycle through to DNA synthesis was dependent on the actions of high glucose. In our laboratory, SV VSMCs were grown and quiesced in medium containing low glucose (5.5 mM). It may be possible that this progression of the cell cycle may have been aided by the presence of glucose.

The mitogenic pathway is a complex network of protein kinases and cascades. The stimulation of growth factor receptors has been found to recruit many kinases to produce the final outcome. These kinases may include ERK, p38 (both MAPKs), P13

kinase and p160 ROCK. A role for these in PDGF-stimulated proliferation of human SV-VSMCs was suggested by using their respective inhibitors to examine their effects on PDGF-induced DNA synthesis. All inhibitors were found to attenuate DNA synthesis at the concentrations used. This may imply that the PDGF mitogenic response is dependent on these kinases. Further experiments using a range of inhibitor concentrations were used to verify this and are shown in the following chapters. ERK has previously been implicated in the PDGF-induced mitogenic pathway (Bornfeldt *et al.*, 1997, Yang *et al.*, 1998, Graves *et al.*, 1996). P13kinase has previously been suggested to play a role in proliferation (Valius & Kazlauskas 1993, Fantl *et al.*, 1992). Other groups have shown inhibition of proliferation using the ROCK inhibitor in VSMCs (Seasholtz *et al.*, 1999, Sawada *et al.*, 2000). However, p38 at present has not been shown to be involved in proliferation, but does play a role in cardiac hypertrophy (Wang *et al.*, 1998).

The importance of P2Y receptors in regulation of proliferation has been suggested in rat VSMCs (Erlinge *et al.*, 1993, Harper *et al.*, 1998), porcine VSMC (Wilden *et al.*, 1998, Wang *et al.*, 1992, Crowley *et al.*, 1994). Erlinge *et al.* (1994) demonstrated for the first time that human VSMC proliferation was also stimulated by ATP. However in our study, nucleotides including ATP, UTP, UTP, 2MeSADP, 2MeSATP and ATPγS were unable to stimulate incorporation of [^{3}H]-thymidine above control levels. The mitogenic capabilities of nucleotides can be debated. In the previous studies in both human and rat VSMCs quiescence was induced by incubating VSMCs for 24 – 48 hours in 0.1 - 0.5% FCS (Erlinge *et al.*, 1993, Erlinge *et al.*, 1994, Wang *et al.*, 1992; Wilden *et al.*, 1998, Crowley *et al.*, 1994). This may not have been sufficient to bring the majority of cells into G_0. In our study, however, cells were brought into G_0 through serum starvation. Results from the our lab (personal communication with Pam White) showed that cells incubated with 0.4% serum for 24 and 48 hours did not induce quiescence, but increased [^{3}H]-thymidine incorporation above control (no serum). This is further supported by studies of cell cycle progression that demonstrated that nucleotides by themselves failed to stimulate the cell cycle beyond G_1 phase (Miyagi *et al.*, 1996). Therefore, it is most likely that the documented ATP and UTP mitogenic responses may in fact be due, in part, to the presence of FCS during 'quiescence'. It is likely that the low levels of FCS in studies by others acted as a progression factors required in the cell cycle enabling ATP to induce progression through to the S and M phase. Our group has previously reported the mitogenic effects of ATP and UTP in cultured rat VSMCs recorded under total serum deprivation (Harper *et al.*,

1998, also further investigated in latter chapter). To ensure the majority of human VSMCs were quiesced in this study, the cells were serum starved for 48 hours in low glucose.

This study also investigates the mitogenic effect of nucleotides in association with PDGF in SV derived VSMC. ATP significantly increased PDGF induced mitogenesis despite being unable to stimulate DNA synthesis alone in 50% of cell lines used. This significant synergistic effect with PDGF has not previously been reported in human VSMCs (not seen in IMA VSMCs – see White *et al.,* 2000) and was not due to the breakdown of ATP to ADP. In porcine aortic smooth muscle cells ATP synergistically increased DNA synthesis with PDGF IGF-1 and EGF (Wang *et al.*, 1992). ATP has also been reported to increase the mitogenic response produced by its sympathetic co-transmitters neuropeptide Y in human VSMCs (Erlinge *et al.,* 1994). Interestingly, ATP is released from activated platelets at the site of vascular injury therefore together with PDGF it may have an important role in the development of pathological conditions such as restenosis following angioplasty *in vivo* (Ross *et al.,* 1993). This data also supports the previous suggestion that nucleotides only move the cell cycle from G_0 to G_1 and require the presence of a progression factor, such as PDGF, to allow DNA synthesis and subsequent cell division to occur (Miyagi *et al.,* 1996, Malam-Souley *et al.*, 1996).

UTP, like ATP, has been shown to be mitogenic (Erlinge *et al.*, 1993, Harper *et al.*, 1998, Satterwhite *et al.*, 1999). An unexpected finding of this study was that both UTP and UDP did not stimulate proliferation or potentiate PDGF-induced response but significantly inhibited the PDGF mediated increase in DNA synthesis in 90% of the cell lines used. UTP also inhibited cell number increases induced by PDGF (White et al, 2000 – inhibition also seen in IMA VSMCs). A concentration response relationship could not be fully established for UTP inhibition of PDGF, however increasing concentrations of UTP alone did not influence basal levels of DNA synthesis. Data from our lab (Roberts *et al.,* 1999) also demonstrated that the transfected human $P2Y_4$ expressed in 1321N1 stimulated by UTP attenuated PDGF mediated increases in DNA synthesis. Other reports in endothelial–derived cells show UTP alone to be antiproliferative (Lemmens *et al.*, 1996). However, the UDP inhibitory response has not been previously reported

Difficulties in receptor characterisation may prove complicated due to interconversion and receptor subtype agonist potency. Nucleotides are easily broken down or inter-converted on the cell surface (Gordon 1986). The commercial supplies of trinucleotides can also be contaminated with small quantities of breakdown products. To avoid mis-interpretation of results nucleotides were HPLC purified to remove contaminants

and experiments were also performed in the presence of regeneration systems such as creatine phosphokinase (White *et al.*, 2000) to regenerate tri-phosphates and hexokinase to regenerate di-phosphates (P. White, unpublished). The UTP response was found not to be dependent its breakdown by ectonucleotidases and the UDP anti-proliferative response was also found not to be dependent on the conversion of UDP to UTP. Taken together with transfected studies, these findings suggest the hypothesis that the inhibitory responses mediated by UTP were via the $P2Y_4$ receptor. UDP is most potent at the $P2Y_6$ receptor, but this subtype was not involved in the mitogenic response mediated by PDGF in studies with transfected receptors (Roberts *et al.*, 1999). This may indicate some differences in signalling of the same P2Y receptors in different cell types. The $P2Y_6$ receptor is the most likely receptor of those currently cloned to mediate UDP responses. Hou *et al.* (2002) have recently suggested that UDP acts on $P2Y_6$ in rat aortic SMCs to stimulate mitogenesis. Others have suggested that a receptor responding to both UTP and UDP may exist (Sak *et al.*, 2001). This proposed pyrimidine receptor is uncloned, but could contribute to the present observations.

$P2Y_1$ and/or $P2Y_2$ receptor subtypes expressed on VSMC have been implicated as possible candidates for ATP-induced mitogenesis (reviewed in Erlinge 1998). Studies using transfected human P2Y subtypes in 1321N1 human astrocytoma cells suggested that $P2Y_1$ was coupled to enhanced DNA synthesis alone and in concert with PDGF (Roberts *et al.*, 1999). Since 2MesADP did not elicit an increase in [^{3}H]-thymidine incorporation, it is unlikely that $P2Y_1$ receptors mediate ATP-induced mitogenesis in hSV VSMCs. Also suramin and PPADS, which are antagonists at $P2Y_1$, did not influence ATP responses (White *et al.*, 2000). $P2Y_2$ subtypes can also be ruled out since both ATP and UTP are equipotent at this receptor but were found to have opposing effects on DNA synthesis. There is no evidence to support that two different agonists acting at the same receptor can have opposite effects. Transfected $P2Y_2$ had no mitogenic effects either (Roberst *et al.*, 1999). ATP is a potent agonist at $P2Y_{11}$, however ATPγS, which is more potent than ATP and 2MeSATP did not stimulate similar responses, thus ATP responses are not mediated via $P2Y_{11}$ receptor. Consistent with this was the effects of suramin, an antagonist at $P2Y_{11}$, which did not attenuate ATP-induced increases in DNA synthesis (White *et al.*, 2000). The ATP-stimulated receptor that mediates proliferative response remains to be elucidated. Recently, Yoshioka *et al.* (2001) demonstrated heteromerisation between $G_{i/o}$ protein-coupled A_1 adenosine receptor and G_q protein-coupled $P2Y_1$ receptor when co-transfected

in HEK293T cells and as a result changes in ligand binding properties and functional changes were observed. Although it remains unclear whether this occurs in living organisms as opposed to overexpression experiments, it suggests a possible mechanism to explain undefined physiological functions of purines. The ATP mitogenic responses observed in this study in hVSMCs may be a result of P2Y receptor heterodimerisation.

The mitogenic synergy between PDGF and ATP may be due to up-regulation of P2Y receptors in response to PDGF. In rat VSMCs the up-regulation of expression of P2Y receptors was stimulated by growth factors such as basic fibroblast growth factor (bFGF) and also by ATP itself (Hou *et al.,* 1999). The up-regulation of the $P2Y_2$ receptor has also been demonstrated in the neointima *in vivo* following experimental balloon angioplasty (Seye *et al.,* 1997) in which growth factors play an important role. PDGF may therefore enhance proliferation by differentially up-regulating the number of P2Y receptors expressed. This together with ATP acting as a competence factor, (Miyagi *et al.,* 1996) may be a possible mechanism by which ATP is able to enhance the response in a synergistic manner to above those levels stimulated by PDGF. On going studies in our lab are investigating the up-regulation of P2Y receptors mRNA expression using semi-quantitative RT-PCR in human VSMCs (Webb *et al.,* 2000). In human SV tissue $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptor subtypes mRNA levels are expressed. In SV VSMC in culture $P2Y_4$ and $P2Y_{11}$ receptor subtypes are up-regulated compared to the tissue from the SV. The expression of $P2Y_{11}$ mRNA is most abundant.

The presence of P2Y receptors was also confirmed by increases in $[Ca^{2+}]_i$ in response to ATP and UTP. This method of measuring calcium increases could not identify which receptor-subtypes were present but did confirm that the increases were P2X independent. UTP is not an agonist for P2X receptor therefore an increase in calcium by this nucleotide could only be possible via a P2Y receptor. In the absence of extracellular calcium nucleotides were still able to stimulate increases in $[Ca^{2+}]i$, which could only occur via release from internal stores rather than influx through ion channels such as P2X receptors. The inability of α,β-methylene-ATP to stimulate increases $[Ca^{2+}]_i$ also verified the absence of certain P2X receptor subtypes in these cells. This supports previous reports of down-regulation of P2X receptors in culture and a transition from contractile to synthetic phenotypes (Pacaud *et al.*, 1995, Erlinge *et al.,* 1998, Kitajima *et al.*, 1994).

Cloned G-protein-coupled P2Y receptors have been shown to couple to the activation of PLC via G_q proteins. This results in the elevation of the second messenger

$Ins(1,4,5)P_3$ and the subsequent release of Ca^{2+} from intracellular stores. ATP elevations of $Ins(1,4,5)P_3$ has been implicated in the stimulation of mitogenesis in VSMC (Erlinge *et al.*, 1995; Yu *et al.*, 1996). Initial experiments to measure the accumulation of total inositol phosphates following stimulation by ATP and UTP in the presence and absence of PDGF proved difficult. Subsequent experiments using the $Ins(1,4,5)P_3$ mass assay (Challis *et al.*, 1990) were unable to detect production of $Ins(1,4,5)P_3$ following stimulation by nucleotides (White *et al.*, 2000). Despite this, the elevation of $[Ca^{2+}]_i$ mediated by both ATP and UTP was found to occur. The phosphoinositide pathway independent increase in $[Ca^{2+}]_i$ is not unusual. $P2Y_1$ receptors in brain endothelial cells have been reported to generate a rise in $[Ca^{2+}]_i$ in response to ADP with little or no $Ins(1,4,5)P_3$ formation (Alberts *et al.*, 1997; Frelin *et al.*, 1993). The elevations in $[Ca^{2+}]_i$ without the concomitant production of $Ins(1,4,5)P_3$ could be a result of two mechanisms. Firstly, only a very small increase in $Ins(1,4,5)P_3$, which may have been to small to be detected using the methods available, may be required to elicit modest increases in $[Ca^{2+}]_i$., which allows Ca^{2+}-induced Ca^{2+} release. Secondly, there may be a $Ins(1,4,5)P_3$-independent stimulated increase in cytosolic $[Ca^{2+}]_i$. For example sphingosine kinase may be activated independently of $Ins(1,4,5)P_3$ production, resulting in a rapid and transient increase in the putative second messenger, sphingosine-1-phosphate (SPP), triggering calcium mobilisation from intracellular stores (Olivera & Spiegel 1993). Another example is via Ca^{2+} sensor proteins (CaBP), which in the presence of calcium bind to $InsP_3$ receptors to increase Ca^{2+} from intracellular stores (Yang *et al.*, 2002). It is still unclear which mechanism may be employed and is discussed further in Chapter 8. In summary there was no difference between the ATP and UTP-induced increase in $[Ca^{2+}]_i$ and thus the differences in regulation of PDGF-induced mitogenesis by UTP and ATP does not occur at the level of $Ins(1,4,5)P_3$ or calcium production.

PDGF stimulates multiple signal transduction pathways that cooperate to generate a mitogenic response. Here we show that the ATP-stimulated P2Y receptor is proliferative in synergy with PDGF and that the UTP-stimulated receptor (most likely $P2Y_4$) is anti-proliferative. This cross talk between P2Y receptor signalling and PDGF receptor signalling pathways is a novel mechanism in human VSMCs. The method of interaction is not known and is further investigated in the following chapters. Studying and understanding these signalling mechanisms is highly relevant to the possible development of prospective drugs. Most significantly are potential anti-proliferative drugs for use in the early treatment of atherosclerosis and to prevent restenosis after angioplasty.

Chapter 4

The role of ERK in mitogenic signalling in hVSMC

4.1 Introduction

The proliferation of VSMCs is a pivotal event in development of atherosclerosis and restenosis (Ross 1993). This proliferation is most likely regulated by growth factors. PDGF is a well-known mitogen regulating cell proliferation and differentiation, which is partly thought to be through the phosphorylation of ERK1/2 (Feig & Schaffhausen 1994, Cowley *et al.*, 1994, Seger *et al.*, 1994). The substrates for ERK1/2 include transcription factors (Davis 1993), which initiate the expression of various genes and proto-oncogenes and whose products are involved in cell proliferation. G-protein coupled receptors are also coupled to mitogenesis through tyrosine kinases and the ERK cascade (Bourne *et al.*, 1995), linking with or overlaping the growth factor signalling pathway. These receptors can activate the ERK cascade via downstream events from their specific class of G-proteins. For example $G\alpha_q$ activates PLC leading to the increase in intracellular calcium concentrations and activation of PKC (Lev *et al.*, 1995, Della Rocca *et al.*, 1997), which then relays messages to PYK2 and Shc, activating Ras and eventually ERK (Bourne 1995). The $G\beta\gamma$ subunit can also instigate the phosphorylation of Shc leading to the activatation of the Ras/MAPK signalling cascade through Grb2/Sos complex (van Biesen *et al*, 1995). Stimulation of ERK1/2 by PDGF has been shown to occur in human VSMCs (Graves *et al.*, 1996, Yang *et al.*, 1998) leading to proliferation. In the previous chapter, the results showed that nucleotides could also regulate proliferation, but little is known of their effects on ERK1/2 in human VSMCs. P2Y receptors were first reported to stimulate ERK1/2 on rat renal mesangial cells (Huwiler *et al.*, 1994) and since then have also been shown to regulate proliferation in animal VSMCs via ERK1/2 activation (Yu *et al.*, 1996, Harper *et al.*, 1998, Wilden *et al.*, 1998).

The signals feeding into this cascade of phosphorylation can be modulated by various other kinases and proteins, thus resulting in a complex network of cross signalling regulating a range of cellular responses. The most important of these responses for this study is that resulting in the proliferation in human VSMCs. The aim of this chapter was to increase our understanding of PDGF induced regulation of ERK1/2 leading to proliferation in these cells and ask whether the modulation of this regulation by the pro-proliferative properties of ATP and the anti-pro-proliferative actions of UTP were acting at the level of ERK regulation.

4.2 Stimulation of ERK by PDGF

4.2.1 Increasing PDGF stimulation time

Yang and colleagues (1998) demonstrated increases in ERK1/2 activation in a time-dependent manner stimulated with PDGF in SV VSMCs. A short period of time such as 5 minutes was sufficient. Initially, 1 nM PDGF was used to determine if this was the case in human VSMC. Cells were grown to confluence in 6-well multi dishes and serum-starved for 48 hours. Agonists were applied to cells for various times (duration of stimulations are indicated in the figure legends) and then the cells were lysed using liquid nitrogen and the addition of lysis buffer. Cell extracts were protein equalised and separated using SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with a phospho-specific ERK1/2 antibody, which in turn was probed with a secondary antibody conjugated to HRP. The results from Western blotting were quantified using a gel documentation system, which measured chemi-illuminescent emission from the blots generated due to HRP metabolising ECL plus reagent (Section 2.6) and the data plotted as the percentage of the basal stimulation as shown (Figure 4.1). The results showed that a 5-minute stimulation was sufficient to significantly increase ERK activation (284 ± 70.7% of basal response, $P<0.05$, one way ANOVA) and there was no significant difference in activation between 5, 7.5 and 10-minute stimulation times. Therefore, the stimulation time of 5 minutes was used for further experiments unless. However, it should be noted that in later studies a longer time course was investigated (Figure 4.9) showing that a later peak of ERK activation occurred at 30-40 minutes.

4.2.2 The effects of increasing PDGF concentrations

The cells were exposed to increasing concentrations of PDGF. ERK phosphorylation was measured and plotted as percentage of basal stimulation (Figure 4.2). The results showed that increasing concentrations of PDGF significantly increased ERK activation in a dose dependent way ($P<0.01$, one way ANOVA). 1nM PDGF was able to sufficiently stimulate ERK phosphorylation 397 ± 42.5% above basal levels ($P<0.01$) and was used at this concentration for the duration of this chapter. The full dose response curve lies to the right of the graph show, but unfortunately higher concentrations of PDGF were

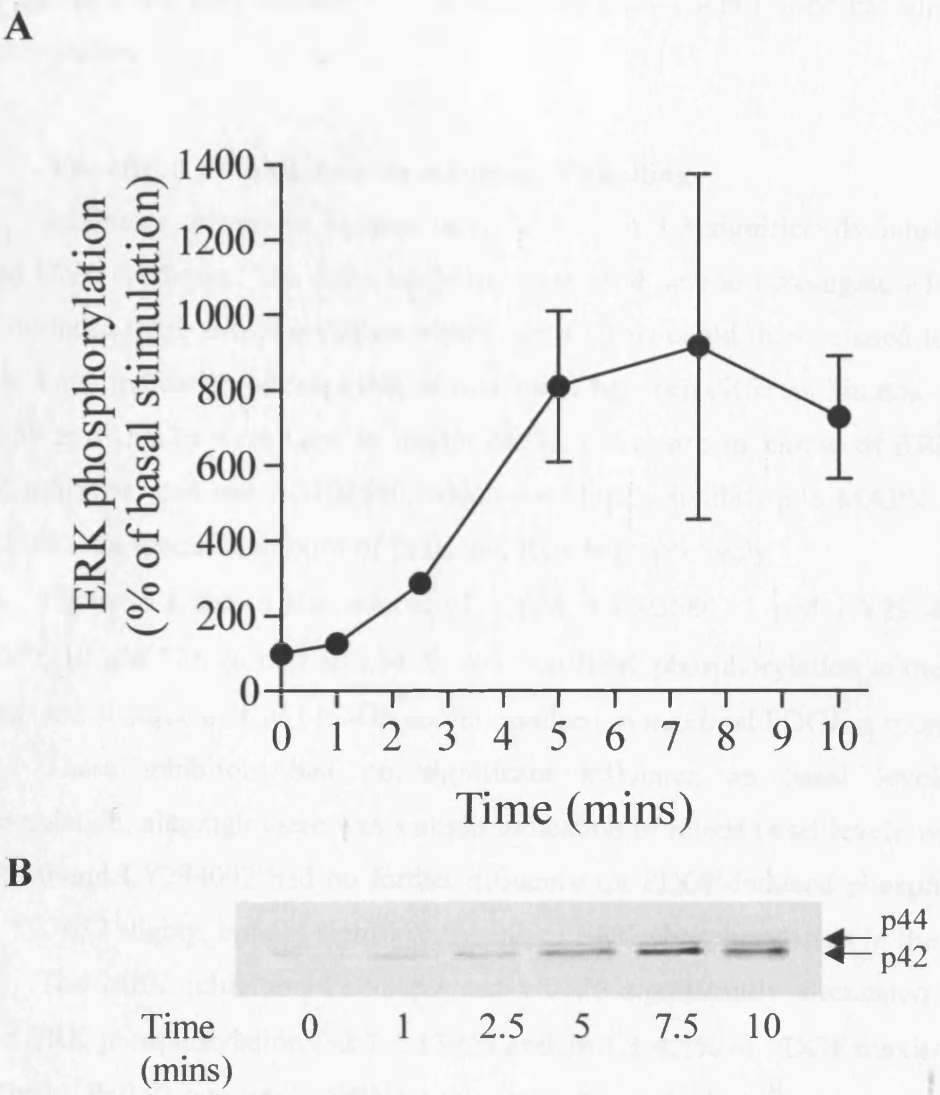


Figure 4.1 PDGF stimulated ERK Time-course

A) Stimulation of ERK1/2 VSMCs with 1 nM PDGF for 0, 1, 2.5, 5, 7.5 and 10 minutes. Data was collected from gel analysis of Western blots probed with phospho-specific ERK antibodies and presented as percentage of the basal for each time point across three experiments (± S.E.M.).

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments.

not used for ERK activation as these studies were performed in 6 well dishes in which PDGF above 2 nM proved costly. As a result the EC_{50} could not be calculated for ERK phosphorylation.

4.2.3 The effects of inhibitors on mitogenic signalling

Inhibitors of various kinases used in Section 3.3 significantly inhibited PDGF-induced DNA synthesis. The same inhibitors were used here to investigate what effects on PDGF-induced ERK phosphorylation would occur. This could then be used to establish if there was any indication of cross talk or regulation between different kinases or pathways. PD98059 and U0126 were used to inhibit MEK, the upstream kinase of ERK. Another MAPK inhibitor used was SB203580, which specifically inhibits p38 MAPK. LY294002 and Y27632 are specific inhibitor of PI3K and ROCK respectively.

Figure 4.3 shows the effects of 1 μM SB203580, 1 μM LY294002, 30 μM PD98059, 10 μM U0126 and 30 μM Y27632 on ERK phosphorylation stimulated in the presence and absence of 1 nM PDGF and normalised to maximal PDGF response in Figure 4.3A. These inhibitors had no significant influence on basal levels of ERK phosphorylation, although there was a slight indication of raised basal levels with Y27632. SB203580 and LY294002 had no further influence on PDGF-induced phosphorylation of ERK. Y27632 slighty, but not significantly, raised ERK phosphorylation in the presence of PDGF. The MEK inhibitors PD98059 and U0126 significantly attenuated the PDGF-induced ERK phosphorylation (32.7 ± 13.6% and 36.1 ± 4.2% of PDGF maximal response respectively, $P<0.01$ one-way ANOVA with Dunnett's multiple comparison post test). The inhibition by the MEK inhibitors was expected since MEK is highly specific for ERK phosphorylation. This was further investigated.

Increasing concentrations of the two inhibitors were used to investigate their inhibitory potency on ERK phosphorylation when stimulated with 1 nM PDGF as before. The effects on basal levels were also measured and compared to PDGF stimulated levels. The results are shown in Figure 4.4 for PD98059 and Figure 4.6 for U0126. Since ERK is an important pathway leading to mitogenesis, the effects of these MEK inhibitors on mitogenesis (measured by [^{3}H]-thymidne incorporation into DNA) was also investigated. Increasing concentrations of the inhibitors were used to pre-incubate cells for 30 minutes and were also present when the cells were stimulated for 1 hour with 1 nM PDGF (as described in Section 2.9). After a further 19 hours incubation in serum-free medium with

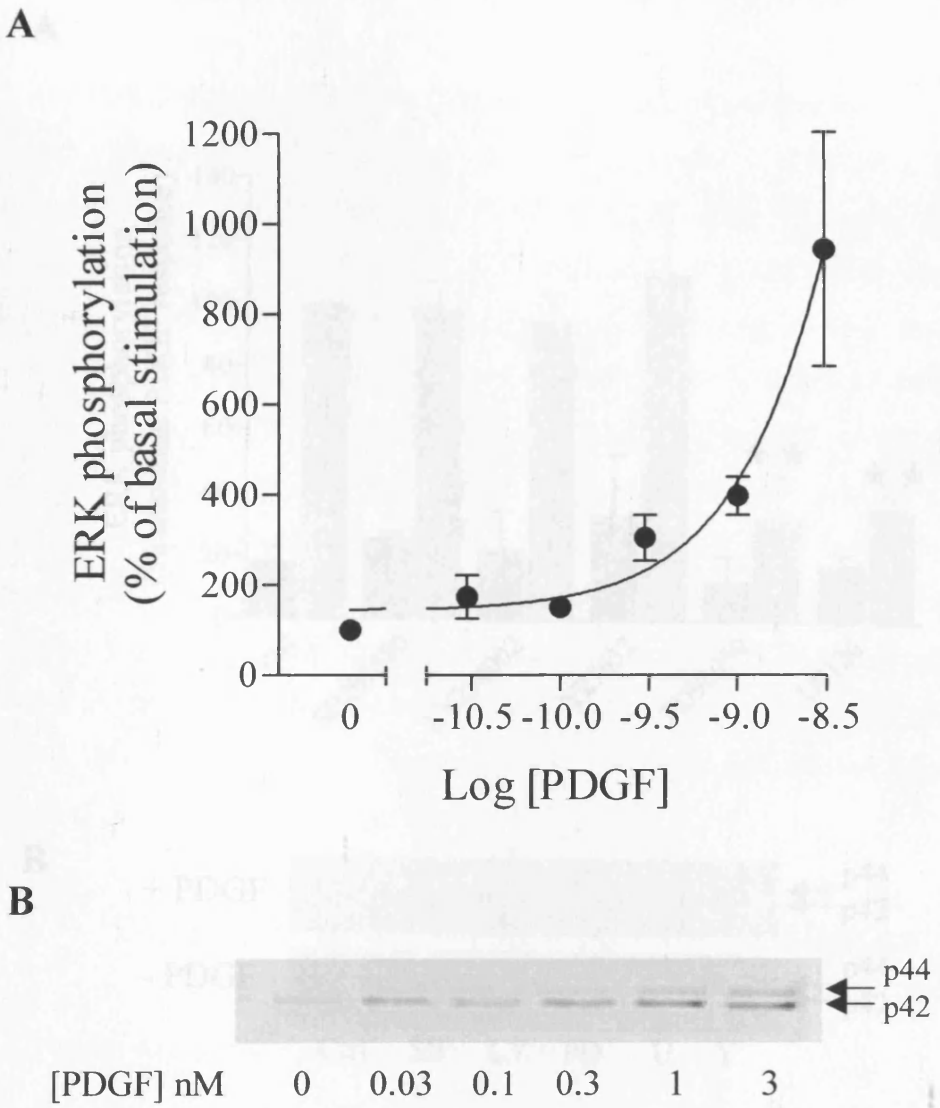


Figure 4.2 The effects of increasing PDGF concentrations on ERK

A) Stimulation of ERK1/2 in human VSMCs stimulated for 5 minutes with increasing concentrations of PDGF. Data was collected from gel analysis of Western blots probed with phospho-specific ERK antibodies and presented as percentage of the basal ± S.E.M. from three experiments.

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments

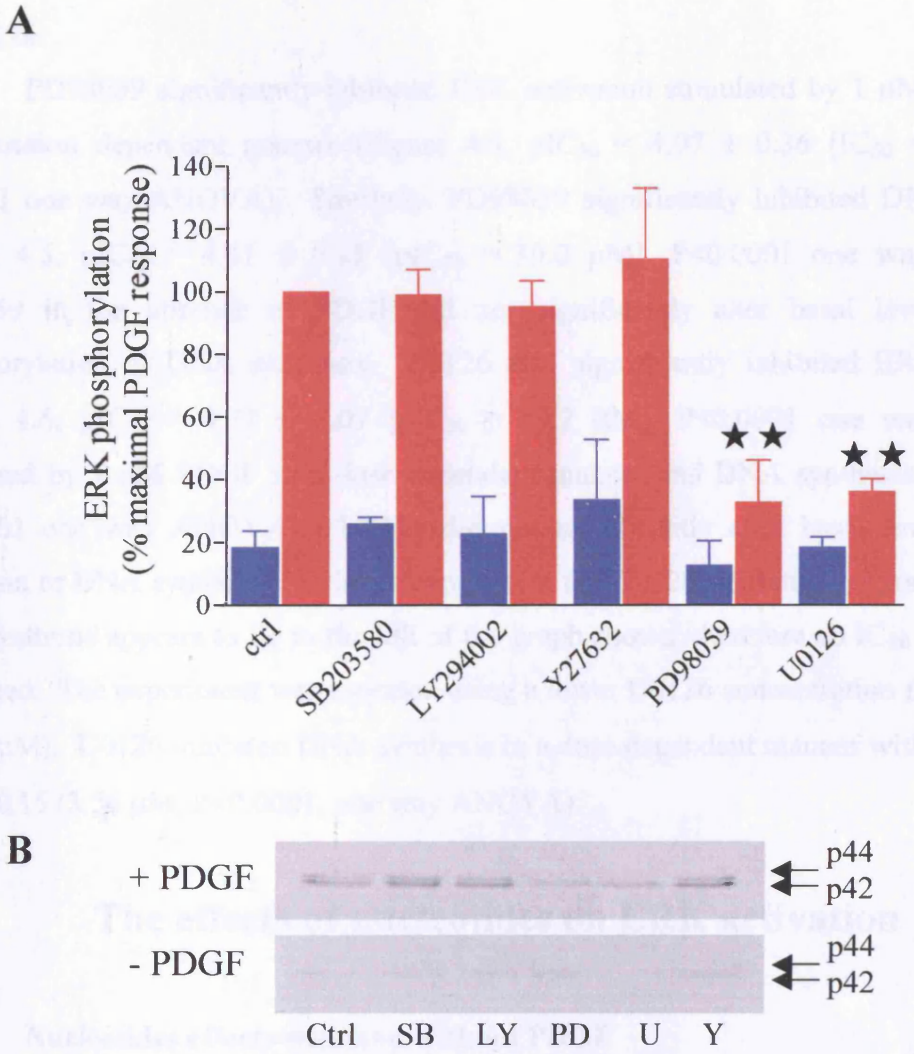


Figure 4.3 The effects of various inhibitors on ERK activation

A) Basal (blue) and 1 nM PDGF (red) stimulation of ERK1/2 activation with 1 μM SB2035880, 1 μM LY294002, 30 μM PD98059, 10 μM U0126 and 30 μM Y27632. Data was collected from gel analysis of Western blots and presented as percentage of the maximal PDGF stimulated response ± S.E.M. from three experiments. ★★ $P<0.01$ one way ANOVA

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments;

no PDGF or inhibitors, [^{3}H] thymidine was added and its incorporation into DNA measured after 4 hours. These results are shown in Figure 4.5 for PD98059 and Figure 4.7 for U0126.

PD98059 significantly inhibited ERK activation stimulated by 1 nM PDGF in a concentration dependant manner (Figure 4.4, pIC_{50} = 4.97 ± 0.36 [IC_{50} = 10.8 μM], P<0.001 one way ANOVA). Similarly, PD98059 significantly inhibited DNA synthesis (Figure 4.5, pIC_{50} = 4.41 ± 0.21 [pIC_{50} = 39.0 μM], P<0.0001 one way ANOVA). PD98059 in the absence of PDGF did not significantly alter basal levels of ERK phosphorylation or DNA synthesis. U0126 also significantly inhibited ERK activation (Figure 4.6, pIC_{50} = 4.72 ± 0.07 [pIC_{50} = 19.2 μM], P<0.0001 one way ANOVA) stimulated by 1 nM PDGF in a dose dependant manner and DNA synthesis (Figure 4.7, P<0.0001 one way ANOVA). U0126 did not significantly alter basal levels of ERK activation or DNA synthesis. In latter experiment the U0126 inhibition response curve for DNA synthesis appears to lie to the left of the graph shown, therefore an IC_{50} could not be calculated. The experiment was repeated using a lower U0126 concentration range (10 nM to 100 μM). U0126 inhibited DNA synthesis in a dose dependent manner with and IC_{50} of 5.45 ± 0.15 (3.56 μM, P<0.0001, one way ANOVA)

4.3 The effects of nucleotides on ERK activation

4.3.1 Nucleotides effects with and without PDGF

ATP, UTP and UDP were initially used to see if they influenced ERK activation. Cells were stimulated for 5 minutes with 300 μM nucleotides either alone or in the presence of 1 nM PDGF. The results are shown in Figure 4.8 and presented as the percentage of basal stimulation. PDGF, as above, significantly stimulated ERK phosphorylation (432.8 ± 13.8% of basal, P<0.01). ATP, UTP and UDP at 300 μM did not significantly influence any increase in basal levels of ERK activation (Figure 4.8A, 191.5 ± 53.5%, 122.6 ± 53.8% and 103.8 ± 3.38% respectively). In the presence of PDGF, nucleotides produced no further influence on PDGF-stimulated ERK phosphorylation. In the presence of 100 μM PD98059 all responses were significantly reduced to basal levels (representative blot shown in Figure 4.8C; P<0.001, one way ANOVA from data pooled across experiments, which is not shown).

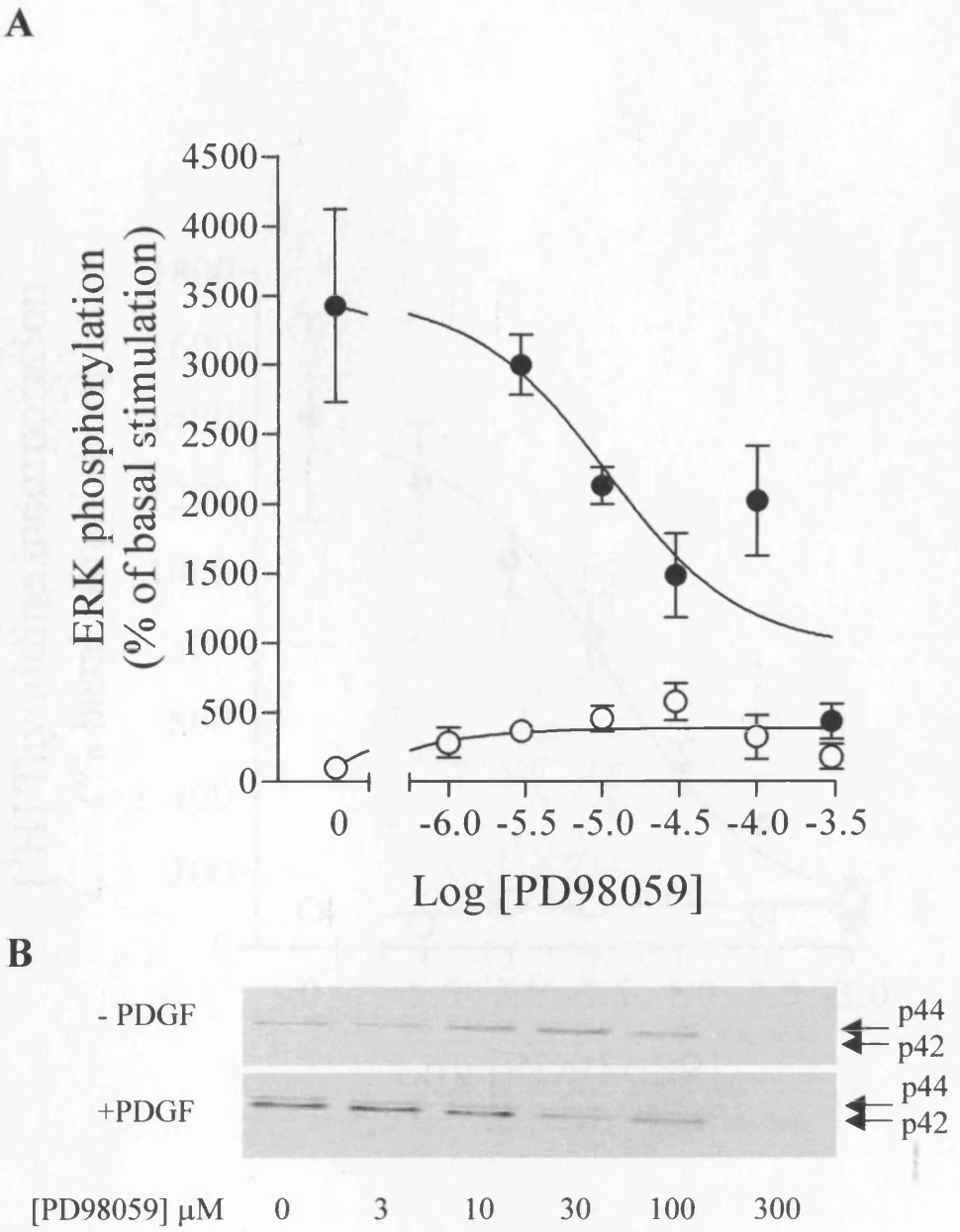


Figure 4.4 The effects of MEK inhibitor PD98059 on ERK activation

A) Stimulation of ERK1/2 with increasing concentrations of MEK inhibitor PD98059 in the presence (•) and absence (o) of 1 nM PDGF. Data was collected from gel analysis of Western blots and presented as percentage of the basal ± S.E.M. from three experiments

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments

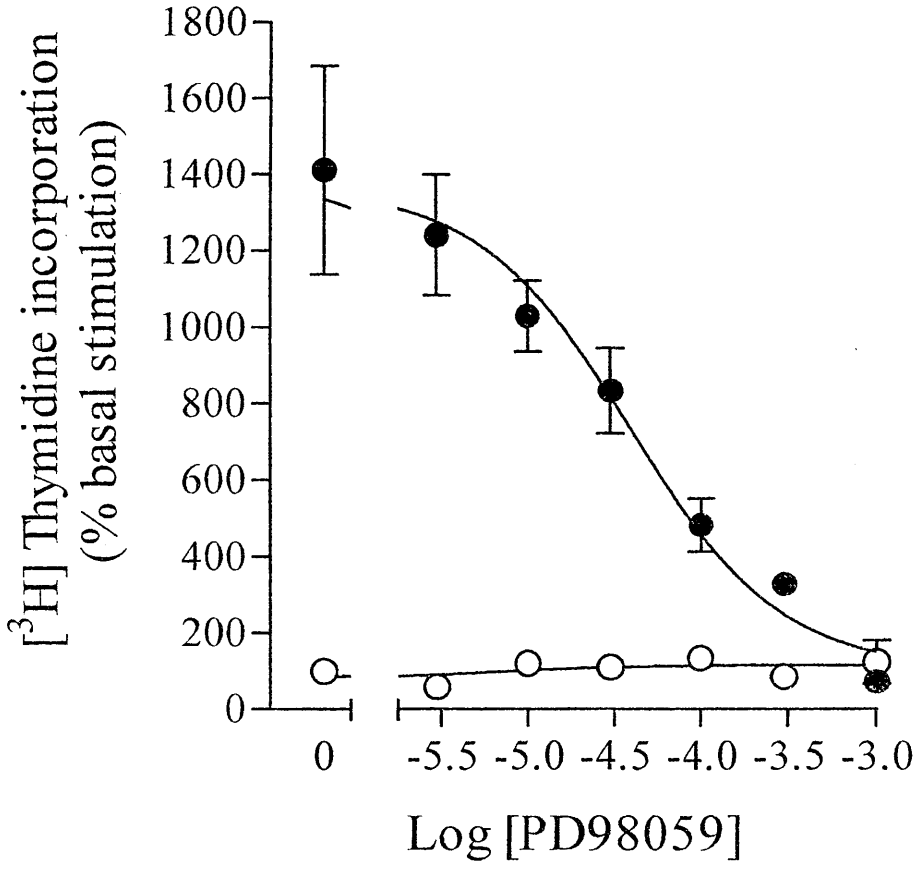


Figure 4.5 The effect of MEK inhibitor PD98059 on DNA synthesis

[^{3}H] Thymidine incorporation into DNA stimulated in the presence (•) and absence (o) of 1 nM PDGF plus increasing concentrations of PD98059. Data presented as percentage of the basal ± S.E.M. from three experiments each carried out in triplicates

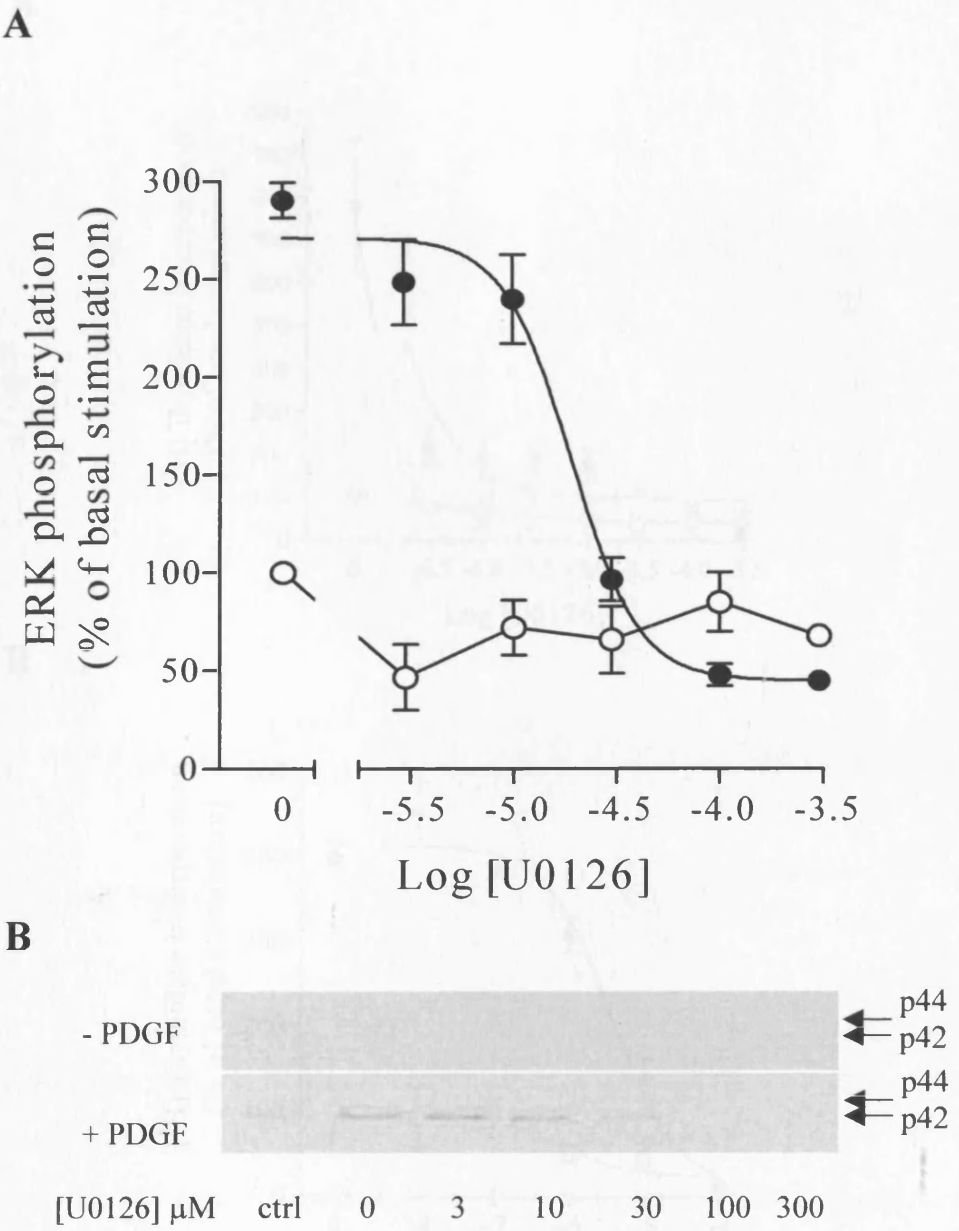
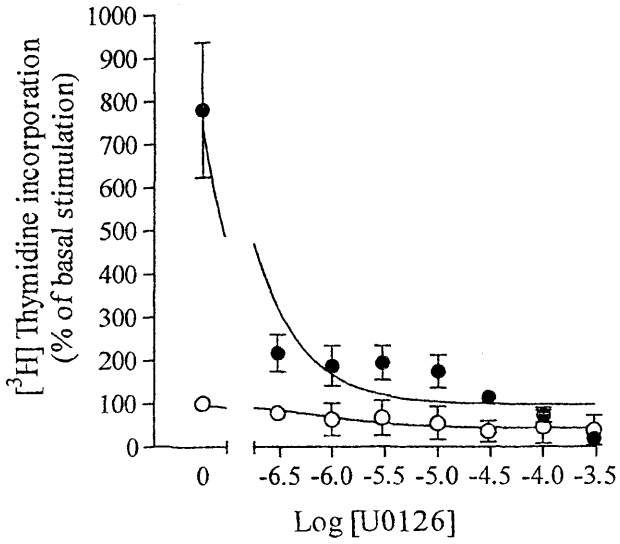


Figure 4.6 The effect of MEK inhibitor U0126 on ERK activation

A) Stimulation of ERK1/2 with increasing concentrations of MEK inhibitor U0126 in the presence (•) and absence (o) of 1 nM PDGF. Data was collected from gel analysis of Western blots and presented as percentage of the basal ± S.E.M. from three experiments

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments

A



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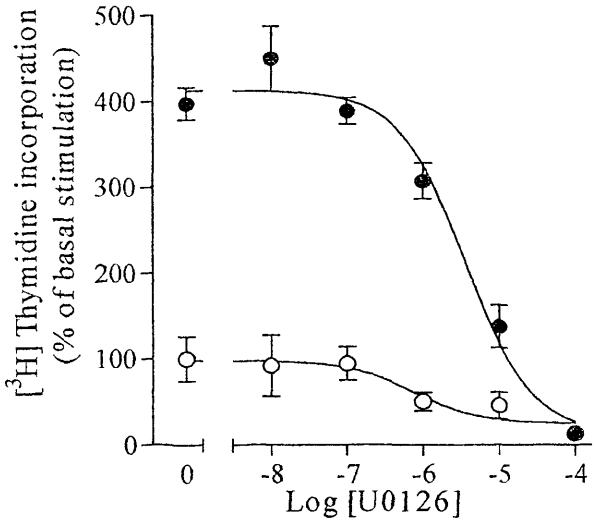


Figure 4.7 The effect of MEK inhibitor U0126 on DNA synthesis

A) [^{3}H] Thymidine incorporation into DNA stimulated in the presence (•) and absence (o) of 1 nM PDGF plus increasing concentrations of U0126. Data presented as percentage of the basal ± S.E.M. from three experiments each performed in triplicates

B) Similar experiment as A but with a lower range of concentrations reproduced with kind permission from Prof. M.R. Boarder. Data normalized to basal levels represented as 100% ± S.E.M. from one experiment (representative of 3) performed in triplicates.

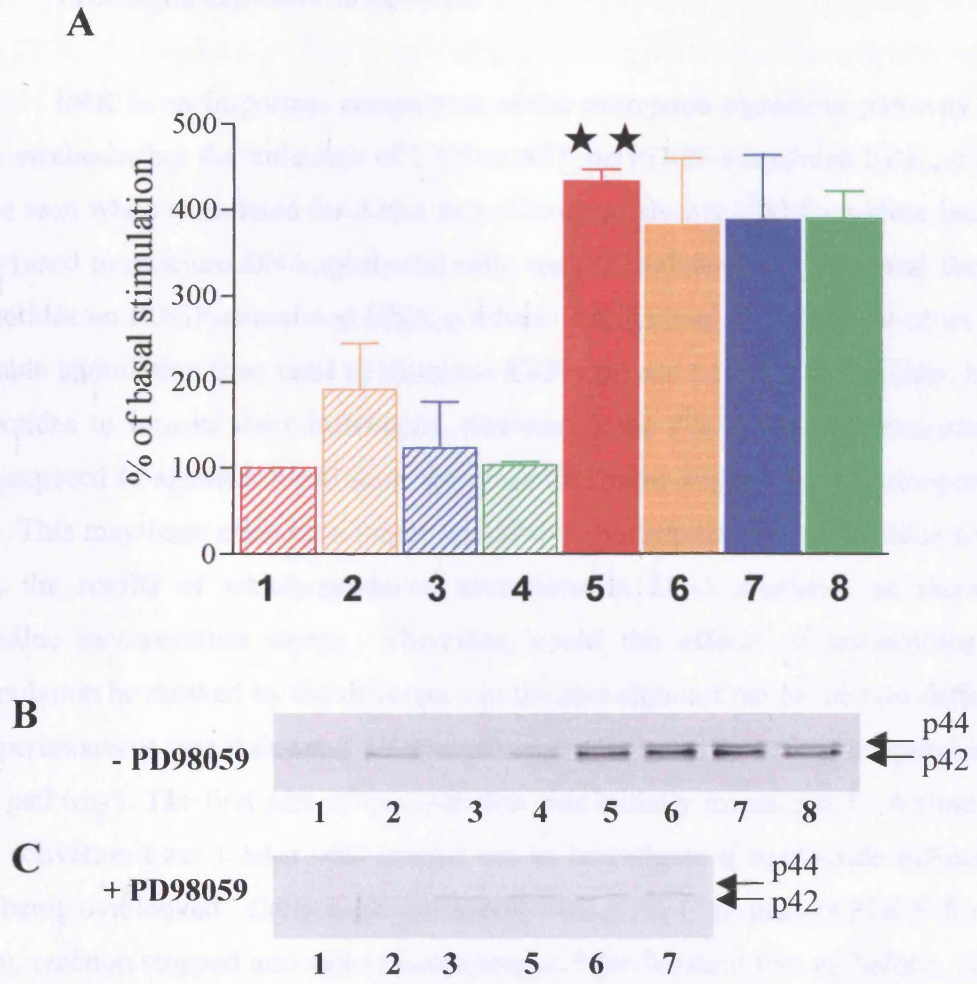


Figure 4.8 The effects of nucleotides and/or PD98059 on ERK stimulation

A) Stimulation of ERK1/2 phosphorylation with nucleotides ± PDGF.

Data was collected from gel analysis of Western blots and presented as percentage of the basal ± S.E.M. from 4 experiments. ★★ $P<0.01$ one way ANOVA

B) Western blot showing phosphorylation of ERK1/2, which is representative of the 4 experiments

C) Western blot showing phosphorylation of ERK1/2 with nucleotides ± PDGF in the presence of 100 μM PD98059, which is representative of the 3 experiments

Labels represented as follows;

1) ctrl ( ), 2) 300 μM ATP ( ), 3) 300 μM UTP ( ), 4) 300 μM UDP ( ),
5) 1 nM PDGF ( ), 6) PDGF+ATP ( ), 7) PDGF+UTP ( ), and
8) PDGF+UDP ( ).

4.3.2 Prolonged exposure to agonists

ERK is an important component of the mitogenic signalling pathway leading to DNA synthesis, but the influence of UTP or ATP on PDGF-stimulated ERK, if any, could not be seen when stimulated for 5 minutes. However, during [^{3}H] thymidine incorporation assay (used to measure DNA synthesis) cells were stimulated for 1 hour and the effects of nucleotides on PDGF-stimulated DNA synthesis could clearly be seen. In other words the 5-minute stimulation time used to stimulate ERK may not have been sufficient in allowing nucleotides to impose their influences, whereas in the [^{3}H] thymidine experiment, cells were exposed to agonists for 1 hour, therefore ERK was stimulated for a longer period of time. This may have allowed a larger window of opportunity for nucleotides to influence ERK, the results of which produced alterations in DNA synthesis as shown by the thymidine incorporation assay. Therefore, could the effects of nucleotides on ERK manipulation be masked by the difference in the stimulation time of the two different types of experiments or was it due to P2Y receptor signalling simply acting independently of the ERK pathway? The first part of this question was initially investigated. A time-course of ERK activation over 1 hour was carried out to investigate if nucleotide influences were truly being overlooked. Cells were stimulated with ATP, UTP plus/or PDGF for the times shown, reaction stopped and cell extracts prepared for Western blot as before. The results for ATP and PDGF are shown in Figure 4.9 and for UTP and PDGF in Figure 4.10.

Activation of ERK by 1 nM PDGF was found to dramatically increase between stimulation times of 30-40 minutes (1299 ± 175% and 1274 ± 473% of basal levels respectively, $P<0.05$ one way ANOVA) compared to 5-minute stimulation (172 ± 19.3% above basal). ATP alone was also able to significantly activate ERK at 30-40 minutes (1294 ± 115% and 1294 ± 417% of basal levels respectively, $P<0.05$ one way ANOVA). Both produced timecourse profiles, which were significant compared to basal levels ($P<0.001$ two way ANOVA) but were not significantly different from each other. In other words ATP was able to stimulate ERK activation in a similar way to PDGF over the duration of an hour. This is not however, reflected in DNA synthesis, as ATP did not increase DNA synthesis with the same magnitude as PDGF.

Surprisingly, ATP in the presence of PDGF significantly potentiated the PDGF response further ($P<0.01$, two way ANOVA comparing PDGF alone with PDGF plus ATP) and increased the response at 30-40 minutes more than 3 fold (3400 ± 694% and 5818 ±

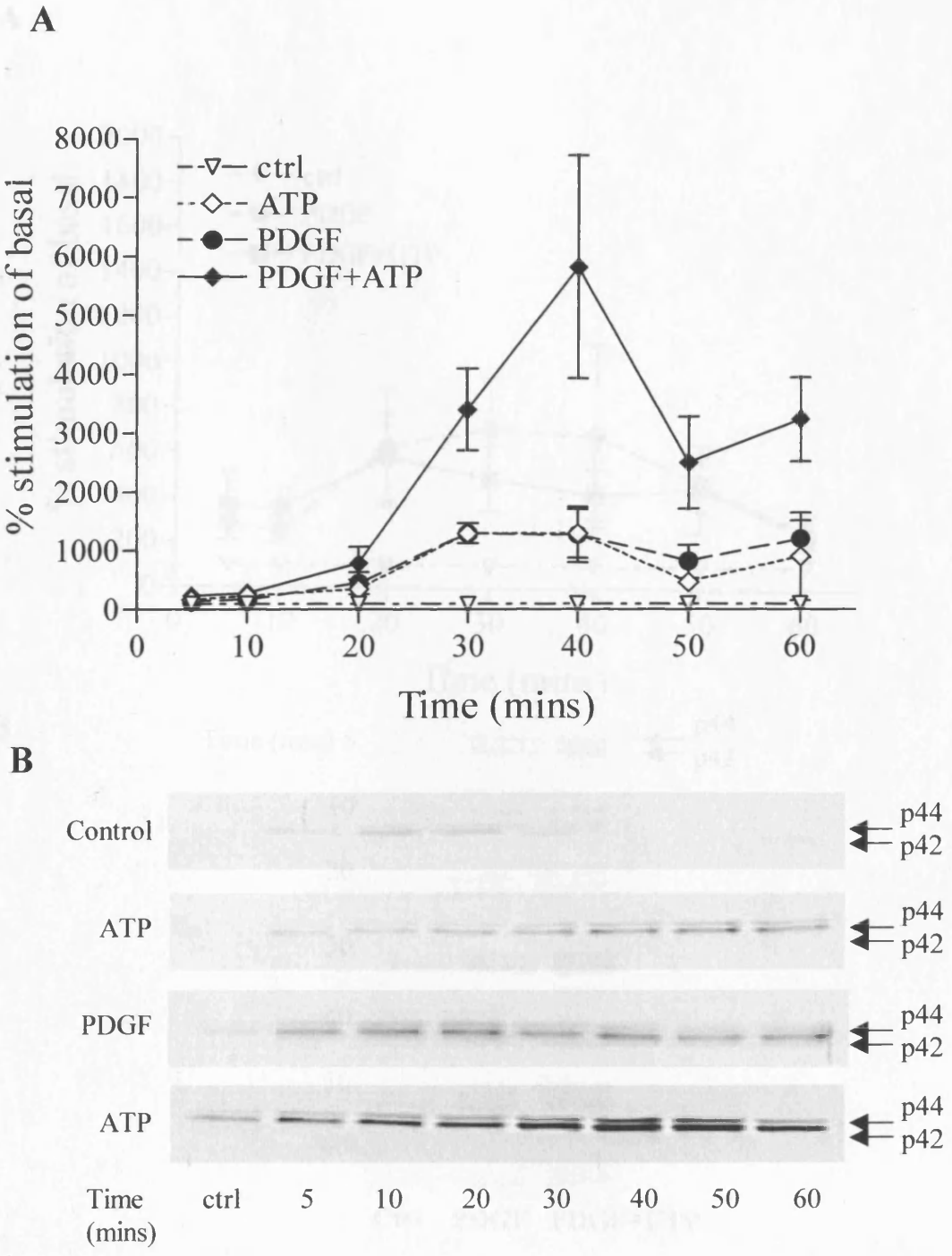


Figure 4.9 Effects of ATP on ERK activation time-course

A) Stimulation of ERK1/2 for 5, 10, 20, 30, 40, 50 and 60 minutes with 300 μM ATP (◇), 1 nM PDGF (●) and 300 μM ATP plus 1nM PDGF (◆). Data was collected from gel analysis of Western blots and presented as % of the basal levels (○) at each time point ± S.E.M. from three experiments; **B**) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments.

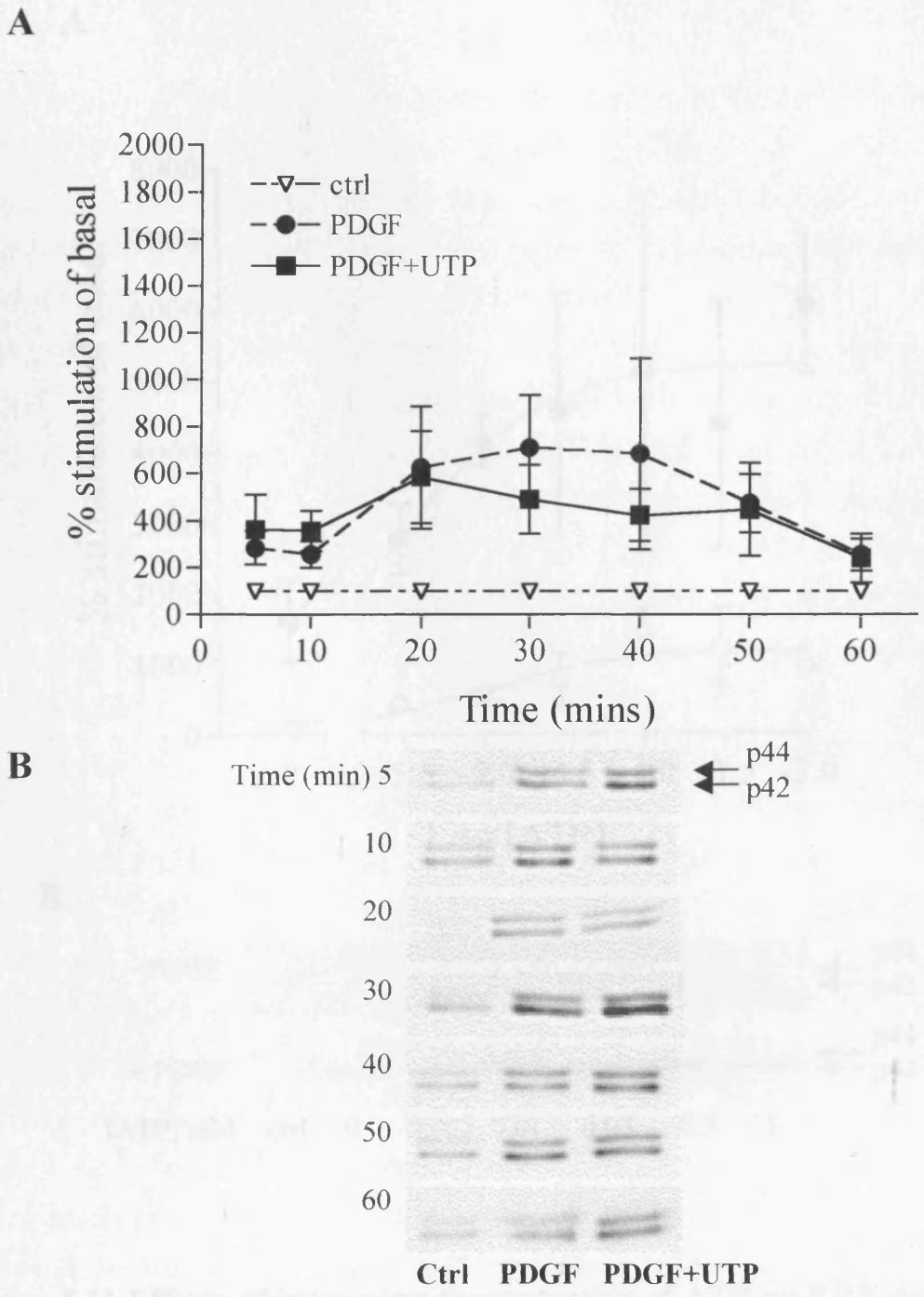


Figure 4.10 Effects of UTP on ERK activation time-course

A) Stimulation of ERK1/2 for 5, 10, 20, 30, 40, 50 and 60 minutes with 1 nM PDGF (●) and 1 nM PDGF ± 300 μM UTP (■). Data was collected from gel analysis of Western blots and presented as percentage of the basal levels (∇) at each time point ± S.E.M. from three experiments; **B**) Western blot showing phosphorylation of ERK1/2 which is representative of the three experiments.

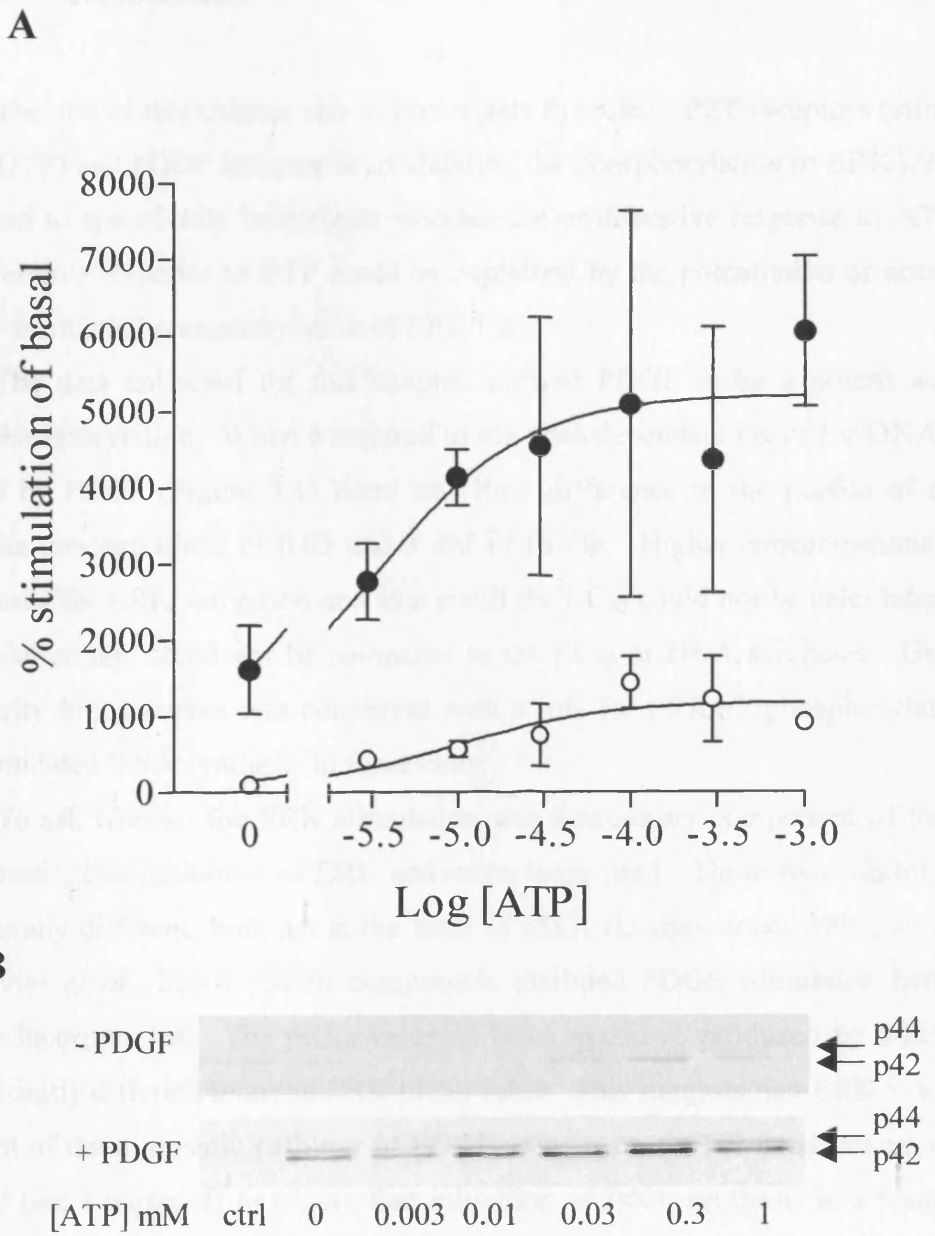


Figure 4.11 Effects of increasing concentration of ATP on ERK activation

A) Stimulation of ERK1/2 for 40 minutes with increasing concentrations of ATP (0.003-1 mM) with (●) and without (○) 1 nM PDGF. Data was collected from gel analysis of Western blots and presented as percentage of the basal levels ± S.E.M. from three experiments;

B) Western blot showing phosphorylation of ERK1/2, which is representative of the three experiments.

4.4 Discussion

The aim of this chapter was to investigate the role of P2Y receptors (stimulated by ATP and UTP) and PDGF receptor in modulating the phosphorylation of ERK1/2 in human VSMCs and to specifically investigate whether the proliferative response to ATP and the anti-proliferative response to UTP could be explained by the potentiation or attenuation of the PDGF-stimulated phosphorylation of ERK1/2.

The data collected for this chapter showed PDGF to be a potent activator of ERK1/2 phosphorylation. When compared to the dose dependent curve for DNA synthesis stimulated by PDGF (Figure 3.1) there was little difference in the profile of the curves between the concentrations of 0.03 and 3 nM of PDGF. Higher concentrations of PDGF were not used for ERK activation and as a result the EC_{50} could not be calculated for ERK phosphorylation and could not be compared to the EC_{50} of DNA synthesis. Despite this, the similarity in the curves was consistent with a role for ERK1/2 phosphorylation in the PDGF-stimulated DNA synthesis in these cells.

To ask whether the ERK stimulation was a necessary component of the pathway to mitogenesis, two inhibitors of ERK activation were used. These two inhibitors, which are structurally different, both act at the level of MEK (Dudley *et al.*, 1995, Favata *et al.*, 1998, Davies *et al.*, 2000). Both compounds inhibited PDGF stimulated ERK and ^3H thymidine incorporation. The pIC_{50} value of DNA synthesis produced by PD98059 was not significantly different than the ERK pIC_{50} value. This suggests that ERK is a necessary component of the mitogenic pathway of PDGF. However, further experiments need to be performed (see Chapter 8) to ensure that inhibition of DNA synthesis is a result of ERK inhibition and not other enzymes such as COX1/2 which can also be inhibited by PD98059. U0126, which does not inhibit COX1/2, also inhibited both ERK and DNA synthesis. However, the U0126 IC_{50} for DNA synthesis was lower in comparison to PD98059 IC_{50} for DNA synthesis (statistical comparison of the two values was not performed due to n=1 for U0126) and also compared to the IC_{50} of ERK phosphorylation by U0126. This ambiguous result could be due to differential responses between cells from different patients. Unfortunately due to the lack of time, experiments using U0126 could not be repeated to verify this. However, these results also suggest the importance of ERK in DNA synthesis induced by PDGF in these cells.

Inhibition of other kinases such as PI3-kinase, p38 and ROCK resulted in an attenuation of DNA synthesis (Figure 3.5). However this was not the case for ERK activation. LY294002, SB203580 and Y27632 did not attenuate PDGF-induced ERK phosphorylation. These results suggest that the ERK pathway is not regulated by these kinases. Conversely, in porcine coronary smooth muscle cells the stimulation of ERK1/2 by PDGF was inhibited in a dose dependent manner by the P13K inhibitors wortmannin and LY294002 (Wilden *et al.*, 1998), which suggests that ERK activation was dependent on PI3-kinase. PD98059 and LY294002 also inhibited DNA synthesis similar to this study. This is an example of the difference in signalling pathways between different species such as human and pigs. In human arterial SMCs, Graves and colleagues (1996) reported increases in ERK phosphorylation stimulated by PDGF. Yang and colleagues (1998) reported increases in ERK activation in human SV VSMCs along with enhanced proliferation and cell cycle inhibitor, $p27^{kip1}$, downregulation in response to PDGF. However in IMA VSMCs only ERK phosphorylation was observed and not proliferation, which indicates that ERK is necessary for proliferation but not sufficient. It was suggested that the different phenotypes of SMCs may display different receptor-mediated signal transduction pathways.

The importance of ERK in the mitogenic signalling pathway of PDGF had now been established, but the influence of UTP or ATP, quite evident at the level of DNA synthesis was not apparent at ERK phosphorylation. There is increasing evidence to show P2Y receptor coupling to ERK1/2 in VSMCs leading to proliferation (Yu *et al.*, 1996, Harper *et al.*, 1998, Wilden *et al.*, 1998), therefore the possibility that nucleotides may have some effect in human cells was further investigated. Since ERK was stimulated by agonists for only 5 minutes, whereas DNA synthesis was stimulated for 1 hour the question was could the effects of nucleotides on ERK activation, if any, be masked by the difference in the stimulation time of the two different types of experiments or was it due to P2Y receptor signalling simply acting independently of the ERK pathway? The former part of this question was found to be true for ATP. After a 5-minute stimulation time ATP did not significantly increase ERK phosphorylation as described in previous chapter (Figure 3.2). By increasing the time of stimulation it was found that not only did the PDGF stimulatory capacity peak at 30-40 minutes, but that ATP also generated responses that were significantly as potent as PDGF. Even more surprisingly ATP in the presence PDGF

generated ERK responses that were greater than the additive effect of the individual agonists. This synergistic ERK activity was most evident at 40 minutes. This was consistent with DNA synthesis studies, which showed synergistic increases in PDGF responses by ATP (Figure 3.2). However, the ATP mitogenic response, which was equal to that of PDGF in phosphorylating ERK, was not consistent with DNA synthesis results as ATP was unable to significantly increase this. It is evident that ATP was able to instigate an ERK response via its G protein-coupled P2Y receptor, however it was unable to sustain the signalling pathway to DNA synthesis independently of the PDGF signalling pathway. The PDGF receptor was able to stimulate more than one pathway leading to mitogenesis, which amalgamated to produce a significant increase in DNA synthesis (supported by data in this study). However, hypothetically the P2Y receptor was only able to generate a signal to ERK and not to the other mitogenic pathways, therefore producing no increase in DNA synthesis. This is shown in Figure 3.2 where ATP alone does not significantly increase DNA synthesis. A similar hypothesis was suggested in rat arterial VSMCs (Malem-Souley *et al.,* 1993) where ATP was able to stimulate early and delayed-early G_1 gene mRNA levels but not late G_1 genes which would have then increased DNA synthesis. ATP was therefore acting as a competence factor (Malem-Souley *et al.,* 1996), which may have induced an semi-activated state where cells could then fully respond to other growth stimuli such as PDGF leading to increased proliferation

As mentioned earlier, PDGF stimulated ERK phosphorylation in human IMA VSMCs but this did not result in proliferation (Yang *et al.,* 1998). The activation of ERK by either a GPCR or TK receptor is not therefore sufficient to instigate proliferation. Other kinases or signalling pathways thus have a role to play together with ERK in stimulating mitogenesis. Wilden and colleagues (1998) reported ATP-induced proliferation in porcine coronary artery SMCs required independent activation of both ERK and PI3K. Alone neither one of these kinases appeared sufficient in produce the signals necessary for proliferation. This data supports the hypothesis that mitogenesis requires more than just the ERK pathway, but also showed that the ATP via its P2Y receptor was capable of utilising two pathways, the ERK and P13K pathway, to stimulate proliferation. This may also have been the case in human VSMCs, but the coupling of ATP-stimulated P2Y receptor to other pathways, such as PI3-kinase, could not be shown since adequate levels of proliferation could not be measured. Wang *et al.* (1992) also reported increases in DNA synthesis in porcine SMCs induced by ATP, which was mediated independently via

arachidonic acid production and PKC. These reports suggest that many different signalling pathways work synergistically to induce mitogenesis.

In this study it appeared that the ATP mitogenic response was dependent on PDGF signalling and synergistically enhanced proliferation. A possible mechanism of this enhancement could occur at the receptor level by P2Y receptor cross talk to the PDGF receptor. Activated GPCR have been shown utilise RTK to induce the ERK pathway. The EGF receptor become active upon LPA-induced GPCR activation, which was mediated via the βγ subunit activation of tyrosine kinase Src which may phosphorylate the EGF receptor (Luttrell *et al.*, 1997, Cunnick *et al.*, 1998). This resulted in the activation of ERK. Angiotensin II also couples to the PDGF receptor activation which resulted in Shc and Grb2 association in rat aortic SMCs (Linseman *et al.*, 1995). Similarly P2Y receptors have been implied to activate RTKs. Further evidence to suggest cross talk between P2Y receptors has been demonstrated by synergistic enhancement of DNA synthesis by ATP and PDGF. Wang *et al.* (1990) reported synergistic enhancement of PDGF, EGF and TGF-α-induced DNA synthesis by ATP and ADP in 3T6 mouse fibroblasts. The same group later showed ATP mitogenic responses to be enhanced by PDGF, EGF and IGF-1 in porcine aortic SMCs (Wang *et al.*, 1992). More recently, ATP stimulated DNA synthesis and ERK activation in coronary artery SMCs by approximately 4 and 7 fold respectively (Agazie *et al.*, 2001). In the presence of insulin, which only stimulated both DNA synthesis and ERK activation by 2 fold, the responses were synergistically enhanced to 17 fold for DNA synthesis and 16 fold for ERK activation. This was not seen at the level of Ras and Raf, which were only enhanced in an additive fashion. ATP inhibited insulin-induced PI3K-PKB activity, which was suggested to relieve the inhibitory action of PKB on the ERK pathway resulting in enhancement of proliferation.

These studies demonstrate that RTK and GPCR signalling pathways can cooperate to in inducing proliferation. Under conditions such as vascular damage ATP released by aggregating platelets may synergistically work with other factors released such as PDGF to induce proliferation in the wound healing mechanism. In this study it is unknown what other pathways, apart from ERK, ATP may be stimulating to induce proliferation and unclear precisely how PDGF receptor signalling is aided by ATP receptor signalling. A possible mechanism may involve P2Y receptor-induced activation of the PDGF receptor. Overall, in human VSMCs the ATP stimulated P2Y receptor did not instigate proliferation, but was able to increase ERK phosphorylation, which in the

presence of PDGF receptor signalling was able to increase PDGF dependent mitogenesis synergistically.

On the other hand, UTP, which also stimulates P2Y receptors, showed no influence on ERK either alone or in the presence of PDGF over an hour. This was consistent with DNA synthesis studies where UTP generated no increase, but was inconsistent with the data in the presence of PDGF, where UTP attenuated DNA synthesis. The UTP inhibitory influence may therefore have been independent of the ERK pathway. This also shows that ATP and UTP were acting at different P2Y receptors and also suggests that the ATP stimulated P2Y receptor couples to ERK and DNA synthesis, whereas the UTP stimulated P2Y receptor does not and therefore has no role in promoting mitogenesis. The human $P2Y_4$ receptor subtype at which both ATP and UTP are equipotent is not involved in mediating the proliferative nor the anti-proliferative effects of ATP or UTP.

Chapter 5

JNK & p38 activation in human VSMCs

5.1 Introduction

MAPKs play an important role in signal transduction modulating many cellular events (Seger & Krebs 1995, Cano *et al.*, 1995). This super family includes ERK, which was discussed in the previous chapter, JNK and p38 MAPK, which will be discussed in this chapter. JNK and p38 MAPK, like ERK, undergo phosphorylation on the threonine and tyrosine residues in the sequence pTXpY upon activation by their upstream MEKs. JNK is phopshorylated by MEKs known as MKK4/7 and p38 MAPK by MEKs known as MKK3/6. The main isoforms of JNK are p46 JNK1, p54 JNK2 and p49 JNK3 (Dérijard *et al.*, 1994, Kyriakis *et al.*, 1994), although up to 10 splice variants have been identified (Gupta *et al.*, 1996). JNK phosphorylates the transcription factor c-jun (AP1). p38 MAPK phosphorylates the activating transcription factor (ATF) family and the isoforms include p38, p38β, p38γ, p38δ and show a slight differences in mobility during SDS-PAGE (Li *et al.*, 1996).

Whereas the ERKs are implicated in cell growth and differentiation, with their signalling pathway partially elucidated, the mechanism of activation of JNK and p38 remain poorly understood. Their main role appears to be involved in inhibiting cell growth and regulating apoptosis (Kyriakis *et al.*, 1996). The JNK and p38 MAPK pathways are activated by ultraviolet light, cytokines, osmotic shock agents such as sorbitol and protein synthesis inhibitors such as anisomycin. Increasing evidence shows JNK and p38 to be regulated by growth factors such as EGF, VEGF and PDGF (Hashimoto *et al.*, 1999, Minden *et al.*, 1994, Pedrum *et al.*, 1998, Yu *et al.*, 2000, Assefa *et al.*, 1999, Lallemand *et al.*, 1998). JNK has also been linked to proliferation (Pedrum *et al.*, 1998, Izumi *et al.*, 2001, Li et al., 1999) although in some reports this has not been shown to be the case (Orsini *et al.*, 1999, Sellers *et al.*, 2001). Constitutively active JNK and p38 MAPK have been shown to play a role in hypertrophy in neonatal rat cardiac myocytes (Wang *et al.*, 1998a & b), although another group reported only p38 MAPK to be involved in hypertrophy whereas JNK had an opposing influence (Nemoto *et al.*, 1998).

GPCR were soon found to be able to regulate the stress activated pathways through the Ras-related small GTP- binding proteins Rac 1 and Cdc42. More recent studies have shown P2Y receptor stimulation of the p38 MAPK and JNK pathways. In rat

glomerular mesangial cells the stimulation of JNK and p38 MAPK with UTP and ATP was shown to be mediated through $P2Y_2$ receptors (Huwiler *et al.*, 1997 & 2000). Similarly, JNK and p38 MAPK were stimulated by UTP in 1321N1 human astrocytoma cells stably expressing recombinant human $P2Y_2$ or $P2Y_4$ receptors (Paul *et al.*, 2000). JNK, but not p38 MAPK, was stimulated by 2MeSADP in cells expressing human $P2Y_1$ receptors (Sellers *et al.*, 2001), whereas ADP activated p38 MAPK kinase by acting at $P2Y_1$ receptors in human platelets (Dangelmaier *et al.*, 2000). Increases in JNK activation was found to be mediated by an autocrine ATP stimulation of P2Y receptors in response to stretch in rat aortic SMCs (Hamada *et al.*, 1998). Conversely, in Eah926 endothelial cells UTP was found to have a negative influence on JNK and inhibited TNFα-induced JNK activation (Paul *et al.*, 2000), which also demonstrates a novel mechanism of P2Y and TNF receptor cross-talk regulation. An example of cross-talk between the ERK and JNK pathways was observed in bovine aortic endothelial cells, where VEGF-induced ERK activation was found to be necessary for JNK activation (Pedrum *et al.*, 1998) and both ERK and JNK were found to be necessary for endothelial cell proliferation. The inhibition of glucose-induced JNK activation in SHSY5Y cells by insulin like growth factor-1 (IGF-1), was found to be mediated via ERK and implicated a role for ERK in inhibitory regulation of JNK (Cheng *et al.*, 1998).

The large diversity in cellular regulation of JNK by growth factors and GPCRs in the examples above demonstrates that the role of these alternative MAPKs depends on the cell type. Little is known about the role of JNK and p38 MAPK in human VSMCs. The aim of this chapter was therefore to determine what functions JNK or p38 MAPKs may play in the PDGF mitogenic signalling in human VSMCs and whether P2Y receptor stimulated by UTP or ATP could also elicit a response. In the previous two chapters the data showed that ATP was able to potentiate PDGF mitogenic responses via ERK. However the anti-proliferative influences of UTP and UDP were found not to be dependent on ERK. This chapter therefore further investigates whether UTP or UDP anti-proliferative actions may be elicited via JNK or p38, which would also demonstrate a cross-talk mechanism between P2Y receptors and PDGF receptors to inhibit mitogenic signalling.

5.2 Activation of JNK in human VSMCs

5.2.1 Anisomycin activation of JNK

Previous studies of JNK activation have shown anisomycin to be potent agonists of JNK activation (Hibi *et al.*, 1993, Cano *et al.*, 1994). This potent JNK stimulator was used to verify positive activation of JNK in human VSMCs. Increasing concentrations of anisomycin (ranging between 0.1-100 ng/ml) were used to stimulate JNK in human VSMC. Cells were grown to confluence in 6-well multi dishes and serum-starved for 48 hours. Agonists were applied to cells for various times (duration of stimulations are indicated in the figure legends) and then the cells were lysed using liquid nitrogen and lysis buffer. Cell extracts were protein equalised and separated using SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with a phospho-specific JNK antibody, which in turn was probed with a secondary antibody conjugated to HRP. The results from Western blotting were quantified using a gel documentation system, which measured chemi-illuminescent emission from the blots generated due to HRP metabolising ECL plus reagent (Section 2.6). The data was plotted as the percentage of the basal stimulation as shown in Figure 5.1. The results showed that increasing concentrations of anisomycin significantly increased JNK activation in a dose dependent way ($pEC_{50} = 7.7 \pm 0.28$, $EC_{50} =$ 20.0 nM, $P<0.01$ One-way ANOVA). 30 ng/ml and 100 ng/ml of anisomycin were able to sufficiently stimulate JNK phosphorylation (786 ± 240% above basal levels, $P<0.05$ and 1110 ± 102% above basal levels, $P<0.01$, respectively). This established that adequate levels of JNK activity in these levels could be efficiently measured using phospho-specific antibodies.

5.2.2 PDGF activation of JNK

PDGF has been shown to stimulate JNK activity (Yu *et al.*, 2000, Assefa *et al.*, 1999). However, little is known about growth factor stimulation of JNK in human VSMCs. 1 nM PDGF was initially used to determine adequate, if any, stimulation times in human VSMC. Cells were stimulated for 10, 20, 30, 40, 50 and 60 minutes and the data was plotted as the percentage of the basal stimulation (Figure 5.2). The results showed that a 20-30 minute stimulation time was sufficient to significantly increased JNK

phosphorylation (427.6 ± 128% and 358.9 ± 33.0% of basal response respectively, $P<0.05$, One way ANOVA) and there was no significant difference in activation between 20 and 30 minutes stimulation times. Therefore, the stimulation time of 30 minutes was used for further experiments unless otherwise stated.

The cells were next exposed to increasing concentrations of PDGF (ranging between 0.1-10 nM). JNK phosphorylation was measured and plotted as percentage of basal stimulation (Figure 5.3). The results showed that increasing concentrations of PDGF significantly increased JNK activation in a dose dependent way (pEC_{50} = 9.49 ± 0.45, EC_{50} = 327 pM, $P<0.01$ One-way ANOVA). 1 nM PDGF was able to sufficiently stimulate ERK phosphorylation 428.2 ± 74.4% above basal levels ($P<0.05$) and was used at this concentration for the following experiments.

JNK activity was also studied by using a recombinant protein fragment of c-jun as a substrate (GST-c-Jun) for immunoprecipitating JNK as described in Section 2.7. Briefly, cells were grown, serum starved as before and stimulated for 30 minutes with 0.3 M sorbitol, 50 ng/ml anisomycin and 1 nM PDGF. Cells were lysed and protein equalised. Glutathione sepharose beads bound to GST-c-Jun were added to the lystates and JNK was affinity purified after incubation. The kinase reaction was initiated by the addition of 40 µl kinase buffer containing 250 µM ATP and 5 µCi [γ^{32}P]ATP to each sample. The reaction was incubated for 20 minutes and stopped by the addition of an equal volume of 2 X Laemmli sample buffer and boiling. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. After overnight exposure no visible detection of phosphorylated c-Jun could be seen (data not shown). An alternative method as described in Section 2.8.3 was also used where JNK was immunoprecipitated using polyclonal JNK antibody rather than incubating with GST-c-Jun beads and then assayed using kinase buffer containing 250 µM ATP, 5 µCi [γ^{32}P]ATP and 5µg/reaction GST-c-Jun. This method also failed to detect phosphorylation of c-Jun. Despite positive phosphorylation of JNK with anisomycin and PDGF using phospho-specific antibodies in the results shown above, assessing the activity of JNK by affinity purification or immunoprecipitation was not possible.

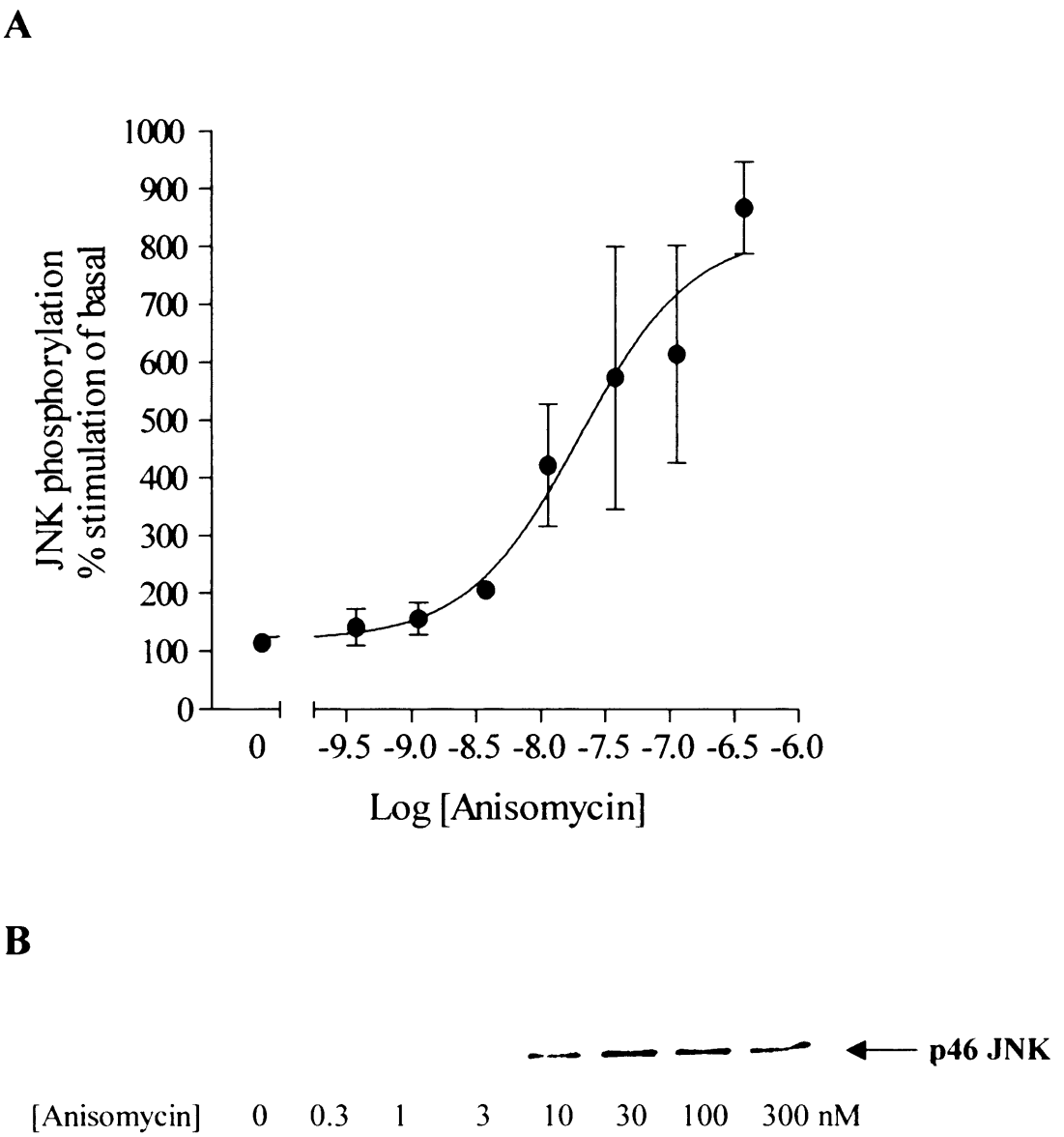


Figure 5.1 The effects of increasing Anisomycin concentrations on JNK

A) Phosphorylation of p46 JNK in human VSMCs stimulated for 30 minutes with increasing concentrations of anisomycin. Data was collected from gel analysis of Western blots probed with phospho-specific JNK 1 antibody and presented as percentage of basal stimulation ± S.E.M. from three experiments.

B) Western blot showing phosphorylation of JNK, which was representative of the three experiments

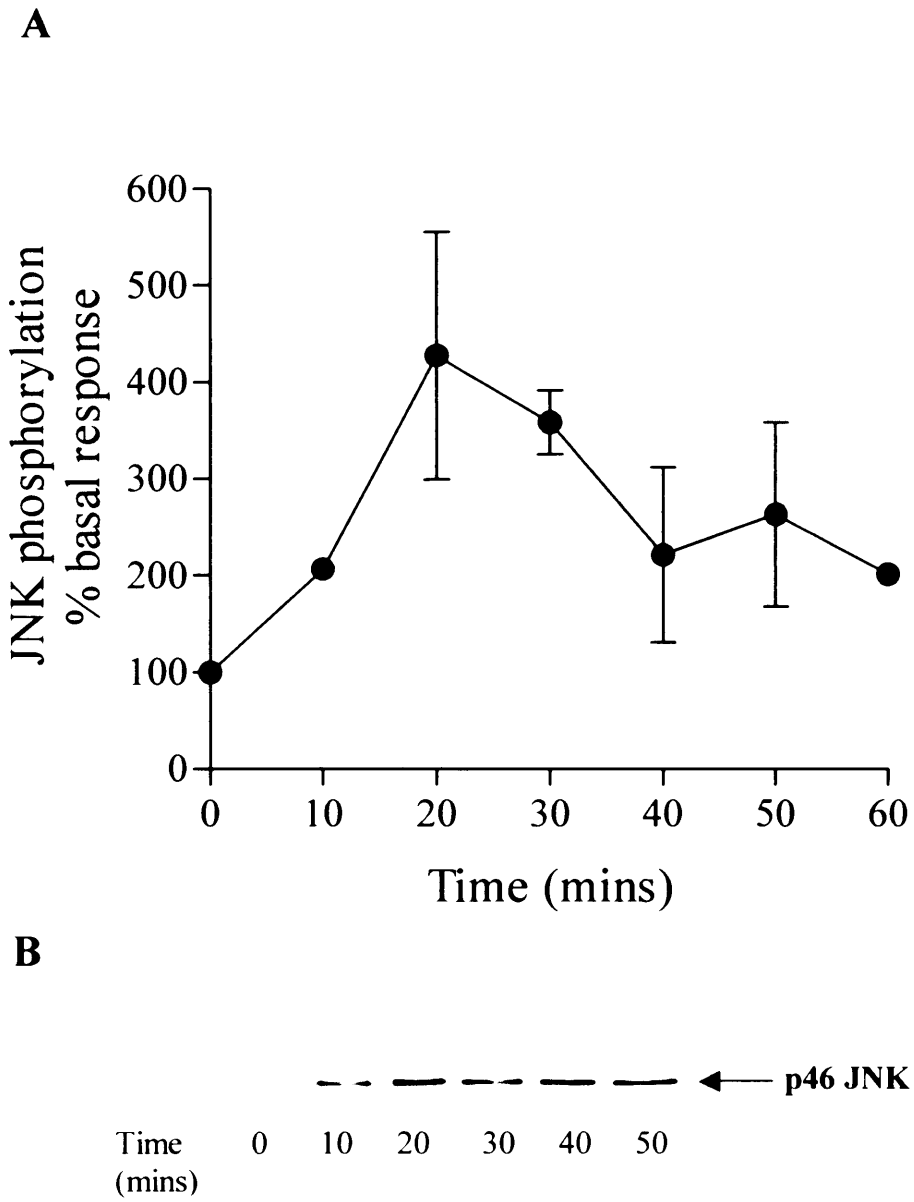
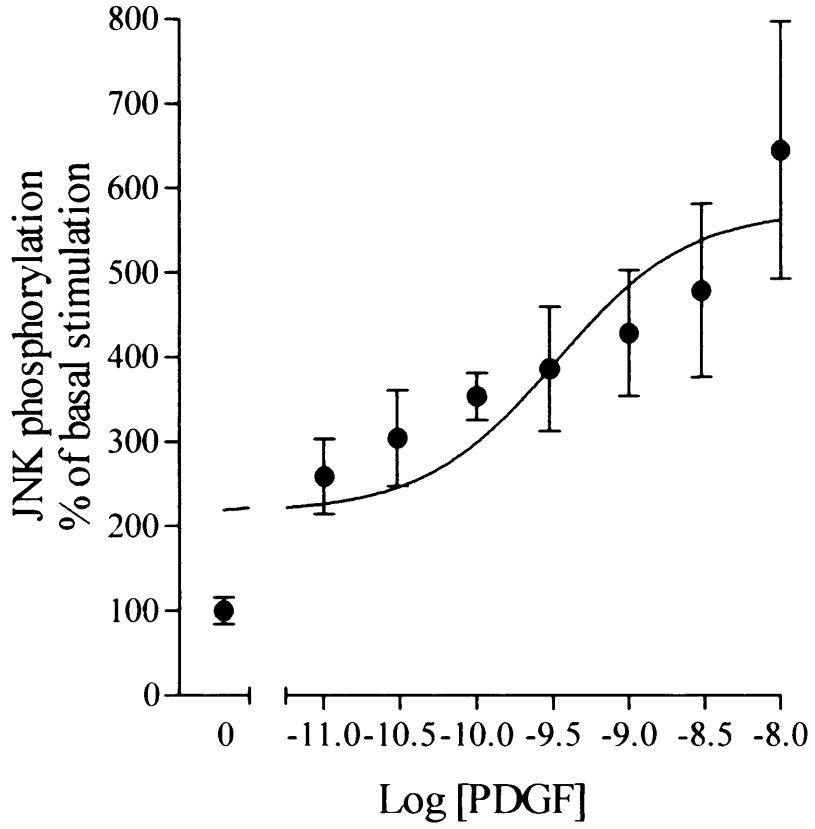


Figure 5.2 Increasing JNK stimulation times with PDGF

A) Phosphorylation of JNK in human VSMCs stimulated with 1 nM PDGF for the times shown. Data was collected from gel analysis of Western blots probed with phospho-specific JNK antibody and presented as percentage of the basal ± S.E.M. from three experiments.

B) Western blot showing timecourse stimulation of p46 JNK with 1 nM PDGF, which was representative of the three experiments

A



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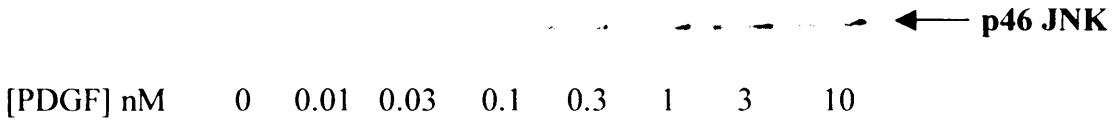


Figure 5.3 The effects of increasing concentrations of PDGF on JNK

A) Stimulation of JNK for 30 minutes with increasing concentrations of PDGF in human VSMCs. Data was collected from gel analysis of Western blots probed with phospho-specific JNK antibody and presented as percentage of the basal response ± S.E.M. from four experiments.

B) Western blot showing phosphorylation of p46 JNK with increasing concentrations of PDGF, which was representative of the four experiments.

5.2.3 The effects of inhibitors on PDGF induced JNK activation

Inhibitors of various kinases were used to investigate their effects on PDGF induced JNK phosphorylation, similar to the experiments performed for ERK activation in the previous chapter. Figure 5.4 shows the effects of 1 μM SB203580, 1 μM LY294002, 30 μM PD98059, 10 μM U0126 and 30 μM Y27632 on JNK phosphorylation stimulated in the presence and absence of 1 nM PDGF and normalised to maximal PDGF response.

The results showed that SB203580, LY294002 and Y27632 inhibitors had no significant influence on basal levels of JNK phosphorylation although there was a slight indication of raised basal levels with SB203580 and LY294002. SB203580, LY294002 and Y27632 also had no significant influence on PDGF induced JNK phosphorylation, which suggests that PDGF was able to stimulate JNK phosphorylation independently of the p38, PI3-kinase and ROCK stimulated pathways. In some individual experiments the inhibition of ROCK appeared to decrease PDGF induced JNK phosphorylation, suggesting that ROCK may play a role in JNK activation induced by PDGF. However, there were varying results between individual experiments and the data was insignificant.

In contrast, the MEK inhibitors PD98059 (30 μM) and U0126 (10 μM) significantly attenuated the PDGF induced JNK phosphorylation reducing the responses to 37.4 ± 18.1% ($P<0.01$ One-way ANOVA with Bonferroni's multiple comparison post test) and 14.5 ± 1.75% ($P<0.001$) of the PDGF maximal response respectively. This suggested that the activation of the ERK pathway by PDGF also resulted in the activation of JNK. This was further investigated by stimulating cells with increasing concentrations of PD98059 (ranging between 3-300 μM) to determine the inhibitory potency on JNK phosphorylation when stimulated with 1nM PDGF. The effects on basal levels were also measured and compared to PDGF stimulated levels. The results are shown in Figure 5.5.

PD98059 significantly inhibited JNK activation stimulated by 1 nM PDGF in a dose dependant manner (pIC_{50} = 4.40 ± 0.51, IC_{50} = 39.4 μM, $P<0.05$ One way ANOVA). PD98059 in the absence of PDGF did not significantly alter basal levels of JNK phosphorylation. The activation by PDGF of JNK, therefore, was apparently dependent on ERK phosphorylation by MEK. This observation is an apparent example of cross talk between the ERK and the JNK pathway and a novel mechanism in human VSMCs stimulated by PDGF.

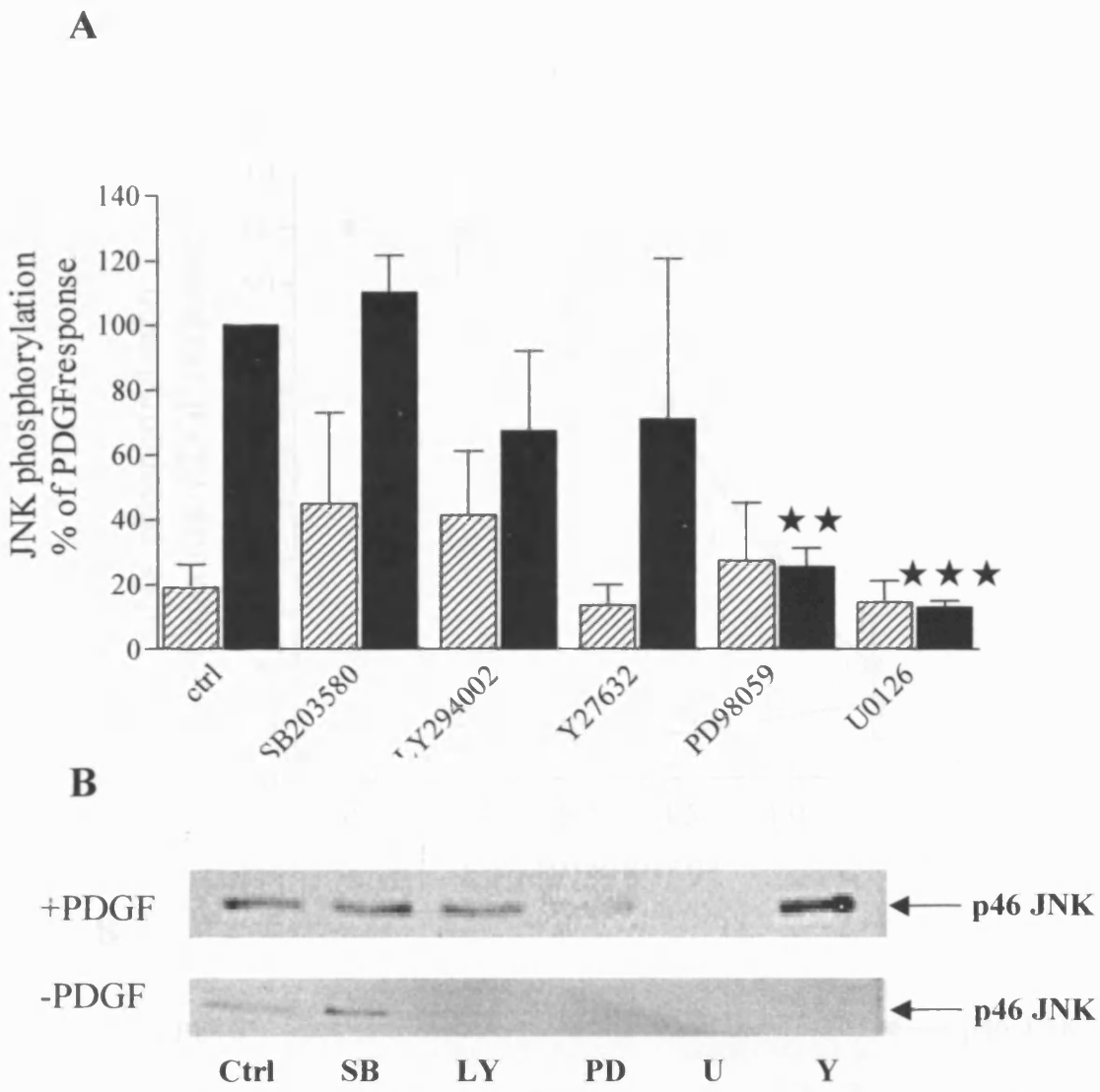


Figure 5.4 The effects of various inhibitors on JNK activation

A) Basal (hatched bars) and 1 nM PDGF (solid bars) stimulation of JNK activation (30 minutes) with 1 μM SB2035880, 1 μM LY294002, 30 μM PD98059, 10 μM U0126 and 30 μM Y27632. Data was collected from gel analysis of Western blots probed with phospho-specific JNK antibody and presented as percentage of the maximal PDGF stimulated response ± S.E.M. from three experiments. Significance from PDGF response on raw data; ★★★ $P<0.001$, ★★ $P<0.01$, One way ANOVA

B) Western blot showing phosphorylation of p46 JNK, which was representative of the three experiments;

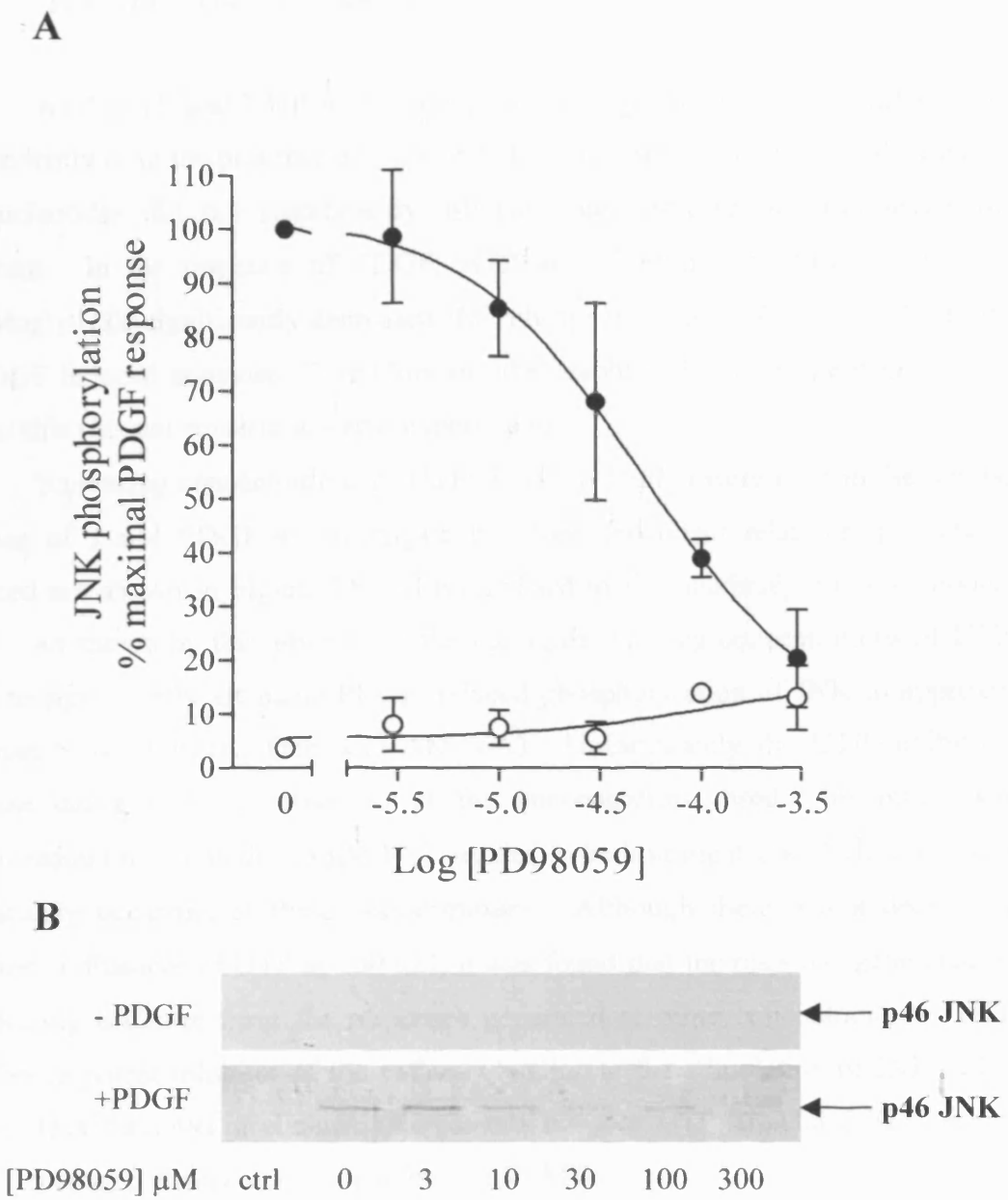


Figure 5.5 The effects of increasing concentrations of PD98059 JNK

A) The effects of increasing concentrations of MEK inhibitor PD98059 on the phosphorylation of JNK in the absence (o) and presence of 1 nM PDGF (•) stimulated for 30 minutes. Data was collected from gel analysis of Western blots probed with phospho-specific JNK antibody and presented as percentage of the basal ± S.E.M. from three experiments

B) Western blot showing phosphorylation of JNK, which was representative of the three experiments.

5.2.4 The influences on nucleotides

ATP, UTP and UDP were used to see if they influenced JNK activation either independently or in the presence of 1 nM PDGF. The results in Figure 5.6 showed that 300 μM nucleotides did not significantly influence any increase in basal levels of JNK activation. In the presence of PDGF, ATP and UDP had no further influence, but surprisingly UTP significantly decreased JNK phosphorylation to 42.1 ± 12.7% ($P<0.05$) of the PDGF induced response. The blots and the graphs indicated some decrease by UDP too, but this was not consistent across experiments.

Increasing concentrations of UTP (3 μM to 1 mM) were used in the absence and presence of 1 nM PDGF to investigate the dose dependent relationship. The results produced are shown in Figure 5.8 and normalised to the maximal response produced by PDGF. As shown by the spline fit of the curve, the varying concentrations of UTP were found to significantly attenuate PDGF induced phosphorylation of JNK to approximately less than 50% ($P<0.001$, One way ANOVA). Unfortunately the UTP inhibitory dose response curve was not observed at the concentrations used. In other words, a concentration range less than 3 μM UTP needed to be investigated as JNK attenuation was significantly occurring at these concentrations. Although there was a decrease in the inhibitory influences of UTP at 100 μM, it was found that the response generated was not significantly different from the responses generated at other concentrations. UTP was, therefore, a potent inhibitor of the pathway leading to the stimulation of JNK induced by PDGF. This is a novel mechanism of cross-talk between UTP-stimulated P2Y receptor and PDGF receptor pathway signalling in human VSMCs.

The simultaneous effects of 100 μM PD98059 and 300 μM UTP on 1 nM PDGF-induced JNK phosphorylation was next investigated. These concentrations were chosen as they produced approximately 50% inhibition of PDGF. Lower concentrations such as 10 μM UTP and 30 μM PD98059 also produced approximately 50% inhibition.

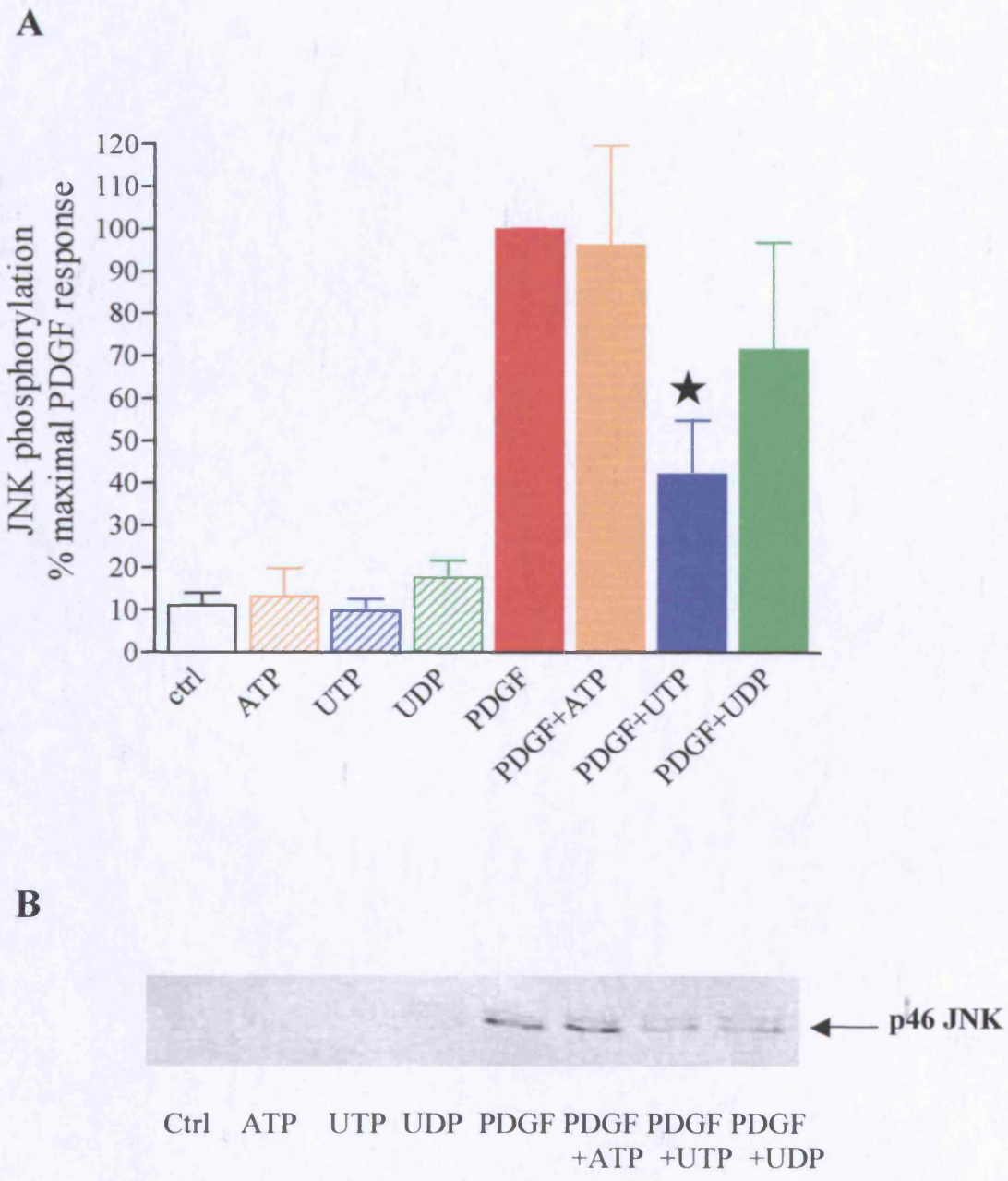
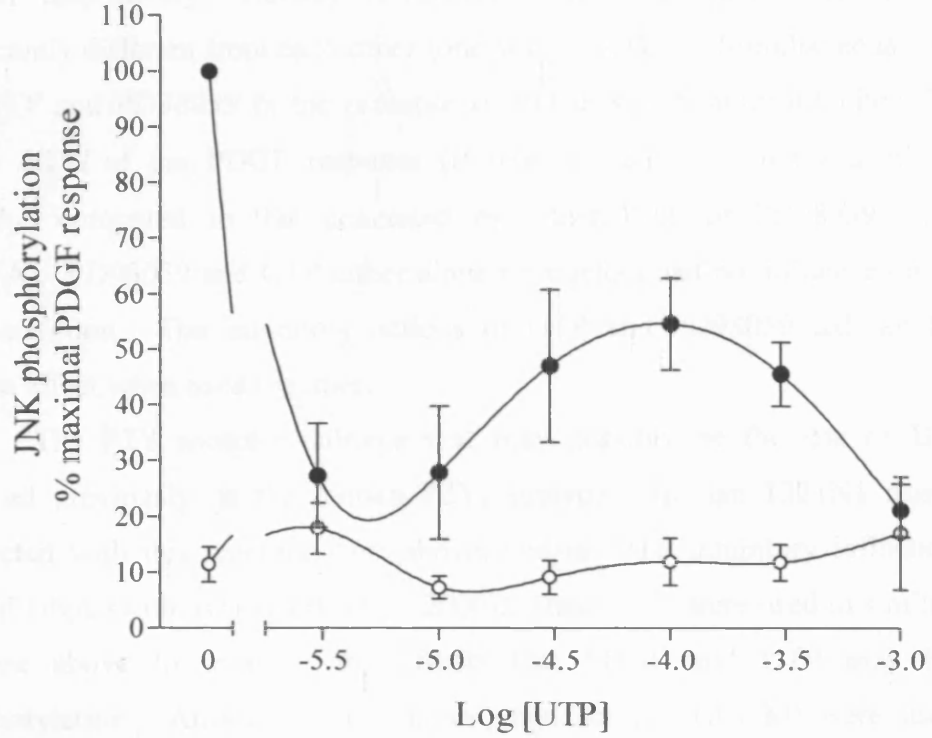


Figure 5.6 The effects of nucleotides on JNK phosphorylation

A) Stimulation of JNK with 300 μM ATP (slashed orange), 300 μM UTP (slashed blue), 300 μM UDP (slashed green), 1 nM PDGF (solid red), PDGF + ATP (solid orange), PDGF + UTP (solid blue), and PDGF + UDP (solid green) for 30 minutes. Data was collected from gel analysis of Western blots and presented as percentage of the basal ± S.E.M. from 4 experiments. Significance from PDGF response on raw data; ★ $P<0.05$, One way ANOVA.

B) Western blot showing phosphorylation of JNK, which is representative of the 4 experiments.

A



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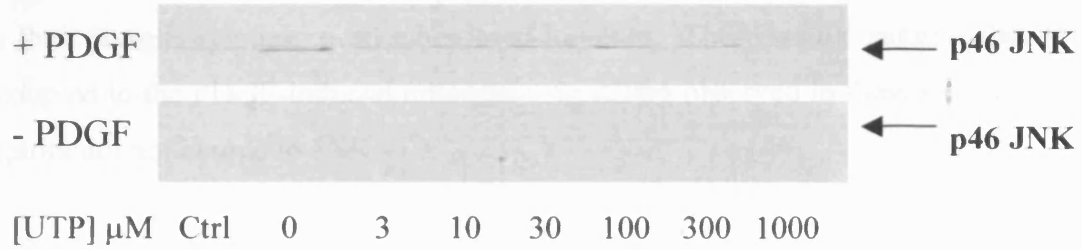
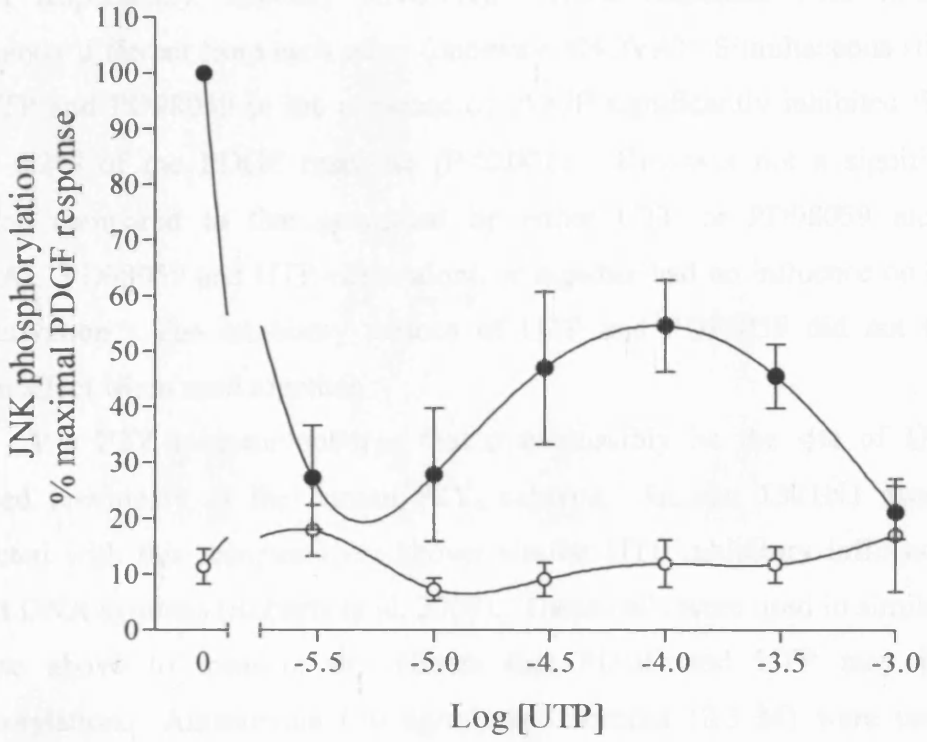


Figure 5.7 Effects of increasing concentration of UTP on PDGF stimulated JNK phosphorylation

A) Stimulation of JNK for 30 minutes with increasing concentrations of UTP ± 1 nM PDGF. Data was collected from gel analysis of Western blots and presented as percentage of the basal levels at each time point ± S.E.M. from 3 experiments

B) Western blot showing phosphorylation of JNK, which is representative of the 3 experiments.

A



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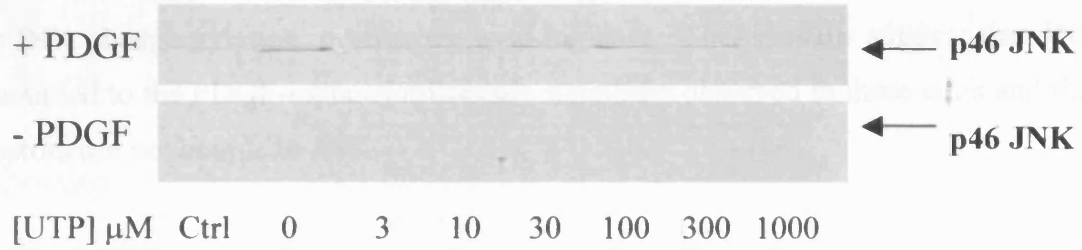


Figure 5.7 Effects of increasing concentration of UTP on PDGF stimulated JNK phosphorylation

A) Stimulation of JNK for 30 minutes with increasing concentrations of UTP ± 1 nM PDGF. Data was collected from gel analysis of Western blots and presented as percentage of the basal levels at each time point ± S.E.M. from 3 experiments

B) Western blot showing phosphorylation of JNK, which is representative of the 3 experiments.

The results are shown in Figure 5.8 and presented as the percentage of maximal PDGF response. Both UTP and PD98059 significantly inhibited JNK phosphorylation by reducing it to 49.0 ± 5.23% and 32.6 ± 6.70% of the PDGF-induced response ($P<0.01$ and $P<0.001$ respectively, one-way ANOVA). These responses were found not to be significantly different from each other (one-way ANOVA). Simultaneous stimulation with both UTP and PD98059 in the presence of PDGF significantly inhibited the response to 29.2 ± 6.2% of the PDGF response ($P<0.001$). This was not a significantly greater inhibition compared to that generated by either UTP or PD98059 alone (one way ANOVA). PD98059 and UTP either alone or together had no influence on basal levels of JNK activation. The inhibitory actions of UTP and PD98059 did not to produce an additive effect when used together.

The P2Y receptor subtype that may possibly be the site of UTP action, as discussed previously, is the human $P2Y_4$ subtype. Human 1321N1 astrocytoma cells transfected with this receptor have shown similar UTP inhibitory influences on PDGF-induced DNA syntheis (Roberts et al, 2000). These cells were used in similar experiments to those above to measure the effects that PDGF and UTP may have on JNK phosphorylation. Anisomycin (50 ng/ml) and sorbitol (0.3 M) were used as positive activators of JNK. The results are shown in Figure 5.9. Both anisomycin and sorbitol were found to stimulate JNK phosphorylation. However, 1 nM PDGF and 300 μM did not stimulate JNK phosphorylation, even when used together. These results suggest that JNK was not coupled to the PDGF-induced mitogenic signalling observed in these cells and that P2Y receptors did not couple to JNK.

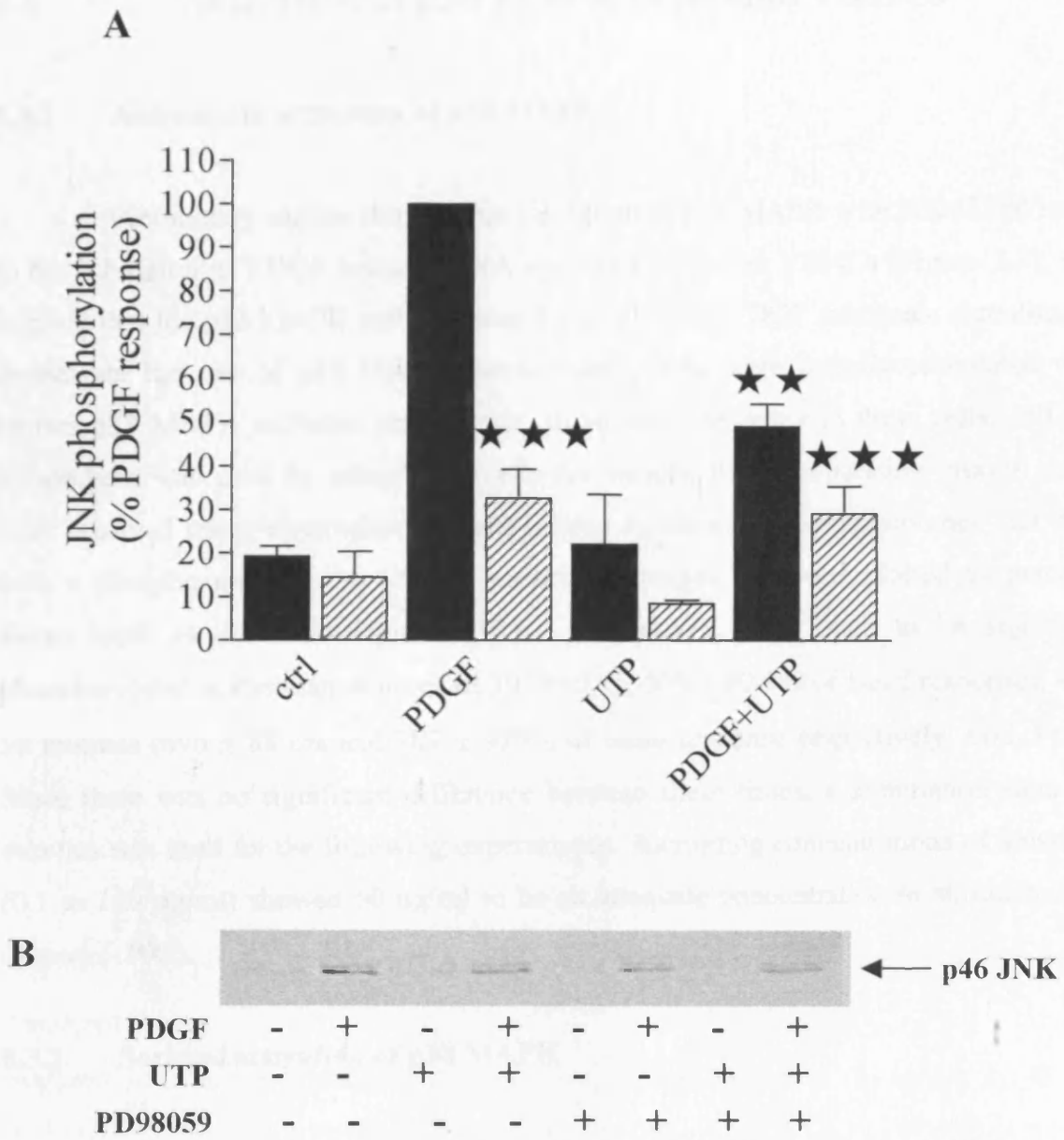


Figure 5.8 The effects of PD98059 and UTP on JNK activation

A) Phosphorylation of JNK stimulated with 1 nM PDGF ± 300 μM UTP in the absence (solid bars) and presence (hatched bars) of 100 μM PD98059. Data was collected from gel analysis of Western blots and presented as percentage of the basal ± S.E.M. from three experiments. Significance from PDGF response on raw data; ★★★ $P<0.001$, ★★ $P<0.01$, one way ANOVA.

B) Western blot showing phosphorylation of JNK, which is representative of the three experiments.

5.3 Activation of p38 MAPK in human VSMCs

5.3.1 Anisomycin activation of p38 MAPK

Preliminary studies showed that inhibition of p38 MAPK with SB203580 resulted in the inhibition of PDGF induced DNA synthesis in human VSMCs (Figure 3.3), which implied that the p38 MAPK pathway may be involved in PDGF mitogenic signalling. To investigate the role of p38 MAPK more closely, cells were initially stimulated with a known p38 MAPK activator, anisomycin, to validate the assay in these cells. 50 ng/ml anisomycin was used by stimulating cells for various times, separating protein extracts were resolved using electrophoresis and blotted to nitrocellulose membranes and probed with a phospho-specific p38 MAPK antibody. Imaged data was plotted as percentage above basal as shown in Figure 5.10A. p38 MAPK was found to be significantly phosphorylated at stimulation times of 30 ($P<0.05$, 539 ± 82.9% of basal response), 40 and 50 minutes (696 ± 88.2% and 787 ± 979% of basal response respectively, both $P<0.01$). Since there was no significant difference between these times, a stimulation time of 40 minutes was used for the following experiments. Increasing concentrations of anisomycin (0.1 to 100 ng/ml) showed 50 ng/ml to be an adequate concentration to stimulate of p38 (Figure 5.10C).

5.3.2 Sorbitol activation of p38 MAPK

Increasing concentrations of sorbitol (ranging between 0.01 to 1 M) were also used to stimulate p38 MPAK (Figure 5.11). Sorbitol significantly increased p38 MAPK phosphorylation in a dose dependent way ($pEC_{50} = 0.48 \pm 0.26$, $EC_{50} = 333$ μM, $P<0.01$).

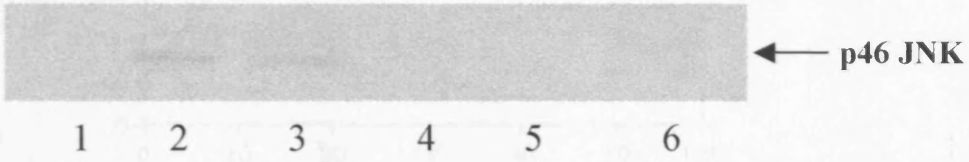


Figure 5.9 JNK stimulation in 1321N1 cells transfected with $hP2Y_4$ receptor

Western blot showing phosphorylation of JNK stimulated with **1**) basal, **2**) 50 ng/ml anisomycin, **3**) 0.3 M sorbitol, **4**) 1 nM PDGF, **5**) 300 μM UTP and **6**) PDGF + UTP. The blot shown is representative of 3 experiments.

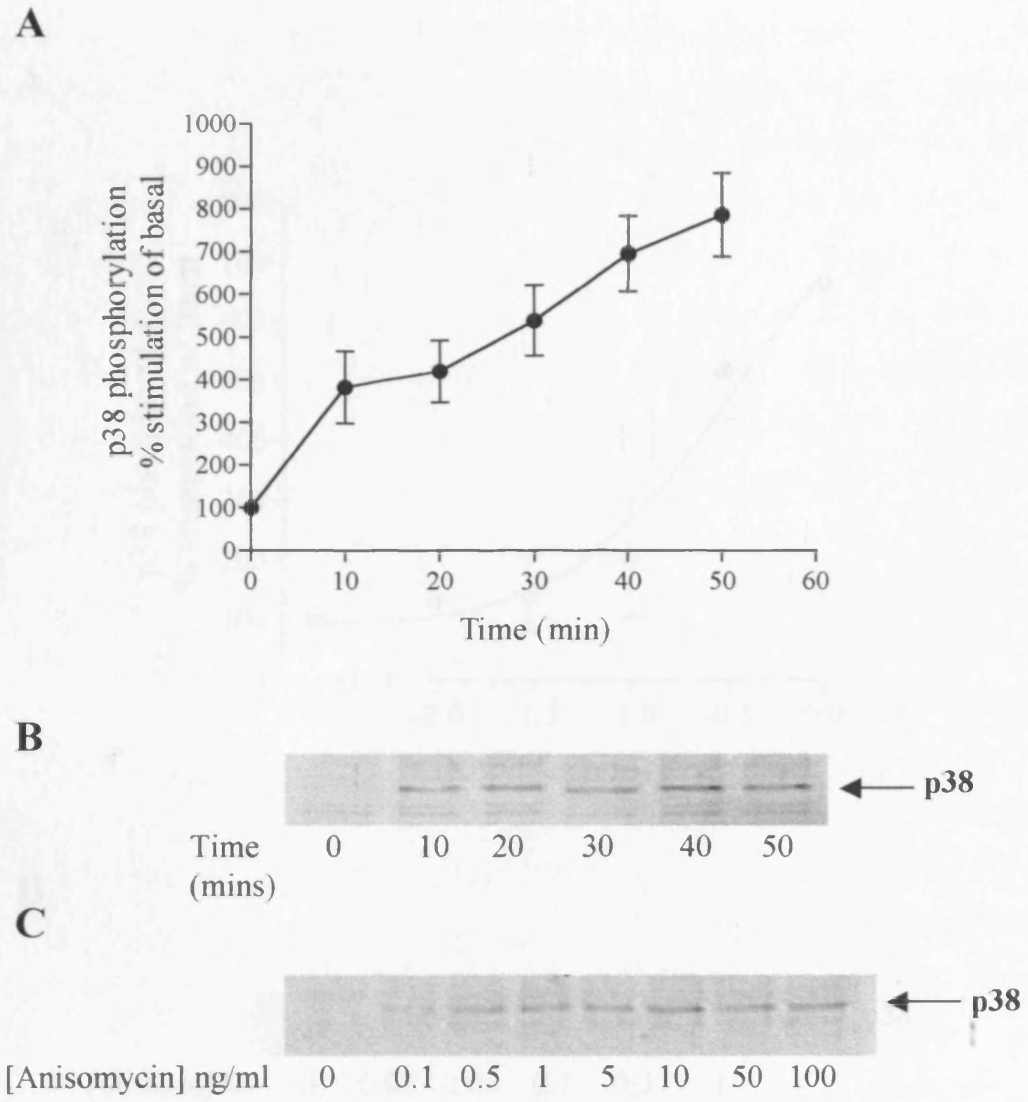


Figure 5.10 Anisomycin activation of p38 MAPK

A) Stimulation of JNK in human VSMCs stimulated with 50 ng/ml Anisomycin for the times shown. Data was collected from gel analysis of Western blots probed with phospho-specific p38 MAPK antibody and presented as percentage of the basal ± S.E.M. from two experiments.

B) Western blot showing timecourse stimulation of p38 MAPK with 50 ng/ml Anisomycin for the times shown which is representative of the two experiments.

C) Western blot showing the effects of p38 MAPK stimulation with increasing concentrations of Anisomycin (blot is representative of the two experiments).

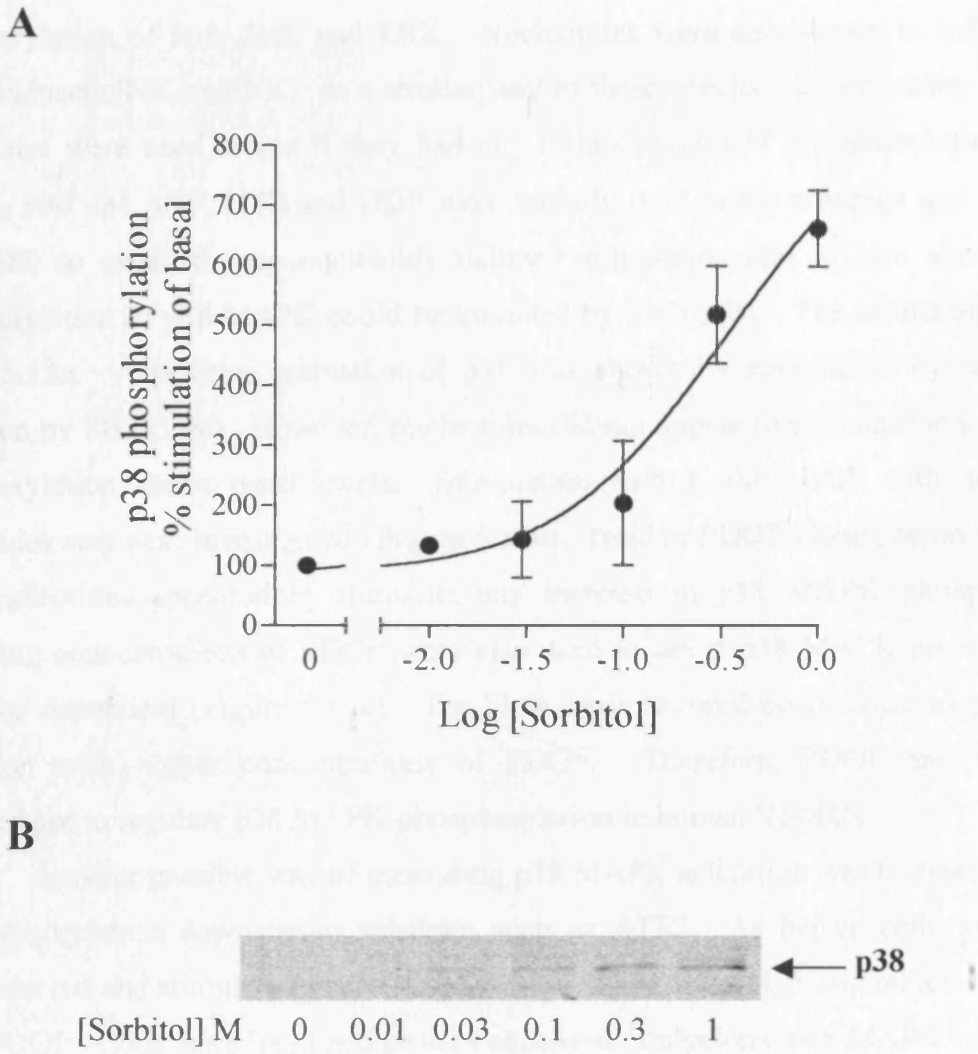


Figure 5.11 Increasing Sorbitol concentration effects on p38 MAPK

A) Stimulation of p38 MAPK in human VSMCs stimulated for 30 minutes with increasing concentrations of Sorbitol. Data was collected from gel analysis of Western blots probed with phospho-specific p38 MAPK antibody and presented as percentage of basal ± S.E.M. from two experiments.

B) Western blot showing phosphorylation of p38 MAPK, which is representative of the two experiments

5.3.3 PDGF & nucleotides

The data presented in this and the previous chapter have shown PDGF to increase phosphorylation of both JNK and ERK. Nucleotides were also shown to influence this PDGF induced JNK or ERK. In a similar way to these previous experiments, PDGF and nucleotides were used to see if they had any influence on p38 phosphorylation. 0.3 M sorbitol, 300 μM ATP, UTP and UDP were initially used in the presence and absence of SB203580 to establish any nucleotide induced stimulation and to also show that the phosphorylation of p38 MAPK could be inhibited by SB203580. The results are shown in Figure 5.12a. A positive activation of p38 was shown by stimulation by sorbitol and inhibition by SB203580. However, nucleotides did not appear to stimulate any increase in phosphorylation above basal levels. Stimulation with 1 nM PDGF with and without nucleotides was next investigated (Figure 5.12b). Neither PDGF alone nor co-stimulation with nucleotides appeared to stimulate any increase in p38 MAPK phosphorylation. Increasing concentrations of PDGF were also used to see if p38 MAPK phosphorylation was dose dependent (Figure 5.12c). The blots again showed no increase in p38 MAPK activation with higher concentrations of PDGF. Therefore, PDGF and nucleotides appeared not to regulate p38 MAPK phosphorylation in human VSMCs.

Another possible way of measuring p38 MAPK activation was to assess its ability to phosphorylate a downstream substrate such as ATF2. As before cells were grown, serum starved and stimulated for 30 minutes with 0.3 M sorbitol, 50 ng/ml anisomycin and 1 nM PDGF. Cells were lysed and protein equalised. Polyclonal p38 MAPK antibody was added to immunoprecipitate p38 MAPK and then kinase buffer containing 5μg/reaction ATF2, 250 μM ATP and 5 μCi [γ^{32}P]ATP was added after washing (see Section 2.7.3). The reaction was incubated for 20 minutes and stopped by the addition of Laemmli sample buffer and boiling. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. After overnight exposure no visible detection of phosphorylated ATF2 could be seen (data not shown), although positive phosphorylation of p38 MAPK with anisomycin and sorbitol was shown in the results above. Therefore, p38 MAPK activity could not be measured by direct phosphorylation of an in vitro substrate ATF2

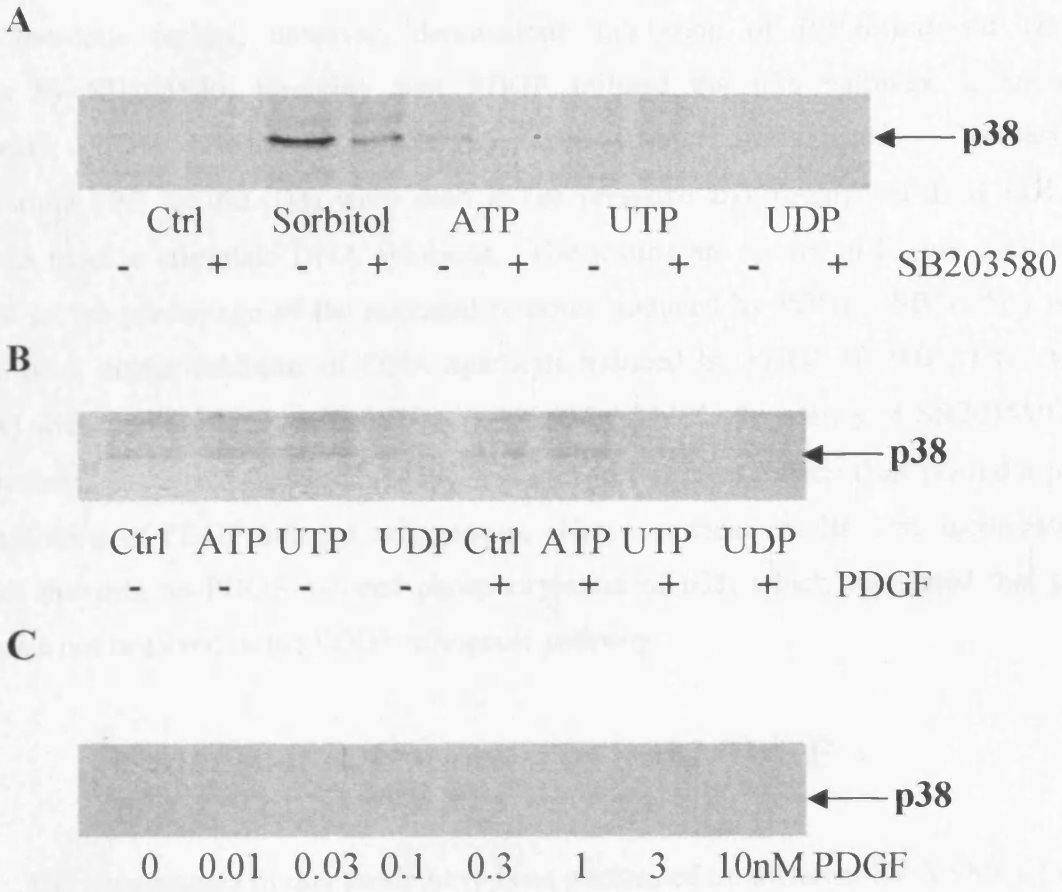


Figure 5.12 The effects of PDGF, nucleotides & SB203580 on p38

A) Western blot showing the effects of 0.3 M Sorbitol, 300 μM ATP, 300 μM UTP, 300 μM UDP ± 1 μM SB203580, which is representative of the 3 experiments.

B) Western blot showing the effects of 300 μM ATP, 300 μM UTP, 300 μM UDP, 1 nM PDGF, PDGF + ATP, PDGF + UTP, and PDGF + UDP, which is representative of the 5 experiments.

C) Western blot showing the effects of increasing concentrations of PDGF on p38 MAPK, which is representative of the 3 experiments.

All stimulated for 40 minutes

5.3.4 p38 MAPK inhibitor SB203580

Previous results, however, demonstrate inhibition of PDGF-induced DNA synthesis by SB203580, implying that PDGF utilised the p38 pathway to induce mitogenesis. The effects of SB203580 were further investigated. Increasing concentrations (0.3 to 100 μM) were used in the presence and absence of 1nM PDGF, which was used to stimulate DNA synthesis. The results are shown in Figure 5.13 and presented as the percentage of the maximal response induced by PDGF. SB203580 was found to be a potent inhibitor of DNA synthesis induced by PDGF ($P<0.01$, One way ANOVA) with a $pIC_{50} = 7.94 \pm 0.29$ ($IC_{50} = 11.6$ nM). This high potency of SB203580 on DNA synthesis suggested that p38 MAPK was indeed inhibited, which then played a part in the inhibition of PDGF induced mitogenesis. However these results were inconsistent with data showing no PDGF-induced phosphorylation of p38, which suggested that p38 MAPK was not involved in the PDGF mitogenic pathway.

5.4 Scatter plot of responses to 1 nM PDGF

The experiments in this thesis have been performed on different SV VSMCs from different patients. Therefore variations in responses may occur. 1 nM PDGF has been shown to stimulate significant increases in DNA synthesis, ERK phosphorylation and JNK phosphorylation. Therefore the responses produced to 1 nM PDGF from all the experiments performed for DNA synthesis were plotted on a scatter graph. Similarly, the increases in ERK phosphorylation and JNK phosphorylation generated by 1 nM PDGF were also plotted (Figure 5.14). The responses are presented as % above basal from each experiment. The mean increase in DNA synthesis was 826 ± 63.0 % above basal, for ERK phosphorylation was 1102 ± 369 % above basal and for JNK phsophorylation was 1179 ± 595

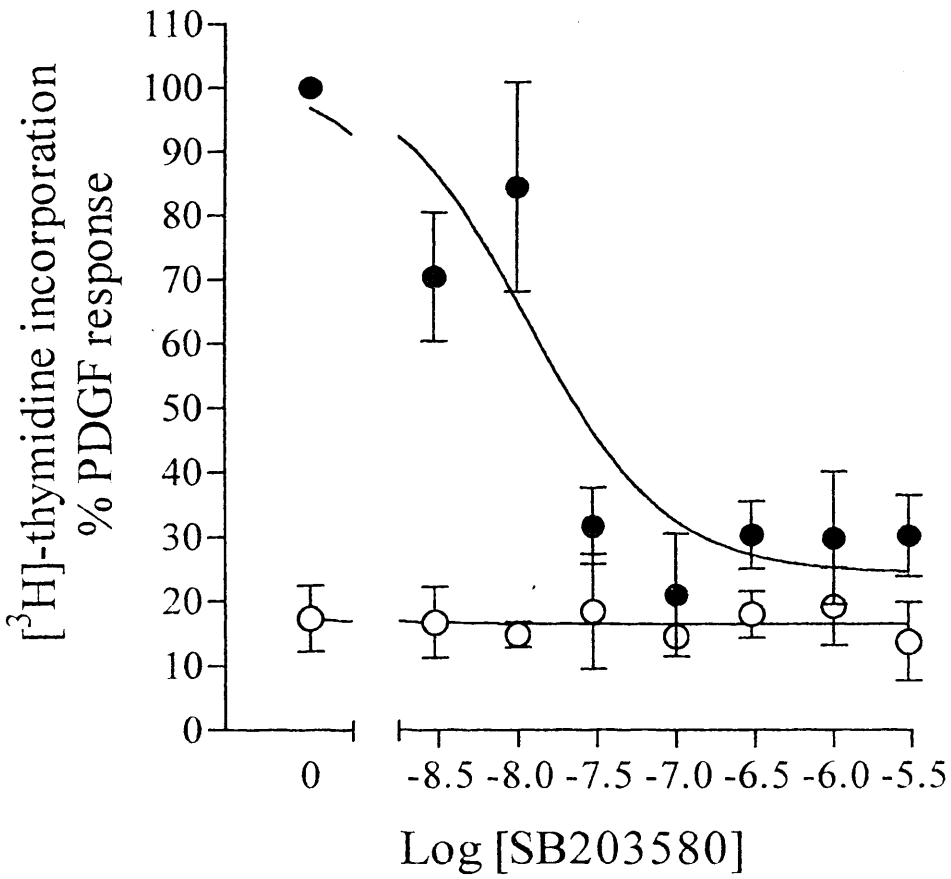


Figure 5.13 The effects of SB203580 on DNA synthesis

The effects of increasing concentrations of SB203580 on [^{3}H] thymidine incorporation into DNA in the presence and absence of 1 nM PDGF stimulated for 1 hour. Data is presented as percentage of the PDGF stimulated response ± SEM from 3 separate experiments each carried out in triplicates.

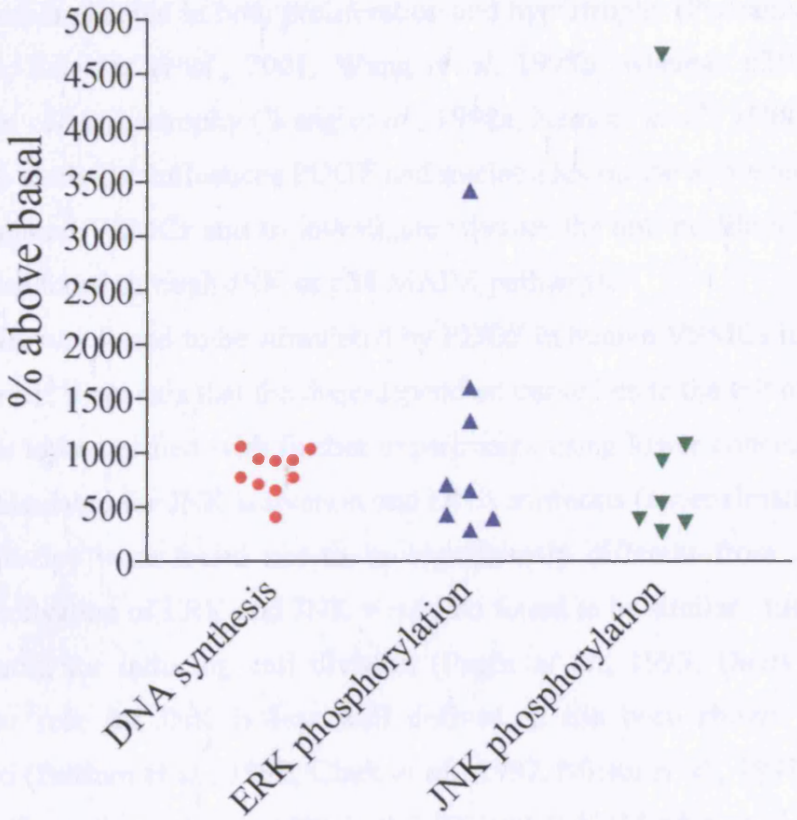


Figure 5.14 Scatter plot of PDGF stimulated responses

Scatter plot showing increases in DNA synthesis (■), ERK phosphorylation (▲) and JNK (▼) phosphorylation in response to 1 nM PDGF from experiments performed throughout this study across SV VSMCs cells from different patients. Data is presented as % above basal of from 10 experiments for DNA synthesis, 8 experiments for ERK phosphorylation and 7 experiments for JNK phosphorylation.

5.5 Discussion

PDGF and P2Y receptors (Minden *et al.*, 1994, Lallemand *et al.*, 1998, Huwiler *et al.*, 1997, Paul *et al.*, 2000) have been shown to stimulate JNK activity in many different types of cell including VSMCs (Li *et al.*, 1999, Hamada *et al.*, 1998). P2Y stimulation of p38 MAPK has also been shown to occur (Huwiler *et al.*, 2000, Dangelmaier *et al.*, 2000). JNK has been implicated in both proliferation and hypertrophy (Pedrum *et al.*, 1998, Izumi et al., 2000, Schmitz *et al.*, 2001, Wang *et al*, 1998b) whereas p38 MAPK has been implicated in cell hypertrophy (Wang *et al.*, 1998a, Nemoto *et al.*, 1998). The aim of this study was to study the influences PDGF and nucleotides on the activation of JNK and p38 MAPK in human VSMCs and to investigate whether the anti-proliferative roles UTP and UDP were mediated through JNK or p38 MAPK pathways.

JNK was found to be stimulated by PDGF in human VSMCs in a dose dependent way. However, it appears that the dose-dependent curve lies to the left of the graph shown, which needs to be verified with further experiments using lower concentrations of PDGF. The EC_{50} calculated for JNK activation and DNA synthesis (approximately 0.3 nM and 3.5 nM respectively) were found not to be significantly different from each other. Dose dependent activation of ERK and JNK were also found to be similar. ERK has been shown to be essential for inducing cell division (Pagés *et al.*, 1993, Davis *et al.*, 1993) and although the role for JNK is less well defined, it has been shown to be involved in proliferation (Pedrum et al., 1998, Clark *et al.*, 1997, Mitsui *et al.*, 1997). Various growth factors have been shown to stimulate both ERK and JNK (Minden *et al.*, 1994, Moxham *et al.*, 1996, Rao *et al.*, 1996, Shapiro *et al.*, 1996) leading to the suggestion that both may contribute to cell proliferation. In this study PDGF has been shown to be mitogenic in human VSMCs leading to increased DNA synthesis (Figure 3.1), ERK stimulation (Figure 4.2), cell cycle progression (unpublished data from M.R. Boarder et al) and cell division (White *et al.*, 2000) along with JNK activation. This data is consistent with the hypothesis that the JNK pathway is necessary for PDGF-induced proliferation in human VSMCs. Immunoprecipitated JNK activity as well as p38 MAPK activity could not be measured in kinase assays in these cells using the methods described. A feasible explanation for this may be due to the low levels of proteins expressed in these cells, which would make immunoprecipitation of a specific protein difficult to achieve and the resultant kinase activity difficult to detect.

Unexpectedly, MEK inhibitors were found to inhibit this PDGF-induced JNK-activation in a dose dependent way. The IC_{50} produced by PD98059 for JNK and DNA synthesis (approximately 36 μM and 39 μM respectively) were not significantly different from each other (students t-test). ERK and JNK inhibition by PD98059 (IC_{50} = approximately 11 μM and 36 μM respectively) were also not-significant from each other (students t-test), which not only supports the hypothesis that JNK is involved in mitogenesis, but also that it plays an important role in ERK-dependent signalling to mitogenesis. Similar observations have been seen in vascular endothelial cells where stimulation of JNK was found to be necessary for proliferation (Pedrum *et al.*, 1998). The down regulation of VEGF-induced JNK activation using dominant negative SEK-1 or JNK-1 resulted in an attenuation of proliferation. MEK inhibitors were also shown to inhibit this JNK activation and that SEK-1 activation was dependent on ERK. This ERK to JNK cross talk via SEK-1 could possibly be a mechanism of the system of cross-talk seen in human VSMCs and would therefore be consistent with the hypothesis that the JNK pathway was necessary for proliferation. ERK cannot directly activate JNK, as JNK is not a substrate of ERK (Songyang *et al.*, 1996). Raf-1 and MEK1/2 have also been shown not to activate JNK (Minden *et al.*, 1994, Sanchez *et al.*, 1994) and Raf-1 does not activate MKK/JNKK/SEK-1 (Lin *et al.*, 1995). MEKK1 can activate MEK1/2 (Lange-Carter *et al.*, 1993), but MKK/JNKK cannot stimulate ERK (Lin *et al.*, 1995). The possibilities that remain are that ERK could regulate one or more of the JNK pathway kinases therefore indirectly activating JNK. This was shown when dominant negative SEK-1 was co-transfected with constitutively active ERK into endothelial cells. JNK activation was inhibited showing that ERK activates SEK-1 through an upstream signalling to this kinase (Pedrum *et al.*, 1998).

The mechanism of crosstalk between the ERK and JNK pathway may engage proteins other than those involved in the ERK/JNK cascade. An example of such a protein is JNK/SAPK-associated protein1 (JSAP1), which was shown to function as a putative scaffold protein in the JNK cascade binding JNK SEK1, and MEKK1 (Ito et al., 1999, Kuboki *et al.*, 2000). In COS-7 cells it was also found to bind unphosphorylated MEK when stimulated with PMA or a constitutively active Raf-1 and in doing so, inhibited the ability of Raf-1 to phosphorylate it, which resulted in the inhibition of ERK phosphorylation (Kuboki *et al.*, 2000). Although this is an example of the inhibitory

influence of JNK on the ERK pathway it shows the increasing evidence for cross talk between the ERK and JNK pathway involving proteins other than kinases.

The increase in JNK activation by PDGF was apparent at 10 minutes which shows rapid induction of JNK by ERK, which was activated as early as 5 minutes. This rapid and potent activation of ERK, which then proceeds to rapid onset of increased JNK activity, cannot be explained by ERK mediating a signal to increase transcription of other growth factor or mediators e.g. PDGF, which would then be secreted to produce an autocrine JNK activation, as this would take several hours (Wilson *et al.*, 1993). Autocrine ATP activation of JNK has been shown to occur within 5 minutes of inducing stretch on rat VSMCs (Hamada *et al.*, 1998). It is possible that in the vasculature stretch may stimulate release of factors including nucleotides, which could act on their P2Y receptor subtypes and induce anti-proliferative actions in the case of UTP. Another possible explanation is that inhibition of JNK by PD98059 may be occurring via an ERK-independent manner. In other words PD98059 may inhibit other kinases such as COX or PKB and JNK may lie downstream of these kinases. This requires further investigation and is discussed in further work in Chapter 8.

In this study a novel mechanism of cross-talk regulation between UTP-stimulated P2Y receptors and PDGF receptors was observed in human VSMCs. UTP exerted a strong attenuation of the PDGF-stimulated JNK activation, which may play a role in the anti-proliferative signalling induced by UTP. P2Y receptors (Huwiler *et al.*, 1997. Hamada *et al.*, 1998, Paul *et al.*, 2000) and G-protein coupled receptors have recently been shown to stimulate JNK in a number of different cell types (Coso *et al.*, 1995b, Zohn *et al.*, 1995, Rao *et al.*, 1996, Shapiro *et al.*, 1996). However, in these experiments UTP alone did not influence JNK basal levels either by attenuating or potentiating it. Neither ATP nor UDP had any effect on JNK activation either in the presence or absence of PDGF. This suggests that P2Y receptors were not coupled to the activation of JNK, but in fact a UTP-sensitive P2Y receptor was indirectly coupled to the inhibition of JNK activation. These results propose that the P2Y receptor subtype involved in JNK inhibition by UTP was not $P2Y_2$, $P2Y_6$ or $P2Y_{11}$ as ATP and UTP are both equi-potent at $P2Y_2$, ATP, but not UTP is a potent agonist at $P2Y_{11}$ and UDP is most potent agonist at $P2Y_6$. At the human $P2Y_4$ receptor neither ATP nor UDP are potent agonists, which therefore implies that the response was mediated via the $P2Y_4$ receptor subtypes. Earlier studies showed that 2MeSADP, which is the most potent agonist at $P2Y_1$ receptors had no influence on mitogenesis (Figure 3.4). Although this agonist was not used in the JNK activation work,

$P2Y_1$ can also be ruled out because UTP is not an agonist. Similar UTP inhibitory observations were also seen in human-derived Eahy926 endothelial cells (Paul *et al.*, 2000). UTP was found to attenuate the TNFα and sorbitol–stimulated JNK and p38 MAPK activity, which was found to mediate through either the $P2Y_2$ or $P2Y_4$ receptor subtypes. However, in 1321N1 cells transfected with human $P2Y_2$ and $P2Y_4$ receptors this group found that TNFα-induced JNK activation was not inhibited by UTP but in fact UTP stimulated JNK activation alone. This suggests that the effects mediated by P2Y receptors are dependent upon cell type. In rat VSMC, thrombin induced DNA synthesis and JNK activation was found to be inhibited by increases in cAMP induced by forskolin, but had no effect on ERK stimulation (Rao *et al.*, 1996). PDGF mitogenic effects were also antagonized by increases in cAMP. These results showed a correlation between JNK activation and its association with thrombin-induced mitogenesis. The hypothesis that the human $P2Y_4$ receptor subtype could be responsible is consistent with previous DNA synthesis inhibition by UTP (Figure 3.2 & 3.3) and also studies performed on 1321N1 human astrocytoma cells transfected with human $P2Y_4$ receptor subtype, which showed inhibition of PDGF-induced DNA synthesis to occur when stimulated with UTP (Roberts *et al.*, 1999). However, in this study JNK phosphorylation was found not to be stimulated by PDGF in 1321N1 cells transfected with human $P2Y_4$. UTP alone or with PDGF also had no affect on JNK phosphorylation.

The site of UTP inhibition was found to be at the level of JNK phosphorylation as there was a decrease in the degree of phosphorylation measured by phospho-specific antibodies. It is unclear as to precisely where the inhibitory actions of UTP may be acting to inhibit JNK phosphorylation. The two possibilities are 1) inhibition of the ERK to JNK pathway cross-talk leading to the stimulation of JNK or 2) ERK-independent inhibition, which could possibly be mediated through a phosphatase or prevention of action of JNK pathway kinases. In the presence of UTP, which alone inhibited PDGF-induced JNK-stimulation, PD98059 was found not to potentiate this inhibition any further. Therefore, the combined inhibitory properties of UTP and PD98059 on JNK phosphorylation was not additive despite them being potent inhibitors individually. However, this data also does not establish which of the two hyothesis above may be true. The inhibitory influences of UTP were also observed at very low concentration than expected (less than 10μM), which was inconsistent with DNA synthesis studies, which showed UTP to significantly inhibit

mitogenesis at 100 μM concentrations and higher. This suggests that the inhibition of JNK was necessary but not sufficient for inhibition of DNA synthesis by UTP.

Nucleotides and PDGF instigated no detectable levels of p38 MAPK phosphorylation. This suggests that p38 MAPK may not have been involved in the proliferative signalling of PDGF, which would be consistent with previous reports showing p38 MAPK to be involved in regulating hypertrophy rather than proliferation (Wang *et al.*, 1998a, Nemoto *et al.*, 1998, Orsini et al., 1999). However inhibition of DNA synthesis by SB203580 was observed, which implied that the p38 MAPK pathway played a crucial role in the PDGF mitogenic signalling. A possible explanation for this inconsistency between DNA synthesis and p38 phosphorylation data could be due SB203580 inhibiting a kinase other than p38. Previous reports have shown SB203580 to inhibit COX1, COX2, GSK and PKB (Börsch-Haubold et al., 1998, Davies *et al.*, 2000), although with lower affinity. It could therefore be possible that the inhibition of these kinases lead to decreased DNA synthesis. In summary, PDGF and P2Y signalling could not be shown to couple to the p38 MAPK pathway, and in this context SB203580 may not have inhibited p38 MAPK but an alternative substrate.

UDP was also shown to attenuate PDGF induced DNA synthesis (Figure 3.2). However unlike UTP it did not attenuate PDGF-induced JNK activation. This suggests that UDP, most likely via $P2Y_6$ for which it is most potent, mediates antiproliferative signalling via a mechanism that is not dependent on the JNK pathway. At present it is unclear how UDP may be mediating this attenuation. As discussed in the previous chapter UTP had no affect on ERK activation. However similar methods were not employed to investigate the effect of UDP on ERK during longer periods of stimulation. As shown in this chapter it is also unclear what role p38 MAPK may have in the PDGF signalling pathway. Therefore it remains unclear whether UDP may be utilising this pathway.

Chapter 6

Investigation of ROCK in hVSMC

6.1 Introduction

The Rho family of small GTP-binding proteins regulate the organization of the actin cytoskeleton and include Rho, Rac and Cdc42 (Hall 1994, Foster *et al* 1996). Both Rac and Cdc42 stimulate JNK and p38 MAPKs (Olson *et al.*, 1995, Bagrodia *et al.*, 1995). Rho on the other hand does not regulate JNK or p38, but controls the assembly of actin stress fibres, focal adhesion complexes and smooth muscle contraction (Hall 1994, Harata *et al.*, 1992, Noda *et al.*, 1994). Like Rac and Cdc42 it is active in the GTP bound form and requires an extracellular signal to activate it, such as lysophosphatidic acid (LPA) (Ridley & Hall, 1992). There is evidence to suggest that activation of Rho down regulates $p27^{kip}$ and increases DNA synthesis, therefore implicating Rho in the regulation of cell growth (Lamarche *et al.*, 1996, Narumiya *et al.*, 1997, Olson *et al.*, 1995, Seasholtz *et al.*, 1999). This was also observed in human VSMCs (Laufs *et al.*, 1999). One of the downstream effectors of Rho is a 160 kDa Ser/Thr kinase called Rho associated coiled-coil kinase (ROCK) or its isoenzyme Rho kinase (Ishizaki *et al.*, 1996, Leung *et al.*, 1995). ROCK increases the formation of stress fibres and recruitment of integrins to focal adhesions and also enhances myosin light chain (MLC) to increase the contractility of actinomyosin, therefore increasing smooth muscle contraction (Reviewed in Narumiya 1996 & Amano *et al.*, 2000). A highly specific inhibitor of ROCK, Y27632, was produced by a Japanese group and found not only to inhibit agonist induced contraction in VSMC, but also reduced the blood pressure in spontaneously hypertensive rats (Uehata *et al.*, 1997). Y27362 has proved a useful tool in determining the functions and involvement of ROCK. In rat aortic, SMCs thrombin-induced DNA synthesis and cell migration was blocked using Y27632 (Seasholtz *et al.*, 1999) as well as endothelin-1 (ET-1) induced hypertrophy in neonatal rat cardiac myocytes (Kuwahara *et al.*, 1999). Interestingly, the stretch-activated stimulation of ERK, which resulted in DNA synthesis, was also inhibited by Y27632 (Numaguchi *et al.*, 1999). This suggests that Rho/ROCK plays an important role in mediating the mechanical stretch signal to ERK activation. The PDGF and LPA-stimulated DNA synthesis in human aortic SMCs and downregulation of $p27^{kip}$ in rat VSMCs were also reduced (Sawada *et al.*, 2000) suggesting that ROCK plays a pivotal role in G_1 cell cycle progression, gene transcription and cell proliferation. Recently $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were shown to stimulate stress fibre formation when transfected into Swiss 3T3 fibroblasts (Sauseau *et al.*, 2000). The same group also showed that ATP,

UTP and UDP stimulated stress fibre formation in vascular myocytes, which was inhibited by Y27632 suggesting that P2Y receptors couple to Rho kinase. Increasing evidence thus suggests that ROCK stimulated by either GPCR or growth factor receptors has a part to play in regulating mitogenesis. The aim of this investigation was to elucidate the potential involvement of Rho/ROCK in the regulation of mitogenesis in human VSMCs. Y27632 was found to inhibit PDGF-induced DNA synthesis in these cells as shown in chapter 3 (Figure 3.5). This was further investigated. Developing an assay to measure ROCK activity was also pursued so that the actions of Y27632 at this kinase could be shown.

6.2 The effects of ROCK inhibitor Y27632

In the previous chapter it was shown that the ROCK specific inhibitor Y27632 significantly inhibited PDGF induced DNA synthesis. An experiment was carried out similar to that performed using MEK inhibitors as described in Section 4.2.3. Briefly, cells were cultured in 96 well dishes, serum-starved for 48 hours and preincubated with increasing concentrations of Y27632 (ranging from 1 μM to 300 μM) prior to stimulation for 1 hour with and without 1 nM PDGF to establish a dose-dependent relationship. After a further 19 hours incubation in serum-free medium with no PDGF or Y27632 [^{3}H]-thymidine was added and its incorporation into DNA measured after 4 hours. The results are shown in Figure 6.1 represented as the % inhibition of the maximal PDGF response. Y27632 was found to significantly inhibit DNA synthesis in a dose dependent way with an IC_{50} = 9.16 ± 5.0 μM ($P<0.01$, one way ANOVA). Alone increasing concentrations of Y27632 had no influence on basal levels of DNA synthesis.

6.3 Measuring ROCK activity

The implication of ROCK in PDGF mitogenic signalling, shown using Y27632, suggests that PDGF stimulates ROCK. To investigate this directly the activity of ROCK needed to be measured when stimulated with PDGF. Three different methods were employed to measure the activity of ROCK; 1) measuring its autophosphorylation, 2) measuring the phosphorylation of its substrates and 3) measuring the translocation of ROCK from cytosol to plasma membrane when activated. Initially, cell lysates were

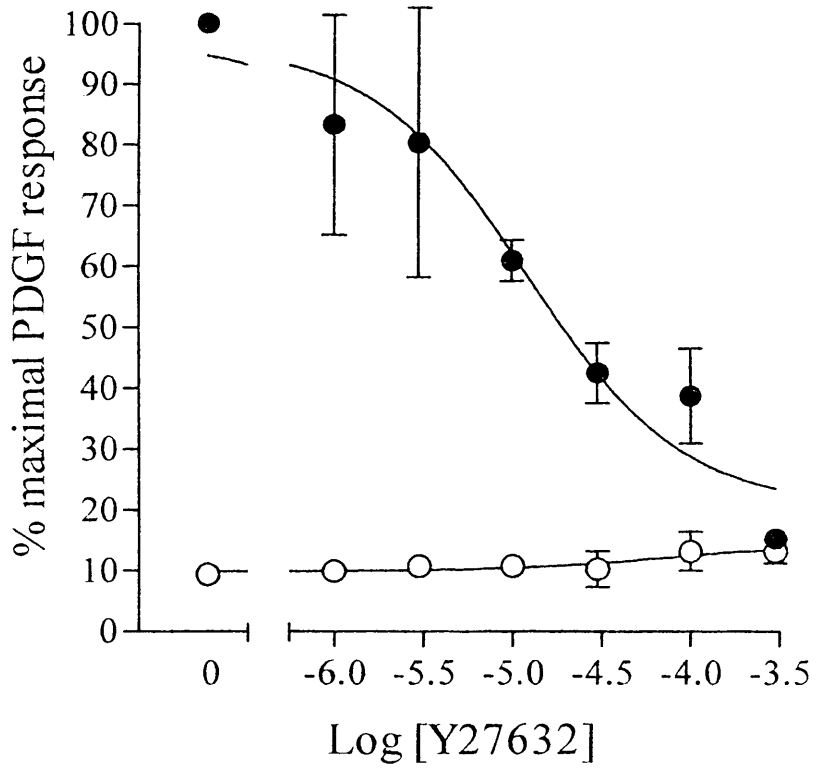


Figure 6.1 The effects of Y27632 on DNA synthesis

The effects of increasing concentrations of ROCK inhibitor Y27632 on [^{3}H] thymidine incorporation into DNA in the presence and absence of 1nM PDGF. Data is presented as percentage of the PDGF stimulated response ± SEM from 3 separate experiments each carried out in triplicates.

separated by electrophoresis and nitrocellulose was probed using a polyclonal ROCK antibody (provided by Dr. L.L Ng). This was to ensure ROCK could be detected using Western blotting and the antibody provided. The results in Figure 6.2A showed that a detectable amount of the ROCK protein was found in SV VSMCs.

6.1.1 Translocation of ROCK

ROCK has been previously been shown to translocate to the cytoskeletal membrane upon activation during platelet aggregation stimulated with thrombin (Fujita *et al.*, 1997). This was detected as early as 20 seconds and peaked at 5 minutes and suggests that this translocation stimulates cytoskeletal reorganization. A similar method of detecting ROCK activation was also employed as described in Section 2.8.4. Briefly, cells were stimulated for 1, 3, 5 or 10 minutes with and without 10 μM LPA or 1 nM PDGF. Cells were lysed and centrifuged at 8,000 X g to precipitate the nuclear membrane fraction. The supernatant was then centrifuged at 100,000 X g to precipitate cytoskeletal membrane fraction and the supernatant represents the soluble cytosolic fraction. Precipitates were washed and resuspended and all samples were separated by electrophoresis and the nitrocellulose membrane was probed with anti-ROCK antibody. The results are shown in Figure 6.2B stimulated with LPA and 6.2C stimulated with PDGF for i) nuclear membrane fraction, ii) cytoskeletal membrane fraction and iii) soluble cytosolic fraction. There was some detectable levels decrease in the relative amounts of ROCK in the cytosolic fraction upon stimulation with LPA at 1, 3 and 5 minute stimulations, but no corresponding increase in the cytoskeletal membrane fraction was seen. In similar experiments, PDGF was found not to influence the levels of ROCK in either the soluble cytosolic fraction or the two membrane fractions. While of interest, the results across three experiments were inconsistent, and it was concluded that no reliable evidence for ROCK activation could be seen with this approach.

6.1.2 In vitro kinase assay

NHE-1 has been shown to be a substrate of ROCK (Kaneko *et al.*, 2000) and MBP is a good substrate for most serine/threonine kinases. Immunoprecipitated ROCK was used in an in vitro assay to measure its ability to phosphorylate MBP and oligopeptide

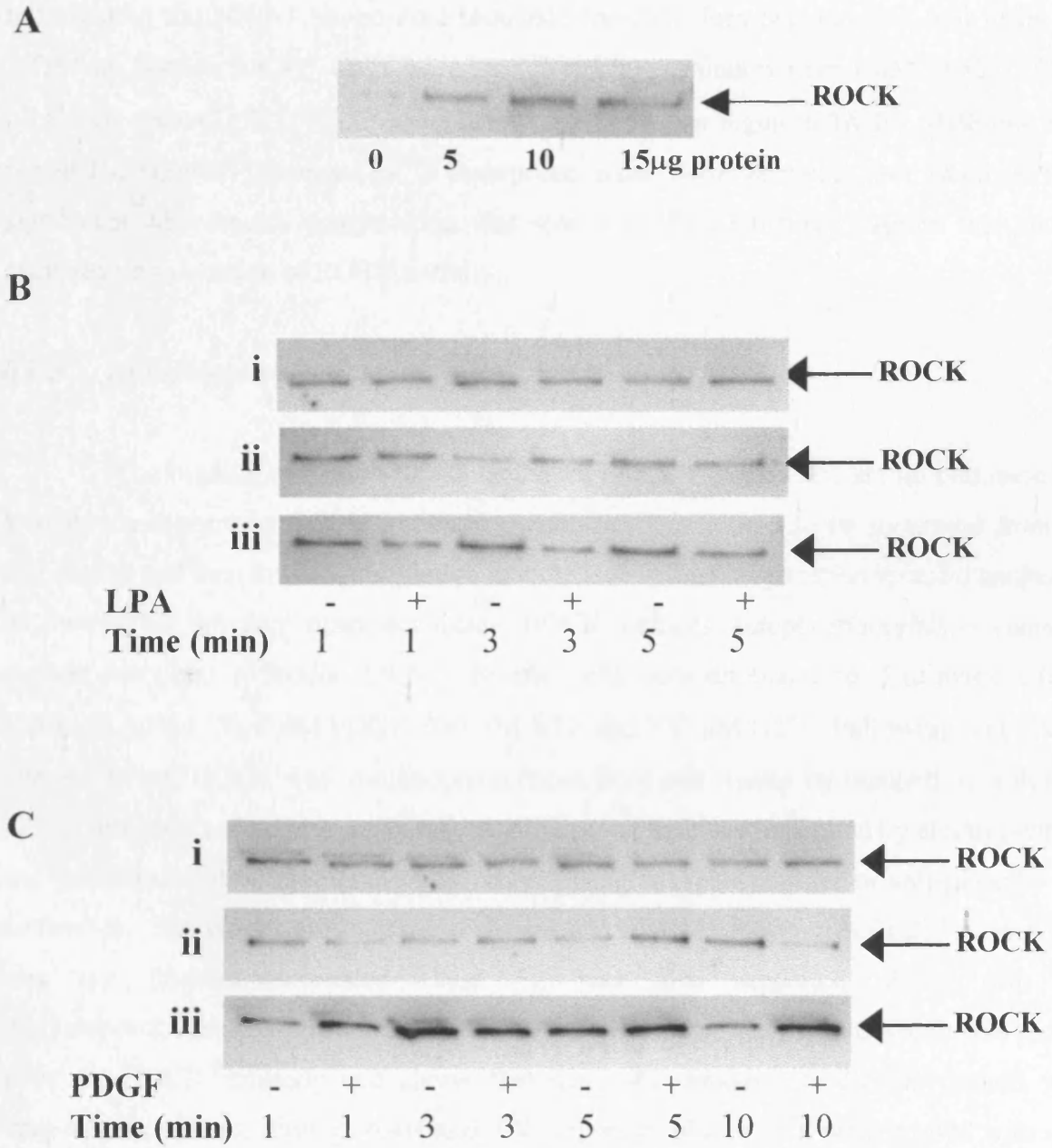


Figure 6.2 Translocation studies of p160^{ROCK}

A) Detection of ROCK in human VSMCs by western blot probed with ROCK polyclonal antibody. Data is representative of two experiments.

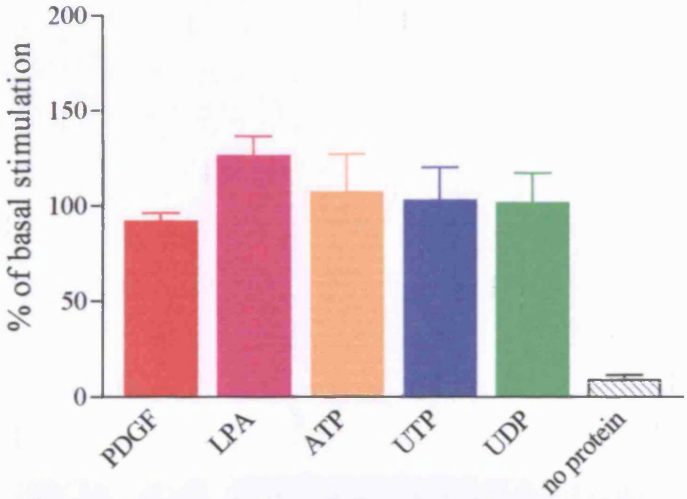
Western blot analysis of **i**) nuclear **ii**) membrane and **iii**) cytosolic cellular fractions probed with ROCK polyclonal antibody when human VSMCs were stimulated for the times shown with **B**) 10 μM LPA and **C**) 1 nM PDGF. Blots shown are represenatative of 3 experiments.

representing the NHE-1 amino acid sequence from residues between 632-656 using ^{32}P-ATP (see Section 2.8.3). Cells were stimulated for 5 minutes with 1 nM PDGF, 10 μM LPA, 300 μM ATP/UTP/UDP. The results are shown in Figure 6.3A for MBP and 6.3B for NHE-1 peptide. Increases in ^{32}P incorporation into MBP were seen, but found not to be significant whereas no incorporation was seen into NHE-1 peptide. Again this method provided no indication of ROCK activity.

6.1.3 Autophosphorylation

The binding of Rho to ROCK activates ROCK. Since there are no commercially available anti-phospho ROCK antibodies available, ROCK had to be separated from the cell lysates and then its phosphorylation detected using Ser/Thr phospho specific antibodies to investigate whether upon activation ROCK induces autophosphorylation (detailed method described in Section 2.8.1-2). Briefly, cells were stimulated for 5 minutes with 10 μM LPA, 10% FCS, 1 nM PDGF, 300 μM ATP and 300 μM UTP. Following stimulation and extraction, ROCK was immunoprecipitated from cell lysates by incubation with anti-ROCK antibody and protein sepharose A. The precipitate was separated by electrophoresis and the nitrocellulose membrane was probed using anti-phospho Ser or anti-phospho Thr antibodies. The results are shown in Figure 6.4A, B, & C. A sample of cell lysate, which was not immunoprecipitated (lane 1), was also separated along with the immunoprecipitated samples (lanes 2-7). Figure 6.4A represents a blot, which was probed with anti-ROCK antibody and shows that detectable amounts of ROCK protein were immunoprecipitated. Figures 6.4B and 6.4C represent blots which were probed with anti-phospho Ser and anti-phospho Thr antibodies respectively. No detectable level of ROCK phosphorylation was seen with either antibody. Lane 1 in each case showed multiple bands of proteins at different molecular weight displaying Ser or Thr phosphorylation, however this was not the case in the other lanes. The band that appears on the blots in the results shown was found not to be ROCK as it was displaced at a slightly higher molecular weight than ROCK. The band also appeared in experiments (not shown) including an internal control subjected to immunoprecipitation without any cell lysate. The band therefore was a non-specified protein in the anti serum being separated during electrophoresis. Autophosphorylation of ROCK using this method did not provide an indication of its state of activation.

A



B

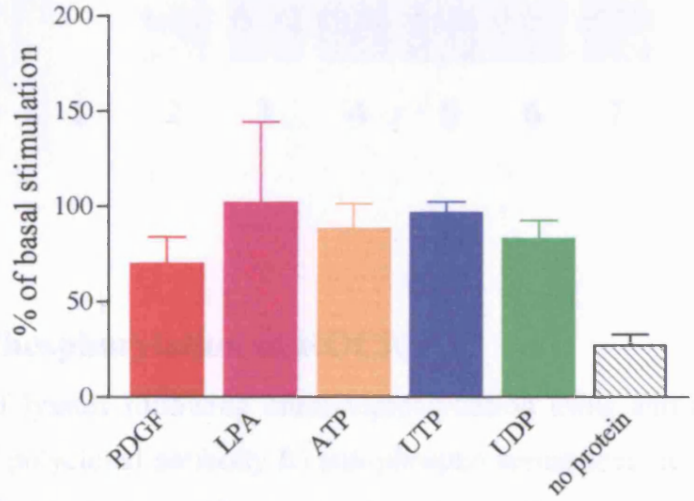


Figure 6.3 Measuring p160^{ROCK} activity

Stimulation of ^{32}P-ATP incorporation into **A)** MBP and **B)** NHE-1 peptide when human VSMCs were stimulated for 5 minutes with 1 nM PDGF (red), 10 μM LPA (purple), 300 μM ATP (orange), UTP (blue) and UDP (green). Data presented as the % of basal stimulation (100%) ± SEM from **A)** three or **B)** two experiments

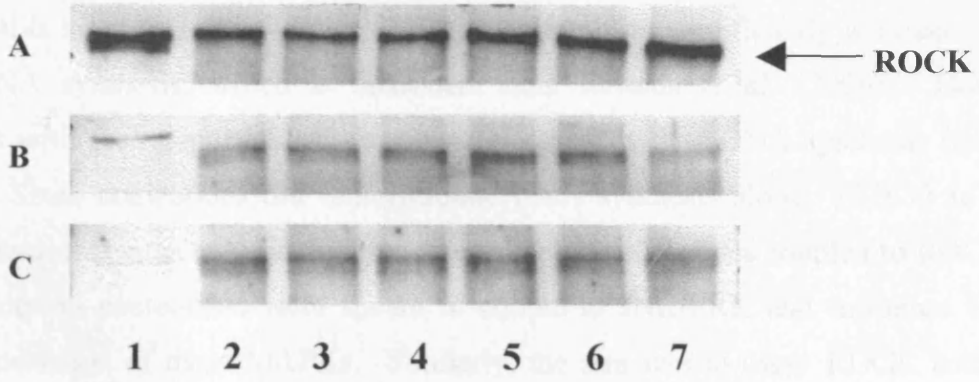


Figure 6.4 Phosphorylation of ROCK

Western blot of lysates following immunoprecipitation using anti-ROCK anibdy probed with **A**) ROCK polyclonal antibody **B**) anti-phospho serine specific antibody, and **C**) anti-phospho specific threonine antibody, stimulated as follows; 1) non immunoprecipitated crude cell lysate, 2) basal, 3) 10% FCS, 4) 1 nM PDGF, 5) 10 μM LPA, 6) 300 μM ATP, 7) 300 μM UTP. Data is representative of three experiments.

6.4 Discussion

Increasing evidence suggests a role for ROCK in control of cell growth stimulated by both growth factors and GPCRs (Kuwahara *et al.*, 1999, Sawada *et al.*, 2000, Seasholtz *et al.*, 1999). The ROCK inhibitor, Y27632, proved a useful tool to show that P2Y receptors couple to ROCK in rat vascular myocytes (Sauseau *et al.*, 2000). Using a similar method another group showed that the PDGF and LPA-stimulated increases in DNA synthesis in human aortic smooth muscle cells were also mediated via ROCK (Sawada *et al.*, 2000). The aim of this investigation was to determine if PDGF-induced mitogenesis was mediated via ROCK in human SV VSMCs and whether P2Y receptors were also coupled to this kinase. In this study Y27632 was found to significantly attenuate PDGF induced DNA synthesis, which is consistent with Sawada *et al.* (2000). However, inconsistent with their report, LPA was found not to stimulate DNA synthesis (data not included). Since nucleotides did not stimulate DNA synthesis alone, Y27632 in DNA synthesis measurements could not be used to establish if nucleotides coupled to ROCK. In previous chapters nucleotides were shown to couple to JNK/ERK and influence PDGF-induced stimulation of these MAPKs. Similarly, the aim was to assay ROCK activation induced by PDGF and to investigate any nucleotide intervention. Unfortunately the three methods used to measure ROCK activity were unsuccessful despite using LPA as a positive stimulation of ROCK. Therefore direct PDGF induced stimulation of ROCK could not be established and nucleotide influence, if any, could not be investigated. The data so far implies that PDGF may couple to ROCK. However, direct activation of ROCK has not been established and it is not known at present whether ROCK is required for mitogenesis. It is possible that Y27632 inhibited other proteins other than ROCK that may have been necessary for mitogenesis and as a result inhibited PDGF-induced DNA synthesis. This needs to be further investigated (Chapter 8). Further improvements in the experimental design of the methods used above also need to be carried out. In particular, measuring ROCK activity using ^{32}P would potentially be the most useful tool for this purpose. A possible way of achieving this may be in changing the conditions of extraction and incubation with substrates to optimise ROCK's phosphorylating capacity. However, there is a possibility that the kinase catalytic activity may be too small to measure under such assay conditions and remains to be investigated.

Chapter 7

Comparison of SHR & WKY Rat VSMC mitogenic responses

7.1 Introduction

The vasculature of animals can exhibit many of the same components that are found in human hypertensive disease, such as hyperplasia and hypertrophy of the smooth muscle cells leading to the thickening of the vascular walls. Cells derived from spontaneously hypertensive rats (SHR) have commonly been used as a model of the hypertensive state. The SHR strain was developed at Kyoto University in Japan in 1963 by inbreeding Wistar rats, which showed persistent high blood pressure (bp.>150mmHg) (Okamoto & Aoki 1963). They are used in comparison to cell lines derived from normotensive Wistar rats (WKY, which showed no abnormal hypertrophy/hyperplasia) which may be used as a control group for the SHR cells.

Enhanced mitogenic responses in SHR VSMC compared to WKY VSMC have been reported in response to stimulation by growth factors via tyrosine kinase receptors (Hamada *et al.*, 1990, Hamet *et al.*, 1988) and other factors acting via G-protein coupled receptors, including angiotensin II (Morton *et al* 1995, Wilkie *et al.*, 1996, Paquet *et al* 1990, Bunkenburg *et al.*, 1992). These responses included elevated DNA synthesis in SHR VSMC in comparison to WKY, and elevated levels of tyrosine kinase activity, tyrosine phosphorylation, and increased PLC and PLD activation (Wilkes *et al.*, 1993). Increased PIP_2 hydrolysis and $[Ca^{2+}]_i$ levels were also shown to occur in SHR cells compared to WKY cells (Osani & Dunn, 1992, Morton *et al.*, 1995, Turla & Webb, 1990), along with a significant rise in cGMP levels (negative regulator of inositol phosphate production) in WKY than SHR (Baines *et al.*, 1996).

The aim of this chapter is to investigate mitogenic cellular signalling by nucleotides in both SHR and WKY VSMCs. Of particular interest is any differences in nucleotide receptor signalling between the two models that may explain the development of hyperplasia and hypertrophy found in the spontaneously hypertensive state, and which may be relevant as a model of the human hypertensive disease. Recently, nucleotides have been shown to ellicit PLC responses in rat SHR and WKY-derived VSMCs (Harper *et al* 1998). UTP was the only full agonist found. The ATP responses were shown to be partial compared to UTP in SHR VSMC. ATP and UTP were also shown to act at the same receptor. At the time of publication this corresponded to the known pharmacology at the cloned $P2Y_4$, which was the human $P2Y_4$ receptor. The response was suramin sensitive, but with unexpected characteristics. Suramin had no effect on the EC_{50} of ATP but lowered the maximum response in a concentration dependent manner. This data was

difficult to reconcile with a $P2Y_4$ receptor. In WKY cells, however, both ATP and UTP were full agonists suggesting responses mediated via $P2Y_2$ receptors.

ADP, 2MeSATP and αβ-methylene ATP either produced a small response or were ineffective. This showed that responses were not elicited through $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ or P2X receptors. UTP is also not an agonist at these receptors. The response to UDP, initially thought to be a partial agonist, was abolished in the presence of hexokinase. It was most likely that the response seen was indirect due to the formation of UTP. So UDP was not an agonist at SHR cells and therefore excludes $rP2Y_6$. RT-PCR analysis of SHR VSMC also showed high levels of mRNA expressing $P2Y_2$, $P2Y_4$ and $P2Y_6$, but not $P2Y_1$.

UTP also increased phosphorylation of ERK and induced cell proliferation, suggesting the involvement of $P2Y_4$ in mitogenesis. However, the involvement of $P2Y_4$ alone in causing mitogenesis was questioned. After publication it became apparent that both UTP and ATP were full agonists at $rP2Y_4$ (Webb *et al.*, 1998b, Bogdanov *et al.*, 1998), although at the $hP2Y_4$ receptor, ATP acts as a partial agonist compared to UTP. The newly cloned $rP2Y_4$ receptor was also reversibly antagonised by RB-2 (IC_{50} = 21.1 ± 2.9 μM), but not by PPADS or suramin. The $hP2Y_4$ receptor is not antagonised by PPADS or suramin (Charlton *et al.*, 1996b), however Communi *et al.* (1996a) showed UTP responses to be PPADS sensitive and RB-2 to have intermediate effects. The $rP2Y_4$ receptor is more like a P2U receptor such as $rP2Y_2$ or $hP2Y_2$ than like $hP2Y_4$, although it shows 83% identity with $hP2Y_4$ (Bogdanov *et al.*, 1998). Recently Kennedy and colleagues (2000) transfected both $hP2Y_4$ and $rP2Y_4$ receptors into 1321N1 cells and compared the increase in intracellular $[Ca^{2+}]$ stimulated by UTP, ATP and ITP. The rank order of agonist potency at the $hP2Y_4$ receptors was UTP (EC_{50}=0.55 μM) >GTP (EC_{50}=6.59 μM) >ITP (EC_{50}=7.38 μM). ATP was inactive at the $hP2Y_4$ receptor. At the $rP2Y_4$ receptor the agonist potency order was UTP (EC_{50}=0.2 μM) > ATP (EC_{50}=0.51 μM) > ITP (EC_{50}=1.82 μM) ≈ GTP (EC_{50}=6.59 μM).

Therefore, the partial agonist affect of ATP shown by Harper and colleagues on SHR VSMCs produced some questions such as why was this occurring despite the evidence to otherwise suggest that both ATP and UTP responses should be equal. This problem needed to be investigated further to avoid any complications in interpreting mitogenic signals as well as ascertaining any differences in P2Y receptor subtype expression on the surface of VSMC. Since both UTP and ATP were shown to be equipotent on both $rP2Y_2$ and $rP2Y_4$, how could the two receptors be distinguished from

each other pharmacologically? Some of the differences between the $rP2Y_2$ and $rP2Y_4$ agonist potency are listed below and were used in this study to try and unravel any differences in $rP2Y_2$ and $rP2Y_4$ receptor expression that may be occurring and to ascertain if one or both receptors were responsible for mitogenic signalling.

1) The $rP2Y_4$ receptor is strongly activated by ITP, whereas the $rP2Y_2$ receptor was weakly activated by ITP. Bogdanov et al. (1998) transfected $rP2Y_4$ in Xenopus oocytes and showed increases in membrane currents evoked by ITP with an EC_{50} = 1.4 μM, compared to EC_{50} = 1.8 μM for ATP and EC_{50} = 2.6 μM for UTP. These results show ITP to be equally potent as UTP or ATP. In sympathetic neurones transfected with $rP2Y_2$ Filippov *et al* (1997) showed nucleotide inhibition of N-type calcium currents. The IC_{50} for UTP and ATP was 0.5 μM, whereas for ITP the IC_{50} = 3.1. This shows ITP to be less potent than ATP or UTP at $rP2Y_2$ receptors
2) The $rP2Y_4$ receptor is weakly activated by purified UDP (EC_{50} = 4.2 μM) although this is a partial agonist compared to UTP (EC_{50} = 2.6 μM) and ATP (EC_{50} = 1.8 μM) (Bogdanov *et al* 1998), whereas purified UDP does not activate $rP2Y_2$ (Filippov *et al* 1997, Nicholas *et al.,* 1996a)
3) ATPγS is a full agonist at $P2Y_2$ receptor but slightly less potent (EC_{50} of 1.72 μM) than UTP or ATP (EC_{50} of 0.14 μM and 0.23 μM respectively) in transfected 1321N1 cells (Lazarowski *et al.,* 1995), but a weak partial agonist at $rP2Y_4$ (EC_{50} = 20.1 μM) (Bogdanov *et al.,* 1998)
4) Suramin is an antagonist at $P2Y_2$ (pA_2 = 4.32, Charlton *et al.,* 1996a), whereas it is ineffective at $P2Y_4$ (Charlton *et al.,* 1996b)

Any differences in receptor expression between SHR and WKY may have physiological relevance for the pathogenesis of hypertension in SHR rats. Pharmacological characterisation of P2Y receptors has been hampered due to inadequate selective antagonists and antibodies to use in binding studies. The cloning of specific P2Y receptors have now made it possible to study the regulation of expression of P2Y receptors subtypes at the mRNA level using RT-PCR. This technique was also employed to identify P2Y subtype mRNA expression in the two cell lines. Therefore not only is the mitogenic signalling of nucleotides being investigated, but also the identification of the receptor subtype involved in this signalling.

7.2 Differences in mitogenic responses between SHR & WKY VSMC

7.2.1 [^{3}H] Thymidine incorporation in response to UTP and ATP

The assay for incorporation of [^{3}H]-thymidine into DNA was used as an index of proliferation. Previous studies have shown ATP to act as mitogen in rat VSMC (Yu *et al.*, 1996, Erlinge *et al.*, 1993 &1995) and UTP in SHR VSMC (Harper *et al.*, 1998). In this study a comparison of mitogenesis in SHR and WKY VSMCs was carried out using the [^{3}H]-thymidine incorporation assay to ascertain any difference between the two cell types. UTP, ATP and ITP (300 μM each) were used and [^{3}H]-thymidine incorporation compared to the basal levels (stimulated with serum free medium) of DNA synthesis. 100 nM angiotensin II, which has previously been reported to be a stronger mitogen in SHR than WKY VSMC (Morton *et al.*, 1996, Wilkie *et al.*, 1996) and 1 nM PDGF were also used for comparison. The results are shown in Figure 7.1.

In SHR VSMC, PDGF gave the largest stimulation (827 ± 146% above basal, $P<0.001$, one way ANOVA) followed by ATP (405 ± 102%, $P<0.001$), UTP (356 ± 123% above basal, $P<0.05$), angiotensin II (281 ± 18.2% above basal, $P<0.001$), and ITP (222 ± 22.4% above basal, $P<0.05$). All these responses were significant at either $P<0.05$ or $P<0.001$. In comparison none of these agonists had any significant influence on WKY VSMCs except PDGF (334 ± 109% above basal, $P<0.05$ paired t test). The PLC response generated by PDGF in SHR was significantly greater than the PDGF response seen in WKY cells ($P<0.001$, one way ANOVA), as were angiotensin II and ATP ($P<0.05$). UTP and ITP gave a larger mean response in SHR than WKY cells, but this difference failed to reach significance in this series of experiments.

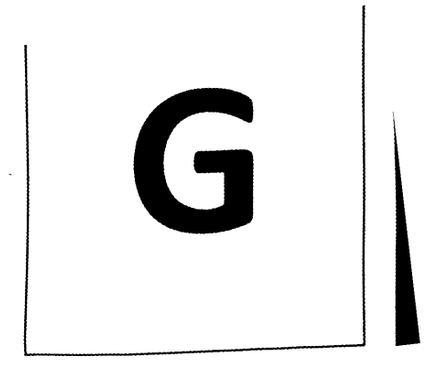
The next step of this investigation was to generate a dose dependent curve using increasing concentrations of ATP. It proved difficult to generate reproducible concentration response curves with the [^{3}H] thymidine incorporation assay. Result for one experiment is shown in Figure 7.2. The responses measured in SHR-derived cells were significantly greater than WKY ($P<0.01$ two way ANOVA).

7.2.2 ERK activation in SHR and WKY VSMCs

Previous reports have shown increased mitogenic response in SHR cells compared to WKY cells using growth factors (Hamada *et al.*, 1990) and agonists acting via G protein-coupled receptors (Paquet *et al.*, 1989, Bunkenburg *et al.*, 1992, Morton *et al.*, 1996, Wilkie *et al.*, 1996) and in response to nucleotides (SHR only, Harper et al., 1998). ERK plays a major role in growth and differentiation signalling in many cell types and was therefore investigated to determine if differences, seen in DNA synthesis in SHR and WKY cells, were feeding through to the activation of this kinase.

SHR and WKY VSMCs were cultured in 6 well multi-well dishes, serum starved for 24 hour and stimulated for 5 minutes. The cells were lysed and samples prepared for western blotting as described previously. Figure 7.3 represents data collected from western blots of lysate samples from SHR and WKY VSMCs stimulated with various agonists and probed with phospho-specific ERK antibody. The data is presented as the percentage above basal stimulation. Angiotensin II was used as a positive control for activation of ERK in these cells (Wilkie *et al*, 1996) and produced a larger mean stimulation of ERK phosphorylation in SHR cells (3039 ± 1160% of basal) than to WKY (701 ± 523%). However these were not significantly different. No difference in ERK phosphorylation stimulated by PDGF was seen. ATP, UTP and ITP also produced larger responses in SHR 5208 ± 2006%, 22274 ± 698% and 2587 ± 1579% of basal respectively) than WKY (1212 ± 530%, 391 ± 298% and 1449 ± 706% of basal respectively). However, no significant difference was displayed between the response in SHR and WKY. The ATP-stimulated response in SHR VSMCs was significant above basal levels ($P<0.05$, one way ANOVA). In general the trend implies greater ERK phosphorylation in SHR than WKY.

MEK inhibitors PD98059 and U0126 were also used to block agonist induced ERK phosphorylation. PD98059 was found to enhance ERK activity rather than inhibit (data not shown). This could be due to insufficient cell permeability of the inhibitor in rat cells (although similar complications were not seen in human VSMCs). U0126, was therefore used as an inhibitor of MEK (Figure 6.3B). 30 μM was found to significantly inhibit ERK activation in both SHR and WKY VSMCs when stimulated with these agonists ($P<0.05$, one way ANOVA).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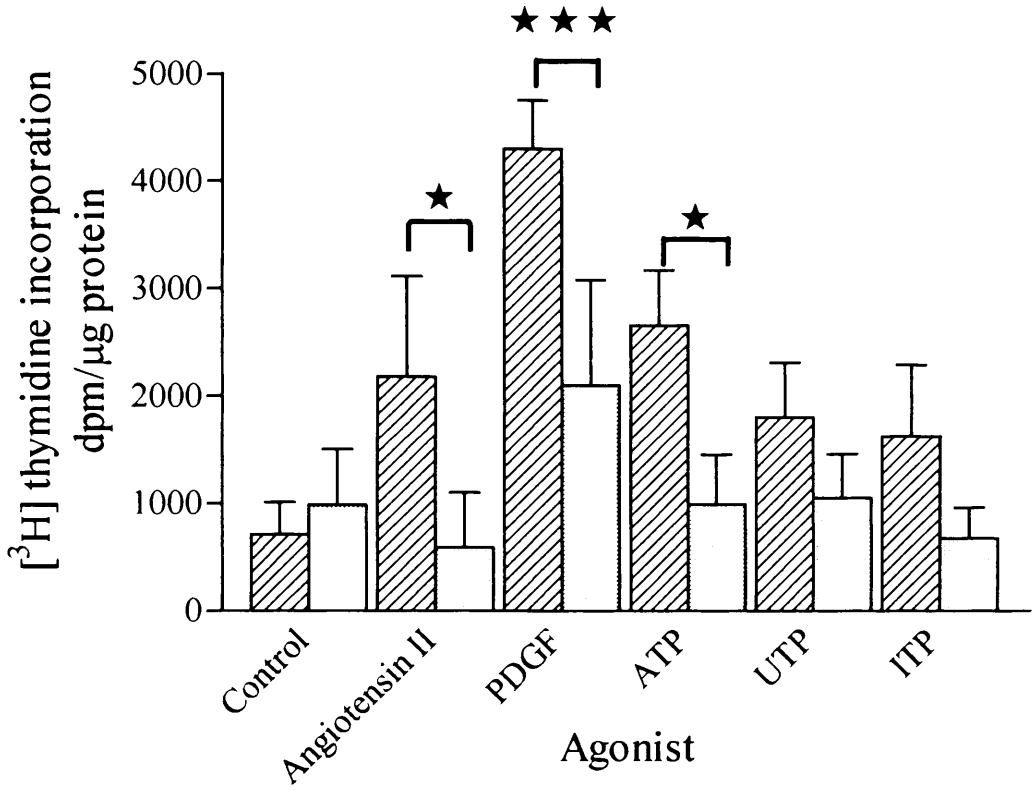


Figure 7.1 [^{3}H] Thymidine incorporation in SHR and WKY VSMCs

[^{3}H] Thymidine incorporation assay showing the differences between SHR (hatched) and WKY (dotted) VSMCs in response to 100 nM angiotensin II, 1 nM PDGF, 300 μM ATP, 300 μM UTP and 300 μM ITP, when stimulated for 1 hour. Data is expressed as the % above unstimulated basal levels for each cell type. Data is representative of the mean ± SEMs of four experiments, each carried out in triplicates. Statistics were performed using one way ANOVA followed by Bonferroni's multiple comparison test on raw data (★★★P<0.001, ★P<0.05).

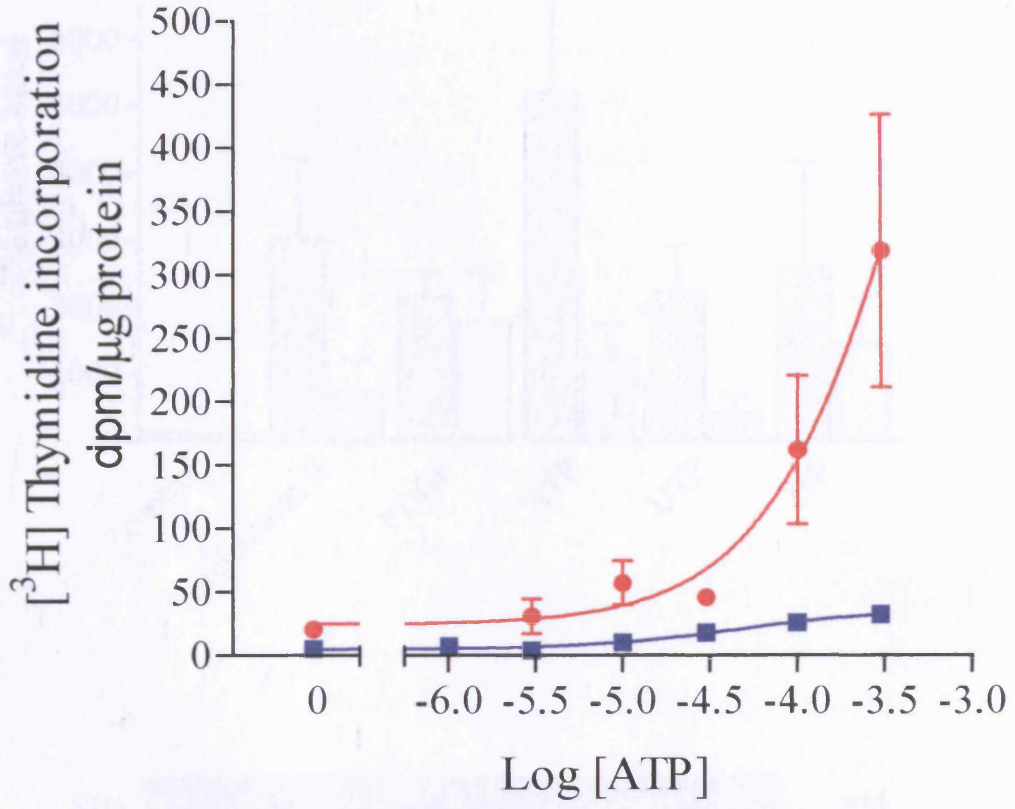


Figure 7.2 ATP-induced DNA synthesis in SHR and WKY VSMCs

[^{3}H] Thymidine incorporation into SHR VSMCs (●) and WKY VSMCs (■), in response to increasing concentrations of ATP. Each point represents the mean of triplicate responses of one experiment.

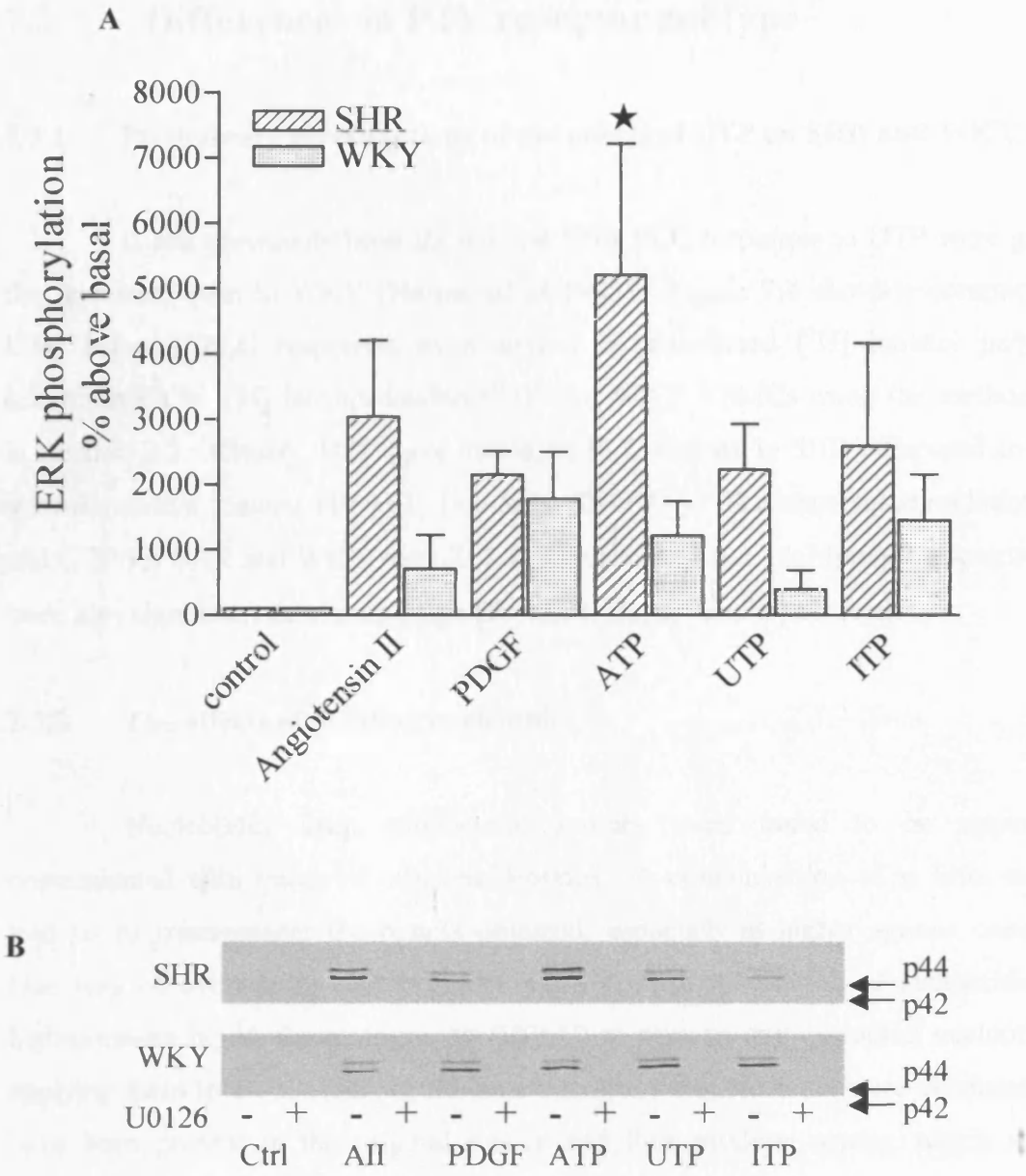


Figure 7.3 ERK Phosphorylation in SHR and WKY VSMCs

A) Phosphorylation of ERK in response to a 5 minute stimulation with 100 nM Angiotensin II, 1 nM PDGF and 300 μM ATP, UTP or ITP in SHR VSMCs (hatched) and WKY VSMCs (dotted). Data was collected from gel analysis of Western blots and presented as percentage of the basal ± SEM from 3 experiments. ★ $P<0.05$ one way ANOVA plus Dunnets post test.

B) Western blot showing phosphorylation of ERK, which is representative of three experiments and also shows inhibition of ERK activation using 30 μM U0126.

7.3 Differences in P2Y receptor subtype

7.3.1 Preliminary investigations of the effects of UTP on SHR and WKY cells

It has previously been shown that SHR PLC responses to UTP were greater than the responses seen in WKY (Harper *et al* 1998). Figure 7.4 shows a comparison of the UTP induced PLC responses as measured by stimulated [^{3}H] inositol polyphosphate accumulation in [^{3}H] inositol-loaded SHR and WKY VSMCs using the method described in Section 2.2. Clearly, UTP gave increased PLC activity in SHR compared to WKY in a dose-dependent manner ($P<0.01$, two way ANOVA). The maximal stimulation with 300 μM UTP for SHR and WKY were 217.7 ± 39.5 and 61.5 ± 14.7dpm/μg respectively, which were also significant from each other ($P<0.001$, Bonferonni's post test).

7.3.2 The effects of purified nucleotides

Nucleotides from commercial sources were found to be impure, usually contaminated with traces of other nucleotides. A contamination of as little as 1% could lead us to misrepresent the results obtained, especially at higher agonist concentrations. One way of overcoming this problem was by rapid purification of nucleotide stocks by high-pressure liquid chromatography (HPLC) to remove any unwanted nucleotides before applying them to cells. This would ensure that any contaminants were eliminated that may have been present in the original source and thus produce results, which reflected the responses more accurately at the concentrations used. This was done for the construction of concentration-response curves. UTP, ATP, ADP, UDP, ITP and ATPγS were HPLC purified by Dr. J. Roberts and were used as before to stimulate cells and PLC activation was then measured. The results generated in SHR and WKY VSMCs are shown in Figure 7.5 and Figure 7.6 respectively. Table 6.1 summarises the maximal responses and EC_{50} values for both SHR and WKY VSMCs.

UTP produced maximal PLC responses in comparison to other nucleotides in both SHR and WKY VSMCs with pEC_{50} of 4.998 ± 0.04 and 4.988 ± 0.04 respectively. ATP induced a maximal PLC response which was partial compared to the maximal response produced by UTP in both SHR and WKY VSMC (72.8%, pEC_{50} = 4.77 ± 0.06, $P<0.01$ in SHR and 72.1%, pEC_{50} = 5.02 ± 0.08, $P<0.01$ in WKY). A similar observation was demonstrated by Harper *et al* (1998) using impure nucleotides in SHR cells.

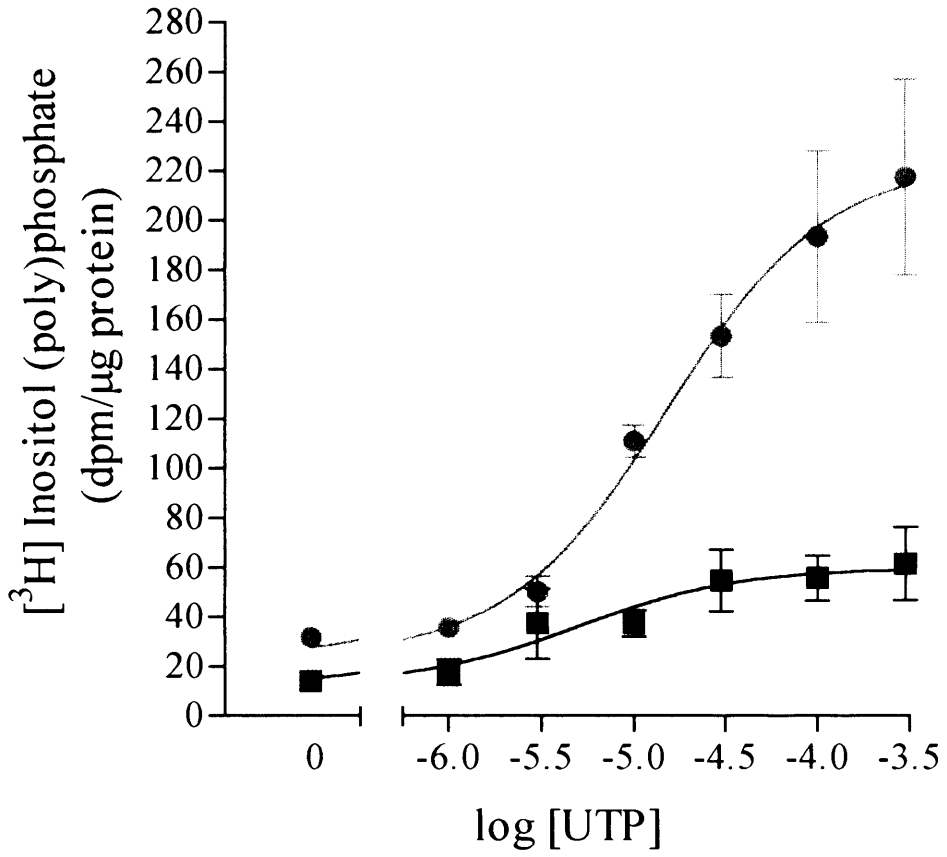


Figure 7.4 UTP-induced PLC activation

Dose response curves of the total [^{3}H]inositol polyphosphate accumulation, as an indication of PLC activation, in SHR (●) and WKY (■) VSMC in response to increasing concentration of UTP. Cells were preincubated with BSS plus 10 mM LiCl for 10 minutes and incubated with a concentration range of 1-300 µM UTP plus 10 mM LiCl for 20 minutes. Each point represents mean of three experiments preformed in triplicates, which have been protein equalised.

ITP and ATPγS were also used to try and distinguish between rat $P2Y_2$ and rat $P2Y_4$ receptors by their differences in selectivity for the two receptors. In SHR cells ITP induced a maximal PLC activation that was 74.0 ± 5.0% of the maximal UTP response ($P<0.001$, pEC_{50} = 4.23 ± 0.05 or EC_{50} = 59.5 μM, Figure 7.5). The maximal responses of ITP and ATP were not significantly different from each other, but the dose dependent curves generated were significantly different ($P<0.01$, two way ANOVA). ITP produced weak partial agonist response, which is consistent with the profile of $rP2Y_2$. ATPγS generated a partial response compared to UTP (50.4 ± 7.4%, $P<0.001$, pEC_{50} = 4.92 ± 0.04 or EC_{50} = 11.9 μM). These responses were also significantly different compared to ATP responses ($P<0.001$, two way ANOVA). The dose response curves generated by ITP and ATPγS were significantly different from each other ($P<0.001$). Here ATPγS acted as a partial, but potent agonist compared to UTP. The highly potent effect is consistent with $P2Y_2$ receptors, however the partial agonist effects are not.

In WKY cells ITP induced a maximal PLC activation that was 61.8 ± 3.5% ($P<0.001$, pEC_{50} = 4.76 ± 0.11 or EC_{50} = 17.8 μM, Figure 7.6). The maximal responses of ITP and ATP were not significant from each other, but the dose dependent curves generated were significant from each other ($P<0.001$, two way ANOVA). ITP produced potent partial agonist responses in WKY VSMC, which is consistent with $P2Y_4$ receptor subtype. ATPγS generated a potent partial response compared to UTP (61.1 ± 3.5%, $P<0.001$, pEC_{50} = 4.66 ± 0.06 or EC_{50} = 22.0 μM in WKY). These responses were also significantly different compared to ATP responses ($P<0.01$, two way ANOVA). The dose response curves generated by ITP and ATPγS in WKY VSMCs the curves were statistically similar.

Purified UDP has also been shown to act as a weak partial agonist at $rP2Y_4$ (Bogdanov *et al* 1998), whereas purified UDP does not activate $rP2Y_2$ (Filippov *et al* 1997, Nicholas *et al*; 1996a,b). Here UDP acted as a partial agonist for both SHR and WKY, however UDP was more potent than UTP and ATP (pEC_{50} = 5.14 ± 0.16 or EC_{50} = 7.2 μM in SHR and pEC_{50} = 5.82 ± 0.16 or EC_{50} = 1.5 μM in WKY). Purified ADP was found to weakly stimulate PLC responses (pEC_{50} = 4.03 ± 0.05 or EC_{50} = 93.3 μM in SHR and pEC_{50} = 3.95 ± 0.13 or EC_{50} = 112 μM in WKY). The responses of UDP and ADP were further investigated in Section 7.3.3. 2MeSATP, α,β-MeATP and 2MeSADP were also used in similar experiments (data not shown), but did not stimulate PLC activation significantly. This was also seen in studies by Harper and colleagues (1998).

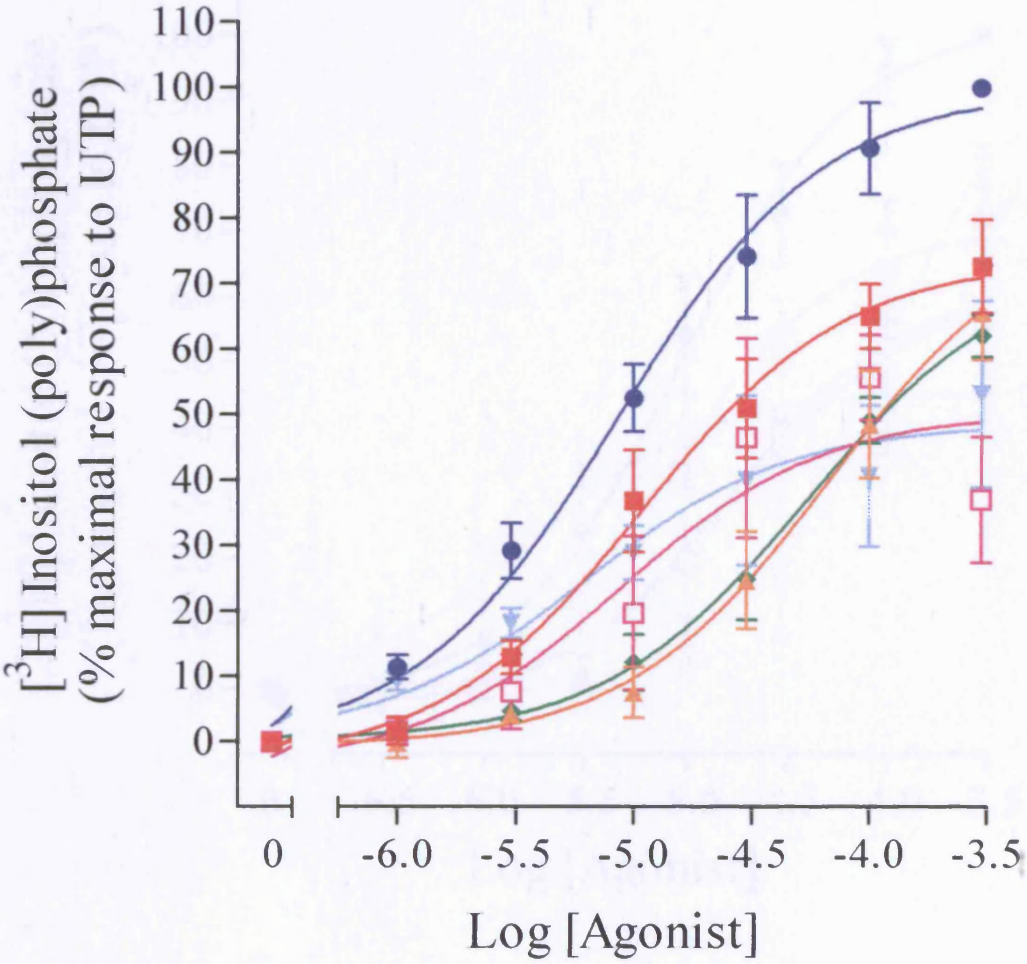


Figure 7.5 PLC activation in SHR VSMCs

Dose response curve of total [^{3}H] inositol polyphosphate accumulation in SHR VSMC to purified UTP (●), UDP (▼), ATP (■), ADP (▲), ITP (♦) and ATPγS (□). Cells were pre-incubated with 10 mM LiCl for 10 minutes and incubated with agonist plus 10 mM LiCl for 20 minutes. Each point represents the mean % maximal response to UTP from 4 experiments each preformed in triplicates.

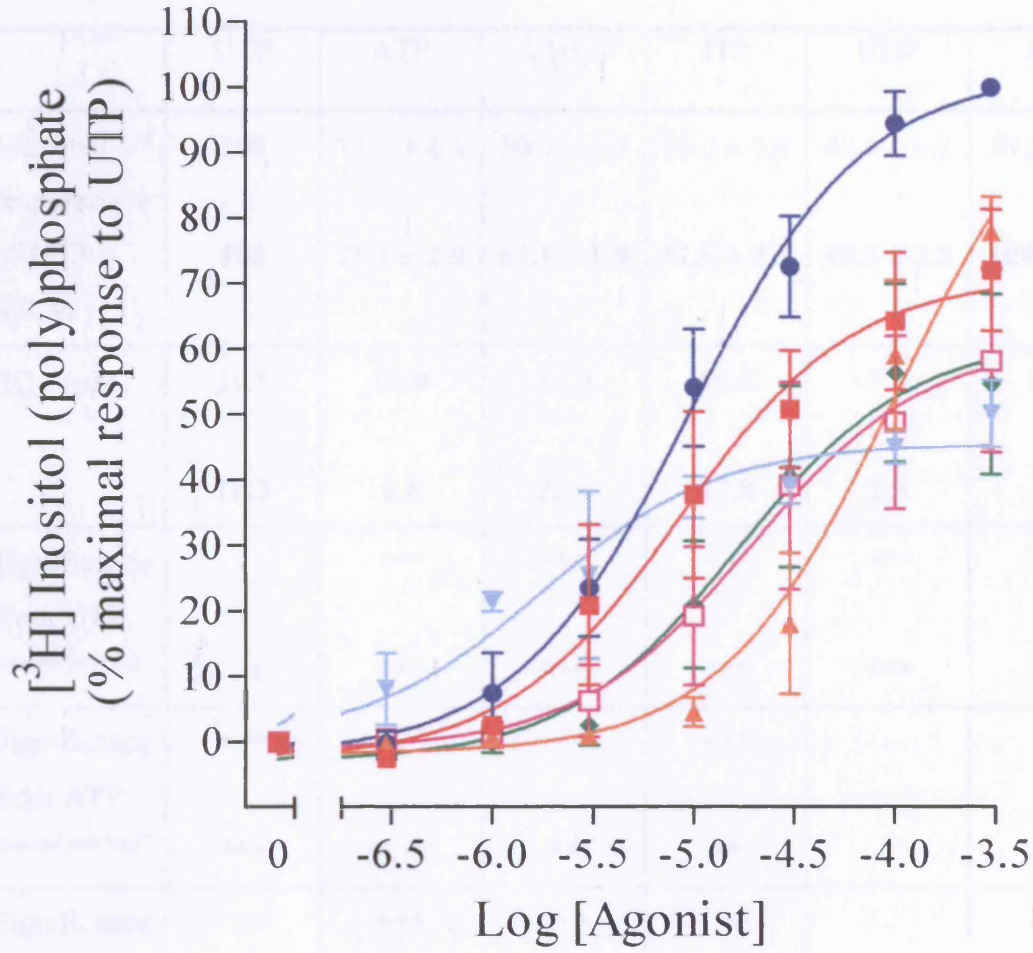


Figure 7.6 PLC activation in WKY VSMCs

Dose response curve of total [^{3}H] inositol polyphosphate accumulation in WKY VSMC to purified UTP (●), UDP (▼), ATP (■), ADP (▲), ITP (♦) and ATPγS (□). Cells were pre-incubated with 10 mM LiCl for 10 minutes and incubated with agonist plus 10 mM LiCl for 20 minutes. Each point represents the mean % maximal response to UTP from 4 experiments each preformed in triplicates.

	UTP	ATP	ATPγS	ITP	UDP	ADP
Maximal response (% of UTP ± S.E.M.)	100 100	73.7 ± 4.4 71.1 ± 2.9	50.4 ± 7.4 61.1 ± 1.8	74.0 ± 5.0 61.8 ± 3.5	48.6 ± 6.2 45.5 ± 2.5	81.2 ± 9.9 108 ± 11.7
EC_{50} (μM)	10.1 10.3	16.9 9.6	11.9 22.0	59.5 17.8	7.3 1.5	93.3 112
Significance from UTP (two way ANOVA)	- -	*** ***	*** ***	*** ***	*** ***	- -
Significance from ATP (two way ANOVA)	*** ***	- -	*** **	*** **	- -	- -
Significance from ITP (two way ANOVA)	*** ***	*** ***	*** n/s	- -	- -	- -

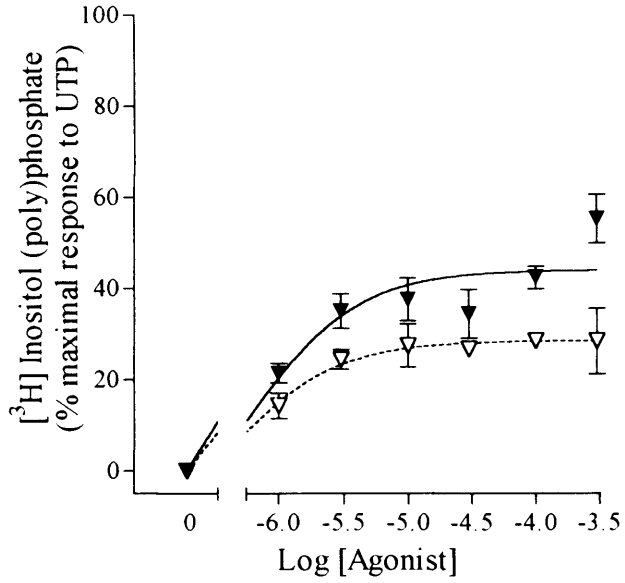
Table 7.1

Table summarising the maximal PLC responses generated by nucleotides as a % of the maximal UTP response and the EC_{50} values generated by increasing concentrations of purified nucleotides in SHR (red) and WKY (blue) VSMCs. *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ using two way ANOVA.

7.3.3 Nucleotide Interconversion

Purified ADP was not very potent in PLC activation in both SHR and WKY (EC_{50} = 93.3 μM and 112.0 μM respectively, Figure 7.5 and 7.6), and individual experiments using ADP produced large variations in PLC activation compared to each other. This could be due to nucleotide inter-conversion occurring on the cells, which may explain variable results. As described before, UDP activated PLC more potently but partially compared to UTP in both SHR and WKY VSMC. However, UDP may also have been susceptible to inter-conversion, which would deviate the results from the true response. Inter-conversion was further investigated for both UDP and ADP by reducing the conversion of di-phosphate to tri-phosphates on the cell surface using hexokinase before and during the stimulation of cells. Stock solutions of 1 mM UDP and ADP were incubated with 50 U/ml hexokinase and 110 mM glucose for one hour at 37°C. Cells were incubated with 1 U/ml hexokinase and 22 mM glucose and 10 mM LiCl for 10 minutes before stimulation and during stimulation. The results for SHR VSMCs are shown in Figure 7.7. Hexokinase treatment of UDP significantly reduced the PLC activation dose response curve indicating that, despite being pure, conversion to UTP was occurring ($P<0.001$, 28.6 ± 0.6% of maximal UTP stimulation, pEC_{50} = 6.39 ± 0.13 μM or EC_{50} = 410 nM). However, UDP was still a potent activator of PLC, which suggests the presence of $P2Y_6$ receptors. The apparent response produced by ADP was also significantly attenuated ($P<0.01$, 46.0 ± 7.5% of maximal UTP stimulation, pEC_{50} = 3.78 ± 0.2 or EC_{50} = 166 μM) indicating that conversion of ADP to ATP was also occurring on the cells and that ADP itself produced a very small and weak PLC response. This is consistent with the very small PLC responses generated with 2MeSADP (data not shown but refer to Harper *et al*, 1998), which indicates that $P2Y_1$ and $P2Y_{12}$ receptors are not expressed in these cultured cells.

A



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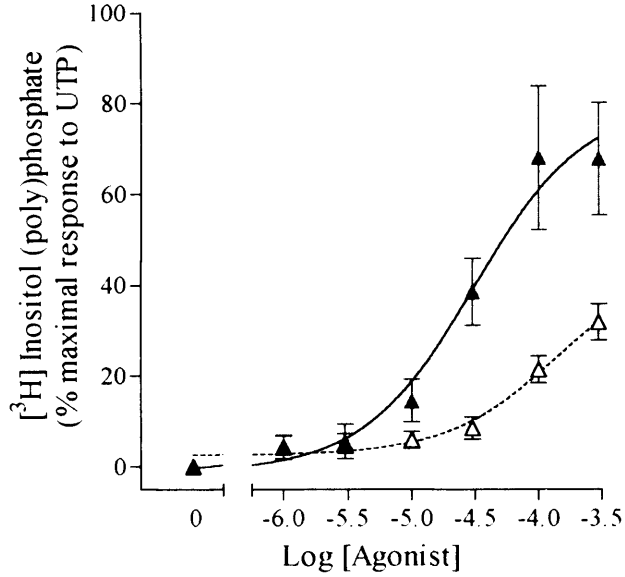


Figure 7.7 PLC activation in the presence of Hexokinase

Dose response curve of total [^{3}H]-inositol polyphosphate accumulation in SHR-VSMC, **A**) to purified UDP (▼) and purified UDP + hexokinase (∇, dotted line) and **B**) to purified ADP (▲) and purified ADP + hexokinase (Δ, dotted line). Cells were pre-incubated with 50 U/ml hexokinase, 110 mM Glucose and 10 mM LiCl for 10 minutes and incubated with agonist plus 1 U/ml hexokinase, 22 mM Glucose and 10 mM LiCl for 20 minutes. Each point represents the mean % maximal response to UTP (not shown) from 3 experiments each performed in triplicates.

Similarly, inter-conversion from tri-phosphate to di-phosphates can also occur. This was reduced by the addition of creatine phospho-kinase (CPK) plus phospho-creatine (PC) in the drug preparation of both UTP and ATP. [^{3}H] Inositol incorporation assay were then carried out for ATP and UDP with and without this re-generation system. Cells were pre-incubated for 10 minutes and stimulated with 10mM LiCl plus 2U/ml CPK and 1mM PC present. The results for SHR VSMCs are shown in Figure 7.8. CPK treatment of UTP was not altered from the response produced by UTP in the absence of CPK, indicating little or no conversion of UTP to UDP. The maximal response produced by ATP plus CPK, however, was significantly greater than produced by ATP alone ($P<0.0001$). This indicates that conversion of ATP to ADP was occurring, which may explain the partial effect of ATP observed in Figure 7.5. ATP in the presence of CPK had a maximal response of 96.0±9.4% ($pEC_{50} = 5.23 \pm 0.14$) of the maximal UTP stimulation ($pEC_{50} = 5.44 \pm 0.07$).

7.3.4 Antagonist Studies: The effects of Suramin on ATP, UTP and ITP

Suramin has been reported to act as an antagonist of $P2Y_2$ receptors, but not $P2Y_4$ (Charlton *et al.*, 1996). It was therefore used as a tool to possibly identify the P2Y receptor subtype present on these cells. Figure 7.9 shows the effects of 300 μM suramin on the dose response curves of UTP and ITP and figure 7.10 shows the effects of suramin on CPK treated and untreated ATP responses. Suramin was found to significantly antagonize the dose response curves produced by UTP ($pEC_{50} = 4.85 \pm 0.25$ without suramin and 4.24 ± 0.21 with suramin, $P<0.01$, two way ANOVA), ITP ($pEC_{50} = 4.25 \pm 0.24$ without suramin and 3.50 ± 0.47 with suramin, $P<0.001$) and CPK treated ATP ($pEC_{50} = 5.17 \pm 0.20$ without suramin and 4.71 ± 0.15 with suramin, $P<0.0001$), but did not significantly antagonize the response produced by untreated ATP. An estimated pA_2 values using the single dose of suramin were calculated according to Furchgott (1972);

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

where r is the dose ratio of EC_{50} value in the presence of antagonist compared to EC_{50} value in the absence of the antagonist and p[A] is the $-\log_{10}$ of the molar antagonist concentration. For suramin the pA_2 was approximately 4.48 in the presence of UTP, 4.45 in the presence of ATP+CPK and 4.75 in the presence of ITP.

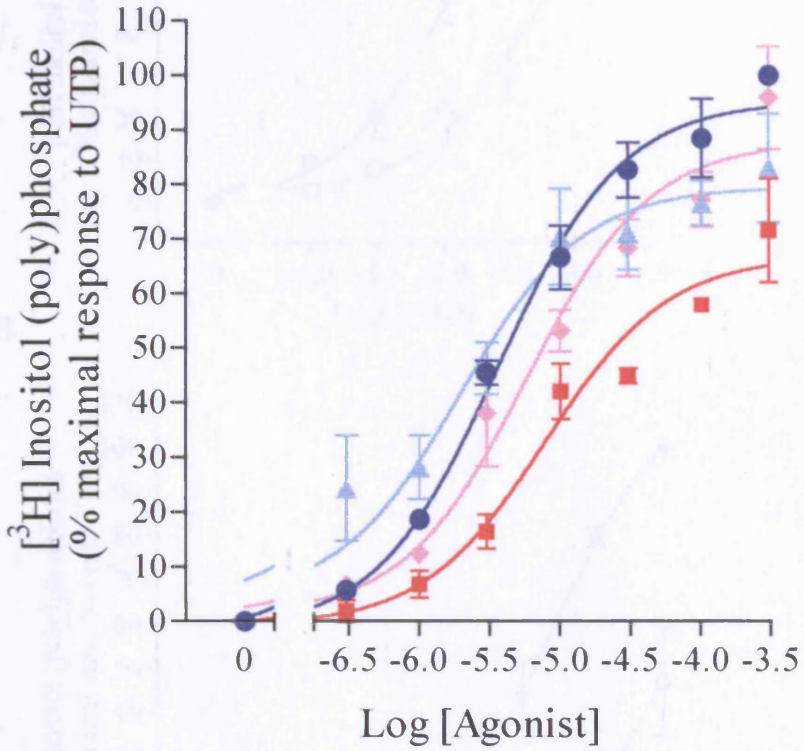


Figure 7.8 PLC activation in the presence of creatine phospho-kinase

Dose response curve of total [^{3}H]-inositol polyphosphate accumulation in SHR-VSMC, stimulated with UTP (●), UTP + creatine phospho-kinase (CPK) (▲), ATP (■) and ATP + CPK (♦). Cells were pre-incubated with 10 mM LiCl for 10 minutes and stimulated with agonist plus 2 U/ml CPK, 1 mM phospho-creatine and 10 mM LiCl for 20 minutes. Each point represents the mean % maximal response from three experiments each performed in triplicate.

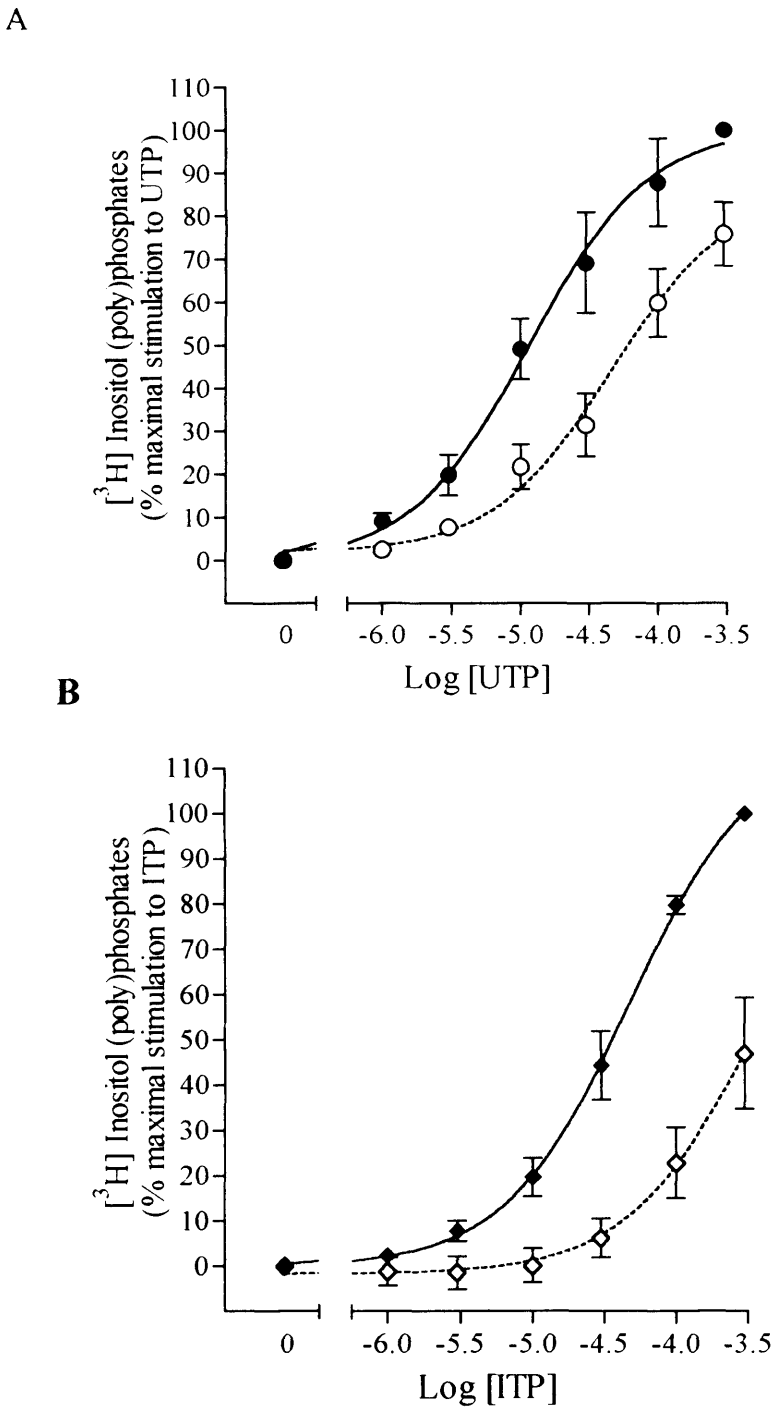
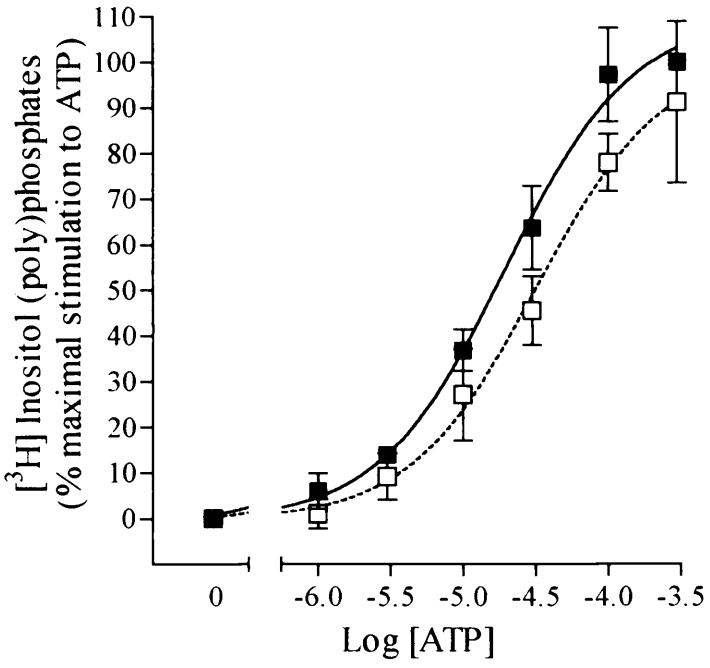


Figure 7.9 The effects of suramin on PLC activation in SHR VSMCs

Dose response curve of Total [^{3}H] Inositol polyphosphate accumulation in SHR-VSMC in response to **A**) UTP (●) or UTP plus 300 μM Suramin (○), **B**) ITP (♦) or ITP plus 300 μM Suramin (◊). Cells were pre-incubated with 10 mM LiCl for 10 minutes and incubated with agonist plus 10 mM LiCl for 20 minutes. Each point represents the mean % maximal response as indicated from 4 experiments each preformed in triplicates.

A



B

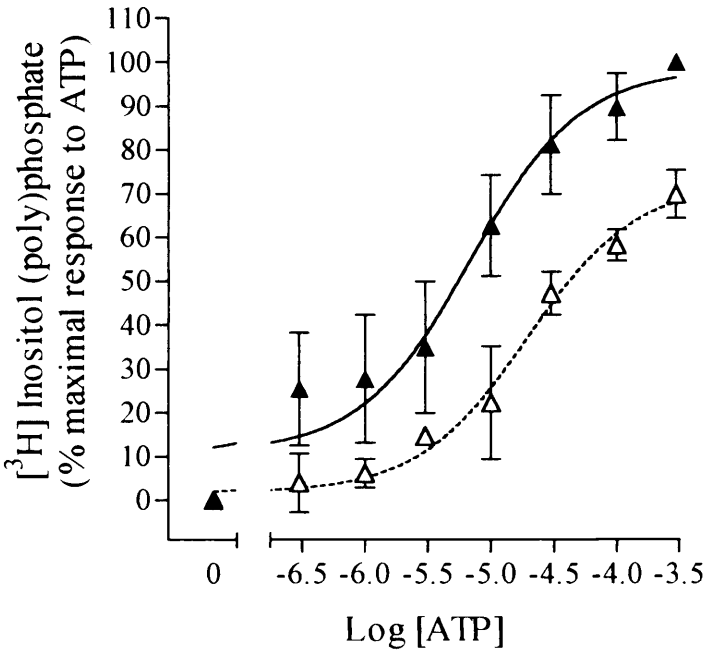


Figure 7.10 The effects of suramin & CPK on PLC activation

Dose response curve of total [^{3}H] inositol polyphosphate accumulation in SHR-VSMC in response to **A**) ATP (■) and ATP plus 300μM suramin (□); **B**) ATP in the presence of CPK with suramin (▲) and without suramin (△).Each point represents the mean % maximal response as indicated from 3/4 experiments each preformed in triplicates.

7.3.5 The effect of simultaneous exposure of SHR VSMCs to UTP and ATP/ITP

The influence of individual nucleotides has so far been investigated and used to identify the presence of certain receptor subtypes. The data so far indicates that both ATP (in the absence of CPK) and ITP act as partial agonists compared to UTP. Simultaneously stimulating cells with UTP and ATP or ITP was also used to determine whether nucleotides were acting at the same receptor. The experiment was designed on the following hypothesis and used as a basis to explain the results;

A) If the PLC responses of UTP (at a near maximal response) were enhanced by a second agonist then this would imply that the second agonist was acting at a different receptor, which was not selective for UTP and the combined activation of PLC by the two receptors would be seen.

B) If there is no change of the UTP induced PLC response by a second agonist then both UTP and the second agonist are full agonist at the same receptor.

C) If there is a decrease in UTP stimulation of PLC activation then this would suggest that the agonist were acting at the same receptor for which UTP was a full agonist and the second agonist was a partial agonist, which antagonises the UTP response.

Figure 7.11 shows the results of SHR cells stimulated simultaneously with 100 μM UTP and increasing concentrations of ATP or ITP. This concentration of UTP was chosen because it was the minimum concentration that produced a near maximal response. This response was greater than the maximal ATP or ITP responses, therefore allowing any antagonist competition between nucleotides to be detected. The results (Figure 7.11A) showed that ATP did not antagonize UTP responses, arguing against C above and inconsistent with similar experimental results reported by Harper *et al.* (1998). ITP has been shown to be a partial agonist, but in this experiment (Figure 7.11B) it did not antagonise the UTP response and is inconsistent with the hypothesis that ITP and UTP act at the same receptor.

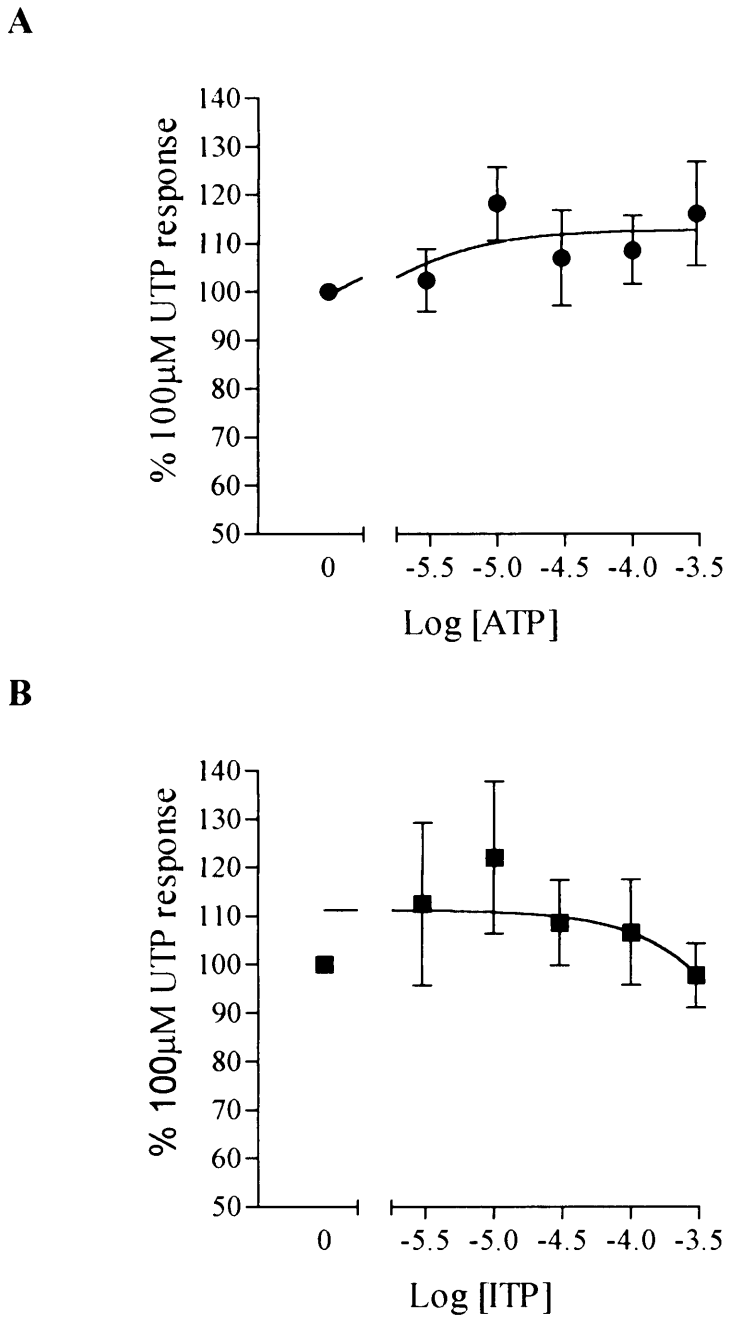


Figure 7.11 Simultaneous agonist stimulation of PLC activation

The effect on UTP induced [^{3}H] inositol accumulation in SHR-VSMC, when simultaneously exposed to ATP/ITP. Cells were pre-incubated with 10 mM LiCl for 10 minutes and stimulated for 20 minutes with 100 μM UTP plus 0, 3, 10, 30, 100 or 300 μM concentrations of either **A**) ATP, (•), or **B**) ITP, (■) in the presence of 10 mM LiCl. Results represent mean of triplicate responses from 3 experiments.

7.4 P2Y receptor subtype mRNA expression

Messenger RNA (ribonucleic acid) is the information-carrying intermediate from DNA to protein synthesis. The synthesis of mRNA is called transcription, where RNA polymerase takes instructions from a DNA template to generate mRNA which is complementary to the DNA template. RT-PCR technique exploits the mRNA produced and was employed here to detect P2Y receptor mRNA expression in SHR and WKY VSMCs. The methods used are described in Section 2.10. Briefly, cytosolic mRNA was extracted with phenol/chloroform extraction and reverse transcribed using reverse transcriptase (RT) to generate a complimentary DNA (cDNA). $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$-specific forward and reverse primers (see table 7.2) and *Taq*-polymerase were used in PCR to amplify regions specific to each receptor. $P2Y_{11}$ forward and reverse primers were not used as the rat $P2Y_{11}$ sequence has not been identified. For each receptor type, several controls were run alongside normal RT-PCR to check the assay conditions. Plasmids containing the sequences of different P2Y receptors were included as templates for the gene-specific PCR amplification to ensure that the primers were designed properly and efficiently amplified the required sequence. RT was omitted from the reverse transcription step so that any bands seen on the agarose gel would thus be due to DNA contamination from external sources or genomic DNA. This control also tests the reaction reagents as well as the effectiveness of mRNA separation from genomic DNA during the phenol/chloroform extraction step. Finally, RT template was omitted from samples and any bands seen here would be due to DNA contamination of the samples or reagents at some point in the procedure, as the primers had no template from which to prime. Primers for GAP were also used with and without RT. The products of the RT-PCR were separated on a 2% agarose gel containing ethidium bromide and visualised under UV illumination. The experiment was repeated three times on separate RNA preps.

The image in Figure 7.12 is representative of the three experiments and shows the PCR products produced from SHR cells (top panel) and WKY cells (bottom panel). The first lane in each receptor subtype represents cDNA from the corresponding mRNA for each receptor. The second lane represents samples with no RT. The third lane represents the positive control from plasmid DNA containing corresponding receptor sequence and the fourth lane represents samples with no template.

From the first lane of GAP product for both SHR and WKY it can been seen that approximately equal amounts of this house keeping gene mRNA was amplified and loaded

onto the gel. This suggests that any difference observed between the two cell types was not due to uneven amounts of total cellular mRNA in one cell type compared to another. From the third lane of each group it can be seen that the primers for each receptor were working effectively. The bands were seen at approximately the correct size of the reaction product for each receptor, when compare to the base-pair ladder. The second and the fourth lanes in each group show that there was no contamination. The first lane in each group for SHR cells shows that no $P2Y_1$, $P2Y_4$ or $P2Y_{12}$ receptor subtype mRNA was found whereas mRNA for $P2Y_2$ and $P2Y_6$ was present. In WKY cells no $P2Y_1$ or $P2Y_{12}$ receptor subtype mRNA was found whereas mRNA for $P2Y_2$, $P2Y_4$ and $P2Y_6$ was present. The images shown are representative of three experiments which showed the same result. Therefore the results are not a result of uneven loading of the samples onto the gel, nor due to variations between different cultures of cells.

Receptor	Primer	Sequence	Product length
Rat $P2Y_1$	Forward	5′-TGGTGGCCATCTCCCCTATTCTCTT-3′	595bp
	Reverse	5′-ATCTCGTGCCTTCACAAACTC-3′	
Rat $P2Y_2$	Forward	5′-TTCCACGTCACCCGCACCCTCTATTACT-3′	539bp
	Reverse	5′-CGATTCCCCAACTCACACATACAAATGATTG-3′	
Rat $P2Y_4$	Forward	5′-CTTCTCTGCCTGGGTGTTTGGTTGGTAGTA-3′	474bp
	Reverse	5′-TCCCCCGTGAAGAGATAGAGCACTGGA-3′	
Rat $P2Y_6$	Forward	5′-GCCAGTTATGGAGCGGGACAATGG-3′	352bp
	Reverse	5′-AGGAACAGGATGCTGCCGTGTAGGTTG-3′	
Rat $P2Y_{12}$	Forward	5′-ATCTGGGCCTTCATGTTCCTGCTGTC-3′	532bp
	Reverse	5′-GTTTCGTTGCCAAAGCCCTCGGTGCTCTC-3′	

Table 7.2 P2Y receptor primer sequences

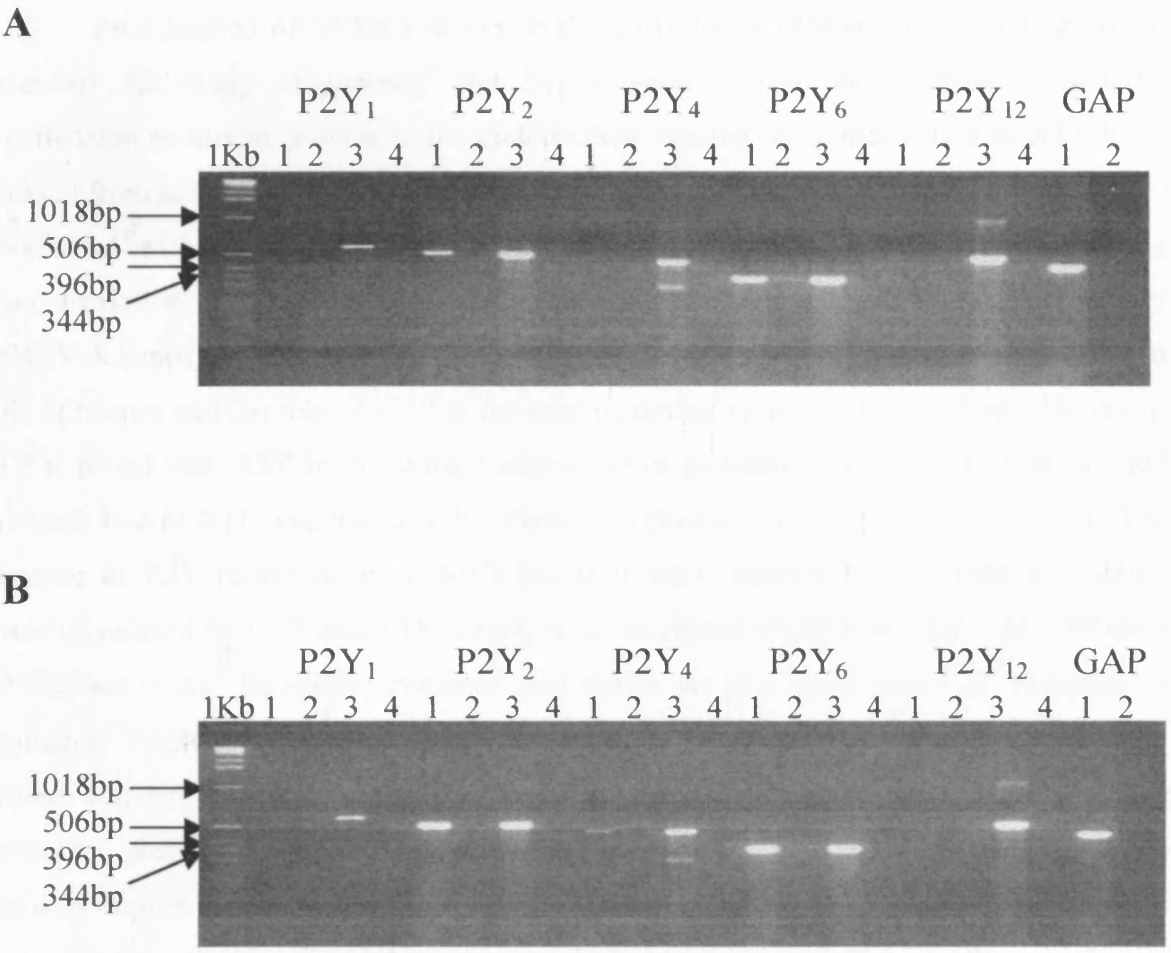


Figure 7.12 RT-PCR of SHR and WKY VSMCs

RT-PCR products from cultured SHR (**A**) and WKY VSMCs (**B**). The lane order is as follows 1) cDNA from rat VSMCs amplified with specific primers as stated in that group 2) no RT 3) positive control generated from plasmid containing the sequence for receptor in each group 4) no template. The primers used were designed to amplify P2Y receptor subtype mRNA including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ and house-keepong gene mRNA GAP. Image representative of three experiments performed on separate cultures.

7.5 Discussion

Proliferation of VSMCs is a crucial step in the development of atherosclerosis, restenosis following angioplasty and hypertension. The sheer stress caused by hypertension results in damage to the endothelium causing nucleotides such as ATP to be released from activated platelets or disrupted cells, which then act on VSMCs. ATP is also co-released with other neurotransmitters from sympathetic nerves in arteries, causing vasoconstriction via P2X receptor ion channel activation such as $P2X_1$ (Evans & Kennedy 1994, Vulchanova et al., 1996). Little is known about the regulated release of UTP from cells or tissues and the role of UTP in the cardiovascular system is still unclear. However, UTP is found with ATP in the storage organelles of platelets with concentrations around one tenth that of ATP, and may also be released at the same time (Goetz *et al.*, 1971). The presence of P2Y receptors on VSMCs has been demonstrated by increases in calcium levels stimulated by UTP and ATP, which is not mediated via P2X receptors as UTP does not activate them. Increasing evidence also shows the involvement of P2Y receptors in regulating VSMCs mitogenesis. P2X receptors in VSMCs were shown to be lost in culture, whereas P2Y were maintained in culture, which correlates with changes from the contractile phenotype to the synthetic phenotype (Pacaud *et al*, 1995, Erlinge *et al.*, 1998) and also implies that any mitogenic responses are mediated via P2Y receptors rather than P2X receptors. Increases in DNA synthesis have been demonstrated in cultured rat cells from the aorta and vena cava stimulated by ATP and ADP (Erlinge *et al.*, 1992, 1993, Yu *et al.*, 1996). Both ATP and UTP have been shown to be involved in the regulation of the cell cycle in rat aortic smooth muscle cells, either alone or with PDGF (Miyagi *et al*, 1996).

In cultured VSMCs from SHRs, Harper and co-workers (1998) showed increases in DNA synthesis stimulated by UTP suggesting the involvement of $P2Y_4$ and $P2Y_2$ in mitogenesis. This group also showed increases in PLC and ERK activation. In a comparison between SHR and WKY, angiotensin II was shown to enhance PLC, PLD and ERK activation. Similarly, the data in this chapter shows enhanced PLC and ERK activation in SHR cells stimulated by ATP. Parallel to this was the enhanced DNA synthesis also observed in SHR cells in response to ATP. Taken together these data suggest that the enhanced P2Y-coupled PLC and ERK activation in SHR cells results in larger levels of mitogenesis. It could therefore be hypothesised that proliferation of hypertensive VSMCs is a result of an enhanced P2Y receptor signalling. Whether this is due to effective coupling of P2Y receptors to mitogenic signalling in SHR than WKY or

whether this is due to differential expression of P2Y receptor subtypes resulting in a discrepancy in mitogenic coupling is unclear.

It must be noted that data collected for ERK phosphorylation did not show any significant difference in stimulation between SHR and WKY cells despite obvious differences in responses generated by angiotensin II, ATP and UTP. Unfortunately large differences between experiments resulted in large S.E.M, the outcome of which was no statistical difference between SHR and WKY.

Harper and colleagues (1998) showed ATP to act as a partial agonist and UTP to act as full agonist. This was also seen using purified UTP and purified ATP in this study. Purification eliminated the possibility of any contaminants that may have been present in the commercial nucleotides, which may have interfered with PLC activation by ATP. This was, however, inconsistent with previous reports, which have shown that both ATP and UTP were equipotent at both $rP2Y_2$ and $rP2Y_4$ receptors (Webb *et al.*, 1998b, Bogdanov *et al.*, 1998). The prospect of nucleotide breakdown on the cells during the experiment was addressed next by adding CPK, which metabolised any di-nucleotides to tri-nucleotides and therefore preventing the deterioration of the final ATP concentration and the accumulation of its breakdown product ADP. Pure ADP had low potency at generating PLC response therefore its accumulation would decrease the maximum response of ATP, resulting in partial agonism. The presence of CPK was found not to increase UTP responses, but increased ATP responses such that they were no longer partial compared to UTP, but stimulating PLC with equal magnitude. The initial experiments, in which ATP produced semi-maximal responses, were therefore misleading due to ATP degradation. These results are consistent with reports showing ATP and UTP to be equally potent at both $rP2Y_2$ (Lustig *et al.*, 1993) and $rP2Y_4$ (Bogdanov *et al.*, 1998, Webb *et al.*, 1998), and therefore suggest that $rP2Y_2$ and/or $rP2Y_4$ receptors are expressed on SHR cells. Partial agonism of ATP was also seen in WKY cells. However, similar CPK experiments were not performed on WKY cells and Harper *et al* (1998) reported ATP and UTP to produce similar responses. It therefore seems likely that ATP breakdown was also occurring on WKY cells to produce a partial effect.

The initial question of whether $P2Y_2$ and/or $P2Y_4$ receptor expression levels varied between SHR and WKY VSMCs and if this variation could explain the difference in mitogenic responses between the two cell types could now be addressed. Both ATP and UTP are equipotent at $rP2Y_2$ and $rP2Y_4$ receptors, which makes it difficult to distinguish between these receptors on the basis of the PLC response generated by these two

nucleotides. A possible method in determining which receptor subtype might be expressed was by studying the effects of other agonists and antagonists at these receptors. ITP has been shown to strongly activate $P2Y_4$ receptors, but weakly activated $P2Y_2$ (Bogdanov *et al.*, 1998, Kennedy *et al.*, 2000, Fillipov *et al* 1998). In the same way ATPγS was found to be a full potent agonist of $P2Y_2$, but partial at $P2Y_4$ (Lazarowski *et al.*, 1995, Bognadov *et al.*, 1998). Purified UDP was found to act as a weak partial agonist at $P2Y_4$, (Bogdanov *et al.*, 1998) but is not and agonist at $P2Y_2$ receptors (Fillipov *et al.*, 1998, Nicholas 1996a). Suramin is an effective antagonist at $P2Y_2$ but not at $P2Y_4$ (Charlton *et al.*, 1996a & 1996b). The responses produced by ITP, ATPγS, pure UDP and suramin in relation to UTP were investigated. Some hypothetical explanations for the results were firstly worked out in order to understand the outcome of the results using previous data described above if we assume similar receptor reserve.

1) If $P2Y_2$ expression levels are far greater than $P2Y_4$, then the ITP potency would be approximately 6 fold less than UTP/ATP, ATPγS would produce maximum responses similar to UTP/ATP, although with lower potency, and suramin would significantly antagonise UTP and ATP. Pure UDP would be ineffective
2) If $P2Y_4$ expression levels are far greater than $P2Y_2$ then ITP would be equi-potent to UTP/ATP, ATPγS would generate partial responses and suramin would be ineffective. Pure UDP would be a weak partial agonist.
3) If both $P2Y_2$ and $P2Y_4$ were expressed then a mixture of the above responses would be observed. For example, ITP would be slighty less potent than UTP/ATP, ATPγS would be a weak partial agonist and suramin would weakly antagonise ATP and UTP responses. UDP would be a very weak partial agonist.

Along with functional assay studies RT-PCR analysis was also employed to give an indication of which receptor subtypes might be expressed in SHR and WKY VSMCs. However, the presence of mRNA for a receptor subtype does not imply that the receptor protein is expressed and functional. Translation is the process by which proteins are synthesised from mRNA and is far more complex than transcription. The mRNA is translated in the 5' to 3' direction simultaneously by many ribosomes. Post transcriptional and post-translational modifications, as well as the structure of the protein, contain signals which decide the ultimate destination of the protein. Receptor expression levels at the membrane may also be too low to detect functionally. In most cases the presence of

mRNA expression has also been confirmed by functional assays. This technique can therefore be useful in corroborating results from functional assays.

In SHR VSMCs the ITP response was approximately 6 fold less than UTP or ATP. This strongly supports hypothesis 1 above and suggests the expression of $rP2Y_2$ receptors. Consistent with this was the suramin-sensitivity of UTP, ITP and ATP (in the presence of CPK) responses. The estimated pA_2 calculated from a single dose of suramin used was found to be similar for that calculated for $P2Y_2$ (Charlton *et al.*, 1996a). RT-PCR analysis of SHR VSMCs showed expression of $P2Y_2$ mRNA but no detectable expression of $P2Y_4$ mRNA. This is contradictory with Harper *et al.* (1998) who demonstrated the presence of $P2Y_4$ mRNA expression in SHR VSMCs. Inconsistent with the presence of $P2Y_2$ subtype expression was that ATPγS potency was similar to UTP and the maximal response was partial. This partial agonism is supported by hypothesis 2 and 3 but the potency is not. Pure UDP potently stimulated responses, but was partial compared to UTP. This is not consistent with the hypothesis above and therefore suggests the expression of $P2Y_6$ receptor subtypes for which it is most potent. This was confirmed by RT-PCR analysis which showed the expression of $P2Y_6$ mRNA. Expression of $P2Y_1$ and $P2Y_{12}$ receptor subtypes was also not seen which is consistent with functional studies where ADP and 2MeSADP (agonists at $P2Y_1$ and $P2Y_{12}$) produced no responses. Expression of $P2X_1$ receptor subtype was also investigated in SHR VSMCs only in a single experiment (data not shown) and found not to be present, which was substantiated by no PLC responses seen by α,β-2MeSATP. This is also consistent with reports of the downregulation of P2X receptors in rat arterial SMCs in culture (Erlinge *et al.*, 1998).

In WKY VSMCs the ITP response was less than two fold less than UTP and ATPγS was a weak partial agonist. This is supported by hypothesis 3 where ITP is slighty less potent that UTP. This suggests a mixed population of $P2Y_2$ and $P2Y_4$ receptor subtype expression. RT-PCR analysis showed expression of $P2Y_2$ and $P2Y_4$ mRNA expression which is consistent with this. Unfortunately, suramin was not used on these cells and cannot contribute to this hypothesis. Similar to SHR cells, UDP produced potent partial responses compared to UTP in WKY cells. This suggested the presence of $P2Y_6$ receptors and again the expression of $P2Y_6$ mRNA was confirmed by RT-PCR analysis. Also similar to SHR cells was that mRNA expression of $P2Y_1$ and $P2Y_{12}$ was not seen in WKY cells. ADP, an agonist at $P2Y_1$ and $P2Y_{12}$, produced a weak response in these cells which would be consisted with RT-PCR results. However, more potent $P2Y_1$ and $P2Y_{12}$ agonist such as

2MeSADP was not used, however it is unlikely that this would have produced a PLC response.

Differences in SHR cells compared to WKY cells were also seen. ATPγS was a more potent partial agonist in SHR than WKY, whereas ITP was more potent in WKY, but weak in SHR. Both ITP and ATPγS produced responses that were partial compared to the maximal UTP response in both SHR and WKY VSMCs. The results favour hypothesis 1 for SHR cells and hypothesis 3 for WKY cells. In other words, the responses stimulated by UTP or ATP in SHR VSMCs is probably mediated by $P2Y_2$ receptors whereas in WKY cells the responses are mediated through a mixed population of $P2Y_2$ and $P2Y_4$ receptors. Both cell types expressed $P2Y_2$ and $P2Y_6$ receptor subtype mRNA levels but did not express $P2Y_1$ and $P2Y_{11}$ mRNA. Most interestingly, RT-PCR analysis revealed that $P2Y_4$ receptor subtype expression was present in WKY cells but not SHR cells, which is consistent with the functional results.

The co-stimulation of cells with UTP and ATP in SHR VSMCs showed no changes of the UTP induced PLC activation. However, Harper and colleagues, who also showed partial agonist properties of ATP, demonstrated that ATP antagonized UTP responses when simultaneously used to stimulate SHR VSMCs. Here we report ITP to also act as a partial agonist, but again could not demonstrate antagonism of UTP when applied together. This data would be consistent with hypothesis B (Section 7.3.5), which suggests that both agonists are full agonists at the same receptor. However in the absence of CPK, ATP and ITP have been shown to be partial agonists compared to UTP. A possible explanation for this discrepancy is that Harper and colleagues used a concentration of 30 μM for UTP, whereas in this study 100 μM was used. A higher concentration of UTP may have saturated the receptor and masked the ATP or ITP effects. However a single experiment using 30 μM UTP was performed parallel with experiments using 100 μM UTP. The results from this single experiment also showed no influence of ATP or ITP on UTP responses. Further experiments using 30 μM UTP need to be carried out to corroborate this. However, any antagonistic properties of ATP or ITP may not have been detected using this experimental model as these nucleotides were partial agonists with a maximal response of approximately 75% of the maximal UTP response. Using an agonist which showed a smaller maximal response such as ATPγS (approximately 50%) would demonstrate if this experiment is a valid method of determining if agonists act at the same receptor or not.

In conclusion differences between SHR and WKY VSMCs were clearly seen in response to nucleotides. ATP and UTP were more efficacious in producing mitogenic responses in the cells derived from hypertensive rats than normotensive. This, therefore, could explain why hypertensive vasculature is prone to increased hyperplasia and hypertrophy. Furthermore P2Y receptor subtype expression was also different between the two cell types in that $P2Y_4$ receptor was not present on hypertensive SMCs whereas it was in normotensive SMCs. The mitogenic responses may therefore be mediated via $P2Y_2$ receptors in SHR VSMCs, whereas in WKY coexpression of $P2Y_4$ may regulate mitogenesis instigated by $P2Y_2$ in an antiproliferative manner. The mechanism of this anti-proliferative regulation is unknown and is considered further in the final discussion (Chapter 8). However, it still remains unclear as to whether there is a quantitative difference in P2Y receptor subtype mRNA expression levels between SHR and WKY (see section 8.1.2). The study here only demonstrates the presence or absence of certain subtypes rather than absolute comparison in numbers. Investigating nucleotide signalling and receptor expression in hypertensive and normotensive VSMC models is of great clinical importance to generate better drugs in dealing with such conditions as hypertension

Chapter 8

Discussion, Conclusion & Further Work

8.1 Discussion & Further Work

Investigating the agents inducing VSM proliferation is important as overgrowth leads to atherosclerosis, blood vessel occlusion and hypertension. Potential anti-proliferative drugs targeted at smooth muscle growth are therefore essential for the treatment of hypertension and atherosclerosis. At present patients with artherosclerosis are treated by bypass grafting, using autologous SV or IMA grafts or percutaneous transluminal coronary angioplasty. However, the failure rate is extremely high and surgical intervention leads to restenosis and intimal hyperplasia. Growth factors and nucleotides have been implicated in VSM proliferation. Platelets contain a large source of ATP, growth factors and other inflammatory mediators. In vascular disease or trauma, where endothelial function is compromised, release of these mediators from platelets and the release of nucleotides from ruptured cells act directly at smooth muscle cells. The effects of extracellular ATP, and its degradation products ADP and adenosine, include vasoconstriction, dilation, platelet aggregation, smooth muscle proliferation and hypertrophy (Ralevic & Burnstock 1991, Erlinge 1998, Boarder and Hourani, 1998).

The aim of this thesis was to investigate the role of P2Y receptors in the regulation of VSMCs from human SV and the hypertensive rat. A growing body of evidence shows that ATP and other nucleotides induce mitogenesis in VSMCS from rat aorta (Erlinge *et al.*, 1992, 1993, Harper *et al.*, 1998), porcine aorta (Wang *et al.*, 1992), bovine aorta (Crowley *et al.*, 1994) and human arteries and vein (Erlinge *et al.*, 1994). The work in this thesis provides further evidence for the role of ATP in proliferation of rat VSMCs, and in concert with PDGF in human cells. In addition the role of UTP as a mitogen in rat VSMCs is also reported. In contrast, UTP acted as an anti-proliferative agent in human VSMCs. UDP was also studied and displayed no mitogenic properties in rat VSMCs, whereas in human cells it also acted as an anti-proliferative agent.

8.1.1 Human VSMCs

In VSMC derived from human SV, nucleotides *per se* were not mitogenic. However in the presence of PDGF, ATP synergistically enhanced proliferation, whereas the pyrimidines, UTP and UDP, inhibited the PDGF-induced mitogenic response. PDGF is a well known mitogen and its signalling pathway is discussed further.

8.1.1.1 Role of PDGF

The mitogenic effects of PGDF observed in this study were consistent with earlier studies by Bornfeldt and co-workers (1997) and Yang and co-workers (1998) who both reported proliferation in response to PDGF in human VSMCs and increases in ERK1/2 phosphorylation. The two inhibitors of ERK pathway, PD98059 and U0126, inhibited PDGF stimulated ERK and DNA synthesis. The use of other protein kinase inhibitors, such as SB203580, LY294002 and Y27632, suggested that many pathways were involved in PDGF stimulated DNA synthesis. They also showed that the ERK pathway was independent of PI3K, p38 and ROCK. All of these pathways were necessary for DNA synthesis, but individually may not have been sufficient to maintain proliferation.

Another MAPK, JNK, was also investigated. PDGF has been shown to stimulate JNK activity in many different types of cell (Minden *et al.*, 1994, Lallemand *et al.*, 1998) including VSMCs (Li *et al.*, 1999a, Hamada *et al.*, 1998). JNK has been implicated in both proliferation and hypertrophy (Pedrum *et al.*, 1998, Izumi et al., 2000, Schmitz *et al.*, 2001, Wang *et al*, 1998b). In this study JNK was stimulated by PDGF in human VSMCs. The stimulation of both ERK and JNK by the same stimulant, as reported here, has previously been shown, for example in rat tracheal SMCs stimulated by endothelin and thrombin (Shapiro *et al.*, 1996), in chicken DT40 B cell line stimulated with EGF (Hasimoto *et al.*, 1999) and in mouse skeletal muscle stimulated with insulin (Moxham *et al.*, 1996). Since PDGF has been shown to be mitogenic in human VSMCs leading to increased DNA synthesis, increased ERK activation, cell cycle progression (unpublished data from M.R. Boarder et al) and cell division (White *et al.*, 2000), the rapid activation of JNK leads to the suggestion that it too may contribute to cell proliferation. This was further corroborated by the attenuation of JNK phosphorylation by MEK inhibitors. This data not only supports the hypothesis that the JNK pathway may be necessary for PDGF-induced proliferation in human VSMCs, but also that its regulation lies downstream of ERK. JNK regulation by ERK has been previously reported in vascular endothelial cells (Pedrum *et al.*, 1998). MEK inhibitors and dominant negative ERK attenuated the VEGF-induced phosphorylation of JNK. Down regulation of JNK activation by dominant-negative SEK-1 and JNK-1 resulted in an attenuation of proliferation suggesting that JNK was necessary for proliferation. The activation of JNK by ERK was also shown to be indirect. ERK cannot directly activate JNK, as JNK is not a substrate of ERK (Songyang *et al.*, 1996). MEK1/2, which are the targets of PD98059 and U0126 have also been shown not to activate JNK

(Minden et al., 1994, Sanchez *et al.*, 1994) therefore MEK signalling directly to JNK does not occur. The possibilities that remain are that ERK could regulate one or more of the JNK pathway kinases. This was shown to be the case when dominant negative SEK-1 was co-transfected with constitutively active ERK into endothelial cells (Pedrum *et al.*, 1998). JNK activation was inhibited which suggested that ERK activates SEK-1 through upstream signalling to this kinase. ERK to JNK cross talk via SEK-1 could possibly be a mechanism of cross-talk seen in human VSMCs, and would be consistent with the hypothesis that the JNK pathway was necessary for proliferation. A simple model of PDGF signalling to ERK and JNK is shown in Figure 8.1A.

Further investigations studying the regulation of JNK by the ERK pathway thus need to be performed. A possible direction in pursuing this could be via JNK inhibitors or dominant negative constructs of JNK and/or upstream kinases such as SEK-1 (MKK4). The recently developed cell permeable inhibitors L-JNK1 and D-JNK1 (Bonny *et al.*, 2001) have been shown to inhibit IL-1β-induced apoptosis of β-cells by competitively blocking the interaction between JNK and c-jun. This inhibitor can be used to verify if JNK is indeed involved in DNA synthesis stimulated by PDGF as measured by the [^{3}H]-thymidine incorporation assay, similar to those experiments performed using the MEK inhibitors (Section 2.9). Dominant negative mutants of JNK and SEK-1 could also be introduced along with constitutively active ERK to investigate if they inhibit JNK activation. Recently Izumi and colleagues (2001) demonstrated that *in vivo* transfection of dominant negative ERK and JNK in rat carotid artery SMC decreased ERK and JNK activation, which would otherwise have increased. Gene transfer of these mutants also prevented neointimal formation and proliferation. Conversely gene transfer of wildtype ERK or JNK enhanced neointimal hyperplasia. As discussed above, Pedrum and colleagues (1998) transfected endothelial cells with dominant negative SEK-1 (or MKK4) which attenuated VEGF ability to stimulate JNK, even in the presence of a constitutively active ERK. A dominant negative MKK4/7 transfected into VSMCs would establish whether the JNK pathway was involved in PDGF-induced DNA synthesis. MKK4/7 activation could also be assayed and inhibition with PD98059 would establish if either of theses MKKs was downstream of MEK1.

The increase in JNK activation by PDGF was rapid and cannot be explained by ERK mediating a signal to increase transcription of other growth factor or mediators e.g. PDGF, which would then be secreted to produce an autocrine JNK activation, as this would take several hours (Wilson *et al.*, 1993). Autocrine ATP activation of JNK has been shown

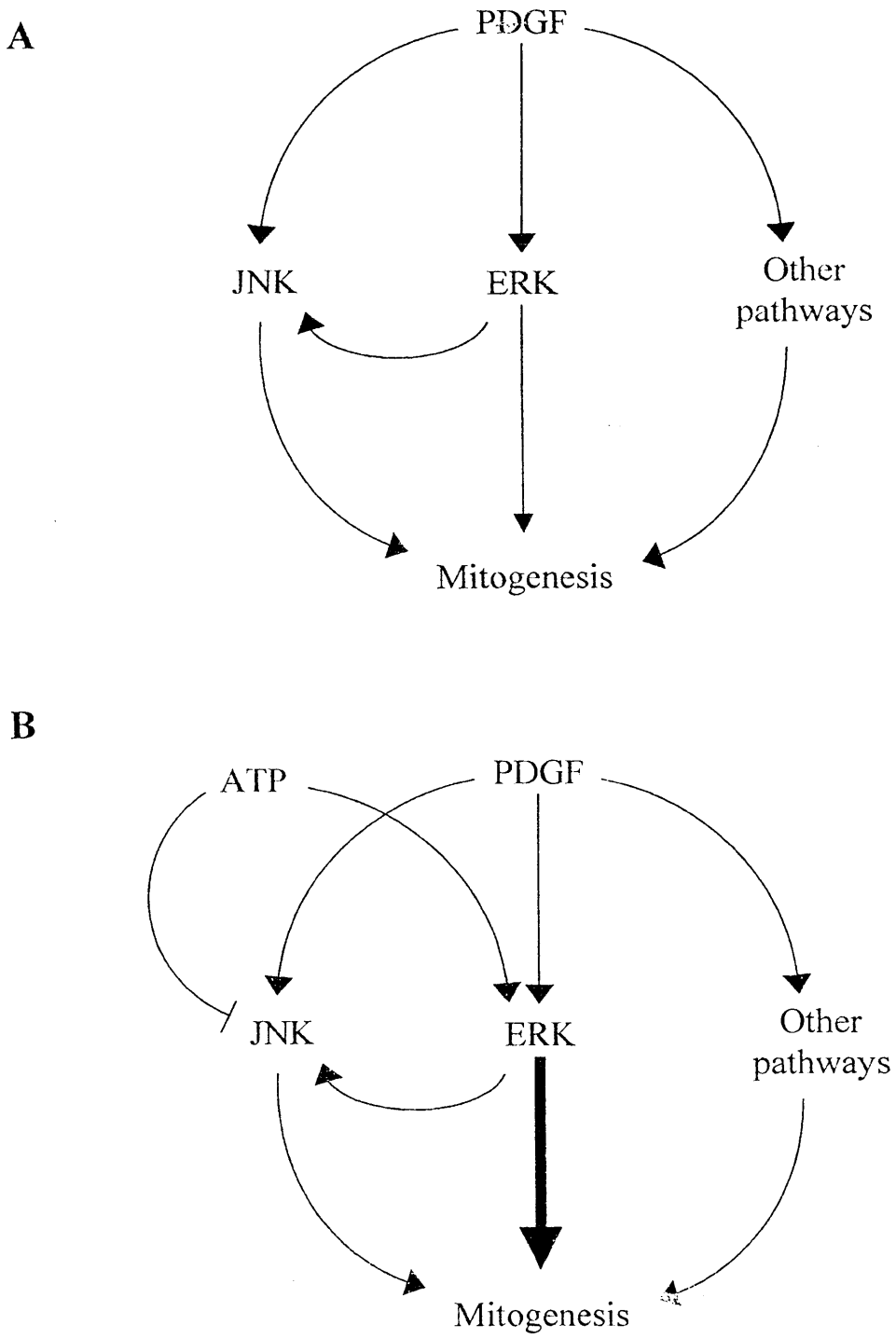


Figure 8.1

Simplified model of signalling pathway to mitogenesis stimulate by A) PDGF and B) PDGF + ATP.

to occur within 5 minutes of inducing stretch on rat VSMCs (Hamada *et al.*, 1998). A similar mechanism where PDGF induces the release of an autocrine JNK activator may be employed in these cells, which is mediated by the ERK pathway. PDGF has been shown to accumulate increases in cAMP and increase PKA activation within 5 minutes in human arterial SMCs (Graves *et al.*, 1996). The addition of indomethacin, a COX inhibitor, inhibited the PDGF-induced cAMP rise consistent with the release of prostaglandins stimulating cAMP. Cytosolic phospholipase A_2 ($cPLA_2$) catalyses the release of arachidonic acid from plasma membrane phospholipids (Dennis 1994), which is then metabolised by COX1/2 to produce prostaglandins. Regulation of $cPLA_2$ is dependent on Ca^{2+} mobilisation, PKC and activation by ERK (Glaser *et al.* 1993, Wightman *et al.*, 1982, Goldberg *et al.*, 1990). This is evidence to suggest that PDGF can regulate, via ERK, signalling pathways which cause the release of autocrine activators of other signalling pathways. Prostaglandin F_2 (PGF_2) and thromboxane have been shown to mediate proliferation in human uterine ULTR cell line used as a model for human VSMCs (Miggins *et al.*, 2001). Stimulation of both ERK and JNK was observed. A simple experiment to verify whether COX1/2 may be involved in PDGF-induced proliferation would be to apply indomethacin to human VSMCs, stimulate with PDGF and measure DNA synthesis. An inhibition of DNA synthesis would suggest that COX1/2 and a prostaglandin signalling pathway is necessary for PDGF mitogenic signalling. Since COX1/2 signalling pathways lie downstream of ERK, this could potentially be a method of signalling to JNK. Again this could be confirmed using indomethacin to block COX1/2 and seeing if this too inhibits JNK phosphorylation induced by PDGF. These experiments would further reveal how PDGF signals to JNK in human VSMCs.

PDGF did not couple to p38-MAPK and the inhibition of PDGF-induced DNA synthesis by SB2035880 was suggested to be as a result of action at a kinase other than p38 MAPK. Other enzymes which may be inhibited by SB203580 include COX1/2 and protein kinase B (PKB) (Davies *et al.*, 2000, Börsch-Haubold *et al.*, 1998). The hypothesis that SB203580 inhibited other enzymes that were involved in mitogenesis, could be tested by using inhibitors of both COX1/2 and PKB. As suggested above indomethacin inhibition of PDGF-induced DNA synthesis would imply COX1/2 is involved in the mitogenic signalling pathway of PDGF. PKB inhibitors can also be used in a similar way to see if they too inhibit DNA synthesis, ERK or JNK activation. These experiments would only suggest that these enzymes were involved in DNA synthesis rather than demonstrate that SB203580 was acting at them. It would be necessary to assay their activities induced by

PDGF and then apply SB203580 to assess their inhibition. If indeed SB203580 were shown to inhibit COX1/2, it would also confirm that JNK was not regulated by COX1/2 because SB203580 was shown not to influence PDGF-induced JNK phosphorylation. Therefore ERK regulation of JNK would be independent of prostaglandin production.

8.1.1.2 Role of ATP

Erlinge *et al.* (1994) demonstrated for the first time that ATP alone could induce human VSMC proliferation. In contrast to this, our studies show that ATP and other nucleotides alone were unable to stimulate an increase in DNA synthesis. This is supported by studies of cell cycle progression that demonstrate that nucleotides by themselves fail to stimulate the cell cycle beyond G_1 phase (Miyagi *et al.*, 1996). It is likely that the low levels of FCS in studies by others acted as a progression factor required in the cell cycle enabling ATP to induce completion of the cell cycle. In our study, human VSMCs were completely deprived of serum for 2 days, therefore eliminating a progression factor and hindering nucleotide-induced mitogenesis. In contrast, in cultured rat VSMCs ATP and UTP stimulated DNA synthesis in the absence of serum, which suggests that nucleotides *per se* may not be mitogenic in human cells.

However, ATP significantly increased PDGF induced mitogenesis despite being unable to stimulate DNA synthesis alone. This significant synergistic effect with PDGF has not previously been reported in human VSMCs (not seen in IMA VSMCs – see White *et al.*, 2000). Experiments involving CPK showed that this was not dependent on the breakdown of ATP to ADP. In porcine aortic smooth muscle cells ATP synergistically increased DNA synthesis with PDGF, IGF-1 and EGF (Wang *et al.*, 1992). ATP has also been reported to increase the mitogenic response produced by its sympathetic co-transmitters neuropeptide Y in human VSMCs (Erlinge *et al.*, 1994). This data also supports the previous suggestion that nucleotides only move the cell cycle from G_0 to G_1 and require the presence of a progression factor, such as PDGF, to allow DNA synthesis and subsequent cell division to occur (Miyagi *et al.*, 1996, Malam-Souley *et al.*, 1996).

ATP-instigated ERK activation was similar to the PDGF-induced response when stimulated for more than 10 minutes. Even more surprisingly, ATP in the presence of PDGF generated ERK responses that were greater than the additive effect of the individual agonists. This was consistent with DNA synthesis studies, which showed synergistic increases in PDGF responses by ATP. However, the ATP mitogenic response, which was equal to that of PDGF in phosphorylating ERK, was not consistent with the lack of DNA

synthesis by ATP. It is evident that ATP was able to instigate an ERK response via its G protein-coupled P2Y receptor. However it was unable to sustain the signalling pathway to DNA synthesis independently of the PDGF signalling pathway. This is consistent with the view that the PDGF receptor was able to stimulate more than one pathway leading to mitogenesis, which amalgamated to produce a significant increase in DNA synthesis. Like ATP, PDGF-induced ERK activation may not always result in proliferation as was seen in human IMA VSMCs by Yang *et al.* (1998). It seems likely, therefore that the activation of ERK by either a GPCR or TK receptor is not sufficient to instigate proliferation. Other kinases or signalling pathways thus have a role to play together with ERK in stimulating mitogenesis. Wilden and colleagues (1998) reported ATP-induced proliferation in porcine coronary artery SMCs required independent activation of both ERK and PI3K. Alone neither one of these kinases appeared sufficient to produce the signals necessary for proliferation. Wang *et al.* (1992) also reported increases in DNA synthesis in porcine SMCs induced by ATP, which were mediated independently via arachidonic acid production and PKC. These reports suggest that many different signalling pathways work synergistically to induce mitogenesis.

In this study it is unknown what other pathways, apart from ERK, ATP may be stimulating to induce proliferation and unclear precisely how PDGF receptor signalling is aided by ATP receptor signalling. As discussed above, JNK activation induced by PDGF was found to be dependent on the ERK pathway. However, an interesting outcome of JNK studies stimulated with nucleotides showed that ATP did not stimulate JNK activation despite stimulating ERK with equal magnitude as PDGF at longer stimulation times. In the presence of PDGF ATP again did not enhance JNK activation further despite potentiating PDGF-induced ERK activation. Therefore, the synergistic enhancement was independent of the JNK pathway. This raises the question why does the PDGF, but not the ATP, signalling pathway to ERK result in JNK activation. It also suggests that the PDGF-induced JNK activation may not exclusively be dependent on the ERK pathway, but must involve a network of different pathways. This combination of different signalling pathways, which incorporates ERK, allows PDGF to signal to JNK, whereas ATP, via its P2Y receptor, may not be able to stimulate all the necessary components to achieve this. Although ATP potentiates the PDGF-induced ERK response, this alone may also be insufficient to potentiate JNK activation. PDGF signalling pathway may also recruit scaffold proteins which complex with the components of the ERK and JNK pathway bringing them in close proximity with each other to allow cross talk. ATP on the other

hand may not employ this mechanism and therefore unable to activate JNK. An example of a scaffold protein that may be involved is JSAP1 (Ito *et al.*, 1999, Kuboki *et al.*, 2000). Another possible explanation is that the signalling pathway of ATP may contain some element that inhibits the activated JNK pathway. Therefore in the presence of PDGF, ATP potentiates ERK but at the same attenuates any further activation of JNK. This mechanism of JNK attenuation by ATP is probably independent of the ERK to JNK crosstalk, because ATP did not attenuate PDGF-induced JNK phosphorylation. The PDGF and the ATP signalling cascade are therefore far more complex than originally envisaged. It would be of further value to study the effects of other growth factors, such as EGF and IGF and to ask if these growth factors (like PDGF) stimulate JNK in an ERK-dependent manner or if this is a mechanism specific for PDGF. Following on from this would be to ask whether ATP synergistically enhances ERK activation and proliferation without further stimulation of the JNK pathway. From these sorts of investigations we can establish whether the ATP-stimulated receptor signals to a variety of growth factor receptors or just PDGF. A simple model of PDGF and ATP signalling is shown in Figure 8.1B.

The human P2Y receptor involved in synergistically enhancing mitogenesis is still unclear. $P2Y_1$ and $P2Y_{11}$ are unlikely candidates as 2MesADP (more potent than ATP at $P2Y_1$) and ATPγS (more potent than ATP at $P2Y_{11}$) did not elicit similar increases in DNA synthesis. $P2Y_2$ receptor subtype is equally activated by ATP and UTP and thus cannot be involved. $P2Y_4$ and $P2Y_6$ receptor subtypes are unlikely candidates because they are stimulated by UTP and UDP respectively.

8.1.1.3 Role of UTP

UTP, like ATP, has been shown to be mitogenic in rat VSMC (Erlinge *et al.*, 1993, Harper *et al.*, 1998, Satterwhite *et al.*, 1999, Chapter 7). An unexpected finding of this study was the opposing influences that UTP had on mitogenic signalling compared to ATP in human VSMC. UTP did not stimulate proliferation or potentiate PDGF-induced response but significantly inhibited the PDGF mediated increase in DNA synthesis. UTP also inhibited cell number increases induced by PDGF (White et al, 2000 – inhibition also seen in IMA VSMCs). Consistent with antiproliferative actions of UTP in human cells is previous data from our laboratory (Roberts *et al.*, 1999) that demonstrated the transfected human $P2Y_4$ expressed in 1321N1 stimulated by UTP to attenuate PDGF mediated increases in DNA synthesis. This was not true of the other UTP receptor, $P2Y_2$. In studies on monkey kidney endothelial cell line LLC-MK_2, UTP alone was shown to be

antiproliferative (Lemmens *et al.*, 1996). The most likely human P2Y receptor candidate for this anti-proliferation is $P2Y_4$. It cannot be $P2Y_1$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ or $P2Y_{13}$ as UTP is not an agonist at these receptors. ATP and UTP are both equi-potent at $P2Y_2$, but this receptor is unlikely to be involved as ATP has been shown to be proliferative.

The ATP influence on PDGF-induced proliferation was found to be mediated at the level of ERK activation. In contrast to this, UTP influence on PDGF was not by modulation of ERK activity. The inhibition of proliferation was independent of the ERK pathway. Another interesting observation was that UTP inhibited the PDGF-induced JNK activation. P2Y receptors have been shown to stimulate JNK activity in many different types of cell including VSMCs (Li *et al.*, 1999, Hamada *et al.*, 1998). This is a novel mechanism of cross-regulation between the UTP-stimulated P2Y receptor and PDGF receptor. The strong attenuation of the PDGF-stimulated JNK activation, may play a role in the anti-proliferative signalling induced by UTP. $P2Y_4$ receptor subtype, which is the most likely mediator of UTP signalling, couples to JNK inhibition. However, using 1321N1 cells transfected with human $P2Y_4$, the effects of UTP on PDGF could not be observed, as PDGF did not stimulate JNK phosphorylation. UTP alone also had no affect on JNK phosphorylation. To elucidate if this receptor subtype does couple to the inhibition of JNK, experiments in 1321N1 cells transfected with human $P2Y_4$ need to be repeated using a growth factor that also stimulates JNK activation.

Similar UTP inhibitory observations have been reported in human-derived Eahy926 endothelial cells (Paul *et al.*, 2000). UTP was found to attenuate the TNFα and sorbitol–stimulated JNK and p38 MAPK activity, which was found to be mediated by either the $P2Y_2$ or $P2Y_4$ receptor subtypes. However, in 1321N1 cells transfected with human $P2Y_2$ and $P2Y_4$ receptors this group found that TNFα-induced JNK activation was not inhibited by UTP but in fact stimulated by UTP alone. This suggests that the effects mediated by P2Y receptors are dependent upon cell type.

There are two possible mechanisms of UTP exerting its effects on JNK. Firstly, UTP may inhibit the ERK to JNK cross-talk. The second mechanism may be inhibition of JNK which is ERK-independent inhibition (possibly be mediated through a phosphatase or inhibition of upstream kinases). We found that the combined inhibitory properties of UTP and PD98059 on JNK phosphorylation was not additive despite them being potent inhibitors individually. This suggests that the UTP inhibitory pathway and the PD98059 inhibitory pathway share some common elements. Alone PD98059 reduced PDGF response to 40%, whereas UTP reduced this to 30%. If they were acting independently of

each other, the outcome of simultaneous stimulation would be additive. As this was not the case it seems likely that their actions merge. This is occurring at a point downstream of ERK as UTP was found not to inhibit it. A possible mechanism is hypothesised in Figure 8.2.

The mechanism for PDGF signalling to JNK is still unclear. This makes it difficult to understand where UTP may be acting to inhibit PDGF-stimulated JNK activity. Further experiments to attempt to determine the mechanism of UTP inhibition need to be performed. But firstly we need to identify the components of the PDGF to JNK signalling pathway and then try to establish where UTP may be acting. The first step would be to investigate MKK4/7, which activates JNK. It is activated by phosphorylation on serine and threonine residues (Derijad *et al.,* 1995) and available phospho-specific antibodies for MKK4/7 can be used in the same way as phospho-specifc JNK antibodies were used (Section 2.4-2.6) to demonstrate if PDGF induces phosphorylation. Next, the addition of UTP or PD98059 alongside PDGF would verify if MKK4/7 phosphorylation is inhibited. This would reveal whether UTP or ERK regulate JNK activation directly or via its upstream kinases. If UTP had no influence on MKK4/7 phosphorylation it would suggest that UTP inhibited JNK not by preventing its phosphorylation but by increasing its dephosphorylation. This may be mediated through increasing the activity of MAPK phosphatases (MKP).

Upstream of MKK4/7 lies the MAPKKKs which include MEKKs, MLKs, ASK and TAK. Measuring their activities are slightly more difficult due to limited tools available such as phospho-specific antibodies. However, they can be shown to be involved in PDGF signalling using dominant-negatives and co-immunoprecipitation studies. A possible method of measuring their activation could be by immunoprecipitating them using specific antibodies, separating proteins by Western blotting and probing the immunoprecipitated proteins using anti-phospho ser/thr antibodies.

The small GTPases Rac1 and Cdc42 can be shown to be involved in the PDGF signalling pathway to JNK by again using dominant negative mutants. The activity of Rac1 and Cdc42 can be measured by their ability to bind to PAK. GST-PAK proteins on sepharose beads can be used to precipitate activated Rac1/Cdc42 from cell extracts, which can then be separated using Western blot. The relative amount of Rac1/Cdc42 precipitated compared to basal levels would act as an indicator of their activity. This method can then be used to assess whether UTP acts at the level of small GTPases.

ROCK and PI3K inhibitors suggest that PDGF utilises these pathways to induce proliferation. These inhibitors, on the other hand, did not interfere with JNK activation by PDGF. Therefore JNK activation is independent of these kinases. Other inhibitors of protein kinases such as PKC, PKB and PKA are available and can be used in a similar way to determine if these kinases have a role to play in JNK activation.

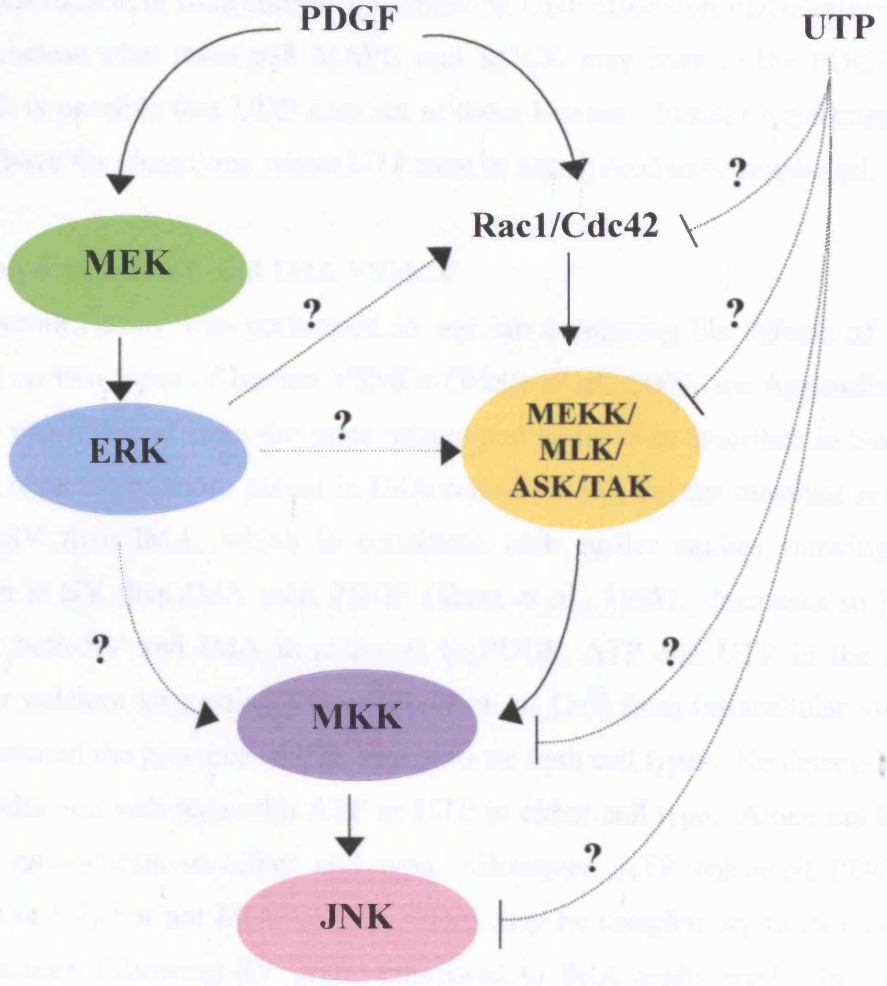


Figure 8.2

Hypothetical model showing the regulation of the JNK signalling pathway PDGF and UTP.

8.1.1.4 Role of UDP

UDP was also found to inhibit PDGF-induced DNA synthesis. This has not been previously reported. However, unlike UTP it did not attenuate PDGF-induced JNK activation. It is most likely that UDP responses were mediated via $P2Y_6$ for which it is the most potent agonist. At present it is unclear how UDP may be mediating this attenuation. The effects of UDP acting at ERK cannot be ruled out and need further investigation. For example construction of time courses investigating UDP effects on PDGF-stimulated ERK. It is also unclear what roles p38 MAPK and ROCK may have in the PDGF signalling pathway. It is possible that UDP may act at these kinases. Similar experiments to those described above for identifying where UTP may be acting need to be employed.

8.1.1.5 Comparison of SV and IMA VSMCs

A parallel study was performed in our lab comparing the effects of PDGF and nucleotides on two types of human VSMCs (White *et al.*, 2000, see Appendix). SV and IMA tissue was obtained from the same patient and cultured as described in Section 2.1.1. PDGF was found to be more potent in IMA cells than SV, but the maximal response was greater in SV than IMA, which is consistent with earlier studies showing increased proliferation in SV than IMA with PDGF (Yang *et al.*, 1998). Increases in $[Ca^{2+}]_i$ was reported in both SV and IMA in response to PDGF, ATP and UTP in the absence of extracellular calcium suggesting the mobilisation of Ca^{2+} from intracellular stores. This also demonstrated the presence of P2Y receptors on both cell types. No detectable increase in $InsP_3$ production was seen with ATP or UTP in either cell type. Alone nucleotides did not induce mitogenesis in either cell type. However, ATP enhanced PDGF-induced mitogenesis in SV, but not IMA VSMCs. This may be contributory to increased intimal proliferation seen following SV grafts compared to IMA grafts used. In contrast, the inhibitory actions of UTP on PDGF-induced mitogenesis was seen to a similar degree in both IMA and SV VSMCs. This study demonstrated that P2Y receptors selective for ATP may play a role in concert with PDGF in intimal hyperplasia of SV grafts following surgery, whereas UTP selective P2Y receptors on both IMA and SV play a role in regulating anti-proliferation.

8.1.2 Rat VSMCs

Proliferation of VSMCs is a crucial step in the development of hypertension, which increases the probability of secondary cardiovascular diseases such as arteriosclerosis, congestive heart disease, atherothrombic brain infarction and nephrosclerosis if left untreated. The sheer stress caused by hypertension results in damage to the endothelium and platelet aggregation, therefore nucleotides such as ATP released from activated platelets or disrupted cells can act on VSMCs. The SHRs are the most commonly used experimental animal model of the human hypertension disease state and are predisposed to develop hypertension spontaneously with a high blood pressure (>150mmHg) seven weeks after birth. The original Wistar strain from Kyoto is used as a normotensive model (WKY). SHR VSMCs display enhanced mitogenic responses compared to WKY VSMC to several G protein-couple receptors (Morton *et al* 1995, Paquet *et al* 1990, Bunkenburg *et al* 1992, Osani & Dunn 1992, Turla & Webb 1990, Harper *et al* 1998).

This study demonstrated enhanced PLC and ERK activation in SHR cells compared to WKY was also shown. Parallel to this was the enhanced DNA synthesis observed in SHR cells in response to UTP and ATP. Taken together these data suggest that the larger P2Y-coupled PLC and ERK activation responses in SHR cells resulted in larger levels of mitogenesis. It could therefore be hypothesised that proliferation of hypertensive VSMCs is a result of an enhanced P2Y receptor signalling.

Another difference between the two cell lines was observed in P2Y receptor subtype expression. It appears that the mitogenic responses stimulated by UTP or ATP in SHR VSMCs were probably mediated by $P2Y_2$ receptors, whereas in WKY cells the responses are mediated through a mixed population of $P2Y_2$ and $P2Y_4$ receptors. Therefore not only did ATP and UTP produced large mitogenic responses in the cells derived from hypertensive rats, but these responses were primarily mediated through the $P2Y_2$ receptor subtype. This implies that the coexpression of $P2Y_4$ in WKY may regulate mitogenesis instigated by $P2Y_2$ in an anti-proliferative manner. The absence of this receptor subtype in SHR allows $P2Y_2$ receptor signalling to proceed to mitogenesis unrestrained. The mechanism of this anti-proliferative regulation is unknown, but would be consistent with the anti-proliferative responses produced by the human $P2Y_4$ receptor also described in this thesis. This down-regulation of $P2Y_4$ could therefore be a mechanism that is employed in hypertensive vasculature, making it more prone to increased hyperplasia and hypertrophy.

Modulation of purinergic receptor expression levels has been shown to occur. VSM cells in culture lose their P2X receptors, whereas P2Y receptors are maintained (Pacaud *et al*, 1995, Erlinge *et al.*,1998). The up-regulation of $P2Y_1$ and $P2Y_2$ mRNA (subtypes implicated in mitogenic signalling mediated by nucleotides) in synthetic phenotype cells was also seen. Furthermore, up-regulation of the $P2Y_2$ receptor has been demonstrated in the neointima *in vivo* following experimental balloon angioplasty (Seye *et al.*, 1997) and the auto-upregulation of expression of P2Y receptors by ATP itself and other growth factors has been demonstrated (Hou *et al.*, 1999).

However, it still remains unclear as to whether there is a quantitative difference in P2Y receptor subtype mRNA expression levels between SHR and WKY. The study here only demonstrates the presence or absence of certain subtypes rather than absolute comparison in numbers. Although not described in this thesis, quantitative RT-PCR methods were designed with Dr. T.E. Webb to quantify absolute mRNA levels in SHR and WKY. This procedure is still under investigation. This method could then be employed to study receptor subtype mRNA levels in VSMCs, not only in culture, but also in the primary tissue from freshly dissected rats. This would show whether $P2Y_4$ receptor expression is absent in SHR vasculature as it is in culture or whether $P2Y_4$ is only down-regulated in culture. However, the presence of mRNA for a receptor subtype does not imply that the receptor is expressed at the plasma membrane and functional. In most cases the presence of mRNA expression has also been confirmed by functional assays, therefore this results from this technique must be corroborated with results from functional assays.

8.1.3 P2Y receptor signalling to MAPKs

P2Y receptors have been shown to couple to the activation of PLC via G_q proteins. This was shown to be the case in rat VSMCs, however in human VSMCs rises in intracellular Ca^{2+} was found to be independent of increases in inositol polyphosphate production. The phosphoinositide pathway independent increase in $[Ca^{2+}]_i$ is not unusual and previously reported in brain endothelial cells (Albert *et al.*, 1997; Frelin *et al.*, 1993). A possible explanation is that only a very small increase in $Ins(1,4,5)P_3$ may have occurred, which may have been to small to detect using the methods available. Binding to $InsP_3$ receptor stimulated release of Ca^{2+} that resulted in Ca^{2+}-induced Ca^{2+} release. The elevations in $[Ca^{2+}]_i$ without the concomitant production of $Ins(1,4,5)P_3$ could also occur. Sphingosine kinase may be activated independently of $Ins(1,4,5)P_3$ production by GPCR,

resulting in a rapid and transient increase in the putative second messenger, sphingosine-1-phosphate (SPP), triggering calcium mobilisation from intracellular stores (Olivera & Spiegel 1993). $P2Y_2$ receptors in HL-60 were recently shown to couple to SPP production (Alemany *et al.*, 2000). Inhibition of sphingosine kinase using DL-threodihydrosphingosine (tDHS) prevented ERK activation, DNA synthesis and Ca^{2+} mobilisation stimulated by ATP and UTP. To ascertain if P2Y receptors are utilising this pathway to increase intracellular Ca^{2+} levels, tDHS can be used to see if it inhibits ERK activation, DNA synthesis and Ca^{2+} mobilisation. If SPP is believed to be involved its formation stimulated by nucleotides can be assayed. If SPP is not involved then the increases in Ca^{2+} can be assumed to be due to undetectable increases in inositol phosphate levels.

G-protein coupled receptors are coupled to mitogenesis through tyrosine kinase and MAPK cascade (Gutkind, 1998; Bourne, 1990, Bourne *et al*, 1995) via their free βγ subunits (Faure *et al.*, 1994, Crespo *et al.*, 1994, Koch *et al.*, 1994). Non-receptor tyrosine kinases such as Src or Src-like tyrosine kinase (Lutterell *et al.*, 1996), Pyk2 (Lev *et al.*, 1995, Dikic *et al.*, 1996, Della Rocca *et al.*, 1997) and PKCs (Pace *et al.*, 1995, Kolch *et al* 1993, Heidecker *et al.*, 1992) have all been shown to play a part.

Though some theoretical pathways have been established for GPCR stimulation of ERK, further work still needs to be carried out for role of the different P2Y subtypes in the activation of ERK. P2Y receptors may employ similar signalling proteins to signal to ERK. In PC12 cells, UTP and ATP stimulation of MAPK were pertussis toxin sensitive, implying signalling via G_i protein, and sensitive to inhibition of Ca^{2+} release (Soltoff *et al.*, 1998a;b). Increased tyrosine phosphorylation in adhesion focal tyrosine kinase (RAFTK also known as Pyk2 and CAKβ), focal adhesion kinase (FAK), Shc, and PKC-δ was also observed. Downregulation of PKC caused only partial reduction in MAPK, suggesting the possibility of both PKC-dependent and independent pathways being involved. P2Y receptor stimulation also increased the association of Shc with Grb2. The results suggest that the $P2Y_2$ receptor-induced activation of MAP kinase was dependent on the elevation of intracellular Ca^{2+} levels and that Pyk2 and PKC mediated the recruitment of Shc and Grb2. Further work established a requirement for the epidermal growth factor receptor activation downstream of Pyk2 (Soltoff *et al.*, 1998b). The EGF receptor has been shown to be activated upon other GPCR activation, such as LPA receptor, which was mediated via the βγ subunit activation of tyrosine kinase Src which may phosphorylate the EGF receptor (Luttrell *et al.*, 1997, Cunnick *et al.*, 1998). Angiotensin II also couples to the PDGF

receptor activation which resulted in Shc and Grb2 association in rat aortic SMCs (Linseman *et al.*, 19995). Similarly P2Y receptors may activate the PDGF receptor.

These studies demonstrate that RTK and GPCR signalling pathways can cooperate to in inducing proliferation. In this study it is unknown what other pathways, apart from ERK, ATP may be stimulating to induce proliferation and unclear precisely how PDGF receptor signalling is aided by ATP receptor signalling. A summary of P2Y receptor activation by ATP and its coupling to ERK in human VSMCs is shown in Figure 8.3A. The signalling via rat P2Y receptor to ERK (hypothesised to be $P2Y_2$) is summarised in Figure 8.3B.

8.2 Conclusion

The growing P2Y receptor family is now one of the largest family of GPCRs. P2Y subtypes are widely distributed in the body. Together this suggests that P2Y receptors have important physiological functions. The role they play in various disease states is a body of work that is also increasing. This thesis focused on the implication that nucleotides are engaged in the regulation of vascular smooth muscle proliferation, which is implicated in the development of atherosclerosis, restenosis and hypertension. Characterising and understanding the P2Y receptor-signalling pathway is of great clinical importance to generate better drugs in dealing with such conditions.

The work in this thesis provides evidence for the role of nucleotides in proliferation of VSMCs. ATP was reported to display proliferative properties and in contrast, UTP and UDP displayed anti-proliferative properties in human VSMCs, mediating its effects through the JNK pathway. ATP is released in close proximity to VSMCs at the site of vascular injury from endothelial cells (during hypoxia and sheer stress), activated platelets (together with PDGF), inflammatory cells and VSMCs themselves (Gordon 1986) and may have an important role in wound healing and repair. However in diseases conditions or following surgery this can also lead to the development of atherosclerosis and pathological conditions such as restenosis following angioplasty *in vivo* (Ross *et al.*, 1993). ATP also stimulates the synthesis of chemoattractant factors such as osteopontin and MCP-1 that are involved in VSMC and monocyte migration (Malam-Souley *et al.*, 1996). UTP is also stored with ATP in platelets and is most likely to be released at the same time. It therefore may function as an anti-proliferative agent

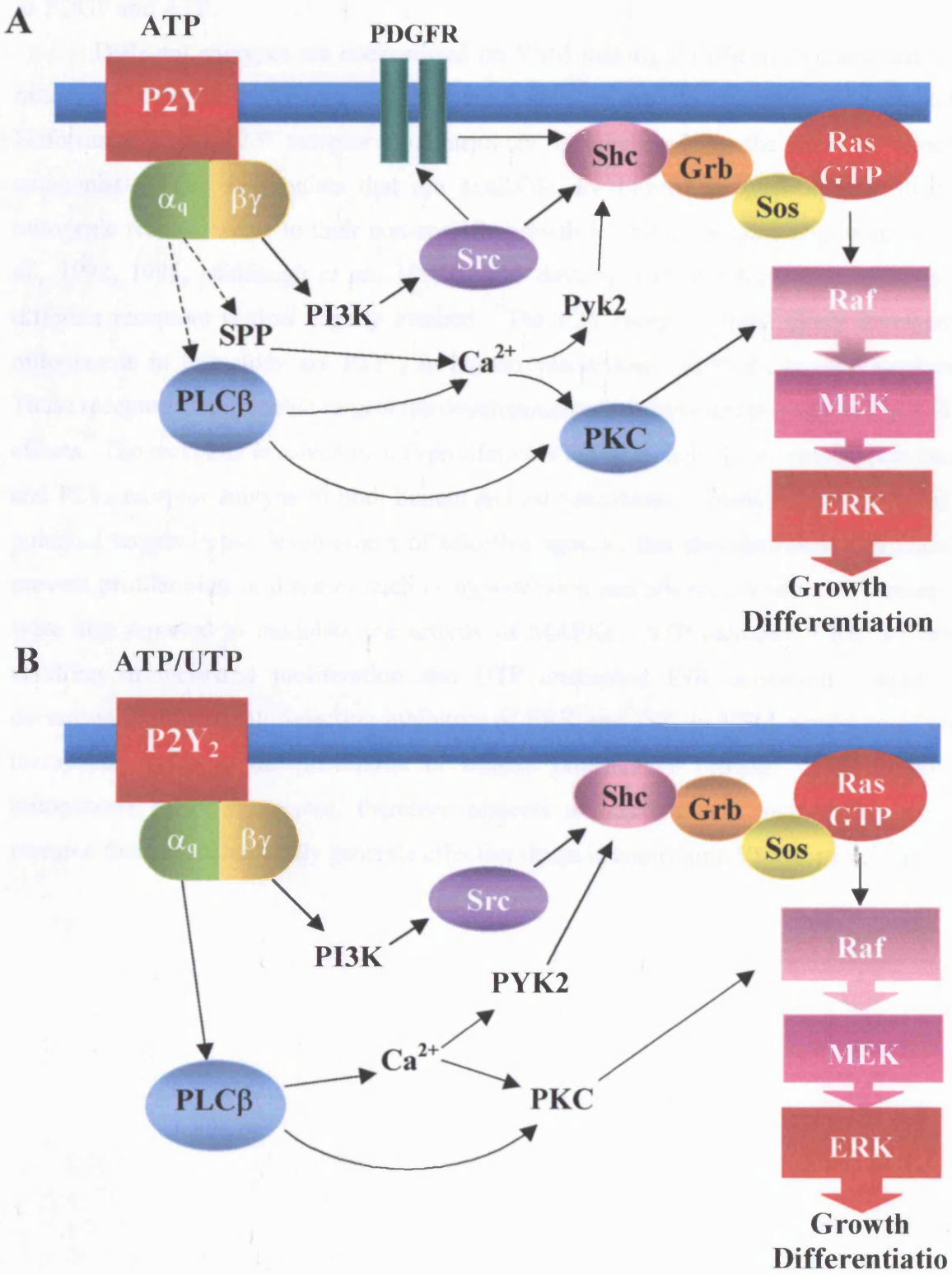


Figure 8.3

P2Y receptor signalling to ERK in A) human and B) rat VSMCs

preventing the proliferation of smooth muscle that may become exposed to mitogens such as PDGF and ATP.

Different subtypes are coexpressed on VSM making it difficult to characterise the mitogenic receptor. This is further confused by the degradation of nucleotides. Unfortunately the P2Y receptor pharmacology is hampered by the lack of selective antagonists. The antagonists that are available are limited in their use in studying mitogenic responses due to their non-specific growth inhibitory or toxic effects (Erlinge *et al.*, 1992, 1995, Middaugh *et al.*, 1992). The development of effective antagonists for different receptors is thus eagerly awaited. The P2Y receptors most likely involved in mitogenesis in this study are $P2Y_{11}$ in human vasculature and $P2Y_2$ in rat vasculature. These receptors are potential targets the development of selective antagonists to block ATP effects. The receptors involved in anti-proliferation are $P2Y_6$ subtype in human vasculature and $P2Y_4$ receptor subtype in both human and rat vasculature. These receptors would be potential targets in the development of selective agonists that stimulate their responses to prevent proliferation in diseases such as hypertension and atherosclerosis. P2Y receptors were also reported to modulate the activity of MAPKs. ATP increased ERK activation resulting in increased proliferation and UTP attenuated JNK activation resulting in decreased proliferation. Selective inhibitors of ERK and JNK in VSM would be of great therapeutic value in the prevention of intimal proliferative disease. The control of mitogenesis by P2Y receptor, therefore presents an exciting and invigorating area of research that would hopefully generate effective drugs in controlling VSMC proliferation.

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Antiproliferative effect of UTP on human arterial and venous smooth muscle cells

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[1]*Cell Signalling Laboratory, Department of Biological Sciences, De Montfort University, Leicester LE1 9BH; and* [2]*Department of Cell Physiology and Pharmacology,*
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White, Pamela J., Rajendra Kumari, Karen E. Porter, Nicholas J. M. London, Leong L. Ng, and Michael R. Boarder. Antiproliferative effect of UTP on human arterial and venous smooth muscle cells. *Am J Physiol Heart Circ Physiol* 279: H2735–H2742, 2000.—We have investigated the hypothesis that responses associated with proliferation are regulated by extracellular nucleotides such as ATP and UTP in cultured human vascular smooth muscle cells (VSMC) derived from internal mammary artery (IMA) and saphenous vein (SV). Platelet-derived growth factor (PDGF), ATP, and UTP each generated an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in both IMA- and SV-derived cells in the absence of detectable inositol 1,4,5-trisphosphate production. ATP alone had no effect on [^{3}H]thymidine incorporation into DNA, but with a submaximal concentration of PDGF it raised [^{3}H]thymidine incorporation in SV- but not IMA-derived cells. UTP alone also was without effect on [^{3}H]thymidine incorporation or cell number. However, in both SV- and IMA-derived cells, UTP reduced the PDGF-stimulated [^{3}H]thymidine response and PDGF-stimulated cell proliferation. This cannot be explained by an inhibitory effect on the p42/p44 mitogen-activated protein kinase (MAPK) cascade, since this response to PDGF was not attenuated by UTP. We conclude that, in human VSMC of both arterial and venous origin, UTP acts as an antiproliferative regulator.

vascular smooth muscle; P2Y receptors; P2 receptors; platelet-derived growth factor; cell proliferation

UNDERSTANDING THE MECHANISMS controlling proliferation of human vascular smooth muscle cells (VSMC) is of considerable importance due to the prevalence of vascular proliferative disease in which intimal smooth muscle cells migrate and proliferate, subsequently occluding the lumen of the blood vessel (26). In coronary artery bypass procedures two vessels are used: internal mammary artery (IMA) and saphenous vein (SV). In a significant number of patients, subsequent problems arise from occlusion of the grafted vessel by this intimal hyperplasia. However, IMA grafts show increased patency over longer periods of time than do SV grafts (4). Interestingly, it has been shown that the serum-stimulated proliferation of smooth muscle cells from SV explants was greater than that for IMA explants (36).

Intimal proliferation of VSMC is controlled by cell surface receptors, which regulate the cell cycle via mitogenic signaling pathways. These include growth factor receptors, G protein-coupled receptors, and integrins (11, 29). Platelet-derived growth factor (PDGF) has been widely studied as a promoter of VSMC proliferation (36). Yang et al. (36) reported greater proliferative responses to PDGF in VSMC derived from SV explants than in those from IMA explants. Rat VSMC in culture are stimulated to proliferate by both ATP and UTP acting via G protein-coupled P2Y receptors (9, 14, 19, 33). Both PDGF and nucleotides are released from activated platelets. In addition, ATP and UTP are known to be released from endothelial cells (17, 27). Nucleotides acting on P2Y receptors, either alone or in conjunction with PDGF, may play an important role in regulating the smooth muscle of blood vessels in both health and disease (3). Currently, there are five cloned and characterized mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) that differ in their agonist profiles. These receptors are each coupled to phospholipase C (PLC) and increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Additional signaling pathways have been studied (2), providing evidence that the ATP-stimulated proliferation of animal VSMC requires activation of p42/p44 mitogen-activated protein kinase (MAPK) pathways (14, 34). Little is known about the regulation of human VSMC by nucleotides. Studies on rings of human SV and coronary artery showed that ATP could regulate vasospasm (12, 25). Furthermore, the responses of human coronary VSMC to ATP and UTP suggested the presence of multiple P2Y receptors (31, 32). Here we investigate the hypothesis that nucleotides regulate proliferative responses in human VSMC, and we report the surprising observation that UTP has an antiproliferative effect.

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METHODS

Isolation and culture of cells. Primary explant cultures were derived from IMA and SV fragments from patients undergoing aortocoronary or peripheral arterial bypass surgery, with the approval of the local Ethical Committee. All comparisons of IMA- and SV-derived cells were made on paired explants from the same patient. Tissue was dissected free of any fat and excess adventitial tissue and then opened along its longitudinal axis, and the endothelial lining was removed by scraping of the luminal surface. Small fragments (~1 mm^3) were transferred to a flask containing culture medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin from GIBCO). After VSMC outgrowth, a confluent monolayer was obtained and passaged. Cells were used between *passages 2* and *6* after 24 h serum free, when they were quiescent and positive for smooth muscle actin immunostaining with monoclonal mouse anti-human *smooth muscle actin* (Dako).

$[Ca^{2+}]_i$ measurements. Preconfluent cells on coverslips were made serum free for 24 h and loaded for 30 min with 4 μM fura 2-AM (Calbiochem) at room temperature in the dark. Cells were washed twice with PBS, and the coverslip was then placed in a multibarrel perfusion apparatus on the microscope stage (Zeiss microscope fitted with ×20 oil-immersion objective). Cells were perfused continuously with the addition of drugs to the perfusate as indicated. Images at 340 and 380 nm were collected, and ratios were calculated by using a microspectrofluorimetry system with Improvision software. Ratios from individual cells were pooled across fields of 8–20 cells.

[^{3}H]inositol phosphate assay. The procedure was essentially as described previously (35). Cells in 96-well plates were incubated in serum-free M199 (GIBCO) with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 3 μCi/ml *myo*-[^{3}H]inositol (specific activity 86 Ci/mmol; Amersham, Amersham, UK). After the 24-h labeling was completed, cells were preincubated with 50 μl of balanced salt solution (BSS; in mM: 125 NaCl, 5.4 KCl, 16.2 $NaHCO_3$, 30 HEPES, 1 NaH_2PO_4, 0.8 $MgSO_4$, 1.8 $CaCl_2$, and 5.5 glucose, buffered to pH 7.4 with NaOH) containing 10 mM lithium chloride for 10 min. Cells were stimulated with 50 μl of PDGF-BB and/or nucleotides (Sigma) at twice the final concentration. The stimulation period was terminated by the addition of 100 μl of cold 1 M trichloroacetic acid, and then cells were neutralized with tri-*n*-octylamine/1,1,2-trichlorofluoroethane extraction and separated for total [^{3}H]inositol phosphates ([^{3}H]InsP_x) on small Dowex chloride AG1X8-400 mesh resin columns (Sigma).

Inositol 1,4,5-trisphosphate mass measurement. The levels of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] were measured in cells cultured serum free for 24 h in 96-well plates. Cells were stimulated for the times indicated, and extracts were obtained as described in *[^{3}H]inositol phosphate assay* but in the absence of lithium chloride. Samples (30 μl) were assayed (5) by the addition of ice-cold assay buffer (30 μl) at 4× concentration (final concentration in mM: 25 Tris·HCl and 1 EDTA, pH 8.0), 3.6 μCi in 30 μl of [^{3}H]Ins(1,4,5)P_3 (specific activity 44 Ci/mmol; Amersham) and 30 μl of bovine adrenal cortical binding protein (~0.6 mg protein/tube), with an incubation period of 30 min. A standard curve of D-Ins(1,4,5)P_3 was generated, and nonspecific binding was defined with 10 μM Ins(1,4,5)P_3. The assay was terminated by rapid separation of bound and free [^{3}H]Ins(1,4,5)P_3 using ice-cold washing buffer (in mM: 25 Tris·HCl, 1 EDTA, and 5 $NaHCO_3$, pH 8.0 at 4°C). The protein-bound [^{3}H]Ins(1,4,5)P_3 was collected onto Whatman GF/B glass fiber filters with the use of a Brandel Cell Harvester and extracted overnight in scintillant before being counted.

[^{3}H]thymidine incorporation. Incorporation of [^{3}H]thymidine (Amersham) into DNA was measured essentially as described previously (35). Cells were cultured close to confluence in 96-well plates and used after they had been serum free for 24 h. Cells in 100 μl of serum-free medium were stimulated for 1 h by the addition of 100 μl of agonist at twice the final concentration. This was followed by incubation for 19 h in serum-free medium and a 4-h incorporation period with [^{3}H]thymidine. DNA was extracted, and [^{3}H]thymidine content was measured by scintillation counting.

Cell proliferation. Cultured cells were seeded at a density of 10^5 cells/well in 24-well plates and left quiescent for 24 h in serum-free medium before pulse stimulation for 1 h daily with 1 nM PDGF, 300 μM UTP, or both. Cells were stimulated for 2, 4, or 7 days, at which time cell numbers were determined by using a hemacytometer.

Creatine phosphokinase treatment. Regeneration of UTP from UDP during incubation of cells with UTP was achieved by the inclusion of 10 mM creatine phosphate and 20 U/ml creatine phosphokinase (Sigma) for the duration of the 1-h incubation. The UTP was purified by high-performance liquid chromatography (HPLC) immediately before use. HPLC was by anion exchange chromatography (Whatman SAX column). We established that the creatine kinase system was capable of converting UDP to UTP by incubating 100 mM UDP with creatine phosphate and creatine phosphokinase and demonstrated by using HPLC that UTP was formed.

Phospho-MAPK Western blots. Cells were cultured close to confluence in six-well plates and were used after they had been made serum free for 24 h. Cells in 900 μl of serum-free medium were stimulated by the addition of 100 μl of agonist at 10-fold final concentration for 5 min. Stimulation was stopped by the addition of liquid N_2, and the cells were extracted into lysis buffer [in mM: 20 Tris·HCl, 250 NaCl, 3 EDTA, 3 EGTA, 1 phenylmethylsulfonyl fluoride, 2 sodium orthovanadate, and 1 β-mercaptoethanol plus 0.5 % (vol/vol) Triton X-100, 20 μg/ml aprotinin, and 5 μg/ml leupeptin, pH 7.6]. The sonicated lysate was cleared by centrifugation at 14,000 *g* for 15 min at 4°C, samples were protein equalized, and Western blots were prepared with the use of an antibody specific for the phosphorylated (activated) forms of p42/p44 MAPK (Promega). The blots were developed with the enhanced chemiluminescence (ECL) procedure (Amersham).

Statistical analysis. Data presented are the means ± SE for three separate experiments performed in triplicate or quadruplicate. Raw data were analyzed with GraphPad Prism (version 3.0) by a one-way analysis of variance (ANOVA) followed by Bonferroni's posttest to compare selected columns or Dunnett's posttest to compare all columns with controls. When two groups were compared, a two-tailed Student's *t*-test was used.

RESULTS

$[Ca^{2+}]_i$ and PLC responses. Fura 2-loaded VSMC derived from both IMA and SV responded to ATP and PDGF with increased $[Ca^{2+}]_i$, as shown in Fig. 1, *A* and *B*. UTP similarly stimulated an increase in $[Ca^{2+}]_i$ in both IMA- (Fig. 1*C*) and SV-derived cells (data not shown). No consistent difference was seen in the response of cells to UTP or ATP over a number of experiments or in the size of the response of IMA-derived cells compared with that of SV-derived cells. The P2X agonist α,β-methylene-ATP (300 μM) did not raise

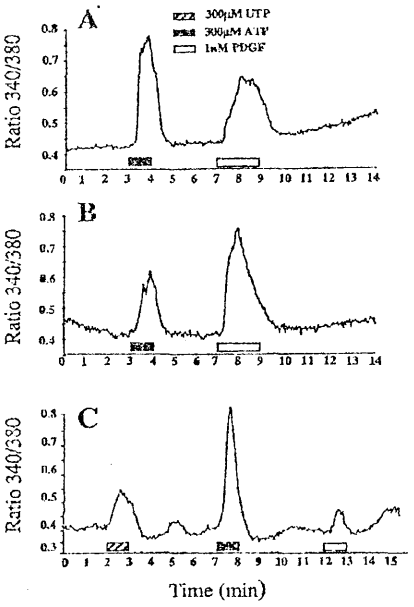

Fig. 1. Intracellular Ca^{2+} responses to platelet-derived growth factor (PDGF), ATP, and UTP in human vascular smooth muscle cells (VSMC). Data are the averaged 340/380 ratios from 8–20 serum-starved cells in response to 1 nM PDGF, 300 μM ATP, or 300 μM UTP perfused over internal mammary artery (IMA) cells (*A* and *C*) or saphenous vein (SV) cells (*B*) as indicated. Traces are representative of at least 3 separate experiments on different cell preparations.

$[Ca^{2+}]_i$, and the removal of extracellular Ca^{2+} with EGTA did not significantly attenuate the response to ATP (data not shown). To determine whether the effects of ATP and UTP were consequent to PLC activation and Ins(1,4,5)P_3 generation, we measured nucleotide-induced accumulation of [^{3}H]InsP_x was measured. As shown in Fig. 2*A*, PDGF stimulation of IMA-derived cells led to [^{3}H]InsP_x accumulation. However, stimulation with ATP or UTP alone did not lead to an increase in [^{3}H]InsP_x accumulation. In addition, in the presence of PDGF, the application of ATP and UTP did not lead to any further increase in [^{3}H]InsP_x accumulation (Fig. 2*A*). These responses were the same for SV-derived cells (data not shown). To investigate the possibility that the nucleotides elicit a transient formation of Ins(1,4,5)P_3 that is not detected with the [^{3}H]InsP_x procedure, we measured the mass of Ins(1,4,5)P_3 formed within seconds of stimulation. As shown in Fig. 2*B*, no increase in Ins(1,4,5)P_3 level was detected between 10 s and 5 min of stimulation in response to PDGF, despite the modest accumulation of [^{3}H]InsP_x levels (Fig. 2*A*). As a positive control for the mass Ins(1,4,5)P_3 procedure, SH-SY5Y neuroblastoma cells were stimulated with carbachol and assayed concurrently with the VSMC; a clear increase in Ins(1,4,5)P_3 accumulation was seen (Fig. 2*B*, *inset*).

[^{3}H]thymidine responses. To compare the mitogenic responses of SV- and IMA-derived cells to PDGF, we used [^{3}H]thymidine incorporation into DNA as an index of proliferation. As shown in Fig. 3*A*, increasing concentrations of PDGF led to increased incorporation of [^{3}H]thymidine. PDGF was significantly more potent in IMA-derived cells than in the paired SV-derived cells (pEC_{50}: -9.22 ± 0.11 for IMA-derived cells and -8.45 ± 0.08 for SV-derived cells; $P < 0.001$, $n = 3$ experiments each in quadruplicate). However, the maximal response was greater in SV-derived cells (SV: 4,828 ± 330 dpm/mg protein; IMA: 1,792 ± 121 dpm/mg protein, $P < 0.05$, $n = 3$). The effect of passage number on [^{3}H]thymidine incorporation was studied by comparing cells from *passages 2–4* in paired IMA- and SV-derived cells from three different patients, with three separate experiments in quadruplicate for each. There was no effect of passage number on the [^{3}H]thymidine incorporation to increasing concentrations of PDGF (data not shown).

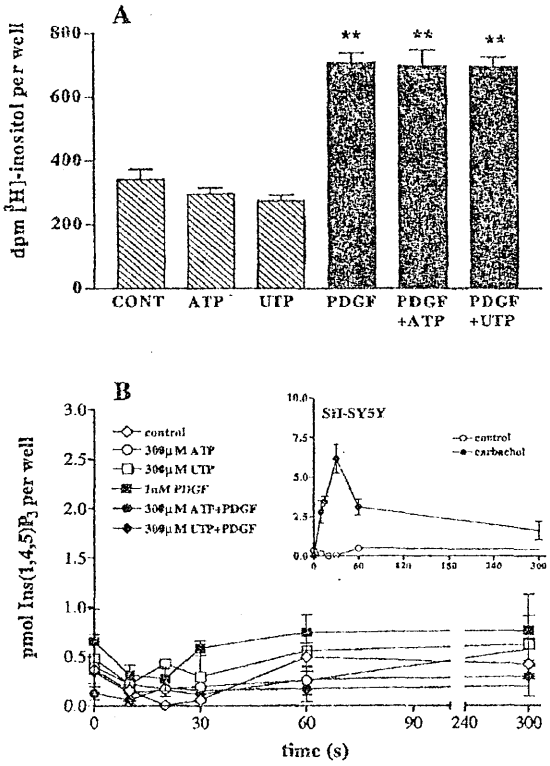

Fig. 2. Phosphoinositide-directed phospholipase C responses in human VSMC. *A*: effect of ATP or UTP (300 μM) in the absence (hatched bars) or presence (solid bars) of PDGF (1 nM) on the accumulation of [^{3}H]inositol phosphates ([^{3}H]InsP_x) in IMA-derived cells. Data are means ± SE; $n = 3$. Cont, control. $^{**}P < 0.01$ compared with equivalent responses in the absence of PDGF, by ANOVA with Dunnett's posttest. *B*: level of Ins(1,4,5)P_3 in response to stimulation with PDGF (1 nM), ATP, or UTP (300 μM) for the times indicated. Data are means ± SE; $n = 3$ separate experiments, each in quadruplicate. One-way ANOVA showed no significant effect of time of incubation with agonists. *Inset*: response of SH-SY5Y cells to 1 mM carbachol (or vehicle control) for the times indicated ($n = 3$).

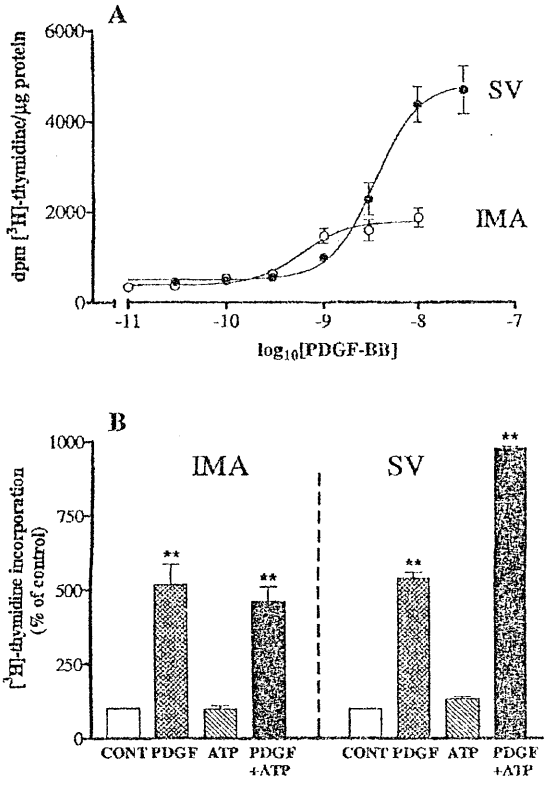

Fig. 3. Stimulated [^{3}H]thymidine incorporation into DNA in human VSMC in response to PDGF (1 nM) and ATP (300 μM). *A*: concentration-response curves to PDGF for SV- and IMA-derived cells. Statistical analysis of data is presented in the text. *B*: [^{3}H]thymidine incorporation (dpm) expressed as a percentage of control values in IMA (*left*) or SV (*right*) cells. Data are means ± SE; $n = 3$ separate experiments, each in quadruplicate. $^{**}P < 0.01$ compared with unstimulated control. PDGF + ATP was also significantly different from PDGF alone for the SV cells ($P < 0.01$, by ANOVA with Bonferroni's posttest).

To investigate the effect of ATP on proliferative responses, we stimulated cells with 300 μM ATP in the presence and absence of PDGF (1 nM). At this submaximal concentration of PDGF, the [^{3}H]thymidine responses of IMA- and SV-derived cells were not substantially different (see Fig. 3, *A* and *B*). ATP, either alone or in the presence of PDGF, did not stimulate [^{3}H]thymidine incorporation in IMA-derived cells. Similarly, in SV-derived cells, ATP alone had no effect. However, when stimulated with ATP in the presence of PDGF, [^{3}H]thymidine incorporation was increased significantly in SV cells but not in IMA cells.

The five cloned and characterized mammalian P2Y receptors have distinct profiles of activation by different nucleotides. A range of nucleotides was therefore tested that would activate each of these receptors: ATP ($P2Y_2$ and $P2Y_{11}$), UTP ($P2Y_2$ and $P2Y_4$), 2-methylthio-ADP (2-MeS-ADP; $P2Y_1$), and UDP ($P2Y_6$). 2-MeS-ADP had no effect on [^{3}H]thymidine incorporation in either IMA- or SV-derived cells whether applied alone or together with 1 nM PDGF (Fig. 4, *A* and *B*).

Application of UTP or UDP alone also had no effect on [^{3}H]thymidine incorporation, but, surprisingly, both UDP and UTP led to a substantial and significant reduction in the PDGF-stimulated level of [^{3}H]thymidine incorporation. This was true for both cell types (Fig. 4). There was no difference in the response to UTP or UDP at 100 μM. The difference in the effect of UTP compared with that of ATP on PDGF-stimulated [^{3}H]thymidine incorporation is depicted in Fig. 5*A*. To determine whether UTP was acting directly, we repeated experiments with UTP purified before use (by high-pressure liquid anion exchange chromatography) and with the inclusion of a creatine phosphokinase-regenerating system to prevent loss of UTP and accumulation of UDP. Figure 5*B* shows the concentration-response curve under these conditions for the antiproliferative effect of UTP on [^{3}H]thymidine incorporation in the presence or absence of PDGF. UTP was effective at concentrations >1 μM, consistent with reports on native P2Y receptors.

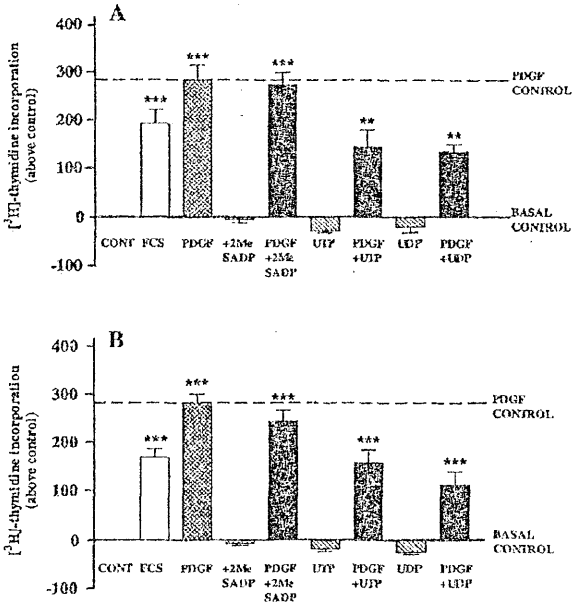

Fig. 4. [^{3}H]thymidine incorporation into DNA of human VSMC in response to PDGF and various nucleotides in IMA (*A*) or SV (*B*) cells, expressed as mean percentages above unstimulated (basal) control values (indicated by the solid line). The broken line indicates the level of [^{3}H]thymidine incorporation evoked by 1 nM PDGF alone (PDGF control). Data are means ± SE; $n = 3$ separate experiments, each in quadruplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with unstimulated control. In *A*, PDGF + UTP and PDGF + UDP were both different from PDGF alone ($P < 0.01$). In *B*, differences from PDGF alone were significant for PDGF + UTP ($P < 0.01$) and PDGF + UDP ($P < 0.001$). Statistics were determined by ANOVA followed by Bonferroni's multiple comparison test.

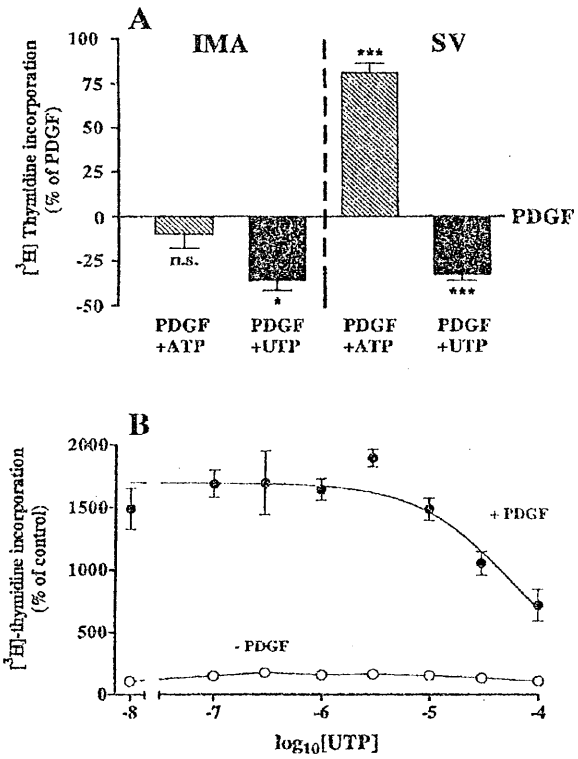

Fig. 5. *A*: effect of ATP or UTP (300 μM) on [^{3}H]thymidine incorporation evoked by 1 nM PDGF, expressed as a percentage of the level of incorporation with PDGF alone, in IMA (*left*) or SV (*right*) cells. Data are means ± SE; $n = 3$. ***$P < 0.001$; *$P < 0.05$ compared with PDGF alone, by ANOVA and Dunnett's posttest. *B*: concentration-response curve for inhibition of PDGF response by UTP. −PDGF, in absence of PDGF; +PDGF, in presence of 1 nM PDGF throughout; ns, not significant. Incubations were carried out in the presence of a creatine phosphokinase-regenerating system as described in METHODS.

The P2 antagonists suramin and pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS), when present at 300 μM for 10 min before and during the 1-h exposure to agonists, had little effect on the responses. Expressed as a percentage of stimulation of [^{3}H]thymidine incorporation by PDGF alone, stimulation by PDGF with 300 μM UTP was 48.8 ± 9.5, PDGF with UTP and suramin was 34.6 ± 5.2, and PDGF with UTP and PPADS was 35.3 ± 2.9 (means ± SE, $n = 3$ separate experiments each in quadruplicate). These results suggest that the response is not at P2X, $P2Y_1$, or $P2Y_2$ receptors (see DISCUSSION).

The effect of 10% FCS on [^{3}H]thymidine incorporation in IMA and SV VSMC is shown in Fig. 4, *A* and *B*. The response was significant but surprisingly smaller than the response to 1 nM PDGF.

Cell proliferation. To measure cell proliferation directly, we counted cells over a number of days in response to repeated stimulation with PDGF in the presence or absence of UTP. Stimulations were daily, over a period of 1 h, at which point the medium was changed for serum-free medium with no agonists present. Table 1 shows results with both SV- and IMA-derived cells pooled across experiments, with stimulation for 2, 4, and 7 days. PDGF stimulation for 2 days alone had no effect, but over 4 or 7 days there was a significant increase in cell number. This increase was greater for SV-derived cells than for IMA-derived cells. UTP alone had no effect on cell number at any time. However, when UTP was present, there was no increase in cell number in response to PDGF. This was true for both cell types. These results show that UTP exerted a powerful antiproliferative effect on both SV- and IMA-derived cells.

Activation of MAPK. Consistent with an earlier report (36), PDGF stimulated phosphorylation of p42/p44 MAPK in both IMA- and SV-derived cells (Fig. 6). Stimulation of SV-derived cells with ATP alone led to an increased phosphorylation of p42/p44 MAPK, but ATP did not stimulate MAPK phosphorylation in IMA-derived cells. When ATP was applied in combination with PDGF, there was no detectable enhancement of p42/p44 MAPK phosphorylation (Fig. 6) over the response to PDGF alone. UTP had no effect on p42/p44 MAPK phosphorylation when added alone to either cell type. In SV-derived cells, but not in IMA-derived cells, UTP enhanced the PDGF-stimulated MAPK phosphorylation.

Table 1. *Stimulation of cell proliferation by PDGF in the presence and absence of UTP*

	Cell Number, ×10^4 cells/well					
	IMA			SV		
	Day 2	*Day 4*	*Day 7*	*Day 2*	*Day 4*	*Day 7*
Control	10.0 ± 0.6	9.4 ± 1.2	9.0 ± 0.7	12.0 ± 0.8	11.6 ± 0.4	11.0 ± 0.5
UTP (300 μM)	8.5 ± 0.1	9.0 ± 0.6	9.0 ± 0.2	10.7 ± 1.2	10.6 ± 0.4	10.5 ± 0.4
PDGF (1 nM)	10.1 ± 1.3	13.5 ± 0.3*	13.2 ± 1.7*	11.3 ± 0.6	15.0 ± 0.8†	17.8 ± 0.9‡
UTP (300 μM) + PDGF (1 nM)	10.4 ± 0.3	10.4 ± 0.3	10.4 ± 0.3	9.8 ± 0.4	12.3 ± 0.1	12.2 ± 0.4

Cells were stimulated for 1 h on 2, 4, or 7 consecutive days with platelet-derived growth factor (PDGF-BB) or UTP alone or in combination, and cells were counted 24 h after the final stimulation. Values are means ± SE from 3 separate experiments each in triplicate. PDGF stimulations were significantly different from controls at *days 4* and *7* (*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ by ANOVA with Bonferroni's posttest), but the response to PDGF + UTP was not significantly different from control at any time. IMA, internal mammary artery; SV, saphenous vein.

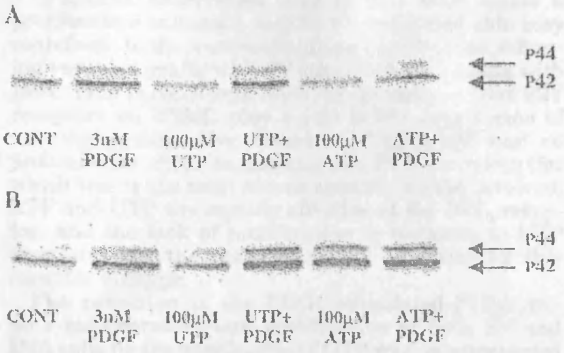

Fig. 6. Phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK) in human VSMC in response to ATP and UTP either alone or in the presence of PDGF in IMA (*A*) or SV (*B*) cells. Incubations were carried out for 5 min with agonists present as indicated. Western blots were specific for the phosphorylated forms of p42/p44 MAPK. Data are representative of 3 separate experiments.

DISCUSSION

This study describes the influence of nucleotides on control of proliferation of human VSMC derived from both veins and arteries. In a healthy blood vessel, the endothelium provides an antiproliferative influence through agents such as nitric oxide and prostacyclin. However, in vascular disease, when endothelial function is compromised, the influence of these agents is diminished. Additionally, proliferative stimuli in the blood may have direct access to exposed smooth muscle cells, leading to intimal proliferation. It has been proposed that nucleotides such as ATP act in this capacity through their cell surface receptors (3). In the diseased vessel, nucleotide release from platelets (together with PDGF) will be enhanced due to downregulation of the anti-platelet influence of endothelium-derived nitric oxide and prostacyclin. In this model the P2Y receptors on VSMC play a critical role in the pathology of intimal proliferation.

The importance of P2Y receptors in this respect has been suggested by previous studies on animal VSMC (9, 14, 19, 33). In intact rat arterial tissue, ionotropic P2X receptors play a role in the contractile response. In culture, these cells lose their P2X receptors, while a P2Y response is found in both intact tissue and cultured cells (21, 24). A recent quantitative PCR study has shown that the transition from the contractile to the synthetic phenotype, which is necessary for intimal proliferation (and also occurs when cells are cultured), corresponds to a loss of mRNA for $P2X_1$ and an upregulation of mRNA for $P2Y_1$ and $P2Y_2$ receptors (8). This is consistent with the hypothesis that P2Y receptors regulate the proliferative response in the synthetic phenotype.

The increases in $[Ca^{2+}]_i$ reported here in both human venous and arterial smooth muscle cells provide clear evidence for the presence of receptors that respond to ATP and UTP. The studies presented do not enable the identification of these receptors, yet the results indicate that the nucleotides are acting at P2Y receptors. The response to UTP and the lack of effect of α,β-methylene-ATP, together with the response to ATP in the absence of extracellular Ca^{2+}, all argue against the involvement of P2X receptors. The demonstration that UTP stimulated PLC and increased $[Ca^{2+}]_i$ in rat VSMC (16, 24) led to the proposal of a P2Y receptor sensitive to UTP, both in the original smooth muscle cells and in culture (24). Because all of the cloned P2Y receptors are coupled to the release of intracellular Ca^{2+} via $Ins(1,4,5)P_3$, the lack of a detectable PLC response reported here was unexpected. However, a similar situation has been reported in other cell types in response to activation of native P2Y receptors (1, 10) and other G protein-coupled receptors (20, 22, 30), in which the $[Ca^{2+}]_i$ increase is apparently independent of $Ins(1,4,5)P_3$.

Mitogenic responses to ATP and UTP have been reported in cultured rat VSMC (9, 14, 19, 33). In some cases nucleotides act as progression factors, stimulating the cell cycle in concert with growth factors such as PDGF. This growth factor has been shown to stimulate the progression of quiescent rat aorta cells from G_0 to G_1 but no further (23). ATP acting on P2Y receptors had no effect on the cell cycle of cells in G_0 but could lead to progression from G_1 to S and M phases. These observations indicate that concomitant stimulation of VSMC with growth factors such as PDGF and agonists acting at P2Y receptors leads to proliferation.

The results of our experiments in which IMA and SV cells were stimulated with PDGF are consistent with previous work (29). We report that the intracellular Ca^{2+} response was of a similar magnitude in SV- and IMA-derived cells, while [^{3}H]thymidine incorporation was greater in the SV cells. The larger maximal response to PDGF seen in SV cells occurred with an EC_{50} that was significantly higher than that occurring in the IMA cells; this may reflect a difference in receptor reserve between these two cell types.

ATP did not act as a full mitogen in either SV or IMA cells; no stimulation of [^{3}H]thymidine incorporation was observed in response to this nucleotide alone. However, in SV cells, ATP acted as a progression factor to enhance the PDGF-stimulated [^{3}H]thymidine incorporation into DNA, consistent with the effects described in rat VSMC (23). However, this comitogenic action of ATP was not seen in IMA cells, despite the observation that the intracellular Ca^{2+} response of IMA cells to ATP is similar to that of SV cells. Differences were also seen in the effects of ATP on p42/p44 MAPK phosphorylation in the two cell types. In SV-derived cells ATP stimulated this index of MAPK activation, but this did not occur in IMA-derived cells; interestingly, this correlates with the ability of ATP to act as a comitogen. However, the addition of ATP in the presence of PDGF failed to further elevate MAPK phosphorylation above that of PDGF alone in either cell type. These results are consistent with the suggestion that activation of MAPK is necessary but not sufficient for the mitogenic response (14, 31).

A salient observation here is that ATP exerts a proliferative influence only in SV cells, and this may contribute to the increased intimal proliferation following vascular grafts with SV compared with grafts with IMA. This is consistent with the hypothesis that P2Y receptors on VSMC play a role in the progression of vascular proliferative disorders. 2-MeS-ADP had no proliferative effect, suggesting that $P2Y_1$ receptors (for which this is the most potent agonist) are not involved. ATP and UTP are equally effective at the $P2Y_2$ receptor, and the lack of proliferation in response to UTP suggests that the response is not mediated by this receptor subtype.

The reduction in the PDGF-stimulated [^{3}H]thymidine incorporation and proliferation of both SV and IMA cells by the application of UTP was an unexpected finding. The conclusions we derive from the thymidine incorporation studies are powerfully confirmed by direct measurement of cell numbers. It takes several days for the cell numbers to be reliably increased by PDGF, but wherever this occurs it is essentially ablated by the presence of UTP. Indeed, the antiproliferative effect of UTP is apparently greater when counting cell number than when looking at [^{3}H]thymidine incorporation.

There was one previous report of an antiproliferative response to UTP in endothelium-derived cells (18), although it was also reported that UTP is mitogenic in cardiac vasculature endothelium (28). Here we have shown that UTP can act directly; its antiproliferative action is not dependent on breakdown by ectonucleotidases. This is demonstrated by experiments in which HPLC-purified UTP was used in the presence of a creatine phosphokinase-regenerating system, which will convert any UDP formed during the incubation back into UTP. This system is usually used as an ATP-regenerating system (15), but we have shown directly that UDP can act as a substrate, although the rate of the kinase reaction is much slower than that with ADP as substrate. These experiments confirm that the antiproliferative P2Y receptor is responsive to UTP. However, the antiproliferative response to UDP observed here may be indirect and dependent on extracellular conversion to UTP. Harden and colleagues (13) showed that the addition of UDP can result in accumulation of extracellular UTP by the action of ectonucleoside diphosphokinase.

Candidates for the receptor responsible for the antiproliferative response to UTP include $P2Y_2$ and $P2Y_4$. Of these, the $P2Y_4$ receptor seems most likely since ATP, which does not elicit an antiproliferative response, is an agonist at $P2Y_2$ receptors but not at human $P2Y_4$ receptors (7). The suramin and PPADS results are consistent with this. Neither compound attenuated the effect of UTP on PDGF-stimulated [^{3}H]thymidine incorporation. Both are effective antagonists at P2X and $P2Y_1$ receptors, suramin is a weak antagonist at $P2Y_2$ receptors, and neither are antagonists at $P2Y_4$ receptors (3, 6).

PDGF-stimulated proliferation was inhibited to a similar degree by UTP in both IMA- and SV-derived cells. This effect was apparently independent of Ca^{2+}, since $[Ca^{2+}]_i$ was elevated by both ATP and UTP, but only UTP had an antiproliferative effect. Similarly, the antiproliferative effect showed no correlation with activation of p42/p44 MAPK, since UTP had no effect on PDGF-mediated MAPK phosphorylation in IMA-derived cells. These results rule out changes in $[Ca^{2+}]_i$ and p42/p44 MAPK phosphorylation state as the mechanism underlying this antiproliferative effect.

Our initial hypothesis was that nucleotides act on vascular smooth muscle P2Y receptors to enhance proliferation of these cells, thus contributing to intimal proliferation in diseased vessels. The results presented here are consistent with this hypothesis with respect to the action of ATP and indicate that differential activation of SV-derived cells by ATP may contribute to greater intimal proliferation seen with these vessels compared with that seen with IMA. However, our results indicate that the effects of nucleotides are more complex than we initially suggested, since they show that UTP is an antiproliferative regulator of human vascular smooth muscle of both arterial and venous origin.

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