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Characterisation of P2Y receptors expressed in neonatal rat cardiac fibroblasts and their role in a model of ischaemic heart disease

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Abstract

Cardiac fibroblasts (CFs) are the predominant cell type in the cardiac tissue and play a vital role in wound healing, hypertrophy and fibrosis. During heart failure there is accumulation of ATP and UTP in the heart, possibly leading to stimulation of P2Y receptors in fibroblasts. However, very little is known about the functional expression and role of P2Y receptors in CFs. Therefore, the aim of this study was to characterise the different subtypes of P2Y receptors expressed in neonatal rat CFs and to investigate their role in an *in vitro* model of ischaemic heart disease.

$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{13}$ were detected by RT-PCR and immunocytochemistry. $P2Y_{11}$-like receptor was identified at the protein level. Adenine (ADP-βS, ATP, ATP-γS, 2MeSADP and 2MeSATP) and uracil (UDP and UTP) nucleotides stimulated inositol phosphate (IP) response in an YM-254890 ($G_{q/11}$-protein inhibitor)-sensitive manner. AMP, ADP-βS, ATP and ATP-γS increased cAMP accumulation, whereas UDP and UTP inhibited forskolin-induced cAMP accumulation, which was abolished by pertussis toxin ($G_{i/o}$-protein inhibitor). The selective $P2Y_1$ antagonist MRS2179 inhibited ADP-βS, ATP-γS and 2MeSADP-induced IP accumulation. The UDP and UTP-mediated IP responses were blocked by MRS2578, a selective $P2Y_6$ antagonist. These data provide strong evidence of the co-expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors coupled to $G_{q/11}$-protein. In addition, $P2Y_2$ and $P2Y_4$ subtypes are also coupled to $G_{i/o}$ whereas $P2Y_{11}$-like to G_s-proteins. CFs may also express a P2Y-like receptor activated by AMP.

The effect of ATP-γS and UTP on cytokine release (IL-1β, IL-6, TNF-α and TGF-β1), cell viability, collagen synthesis and protein kinase activation (MAPK and Akt/PKB) was determined in CFs exposed to hypoxia and angiotensin-II as a model of ischaemic heart disease. IL-1β production was regulated by both ATP-γS and UTP whereas IL-6 release was induced by ATP-γS. ATP-γS and UTP did not effect the TNF-α and TGF-β1 production. ATP-γS mediated the deposition of collagen whereas UTP inhibited the collagen accumulation. Both the nucleotides did not affect CF viability or activate MAPK and Akt/PKB.

In conclusion, neonatal rat CFs functionally express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors. The data also suggest that P2Y receptors activated by ATP-γS induce cardiac fibrosis and hypertrophy whereas P2Y receptors stimulated by UTP inhibit fibrosis, during ischaemic heart disease. In addition, this study showed the importance of P2Y receptors on CFs in the context of heart disease however, their role in myocardial remodelling requires further studies.

Dedicated

to my

Parents and Gurus

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Abbreviations and Chemical names

2-MeSADP	2-(methylthio) adenosine 5'-diphosphate trisodium salt
2-MeSATP	2-(methylthio) adenosine triphosphate tetrasodium salt
AC	Adenylyl cyclase
ADP-βS	Adenosine 5′-[β-thio]diphosphate trilithium salt
AMP	adenosine 5'-monophosphate sodium salt
ANG-II	Angiotensin-II
AT_1	Angiotensin type-I receptor
ATP	Adenosine 5'-triphosphate
ATP-γS	Adenosine 5'-[γ-thio] triphosphate tetralithium salt
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
DDR2	Discoidin domain receptor 2
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotide mix
DTT	Dithiothreitol
ECM	Extracellular matrix
EPO	Erythropoietin
ERK1/2	Extracellular regulatory kinase1/2
FCS	Foetal calf serum
FSK	Forskolin
GPCR	Gaunine –protein coupled receptor
GbR	γ-aminobutyric acid b receptor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H_2SO_4	Sulphuric acid
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
Hx	Hypoxia

KT5720	(9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid
L-15 medium	Leibovitz's medium
LDH	Lactate dehydrogenase
LIF	leukaemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby kidney cell
M-MLV RT	Moloney murine leukaemia virus reverse transcriptase
MMP	Matrix metalloproteinase
MRS 2179	2-deoxy-N^6-methyl adenosine 3',5'-diphosphate diammonium salt
MRS2578	N,N''-1,4 butanediylbis [N'-(3-isothiocyanatophenyl) thiourea
NaOH	Sodium hydroxide
Nx	Normoxia
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IP	Inositol Phosphate
PBS	Phosphate buffered solution
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PTX	Pertussis toxin
RAS	Renin-angiotensin system
RB-2	Reactive blue 2
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TIMP	Tissue inhibitors of metalloproteinase

U73122	1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17yl] amino] hexyl]-1H-pyrrole-2,5-dione
UDP	uridine 5'-diphosphate sodium salt
UTP	uridine 5'-triphosphate trisodium salt hydrate
YM	YM254890

Chapter 1

Introduction

Chapter 1.0 – Introduction

1.1 G-Protein coupled receptors (GPCRs)

Multicellular organisms have evolved successfully because of their cells ability to communicate with each other and with the surrounding environment. This process is mediated by the release of chemical messengers from one cell that can initiate specific response(s) in others via specialised receptors (Nelson and Cox, 2005). Based on their structure and function the receptors can be divided into six group: (1) ligand-gated ion channel receptors which regulate membrane potential and signalling in neurons (e.g: nicotinic acetylcholine receptor), (2) tyrosine kinase receptors which control phosphorylation of proteins involved in cell proliferation and differentiation (e.g.: insulin receptor), (3) receptors coupled to guanine nucleotide binding protein (e.g.: Guanine-protein coupled receptors, GPCRs) which initiate various intracellular responses (e.g.: β_2-adrenergic receptor), (4) Nuclear receptors which act within the nucleus to alter gene expression (e.g.: steroid receptors), (5) cytokine receptors which activate a cascade of cytoplasmic enzymes that stimulate gene regulators (e.g.: glycoprotein 130) and (6) adhesion receptors which interacts with the extracellular matrix and cytoskeletal system (e.g.: integrin receptor; Schlyer and Horuk, 2006; Nelson and Cox, 2005; Pierce *et, al.*, 2002; Marinissen and Gutkind, 2001).

GPCRs are the most common and widely distributed receptor family in vertebrates. Sequencing of the human genome has identified more than 800 genes encoding GPCRs (Schlyer and Horuk, 2006). The diversity of GPCRs is evident from the 200 endogenous ligands (like neurotransmitters, hormones, amino acids, nucleotides, peptides, odorants, light, taste ligands, steroids and fatty acids) that activate them (Table 1.1). However, GPCRs for which no endogenous ligands have been identified are referred to as "orphan" GPCRs. GPCRs are a major target for drug discovery in the pharmaceutical industry, as more than 30% of marketed therapeutics act on GPCRs. The GPCR superfamily in both vertebrates and invertebrates can be divided into six families – A, B, C, D, E, and F; based on their sequence similarity (Fredriksson *et al.,* 2003; Foord *et al.,* 2005). Families D, E and F are not expressed in humans. Families D, E and F represent fungal pheromone receptors, cAMP receptors and archaebacterial opsin receptors, respectively (Fredriksson *et al.,* 2003; Foord *et al.,* 2005).

Table 1.1: Some of the endogenous ligands of G-protein coupled receptors (GPCRs)

Endogenous Ligands	Receptor	Coupling to Gα-protein subclass
Amino acids		
GABA	$GABA_{B1}$, $GABA_{B2}$	$G_{q/11}$
Biogenic Amines		
Acetylcholine	M_1, M_3,M_5	$G_{q/11}$
	M_2, M_4	$G_{i/o}$
Adrenaline and	α_{1A}, α_{1B}, α_{1D}	$G_{q/11}$
Noradrenaline	α_{2A}, α_{2B}, α_{2C}	$G_{i/o}$
	β_1, β_2,	G_s
	β_3	$G_{i/o}$
Dopamine	D_1,D_5	G_s
	D_2, D_3, D_4	$G_{i/o}$
Histamine	H_1	$G_{q/11}$
	H_2	G_s
	H_3, H_4	$G_{i/o}$
Ions		
Calcium	CaSR	$G_{q/11}$, $G_{i/o}$
Nucleosides/Nucleotides*		
Adenosine	A_1,A_3	$G_{i/o}$
	A_{2A},A_{2B}	G_s
Peptides/Proteins (Hormones)		
Angiotensin II	AT_1	$G_{q/11}$, $G_{i/o}$, $G_{12/13}$
Bradykinin	B_1,B_2	$G_{q/11}$
Opioids	δ, κ, μ	$G_{i/o}$
Vasopressin	V_{1a},V_{1b}	$G_{q/11}$
	V_2	G_s

* See Table: 1.5, CaSR – calcium sensing receptor

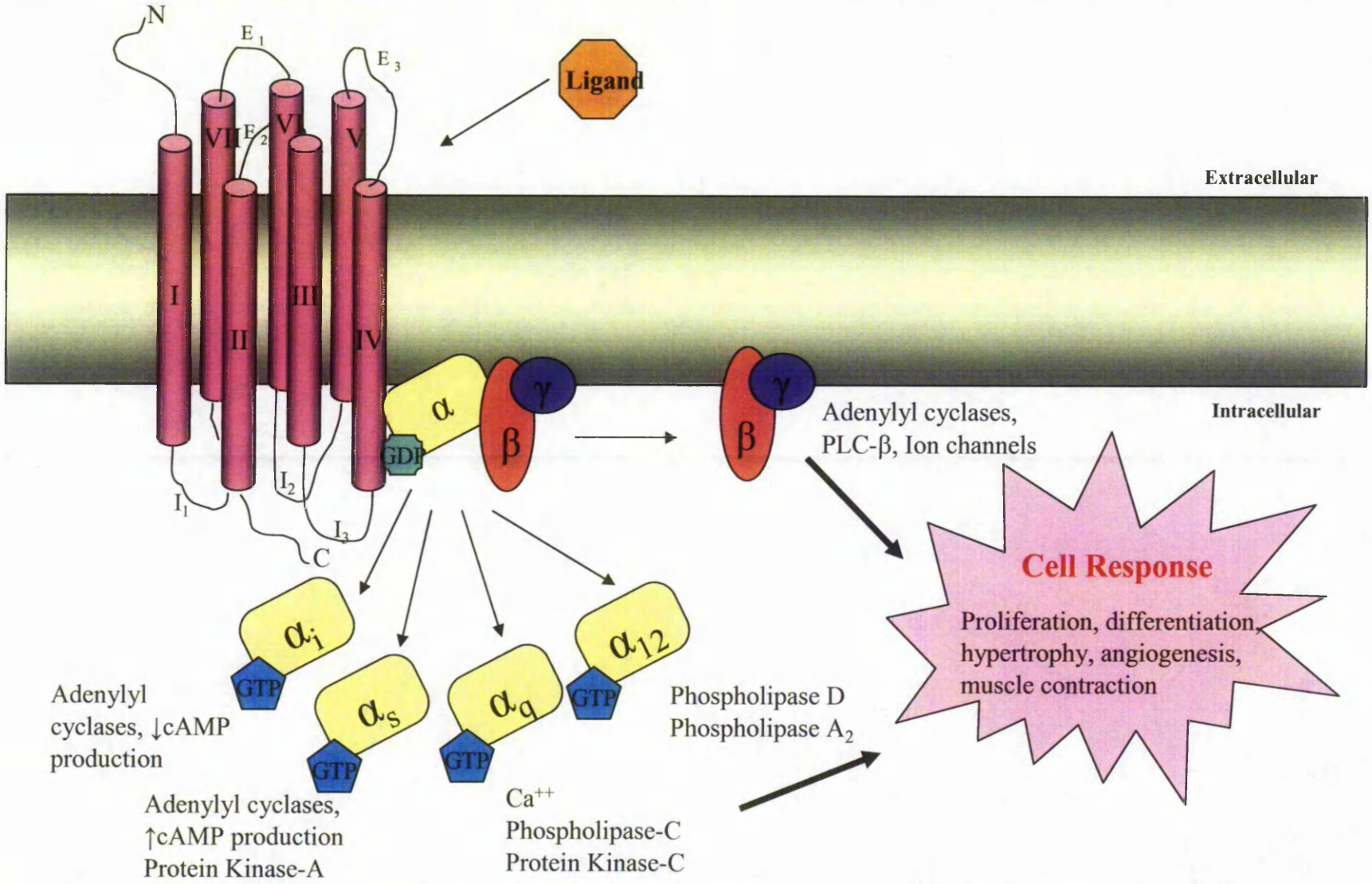

Figure 1.1: Schematic representation of G protein-coupled receptor (GPCR) signal transduction. GPCRs have seven transmemebrane domains (I – VII), three extracellular loops (E_1,E_2,E_3) and three intracellular loops (I_1,I_2,I_3). G-proteins consist of α,β,γ subunits. N, amino terminal; C, carboxylic group; GDP, guanosine diphosphate; GTP, guanosine triphosphate

Family A or rhodopsin family is the largest GPCRs family with 701 receptors (450 – olfactory receptors and 241 – non-olfactory receptors; Table 1.2). Some of the salient features of this family is a NSxxNPxxY motif at transmembrane domain (TM) VII, a tripeptide DRY between TMII and intracellular loop 2 (I_2) and a disulfide bond between extracellular loop 1 and 2. Adrenergic receptors, histamine receptors, serotonin receptors and P2Y receptors are some of the receptors which belong to this family. Family B, also known as the secretin receptor family binds high molecular-weight hormones/peptides like secretin, glucagons, vasoactive intestinal peptide (VIP; Table 1.2). The secretin receptor is the first cloned receptor of this family, therefore the term secretin receptor family. There are fifteen different receptors under this family such as calcitonin receptor, corticotropin-releasing hormone receptors and parathyroid hormone receptors. Family C or glutamate receptor family consists of eight metabotropic glutamate receptors, GABA receptors, one calcium-sensitive receptor (CASR) and five taste receptors (TAS1; Table 1.2). The N-terminus is responsible for ligand binding which comprises of 280 to 580 amino acids.

The central core of the GPCRs is composed of seven transmembrane helical domains (TMI-TMVII) with an extracellular N-terminal domain and an intracellular C-terminal domain (Figure 1.1). The transmembrane helices are connected by three extracellular (E_1, E_2 and E_3) and three intracellular loops (I_1, I_2 and I_3; for detailed reviews see Schlyer and Horuk, 2006; Milligan 2004; Bai, 2004; Hermans, 2003; Offermanns, 2003; Kiselyov *et al.,* 2003; Pierce *et, al.*, 2002; Rana and Insel 2002; Neves *et, al.,* 2002; Hur and Kim, 2002; Albert and Robillard, 2002; Bockaert, 2001).

The third intracellular (I_3) loop is more important in coupling GPCRs to G-proteins than C-terminal domain (Heydorn *et al.,* 2004; Havlickova *et al.,* 2003; Lai *et al.,* 2002). The first four G-proteins (G_s, G_t, G_i and G_o) were identified by biochemical methods, after which a large number of G-proteins and their subunits were documented by cDNA cloning (Offermanns, 2003; Pierce *et al.,* 2002; Rana and Insel 2002; Neves *et, al.,* 2002). The α subunits of the heterotrimeric G-protein are divided into $G\alpha_s$ ($G\alpha_s$, $G\alpha_{sXL}$, $G\alpha_{olf}$), $G\alpha_i$ ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_Z$), $G\alpha_q$ ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$) and $G\alpha_{12}$ ($G\alpha_{12}$ and $G\alpha_{13}$) sub-families based on structural and functional homologies (Offermanns, 2003; Neves *et, al.,* 2002; Simon *et, al.*, 1991). Currently there are 20 Gα, 5 G_β and 11G_γ subunits.

Table 1.2: G-protein coupled receptor families.

Family	Agonist	Receptor
Family-A/Rhodopsin receptor family		
5-hydroxytryptamine (5-HT)	5-HT	5-HT_1, 5-HT_2, 5-HT_4, 5-HT_{5A}, 5-HT_6, 5-HT_7
Muscarinic	Acetylcholine	M_1, M_2, M_3, M_4, M_5
Adenosine	Adenosine	A_1, A_{2A}, A_{2B}, A_3
Histamine	Histamine	H_1, H_2, H_3, H_4
Vasopressin	Vasopressin	V_{1A}, V_{1B}, V_2
P2Y	ATP, UTP	$P2Y_1$, $P2Y_2$, $P2Y_4$
Adrenergic	Noradrenaline	β_1, β_2, β_3
Family-B/Secretin receptor family		
Calcitonin	Calcitonin	CT
	Amylin	AMY_2
	Amylin, CGRP	AMY_1, AMY_3
Corticotropin releasing factor (CRF)	CRF, urocortin	CRF_1
	Urocortin	CRF_2
Glucagon	GHRH	GHRH
	GIP	GIP
	Secretin	Secretin
Family-C/Glutamate receptor family		
Metabotropic glutamate	Glutamate	$mGlu_1$, $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$, $mGlu_6$
GABA	GABA	$GABA_B$
Calcium sensor	Calcium	CaS

GHRH – Growth hormone-releasing hormone, GIP – Gastric inhibitory polypeptide, GABA – γ-aminobutyric acid.

The βγ-complex of the heterotrimeric G-protein also plays an important role in regulating various effectors (Table 1.3) including certain isoforms of adenylyl cyclase (AC), phospholipase C-β (PLC-β), G-protein regulated kinases (GRK2 and GRK3), inhibition of voltage-dependent Ca^{2+}-channels and activation of G-protein regulated inwardly rectifying K^{+}-channels (GIRKs) (Offermanns, 2003; Yamada, *et, al.,* 1998; Sunahara, *et, al.*, 1996).

1.1.1 Signal transduction of GPCRs

Activation:

Heterotrimeric G-proteins consist of three subunits α, β and γ. In the ground or basal state the G-protein subunits are closely associated with GDP attached to the α subunit (Figure 1.2; Stage I). Upon activation, GPCRs undergo a conformational change which promotes G-protein coupling via the regions within the third intracellular loop. G-protein coupling then causes a conformational change within the α subunit, which leads to the exchange of GDP for GTP (the concentration of GTP is higher than GDP in the cytoplasm). Following GDP/GTP exchange the G-protein dissociates into the Gα subunit and the Gβγ dimer complex. The activation of receptor ultimately causes the release of GDP from the α subunit and a tight interaction between GPCR and G-protein (Stage-II, Figure-1.2). GTP binds to the free α subunit rapidly resulting in the dissociation from βγ subunits. α-GTP and βγ subunit activate their respective effectors, which are either similar or different (Stage-III, Figure-1.2). G-proteins return to Stage-I by following the hydrolysis of GTP back to GDP via the GTPase activity of the α subunits (Offermanns, 2003; Bockaert, 2001). Regulators of G-protein signalling (RGS) enhance the GTPase activity of certain Gα subunits ($G\alpha_i$ and $G\alpha_q$, De Vries *et al.,* 2000; Neubig and Siderovski, 2002), thus promoting the reassociation of αβγ subunits (Bockaert, 2001).

Desensitisation:

The activation and signal transduction systems are tightly regulated by a phenomenon termed "desensitisation" (dampening the signal), even in the presence of continuous agonist stimulation at the receptor (Ferguson, 2001; Figure1.3). This mechanism is mediated by phosphorylation of the receptor by second-messenger kinases (such as protein kinase C, PKC; protein kinase A; PKA), or by G-protein-coupled receptor kinases (GRKs; Pitcher *et al.,* 1998).

Table 1.3: Effectors regulated by Gα and Gβγ subunits

Effector	Subtype	G-protein regulation
AC	1	↑ G_s; ↓ Gβγ
	2	↑ G_s; ↓ Gβγ
	3	↑ G_s; ↓ G_i
	4	↑ G_s; ↓ Gβγ
	5	↑ G_s; ↓ $G_{i/Z}$
	6	↑ G_s; ↓ $G_{i/Z}$
	7	↑ G_s; ↓ Gβγ
	8	↑ G_s; ↓ G_i
	9	↑ G_s; ↓ G_i
PLC	β1	↑ $G_{q/11}$
	β2	↑ $G_{q/11}$, ↑Gβγ
	β3	↑ $G_{q/11}$, ↑Gβγ
	β4	↑ $G_{q/11}$
GIRK	GIRK1	↑Gβγ
	GIRK2	↑Gβγ
	GIRK3	↑Gβγ
	GIRK4	↑Gβγ
VDCC	P/Q-type	↓ Gβγ
	N-type	↓ Gβγ
	R-type	↓ Gβγ
PI3-K	PI3-Kβ	↑Gβγ
	PI3-Kγ	↑Gβγ
GRK	GRK2	↑Gβγ
	GRK3	↑Gβγ
RhoGEF	Lsc/p115RhoGEF	↑G_{13}
	PDZ-RhoGEF	↑$G_{12/13}$
	LARG	↑$G_{12/13}$

AC, adenylyl cyclase; PLC, phospholipase C; GIRK, G-protein regulated inward rectifier potassium channel; VDCC, voltage-dependent Ca^{++}-channel; PI3-K, phosphoinositide-3-kinase; GRK, G-protein regulated kinase; RhoGEF, Rho guanine nucleotide exchange factor

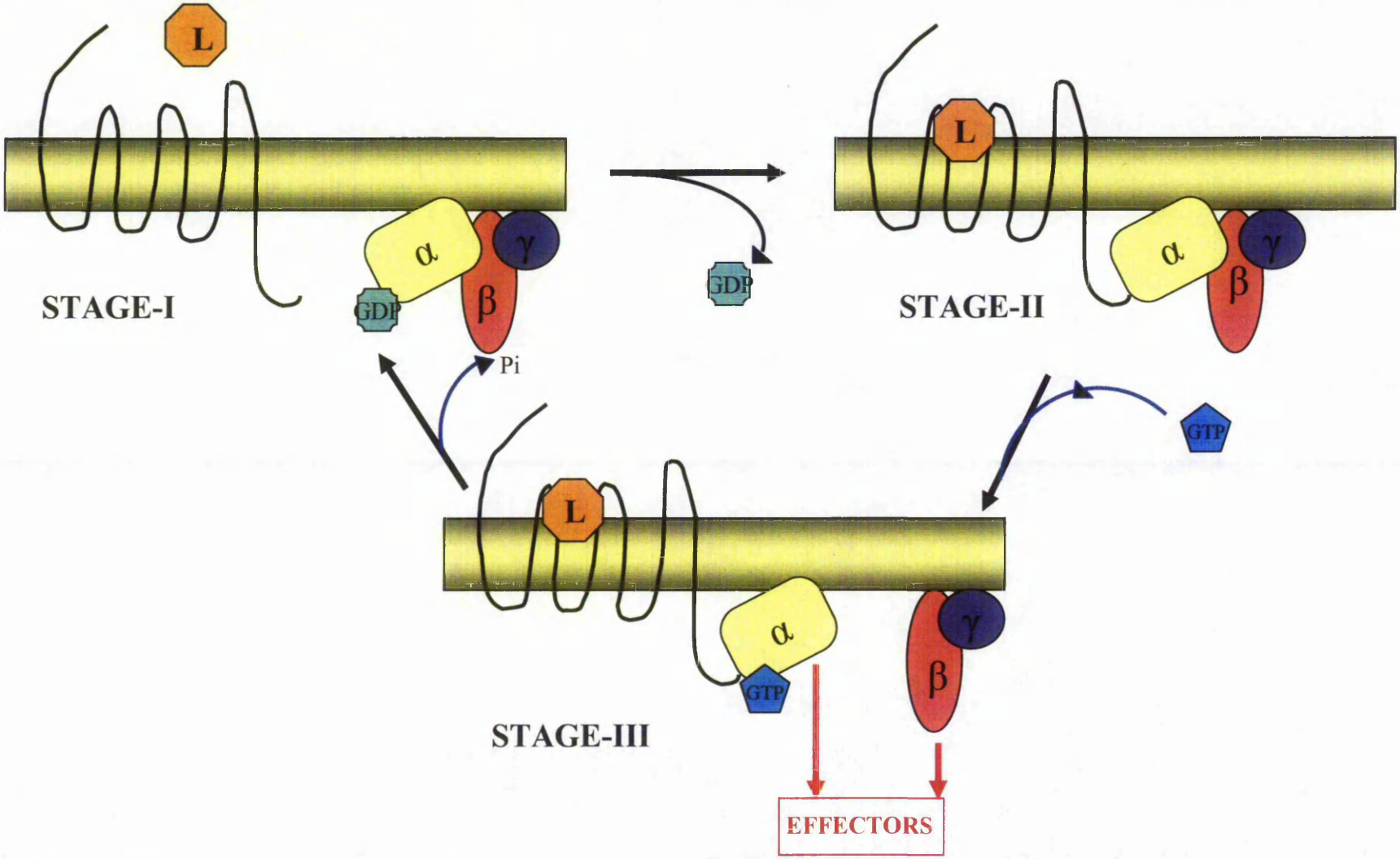

Figure 1.2: Diagrammatic representation of the G-protein coupled receptor (GPCR) activation cycle. In the basal state the βγ-guanosine diphosphate (GDP)-α subunit are associated. When activated by ligand (L) GDP is replaced by GTP (guanosine triphosphate) causing conformational changes within the α-subunit and dissociation from the βγ-complex. During this state the subunits interact with effector proteins and regulate the production of second messengers.

PKA and PKC can phosphorylate the receptor at the carboxyl-terminal and facilitate the uncoupling of respective G-proteins (PKA – G_s; PKC – G_q) from the receptor.

Another mechanism mediated by these kinases is the heterologous desensitization, where the kinase stimulated by one receptor can desensitise another receptor (Pierce *et al.,* 2002). It is well known that PKA mediates phosphorylation of the β_2-adrenergic receptor and regulates its coupling to G_s- and G_i- proteins (Daaka *et al.,* 1997; Zamah *et al.,* 2002). Moreover, PKA phosphorylates the prostacyclin receptor at serine 357 and regulates its coupling to G_s, G_i and G_q proteins (Lawler *et al.,* 2001). The common mechanism of receptor desensitisation is by GRK-β-arrestin system. At least five different GRK genes are identified in human genome. GRK2 (β-adrenergic receptor kinase; β-ARK) is one of the common GRKs. GRK1 (rhodopsin kinase) and GRK7 are localised in retina and GRK4 is only expressed in testis, brain and kidney (Pitcher *et al.,* 1998; Sallese *et al.,* 2000). These kinases are located in the cytosol and when activated are drawn towards the plasma membrane and receptors. The mechanisms which modulate this process are receptor activation and interaction of βγ subunits with GRKs (Pierce *et al.,* 2002). It is noteworthy that mRNA and protein levels of GRK2 and GRK5 were increased in experimental models of congestive heart failure, suggesting that GRK regulate GPCRs in heart failure (Vinge *et al.,* 2001). Receptor phosphorylation by GRKs creates a binding site for β-arrestin and thereby preventing the interaction of receptor and the G-protein (Figure 1.3). There are four arrestin genes identified so far they are visual arrestin, cone arrestin, β-arrestin-1 and β-arrestin-2. β-arrestin-1 and β-arrestin-2 are ubiquitously expressed in all tissues expect retina (Krupnick and Benovic, 1998). The binding of β-arrestin to the receptor also assists in the receptor desensitization and receptor endocytosis into vesicles (internalization).

Internalisation:

Receptor internalisation promotes receptor resensitisation and thus positively regulates receptor signalling (Figure 1.3). Some of the internalising pathways are clathrin-coated pits, caveolae-mediated receptor internalization and uncoated vesicles. The well studied internalisation pathway of receptors is β-arrestin-dependent internalisation by clathrin-coated vesicles (Pierce *et al.,* 2002). β-arrestin regulates this process by interacting with clathrin and clathrin adaptor protein 2 alongside with phosphorylated receptors.

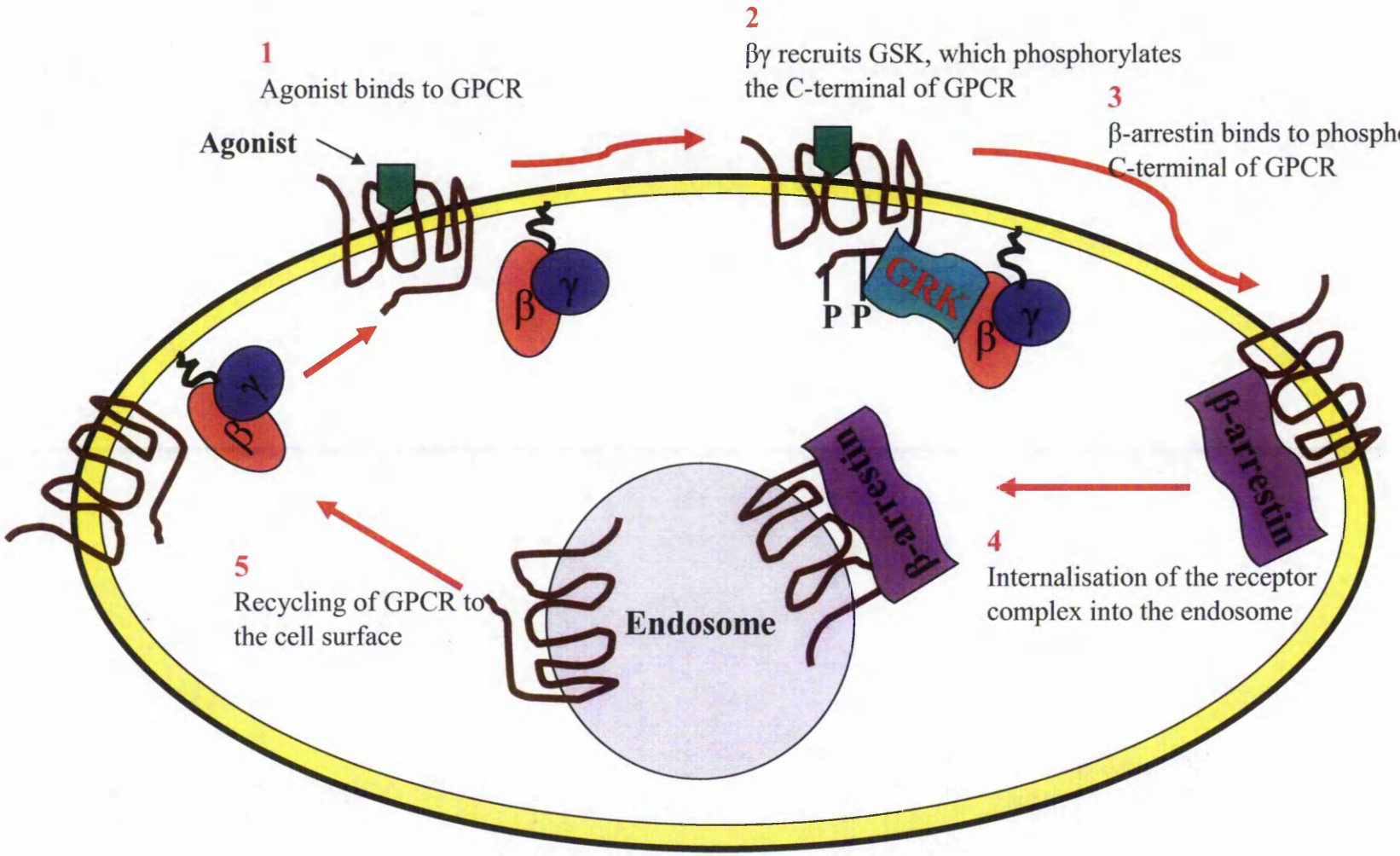

Figure 1.3: Desensitisation and internalisation of G-protein coupled receptor s (GPCR). Activation of a GPCR by an agonist leads to The dissociation of Gα and βγsubunits (see figure1.2). The process of desensitisation and internalisation is regulated by G-protein receptor kinases (GSK) and β-arrestin

β-arrestin also mediates the trafficking of GPCRs by controlling the rate of recycling (Figure 1.3). β-arrestin-1 and β-arrestin-2 possess different affinities for GPCRs, for example β-arrestin-2 translocates more readily β_2-adrenergic receptor than β-arrestin-1 resulting in rapid recycling (Oakley *et al.,* 2000). The most recent pathway by which β-arrestin can regulate GPCRs internalisation is by ubiquitylation (addition of ubiquitin to the target proteins; Shenoy *et al.,* 2001). β-arrestin-2 interacts with one of the enzymes which promotes ubiquitylation (ubiquitin ligase) and modifies internalisation of β_2-adrenergic receptor (Shenoy *et al.,* 2001) .

1.1.2 Different types of G-proteins

G_s protein:

G_s pathway is a well-defined effector pathway leading to the activation of AC and PKA (Figure 1.4). This pathway activates all the known AC isoforms (Offermanns, 2003; Cordeaux and Hill, 2002). AC converts ATP to cyclic AMP (cAMP) and there are nine isoforms of AC (AC-1 – AC-9; see Table: 1.3). All the nine isoforms have similar sequence in their catalytic sites. Each AC isoform comprises of two hydrophilic domains and two cytoplasmic domains (C1 and C2; Hanoune and Defer, 2001). The isoforms are widely distributed in the human body. AC isoforms can also be modulated by protein kinase C (PKC) which stimulates AC-2, AC-3, and AC-5 and by increases in intracellular Ca^{2+} which can either stimulate (AC-1, AC-3 and AC-8) or inhibit (AC-5, AC-6) certain isoforms of AC (Tang and Hurley, 1998; Cooper *et al.,* 1995). Forskolin (FSK), a plant extract activates all AC isoforms, except AC-9 because of the alteration of Ser→ Ala and Leu→Tyr amino acids in the binding pocket (Hanoune and Defer, 2001). FSK binds to the cytoplasmic domains of AC by hydrophobic and hydrogen bonding interactions (Hanoune and Defer, 2001). AC and cAMP play an important role in regulating cell differentiation, maturation of spermatozoa, drug and alcohol dependency and in learning and memory (Hanoune and Defer, 2001).

Cholera toxin, a bacterial toxin from *Vibrio cholerae* is used to study coupling to G_s proteins. Cholera toxin blocks the GTPase activity by catalysing the transfer of ADP-ribose from NAD^+ to G_s and subsequently activating AC (Fujinaga, 2006). Vasopressin V_2 receptor, adenosine $A_{2A,B}$ receptors, adrenergic $\beta_{1,2}$ receptors, histamine H_2 receptor are some examples of receptors coupled to G_s protein.

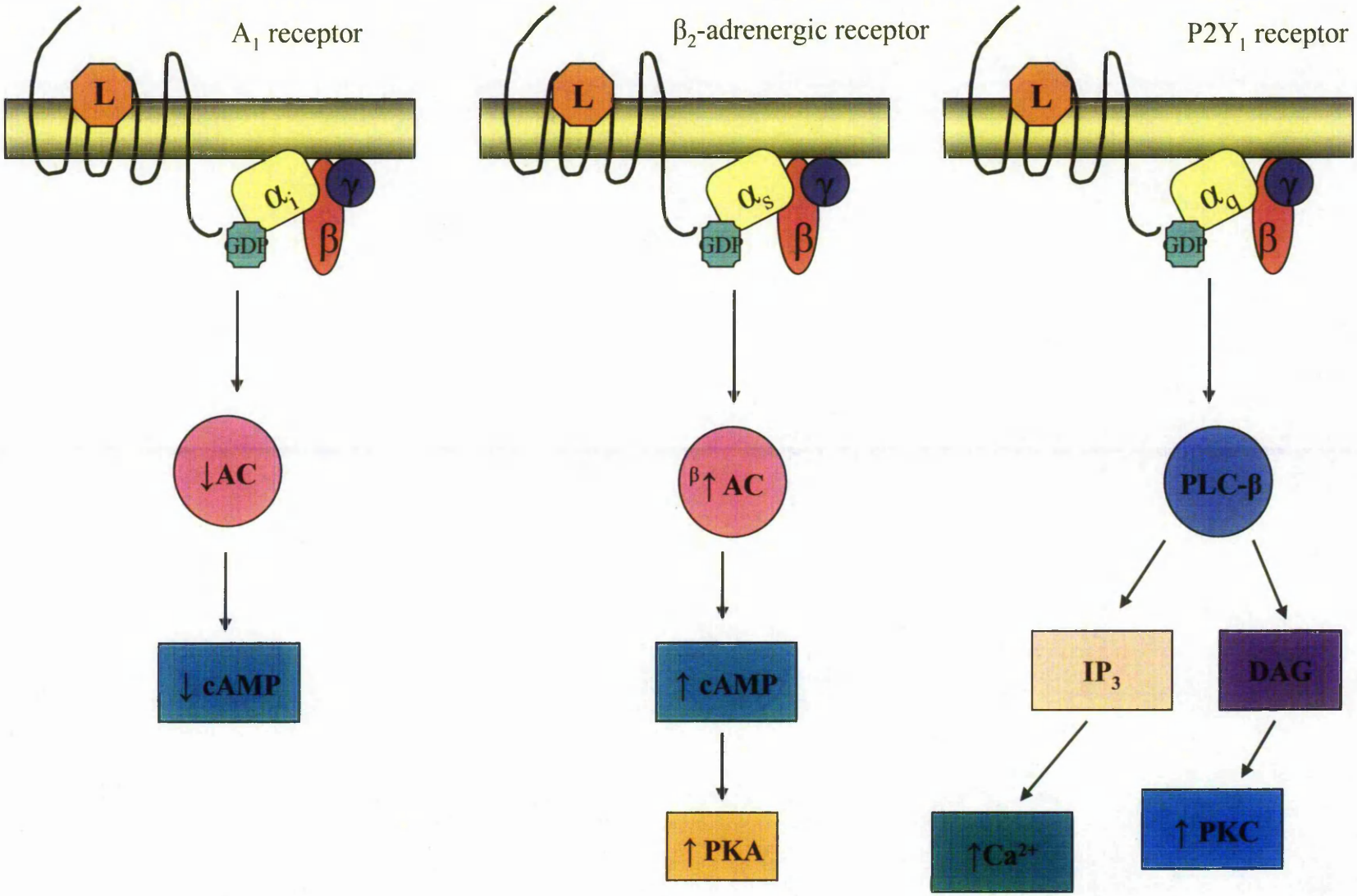

Figure 1.4: A schematic representation of different G-protein signal transduction pathways. L, Ligand; AC, Adenylyl cyclase; cAMP, cyclic AMP; PKA, protein kinase A, PLC, phospholipase C; IP3, inositol triphosphate; DAG diacylglycerol; PKC, protein kinase C.

$G_{i/o}$ protein:

There are three members of the G_i protein family, G_{i1}, G_{i2} and G_{i3} which mediate receptor-dependent inhibition of various types of AC and in turn decrease the activity of PKA (Figure 1.4). $G\alpha_{i1}$, G_{i2} and G_{i3} inhibit all isoforms of AC; expect AC-2 and AC-7 (Table 1.3; Watts and Neve, 2005). In addition, the $G\beta\gamma$ subunits released following G_i/G_o protein activation modulate a variety of other effectors, for example stimulation of PLC-β, activation of G-protein regulated inward rectifier potassium channels (GIRK), inhibits AC (AC-2 and AC-7) and finally stimulates phosphatidylinositol 3- kinase (PI3K). Adenosine A_1 receptor not only inhibits AC through G_i but also activates PLC via $G_i\beta\gamma$ subunits (Gerwins and Fredholm, 1992; Dickenson and Hill, 1998). G_o is highly expressed G-protein in the nervous system. However, its function is poorly understood and its effects are mediated by βγ-subunits (Wettschureck and Offermanns, 2005). Pertussis toxin (PTX), a bacterial toxin from *Bordetella pertussis* is frequently used to study coupling to $G_{i/o}$ proteins. PTX catalyzes adenosine diphosphate (ADP)-ribosylation of the Gα subunit at a cysteine residue near its carboxylic terminal thereby preventing the displacement of GDP by GTP (Bokoch *et al.,* 1983; Locht and Anotoine, 1997; Nurnberg, 1997; Neves *et, al.,* 2002; Watts and Neve, 2005). Muscarinic $M_{2,4}$ receptors, adrenergic β_3 receptor, dopamine receptors $D_{2,3,4}$ receptors and adenosine $A_{1,3}$ receptors are some examples of $G_{i/o}$-protein coupled receptors.

G_q protein:

There are four members of the G_q protein family, G_q, G_{11}, G_{14} and $G_{15/16}$ which mediate PTX-insensitive regulation of PLC-β to produce inositol triphosphate (IP_3) and diacylglycerol (DAG) (see Figure 1.4, Figure 1.5, Table 1.3; Hubbard and Helper, 2006). Nearly 40% of all GPCRs are coupled to the G_q protein family. Large amount of studies are focussed on G_q and G_{11}-coupled receptors and little is known about G_{14} and $G_{15/16}$-coupled receptors. A study carried out on G_{11} and G_q -deficient mice revealed the importance of G_q proteins in cardiac growth and development. Indeed, the G_q-deficient mice showed high incidence of cardiac malformation and craniofacial defects, while G_{11}-deficient mice were normal (Offermanns *et al.,* 1998).

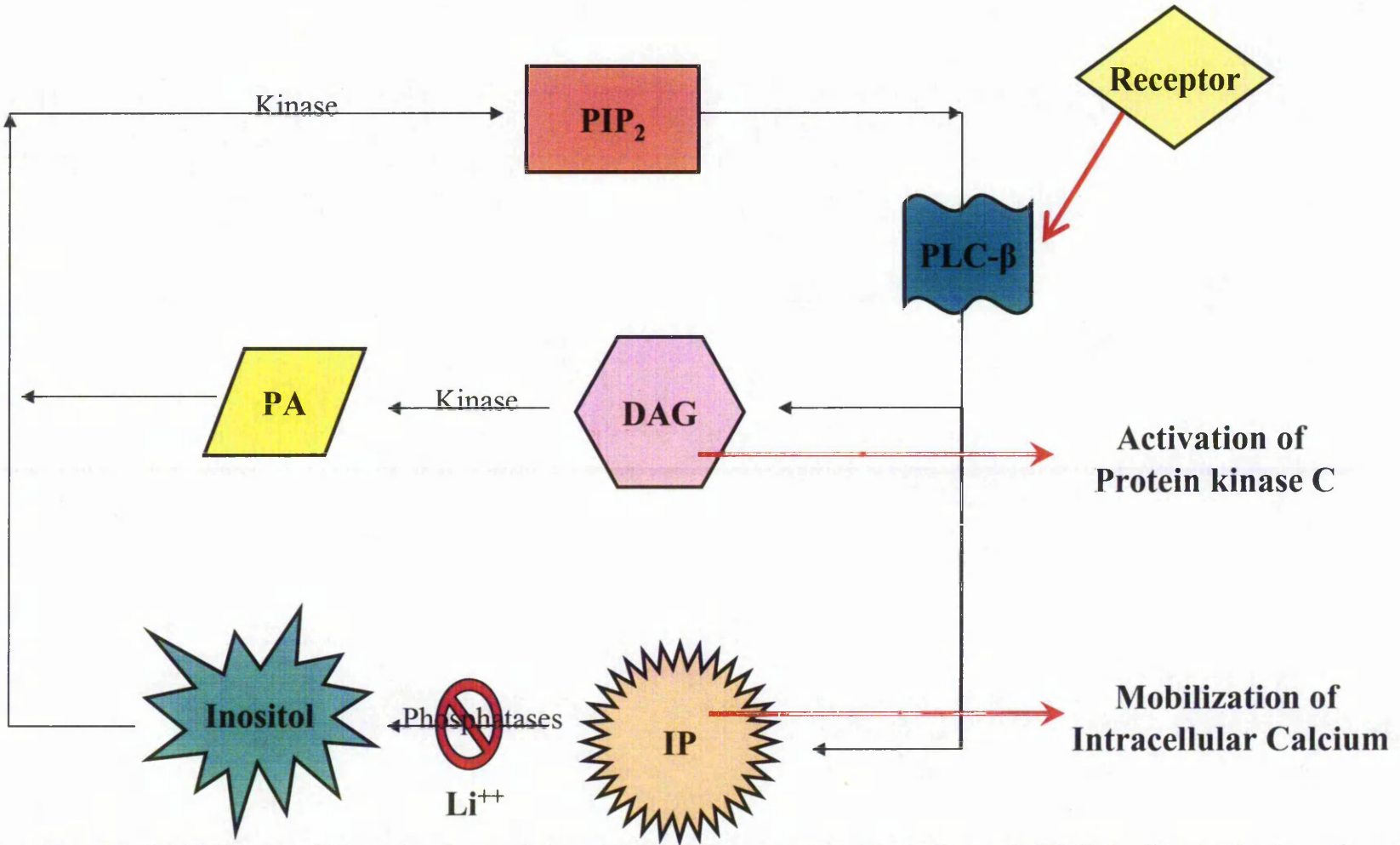

Figure 1.5: The phosphatidylinositol (PI) cycle. G_q-coupled protein receptor activates phospholipase C (PLC) leading to the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) via PIP_2 breakdown. DAG is converted to phosphatidic acid (PA). Inositol and PA are used in the regeneration of PIP_2. During the IP_3 accumulation assay the cells are incubated with radio-labelled inositol ([^{3}H]-inositol) and total [^{3}H]-IP accumulation (Li^{++} prevents the breakdown of IP to inositol) is measured.

YM-254890, a novel cyclic depsipeptide isolated from *Chromobacterium* sp. QS3666 selectively blocks the activation of $G_{q/11}$ protein (Taniguchi *et al.,* 2004). YM-254890 inhibits exchange of GDP for GTP in the $G_{q/11}$ activation stage thereby preventing the $G_{q/11}$ signalling pathway (Takasaki *et al.,* 2004).

IP_3 triggers the release of calcium from intracellular stores and DAG activates PKC (Rhee, 2000). The intracellular calcium also activates calcium channels at the cell surface to allow influx of extracellular calcium. An increase in intracellular calcium initiates proliferation, stimulates fluid and electrolyte secretion, controls muscle contraction, triggers fertilization and initiates apoptosis (Kiselyov *et al.,* 2002). Histamine H_1 receptor, $P2Y_1$ receptor, oxytocin receptor, angiotensin type-I AT_1 receptor and Vasopressin $V_{1a,1b}$ receptors are some examples of G_q-protein coupled receptors.

G_{12} and G_{13} proteins:

$G\alpha_{12}$ and $G\alpha_{13}$-proteins constitute the $G\alpha_{12}$ family. Individual cellular responses regulated through $G\alpha_{12}$ and $G\alpha_{13}$-proteins were difficult to study because $G\alpha_{12}/G\alpha_{13}$-coupled receptors also appear to activate $G\alpha_q$ family members. $G\alpha_{12}$ is known to interact directly with GTPase-activating protein for Ras, RasGAP and Bruton's tyrosine kinase (Btk) (Jiang, *et al.,* 1998; Table 1.3). The effect of this pathway on physiological activities is not fully understood. $G\alpha_{13}$ is reported to directly activate p115RhoGEF (a RhoGEF subtype) (Table: 1.3) and thus stimulates Rho. Downstream targets of activated Rho include the Na^+-H^+-exchanger and phospholipase D (PLD; Offermanns, 2003; Neves *et al.,* 2002). AT_1 receptor, lysophosphatidic acid (LPA) receptor, CXC chemokine receptor and CCK-A receptor are some examples of $G_{12/13}$-protein coupled receptors.

1.1.3 Dimerisation of GPCRs

In the last couple of years, the classical idea that GPCRs function as a monomeric unit has been challenged by numerous studies, suggesting that GPCRs exists as homodimers or heterodimers. GPCRs can form dimers at any stage of their life cycle (Figure 1.6). For detailed reviews on dimerisation and GPCRs refer to Milligan, 2006; Terrillon and Bouvier, 2004; Milligan, 2004; Bai, 2004; Angers *et al.,* 2002; Devi, 2001.

Ontogeny:

GPCRs are synthesized in the endoplasmic reticulum (ER) and only the correctly folded receptors are allowed to exit the ER and be expressed at the cell surface (Petaja-Repo *et al.,* 2000). Margeta-Mitrovic *et al.,* (2000) showed that dimerisation of the metabotropic γ-aminobutyric acid b receptor (GbR) is essential to escape from ER quality control and express functionally at the cell surface. GbR consists of two subunits GbR_1 and GbR_2. When expressed alone, GbR_1 is retained inside ER as an incomplete protein whereas GbR_2 exits ER and reaches plasma membrane, but it is not functional (Margeta-Mitrovic *et al.,* 2000). The C-terminal RXR(R) motif of GbR_1 acts as a retention signal and is retained in ER. Interaction between GbR_1 and GbR_2 masks the retention motif signal and the assembly is expressed at the cell surface (Margeta-Mitrovic *et al.,* 2000). Studies, using cellular fractionation and bioluminescence resonance energy transfer approaches like FRET and BRET showed that C5a and oxytocin, vasopressin V_{1a} and V_2 form homo and heterodimers in ER (Floyd *et al.,* 2003; Terrillon *et al.,* 2003).

Ligand-promoted dimerisation:

Several studies reported that ligand binding can promote (Roess and Smith, 2003; Hunzicker-Dunn *et al.,* 2003) or inhibit (Latif *et al.,* 2002; Cheng and Miller, 2001) dimerisation; however other researchers reported that ligand binding did not modulate the homodimerisation or heterodimerisation (Floyd *et al.,* 2003; Terrillon *et al.,* 2003). Previous studies reported that chemokine SDF-1α promoted dimerisation of CXCR4 receptor that existed as a momoner in the absence of ligand (Vila-Coro *et al.,* 1999). However, recent studies using BRET and sedimentation methods indicated that CXCR4 receptor existed as a dimer and was unaffected by its ligand – SDF-1α (Babock *et al.,* 2003). The techniques used to determine dimerisation process like co-immunoprecipitation, bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) may lead to ambiguous results (Angers *et al.,* 2002). The efficacy of BRET and FRET is highly dependent on molecular proximity (<100Å), orientation of acceptor and donor fluorophores and due to these properties FRET or BRET is a better choice to study protein-protein interactions (Angers *et al.,* 2002). In addition, other experimental approaches like crystallisation or atomic force microscopy will be needed to state the formation of dimers (Kunishima *et al.,* 2000; Dann *et al.,* 2001; Liang *et al.,* 2003).

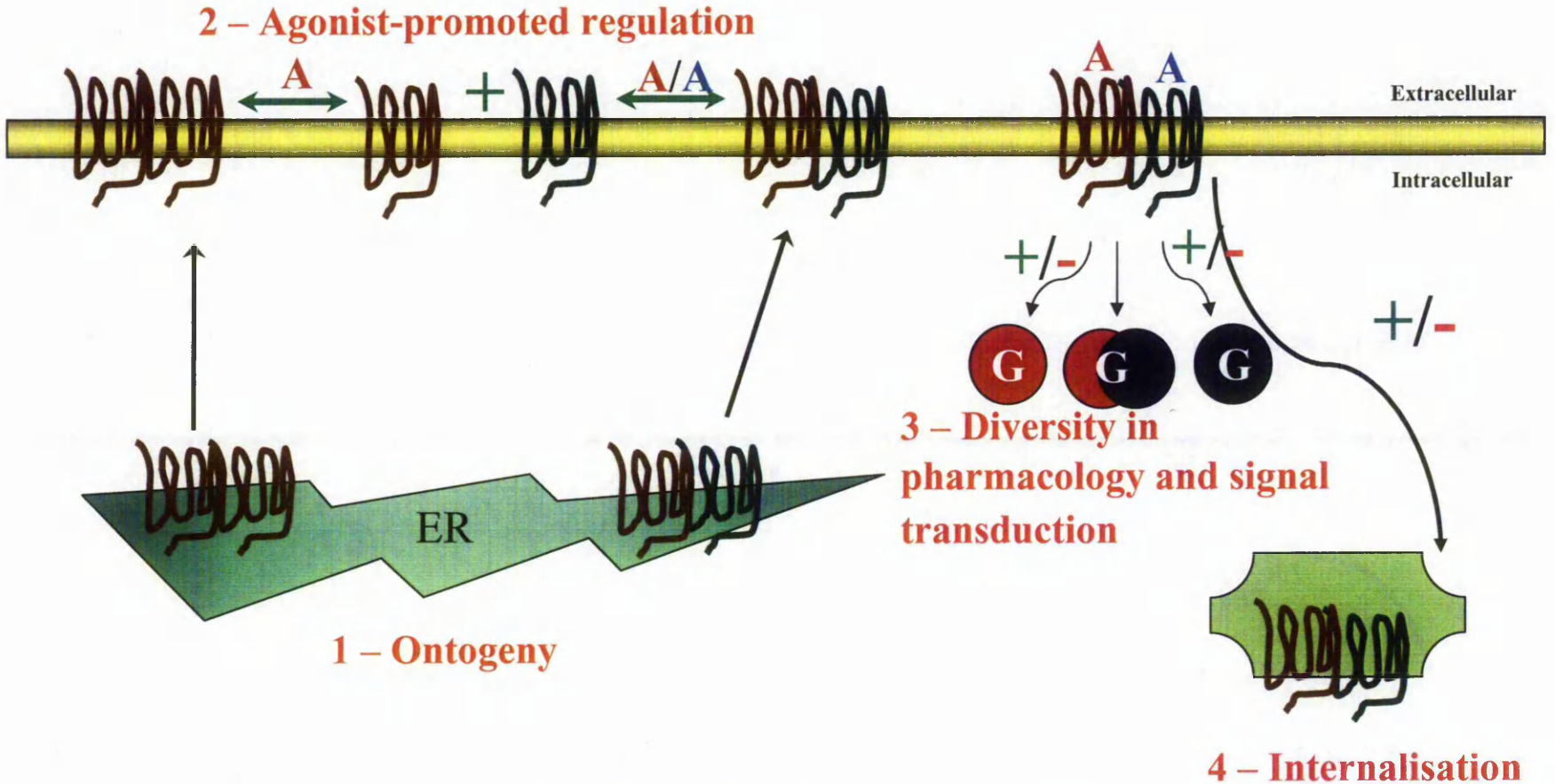

Figure 1.6: G-protein coupled receptors (GPCRs) can form dimers at any stage of their life cycle. 1 – In some GPCRs, dimerisation is necessary for receptor maturation and functional expression at cell surface; 2 – Agonists (A) can promote formation of dimers at the plasma membrane; 3 – heterodimerisation can potentiate (+) or attenuate (-) signalling or change G-protein (G) selectivity and can also result in ligand binding cooperativity; 4 – receptor internalisation is affected by heterodimerisation. Stimulation of one protomer can be sufficient to promote internalisation of two receptors (dimer) or inhibit the internalisation of both receptors.

Diversity in pharmacology and signal transduction and internalisation of dimers:

The first indication of pharmacological diversity in dimers was revealed by the studies on δ- and κ-opioid receptors (Jordan and Devi, 1999). Jordan and Devi reported that the dimer had very low affinity for either δ- or κ-opioid selective agonists alone, but the combination of these agonists restored their affinity at the dimer complex. Such a ligand-binding cooperativity occurs as a result of receptor heterodimerisation; which is also seen at opioid δ/μ (Gomes *et al.,* 2000), adenosine A_{2A}/dopamine D_1 (Franco *et al.,* 2000), and muscarinic m_2/m_3 (Terrillon and Bouvier, 2004) receptors. These distinct pharmacological features provide new opportunities for the development of novel compounds to target specific heterodimers.

In case of GbR signal transduction both GbR_1 and GbR_2 are necessary as GbR_1 posses the binding site for γ-aminobutyric acid (GABA) and GbR_2 couples to the G-protein (Galvez *et al.,* 2001). In the classical model of GPCR activation, one receptor interacts with one heterotrimeric G protein. This notion must be re-visited in context of GPCR dimerisation. The structural studies have identified several points of contact between the receptor and G-protein on both α and βγ subunits (Hamm, 2001). In fact, the leukotriene B_4 receptor dimer and a G-protein formed a novel pentameric assembly (Baneres and Parello, 2003). In addition, dimerisation can also alter the selectivity of some GPCRs towards the G-proteins – G_s, G_i, G_q. Co-expression of μ- and δ- opioid receptors and CCR_5 and CCR_2 receptors resulted in the loss of G_i coupling, respectively (Charles *et al.,* 2003; Mellado *et al.,* 2001). Receptor internalisation, a process which attenuates the signal transduction could also be affected by dimerisation. Studies on δ-opioid/β_2 adrenergic (Jordan *et al.,* 2001), α_{1a}/α_{1b} adrenergic (Stanasila *et al.,* 2003), A_{2A} adenosine/ D_2 dopamine (Hillion *et al.,* 2002) receptors have suggested that stimulation of one receptor was sufficient to promote internalisation dimer complex. On the other hand, the κ-opioid receptor inhibited the internalisation of both δ-opioid and β_2 adrenergic receptors (Jordon and Devi, 1999; Jordan *et al.,* 2001). Moreover, studies have shown that P2Y receptors can form dimmers (Suzuki *et al.,* 2006; Ambrosi *et al.,* 2006; Nakata *et al.,* 2005; Yoshioka *et al.,* 2001). Ambrosi and associates (2006) documented that $P2Y_4$ receptor forms homodimers whereas $P2Y_1$ and $P2Y_2$ receptors form heterodimers with the adenosine A_1 receptor, respectively (Yoshioka *et al* 2001; Suzuki *et al.,* 2006).

1.2 Purine Receptors

Extracellular purines (adenosine, ADP and ATP) and pyrimidines (UDP and UTP) are ubiquitous signalling molecules, which induce a wide spectrum of biological actions via specific receptors termed purine receptors. Purine receptors consist of two main families; adenosine/P1 receptors and P2 receptors. Based on molecular, biochemical and pharmacological evidence P1 receptors are further divided into A_1, A_{2A}, A_{2B} and A_3 receptors. P2 receptors are also subdivided according to their molecular structure and signal transduction mechanism into ligand-gated ion channels P2X and G-protein coupled P2Y receptors (Ralevic and Burnstock, 1998; Abbracchio *et al.*, 2006).

1.2.1 P2X receptors

P2X receptors represent the third largest family of inotropic receptors and comprise of seven subtypes ($P2X_1$ – $P2X_7$; Volonte *et al.*, 2006; North, 2002). P2X receptors are ATP-gated ion channel receptors which mediate rapid excitatory neurotransmission and selective permeability to Na^+, K^+, Ca^{2+} cations in excitatory cells like neurons, glia and smooth muscle cells (Jacobson *et al.*, 2002; North, 2000). Activation of P2X receptors can also stimulate L-type Ca^{2+} channels and the influx of calcium activates mitogen-activated protein (MAP) kinases via calcium sensitive tyrosine kinases (Swanson *et al.*, 1998). There is ample evidence that functional P2X receptors form a trimeric motif (Torres *et al.*, 1999; Nicke *et al.*, 1998). The topology of a subunit consists of two transmembranal domains (TM1 and TM2) which are connected together by a hydrophilic extracellular loop made up of 270 amino acids (North 2002). P2X receptors form functional homomers, and heteromers like $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$ (Jacobson *et al.*, 2002; North 2002). The properties of the various P2X receptor subtypes are summarised in Table 1.4 and for detailed reviews refer to Ralevic and Burnstock, 1998; North and Surprenant, 2000; North, 2002; Jacobson *et al.*, 2002; Burnstock and Knight, 2004; Volante *et al.*, 2006.

Table 1.4: Overview of P2X receptors

Nomenclature	Signalling Transduction	Agonist	Antagonist	Desensitisation Kinetics
$P2X_1$	Intrinsic cation channel (Ca^{2+}, Na^{+})	α,β-MeATP=ATP=2-MeSATP	TNP-ATP, IP_5I, NF023	Rapid
P2X2	Intrinsic cation channel (Ca^{2+})	ATP≥ATPγS≥2-MeSATP>>α,β-MeATP	Suramin, PPADS	Slow
$P2X_3$	Intrinsic cation channel	2-MeSATP≥ATP≥ α,β-MeATP	TNP-ATP, Suramin, PPADS	Rapid
$P2X_4$	Intrinsic cation channel (Ca^{2+})	ATP>> α,β-MeATP	-	Slow
$P2X_5$	Intrinsic cation channel	ATP>> α,β-MeATP	Suramin, PPADS	Slow
$P2X_6$	Intrinsic cation channel	ATP>2-MeSATP> ADP	Suramin, PPADS	Slow
$P2X_7$	Intrinsic cation channel	Bz-ATP>ATP≥ 2-MeSATP>> α,β-MeATP	Coomassie brilliant blue, KN-62	Slow

Modified and adapted from Jacobson *et al.,* 2002; Burnstock and Knight, 2004.

1.2.2 P2Y receptors

P2Y receptors belong to G-protein coupled receptor superfamily. Currently there are eight subtypes - $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio *et al.*, 2006; Kugelgen, 2005; Sak and Webb, 2002; Communi *et al.*, 2000; Kugelgen and Wetter, 2000). The missing numbers represent the receptors which are misplaced in the family (e.g.: $p2y_7$) or receptors cloned from non-mammalian vertebrates (e.g.: $p2y_3$) (Jacobson *et, al.*, 2002). P2Y receptors are activated by purines and pyrimidines nucleotides. Based on this they are divided into three groups; group-I ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$) activated by purine nucleotides; group-II ($P2Y_4$, $P2Y_6$ and $P2Y_{14}$) stimulated by pyrimidine nucleotides; group-III ($P2Y_2$) activated by both nucleotides (Kugelgen, 2005). $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors couple to $G_{q/11}$ and trigger phospholipase C activation (Kugelgen and Wetter, 2000), whereas $P2Y_{12}$ and $P2Y_{13}$ receptors are coupled to $G_{i/o}$ and inhibit adenylyl cyclase (Communi *et al.*, 2001; Hollopeter *et al.*, 2001; Chambers *et al.*, 2000; Scrivens and Dickenson, 2005). The $P2Y_{11}$ receptors couple positively to both phospholipase C and adenylyl cyclase (via $G_{q/11}$ and G_s; Communi *et al.*, 1997). $P2Y_{14}$ also couples to $G_{q/11}$ and $G_{i/o}$ proteins. The properties of the various P2Y receptor subtypes are summarised in Table 1.5.

1.2.2.1 Structure of P2Y receptors:

P2Y receptors are made up of 308 to 377 amino acids corresponding to a weight of 41 to 53 KDa (after glycosylation; Volonte *et al.*, 2006). The structure of P2Y receptor is similar to other GPCRs (Figure 1.1) with an exception of an essential disulfide bridge between the N-terminal domain and extracellular loop (E) 3 which is characteristic of P2Y receptors (Hoffmann *et al.*, 1999; Ding *et al.*, 2003). The P2Y receptor model was designed by structural comparison of sequence analysis, mutagenesis and homology modelling studies. Two structurally distinct P2Y receptor subgroups were identified using homology modelling with high-resolution structure of bovine rhodopsin as a template. Firstly, the G_q-coupled subgroup – $P2Y_{1,2,4,6,11}$ and secondly, the G_i-coupled subgroup – $P2Y_{12,13,14}$ (Abbracchio *et al.*, 2006). Most of the site-directed mutagenesis work was carried out on the human $P2Y_1$ receptor. In order, to identify the residues involved in ligand recognition in $P2Y_1$ receptor, individual residues on transmembrane domains (TM) 3, 5, 6 and 7 were mutated to alanine and other amino acids (Moro *et al.*, 1998; Hoffmann *et al.*, 1999).

Table 1.5: Overview of P2Y receptors

Nomenclature	Tissue distribution	Signalling transduction	Principal agonists	Antagonists
$P2Y_1$	Platelets, heart, brain, skeletal muscles	$G_{q/11}$	2-MeSADP=2-MeSATP>ADP>ATP	MRS2179
$P2Y_2$	Lung, heart, spleen, kidney	$G_{q/11}$, $G_{i/o}$	UTP=ATP	Suramin
$P2Y_4$	Placenta, vascular smooth muscle	$G_{q/11}$, $G_{i/o}$	ITP=ATP=UTP=ATPγS >UDP	Reactive blue-2
$P2Y_6$	Lung, heart, aorta, brain	$G_{q/11}$	UDP>UTP>ADP>2-MeSATP	MRS2578
$P2Y_{11}$	Spleen, intestine, immunocytes	G_s, $G_{q/11}$	ATPγS >ATP>ADPβS	-
$P2Y_{12}$	Platelets, brain	$G_{i/o}$	2-MeSATP>ADP>ATP	ATP,ARL66096* ARC69931*
$P2Y_{13}$	Spleen, lymphocytes, monocytes	$G_{i/o}$	2-MeSADP=ADPβS =2-MeSATP>ADP	ARC69931*
$P2Y_{14}$	Spleen, thymus	$G_{i/o}$,$G_{q/11}$	UDP-glucose	-

ATP, adenosine 5'-triphosphate; ATP-γ-S adenosine 5'-[γ-thio] triphosphate; 2-MeSATP, 2-methylthioadenosine triphosphate; 2-MeSADP, 2-(methylthio)adenosine 5'-diphosphate; UDP, uridine 5'-diphosphate; UTP, uridine 5'-triphosphate; UDP-glucose, uridine 5'-diphosphate-glucose; MRS, ^{6}N-methyl-2'-deoxyadenosine-3',5'-biphosphate; MRS2567, . 1,2-di-(4-isothiocyanatophenyl)ethane; ARL66096, 2-propylthio-βγ-difluoromethylene ATP; ARC69931, N^6-(2-prasugrel) also called Cangrelor. * - Commercially not available.Adapted and modified from Abbracchio *et al.,* 2006; Kugelgen, 2005; Sak and Webb, 2002.

These studies revealed that positively charged lysine and arginine residues mainly on TM3 and TM7, and to a lesser extent TM6, bind to negatively charged phosphate moiety of nucleotides (Moro *et al.,* 1998; Hoffmann *et al.,* 1999). Indeed, converting the positively charged amino acids to neutral amino acids on TM6 and TM7 of $P2Y_2$ receptor incredibly decreased the potencies of ATP and UTP at the receptor (Erb *et al.,* 1995). In addition, several amino acid residues of E1 and E2 are involved in receptor activation (Moro *et al.,* 1998; Hoffmann *et al.,* 1999). The computational models of $P2Y_1$ and $P2Y_{12}$ receptors derived from multiple-sequence alignment revealed that TM7 cytoplasmic end was folded at an angle approximately 90° forming a helical segment homologous to H8 in rhodopsin and runs parallel to cytoplasmic membrane (Abbracchio *et al.,* 2006; Hoffmann *et al.,* 1999).

1.2.2.2 Classification of P2Y receptor subtypes:

Group-I

$P2Y_1$: $P2Y_1$ was cloned and pharmacologically characterised from mouse, rat and human (Von kugelgen, 2005). The $P2Y_1$ receptor is selective for the endogenous ligands ADP, ATP and certain diadenosine polyphosphates (e.g: diadenosine tetraphosphate, AP4A; diadenosine pentaphosphate, AP_5A), but not for uracil nucleotides (Abbracchio *et al.,* 2006). It is strongly activated by 2-MeSADP, 2-MeSATP, ADP, ADPβS and adenosine-5'-O-(2-fluoro)-diphosphate($ADP_\beta F$). $P2Y_1$ is solely coupled to $G_{q/11}$ proteins and its activation mediates inositol phosphate production and increases intracellular Ca^{2+} release (Tulapurkar *et al.,* 2004; Calvert *et al.,* 2004; Czajkowski *et al.,* 2003). MRS2179 is the most potent $P2Y_1$ receptor antagonist reported to date (Jacobson *et al.,* 2002). However, previous studies have also shown that MRS2179 can block $P2X_1$ and $P2X_3$ ion-channel receptors are activated by ATP and UTP (McLaren *et al.,* 1998; Rae *et al.,* 1998 Brown *et al.,* 2000). $P2Y_1$ mRNA is widely distributed in the human brain, platelets, heart, placenta, stomach, kidneys, lungs, liver, spleen and skeletal muscles (Burnstock and Knight, 2004). $P2Y_1$ regulates the shape of platelets and initiates platelet activation and aggregation, which is well documented in $P2Y_1$ receptor-deficient mice (Wood, 2006). The mRNA levels of $P2Y_1$ receptor within the brain are highest in the nucleus accumbens, putamen, striatum and caudate nucleus (Burnstock and Knight, 2004).

$P2Y_{11}$: The $P2Y_{11}$ receptor was characterised and cloned from human and canine (Communi *et al.,* 1997; Zambon *et al.,* 2001). Contrary to other P2Y receptors, the $P2Y_{11}$

gene contains an intron in the coding sequence and is dual coupled to G_s and $G_{q/11}$-proteins (Qi *et al.,* 2001; Conigrave *et al.,* 2000; Communi *et al.,* 1999). $P2Y_{11}$ is a purine nucleotide specific receptor with greater selectivity for ATP over ADP. The rank order of various agonists to stimulate inositol phosphate production or cAMP accumulation at the human $P2Y_{11}$ is ATP-γS ≈ BzATP > dATP > ATP > ADP-βS > 2-MeSATP (Communi *et al.,* 1999). At present there are no known selective $P2Y_{11}$ receptor antagonists (Abbracchio *et al.,* 2006). $P2Y_{11}$ receptor is not cloned in rodents. However, recently Balogh *et al.,* (2005) have shown that ATP activates inositol phosphate production and cAMP accumulation in mouse cardiomyocytes via $P2Y_{11}$-like receptors. Moreover, extracellular ATP in mouse neuroblastoma neuro2a cells stimulated neurite outgrowth through a receptor with a $P2Y_{11}$ pharmacological profile (Lakshmi and Joshi 2006). Chootip and associates (2005) have also identified a $P2Y_{11}$-like receptor in smooth muscle cells of the rat pulmonary artery which was pharmacologically similar to human $P2Y_{11}$ receptor. There are no known selective antagonists at $P2Y_{11}$ receptor, however, the non-selective P2 antagonist suramin behaves as a competitive antagonist and PPADS and RB2 do not block the receptor (Abbracchio *et al.,* 2006). $P2Y_{11}$ mRNA is detected in human spleen, brain, pituitary gland, lymphocytes, monocyte-derived dendritic cells, liver and intestine (Abbracchio *et al.,* 2006). In human monocyte-derived dendritic cells, ATP via $P2Y_{11}$ receptor modified the secretion of IL-12, IL-10, expression of chemokine receptor and altered the migratory behaviour to dendritic cells (Abbracchio *et al.,* 2006).

$P2Y_{12}$: The $P2Y_{12}$ receptor represents the ADP-sensitive P2 receptor, previously termed P_{2T}, $P2Y_{ADP}$ and P2YT and has been cloned and pharmacologically characterised in mouse, rat and human (Zhang *et al.,* 2002; Foster *et al.,* 2001; Hollopeter *et al.,* 2001). $P2Y_{12}$ receptor is activated by diphosphate nucleotides and has a rank order of agonist potency: 2-MeSADP >> ADP > ADP-βS (Abbracchio *et al.,* 2006). ATP and its analogs like ARL 67085 and AR-C69931 MX are reported to antagonise the human and mouse $P2Y_{12}$ receptor in platelets (Hollopeter *et al.,* 2001; Ingall *et al.,* 1999). Interestingly, in brain capillary endothelial cells, neuronal cells and in some heterologous transfected cells ATP and its triphosphate analogs activated the $P2Y_{12}$ receptor (Unterberger *et al.,* 2002; Simon *et al.,* 2001; Abbracchio *et al.,* 2006). The $P2Y_{12}$ receptor is coupled to $G_{i/o}$ proteins and thus inhibits adenylyl cyclase (Simon *et al.,* 2001; Abbracchio *et al.,* 2006). $P2Y_{12}$

receptor is highly expressed in megakaryocyte/platelet lineage and plays an important role in thrombogenesis and also maintains platelet shape (Cattaneo, 2007). The active metabolite of clopidogrel, a well known anti-platelet drug binds covalently to the cysteine residues of the $P2Y_{12}$ extracellular loops and thereby inhibits the ligand binding (Cattaneo, 2007). The $P2Y_{12}$ knockout mice show signs of prolonged bleeding time and reduced sensitivity to ADP, thrombin and collagen (Foster *et al.,* 2001). Besides platelets, $P2Y_{12}$ receptor is also expressed in glial cell, brain capillary endothelial cells, chromaffin cells and subregions of the brain (Abbracchio *et al.,* 2006).

$P2Y_{13}$: The $P2Y_{13}$ receptor was identified, cloned and characterised in mouse, rat and human. The putative rat $P2Y_{13}$ is 79% and 87% identical to the human and mouse orthologs, respectively (Fumagalli *et al.,* 2004). There is 48% amino acid homology between human $P2Y_{12}$ and $P2Y_{13}$ which explains their similar pharmacological profile (Marteau *et al.*, 2003). The $P2Y_{13}$ receptor is stimulated by ADP and 2-MeSADP, the potency of 2-MeSADP being higher or equal to ADP (Fumagalli *et al.,* 2004; Communi *et al.,* 2001). ADP is the endogenous ligand for $P2Y_{13}$ receptor. AR-C67085MX ($P2Y_{12}$ antagonist), is a weak antagonist for the $P2Y_{13}$ (Vasiljev *et al.,* 2001). ADP induced increases in GTPγ[^{35}S] binding, inhibition of cAMP accumulation and ERK1/2 phosphorylation of the $P2Y_{13}$ receptor was sensitive to pertussis toxin, indicating that $P2Y_{13}$ is coupled to $G_{i/o}$ protein. $P2Y_{13}$ receptor has been detected in spleen, brain, placenta, liver, lung, dendritic cells, monocytes, erythrocytes by RT-PCR, Northern blotting and dot blot analysis. In humans, within the brain $P2Y_{13}$ receptors are highly expressed in nucleus accumbens, putamen, striatum and caudate nucleus (Burnstock and Knight, 2004). Wang *et al.,* (2005) reported that stimulation of $P2Y_{13}$ in red blood cells activates a negative feedback pathway which may control the concentration of ATP in plasma and tissues. Indeed, 2-MeSADP reduced the plasma ATP concentration in an *in vivo* pig model (Wang *et al.,* 2005).

Group II

$P2Y_4$: The $P2Y_4$ receptor was cloned and characterised in mouse, rat and human. Amino acid sequencing studies showed that the human $P2Y_4$ is 51% and 40% identical to the human $P2Y_2$ and $P2Y_6$ receptors (Communi and Boeynaems, 1997). It is a uridine-

nucleotide specific receptor (Kumari *et al.,* 2003; Kennedy *et al.,* 2000). UTP is the potent agonist at the human $P2Y_4$ receptor and ATP is an antagonist; however in rat UTP and ATP are equipotent in activating the $P2Y_4$ receptor (Kennedy *et al.,* 2000; Bogdanov *et al.,* 1998). $P2Y_4$ receptor is coupled to $G_{q/11}$ proteins and activates PLC-β. However, the UTP-induced increases in inositol phosphate production were partially sensitive to pertussis toxin (Communi et al., 1996), indicating that human $P2Y_4$ interacts with $G_{i/o}$ protein along with $G_{q/11}$ protein. Although there are no known selective $P2Y_4$ antagonists reported. Pyriodoxal-5-phosphate-6-azophenyl-2-4-disulfonate (PPADS) is a weak antagonist, with suramin being insensitive for the human $P2Y_4$ receptor (Kumari *et al.,* 2003). $P2Y_4$ mRNA is found in human placenta, intestine, and cell lines derived from lung, peripheral blood cells and in rat heart, brain, inner ear and kidney (Abbracchio *et al.,* 2006).

$P2Y_6$: The $P2Y_6$ receptor is characterised and cloned from mouse, rat and human (Lazarowski *et al.,* 2001; Nicholas *et al.,* 1996; Communi *et al.,* 1996). Amino acid sequencing studies showed that the human $P2Y_6$ is 37% and 40% identical to the human $P2Y_2$ and $P2Y_4$ receptors (Communi *et al.,* 1996). The most potent agonist for $P2Y_6$ receptor is UDP whereas UTP, ATP, ADP, 2-MeSATP have weak or no effect on $P2Y_6$ receptor (Hoffmann *et al.,* 2004). The potency rank order of various agonist at the human $P2Y_6$ receptor is UDP > UTP > ADP > 2-MeSATP >> ATP (Communi *et al.,* 1996). UDP mediated PLC-β and inositol phosphate production via $P2Y_6$ was resistant to PTX toxin, indicating $G_{q/11}$ protein coupling (Abbracchio *et al.,* 2006). In addition, UDP was reported to increase cAMP levels and mediate secretion of NaCl indirectly by stimulating prostaglandin release via $P2Y_6$ receptor, which was blocked by indomethacin (Kottgen *et al.,* 2003). Mamedova *et al.,* (2004) reported that MRS2578 selectively antagonised rat $P2Y_6$ receptor. $P2Y_6$ mRNA is widely distributed and is found in human spleen, thymus, intestine, placenta, dendritic cells and lymphocytes. $P2Y_6$ receptor is involved in the release of interleukin-8 (IL-8) from human monocytic THP-1 cells and is expressed in activated $CD4^+$ and $CD8^+$ T cells (Somers *et al.,* 1998). UDP stimulates secretion of chloride from human nasal epithelial cells, rat colonic epithelial cells and in mouse gallbladder via $P2Y_6$ receptor (Kottgen *et al.,* 2003; Lazarowski *et al.,* 2001; Kim *et al.,* 2004).

$P2Y_{14}$: The $P2Y_{14}$ receptor is 47% identical to the $P2Y_{12}$ and $P2Y_{13}$ receptors (Abbracchio *et al.,* 2006). UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine are recognized agonists for $P2Y_{14}$ receptor, previously called the UDP-glucose receptor (Abbracchio, *et al.,* 2003). Cell membranes expressing this receptor showed no response to ATP, ADP, UTP, UDP and other sugar-nucleotides (Chambers *et al.,* 2000). UDP-glucose is the only endogenous ligand reported to be released from different cell lines (Lazarowski *et al.,* 2003). The $P2Y_{14}$ receptor is coupled to $G_{q/11}$ and G_i-proteins (Scrivens and Dickenson, 2005; Jacobson *et al.,* 2004; Abbracchio, *et al.,* 2003; Freeman *et al.,* 2001). There are no known selective $P2Y_{14}$ antagonists reported (Abbracchio *et al.,* 2006). However, the effect of the P2 receptor antagonists, suramin, PPADS and RB 2 at the $P2Y_{14}$ receptor is not reported. The receptor is cloned in mouse, rat and in humans and possess similar pharmacological profiles (Freeman *et al.,* 2001). $P2Y_{14}$ mRNA is highly expressed in glial cells, neutrophils and lymphocytes. Physiologically $P2Y_{14}$ receptor may have an active role in humoral immunity as the levels of $P2Y_{14}$ receptor expression increase after immunological challenge (Moore *et al.,* 2003).

Group III:

$P2Y_2$: $P2Y_2$ receptor was cloned and characterised from mouse, rat, canine and human (Abbracchio *et al.,* 2006). Both ATP and UTP are equipotent in activating the $P2Y_2$ receptor and other agonists like ADP, UDP, 2-MeSATP and α,βMe-ATP display weak to no activity (Tulapurkar *et al.,* 2004; Abbracchio *et al.,* 2006). The $P2Y_2$ receptor is coupled to G_i and G_q proteins (Huwiler *et al.,* 2002; Soltoff *et al.,* 1998; Mosbacher *et al.,* 1998; Communi *et al.,* 1995). There is no known selective $P2Y_2$ antagonist. However, the responses of the $P2Y_2$ receptor are blocked by the non-selective P2 antagonists, suramin and PPADS (Von Kugelgen, 2005). Studies on blockade effects of suramin and PPADS on $P2Y_2$ receptors provided the evidence for a suramin-sensitive and PPADS-insensitive responses and a PPADS-sensitive and suramin-insensitive responses, suggesting subtypes of the $P2Y_2$ receptors (Insel *et al.,* 2001). The $P2Y_2$ receptor is functionally expressed on cardiac myocytes and fibroblasts, smooth muscle cells, endothelial cells, oesteoblasts, astrocyes and cells derived from nervous systems (Abbracchio *et al.,* 2006). The activation of $P2Y_2$ receptors in the airway epithelia leads to the opening of Ca^{2+}-sensitive Cl^- channels, and increases epithelial fluid secretion (Chinet *et al.,* 1999). $P2Y_2$ receptors

activate a wide range of cell signalling cascades like ERK1/2, p38, JNK, PKC, synthesis of arachidonic acid, protaglandins and nitric oxide synthesis (Abbracchio *et al.,* 2006). Activation of $P2Y_2$ receptor regulates the cell cycle and induces cell proliferation in various cell types like human epidermal keratinocytes, lung epithelial tumour cells, glioma cells and smooth muscle cells (Abbracchio *et al.,* 2006).

1.2.2.3 Source of Naturally Occurring Nucleotides:

The concentration of nucleotides ATP, ADP, UTP, UDP, UDP-glucose in the extracellular space required to activate the P2Y receptors ranges from 0.1 to 10μM (Abbracchio *et al.,* 2006). The release of extracellular nucleotides by various mechanisms is summarised below.

Basal unstimulated release of nucleotides:

Various studies have detected extracellular nucleotides in multiple cell types under basal conditions (Lazarowski *et al.,* 2000, 2001; Schwiebert *et al.,* 2002; Joseph *et al.,* 2003). The levels of nucleotides are maintained by steady-state equilibrium between their release and metabolism. Schwiebert *et al* (2002) showed that by inhibiting the metabolism of ATP resting levels of ATP gradually increased in endothelial cells. In Madin-Darby kidney cell (MDCK) basal ATP levels activated P2Y receptors and thereby promoted the formation of prostaglandins, indicating that constitutive release of ATP regulates other signalling mechanisms in an autocrine/paracrine manner (Ostrom *et al.,* 2000). 1321N1 astrocytes, COS-7, CHO-K1, C6 glioma cells and human airway epithelial cells constitutively release UDP-glucose in similar or higher levels than ATP (Abbracchio *et al.,* 2006).

Release of ATP from excitable and secretory tissues:

In neurons, platelets, adrenal medullary chromaffin cells and mast cells ATP is stored in vesicles. When these cells are stimulated, ATP is released into the extracellular environment by exocytosis. The concentration of ATP can reach up to 150 to 1000μM in the vesicles (Abbracchio *et al.,* 2006; Burnstock, 2006).

Release of ATP from nonexcitory cells:

Extracellular nucleotides are also released from fibroblasts, endothelial and epithelial cells, smooth muscle cells, astrocytes, red blood cells and monocytes. Hypoxia, mechanical stimulation, vesicular trafficking can induce the release of nucleotides from these cells (Abbracchio *et al.,* 2006). Gerasimovskaya and associates (2002) reported that hypoxia

induced ATP release from adventitial fibroblasts and endothelial cells, resulted in proliferation of fibroblasts via activation of P2 receptors. Furthermore, erythrocytes also released ATP during hypoxic conditions which induced vasodilation in skeletal muscles and pulmonary artery (Sprague *et al.*; 2001; Gonzalez-Alonso *et al.*, 2002). Activation of bradykinin, acetylcholine and serotonin receptors in endothelial cells mediated the release of ATP (Yang *et al.*, 1994). In addition, thrombin promoted ATP release in endothelial cell was mediated by Ca^{2+} mobilisation and activation of Rho by G_{12} pathway (Ostrom *et al.*, 2000; Koyama *et al.*, 2001). Pathogenic bacteria like *Shigella* and *Escherichia coli* also induce ATP release from gut epithelial cells by cell lysis, thus resulting in diarrhea (Crane *et al.*, 2002; Tran Van Nhieu *et al.*, 2003).

Metabolism of Extracellular Nucleotides:

Ecto-nucleotidases rapidly metabolise nucleotides leading to initiation or potentiation or termination of their signalling pathways (Figure 1.7). Extracellular ecto-nucleotidases are expressed in most cell types. Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase /phosphodiesterase (E-NPP), ecto-5'-nucleotidase, alkaline phosphatase and adenylate kinase are the predominant ectoenzymes. For detailed reviews refer to Abbracchio *et al.*, 2006; Goding *et al.*, 2003 and Zimmermann, 2000.

1.2.2.4 Therapeutic significance of P2Y receptors:

P2Y receptors can be therapeutic targets for treatment of diseases. Indeed, INS-365, a $P2Y_2$ and $P2Y_4$ agonist is being tested for the treatment of dry eye disease. Activation of these receptors causes a significant increase in tear fluid secretion in normal rabbit model (Murakami *et al.*, 2000). Another stable $P2Y_2$ agonist INS-3717 increases subretinal fluid reabsorption relative to controls in a rat model (Maminishkis *et al.*, 2000). AR-C69931MX, an antagonist for $P2Y_{12}$ receptor found on platelets shows potent anti-thrombotic effects. This compound is now subjected to phase-II clinical trials. The widely used clopidogrel acts by inactivating the $P2Y_{12}$ receptors on platelets (Savi *et al.*, 2001). A study on aerosolized INS-365 for chronic obstructive pulmonary disease (COPD) showed enhanced mucociliary clearance compared to placebo (Kellerman, 2002). As P2Y receptors are found throughout the body, the knowledge gained on their functional pathways may be of use for the treatment of a wide range of human diseases (Burnstock and Knight, 2004).

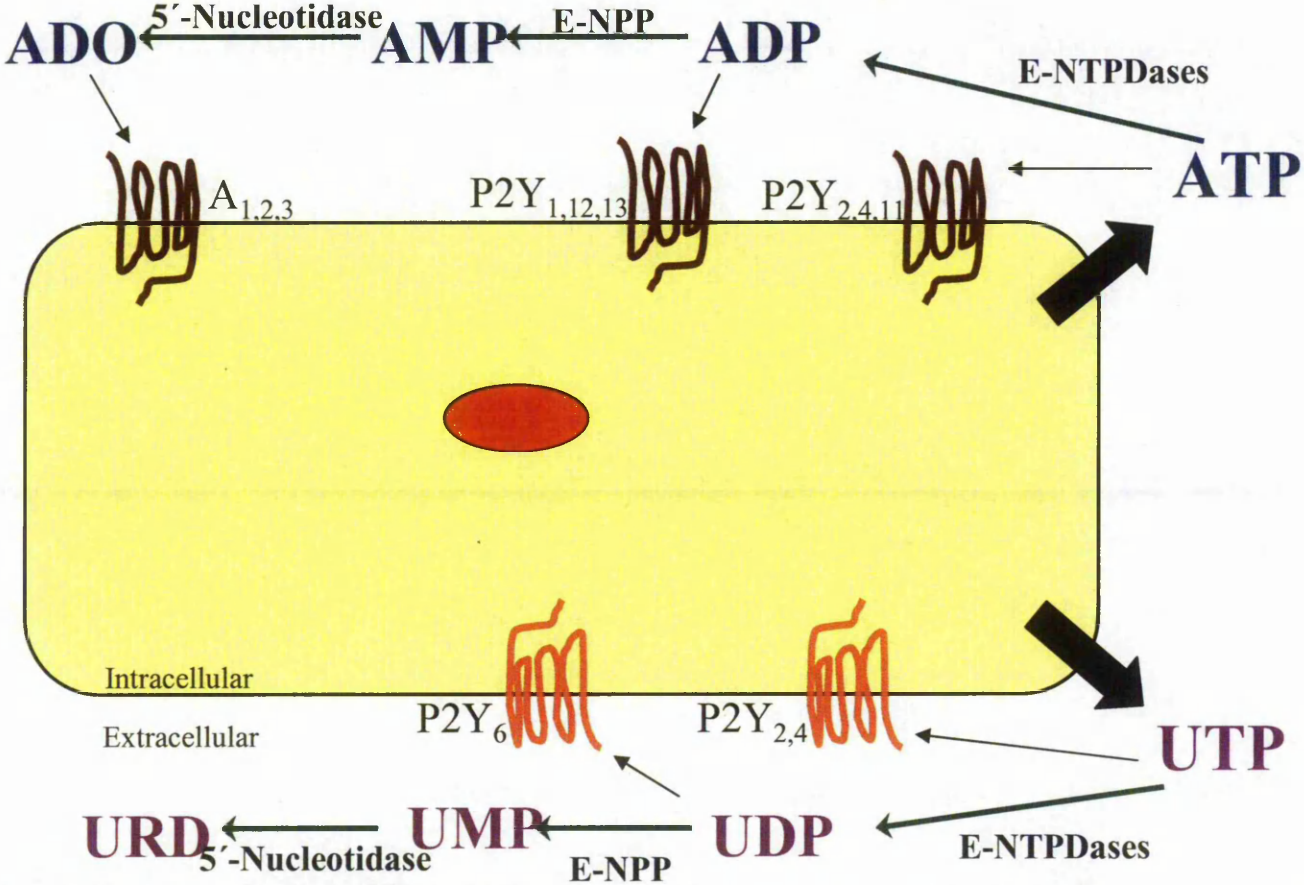

Figure1.7: Extracellular nucleotide metabolism. Several cell types are known to secrete extracellular nucleotides. The released ATP and UTP are subjected to metabolism by ecto-nucleotidases. The ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) can hydrolyse both triphosphates and diphosphates. Furthermore, ecto-nucleotide phosphophosphates (E-NPP) convert the diphosphate into monophosphate or can directly hydrolyse triphosphates into monophosphate. 5′-nucleotidase hydrolyses the monophosphates into the corresponding nucleotides. The metabolites of nucleoside triphosphates can stimulate different subtypes of P2Y and adenosine receptors leading to activation of different signalling pathways. ADO – adenosine, URD – uridine.

1.3 Cardiac Fibroblasts

The heart constitutes of cardiac myocytes and non-myocytes including fibroblasts, vascular smooth muscle cells and endothelial cells (Baudino *et al.,* 2006; Camelliti *et al.*, 2005; Brown *et al.*, 2005). Fibroblasts are the predominant cell type (two-thirds) in the cardiac tissue and play a vital role in wound healing, hypertrophy and fibrosis. Cardiac fibroblasts perform three important functions: (i) synthesis of extracellular matrix (ECM) proteins, (ii) synthesis and release of hormones (e.g.: angiotensin-II) which maintain the ECM, (iii) create a stress tolerant connecting network. Components of ECM are involved in the regulation of events related to cardiac development, maintenance of normal adult phenotype and response to physiological signals associated with onset of disease (Shalini *et al.,* 1998; Weber *et al.,* 1991). Several studies have been carried out on the organisation and function of the connective tissue network, but little is known about the cells that regulate this network. Research into cardiac hypertrophy heart revealed that fibroblasts are involved in the deposition of collagen and maintenance of cardiac function. Recently, the organization of fibroblasts in the heart was characterized by using the specific marker, the discoidin domain receptor 2 (DDR2), a collagen-specific receptor tyrosine kinase (Goldsmith *et al.*, 2004). Confocal and high voltage transmission electron microscopy revealed that fibroblasts interact with each other as well as with cardiac myocytes with the help of their long filopodia (Goldsmith *et al.*, 2004). During cardiovascular diseases, fibroblasts play an important role in remodelling of myocardium, resulting in hypertrophy of cardiomyocytes, migration and proliferation of fibroblasts and excessive deposition of ECM (Camelliti *et al.*, 2005; Brown *et al.*, 2005a). All these events induce stiffening of the myocardium which is an important patho-physiological feature of cardiac dysfunction (Sun and Weber, 2000).

Cardiac fibroblasts are not only involved in structure and biochemical aspects of the heart, but also contribute to the cardiac electrophysiology (Camelliti *et al.*, 2005; Brown *et al.*, 2005a). Fibroblasts can disrupt the electrical impulses or reduce the electrical conductance between myocytes by interstitial fibrosis and collagen accumulation resulting in cardiac arrhythmogenesis (Spach and Boineau, 1997). In addition, fibroblasts are efficient mechano-electrical transducers by forming functional gap-junctions between fibroblasts and myocytes. These junctions support synchronised electrophysiological activity in distant myocytes inter-linked by fibroblasts (Gaudesius *et al.,* 2003).

1.3.1 Cardiac Fibroblasts and Purinoceptors

Extracellular nucleotides, mainly ATP mediate a variety of physiological responses in cardiovascular system including dilation, vasoconstriction and platelet aggregation (Vassort, 2001). Using reverse transcriptase-polymerase chain reaction (RT-PCR) and northern analysis, four subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$) were identified in neonatal and adult rat hearts with the $P2Y_6$ being the most abundant ($P2Y_6 > P2Y_1 > P2Y_2 = P2Y_4$; Hou *et al.,* 1999; Zheng *et al.,* 1998; Webb *et al.*, 1996). In rat neonatal myocytes, $P2Y_1$ is expressed in higher levels than $P2Y_2$, $P2Y_4$ and $P2Y_6$. In neonatal rat cardiac fibroblasts, $P2Y_1$ and $P2Y_6$ are expressed at higher levels than $P2Y_2$ and $P2Y_4$ (Webb *et al.*, 1998). $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors are expressed in human heart (Hou *et al.,* 1999; Wihlborg *et al.,* 2006). Interestingly, the mRNA levels for the $P2Y_2$ receptor are up-regulated in patients with congestive heart failure (CHF),–indicating a possible pathological role for this receptor (Hou *et al.,* 1999).

Evidence gathered over the last decade shows that extracellular ATP in the heart may come from a variety of sources. Platelet degranulation, cell lysis, and secretion from sympathetic nerve terminals may contribute to the local accumulation of extracellular ATP (Ingerman *et al.,* 1979; Gordon, 1986). ATP is also released from cardiomyocytes during hypoxia (Forrester and Williams, 1977). Recent studies have shown that cultured neonatal rat cardiac fibroblasts responded to extracellular ATP through $P2Y_1$ and $P2Y_2$ receptors by inducing the expression of the immediate early gene c-*fos* (Zheng *et al.,* 1998). This increase in c-*fos* expression is mediated by PKC but not by the ATP induced increase in intracellular calcium (through G_q pathway). Zheng *et al.,* (1998) indicated that ATP analogues also enhance c-*fos* mRNA through the $P2Y_2$ receptor and inhibit [^{3}H]-thymidine incorporation into DNA by $P2Y_1$ receptor. As fibroblasts perform vital functions (as mentioned above), extracellular ATP acting through P2Y receptors may play an important role in maintaining and remodelling the extracellular matrix during diseased states. Studies carried out by Dubey *et al.,* (2001) strongly support the hypothesis that adenosine causes inhibition of cardiac fibroblast growth by activating adenosine A_{2B} receptor coupled to inhibition of MAP kinase activity. Thus, A_{2B} receptors may play a pivotal role in regulating cardiac remodelling associated with fibroblast proliferation.

Stimulation of $P2Y_2$ and β_2-adrenergic receptors in cardiac fibroblasts potentiates the cAMP production over that of β_2-adrenergic activation, proposing an interaction

between P2Y and β_2-adrenergic receptors – a cross talk between the G_q/G_s. This interaction is unidirectional because the inositol production was not increased by β_2-adrenergic agonists (Meszaros *et al.,* 2000). The interaction between G_q/G_s pathways may involve $\beta\gamma$ subunits released when the G_q pathway is stimulated (Selbie *et al.*, 1998).

1.3.2 Regulation of cardiac fibroblast function by GPCRs

Cardiac fibroblasts also express other GPCRs such as adenosine A_{2B}, β_2-adrenergic, angiotensin II (ANG-II), B_2, and 5-HT_{2B} receptors (Tang and Insel, 2004; Ostrom *et al.,* 2003; Meszaros *et al.,* 2000; Kim *et al.,* 1999). These receptors can modulate the function of cardiac fibroblasts by altering the balance between the synthesis and degradation of extracellular matrix leading to abnormal accumulation of ECM causing cardiac fibrosis (Ostrom *et al.,* 2003). The GPCRs coupled to G_q protein have been implicated in pathogenesis of cardiac hypertrophy (Adams *et al.*, 1998; Ramirez *et al.,* 1997). On the other hand, G_s coupled receptors inhibit collagen deposition by fibroblasts (Dubey *et al.,* 1998).

Ostrom *et al.,* (2003) characterised a cross talk between G_q (ANG-II) and G_s (β-adrenergic receptor) signalling pathways and its impact on collagen production in cardiac fibroblasts. ANG-II stimulated cell proliferation, collagen synthesis and potentiated cAMP accumulation stimulated by the β-adrenergic receptor (Ostrom *et al.,* 2003). ANG-II potentiated cAMP formation via Ca^{2+}-dependent activation of AC3, which in turn attenuated collagen synthesis (Ostrom *et al.,* 2003). ANG-II mediates fibroblast proliferation, ECM accumulation in rat cardiac fibroblasts via the angiotensin I receptor (AT_1; Villarreal *et al.,* 1993). Indeed, blocking the AT_1 receptor in human cardiac fibroblasts inhibited the fibrotic properties of ANG-II like activation of MAPK, DNA synthesis, stimulation of transforming growth factor-β1 (TGF-β1) and adhesion of fibroblasts to collagen I and III (Kawano *et al.,* 2000). In neonatal rat cardiac fibroblasts, pro-inflammatory cytokines like interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) up-regulated the expression of the AT_1 receptor through .NF-κB activation (Cowling *et al.,* 2002; Gurantz *et al.,* 2005). These studies indicate that AT_1 receptor plays a potential role during heart failure and ventricular remodelling in post-myocardial infarction (MI).

Rat cardiac fibroblasts, express mRNA for all four subtypes of adenosine receptor – A_1, A_{2A}, A_{2B} and A_3, however fibroblasts do not express functional A_1 and A_3 receptors

(Chen *et al.,* 2004). Adenosine is released during ischaemia or ischaemia/reperfusion and exerts its cardioprotective properties on heart cells (Mubagwa *et al.,* 2001, Germack and Dickenson 2005). Chen and associates (2004) provided evidence that stimulation of A_{2B} receptors leads to inhibition of cardiac fibroblast proliferation, protein and collagen synthesis. Grden *et al.,* (2006) showed that mRNA expression levels of different adenosine receptor subtypes altered during diabetic conditions. Cardiac fibroblasts cultured in elevated levels of glucose up-regulated the expression of A_1, A_{2A} receptors, down-regulated the expression of A_3 and had no effect of A_{2B} receptor levels Grden *et al.,* 2006). Alternately, fibroblasts exposed to insulin decreased the expression of A_1, A_{2A} receptors and had no effect on A_{2B} and A_3 receptor expression (Grden *et al.,* 2006).

5-hydroxytryptamine (5-HT) and isoproterenol increased the production of interleukin-6 (IL-6), IL-1β and TNF-α through the 5-HT_{2B} receptor (Callebert *el al.,* 2004). Release of these cytokines in the heart causes hypertrophy which was prevented by the 5-HT_{2B} receptor antagonists SB206553 and SB215505 (Callebert *el al.,* 2004). Also 5-HT_{2B} receptor-knockout mice did not develop cardiac hypertrophy when exposed to isoproterenol and 5-HT, confirming the role of the serotonergic 5-HT_{2B} receptor (Callebert *el al.,* 2004).

Bradykinin, a nonapeptide is involved in the regulation of cardiac fibroblast function (Innis *et al.*, 1981; Nolly *et al.*, 1994; Rhaleb *et al.*, 1992; Kim *et al.,* 1999). Out of the four identified subtypes of bradykinin receptor (B_1, B_2, B_3 and B_4), only the B_2 subtype is present in cardiac tissue (Minshall *et al.,* 1995). Fibroblasts exposed to bradykinin showed down-regulation of mRNA levels for fibronectin and collagen type I and type III (Kim *et al.,* 1999).

Urotensin II (UII), a vasoconstrictor peptide and its receptor were identified in the heart (Matsushita *et al.,* 2001). UII peptide and its receptor expression were increased in a rat model of MI (Tzanidis *et al.,* 2003). In addition, neonatal rat cardiac fibroblasts stimulated with UII increased the levels of mRNA transcripts for procollagen I, III and fibronectin in correlation with increases in collagen synthesis (Tzanidis *et al.,* 2003).

Adrenomedullin is a 52-amino acid peptide which is released by myocytes and non-myocytes (mainly fibroblasts) stimulates cAMP production in fibroblasts and also increases collagen synthesis (Fujisaki *et al.,* 1995; Kitamura *et al.,* 1993; Nishikimi *et al.,* 1998).

1.4 Heart failure and Cytokines

Heart failure is the leading cause of death in the developed world. Coronary artery disease, pressure-volume overload, infectious myocarditis, alcohol abuse, genetic abnormalities result in diminished contractile performance and pathophysiological remodelling leading to heart failure. The hypertrophy of cardiac myocytes and the alteration in Ca^{2+} movements result in loss of myocardial contractility. In addition, hyperplasia of cardiac fibroblasts and altered secretion of extracellular matrix (ECM) leads to myocardial remodelling. The heart initiates a sequence of inflammatory events and wound healing processes in response to myocardial injury (Ertl and Frantz, 2005; Brown *et al.,* 2005a; 2005b; Nian *et al.,* 2004; Manabe *et al.,* 2002; Frangogiannis *et al.,* 2002). Initial steps of wound healing are dominated by blood cells like platelets, neutrophils, macrophages and lymphocytes (Ertl and Frantz, 2005; Nian *et al.,* 2004; Manabe *et al.,* 2002). However, the later phase of wound healing is dictated by cardiac fibroblasts, to repair and rebuild the myocardial architecture (Brown *et al.,* 2005a; 2005b). These events are synchronized by release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and by the secretion of fibrotic factors like ANG-II, TGF-β and aldosterone (Brown *et al.,* 2005a; 2005b).

The mRNA levels of IL-1, IL-6 and TNF-α are up-regulated within the infarction area and the surrounding myocardium (Irwin *et al.,* 1999; Deten *et al.,* 2002). This robust increase in pro-inflammatory cytokine levels may return to normal levels if the infarction area is small. There can be a second wave of cytokine up-regulation if the infarction zone is large, or if the host local immune system is still active. The following are some of the factors inducing cytokine production in the heart:

Mechanical Stress:

TNF-α and IL-6 are released at the core of ischaemic injury but their levels are usually maximal at the border areas (Irwin *et al.,* 1999; Gwechenberger *et al.,* 1999). Ischaemic stress along with myocardial mechanical stress is a potent regulator of cytokine production. Kapadia and associates (1997) in a feline model have documented that haemodynamic pressure induced *de novo* TNF-α mRNA expression and production within 30 and 60 minutes, respectively. Mechanical stimulus via integrins, cytoskeleton and sacrolemmal protein activates mitogen-activated protein kinase (MAPK), JAK-signal transducer and activator of transcription (STAT) pathways which are essential to induce cytokine genes like, IL-6 and TNF-α (Nian *et al.,* 2004).

Intrinsic response to injury:

Ischaemic conditions induce the expression of the C/AATT-enhancer binding protein-beta and enhance STAT3 activity both of which are linked with the increase in gp130 receptor expression and IL-6 production (Deten *et al.,* 2002). These conditions are involved in the formation of cardiac fibrosis and hypertrophy (Deten *et al.,* 2002). Peroxisome proliferator-activated receptor (PPAR-γ) is a stress-induced inflammatory regulator which controls cell proliferation and host immune responses including cytokine expression. Indeed, pre-treatment with pioglitazone, a PPAR-γ agonist improved the left ventricular remodelling and function and attenuated the expression of inflammatory cytokines (TNF-α, TGF-β) and chemokines (monocyte chemoattractant protein-1, MCP-1; Shiomi *et al.,* 2002).

Reactive oxygen Species:

Reactive oxygen species (ROS) can also induce the production of cytokines; however cytokines can also mediate ROS release (Meldrum *et al.,* 1998; Nakamura *et al.,* 1998). Infusion of rat heart with hydrogen peroxide mediated the production of TNF-α and caused cardiomyocyte death and myocardial dysfunction via activation of the p38 MAP kinase pathway (Meldrum *et al.,* 1998). Conversely, TNF-α induced hypertrophy of neonatal rat cardiomyocytes was inhibited by anti-oxidants like butylated hydroxyanisole (BHA), vitamin E, and catalase, indicating the involvement of ROS (Nakamura *et al.,* 1998).

Self-amplifying pathways by cytokines:

Cytokines have the capacity to self-amplify their production via a positive feedback mechanism involving NF-κB activation. Indeed, Irwin *et al.,* (1999) noted that TNF-α localised in the infarct and peri-infarct areas can induce TNF-α up-regulation in the surrounding normal areas. These observations were completely blocked by the infusion of Etanercept, a TNF-α antagonist (Nakamura *et al.,* 2003).

The localised increase in cytokines can also be induced by the direct recruitment of inflammatory cells like monocytes, macrophages, neutrophils and mast cells (Nian *et al.,* 2004). Kumar *et al.,* (1997) and Kakio *et al.,* (2000) noted that MCP-1 expression was increased in both canine and rodent ischaemia reperfused hearts, enhancing the transmigration of macrophages to the infarct zone.

1.4.1 Effect of cytokines on cardiac cell survival and apoptosis

The pro-inflammatory cytokines are consistently released in response to myocardial injury, which is analogous to the innate immune system. There is a growing body of evidence that short-term activation of pro-inflammatory cytokines is beneficial whereas long-term activation is detrimental. Indeed, pre-treatment with IL-1 or TNF-α protected the heart from ischaemia reperfusion injury and increased the recovery of left ventricular pressure compared to the control hearts (Mann, 2003). The cardioprotective properties of IL-1 and TNF-α are mediated by up-regulation of manganese superoxide dismutase (MnSOD; a free radical scavenger) and heat shock protein expression (Wong *et al.,* 1988; Nakano *et al.,* 1996). In addition, IL-1β and TNF-α can also activate NF-κB, Bcl-2, Bfl-1, Bcl-xL and cellular inhibitors of apoptosis 1 and 2. TNF-α induces its effects via TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) (Mann, 2003; Nian *et al.,* 2004). Conversely, TNF-R1 belongs to the 'death receptor' family of cell surface receptors which when activated stimulates the caspase-dependent apoptotic pathway and ROS leading to cell death (Nian *et al.,* 2004; Pabhu, 2004).

IL-6 family of cytokines, interleukin-6 (IL-6), cardiotrophin-1 (CT-1), leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and IL-11, also possess cytoprotective properties (Mann, 2003). The effects of IL-6-related cytokines are mediated by the gp130 receptor (Giese *et al.,* 2005). LIF protected neonatal rat cardiomyocytes from apoptosis by inducing cytoprotective genes, like Bcl-x via STAT signalling pathway (Fujio *et al.,* 1997). In addition, LIF decreases the lactate dehydrogenous levels in reperfused rabbit hearts by activating the MnSOD via IL-1 and TNF-α release (Nelson *et al.,* 1995).

The bone marrow-derived cytokines like erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) possess direct cardioprotective effects. EPO and G-CSF improved haemodynamics and ventricular remodelling and function in reperfused hearts, respectively (Nian *et al.,* 2004).

1.4.2 Effect of cytokines on cardiac myocyte contractility:

Pro-inflammatory cytokines regulate different signalling pathways that can modulate the contractile function of myocytes. The contraction of myocytes is mediated by the influx of Ca^{2+} through L-type Ca^{2+} channels. IL-2 reduced the Ca^{2+} transient levels and increased the Ca^{2+} levels in rat myocytes (Cao *et al.,* 2003). The decreased in intracellular Ca^{2+} transient probably reflects the reduced SR Ca^{2+} release or Ca^{2+} influx

(Cao *et al.,* 2003). Alternatively, myocytes treated with IL-1β showed a decrease in L-type Ca^{2+} current in a PTX-insensitive manner (Schreur *et al.,* 1997). In isolated guinea-pig myocytes IL-6 and TNF-α exerted a negative inotropic action through a NO-dependent pathway and by the activation of sphingomyelinase (Sugishita *et al.,* 1999). These studies reveal that IL-2, IL-6 and TNF-α modulate Ca^{2+} homeostasis and alter excitation-contraction (E-C) coupling.

Nitric oxide (NO) regulates Ca^{2+} transient levels, desensitises myofilaments to Ca^{2+} in myocytes and affected myocardial contraction in a dose-dependent manner (Pradhu, 2004a). These effects of NO are mediated by two pathways, production of cGMP and direct protein nitrosylation of thiol residues (Pradhu, 2004a, b). A number of studies have reported that cytokine-mediated myocardial contractile dysfunction is via NO derived from constitutive NO synthase (cNOS). Finkel and associates (1992) first reported that pro-inflammatory cytokines like IL-2, IL-6 and TNF-α decreased the contractions of papillary muscles in a concentration-dependent manner. Moreover, these negative inotropic effects were blocked by non-specific NOS inhibitor L-NMMA and the presence of L-arginine reversed the L-NMMA affects (Finkel *et al.,* 1992). Similarly, recent studies on IL-1β, IL-2, IL-6, and TNF-α in different mammalian heart preparations such as guinea pig, rabbit and rat and in isolated myocytes further strengthened the negative inotropic role of cNOS-derived NO (Pradhu, 2004a, b). Yokoyama *et al.,* (1993) have reported that in ventricular and isolated feline myocytes TNF-α induced negative inotropic affects and decreased intracellular Ca^{2+} transient levels in a NO and cGMP-independent manner. Interestingly, blocking NO by L-NMMA did not inhibit the inotropic affects, but TNF-α antibodies inhibited the inotropic effects, indicating a direct affect of TNF-α (Yokoyama *et al.,* 1993).

The pro-inflammatory cytokines can also affect the myocardial contractility via sphingomyelinase-dependent signalling, activation of phospholipase A_2 (PLA_2) and arachidonic acid (Pradhu, 2004a, b).

1.4.3 Effect of cytokines on cardiac fibroblast function

In the following sections, the effects of pro-inflammatory cytokines on cardiac fibroblast functions such as proliferation, migration and ECM maintenance are summarised (Table 1.6).

Proliferation:

Fibroblasts play an important role in wound healing and their population is increased at the site of injury by proliferation. However, uncontrolled proliferation leads to the formation of fibrotic tissue. Plamer and associates (1995) first reported the anti-proliferative properties of IL-1β in cardiac fibroblasts. Investigations into the cell cycle revealed that IL-1β interfered with the G1/S interphase by preventing the phosphorylation and post-translational modification of retinoblastoma (Rb) protein, inhibiting the expression and activity of cyclins and cyclin-dependent kinases and increasing the expression of cyclin kinase inhibitor p27/KIP1 (Koudssi *et al.,* 1998). These anti-proliferative properties of IL-1β on cardiac fibroblasts are mediated directly via the IL-1 receptor and did not involve prostaglandins and NO (Koudssi *et al.,* 1998). IL-6, LIF and CT-1 are secreted by cardiac fibroblasts and may act as autocrine factors and affect fibroblast function (Fredj *et al.,* 2005; King *et al.,* 1996; Kuwahara *et al.,* 1999). Wang and associates (2002) noted that murine cardiac fibroblasts treated with LIF for 72 hours resulted in an increase in DNA synthesis which was completed blocked by antibodies against LIF. Similarly, CT-1 stimulated the proliferation of adult rat cardiac fibroblasts (Tsuruda *et al.,* 2002). Norepinephrine (NE) increased the mRNA expression of IL-6 and stimulated the proliferation of rat cardiac fibroblasts (Leicht *et al.,* 2003). Moreover, application of IL-6 antibody inhibited the NE-mediated proliferation to 34%, and completely blocked the exogenous IL-6 induced proliferation (Leicht *et al.,* 2003). This study, suggests that NE induced proliferation of fibroblasts is partly due to the increase in expression of IL-6. TNF-α was primarily identified to exhibit anti-proliferative and cytotoxic properties on tumour cells. However, recent studies have showed that the action of TNF-α on proliferation and differentiation differs widely in different cell types. In a rat model of myocardial infarction, TNF-α expression and proliferating cardiac fibroblasts were detected at the border zone of the infarct region (Jacobs *et al.,* 1999). Indeed, TNF-α induced proliferation of isolated fibroblasts from both the infarct zone and remote myocardium in a dose-dependent manner. In addition, Jacobs *et al.,* (1999) also reported an increase in fibronectin production when cells are treated with TNF-α which correlates with the increase in fibroblast number. This proliferatory affect of TNF-α was blocked in the presence of neutralising TNF-α antibodies (Jacobs *et al.,* 1999).

Migration:

Cell migration is a multi-step process that contributes to wound healing and tissue remodelling (Ridley *et al.,* 2003). Following a cardiac injury, the initial healing process is dominated by infiltration and migration of immune cells and cardiac fibroblasts to the site of injury (Brown *et al.,* 2005a, b). These events are regulated by cytokines and growth factors. Cytokines are well known to initiate the mitogen-activated protein kinases (MAPKs) which are associated with cellular migration (Ridley *et al.,* 2003; Huang *et al.,* 2004; Mitchell *et al.,* 2007).

Mitchell and associates (2007) using modified Boyden chambers noted that IL-1β and TNF-α, but not IL-6, stimulated the migration of rat cardiac fibroblasts. Furthermore, the migration of fibroblasts induced by IL-1β was blocked by MAPK inhibitors (ERK ≥ JNK > p38; Mitchell *et al.,* 2007). Brown *et al.* (2005b) also noted that IL-1β stimulated a robust increase in fibroblast migration whereas INF-γ inhibited the migration of cardiac fibroblasts. In addition, to pro-inflammatory cytokines fibroblast growth factor-2 and TGF-β1 also induce migration of cardiac fibroblasts (Detillieux *et al.,* 2003; Stawowy *et al.,* 2004). However, the fibroblasts isolated from a rat model of myocardial infarction showed decreased migration compared to the sham operated rats (Squires *et al.,* 2005). Cellular migration is the least studied function of cardiac fibroblasts.

Table 1.6: Effect of pro-inflammatory cytokines on cardiac fibroblast function

	IL-1	**IL-6**	**TNF-α**	**IFN-γ**
Proliferation	↓	↑	↓↑	↓
Chemotaxis	↑	n.d.	↑	↓
ECM Production	↓	↑	↓	↓
MMP Expression	↑	↔	↑	↔
TIMP Expression	↓	n.d.	↓	n.d.

↓ - decrease, ↑ - increase, ↔ - no effect, n.d. – no data available. ECM – extracellular matrix; MMP – matrix metalloproteinase, TIMP – tissue inhibitors of matrix metalloproteinase. Adapted and modified from Brown *et al.,* 2005b.

Regulation of extracellular matrix (ECM):

Cardiac fibroblasts are the predominant cell type in heart and their main function is the deposition of extracellular matrix (ECM; Baudino *et al.,* 2006; Camelliti *et al.,* 2005; Brown *et al.,* 2005a). The ECM comprises of collagen (90%; Type-I, III, IV, V, VI), fibronectin, elastin, laminin, proteoglycans and glycosaminoglycans (Bosman and Stamenkovic, 2003; Jugdutt, 2003a). During patho-physiological conditions fibroblasts are activated and therefore secrete excess ECM leading to cardiac fibrosis and left ventricular hypertrophy (Baudino *et al.,* 2006; Brown *et al.,* 2005a).

In isolated rat cardiac fibroblasts, IL-1β and TNF-α decreased collagen synthesis which was associated with an increase in the mRNAs for pro matrix metalloproteinase (MMP)-2 and proMMP-3 and total MMP activity (Siwik *et al.,* 2000). In addition, fibroblasts exposed to IL-1β decreased the expression of major fibrillar procollagen α_1(I), α_2(I), and α_1(III) mRNA and increased the mRNA expression of non-fibrillar procollagen α_1(IV), α_2(IV) and fibronectin (Siwik *et al.,* 2000). Siwik and researchers (2000) reported that the affects of IL-1β on collagen synthesis are not mediated by NO production. In addition, IL-1β and TNF-α were documented to inhibit the expression of tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-1 and TIMP-3 (Li *et al.,* 2000). Like other pro-inflammatory cytokine, IFN-γ also decreased the collagen accumulation in rat cardiac fibroblasts (Grimm *et al.,* 2001).

Osteopontin (OPN) also known as cytokine Eta-1 is a novel inflammatory mediator and promotes collagen synthesis and accumulation in post-myocardial infarction remodelling (Trueblood *et al.,* 2001). OPN is a multifunctional matrix protein that interacts with integrins $\alpha_v\beta1$, $\alpha_v\beta3$ and $\alpha_v\beta5$ and CD44 receptors in addition to fibronectin and collagen (Singh *et al.,* 2007). Xei and associates (2003) have documented that in adult rat cardiac fibroblasts OPN via β3 integrin inhibited the IL-1β stimulated MMP-2 and MMP-9 activity.

All these studies indicate that inhibition of MMP is a novel strategy for the treatment of post-myocardial infarction remodelling. Indeed, selective blocking of MMP in a rabbit model of myocardial infarction inhibited the left ventricular remodelling and increased the neovascularisation at the infarction zone and decreased the scar (Lindsey *et al.,* 2002). This correlates with a previous study where inhibiting elastase reduced inflammation, necrosis and fibrosis without affecting the ventricular function in a model of murine myocarditis (Lee *et al.,* 1998).

1.5 Role of cardiac fibroblasts in ventricular remodelling

The heart's primary function to pump blood mainly depends on cardiac myocytes, whose structural and functional integrity largely depends on cardiac fibroblasts (Jugdutt 2003a, b). 75% of the cells in a healthy heart are nonmyocytes of which 90-95% are fibroblasts, which play a major role in ECM production and deposition (Jugdutt 2003a, b). Hypertension, heart failure and myocardial infarction result in death due to changes in structure, shape and topography of the left ventricle.

1.5.1 Extracellular Matrix (ECM)

ECM acts as scaffolding and supports the physical structure of the cells; regulates their development, migration and proliferation in all kinds of tissues (Trackman, 2005; Bosman and Stamemkovic, 2003). ECM is composed of two main macromolecules namely, (a) fibrous proteins, which provide support (collagen and elastin) or attachment (fibronectin and laminin) and (b) glycosaminoglycans (GAGs) which form proteoglycans with proteins (Bosman and Stamemkovic, 2003). Collagen is the major component of ECM and provides structural support and resistance under stress while elastin promotes flexibility to the cells. Fibronectin attaches fibroblasts and other cell types to the ECM whereas laminin binds the cells to the basement membrane. Proteoglycans mediate the activity of growth factors, proteases and protease inhibitors. GAG and proteoglycans associate with collagen to form polymeric complexes (Trackman, 2005; Jugdutt, 2003a, b; Bosman and Stamemkovic, 2003).

Collagen

Collagen is the most common protein which accounts for 25% of the total protein content in mammals (Jugdutt, 2003b; Brodsky and Shah, 1995). The collagen molecule comprises of glycine, proline and hydroxyproline amino acids which are coiled together to form a central, long, stiff and triple helical structure (Brodsky and Shah, 1995). There are 19 different collagen molecules (collagen type I-XIX) identified so far which are expressed in different combinations in various tissues and are encoded by separate genes (Jugdutt 2003b). Collagen type I is the most common collagen molecule. The secreted collagen molecules polymerises to form different varieties of collagen such as fibrillar collagens (Types I, II, III, V, IX, XII), network-forming collagens (Types IV, VII) and meshwork like type IV or assemble into lattice (Type VIII).

Collagen synthesis involves several enzymatic steps including the synthesis of pro-α chains, hydroxylation of prolines and lysines, glycosylation of hydroxyl serines, formation and secretion of procollagen helixes and conversion of procollagen into fibrils (Jugdutt, 2003b; Brodsky and Shah, 1995; Figure 1.8). The formation of 4-hydroxyproline from proline is catalysed by prolyl-4-hydroxylase and is a vital enzymatic step in collagen biosynthesis. Hydroxyproline amino acid residues are crucial for folding of procollagen chain into triple-stranded helices (Jugdutt, 2003b; Brodsky and Shah, 1995). Table 1.7 summarises the different cytokines, growth factors, hormones which regulate collagen synthesis. Collagen is systematically degraded by two types of extracellular proteolytic enzymes, matrix metalloproteinases (MMPs) and serine proteases. The action of MMPs depends on Ca^{2+} and Zn^{2+} ions. MMPs mainly consist of collagenase, gelatinases and stromelysins which degrade collagen, laminin and fibronectin (Ahmed *et al.,* 2006; Jugdutt, 2003b; Table 1.7).

Collagen and Heart

The structure and function of the heart greatly depends on the fibrillar collagen. The collagen content differs between the left and right ventricle in that the right ventricle has 30% more collagen than the left ventricle (Bonnin *et al.,* 1981; Jugdutt, 2003b). Collagen biosynthesis is much slower than other proteins because the former has a longer half-life (80-120 days), which is ten times greater than the non-collagen protein (Weber *et al.,* 1988). Collagen type I and III are the major fibrillar collagen in the cardiac ECM and account for nearly 85% and 11% of the total myocardial collagen respectively (Brown *et al.,* 2005a). Left ventricular (LV) volume overload causes LV hypertrophy by increasing the size of collagen fibres and not by altering the concentration of collagen (Weber *et al.,* 1988). Furthermore, Mukherjee and Sen (1990) noted that during ventricular remodelling and adaptation to the pathophysiological changes, the ratio of collagen type I and type III are altered. Hearts isolated from spontaneously hypertensive rats (SHR) have the same total collagen concentration as the normotensive hearts however an increase in collagen type III and a decrease in type I/III ratio was observed in SHR (Mukherjee and Sen, 1990). Interestingly, captopril, an angiotensin-converting enzyme (ACE) inhibitor decreased the hypertensive hypertrophy in SHR by normalising the collagen type I/III ratio and decreasing the collagen content (Mukherjee and Sen, 1990). Similarly, in patients suffering from ischaemic cardiomyopathy the collagen type I/III ratio decreased by 35% compared to normal individuals (Mukherjee and Sen, 1991).

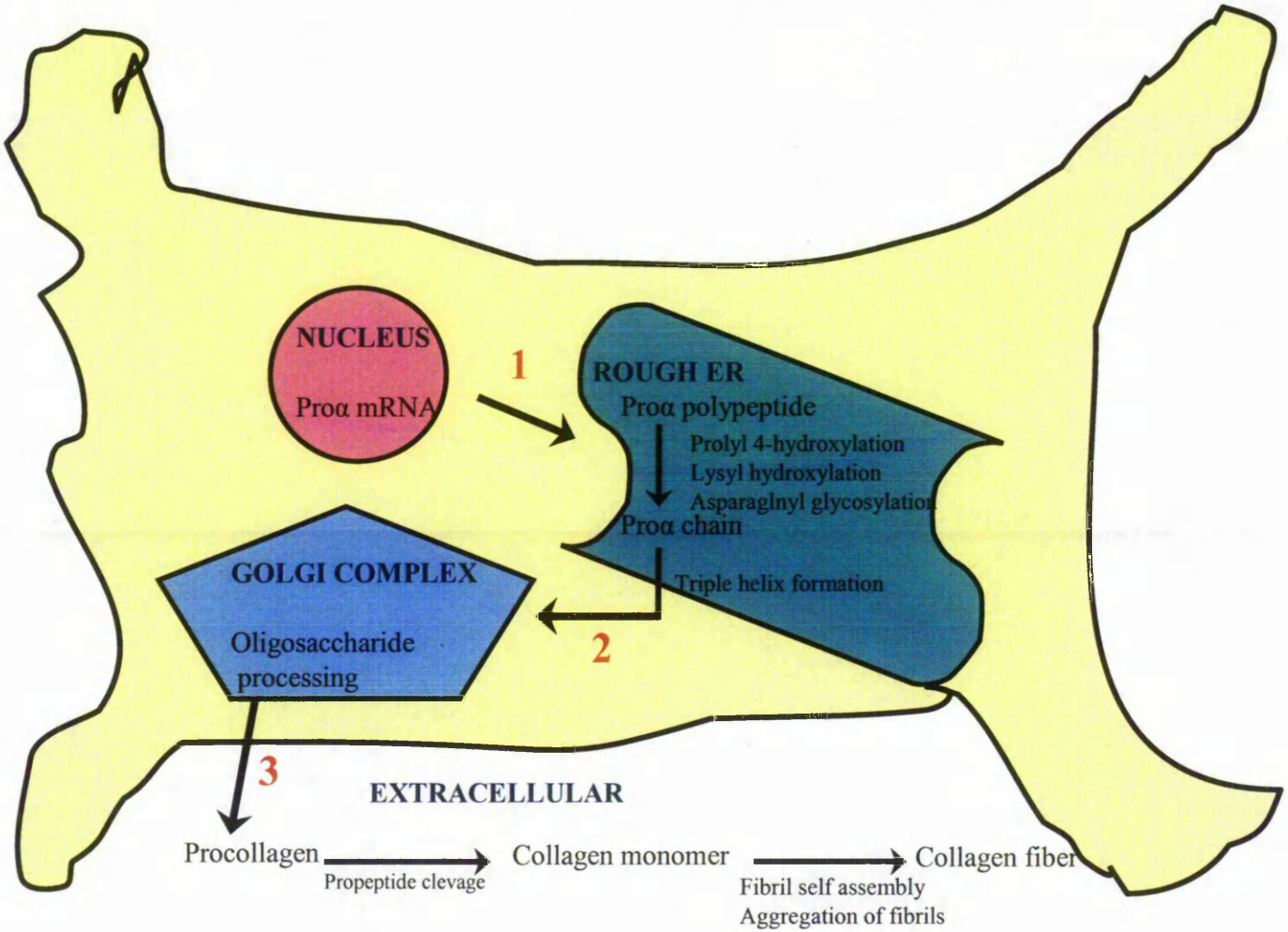

Figure 1.8: Schematic representation of collagen biosynthesis. Collagen synthesis involves several enzymatic steps like, synthesis of pro-α chains, hydroxylation of prolines and lysines, glycosylation of hydroxyl serines, formation and secretion of procollagen helixes and conversion of procollagen into fibrils. In the extracellular space the procollagen triple helixes are aggregated and rearranged to form collagen fibrils. Cytokines, hormone and growth factors can modulate the collagen synthesis (Table 1.7).

Table 1.7: Factors influencing collagen synthesis

Factor	Collagen Synthesis
Hormones	
Angiotensin-II	↑
Aldosterone	↑
Bradykinin	↓
Catecholamines	↓
Progesterone	↑
Glucocorticoids	↓
Growth hormone	↑
Enzymes	
Angiotensin-converting enzyme	↑
Prolyl 4-hydroxylase	↑
Matrix metalloproteinase	↓
Tissue inhibitor metalloproteinase	↑
Cytokines	
Interleukin-1	↑↓
Tumour necrosis factor-α	↓
Growth Factors	
Transforming growth factor-β	↑
Basic fibroblast growth factor	↑
Insulin-like growth factor	↑
Epidermal growth factor	↑
Platelet derived growth factor	↑

↑ - increase and ↓ - decrease. Adapted and modified from Jugdutt 2003b.

It is notable that collagen type I content did not change but the type III collagen increased by 58% over the normal level (Mukherjee and Sen, 1991). The concentration of type III collagen was significantly lowered in cardiomyopathy patients receiving captopril (Mukherjee and Sen, 1991). These studies along with others suggest that increased deposition of collagen type III may contribute to heart failure (Marijianowski *et al.,* 1995 and Brooks *et al.,* 2003).

Cardiac fibrosis can lead to the following consequences: a) systolic dysfunction and LV hypertrophy; b) disruptions in myocyte electrical conductance leading to arrhythmogenesis and c) perivascular fibrosis around the coronary arteries causing a decrease in myocyte oxygen availability and potential ischaemic conditions (Manabe *et al.,* 2002; Jugdutt, 2003a; Brown *et al.,* 2005a; Berk *et al.,* 2007).

MMPs and Heart

The different types of MMPs are identified in fibroblasts, endothelial cells and polymorphonuclear cells (Table 1.8). In the heart, MMPs are co-expressed with TIMP (Tyagi, 1997). Cytokines, growth factors and hormones regulate the synthesis and secretion of pro-MMPs and TIMPs. MMP-1, MMP-8 and MMP-13 initiate the degradation of ECM by disrupting the α-chain in collagen type I and type II molecules and MMP-2 and MMP-9 further cleavage the collagen fragments (Ahmed *et al.,* 2006; Nagase *et al.,* 2006; Table 1.8). The proteolytic activity of MMPs is controlled at a) the transcriptional level, TNF-α activates MMP mRNA; b) the activation level, MMP is secreted in a latent zymogen form which is activated by proteolytic reactions; c) inhibition by TIMP (Tyagi, 1997; Li *et al.,* 1998; Spinale *et al.,* 2000; Feldman *et al.,* 2001; Jugdutt, 2003b; Nagase *et al.,* 2006). Spinal and researchers (2000) have documented that MMP-9 levels are increased in both ischaemic and idiopathic dilated cardiomyopathy (DCM) whereas levels of MMP-1 decreased in both types of DCM. In addition, a local MMP induction/activation system is identified in isolated human myocytes which can regulate ECM (Spinale *et al.,* 2000). Iwanaga and associates showed that in Dahl salt-sensitive hypertensive rats the TIMP-2, TIMP-4 and MMP-2 activity and expression levels increased with the development of LVH. The role of MMP-2 in ventricular remodelling was further strengthened by using of MMP-2 knockout mice. Matsusaka *et al* (2006) studied the progress of cardiac hypertrophy induced by chronic pressure overload in these knockout mice.

Table 1.8: Main components of matrix metalloproteinases and their substrates

Type of Enzyme	Matrix metalloproteinases (MMPs)	Substrates
Collagenases	MMP-1	Collagen type – I, II, III, VII and X; gelatins, proteoglycans
	MMP-8	Collagen type – I, II, III
	MMP-13	Collagen type – I, II, III
Gelatinases	MMP-2	Gelatin type I, collagen type – I, II, III, IV, V and VII; fibronectin, elastin, laminin
	MMP-9	Gelatin type I and V; collagen type – I, II, III, IV and V; fibronectin, elastin and laminin
Stromelysins	MMP-3	Gelatin type I, III, IV and V; collagen type III, IV, IX and X; proteoglycans, fibronectin and laminin
	MMP-10	Collagen type IV, laminin, fibronectin

Adapted and modified from Jugdutt 2003a.

These experiments revealed that MMP-2 deficient mice had significantly lower LV weight/body weight, LV end-diastolic pressure, interstitial fibrosis and myocyte hypertrophy when compared to the wild type mice (Matsusaka *et al.,* 2006). Furthermore, the zymographic activity of MMP-2 was increased by 2.4 fold in the pressure overload induced wild type mice (Matsusaka *et al.,* 2006). Similarly, mice deficient in MMP-9 had minimal cardiac fibrosis and LV dilation, which correlates with the observation that plasma MMP-9 levels increased with the deterioration of LV function (Heymans *et al.,* 2005; Shen *et al.,* 2006).

The activity of MMPs is regulated by general protease inhibitors and TIMPs (Weber *et al.,* 1994; Brown et al., 2005; Berk *et al.,* 2007). α2-macroglobulin, a protease inhibitor irreversibly blocks the MMPs activity whereas TIMPs reversibly blocks specific MMPs. Vertebrates express four different types of TIMPs, TIMP-2 and TIMP-4 are commonly expressed whereas TIMP-1 and TIMP-3 expression is induced at transcriptional level by growth factors and cytokines (Kassiri and Khokha, 2005). All these studies highlight the fact that disruption of balance between ECM biosynthesis and MMPs-TIMPs leads to the development of cardiac fibrosis and hypertrophy.

1.6 Renin-Angiotensin System (RAS)

Angiotensin II (ANG-II) is an octapeptide which exerts haemodynamic and renal effects and plays an important role in the development of cardiovascular disorders like hypertension, hypertrophy and renal diseases (Rosenkranz, 2004; Levy, 2005; Mehta and Griendling, 2007). It is produced by the circulating plasma renin-angiotensin system (RAS) and locally through tissue RAS. ANG-II is synthesised by the enzymatic cleavage of hepatic-derived angiotensinogen to angiotensin I (ANG-I) by renin followed by subsequent conversion of ANG-I to ANG-II by angiotensin converting enzyme (ACE; Figure 1.9). Angiotensinogen can be converted into ANG-I by non-renin enzymes such as tonin or cathepsin and ANG-II can be formed by enzymes like trypsin, cathepsin and chymase (Urata *et al.,* 1990; Levy, 2005; Kramkowski *et al.,* 2006; Figure 1.9). Crackower and researchers (2002) have shown that ANG (1-7) is produced by the enzymatic cleavage of ANG-I or ANG-II by ACE2. ACE is the main metabolizing enzyme for ANG (1-7), therefore ACE inhibitors cause an increase in the levels of ANG (1-7) and its precursor ANG-I (Santos *et al.,* 2000). ANG (1–7) has contradictory pharmacological and physiological actions to ANG-II such as vasodilation, improvement of cardiac function, and inhibition of cell proliferation (Igase *et al.,* 2005; Dantas and Sandberg, 2005; Mehta and Griendling, 2007).

Most of the affects of ANG-II are mediated by type I (AT_1) and type II (AT_2) receptors. Both AT_1 and AT_2 receptors are cloned and characterised in rodents and humans (Griendling *et al.,* 1994; Mehta and Griendling, 2007). Other subtypes of ANG-II receptors have also been identified such as AT_3 and AT_4 receptors; however the physiological role and pharmacology of these receptors are still unclear (Unger *et al.,* 1996; Stanton, 2003; Mehta and Griendling, 2007). AT_1 receptor is widely distributed in various organs such as liver, adrenals, brain, heart, lungs, kidneys and blood vessels.

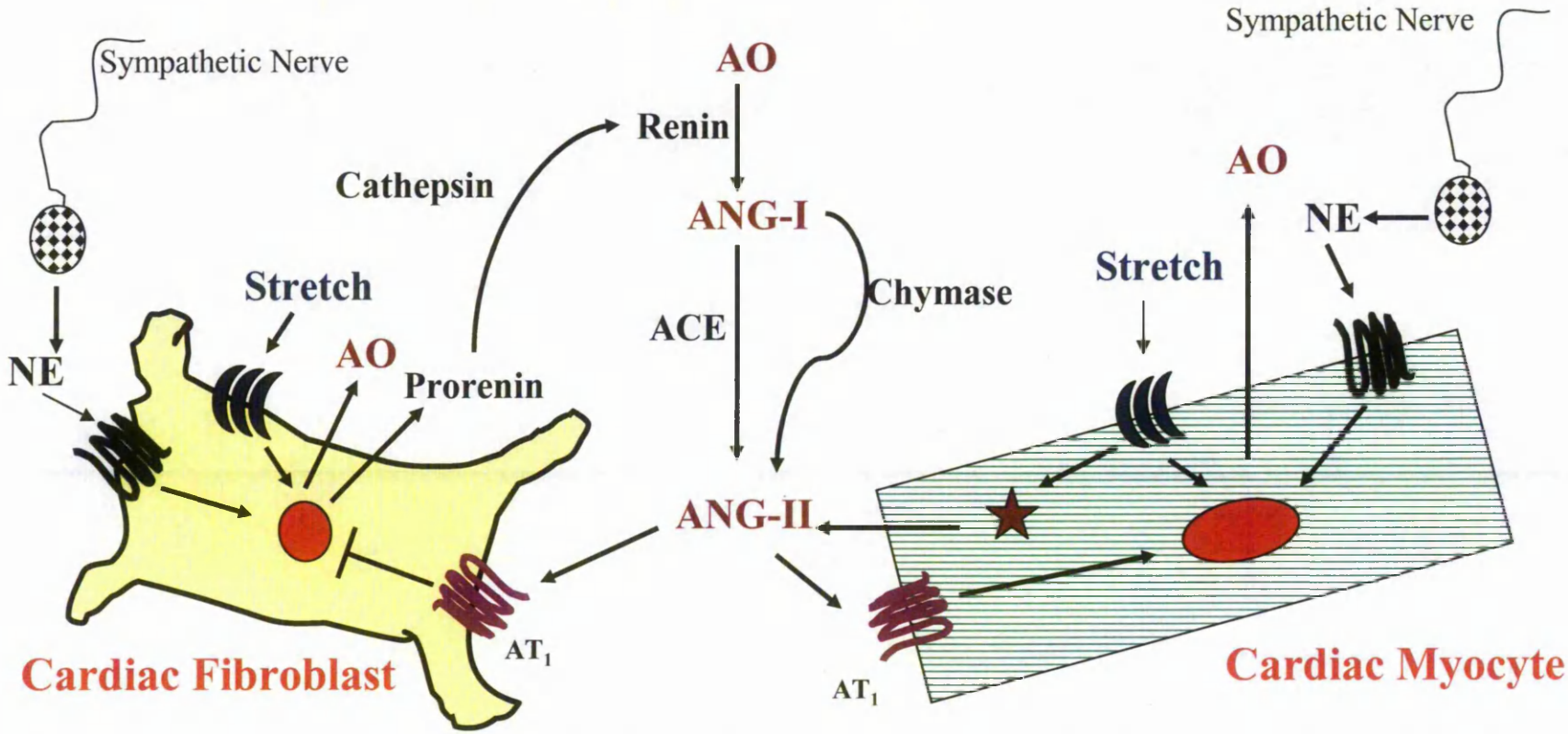

Figure 1.9: Local cardiac regulation of angiostensin-II (ANG-II) production. Mechanical stretch stimulates expression and synthesis of angiotensinogen (AO) in cardiac fibroblasts and cardiac myocytes. Activation of β-adrenergic receptors by norepinephrine (NE) also stimulates the production of AO in fibroblasts and myocytes and renin from fibroblasts. ANG-II is synthesised by the enzymatic cleavage of AO to angiotensin I (ANG-I) by renin followed by subsequent conversion of ANG-I to ANG-II by angiotensin converting enzyme (ACE). AO can be converted into ANG-I by non-renin enzymes such as cathepsin and ANG-II can be formed by enzymes like chymase. In cardiac myocytes ANG-II via AT_1 receptors increases the AO gene expression. On the other hand, in fibroblasts ANG-II induces feedback inhibition of AO and renin production. Adapted and modified from Dostal and Baker, 1999.

ANG-II mediates vascoconstriction, increased cardiac contractility, renal tubular sodium reabsorption, proliferation, oxidative stress and cardiac and vascular hypertrophy via AT_1 (Nickenig *et al.,* 2006; Crowley *et al.,* 2007; Mehta and Griendling, 2007). AT_1 is coupled to $G_{q/11}$ and $G_{12/13}$ proteins and when activated by ANG-II stimulates down-stream targets like PLC, PLA_2, PLD (Ushio-Fukai *et al.,* 1998; Mehta and Griendling, 2007; Oro *et al.,* 2007). In addition, ANG-II via the AT_1 receptor activates and interacts with various receptor tyrosine kinases such as EGFR, PDGF and insulin receptor; non-receptor tyrosine kinases involving the c-Src kinase family, focal adhesion kinase (FAK) and Janus kinase (JAK); and activates NAD(P)H oxidases and ROS (Hunyady and Catt, 2006; Oro *et al.,* 2007; Mehta and Griendling, 2007).

1.6.1 Role of Angiotensin-II in cardiac diseases

ANG-II induces phenotypic and morphological changes in cells, alteration in gene expression in virtually in all cardiac cell types such as endothelial cells, smooth muscle cells, cardiomyocyte, fibroblasts and monocytes and macrophages (Mehta and Griendling, 2007; Figure 1.9). Rajagopalan *et al* (1996) and Schena *et al.,* 1999 noted that in human endothelial cells, ANG-II potentiated the formation of NO and mRNA levels of eNOS subsequently leading to oxidative stress and endothelial dysfunction. In addition, ANG-II enhances the formation of atherosclerotic lesions by inducing the expression of cell adhesion molecules like VCAM-1 and ICAM-1 and LDL receptor in vascular smooth muscle cells and endothelial cells (Pueyo *et al.,* 2000; Li *et al.,* 1999). Several lines of evidence revealed the presence of ANG-I and ANG-II in atria and/or ventricles of rat, dog and pig (Dostal and Baker, 1999). Moreover, identification of angiotensins in isolated cardiomyocytes, fibroblasts and endothelial cells indicated that these cardiac cells are involved in the local production of ANG-II (Dostal *et al.,* 1992; Dostal and Baker, 1999; Figure 1.9). Serneri *et al.,* (1996) using ^{125}I-ANG-I and ^{125}I-ANG-II and enzyme kinetics documented that healthy human cardiac tissues continuously synthesised and produced ANG-II. Furthermore, the ANG-I and ANG-II protein levels and mRNA levels of angiostensiogen and ACE were increased in patients suffering from heart failure which strengthened the role of ANG-II in ventricular dysfunction and heart failure (Serneri *et al.,* 2001). This increase in ANG-II coincides with the decrease in AT_1 receptor density on isolated cardiomyocytes from heart failure patients compared to normal heart (Serneri *et al.,* 2001). Moreover, Sun and Weber (1996) showed that in a model of rat myocardial infarction (MI) ACE was colocalised

with myofibroblasts at the site of MI and pericardial fibrosis, suggesting the involvement of RAS in ventricular remodelling. In another study, Higaki *et al.,* (2000) demonstrated that local over-expression of cardiac ACE causes cardiac hypertrophy and increase in collagen content without altering the systemic factors such as blood pressure, heart rate and circulating ACE activity. The role of ANG-II in development of cardiac hypertrophy was further supported by the fact that perindopril (ACE inhibitor), blocked both the hypertrophy and increase in collagen content (Higaki *et al.,* 2000). McEvans *et al.,* (1998) showed that infusion of ANG-II for two weeks increased the proliferation of adeventitial/interstitial fibroblasts from coronary arterioles and VSMC. However, infusion of losartan attenuated the affects of ANG-II-induced cell proliferation (McEvans *et al.,* 1998). The direct cell proliferation affect of ANG-II on fibroblasts was observed using the proliferating cell nuclear antigen (PCNA), an S-phase marker (Campbell *et al.,* 1995). Chronic infusion of ANG-II or an increase in endogenous plasma ANG-II concentration induced by a unilateral renal ischaemia model caused necrosis of cardiomyocytes (Campbell *et al.,* 1995). Immunohistochemistal analysis of the hearts showed that there is increased proliferation of fibroblasts and infiltration of immune cells at the site of necrosis (Campbell *et al.,* 1995). Several *in vitro* studies assessing cell number, [^{3}H]-thymidine or BrdU incorporation in cardiac fibroblasts obtained from humans, neonatal or adult rats also showed that ANG-II induced proliferation of cardiac fibroblasts (Kawano *et al.,* 2000; Bouzegrhane and Thibault, 2002; Rosenkranz, 2004; Wang *et al.,* 2006).

1.6.2 ANG-II and TGF-β1

Recent studies have shown that at molecular level both RAS and TGF-β1 act together in promoting cardiac remodelling (Rosenkranz, 2004; Agrotis *et al.,* 2005; Figure 1.10). TGF-β1 is a profibrotic cytokine and mediates the synthesis of ECM in various organs like heart, kidney, liver, lungs, colon and intestine (Khan and Sheppard, 2006; Bataller and Brenner, 2005; Agrotis *et al.,* 2005; Rosenkranz, 2004). In mammals, TGF-β exits in three different isoforms TGF-β1, TGF-β2 and TGF-β3 (Agrotis *et al.,* 2005). TGF-β1 is primarily secreted by cardiac fibroblast. VSMC, endothelial cells and macrophages also contribute to the TGF-β1 production in heart (Lijnen and Petrov, 2002; Khan and Sheppard, 2006). TGF-β1 plays an important role in stabilising the atherosclerotic plaques by inhibiting the local inflammation response (Cipollone *et al.,* 2004).

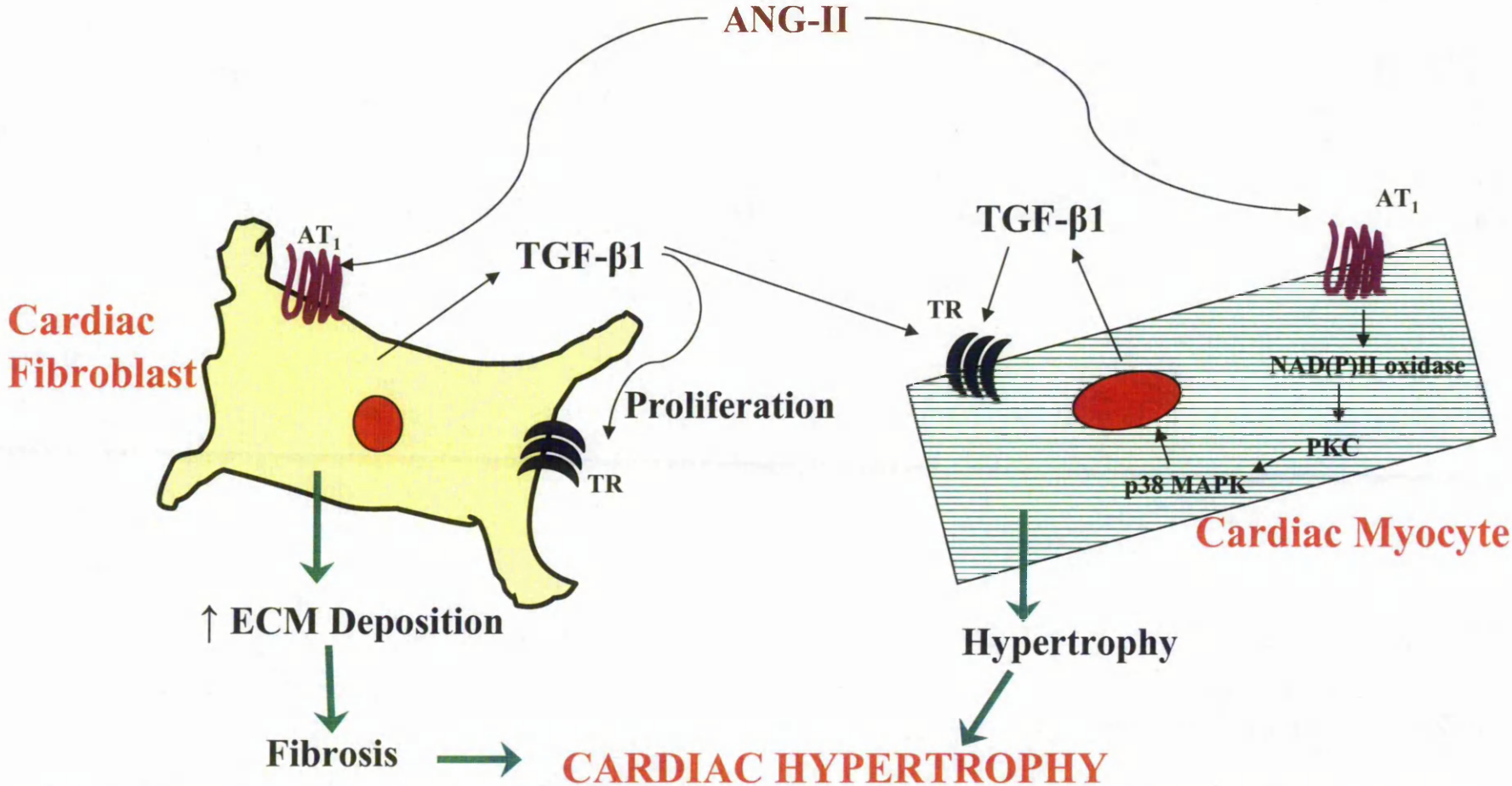

Figure 1.10: Cross-link between transforming growth factor-β1 (TGF-β1) and angiotensin-II (ANG-II) in inducing cardiac hypertrophy. ANG-II induces the release of TGF-β1 from fibroblasts and leading to their proliferation, increased deposition of extracellular matrix (ECM) and fibrosis. In cardiac myocytes, ANG-II up-regulates the expression of TGF-β1 by activating NAD(P)H oxidase and subsequent stimulation of protein kinase C (PKC) and p38 mitogen-activated protein kinase (p38 MAPK). Overall, these events cause cardiac hypertrophy. AT_1 – Angiotensin type-I receptor, TR – TGF-β1 receptor. Adapted and modified from Rosenkranz, 2004.

Additionally, TGF-β1 is involved in wound healing and stimulates the migration of neutrophils, monocytes/macrophages and fibroblasts to the site of injury (Faler *et al.*, 2006). However, over-expression of TGF-β1 results in excess ECM deposition and eventually leads to fibrosis (Khan and Sheppard, 2006). Several research studies in humans and animal models have revealed that the cardiac expression of TGF-β1 increases during fibrosis and hypertrophy. Indeed, Boluyt and researchers (1994) reported that the hypertrophic myocardium expresses high levels of TGF-β1 mRNA and that the increase in TGF-β1 is in conjunction with the upregulation of fibronectin and collagen genes. Similarly, expression of TGF-β1 is increased in the left ventricular myocardium from patients with hypertrophic cardiomyopathy and idiopathic hypertrophic cardiomyopathy and also in myocardial infarction animal models (Pauschinger *et al.*, 1999; Takahashi *et al.*, 1994; Thompson *et al.*, 1988). Transgenic mice over-expressing TGF-β1 developed cardiac hypertrophy, which was characterised by an increase in heart weight, heart weight: body weight ratio and interstitial fibrosis (Rosenkranz *et al.*, 2002). Finally, the administration of anti-TGF-β1 neutralising antibody prevented myocardial fibrosis and diastolic dysfunction by inactivating the fibroblast in pressure-overloaded rat hearts (Kuwahara *et al.*, 2002). In isolated cardiac fibroblasts, TGF-β1 also induces the characteristics of cardiac hypertrophy such as cell proliferation, increased synthesis of collagen, fibronectin and proteoglycans, and conversion of fibroblasts to myofibroblasts (Bouzegrhane and Thibault, 2002; Khan and Sheppard, 2006; Mehta and Griendling, 2007).

TGF-β can be activated by RAS components such as ANG-II, ANG III, renin, and aldosterone (Wolf, 2006). In fact, ANG-II-induced increase in TGF-β1 expression correlates with cardiac fibrosis, hypertrophy and atrial natriuretic factor (ANF) expression. Campbell and Katwa (1997) documented that ANG-II-induced expression of TGF-β1 at mRNA and protein levels in cardiac fibroblasts was blocked by the AT_1 antagonist, losartan. Studies in several animal models have also noted that blockade of ANG-II by an AT_1 antagonist inhibited the TGF-β1 expression and cardiac fibrosis and hypertrophy (Tomita *et al.*, 1998; Wenzel *et al.*, 2002; Tokuda *et al.*, 2004; Khan and Sheppard, 2006). Finally, deficiency of TGF-β1 expression in transgenic mice completely prevented the onset of cardiac fibrosis and hypertrophy induced by ANG-II (Diebold *et al.*, 1995).

Taken together, these *in vivo* and *in vitro* studies suggest that RAS and TGF-β1 are linked together in the development of cardiac fibrosis and hypertrophy.

1.7 Aims of the study

Neonatal rat cardiac fibroblasts are known to expression $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ at mRNA level; however the functional expression of these receptors is poorly studied (Webb *et al.,* 1996). In addition, several receptors like $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ were identified and added to the P2Y receptor family after this initial study. Therefore, the primary aim of the project is to further identify and pharmacologically characterise the P2Y receptors expressed in neonatal rat cardiac fibroblasts. The characterisation of P2Y receptors will be carried out by identifying the different P2Y receptor subtypes at mRNA and protein levels using RT-PCR analysis and immunocytochemistry, respectively. The functional expression of P2Y receptors will be evaluated by stimulating the receptors with adenine (AMP, ADP-βS, ATP, ATP-γS, 2-MeSADP and 2-MeSATP) and uracil (UDP and UTP) nucleotides. cAMP and inositol phosphate accumulation will be measured to identify the G-protein(s) pathways coupled to P2Y receptors. Pertussis toxin (inhibits $G_{i/o}$-protein) and YM-254890 (blocks $G_{q/11}$-protein) will be used to confirm the G-protein coupling. The use of non-selective P2 antagonists (suramin, PPADS and reactive blue 2) and selective antagonists of $P2Y_1$ and $P2Y_6$ will further strengthen the functional expression of P2Y receptor subtypes.

The secondary aim of the study is to evaluate the role of P2Y receptors in cardiac fibroblasts in ischaemic heart disease by developing an *in vitro* model associating hypoxia and angiotensin II. Cardiac fibroblasts are known to release cytokines which can modulate myocyte and fibroblast functions. Moreover, fibroblasts are the predominant cell type in heart and their main function is the deposition of extracellular matrix (Brown *et al.,* 2005a). P2Y receptors are activated by either ATP or UTP; therefore these nucleotides will be used to study the role of P2Y receptors in regulating cytokine releases like IL-1β, IL-6, TNF-α, and TGF-β1 and collagen accumulation. Ischaemic conditions induce cell death and activation of mitogen-activated protein kinases and protein kinase B/Akt in different cell types. Therefore, the effect of P2Y receptors on fibroblast viability will be investigated by lactate dehydrogenase (LDH) assay and activation of protein kinases by western blotting, respectively.

Overall, this study will characterise the different P2Y receptor subtypes in neonatal rat cardiac fibroblasts and provide important insights into the role of these receptors during ischaemic heart disease.

Chapter 2

Materials and Methods

CHAPTER 2: Materials and Methods

2.1 Chemicals and Reagents

2.1.1 General chemicals and reagents:

Fisher Scientific UK Ltd, Loughborough, UK

Acetone, ethanol, glacial acetic acid, hydrochloric acid (HCl), isopropanol, methanol, sulphuric acid (H_2SO_4)

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

Bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), goat serum, formic acid, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS), lithium chloride (LiCl), phosphate buffered solution (PBS) tablets, sodium hydroxide (NaOH), tris-HCl, trichloroacetic acid, trypan blue solution, Tween 20®,

Vector laboratories, Orton Southgate, Peterborough, UK

Vectorsheild® (mounting medium with propidium iodide)

R and D Systems Europe Ltd., Barton Lane, Abingdon, UK

Delipidized bovine serum albumin, substrate reagent pack for ELISA

2.1.2 Reagents for cell culture:

Reagents were obtained from the following suppliers:

Cambrex Bioscience Wokingham Ltd, Workingham, Berkshire, UK

Hank's Balanced Salt Solution (HBSS), Leibovitz's (L-15) medium, Dulbecco's Modified Eagle's Medium (DMEM), foetal calf serum (FCS), penicillin-streptomycin mixture, L-glutamine, trypsin with versene 10X.

Worthington Biochemical Corporation, Lakewood, USA

Trypsin, soyabean trypsin inhibitor and collagenase

2.1.3 Reagents for molecular biology:

Molecular biology reagents were obtained from:

Ambion (Europe) Ltd. Huntingdon, Cambridgeshire, UK

RNA-Bee™

Promega Uk, Chilworth, Southampton, UK

100bp DNA ladder, dithiothreitol (DTT), moloney murine leukaemia virus reverse transcriptase (M-MLV RT), blue/orange 6X loading dye, random primers, RNasin ribonuclease inhibitor, *Taq* DNA polymerase in storage buffer B, transcription optimized 5X buffer.

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

Deoxynucleotide (dNTP) mix, diethyl pyrocarbonate (DEPC), ethidium bromide, sodium acetate

2.1.4 Antibodies for immunocytochemistry:

Primary and secondary antibodies were purchased from:

Almone Laboratories, Buckingham Bucks, UK

Anti-$P2Y_{1,2,4,6,11,12,13}$ receptor subtypes and respective peptides

Vector laboratories, Orton Southgate, Peterborough, UK

Anti-rabbit immunoglobulin-FITC

2.1.5 Antibodies for western blotting analysis:

Primary and secondary antibodies were obtained from:

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

Phospho-specific ERK1/ERK2 (Thr^{202}/Tyr^{204}) and non-phospho-specific total ERK1/2 antibodies.

New England Biolabs (UK) Ltd., Wilbury way, Hitchin, Hertfordshire, UK

Phospho-specific PKB (Ser^{473}) and non-phospho-specific total PKB antibodies, Phospho-p38 MAP Kinase (Thr^{180}/Tyr^{182}) mouse monoclonal and non-phospho-specific total p38 antibodies and non-phospho-specific total JNK.

Santa Cruz Biotechnology, Autogen Biochem UK Ltd., Holly ditch farm, Calne, UK

Phospho-specific JNK1 (Thr^{183}/Tyr^{185}) mouse monoclonal antibody.

2.1.6 Radioactive chemicals:

Radioactive chemicals were purchased from:

GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK

8-[^{3}H]-adenine

MP Biomedicals, Radiochemical Division, Irvine, California, USA

2-[^{3}H]-*myo*-inositol

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

2, 3-[^{3}H]-L-proline

2.1.7 Agonist and antagonists:

Agonist and antagonists were obtained from following suppliers:

Sigma-Aldrich Company Ltd., The old brickyard, Gillingham, UK

Adenosine, adenosine 5'-monophosphate sodium salt (AMP), adenosine 5'-[β-thio]diphosphate trilithium salt (ADP-βS), adenosine 5'-triphosphate (ATP), adenosine 5'-[γ-thio] triphosphate tetralithium salt (ATP-γS), 2-(methylthio) adenosine 5'-diphosphate trisodium salt (2-methylthioADP), MRS 2179 (2-deoxy-N^6-methyl adenosine 3',5'-diphosphate diammonium salt), reactive blue 2 (RB 2), uridine 5'-diphosphate sodium salt (UDP) and uridine 5'-triphosphate trisodium salt hydrate (UTP)

Tocris Cookson Ltd., North Point, Avonmouth, UK

2-methylthioadenosine triphosphate tetrasodium salt (2-methylthioATP), MRS2578 (N,N''-1,4 butanediylbis [N'-(3-isothiocyanatophenyl) thiourea), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) tetrasodium salt and suramin hexasodium salt,

2.1.8 Inhibitors of the cell signalling pathways:

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

Pertussis toxin,

Tocris Cookson Ltd., North Point, Avonmouth, UK

KT5720 and U73122

Yamanouchi Pharmaceutical Co., Ltd, Tsukuba-shi, Ibaraki, Japan

YM-254890 was a generous gift from Professor Taniguchi

2.1.9 Other chemicals used in investigating cell signalling pathways:

Sigma-Aldrich Company Ltd., The old brickyard, New road, Gillingham, UK

Aluminium oxide type WN-3, dowex 1X8 dowex 50WX4-400, forskolin, glucose, hexokinase type C-300, rolipram,

Packard Bioscience B.V, Rigaweg 22, Groningen, Netherlands

Ultima Gold™ (scintillation counting fluid)

2.1.10 Assay kits

Assay kits were purchased from the following suppliers:

R and D Systems Europe Ltd., Barton Lane, Abingdon, UK

ELISA development kits for IL-1β, IL-6, TNF-α and TGF-β1

Bio-Rad Laboratories Ltd., Maxted road, Hemel Hempstead, UK

DC protein assay reagent package

Promega UK, Chilworth, Southampton, UK

Non-radioactive cytotoxicity assay kit

Geneflow Limited, Fradley, Staffordshire, UK

EZ-ECL Chemiluminescence Detection kit for HRP

2.2 Materials

Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted road, Hemel Hempstead, UK.

Poly-prep chromatography columns

Fahrenheit Laboratory Supplies, Rotherham , UK

Cell strainer (70μM)

Sarstedt Ltd., Leicester, UK

6-well plates, 5, 10, 25 ml pipettes, pipette tips, disposable filters (0.20μm)

Scientific Laboratory Supplies (SLS) limited, Wilford industrial estate, Nottingham, UK

4-well chamber slides, 75 and 150 sq.cm tissue culture flasks, cover slips (No: 0), scalpels No: 22, scintillation vials, sterile filter tips, pertridishes, burner, haemocytometer, pipette controller.

Corning Life Sciences, Schiphol-Rijk, Netherlands

EIA/RIA strip-well paltes

2.3 Instruments

Flow Laboratories, High Wycombe, UK

Laminar flow cabinet class II

Leica Microsystems (UK) Ltd., Knowhill, Milton Keynes, UK

Leica TCSNT confocal laser microscope system

Olympus Opitical Co (UK), Ltd, London, UK

Culture microscope CK30/CK40

Packard Instruments, Meriden, Connecticut, USA

Tri-carb 300 liquid scintillation counter

Sanyo Gallenkamp PLC, Loughborough, UK

Harrier 18/80 centrifuge, multi-gas incubator M

2.4 Methods for cell culture

2.4.1 Animals:

Adult Wistar rats (Charles River Laboratories, UK) were maintained *ad libitum* with access to regular rat chow and water in the Nottingham Trent University animal house according to regulations of the Animal (scientific procedures) Act 1986. 1-4 day old neonatals born to these animals were used to obtain cardiac fibroblasts.

2.4.2 Isolation and culturing of neonatal rat cardiac fibroblasts:

Neonatal rats were euthanized by cervical dislocation. Under sterile conditions, thoracotomy was performed and the isolated hearts were maintained in Hank's Balanced Salt Solution (HBSS) on ice. The hearts were rinsed with HBSS twice to remove blood. Neonatal cardiac fibroblasts were isolated from the hearts using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, USA). Briefly, trypsin was reconstituted with 2ml HBSS and used at 185U for 10-12 hearts. Neonatal ventricles were minced using a scalpel (number 22) in 9ml HBSS and subjected to trypsin digestion overnight at 4°C. Next day, the tissue mixture was transferred to a $75cm^2$ tissue culture flask. Soyabean trypsin inhibitor was reconstituted in 1ml of HBSS (1850U/10-12 hearts) and added to terminate the trypsin digest. The culture flasks were incubated for 15 minutes in a humidified incubator (95% air / 5% CO_2 at 37°C). The collagen in the tissues was broken down by adding 2.5ml collagenase (1040U/10-12 hearts) solubilised in Leibovitz's (L-15) medium. The flasks were then agitated in a shaking water bath for 60 minutes at 37°C. After enzymatic digestion of the ventricles, the cells were filtered through a cell strainer (70μm), and centrifuged at 1000rpm for 5 minutes at room temperature. The resulting pellet was re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v heat inactivated foetal calf serum, 2mM L-glutamine, and penicillin/streptomycin ($100Uml^{-1}$) and seeded in 75 cm^2 flasks and left for 50 minutes in a humidified incubator (95% air / 5% CO_2 at 37°C). This allows cardiac fibroblasts to be separated from cardiac myocytes by cellular attachment. The medium was then removed (containing mainly myocytes) and fresh fully supplemented DMEM added and cardiac fibroblasts were cultured for six days until confluence.

2.4.2.1 Passaging cardiac fibroblasts:

Trypsin (10X; 5mM EDTA) was thawed to room temperature and the normality of EDTA was increased to 50mM. The working dilution of trypsin was $0.5gL^{-1}$ trypsin with 5mM EDTA. 2ml of trypsin (10X) solution was added to 18ml of phosphate buffered solution (PBS) and sterilized by filtering through 0.2μm filter and stored at -20°C.

On the sixth day, the cell culture supernatant was collected and fibroblasts were trypsinised (5ml; $0.5gL^{-1}$ trypsin with 5mM EDTA) for 3 minutes in a humidified incubator (95% air / 5% CO_2 at 37°C). The trypsin activity was neutralised by adding 5ml fresh DMEM medium and cells were centrifuged at 1000rpm for 5 minutes at room temperature. The pellet was re-suspended in 5ml DMEM medium and fibroblasts were counted. 10μl of cell suspension was mixed with 10μl of trypan blue (0.4%) solution and loaded on to a haemocytometer. The cells were counted in four separate fields using light microscope under 10X magnification. The following equation was used to determine the cell number:

$$N / 4 \times 2 \times V \times 10^4$$

N – total number of cells counted in four fields

V – volume of medium cells suspended

Fibroblasts ($4 - 5 \times 10^6$ cells) were seeded in 175 cm^2 flasks and cultured for five days. Two passages were carried out to obtain fibroblast rich cultures.

2.5 Reverse transcription polymerase chain reaction (RT-PCR) analysis for the mRNA expression of P2Y receptor subtypes

2.5.1 Isolation of total RNA from neonatal rat cardiac fibroblasts:

After the first passage the cardiac fibroblasts were plated in a 6-well plate at a final density of 0.8×10^6 cells/well and cultured for two days. The cells were serum-starved overnight with DMEM containing 2mM L-glutamine, and penicillin/streptomycin ($100Uml^{-1}$). The medium was removed and the fibroblasts were washed twice with 2ml sterile PBS. Total RNA was isolated from the neonatal rat cardiac fibroblasts using RNA-Bee™ (Ambion). The serum-starved cells were lysed by adding 1ml of RNA-Bee (10^6 cells/1 ml). 0.2ml of

chloroform was added and shaken vigorously for few seconds. The sample mixture was stored on ice for 5 minutes and then centrifuged at 12,000g for 15 minutes at 4°C. Centrifugation separates the mixture into three phases, the lower blue phenol-chloroform phase (proteins and DNA), interphase (mostly DNA) and upper colourless aqueous phase (RNA). The aqueous layer was collected into a new eppendorf and 1X volume of isopropanol was added and incubated for 10 minutes at room temperature. The precipitated RNA was obtained by centrifugation at 12,000g for 5 minutes at 4°C. The supernatant was discarded; the RNA pellet was washed with 80%v/v ethanol and solubilised in 60µl diethyl pyrocarbonate (DEPC) water in order to inactivate RNAse. The RNA was measured using ultra-violet (UV) spectrophotometer at 260/280nm wavelength ratio. The concentration of RNA was determined by using the formula:

A_{260} nm x dilution faction x 40 = µg / ml

2.5.2 Synthesis of single-stranded DNA (ssDNA):

The following reagents were added to RNA to remove genomic DNA:

Total RNA (35-55µg)	55µl	
5 x Transcription optimized buffer	16µl	
RNasin (40U/µl)	1µl	Ribonuclease inhibitor
Dithiothreitol (DTT; 100mM)	5µl	Antioxidant to stabilize enzymes
RQ1 (RNA qualified) RNase free DNase (1U/µl)	3µl	Removes template DNA from RNA preparations

The sample mixture was incubated at 37°C for 20 minutes. This was followed by extraction with RNA-Bee. The procedure as described in section *2.2.1* was carried out, but the RNA pellet was suspended in 54µl DEPC treated water and heated for 5 minutes at 65°C to break any RNA secondary structures.

2.5.3 cDNA synthesis:

The following reagents were added to the ssDNA and incubated at 37°C for 90 minutes.

RNA after DNA digestion	54µl	
RNasin (40U/µl)	2µl	Ribonuclease inhibitor
5X Moloney Murine Leukemia Virus (M-MLV) buffer	20µl	
Deoxynucleotide (dNTP) mix (5mM)	20µl	Source of nucleotides
Random primers (540ng/µl)	2µl	
M-MLV Reverse. Transcriptase (M-MLV RT; 200U/ml)	2µl	RNA-dependent DNA polymerase

The cDNA was precipitated by adding 100µl of DEPC treated water, 20µl of 3M sodium acetate (pH 5.2) and 120µl isopropanol then incubated for 15 minutes on ice. cDNA pellet was obtained by centrifugation at 12,000g for 5 minutes at 4°C. This step was performed at least two times. cDNA pellet was then washed with 80% v/v ethanol and re-suspended in 25µl DEPC-water. The cDNA was measured using UV spectrophotometer at 260/280nm wavelength ratio. The concentration of cDNA was determined by using the formula:

A_{260} nm x dilution faction x 20 = µg / ml

2.5.4 Polymerase chain reaction (PCR):

Specific primers were synthesised for the rat β-actin (control) and $P2Y_{1,2,4,6,12,13,14}$ receptors (Sigma-Genosys Ltd., Cambridge UK). The primers were dissolved in 100µl DEPC-water. The concentration of primers was measured using UV spectrophotometer at 260/280nm wavelength ratio and adjusted to 200ng/µl. Primer sequences and expected product size (base pair, bp) are given in Table 2.1.

Table 2.1: Sequences of forward (Fw) and reverse (Rw) oligonucleotide primers, annealing temperatures and expected product length (base pairs, bp) of amplification products for RT-PCR analysis of rat β-actin and P2Y $_{1,2,4,6,12,13,14}$ receptors.

Accession number	Primer	Sequences	cDNA (bp)	Annealing Temp. (°C)	References
	β-actin	Fw: 5'-CGTAAAGACCTCTATGCCAA-3' Rw: 5'-GGTGTAAAACGCAGCTCAGT-3	301	57	Germack and Dickenson (2005)
NM-012800	**P2Y$_1$**	Fw: 5'-CATCTCCCCCATTCTCTT-3 Rw: 5'-GTTGCTTCTTCTTGACCTGT-3'	663	57	Hou *et al* (1999)
NM-017255	**P2Y$_2$**	Fw: 5'-ACCCGCACCCTCTATTACT-3' Rw: 5'-CTTAGATACGATTCCCCAACT-3'	538	57	Hou *et al* (1999)
NM-031680	**P2Y$_4$**	Fw: 5'-TGGGTGTTTGGTTGGTAGTA-3' Rw: 5'-GTCCCCCGTGAAGAGATAG-3'	464	57	Hou *et al* (1999)
NM-057124	**P2Y$_6$**	Fw: 5'-GTTATGGAGCGGGACAATGG-3' Rw: 5'-AGGATGCTGCCGTGTAGGTT-3'	347	57	Hou *et al* (1999)
NM-022800	**P2Y$_{12}$**	Fw: 5'-TCCCATTGCTCTACACTGTCCTG-3' Rw: 5'-AAGAACATTTCGTTATGTCC-3'	458	57	
NM-001002853	**P2Y$_{13}$**	Fw: 5'-CAGGGACACTCGGATGACA-3' Rw: 5'-TGTTCGGCAGGGAGATGA-3'	424	57	
NM-133577	**P2Y$_{14}$**	Fw: 5'-TGTCTGCCGTGATCTTCT-3' Rw: 5'-GGGTCCAGACACACATTG-3'	589	57	Fumaggalli *et al* (2003)

The following reagents were added to start the PCR reaction:

10 x *Taq* buffer	5μl
Magnesium chloride ($MgCl_2$; 25mM)	3μl
dNTPs (1.25mM)	8μl
Forward primer (200ng/μl)	1μl
Reverse primer (200ng/μl)	1μl
cDNA template	200ng (β-actin) 600ng (P2Y receptors)

The volume of PCR mixture was adjusted with DEPC treated water to 50μl. In the negative control the volume of cDNA template was replaced with DEPC treated water.

The samples and controls were subjected to the following conditions in the PCR thermocycler:

- 95°C for 5 minutes. Followed by addition of 0.3μl *Taq* polymerase.
- 95°C for 1 minute – denaturation step
- 57°C for 1 minute – annealing step
- 72°C for 1 minute – extension step

} 30 cycles

The aliquots were then stored at -20°C.

2.5.5 Agarose gel electrophoresis:

1.5% (w/v) agarose gels were prepared by dissolving 1.5g of agarose in 100ml of tris-acetate EDTA (TAE-EDTA; 50 X TAE: 242g tris-base, 5.7% v/v glacial acetic acid, 100ml 0.5M EDTA, pH 8.0). Agarose was solubilised in TAE-EDTA by heating in the microwave at medium power for 1-2 minutes and shaken occasionally. Once cooled, 3μl (10μg/ml) ethidium bromide was added to the solution and the gel was cast in Bio-Rad DNA-sub electrophoresis tray (Bio-Rad, Watford, Hertfordshire, UK) and allowed to set for 30 minutes. DNA samples (20μl) were mixed with 3μl blue/orange 6X loading dye [15% Ficoll®400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA] in order to increase the density and track the

migration of samples during electrophoresis. Samples along with 5μl 100bp DNA ladder were loaded on the gel and ran for 30-40 minutes at 90V in TAE-EDTA buffer supplemented with 3μl (10μg/ml) ethidium bromide. The DNA fragments were visualised by ethidium bromide staining and observed under U.V using a transilluminator (Syngene). The bands were quantified by densitometry using GeneGenius BioImaging System (Syngene, Synoptics Ltd., UK).

2.6 Immunocytochemistry

After the first passage the cardiac fibroblasts were plated in a 4-well chamber slide at a final density of 60,000 cells/well and cultured for two days with 700μl/well fully supplemented DMEM medium. The cells were serum-starved overnight with 500μl/well DMEM containing 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}). P2Y receptors were visualised by indirect immunofluorescence method. The serum-free medium was removed and the fibroblasts were washed three times with 1ml PBS. The cells were fixed with ice cold 200µl acetone for two minutes at -20°C followed by three washings with 1ml PBS. Anti-P2Y$_{1,2,4,6,11,12,13}$ receptor rabbit antibodies and their corresponding control antigen peptides were used to identify P2Y receptors expressed in neonatal rat cardiac fibroblasts. The primary antibodies were diluted in reagent buffer [3% w/v bovine serum albumin (BSA), 1:10,000 v/v Tween 20® in PBS, see Table 2.2]. For the control peptide antigen, primary antibodies and respective peptides were pre-incubated for one hour at 37°C in reagent buffer. Fibroblasts were then incubated with 200µl P2Y$_{1,2,4,6,11,12,13}$ receptor antibodies or antibody-antigen mixtures (negative control) for one hour at 37°C in a humidified chamber. The unbound antibodies were removed and cells were washed with 1ml PBS three times. Secondary anti-rabbit immunoglobulin-FITC was diluted in reagent buffer at 1:80 concentrations. The cells were incubated with 200µl secondary antibody for one hour at 37°C in a humidified chamber. The unbound secondary was removed and cells were washed with 1ml PBS three times. The slides were allowed to dry for 3-4 minutes at room temperature. One drop of Vectorshield® mounting medium (to preserve fluorescence) with propidium iodide (stains the nucleus red) was added per well. The chamber slides were covered with cover slips (number 0) and the edges were sealed

with nail varnish and stored at 4°C protected from light. Cardiac fibroblasts were analysed by using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon krypton laser (FITC: E_{495}/E_{278}; propidium iodide: E_{535}/E_{615}).

Table 2.2: Rat P2Y receptor subtypes' primary antibodies and peptide used in the immunocytochemistry.

Antibodies	Epitope	Epitope Location	Antibody Conc.(mg/ml)	Peptide Conc.(mg/ml)
Anti-$P2Y_1$	242-258	I_3	0.8	0.4
Anti-$P2Y_2$	227-244	I_3	0.8	0.4
Anti-$P2Y_4$	337-350	C-terminal	0.3	0.4
Anti-$P2Y_6$	311-328	C-terminal	0.8	0.4
Anti-$P2Y_{11}$	357-373	C-terminal	0.8	0.4
Anti-$P2Y_{12}$	125-142	I_2	0.8	0.4
Anti-$P2Y_{13}$	119-134	E_2	1.0	0.4

Conc – concentration, I_3 – Intracellular loop 3, I_2 – Intracellular loop 2 and C-terminal – Carboxyl terminal.

2.7 Total Inositol phosphate (IP) accumulation assay

2.7.1 Generation of [^{3}H]-inositol phosphate ([^{3}H]-IPs):

After the first passage the cardiac fibroblasts were plated in a 24-well plate at a final density of 0.15 x 10^6 cells/well and cultured for two days in fully supplemented DMEM medium. The cells were serum-starved and labelled overnight with 500μl/well [2,^{3}H]-*myo*-inositol (3μCi/well) in serum-free L-15 medium containing 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}) in a humidified incubator (95% air/5% CO_2 at 37°C). L-15 medium was used because it contains low concentration of inositol (2mg/L). Excess

[^{3}H]-*myo*-inositol was removed by washing twice with 500μl/well HBBS and cells incubated with 500μl/well serum-free DMEM containing 20mM LiCl (inositol-1 phosphatase inhibitor; see Figure 1.4) for 30 minutes, followed by incubation with agonists (10μl/well) for 30 minutes. The incubation was terminated by aspirating the medium and adding 1000μl/well of ice cold methanol / 0.1M HCl (1:1vv^{-1}). The plates were stored at -20°C before isolation of [^{3}H]-inositol phosphate ([^{3}H]-IPs).

UDP stocks were treated with hexokinase to eliminate any possible contamination with UTP. UDP was incubated with 250U/ml hexokinase and 25mM glucose for 2 hours in serum-free DMEM medium for 2 hours at 37°C.

Antagonist studies were carried out by treating the cells with the indicated concentrations of antagonists for 30 minutes prior to stimulation with agonists. To investigate the involvement of G_i protein in inositol phosphate accumulation, fibroblasts were pre-incubated with or without 100ng/ml pertusiss toxin (PTX; G_i protein inhibitor) for 18 hours ((Bokoch *et al.,* 1983, Germack and Dickenson, 2004). The involvement of $G_{q/11}$ protein in inositol phosphate production was evaluated in the presence or absence of 1μM YM-254890 (YM; $G_{q/11}$ protein inhibitor; Takasaki *et al.,* 2004) for 30 minutes before activation with agonists.

2.7.2 Isolation and qualification of [^{3}H]-inositol phosphates:

[^{3}H]-IPs were isolated, using Bio-Rad polyprep columns containing 1ml Dowex 1X8-200 resin and water in 1:1 ratio. The resin was pre-washed with 10ml 1M HCl, followed by 20ml of distilled water. The well contents were carefully transferred to vials containing 4.2ml of neutralizing solution (15ml 0.5M NaOH, 110ml 25mM Tris buffer and 340ml water). This mixture was then added to the columns followed by 20ml of distilled water and 10ml 25mM ammonium formate. [^{3}H]-IPs were eluted into scintillation vials by 3ml 1M HCl containing 10ml of Ultima Gold^{TM} scintillation fluid and counted in a Packard Instruments Tri-carb 300 liquid scintillation counter for 3 minutes. The dowex columns were regenerated by washing with 10ml 1M HCl, followed by 20ml of distilled water.

2.8 cyclic AMP (cAMP) accumulation assay

2.8.1 Generation of [^{3}H]-cAMP:

After the first passage the cardiac fibroblasts were plated in a 24-well plate at a final density of 0.15 x 10^6 cells/well and cultured for two days. The cells were serum-starved overnight with 500μl/well DMEM containing 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}). Serum-starved fibroblasts were incubated with 500μl/well of serum-free DMEM containing [^{3}H]-adenine (2μCi/well) for 3 hours in a humidified incubator (95% air / 5% CO_2 at 37°C). The radio-labelled cells were washed twice with 500μl/well HBBS buffer and then incubated with 500μl/well serum-free DMEM containing 10μM rolipram, a phosphodiesterase inhibitor, for 15 minutes. The indicated concentrations of agonists (10μl/well) were added and cells incubated for 5 minutes prior to incubation for 10 minutes with 1.5μM forskolin or 15 minutes without forskolin. The reaction was terminated by aspirating the medium and adding 500μl/well of 5% (w/v) trichloroacetic acid. The plates were stored at -20°C before they were quantified.

UDP stock solution (10mM) was treated with hexokinase to eliminate any possible contamination with UTP. UDP was incubated with 250U/ml hexokinase and 25mM glucose in serum-free DMEM medium for 2 hours at 37°C.

Antagonist studies were carried out by treating the cells with the indicated concentrations of antagonists for 30 minutes prior to stimulation with agonists. To investigate the involvement of G_i protein in cAMP accumulation, fibroblasts were pre-incubated with or without 100ng/ml pertusiss toxin (PTX; G_i protein inhibitor) for 18 hours (Bokoch *et al.*, 1983, Germack and Dickenson, 2004). The involvement of $G_{q/11}$ protein in cAMP production was evaluated in the presence or absence of 1μM YM-254890 (YM; $G_{q/11}$ protein inhibitor; Takasaki *et al.*, 2004) for 30 min before activation with agonists.

2.8.2 Isolation and qualification of [^{3}H]-cAMP:

[^{3}H]-cyclic AMP was isolated by sequential dowex-alumina column chromatography and quantified by liquid scintillation spectrometry. Bio-Rad polyprep dowex columns containing 2.4ml of AG 50W-X4 resin and water in 1:1 ratio (dowex columns) or 0.6g neutral alumina (alumina columns) were prepared. Dowex columns were pre-washed with

5ml 1M HCl, followed by 20ml of distilled water and alumia columns pre-washed with 20ml 0.1M imidazole. The well contents [500μl of 5% (w/v) trichloroacetic acid] were transferred carefully to the corresponding dowex resin columns and washed with 3ml of distilled water. After the water had drained the dowex columns were placed on top of the alumina columns and [^{3}H]-cyclic AMP eluted from the dowex to the alumina by washing with 4ml of distilled water. 5ml 0.1M imidazole was used to elute [^{3}H]-cAMP into the scintillation vials. The levels of [^{3}H]-cAMP were determined by adding 10ml of Ultima Gold^{TM} scintillation fluid and counted in a Packard Instruments Tri-carb 300 liquid scintillation counter for 3 minutes.

The dowex and alumina columns were regenerated by washing with 5ml 1M sodium hydroxide (NaOH) followed by 20ml of distilled water. Dowex columns were then washed with 5ml 1M HCl, followed by 20ml of distilled water whereas the alumia columns were washed with 20 ml 0.1M imidazole.

2.9 Estimation of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and transforming growth factor beta 1 (TGF-β1) by enzyme linked immunosorbent assay (ELISA)

2.9.1 Preparation of cell culture supernates:

After the first passage the cardiac fibroblasts were plated in a 24-well plate at a final density 0.20 x 10^6 cells/well and cultured for two days in 1.5ml/well DMEM supplemented with 10% heat inactivated foetal calf serum, 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}). On the third day the fully supplemented DMEM medium was replaced by serum and glucose free DMEM containing 2mM L-glutamine and penicillin/streptomycin (100Uml^{-1}). Cardiac fibroblasts were stimulated with 32μM ATP-γS and 10μM UTP alone or in combination with lipopolysaccharide (100ng/ml; LPS) to simulate septic conditions or in combination with angiotensin-II (50nM; ANG-II) to simulate cardiac hypertrophy under normoxic and hypoxic conditions for 1, 2, 4, 8, 18 hours. The hypoxic conditions were achieved by using serum and glucose free DMEM and exposure to hypoxia using a hypoxic incubator (5% CO_2/0.5% O_2 at 37°C), where oxygen

was replaced by nitrogen gas (94.5%). Following the various conditions and treatments the cell supernates were collected and stored at -80°C before measuring the cytokine levels. To evaluate the levels of TGF-β1 the latent TGF-β1 must be activated to immunoreactive TGF-β1. For this 0.1ml 1N HCl was added to the cell supernates (500µl) and incubated for 10 minutes at room temperature. The assay samples were later neutralised by adding 100µl 1.2N NaOH/0.5M HEPES.

2.9.2 Measurement of IL-1β, IL-6, TNF-α and TGF-β1 by ELISA:

2.9.2.1 Preparation of ELISA plate:

The capture antibodies (IL-1β, IL-6, TNF-α and TGF-β1) were reconstituted with 1.0ml PBS and diluted to working concentrations (see Table2.3) in PBS. 96-well flat bottom microplates (high binding type-I) were coated with 100µl per well of the diluted capture antibody and incubated overnight at room temperature. The unbound capture antibody was aspirated and the wells washed three times with 400µl wash buffer (0.05% v/v Tween® 20 in PBS, pH 7.2 – 7.4). To prevent unspecific binding the wells were blocked with respective reagent diluent (300µl) and incubated at room temperature for a minimum of one hour. Following the blocking, the reagent diluent was removed and the washing steps were repeated.

Table 2.3: Concentration of capture antibody, detection antibody and reagent diluent compositions used in ELISA.

Cytokine	Capture Antibody Conc.(µg/ml)		Detection Antibody Conc.(µg/ml)		Reagent Diluent
	Stock	Working	Stock	Working	
IL-β1	144	0.8	63	0.35	1% w/v BSA in PBS, pH 7.2
IL-6	720	4	63	0.35	1% w/v BSA in PBS, pH 7.2
TNF-α	720	4	63	0.35	1% w/v BSA in PBS, pH 7.2
TGF-β1	720	4	36	0.2	1.4% v/v dBS, 0.05% v/v Tween® 20 in PBS, pH7.2

BSA - bovine serum albumin, dBS – bovine serum, Conc. – concentration, PBS – phosphate buffered solution

2.9.2.2 ELISA procedure:

100µl of sample or standard diluted in appropriate diluent were added to each well and incubated for 2 hours at room temperature. The cytokine of interest was bound to the immobilised antibody. Subsequently the unbound samples were aspirated and wells washed. Detection antibodies were reconstituted in suitable reagent diluent and diluted to the appropriate working concentration (see Table 2.3). 100µl of diluted detection antibody was added to each well and incubated for 2 hours at room temperature. The unbound detection antibody was aspirated and the wells were washed. Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP) was diluted 1:200 in reagent diluent and 100µl was added to each well for 20 minutes at room temperature and plates protected from direct light. Following the wash to remove any unbound enzyme reagent, 100µl substrate solution was added to the wells. The substrate solution comprised of 1:1 mixture of colour reagent A (hydrogen peroxide) and colour reagent B (tetramethylbenzidine). After 20 minutes, the enzymatic reaction was terminated by adding 50µl stop solution (2N sulphuric acid) and the colour changes from blue to yellow. The intensity of yellow colour is proportional to the amount of cytokine present in the samples. The optical density of each well was read using a microplate reader set at 450nm. To correct for optical imperfections the samples were also read at 540 nm and values were subtracted from the 450nm readings.

2.10 Various conditions and treatment of cardiac fibroblasts

Neonatal rat cardiac fibroblasts were stimulated with 32µM ATP-γS and 10µM UTP alone or in combination with angiotensin-II (ANG-II; 50nM) to simulate cardiac hypertrophy under normoxic and hypoxic conditions for 4 and 18 hours. The hypoxic conditions were achieved by using serum and glucose free DMEM and exposure to hypoxia using a hypoxic incubator (5% CO_2/0.5% O_2 at 37°C), where oxygen was replaced by nitrogen gas.

2.11 Lactate Dehydrogrenase (LDH) assay

Lactate dehydrogenase (LDH), a stable cytosolic enzyme was measured using the CytoTox96 non-radioactive cytotoxicity assay kit (Promega, Southampton, UK). LDH in the cell supernatants catalyses the enzymatic reaction between NAD^+ and lactate to form NADH and pyruvate, NADH in turn reacts with tetrazolium dye (INT) in conjunction with diaphorase to form a red formazan product. The amount of red colour formed is proportional to the number of cells dead.

After the first passage, cardiac fibroblasts were plated in a 96-well flat bottomed plate at a final density of 35,000 cells/well in quadruplets and cultured for two days. On the third day, the fully supplemented DMEM medium was replaced with glucose- and serum-free DMEM containing 2mM L-glutamine and penicillin/streptomycin ($100Uml^{-1}$). Fibroblasts were then treated as described in section 2.10. Following the treatments and conditions the plates were centrifuged for 5 minutes at 250g. 50µl of cell supernatant was removed from each well and added to a new 96-well plate. Following this 60µl supernatant was discarded from each well and the cells were lysed with 10µl of lysis solution 10X (9% v/v Triton®X-100) for 45 minutes at 37°C. The cellular debris was pelleted by centrifuging the plate for 5 minutes at 250g. 50µl of cell supernatant was removed from each well and added to a new 96-well plate. The enzymatic reaction was started by adding 50µl assay substrate (diaphorase, lactate, NAD^+ and tetrazolium dye) solution to each well and incubated for 30 minutes on a shaker at room temperature and protected from direct light. The colorimetric reaction was terminated by adding stop solution (50µl, 1M acetic acid) and the optical density of the colour measured at 490nm using a SpectraFluor 96-well plate reader.

2.12 Western blotting

2.12.1 Preparation of protein samples for Western blot analysis:

After the first passage, the cardiac fibroblasts were plated in a 6-well plate at a final density 1.0×10^6 cells/well and cultured for two days in 5ml/well DMEM supplemented with 10% heat inactivated foetal calf serum, 2mM L-glutamine, and

penicillin/streptomycin ($100Uml^{-1}$). On the third day, the fully supplemented DMEM medium was replaced by serum and glucose free DMEM containing 2mM L-glutamine and penicillin/streptomycin ($100Uml^{-1}$). Cardiac fibroblasts were treated as described in section 2.10. Following the various conditions and treatments the cell supernates were removed and the fibroblasts were washed twice with ice-cold 2ml PBS. The cells were lysed by scrapping the well using 100µl lysis buffer (see Table 2.4). The proteins were separated from the cell debris by centrifugation of the cell lysate at 13,000g for 10 minutes at 4°C. 70µl of supernatant was collected and mixed with 70µl of sample buffer in a 1.5ml eppendorf tube (see Table 2.4). In addition, 10µl of the supernatant was collected in a 0.5ml eppendorf tubes for determination of protein content. All samples were stored at -80°C.

Table 2.4: Composition of buffers used in the preparation of cell lysates for western blotting analysis.

RIP BUFFER

Reagents	Quantity
Sodium chloride (150mM)	4.38g
Tris-HCl (50mM)	3.027g
EDTA (5mM)	0.931g
Distilled water	up to 500ml

LYSIS BUFFER

Reagents	Quantity	Purpose
RIP buffer	2.5ml	Buffering agent
Igepal CA-630	25µl	Non-ionic detergent
Deoxycholate acid	12.5mg	Detergent
10% w/v SDS	25µl	Anionic detergent
500mM Benzamidine	5µl	Peptidase inhibitor
100mM PMSF	2.5µl	Protease inhibitor
200mM Sodium orthovanadate	12.5µl	ATPase, alkaline and tyrosine phosphatase inhibitor
1M Sodium fluorate	2.5µl	Alters membrane permeability and inhibitors phosphatase

SAMPLE BUFFER

Reagents	Quantity
1M Tris-HCl, pH 6.8	2.5ml
Glycerol	6.0ml
10% w/v SDS	8.0ml
2-Mercaptoethanol	2.0ml
0.5% w/v Bromophenol blue	4.0ml
Deionised water	17.5ml

2.12.1.1 Determination of protein concentration by modified Lowry Assay:

The total protein content in cell lysates was determined by using the Bio-Rad *DC* Protein Assay. The assay is similar to the Lowry assay, but with a few modifications. The colourimetric protein assay is based on the reaction between proteins and alkaline copper tartrate solution followed by reduction of copper treated proteins by Folin reagent (acidic mixture of molybdenum and tungsten ions). The characteristic blue colour is due to the formation of heteropolymolybdenum and has the absorbance at 750nm.

25µl of reagent A (alkaline copper tartrate solution) and 200µl of reagent B (folin reagent) were added sequentially to 5µl of samples in a flat-bottomed 96-well plate. A standard curve was constructed by using 5µl of 0, 1, 2, 2.5, 4, 5, 7.5, 10µg of bovine serum albumin. The plate was incubated for 15 minutes at room temperature before reading the absorbance at 750nm using a SpectraFluor 96-well plate reader.

2.12.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting:

2.12.2.1 Polyacrylamide gel electrophoresis (PAGE):

The polyacrylamide gel was prepared by polymerisation of monomeric acrylamide into polymeric acrylamide chains and the cross-linking of these chains by N,N'-methylene bisacrylamide. The polymerisation reaction was initiated by the addition of ammonium persulphate and catalysed by N,N,N,N-tetramethylethylenediamine (TEMED). TEMED accelerates the formation of free radicals from ammonium persulphate. The gels consisted of 12% w/v separating/resolving gel and 5% w/v stacking gel. The acrylamide stock solution used for the gels consisted of 30% w/v acrylamide and 0.8% w/v N,N'-methylene bisacrylamide. Separating gels were made using 1.5M Tris-HCl (pH 8.8) whereas stacking gels contained 1.0M Tris-HCl (pH 6.8). The components for the resolving and stacking gels are detailed in Table 2.5. The separating gel was cast in the Bio-Rad mini-Protein II gel systems and covered with a layer of water. After polymerisation of the resolving gel, the stacking gel was over layered and a 10-well comb inserted. The comb was removed after polymerisation of the stalking gel and the wells were washed with distilled water. The protein samples (ERK1/2 and Akt/PKB – 15µg, p38MAPK - 30µg and JNK - 35µg of protein) were loaded onto the wells using protein electrophoresis tips and electrophoresis

performed at 200V for 45 minutes in the running buffer (24.8mM Tris-HCl, 0.192M glycine and 3.5mM SDS).

Table 2.5: Composition of resolving and stacking polyacrylamide gel

Reagents	**Separating/ Resolving gel (ml)**	**Stacking gel (ml)**
Distilled water	9.9	8.2
30% w/v acrylamide	12.0	2.0
1.5M Tris-HCl (pH 8.8)	7.5	-
1.0M Tris-HCl (pH 6.8)	-	1.5
10% w/v SDS	0.3	0.12
10% w/v ammonium persulphate	0.3	0.12
TEMED	0.03	0.012

2.12.2.2Western blotting of proteins:

The proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes using a wet blot system. The stalking gel was removed and the resolving gel was washed with transfer buffer (25mM Tris-HCl, 192mM glycine, 20% v/v methanol). The electrophoresis apparatus was assembled with the fibre pad on the bottom followed by 2 sheets of whatman filter paper (No: 1), resolving gel, nitrocellulose paper, another 2 sheets of whatman filter paper and the fibre pad. While assembling the unit care was taken to prevent air bubbles. Western blotting was performed using Bio-Rad apparatus at 100V for 60 minutes in ice-cold transfer buffer. The transfer of proteins onto the nitrocellulose paper was visualised with Ponceau Red solution (0.2% w/v Ponceau and 0.4% v/v glacial acetic acid). The stain was removed by washing with Tris-buffered saline (TBS; 200mM Tis-HCl, 1.37M sodium chloride and pH adjusted to 7.6 with concentrated HCl). The membranes were subsequently incubated for an hour with 5% w/v fat-free dried milk powder (marvel) in 0.1% v/v Tween-20®-TBS at room temperature to prevent non-specific binding of primary antibody.

2.11.2.3 Immuno-probing of western blots:

The primary antibody was diluted to the working concentration in 5% w/v fat-free dried milk powder (marvel) in 0.1% v/v Tween-20® and TBS (Table 2.6).

Table 2.6: Primary and secondary antibody concentrations used in western blotting

Antibody	Working dilution
Phospho-p44/42 MAP Kinase [ERK1/2; (Thr^{202}/Tyr^{204})] mouse monoclonal	1 in 1000
Phospho-p38 MAP Kinase (Thr^{180}/Tyr^{182}) mouse monoclonal	1 in 1000
Phospho-specific JNK1 (Thr^{183}/Tyr^{185}) mouse monoclonal	1 in 500
Phospho-specific Akt (Ser^{473}) polyclonal	1 in 500
Non-phospho-specific total ERK1/2 polyclonal	1 in 500
Non-phospho-specific total p38 polyclonal	1 in 500
non-phospho-specific total JNK polyclonal	1 in 500
non-phospho-specific total Akt polylonal	1 in 500
Polyclononal goat anti-rabbit immunoglobulin/HRP	1 in 1000
Polyclononal goat anti-mouse immunoglobulin/HRP	1 in 1000

After blocking the blots with milk, the membranes were incubated with primary antibodies overnight at 4°C. Following three washings for 5 minutes in 0.1% v/v Tween-20®-TBS, the membranes were incubated with secondary antibody for at least one hour at room temperature. Another series of washings with 0.1% v/v Tween-20®-TBS were performed for 10 minutes three times.

The protein bands were revealed by using Enzymatic Chemiluminescence (ECL) detection kit for horseradish peroxidase (HRP). In the presence of peroxides HRP catalyses the

oxidation of luminol from ground state to excited state. The excited luminal returns to ground state by emitting light. The kit consists of reagent A (luminol) and reagent B (peroxide solution) which were mixed in equal amounts. 2ml of this mixture is then applied to the membrane for 1 minute and the membrane was carefully wrapped in the cling film and placed in the auto-radiograph cassette. The Kodak films were exposed to the membranes in the dark room for varying lengths of time. The film was then developed and fixed using Kodak developer and Kodak fixer. The bands were quantified by densitometry using GeneGenius BioImaging Systems (Syngene, Synoptics Ltd., UK).

2.11.3 Stripping and reprobing the membranes:

The western blotting membranes can be stripped of the antibodies and reprobed with other antibodies. The removal of antibodies was performed by incubating the membrane in stripping buffer (100mM 2-mercaptoethanol, 2% w/v SDS, 62.5mM Tris-HCl at pH 6.7) at 50°C for 30 minutes with occasional shaking. The membrane was then washed for three times with a large volume of 0.1% v/v Tween-20®-TBS. Finally the membrane can be immunoprobed as described in the above section after blocking with 5% w/v fat-free dried milk powder (marvel) in 0.1% v/v Tween-20®-TBS.

2.13 Collagen Assay

2.13.1 Measurement of collagen by [^{3}H]-L-proline incorporation:

After the first passage cardiac fibroblasts were plated in a 12-well plate at a final density of 0.4 x 10^6 cells/well and cultured for overnight in 3ml/well DMEM supplemented with 10% heat inactivated foetal calf serum, 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}). Next day, the fibroblasts were pulsed with 5μCi/ml of 2,3-[^{3}H]-L-proline in DMEM supplemented with 2% heat inactivated foetal calf serum, 2mM L-glutamine, and penicillin/streptomycin (100Uml^{-1}). On the second day, following the washing with 2ml HBBS buffer the cell supernatant was replaced by serum- and glucose-free DMEM containing 2mM L-glutamine and penicillin/streptomycin (100Uml^{-1}). Cardiac fibroblasts were stimulated as mentioned in section 2.10. Following the various conditions and treatments the cell supernatants were collected in scintillation insert vials and the

fibroblasts were washed twice with 1ml PBS. To extract the soluble collagen, the fibroblasts were incubated for 10 minutes at room temperature with 150µl soluble collagen buffer (12mM sodium deoxycholate, 150mM sodium chloride, 50mM Tris-HCl and 5mM EDTA). The buffer was later collected into a separate scintillation insert vials. After washing the wells with 1ml PBS the non-soluble collagen was obtained by scrapping the well with 100µl of non-soluble collagen buffer (4% w/v SDS, 50mM Tris-HCl). The viscous solution was again collected into a new scintillation insert vials. The levels of [^{3}H]-collagen were determined in the different fractions by adding 2ml of Ultima Gold^{TM} scintillation fluid and counting in a Packard Instruments Tri-Carb 300 liquid scintillation counter for 3 minutes.

2.13.2 DNA Assay:

DNA levels were measured to determine if, nucleotides, ANG-II and the conditions described in section 2.10 stimulated the cell division of fibroblasts. 200µl of ethidium bromide (2.5mg/ml) was added to 20µl of soluble collagen samples in a 96-flat well chimmey base black coloured plate. The plate was protected from direct light and the fluorescence was read using a SpectraFluor 96-well plate reader (Excitation wavelength: 540nm, Emission wavelength: 595nm, lag time: 20µsec. and integration time: 40µsec.)

2.14 Data analysis

EC_{50} and IC_{50} (drug concentration that produces 50% of maximal stimulatory or inhibitory response) values were obtained by computer-assisted curve (sigmoidal dose response) using the computer program PRISM (GraphPAD software, San Diego, U.S.A). Statistical significance was determined by analysis of variance (ANOVA) followed by Bonferroni's test and $p < 0.05$ was considered as the limit of statistical significance. All data are presented as mean ± S.E.M. and n in the text refers to the number of separate experiments.

Chapter 3

Characterisation of P2Y receptors

Chapter 3: Results – Characterisation of P2Y receptors in neonatal rat cardiac fibroblasts

3.1 Expressions of P2Y receptors in neonatal rat cardiac fibroblasts

The expression of mRNA encoding for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors was investigated in serum-starved neonatal rat cardiac fibroblasts using RT-PCR analysis. β-actin was used as a house-keeping gene and the expression of P2Y receptor mRNAs was expressed as a percentage of the β-actin transcript. Since the rodent $P2Y_{11}$ receptor has not been cloned, the investigation of this particular receptor was hindered. mRNA coding for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$ was detected in neonatal rat cardiac fibroblasts (Figure 3.1A). No evidence was obtained for the expression of $P2Y_{12}$ and $P2Y_{14}$ receptors (Figure 3.1A). $P2Y_2$ and $P2Y_6$ receptors were expressed in higher levels compared to $P2Y_{1,4,13}$ receptors (Figure 3.1B). The expression of P2Y receptors at the protein level was carried out by immunocytochemistry using $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ receptor antibodies. The human $P2Y_{11}$ receptor antibody displays cross-reactivity with rodent ortholog as previously shown in mouse neuroblastoma neuro2a cells and in rat spleen and lung tissues (Lakshmi and Joshi, 2006; Almone Laboratories). The immunofluorescence was detected for all the receptors, except for $P2Y_{12}$ with higher intensity for $P2Y_6$, $P2Y_2$, and $P2Y_{11}$ (Figure 3.2). The protein expression of P2Y receptor subtypes by confocal microscopy correlated well with the RT-PCR analysis. No staining was observed in the absence of primary antibody or in the presence of the respective immunogenic peptides (Figure 3.2). Webb *et al* (1997) have shown that mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ were expressed in neonatal rat cardiac fibroblast which is consistent with the present study. In addition, for the first time $P2Y_{11}$ and $P2Y_{13}$ receptors were detected in neonatal rat cardiac fibroblasts.

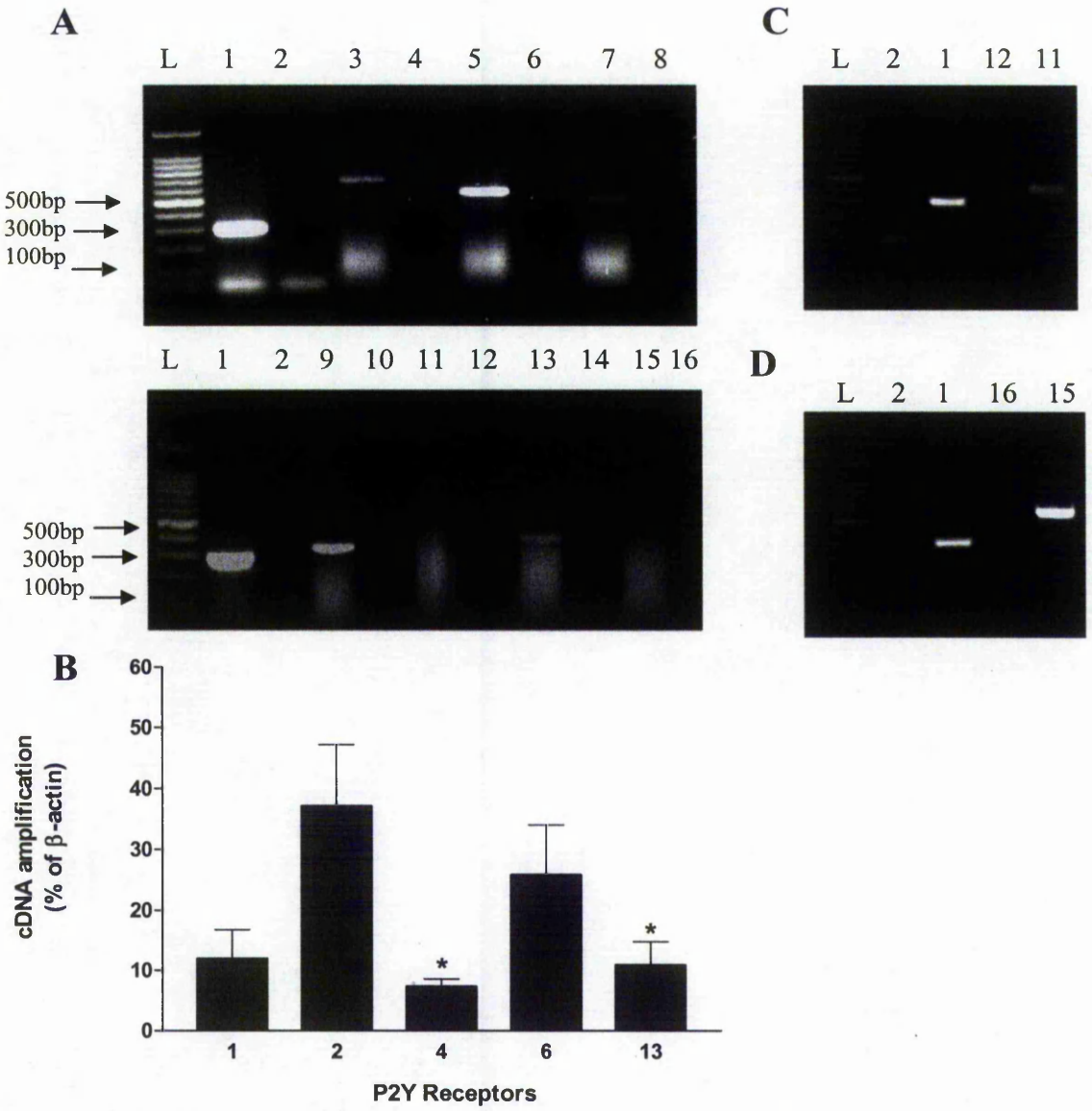

Figure 3.1: Expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptor mRNA in neonatal rat cardiac fibroblasts. Total RNA was prepared and semi-quantitative RT-PCR was carried out as described in section 2.5. **Panel A** displays 1.5% agarose gel electrophoresis of β-actin (lane 1-2) and $P2Y_1$ receptor mRNA, (lanes 3-4), $P2Y_2$ (lanes 5-6), $P2Y_4$ (lanes 7-8), $P2Y_6$ (lanes 9-10), $P2Y_{12}$ (lanes 11-12), $P2Y_{13}$ (lanes 13-14) and $P2Y_{14}$ (lanes 15-16). Lanes 2, 4, 6, 8, 10, 12, 14 and 16 correspond to the primer control without cDNA and lane L to the ladder (100bp). **Panel B** displays the percentage of P2Y receptor mRNA expressed as a percentage of β-actin (100%). Values were obtained by densitometric analysis (GeneGenius BioImaging System; Syngene, Synoptics Ltd., UK) of RT-PCR reaction products and normalized using the β-actin signal (100%). Each point represents the mean ± S.E.M of 7 independent experiments. * $P<0.05$ versus $P2Y_2$ receptor mRNA expression. **Panel C** and **Panel D** represent the expression of $P2Y_{12}$ and $P2Y_{14}$ receptor

mRNA in rat brown adipose tissue and in rat spleen as a possitve control for $P2Y_{12}$ and $P2Y_{14}$ primers, respectively.

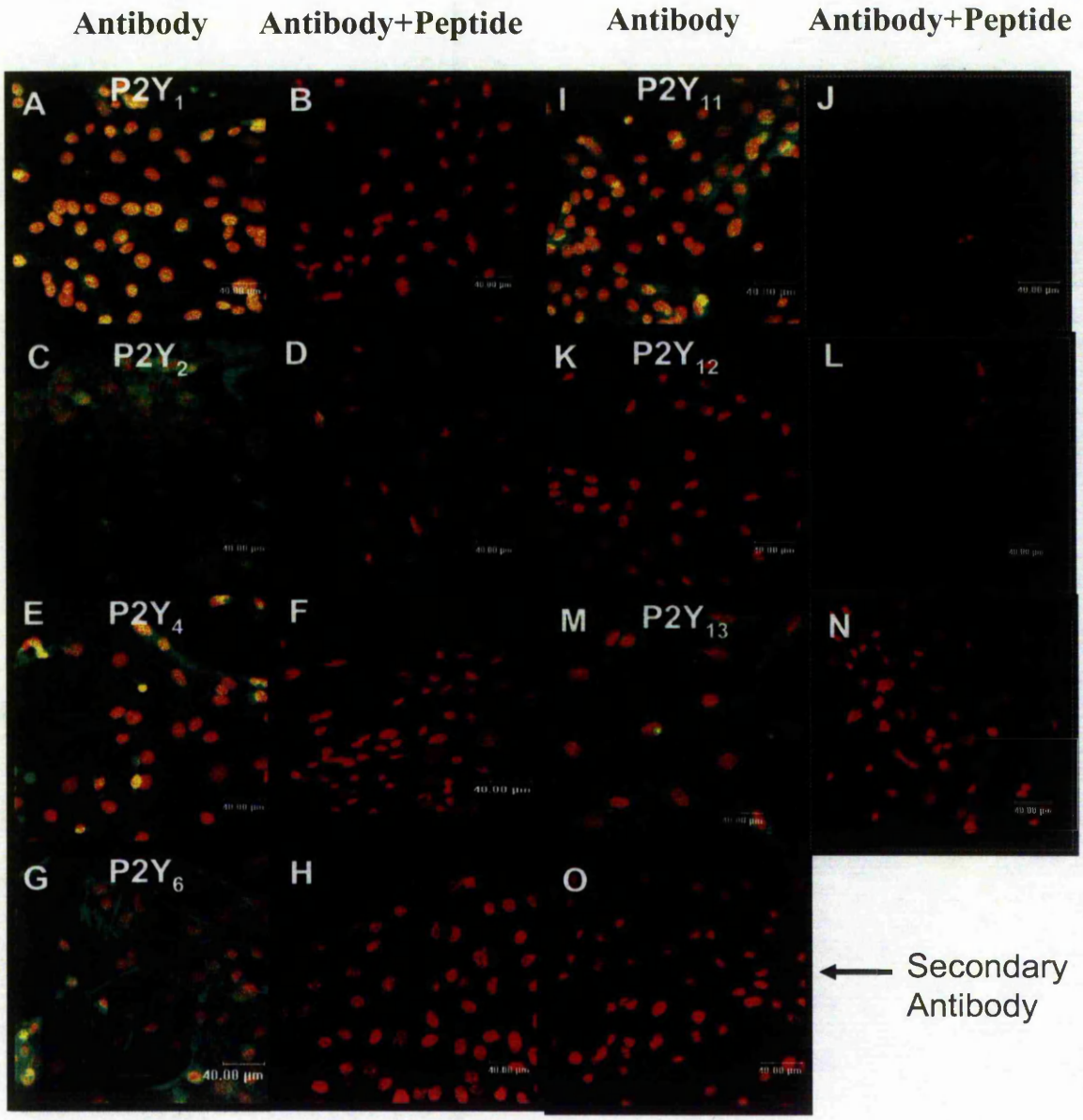

Figure 3.2: Expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors in neonatal rat cardiac fibroblasts by immunocytochemistry. The P2Y receptor protein expressions were evaluated by immunocytochemistry as mention in section 2.6. The green colour represents the P2Y receptors and the nuclei were stained red with propidium iodide. **Panels A, C, E, G, I, K** and **M** show $P2Y_{1,2,4,6,11,12,13}$ expression; in **Panels B, D, F, H, J, L** and **N** the cells were incubated with respective peptide and antibody mixture and **Panel O** illustrates fibroblasts incubated with secondary antibody. Cardiac fibroblasts were analysed by using a Leica TCSNT confocal laser microscope system (Leica) equipped with an argon krypton laser (FITC: E_{495}/E_{278}; Propidium iodide: E_{535}/E_{615}). Images presented are from one experiment and representative of four.

3.2 Effect of extracellular nucleotides on total inositol phosphate production in neonatal rat cardiac fibroblasts

Most of the P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) are G_q protein coupled receptors linked to phospholipase-C (PLC) (Abbracchio *et al.,* 2006). Therefore, [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation was investigated using adenine (AMP, ADP-βS, ATP, ATP-γS, 2-MeSADP and 2-MeSATP) and uracil (UDP and UTP) nucleotides. Both adenine (except AMP) and uracil nucleotides increased [^{3}H]-IP production in a concentration-dependent manner (Figure 3.3, Table 3.1). ATP and ATP-γS (stable analogue of ATP) elicited concentration-dependent increases in [^{3}H]-IP production (Figure 3.3A). The potencies and maximal responses of ATP and ATP-γS are shown in Table 3.1. It is notable that ATP-γS was more potent than ATP and with a higher maximal response (Table 3.1). ATP can be broken down into adenosine by ecto-nucleotidases, which is of importance since rat cardiac fibroblasts express the adenosine A_{2B} receptor (Dubey *et al.,* 2001; 1998; Chen *et al.,* 2004). Therefore, [^{3}H]-IP accumulation was performed following adenosine stimulation (Figure 3.3B). Interestingly, an inhibition of basal [^{3}H]-IP production was observed with adenosine, which may explain the difference between the maximal responses obtained with ATP and ATP-γS (Table 3.1). AMP also induced an inhibition of [^{3}H]-IP generation, indicating that AMP dose not stimulate G_q coupled receptors. (Figure 3.3B, Table 3.1). ADP-βS, 2-MeSADP and 2-MeSATP all induced small but significant increases in [^{3}H]-IP production (Figure 3.3A, 3.3C; Table 3.1).

Both uracil nucleotides UDP and UTP stimulated increases in [^{3}H]-IP production (Figure 3.4, Table 3.1). Interestingly, UDP-induced [^{3}H]-IP production was significantly biphasic and both components of the concentration-response curve elicited a similar maximal response. The data suggests the involvement of two different P2Y receptors in the [^{3}H]-IP accumulation induced by UDP. The [^{3}H]-IP production with UDP-glucose, a $P2Y_{14}$ receptor agonist was not performed, since this subtype was not detected at mRNA level on cardiac fibroblasts (Figure 3.1).

The rank order of agonist potency to induce total inositol phosphate accumulation was 2-MeSADP > 2-MeSATP ≈ ADP-βS > UDP (Receptor-I) > UTP > ATP-γS > UDP (Receptor-II) > ATP, indicating that the $P2Y_1$ receptor is the predominant G_q coupled P2Y receptor in neonatal rat cardiac fibroblasts.

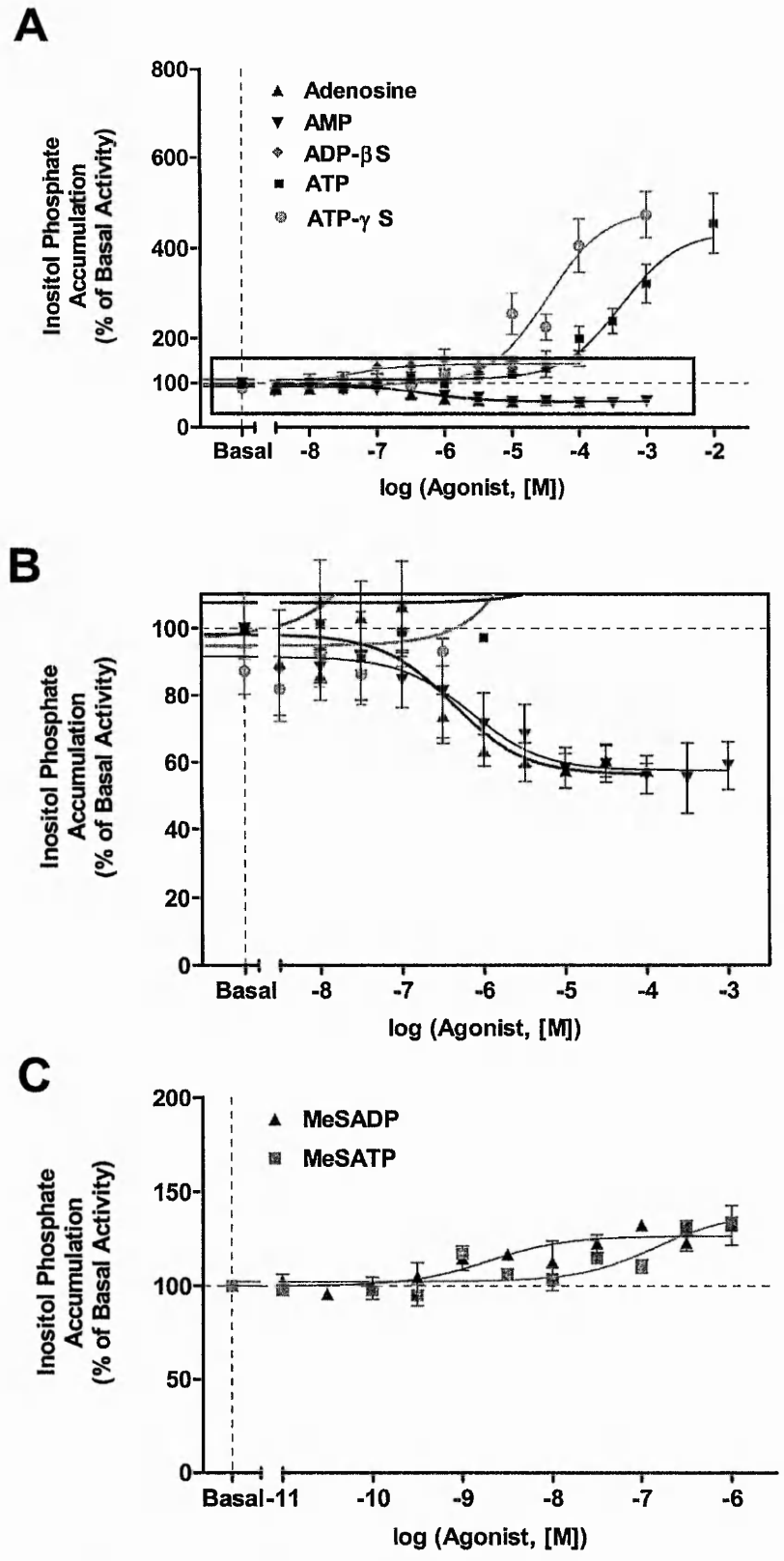

Figure 3.3: Effect of adenine nucleotides on inositol phosphate accumulation in isolated neonatal rat cardiac fibroblasts **(Panels A-C)**. Panel B represents the enlargement of the frame in panel A. [^{3}H]-inositol labelled and serum-starved fibroblasts were pre-incubated with 20mM LiCl for 30 minutes and then incubated

with indicated concentrations of adenine nucleotides for 30 minutes. Data were expressed as the percentage of the basal level of inositol phosphate accumulation (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate.

Table 3.1: Ligand potencies and maximal responses of adenine and uracil nucleotides at multiple effector pathways of the P2Y receptors on neonatal rat cardiac fibroblasts.

Agonist	G_s				G_i				G_q				Forskolin-induced cAMP accumulation (G_s pathway)			
	n	EC_{50}	E_{max}		n	*IC_{50}*	*I_{max}*		n	*IC_{50}*/EC_{50}	*I_{max}*/E_{max}		n	EC_{50}	E_{max}	
Adenosine	3	5.2±0.4 (6.3)	112±23	↑					4	*6.1±0.1 (0.79)*	*39±10*	↓	-	-	-	-
AMP	4	4.9±0.1 (12.5)	676±152	↑↑↑					4	*6.2±0.4 (0.63)*	*36±4*	↓	3	5.6±0.2 (2.51)	1188±172	↑↑↑
ADP-βS	4	5.3±0.4 (5.0)	94±39	↑					3	7.2±0.2 (0.06)	39±7	↑	4	4.7±0.4 (19.95)	933±131	↑↑↑
ATP	5	4.7±0.4 (19.9)	128±37	↑					6	3.5±0.2 (316.2)	322±50	↑↑	4	5.9±0.1 (1.26)	467±68	↑
ATP-γS	4	5.4±0.2 (3.9)	247±53	↑↑					5	4.5±0.1 (31.6)	391±35	↑↑	4	5.7±0.1 (1.99)	659±180	↑↑
2-MeSATP	3	NR	NR	-					3	7.1±0.4 (0.07)	34±4	↑	3	NR	NR	-
2-MeSADP	3	NR	NR	-					3	8.5±0.8 (0.003)	33±7	↑	3	NR	NR	-
UDP	4	NR	NR	-	4	*4.9±0.5 (10)*	*51±15*	↑	5	Receptor-I* 5.8±0.2 (1.5) Receptor-II* 3.6±0.0 (251.1)	Receptor-I*: 329±29 Receptor II* 291±31	↑↑				
UTP	4	NR	NR	-	3	*5.4±0.3 (3.98)*	*82±16*	↑↑	5	5.2±0.2 (6.3)	345±110	↑↑				

Values given for EC_{50}/IC_{50} and E_{max}/I_{max} are expressed as mean value ± S.E.M from n independent experiments, performed in duplicate. Values in parentheses are EC_{50}/IC_{50} expressed in μM and E_{max}/I_{max} in % on basal or forskolin activity. Values in italics represent the IC_{50} and I_{max}. ↑ - increase, ↓ - decrease, NR – no response, * Receptor-I and Receptor-II represent the efficacy and maximal responses obtained with the two different components of the concentration-response curve.

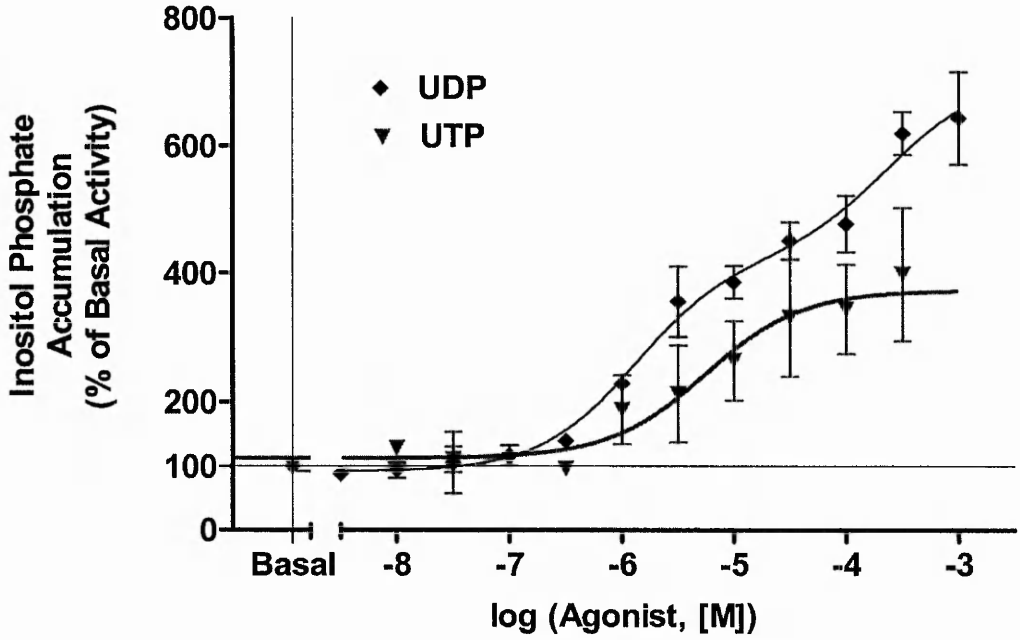

Figure 3.4: Effect of uracil nucleotides on inositol phosphate accumulation in isolated neonatal rat cardiac fibroblasts. [^{3}H]-inositol labelled and serum-starved fibroblasts were pre-incubated with 20mM LiCl for 30 minutes and then incubated with indicated concentrations of uracil nucleotides for 30 minutes. Data were expressed as the percentage of the basal level of inositol phosphate accumulation (100%). Each point represents the mean ± S.E.M for 5-6 experiments from separate cell cultures, performed in duplicate.

3.3 Effect of extracellular nucleotides on cAMP production in neonatal rat cardiac fibroblasts

The $P2Y_{11}$ receptor is known to induce cAMP production through G_s coupling whereas $P2Y_2$, $P2Y_4$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors inhibit cAMP accumulation via G_i coupling (Abbracchio *et al.,* 2006). In order, to determine the effect of adenine and uracil nucleotides on adenylyl cyclase activity, cAMP accumulation was measured in neonatal rat cardiac fibroblasts. All adenine nucleotides, except 2-MeSADP and 2-MeSATP induced significant increases in [^{3}H]-cAMP accumulation (Figures 3.5). As seen with [^{3}H]-IP accumulation measurements the potencies and maximal responses of ATP and ATP-γS were different. ATP-γS was more potent than ATP, and induced a significantly two-fold higher maximal response (P<0.01; Table 3.1), indicating the breakdown of ATP into adenosine as shown in the [^{3}H]-IP production study. Adenosine also induced an increase in [^{3}H]-cAMP production (Figure 3.5A) and the combined maximal responses of adenosine and ATP (E_{max}; 240% over basal) were similar to ATP-γS alone. ADP-βS induced a small rise in [^{3}H]-cAMP accumulation, whereas AMP mediated a higher response on [^{3}H]-cAMP accumulation compared to the other adenine nucleotides (Table 3.1). No [^{3}H]-cAMP accumulation was observed in response to 2-MeSADP, 2-MeSATP, UDP and UTP (Figure 3.5B, 3.6), indicating these nucleotides do not activate the G_s pathway.
The rank order of potency of the agonists for stimulating [^{3}H]-cAMP accumulation was ATP-γS ≈ ADP-βS ≈ Adenosine > AMP > ATP.

The effects of 2-MeSADP, 2-MeSATP and uracil nucleotides on forskolin-stimulated [^{3}H]-cAMP accumulation were investigated to evaluate the possible coupling of P2Y receptor subtypes to G_i-proteins. This assay is widely used to monitor the negative coupling of G_i-protein coupled receptors to adenylyl cyclase (De Souza *et al.,* 1983; Germack and Dickenson 2004). The concentration of forskolin (1.5μM) used in these experiments was obtained by performing a concentration-dependent curve on cAMP synthesis in cardiac fibroblasts (Figure 3.7).

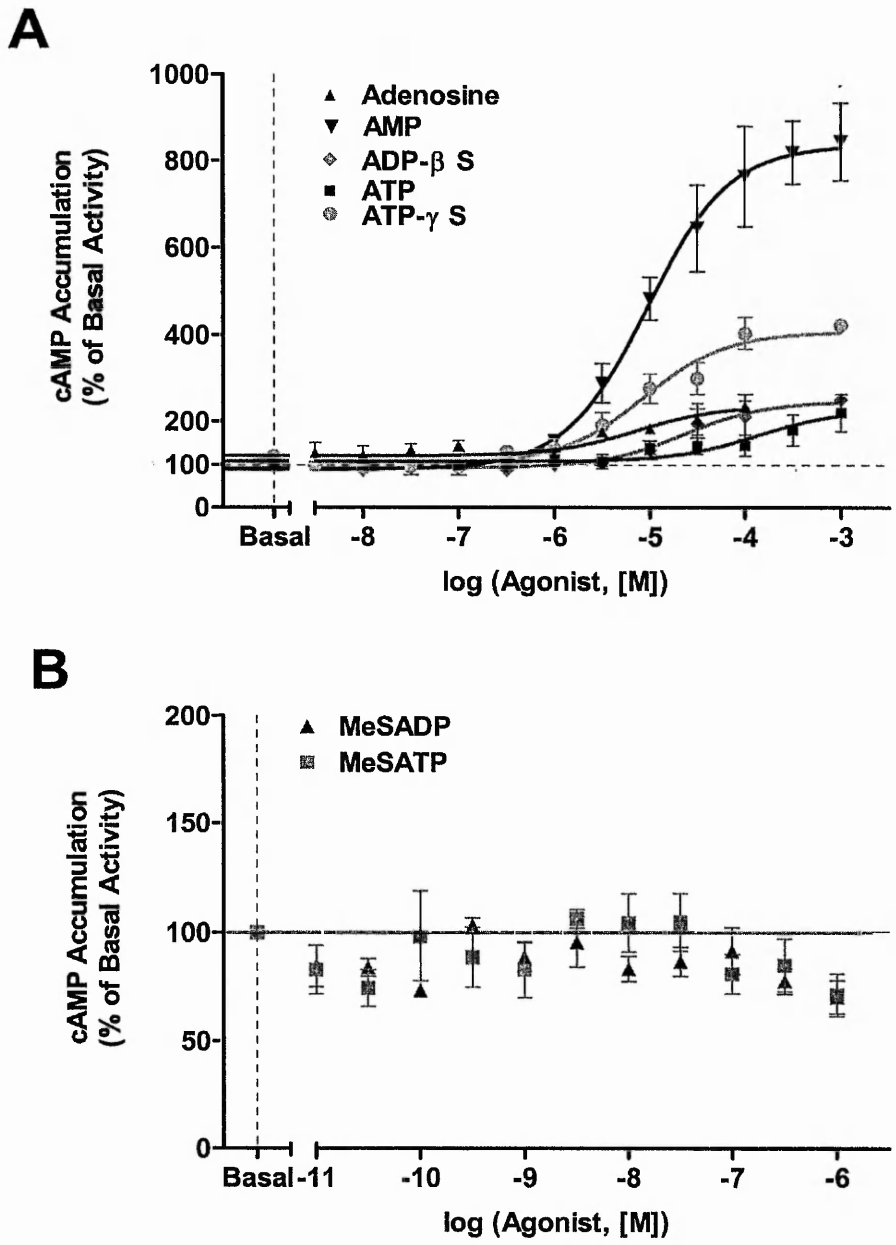

Figure 3.5: Effect of adenine nucleotides on cAMP accumulation in isolated neonatal rat cardiac fibroblasts (**Panels A-B**). Serum-starved fibroblasts were labelled with [^{3}H]-adenine and pre-incubated with phosphodiesterase inhibitor, 10μM rolipram for 15 minutes. Fibroblasts were stimulated with the indicated concentrations of adenine nucleotides for 15 minutes. Data was expressed as the percentage of the basal cAMP accumulation (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate.

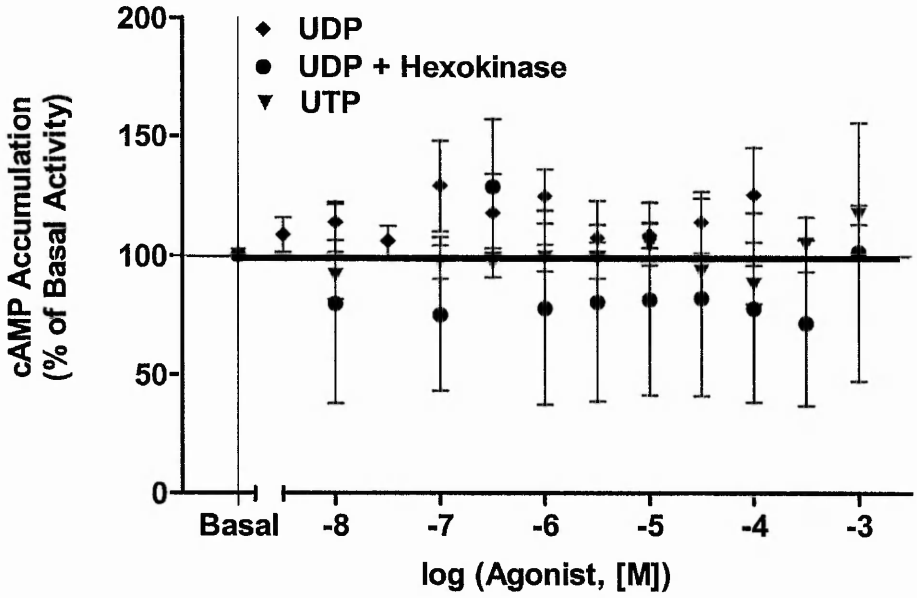

Figure 3.6: Effect of uracil nucleotides on cAMP accumulation in isolated neonatal rat cardiac fibroblasts. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and pre-incubated with phosphodiesterase inhibitor, 10μM rolipram for 15 minutes. Fibroblasts were stimulated with the indicated concentrations of adenine nucleotides for 15 minutes in the absence of forskolin. Data was expressed as the percentage of the basal cAMP accumulation (100%). Each point represents the mean ± S.E.M for 3-4 experiments from separate cell cultures, performed in duplicate.

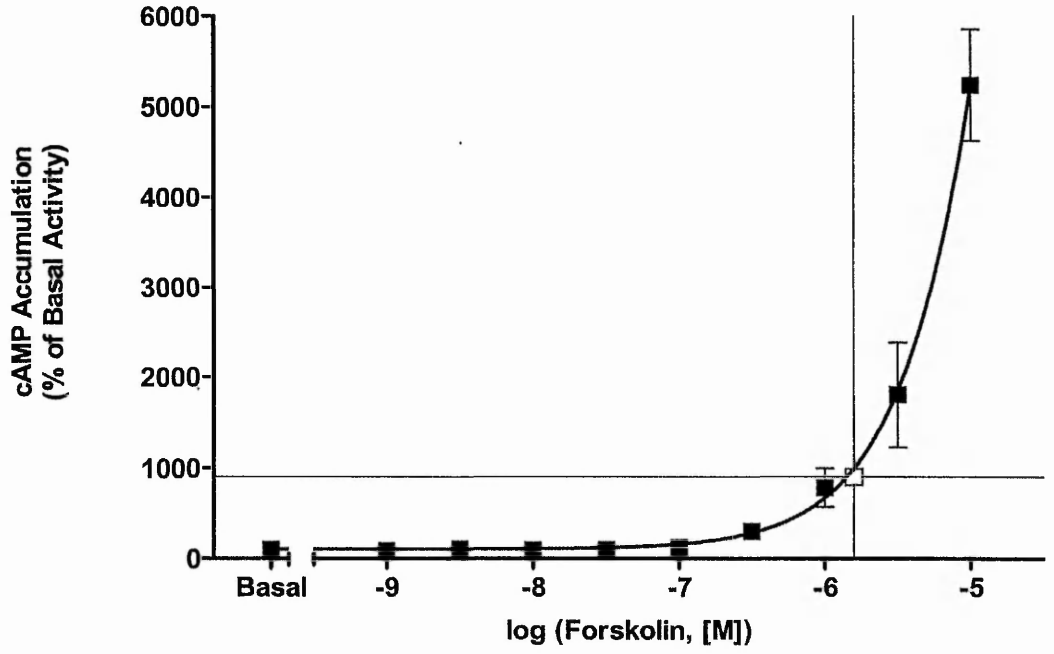

Figure 3.7: Effect of forskolin on cAMP accumulation in isolated neonatal rat cardiac fibroblasts. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and pre-incubated with phosphodiesterase inhibitor, 10μM rolipram for 15 minutes. Fibroblasts were stimulated with the indicated concentrations of FSK. Data was expressed as the percentage of the basal cAMP accumulation (100%). The open square represents the forskolin concentration (1.5μM) used in the cAMP assays. Each point represents the mean ± S.E.M for 3 experiments from separate cell cultures, performed in duplicate.

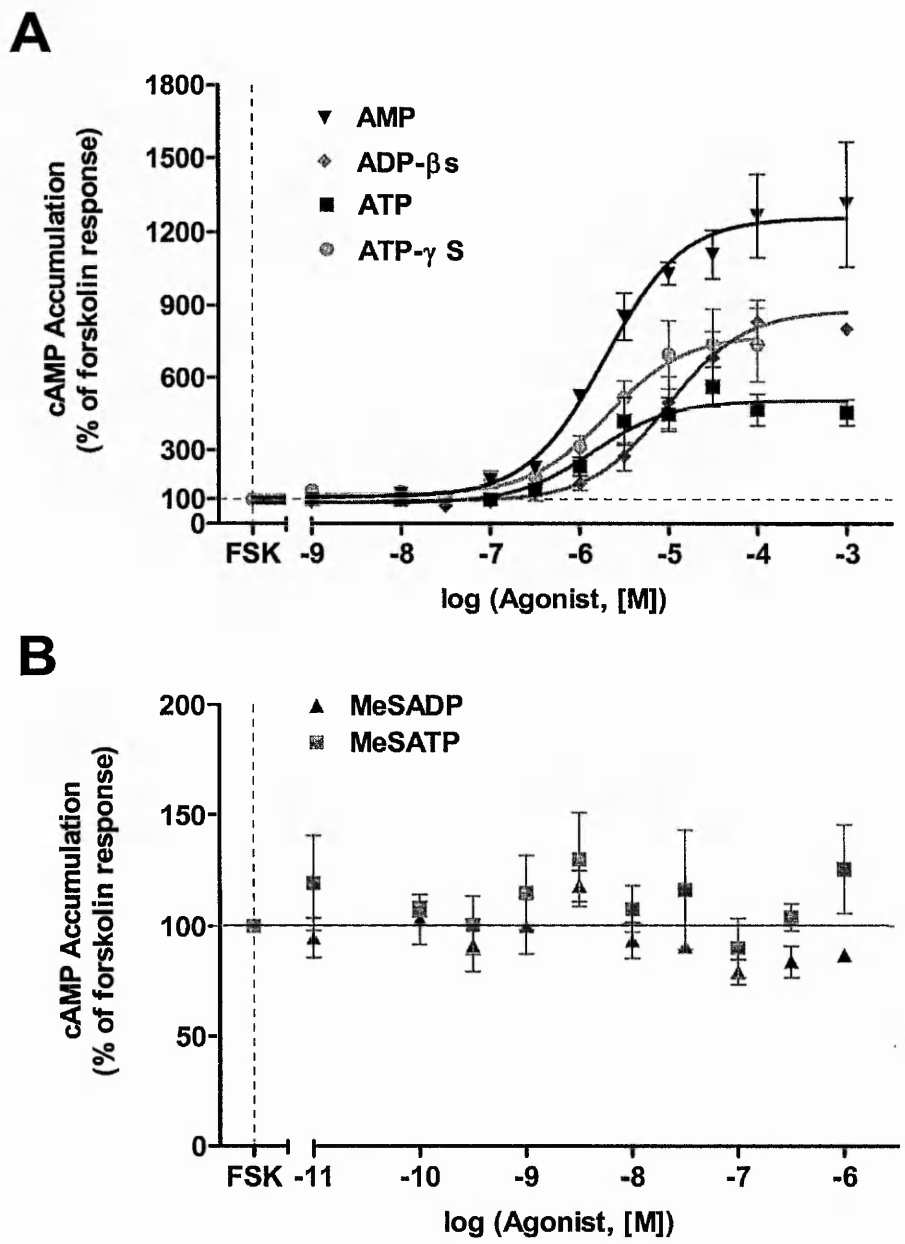

Figure 3.8: Effect of adenine nucleotides on forskolin (FSK)-stimulated cAMP accumulation in isolated neonatal rat cardiac fibroblasts **(Panels A-B)**. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and pre-incubated with phosphodiesterase inhibitor, 10μM rolipram for 15 minutes. Fibroblasts were initially pre-stimulated for 5 minutes with indicated concentrations of adenine nucleotides, prior to stimulation with 1.5μM FSK for 10 minutes. Data were expressed as the percentage of the forskolin response (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate.

The maximal response of [^{3}H]-cAMP accumulation with 1.5μM forskolin (900% over basal) was not near saturation value (Figure 3.7). AMP, ADP-βS, ATP and ATP-γS potentiated forskolin stimulated [^{3}H]-cAMP production by 43%, 90%, 72% and 63%, respectively (Figure 3.8A). In contrast, 2-MeSADP and 2-MeSATP did not augment or inhibit forskolin-induced [^{3}H]-cAMP accumulation (Figure 3.8B) indicating that the receptor(s) stimulated by these agonists are not coupled to G_s or G_i proteins. The rank order for adenine nucleotide mediated potentiation of forskolin-induced [^{3}H]-cAMP accumulation was ATP ≈ ATP-γS ≥ AMP >> ADP-βS. In marked contrast, UDP and UTP produced an inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation (Figure 3.9). The response induced by UTP was 60% higher than UDP-mediated inhibition of forskolin mediated [^{3}H]-cAMP accumulation. The UDP stock solution was treated with hexokinase to remove any possible contamination with UTP. However, hexokinase pre-treatment of UDP stocks failed to effect the maximal activity (I_{max} 51 ± 15), but decreased its potency in an insignificant manner (IC_{50} 4.9 ± 0.5) (Table 3.1 and Figure 3.9). Thus UDP stocks were not significantly contaminated with UTP, therefore further experiments were carried out in the absence of hexokinase.

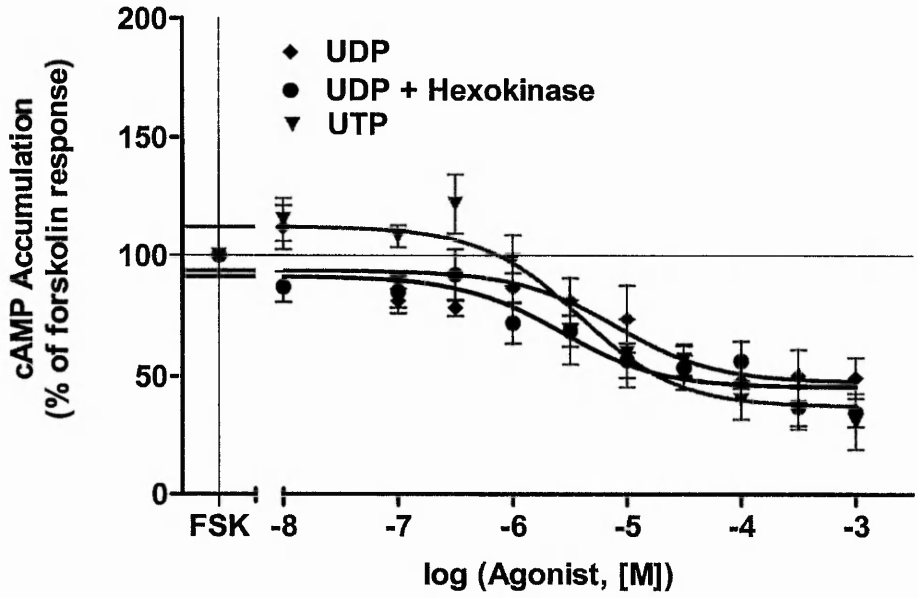

Figure 3.9: Effect of uracil nucleotides on forskolin (FSK)-stimulated cAMP accumulation in isolated neonatal rat cardiac fibroblasts. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and pre-incubated with phosphodiesterase inhibitor, 10μM rolipram for 15 minutes. Fibroblasts were initially pre-stimulated for 5 minutes with indicated concentrations of uracil nucleotides, prior to stimulation with 1.5μM FSK for 10 minutes. Data were expressed as the percentage of the forskolin response (100%). Each point represents the mean ± S.E.M for 4-7 experiments from separate cell cultures, performed in duplicate.

3.4 Effect of antagonists on [^{3}H]-IP and [^{3}H]-cAMP accumulation induced by extracellular nucleotides

Antagonists were used to further characterise pharmacologically the expression of P2Y receptors. Only a few P2Y subtype-selective antagonists are commercially available. MRS2179, a competitive antagonist at the $P2Y_1$ receptor; MRS2578, a selective blocker of the $P2Y_6$ receptor and non-selective P2 antagonists namely reactive blue 2 (RB 2), suramin and PPADS, which also antagonise P2X receptors in addition, to P2Y receptor subtypes. (Boyer *et al.,* 1998; Mamedova *et al.,* 2004; Burnstock and Ralevic, 1998).

3.4.1 Effect of antagonists on [^{3}H]-IP accumulation induced by extracellular nucleotides in neonatal rat cardiac fibroblasts

100μM suramin, 100μM PPADS and 100μM RB 2 had no effect on basal [^{3}H]-IP accumulation (Figure 3.10). The [^{3}H]-IP accumulation induced by 10μM ADP-βS, 0.1μM 2-MeSADP and 1μM 2-MeSATP were completely inhibited by suramin, PPADS and RB 2. (Figure 3.11), suggesting the involvement of the $P2Y_1$ receptor. ATP-γS (100μM)-induced increase in [^{3}H]-IP production was partially blocked by suramin (18%, $P<0.01$) and RB 2 (60%, $P<0.001$), implying $P2Y_{11}$ receptor activation by ATP-γS. On the other hand, PPADS potentiated the ATP-γS-induced [^{3}H]-IP synthesis (16%, $P<0.01$, Figure: 3.11). AMP-induced inhibition of basal [^{3}H]-IP production was blocked and reversed by suramin (81%, $P<0.001$), PPADS (70%, $P<0.001$) and RB 2 (46%, $P<0.01$; Figure 3.11). Finally, uracil nucleotide-induced [^{3}H]-IP accumulation was moderately abolished by suramin (UDP: 19%, $P<0.001$) and RB 2 (UDP: 70%, $P<0.001$; UTP: 60%, $P<0.001$; Figure 3.12). As shown in figure 3.12, PPADS potentiated the UDP-induced [^{3}H]-IP production by 20% ($P<0.001$), however it was ineffective on UTP.

ADP-βS, ATP-γS and 2-MeSADP activate the G_q-coupled $P2Y_1$ receptor. Therefore, inhibition curves were constructed with ADP-βS (10μM), ATP-γS (100μM) and 2-MeSADP (0.1μM) in the presence of MRS2179. MRS2179 antagonised [^{3}H]-IP accumulation induced by ADP-βS (40%), ATP-γS (23%) and 2-MeSADP (40%) in a concentration-dependent manner (Figure 3.13, Table 3.2).

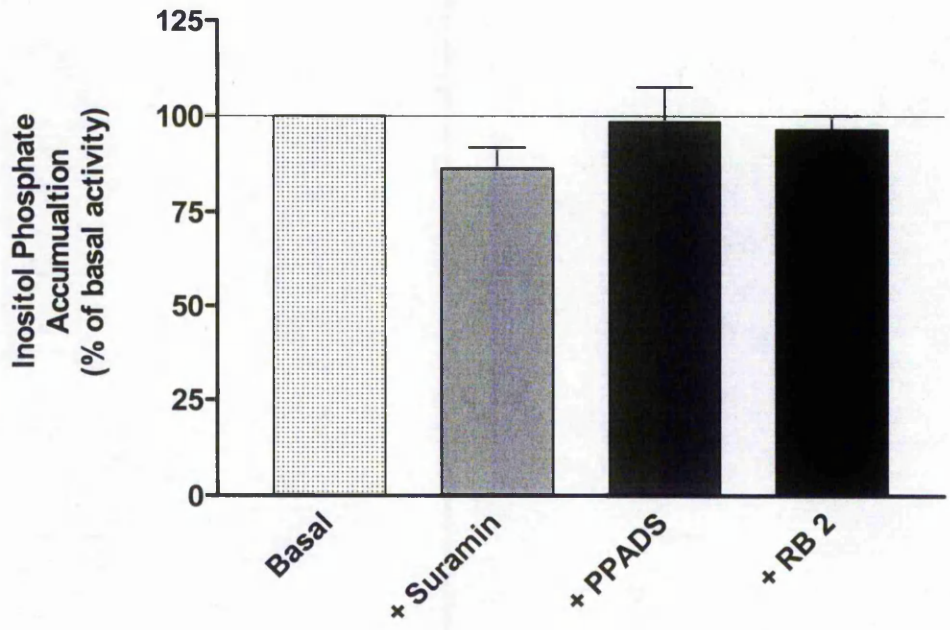

Figure 3.10: Effect of classical non-selective P2 receptor antagonists on inositol phosphate accumulation. [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with 20mM LiCl and the antagonists suramin (100μM), PPADS (100μM) and RB 2 (100μM) for 60 minutes. Data was expressed as the percentage of basal activity (100%). Each point represents the mean ± S.E.M for 3-4 experiments from separate cell cultures, performed in duplicate.

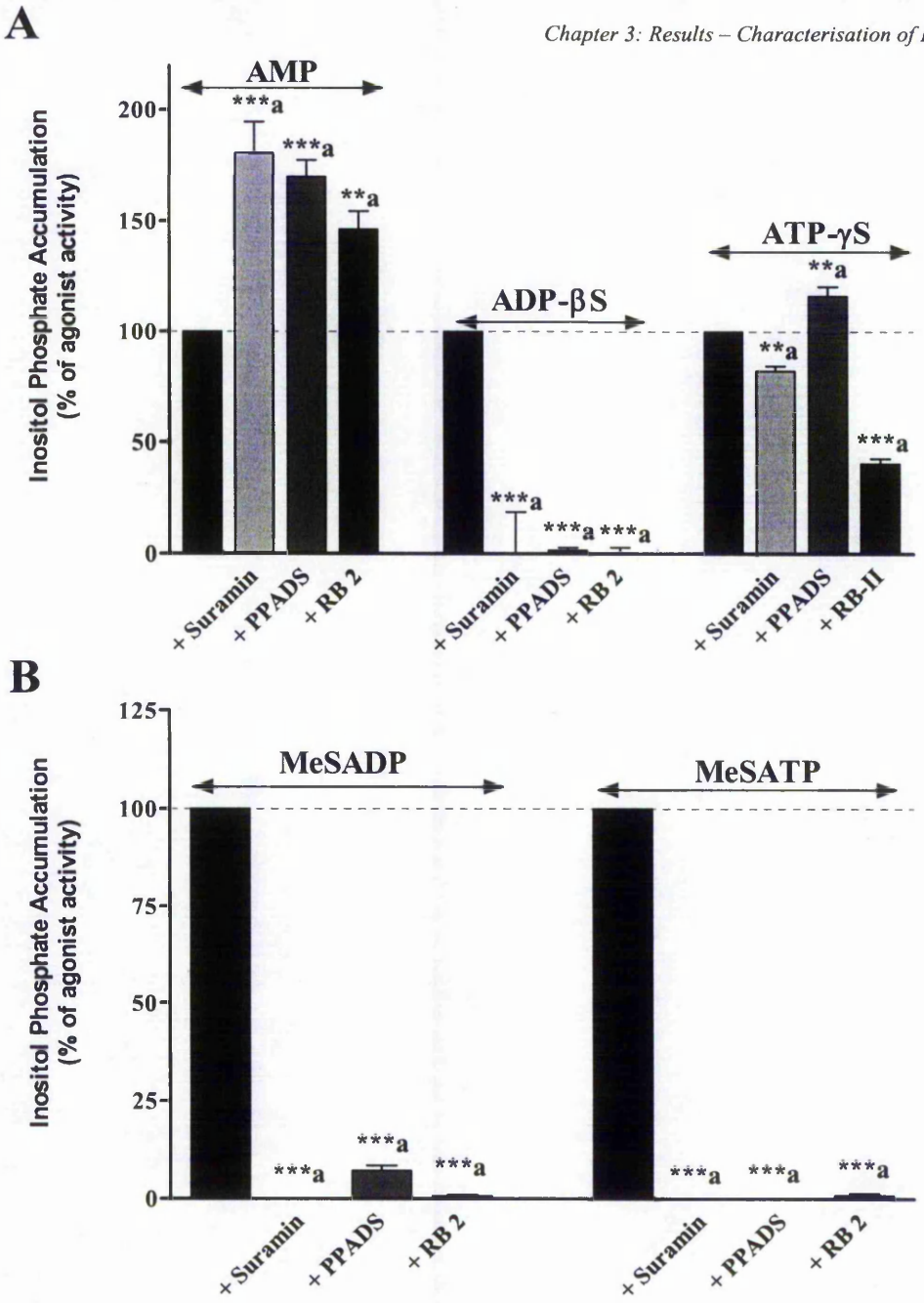

Figure 3.11: Effect of classical non-selective P2 receptor antagonists on inositol phosphate accumulation observed with AMP (10μM), ADP-βS (10μM) and ATP-γS (100μM, **Panel A**) and 2-MeSADP (0.1μM) and 2-MeSATP (1μM, **Panel B**). [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with suramin (100μM), PPADS (100μM) and RB 2 (100μM) for 30 minutes before stimulating with adenine nucleotides. ADP-βS, ATP-γS, 2-MeSADP and 2-MeSATP induced an increase in [^{3}H]-IP production by 40%, 440%, 33% and 33% over the basal activity, whereas AMP induced an inhibition of basal [^{3}H]-IP accumulation by 30%. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 4-6 experiments from separate cell cultures, performed in duplicate. ** P<0.01 and *** P<0.001; a versus the agonist response in absence of antagonist.

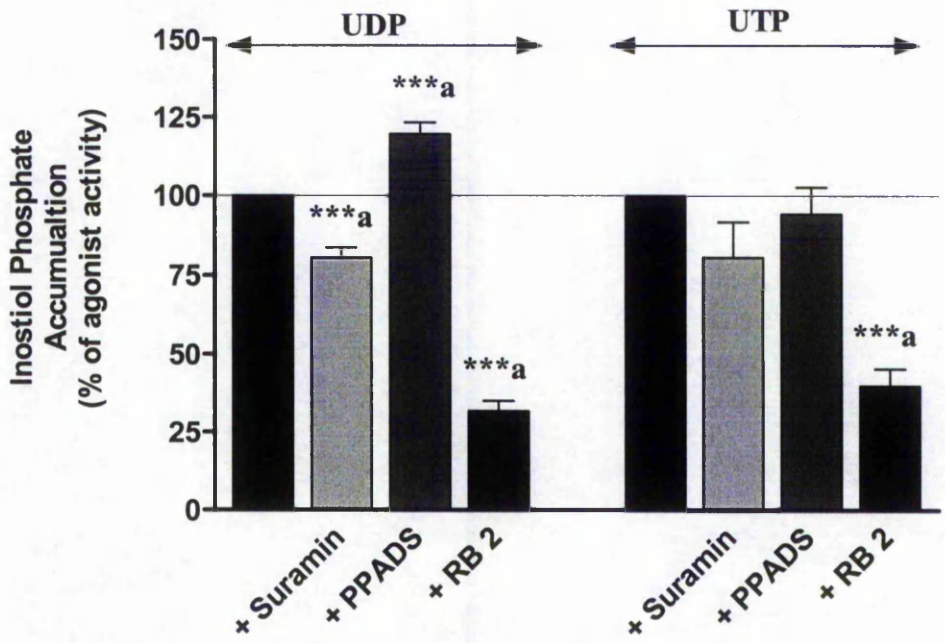

Figure 3.12: Effect of classical non-selective P2 receptor antagonists on inositol phosphate accumulation observed with uracil nucleotides. [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with suramin (100μM), PPADS (100μM) and RB 2 (100μM) for 30 minutes before stimulating with uracil nucleotides. UDP (100μM) and UTP (100μM) produced an increase in [^{3}H]-IP production by 450% and 375% over the basal activity. Data was expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate. a versus the agonist response in absence of antagonist, ** P<0.01 and *** P<0.001

However, the 1μM 2-MeSATP-induced [^{3}H]-IP increase was not antagonised by MRS2179 (Figure 3.13B, Table 3.2). As shown in Figure 3.13A, the effect of AMP (10μM) on [^{3}H]-IP production was not blocked by MRS2179. These data strongly suggest that the $P2Y_1$ receptor is functionally expressed on neonatal rat cardiac fibroblasts.

Previous studies have shown that MRS2179 also blocks the response at $P2X_1$ and $P2X_3$ ion-channel receptors (Brown *et al.,* 2000). It is noteworthy that the [^{3}H]-IP accumulation observed with UTP (100μM) was partially inhibited by MRS2179 (20%) in a concentration-dependent manner (Figure 3.14, Table 3.2), suggesting that UTP (100μM) activates $P2X_{1,3}$ receptors (McLaren *et al.,* 1998; Rae *et al.,* 1998). However, the [^{3}H]-IP stimulation with UDP was unaffected by MRS2179 (Figure 3.14, Table 3.2). The rank order of MRS2179 potencies to antagonise agonist-induced [^{3}H]-IP accumulation was ADP-βs $\approx$ ATP-γS > 2-MeSADP > UTP.

Concentration-inhibition curves were also constructed with ATP-γS, UTP and UDP in the presence of MRS2578 to further pharmacologically characterise the expression of the $P2Y_6$ receptor. ATP-γS, UDP and UTP-mediated [^{3}H]-IP production was antagonised by the MRS2578 (Figure 3.15, Table 3.3). The inhibition of ATP-γS and UDP-induced [^{3}H]-IP accumulation by MRS2578, produced bell-shaped inhibition curves which are a characteristic feature of positive cooperative interactions (Figure 3.15; Swillens *et al.,* 1995), suggesting an interaction between different P2Y receptor subtypes. Indeed, such an interaction has been reported for $P2Y_1$ and $P2Y_2$ receptors with the adenosine A_1 receptor (Yoshioka *et al.,* 2001, 2004; Suzuki *et al.,* 2006). These data further strengthen the idea that UDP activates two different P2Y receptors (Table 3.3).

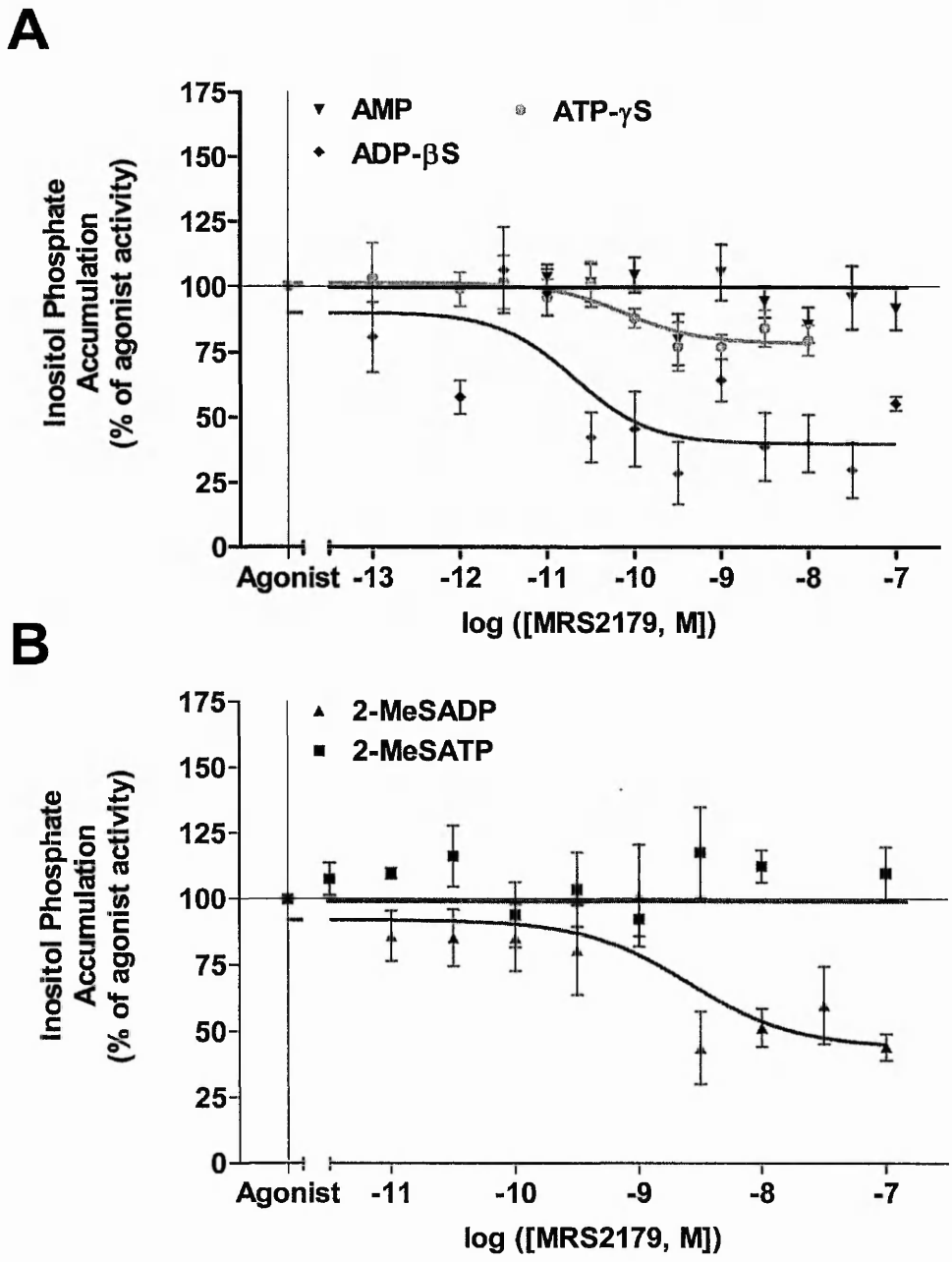

Figure 3.13: Effect of the $P2Y_1$ receptor selective antagonist MRS2179 on inositol phosphate (IP) accumulation induced by adenine nucleotides. [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with the indicated concentrations of MRS2179 for 30 minutes before stimulating with AMP (10μM), ADP-βS (10μM), ATP-γS (100μM, **Panel A**) and 2-MeSADP (0.1μM) and 2-MeSATP (1μM, **Panel B**) induced an increase in [^{3}H]-IP production by 40%, 440%, 33% and 33% over the basal activity, whereas AMP induced inhibition of basal [^{3}H]-IP accumulation by 30%. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate.

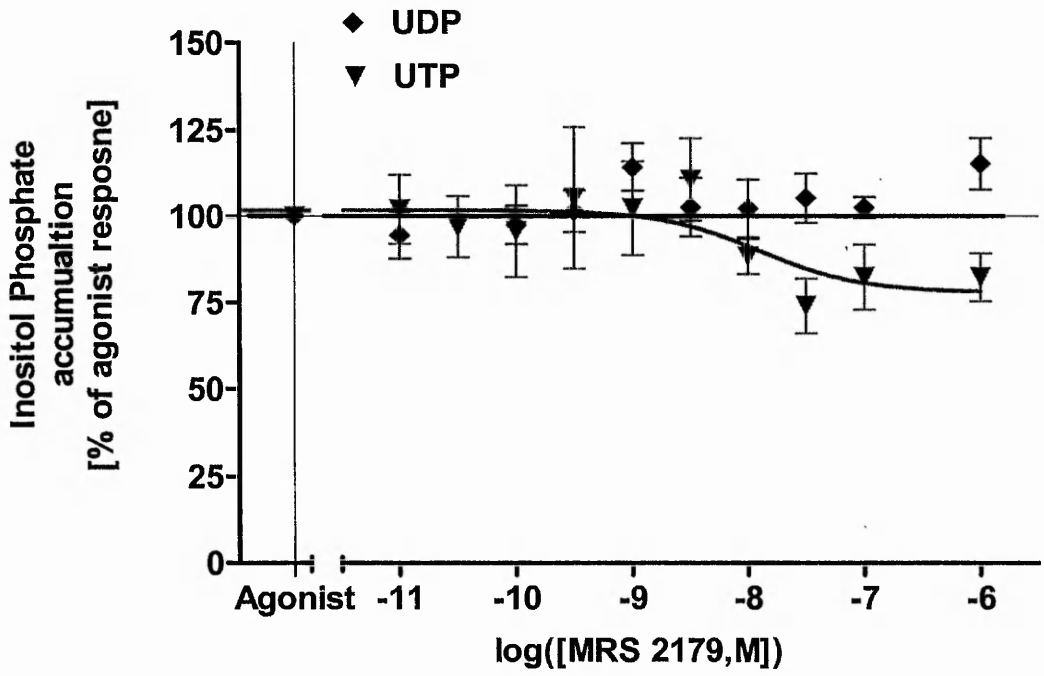

Figure 3.14: Effect of the $P2Y_1$ receptor selective antagonist MRS2179 on inositol phosphate (IP) accumulation induced by uracil nucleotides. [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with the indicated concentrations of MRS2179 for 30 minutes before stimulating with UDP (100μM) and UTP (100μM). UDP and UTP induced an increase in [^{3}H]-IP production by 450% and 300% over the basal activity. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 4 experiments from separate cell cultures, performed in duplicate.

Table 3.2: Adenine and uracil nucleotide induced potencies and maximal responses in the presence of MRS2179 ($P2Y_1$ receptor antagonist) on P2Y receptors at multiple effector pathways in cardiac fibroblasts.

Agonist	**G_s**				**G_i**				**G_q**				**Forskolin-induced cAMP accumulation** (Gs pathway)			
	n	IC_{50}	I_{max}		n	IC_{50}	I_{max}		n	IC_{50}	I_{max}		n	IC_{50}	I_{max}	
AMP	3	NR	NR	-					4	NR	NR	-	3	NR	NR	-
ADP-βS	3	NR	NR	-					4	10.7±0.4 (0.003)	48±5	↓↓	3	NR	NR	-
ATP-γS	3	NR	NR	-					3	10.1±0.4 (0.1)	23±1	↓	3	NR	NR	-
MeSADP	-	-	-	-					3	8.8±0.6 (10)	40±9	↓↓	-	-	-	-
MeSATP	-	-	-	-					3	NR	NR	-	-	-	-	-
UDP					*3*	*NR*	*NR*	-	3	NR	NR	-				
UTP					*3*	*NR*	*NR*	-	3	7.6±0.7 (10)	20±2	↓				

Values given for IC_{50} and I_{max} are expressed as mean ± S.E.M from n independent experiments, performed in duplicate. Values in parentheses are IC_{50} values expressed in nM. Values in italics are potencies and maximal responses (percentage of that obtained with forskolin/agonist – decrease in adenylyl cyclase activity) of uracil nucleotides through the $G_{i/o}$ pathway. NR- no response, ↓ - decrease.

Table 3.3: ATP-γS and uracil nucleotide induced potencies and maximal responses in the presence of MRS2578 ($P2Y_6$ receptor antagonist) on P2Y receptors at multiple effector pathways in neonatal rat cardiac fibroblasts.

Agonists	G_s				G_i				G_q						
	n	I_{max}	IC_{50}		n	I_{max}	IC_{50}		n	E_{max}	EC_{50}		I_{max}	IC_{50}	
ATP-γS	3	NR	NR	-					4	18±2	7.6±0.1 (25.1)	↑	84±7	5.4±0.1 (3980)	↓↓↓
UDP					*5*	*NR*	*NR*	-	4	35±6	7.3±0.1 (50.1)	↑↑	71±6	5.3±0.2 (5010)	↓↓↓
UTP					*5*	*NR*	*NR*	-	4				87±11	5.1±0.1 (7940)	↓↓↓

Values given for IC_{50} or EC_{50} and I_{max} or E_{max} are expressed as mean ± S.E.M from n independent experiments, performed in duplicate. Values in parentheses are IC_{50} values expressed in nM. Values in italics are potencies and maximal responses (percentage of that obtained with forskolin/agonist – decrease in adenylyl cyclase activity) of uracil nucleotides through the G_i pathway. NR- no response, ↑ - increase, ↓ - decrease.

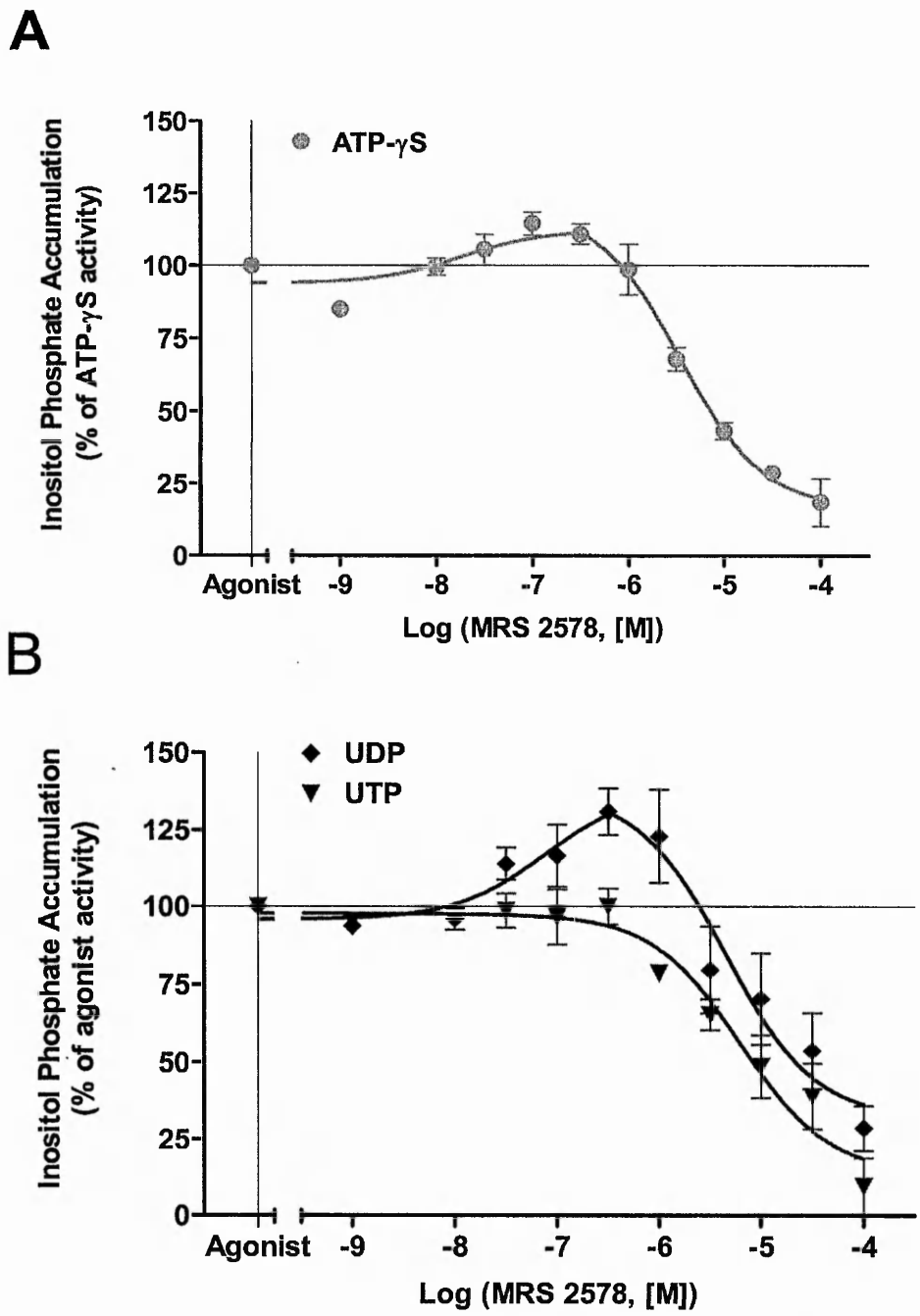

Figure 3.15: Effect of the $P2Y_6$ receptor selective antagonist MRS2578 on inositol phosphate (IP) accumulation induced by ATP-γS **(Panel A)** and uracil nucleotides **(Panel B)**. [^{3}H]-inositol labelled and serum-starved fibroblasts were incubated with the indicated concentrations of MRS2578 for 30 minutes before stimulating with ATP-γS (100μM), UDP (100μM) and UTP (100μM). ATP-γS, UDP and UTP induced an increase in [^{3}H]-IP production by 420%, 450% and 300% over the basal activity. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 4 experiments from separate cell cultures, performed in duplicate.

Figure 3.3

3.4.2 Effect of antagonists on [^{3}H]-cAMP accumulation induced by extracellular nucleotides

Figure 3.16 illustrates the effect of the non-selective P2 antagonists on basal levels of [^{3}H]-cAMP accumulation and forskolin-induced [^{3}H]-cAMP production. 100μM RB 2 inhibited both the basal level of [^{3}H]-cAMP production (39%, $P<0.001$) and forskolin-induced [^{3}H]-cAMP synthesis (52%, $P<0.001$), whereas 100μM PPADS only blocked the forskolin-induced [^{3}H]-cAMP production (30%, $P<0.001$). 100μM suramin had no effect on cAMP accumulation (Figure 3.16). The inhibitions seen with PPADS and RB 2 were removed and the response with agonists corrected accordingly. Suramin, PPADS and RB 2 had no effect on AMP-induced [^{3}H]-cAMP accumulation and also on forskolin augmented [^{3}H]-cAMP responses. Suramin and RB 2 partially blocked the ADP-βS-mediated [^{3}H]-cAMP synthesis ($P<0.001$ and $P<0.001$), yet these antagonists had no effect on ADP-βS induced forskolin-potentiated [^{3}H]-cAMP (Figure 3.17). PPADS did not antagonise the ADP-βS response on basal [^{3}H]-cAMP production, however potentiated the forskolin-stimulated [^{3}H]-cAMP production by ADP-βS ($P<0.001$, Figure 3.17). Suramin partly inhibited the ATP-γS response on basal ($P<0.05$) and forskolin-potentiated [^{3}H]-cAMP production ($P<0.01$). PPADS and RB 2 only blocked the basal [^{3}H]-cAMP production ($P<0.01$, $P<0.001$) and were ineffective on forskolin-mediated [^{3}H]-cAMP synthesis by ATP-γS (Figure 3.17). These observations support the functional expression of a rat $P2Y_{11}$-like receptor since the human $P2Y_{11}$ receptor is sensitive to suramin and RB 2 but not to PPADS (Communi *et al.*, 1999).

The non-selective P2 antagonists had no effect on UDP induced inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation (Figure 3.18). However, UTP mediated inhibition of forskolin-triggered [^{3}H]-cAMP accumulation was sensitive to PPADS ($P<0.05$) and resistant to RB 2. It is noteworthy, that suramin did not antagonise but in fact potentiated the inhibition of forskolin-stimulated [^{3}H]-cAMP production by UTP ($P<0.05$, Figure 3.18).

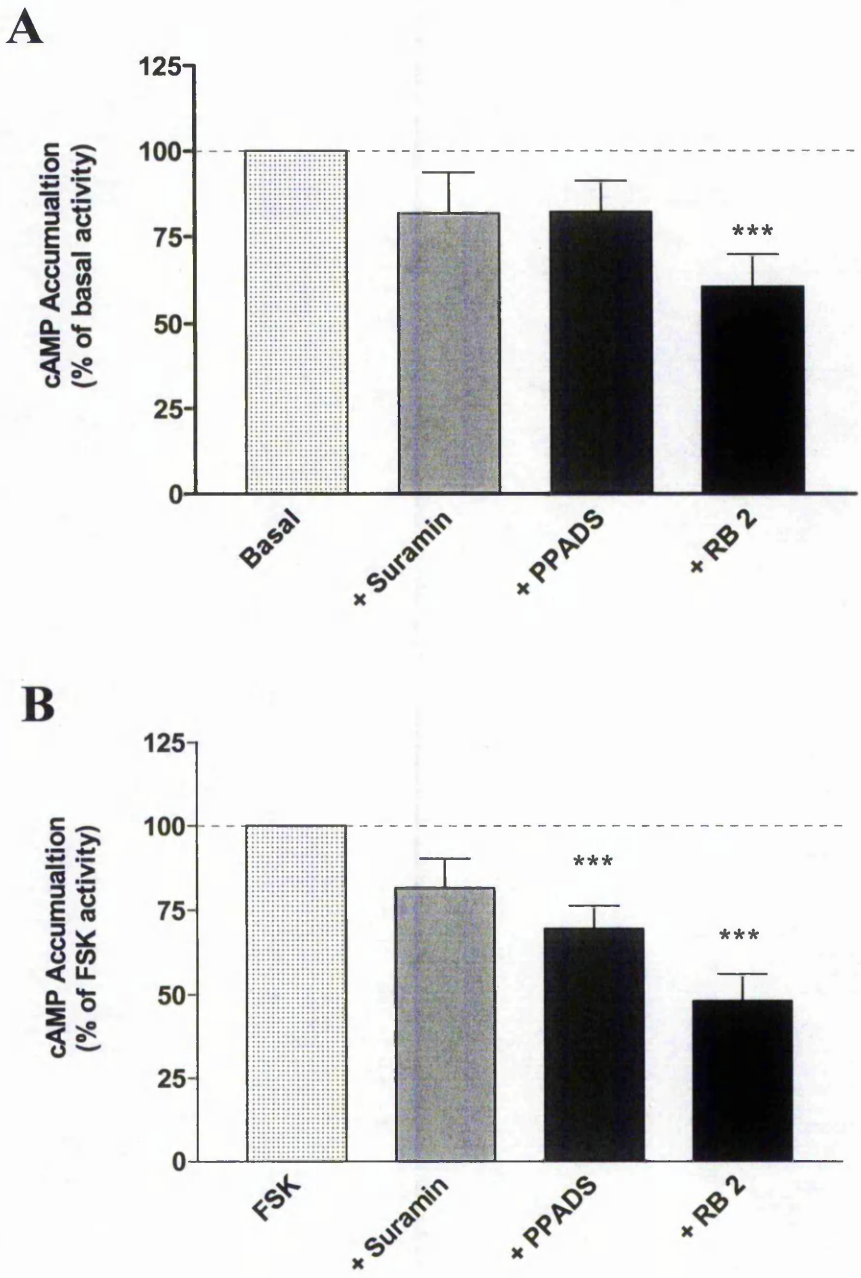

Figure 3.16: Effect of non-selective P2 receptor antagonists on cAMP accumulation. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and incubated with 10μM rolipram and suramin (100 μM), PPADS (100μM) and RB 2 (100μM) with **(Panel A)** or without stimulation with 1.5μM forskolin (FSK; **Panel B**). Data were expressed as the percentage of the basal (A) or forskolin (B) response (100%). Each point represents the mean ± S.E.M for 4-6 experiments from separate cell cultures, performed in duplicate. *** P<0.001 versus basal or forskolin response.

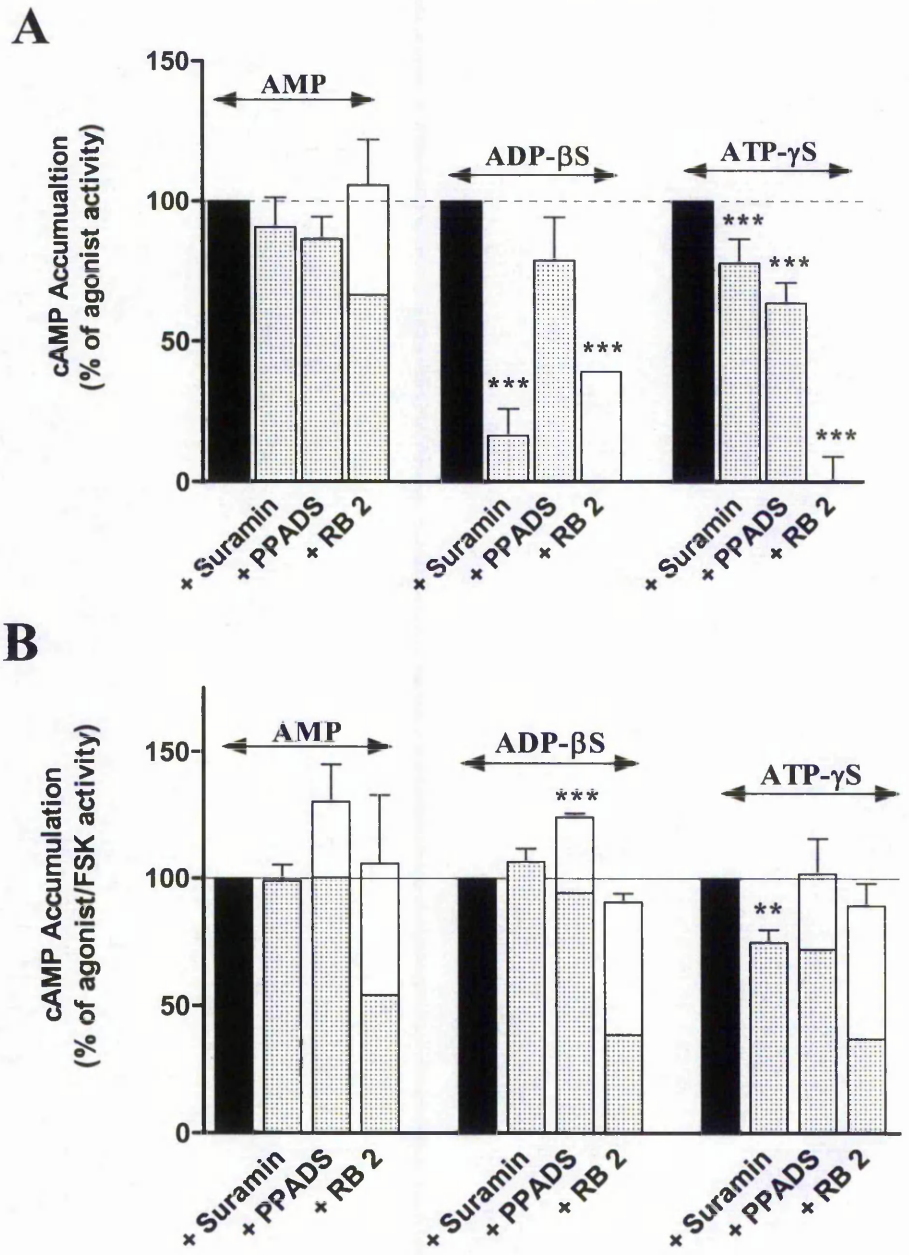

Figure 3.17: Effect of non-selective P2 receptor antagonists on cAMP accumulation induced by adenine nucleotides in the absence **(Panel A)** and presence of 1.5μM forskolin **(Panel B)**. Serum-starved fibroblasts were labelled with [^{3}H]-adenine and incubated with suramin (100μM), PPADS (100μM) and RB 2 (100μM) for 30 minutes before stimulating with adenine nucleotides. AMP (100μM), ADP-βS (100μM) and ATP-γS (100μM) induced cAMP production by 600%, 90% and 200% over the basal activity. AMP, ADP-βS and ATP-γS augmented forskolin-induced cAMP accumulation by 1000%, 900% and 600% over the forskolin activity. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 3-6 experiments from separate cell cultures, performed in duplicate. Filled

bars indicate the data from agonists in combination with antagonists and open bars represents the data after removing the effect of the antagonists on basal or forskolin-stimulated cAMP accumulation. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ versus the agonist response in the absence of antagonist.

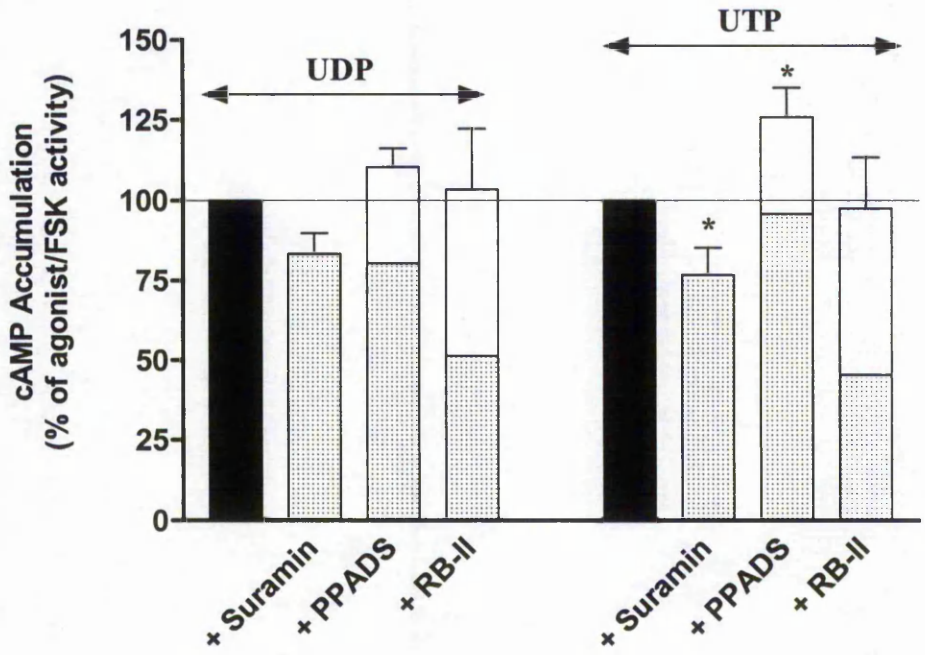

Figure 3.18: Effect of non-selective P2 receptor antagonists on inhibition of forskolin (FSK)-stimulated cAMP accumulation observed with uracil nucleotides. Serum-starved cardiac fibroblasts were labelled with [^{3}H]-adenine and incubated with suramin (100μM), PPADS (100μM) and RB 2 (100μM) for 30 minutes before stimulating with uracil nucleotides. UDP (100μM) and UTP (100μM) produced an inhibition of forskolin-induced cAMP production by 50% and 80%. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 4-5 experiments from separate cell cultures, performed in duplicate. Filled bars indicate the data from agonists in combination with antagonists and open bars represents the data after removing the effects of the antagonists on the forskolin-stimulated cAMP accumulation. * P<0.05 versus the agonist response in the absence of antagonist.

MRS2179, the $P2Y_1$ antagonist had no effect on 100μM AMP, 100μM ADP-βS and 100μM ATP-γS induced [^{3}H]-cAMP accumulation (Figure 3.19A, Table 3.2). Similarly, AMP (100μM), ADP-βS (100μM) and ATP-γS (100μM) mediated augmentation of forskolin-induced [^{3}H]-cAMP production was not antagonised by MRS2179 (Figure 3.19B, Table 3.2). Moreover, MRS2179 did not block UDP (100μM) and UTP (100μM) induced inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation (Figure 3.20, Table 3.2). These observations indicate that the $P2Y_1$ receptor was not involved in [^{3}H]-cAMP accumulation mediated by AMP, ADP-βS, ATP-γS or inhibition mediated via UDP and UTP. As depicted in figure 3.21A, the $P2Y_6$ receptor antagonist MRS2578 did not antagonise the 100μM ATP-γS induced [^{3}H]-cAMP accumulation. UDP (100μM) and UTP (100μM)-induced inhibitions of forskolin-response were unaffected by MRS2578 (Figure 3.21B and Table 3.3). In addition, the $P2Y_6$ antagonist inhibited by 36% forskolin-induced cAMP accumulation indicating that this antagonist has a direct effect on adenylyl cyclase activity (Figure 3.21B). These results suggest that $P2Y_1$ and $P2Y_6$ receptor-induced responses involve only G_q protein pathway(s).

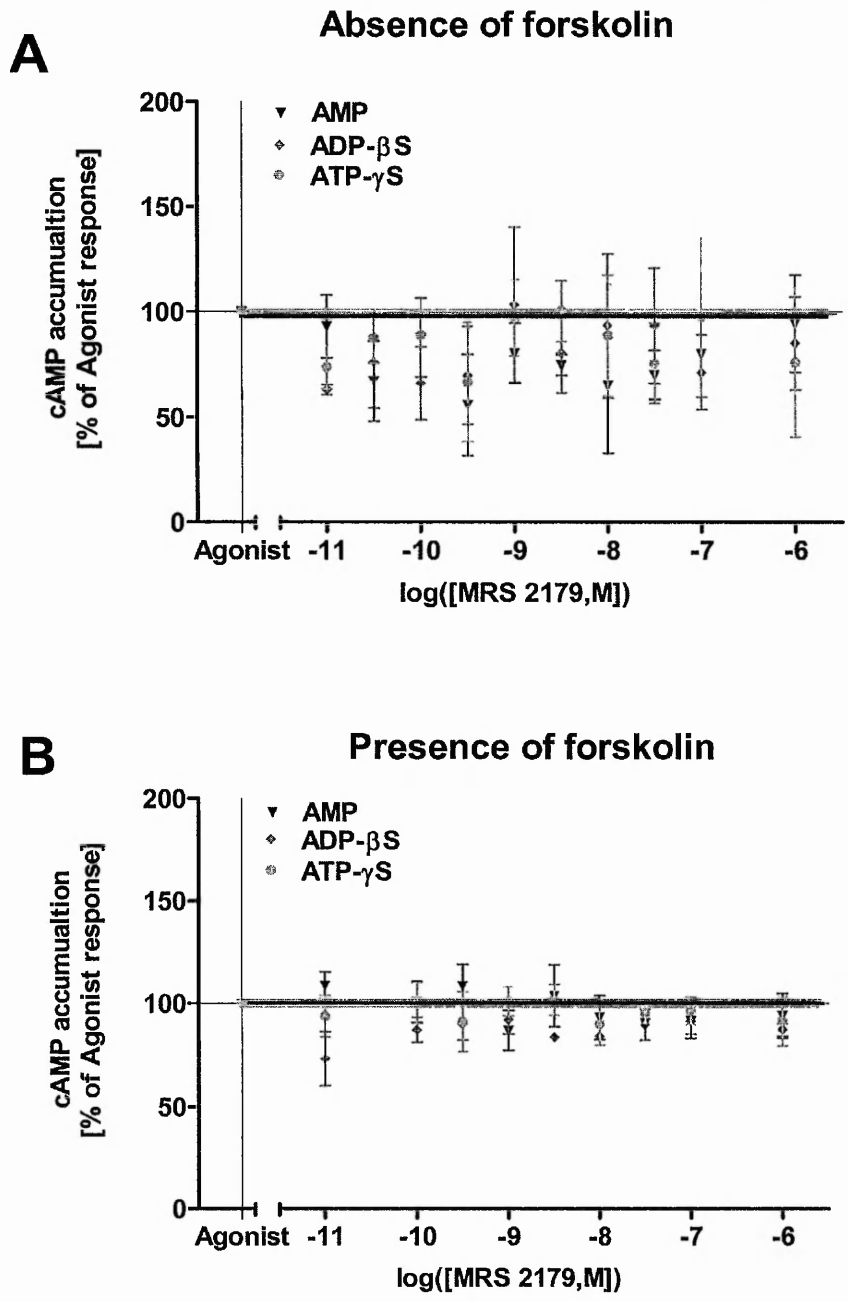

Figure 3.19: Effect of the $P2Y_1$ receptor selective antagonist MRS2179 on cAMP accumulation induced by adenine nucleotides - AMP (100μM), ADP-βS (100μM) and ATP-γS (100μM) in the absence **(Panel A)** and in the presence of 1.5 μM forskolin **(Panel B)**. Serum-starved cardiac fibroblasts were incubated with the indicated concentrations of MRS2179 for 30 minutes before stimulating with adenine nucleotides. AMP, ADP-βS and ATP-γS induced cAMP production by 600%, 90% and 200% over the basal activity. AMP, ADP-βS and ATP-γS augmented forskolin-induced cAMP accumulation by 1000%, 900% and 600% over the forskolin activity. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 3-4 experiments from separate cell cultures, performed in duplicate.

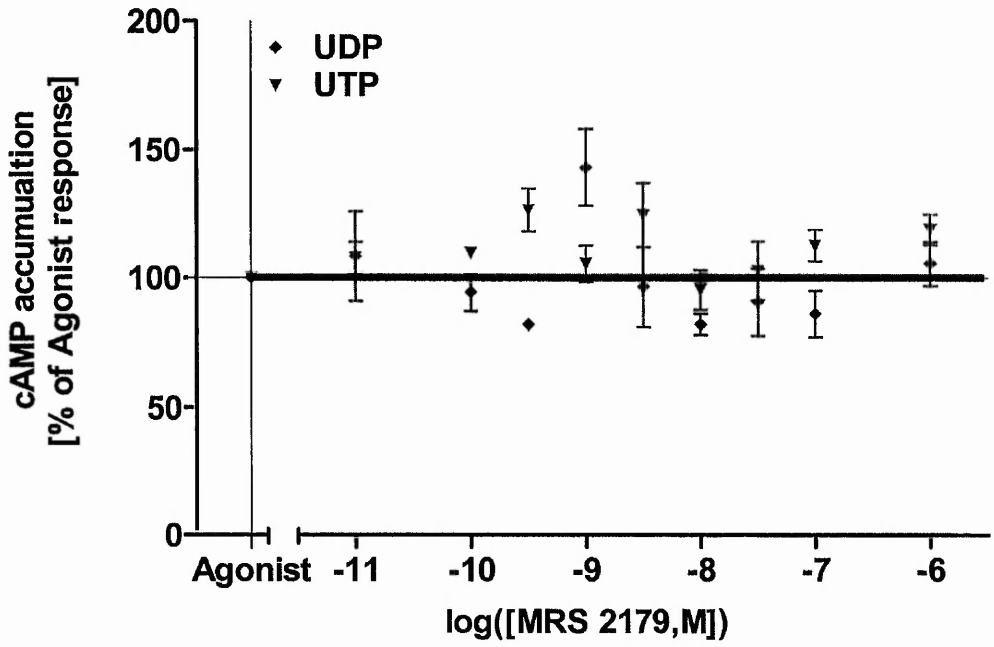

Figure 3.20: Effect of the $P2Y_1$ receptor selective antagonist, MRS2179 on inhibition of forskolin-stimulated cAMP accumulation observed with uracil nucleotides. UDP (100μM) and UTP (100μM) produced an inhibition of forskolin-induced cAMP production by 50% and 80%. Data were expressed as the percentage of agonist activity (100%). Each point represents the mean ± S.E.M for 3 experiments from separate cell cultures, performed in duplicate.

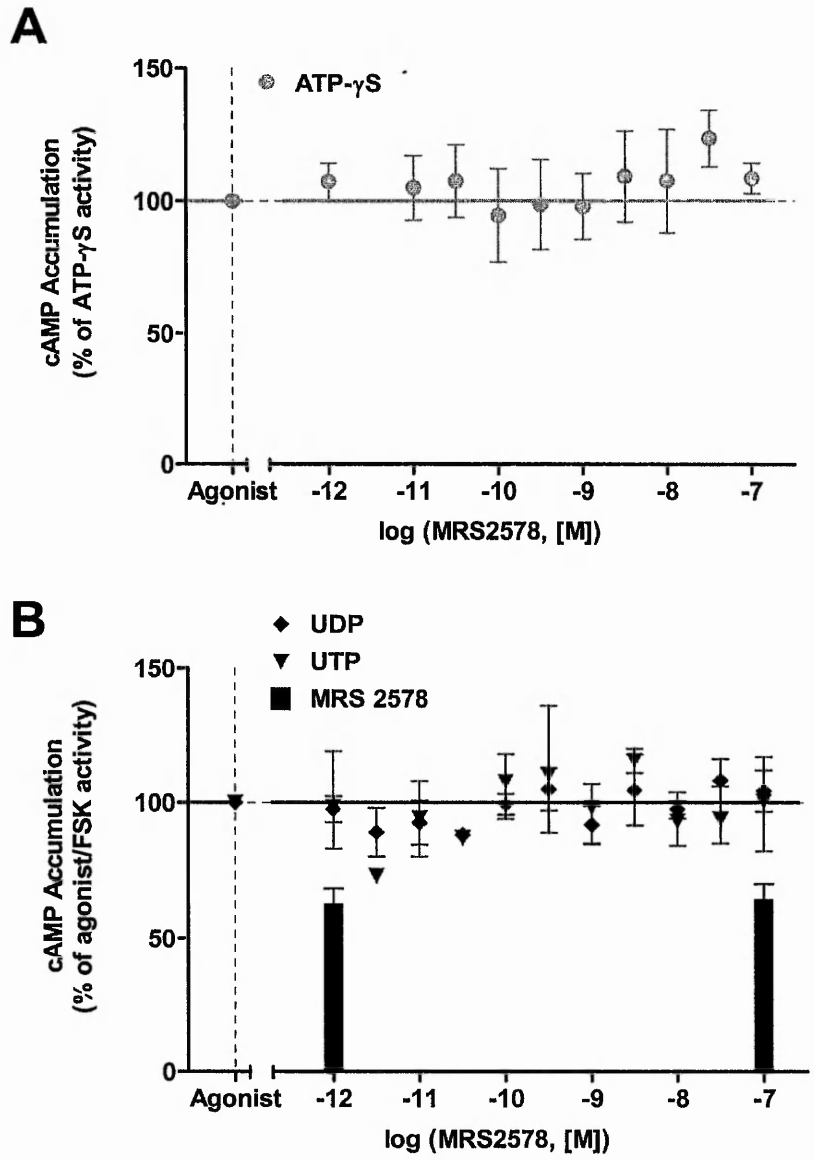

Figure 3.21: Effect of the $P2Y_6$ receptor selective antagonist, MRS2578 on ATP-γS induced cAMP accumulation **(Panel A)** and uracil nucleotide mediated inhibition of forskolin-stimulated cAMP accumulation **(Panel B)**. Serum-starved fibroblasts were incubated for 30 minutes with the indicated concentrations of MRS2578 before stimulating for 15 minutes with 100μM ATP-γS or for 5 minutes either with 100μM UDP and 100μM UTP prior to stimulation with 1.5μM forskolin (FSK). The bar graphs in panel B represent the inhibition of forskolin-induced [^{3}H]-cAMP accumulation by 100nM and 0.001nM MRS2578 (35%). The response of UDP and UTP were corrected accordingly. ATP-γS induced an increase in cAMP accumulation by 380% above basal activity and UDP and UTP produced an inhibition of forskolin-induced cAMP production by 50% and 80%. Data were expressed as the percentage of the agonist-

induced cAMP production in the absence of antagonist (100%) and represent the mean ± S.E.M of 3-5 independent experiments each performed in duplicate.

3.5 Effect of signal transduction pathway inhibitors on adenine and uracil nucleotide-induced responses in rat neonatal cardiac fibroblasts

The results from the concentration-response curves (Figures 3.3 – 3.6, 3.8 – 3.9), indicate that P2Y receptors are coupled to G_q (activation of IP), G_s (stimulation of cAMP accumulation) and G_i (inhibition of forskolin-induced cAMP response) proteins. In order, to investigate the mechanisms involved in P2Y receptor induced [^{3}H]-IP and [^{3}H]-cAMP activation, neonatal rat cardiac fibroblasts were pre-treated with pertussis toxin (PTX; 100ng/ml, 18 hours) and YM-254890 (YM; 1μM, 30 minutes). PTX is used to characterise the functional response of receptors coupled to $G_{i/o}$ protein. PTX catalyses the ADP-ribosylation of $G_{i/o}$ protein (PTX sensitive G-proteins) and thereby prevents them from interacting with the receptors (Bokoch *et al.,* 1983). However G_s, and G_q proteins are resistant to PTX. YM is a novel *Chromobacterium sp.,* toxin which selectively inhibits $G_{q/11}$ coupled receptors by acting on the exchange of GDP to GTP in $G_{q/11}$ receptor activation (Taniguchi *et al.,* 2004).

3.5.1 Effect of signal transduction pathway inhibitors on [^{3}H]-IP production

As shown in Figure 3.22A, YM completely blocked [^{3}H]-IP accumulation induced by 10μM ADP-βS (P<0.01, n=4), 100μM ATP-γS (P<0.001, n=4), 0.1μM 2-MeSADP (P<0.01, n=6) and 1μM 2-MeSATP (P<0.01, n=4). Moreover, YM blocked the inhibition of [^{3}H]-IP production observed with 10μM AMP in a significant manner (P<0.05, n=4; Figure 3.22A). Several studies have shown that $\beta\gamma$ subunits released following G_i-protein coupled receptor activation, stimulate PLC and potentiate second messengers mediated by G_q such as IP and Ca^{2+} mobilisation (Bakker *et al.,* 2004; Quitterer and Lohse 1999; Tomura *et al.,* 1997). In order, to investigate the direct activation of G_q-coupled P2Y receptors experiments were carried out in presence of the G_i blocker PTX. Pretreatment of fibroblasts with PTX had no significant effect on [^{3}H]-IP production induced by AMP, ADP-βS, ATP-γS and 2-MeSATP (Figure 3.22A), confirming the involvement of only G_q-proteins in [^{3}H]-IP production.

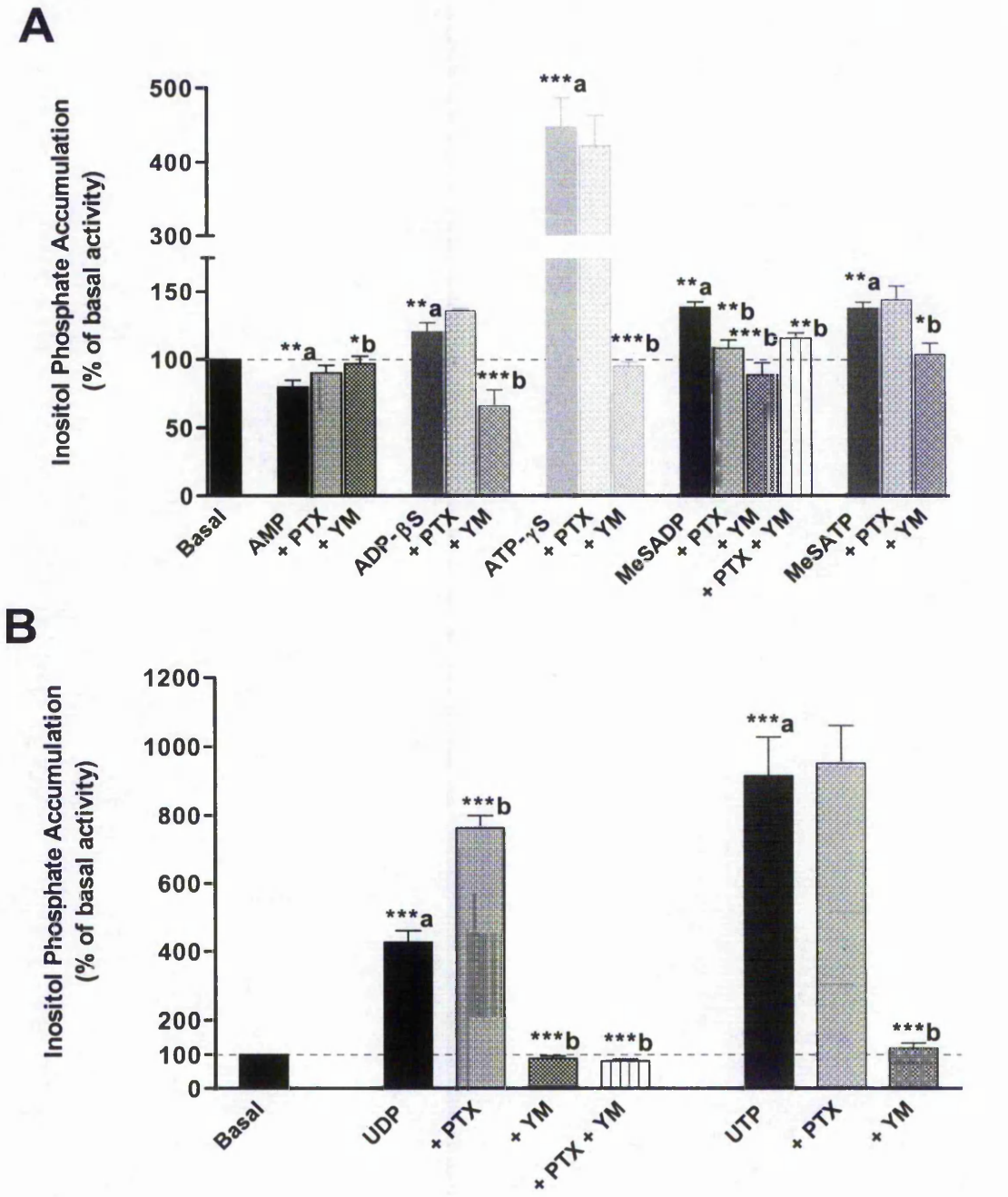

Figure 3.22: Effect of $G_{i/o}$ (pertussis toxin; PTX) and $G_{q/11}$ (YM-254890; YM) protein inhibition on adenine and uracil nucleotide induced inositol phosphate accumulation (G_q pathway). [^{3}H]-inositol labelled and serum-starved fibroblasts were pre-treated for 18 hours with 100ng/ml PTX ($G_{i/o}$ inhibitor) and for 30 minutes with 1μM YM ($G_{q/11}$ blocker) before stimulating for 30 minutes with 10μM AMP, 10μM ADP-βS, 100μM ATP-γS, 0.1μM MeSADP and 1μM MeSATP **(Panel A)** or 32μM UDP and 100μM UTP **(Panel B)**. Data were expressed as a percentage of basal activity (100%). Each point represents the mean ± S.E.M of 4 to 7 independent experiments, performed in duplicate. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$; a versus basal activity, b versus agonist activity in the absence of PTX or YM.

Interestingly, the accumulation of [^{3}H]-IP observed with 2-MeSADP was significantly inhibited by PTX (P<0.01, n=6; Figure 3.22A), suggesting a possible cross-talk between $G_{i/o}$ and $G_{q/11}$-coupled receptors. [^{3}H]-IP accumulation induced by 32μM UDP (P<0.001, n=7) and 100 μM UTP (P<0.001, n=4) was significantly blocked by the $G_{q/11}$ inhibitor, YM (Figure 3.22B). No significant effect on [^{3}H]-IP accumulation was observed with UTP in PTX treated fibroblasts (Figure 3.22B). In marked contrast, UDP induced 2-fold increase in [^{3}H]-IP production (P<0.001, n=7; Figure 3.22B) in fibroblasts pre-treated with PTX, suggesting a possible interaction between $G_{i/o}$ and $G_{q/11}$-proteins, which may also explain the biphasic concentration-response curve obtained for UDP (Figure 3.4). To further investigate this potential interaction between $G_{i/o}$ and G_q, measurements of [^{3}H]-IP accumulation were performed in the presence of both PTX + YM. UDP induced accumulation of [^{3}H]-IP was completely blocked by combination of PTX and YM (P<0.001, n=6; Figure 3.22B).

3.5.2 Effect of signal transduction pathway inhibitors on [^{3}H]-cAMP production

As shown in Figure 3.23A, PTX and YM had no significant effect on 100μM AMP, 100μM ADP-βS and 100μM ATP-γS induced [^{3}H]-cAMP production in cardiac fibroblasts. Similarly, the augmentation of forskolin-induced [^{3}H]-cAMP accumulation observed with 100μM AMP, 10μM ADP-βS and 100μM ATP-γS was not altered by PTX and YM toxins (Figure 3.23B). cAMP accumulation assay in the presence of PTX and YM was not carried out for 2-MeSADP and 2-MeSATP since these nucleotides did not induce [^{3}H]-cAMP accumulation (see Figures 3.5B and 3.8B, Table 3.1) .

Cardiac fibroblasts are known to express calcium-sensitive AC isoforms (AC-5 and AC-6; Swaney *et al.,* 2003). The inhibition of forskolin-stimulated cAMP accumulation observed with uracil nucleotides could be a result of an increase in intracellular Ca^{2+} through G_q activation. However, as shown in Figure 3.25 PTX pre-treatment completely abolished the inhibition of forskolin mediated [^{3}H]-cAMP production induced by both uracil nucleotides (UDP: P<0.001, n=8; UTP: P<0.001, n=5). Interestingly, 100μM UDP induced inhibition of forskolin-stimulated [^{3}H]-cAMP production was also sensitive to YM (P<0.01, n=8, Figure 3.24). Moreover, the UDP-mediated inhibition of forskolin response was significantly blocked by PTX and YM (P<0.001, n=5; Figure 3.24). The G_q inhibitor had no significant effect on UTP induced inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation (see Figure 3.24).

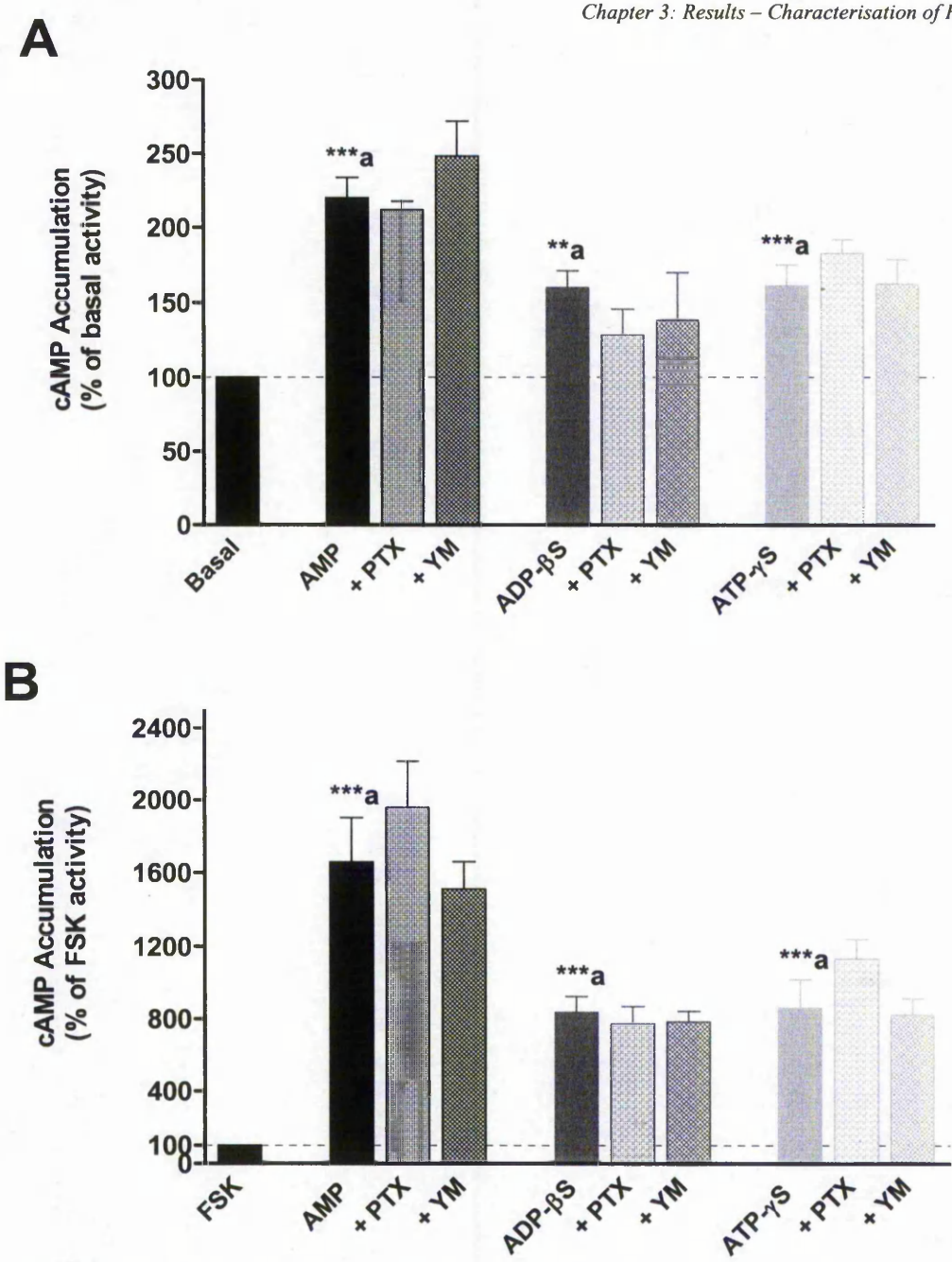

Figure 3.23: Effect of $G_{i/o}$ (pertussis toxin; PTX) and $G_{q/11}$ (YM-254890; YM) protein inhibition on adenine nucleotide induced cAMP accumulation and forskolin-induced cAMP accumulation. Serum-starved cardiac fibroblasts were pre-treated for 18 hours with 100ng/ml PTX ($G_{i/o}$ inhibitor) and for 30 minutes with 1μM YM ($G_{q/11}$ blocker) before stimulating for 15 minutes with 100μM AMP, 10μM ADP-βS and 100μM ATP-γS **(Panel A)** or for 5 minutes with 100μM AMP, 10μM ADP-βS and 100μM ATP-γS followed by 10 minutes stimulation with 1.5μM forskolin (FSK; **Panel B).** Data were expressed as a percentage basal activity (Panel A) or FSK activity (Panel B). Each point represents the mean ± S.E.M of four to five independent experiments performed in duplicate. ** P<0.01, *** P<0.001; a versus basal or FSK activity.

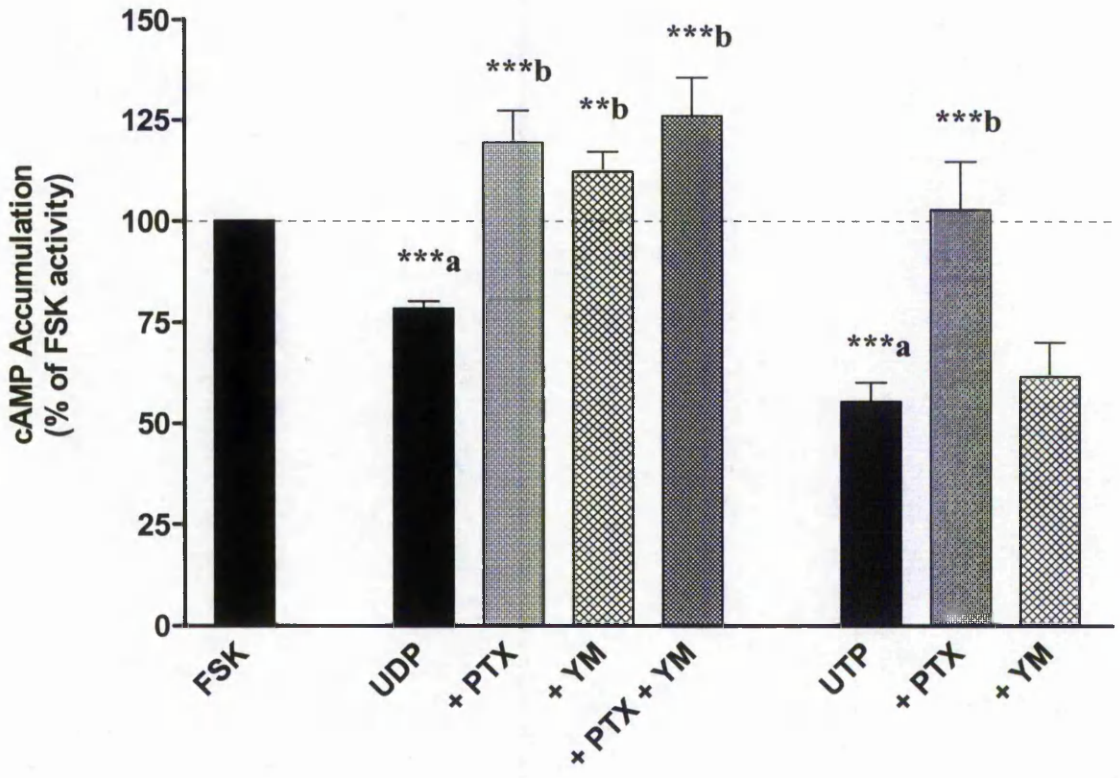

Figure 3.24: Effect of $G_{i/o}$ (pertussis toxin; PTX) and $G_{q/11}$ (YM-254890; YM) protein inhibition on uracil nucleotide induced inhibition of forskolin (FSK) stimulated cAMP production (G_i pathway). Serum-starved cardiac fibroblasts were pre-treated for 18 hours with 100ng/ml PTX ($G_{i/o}$ inhibitor) and for 30 minutes with 1μM YM ($G_{q/11}$ blocker) before stimulating for 5 minutes with 100μM UDP, 100μM UTP followed by 10 minutes stimulation with 1.5μM FSK. Data were expressed as a percentage of FSK activity (100%). Each point represents the mean ± S.E.M of five to eight independent experiments performed in duplicate. ** P<0.01, *** P<0.001; a versus FSK activity and b versus agonist activation in the absence of inhibitors.

These observations indicate that UTP-mediated inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation involves only $G_{i/o}$-coupled P2Y receptors, whereas responses to UDP involve receptor(s) coupled to G_q and G_i proteins in neonatal rat cardiac fibroblasts.

3.5.3 Role of PLC and PKA in AMP induced responses in neonatal rat cardiac fibroblasts

As described in section 3.3, AMP induced a significantly higher [^{3}H]-cAMP accumulation response compared to the other adenine nucleotides. In addition, AMP also inhibited basal [^{3}H]-IP generation. Lagalia *et al.,* (1996) and Yue *et al.,* (1998) have shown that cAMP and PKA can inhibit IP accumulation. Indeed, the PKA inhibitor (KT5720) reversed the inhibition of basal [^{3}H]-IP production mediated by AMP (P<0.05, Figure 3.25). Moreover, the ability of AMP to directly stimulate [^{3}H]-cAMP and potentiate forskolin-induced [^{3}H]-cAMP accumulation were sensitive to the PLC blocker, U73122 (P<0.001, Figure: 3.26). These data suggest a possible interaction between PLC and PKA-dependent pathways.

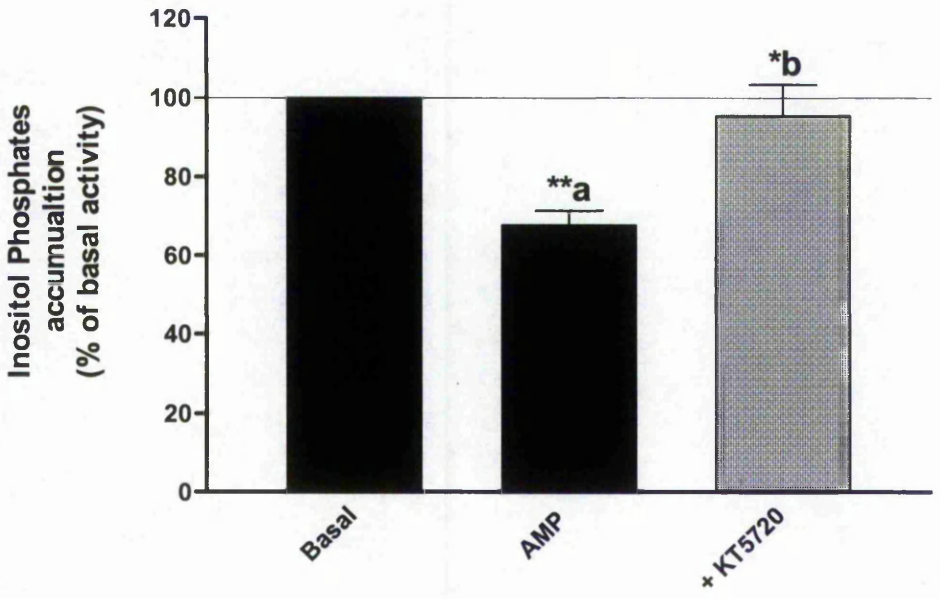

Figure 3.25: Effect of the PKA inhibitor (KT5720) on AMP induced inhibition of basal inositol phosphate accumulation (G_q pathway). [^{3}H]-inositol labelled and serum-starved fibroblasts were pre-treated for 30 minutes with 1μM KT5720 before stimulating for 30 minutes with 10μM AMP. Data were expressed as a percentage of basal activity (100%). Each point represents the mean ± S.E.M of four independent experiments, performed in duplicate. * $P<0.05$ and ** $P<0.01$; a versus basal activity, b versus agonist activity in the absence of KT5720.

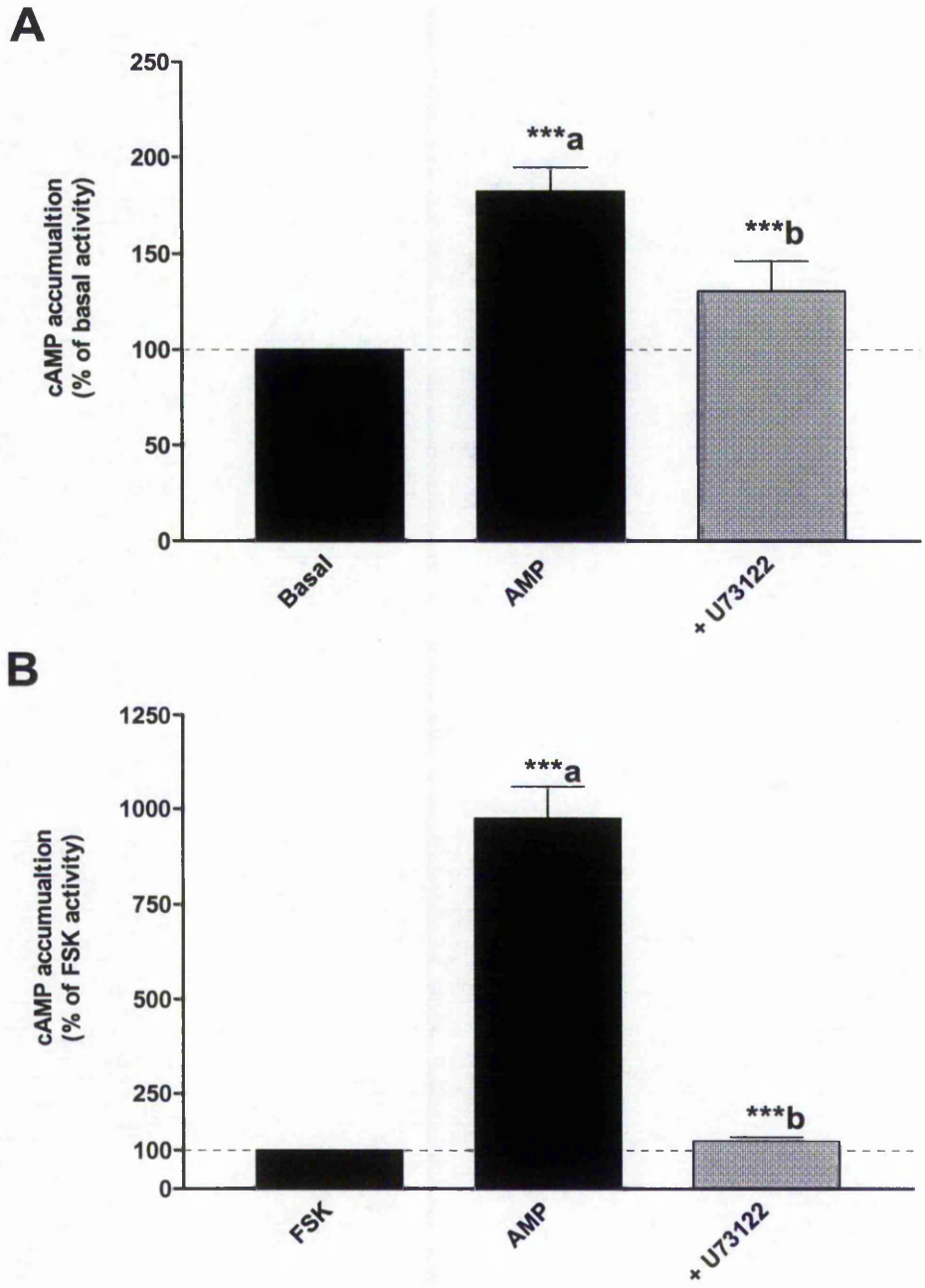

Figure 3.26: Effect of the PLC inhibitor U73122 on AMP induced cAMP accumulation. Serum-starved cardiac fibroblasts were pre-treated for 30 minutes with 10μM U73122 before stimulating for 15 minutes with 100μM AMP **(Panel A)** and for 5 minutes with 100μM AMP followed by 10 minutes stimulation with 1.5μM forskolin (FSK; **Panel B**). Data were expressed as a percentage of basal activity (Panel A) or FSK activity (Panel B). Each point represents the mean ± S.E.M of four to five independent experiments performed in duplicate. *** $P<0.001$; a versus basal or FSK activity and b versus AMP response.

Chapter 3: Discussion – Characterisation of P2Y receptors in neonatal rat cardiac fibroblasts

3.6 P2Y receptors expressed in neonatal rat cardiac fibroblasts

The P2Y receptors belong to the superfamily of G-protein coupled receptors (GPCR). The $P2Y_1$ receptor was the first identified receptor for purine nucleotides (Webb *et al.*, 1993). Since then more P2Y receptor subtypes have been identified and currently in mammals there are eight subtypes - $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio *et al.*, 2006; Von Kugelgen, 2005; Sak and Webb, 2002; Communi *et al.*, 2000; Kugelgen and Wetter, 2000). The missing numbers represent the receptors which are misplaced in the family ($p2y_7$) or receptors cloned from non-mammalian vertebrates ($p2y_3$) (Abbracchio *et al.*, 2006; Jacobson *et al.*, 2002). $P2Y_{1,2,4,6}$ couple predominantly to G_q protein; $P2Y_{11}$ couples to G_s protein whereas $P2Y_{12,13,14}$ are coupled to G_i protein. Interestingly, $P2Y_{2,4}$ also couple to G_i protein and $P2Y_{11}$ also couples to G_q protein (Abbracchio *et al.*, 2006, Von Kugelgen, 2005; Scrivens and Dickenson, 2005; Costanzi *et al.*, 2004; Jacobson *et al.*, 2004, 2002).

3.6.1 Expression of adenine nucleotide activated P2Y receptors:

ADP is generally considered to be generated following nucleotidase-mediated breakdown of ATP release during hypoxia, cell lysis and tissue damage (Gordon, 1986; Ingerman *et al.*, 1979). $P2Y_{1,11,12,13}$ receptors are ADP-sensitive receptors (Fumagalli *et al.*, 2004; Calvert *et al.*, 2004; Czajkowski *et al.*, 2003). However, among the ADP-sensitive receptors only $P2Y_{1,13}$ mRNA expression was found in neonatal rat cardiac fibroblasts. Unfortunately, there is no information available about the expression of the $P2Y_{11}$ receptor in mouse or rat heart, since the gene has not been cloned in rodent. $P2Y_{11}$ receptor has only been cloned in man and canine (Communi *et al.*, 1997; Zambon *et al.*, 2001). However, in this study for the first time the protein expression of $P2Y_{11}$ receptor in addition to $P2Y_1$ and $P2Y_{13}$ receptors on cardiac fibroblasts was reported (Figure 3.2). The present work also showed that ADP-βS stimulated inositol phosphate and cAMP production indicating the activation of G_q- and G_s-protein-dependent signalling pathways. The induction of cAMP and IP production by ADP-βS was antagonised by suramin, but not by PPADS which corresponds to the pharmacological profile of the human $P2Y_{11}$. However, the thiophosphorylated

agonists, 2-MeSADP and 2-MeSATP had no effect on cAMP production in the absence or presence of forskolin. $P2Y_{11}$ is dual coupled to G_s and G_q proteins (Qi *et al.,* 2001; Conigrave *et al.,* 2000; Communi *et al.,* 1999), whereas the $P2Y_{13}$ receptor is mainly coupled to G_i protein (Fumagalli *et al.,* 2004; Communi *et al.,* 2001). The lack of response on cAMP accumulation when P2Y receptors are stimulated by 2-MeSADP and 2-MeSATP may be a consequence of activating $P2Y_{11}$ (G_s) and $P2Y_{13}$ (G_i) receptors simultaneously.

2-MeSADP and 2-MeSATP induced total inositol phosphate production indicating a coupling to G_q protein (Sak and Webb *et al.,* 2002). The accumulation of inositol phosphates observed with 2-MeSADP and 2-MeSATP was virtually abolished by all non-selective P2 antagonists and totally blocked by the $G_{q/11}$ protein inhibitor YM-254890 (YM). Interestingly, the $G_{i/o}$-protein inhibitor pertussis toxin (PTX) partially blocked inositol phosphate production induced by 2-MeSADP. Three possible mechanisms may explain the effect of PTX on 2-MeSADP response which is presumably mediated by βγ subunits released following G_i activation (Figure 3.27). PLC-β acts as a timer to limit the duration of G_q activation by interacting with the GTPase *A*ccelerating Protein domain (GAP) and increasing the GTPase activity of G_q (Paulssen *et al.,* 1996; Berstein *et al.,* 1992). GAP/PLC-β interaction can be strongly inhibited by βγ subunits (Chidiac *et al.,* 1999). Secondly, βγ subunits can directly stimulate PLC-β activity (Tomura *et al.,* 1997). Finally, βγ subunits can increase the duration of G_q activity by inhibiting the phosphorylation and inactivation of PLC-β by PKC (Cordeaux *et al.,* 2000). Moreover, 2-MeSADP is known to activate the G_i-coupled $P2Y_{13}$ receptor (Fumagalli *et al.,* 2004) which may stimulate PLC via βγ subunits released following G_i activation. These observations indicate that the inositol phosphate accumulation induced by 2-MeSADP may involve $P2Y_1$ (G_q) and $P2Y_{13}$ (G_i) receptors (Figure 4.2). Unfortunately due to lack of selective $P2Y_{13}$ receptor agonist and antagonist the signal transduction study on this receptor was hindered (Figure 3.28).

The role of the $P2Y_1$ receptor was explored using MRS2179, a competitive antagonist of the $P2Y_1$ receptor (Boyer *et al.,* 1998). In this study, IP accumulation induced by ADP-βS and 2-MeSADP was blocked by MRS2179 as observed in previous studies (Baurand *et al.,* 2001, Boyer *et al.,* 1998). 2-MeSADP induced increases in inositol phosphate production were moderately blocked (60% inhibition) by MRS2179, indicating the activation of $P2Y_1$ and other P2Y receptor subtypes by this nucleotide.

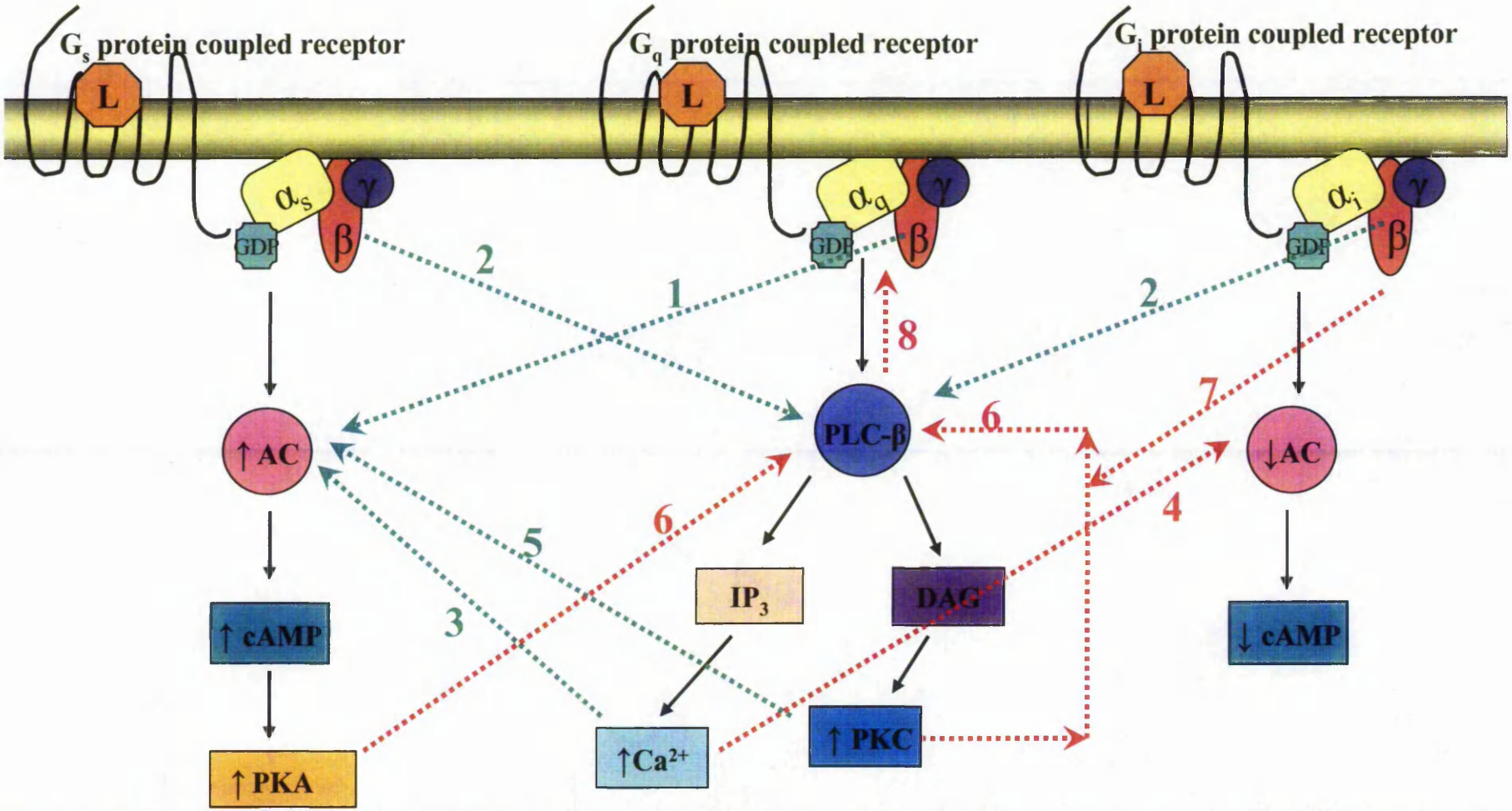

Figure 3.27: Interaction between G_s, G_i and G_q signalling pathways. Activation of G_s, G_i and G_q pathways leads to the stimulation of adenylyl cyclase (AC) , inhibition of AC and activation phospholipase C-β (PLC-β) and the production of second messengers (solid arrows). (1) βγ subunits from G_q may stimulate particular forms of AC, (2) βγ subunits from G_s and G_i can also stimulate PLC-β, (3) intracellular Ca^{2+} activates some AC isoforms, (4) intracellular Ca^{2+} can also inhibit some AC isoforms, (5) Protein kinase C (PKC) stimulates isoforms of AC, (6) Protein kinase A (PKA) and PKC phosphorylate and inhibit PLC activity, (7) βγ subunits from G_i can inhibit the inactivation of PLC-β by PKC and (8) PLC-β inactivates G_q activation by interacting with GTPase *A*ccelerating Protein domain (G*A*P).

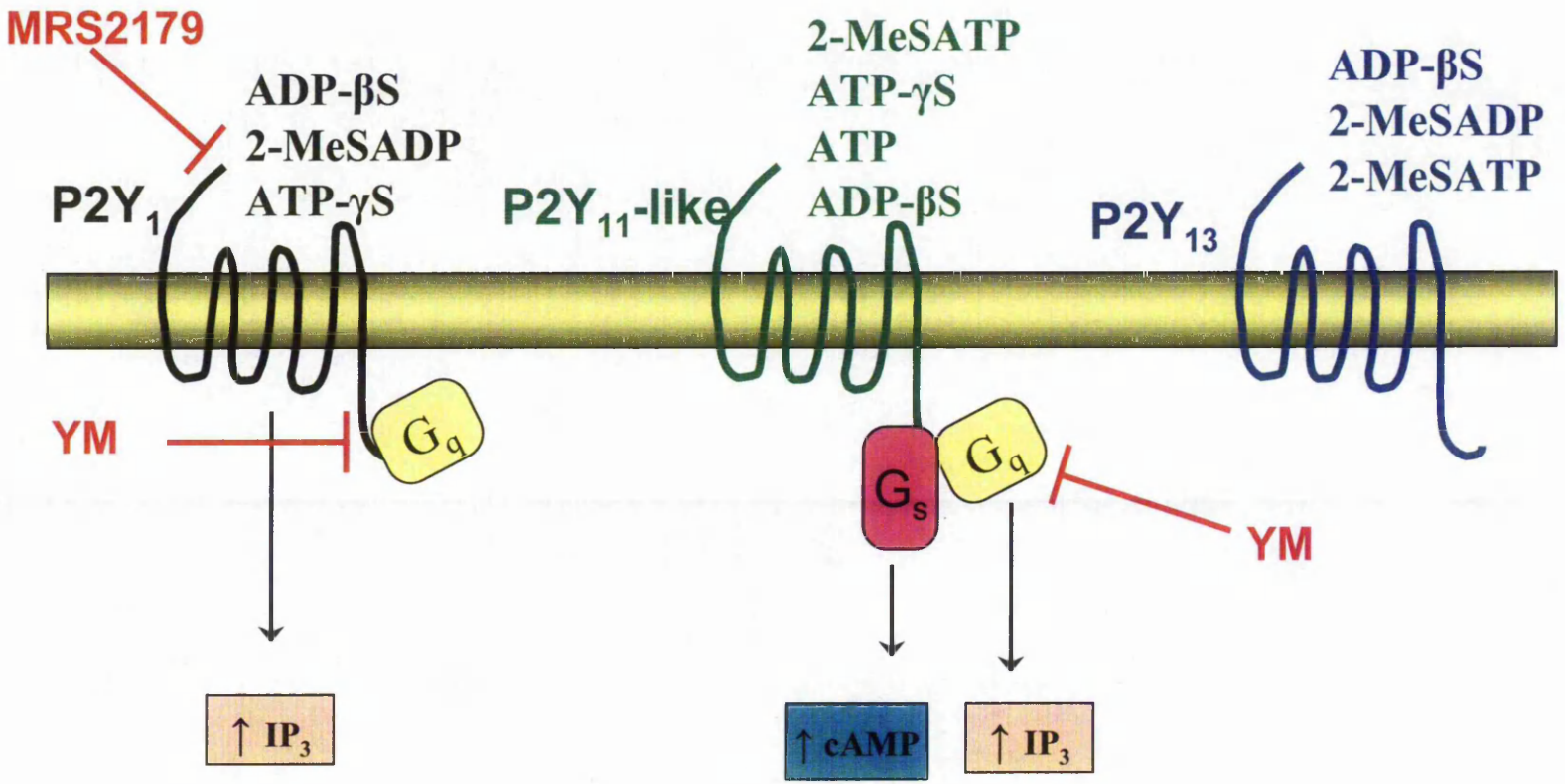

Figure 3.28: Expression of P2Y receptors activated by adenine nucleotides. Neonatal rat cardiac fibroblasts expression $P2Y_1$, $P2Y_{11}$-like and $P2Y_{13}$ receptors. $P2Y_1$ receptor is coupled to G_q-protein in a YM-254890 (YM; G_q-protein inhibitor)-sensitive manner and is stimulated by ADP-βS, 2-MeSADP and ATP-γS. The responses to these nucleotides were blocked by the $P2Y_1$ receptor antagonist MRS2179. $P2Y_{11}$-like receptor is dual coupled to G_s and G_q-proteins and is activated by ATP, ATP-γS, 2-MeSATP and ADP-βS. The G_q pathway of $P2Y_{11}$-like receptor is sensitive to YM. $P2Y_{13}$ receptor is detected at both mRNA and protein levels, however the functional activity of this receptor could not be determined.

Overall the mRNA, protein analysis and the rank order of the potency to induce total inositol phosphate accumulation [2-MeSADP > 2-MeSATP ≈ ADP-βS > UDP (Receptor-I) > UTP > ATP-γS > UDP (Receptor-II) > ATP], all strongly indicates the expression of the $P2Y_1$ receptor on neonatal rat cardiac fibroblasts. However, MRS2179 had no affect on 2-MeSATP induced inositol phosphate accumulation, suggesting the involvement of other P2Y-like receptors, probably $P2Y_{11}$ receptor. The human $P2Y_{11}$ is also activated by ADP-βS, ATP and its analogue ATP-γS (Communi *et al.,* 1999). It is noteworthy that the stimulation of inositol phosphate production observed with ATP and ATP-γS was inhibited by YM, strongly supporting the involvement of G_q protein. MRS2179 also partially antagonised the G_q activation induced by ADP-βS and ATP-γS, indicating the stimulation of other P2Y receptor subtypes. Overall, these data suggest functional expression of the $P2Y_1$ and possibly a $P2Y_{11}$-like receptor on neonatal rat cardiac fibroblasts (Figure 3.28).

ADP-βS, ATP and ATP-γS all induced cAMP accumulation and potentiated forskolin-stimulated cAMP production, indicating activation of the G_s pathway. It is possible that cAMP accumulation induced by ADP-βS, ATP and ATP-γS could involve cross-talk between G_q/G_s-protein dependent pathways (Figure 3.27). Indeed, Ca^{2+} mobilization and the activation of PKC following PLC activation has been shown in several cell types to potentiate cAMP accumulation (Heydorn *et al.,* 2004; Ostrom *et al.,* 2003; Cordeaux and Hill, 2002). It has been suggested that PKC can activate AC1, 3, 5, 7 isoforms (Cordeaux and Hill, 2002; Cooper *et al.,* 1995). In addition, an increase in intracellular Ca^{2+} levels can also stimulate AC1, 3, 8 isoforms (Tang and Hurley, 1998; Sunahara *et al.,* 1996). Finally, $G_q\beta\gamma$ subunits activate AC5 and AC6 isoforms (Cordeaux and Hill, 2002; Bayewitch *et al.,* 1998). All these mechanisms may contribute to the activation of the G_s pathway, via G_q-protein coupled receptors particularly since AC2, AC3, AC4, AC5, AC6, AC7 and AC8 isoforms are expressed in rat cardiac fibroblasts (Swaney *et al.,* 2003). However in the present study, blocking the G_q pathway by YM did not affect the cAMP accumulation induced by adenine nucleotides, suggesting the direct activation of G_s-protein coupled receptors. The increase in cAMP accumulation induced by ADP-βS and ATP-γS was not blocked by the $P2Y_1$ antagonist MRS2179. Taken together these nucleotides are either activating a P2Y receptor subtype coupled to G_s/G_q proteins ($P2Y_{11}$-like) or stimulating two different P2Y receptors one coupled to G_s and other to G_q protein. Suramin is a more potent antagonist than RB 2 at both the human and canine $P2Y_{11}$ receptor, whereas

PPADS is inactive (Communi *et al.,* 1999 and Torres *et al.,* 2002). In the present study, suramin and RB 2 blocked ADP-βS and ATP-γS-induced increase in inositol phosphate accumulation, probably through $P2Y_{11}$ receptor in neonatal rat cardiac fibroblasts. The immunocytochemistry in association with the functional studies suggest that a $P2Y_{11}$-like receptor is involved in the adenine nucleotide-induced responses in neonatal rat cardiac fibroblasts. Balogh *et al.,* (2005) have recently shown that ATP activates inositol phosphate production and cAMP accumulation in mouse cardiomyocytes via $P2Y_{11}$-like receptors. Moreover, extracellular ATP in mouse neuroblastoma neuro2a cells stimulated neurite outgrowth through a receptor with a $P2Y_{11}$ pharmacological profile (Lakshmi and Joshi 2006). Chootip and associates (2005) have also identified a $P2Y_{11}$-like receptor in smooth muscle cells of the rat pulmonary artery which was suramin-sensitive and PPADS-resistant. These recent observations along with the present study suggest the existence of a novel rodent $P2Y_{11}$-like receptor.

3.6.2 Expression of P2Y receptors activated by uracil nucleotides:

3.6.2.1 Effect of UDP and UTP on inositol phosphate and cAMP accumulation

Identification of $P2Y_{2,4,6}$ receptor mRNA by RT-PCR analysis and the protein expression by immunostaining was consistent with a previous study on neonatal rat cardiac fibroblasts (Figure 3.1 and 3.2; Webb *et al.,* 1998). $P2Y_{2,4,6}$ receptors are activated by uracil nucleotides like UDP and UTP (Abbracchio *et al.,* 2006). In addition, $P2Y_{2,4}$ receptors are coupled to both G_q and G_i proteins and therefore would be expected to stimulate inositol phosphate accumulation and inhibit forskolin-stimulated cAMP accumulation (Soltoff *et al.,* 1998; Filippov *et al.,* 1997). Kumari *et al.,* (2003) reported that stimulation of $P2Y_2$ receptor in rat vascular smooth muscle cells by UTP induced activation of inositol phosphate accumulation. Similarly, UTP mediated inositol phosphate generation and increased intracellular Ca^{2+} levels in adult rat cardiac fibroblasts by acting through $P2Y_2$ receptors (Meszaros *et al.,* 2000). When stimulated with UTP, rat $P2Y_4$ receptor transfected in 1321N1 cells also increased intracellular Ca^{2+} levels (Kennedy *et al.,* 2000). In *Xenopus oocytes* transfected with the $P2Y_2$ receptor pertussis toxin blocked UTP-induced MAP kinase activation suggesting G_i-coupling (Soltoff *et al.,* 1998). Similarly, UTP can also mediate mobilization of intracellular Ca^{2+} in a pertussis toxin manner by $P2Y_2$ and $P2Y_4$ receptors in rat cervical ganglia neurones (Filippov *et al.,* 1997; Communi *et al.,* 1995). Although

these studies have reported $P2Y_{2,4}$ receptors coupling to G_i and G_q proteins at present there are no reports describing the activation of both pathways in the same cell type (Kumari *et al.,* 2003; Jacobson *et al.,* 2000; Meszaros *et al.,* 2000; Kennedy *et al.,* 2000; Soltoff *et al.,* 1998; Filippov *et al.,* 1997).

In this study, UTP inhibited forskolin stimulated cAMP accumulation, through G_i protein coupling and induced total inositol phosphate accumulation via G_q coupling.

UDP is the preferred ligand at the $P2Y_6$ receptor (Nicholas *et al.,* 1996; Abbracchio *et al.,* 2006). In this study, UDP was more potent than UTP in activating inositol phosphate production. This is in agreement with a previous study using NG108-15 cells expressing the $P2Y_6$ receptor (Sak *et al.,* 2001). Additionally, Calvert *et al.,* (2004) also reported that the $P2Y_6$ increased intracellular Ca^{2+} in superior cervical ganglion neurons and glia cells in response to UDP indicating a coupling to G_q pathway. It is notable that the uracil nucleotides, UDP and UTP were the only nucleotides used in this study, which inhibited forskolin-induced cAMP accumulation in a PTX-sensitive manner indicating G_i protein coupling. UDP is a partial agonist in *Xenopus oocytes* transfected with the rat $P2Y_4$ receptor (Bogdanove *et al.,* 1998). In the present study, the inhibition of cAMP production and stimulation of inositol phosphate accumulation by UDP and UTP suggests the functional expression of $P2Y_2$ and $P2Y_4$ receptors on fibroblasts. Moreover, the maximal inhibition of forskolin-response by UTP was greater than UDP-mediated inhibition, suggesting the expression of the $P2Y_4$ receptor in cardiac fibroblasts. In conclusion, these results of this study indicate that $P2Y_{2,4}$ and $P2Y_6$ receptors are functionally expressed in neonatal rat cardiac fibroblasts and are coupled to G_i and G_q proteins.

3.6.2.2 Effect of G_i and $G_{q/11}$-protein inhibitors on uracil nucleotide-induced responses

The activation of G_q and G_i pathways by the uracil nucleotides was further investigated in the presence of G_i blocker PTX and $G_{q/11}$ inhibitor YM. The capacity of UDP to promote inositol lipid hydrolysis in cardiac fibroblasts was markedly sensitive to G_q. Interestingly, in fibroblasts the accumulation of inositol phosphate induced by UDP was increased by 2-fold in the presence of PTX suggesting an inhibitory effect on inositol phosphate production (G_q) through G_i protein. It is well documented that $G\beta\gamma$ subunits released from stimulation of G_i protein coupled receptors activate PLC to

generate inositol phosphates (Rebecchi and Pentyala, 2000; Cordeaux and Hill, 2002). However, Misawa *et al* (1995) demonstrated that opioid κ agonist; U-50488H inhibits PLC in guinea pig cerebellar membranes through a coupling to PTX-sensitive G_i protein. Similarly, Litosch *et al* (1996) reported that βγ subunits from G_i protein mediate a rapid and transient inhibition of PLC-β1. These observations may explain the PTX-sensitive inositol phosphate production mediated by UDP. Alternatively, the most likely explanation is that the receptor activated by UDP mediates distinct signalling pathways through direct interaction with multiple G-proteins (G_q and G_i). By removal of one G-protein using PTX (G_i) the receptor is more efficiently coupled to G_q protein, resulting in increased inositol phosphate accumulation. Moreover, the inhibition of forskolin-stimulated cAMP accumulation by UDP was sensitive to both PTX and YM suggesting the participation of G_i and G_q-proteins in the cAMP pathway. Angiotensin-II suppressed renin secretion from justaglomerular cells by inhibiting AC5 and AC6 through a Ca^{2+}-dependent mechanism (Grunberger *et al.*, 2006). Both AC5 and AC6 are expressed in rat cardiac fibroblasts (Ostrom *et al.*, 2003), which may explain YM-sensitive inhibition of forskolin response mediated by UDP. Taken together these data strongly support the existence of cross-talk between G_q and G_i pathways either via a single receptor subtype coupling to G_q/G_i or two different receptors (coupling to G_q or G_i) both activated by UDP.

Inositol phosphate production stimulated by UTP did not show such an interaction. Indeed, UTP induced inositol phosphate response was solely through the G_q pathway since PTX did not affect the inositol phosphate accumulation whereas, the functional response was blocked by YM. As a whole, these findings illustrate that UDP stimulates a P2Y receptor coupled to G_q and G_i proteins or activates at least two receptors one coupled to G_q and the other to G_i proteins, which is strengthened by the biphasic curves observed with UDP-induced inositol phosphate accumulation. On the other hand, UTP mediated YM-sensitive inositol phosphate accumulation and PTX-sensitive cAMP production, indicating that UTP stimulates two different receptors one coupled to G_q and other to G_i pathways.

3.6.2.3 Effect of antagonists on uracil nucleotide-induced responses

Suramin, PPADS and RB 2 are non-selective P2 antagonists which also inhibit ecto-nucleotidases (Lambrecht *et al.,* 2002; Muller 2002; Tuluc *et al.,* 1998) and cAMP accumulation as shown in figure 3.16. $P2Y_2$ receptor is resistant to PPADS whereas suramin has no effect on $P2Y_4$ receptor mediated function (Von Kugelgen, 2005;

Abbracchio *et al.,* 2006). In the present study, suramin had no effect on UDP and UTP-inhibited forskolin response, indicating the functional expression of suramin resistant $P2Y_4$ receptor. PPADS counteracted the UTP-induced inhibition of forskolin and had no affect on inositol phosphate production mediated by UTP. RB 2 behaves as a competitive antagonist at recombinant rat $P2Y_4$ receptor (Wildman *et al.,* 2003, Bogdanov *et al.,* 1998). Moreover, the $P2Y_6$ receptor mediated effect of UDP on PLC response was antagonised by RB 2 in the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 (Sak *et al.,* 2001). Indeed, RB 2 blocked the UDP and UTP-induced inositol phosphate production, suggesting the involvement of $P2Y_4$ and/or $P2Y_6$ receptors in G_q-protein activation. Interestingly, UDP mediated inhibition of forskolin-response was resistant to suramin, PPADS and RB 2. Nevertheless, UDP-mediated inositol phosphate accumulation in fibroblasts was potentiated in the presence of PPADS. These data suggest that the UDP and UTP-induced effects on cAMP and inositol phosphates in rat neonatal cardiac fibroblasts involved $P2Y_{2,4,6}$ and possibly an uncloned P2Y receptor or a non-P2Y receptor. Indeed, Mellor and researchers (2001, 2002) documented that UDP can stimulate cysteinyl leukotrienes receptor I in human mast cells. It is difficult to functionally distinguish between $P2Y_2$ and $P2Y_4$ receptors due to the lack of selective antagonists.

To investigate the role of $P2Y_1$ receptors in G_i activation by UDP and UTP, functional studies were performed in the presence of MRS2179. As expected the $P2Y_1$ receptor was not involved in the inhibition of forskolin-response induced by UDP. However, UTP-induced inositol phosphate production was partially sensitive to MRS2179, indicating the involvement of $P2Y_1$ subtype. However, previous studies have also shown that rat $P2X_1$ and $P2X_3$ ion-channel receptors are activated by UTP (McLaren *et al.,* 1998; Rae *et al.,* 1998). In addition, MRS2179 is known to inhibit ATP-evoked responses at recombinant rat $P2X_1$ and $P2X_3$ receptors (Brown *et al.,* 2000).

MRS2578, a selective rat $P2Y_6$ antagonist was used to further investigate the functional expression of the $P2Y_6$ receptor (Mamedova *et al.,* 2004). The putative $P2Y_6$ antagonist did not block the uracil nucleotide mediated inhibition of cAMP levels, suggesting that UDP and UTP do not stimulate G_i pathway via $P2Y_6$ receptor. Therefore, the UTP-mediated inhibition of forskolin-triggered cAMP is solely via $P2Y_{2,4}$ subtypes. It is noteworthy that MRS2578 produced a concentration-dependent biphasic inhibition of IP induced by UDP and ATP-γS. Indeed, such a bell-shaped inhibition curve is

characteristic feature of positive cooperative interaction between two receptors and also related to dimerisation as shown with vasopressin and oxytocin receptors (Albizu *et al.*, 2006; Swillens *et al.*, 1995). Moreover, previous studies have shown that P2Y receptors can form dimers (Suzuki *et al.*, 2006; Ambrosi *et al.*, 2006; Nakata *et al.*, 2005; Yoshioka *et al.*, 2001). Ambrosi and associates (2006) documented that $P2Y_4$ receptor forms homodimers whereas $P2Y_1$ and $P2Y_2$ receptors form heterodimers with the adenosine A_1 receptor, respectively (Yoshioka *et al* 2001; Suzuki *et al.*, 2006). These data further strengthen the idea that UDP activates two different P2Y receptors in neonatal rat cardiac fibroblasts. In the present study, MRS2578 also inhibited UTP-mediated inositol phosphate accumulation. Overall, these studies with $P2Y_6$ antagonist indicate that the $P2Y_6$ receptor is activated by UDP and UTP through G_q-protein coupling, as expected (Abbracchio *et al.*, 2006).

This study displays a complex activation of multiple G-proteins and P2Y receptors by uracil nucleotides via $P2Y_{2,4}$ and $P2Y_6$ receptors in neonatal rat cardiac fibroblasts.

3.6.2.4 ATP, ATP-γS and cAMP accumulation

It is noteworthy that ATP is also an agonist ligand at both the $P2Y_2$ and $P2Y_4$ receptors. As discussed previously in this study ATP directly stimulated cAMP accumulation and potentiated forskolin-stimulated cAMP production suggesting the activation of the G_s pathway. However, previous research showed that ATP like UTP promoted activation of MAP kinase via G_i-protein coupling in *Xenopus oocytes* transfected with the $P2Y_2$ receptor (Soltoff *et al.*, 1998). Likewise, ATP-induced mobilization of intracellular Ca^{2+} was blocked by PTX in rat cervical ganglia neurones expressing $P2Y_2$ and $P2Y_4$ receptors (Filippov *et al.*, 1997). Based on these observations it would be predicted that ATP and ATP-γS would inhibit (via G_i-protein coupling) forskolin-stimulated cAMP accumulation. One possible explanation would be that ATP can activate other P2Y receptors ($P2Y_{11}$) and P2X receptors (Weidema *et al.*, 2001). Activation of P2X ligand-gated channels by ATP can mediate Ca^{2+} influx into the cells (Vial *et al.*, 2004; Weidema *et al.*, 2001). The increase in the intracellular Ca^{2+} levels may stimulate AC1, 3, 8 isoforms and consequently raise cAMP levels (Tang and Hurley, 1998). The maximal response of ATPγS induced cAMP and inositol phosphate accumulation was higher than ATP (Figures 3.3A and 3.5A), suggesting a breakdown of ATP into adenosine by ectonucleotidases and 5'nucleotidases. Fibroblasts are known to functionally express the G_s protein coupled adenosine A_{2B} receptor (Queiroz *et al.*, 2004; Dubey *et al.*, 2001; Dubey *et al.*, 1998). Indeed the results presented here have

shown that adenosine activates cAMP production and inhibits IP accumulation, which may explain the difference between ATP and ATPγS responses.

3.6.3 AMP and P2Y receptor:

Inbe and colleagues (2004) recently deorphanised GPR80/99 which when transfected in HEK293 cells was selectively activated by adenosine and AMP. At that time this receptor was named the $P2Y_{15}$ receptor (Inbe *et al.,* 2004). Following this discovery, it was reported that GPR80 was activated by citric acid cycle intermediate α-ketoglutaric acid (He *et al.,* 2004). Furthermore, Qi and his researchers demonstrated that GPR80 when stably expressed in CHO and COS-7 cells was not activated by adenosine and AMP (Qi *et al.,* 2004). Thus, the P2Y Receptor Nomenclature Subcommittee concluded that GPR80/99 was not a P2Y receptor (Abbracchio *et al.*, 2005; 4th International Symposium on Nucleosides and Nucleotides, 2004). In the present study, AMP induced a robust stimulation of cAMP production in cardiac fibroblasts. Moreover, AMP caused the maximal cAMP accumulation compared to other the nucleotides (adenosine, ATP, ATP-γS and ADP-βS). Communi *et al.,* (1999) documented that AMP-αS behaved as a partial agonist at the human $P2Y_{11}$ receptor by stimulating cAMP and inositol phosphate pathways. However, in neonatal rat cardiac fibroblasts AMP induced an inhibition of basal inositol phosphate accumulation. It has been shown that PKA activation induced the inhibition of the G_q pathway by inhibiting PLC-β_3 activity (Laglia *et al.,* 1996; Yue *et al.,* 1998). Indeed, as seen in figure 3.25 inhibiting PKA activity reversed the inhibition of G_q mediated by AMP. In addition, the PLC inhibitor U73122 attenuated AMP-induced cAMP accumulation. Overall, these observations suggest a cross-talk between PLC and PKA pathways in AMP induced responses. The accumulation of cAMP by AMP was resistant to PTX and YM and only the inhibition of basal inositol phosphate accumulation was sensitive to YM. Furthermore, the non-selective P2 antagonists potentiated the inositol phosphate inhibition induced by AMP. Suramin, PPADS and RB 2 were ineffective on AMP-induced cAMP response. In addition, the $P2Y_1$ antagonist MRS2179 had no effect on AMP-induced cAMP accumulation and AMP-mediated inhibition of basal inositol phosphate accumulation. Overall, these pharmacological observations indicate that AMP may activate a novel P2Y-like receptor or a non-P2Y receptor coupled to G_s and probably to G_q pathways.

3.6.4 Conclusion

Investigating the functional expression of P2Y receptors is complicated due to the lack of specific agonists and antagonists. The presence of multiple P2Y receptor subtypes and G-proteins on cardiac fibroblasts may also lead to the formation of heterodimers and infidelity in G-protein(s) coupling. In general, this study revealed that neonatal rat cardiac fibroblasts express five functional P2Y receptors: $P2Y_1$ (2-MeSADP/ADP-βS responsive; G_q pathway), $P2Y_{2,4}$ (UTP/UDP/ATP responsive; G_q/G_i pathway), $P2Y_6$ (UDP/UTP responsive; G_q pathway) and $P2Y_{11}$-like (ATP, 2-MeSADP/2-MeSATP sensitive; Figure 3.29). $P2Y_{13}$ receptor was identified at mRNA and protein levels however; the functional expression is hindered due to the lack of selective agonist and antagonists. Moreover, this study suggests the expression of a novel P2Y-like receptor activated by AMP.

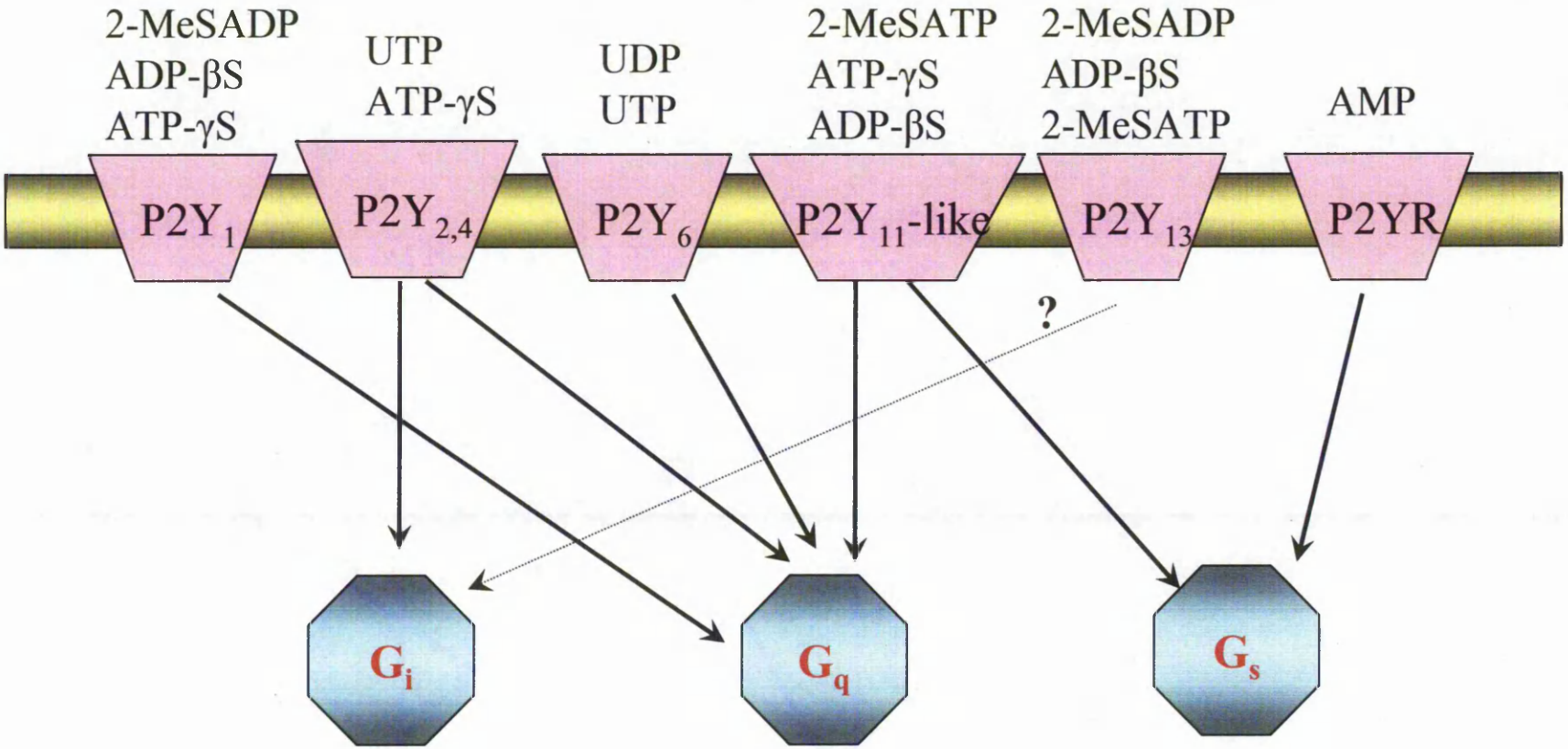

Figure 3.29: P2Y receptor subtypes functionally expressed on neonatal rat cardiac fibroblasts. The $P2Y_1$ receptor is coupled to G_q protein and activated by 2-MeSADP and ADP-βS; $P2Y_{2,4}$ activated by both UTP and ATP-γS and dual coupled to G_i and G_q proteins; $P2Y_6$ is coupled to G_q and is activated mainly by UDP; the $P2Y_{11}$-like is dual coupled to G_s and G_q and is activated by 2-MeSATP, ATP-γS, 2-MeSADP; the signal transduction studies were hindered for $P2Y_{13}$ (broken arrow) which is mainly stimulated by 2-MeSADP and coupled to G_i protein and finally the possible presence of P2Y-like receptor (P2YR) stimulated by AMP coupled to G_s pathway. ? – not known.

Chapter 4

Role of P2Y receptors in an *in vitro* model of ischaemic heart disease

Chapter 4: Results – Role of P2Y receptors in a model of ischaemic heart disease

4.1 Establishing the model of ischaemic heart disease

ATP and UTP are released from the heart during ischaemic conditions (Clemens and Forrester, 1981; Gordon, 1986; Vial *et al.,* 1987; Erlinge *et al.,* 2005 Wihlborg *et al.,* 2006) and can mediate their effects by stimulating P2Y receptors expressed in cardiac fibroblasts. An *in vitro* model of ischaemic heart disease was developed, in association with angiotensin-II (ANG-II) and hypoxia, to study the effects of extracellular nucleotides on neonatal rat cardiac fibroblasts.

4.1.1 Optimizing the parameters for the model of ischaemic heart disease

ANG-II is continuously released from the myocardium in healthy subjects and is increased during heart failure (Neri *et al.,* 1996; 2001). The secretion of ANG-II can activate the angiotensin-1 (AT_1) receptor expressed on rat cardiac fibroblasts leading to cardiac fibrosis, increase in collagen production and release of cytokines (Levy, 2005; Lijnen *et al.,* 2000). Cardiac myocytes and fibroblasts were stimulated for varying intervals of time (16 – 24 hours) in different studies to induce these effects (Lijnen *et al.,* 2000; Sarkar *et al.,* 2004; Yokoyama *et al.,* 1999; Sano *et al.,* 2000). Several studies have shown the functional link between ANG-II and transforming growth factor- beta1 (TGF-β1; Schultz *et al.,* 2002; Rosenkranz, 2004). Indeed, TGF-β1-deficient mice when exposed to chronic levels of ANG-II did not develop hypertrophy (Schultz *et al.,* 2002). In the present study, the release of TGF-β1 from fibroblasts stimulated by various concentrations of ANG-II for 24 hours was monitored by ELISA. ANG-II induced a concentration-dependent increase in TGF-β1 with a maximal response of 21% over the basal levels with an EC_{50} value of 2.5nM (Figure 4.1). The concentration of ANG-II (50nM) used in the development of the ischaemic heart disease model was ascertained from its potency to stimulate the release of TGF-β1. The hypoxic conditions were simulated by exposing cardiac fibroblasts to 0.5% O_2 and maintaining the fibroblasts in serum and glucose-free medium (Germack and Dickenson, 2005; Rocha-Singh *et al.,* 1991).

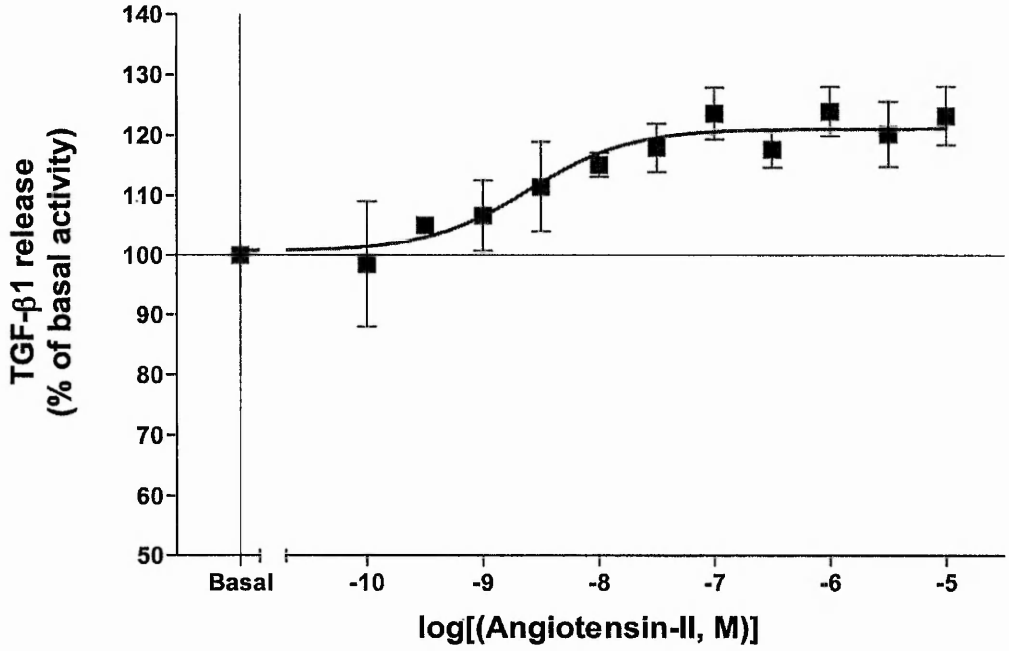

Figure 4.1: Effect of angiostensin-II (ANG-II) on the release of transforming growth factor-beta1 (TGF-β1) in neonatal rat cardiac fibroblasts. Cardiac fibroblasts were stimulated with indicated concentrations of ANG-II in serum- and glucose-free DMEM media for 24 hours under normoxic conditions. The cell culture supernates were collected and evaluated for TGF-β1 by ELISA. Data were expressed as the percentage of the basal level of TGF-β1 (100%). Each point represents the mean ± S.E.M for 5 experiments from separate cell cultures, performed in duplicate.

ATP-γS (a stable and less hydrolysable form of ATP; 32μM) and UTP (10μM) were used to stimulate P2Y receptors in this model of ischaemic heart disease. The concentrations of ATP-γS and UTP were determined from the functional studies and correspond to ten times their potencies (see Table 3.1).

4.1.1.1 Effects of ATP-γS and UTP on cytokine releases in the model of ischaemic heart disease

The heart initiates a sequence of inflammatory events and wound healing processes in response to myocardial injury (Ertl and Frantz, 2005; Brown *et al.,* 2005a; 2005b; Nian *et al.,* 2004; Manabe *et al.,* 2002; Frangogiannis *et al.,* 2002). Initial steps of wound healing are dominated by blood cells like platelets, neutrophils, macrophages and lymphocytes (Ertl and Frantz, 2005; Nian *et al.,* 2004; Manabe *et al.,* 2002). However, the later phase of wound healing is dictated by cardiac fibroblasts, to repair and rebuild the myocardial architecture (Brown *et al.,* 2005a; 2005b). These events are synchronized by release of inflammatory cytokines such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) and by the secretion of fibrotic factors like ANG-II, TGF-β and aldosterone (Brown *et al.,* 2005a; 2005b). Therefore, the release of IL-1β, IL-6, TNF-α and TGF-β1 from cardiac fibroblasts was evaluated following the stimulation with ATP-γS and UTP in presence or absence of ANG-II under normoxic (Nx) or hypoxic (Hx) conditions at different time points 1, 2, 4, 8 and 18 hours to study the involvement of P2Y receptors.

Effects of ATP-γS and UTP on IL-1β release in the model of ischaemic heart disease:

Cardiac fibroblasts exposed for 4 hours Hx significantly inhibited the basal release of IL-1β by 20% ($P<0.05$, Figure 4.2A). ATP-γS and UTP did not induce the release of IL-1β under Nx or Hx conditions (Figure 4.2B – C). ANG-II did not stimulate the secretion of IL-1β (Figure 4.3A). However, in the presence of ATP-γS and UTP a small but significant increase of IL-1β production was observed (ATP-γS: $P<0.01$, 13% over ANG-II response; UTP: $P<0.001$, 14% over ANG-II response; Figure 4.3B – C) at 4 hours Hx exposure. Interestingly, fibroblasts treated for 18 hours with UTP and ANG-II in Hx inhibited IL-1β release. Nx environment did not induce IL-1β accumulation by ATP-γS and UTP in combination with ANG-II (Figure 4.3B – C).

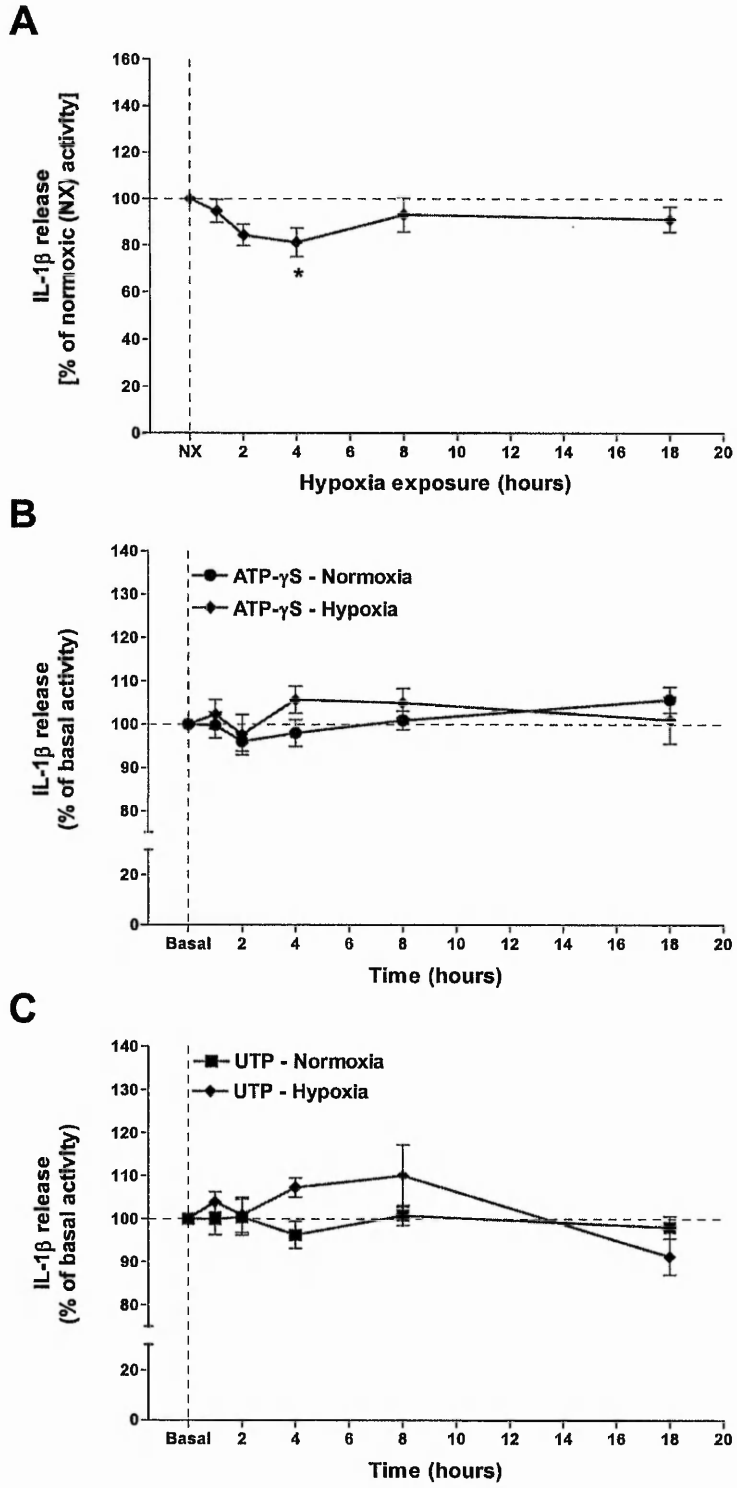

Figure 4.2: Effect of ATP-γS and UTP on interleukin-1β (IL-1β) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points.

Cardiac fibroblasts were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions, respectively (**Panels B** and **C**). IL-1β released into the medium was asserted by ELISA. Data were expressed as the percentage of basal IL-1β level (100%) for each time point. Each point represents the mean ± S.E.M for 7 – 13 experiments from separate cell cultures, performed in duplicate. * $P<0.05$ versus the respective normoxic response.

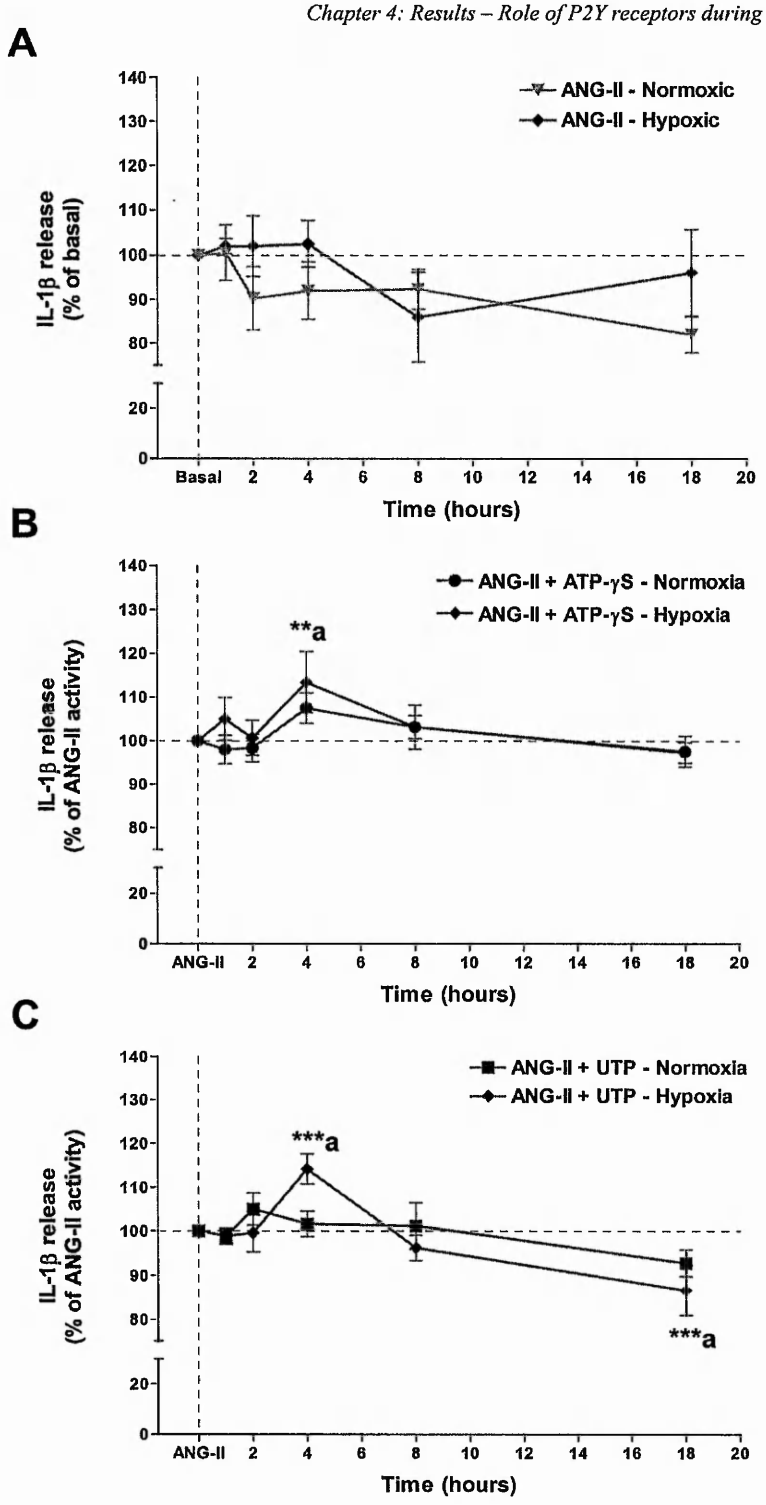

Figure 4.3: Effect of ATP-γS and UTP in presence of angiotensin-II (ANG-II) on interleukin-1β (IL-1β) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts treated with ANG-II

(50nM) were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were stimulated with ANG-II in combination with either ATP-γS (32μM) or UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions (**Panels B** and **C**). The cell culture supernates were collected and evaluated for IL-1β by ELISA. Data were expressed as the percentage of respective basal (100%) for each time point in panel A whereas in panels B and C data were expressed as the percentage of ANG-II response (100%) for each time point. Each point represents the mean ± S.E.M for 4 – 6 experiments from separate cell cultures, performed in duplicate.

These results indicate a possible role of ATP-γS and UTP in stimulating the release of IL-1β during ischaemic heart diseases and the presence of ANG-II is essential to observe the IL-1β release by nucleotides in Hx (Table 4.1).

Effects of ATP-γS and UTP on IL-6 release in the model of ischaemic heart disease:

4 hours Hx significantly inhibited the basal production of IL-6 by fibroblasts ($P<0.01$, 23% below the Nx levels; Figure 4.4A). ATP-γS significantly increased the release of IL-6 in both, Nx and Hx conditions in a time-dependent manner (Figure 4.4B). It is noteworthy that Hx did not alter the release of IL-6 induced by ATP-γS. In contrast, UTP did not induce the release of IL-6 in Nx and Hx treatments (Figure 4.4C). ANG-II did not induce IL-6 release in Nx and Hx (Figure 4.5A). However, the combination of ATP-γS and ANG-II mediated the secretion of IL-6 from fibroblasts in both Nx and Hx conditions (Figure 4.5B). It is notable that ANG-II potentiated significantly the release of IL-6 mediated by ATP-γS at 2 hours by 21% ($P<0.01$) and 18 hours by 29% ($P<0.01$) in Nx and at 18 hours in Hx by 22% ($P<0.05$). However, the combination of UTP and ANG-II did not induce IL-6 production (Figure 4.5C). These observations suggest that P2Y receptors activated by ATP-γS are involved in the release of IL-6 under ischaemic conditions and UTP stimulated P2Y receptors are probably not involved in IL-6 release (Table 4.1).

Effects of ATP-γS and UTP on TNF-α release in the model of ischaemic heart disease:

Like the other cytokines IL-1β and IL-6, the basal production of TNF-α was significantly inhibited at 4 hours Hx by 20% ($P<0.01$, Figure 4.6A). ATP-γS, UTP and ANG-II did not mediate the release of TNF-α in Nx and Hx conditions (Figure 4.6B – C, 4.7A). In presence of ANG-II, UTP induced TNF-α synthesis in Nx (17% over ANG-II response; $P<0.001$) and had no effect in Hx, whereas ATP-γS did not stimulate TNF-α release in Nx and Hx (Figure 4.7B – C). These sets of experiments indicate that during ischaemic conditions ATP-γS and UTP are not involved in the production of TNF-α (Table 4.1).

Table 4.1: Regulation of cytokine production by P2Y receptors in neonatal rat cardiac fibroblasts during ischemic heart disease

Cytokine	Normoxia (Nx)					Hypoxia (Hx)						
				+ ANG-II						+ ANG-II		
	ATP-γS	UTP	ANG-II	ATP-γS	UTP	Hx	ATP-γS	UTP	ANG-II	ATP-γS	UTP	
IL-1β	↔	↔	↔	↔	↔	↓	↔	↔	↔	↑	↑	4 Hours
IL–6	↑	↔	↔	↑	↔	↓	↑	↔	↔	↑	↔	
TNF-α	↔	↔	↔	↔	↑	↓	↔	↔	↔	↔	↔	
TGF–β1	↔	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔	
IL-1β	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	18 Hours
IL–6	↑	↔	↔	↑	↔	↔	↑	↔	↔	↑	↔	
TNF-α	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	
TGF–β1	↔	↔	↔	↔	↔	↓	↔	↔	↑	↔	↔	

The *in vitro* model of ischemic heart disease was developed in associating with angiotensin-II (50nM; ANG-II) and hypoxia (0.5% O_2). Cardiac fibroblasts were stimulated with ATP-γS (32μM) or UTP (10μM) in absence or presence of ANG-II in serum- and glucose-free DMEM media for 4 hours and 18 hours. □ - no change; □- increase; □- decrease in cytokine secretion.

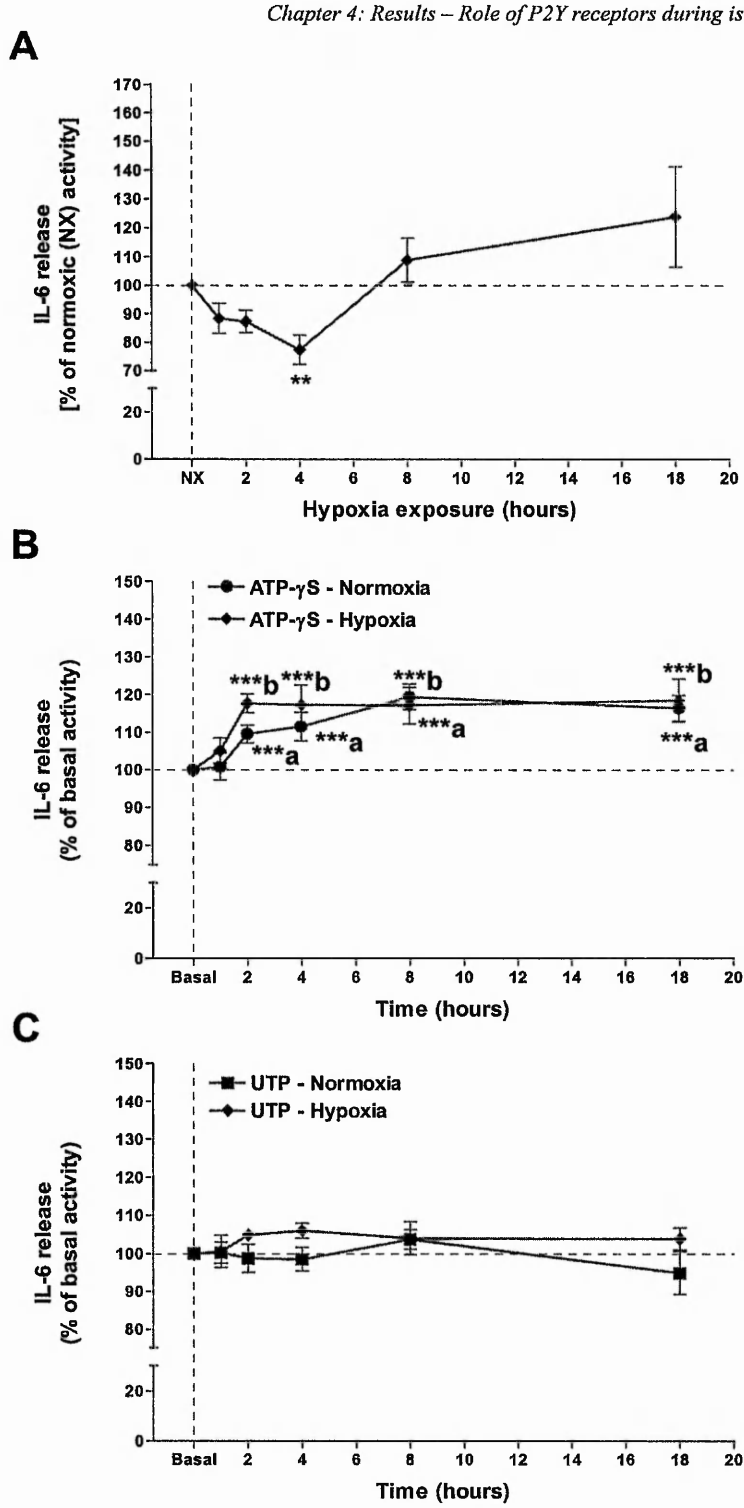

Figure 4.4: Effect of ATP-γS and UTP on interleukin-6 (IL-6) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were

stimulated with ATP-γS (32μM) and UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions, respectively (**Panels B** and **C**). IL-6 released into the medium was asserted by ELISA. Data were expressed as the percentage of basal IL-6 level (100%) for each time point. Each point represents the mean ± S.E.M for 7 – 13 experiments from separate cell cultures, performed in duplicate. ** $P<0.01$, *** $P<0.001$; a (ATP-γS) versus basal levels and b (UTP) versus basal levels.

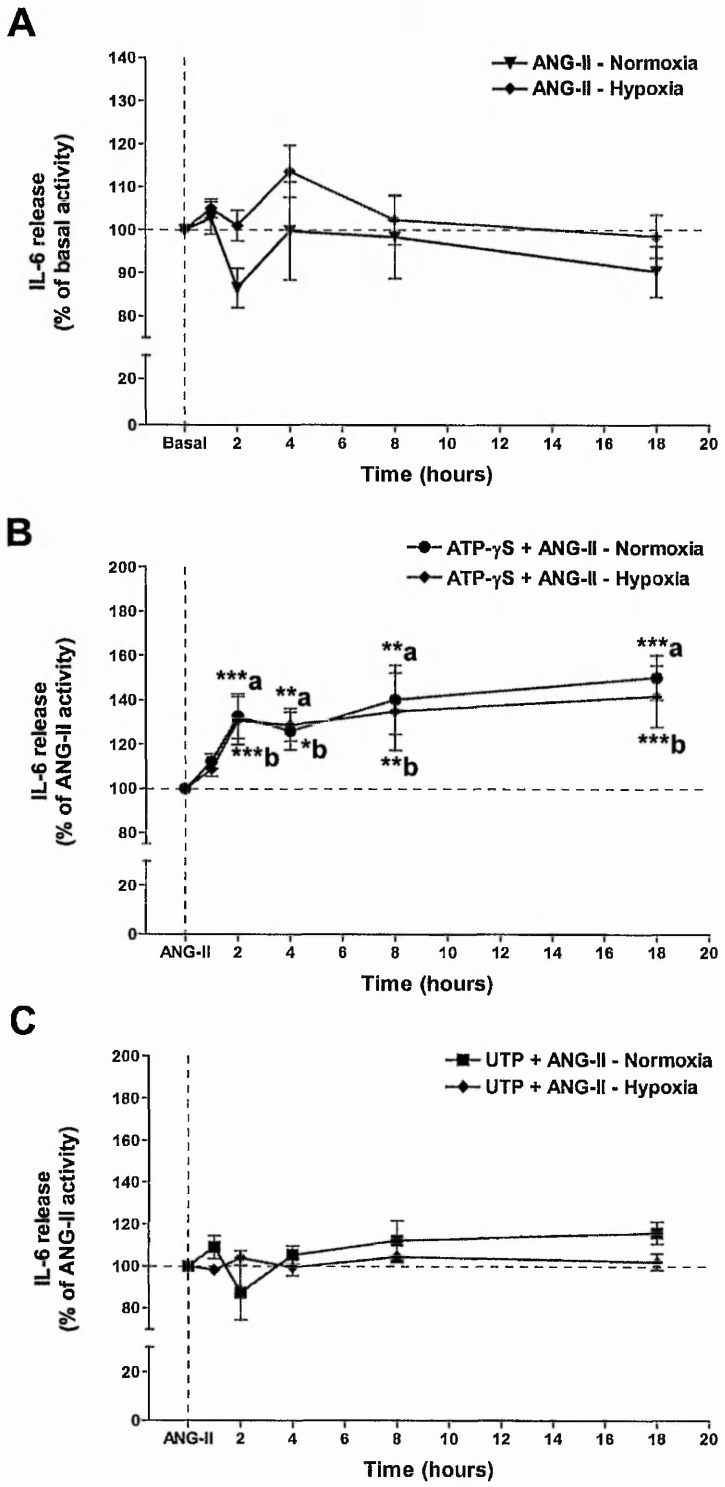

Figure 4.5: Effect of ATP-γS and UTP in presence of angiotensin-II (ANG-II) on interleukin-6 (IL-6) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts treated with ANG-II

(50nM) were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were stimulated with ANG-II in combination with either ATP-γS (32μM) or UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions (**Panels B** and **C**). The cell culture supernates were collected and evaluated for IL-6 by ELISA. Data were expressed as the percentage of respective basal (100%) for each time point in panel A whereas in panels B and C data were expressed as the percentage of ANG-II response (100%) for each time point. Each point represents the mean ± S.E.M for 4 – 6 experiments from separate cell cultures, performed in duplicate. ** $P<0.01$, *** $P<0.001$; a (ATP-γS + ANG-II) versus basal levels and b (UTP + ANG-II) versus basal levels.

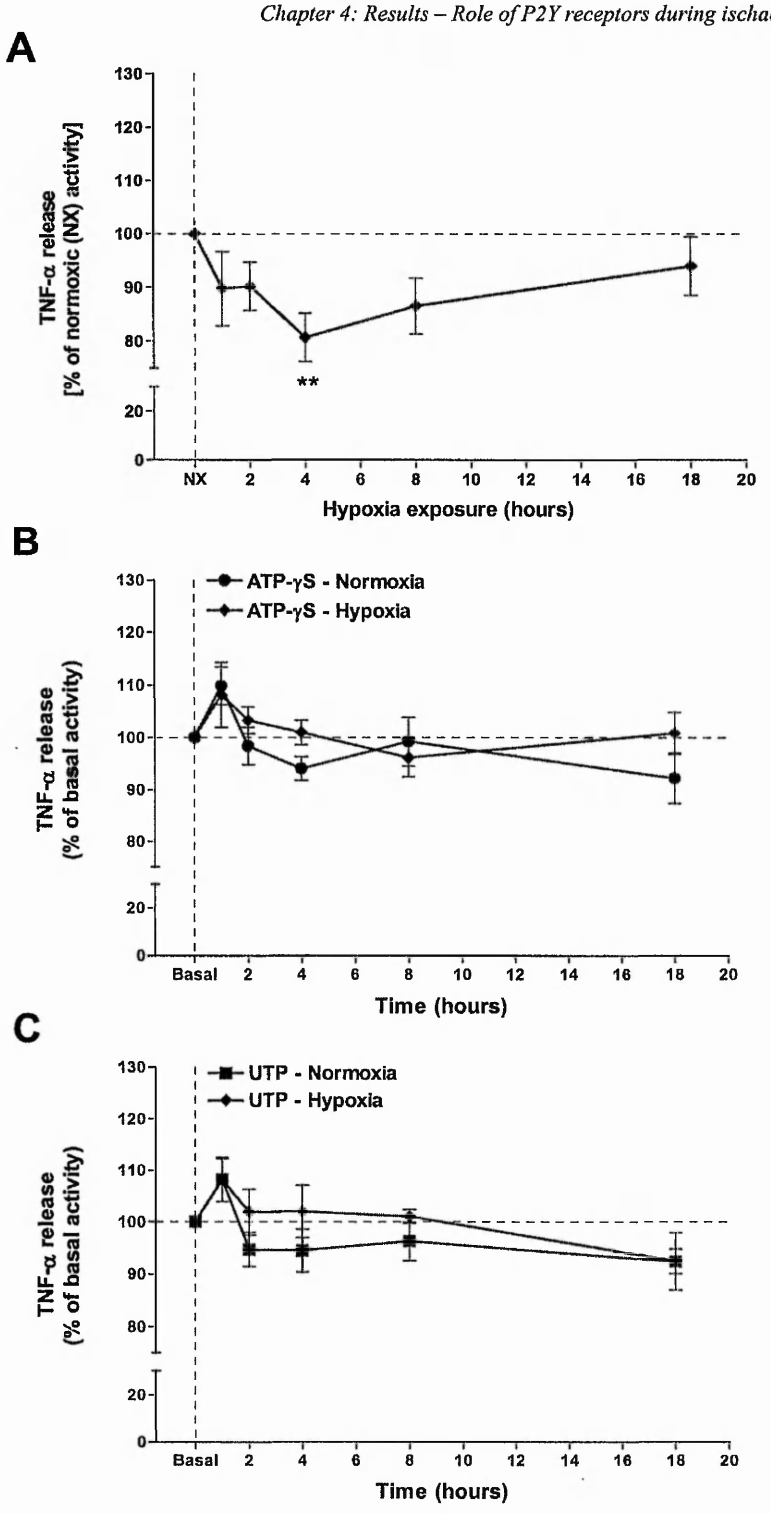

Figure 4.6: Effect of ATP-γS and UTP on tumour necrosis factor-α (TNF-α) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts

were stimulated with ATP-γS (32μM) and UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions, respectively (**Panels B** and **C**). TNF-α released into the medium was asserted by ELISA. Data were expressed as the percentage of basal TNF-α level (100%) for each time point. Each point represents the mean ± S.E.M for 6 – 12 experiments from separate cell cultures, performed in duplicate. ** $P<0.01$.

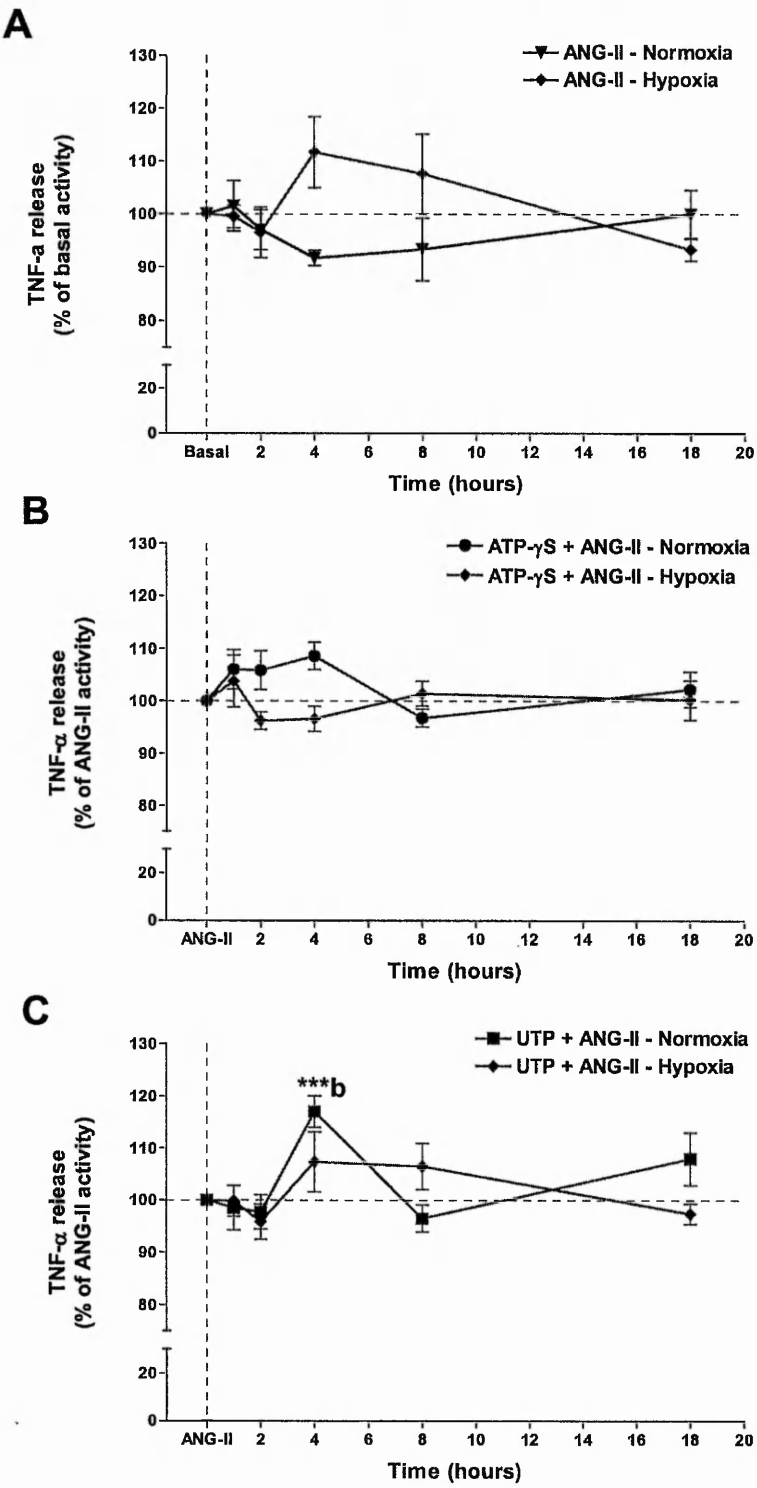

Figure 4.7: Effect of ATP-γS and UTP in presence of angiotensin-II (ANG-II) on tumour necrosis factor-alpha (TNF-α) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts treated with

ANG-II (50nM) were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were stimulated with ANG-II in combination with either ATP-γS (32μM) or UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions (**Panels B** and **C**). TNF-α released into the medium was asserted by ELISA. Data were expressed as the percentage of respective basal (100%) for each time point in panel A whereas in panels B and C data were expressed as the percentage of ANG-II response (100%) for each time point. Each point represents the mean ± S.E.M for 4 – 6 experiments from separate cell cultures, performed in duplicate. *** $P<0.001$; b (UTP + ANG-II; Nx) versus ANG-II response.

Effects of ATP-γS and UTP on TGF-β1 release in the model of ischaemic heart disease:

As illustrated in Figure 4.8A, TGF-β1 production was significantly inhibited by Hx at 18 hours (36%, $P<0.001$). At 4 hours of UTP stimulation the secretion of TGF-β1 was increased by 19% in Nx but not in Hx (Figure 4.8C). ANG-II induced TGF-β1 release at 18 hours Hx but not in Nx (Figure 4.9A). Furthermore, no TGF-β1 release was observed with ATP-γs and UTP in combination in ANG-II under both Nx and Hx (Figure 4.9B-C) (Table 4.1).

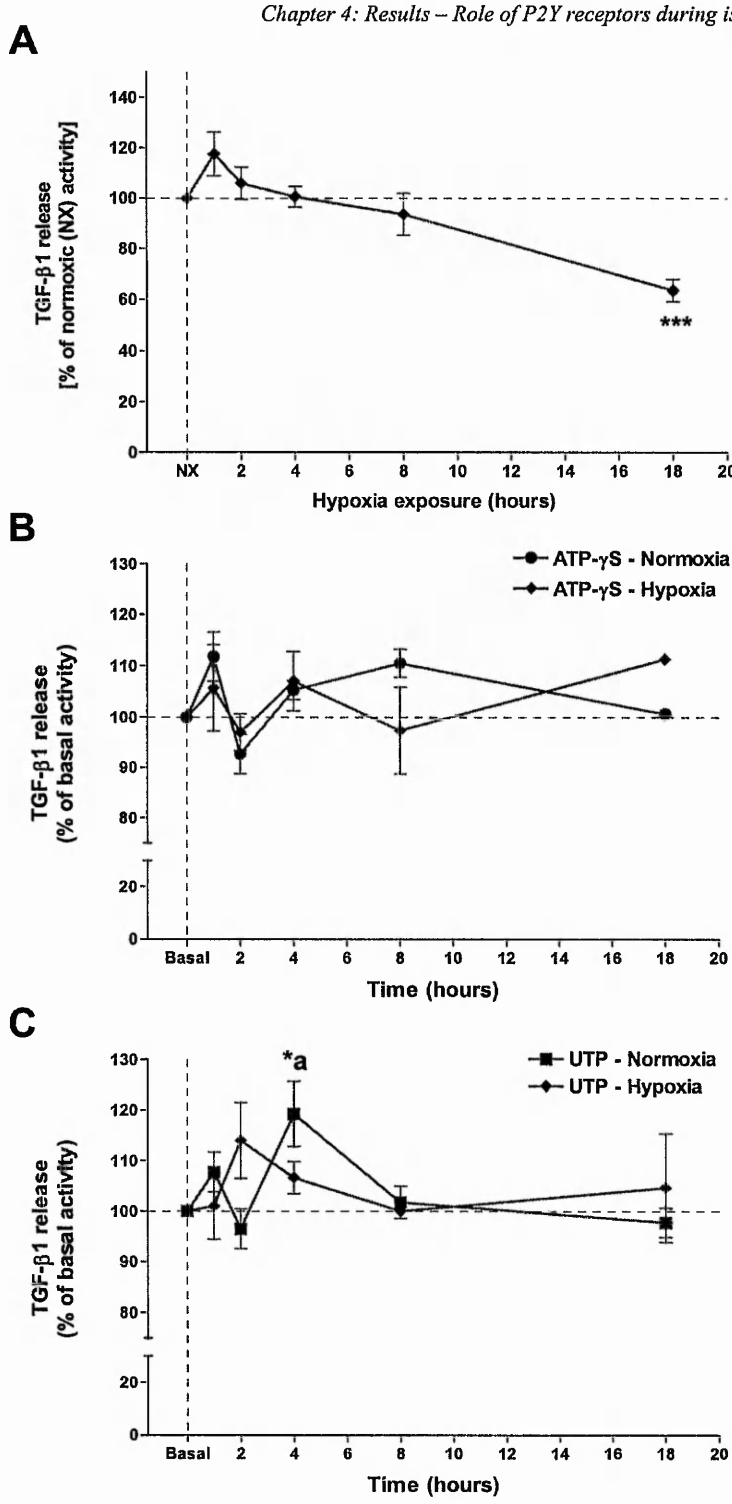

Figure 4.8: Effect of ATP-γS and UTP on transforming growth factor-beta1 (TGF-β1) release in neonatal rat cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts were exposed to hypoxia (0.5% O_2; **Panel A**).

Fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions, respectively (**Panels B** and **C**). TGF-β1 released into the medium was asserted by ELISA. Data were expressed as the percentage of basal TGF-β1 level (100%) for each time point. Each point represents the mean ± S.E.M for 6 – 12 experiments from separate cell cultures, performed in duplicate. * $P<0.05$, ** $P<0.01$; a versus basal response.

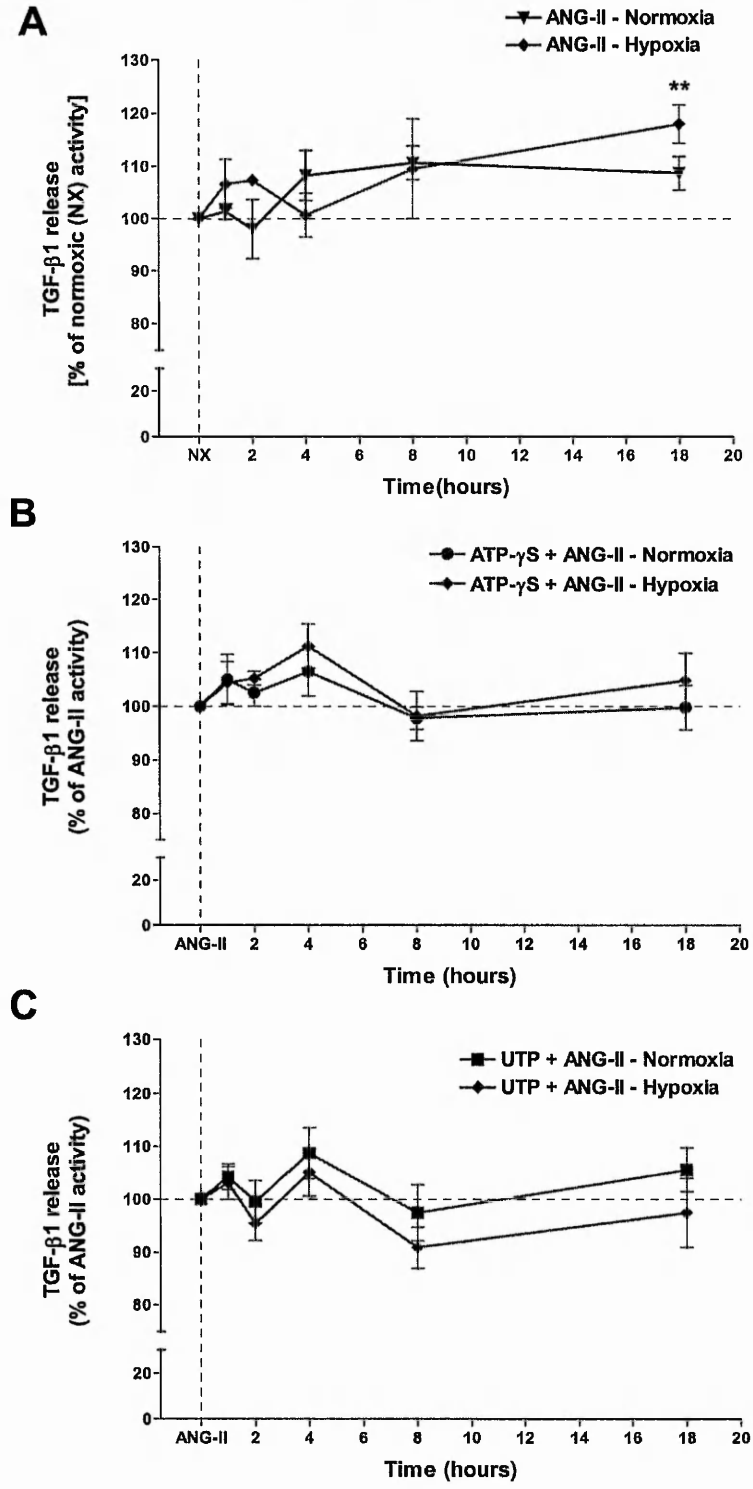

Figure 4.9: Effect of ATP-γS and UTP in presence of angiotensin-II (ANG-II) on transforming growth factor-beta1 (TGF-β1) release in rat neonatal cardiac fibroblasts exposed to normoxic and hypoxic conditions for different time points. Cardiac fibroblasts

treated with ANG-II (50nM) were exposed to hypoxia (0.5% O_2; **Panel A**). Fibroblasts were stimulated with ANG-II in combination with either ATP-γS (32μM) or UTP (10μM) in serum- and glucose-free DMEM media under normoxic and hypoxic conditions (**Panels B** and **C**). TGF-β1 released into the medium was asserted by ELISA. Data were expressed as the percentage of respective basal (100%) for each time point in panel A whereas in panels B and C data were expressed as the percentage of ANG-II response (100%) for each time point. Each point represents the mean ± S.E.M for 4 – 6 experiments from separate cell cultures, performed in duplicate. ** $P<0.01$.

Effects of ATP-γS and UTP on LPS induced cytokine production:

The gram-negative bacterial endotoxin lipopolysaccharide (LPS) is widely used to induce inflammatory properties in a variety of immune cells. During embryonic developmental stages cardiac fibroblasts constituted the innate immune system (Brown *et al.,* 2005a; Nemoto, *et al.,* 2002). LPS stimulates excessive release of proinflammatory cytokines and lipid mediators which lead to septic shock and organ failure (Tracey and Lowry, 1990; Nemoto *et al.,* 2002). Elevated levels of LPS are found in chronic heart failure patients with oedematous exacerbation and LPS induces left ventricular dysfunction and septicemia (Zeni *et al.,* 2001).

LPS induced an increase in basal IL-1β (1000% over basal), IL-6 (2894% over basal) and TNF-α (246% over basal) in a concentration-dependent manner (Figure 4.10). Neonatal rat cardiac fibroblasts were stimulated with LPS in order to determine the pro/anti-inflammatory properties of ATP-γS and UTP. The concentration of LPS (100ng/ml) used in these studies corresponds to the near-saturation point to stimulate IL-1β, IL-6 and TNF-α (Figure 4.10).

LPS induced the production of IL-1β, IL-6 and TNF-α in a time dependent manner (Figure 4.11A, 4.12A, 4.13A). ATP-γS potentiated LPS-stimulated IL-1β release at 2 hours (18%, $P<0.05$) and 4 hours (25%, $P<0.001$; Figure 4.11B). At 18 hours, LPS-induced IL-1β release was inhibited by ATP-γS (32%, $P<0.001$, Figure 4.11B). UTP also augmented LPS-activated IL-1β release at 4 hours (18%, $P<0.05$, Figure 4.11B). The production of IL-6 induced by LPS was amplified by ATP-γS from 2 hours (34%, $P<0.001$) and reached a plateau at 4 hours (39%, $P<0.001$) and decreased at 8 hours (19%, $P<0.05$, Figure 4.12B). UTP had no effect on IL-6 production (Figure 4.12B). LPS-activated TNF-α release was potentiated by ATP-γS but not by UTP at 4 hours (40%, $P<0.05$, Figure 4.13B). These results imply that ATP-γS, probably via P2Y receptors, potentiates the release of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α while UTP only stimulates the release of IL-1β. This indicates that ATP-γS and UTP stimulate different P2Y receptors during infectious myocarditis (simulated by LPS; Table 4.2).

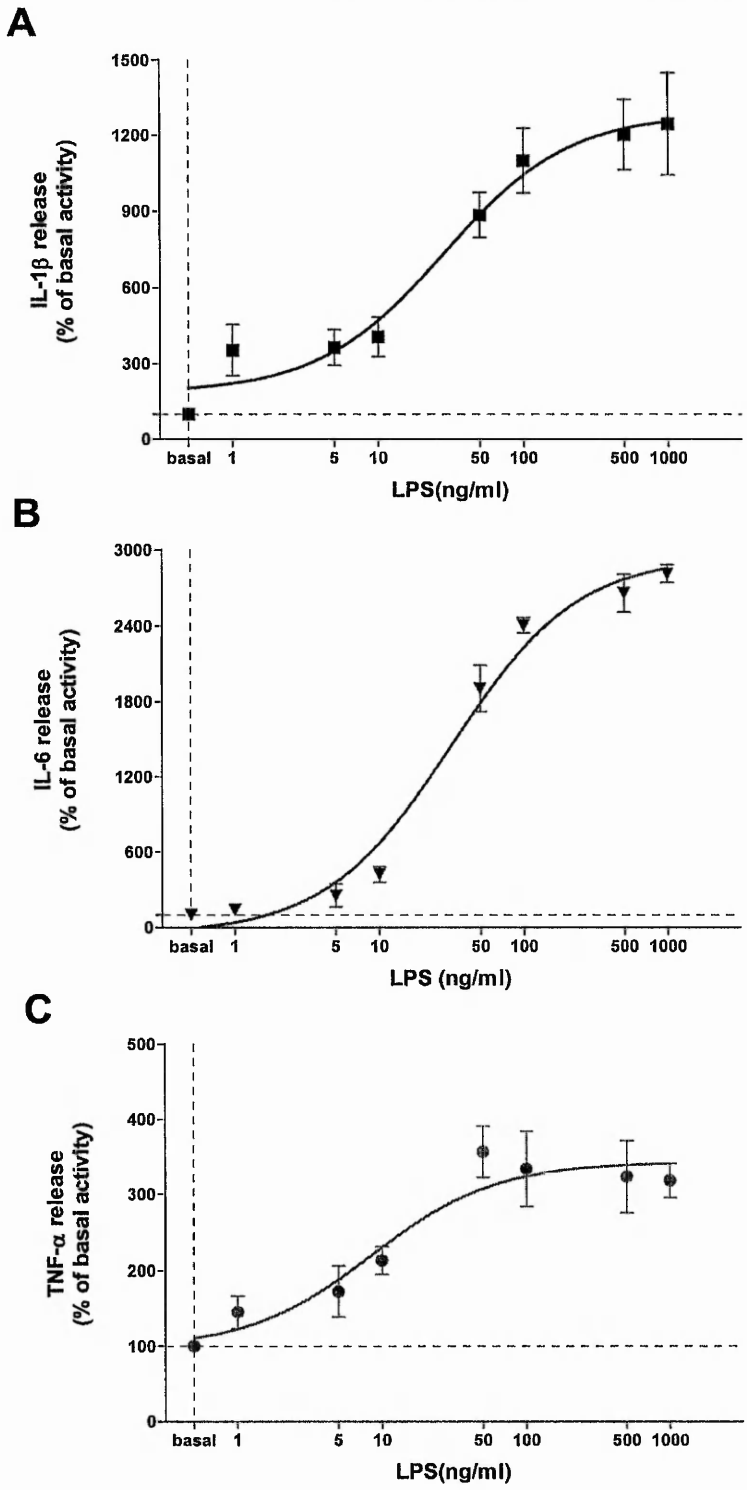

Figure 4.10: Effect of lipopolysacharide (LPS) on the release of interleukin-1β (IL-1 β, Panel A), interleukin-6 (IL-6, Panel B), and tumour necrosis factor-alpha (TNF-α, Panel C) in neonatal rat cardiac fibroblasts. Cardiac fibroblasts were stimulated with indicated

concentrations of LPS in serum and glucose-free DMEM media for 8 hours under normoxic conditions. The cell culture supernates were collected and evaluated for IL-1β, IL-6 and TNF-α by ELISA. Data were expressed as the percentage of the basal level of respective cytokine (100%). Each point represents the mean ± S.E.M for 3 experiments from separate cell cultures, performed in duplicate.

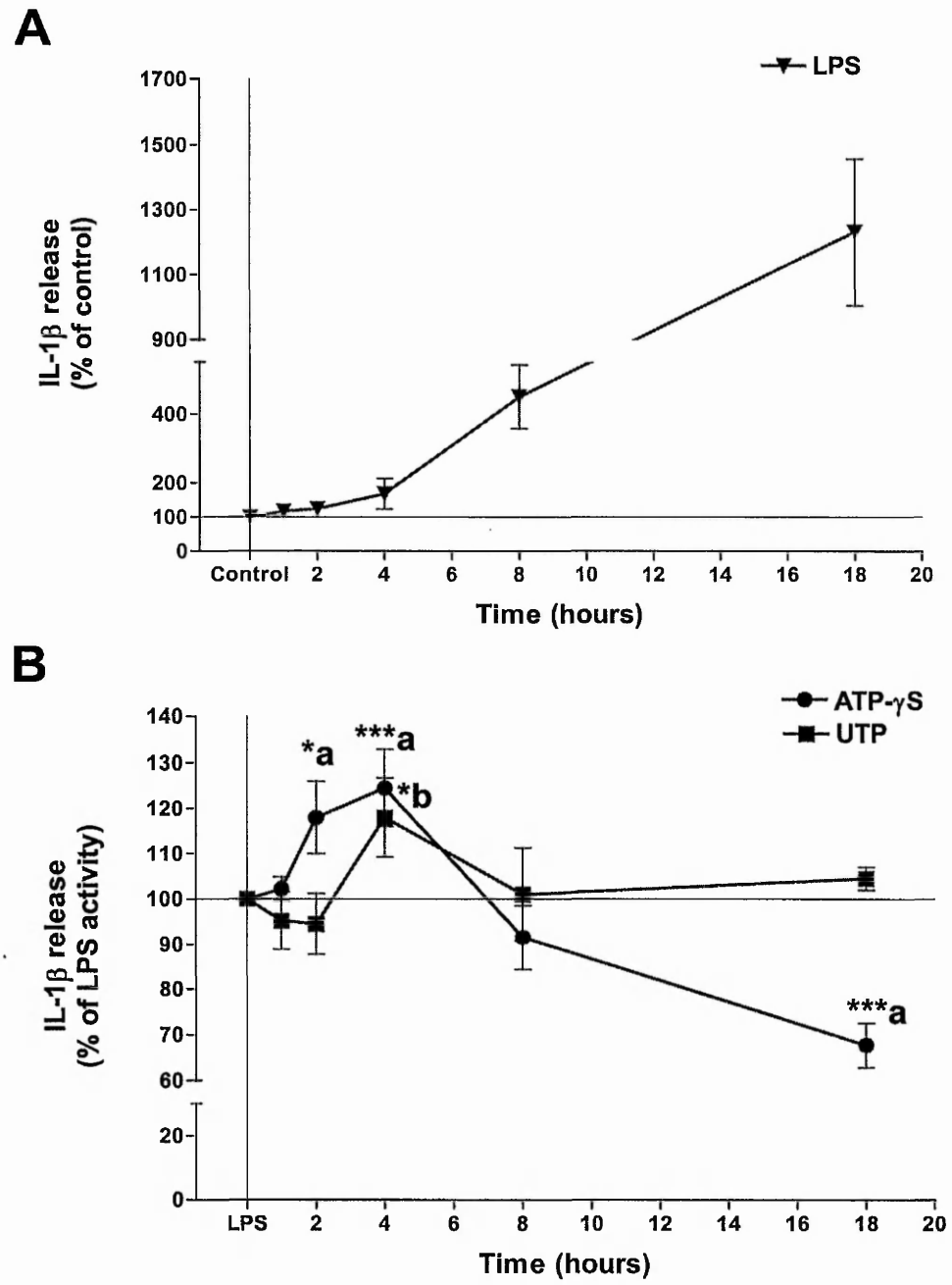

Figure 4.11: Effect of ATP-γS and UTP on lipopolysacharide (LPS)-induced release of interleukin-1β (IL-1β) in rat neonatal cardiac fibroblasts at different time points. **Panel A** represents the 100ng/ml LPS-augmented IL-1β release. Fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in the presence of LPS in serum- and glucose-free DMEM media under normoxic conditions (**Panels B**). IL-1β released into the medium was asserted by ELISA. Data were expressed as the percentage of respective basal (100%) for

panel A whereas in panel B data were expressed as the percentage of LPS response (100%) at each time point. Each point represents the mean ± S.E.M for 3 – 4 experiments from separate cell cultures, performed in duplicate. * $P<0.05$, *** $P<0.001$; a (ATP-γS), b (UTP) versus LPS response.

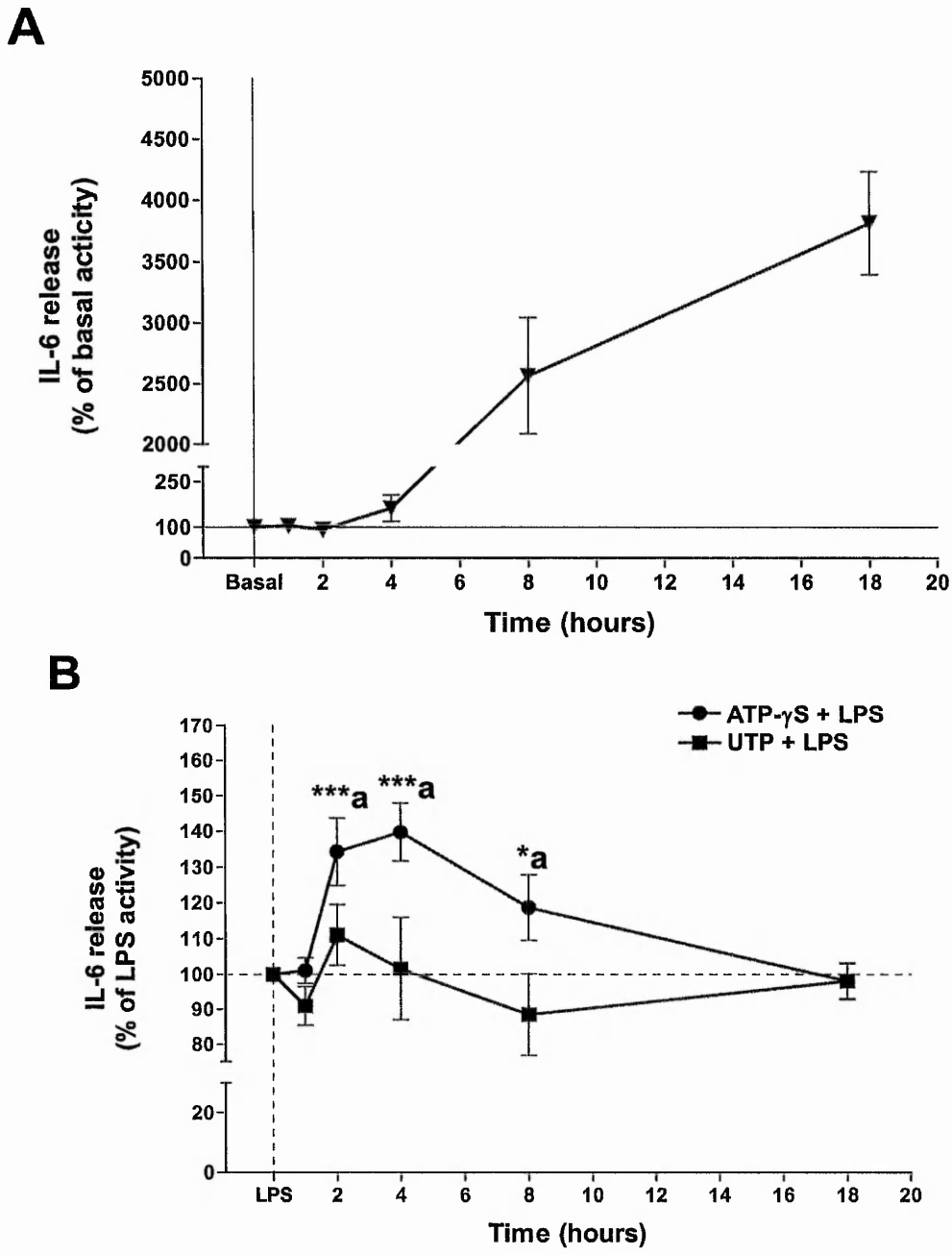

Figure 4.12: Effect of ATP-γS and UTP on lipopolysacharide (LPS)-induced release of interleukin-6 (IL-6) in neonatal rat cardiac fibroblasts at different time points. **Panel A** represents the 100ng/ml LPS-augmented IL-6 release. Fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in the presence of LPS in serum- and glucose-free DMEM media under normoxic conditions (**Panels B**). IL-6 released into the medium was asserted by ELISA. Data were expressed as the percentage of respective basal (100%) for

panel A whereas in panel B data were expressed as the percentage of LPS response (100%) at each time point. Each point represents the mean ± S.E.M for 3 – 4 experiments from separate cell cultures, performed in duplicate. * $P<0.05$, *** $P<0.001$; a (ATP-γS), b (UTP) versus LPS response.

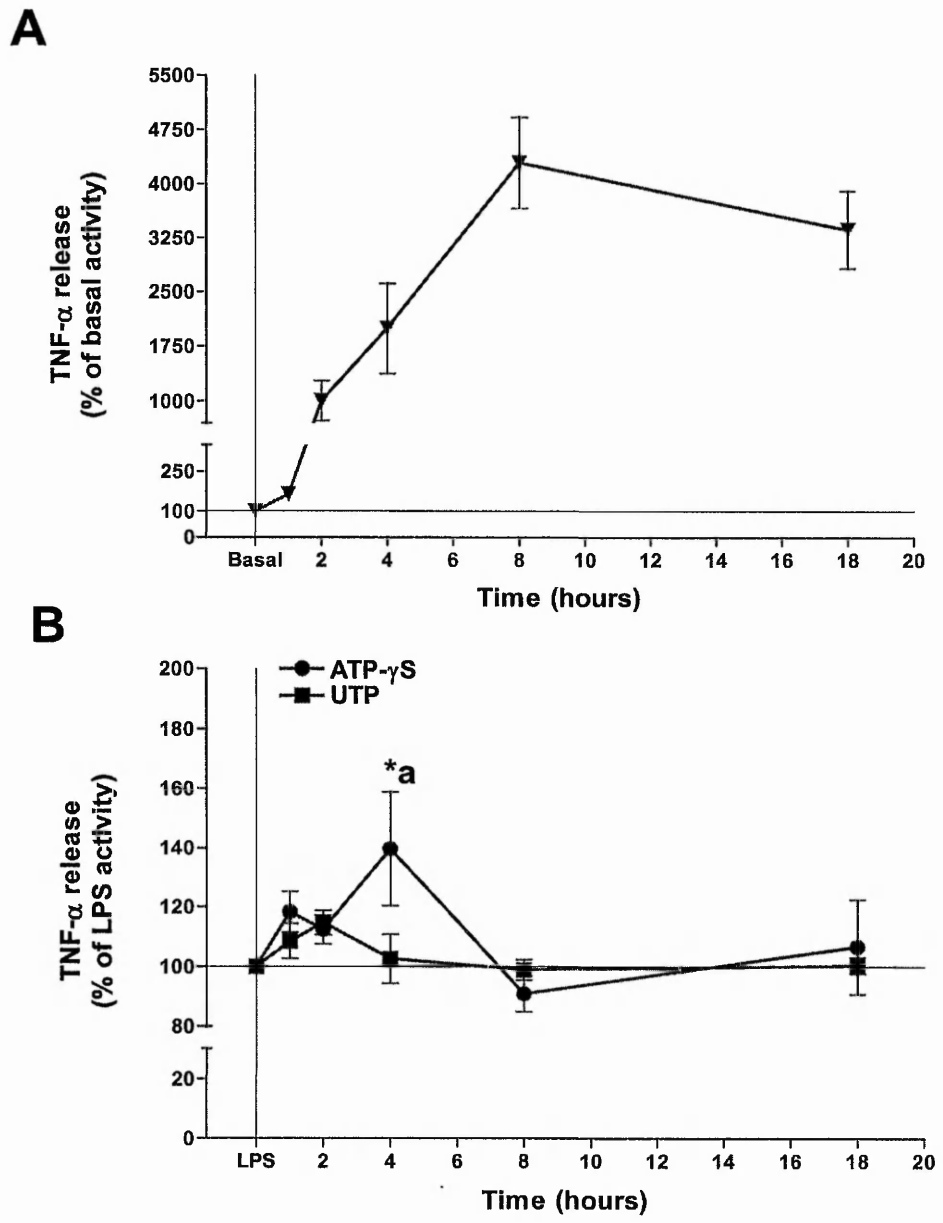

Figure 4.13: Effect of ATP-γS and UTP on lipopolysacharide (LPS)-induced release of tumour necrosis factor-alpha (TNF-α) in neonatal rat cardiac fibroblasts at different time points. **Panel A** represents the 100ng/ml LPS-augmented TNF-α release. Fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in the presence of LPS in serum- and glucose-free DMEM media under normoxic conditions (**Panels B**). TNF-α released into the medium was asserted by ELISA. Data were expressed as the percentage of respective basal (100%) for panel A whereas in panel B data were expressed as the percentage of LPS

response (100%) at each time point. Each point represents the mean ± S.E.M for 3 – 4 experiments from separate cell cultures, performed in duplicate. * $P<0.05$; a (ATP-γS) versus LPS response.

Table 4.2: Effect of P2Y receptors on lipopolysaccharide (LPS)-induced cytokine release.

Cytokine	Normoxia					
				+ LPS		
	ATP-γS	UTP	LPS	ATP-γS	UTP	
IL-1β	↔	↔	↑	↑↑	↑	4 Hours
IL–6	↑	↔	↑	↑↑	↔	4 Hours
TNF-α	↔	↔	↑	↑↑	↔	4 Hours
IL-1β	↔	↔	↑↑↑	↓↓	↔	18 Hours
IL–6	↑	↔	↑↑↑	↔	↔	18 Hours
TNF-α	↔	↔	↑↑↑	↑	↔	18 Hours

Neonatal rat cardiac fibroblasts were stimulated with ATP-γS (32μM) or UTP (10μM) in absence or presence of lipopolysacharide (LPS; 100ng/ml) in serum- and glucose-free DMEM media for 4 hours and 18 hours under normoxic conditions. □ - no change; □- increase; □- decrease in cytokine secretion.

Overall, it is notable from the cytokine studies that ATP-γS and UTP either activated or inhibited the cytokine release (IL-1β, IL-6, TNF-α and TGF-β1) at 4 and 18 hours. Therefore, further investigations for the effects of nucleotides on rat neonatal cardiac fibroblasts were carried out at 4 and 18 hours.

4.2 Effect of ATP-γS and UTP on cell viability in cardiac fibroblasts

Cardiac fibroblasts are active and resistant to hypoxic stress which generally threatens the survival of other cell types and fibroblasts from lung and skin (Mayorga *et al.,* 2004). Previous research has shown that stimulation of $P2Y_{1,2,12}$ receptors regulates death and survival mechanisms in astrocytes (Mamedova *et al.,* 2006; Chorna *et al.,* 2004). In addition, UTP via $P2Y_{2,4}$ receptors protect rat cardiomyocytes from hypoxic stress, however activation of $P2Y_4$ induced death in human neuroblastoma SH-SY5Y cells (Yitzhaki *et al.,* 2005; Cavaliere *et al.,* 2005). In view of these studies and the fact that ATP-γS and UTP mediated the production of inflammatory cytokines (section 4.1); it is hypothesized that these nucleotides will modulate cardiac fibroblast viability. In the present study, fibroblast viability was investigated by measuring lactate dehydrogenase (LDH) released into the cell culture media. Unfortunately, the necrotic and apoptotic types of cell death can not be differentiated by LDH assay, which mainly measures cell necrosis. Cell death was measured at 4 and 18 hours after different treatments and conditions (section 2.10) and compared with LDH release from normoxic controls.

The exposure of cardiac fibroblasts to 4 hours Hx did not modify cell viability (Figure 4.14A) whereas, ANG-II treatment significantly decreased the LDH release by 29% in Hx ($P<0.05$, Figure 4.14B). In contrast, 18 hours of Hx condition induced cell death by 258±28% of normoxic control ($P<0.001$, Figure 4.15A), while ANG-II did not modify LDH release in 18 hours Hx (Figure 4.15B). ATP-γS and UTP in absence or presence of ANG-II under Nx and Hx environments did not affect the viability of fibroblasts at 4 hours and 18 hours (Figures 4.14, 4.15). In this study, ATP-γS and UTP did not affect the cell viability, suggesting that P2Y are not involved in regulating survival mechanisms in neonatal rat cardiac fibroblasts.

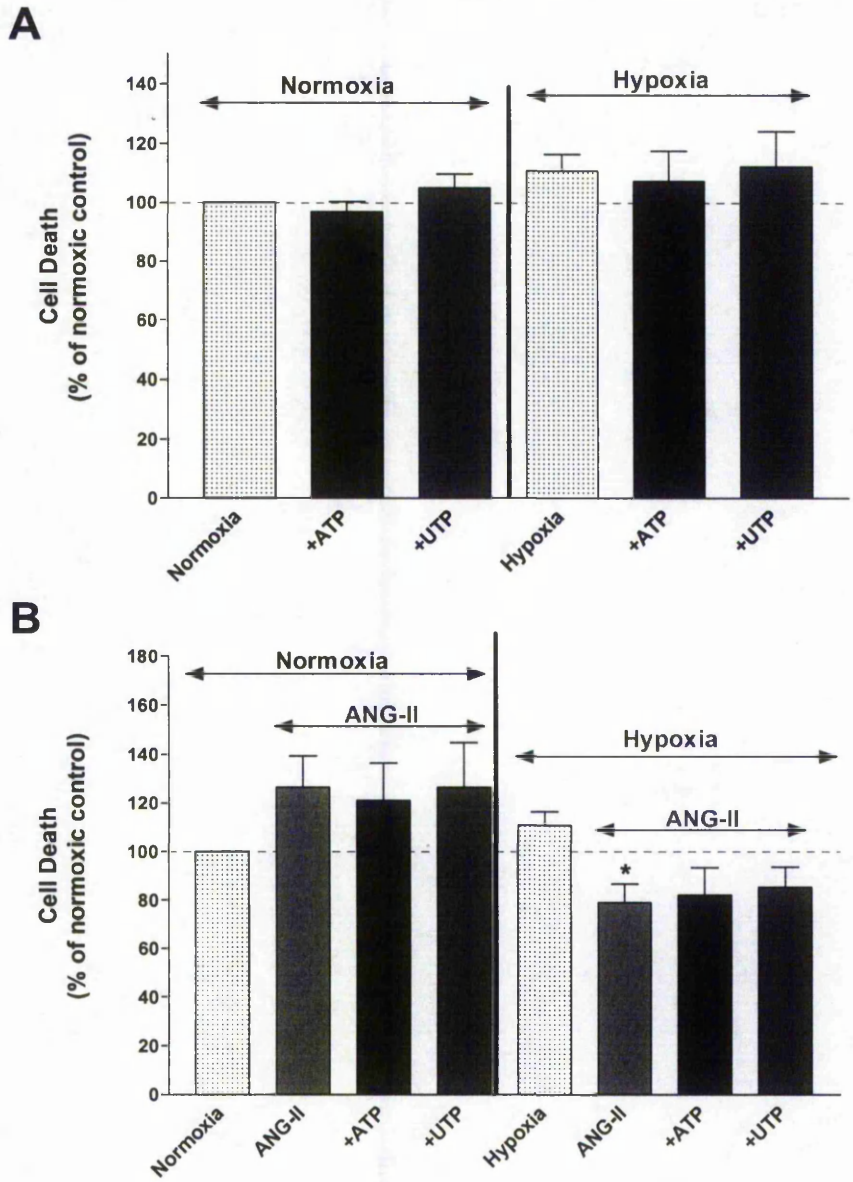

Figure 4.14: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on cell death in neonatal rat cardiac fibroblasts exposed for 4 hours in normoxia and hypoxia. Cardiac fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of ANG-II (50nM; **Panel B**) and exposed for 4 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell culture supernates were evaluated for LDH activity. Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 3 – 4 experiments from separate cell cultures, performed in duplicate. * $P<0.05$ versus hypoxic response.

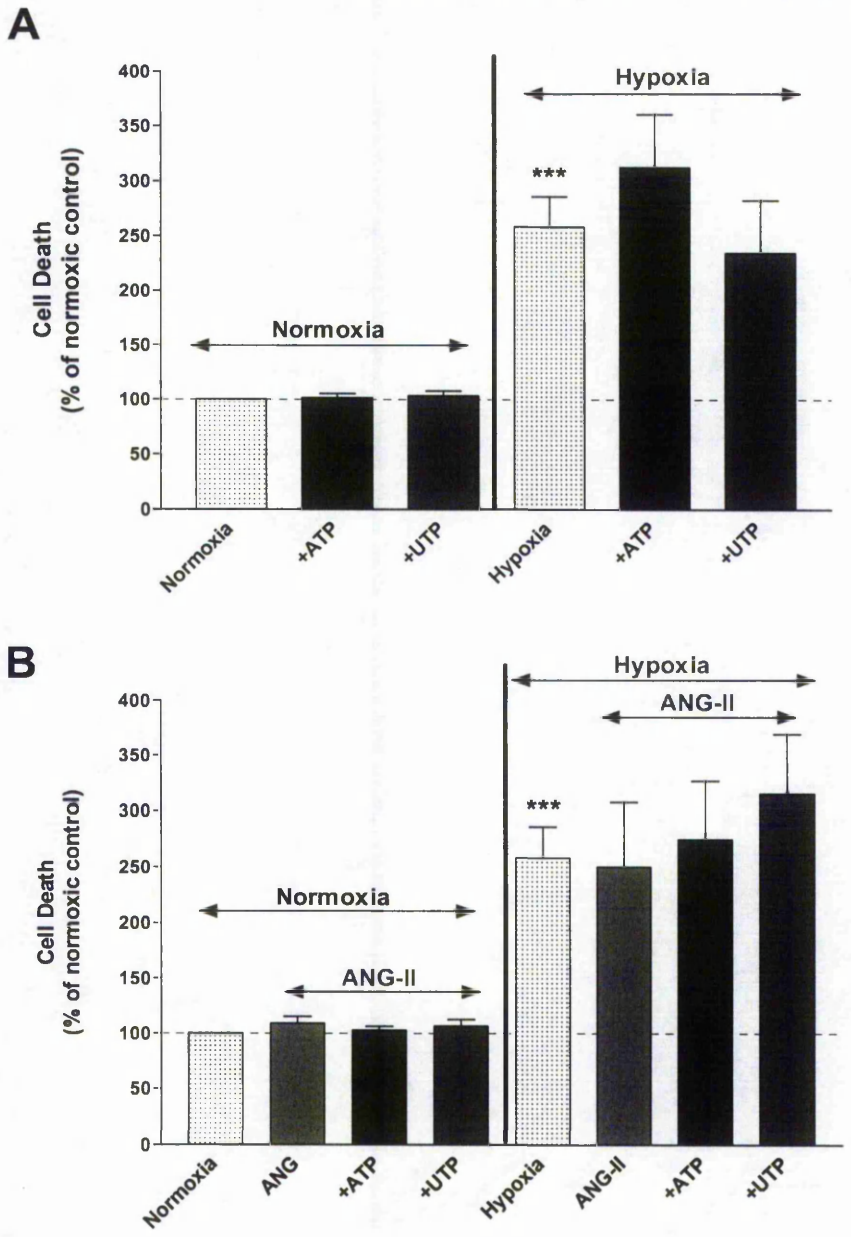

Figure 4.15: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on cell death in neonatal rat cardiac fibroblasts exposed for 18 hours in normoxia and hypoxia. Cardiac fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of angiotensin-II (50nM; **Panel B**) and exposed for 18 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell culture supernates were evaluated for LDH activity. Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 3 – 4 experiments from separate cell cultures, performed in duplicate. *** $P<0.001$ versus normoxic response.

4.3 Effect of ATP-γS and UTP on collagen synthesis in cardiac fibroblasts

Cardiac fibroblasts are the predominant cell type in heart and their main function is the deposition of extracellular matrix (ECM; Camelliti *et al.,* 2005; Brown *et al.,* 2005a). The ECM comprises of collagen (90%; Type-I, III, IV, V, VI), fibronectin, elastin, laminin, proteoglycans and glycosaminoglycans (Bosman and Stamenkovic, 2003; Jugdutt, 2003a, b). During, patho-physiological conditions fibroblasts are activated and secrete excess ECM leading to cardiac fibrosis and left ventricular hypertrophy (Brown *et al.,* 2005a). Since collagen is the major constituent of ECM, its production was monitored by [^{3}H]-proline incorporation assay in different fractions – ECM fraction (insoluble collagen), cellular fraction (soluble collagen) and cell culture medium.

4 hours Hx induced an inhibition of insoluble collagen production by 26% compared to Nx control (P<0.01, Figure 4.16A). Fibroblasts treated with ATP-γS stimulated insoluble collagen synthesis by 53% in Nx (P<0.05, Table 4.3), whereas no increase in insoluble collagen was observed in Hx (Figure 4.16A). The profibrotic factor ANG-II in our study did not potentiate insoluble collagen synthesis in Nx and Hx conditions (4 hours, Figure 4.16A, Table 4.3). ATP-γS in combination with ANG-II potentiated insoluble collagen synthesis in Hx (4 hours), whereas Nx conditions did not mediate the insoluble collagen secretion (Figure 4.16A). The uracil nucleotide UTP, alone or in presence of ANG-II did not induce collagen synthesis in the ECM fraction when treated for 4 hours in Nx and Hx (Figure 4.16A, Table 4.3). ATP-γS and UTP in absence or presence of ANG-II did not stimulate collagen production in the cellular and cell culture medium fractions (Figure 4.16B – C). 4 hours of various treatments and conditions on cardiac fibroblasts did not alter the total (insoluble + soluble + medium) collagen synthesis (Figure 4.17).

Human cardiac fibroblasts induced collagen Type-I production when exposed to prolonged hypoxic conditions for 24 hours (Agocha *et al.,* 1997). Indeed in the present study, neonatal rat cardiac fibroblasts exposed for 18 Hx stimulated synthesis of insoluble collagen (37%, P<0.01), however the soluble (cellular) collagen was inhibited by 28% (P<0.001, Figure 4.18A – B). Surprisingly, fibroblasts treated with ANG-II for 18 hours Nx did not induce insoluble collagen secretion but Hx exposure appears to induce collagen synthesis, although this was not statistical significant (Figure 4.18A).

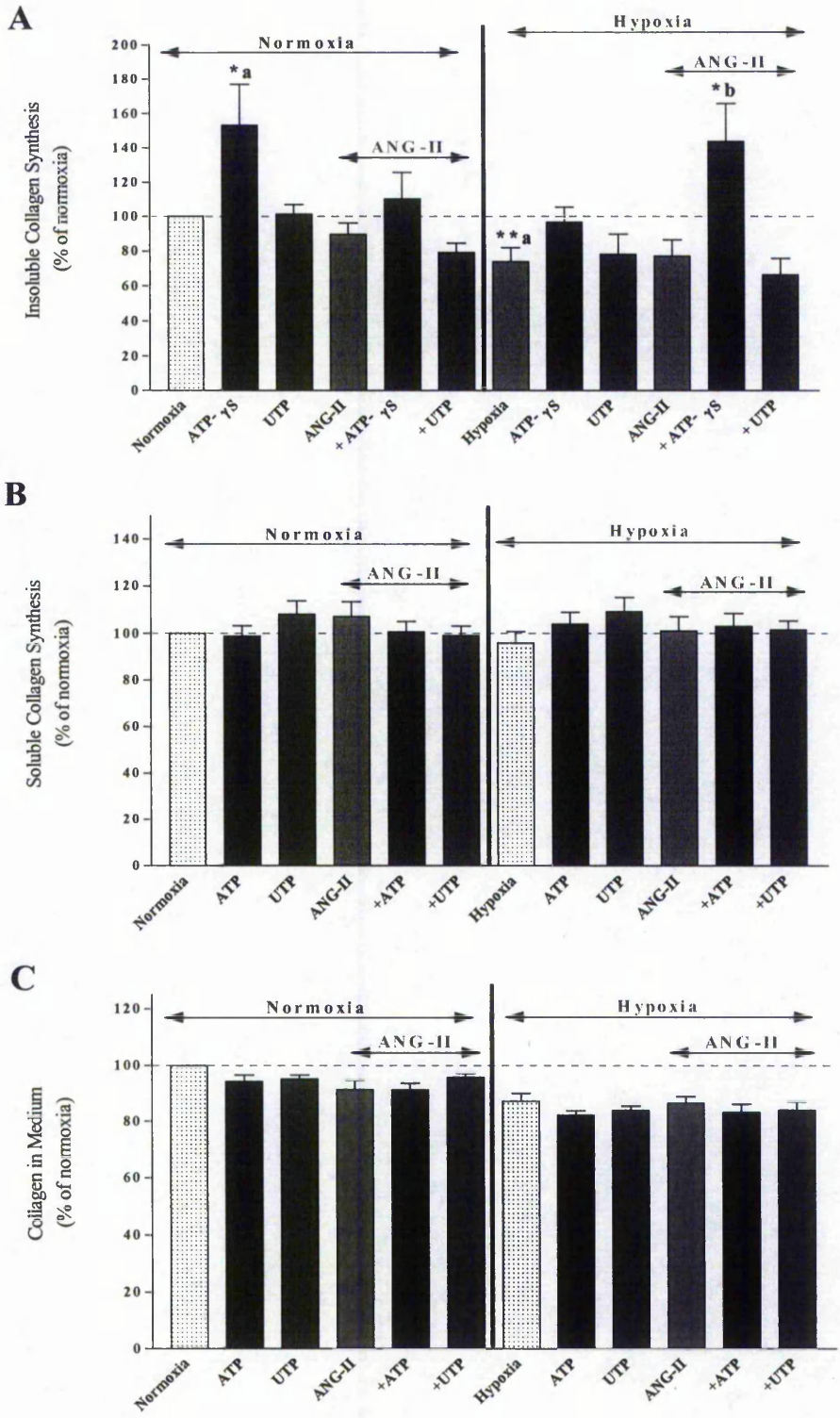

Figure 4.16: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on collagen synthesis in neonatal rat cardiac fibroblasts exposed to normoxia and hypoxia

for 4 hours. Cardiac fibroblasts were radio-labeled with [^{3}H]-proline in DMEM supplemented with 2% v/v FCS for 24 hours. The labeled fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence or presence of ANG-II (50nM) in serum- and glucose-free medium and exposed for 4 hours in normoxic (21% O_2) and hypoxic (0.5% O_2) conditions. The collagen content was measured in extracellular fraction (insoluble collagen, **Panel A**), cellular fraction (soluble collagen, **Panel B**) and cell culture medium (**Panel C**). Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 6 – 8 experiments from separate cell cultures, performed in duplicate. * $P<0.05$, ** $P<0.01$; a versus normoxic response and b versus ANG-II response in hypoxia.

Table 4.3: Regulation of insoluble collagen accumulation by P2Y receptors in neonatal rat cardiac fibroblasts during ischaemic heart disease

Insoluble Collagen Accumulation	Normoxia (Nx)					Hypoxia (Hx)					
				+ ANG-II						+ ANG-II	
	ATP-γS	UTP	ANG-II	ATP-γS	UTP	Hx	ATP-γS	UTP	ANG-II	ATP-γS	UTP
4 Hours	↑↑↑	↔	↔	↔	↔	↓↓	↔	↔	↔	↑↑	↔
18 Hours	↔	↑↑	↔	↔	↓↓↓	↑↑	↔	↓↓	↔	↔	↓↓

The *in vitro* model of ischaemic heart disease was developed in associating with angiotensin-II (ANG-II; 50nM) and hypoxia (0.5% O_2). Cardiac fibroblasts were stimulated with ATP-γS (32μM) or UTP (10μM) in absence or presence of ANG-II in serum and glucose-free DMEM media for 4 hours and 18 hours. □ - no change; □- increase; □- decrease in insoluble collagen accumulation.

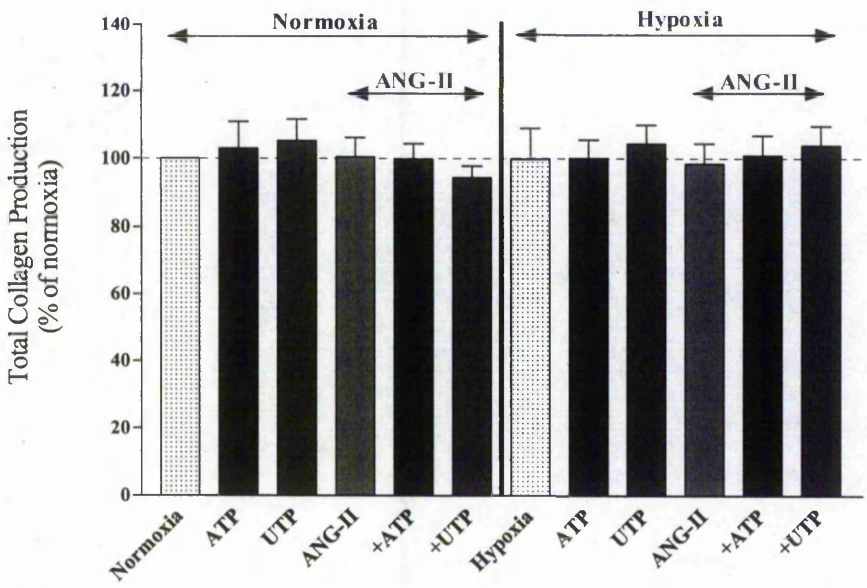

Figure 4.17: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on total collagen synthesis in neonatal rat cardiac fibroblasts exposed to normoxia and hypoxia for 4 hours. Cardiac fibroblasts were radio-labeled with [^{3}H]-proline in DMEM supplemented with 2% v/v FCS for 24 hours. The labeled fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence or presence of ANG-II (50nM) in serum and glucose-free medium and exposed for 4 hours in normoxic (21% O_2) and hypoxic (0.5% O_2) conditions. The total collagen content was obtained by adding the insoluble collagen, soluble collagen and collagen in cell culture medium (Figure 4.16). Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 6 – 8 experiments from separate cell cultures, performed in duplicate.

Contrary to 4 hours exposure, 18 hours of treatment with ATP-γS or combination with ANG-II in Nx and Hx did not modify the collagen synthesis in the ECM fraction (Figure 4.18A). UTP mediated insoluble collagen production in Nx conditions (18 hours, 21%, $P<0.001$), however in the presence of ANG-II insoluble collagen secretion was inhibited by around 30% compared to ANG-II response ($P<0.01$, Figure 4.18A). UTP also attenuated the increases in insoluble collagen production potentiated by 18 hours of Hx (37%, $P<0.05$) and ANG-II (24%, $P<0.05$) treatments in fibroblasts (Figure 4.18A). ATP-γS and UTP in absence or presence of ANG-II did not mediate collagen production in the cellular and cell culture medium fractions (Figure 4.18B – C). 18 hours of various treatments and conditions did not alter the total (insoluble + soluble + medium) collagen synthesis in neonatal rat cardiac fibroblasts (Figure 4.19; Table 4.3).

DNA was quantified by ethidium bromide to determine if the modification of collagen synthesis induced by ATP-γS and UTP involved proliferation of the cells. It is well known that ANG-II stimulates proliferation of fibroblasts and hypoxia regulates cell cycle and induces proto-oncogenes expression (Bouzegrhane and Thibault, 2002; Agocha *et al.*, 1997). Moreover, collagen synthesis is regulated by transcriptional and post-transcriptional factors (Jugdutt, 2003b, Laurent, 1987). However, as illustrated in figure 4.20 there were no alterations in DNA levels when cardiac fibroblasts were exposed to various conditions and treatments for 4 and 18 hours, indicating the absence of cell proliferation.

Overall, ATP-γS stimulates insoluble collagen secretion during early stage ischaemic conditions (4 hours) whereas UTP prevents insoluble collagen accumulation during prolonged ischaemic conditions (18 hours; Table 4.3).

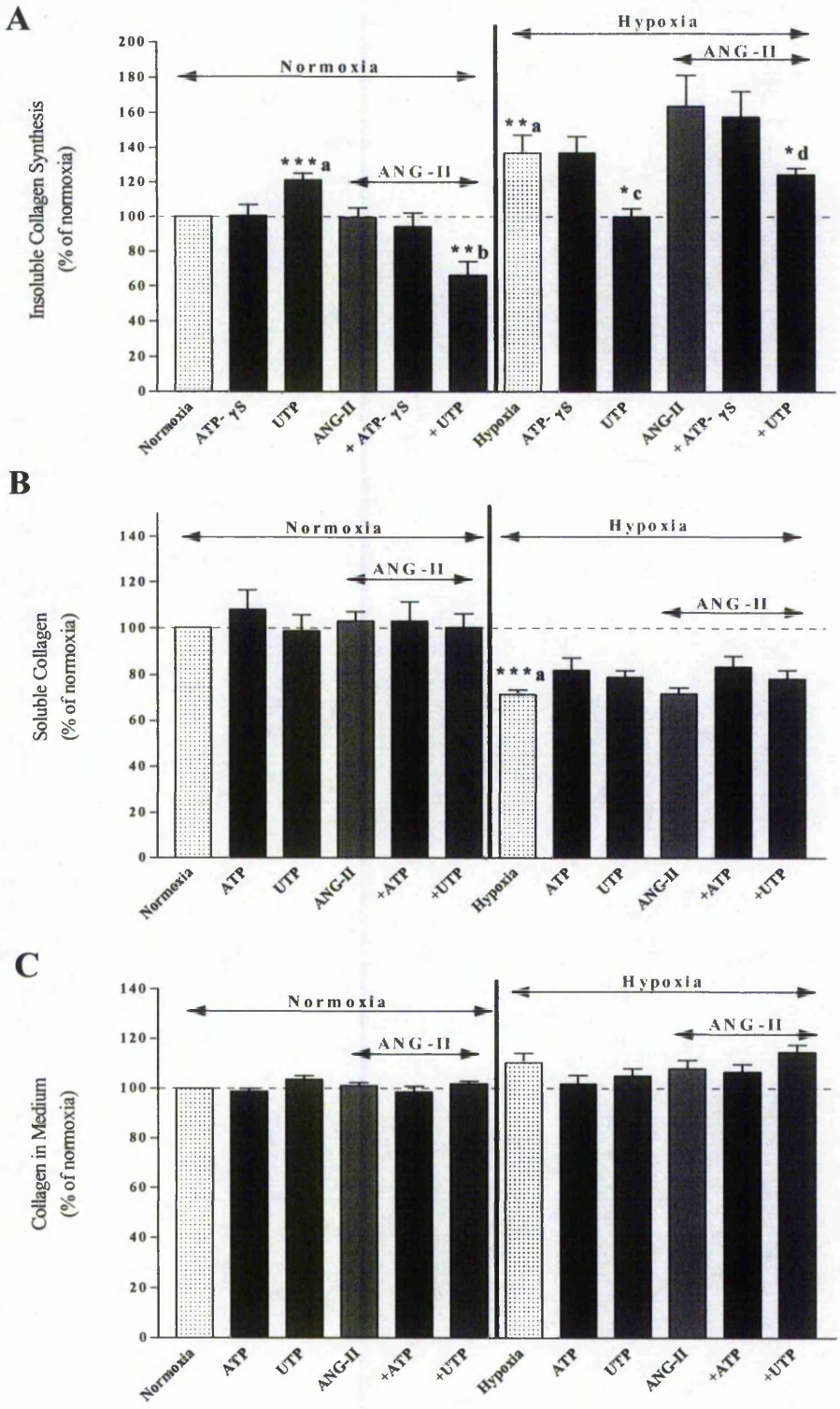

Figure 4.18: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on collagen synthesis in neonatal rat cardiac fibroblasts exposed to normoxia and hypoxia

for 18 hours. Cardiac fibroblasts were radio-labeled with [^{3}H]-proline in DMEM supplemented with 2% v/v FCS for 24 hours. The labeled fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence or presence of ANG-II (50nM) in serum and glucose-free medium and exposed for 18 hours in normoxic (21% O_2) and hypoxic (0.5% O_2) conditions. The collagen content was measured in extracellular fraction (insoluble collagen, **Panel A**), cellular fraction (soluble collagen, **Panel B**) and cell culture medium (**Panel C**). Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 5 – 8 experiments from separate cell cultures, performed in duplicate. * $P<0.05$, ** $P<0.01$; a versus normoxic response, b versus ANG-II response in normoxia, c versus hypoxic response and d versus ANG-II response in hypoxia.

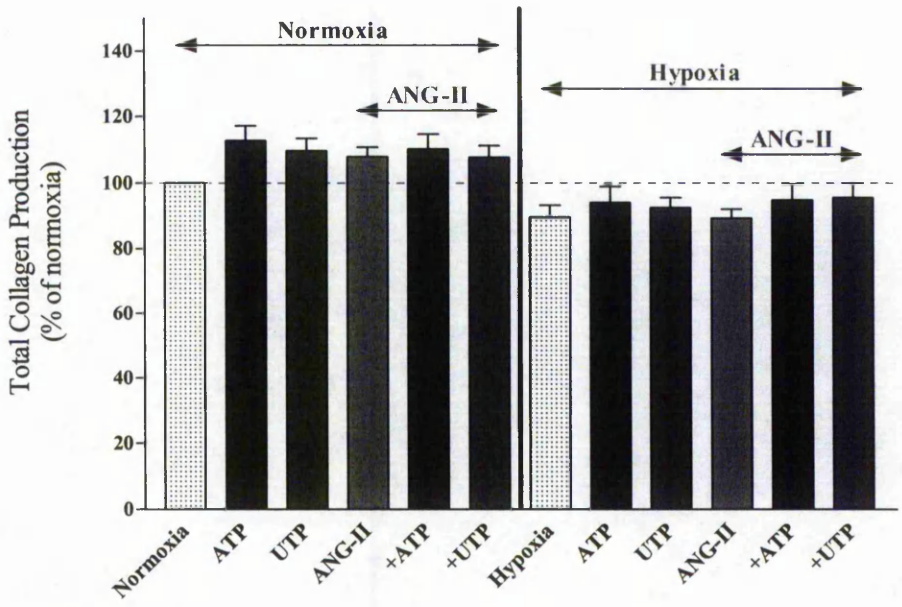

Figure 4.19: Effect of ATP-γS and UTP in absence or presence of angiotensin-II (ANG-II) on total collagen synthesis in neonatal rat cardiac fibroblasts exposed to normoxia and hypoxia for 18 hours. Cardiac fibroblasts were radio-labeled with [^{3}H]-proline in DMEM supplemented with 2% v/v FCS for 24 hours. The labeled fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence or presence of ANG-II (50nM) in serum- and glucose-free medium and exposed for 18 hours in normoxic (21% O_2) and hypoxic (0.5% O_2) conditions. The total collagen content was obtained by adding the insoluble collagen, soluble collagen and collagen in cell culture medium (Figure 4.18). Data were expressed as the percentage of the normoxic control (100%). Each point represents the mean ± S.E.M for 5 – 8 experiments from separate cell cultures, performed in duplicate.

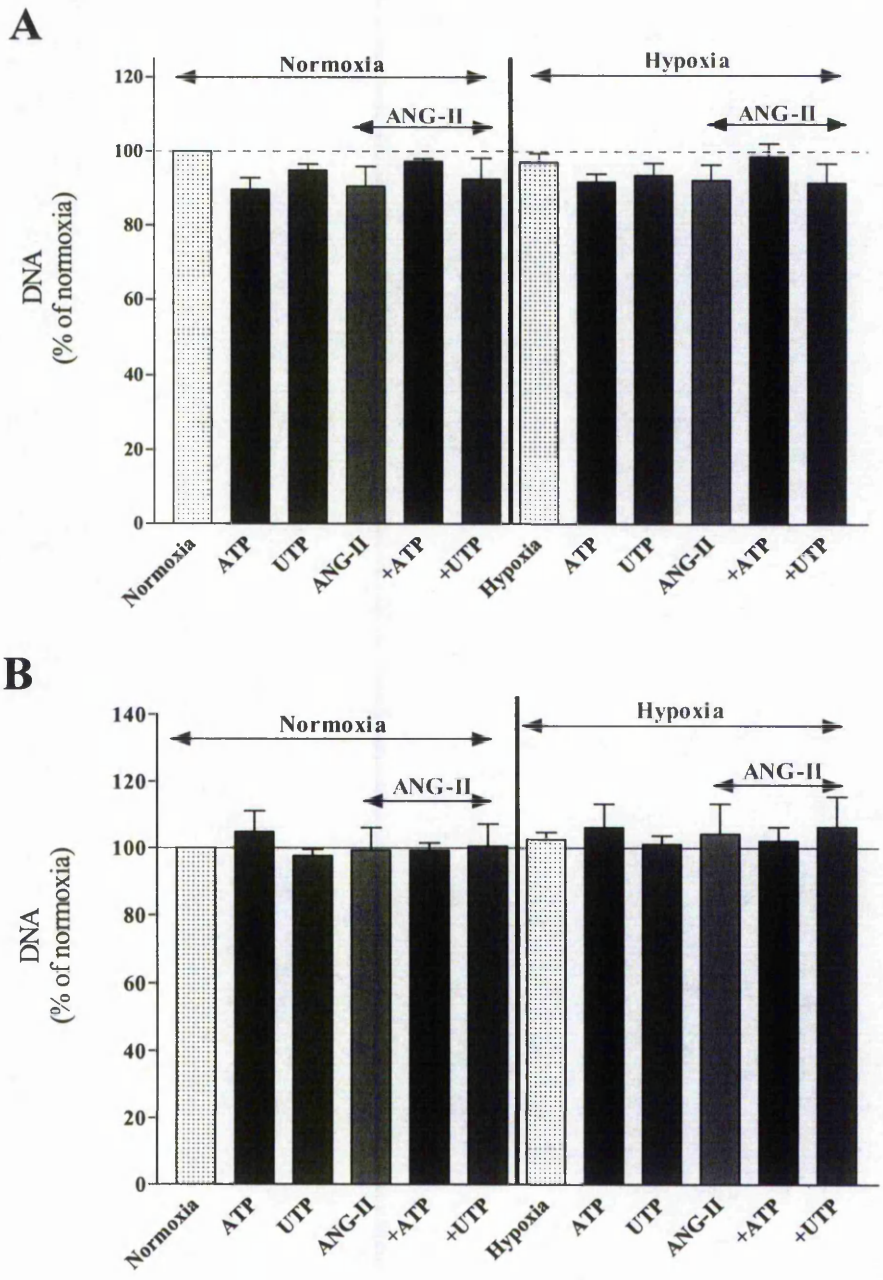

Figure 4.20: Effect of ATP-γS and UTP in absence or presence of angiotensin-II on DNA synthesis in neonatal rat cardiac fibroblasts exposed to normoxia and hypoxia for 4 (**Panel A**) and 18 hours (**Panel B**). 2.5mg/ml ethidium bromide was added to 20μl of soluble collagen samples and the DNA was quantified by measuring the fluorescence using a SpectraFluor 96-well plate reader (Excitation wavelength: 540nm, Emission wavelength: 595nm, lag time: 20 μsec. and integration time: 40 μsec.)

4.4 Effect of ATP-γS and UTP on ERK1/2, p38 MAPK, JNK and Akt/PKB phosphorylation in cardiac fibroblasts

The heart is a dynamic organ which undergoes molecular reprogramming, cellular adaptation and remodeling in pathological conditions. All these events are initiated and coordinated by stress-responsive signaling pathways such as extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun NH_2-terminal kinase (JNK), protein kinase C (PKC), Akt/protein kinase B (PKB) and janus kinase (JAK; Sugden and Bogoyevitch, 1995; Petrich and Wang, 2004). Therefore the activation of kinases was investigated by western blotting following the treatment of cardiac fibroblasts with ATP-γS, UTP and ANG-II in Nx and Hx.

ERK1 and ERK2 are the most widely studied and abundantly expressed isoforms of ERK (Baines and Molkentin, 2005). Several studies have unequivocal suggested that ERK1/2 activation protects the cells by inhibiting apoptosis (Yue *et al.,* 2000, Iwai-Kanai *et al.,* 2002, Lips *et al.,* 2004; Germack and Dickenson, 2005). As shown in figure 4.21, 4 hours UTP treatment in NX down-regulated the ERK1/2 activity (72±5%, $P<0.001$), whereas ATP-γS did not affect ERK1/2 phosphorylation. In cardiomyocytes and adventitial fibroblasts hypoxia stimulated ERK1/2 activity (Germack and Dickenson, 2005; Gerasimovskaya *et al.,* 2002), however Hx induced an inhibition of ERK1/2 activity in cardiac fibroblasts (48±7%, $P<0.001$). ATP-γS and UTP did not modulate the inhibition of ERK1/2 activity by Hx. In addition, ATP-γS and UTP in combination with ANG-II did not modify the ERK1/2 levels in Nx and Hx conditions (Figure 4.21). These results suggest that ATP-γS and UTP via P2Y receptors are not involved in activation of ERK1/2 in rat neonatal cardiac fibroblasts when exposed to Hx and ANG-II for 4 hours.

p38 MAPK can exhibit both pro- and anti-apoptotic properties depending on the cell types, signaling pathways and experimental conditions (Baines and Molkentin, 2005; Petrich and Wang 2004; Steenbergen, 2002). In the present study, Hx had no effect on p38 phosphorylation. ATP-γS and UTP in Nx and Hx did not modify p38 activation (Figure 4.22).

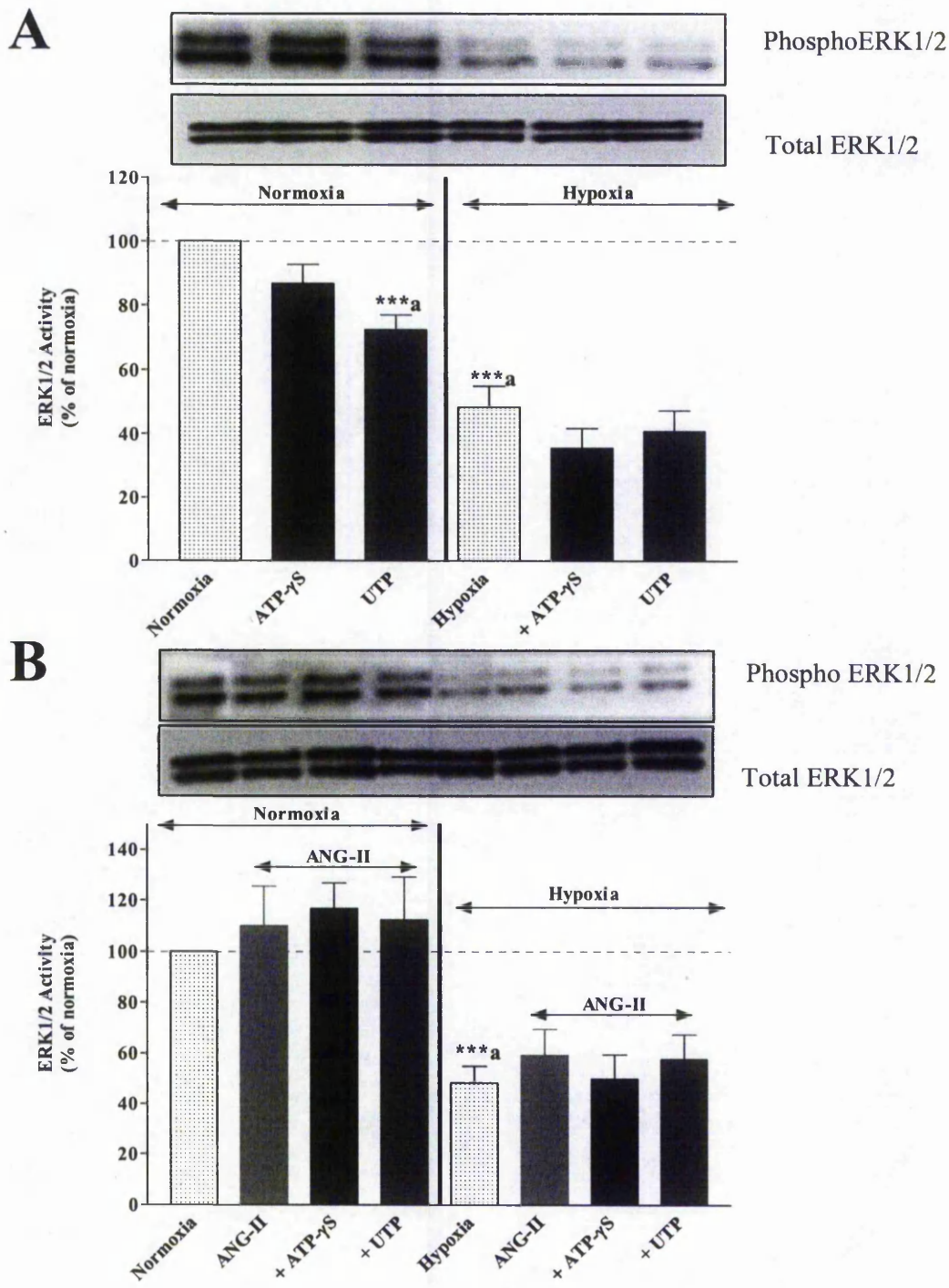

Figure 4.21: Effect of ATP-γS and UTP on extracellular signal-regulated protein kinases 1/2 (ERK1/2) in absence or presence of angiotensin-II (ANG-II) in neonatal rat cardiac fibroblasts exposed for 4 hours in normoxia and hypoxia. Cardiac fibroblasts were

stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of angiotensin-II (50nM; ANG-II; **Panel B**) and exposed for 4 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell lysates were analysed by western blotting using a phospho-specific ERK1/2 antibody. The same blots were stripped and analysed using an antibody that recognizes total ERK1/2 to confirm equal loading on each lane. The immunoblots presented are from one experiment and representative of 5 – 6 experiments. The values were obtained from densitometric analysis of blots and each point represents the mean ± S.E.M for 5 – 6 experiments from separate cell cultures. Data were expressed as the percentage of the normoxic control (100%). *** $P<0.001$, a versus normoxic response.

As depicted in figure 4.22B, ANG-II did not change p38 activity in Nx whereas in the presence of ATP-γS and UTP p38 phosphorylation was decreased by around 40 %. No such modifications were observed in Hx (Figure 4.22). These data indicate that ATP-γS and UTP are not involved in the activation of p38 MAPK, however in combination with ANG-II these nucleotides inhibit p38 phosphorylation in neonatal rat cardiac fibroblasts exposed to Nx.

JNK activity was not altered by Hx (Figure 4.23A). As presented in figure 4.23A, ATP-γS and UTP did not induce JNK phosphorylation in Nx and Hx conditions. Cells stimulated with ANG-II and nucleotides also did not affect the JNK activity under Nx and Hx conditions (Figure 4.23B). These observations indicate that ATP-γS and UTP via P2Y receptors are not involved in JNK activity.

Akt or protein kinase B (PKB) is the best characterised downstream messenger in the phosphoinositide 3-kinase (PI3K) pathway involved in cell proliferation and survival (Song *et al.,* 2005). As shown in figure 4.24A, Akt/PKB activation was significantly down-regulated by Hx (60%, $P<0.001$). ATP-γS and UTP did not modify the Akt/PKB phosphorylation in Nx, whereas UTP induced an increase in Akt/PKB activity by 31% ($P<0.05$) only in Hx. ANG-II in Nx mediated an inhibition of Akt/PKB phosphorylation by 30% ($P<0.001$; Figure 4.24B), whereas the nucleotides did not alter the kinase activity in Nx and Hx (Figure 4.24B). These novel findings indicate that UTP activated P2Y receptors may regulate the survival pathways during hypoxic stress in cardiac fibroblasts.

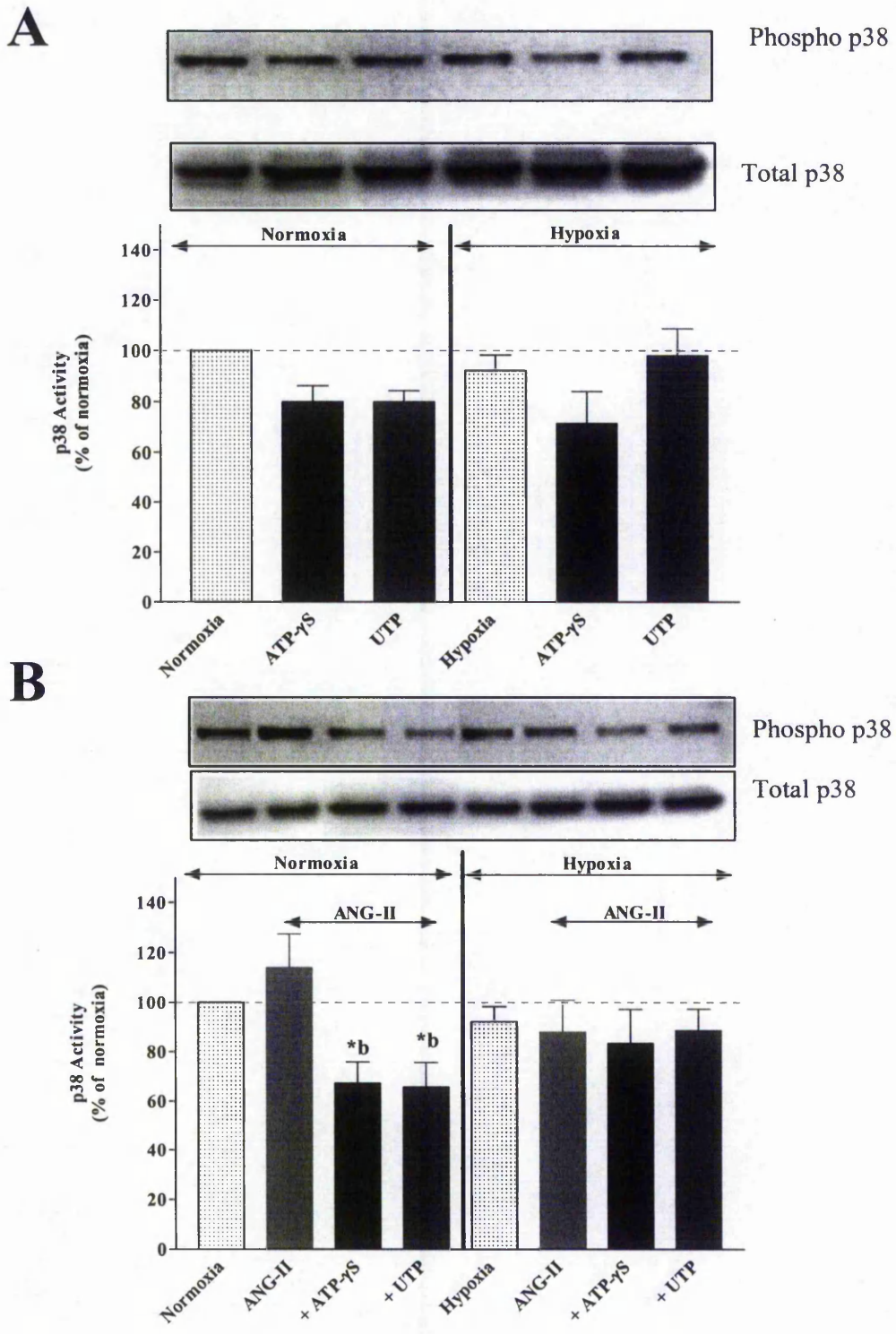

Figure 4.22: Effect of ATP-γS and UTP on p38 mitogen-activated protein kinases (MAPK) in absence or presence of angiotensin-II in neonatal rat cardiac fibroblasts

exposed for 4 hours in normoxia and hypoxia. Cardiac fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of angiotensin-II (50nM; ANG-II; **Panel B**) and exposed for 4 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell lysates were analysed by western blotting using a phospho-specific p38 antibody. The same blots were stripped and analysed using an antibody that recognizes total p38 to confirm equal loading on each lane. The immunoblots presented are from one experiment and representative of 6 – 8 experiments. The values were obtained from densitometric analysis of blots and each point represents the mean ± S.E.M for 6 – 8 experiments from separate cell cultures. Data were expressed as the percentage of the normoxic control (100%). * $P<0.05$, b versus ANG-II response in normoxia.

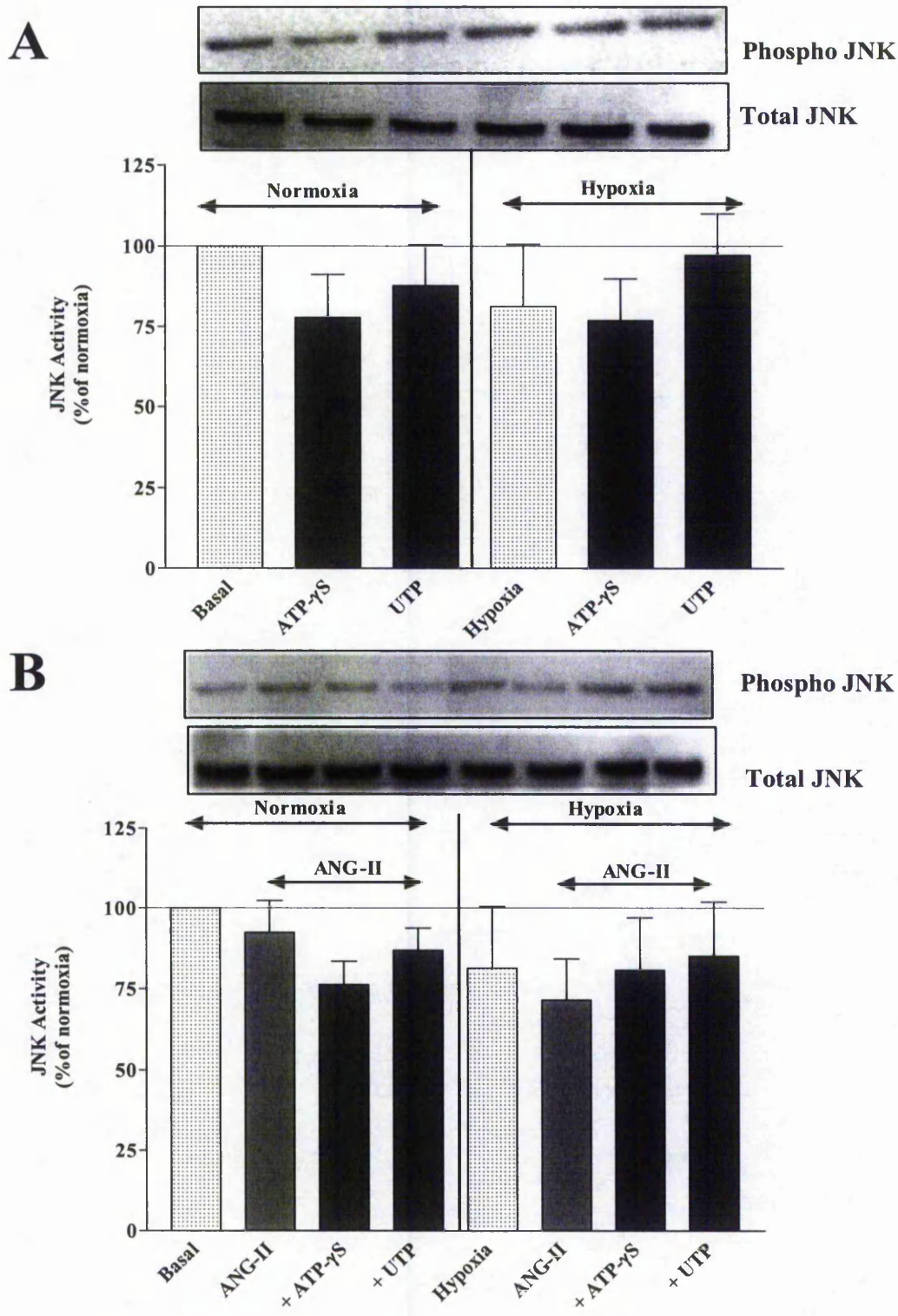

Figure 4.23: Effect of ATP-γS and UTP on c-Jun NH_2-terminal kinase (JNK) in absence or presence of angiotensin-II in neonatal rat cardiac fibroblasts exposed for 4 hours in

normoxia and hypoxia. Cardiac fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of angiotensin-II (50nM; ANG-II; **Panel B**) and exposed for 4 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell lysates were analysed by western blotting using a phospho-specific JNK antibody. The same blots were stripped and analysed using an antibody that recognizes total JNK to confirm equal loading on each lane. The immunoblots presented are from one experiment and representative of 6 – 8 experiments. The values were obtained from densitometric analysis of blots and each point represents the mean ± S.E.M for 6-8 experiments from separate cell cultures. Data were expressed as the percentage of the normoxic control (100%). * $P<0.05$, ** $P<0.01$; a versus normoxic control and b versus ANG-II response in normoxia.

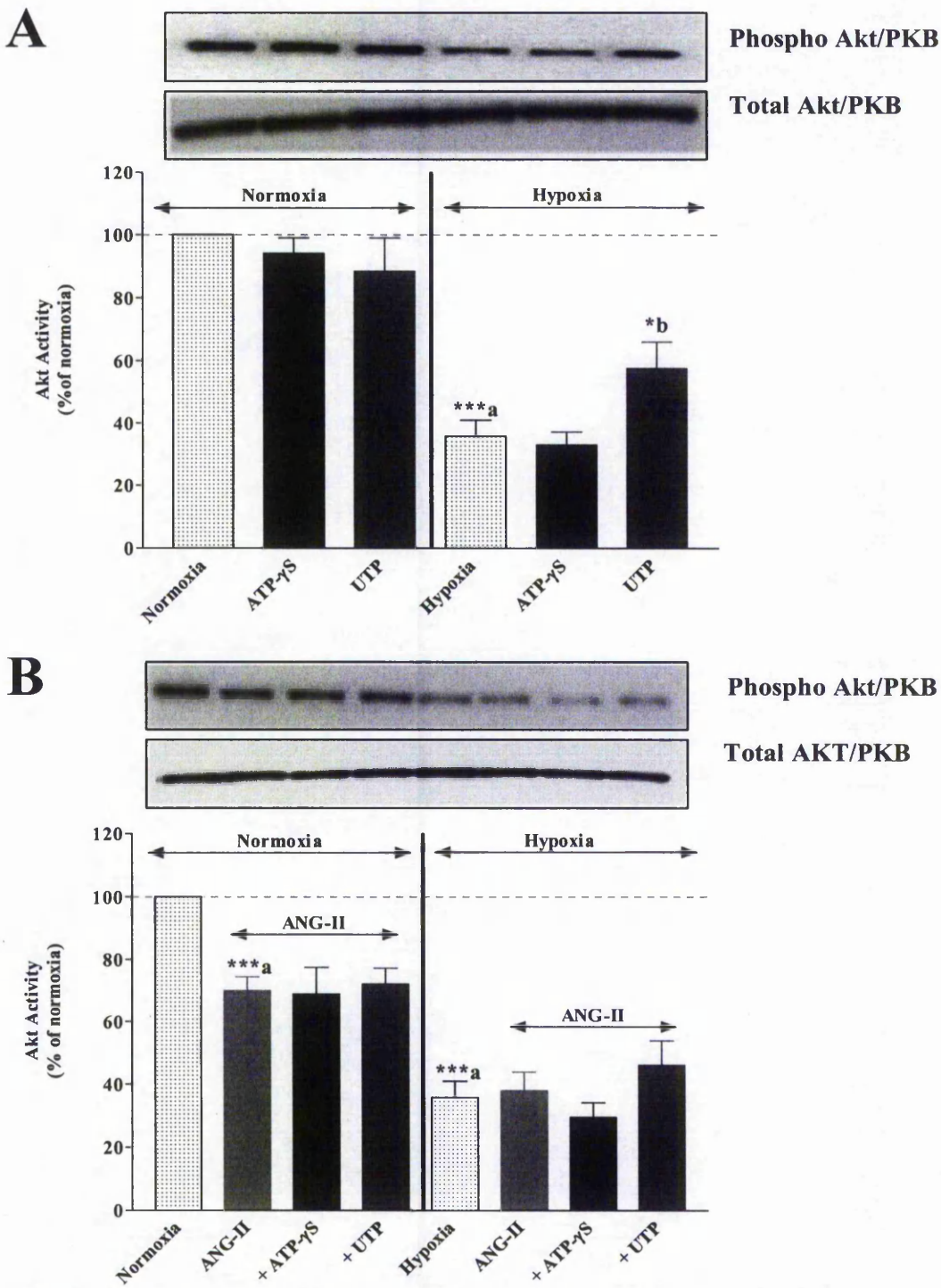

Figure 4.24: Effect of ATP-γS and UTP on protein kinase B (PKB)/Akt in absence or presence of angiotensin-II in neonatal rat cardiac fibroblasts exposed for 4 hours in normoxia and hypoxia. Cardiac fibroblasts were stimulated with ATP-γS (32μM) and UTP (10μM) in absence (**Panel A**) or presence of angiotensin-II (50nM; ANG-II; **Panel B**) and

exposed for 4 hours in normoxia (21% O_2) and hypoxia (0.5% O_2). The cell lysates were analysed by western blotting using a phospho-specific Akt antibody. The same blots were stripped and analysed using an antibody that recognizes total Akt to confirm equal loading on each lane. The immunoblots presented are from one experiment and representative of 5 – 6 experiments. The values were obtained from densitometric analysis of blots and each point represents the mean ± S.E.M for 5-6 experiments from separate cell cultures. Data were expressed as the percentage of the normoxic control (100%). * $P<0.05$, a versus normoxic control; b versus ANG-II response in normoxia.

Chapter 4: Discussion – Role of P2Y receptors in an *in vitro* model of ischaemic heart disease

Recent studies have shown that ATP and UTP are released during myocardial infarction in human (Wihlborg *et al.,* 2006). Similarly, Erlinge and researchers (2005) have shown that the level of UTP increased in porcine heart following cardiac ischaemia. Furthermore, ATP is released from cardiac myocytes and pulmonary artery advential fibroblasts exposed to ischaemia (Dutta *et al.,* 2004; Gerasimovskaya *et al.,* 2002). These observations suggest that ATP and UTP released during cardiac ischaemia can mediate their effects by stimulating P2Y receptors and influence fibroblast function. Indeed, neonatal rat cardiac fibroblasts functionally express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$-like receptors as shown earlier in chapter 3. Therefore this study aimed to develop an *in vitro* model of ischaemic heart disease associating angiotensin-II (ANG-II) and hypoxia in order to investigate the involvement of P2Y receptors in such pathological conditions. Cytokine release, cardiac protection, collagen secretion and activation of mitogen-activated protein kinase (MAPK) and Akt/protein kinase B (PKB) were determined in fibroblasts exposed to normoxic and ischaemic conditions. ATP activates $P2Y_1$ and $P2Y_{11}$-like receptors whereas UTP activates $P2Y_6$ receptor and $P2Y_2$ and $P2Y_4$ receptors are activated by both the nucleotides (Abbracchio *et al.,* 2006). ATP-γS (a less hydrolysable form of ATP) and UTP were used to stimulate the different subtypes of P2Y receptors expressed on neonatal rat cardiac fibroblasts.

4.5 P2Y receptors in ischaemic heart disease and cytokine release:

In heart, myocytes and non-myocytes such as fibroblasts, endothelial cells, smooth muscle cells release cytokines in response to patho-physiological changes (Mann, 2003; Prabhu, 2004). Cardiac diseases like heart failure, hypertrophy, cardiomyopathy, myocarditis, cardiac allograft rejection and sepsis-associated cardiac dysfunction are all associated with cytokine activation (Mann, 2003; Prabhu, 2004). Therefore, to establish the parameters for the model of ischaemic heart disease the release of IL-1β, IL-6, TNF-α and TGF-β1 cytokines from cardiac fibroblasts was evaluated following stimulation with ATP-γS and UTP in presence or absence of ANG-II under normoxic (Nx) or hypoxic (Hx) conditions at different time points (1,2,4,8 and 18 hours).

In the present study, the ischaemic conditions were simulated by a combination of ANG-II and hypoxia (0.5% O_2). Cardiac fibroblasts were maintained in serum- and glucose-free media. Malhotra and Brosius III (1999) have shown that glucose uptake and glycolysis protected the neonatal rat cardiomyocytes from the hypoxic stress and apoptosis. Indeed, ischaemic conditions induced the translocation of glucose transporter (GLUT)-4 and GLUT-1 to sacrolemma and subsequently increased the uptake of glucose (Sun *et al.,* 1994; Brosius *et al.,* 1997). Furthermore, during ischaemic heart disease there is decreased blood flow and nutrients to the cardiac cells (Lee *et al.,* 2004; Downward, 2003). For these reasons in this study neonatal rat cardiac fibroblasts were maintained in serum- and glucose-free media.

Neonatal rat cardiomyocytes and human monocytes exposed to hypoxia stimulated the production of IL-6 and TNF-α, respectively (Yamauchi-Takihara *et al.,* 1995; Guida and Stewart, 1998). Hypoxia induces transcription factors like *c-fos*, *c-jun*, activating protein-1 (AP-1) and NF-κB, which in turn increased the production of cytokines in myocytes (Webster *et al.,* 1993). However, in this study 4 hours of hypoxic conditions inhibited the production of IL-1β, IL-6 and TNF-α by cardiac fibroblasts. Normoxic conditions did not induce the release of cytokines at any time point. This difference in cytokine release may be due to the combination of glucose deprivation and hypoxia. Indeed, the TNF-α mRNA expression increased during hypoxic in presence of glucose conditions but combination of hypoxia and glucose deprivation decreased the TNF-α expression in human monocytes (Guida and Stewart, 1998). Moreover, treatment of cardiac fibroblasts with ANG-II in normoxic and hypoxic conditions did not induce the production of proinflammatory cytokines like IL-1β, IL-6 and TNF-α. However, fibroblasts exposed to 18 hours ANG-II and hypoxia induced the production of profibrotic cytokine TGF-β1. Campbell and Katwa (1997) reported that ANG-II-induced the expression of TGF-β1 at mRNA and protein levels in cardiac fibroblasts. It is suggested that ANG-II dependent up-regulation of TGF-β1 can be mediated via NAD(P)H oxidase and activation of PKC (Rosenkranz, 2004). Furthermore, ANG-II can also stimulate the production of TGF-β1 via the transactivation of epidermal growth factor receptor on cardiac fibroblasts (Moriguchi *et al.,* 1999).

P2Y receptors and IL-6 release

Neonatal rat cardiac fibroblasts treated with ATP-γS induced the production of hypertrophic cytokine IL-6 in both normoxic and hypoxic conditions. It is notable that hypoxia did not affect the induction of IL-6 by ATP-γS. These results suggest that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$-like receptors which are activated by ATP-γS can be involved in the production of IL-6. However, due to the lack of IL-6 release by fibroblasts when stimulated by UTP, indicates that $P2Y_2$ and $P2Y_4$ receptors are not involved in ATP-γS-induced IL-6 release. In addition, the lack of UTP effect on IL-6 release indicates that uracil nucleotide activated $P2Y_6$ receptor is not implicated in IL-6 release. The stimulation of P2Y receptors by adenine or uracil nucleotides induced the formation of IP and DAG by PLC and subsequent activation of PKC and increase in intracellular Ca^{2+} levels (Abbracchio *et al.,* 2006). Several studies have shown that ATP stimulated IL-6 release was mediated by PLC/PKC pathway (Shigemoto-Mogami *et al.,* 2001; Ihara *et al.,* 2005; Yoshida *et al.,* 2006; Gabel, 2007). ATP acting via PLC-linked P2Y receptor leads to the formation of IP and DAG. Shigemoto-Mogami *et al.,* (2001) reported that ATP-induced IL-6 release in microgila MG-5 cells was inhibited by Gö6976, a blocker of Ca^{2+}-dependent PKC isoforms. Similarly, in human osteoblasts ATP-stimulated IL-6 synthesis was inhibited by PLC inhibitor (U73122; Ihara *et al.,* 2005).

Interestingly, ANG-II potentiated the release of IL-6 mediated by ATP-γS in both normoxia and hypoxia (Figure 4.25). This augmentation of IL-6 levels can be due to a cross-talk between G_s-coupled $P2Y_{11}$-like receptor and G_q-coupled AT_1 receptor. Indeed, such interaction has been reported between β-adrenergic receptor and AT_1 in cardiac myocytes (Barki-Harrington *et al.,* 2003). Similarly, Jaffre *et al.,* (2004) reported that 5-HT_{2B} receptors are essential for the isoproterenol-mediated release of IL-6 in murine cardiac fibroblasts. The contribution of ATP-activated $P2X_7$ receptors can be very negligible as these receptors are activated at high concentrations of ATP (100μM - 1000μM) and in the present study the concentration of ATP-γS used was 32μM. The precise mechanism for the ANG-II potentiated the release of IL-6 mediated by ATP-γS needs to be established. Overall, these data suggest that $P2Y_1$ and $P2Y_{11}$-like in cardiac fibroblasts receptors play an important role in IL-6 production during ischaemic conditions.

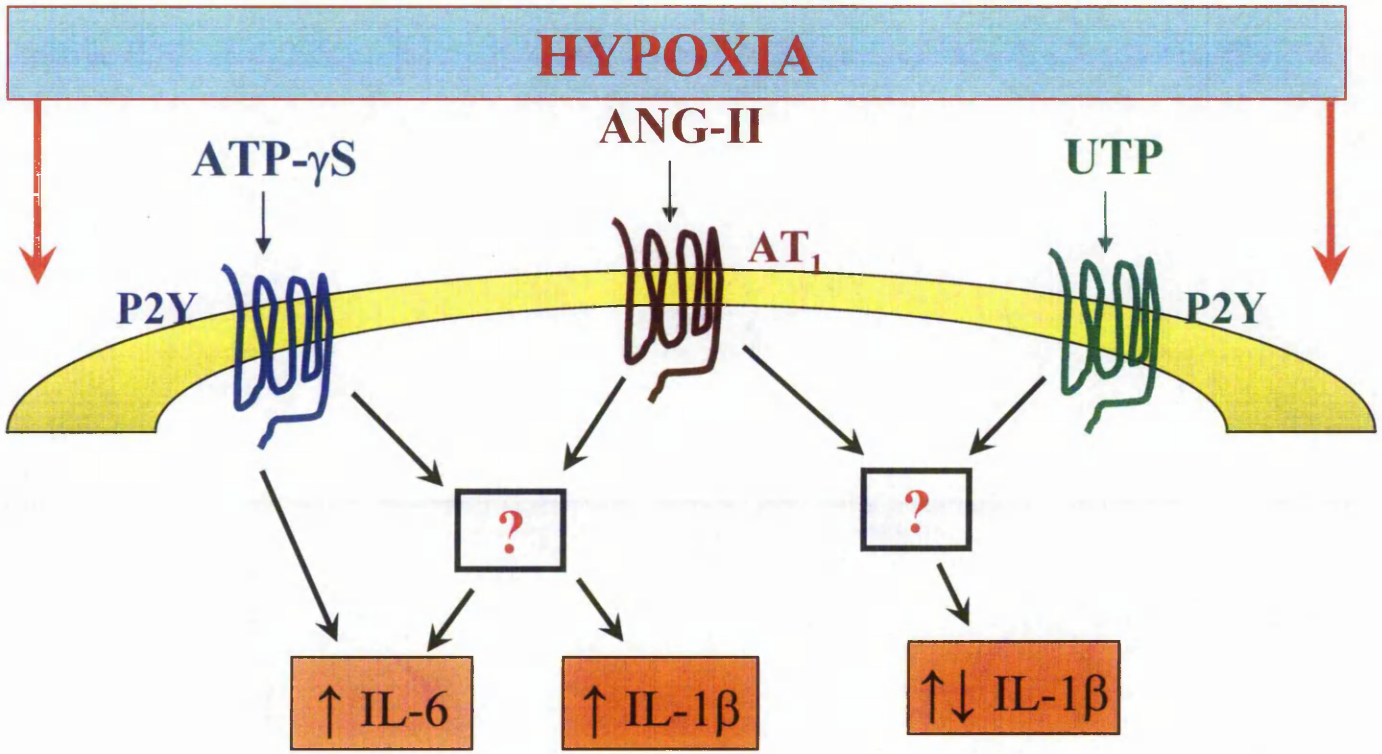

Figure: 4.25: Regulation of IL-1β and IL-6 cytokine release by P2Y receptors in an *in vitro* model of ischemic heart disease. The *in vitro* model of ischemic heart disease in neonatal rat cardiac fibroblasts was developed in association with angiotensin-II (50nM; ANG-II) and hypoxia (0.5% O_2). P2Y receptors activated by ATP-γS ($P2Y_1$ and $P2Y_{11}$-like) induced the release of IL-6 in hypoxia, whereas UTP had no affect. An interaction was observed between angiotesin type 1 (AT_1) receptor and the P2Y receptors. Combination of both ANG-II and hypoxia induced the release of IL-1β by ATP-γS and UTP. It is noteworthy that under ischemic conditions UTP induced a biphasic production of IL-1β. The IL-1β release can be probably via $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors in neonatal rat cardiac fibroblasts. ↑ - increase, ↓ - decrease, ? – unknown.

P2Y receptors and IL-1β release

ATP-γS and UTP did not induce the production of IL-1β under normoxic or hypoxic conditions. However, in presence of ANG-II both ATP-γS and UTP stimulated IL-1β release following 4 hours hypoxia (Figure 4.25). IL-1β is secreted in the form of pro-IL-1β, which is cleaved and activated by caspase-1 in association with Nalp3, an inflammasome (Lich *et al.,* 2006; Meylan *et al.,* 2006). Extracellular ATP treatment in macrophages resulted in activation of caspase-1 and release of IL-1β and IL-18 (Cruz *et al.,* 2007). In addition, ATP also stimulated the production of reactive oxygen species (ROS) which activates the phosphatidylinositol 3-kinase (PI3K) pathway. This ATP-induced ROS-dependent PI3K pathway is also essential to stimulate caspase-1 and release of IL-1β (Cruz *et al.,* 2007). Previous studies also showed that alteration in intracellular Ca^{2+} levels contributes to IL-1β post-translational processing (Gabel, 2007). In rat vascular smooth muscle cells, UTP up-regulated the IL-1β mRNA through nuclear factor of activated T-cells (NFAT) and NF-κB by activating $G_{q/11}$-coupled P2Y receptors (Abbott *et al.,* 2000). In this study, ATP-γS and UTP through the activation of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptor, all coupled to $G_{q/11}$-protein can stimulate IL-1β release. From these studies, it can be hypothesized that ATP-γS modulates IL-1β release directly by activating caspase-1 and increasing intracellular Ca^{2+} levels. Likewise, UTP can primarily stimulate IL-1β by elevating intracellular Ca^{2+} levels.

It is noteworthy that in the present study combination of ANG-II with ATP-γS or UTP was necessary to induce IL-1β release (Figure 4.25). IL-1β release in post-myocardial infarction heart increased the density of angiostensin-II type 1 (AT_1) receptor by activating NF-κB (Cowling et al., 2002). Furthermore, neonatal rat cardiac fibroblasts treated with IL-1β increased AT_1 receptor mRNA levels (Gurantz *et al.,* 2005). Interestingly, cardiac fibroblasts treated for 18 hours with UTP and ANG-II in hypoxia inhibited IL-1β release. The activation and signal transduction pathways are tightly regulated by a phenomenon termed "desensitisation" in the presence of continuous agonist stimulation at the receptor (Ferguson, 2001). This mechanism is mediated by phosphorylation of the receptor by second-messenger kinases like PKC and PKA, or by G-protein-coupled receptor kinases (GRKs; Pitcher *et al.,* 1998). The inhibition of IL-1β release may involve the desensitization and internalization of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Otero *et al.,* 2000;

Brinson and Harden, 2001) or enzymatic breakdown of UTP into UDP by ecto-nucleotidases. Taken together, these results indicate that in cardiac fibroblasts ATP-γS and UTP regulate the release of IL-1β during ischaemic heart diseases probably via $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors.

P2Y receptors and TNF-α and TGF-β1 release

Neonatal rat cardiac fibroblasts treated with ATP-γS did not induce the production of TNF-α or TGF-β1 cytokines in combination with ANG-II in both normoxic and hypoxic conditions, excluding $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$-like receptors. UTP in presence of ANG-II mediated the release of TNF-α only in normoxic conditions. For the first time, this report shows that UTP induced the production of TGF-β1 in the cardiac fibroblasts under normoxia probably via the $P2Y_6$ receptor. The possible mechanism by which UTP induced the release of TGF-β1 is not known. However, UTP did not induce TGF-β1 release in presence of ANG-II and hypoxia. These findings indicate that $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors stimulated by ATP-γS and UTP are not involved in the production of TNF-α and TGF-β1 under ischaemic conditions.

4.5.1 P2Y receptor activation regulates LPS-potentiated cytokine release:

The gram-negative bacterial endotoxin lipopolysaccharide (LPS) is widely used to induce inflammatory properties in a variety of immune cells. LPS activates a wide range of pro-inflammatory process via Toll-like receptors (TLRs; Beutler, 2000). The mammalian myocardium expresses CD14, TLR2, TLR4 and TLR6 receptors of which CD14/TLR4 interacts with LPS. Frantz and researchers (1999) have shown that the expression of TLR4 was increased in a murine myocardium remodelling model and in patients suffering from idiopathic dilated cardiomyopathy, suggesting the activation of innate immune system during myocardial injury. Moreover, mice injected with LPS developed left ventricular dysfunction via TLR4 receptors (Nemoto *et al.,* 2001). During embryonic developmental stages cardiac fibroblasts constituted the innate immune system (Brown *et al.,* 2005a; Nemoto, *et al.,* 2002). LPS stimulates excessive release of proinflammatory cytokines and lipid mediators which lead to septic shock and organ failure (Tracey and Lowry, 1990; Nemoto *et al.,* 2002).

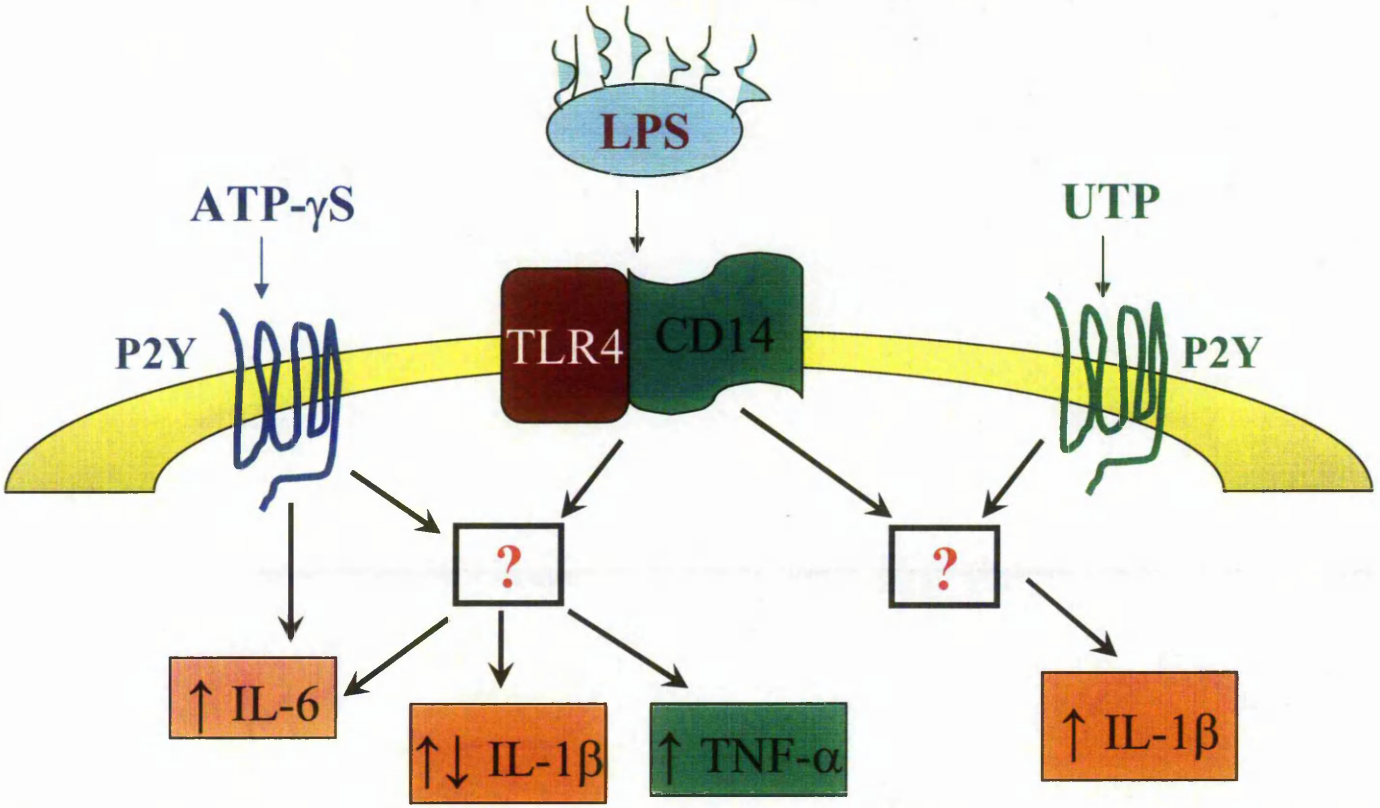

Figure: 4.26: Regulation of lipopolysaccharide (LPS)-induced cytokine release by P2Y receptors in neonatal rat cardiac fibroblasts. LPS mediates its inflammatory responses via toll-like receptors 4 (TLR4) and CD14 and induced the production of IL-1β, IL-6 and TNF-α. ATP-γS alone induced the IL-6 release whereas UTP did not induce any cytokine production. LPS-induced IL-6 and TNF-α secretions were potentiated by ATP-γS. Interestingly, ATP-γS induced a biphasic production of LPS-potentiated IL-1β release whereas UTP only augmented IL-1β release. An interaction was observed between LPS-mediated inflammatory pathway and the P2Y receptors in cardiac fibroblasts. During septicaemia, $P2Y_1$ and $P2Y_{11}$-like (ATP-γS activated) receptors may be involved in the IL-1β, IL-6 and TNF-α production. IL-1β is probably released following $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like (ATP-γs/UTP stimulated) receptors activation in neonatal rat cardiac fibroblasts. ↑ - increase, ↓ - decrease, ? – unknown.

P2Y receptors are known to modulate the secretion of cytokine-induced by LPS in glial cells, astrocytes, and macrophages (Bianco *et al.,* 2005; Kucher *et al.,* 2005; Gabel, 2007). Therefore, in the present study, the effect of P2Y receptors during septicaemia was studied on neonatal rat cardiac fibroblasts.

Cardiac fibroblasts treated with LPS stimulated the release of IL-1β, IL-6 and TNF-α in a concentration- and time-dependent manner (Figure 4.27). Several studies have shown that LPS stimulates nitric oxide production and inducible nitric oxide synthetase (iNOS) expression, NAD(P)H oxidase , ROS production, and activation of NF-κB, ERK1/2, and p38 MAPKs all leading to the release of proinflammatory cytokines (Guerra, *et al.*, 2007; Chen and Wang, 1999, Beckman and Koppenol, 1996). Fibroblasts treated with ATP-γS from one to eight hours did not stimulate the release of IL-1β or TNF-α; however ATP-γS potentiated LPS-mediated IL-1β, IL-6 and TNF-α production. Likewise, UTP alone did not induce any cytokine production but augmented LPS-induced IL-1β secretion at 4 hours. The nucleotide-mediated LPS-dependent cytokine responses can be explained as follows. Firstly, LPS may alter the expression of P2Y receptor subtypes. Indeed, Bianco and associates (2005) reported that LPS in N9 microglia cells increased $P2Y_6$ mRNA and decreased $P2Y_{14}$ mRNA, whereas the $P2Y_1$, $P2Y_2$ and $P2Y_4$ mRNA levels were not altered. In contrast, $P2Y_2$ mRNA expression was up-regulated in vascular smooth muscle cells treated with LPS (Hou *et al.,* 2000). Secondly, treatment of cells with LPS induces the release of nucleotides (Kukukski *et al.,* 2007; Warny *et al.,* 2001). Indeed, treatment of LPS-stimulated monocytes and neutrophils with apyrase, a nucleotide scavenger partially inhibited the LPS-induced IL-8 release and cell migration, respectively (Warny *et al.,* 2001; Kukukski *et al.,* 2007). Thirdly, NF-κB which is associated with the induction of cytokine genes can be activated by both LPS and calcium (Dolmetsch *et al.,* 1998; Beutler, 2000). Thus, the potentiation of cytokines release following the activation of P2Y receptors by ATP-γS and UTP in combination with LPS probably involves increases in intracellular calcium levels in neonatal rat cardiac fibroblasts. All these aforementioned studies suggest an interaction between the LPS-mediated inflammatory pathways and P2Y-receptor activated signal transduction in neonatal rat cardiac fibroblasts (Figure 4.27). Furthermore, Chen and associates have reported that UTP alone in J774 murine macrophages did not active NF-κB and iNOS, however potentiated LPS-mediated NF-κB,

AP-1 and iNOS induction via Ca^{2+}/calmodulin-dependent protein kinase. This is consistent with the UTP-induced potentiation of IL-1β release by LPS observed in the present study. Interestingly, from 4 hours onwards fibroblasts treated with ATP-γS inhibited LPS-induced IL-1β release. This finding can be supported by the fact that LPS-induced cardiac IL-6 expression suppressed the expression of IL-1β (Saito *et al.,* 2000). Alternatively, the inhibition of IL-1β may involve the desensitization and internalization of $P2Y_2$ and $P2Y_4$ receptors (Otero *et al.,* 2000; Brinson and Harden, 2001).

In conclusion, the results from the cytokine release studies indicate that $P2Y_1$ and $P2Y_{11}$-like receptors are involved in IL-6 release. In addition, the production of IL-1β appears to be regulated by $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors expressed in neonatal rat cardiac fibroblasts during ischaemic conditions. Finally, it is noticeable that P2Y receptors predominantly modified cytokine release at 4 and 18 hours. Therefore, the investigations for the role of nucleotides in ischaemic heart disease were carried out at 4 and 18 hours on neonatal rat cardiac fibroblasts.

4.6 P2Y receptors and cardioprotection in neonatal rat cardiac fibroblasts

Myocardial infarction or ischaemia leads to cardiac remodeling involving changes in the ventricular size, shape and thickness (Jugdutt 2003a, b). Ventricular remodelling causes decrease in heart function and ultimately lead to heart failure due to cardiomyocyte death and excessive deposition of ECM by cardiac fibroblasts. During these stressful conditions fibroblasts are functionally active, undergo proliferation and secrete disproportionate amounts of ECM (Brown *et al.,* 2005a). Cardiac fibroblasts are resistant to cell death induced by simulated ischaemia (hypoxia and glucose deprivation) compared to dermal fibroblasts (Mayorga *et al.,* 2004). Therefore, in the present study the effect of ischaemic conditions and extracellular nucleotides on cardiac fibroblast viability was evaluated by measuring lactate dehydrogenase (LDH) release. Unfortunately, the necrotic and apoptotic types of cell death can not be differentiated by the LDH assay, which mainly measures cell necrosis.

Cardiac fibroblasts were resistant to 4 hours of hypoxic treatment however 18 hours did induce cell death. ANG-II, ATP-γS and UTP did not affect the cell death induced by hypoxia. ANG-II is known to regulate fibroblast proliferation and function during myocardial injury via the release of TGF-β1 (Kawano, *et al.,* 2000; Bouzegrhane and Thibault, 2002). ANG-II has been shown to protect fibroblasts against the detrimental affects of IL-1β by inhibiting iNOS protein expression and NO production and also by activation PI3K/Akt-induced cell survival pathways (Ti an *et al.,* 2003). However, the present study cannot be compared to Tian's (2003) because of the difference in experimental conditions.

Moreover, several studies have shown that P2Y receptors are involved in regulating cell death (Chorna *et al.,* 2004; Mamedova *et al.,* 2006; Sellers *et al.,* 2001; Kim *et al.,* 2003). It is noteworthy that these studies were carried out using the 1321N1 astrocyte cell line expressing transfected P2Y receptors. These investigations revealed that activation of $P2Y_1$ receptors decreased the cell viability (Sellers *et al.,* 2001; Mamedova *et al.,* 2006), stimulation of $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors prevented the cell death (Mamedova *et al.,* 2006; Chorna *et al.,* 2004; Kim *et al* 2003) and finally $P2Y_4$ had no affect in cell survival (Kim *et al* 2003). However, activation of $P2Y_4$ induced death in human neuroblastoma SH-SY5Y cells (Cavaliere *et al.,* 2005). Neonatal rat cardiac fibroblasts express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-like receptors and the activation of these multiple P2Y receptors by ATP-γS and UTP could result in stimulation of different cell survival pathways leading to the absence of effect on cardiac fibroblast viability in ischaemic conditions.

Recent studies have demonstrated that UTP protected cardiomyocytes from hypoxic stress and reduced the infarction size and improved the heart function in rat (Yitzhaki *et al.,* 2005; 2006). However, in the above experiments myocytes and the rats were preconditioned with UTP before the exposure to hypoxia or in the rat model of myocardical infarction which is again different from the present experimental model. Taken together, P2Y receptors are not involved in regulating cell death mainly induced by necrosis in cardiac fibroblast during ischaemic conditions.

4.7 P2Y receptors modulate collagen accumulation during ischaemic heart disease

Cardiac fibroblasts are the predominant cell type in heart and their main function is the secretion of ECM (Baudino *et al.*, 2006; Camelliti *et al.*, 2005; Brown *et al.*, 2005a). Collagen is the major constituent which accounts for 90% of ECM (Jugdutt 2003a, b). During myocardial injury cardiac fibroblasts secrete disproportionate amounts of collagen, resulting in cardiac fibrosis (Jugdutt 2003a, b; Brown *et al.*, 2005a). Fibrosis results in dysfunction of the heart leading to left ventricular hypertrophy, arrhythmogenesis, perivascular fibrosis and eventually leading to heart failure (Brown *et al.*, 2005a). In this study, collagen accumulation was assayed in different fractions, ECM fraction (insoluble collagen), cellular fraction (soluble collagen) and cell culture medium.

Previous studies have shown that chronic hypoxic induces the collagen synthesis in different cell types (Agocha *et al.*, 1997; Papakonstantinou *et al.*, 2003). Indeed, in the present study, hypoxic conditions (18 hours) increased the insoluble collagen production and decreased the soluble collagen accumulation in neonatal rat cardiac fibroblasts. The collagen content in cell culture media reminded unaltered. The enhanced collagen production may involve the increase in prolyl-4-hydroxylases and lysylhydroxylases activity. These hydroxylase enzymes catalyse the formation of 4-hydroxyproline from proline which is a vital step in collagen biosynthesis (Jugdutt, 2003b; Brodsky and Shah, 1995). In fact, hypoxic conditions stimulated the gene expression of prolyl-4-hydroxylases and lysylhydroxylases enzymes in rat smooth muscle A7r5 cell line (Hofbauer *et al.*, 2003). Conversely, the insoluble collagen synthesis was inhibited in cardiac fibroblasts exposed to 4 hours hypoxia with no changes in the soluble or media collagen fractions. The possible explanation may be related to the decrease in cellular protein synthesis or increase in the matrix metalloproteinase (MMP) activity.

ANG-II is known to induce cardiac fibrosis by stimulating collagen gene expression and protein turnover in cardiac fibroblasts (Lijnen *et al.*, 2000; Zhou *et al.*, 1996). However, the results from the present study show that ANG-II did not induce the production of collagen in neonatal rat cardiac fibroblasts under normoxic or hypoxic conditions. This observation is consistent with the previous studies where ANG-II did not induce the

transcription of collagen genes but did increase TGF-β1 mRNA expression in neonatal and adult rat cardiac fibroblasts (Pathak *et al.*, 2001; Sarkar *et al.*, 2004). Similarly, isolated human cardiac fibroblasts treated with ANG-II from 4 to 48 hours did not modulate the collagen type I and type III mRNA or protein expression (Agocha *et al.*, 1997; Kawano *et al.*, 2000; Kupfahl *et al.*, 2000). These investigations and ours suggest that ANG-II-mediated collagen synthesis may involve a cross-talk between fibroblasts and other cardiac cells like myocytes and/or endothelial cells including their paracrine factors. Indeed, ANG-II induced the expression of collagen type I and type III mRNA when the fibroblasts were co-cultured with myocytes (Pathak *et al.*, 2001; Sarkar *et al.*, 2004).

In the current study, ATP-γS triggered insoluble collagen accumulation only at 4 hours normoxic condition and in the presence of ANG-II in hypoxia (Figure4.27). Both the soluble and cell culture collagen fractions were not altered. This suggests that G_q-coupled $P2Y_1$, $P2Y_2$, $P2Y_4$ receptors and $P2Y_{11}$-like receptor dual coupled to G_s/G_q are involved in collagen production in normal and ischaemic conditions. However, due to the lack of collagen deposition by cardiac fibroblasts when stimulated by UTP, it indicates that $P2Y_2$ and $P2Y_4$ receptors are not involved in ATP-γS-induced collagen accumulation. This also suggests that uracil nucleotide activated $P2Y_6$ receptor is not implicated in collagen production. One possible explanation for the ATP-γS-induced collagen synthesis is via ATP-γS-mediated IL-6 release (Figure 4.27). In fact, IL-6 promotes ECM deposition by enhancing the collagen biosynthesis and inducing the synthesis of tissue inhibitor of metalloproteinases (TIMPs) in fibroblasts derived from skin and lung, respectively (Brown *et al.*, 2003a). Likewise, TIMPs production was enhanced by IL-6 in chondrocytes, synoviocytes and fibroblasts originated from human cervix and skin (Silacci *et al.*, 1998; Sato *et al.*, 1990).

Recent studies have demonstrated that activation or over-expression of adenylyl cyclases (AC) inhibited collagen synthesis in rat cardiac fibroblasts (Swaney *et al.*, 2005; Liu *et al.*, 2006). Indeed, activation of the cAMP/PKA/CREB pathway either directly via forskolin or indirectly via β-adrenergic receptor activation inhibited collagen synthesis by attenuating TGF-β1 signalling (Swaney *et al.*, 2005; Liu *et al.*, 2006). Similarly, adenosine via adenosine A_{2B} receptors inhibited FCS-induced collagen synthesis in rat cardiac fibroblasts probably through the cAMP pathway (Dubey *et al.*, 1998; Dubey *et al.*, 2000). These previous studies suggest that G_s-coupled receptors inhibit collagen synthesis.

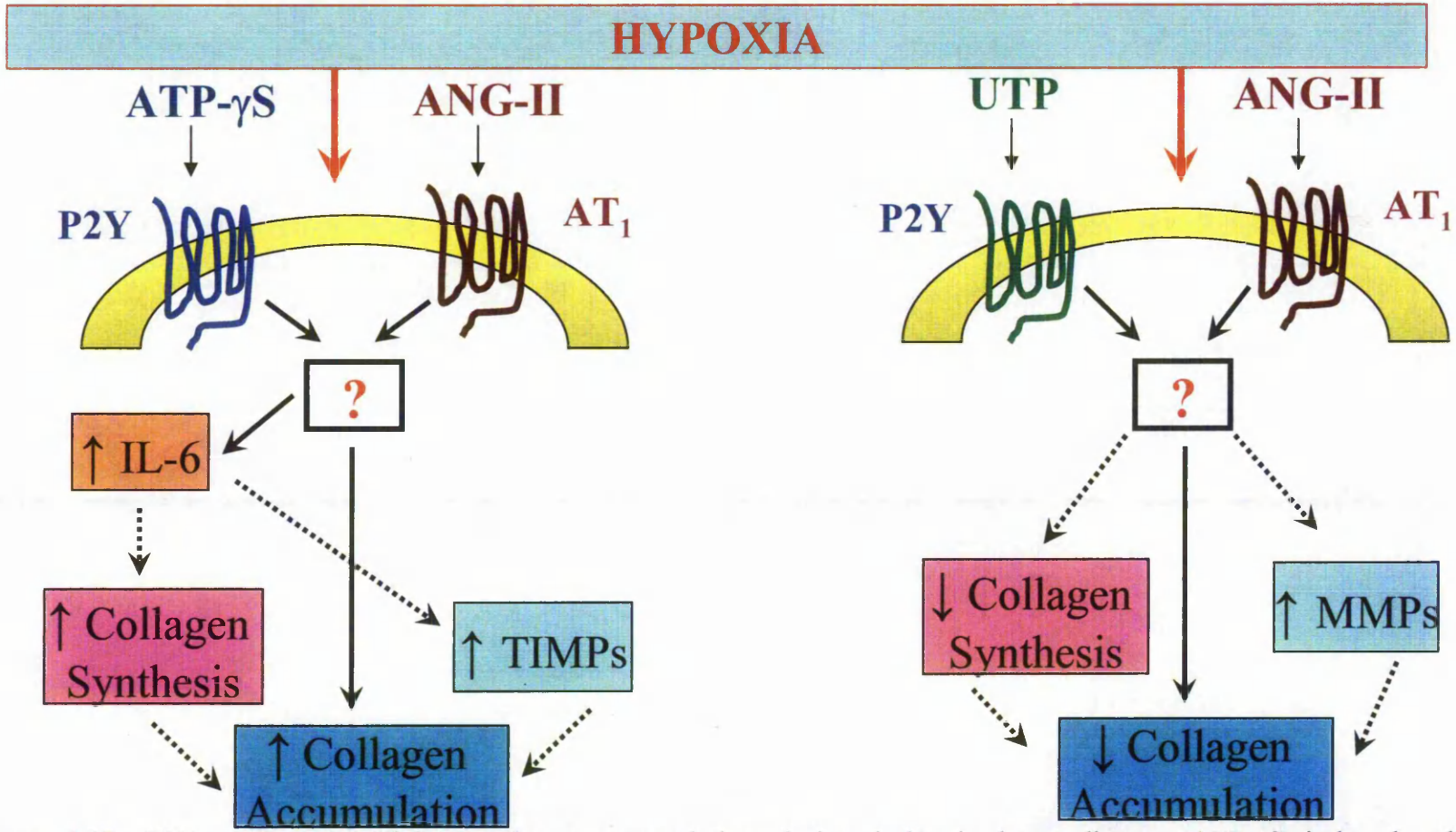

Figure 4.27: P2Y receptors modulate collagen accumulation during ischemic heart disease. ATP-γS induced collagen accumulation in neonatal rat cardiac fibroblasts probably via $P2Y_1$ and $P2Y_{11}$-like receptor mediated IL-6 production. IL-6 is known to increase collagen biosynthesis and stimulate the expression of tissue inhibitor of metalloproteinases (TIMPs) subsequently leading to increased collagen accumulation. Conversely, UTP inhibited collagen deposition possibly by inhibiting collagen synthesis or by increasing the matrix metalloproteinases (MMPs) activity via the $P2Y_6$ receptor in cardiac fibroblasts. Bold arrow – direct action, broken arrow – indirect action, ↑ - increase, ↓ - decrease, ? – unknown

It is notable that ATP induced mRNA expression of collagen type IVα1, laminin and fibronectin via TGF-β1 release was resistant to MRS2179, a $P2Y_1$ antagonist in rat mesengial cells expressing $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Solini *et al.*, 2005). In addition, ATP stimulated the collagen accumulation in bovine articular chondrocytes (Croucher *et al.*, 2000). Overall, these observations suggest that adenine nucleotide-activated P2Y receptors are involved in collagen production during 4 hours of ischaemic conditions. However, the precise P2Y receptor(s) and mechanism involved is not yet established.

Cardiac fibroblasts treated with UTP for 18 hours inhibited the hypoxia-induced insoluble collagen production and surprisingly increased the insoluble collagen production under normoxic conditions. However, ATP-γS did not alter the collagen in normoxia or hypoxia-induced collagen synthesis in the presence of ANG-II. Moreover, both nucleotides under normal and ischaemic conditions did not regulate the soluble and cell medium portion of collagen. It can be hypothesized that UTP could directly inhibit the hydroxylase enzymes and subsequently block the collagen synthesis or increase the degradation of collagen by enhancing the expression of MMPs (Figure 4.27). In accordance with the present study UTP inhibited mRNA expression of collagen type IVα1 induced by glucose (Solini *et al.*, 2005). The lack of collagen production by cardiac fibroblasts when stimulated by ATP-γS suggests that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$-like receptors are not involved in regulating collagen accumulation during 18 hours of ischaemic conditions. The anti-fibrotic property of UTP is probably via the $P2Y_6$ receptor.

4.8 Mitogen-activated protein kinases, Akt/protein kinase B (PKB) and P2Y receptors in ischaemic heart disease condition

Cells are constantly exposed to a variety of stress responses like physical (mechanical stretch, heat, osmotic shock), chemical (superoxides), metabolic (ischaemia, starvation) and biological (cytokines, bacterial and viral proteins) agents. These events lead to the activation of signal transduction pathways which control the cell functions including metabolism, proliferation, differentiation, migration and survival. Mitogen-activated protein kinases (MAPK) and Akt/protein kinase B (PKB) signalling cascades participate in such events. MAPKs comprises of extracellular signal-regulated protein kinase 1/2

(ERK1/2), p38 MAPK and c-Jun NH_2-terminal kinases (JNK). All of the three kinases are directly implicated in cellular physiology and cell survival.

Indeed, ERK1/2 cascade initiates cell survival via the direct activation of p90 ribosomal S6 kinase (90RSK) and subsequently phosphorylates and inactivates Bad, a pro-apoptotic family member of Bcl-2 (Baines and Molkentin, 2005; Klumpp and Krieglstein, 2002). In addition, 90RSK phosphorylates the transcription factor CREB and promotes cell survival through initiating anti-apoptotic genes (Bonni *et al.,* 1999). For example, 2-MeSADP via the ERK1/2 pathway protected 1321N1 cells expressing the $P2Y_{12}$ receptor against cell death induced by TNF-α (Mamedova *et al.,* 2006). In the present study, neonatal rat cardiac fibroblasts treated with UTP for 4 hours in normoxia inhibited basal ERK1/2 activation whereas ATP-γS had no affect. Several studies have shown that ATP and UTP via P2Y receptors activated ERK1/2 in different cell types like neonatal cardiomyocytes, endothelial cells, macrophages and keratinocytes (Pham *et al.,* 2003; Morris *et al.,* 2004; Kobayashi *et al.,* 2006). Previous reports have stated that stress-induced ERK1/2 is related to cell survival and protection (Baines and Molkentin, 2005). In this study exposure of cardiac fibroblasts to hypoxic conditions (0.5% O_2) inhibited basal ERK1/2 activity. In marked contrast, hypoxia (3% O_2) induced ERK1/2 phosphorylation in adventitial fibroblasts and endothelial cells (Gerasimovskaya *et al.,* 2002). Similarly, neonatal rat cardiomyocytes exposed to hypoxia (0.5% O_2) mediated the activation of ERK1/2 (Germack and Dickenson, 2005). ANG-II is known to activate ERK1/2 in different cell types via AT_1 receptors (Mehta and Griendling, 2007). Indeed, Sano *et al.,* (2001) and Zou *et al.,* (1998) have shown that ANG-II in cardiac fibroblasts induced ERK1/2 phosphorylation. However, in the current study ANG-II did not affect ERk1/2 activation in either normoxic or hypoxic conditions. Furthermore, ATP-γS and UTP in the presence of ANG-II under normoxic and hypoxic conditions did mediate ERK1/2 phosphorylation.

Taken together, these data suggest that during 4 hours of ischaemic conditions P2Y receptors are not involved in ERK1/2 activation in neonatal rat cardiac fibroblasts.

p38 and JNK are also called stress-activated MAP kinases because of their association with different cardiac pathology like hypertrophy, ECM remodeling, metabolic regulation and cell death (Petrich and Wang, 2004). However, in the present study hypoxia did not induce the phosphorylation of p38 and JNK. Similarly, under normoxic and hypoxic conditions

cardiac fibroblasts treated with ANG-II did not activate p38 and JNK. ATP-γS and UTP did not modify p38 MAPK and JNK activation in neonatal rat cardiac fibroblasts under both normoxic and hypoxic conditions. It is noteworthy that these nucleotides inhibited the p38 activity in presence of ANG-II only in normoxic conditions. However, earlier studies have shown that ANG-II stimulated p38 and JNK levels in rat cardiac fibroblasts (Sano *et al.,* 2001, Omura *et al.,* 2005). ATP and ATP-γS in MG-5 microgila cell line induced the release of IL-6 cytokine via the activation of p38, but not JNK pathway (Shigemoto-Mogani *et al.,* 2001). Likewise, ANG-II mediated the expression of IL-6 mRNA via p38 and JNK cascade in cardiac fibroblasts (Sano *et al.,* 2001). These, two studies suggest that ATP-γS-induced IL-6 production in neonatal rat cardiac fibroblasts is not via p38 pathway. On the other hand, Pham *et al.,* (2003) reported that p38 and JNK are not involved in the UTP-mediated hypertrophy of cardiac myocytes. ATP also did not activate p38 and JNK in cardiac myocytes (Pham *et al.,* 2003). These studies point out the fact that p38 and JNK phosphorylation regulate different cellular process and their activation varies from one cell type to another and also depends upon the stimulus (Davis, 2000; Baines and Molkentin, 2005). Overall, these data suggest that P2Y receptors do not activate p38 and JNK in neonatal rat cardiac fibroblasts during four hours of ischemic conditions.

The serine/threonine protein kinase Akt/PKB stimulates cell proliferation and inhibits apoptosis (Lawlor and Alessi, 2001). Previous studies using neonatal rat cardiac myocytes have reported increases in Akt/PKB phosphorylation during 4 hours of simulated ischaemia (Germack and Dickenson, 2004, 2005). However, Punn *et al.,* (2000) reported that detectable Akt/PKB activity was observed at 18-24 hours hypoxia but not at 4 hours. Furthermore, an increase in Akt/PKB activity was observed in rat hearts subjected to ischaemic conditions for 1-2 hours (Kim *et al.,* 2002). In the present model of ischaemic heart disease, cardiac fibroblasts inhibited the activity of Akt/PKB during 4 hours of hypoxic exposure. Interestingly, UTP reversed the hypoxia-inhibited Akt/PKB activity in cardiac fibroblasts. It is very tempting to conclude that UTP possess anti-apoptotic properties. However, no such conclusion can be drawn as hypoxia (4 hours) did not induce cell death in cardiac fibroblasts. ATP-γS did not modulate the phosphorylation of Akt/PKB under normal or hypoxic conditions. Likewise, ATP-γS and UTP in combination with ANG-II under normal and hypoxic conditions did not activate Akt/PKB. Taken together,

these results suggest that P2Y receptors do not activate PKB/Akt in neonatal rat cardiac fibroblasts during ischaemic conditions.

In conclusion, P2Y receptors on neonatal rat cardiac fibroblasts are not involved in activation of MAPK and PKB/Akt pathways during 4 hours of ischaemic conditions.

4.9 Conclusion

In general, these studies have revealed that there are two groups of P2Y receptors functionally expressed on neonatal rat cardiac fibroblasts, one activated by ATP-γS and the other one stimulated by UTP. The P2Y receptors activated by ATP-γS are involved in proinflammatory processes since they release IL-1β and IL-6 cytokines in ischaemic conditions. Furthermore, these P2Y receptors are also involved in collagen deposition during initial ischaemic conditions (4 hours). P2Y receptors stimulated by UTP only regulate IL-1β secretion in cardiac fibroblasts. In addition, these receptors activate anti-fibrotic pathways during ischaemic conditions. P2Y receptors are not involved in the cardiac fibroblast survival and do not activate MAPK and Akt/PKB pathways during the initial stages of ischaemia.

Chapter 5

General Conclusion and Future Work

Chapter 5: General Conclusion and Future Work

5.1 General Conclusion

P2Y receptors belong to the G-protein coupled receptor (GPCR) superfamily and eight mammalian subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) have been cloned and characterised in different cell types (Abbracchio *et al.,* 2006; Von Kugelgen, 2006). This study revealed the functional expression of five different P2Y receptors in neonatal rat cardiac fibroblasts: $P2Y_1$ (2-MeSADP/ADP-βS responsive; G_q pathway), $P2Y_{2,4}$ (UTP/UDP/ATP responsive; G_q/G_i pathway), $P2Y_6$ (UDP/UTP responsive; G_q pathway), $P2Y_{11}$-like (ATP, 2-MeSADP/2-MeSATP sensitive). The $P2Y_{13}$ receptor was identified at mRNA and protein levels; however the functional characterisation of this subtype was hindered by the lack of specific agonists and antagonists. Moreover, the expression of a P2Y-like receptor activated by AMP and coupled to G_s-protein has been identified in neonatal rat cardiac fibroblasts. Cardiac fibroblasts are the predominant cell type found in the heart (Brown *et al,* 2005a). Fibroblasts play an important role in myocardial remodelling process observed in cardiovascular diseases such as ischaemic heart disease, which involves an increase in ECM, cardiomyocyte hypertrophy, migration and proliferation of fibroblasts (Brown *et al.,* 2005a). Recent studies have shown that ATP and UTP are released during myocardial infarction in human (Wihlborg *et al.,* 2006), possible leading to the stimulation of P2Y receptors and affecting fibroblast functions. In the current study, the role of P2Y receptors was investigated using an *in vitro* model of ischaemic heart disease associating angiotensin-II and hypoxia. IL-1β production was regulated by both ATP-γS and UTP whereas IL-6 release was induced by ATP-γS. ATP-γS and UTP did not affect the TNF-α and TGF-β1 production. ATP-γS mediated the deposition of collagen whereas UTP inhibited the collagen accumulation. Both nucleotides did not affect the fibroblast viability or MAPK and Akt/PKB activity during ischaemic heart disease conditions. These data suggest that P2Y receptors activated by ATP-γS induce cardiac fibrosis and hypertrophic responses whereas P2Y receptors stimulated by UTP inhibit fibrosis, during ischaemic conditions. These results indicate that blockade of P2Y receptors stimulated by ATP and agonists selective for UTP-sensitive P2Y receptors may have a therapeutic role in

myocardial remodeling during ischaemia. However, the precise P2Y receptors involved in these processes are not yet established in cardiac fibroblasts.

5.2 Future Work

Cardiac fibroblast is the major cell type in the myocardium and plays a vital role in wound healing, hypertrophy and fibrosis. Fibroblasts perform three important functions: (i) synthesis of extracellular matrix (ECM) proteins, (ii) synthesis and release of hormones which maintain the ECM, (iii) create a stress tolerant connecting network. During ischaemic heart disease fibroblasts are functionally activated, undergo proliferation and secrete disproportionate amounts of ECM (Brown *et al.,* 2005a). Indeed, cardiac fibroblasts isolated from myocardial infarction region have increased rate of proliferation and collagen synthesis compared with the fibroblasts isolated from non-myocardial infarction regions (Squires *et al.,* 2005). There are several lines of evidence that ATP and UTP are released during myocardial infarction in human and also from isolated cardiac myocytes, pulmonary artery advential fibroblasts exposed to hypoxia (Wihlborg *et al.,* 2006; Dutta *et al.,* 2004; Gerasimovskaya *et al.,* 2002;). These observations suggest that ATP and UTP released during cardiac ischaemia can mediate their effects by stimulating P2Y receptors and influence fibroblast function. Furthermore, as shown in the present study P2Y receptors stimulated by ATP-γS and UTP regulate cytokine release and collagen deposition during ischaemic heart disease conditions. To understand further the involvement of P2Y receptors in modulating fibroblast functions the following studies have to be performed.

In the current study, the role ATP-γS and UTP on ERK1/2, p38 MAPK and JNK and PKB/Akt activity is not evaluated at 18 hours of ischaemic treatment. Cardiac fibroblasts treated with ATP-γS and UTP did not active the MAPK and PKB/Akt phosphorylation during 4 hours of ischaemic conditions. It is noteworthy that 18 hours of hypoxia induced the release of LDH and caused cell death in fibroblasts. According to the cytokine release and collagen accumulation at 18 hours determining the MAPK and Akt/PKB activity will help to identify the signalling pathways involved in the effects of P2Y receptors in fibroblasts.

All the P2Y receptors functionally expressed in neonatal rat cardiac fibroblasts were coupled to $G_{q/11}$-protein and their stimulation lead to the production of inositol phosphates and subsequent activation of protein kinase C (PKC). Wang *et al.,* (2003) have demonstrated that PKC levels were elevated in cardiac hypertrophy and in heart failure following myocardial infarction. In addition, ANG II released during heart disease induces cardiac gene expression, cell growth and remodelling of the myocardium via PKC pathway (Sugden *et al.,* 1995; Mehta and Griendling, 2007). PKC seems to be a key kinase involved in the changes observed in heart diseases. Therefore, evaluating PKC activity in neonatal rat cardiac fibroblasts following the stimulation of P2Y receptors with ATP-γS and UTP during ischaemia will give more information about the different subtypes of P2Y receptors. In addition, inhibition of PKC activity attenuated the cardiac fibrosis and improved the cardiac function in rats subjected to myocardial infarction (Boyle *et al.,* 2005).

Neonatal rat cardiac fibroblasts treated with UTP inhibited collagen accumulation during ischaemic conditions. The lack of collagen production by cardiac fibroblasts when stimulated by ATP-γS suggests that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$-like receptors are not involved in regulating collagen accumulation during 18 hours of ischaemic conditions. The anti-fibrotic property of UTP probably involves $P2Y_6$ receptor but not $P2Y_2$ and $P2Y_4$ receptors. The role of $P2Y_6$ receptor in the modulation of collagen production can be investigated by RNA interference which "knocks out" the $P2Y_6$ expression. ATP-γS induced collagen accumulation by a mechanism which is not well established. Matrix metalloproteinases (MMPs) degrade the collagen and regulate the collagen deposition (Jugdutt *et al.,* 2003a, b). Therefore, measuring the MMP activity by zymography following the stimulation of P2Y receptors with ATP-γS and UTP during ischaemia will **??** precise the role of P2Y receptors in regulating of collagen secretion.

Hypoxia is known to alter the G-protein coupled receptor expression in several cell types (Feoktistov *et al.,* 2004; Franke and Illes, 2006; Lee *et al.*, 2007). In human umbilical vein endothelial cells (HUVECs) exposed for 3 hours hypoxia (0% O_2) adenosine A_{2A} receptor mRNA and functional expression were decreased whereas adenosine A_{2B} receptor were up-regulated (Feoktistov *et al.,* 2004). In addition, hypoxia increased the protein expression of A_1 receptor protein expression in neonatal rat cardiac myocytes (Germack

and Dickenson unpublished data) and DDT_1-MF_2 cells (Hammond *et al.,* 2004). Furthermore, functional expression of β_1-adrenergic receptor was decreased in neonatal rat cardiac myocytes when exposed to hypoxia (0.5% O_2; 18 hours; Ampolu and Germack, 2007). Interestingly, Lammer *et al.,* (2004) demonstrated that cerebral ischaemia in spontaneously hypertensive rats increased the expression of $P2Y_1$ receptor at the peri-infarct area. Furthermore, glucose can also influence the expression of receptors. Indeed, cardiac fibroblasts cultured in elevated levels of glucose up-regulated the expression of A_1, A_{2A} receptors, down-regulated the expression of A_3 and had no effect of A_{2B} receptor levels Grden *et al.,* 2006). Alternately, fibroblasts exposed to insulin decreased the expression of A_1, A_{2A} receptors and had no effect on A_{2B} and A_3 receptor expression (Grden *et al.,* 2006). Therefore, determining the expression of different subtypes of P2Y receptors in cardiac fibroblasts exposed to ischaemic conditions can help to identify the subtypes involved in modulating fibroblast functions.

In the present study, the characterisation and functional role of P2Y receptors was determined in neonatal cardiac fibroblasts. Webb and associates (1996) have reported that the expression levels of P2Y receptor subtypes in heart vary with age. Using reverse transcriptase-polymerase chain reaction (RT-PCR) and northern analysis, four subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$) were identified in adult rat hearts with the $P2Y_6$ being the most abundant ($P2Y_6 > P2Y_1 > P2Y_2 = P2Y_4$; Hou *et al.,* 1999; Zheng *et al.,* 1998;). However, in neonatal rat cardiac fibroblasts, $P2Y_1$ and $P2Y_6$ are expressed at higher levels than $P2Y_2$ and $P2Y_4$ (Webb *et al.*, 1998). Therefore further experiments are needed to confirm the expression and role of P2Y receptors in an adult rat model.

Chapter 6

References

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