

Pharmacological and Molecular Characterisation of P2Y Receptors in Endothelial and Epithelial Cells

Item Type	Thesis or dissertation
Authors	D'Souza, Vijay Kenneth
Publisher	University of Wolverhampton
Download date	2026-04-15 06:59:20
Link to Item	http://hdl.handle.net/2436/20512

**PHARMACOLOGICAL AND MOLECULAR
CHARACTERISATION OF P2Y RECEPTORS IN ENDOTHELIAL
AND EPITHELIAL CELLS**

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A thesis submitted in partial fulfilment of the
requirements of the University of Wolverhampton
for the degree of Doctor of Philosophy

2008

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ABSTRACT

In light of the significant modulation of receptor activity previously shown by a peptide (designated L247), designed to mimic the third extracellular loop of the human P2Y₂ receptor, the aim of this study was to use this peptide as an immunogen to generate and fully characterise polyclonal rabbit antibodies to the P2Y₂ receptor. Other aims of this study were to characterise epithelial and endothelial cells for a thorough expression profile of P2Y receptor mRNA transcripts in order to provide a rapid screen for the molecular determinants of these receptors in these cells. These studies also aimed to confirm previously published pharmacology, thus, to set the basis for western blot studies using P2Y receptor antibodies.

Bovine aortic endothelial cells that co-express P2Y₁ and P2Y₂ receptors; EAhy926, a human endothelial fusion cell line, that express P2Y₂ receptors; and ECV304 human bladder cancer cell line, known to express P2Y₂-like and P2Y₁₁-like receptors were used in this study. The dose dependent accumulation of inositol phosphates and cAMP response to potent P2Y₁₁ agonists and RT-PCR studies confirmed the functional expression of both P2Y₂ and P2Y₁₁ receptors in ECV304 cells. Likewise, the dose dependent accumulation of inositol phosphates in response to potent P2Y₂ and P2Y₆ agonists and the presence of mRNA transcripts confirmed the expression of functional P2Y_{2/4}- like and P2Y₆- like receptors in EAhy926 cells.

Polyclonal antiserum raised against L247 peptide was affinity purified and the purified fractions showed strong immunoreactivity with immobilised immunogenic antigen in ELISA. In western blot analysis L247 rabbit polyclonal anti-P2Y₂ antibody detected strong bands in ECV304 and EAhy926 cells. On pre-absorption with the immunogenic peptide these responses were abolished suggesting that this antibody is antigen specific. Agonist induced P2Y₂ receptor desensitisation studies in ECV304 cells showed that prolonged agonist incubation caused the receptor sequestration. The loss of bands caused by P2Y₂ receptor desensitisation and sequestration in membrane enriched fractions of agonist incubated ECV304 cells confirmed the specificity of L247 antibody. This antibody also showed no immunoreactivity in 1321N1 human brain astrocytoma cells devoid of any P2Y receptor subtypes cells. Deglycosylation studies revealed that the P2Y₂ receptors are glycosylated in ECV304 cells.

The polyclonal rabbit anti-P2Y₂ receptor antibodies obtained from commercial sources produced completely different immunoreactive profiles with multiple bands even in 1321N1 cells. Furthermore, in comparison to L247 anti-P2Y₂ antibody the commercial antibody showed no difference between normal and agonist incubated cells suggesting that this antibody may not be recognising the P2Y₂ receptors in ECV304 cells. Likewise polyclonal rabbit antibodies to other P2Y receptors either showed no response or showed strong immunoreactive profile with multiple bands even in 1321N1 cells suggesting that these antibodies may not have been extensively characterized. Furthermore, immunofluorescence studies with commercial anti-P2Y₂ antibodies showed that they may be only recognising non-denatured receptors. These studies suggest that the L247 anti-P2Y₂ antibody raised against peptide designed to mimic specific region in the third extracellular loop of human P2Y₂ receptor is highly specific and sensitive and provides an important tool to study endogenously expressed P2Y₂ receptors in both non-denatured and denatured state. These studies indicate that this strategy of generating antibodies may be used to generate highly specific antibodies to other P2Y receptor subtypes.

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ACKNOWLEDGEMENTS

I am deeply grateful for the support and encouragement of my Director of Studies Dr. Colin Brown and for his patience and help in preparation of this thesis.

I am also indebted to Dr. Julia Brown of Aston University for her interest in the project and for all the discussion and inputs.

I also thank Dr. Paul Nelson, my second supervisor for his inputs in my project.

Gratitude is extended to the fellow members of the Diabetes group at University of Wolverhampton especially Graham Freimanis, Dr. James Brown, Kenya Ward, Dr. Rachel Baker and Richard Smith for their assistance and friendship. I also thank Dr. Sarah Brown for her help in confocal microscopy and Dr. William Simmons for his insights into antibody purification. I am also grateful to the technicians in the Institute of Healthcare sciences for their technical assistance. Special thanks are extended to Dr. Weiguang Wang for his help in western blot studies.

The work presented in this thesis is not supported by grants. The equipment and consumables used for this work has been funded entirely by the University of Wolverhampton. I owe a special debt of gratitude to Prof. John Darling, Director of Research Institute in Healthcare Science for making this work possible.

I would like to thank my parents, my brother Vinod and sister Veera for their love and support. I also thank my wife, Reshma for being so nice and for listening to western blot stories.

DEDICATION

This thesis is dedicated to my brother Vinod

ABBREVIATIONS

AC	Adenylate cyclase
ADP	Adenosine 5'-diphosphate
ADP β S	Adenosine 5'-O-2-(thio) diphosphate
AES	Amino-terminal enhancer of split proteins
Akt/PKB	Akt/protein kinase B
AMI	Acute myocardial infarction
AML-193	Acute myeloid leukemia-193
AMP	Adenosine 5'-monophosphate
AMV	Avian Myeloblastosis Virus Reverse transcriptase
Ap4A	Diadenosine-tetraphosphate
APCs	Antigen-presenting cells
AR-C67085	2-propylthio-D- β γ -dichloromethylene adenosine 5'-triphosphate
AR-C69931MX	N^6 -(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)- β β , γ -dichloromethylene ATP
ATP	Adenosine 5'-triphosphate
ATP γ S	Adenosine 5'-O-(3-thiotriphosphate
BAEC	Bovine aortic endothelial cells
BCA	Bicinchoninic Acid
<i>bcl-2</i>	B-cell leukemia/lymphoma 2
<i>bcl-xl</i>	Antiapoptotic Bcl-2 family member, a death antagonist
β , γ -meATP	β , γ -methylene-ATP
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin

BRIN-BD11	Electrofusion cell line of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells
BSS	Balanced salt solution
BzATP	Benzoyl-Benzoyl-ATP
Ca ⁺⁺	Calcium ions
[Ca ²⁺] _c	Cytosolic free calcium concentration
[Ca ²⁺] _i	Intracellular calcium
CaCC	Ca ²⁺ -activated chloride (Cl ⁻) channels
Caco-2	Human Caucasian colon adenocarcinoma
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CASMC	Coronary artery smooth muscle cells
CB1	Cannabinoid receptor 1
CBF	Ciliary beat frequency
CCR5 receptor	CC motif, receptor 5
cDNA	Complementary DNA
CFPAC-1	Cystic fibrosis pancreatic adenocarcinoma cell line
CFTR	Cystic Fibrosis (CF) transmembrane regulator
cGMP	Guanosine 3',5' cyclic monophosphate
CGP 12177	4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride
CHO	Chinese hamster ovary
CHO-K1 cells	A subclone of the parental CHO cell line
Cl ⁻	Chloride
CO ₂	Carbon dioxide
COX-2	Cyclo-oxygenase -2
CREB	cAMP response element-binding

CSMC	Coronary smooth muscle cells
CTL	Cytotoxic T lymphocytes
CTNNB1	Catenin (cadherin-associated protein), beta 1,
CXCL8	CXC-chemokine 8
CysLT1	Cysteinyl leukotriene receptor
DAG	Diacylglycerol
DAPI	4',6 diamidino-2-phenylindole
DCs	Dendritic cells
DM2	Type 2 diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EAh926	Cell line derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549
EC	Extracellular region
ECACC	European collection of animal and cell cultures
ECP	Eosinophil cationic protein
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EL	Extracellular loop
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal related protein kinase
Fab	Fragment antigen binding
FCS	Foetal calf serum

GAM	gp130-associated-molecule
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GPR	G-protein-coupled receptor
GRK	G protein-coupled receptor kinase
³ H	Tritium, a radioactive isotope of hydrogen
HA	Human influenza hemagglutinin
HT-29	Human Caucasian colon adenocarcinoma grade II
HCAEC	Human coronary artery endothelial cells
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HERV	Human endogenous retrovirus
hFSHR	Human follicle-stimulating hormone receptor
hKvLQT1	Human KvLQT1 (potassium voltage-gated channel, KQT-like subfamily, member 1)
HLMC	Human lung mast cells
hMCs	Human cord-blood-derived mast cells
HRP	Horseradish peroxidase
hSK4	Human small conductance, Ca ²⁺ -activated K ⁺ channel
5-HT(4)	5-hydroxytryptamine 4
HUVEC	Human umbilical vein endothelial cell
IDP	Inosine (5'-)diphosphate
IgE	Imuunoglobulin E
IgM	Imuunoglobulin M
IL-1β	Interleukin-1beta
IL-6	Interleukin-6

IL-8	Interleukin-8
Inos	Inducible nitric oxide synthase
InsP1- 4	Inositol triphosphate (Ins 1,4,5)
INS365	Diquafosol
INS37217	Denufosol tetrasodium
IP ₃	Inositol triphosphate (Ins 1,4,5)
I/R	Ischemia-reperfusion (I/R)
JNK	Jun kinase
KATP	ATP-activated K ⁺ (KATP) channels
KLH	Keyhole Limpet Hemocyanin
LAK	Lymphokine-activated killer cells
LIMA	Left internal mammary artery
LPS	Lipopolysaccharide
LTB(4)-	Proinflammatory chemokine, LTB(4)
lysoPS	Lysophosphatidyl-L-serine (lysoPS)
MAbs	Monoclonal Antibodies
MCAs	Middle cerebral arteries
MDCK(II)	Madin-Darby canine kidney (MDCK) cells
MEK	MAPK/ERK kinase
2-MeSADP	2-Methylthio-ADP
2-MeSAMP	2-Methylthio-AMP
2-MeSATP	2-methylthio-ATP
MKK	Mitogen-activated protein (MAP) kinase kinase
mRNA	Messenger RNA
MRS2179	2'-deoxy- <i>N</i> ₆ -methyladenosine-3',5'-diphosphate

MRS2279	2-chloro- <i>N</i> ⁶ -methyl-(<i>N</i>)-methanocarpa-2'-deoxyadenosine-3',5' bisphosphate
MRS2567	1,2-di-(4-isothiocyanatophenyl)ethane
MRS2575	1,3-Di-[(4-isothiocyanatophenyl)-thioureido] propane
MRS2578	1,4-Di-[(3-isothiocyanato phenyl)-thioureido] butane
1321N1	Human brain astrocytoma cells
NaCl	Sodium Chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium Hydroxide
NF- κ B	Nuclear factor-kappa B
(NH ₄) ₂ SO ₄	Ammonium sulphate
NO	Nitric oxide
NOS	Nitric oxide synthase
³³ P	Phosphorus isotope
pA ₂	Negative logarithm of the concentration of antagonist needed to shift the dose response curve by a factor of 2
p38–MAPK	p38 isoform of Mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
pEC ₅₀	Negative logarithm of the EC ₅₀
PGE ₂	Prostaglandin E ₂
PI3K	Phosphatidylinositol 3-kinase (PI3-kinase)
PIP2	Phosphatidyl inositol bisphosphate (PIP2)
pKB	Antagonist affinity estimates
PKC	Protein kinase C
PLC beta	Phospholipase C beta

PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PPADS	Pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid
PTX	Pertussis toxin
PVDF	Polyvinylidene fluoride
RAFTK	Related adhesion focal tyrosine kinase
RGD	Arginine-glycine-aspartic acid
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RT-PCR-	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH3	Src homology 3
SMCs	Smooth muscle cells
SSF1 gene	Saccharomyces cerevisiae nuclear protein
TBS-T	Tris-Buffered Saline Tween-20
TCA	Trichloroacetic acid
TCF	Transcriptional co-activator of T cell factor
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
Th2	Type 2 helper T cell
TLE3	Transducin-like enhancer of split 3
TM	Transmembrane
TMB	3, 3', 5, 5' – Tetramethyl Benzidine
TMV	Tracheal mucus velocity
TNF-alpha	Tumor necrosis factor α

TRI	Trizol reagent
UDP	Uridine 5'-diphosphate
UDP β S	Uridine 5'-O-thiodiphosphate
UDP γ S	Uridine-thiodiphosphate.
Up(4)A	Uridine adenosine tetraphosphate
UTP	Uridine 5'-triphosphate
VCAM-1	Vascular cell adhesion molecule-1

AMINO ACID CODES

Amino Acid	Triple letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Functional human P2Y receptor subtypes

The role of purinergic receptors and the description of their pharmacology were originally proposed by Burnstock and colleagues (Burnstock *et al.*, 1970) by way of describing responses that previously had been defined as nonadrenergic and noncholinergic. Adenosine triphosphate (ATP) was proposed as the natural ligand of these receptors. On further study, it was proposed that purinergic receptors should be subdivided into P1 receptors, which respond to adenosine and are coupled to adenylate cyclase, and P2 receptors, which respond to ATP and adenosine diphosphate (ADP). The P2 class has subsequently been divided further into P2X and P2Y receptors. P2X receptors are ligand gated receptors containing an intrinsic ion channel whereas metabotropic P2Y receptors belong to the super family of G protein coupled receptors.

To date, eight functional human P2Y receptor subtypes have been identified (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄) that cannot be distinguished by their specific pharmacological selectivity for different nucleotides. The P2Y₁ receptor is highly responsive to both ADP and ATP (Ayyanathan *et al.*, 1996; Leon *et al.*, 1996; Schachter *et al.*, 1996; Palmer *et al.*, 1998). The triphosphate-preferring P2Y₂ receptor is activated equipotently by ATP and UTP (Parr *et al.*, 1994; Nicholas *et al.*, 1996). The P2Y₄ receptor is activated preferentially by UTP, while both UDP and ADP have little or no effect (Nguyen *et al.*, 1995; Communi *et al.*, 1996a; Nicholas *et al.*, 1996). UDP potently activates the pyrimidine-preferring P2Y₆ receptor, while UTP and ADP are low potency agonists (Communi *et al.*, 1996b; Nicholas *et al.*, 1996).

The P2Y₁₁ receptor is potently activated by ATP and certain ATP analogues e.g. BzATP and ATP γ S and this receptor couples to both phospholipase C and adenylyl cyclase stimulation (Communi *et al.*, 1997; Communi *et al.*, 1999a). Some of the functional P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁) are coupled to phospholipase C. Agonist stimulation of these receptor subtypes results in mobilisation of intracellular Ca²⁺ ions from internal stores via G_{q/11}-mediated phospholipase C activation but some may couple also to adenylyl cyclase either through G_i (P2Y₂, P2Y₄) or G_s (P2Y₁₁) (Morse *et al.*, 2001). Upon activation, phosphatidyl inositol bisphosphate

(PIP₂) is hydrolysed leading to the generation of inositol triphosphate (Ins 1,4,5), which in turn releases calcium from intracellular stores (Lytton *et al.*, 1991).

ADP is a potent agonist at the P2Y₁₂ receptor (Hollopeter *et al.*, 2001; Zhang *et al.*, 2001). The P2Y₁₂ receptor which was formerly called P2Y (ADP), P2Y (AC), P2Y_{cyc} or P2T (AC) is activated by 2MeSADP and ADP and coupled to the inhibition of adenylyl cyclase through G_i (Hollopeter *et al.*, 2001). The P2Y₁₃ receptor shares high affinity for ADP and is coupled to inhibition of adenylyl cyclase and the stimulation of MAP kinases (ERK1 and ERK2) and is linked to the G_{αi} class of G proteins (Communi *et al.*, 2001a; Zhang *et al.*, 2002). The P2Y₁₄ receptor is potently activated by UDP-glucose and related sugar nucleotides such as UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine and is coupled to G proteins of the G_{i/o} class (Chambers *et al.*, 2000a; Abbracchio *et al.*, 2003).

The activation of a receptor by different nucleotides and the ability of a single nucleotide to activate more than one receptor makes it difficult to determine the identity of functionally expressed receptors. Therefore, there is a need for selective agents which are able to bind in an agonistic or antagonistic manner. The development of such agents would be beneficial to not only determine the functional expression but also to study the pharmacology of P2Y receptor subtypes.

1.2 Ligands for P2Y receptors

Table 1.1 Human P2Y receptor agonists

P2Y subtypes expressed in human tissues	Agonists	References
P2Y ₁	2-MeSADP > ADP > 2-MeSATP > ATP	(Ayyanathan <i>et al.</i> , 1996; Janssens <i>et al.</i> , 1996; Leon <i>et al.</i> , 1996; Leon <i>et al.</i> , 1997; Jin <i>et al.</i> , 1998a; Palmer <i>et al.</i> , 1998)
P2Y ₂	UTP = ATP > Ap4A > ATP	(Parr <i>et al.</i> , 1994; Lazarowski <i>et al.</i> , 1995; Nicholas <i>et al.</i> , 1996)
P2Y ₄	UTP > UTP γ S >> ATP, UDP	(Communi <i>et al.</i> , 1995; Nguyen <i>et al.</i> , 1995; Communi <i>et al.</i> , 1996a; Nicholas <i>et al.</i> , 1996)
P2Y ₆	UDP = 5-Br-UDP >> UTP > 2-MeSADP	(Communi <i>et al.</i> , 1996b; Southey <i>et al.</i> , 1996; Maier <i>et al.</i> , 1997)
P2Y ₁₁	ATP γ S = BzATP > ATP > 2-MeSATP	(Communi <i>et al.</i> , 1997; Communi <i>et al.</i> , 1999b)
P2Y ₁₂	2-MeSADP > ADP	(Hollopeter <i>et al.</i> , 2001; Takasaki <i>et al.</i> , 2001; Zhang <i>et al.</i> , 2001)
P2Y ₁₃	2-MeSADP = ADP > ADP β S > IDP	(Communi <i>et al.</i> , 2001a; Zhang <i>et al.</i> , 2002)
P2Y ₁₄	UDP-Glucose	(Chambers <i>et al.</i> , 2000a; Abbracchio <i>et al.</i> , 2003)

All the available agonists have been shown to activate more than one P2Y receptor. However, the main discriminating features in the profiles of agonist activating receptors are that P2Y₁ and P2Y₁₁ are selective for adenine nucleotides and all other P2Y receptors can be activated also by uracil nucleotides though some of these receptors show weak selectivity between uracil or adenine nucleotides (e.g. P2Y₂, P2Y₄). Also there is no preference between nucleotide diphosphate or nucleotide triphosphate as agonists (e.g. P2Y₁, P2Y₆). The agonist of P2Y₁ receptor 2-methylthio adenosine triphosphate (2MeSATP) can also activate P2Y₁₁, and uridine 5'-triphosphate (UTP) can activate P2Y₂, P2Y₄ and P2Y₆ receptors (Lazarowski *et al.*, 1995; Communi *et al.*, 1996a; Communi *et al.*, 1996b).

In addition, in the case of antagonists, the inhibition of agonist activity is not subtype selective for P2Y receptors (Charlton *et al.*, 1996a). However in recent years a few agents have been reported to be selective antagonists like MRS2279 specific for the P2Y₁ receptor with no effect on activation of the human P2Y₂, P2Y₄, P2Y₆, or P2Y₁₁ receptors by their cognate agonists (Boyer *et al.*, 2002). MRS2567 and MRS2578 have been shown to block agonist effects at both human and rat P2Y₆ receptors and MRS2575 was shown to selectively block human P2Y₆ but not rat P2Y₆ receptors (Mamedova *et al.*, 2004). AR-C67085 is also reported as the selective antagonist at the platelet ADP receptor, P2Y₁₂ (Ingall *et al.*, 1999). There are at present, no commercially available selective antagonists for P2Y₂, P2Y₄, P2Y₁₁, P2Y₁₃, P2Y₁₄ receptors (see Table 2).

Table 1.2 Human P2Y receptor antagonists

P2Y subtypes expressed in human tissues	Antagonists (pA ₂ or pK _B values or antagonistic effect at highest concentration)	References
P2Y ₁	MRS2279 MRS2179 (6.75) Reactive blue 2 (6.1) Suramin (5.5) PPADS (5.4-4.9)	(O'Grady <i>et al.</i> , 1996; Schachter <i>et al.</i> , 1996; Moro <i>et al.</i> , 1998; Boyer <i>et al.</i> , 2002)
P2Y ₂	Suramin (4.3)	(Charlton <i>et al.</i> , 1996a; 1996b)
P2Y ₄	PPADS (decrease by 30-73 % at 100 μM) Reactive blue 2 (decrease by 33 % at 100 μM)	(Charlton <i>et al.</i> , 1996b; Communi <i>et al.</i> , 1996a)
P2Y ₆	MRS2575, MRS2567, MRS2578 Reactive blue 2 (6.0) PPADS (decrease by 69 % at 100 μM)	(Robaye <i>et al.</i> , 1997; Mamedova <i>et al.</i> , 2004)
P2Y ₁₁	Suramin (6.09) Reactive blue 2 (decrease by 80 % at 100 μM)	(Communi <i>et al.</i> , 1999b)
P2Y ₁₂	AR-C69931MX >> 2-MeSAMP	(Hollopeter <i>et al.</i> , 2001; Takasaki <i>et al.</i> , 2001; Zhang <i>et al.</i> , 2001)
P2Y ₁₃	No blockade by MRS2179 at 3μM	(Zhang <i>et al.</i> , 2002)
P2Y ₁₄	No data	

1.3 Molecular biology of P2Y receptors

Table 1.3 Chromosomal locations of the genes encoding for the P2Y-receptors

Receptor Type	Species	GenBank Access. No.	Chromosomal Location
P2Y ₁	Human	U42029, U42030, S81950	3q25.2
	Bovine	X87628	
	Rat	U22830	2q31
	Mouse	U22829	3E1
P2Y ₂	Human	NM_002564	11q13.5
	Canine	XM_542321	21
	Porcine	AY620400	
	Rat	L46865, U09402	1q32
	Mouse	L14751	7F1
P2Y ₄	Human	U40223, X91852, X96597	Xq13
	Rat	Y11433, Y14705	Xq31
	Mouse	AJ277752	XC2
P2Y ₆	Human	U52464, AF007891	11q13.5
	Rat	D63665	1q32
	Mouse	NM_183168	7E1
P2Y ₁₁	Human	NM_002566	19p13.2
	Canine		
P2Y ₁₂	Human	AF313449, AF321815, AB052684	3q25.1
	Bovine	AJ623293	
	Rat	AF313450	2q31
	Mouse	AJ312130, NM_027571	3D
P2Y ₁₃	Human	NM_023914, NM_176894	3q24
	Rat	AY639875	2q31
	Mouse	NM_028808	3D
P2Y ₁₄	Human	NM_014879	3q21-25
	Rat	U76206	2q31
	Mouse	NM_133200	3D

P2Y receptors are highly diverse in their amino acid sequence as shown by P2Y₁ and P2Y₁₁ receptors, which share only 33% of amino acid sequence despite similarity in their agonist profiles (von Kügelgen and Wetter, 2000). Similarly P2Y₁ and P2Y₁₂ receptor share only 18% similarity in amino acid sequence (von Kugelgen, 2006). However, the highly conserved amino acid residues in the predicted TM3 (transmembrane 3), TM6 and TM7 regions of P2Y receptors close to the intracellular loop, is thought be responsible for their ligand specificity (von Kügelgen and Wetter, 2000). All P2Y receptors pocess 4 cysteine residues at their extracellular domain and these residues form 2 disulfide bridges. The first one between the N-terminal domain and ECIV (extracellular region IV) and the second bridge between ECII and ECIII (Hoffmann *et al.*, 1999; Ding *et al.*, 2003). P2Y₂ and P2Y₁₂ receptors expressed at the level of the cell membrane are shown to be modified by N-linked glycosylation (Erb *et al.*, 1993; Zhong *et al.*, 2004). Furthermore, N-linked glycosylation has been shown to be essential for signal transduction, but not for ligand binding or cell surface expression of P2Y₁₂ refeptors (Zhong *et al.*, 2004).

Many orphan G-protein coupled receptors have a structural similarity with P2Y receptors e.g. the sequence similarity of pseudogene, psi GPR79 with the P2Y₂ gene and GPR80 with the P2Y₁ gene (Lee *et al.*, 2001). Identification of closely related orphan G-protein coupled receptors (no identified natural ligand) at chromosomal location 3q24-3q25 along with P2Y₁ and uridine 5'-diphosphoglucose receptor KIAA0001, suggest that these receptors may have evolved from a common ancestor and may be activated by related ligands (Wittenberger *et al.*, 2001). Phylogenetic analysis of human G-protein-coupled receptors found that the orphan receptor GPR87 is closely related to the receptor for UDP-glucose KI01 and to the ADP-binding receptors P2Y₁₂ and GPR86 (Joost and Methner, 2002).

With the help of phylogenetic analyses and sequence comparisons of GPCRs it is possible to group receptors based on their ligand preference, in so doing, able to predict ligand types for orphan receptors. Furthermore, GPCRs activated by the signals from endogenous sources such as peptides, lipids, neurotransmitters, or nucleotides are well conserved between human and mouse; and the genetic polymorphism and loss of such an GPCR may only cause a selective defect in particular physiological function it is involved in (Vassilatis *et al.*, 2003). Orphan G-protein-coupled receptor 34 (GPR34)

which showed evidence for common evolutionary origin to ADP-like receptors (P2Y₁₂ and P2Y₁₃) did not show any response to ADP and has been reported as the functional mast cell lysoPS receptor activated by Lysophosphatidyl-L-serine (lysoPS) (Schoneberg *et al.*, 1999; Schulz and Schoneberg, 2003; Sugo *et al.*, 2006).

Diadenosine-polyphosphates which are known to activate P2Y₁, P2Y₂ and P2Y₁₃ have also been reported to activate yet unidentified receptors in rat midbrain cholinergic synaptic terminals (Diaz-Hernandez *et al.*, 2001; Patel *et al.*, 2001; Marteau *et al.*, 2003; Diaz-Hernandez *et al.*, 2004). Diadenosine polyphosphates have also been shown to selectively activate an unidentified P2Y-like receptor in lung epithelial cells (Laubinger *et al.*, 2003; Miras-Portugal *et al.*, 2003).

However, sequence similarity to P2Y receptor does not predict a P2Y receptor related response. Orphan receptor GPR80/GPR99, previously named the P2Y₁₅ receptor, is responsive to AMP and adenosine and thought to resemble P2Y nucleotide receptors more than P1 adenosine receptors and is also thought to be coupled to G_q and G_s (Inbe *et al.*, 2004). However, this receptor responds predominantly to citric acid cycle intermediate α -ketoglutarate and therefore is not a P2Y receptor (He *et al.*, 2004; Abbracchio *et al.*, 2005).

Genetic variations such as gene polymorphism of the P2Y gene family is thought to cause variability in P2Y receptor mediated responses. Interindividual variation of functional effects in terms of platelet response to ADP has been attributed partly to gene polymorphisms. Carriers of more than one G allele (frequency 0.15) at 1622 position of P2Y₁ receptor was found to be associated with increased platelet response to ADP. However, this A>G polymorphism at position 1622 in the coding sequence of the P2Y₁ gene was silent without affecting the amino acid structure of P2Y₁ receptor (Hetherington *et al.*, 2005). A recent study also has reported no association between response to aspirin or clopidogrel in cardiac patients and PIA₂ gene polymorphism of glycoprotein IIIa, T744C polymorphism of the P2Y₁₂ gene and the 1622A>G polymorphism of the P2Y₁ gene, indicating that the variability in anti-platelet drug response may not be only caused by single gene mutations but also could be due to other factors (Lev *et al.*, 2007).

Chromosomal clustering and phylogenetic analysis has provided insights into diverse evolutionary arrangements of P2Y receptor gene family. The close arrangements of the chromosomal location of the genes encoding for P2Y₂ and P2Y₆ (11q13.5) has been explained on the premise that gene development occurred with local gene duplication before chromosomal duplication (Somers *et al.*, 1997; Fredriksson *et al.*, 2003). For human P2Y₂ receptors gene polymorphism has been reported from European populations characterized by an Arg334 - Cys334 transition (arginine 334 to cysteine 334) with frequency distribution of R (Arg) allele 0.8 versus 0.2 for the C (Cys) allele (66% of the tested persons were homozygotes R/R (Arg/Arg), 29% were heterozygotes R/C (Arg/Cys) and 5% were homozygotes C/C (Cys/Cys) (Janssens *et al.*, 1999). This polymorphism, however, did not affect the agonist affinity but the stimulation of P2Y₂ receptor with Cys variant resulted in a slower time course (Janssens *et al.*, 1999).

Analysis of the coding region of P2Y₂ gene in CF (Cystic Fibrosis) patients revealed three frequent nonsynonymous P2Y₂ receptor polymorphisms (Leu46Pro; Arg312Ser and Arg334Cys). The intracellular Ca²⁺ - release was significantly increased in cells expressing the homozygous Cys334 variant compared to wild type and this effect was more pronounced in cells carrying the homozygous Ser312 variant. These studies indicate that the P2Y₂ receptor gene haplotypes resulted from gene polymorphism influence intracellular Ca²⁺ - release (Buscher *et al.*, 2006).

The extremely rare case of intergenic splicing seen in mammals has been found between the P2Y₁₁ and SSF1 gene (*Saccharomyces cerevisiae* nuclear protein, which plays an important role in mating) in cDNA libraries. The gene encoding P2Y₁₁ and the human orthologue of SSF1 are located on chromosome 19. The chimeric SSF1-P2Y₁₁ receptor fusion protein, when stably expressed in CHO-K1 cells generated a cAMP response to a P2Y₁₁ agonist which was qualitatively similar to that observed for P2Y₁₁ receptor (Communi *et al.*, 2001b). The common P2Y₁₁ gene polymorphism of Ala87Thr has been reported to be associated with acute myocardial infarction (AMI) with patients carrying homozygous Thr87 posing greater risk with increased levels of C-reactive protein (Amisten *et al.*, 2007).

The polymorphism of P2Y₁₂ receptor gene expressed in platelets from a person with congenital bleeding disorder was characterized by a G-to-A transition in one allele (changing the codon for 256Arg-256Gln in the sixth transmembrane domain) and a C-to-T transition in the other allele (changing the codon for Arg265- 256Trp in the beginning of the third extracellular loop) (Cattaneo *et al.*, 2003). These mutations did not interfere with ADP analogue binding to the receptor but altered the function of the receptor thought to be caused by defective signalling. The binding of the receptor with nucleotides was previously proposed to be caused by the interaction of positive charged residue on the external domain with the phosphate groups of nucleotides. However, this positive charge was lost in this case by the mutation to Trp (Moro *et al.*, 1999; Cattaneo *et al.*, 2003). These reports underscore the role of molecular characterisation of P2Y receptors in designing receptor subtype selective ligands, identifying novel targets and analysing the altered response caused by genetic variation in receptor mediated therapy.

1.4 P2Y Receptors and binding domains

Since the fully ionized form of nucleotides have been shown to be an activating ligand for both P2Y₁ and P2Y₂ receptors the positively charged amino acids conserved in TM3, TM6, TM7 are involved in binding of the negatively charged phosphate groups of nucleotides. Mutational analysis of the P2Y₁ receptor suggested that positively charged residues on the exofacial side of TM3 and TM7 are critical determinants of the ATP binding pocket (Jiang *et al.*, 1997). Site-directed mutagenesis of His¹³² and Tyr¹³⁶ in TM3, Thr²²² and Phe²²⁶ in TM5, Lys²⁸⁰ in TM6 and Gln³⁰⁷ and Ser³¹⁴ in TM7 of the human P2Y₁ receptor decreased agonist induced responses and antagonistic potency of MRS 2179 suggesting their role in nucleotide binding (Guo *et al.*, 2002). The basic amino acid lysine appeared to be more important in P2Y₁. Other than its critical role for the activation of the receptor, it has been shown to be important in the binding of P2 receptor antagonists. The antagonistic potencies and affinities of chemically unrelated agents such as PPADS, NF023 and reactive blue 2 were affected by the mutation of Lys²⁸⁰ on TM6 of the human P2Y₁ receptor (Guo *et al.*, 2002).

Site-directed mutagenesis of the P2Y₂ receptor suggested that positively charged amino acids on TM6 and TM7 are involved in ligand binding. The mutation of histidine²⁶² and arginine²⁶⁵ to leucine decreased the potency of ATP and UTP with no effect on the ability to select between tri- and diphosphates (Erb *et al.*, 1995). Recent studies have revealed information about the conserved residues in the transmembrane regions of the human P2Y receptor subtypes. The P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors have an H-X-X-R/K motif in TM6. P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors have a conserved Y-Q/K-X-X-R motif in TM7 whereas P2Y₁₂, P2Y₁₃ and P2Y₁₄ have K-E-X-X-L motif in TM7 (Abbracchio *et al.*, 2003).

The diphosphates selectivity of canine P2Y₁₁ receptor unlike the triphosphate selectivity of human P2Y₁₁ receptor was attributed partly to the mutation of arginine²⁶⁵ in human P2Y₁₁ receptor to glutamine in the canine P2Y₁₁ receptor. The positively charged arginine residue appears to be crucial to the recognition of extracellular nucleotides by the P2Y₁₁ receptor (Qi *et al.*, 2001). In platelet P2Y₁₂ receptors from a patient with a congenital bleeding, the polymorphism of Arg-256-Gln at one allele in the sixth

transmembrane domain, and an Arg-265-Trp at the other allele in the third extracellular loop has been reported (Cattaneo *et al.*, 2003).

In the P2Y₁ receptor the presence of two critical disulfide bridges and involvement of charged residues in ligand recognition has indicated the involvement of second and third extracellular loops and transmembrane regions (Hoffmann *et al.*, 1999). In addition, low affinity binding sites termed “meta-binding sites” have been suggested for the nucleotide binding of the P2Y₁ receptor in the second and third extracellular loop (Moro *et al.*, 1999). In a study involving the P2Y_{1/6} chimeric receptor (chimera of human P2Y₁ with rat P2Y₆ receptor), Tyr¹¹⁰ residue was identified to play an important role in the recognition of the nucleobase in the P2Y₁ and P2Y₆ receptors. Furthermore this study ruled out the possibility of recognition of uracil nucleotides exclusively by extracellular regions, but indicated that ligand recognition must involve a combination of extracellular and transmembrane domains. Thus it was believed that the initial binding of the ligand takes place to the exofacial region and the subsequent movement of it to a deeper binding site in the transmembrane regions (Hoffmann *et al.*, 2004). Analysis of receptor-ligand complexes of the human P2Y₁ receptor with its ligand ATP in a homology modeling using the 2.6 Å crystal structure of bovine rhodopsin suggests that the triphosphate moiety is tightly bound by a multitude of interactions (Major and Fischer, 2004).

Studies involving human/rat P2Y₄ receptor chimeras revealed that the second extracellular loop and the N-terminus play a key role in determining agonistic activity of ATP at mammalian P2Y₄ receptors. According to the mutational analysis, Asn¹⁷⁷, Ile¹⁸³, and Leu¹⁹⁰ were crucial residues within the EL2 (extracellular loop 2) which impart ATP agonism (Herold *et al.*, 2004). The disulphide bridge between the second extracellular loop (EL2) and third transmembrane region (TM3) and the residues within EL2 are considered to be essential for activation for most GPCR (Klco *et al.*, 2005).

The P2Y₂ nucleotide receptor contains the integrin-binding domain arginine-glycine-aspartic acid (RGD) in its first extracellular loop which interacts with integrins and the integrin-associated thrombospondin receptor, CD47. The RGD sequence associated interaction of P2Y₂ receptor with integrins and CD47 was considered necessary for coupling the P2Y₂ with G_o protein (Erb *et al.*, 2001). SH3 binding sites (PXXP motifs)

serve important functional roles including protein interaction in many GPCRs (Mayer, 2001). The human P2Y₂ nucleotide receptor has two proline-rich, SH3 binding sites (PXXP) in the carboxyl-terminal tail and these SH3 domains directly associate with the tyrosine kinase Src and activate Src (Liu *et al.*, 2004b).

The dimer formation of P2Y₁ with A₁ receptor has given new insights into purine signalling and P2Y receptor interaction with other GPCRs (Yoshioka *et al.*, 2001). These studies suggested that A₁ and P2Y₁ receptors can form constitutive hetero-oligomers caused by simultaneous agonist promoted activation in living cells (Yoshioka *et al.*, 2002b). The composition of ligand binding domains of P2Y receptors, therefore, is valuable in understanding the mechanism of ligand recognition and receptor activation. It also facilitates the analysis and regulation of the multiple signalling pathways that are then activated by receptor complex formation with growth factors, receptors and other proteins. Comparison of the binding domains and their structural homologues in other P2Y receptors will help in understanding their allosteric regulation and also in designing selective ligands to alter their function.

1.5 Antibodies to P2Y receptors

Antibodies, which are raised against the receptor protein or against peptide sequences, are commonly used to localize receptor proteins at the microscopic levels and they present a potential tool to study the target protein (Mijares *et al.*, 2000). Polyclonal antibodies are directed against different epitopes on the target protein and interact with different affinities. Therefore they detect multiple epitopes and recognise the antigen from different orientations. Monoclonal antibodies, on the other hand, are monospecific as they are specific for a single epitope. Moreover, polyclonal antibodies are less time consuming simple and cheaper to produce than monoclonal antibodies. However, polyclonal antibodies are susceptible to differences in reactivity and titre that might be caused by batch to batch variation. The continuous production of monoclonal antibodies in culture with B cell hybridomas can produce unlimited supply of antibodies of unique specificity but their production is time consuming (Nelson *et al.*, 2000).

The lack of sensitive and selective antibodies to P2Y receptors, which belong to the rhodopsin-like/beta adrenergic receptor family, has hindered the task of studying the biochemical and cell biological properties of endogenous P2Y receptors. To date, the study of biochemical properties of GPCRs has mainly resorted to the use of receptor cDNAs to introduce an epitope tag at the N-terminus. Antibodies directed against this tag are then used to investigate epitope-tagged receptors expressed in heterologous cells. On the other hand, generation of antibodies to closely related receptors advocates the use of peptides derived from the N-terminal region as this region is comparatively diverse between closely related receptors (Gupta and Devi, 2006).

However, suitable antibodies have been generated - using sequences taken from other regions- which have shown specific interaction with the native receptor belonging to GPCR superfamily. Antibodies directed against the second extracellular loop of the human β_1 -adrenoceptor reacted strongly with non-denatured receptors and decreased the affinity of the radiolabelled antagonist ($[^3\text{H}]\text{CGP 12 177}$). However, antibodies directed against carboxy- and amino-terminal receptor domains reacted strongly with denatured and non-denatured receptors but did not interfere with binding of antagonist (Jahns *et al.*, 1996).

These subtype-specific antibodies to β_1 and β_2 -adrenoceptors (AR) have been used to map the distribution of subtypes in the rat nephron using immunofluorescence techniques (Boivin *et al.*, 2001). Peptides derived from N-terminal region have been successfully used to raise antibodies to μ , δ and κ subtypes of opioid receptors which recognize recombinant as well as endogenous receptors (Gupta and Devi, 2006). A recent study has reported the successful generation of antibodies to GPCR that could detect conformational changes in N-terminal region following receptor activation. These antibodies differentially recognized inactive and agonist-activated μ opioid receptors. Furthermore, antibodies generated to the mid-portion of the N-terminal tail exhibited enhanced recognition of activated receptors whereas those to the distal regions did not. Targeting the N-terminal region to generate receptor conformation specific antisera has been successfully applied to other $G\alpha_i$ -coupled (δ opioid, CB1 cannabinoid, α_{2A} adrenergic) as well as $G\alpha_s$ - (β_2 adrenergic) and $G\alpha_q$ -coupled (AT1 angiotensin) receptors (Gupta *et al.*, 2007). Receptor conformation change affecting the ligand binding has also been seen in antibodies raised against synthetic peptides of extracellular domain of human follicle-stimulating hormone receptor (hFSHR) (Schmidt *et al.*, 2001).

Monoclonal antibodies produced against a peptide corresponding to the second extracellular loop of the human β_2 -adrenoceptor exerted a dose-dependent “agonist-like” effect on the spontaneous beating rate of neonatal rat cardiomyocytes. This agonist effect was blocked by preincubation with the target peptide and with the β_2 -antagonist indicating the receptor specificity of the antibody. However, this antibody which recognised the receptor in its native form failed to recognise denatured receptor on western blot analysis (Lebesgue *et al.*, 1998). The monomeric Fab fragments of this monoclonal antibody to human β_2 -adrenoceptor, however, inhibited the agonist induced receptor activation. Furthermore, the cross-linking of Fab fragment with rabbit anti-mouse IgG restored the agonist-like effect to Fab fragments thought to be caused by dimerization of receptor conformation. Therefore these studies suggested that anti-receptor antibodies can thus act both as agonist in the dimeric state and as antagonist in the monomeric state (Mijares *et al.*, 2000). Monoclonal antibody directed against the second extracellular loop of the human 5-HT₄ receptor showed an antagonist like activity at higher concentration of 50 and 500 pM; and facilitated the agonist action at

lower concentration range of 5 pM. This study suggested that the high affinity antibody at higher concentrations favours monovalent binding resulting in antagonist like activity and which at lower concentration results in agonist action because of bivalent recognition of the receptor (Kamel *et al.*, 2005).

Polyclonal antibodies raised against synthetic peptide corresponding to the second extracellular loop of human cardiac 5-HT₄ receptor, a GPCR has been shown to exert "inverse agonist-like" (ligands with higher affinity favouring the resting conformation than the active conformation of the receptor) activities. This antibody was found to allosterically inhibit receptor activation by blocking the induction of an active conformation by the agonist. Furthermore, the antibody also recognized the denatured 5-HT₄ receptor in immunoblots (Salle *et al.*, 2001). The same antibody when analysed in cell systems expressing a larger amount of human 5-HT₄ receptors has shown "agonist-like" effects indicating that the activity of the antibody may also depend on the molecular density and/or the cellular environment of the target receptors in addition to epitopic region recognised by the antibody (Bozon *et al.*, 2002).

A monoclonal antibody mapping distinct epitopes of the CCR5 receptor have shown existence of multiple active conformation states of receptors. Amongst them, a monoclonal antibody mapping epitopes of second extracellular loop has been shown to compete for gp120 binding and promote efficient internalization of the receptor without triggering intracellular signaling (Blanpain *et al.*, 2002). A scFv fragment (single chain variable fragment), of a monoclonal antibody directed against the second extracellular loop of the β 2 adrenergic receptor recognised the receptor having shown inverse agonist like properties both *in vitro* and *in vivo*. Inverse agonism is observed when molecules specifically recognize the resting conformation of the receptor and shift the active \rightarrow resting conformational equilibrium to the right (Peter *et al.*, 2003).

On the basis of these published studies, the idea of generating antibodies to P2Y receptors (family A GPCRs), directed not only to the N-terminal region but also to transmembrane and extracellular domains involved in ligand recognition and receptor activation seems realistic. Antibodies so generated could be used to examine the localization of active receptors as well as the extent of modulation of receptor activity

by cross-talk between receptors. Therefore, antibodies to P2Y receptors could provide powerful tools to examine the duration and extent of activation of endogenous receptors as well as to screen for drugs of potential therapeutic value that act as allosteric modulators.

1.6 P2Y receptors and intracellular signaling transduction pathways

The P2Y receptor subtypes can be classified in two principal signaling transduction pathways. The receptors subtypes belonging to the first group (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁) are coupled to phospholipase C (PLC) via G_q- proteins. Agonist stimulation of these receptor subtypes results in mobilisation of intracellular Ca²⁺ ions from internal stores via G_{q/11}-mediated phospholipase C. Upon activation, phosphatidyl inositol bisphosphate (PIP₂) is hydrolysed leading to the generation of inositol triphosphate (Ins 1,4,5), which in turn releases calcium from intracellular stores (Lytton *et al.*, 1991). In addition, P2Y₁₁ is also coupled to adenylyl cyclase via G_o through UTP stimulation and via G_q and G_s through ATP stimulation (Morse *et al.*, 2001; White *et al.*, 2003). Upon activation, phosphatidyl inositol bisphosphate (PIP₂) is hydrolysed leading to the generation of inositol triphosphate (Ins 1,4,5), which in turn releases calcium from intracellular stores (Lytton *et al.*, 1991). The second group which consists of P2Y₁₂, P2Y₁₃ and P2Y₁₄ all couple to the inhibition of adenylyl cyclase through G_i- proteins which results in a decrease in intracellular cAMP (Chambers *et al.*, 2000a; Communi *et al.*, 2001a; Hollopeter *et al.*, 2001; Abbracchio *et al.*, 2003).

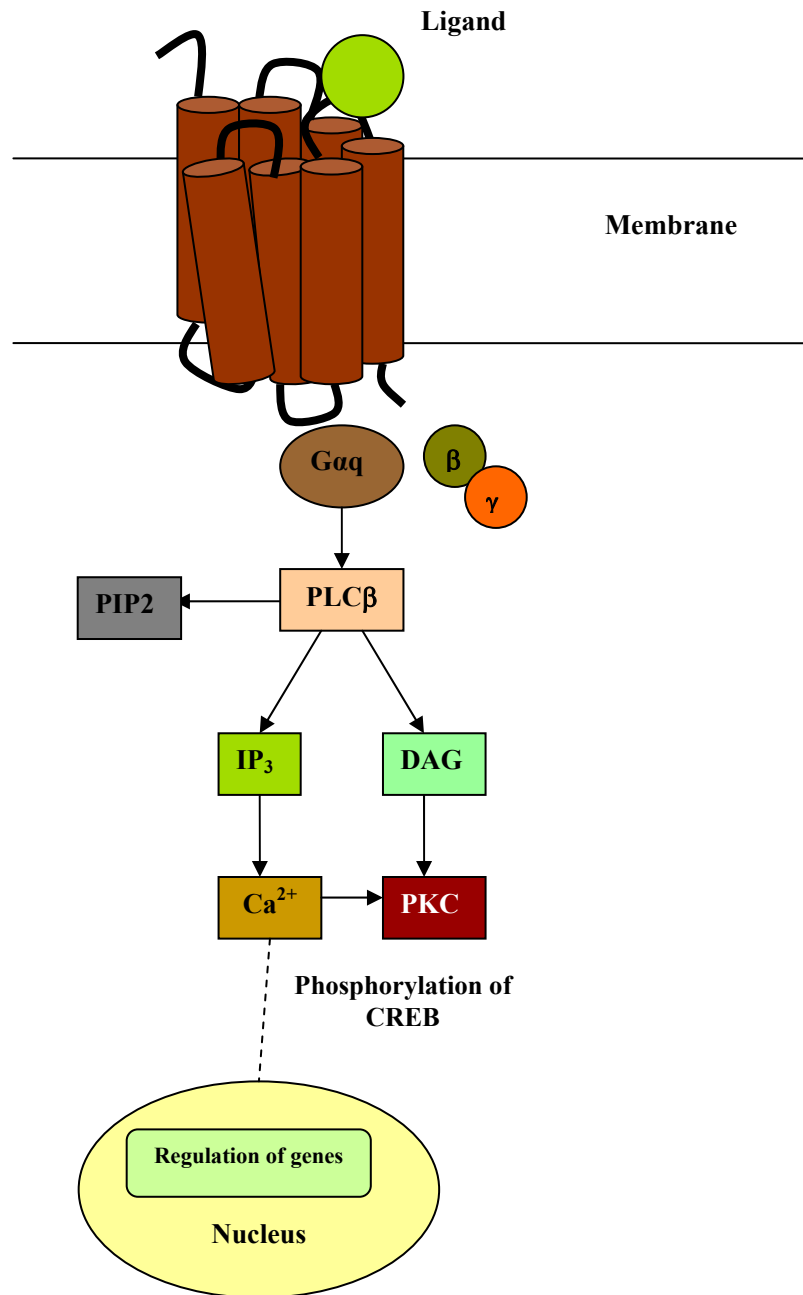


Figure 1.1 IP₃ signal transduction pathway: Agonist stimulation of P2Y receptors leads to activation of heterotrimeric G proteins and their dissociation into α and βγ subunits. The activation of P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors coupled to G_{q/11}-mediated phospholipase C cause the hydrolysis of the phosphatidyl inositol bisphosphate (PIP₂) leading to the generation of inositol triphosphate (IP₃) and DAG (Diacylglycerol) to cause the mobilization of intracellular Ca²⁺.

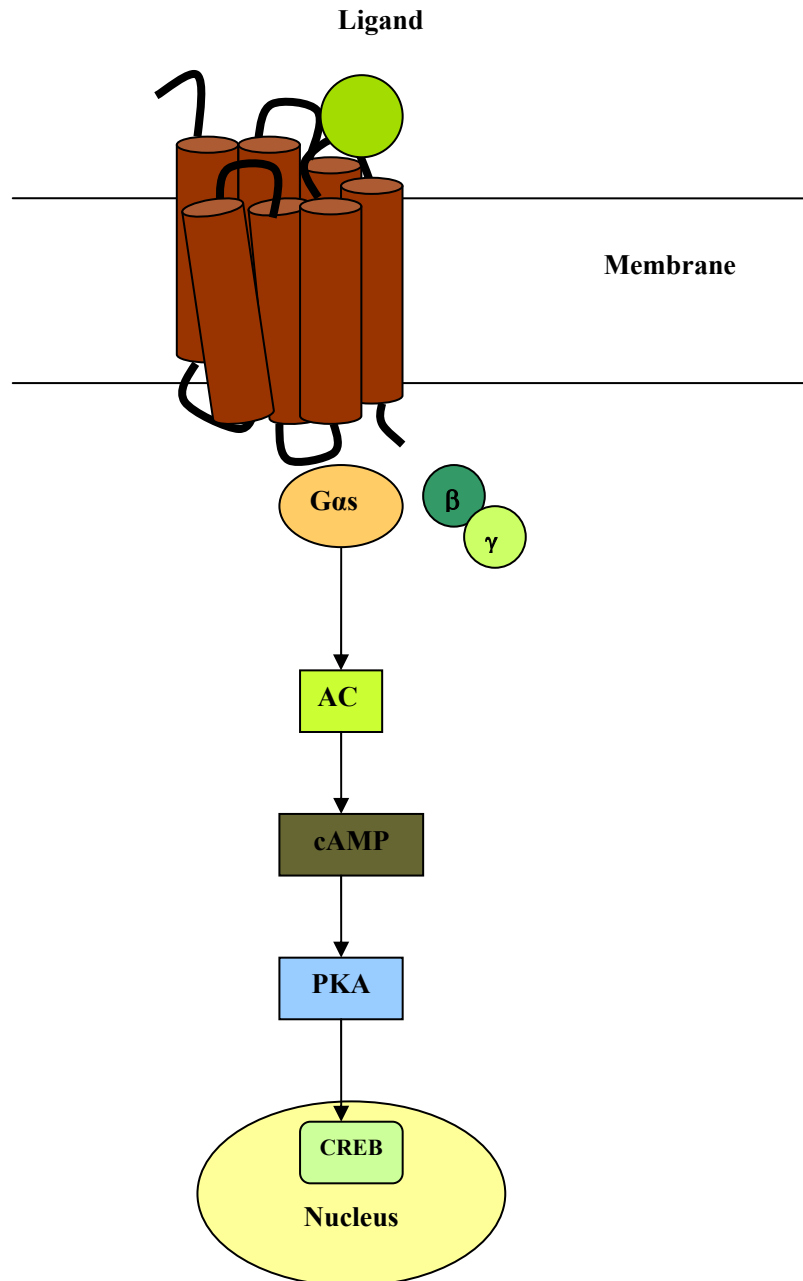


Figure 1.2 cAMP signal transduction pathway: Agonist stimulation of P2Y receptors leads to activation of heterotrimeric G proteins and their dissociation into α and $\beta\gamma$ subunits. The P2Y₁₁ receptor activates both phospholipase C and adenylyl cyclase. The activation of the P2Y₁₁ receptors coupled to G_s stimulate adenylyl cyclase leading to a rise in cAMP whereas P2Y₁₂ and P2Y₁₃ receptors are coupled solely to G_i and inhibition of adenylyl cyclase.

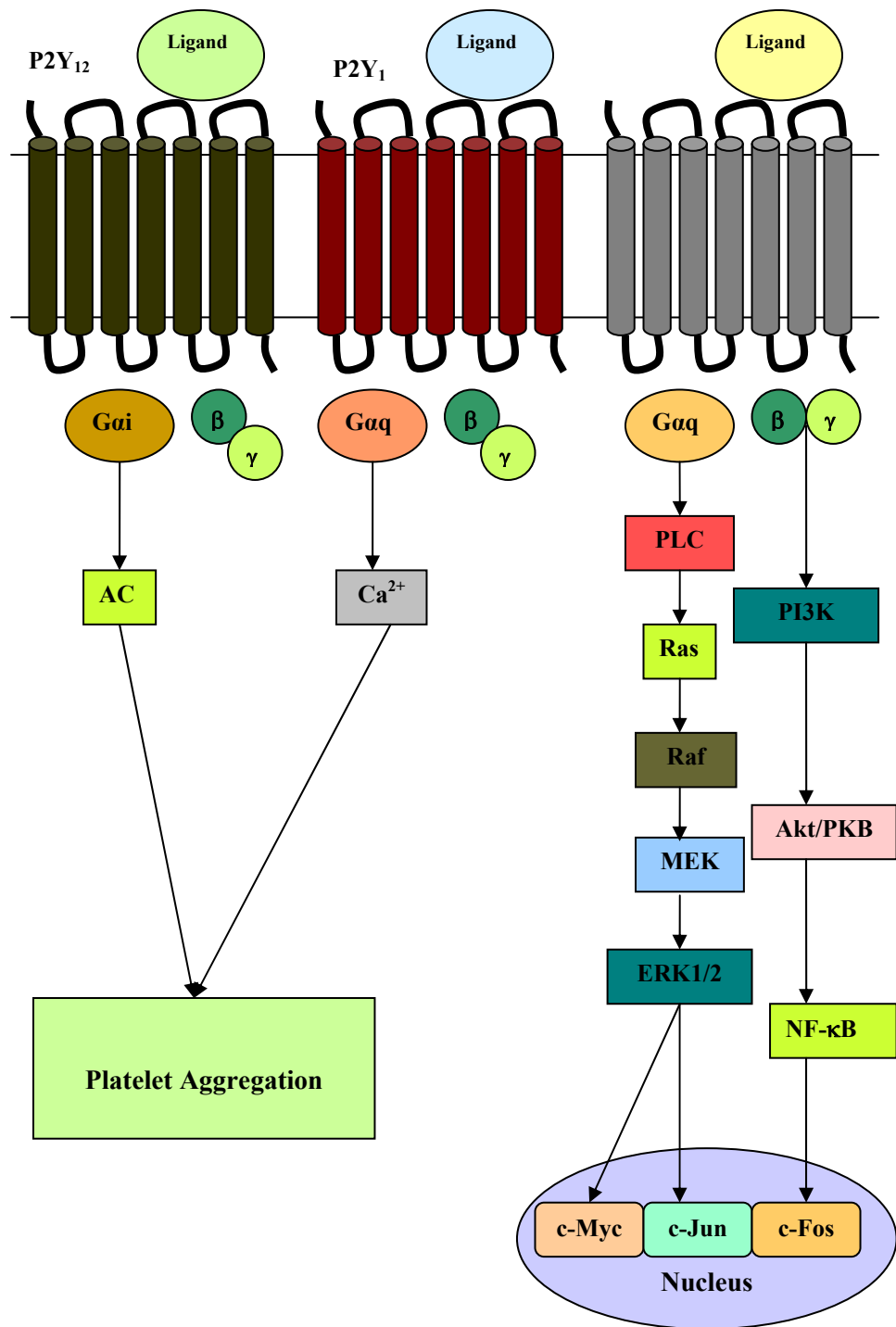


Figure 1.3 Other signal transduction pathways coupled to P2Y receptors. Concomitant activation of P2Y₁ (causes platelet shape change) and P2Y₁₂ receptor initiates signaling pathways that trigger the activation of Glycoprotein-IIb/IIIa, which then promote high-affinity binding to fibrinogen and platelet aggregation. P2Y-regulated activation of RTKs (Receptor Tyrosine Kinases) coupled to PLC activate the Ras-MEK cascade whereas P2Y receptor activation mediated dissociated G-Protein βγ subunits initiate PI3K (Phosphatidylinositol- 3-Kinase) and activate NF-KappaB (Nuclear Factor-KappaB) pathway.

The selectivity of signaling pathways between these two groups of P2Y receptors has been confirmed by various studies. The purified P2Y₁ receptor showed receptor activity when reconstituted with G α_q / $\beta_1\gamma_2$ or G α_{11} / $\beta_1\gamma_2$ proteins (its protein-signaling cohorts) (Waldo and Harden, 2004). Similarly, the purified P2Y₁₂ receptor protein coupled more effectively with G α_{i2} / $\beta_1\gamma_2$ than G α_{i1} and G α_{i3} and did not couple with G α_o or G α_q / $\beta_1\gamma_2$ proteins when reconstituted with $\beta_1\gamma_2$ and G α - subunits in lipid vesicles (Bodor *et al.*, 2003). Later studies reported that two arginine residues (R333 and R334) in the conserved BBXXB (B for basic and X for non-basic residue) region of the carboxyl (COOH) terminus of the human P2Y₁ receptor are essential for the coupling of G $_q$ -protein (Ding *et al.*, 2005). G $_i$ -coupled P2Y₁₂ and P2Y₁₃ receptors have been shown to cause an inhibition of voltage dependent N-type calcium channels in neuronal cells mediated by $\beta\gamma$ - subunit release from a heterotrimeric G-protein (Kulick and von Kugelgen, 2002; Simon *et al.*, 2002; Wirkner *et al.*, 2003). An inhibition of N-type calcium channels has also been demonstrated for the P2Y₁ receptor suggesting that this may interfere in the regulation of neurotransmitter release or neuronal excitability (Filippov *et al.*, 2000; Gerevich *et al.*, 2004).

Evidence for the activation of two distinct signaling pathways by P2Y receptors diverging at the G protein level has been provided by human erythroleukemia (HEL) cells. In these cells the activation of phospholipase C (PLC) by the P2Y₂ receptor was inhibited completely by G α_{16} antisense RNA and partially by pertussis toxin (PTX) (Baltensperger and Porzig, 1997). Furthermore, studies have suggested that P2Y₂ receptors in visceral smooth muscle and vascular smooth muscle are coupled to PLC- β_1 via G $\alpha_{q/11}$ and to PLC- β_3 via G α_{i3} / $\beta_1\gamma_2$ derived $\beta\gamma$ subunits (Murthy and Makhoulf, 1998). RGD-dependent interaction between the P2Y₂ and α_v integrins has been shown to be vital for the receptor to initiate G $_o$ -mediated signaling events leading to chemotaxis (cell migration) (Bagchi *et al.*, 2005).

P2Y₁₂ receptors that regulate platelet activation, couple to inhibition of adenylate cyclase (Hollopeter *et al.*, 2001). However, the involvement of other signaling transduction pathways including the activation of phosphatidylinositol 3-kinase (PI3-kinase) via G α_i and RhoA and Rho-kinase may also play a major role in platelet aggregation (Resendiz *et al.*, 2003; Soulet *et al.*, 2004). In addition, G α_{i3} but not G α_{i2}

deficiency in platelets reduces activation of RhoA and $G\alpha_{13}$ mediated signaling processes are required for normal hemostasis and thrombosis (Moers *et al.*, 2003). In heterologously-expressed human P2Y₁₁ receptors, UTP was found to be a Ca^{2+} -mobilizing agonist without inositol triphosphate (IP₃) or cAMP increase and stimulation with ATP resulted in elevated IP₃, cAMP and cytosolic calcium. Thus, ATP and UTP acting at the same receptor work through distinct signaling pathways suggesting the possibility of agonist-specific signaling (White *et al.*, 2003). Multiple active (ligand-specific) conformations have been proposed for the P2Y₁₃ receptor depending on variation in the concentration ratio of ADP/2MeSADP which affects its coupling to $G\alpha_{16}$, G_i and G_s (at high concentration of ADP) coupled pathways, thus resulting in distinct receptor conformations based on structure and concentration of the receptor ligand (Marteau *et al.*, 2003).

Activation of P2Y receptor subtypes is associated with stimulation of mitogen-activated protein (MAP) kinase, in particular extracellular signal related protein kinase (ERK) 1/2. However, other classes of MAP kinases (MKK3/6- p38-MAPK cascade), related adhesion focal tyrosine kinase (RAFTK), protein kinase C (PKC), calcium and phosphatidylinositol 3-kinase (PI3-K) are also involved (Soltoff, 1998; Huwiler *et al.*, 2000; Communi *et al.*, 2001a; Santiago-Perez *et al.*, 2001).

1.7 Tissue distribution of P2Y receptor subtypes

P2Y receptors are a major class of receptors ubiquitously expressed throughout the tissues of the body. The P2Y₁ receptor is expressed in placenta, prostate and brain tissue (Ayyanathan *et al.*, 1996; Moore *et al.*, 2001). P2Y₂ receptors have been detected in skeletal muscle and heart and they were present in spleen, lymphocytes, macrophages, bone marrow and lung (Parr *et al.*, 1994; Moore *et al.*, 2001). Intestine, pituitary, placenta and brain were reported to have P2Y₄ receptors (Communi *et al.*, 1995; Moore *et al.*, 2001). P2Y₆ mRNA has been detected in spleen, placenta and kidney, lung, intestine, adipose, bone and heart (Maier *et al.*, 1997; Moore *et al.*, 2001). P2Y₁₁ receptors have been detected in brain, pituitary, lymphocytes as well as spleen (Communi *et al.*, 1997; Moore *et al.*, 2001). P2Y₁₂ receptor mRNA has been shown to be expressed in spinal cord and brain (Hollopeter *et al.*, 2001; Zhang *et al.*, 2001). *In situ* hybridisation data had suggested that P2Y₁₂ is expressed in astrocytes and not neurons (Hollopeter *et al.*, 2001) and expression of this receptor has been located in substantia nigra, caudate-putamen, thalamus, and temporal cortex (Hollopeter *et al.*, 2001; Zhang *et al.*, 2001). Analysis of P2Y₁₃ distribution revealed that these receptors are expressed in brain tissue and the immune system (Zhang *et al.*, 2002). Cells of the placenta, adipose tissue, stomach and intestine express the P2Y₁₄ (UDP- glucose) receptor (Chambers *et al.*, 2000a) (See Table 1.4).

Studies have reported the location of P2Y receptor expression particularly in the epithelial cells. The polarised expression of human P2Y receptor subtypes (P2Y₁, P2Y₁₁, P2Y₁₂, and P2Y₁₄ receptors at the basolateral membrane and P2Y₂, P2Y₄, and P2Y₆ receptors at the apical membrane) has been reported in epithelial cells (Wolff *et al.*, 2005). Furthermore, the G_q-coupled P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors) were targeted in identical manner in MDCK(II), 16HBE14o (derived from bronchial epithelial cells transformed with the SV40 virus) and Caco-2 (derived from a colonic adenocarcinoma), the three epithelial cells suggesting that polarized expression of these P2Y receptor subtypes does not depend on the type of epithelial cell in which they are expressed (Wolff *et al.*, 2005). Further studies showed that the apical targeting signal in the P2Y₂ receptor located in its first extracellular loop and that four amino acids (Arg⁹⁵, Gly⁹⁶, Asp⁹⁷, and Leu¹⁰⁸) within this loop play a major role in directing apical targeting (Qi *et al.*, 2005).

Table 1.4 Tissue distribution of P2Y receptor subtypes

P2Y Receptor	Tissue distribution
P2Y ₁	Placenta, prostate and brain tissue
P2Y ₂	Skeletal muscle, heart, spleen, lymphocytes, macrophages, bone marrow and lung
P2Y ₄	Intestine, pituitary, placenta and brain
P2Y ₆	Spleen, placenta, kidney, lung, intestine, adipose, bone and heart
P2Y ₁₁	Brain, pituitary, lymphocytes and spleen
P2Y ₁₂	Astrocyte cell line, substantia nigra, caudate-putamen, thalamus, and temporal cortex
P2Y ₁₃	Brain tissue and the immune system
P2Y ₁₄	Placenta, adipose tissue, stomach and intestine

1.8 Diagnostic and therapeutic potential for P2Y receptors

1.8.1 Cystic fibrosis and chronic obstructive lung diseases

P2Y receptors mediate a broad spectrum of signaling events, such as sensory perception, cell growth, allergic responses in asthma, regulation of insulin secretion and microvascular tone (Erlinge, 1998; Hazama *et al.*, 1998; Forsythe and Ennis, 1999; Burnstock, 2000; Lewis *et al.*, 2000). Cystic fibrosis (CF) is characterized by abnormal electrolyte transport across the epithelia of the airways. In particular, there is excessive sodium absorption and deficient chloride secretion. In this condition and in chronic bronchitis the mucociliary clearance is impaired. P2Y receptors, which are found on the apical surface of airway epithelia, which are thought to contribute to the mucociliary clearance mechanism in the lung have been extensively studied (Yerxa, 1999). The natural ligand of P2Y₂ receptors, uridine triphosphate (UTP), stimulates serosal-to-mucosal chloride transport and fluid transport via a non- Cystic Fibrosis (CF) transmembrane regulator (CFTR) mechanism in isolated normal and CF epithelial cells and may be useful in the hydration of mucus and respiratory mucosa (Knowles *et al.*, 1991; Mason *et al.*, 1991; Benali *et al.*, 1994). Subsequently, it was found that UTP stimulates mucin secretion from goblet cells, increased surfactant release from type II alveolar cells, and increased the beat frequency of cilia in isolated normal and CF epithelial cells (Gobran *et al.*, 1994; Kim *et al.*, 1996; Morse *et al.*, 2001). This provides evidence for the presence of P2Y₂ receptors that can stimulate the major components of the mucociliary clearance system.

Chloride and fluid transport and mucin secretion in the airways have been shown to be regulated in fundamentally important ways by ATP and UTP acting from the lumen via apical membrane P2Y₂ purinoceptors (Boucher, 1994; Knowles *et al.*, 1995; Kim *et al.*, 1997; Ralevic and Burnstock, 1998). UTP has been shown to stimulate mucociliary clearance whereas ATP has been shown to stimulate ciliary activity directly (Wong and Yeates, 1992; Geary *et al.*, 1995; Bennett *et al.*, 1996; Sabater *et al.*, 1999). A concentration-dependent relationship between ATP and ciliary beat frequency (CBF) and a positive correlation between the ATP-induced responses in intercellular Ca²⁺ and CBF has been reported in rabbit trachea epithelial cells (Korngreen and Priel, 1996).

ATP and UTP are equipotent in their stimulatory effects on chloride and, fluid secretion and the agonists are equipotent and cross-desensitize in their activation of phospholipase C (PLC) (Brown *et al.*, 1991; Mason *et al.*, 1991; Parr *et al.*, 1994; Cressman *et al.*, 1999). ATP and UTP effect these responses selectively through P2Y₂ receptors. This was indicated in a study showing >85% of the chloride secretory response to each agonist was lost in the trachea of the P2Y₂-R (-/-) mouse (Cressman *et al.*, 1999). In addition to its actions at P2Y receptors, ATP also acts to effect responses through a family of ligand-gated P2X purinoceptors (Ralevic and Burnstock, 1998). Adenosine also stimulates chloride secretion in airways a response mediated by the P1 purinoceptor A_{2B}AR (Pratt *et al.*, 1986; Lazarowski *et al.*, 1992; Stutts *et al.*, 1994; Ralevic and Burnstock, 1998).

Northern blotting studies in cell lines derived from the human lung revealed the co-expression of P2Y₂, and P2Y₆ mRNA in the epithelial cell lines derived from the airway epithelium and type II alveolar epithelial cells (Communi *et al.*, 1999a). mRNA for P2Y₄ receptors was detected in epithelial cell lines derived from the airway epithelium, submucosal cells and airway smooth muscle cells and P2Y₁ mRNA was detected in serous cells (Communi *et al.*, 1999a). Furthermore, the inositol trisphosphates assays have identified responses typical of the P2Y₂ receptors in the airway epithelial cell lines and P2Y₄ receptors in submucosal cells (Communi *et al.*, 1999a).

The Ca²⁺-activated chloride (Cl⁻) channels (CaCC) expressed in human airway epithelia are activated by extracellular nucleotides (ATP and UTP). These Cl⁻ channels are up-regulated in the airways of cystic fibrosis (CF) patients. Activation of the cAMP pathway has been shown to increase CaCC-mediated secretion in CF. Moreover, the activation of Ca²⁺-dependent basolateral K⁺ channels has also been shown to improve CaCC mediated Cl⁻ secretion. The same study showed that the co-activation of cAMP- and Ca²⁺-dependent basolateral K⁺ channels (hKvLQT1 and hSK4) participate in CaCC-mediated Cl⁻ secretion and cystic fibrosis transmembrane conductance regulator (CFTR) in human airway epithelia. Therefore, both K⁺ channels form novel targets to improve mucociliary clearance in the CF airway disease (Mall *et al.*, 2003). Furthermore, P2Y₂ receptor agonists have shown to mediate Cl⁻ secretory response through Ca²⁺-dependent stimulation of CaCC, and PKC-dependent stimulation of CFTR (Paradiso *et al.*, 2000). Therefore, the impaired chloride transport in airway disease

caused by dysfunctional CFTR can be overcome by activating alternative (CaCC activated) chloride channels.

Recent studies have attempted to identify the cause responsible for the termination of aerosolized nucleotide-mediated mucociliary clearance (MCC). The rapid elimination of P2 receptor agonists from human airway epithelial surfaces by epithelial ectonucleotidases with an enhanced efficiency in chronic inflammatory lung diseases, including CF, limits the nucleotide-mediated mucociliary clearance (MCC) capacity of these agents. The nucleotides are not cleared by permeation through the epithelial layer as human bronchial epithelial cells seem impermeable to extracellular nucleotides. Rather, the nucleotides are cleared by extracellular metabolism by cell-associated ectonucleotidases. These enzymes have been reported to show higher activities on mucosal epithelial surfaces and act with increasing efficiency in areas most susceptible to airway obstruction. The enzyme, non-specific alkaline phosphatase, is the major ectonucleotidase responsible for the termination of aerosolized nucleotide-mediated MCC in the lungs of CF patients (Picher *et al.*, 2004).

Metabolically stable dinucleotides present a promising therapeutical approach to the treatment of CF. P2Y₂ receptor agonist, Denufosol tetrasodium (INS37217) is a deoxycytidine-uridine dinucleotide, which resists metabolism by airway cells and sputum enzymes. This compound lead to an increased chloride and water secretion, increased cilia beat frequency, and increased mucin release. At micromolar concentrations in an animal model this compound produced greater peak increases in tracheal mucus velocity (TMV) with a single administration significantly enhancing mucus transport for at least 8 hours. This type of P2Y₂ agonist attempts to enhance mucociliary clearance components by activating alternative chloride channels not dependent on CFTR to provide effective treatment for CF lung disease (Yerxa *et al.*, 2002). Phase 1 and Phase 2 clinical trials for this compound have been completed where doses up to 60 mg of denufosol inhalation solution were well-tolerated in most subjects with CF (Deterding *et al.*, 2005).

UTP and ATP induced chloride secretory response was abolished in the jejunal epithelium of P2Y₄-null mice. An oral P2Y₄ receptor agonist has been suggested to stimulate the jejunal secretion of electrolytes and water as a therapeutic strategy to treat

gastrointestinal abnormalities associated with CF including meconium ileus and chronic malabsorption in newborns (Robaye *et al.*, 2003). Treatment with topical application of P2Y₂ receptor agonist has been shown to acutely stimulate MCC and enhance sputum expectoration in mild chronic bronchitics (Bennett *et al.*, 2001; Donnelly and Rogers, 2003). The involvement of P2Y receptor in cystic fibrosis and chronic obstructive lung disease make them the ideal targets to devise novel therapies not only to improve the health status but also to modify the natural course of the disease.

1.8.2 P2Y receptors and cancer

The growth inhibition and programmed cell death (apoptosis) observed in various tumour models induced by ATP was thought to be mediated by ionotropic P2- receptors alone. Evidence for this was shown by the requirement of extracellular ATP for efficient interleukin 2-activated peripheral blood lymphoid cells (LAK) cell-mediated killing of tumour cells through the activation of P2X receptors (Correale *et al.*, 1997). However, growing evidence suggests that by altering intracellular calcium concentrations, metabotropic P2Y-receptors may be involved in both growth inhibition and apoptosis. Earlier reports show that ATP and hydrolysis-resistant ATP analogues induced greater than 90% inhibition of the growth of human androgen-independent prostate carcinoma cell lines (Fang *et al.*, 1992). However, later studies involving prostate carcinoma cells showed variability in the activity of nucleotides in the mediation of the P2Y₂ receptor in inducing inhibition of cell growth and apoptosis. Janssens and Boeynaems (2001), reported that ATP caused the inhibition of cell growth and caused cell death either by apoptosis or by necrosis; whereas UTP failed to produce any effect on growth eventhough it was equipotent to ATP in accumulation of Ins(1, 4, 5)P₃. Furthermore, the Ins(1, 4, 5)P₃ accumulation in response to nucleotides involved the P2Y₂ receptor but the growth inhibitory effect was shown to be unrelated to Ins(1, 4, 5)P₃ or cyclic AMP (cAMP) accumulation suggesting the possible involvement of P2X receptors in inhibition of cell growth and cell death (Janssens and Boeynaems, 2001).

In studies employing a rabbit lens cell line, agonists that mobilise calcium also modulated lens cell growth, and the inactivation of the same calcium stores by agents such as thapsigargin and caffeine, inhibited growth (Duncan *et al.*, 1996). In another study, P2Y₂ receptor agonists induced a time-dependent increase in apoptosis and dose-

dependent inhibition of cell proliferation in two colorectal carcinoma cell lines (Hopfner *et al.*, 2001). Reports have suggested that ATP is released from malignant cells in response to chemo or radiotherapy. Extracellular ATP is known to inhibit growth of Ehrlich ascites tumour cells, colon cancer, ovarian cancer, endometrial cancer, breast cancer and fibroblast cell lines (Estrela *et al.*, 1995; Correale *et al.*, 1997; Hopfner *et al.*, 1998; Katzur *et al.*, 1999; Li *et al.*, 2000; Schultze-Mosgau *et al.*, 2000). ATP is thought to inhibit proliferation and induce anti-apoptotic effects by emptying the calcium pool in human breast tumor cell lines, which in turn causes cell growth inhibition and induction of apoptosis (Vandewalle *et al.*, 1994). It has also been shown that the ovarian epithelial cell lines which express functional P2Y₂ receptors inhibit cell proliferation when activated by extracellular ATP (Schultze-Mosgau *et al.*, 2000).

In endometrial carcinoma cell lines, activation of P2Y₂ receptors by a slowly degradable ATP analogue, ATP γ S, was associated with a significant suppression of cell proliferation without affecting cellular apoptosis (Katzur *et al.*, 1999). The antiproliferative and apoptotic effects of P2Y₂ receptor stimulation by nucleotides have also been reported in the human colorectal cancer cell line (Hopfner *et al.*, 1998). However, the combined antiproliferative effects of P2Y₂ receptors and the apoptosis inducing effects of nucleotides through P2Y receptors has been shown in relatively few cell lines (Vandewalle *et al.*, 1994; Hopfner *et al.*, 2001; Maaser *et al.*, 2002). The stimulation of P2Y₂ receptors by ATP in oesophageal cancer cells leads to cell cycle arrest and apoptosis (Vandewalle *et al.*, 1994; Hopfner *et al.*, 2001; Maaser *et al.*, 2002). These cells responded to both purine and pyrimidine nucleotides and the increase of [Ca²⁺]_i was shown to be PLC-dependent, indicating that the induction of [Ca²⁺]_i was mediated by G_{q/11}-coupled and PLC-linked metabotropic P2Y₂ receptors (Maaser *et al.*, 2002). The ionotropic P2X receptor subtypes P2X₁, and P2_Z/P2X₇ implicated in apoptosis induced by extracellular ATP were, however, not found to be functionally expressed in both primary oesophageal cancer cells and oesophageal cancer cell lines (Chvatchko *et al.*, 1996; Ferrari *et al.*, 1999; Maaser *et al.*, 2002).

ATP has also been studied as an anti-cachexia and antiproliferative agent in advanced lung cancer. ATP was shown to inhibit loss of skeletal muscle mass, muscle strength and a temporary dose-dependent increase in whole-body glucose turnover during high

doses of ATP infusion in patients with advanced lung cancer (Agteresch *et al.*, 1999; Agteresch *et al.*, 2000a; Agteresch *et al.*, 2000b). However, glucose turnover is inhibited with long term exposure to ATP (Agteresch *et al.*, 2000b). It is proposed that the cytotoxic cell lysis effect of CTL (Cytotoxic T lymphocytes) and LAK (Lymphokine-activated killer cells) in immune defence against tumour cells and virus infected cells could be mediated by ATP and that LAK and CTL cells themselves act as a putative source of ATP (Di Virgilio *et al.*, 1990). Surprisingly these cells themselves were resistant to the lytic effects of ATP (Di Virgilio *et al.*, 1989; Zanovello *et al.*, 1990). This was attributed to the possibility of the absence of purinergic receptors and presence of ectonucleotidases in these cells (Correale *et al.*, 1995).

In contrast to these inhibitory effects, ATP has also been implicated in proliferation of a breast cancer cell line through elevation of intracellular calcium $[Ca^{2+}]_i$ (Dixon *et al.*, 1997). At micromolar concentrations ATP promoted a biphasic rise in $[Ca^{2+}]_i$ and stimulated growth of the human ovarian cancer cell line (Popper and Batra, 1993). A similar pattern of biphasic increase in $[Ca^{2+}]_i$ was also observed following stimulation with micromolar concentrations of ATP in another human ovarian cancer cell line. Here the pattern was a rapid rise to a peak level followed by a smaller but more sustained phase. ATP induced a stimulatory effect on cell proliferation at micromolar concentration and at higher concentration (100 μ M-1mM) significantly decreased cellular proliferation (Batra and Fadeel, 1994).

Cell proliferation of many other tissues stimulated by nucleotides through the activation of P2Y receptors-mainly P2Y₂- has been reported. Recent studies have reported that agonist-induced binding of Src to two Src homology 3 (SH3) binding sites (PXXP motifs) in carboxyl-terminal tail of the human P2Y₂ nucleotide receptors transactivate epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor in 1321N1 astrocytoma cells (Liu *et al.*, 2004b). Agonist induced P2Y₂ receptor activation through the mediation of PKC, rise of intracellular Ca^{2+} and tyrosine kinase pathway has been associated with cell proliferation. Here, P2Y₂ receptor agonist increased mitogen-activated protein kinase (MAPK) activation through the activation of Ras/Raf/MEK/MAPK pathway in the proliferation of C(6) glioma cells (Tu *et al.*, 2000). P2Y₁ receptors were found in keratinocytes of human foetal epidermis positive

for PCNA (proliferating cell nuclear antigen) and Ki-67, suggesting a role in proliferation (Greig *et al.*, 2003).

ATP γ S (ATP gamma S) and UTP treatment of MCF-7 cells activated transcription of the immediate early gene *c-fos* leading to elevated intracellular calcium levels and increased proliferation (See Figure 1.3) (Wagstaff *et al.*, 2000). Furthermore, ATP γ S and epidermal growth factor (EGF) together synergistically activated *c-fos* involving the ERK cascade with notably increased levels of ERK, CREB and EGF receptor phosphorylation, as well as hyperphosphorylation of ternary complex factor (Wagstaff *et al.*, 2000). The studies also reported that the activation of P2Y₂ receptor by extracellular ATP and UTP brings about PI3K (phosphoinositide 3-kinases) and downstream PKC- epsilon and ERK1/2 activation to induce transcription of *c-Fos* protein and cell proliferation in HeLa cells (Muscella *et al.*, 2003).

However, this is not an exclusive signaling transduction pathway associated with P2Y receptor regulated proliferation. Proliferative activity of extracellular nucleotides (ATP/UTP/UDP) on human lung epithelial cell line, A549, has been attributed to the activation of the P2Y₂ and P2Y₆ receptors (Schafer *et al.*, 2003). Moreover, the P2Y₂ receptor mediated proliferative activity downstream of phospholipase C has been shown to be dependent on Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and nuclear factor-kappaB (NF- κ B). Furthermore, inflammatory cytokines IL-1 β (interleukin-1beta) and IL-6 (interleukin-6) attenuated UTP but not ATP stimulated cell proliferation (Schafer *et al.*, 2003). The P2Y₆ agonist induced cell proliferation in HeLa cells activated multiple signalling pathways where conventional and atypical PKCs were responsible for the induction of *c-Fos* and ERK was responsible for cell proliferation which was dependent on both novel and atypical PKCs and PI3K activities (Muscella *et al.*, 2004).

In addition to acting like proliferative agents, P2Y receptor agonists have promoted tumour aggression by cell invasion. P2Y receptors and their down-stream ERK1/2 and p38 protein kinases have been shown to enhance prostate cancer cell invasion in two human prostate carcinoma cell lines (Chen *et al.*, 2004). Furthermore, extracellular nucleotides have been reported to exert differential effects on anticancer drugs. A study

reported that ATP antagonized the antiproliferative effect of paclitaxel and etoposide, the cytostatic drugs which inhibit cell cycle progression and are used in chemotherapeutic regimens in lung cancer treatment. Whereas ATP enhanced the activity of cisplatin, also a cytostatic drug, thus suggesting the differential control of proliferation of lung epithelial tumor cells by extracellular nucleotides (Schafer *et al.*, 2003). Extracellular nucleotides acting on P2Y receptors play a complex role in regulating cell growth, they aid growth inhibition and apoptosis and conversely they act as proliferative agents to promote tumour progression. A comprehensive understanding of distinct signaling pathways employed by the P2Y receptors in promoting apoptosis and cell proliferation in different tissue types would make them ideal targets for therapeutic intervention.

1.8.3 P2Y receptors and the vascular system

Vascular endothelial cells have been shown to release ATP, an endogenous P2Y receptor agonist not only in response to numerous stimuli, such as shear stress and vasoactive agonists but also in response purine nucleotide analogues (Yang *et al.*, 1994). The P2Y receptor mediated actions of ATP on the endothelium in coronary vessels of guinea pigs resulted in the Nucleotide Axis Hypothesis (Buxton *et al.*, 2001). According to this hypothesis, ATP stimulates the release of vasoactive mediators from endothelium, including ATP which in turn propagates the actions of ATP in blood vessels (Buxton *et al.*, 2001). P2Y receptors in endothelial cells are the mediators to act on contraction and relaxation of the glomeruli (Jankowski *et al.*, 2001a). The receptor ligand, in the form of extracellular ATP was stated to be involved in the activation of P2Y-receptors in regulating the volume of renal glomeruli and influencing glomerular filtration rate of rats. ATP-dependent glomerular relaxation in the initial phase and glomerular contraction caused by either ATP itself or adenosine formed from ATP hydrolysis in the maintenance phase was the likely mechanisms of this action (Jankowski *et al.*, 2001b). P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors have been shown to be expressed in human umbilical vein endothelial cells (Jin *et al.*, 1998b; Wang *et al.*, 2002).

Stimulation of endothelial P2Y₂ receptors in rat-isolated mesenteric artery by P2Y₂ agonist ATPgammaS (ATP γ S) showed potentiation of relaxation and hyperpolarization

(Mistry *et al.*, 2003). The relaxation of mesenteries by ATP and analogues was mediated by endothelial P2Y₁ and P2Y₂ receptors coupled to the NO/cGMP (nitric oxide and guanosine 3',5' cyclic monophosphate) pathway (Buvinic *et al.*, 2002). Only the presence of ATP and UTP facilitated a prolonged and stable flow-induced vasodilatation in rat small mesenteric arteries suggesting the physiological importance of nucleotides released by endothelial cells during intraluminal flow in blood vessels (Liu *et al.*, 2004a). The activation of endothelial P2 receptors in the cerebral circulation are known to dilate the vessel through releasing NO, prostanoid, and/or EDHF in the vasculature with the variation of these relaxing factors depending on species, size, and location of vessels (Ralevic and Burnstock, 1998). The vasodilation induced by P2Y₁ agonist was nitric oxide (NO) dependent and that produced by P2Y₂ agonist was endothelium-derived hyperpolarizing factor (EDHF) and NO-dependent in rat middle cerebral arteries (Marrelli, 2001; Wang *et al.*, 2002). However, in rat intracerebral arterioles the P2Y₁ receptor activation caused the dilation via both NO and potassium channels (probably EDHF), whereas the P2Y₂ receptor activation dilated the cerebral arteriole via potassium channel activation independently of NO (Horiuchi *et al.*, 2003).

In humans, the P2Y₁ receptor selective agonist 2-MeSADP reported to induce potent dilation of left internal mammary artery (LIMA) obtained from patients undergoing coronary artery bypass surgery. The vasodilatation was higher than that observed for the P2Y₂ and P2Y₄ agonist UTP γ S and selective P2Y₆ agonist UDP γ S and mediated through release of EDHF, NO and prostaglandins (Wihlborg *et al.*, 2003). The pathological condition of ischemia-reperfusion (I/R) – worsening of a hypoxic injury after reperfusion of ischemic tissues- attenuated the dilation induced by P2Y₁-selective agonist 2MeS-ATP in the rat middle cerebral arteries (MCAs). However, the dilations to the P2Y₂-selective agonist UTP were potentiated due to the upregulation of the EDHF mechanism which relaxes the vessel by hyperpolarizing the vascular smooth muscle (Marrelli *et al.*, 1999). In guinea-pig thoracic aorta (endothelial cells) UTP stimulated a robust P2Y₄ mediated vasodilation (Kaiser and Buxton, 2002).

P2Y receptor stimulation on smooth muscle cells has been shown to cause vasoconstriction. ADP acting on P2Y₁₂ receptors has been reported not only important for platelet activation but also found to stimulate vasoconstriction in internal mammary

artery. Drugs with P2Y₁₂ receptor antagonistic effects affecting both platelets and vascular smooth muscle cells could be beneficial for prevention of both thrombosis and vasospasm (Wihlborg *et al.*, 2004). The stable nucleotide uridine adenosine tetraphosphate (Up(4)A) obtained from mechanically stimulated human endothelial cells was reported to be the cause of vasoconstriction mediated through P2 receptors in an isolated perfused rat kidney (Jankowski *et al.*, 2005). P2Y receptor mediated vasoconstriction have also been reported from smooth muscle cells of rat pial (cerebral) arterioles suggesting that nucleotides circulating in the blood stream probably act through vasoconstrictor P2X and P2Y receptors in case of endothelial dysfunction (Lewis *et al.*, 2000). In rat cerebral microvessels, UTP stimulation of two different classes of P2Y receptors resulted in competition between endothelial and smooth muscle receptor stimulation. UTP-induced constriction was mediated via P2Y₄ receptors in the smooth muscle and dilation mediated possibly by endothelial P2Y₂ receptor (Horiuchi *et al.*, 2001). P2Y₆ mediated vasoconstriction has been observed in endothelium-denuded human cerebral arteries in response to UDPβS (Malmsjo *et al.*, 2003b). The potent contractile effects mediated by P2Y₆ receptors have also been reported in rat basilar artery smooth muscle cells in response to stable nucleotide analogue, uridine 5'-O-thiodiphosphate (UDPβS) (Malmsjo *et al.*, 2003a).

Rho kinase signaling reported to play an important role in eliciting vasoconstriction in rat cerebral arteries induced by pyrimidine nucleotides (Luykenaar *et al.*, 2004). The vasoconstriction of rat afferent arterioles mediated by activation of P2Y receptor was showed to be largely through L-type calcium channel-independent mechanisms (Inscho and Cook, 2002). Later studies in vascular smooth muscle cells of rat aorta revealed that the responses generated by UTP and to ATP were predominantly mediated by P2Y₂ receptor (Kumari *et al.*, 2003). In endothelium-denuded human coronary arteries, extracellular nucleotides elicited contraction was primarily by activation of P2Y₂ receptors (Malmsjo *et al.*, 2000). The strong vasoconstriction elicited by the selective P2Y₆ receptor agonist, UDPβS (uridine 5'-O-thiodiphosphate) in saphenous vein (smooth muscle cells) suggested a therapeutic role for P2Y₆ antagonists in preventing vasospasm and restenosis during and after revascularization surgery (Borna *et al.*, 2003). Extracellular nucleotides acting on the P2Y receptors on the smooth

muscle cells also induced vasoconstriction in canine and rat coronary vessels (Matsumoto *et al.*, 1997; Welsh and Brayden, 2001).

Besides vasoconstriction, the stimulation of P2Y receptors in smooth muscle cells regulates development of inflammatory vascular disease and cell proliferation. P2Y₂ and P2Y₆ agonists have shown a concentration-dependent chemotactic (cell migration) effect on cultured rat aortic smooth muscle cells (SMCs) via the action of osteopontin involving Rho and mitogen-activated protein (MAP) kinase pathways (Chaulet *et al.*, 2001; Pillois *et al.*, 2002). ATP-stimulated coronary artery smooth muscle cells (CASMC) proliferation shown to be dependent on independent activation of both the ERK and PI3K signaling pathways (Wilden *et al.*, 1998). P2Y₂ receptor agonists were involved in monocyte recruitment by expression of vascular cell adhesion molecule-1 (VCAM-1) in human coronary artery endothelial (HCAEC) cells (Seye *et al.*, 2003). In rabbit carotid arteries P2Y₂ receptors promoted the development of intimal hyperplasia associated with atherosclerosis and restenosis (Seye *et al.*, 2002). P2Y₂ receptor was found to be up-regulated in porcine Helloof stented coronary arteries to mediate the mitogenic effects of nucleotides (Shen *et al.*, 2004).

These studies suggest the importance of P2Y receptors as a therapeutic target by designing drugs to modulate their function to attenuate disease process. To that end, P2Y₆ receptor has been suggested as a suitable target for the treatment of cerebral vasospasm (Malmsjo *et al.*, 2003b). Similarly, targeting P2Y₂ receptor with specific antagonists has been suggested in the treatment of coronary vasospastic disorders such as angina pectoris (Malmsjo *et al.*, 2000).

P2Y₁, P2Y₂, P2Y₆, and P2Y₁₁ receptor mRNA transcripts have been detected in human myocardium in both atria and ventricles (Hou *et al.*, 1999). P2Y₂ receptors, in particular, have been found to be up-regulated in CHF (congestive heart failure) heart (Hou *et al.*, 1999). P2Y₂ and P2Y₁₁ receptors are expressed in Human coronary artery endothelial (HCAEC) cells (Moore *et al.*, 2001). In blood, P2Y₁, P2Y₂, P2Y₆ and P2Y₁₁ were reported to be expressed by monocytes, B lymphocytes (Di Virgilio *et al.*, 2001). Platelets co-express P2Y₁, P2Y₁₂ and P2X₁ receptors (Hollopeter *et al.*, 2001). P2Y₁ and P2Y₁₂ receptors are differentially involved in generation of thrombin which in turn activates platelets (Leon *et al.*, 2003). Ticlopidine and Clopidogrel which irreversibly

inhibit platelet P2Y₁₂ receptor function have been used for the prevention of secondary events in acute cardiovascular disease (Quinn and Fitzgerald, 1999; Bhatt and Topol, 2003). Thus expression of P2Y receptors in heart, blood and blood vessels are involved in regulation of physiological process, therefore, may influence the disease process.

1.8.4 P2Y receptors and diabetes

ATP and its analogues have been shown to increase glucose-induced insulin secretion through activation of P2Y receptors present on isolated pancreatic β -cells of rats (Bertrand *et al.*, 1987). ADP β S was reported to be a potent insulin inducing agent *in vivo* in rats and dogs dependent upon the nutritional status of the animals and the plasma level of glucose. In addition, both ADP β S and ATP α S have been shown to amplify glucose-induced insulin secretion by activating beta-cell adenylyl cyclase and the subsequent cAMP/protein kinase A signaling pathway in rat pancreatic islets and in the INS-1 secreting cell line (Chevassus *et al.*, 2002). Furthermore, ADP β S improved glucose tolerance and was effective on oral administration signifying the therapeutic importance of the P2Y receptor expressed in the beta cells as a target for antidiabetic drugs (Hillaire-Buys *et al.*, 1993). Similarly, glucose dependent insulin release stimulated by ADP β S is also reported in human pancreatic islets (Fernandez-Alvarez *et al.*, 2001).

In the absence of extracellular Ca²⁺, ATP was reported to stimulate insulin as an intracellular Ca²⁺ mobilisation agent involving inositol 1,4,5-trisphosphate (IP₃) (Arkhammar *et al.*, 1990). Extracellular ATP was also shown to raise the cytoplasmic free Ca²⁺ concentration in human pancreatic islets and isolated human insulin-secreting cells (Kindmark *et al.*, 1991). Recent studies in rat isolated pancreas has ruled out the direct inhibitory effect on ATP-activated K⁺ (KATP) channels in the P2Y receptor activated insulin secretion caused by beta-cell metabolism and a rise in intracellular calcium (Farret *et al.*, 2004).

Defective P2Y (P2Y₂ or P2Y₄) receptor function causing reduced uptake of glucose by GLUT1 (Glucose transporter) has been proposed in type II diabetics. A high level of extracellular ATP is thought to cause desensitisation of P2Y receptors and subsequent blocking of GLUT1 in this condition leading to hyperglycaemia (Solini *et al.*, 2003). The loss of responsiveness of platelets in patients with type 2 diabetes mellitus (DM2) to insulin and increased signaling through P2Y₁₂ has been credited to the hyperactivity of platelets observed in patients with DM2 (Ferreira *et al.*, 2006).

1.8.5 P2Y receptors and hepatocytes

In rat hepatocytes, increases in cytosolic free calcium concentration [Ca²⁺]_c in response to extracellular nucleotides acting through the phosphoinositide signaling pathway were reported to mediate by P2Y₁ and P2Y₂ receptors (Dixon, 2000; Dixon *et al.*, 2000). Later studies in hepatocytes revealed that stimulation with ADP caused an increase in inositol (poly) phosphates following its conversion to ATP suggesting the expression of P2Y₂ receptor. However, the activation of P2Y₁ did not appear to be coupled to inositol 1,4,5-trisphosphate production (Dixon *et al.*, 2003).

The stimulation of glycogen phosphorylase in freshly isolated rat hepatocytes by 2-MeSADP has been attributed to the action of P2Y₁ receptors coupled to raised [Ca²⁺]_c, and by inhibiting cyclic AMP levels by an unknown G_i-coupled receptor subtype, other than P2Y₁, P2Y₁₂, or P2Y₁₃ receptors (Dixon *et al.*, 2004). Recent studies, however, have reported an absence of responses mediated by P2Y₁ receptors in rat hepatocytes. Furthermore, in human hepatocytes P2Y receptors control both glycogen metabolism and proliferation-associated responses such as increased [Ca²⁺]_c and mitogen-activated protein kinase cascades through P2Y₂ receptors (Dixon *et al.*, 2005). These studies predict an important role for P2Y receptors in regulating hepatocytes and their functions including the release of glucose from glycogen stores into the blood stream controlled by glycogen phosphorylase enzyme.

1.8.6 P2Y receptors and dry eye syndrome

The identification of functional P2Y₂ nucleotide receptors that govern mucin secretion in rabbit and human conjunctival cells suggested these receptors might be useful

pharmacological targets for therapeutic modulation of tear film mucins in dry-eye disorders and/or corneal wound healing (Jumblatt and Jumblatt, 1998). The involvement of nucleotides in stimulating Cl⁻ (chloride) secretion by activating P2Y₂ and/or the P2Y₄ receptor has been documented in the pigmented rabbit conjunctiva (Hosoya *et al.*, 1999; Li *et al.*, 2000).

As a potential therapeutic agent in the treatment of dry eye, the P2Y₂ agonist INS365 was found to increase tear fluid secretion, corneal epithelial resistance, and release of glycoprotein-containing moieties from goblet cells as a mark of improved surface health in a rat dry eye model (Fujihara *et al.*, 2001). INS365 ophthalmic solution was well-tolerated when administered as eye drops in normal human subjects (Mundasad *et al.*, 2001). Diquafosol (INS365), which is a stable derivative of UTP, was found to stimulate chloride (Cl⁻) secretion and increase tear production through stimulation of P2Y₂ receptors in the rabbit conjunctiva (Murakami *et al.*, 2004). INS365 was well tolerated and found to reduce corneal staining and relieve certain patient symptoms in a double-blind, placebo-controlled safety and efficacy trial (Tauber *et al.*, 2004). This ophthalmic formulation has been undergoing an additional confirmatory clinical phase III as a 1% and 2% solution and expected to be approved soon (Nichols *et al.*, 2004). Therefore, synthesis of better nucleotide analogues which can manipulate P2Y receptor signaling in the eye could help in understanding the ocular physiology and in developing better therapeutic strategy in dry eye related disorders.

1.8.7 P2Y receptors and host defence

Studies have shown that stimulation with ATP and UTP enhanced anti-IgE-induced histamine release in human lung mast cells (HLMC) implicating the involvement of P2Y₂ receptors in human allergic and asthmatic reactions (Schulman *et al.*, 1999). The expression of the cysteinyl leukotriene (CysLT₁) receptor in human cord-blood-derived mast cells (hMCs) with dual ligand specificity to inflammation-derived cys-LTs and P2Y receptor ligand UDP is considered to provide link between the inflammatory and neurogenic elements of bronchial asthma (Mellor *et al.*, 2001). Acting on the P2Y₂ receptor extracellular nucleotides induce stimulation and activation of neutrophil and act as chemoattractants inducing chemotaxis (Verghese *et al.*, 1996). Activation of P2Y₂ subsequently generates leukotrienes like the potent proinflammatory chemokine,

LTB₄ which intensifies inflammatory response. In turn the leukotrienes act in both autocrine and paracrine manner to amplify the process of chemotaxis in PMN (polymorphonuclear neutrophils) by upregulation of Mac-1 expression (Kannan, 2003). Furthermore, in the presence of exogenous soluble fibrinogen both ATP and UTP cause primary granule release (degranulation) from human neutrophils through the P2Y₂ receptor and through activation of p38 MAPK and ERK1/2 pathway (Kannan, 2004; Meshki *et al.*, 2004).

ATP acting on P2Y₂ receptor has been reported to promote transcription of IL-6 in macrophages (Hanley *et al.*, 2004). Studies have shown that co-ordinated action of P2Y and P2X receptors can modulate macrophage production of TNF- α , IL-1 β and nitric oxide (NO) following lipopolysaccharide (LPS) exposure on macrophages (Guerra *et al.*, 2003). Studies in mouse J774 macrophages have proposed a regulatory role for UTP at inflammatory sites. These studies showed that UTP acting at the P2Y₆ receptor, the major P2Y receptors in J774 macrophages, potentiates lipopolysaccharide (LPS)-induced cyclo-oxygenase -2 (COX-2) protein expression and subsequently enhances prostaglandin E₂ (PGE₂) release. Cyclo-oxygenase (COX) is the rate-limiting enzyme in the biosynthesis of eicosanoids, which along with arachidonic acid (AA) are the early events in macrophage activation by inflammatory stimuli (Chen and Lin, 2000). Earlier studies in mouse J774 macrophages found UTP to potentiate the effect of lipopolysaccharide (LPS) on inducible nitric oxide synthase (iNOS) induction and nitric oxide (NO) production (Chen *et al.*, 1998). UTP also caused iNOS induction independent of arachidonic acid metabolites via activation of Ca²⁺/calmodulin-dependent protein kinase and NF- κ B (Chen and Lin, 2000).

The activation of P2Y₆ receptor by UDP has been shown to stimulate interleukin (IL)-8 release in human THP-1 monocytic cells (Warny *et al.*, 2001). Further studies suggested that human eosinophils stimulated with extracellular nucleotides trigger secretion of eosinophil cationic protein (ECP) by possibly stimulating a P2Y₂ receptor and that the secretion of IL-8 can be due to activation of P2X₁ and P2X₇ in addition to P2Y₆ receptors (Idzko *et al.*, 2003). Thus, P2Y receptors could play an important role in inflammation and innate immune defences and also in the modulation of eosinophil functions.

Dendritic cells (DCs) are heterogeneous antigen-presenting cells (APCs) that are specialized to activate naïve T lymphocytes and play a central role in the initiation and regulation of immune responses (Banchereau and Steinman, 1998). UDP has shown to regulate dendritic cell functions by stimulating Ca^{2+} transients, actin polymerization, and chemotaxis in immature dendritic cell (DCs). UDP also stimulated the release of the CXC-chemokine 8 (CXCL8) from mature DCs (Idzko *et al.*, 2004). UTP acting on P2Y nucleotide receptor expressed in CD11c^+ murine dendritic cells stimulated an increase in intracellular calcium and induced cytokine production (Marriott *et al.*, 1999). In immature human dendritic cells ATP was reported to induce chemotactic activity mediated by $G_{i/o}$ -protein-coupled P2Y receptors. However, functional studies in mature DCs revealed the down-regulation of $G_{i/o}$ -coupled P2Y receptor mediated chemotactic responsiveness, possibly to facilitate them to migrate to secondary lymphoid organs to establish contact with T lymphocytes and to initiate immune responses (Idzko *et al.*, 2002).

P2Y₂ receptor activation on human peripheral blood neutrophils has been shown to induce a typical chemotactic response suggesting that P2Y receptor agonists could play a regulatory role in inflammation (Verghese *et al.*, 1996; Kannan, 2003). ATP acting on the P2Y₁₁ receptor has been shown to mediate the activation, maturation and modulation of the functions of human monocyte-derived dendritic cells (Wilkin *et al.*, 2001; Marteau *et al.*, 2004). P2Y₁₁ receptor agonist synergised with other dendritic cell stimuli to modulate cytokine production to favour type 2 helper T cell (Th2) response, which play a triggering role in the activation and/or recruitment of IgE antibody-producing B cells, mast cells and eosinophils, the cell types involved in the allergic inflammation (Romagnani, 1997; Maggi, 1998; Wilkin *et al.*, 2002). In mediating chemotactic mechanism of dendritic cells, different P2Y receptor subtypes have been implicated for a diverse role. At low concentration gradient, the nucleotides stimulate the migration of dendritic cells mediated by P2Y₂ receptors. However, high concentration gradient of nucleotides formed at sites of inflammation inhibit the migration of local dendritic cells is mediated by P2Y₁₁ receptor (Schnurr *et al.*, 2003).

Human immature dendritic cells (DCs) have shown to express the UDP-Glucose receptor P2Y₁₄. UDP-glucose and other glycolytic metabolites found at high levels in

tumour cells and supernatants from necrotic cancer cells have been shown to induce maturation characteristics in immature DCs to suggest that increased levels of UDP-glucose released from necrotic tumor cells might reach a danger threshold and might initiate an immune response through P2Y₁₄ receptor mediation together with other signaling via the induction of DC maturation (Sauter *et al.*, 2000; Skelton *et al.*, 2003). Thus UDP-glucose could be acting as a tumour specific danger signal.

P2Y₂ receptor mediated inhibition of inducible NO synthase (iNOS) in rat mesangial cells lead the authors to postulate that extracellular nucleotides may regulate the generation of nitric oxide (NO) during the glomerular inflammatory response by limiting activation of iNOS to prevent excessive tissue damage (Mohaupt *et al.*, 1998). Nucleotides induced cell proliferation and functional expression of P2Y₂ receptor in human keratinocytes suggested a major role for these receptors in epidermal homeostasis and as a target for therapy of proliferative disorders of the epidermis such as psoriasis (Dixon *et al.*, 1999). Thus, P2Y receptors in immune cells exert their influence in host defence mechanism, inflammatory process and in modulating immune response.

1.8.8 P2Y receptor and bone

P2Y₁, P2Y₂, P2Y₄, P2Y₆ receptor mRNA transcripts have been detected in human bone and osteoblastic cell lines (Maier *et al.*, 1997). Functional P2Y receptors, in particular P2Y₂, have been shown to be expressed in human osteoclastoma, osteoblasts and chondrocytes (Hoebertz *et al.*, 2000). Nucleotides acting via P2Y receptors have been shown to be potent potentiators of parathyroid hormone-induced signaling and transcriptional activation in osteoblasts (Bowler *et al.*, 2001).

Extracellular nucleotides acting on different P2Y receptors have shown to increase bone resorption and inhibit bone formation. The powerful stimulatory action of ADP on osteoclast (bone-resorbing cells) formation suggested that the activity is mediated by P2Y₁ receptor, whereas UTP, through the activation of P2Y₂ receptor blocks bone formation by osteoblasts. Furthermore, ATP has been reported to simultaneously stimulate resorption and inhibit bone formation. These studies suggest a role for P2Y receptors in bone metabolism and provide novel drug targets for pathological conditions

associated with bone loss such as osteoporosis, characterized by low bone mass and disruption of bone architecture caused by increased bone resorption over bone formation (Hoebertz *et al.*, 2002; Hoebertz *et al.*, 2003).

Nucleotides acting on P2Y₂ purinoceptors are found to be responsible for oscillatory fluid flow-induced [Ca²⁺]_i mobilization in osteoblastic cells (You *et al.*, 2002). The nucleotide stimulation of P2Y₆ receptors in osteoclasts, which also express P2Y₁, P2Y₂ receptor initiated NF-kappaB signaling, thereby enhancing the survival of osteoclasts and enhancing bone resorption. P2Y₆ receptor antagonists, therefore, may be useful in inflammatory bone diseases such as rheumatoid arthritis and periodontitis, where activation of P2Y₆ receptors might enhance bone destruction (Korcok *et al.*, 2005).

1.8.9 P2Y receptors and neuroinflammation

P2Y receptor expression coupled to an increase in intracellular calcium has been reported in neurons and glia of superior cervical ganglion (SCG) implicating the P2Y receptors in the nervous system (Calvert *et al.*, 2004). Neuroprotective properties have been attributed to P2Y₂ receptors expressed in astrocytes, where activation of P2Y₂ receptors induced anti-apoptotic genes, *bcl-2* and *bcl-xl* expression in to trigger survival-signaling cascades in human astrocytic cells (Chorna *et al.*, 2004). P2Y₂ receptors also mediate the up-regulation of the expression of genes for neurotrophins, neuropeptides and growth factors and extracellular matrix proteins through the activation of the CREB/*bcl-2* pathway. In addition, the conditioned media from UTP treated 1321N1 cells promoted neurite outgrowth in PC-12 cells. Thus, P2Y₂ receptors may regulate important physiological or neuroprotective mechanisms in astrocyte signaling and nerve tissue regeneration under pathological conditions in the human brain (Chorna *et al.*, 2004).

Extracellular ATP through the mediation of P2 receptors has been reported to cause cell death of fully differentiated and mature neurons, dissociated primary cells, and organotypic cultures possibly comprising both apoptotic and necrotic features of degeneration (Volonte *et al.*, 2003). ATP analogues also resulted in an increase of inducible cyclooxygenase-2 (COX-2), a pathological feature common in a variety of neurodegenerative diseases characterized by inflammation and astrocytic activation

(Brambilla and Abbracchio, 2001). Studies in rat astrocytes implicated activation of a calcium-independent G-protein-coupled P2Y receptor linked to ERK1/2 and COX-2 pathway behind reactive gliosis (Brambilla *et al.*, 2002).

The ADP β S-induced proliferation has been inhibited by P2Y₁ receptor antibody suggesting a regulatory role for P2Y receptors in pathophysiological processes in glial cells (Franke *et al.*, 2004). P2Y₁₂ receptor mediation has been implicated in the autoinhibition of transmitter release from neuronal cells through an inhibition of voltage-gated Ca²⁺ channels (Lechner *et al.*, 2004). Therefore, selective P2Y receptor subtype antagonists that are able to permeate the blood-brain barrier could be very useful agents in preventing neurodegenerative processes (Franke and Illes, 2006). A possible involvement of P2Y receptors in generating or modulating pain perception is also being investigated in animal models (Kennedy *et al.*, 2003).

1.9 Cell types used in this study

1.9.1 1321N1

The lack of response of the wild type 1321N1 cells to extracellular nucleotides have made them useful a host cell line for purinergic receptor expression (Filtz *et al.*, 1994; Schachter *et al.*, 1996). They are reported to express a P1 receptor which mediates a relatively weak response by intracellular second messengers (Hughes and Harden, 1986). 1321N1 human astrocytoma cells have been extensively used for the recombinant expression of P2Y receptor subtypes and were used in cloning and subsequent stable expression to study the receptor pharmacology of P2Y₂, P2Y₄ and P2Y₆ receptors and other subtypes belonging to this receptor class (Parr *et al.*, 1994; Communi *et al.*, 1995; Communi *et al.*, 1996b). These cells have also been used to generate stable transfectants expressing the mutant P2Y₂ receptors in site-directed mutagenesis and also to express receptor chimera's (Erb *et al.*, 1995; Sak and Illes, 2005).

1.9.2 Human bladder cancer cell line, ECV304

ECV304 is a commercially available bladder cancer epithelial cell line that has been purchased from ECACC. Early studies indicated that ECV304 cells express P2Y₁, P2Y₂ and a third receptor responding to AMP with increases in [Ca²⁺]_i (Conant *et al.*, 1998). ECV304 has been shown to express a P2Y₂ - like receptor and therefore proves useful for the study of P2Y receptor pharmacology (Brown *et al.*, 2000). Further studies on this cell line have shown that P2Y₂ receptors through activation of PLC (phospholipase C) and mobilisation of intracellular calcium stimulates production of nitric oxide. Further studies have shown evidence for the presence of second P2Y receptor coupled to adenylate cyclase (AC) stimulation (Brown, 2001). Therefore, ECV304 cells appear to be a very useful research tool for studying P2Y receptors that activate two distinct signaling pathways.

1.9.3 EAhy926

EAhy926 is an established permanent human cell line expresses factor VIII-related antigen, a highly differentiated function of vascular endothelium. This cell line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549 (Edgell *et al.*, 1983). Studies in EAhy926 cells have reported the nucleotides stimulated activation of MAP kinase in a protein kinase C-dependent manner through interaction with a P2Y₂ receptor (Graham *et al.*, 1996a). P2Y₂ mediated desensitization of the MAP kinase signal in EAhy926 cells appear to involve multiple regulatory mechanisms involving the induction and regulation of other proteins (Graham *et al.*, 1996b). This finding was further supported by the fact that UTP stimulated inhibitory effect on tumor necrosis factor α (TNF α) - stimulated stress activated protein kinases in EAhy926 cells involved Ca²⁺ independent PKC isoforms and mediation of P2Y₂ receptor (Paul *et al.*, 2000). The expression of P2Y receptors makes this useful research tool for the study of P2Y receptor pharmacology in endothelial cells.

1.9.4 Bovine aortic endothelial cells (BAEC)

Since these cells were first cultured in 1975 (Booyse *et al.*, 1975) they have been extensively studied. The rank order of agonist potency of 2-methylthio-ATP (2MeSATP) > adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) > ADP > ATP > β,γ -imido-ATP > β,γ -methylene-ATP (β,γ -meATP) in accumulating total inositol phosphates was reported to be consistent with action at receptors of the P2Y subtype (Allsup and Boarder, 1990). On further studies it was suggested that the effect of P2Y receptor agonists 2MeSATP and UTP on the accumulation of inositol phosphates were additive, whereas the effects of ATP and either UTP or 2MeSATP were not. This together with competitive antagonism of suramin for the response to ADP β S and 2MeSATP but not the response to UTP indicated the co-existence of two separate receptors expressed. P2Y-purinceptors (responding to purines) and nucleotide receptors (responding to both purines and pyrimidines) on bovine aortic endothelial cells (Motte *et al.*, 1993). It was thought that purines such as ATP/ADP may regulate aortic endothelial cells by interacting with two phospholipase C (PLC)-linked receptors (Wilkinson *et al.*, 1993). This activation of PLC caused by these two co-existing receptors was through different

routes as it was shown that pattern of Ins (1,4,5)P₃ accumulation was dependent on the type of receptor stimulated and stimulation was differentially regulated by protein kinase C (PKC) (Purkiss *et al.*, 1994). Further, in BAE cells the agonistic activity of P2Y₁ but not P2Y₂ receptor was antagonised by Pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) (Brown *et al.*, 1995). Further studies showed that both P2Y₁ and P2Y₂ purinoceptors stimulate endothelial NOS in a manner that is dependent on PKC activity (Brown *et al.*, 1996). Investigation of increases in intracellular calcium [Ca²⁺]_i in a single BAE cell in primary culture in response to ATP, ADP, AMP, adenosine, UTP, 2-MeSATP, 2-methylthio ADP (2-MeSADP) and α,β -methylene ATP (α,β -meATP) further supported that different intracellular signalling pathways could be involved in increasing [Ca²⁺]_i (Duchene and Takeda, 1997). Recent studies have reported the accumulation of inositol phosphates by 2MeSATP, 2MeSADP and UTP suggesting the native expression of P2Y₁ and P2Y₂ receptors (Brown and Brown, 2002) which makes these cells a useful research tool for studying P2Y receptors.

1.9.5 BRIN-BD11

BRIN-BD11 cell line represents an important stable glucose-responsive insulin-secreting beta-cell line, established after electrofusion of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells (McClenaghan *et al.*, 1996). Although there are no reports of P2Y receptor studies involving BRIN-BD11 cells, these receptors have been studied in insulin secreting B cells of rat. In isolated perfused rat pancreas, ADP β S has been found to be a potent secretory agent (Hillaire-Buys *et al.*, 1994). P2Y receptors have been reported to potentiate glucose-induced insulin secretion in rat pancreatic B cells (Petit *et al.*, 1998). ADP β S have been shown to augment glucose-induced insulin secretion by activating beta-cell adenylyl cyclase and the subsequent cAMP/protein kinase A signaling pathway in rat pancreatic islets (Chevassus *et al.*, 2002). Extracellular ATP has been shown to induce intracellular calcium release by activating P2Y receptors in rat pancreatic beta-cells (Zhao *et al.*, 2003). Both P2Y₁ and P2Y₂ receptors have been shown to be present in rat pancreas (Coutinho-Silva *et al.*, 2001). Rat pancreatic beta cells have been shown to express P2Y₄ receptors (Coutinho-Silva *et al.*, 2003).

Moreover, ATP has been shown to stimulate insulin release in RINm5F cells by phospholipase C (PLC) dependent generation of inositol trisphosphate mediated Ca^{2+} mobilization and PLC- independent mechanisms (Li *et al.*, 1991; Cao *et al.*, 1997). Rat P2Y_1 receptor has been cloned from RINm5F cells (Tokuyama *et al.*, 1995). Furthermore, BRIN-BD11 cells have retained attributes of normal insulin-secreting cells including two enzymes responsible for glucose phosphorylating activity and PLC/PKC and adenylate cyclase/protein kinase A, the regulatory elements of late stages of insulin secretory pathway (McClenaghan and Flatt, 1999). However, whether the genetic modification caused by electrofusion has retained the characteristics of functional P2Y receptors as observed in rat pancreas and RINm5F cells cannot be ascertained without pharmacological evidence. Nevertheless, given the association of P2Y receptors in insulin release, BRIN-BD11 cells will be useful to study P2Y receptors.

1.10 The basis for this research project

Previous work in our lab had lead to the design and synthesis and subsequent evaluation of peptides specifically mimicking the extracellular regions of the human P2Y₂ receptor. These peptides were designed with predominantly hydrophilic residues to enable them to enter the nucleotide binding pocket. These tetradecapeptides were designed to represent and span the entire extracellular region of the human P2Y₂ receptor. Thus, the peptide RH1 was made from residues 14-27 of ECI (extracellular region 1), L245 from ECII (94-107), L246 from ECIII (174-187), RH2 from ECIII (180-193), L247 from ECIV (271-284) and a scrambled version of L247 was synthesised as a control.

Peptides were analysed in second messenger assays where they exhibited “agonist-like” properties. Moreover, peptide L247, mimicking the third extra cellular loop of the human P2Y₂ receptor was shown to be a weak agonist of IP₃ production in ECV304 cells and stimulate stimulate nitric oxide synthase activity in BAE cells. In addition, when added simultaneously with ATP, it has shown synergistic effects on inositol phosphate production and intracellular calcium release in ECV304 cells (this synergistic effect was specific to P2Y agonists with no such effects being observed upon the simultaneous addition of L247 and histamine). L247 was equipotent to 100μM ATP in activating phospholipase D in porcine aortic endothelial cells. These results suggested that L247 acts as allosteric modulator of the P2Y₂ receptor (Brown, 2001).

1.11 Aims of this project

The above results suggest that the L247 peptide, mimicking the third extracellular loop of human P2Y₂ receptor, has an effect on modulation of receptor activity when used in conjunction with endogenous ligands. This is significant in light of the lack of subtype-specific agonists and antagonists available and the problems associated with using radioligands in studying P2Y receptor pharmacology. The aims of this project are to use L247 peptide as an immunogen to generate polyclonal rabbit antibodies to the P2Y₂ receptor. Furthermore, to evaluate EAhy926 and further evaluate ECV304 cells for the functional expression of P2Y subtypes with assays for second messengers in addition to already characterized BAE cells as research tools. The aim is also to generate a RT-PCR screen for P2Y receptor subtype expression by human cells and to use this to confirm the proposed P2Y receptor expression derived from pharmacology studies. Finally, to purify and fully characterise the polyclonal antibody raised to the L247 peptide.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

i) Equipment

BioLogic HR Chromatography System	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK
BioVision Imaging Technology	BioGene Ltd., Kimbolton, UK
Block heater	Wolf Laboratories York
Centrifuges	M.S.E Mistral 2000, Fisons Instrumentation, U.K. Mikro 20, Hettich Zentrifugen, D78532 Tutlingen. MIkro 22R, Hettich Zentrifugen, D78532 Tutlingen. Spectrofuge 16M, National Labnet Co. Edison, NJ. USA International Equipment Company Needham Heights, MA, USA
Class II cabinet	Clean Air Techniek b.v., The Netherlands
CO ₂ incubator	Jensons Scientific Inc. Bridgeville, PA 15017, USA
DNA Analyzer	Applied Biosystems, USA
Genequant	Pharmacia Biotech Inc NJ, USA
Hybaid PCR Express thermocycler	Hybaid, Action Court, Middlesex, UK

ImageQuant™ TL image analysis software	GE Healthcare Life Sciences Amersham, Bucks, UK
Inverted microscope	Celti, Optical Instruments, Belgium
Labsystems Multiskan MS	Labsystems, Finland
Magnetic Stirrer Hotplate	Stuart Scientific, Beacon Road, Staffordshire, UK
Mini-PROTEAN 3 cell Electrophoresis system	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK
Mini Trans-Blot cell	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK
Monomixer	Sarstedt, Nümbrecht, Germany
PhosphorImager™ technology	GE Healthcare Life Sciences Amersham, Bucks, UK
Platform Shaker STR6	Stuart Scientific, Beacon Road, Staffordshire, UK
PowerPac 1000 Power Supply	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK
LC 494 Protein Sequencing System	Applied Biosystems, USA
Rocker	Wolf Laboratories, York, UK
Scintillation counter	Packard, Berkshire, U.K

Vortex

Sigma-Aldrich Company Ltd.,
Dorset, UK

Water baths

Grant Instruments (Cambridge)
Ltd., Cambridge UK

Well washer 4 MK 2

Labsystems, Finland

ii) Chemicals

Acetone	BDH Laboratories Supplies, Poole, England
Ammonium formate	Sigma-Aldrich Company Ltd., Dorset, UK
Ammonium sulfate	Sigma-Aldrich Company Ltd., Dorset, UK
AMV reverse transcriptase	Promega UK, Southampton, UK
AMV RT buffer	Promega UK, Southampton, UK
BSA (Bovine Serum Albumin)	Sigma-Aldrich Company Ltd., Dorset, UK
Calcium chloride	BDH Laboratories Supplies, Poole, England
Collagenase (Sigma Type IA)	Sigma-Aldrich Company Ltd., Dorset, UK
Complete, Mini Protease Inhibitor Cocktail Tablets	Roche Diagnostics GmbH, Mannheim, Germany
DAPI	Vector Laboratories, Ltd., Peterborough, England
DC Protein Assay Kit	Bio-Rad Laboratories Ltd., Hemel Hempstead, UK
Decon	BDH Laboratories Supplies, Poole, England
Diethyl Ether	BDH Laboratories Supplies, Poole, England
DMSO	Sigma-Aldrich Company Ltd., Dorset, UK

dNTP mix	Promega UK, Southampton, UK
Dowex 1X8-400	Sigma-Aldrich Company Ltd., Dorset, UK
Dulbecco's Modified Eagle's Medium high glucose	Sigma-Aldrich Company Ltd., Dorset, UK
Earles Balanced Salts solution	Sigma-Aldrich Company Ltd., Dorset, UK
ECL Plus™ Western Blotting Detection Reagent	GE Healthcare UK Ltd Amersham, Bucks, UK
Ethanol	BDH Laboratories Supplies, Poole, England
Ethidium bromide	Sigma-Aldrich Company Ltd., Dorset, UK
Ethylene glycol	BDH Laboratories Supplies, Poole, England
Fetal calf serum	PAA Laboratories Ltd, Yeovil, Somerset, UK
GenElute™ PCR Clean-Up Kit	Sigma-Aldrich Company Ltd., Dorset, UK
Gentamicin	Sigma-Aldrich Company Ltd., Dorset, UK
Glucose	BDH Laboratories Supplies, Poole, England
Hydrochloric Acid	BDH Laboratories Supplies, Poole, England
HEPES	Sigma-Aldrich Company Ltd., Dorset, UK

'Hi-Pure' Low EEO agarose gels	BioGene Ltd., Kimbolton, UK
Hyper Ladder IV – 100bp ladder	Bioline Ltd. Humber Road, London
Lithium chloride	Fluka, Neu-Ulm, Switzerland
L-glutamine	Sigma-Aldrich Company Ltd., Dorset, UK
Magnesium sulphate	BDH Laboratories Supplies, Poole, England
Medium 199 with Earle's Salts with L-Glutamine	PAA Laboratories Ltd, Somerset, UK
Methanol	BDH Laboratories Supplies, Poole, England
Minimal essential medium with D-Valine	PAA Laboratories Ltd, Somerset, UK
N-Glycosidase F Deglycosylation Kit	Roche Diagnostics GmbH, Mannheim, Germany
Nuclease-Free Water	Promega UK, Southampton, UK
Oligo(dT) ₁₅ Primer	Promega UK, Southampton, UK
Penicillin–Streptomycin solution Hybri-Max [®]	Sigma-Aldrich Company Ltd., Dorset, UK
Potassium chloride	BDH Laboratories Supplies, Poole, England
Pre-Aliquoted Reddy Mix [™] PCR Master-Mix	Abgene Limited, Epsom, UK

PROSEP-Thiosorb	Millipore (U.K.) Limited, Watford, UK
Protein standard	Sigma-Aldrich Company Ltd., Dorset, UK
ProteoExtract™ Native Membrane Protein Extraction Kit	Merck Chemicals Limited, Nottingham, UK
RNaseZap® RNase Decontamination Solution	Applied Biosystems, Warrington, UK
RNasin Ribonuclease Inhibitor	Promega UK, Southampton, UK
RPMI 1640 without L-Glutamine, with Phenol Red	PAA Laboratories Ltd, Yeovil, Somerset, UK
RQ1-Rnasin Free DNase	Promega UK, Southampton, UK
SeeBlue® Plus2 Pre-Stained Standard	Invitrogen Ltd., Paisley, UK
Sodium bicarbonate	BDH Laboratories Supplies, Poole, England
Sodium Chloride	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium phosphate monobasic dihydrate	BDH Laboratories Supplies, Poole, England
Swine Anti-Rabbit-peroxidase conjugate	Dako UK Ltd. Cambridgeshire, UK
TEMED	Sigma-Aldrich Company Ltd., Dorset, UK
TMB	Sigma-Aldrich Company Ltd., Dorset, UK
TRI reagent	Sigma-Aldrich Company Ltd., Dorset, UK

Tris-Borate-EDTA buffer	Sigma-Aldrich Company Ltd., Dorset, UK
Trichloroacetic acid (TCA)	BDH Laboratories Supplies, Poole, England
Tris/Glycine/SDS running buffer	Geneflow Ltd., Fradley Staffordshire, UK
Tris/Glycine transfer buffer	Geneflow Ltd., Fradley Staffordshire, UK
Trizma [®] hydrochloride solution	Sigma-Aldrich Company Ltd., Dorset, UK
Trypsin/EDTA	Sigma-Aldrich Company Ltd., Dorset, UK
Tween – 20	Pharmacia Biotech, Uppsala, Sweden
Ultima Flo AF scintillation fluid	PerkinElmer, Inc. Waltham, Massachusetts, USA
Urea	Sigma-Aldrich Company Ltd., Dorset, UK

iii) P2 receptor agonists

The following P2 receptor agonists were supplied by Sigma-Aldrich Company Ltd.: ADP, ADP β S, 2MeSADP, ATP, 2MeSATP, ATP γ S, BzATP, UDP, and UTP

iv) Radioisotopes

The radiochemical [^3H]-*myo*-inositol was supplied by Amersham Pharmacia Biotech UK Ltd.

v) Primary cultures and cell lines

Human cell lines, ECV304 and 1321N1, were supplied by European Collection of Animal and Cell Cultures (ECACC). EAhy926 human cell line was a gift from Professor Helen Griffiths, Professor of Biomedical Sciences, School of Life and Health Sciences, Aston University, UK. Bovine aortic endothelial (BAE) cells were isolated preparations from bovine aortae. BRIN-BD11 cell line was a gift to Diabetes Research Group from Professor Peter Flatt, University of Ulster, Coleraine.

vi) Culture reagents

Medium 199 with Earle's salts with L-glutamine, Minimal essential medium with D-Valine and RPMI 1640 (without L-glutamine, with phenol red) and Fetal calf serum were supplied from PAA Laboratories Ltd, Yeovil, UK. The following culture reagents were supplied by Sigma-Aldrich Company Ltd., Dorset, UK.:

Dulbecco's modified eagle's medium high glucose, Earles balanced salts solution, Amphotericin B, Gentamicin, L-glutamine and Penicillin–Streptomycin solution Hybri-Max[®].

vii) Antibodies

L247 rabbit polyclonal anti-P2Y₂ antibody is an in-house antibody. Anti-purinergic receptor P2Y₁₂ and P2Y₁ antibodies were supplied by Sigma-Aldrich Company Ltd., Dorset, UK. Anti-P2Y₁₁ rabbit polyclonal antibody was supplied by Affinity BioReagents, Inc. USA. Anti-P2Y₁₃/GPR86/GPR94 antibody was supplied by Acris Antibodies GmbH, Hiddenhausen, Germany. Rabbit anti-P2Y₂ receptor antibody was supplied by Zymed Laboratories Inc. USA. The following anti-purinergic receptor anti-P2Y₂, anti-P2Y₄ and anti-P2Y₆ rabbit polyclonal antibodies were supplied by Santa Cruz Biotechnology, Inc. USA. Polyclonal Swine anti-rabbit-peroxidase conjugate and polyclonal Swine anti-rabbit Immunoglobulins/FITC antibody were supplied by Dako UK Ltd. Cambridgeshire, UK.

2.2 Cell culture

2.2.1 Isolation of bovine aortic endothelial cells

Bovine aortae, obtained from a local abattoir were transported to the laboratory on ice in sterile balanced salt solution (BSS) containing 100U/ml pencillin, 100µg/ml streptomycin, 100µg/ml gentamicin and 2.5µg/ml amphotericin B. Isolation of endothelial cells was carried out not more than one hour after collection of the aortae, as originally described (Booyse et al, 1975), in sterile conditions in a class II cabinet. The aortae were washed in sterile BSS. Collateral arteries were closed off using cotton, prior to addition of 0.2% (w/v) collagenase (Sigma Type IA) solution at 37⁰C. The aortae were incubated with collagenase for 20 minutes at room temperature to digest the endothelial cells away from the arteries. Collagenase was removed from the aortae and they were washed in sterile BSS. The detaching endothelial cells were collected in minimal essential medium with D-valine, containing 10% (v/v) fetal calf serum (PAA), 2mM L-glutamine (Sigma), 100µg/ml streptomycin, 100U/ml pencillin, 2.5µg/ml amphotericin B and 100µg/ml gentamicin into 25cm² flask (Sarstedt).

Table 2.1 The Composition of balanced salts solution pH 7.4

NaCl	125mM
KCl	5.4mM
NaHCO₃	16.2mM
HEPES	15mM
NaH₂PO₄ 2H₂O	1mM
MgSO₄ 7H₂O	0.8mM
CaCl₂ 6H₂O	1.8mM
Glucose	5.5mM

2.2.2 Tissue culture of 1321N1, ECV304, EAhy926, Bovine aortic endothelial (BAE) and BRIN-BD11 cells

ECV304 cells (ECACC) were maintained in M199 medium with L-glutamine (Sigma) supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin. Bovine aortic endothelial (BAE) cells were maintained in Minimal essential medium with D-Valine (PAA) supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin and 2 mM L-Glutamine (Sigma). Human brain astrocytoma cells (1321N1) were maintained in Dulbecco's Modified Eagle's Medium high glucose (Sigma) supplemented with 2mM Glutamine (Sigma) and 10% Foetal Bovine Serum (PAA). BRIN-BD11 cells, produced by electrofusion of normal rat pancreatic β-cells and RINm5F cells were cultured in RPMI medium (PAA) supplemented with 10% FCS (PAA), 100 units/ml penicillin and 100 µg/ml streptomycin.

The cells were subcultured using a standard trypsin/EDTA (Sigma) method once the cells were grown into a confluent monolayer either in 2x75 cm² or 2x175 cm² tissue culture flasks (Sarstedt). Briefly, the growth medium was removed from the cells and the cells were washed (3X5ml) with Ca⁺⁺ and Mg⁺⁺ free Earles Balanced Salts solution (Sigma) to remove all traces of serum. Trypsin /EDTA solution was dispensed into culture vessels to completely cover the monolayer and the cells were incubated at 37⁰C for 5 minutes to ensure that the cells were detached from the base of the flask. 10 ml of medium was added to the flask and the total volume of cell suspension was made up to the volume required for next passage in new culture vessels. The cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37⁰C until they were confluent.

2.3 Measurement of second messengers in cells

2.3.1 Measurement of total inositol (poly) phosphates

Cells grown to confluence in 24 well plates were labelled for 24 hours with 0.5µCi (17.0 Ci/mmol) (Amersham) per well in 0.5ml serum free medium. The 24 well plates containing cells were transferred to a 37⁰C water bath and lithium chloride was added at a final concentration of 10mM for 10 minutes. Following incubation with lithium

chloride the cells were stimulated for 15 minutes with agonists or incubated with antagonists for 15 minutes and then stimulated with with agonists for 15 minutes. The stimulation was terminated by removal of stimulation medium and immediate addition of 0.5M ice-cold trichloroacetic acid (TCA). The cells were then incubated on ice for one hour and extracts transferred to polypropylene tubes and washed with (3X 4 volumes) water-saturated diethyl ether and then neutralised to pH 7 with 0.6M NaHCO₃. After the washings, the negatively charged samples were added to positively charged 1ml Dowex 1X8-400 (Sigma) columns and allowed to drain before adding 10 ml of distilled water to remove unincorporated [³H] inositol. After allowing the water to run through completely the columns were transferred into serially numbered scintillation vials. The fractions containing [³H] IP₁-IP₄ were recovered by extraction using 5ml of 2M ammonium formate. 5 ml of Ultima Flo AF scintillation fluid (i.e. at ratio 1:1) was added to the vial containing the collected fraction and mixed for 10 seconds before counting on a scintillation counter.

2.3.2 Measurement of cAMP production

Cells were grown to confluence in 24 well plates in a CO₂ incubator and then transferred to a 37⁰C water bath washed twice with warm medium and incubated for 15 minutes at 37⁰C in BSS. Agonists were added for a further period of 20 minutes, then reactions were terminated by washing cells twice in 1 ml of 70% (v/v) ice cold ethanol. Cytoplasmic contents were extracted by scraping cells with a pipette tip in 0.5ml/well ice cold 70% (v/v) ethanol. Samples were stored on ice for 60 minutes and supernatants separated from cell debris by centrifugation. Pelleted material was extracted further and the combined supernatants dried under vacuum. Cytoplasmic extracts were dissolved in buffer (50mM Tris, 4mM EDTA, pH 7.5) and cAMP measured using an Amersham Biotrak assay kit according to the manufacturer's instructions.

2.4 RT-PCR studies

2.4.1 Primer design

The primer sequences for human P2Y₁₋₁₁ receptors were taken from previously published work (Adrian *et al.*, 2000). Primers for human P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors were designed and analysed using a program called PrimerSelect (DNASTAR Lasergene software). The primers were selected from a list of suggested primers evaluated on the thermodynamic properties for annealing reactions.

2.4.2 RT-PCR studies for P2Y (P2Y₁₋₁₄) receptor mRNA transcripts

Total RNA was isolated from cultured cells by adding the appropriate amount of TRI reagent, RNasin Ribonuclease Inhibitor and RQ1-Rnasin Free DNase. The quantity of RNA was determined photometrically at 260nm using Genequant. 1µg total RNA was reverse transcribed to cDNA in a 50µl reaction volume containing nuclease free water, AMV RT buffer, Oligo_{dt} primer, dNTP mix and AMV reverse transcriptase (all reagents from Promega). Nuclease free water was used as negative control. The PCR reactions were performed in 50µl final volume containing Pre-Aliquoted Reddy MixTM PCR Master-Mix, 3 µl cDNA from RT stage/ nuclease free water as negative control, 7.5 pmol of primers GAPDH, P2Y₁, P2Y₂, P2Y₄, P2Y₆ (10 pmol in the case of P2Y₁₁). After initial denaturation for 2 min at 94⁰C, amplifications were carried out for 35 cycles as follows: denaturation at 94⁰C for 45 seconds, annealing at primer specific temperature for 45 seconds, and extension at 72⁰C for 90 seconds. After the final PCR cycle the final extension was at 72⁰C for 15 minutes. Ethidium bromide stained 'Hi-Pure' Low EEO agarose gels (Bio/Gene) of PCR products were analysed against DNA Markers (Hyper Ladder IV – Bioline, 100bp ladder) and by scanning with a BIO VISIONTM UV imager.

2.4.3 PCR product purification

The PCR amplification products were purified using GenElute™ PCR Clean-Up Kit (Sigma). GenElute Miniprep Binding Column (with a blue o-ring) was inserted into the collection tube provided. 0.5 ml of the Column Preparation Solution (which maximizes binding of the DNA to the membrane resulting in more consistent yields) was added to each miniprep column which was centrifuged at 12,000 x g for 1 minute and the eluate was discarded. 5 volumes of Binding Solution were added to 1 volume of the PCR reaction and mix. The solution was transferred into the binding column and the column centrifuged at 16,000 x g for 1 minute. The eluate was discarded and the collection tube was retained. The binding column was replaced into the collection tube. 0.5 ml of diluted Wash Solution was applied to the column and it was centrifuged at maximum speed (16,000 x g) for 1 minute. The eluate was discarded retaining the collection tube. The column was replaced into the collection tube and the column was centrifuged at maximum speed (16,000 x g) for 2 minutes, without any additional wash solution, to remove excess ethanol. The collection tube was retained and any residual eluate was discarded. The column was transferred to a fresh 2 ml collection tube. 50 µl of Elution Solution was applied to the centre of each column and they were incubated at room temperature for 1 minute. The column was centrifuged at maximum speed (16,000 x g) for 1 minute to elute the DNA. The PCR amplification product present in the eluate was stored at -20°C.

2.4.4 DNA sequencing

Depending on the template type (i.e. the size of PCR product) 5-20 ng of the purified PCR product per reaction with a minimum of 30µl of template (enough for two reactions) and 3.2 picomoles (a minimum of 10µl) of Primer per reaction were prepared in dilution in Nuclease-Free Water (Promega). DNA sequencing was performed by the Sequencing Service (School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.5 Purification of antibody

2.5.1 L247 polyclonal antibody production

The polyclonal antisera was produced in rabbit by Severn Biotech Ltd. using L247 peptide (RSLDLSCHTLNAIN) designed to mimic the specific region of the third extracellular loop of human P2Y₂ receptor. 2.5ml of test bleed, 10ml of production bleed and 50ml of terminal bleed were obtained as the final volumes of anti sera.

2.5.2 Purification of L247 polyclonal antibodies

The polyclonal antisera was purified by affinity chromatography using a BioLogic HR Chromatography System (Bio-Rad) and PROSEP-Thiosorb (Millipore) affinity adsorbent columns. Sufficient reagent was added to the starting material (serum or cell culture supernatant) so that it contained 20 mM HEPES, 7.5% (NH₄)₂ SO₄ and 0.5M NaCl. The pH of the solutions was adjusted to 7.5 using 5M NaOH and the solution was filtered through a 0.45 or a 0.2 µm filter. After loading the sample on to the column the bound antibody fractions were eluted using three elution conditions including elution buffer 1 (20mM HEPES, 1M NaCl, pH 7.5), elution buffer 2 (20mM HEPES, 2M NaCl, pH 7.5) and elution buffer 3 (60% Ethylene glycol).

2.5.3 Dialysis

The purified fractions were then dialysed for 24 hours against multiple buffer (Phosphate Buffered Saline, pH 7.5) changes using SnakeSkin™ Dialysis Tubing (Pierce) before storing them in aliquots at -20⁰C.

2.5.4 Protein quantification

Antibody concentrations in the purified fractions of terminal bleed of L247 polyclonal antisera were quantified using a Bicinchoninic Acid (BCA) Protein Assay Kit (BCA1-Sigma). The concentration of the unknown sample (L247 Ab fraction I and II) was determined by comparing its absorbance at 562 nm to the standard curve prepared using

BSA (Bovine Serum Albumin) protein standards. This standard assay was performed in a test tube. The required amount of BCA Working Reagent needed for the assays was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. The BCA Working Reagent was mixed until it was light green in colour. BSA protein standards of different concentrations ranging from 200-1,000 $\mu\text{g/ml}$ (20-100 μg of total protein) were prepared by making serial dilutions starting from the 1 mg/ml standard, and then using 0.1 ml of each diluted standard in the assay. Dilutions were carried out in the same buffer as the unknown sample. For protein samples with unknown concentrations, a dilution scheme was prepared to ensure the concentration fall within the linear range of 200-1,000 $\mu\text{g/ml}$. 2 ml of the BCA Working Reagent was added to 0.1 ml of each BSA protein standard, blank, and unknown sample (Antibody fractions). The contents of the test tube were thoroughly mixed and incubated at 37⁰C for 30 minutes. The absorbance of the solution was recorded at 562 nm immediately after incubation. The assay table and a standard curve based on either the BSA protein standard concentration or on the amount of protein present in the BSA protein standard was created (Figure 2.1). The protein concentrations were determined by comparison of the absorbance of the unknown samples to the standard curve prepared using the BSA protein standards.

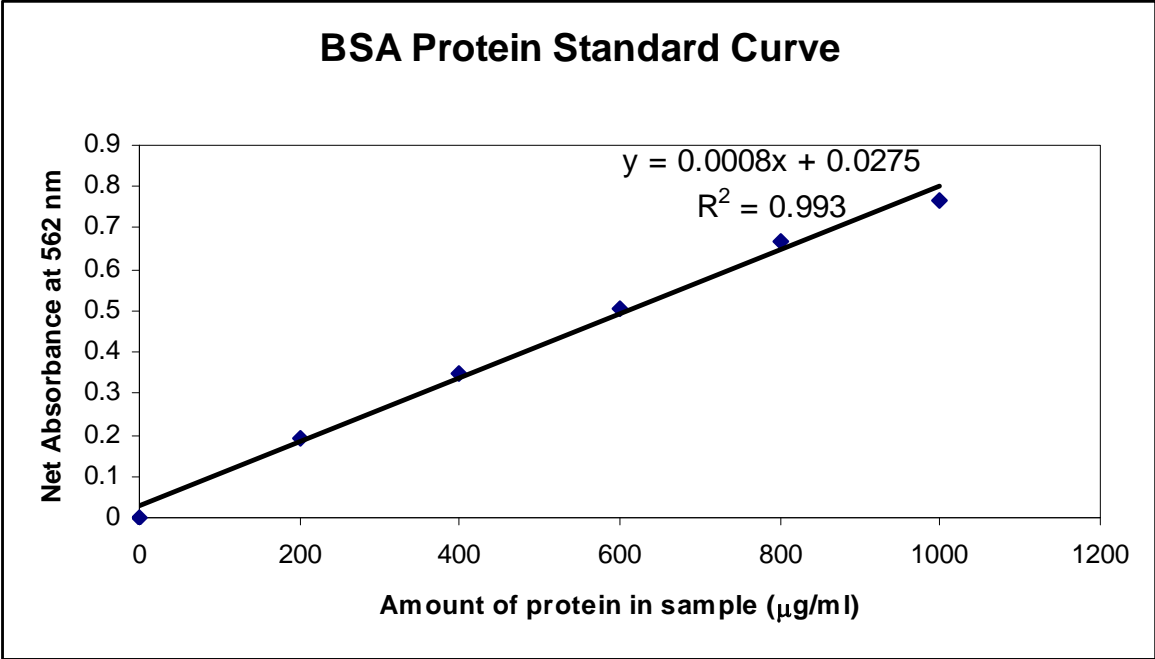


Figure 2.1 Bovine Serum Albumin (BSA) protein standard curve

2.5.5 Indirect ELISA

Each well of a 96-well plate was coated with 50µl of L247 peptide (RSLDLSCHTLNAIN) diluted in distilled water at the required concentration and incubated overnight at 37⁰C (24 hrs) without a lid to enable water to evaporate which enabled maximum peptide binding per well. The wells were washed in phosphate buffered saline (Well washer 4 MK 2- Labsystems). Remaining sites in the well were blocked with 2% BSA in PBS 50µl per well and incubated for 1 hour at 37⁰C. After washing three times with 0.1% Tween – 20 in PBS, primary antibody in diluant (0.05% Tween- 20/ 1% BSA in PBS) was added at required dilution (50µl per well) and incubated for 1 hour at 37⁰C. After washing three times with 0.1% Tween- 20 in PBS, secondary antibody was added to all wells at the required dilution (0.1% Tween – 20 / 2% BSA in PBS) and incubated for 1 hour at 37⁰C (in dark if light sensitive). After washing 3 times with 0.1% Tween- 20 in PBS 50µl of 3, 3', 5, 5' – Tetramethyl Benzidine (TMB) Liquid Substrate system for ELISA was used at the required incubation/development period. The reaction was stopped using 2N HCl. The absorbancy was read using a Labsystems Multiscan MS plate reader.

2.6 Western blot studies

2.6.1 Protein extraction

The extraction of membrane proteins from ECV304, EAhy926, BAEC, 1321N1 and BRIN-BD1 cells was carried out using ProteoExtract™ Native Membrane Protein Extraction Kit (Calbiochem). The cells were grown to approximately 80 % confluency and the growth medium was removed carefully without disturbing the cell monolayer. The cells were washed carefully overlaying the cell monolayer with 2 ml ice cold Wash Buffer. The Wash Buffer was aspirated completely without disturbing the cell monolayer. The washing step was repeated to remove contaminating media components. 10 µl of Protease Inhibitor Cocktail was added to the wall of the cell container and immediately 2 ml ice cold Extraction Buffer I was added. The components were mixed by swirling the flask without disturbing the monolayer and incubated for 10 min at 4°C under gentle agitation. The supernatant (enriched in “soluble” proteins) was set aside using a pipette without disturbing the cell layer. 5 µl

Protease Inhibitor Cocktail was added to the wall of the cell container and immediately 1 ml ice cold Extraction Buffer II was added. The components were mixed by swirling the flask without disturbing the monolayer and incubated for 30 min at 4°C under gentle agitation. The supernatant (membrane fraction) enriched in integral membrane and membrane associated proteins were transferred into a sample tube using a pipette without disturbing the cell debris. The insoluble material was centrifuged at 16,000 x g and 4°C for 15 min. The extract was quantified using DC Protein Assay Kit (BIO-RAD). For long-term usage samples were stored at or below -20° C in aliquots of convenient size.

2.6.2 Protein quantification

Protein concentrations from extracted membrane protein fractions were quantified using a DC Protein Assay Kit (BIO-RAD). The concentration of the unknown sample was determined by comparing its absorbance at 690 nm to the standard curve prepared using BSA (Bovine Serum Albumin) protein standards.

2.6.3 SDS-PAGE and western blot analysis

The protein extracts from cultured cells were mixed with SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 5% β-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v)) and heated at 95°C for 5 minutes. The protein samples were analyzed in a 10% sodium dodecyl sulfate-polyacrylamide gel in the Mini-PROTEAN 3 cell Electrophoresis system (Bio-Rad) using Tris/Glycine/SDS running buffer (Geneflow). 40µg of total protein was loaded in each well. After electrophoresis the proteins were transferred electrophoretically to nitrocellulose membranes using Tris/Glycine transfer buffer (Geneflow) in Mini Trans-Blot cell (Bio-Rad) for 1hour. Following the transfer the nitrocellulose membrane was blocked for 1 h with 5% nonfat dry milk in TBS-0.2% Tween 20 (TBS-T), followed by three washes with TBS-T for 15 minutes each. The membrane was then incubated with Primary (anti-P2Y receptor subtype) antibody in 5% nonfat dry milk in TBS-T overnight at 4°C with gentle agitation. The membrane was washed three times for 15 minutes and incubated with secondary antibody (Swine Anti-Rabbit-peroxidase (Dako) conjugate) at 1:3000 dilutions for an hour at room temperature. The membrane was washed three times for 15 min with TBS-T before adding the ECL Plus™ Western Blotting Detection Reagent

(GE Healthcare). The chemifluorescent signal was detected on Storm™ imaging system based on PhosphorImager™ technology (GE Healthcare) and analysed using ImageQuant™ TL image analysis software (GE Healthcare).

2.6.4 Antibodies

The immunogenic peptide sequences of L247 anti-P2Y₂ antibody and all anti-P2Y receptor rabbit polyclonal antibodies used in this study were compared with protein sequence databases using blastp (protein-protein BLAST) program of the Basic Local Alignment Search Tool (BLAST) to find regions of local similarity and to find significance of such matches.

Anti- P2Y₁ antibody (P6487-Sigma) is developed in rabbit using a highly purified peptide (C)RALIYKDLDNSPLRRKS corresponding to amino acid residues 242-258 of the rat or human P2Y₁ receptor protein, with an additional N-terminal cysteine, conjugated to KLH as the immunogen. The antibody is affinity isolated using immobilized immunogen. According to the data sheet this antibody recognizes P2Y₁ protein (66 kDa) from human platelets and rat brain membranes by immunoblotting.

The immunogenic peptide used to raise this anti-P2Y₁ antibody corresponds to amino acid residues 242-258 of the human P2Y₁ receptor protein. Likewise, the immunogenic sequence also corresponds to P2Y₁ receptor amino acid residues 129-145 of Chinese hamster (*Cricetulus griseus*-gi:20384813), 233-249 of pig (*Sus scrofa*-gi:58618069), 242-258 of cattle (*Bos taurus*-gi:27806041), 242-258 of domestic guinea pig (*Cavia porcellus*-gi:33413446), 242-258 of Norway rat (*Rattus norvegicus*-gi:25742733), 231-247 of chicken (*Gallus gallus*-gi:45384488), 242-258 of dog (*Canis familiaris*-gi:52788591), 242-258 of house mouse (*Mus musculus*-gi:6679193), 230-246 of African clawed frog (*Xenopus laevis*-gi:16660636).

Anti-P2Y₂ polyclonal antibody raised in rabbit using L247 peptide as an immunogen and affinity purified from rabbit antiserum. L247 peptide, H-RSLDLSCHTLNAIN has been shown to mimic the third extracellular loop of human P2Y₂ receptor. The immunogenic peptide sequence also corresponds to P2Y₂ receptor amino acid residues 272- 285 of house mouse (*Mus musculus*-gi:13879282), 271-284 of Norway rat (*Rattus*

norvegicus-gi:38197686) and 144-157 of Chinese hamster (*Cricetulus griseus*-gi:20450943).

Anti- P2Y₂ (H-70) rabbit polyclonal antibody (sc20124-Santa Cruz Biotechnology) raised against amino acids 308-370 mapping at the C-terminus of P2Y₂ of human origin. This antibody has been shown to recognise P2Y₂ expression in AML-193 whole cell lysate. The immunogenic sequence mapping the C-terminus of P2Y₂ receptor closely matches the sequence of P2Y₂ receptors of chimpanzee and rhesus monkey.

Anti- P2Y₂ polyclonal antibody (34-7600-Zymed) is epitope-affinity-purified from rabbit antiserum developed using a synthetic peptide derived from the C-terminal region of the human P2Y₂ receptor protein. This antibody reacts with the human P2Y₂ receptor protein and has been reported to exhibit a strong single band ~45-47 kDa in western blots. According to notes from the supplier this antibody is not expected to react with mouse or rat based on amino acid sequence homology.

Anti-P2Y₄ (H-60) rabbit polyclonal antibody (sc20125-Santa Cruz Biotechnology) raised against amino acids 306-365 mapping at the C-terminus of P2Y₄ of human origin. This immunogenic peptide sequence also corresponds to P2Y₄ receptor amino acid residues 322-381 of chimpanzee (*Pan troglodytes*-gi:55663355), 96% of amino acid residues 306-365 of predicted sequence of P2Y₄ receptor of rhesus monkey (*Macaca mulatta*-gi:109131105), 70% of amino acid residues 306-365 of cattle (*Bos taurus*-gi:61868522), 68% of amino acid residues of 302-361 of Norway rat (*Rattus norvegicus*-gi:13928944), 60% of amino acid residues of 302-361 of house mouse (*Mus musculus*-gi:111600884), 59% of amino acid residues of 109-135 of Mongolian gerbil (*Meriones unguiculatus*-gi:12751143).

Anti-P2Y₆ (H-70) rabbit polyclonal antibody (sc20127-Santa Cruz Biotechnology) raised against amino acids 1-70 mapping at the N-terminus of P2Y₆ of human origin. This immunogenic peptide sequence also corresponds to hypothetical protein amino acid residues 1-70 of orangutan (*Pongo pygmaeus*), 1-70 of predicted pyrimidinerbic P2Y₆ receptor of chimpanzee (*Pan troglodytes*), 97% amino acid residues of 1-70 of predicted pyrimidinerbic receptor P2Y₆ isoform 8 of rhesus monkey (*Macaca mulatta*), 97% amino acid residues of 1-70 of predicted sequence similar to P2Y₆ purinoceptor of

dog (*Canis familiaris*), 81% amino acid residues of 1-70 of Norway rat (*Rattus norvegicus*-gi:47938969), 81% amino acid residues of 1-70 of house mouse (*Mus musculus*-gi:39795717), 55% amino acid residues of 1-70 of zebrafish (*Danio rerio*-gi:68366846), 39% of amino acid residues 1-89 of cattle G protein-coupled receptor 8 cattle (*Bos taurus*-gi:27806597).

Anti-P2Y₁₁ rabbit polyclonal antibody (OPA1-15632 –ABR) was developed using a synthetic peptide (KLH conjugated) corresponding to the 3rd extracellular loop and detects P2Y₁₁ from human samples.

Anti-Purinergic Receptor P2Y₁₂ antibody (P4871-Sigma) is developed in rabbit using a highly purified peptide KTTRPFKTSNPKNLLGAK, corresponding to amino acid residues 125-142 of human P2Y₁₂ with an additional N-terminal cysteine as the immunogen. The epitope is highly conserved in rat and mouse (16/18 residues identical). The antibody was affinity isolated on immobilized immunogen. Anti-Purinergic Receptor P2Y₁₂ specifically recognizes P2Y₁₂ protein in rat brain membranes and human platelets by immunoblotting.

This immunogenic peptide sequence also corresponds to hypothetical protein amino acid residues 125-142 of crab-eating macaque (*Macaca fascicularis*-gi:14388397), similar to 125-142 amino acid residues of purinergic receptor P2Y₁₂ isoform 4 of rhesus monkey (*Macaca mulatta*-gi:109048722), 94% amino acid residues 75-92 of pig (*Sus scrofa*-gi:58618077), 88% amino acid residues 122-139 of cattle purinergic G-Protein coupled receptor (*Bos taurus*-gi:46019617), 88% amino acid residues 122-139 of dog (*Canis familiaris*-gi:50979232), 88% amino acid residues 131-148 of unnamed protein product of house mouse (*Mus musculus*-gi:26329111), 88% amino acid residues 131-148 of P2ry12 protein of house mouse (*Mus musculus*-gi:20073303), 88% amino acid residues 131-148 of Norway rat (*Rattus norvegicus*-gi:12248187).

Anti-P2Y₁₃/GPR86/GPR94 antibody (SP4616P-Acris Antibodies) which was developed in rabbit using a synthetic peptide (KLH conjugated) corresponds to the second extracellular domain of the receptor. This antibody is peptide immunogen affinity purified immunoglobulin fraction and the immunizing peptide sequence shows 93% identity (93% homology) with mouse P2Y₁₃ receptor.

2.6.5 Enzymatic N- deglycosylation

5 µl of reduced denaturation buffer was added to each vial containing 5 µl of glycoprotein or control glycoprotein. After mixing the contents the protein samples were incubated for 3 min at 95°C. After heating the contents were centrifuged down. 10 µl of Reaction buffer was added to each vial and mixed. 10 µl of reconstituted N-glycosidase F was added to all vials containing glycoproteins (both test and control) to be deglycosylated. 10 µl of Reaction buffer was added to all vials containing glycoproteins not to be deglycosylated. All the vials were incubated for 1 hour at 37°C. The glycoprotein samples were mixed with an equal amount of SDS-sample buffer and heated for 3 min to 95°C. After the heating all the protein samples were analyzed in a 10% sodium dodecyl sulfate-polyacrylamide gel in the Mini-PROTEAN 3 cell Electrophoresis system (Bio-Rad) using Tris/Glycine/SDS running buffer (Geneflow).

2.6.6 Protein sequencing

The deglycosylated protein fractions were analysed in a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred electrophoretically to Sequi Blot PVDF membranes (Bio-Rad). The Sequi Blot PVDF membrane was divided in two halves and one half is used in western blotting analysis using anti P2Y₂ primary antibody. On detecting the chemifluorescent signal the corresponding region on the non used membrane was cut and placed in a vial and sent for protein sequencing. The protein sequencing was performed by Alta Biosciences, University of Birmingham using an Applied Biosystems 'Procise' 494HT machine by the established technique of Edman degradation.

2.6.7 Receptor desensitisation

ECV304 cells were grown to 90% confluency in 75cm² flasks under conditions described in section 2.2.2. The cells were incubated with 300µM UTP or histamine for 1 hour and 24 hour time periods at 37°C. Control cells were incubated without UTP. Cells were removed from the flasks and the protein was extracted and quantified as described in section 2.6.1 and 2.6.2 respectively.

2.7 Immunofluorescence analysis

Autoclaved coverslips were placed in 6-well plates and cultured cells were seeded on coverslips at approximately 5×10^6 cells per well and allowed to attach overnight. The media was removed and the cells were rinsed with PBS (Phosphate buffered saline). The slides were fixed (immobilising the antigens by removing lipids to dehydrate the cells and precipitate the proteins on the cellular architecture) with 1:1 acetone:methanol for 5-6 minutes. The coverslips were washed twice with PBS and blocked by adding 3% BSA in PBS-T for 30 minutes. Primary antibody was diluted in 1% BSA PBS-T in appropriate dilution range and added to each well containing coverslips and incubated at room temperature for 1 hour. The antibody was removed and the coverslips were washed four times with 1% BSA PBS-T on rocker. Fluorescein (FITC) conjugated secondary antibody was diluted as per instructions in 1% BSA PBS-T and incubated for 1 hour in the dark and on a shaker. The secondary antibody was removed and the coverslips were washed 4 times with 1% BSA PBS-T in the dark on a shaker. The excess fluid was removed by blotting and a small drop (25 μ l) of VECTASHIELD[®] Mounting Medium with DAPI (4',6 diamidino-2-phenylindole) was placed on each slide. The coverslips were then inverted and gently laid on top of vectorshield. The edges of the coverslips were sealed using a clear nail varnish and allowed to dry in the dark for 10-15 minutes before analysing them using a confocal microscope.

CHAPTER 3

PHARMACOLOGICAL CHARACTERISATION OF P2Y RECEPTORS IN ECV304 AND EAhy926 CELLS

3.1 Introduction

Analysis of P2Y receptors has been compromised by the lack of selective agents including radioligands. Studies with radiolabelled nucleotides have shown that they bind to non P2 receptor proteins and are metabolically unstable (Laubinger and Reiser, 1998; Chambers *et al.*, 2000b). In addition, until recently, a lack of P2Y subtype selective antibodies has complicated the study of the P2Y receptor family. Therefore functional studies, e.g., measurement of inositol phosphates, measurement of intracellular calcium concentration (Erb *et al.*, 1995; Czubayko and Reiser, 1996) and determining agonist stimulated cAMP production in receptors coupled to adenylate cyclase (Chambers *et al.*, 2000b) has been the predominant method for studying P2Y receptors. Recent binding studies have employed solubilised and purified receptor proteins as they are more suitable due to the way they bind to P2- receptor ligands in a GTP γ S sensitive manner (Bodor *et al.*, 2003; Waldo and Harden, 2004). More recently, receptor-specific, high affinity radioligands have been utilised to study P2Y₁ and P2Y₁₂ receptor activity. CHO cells transfected with human P2Y₁₂ receptors displayed a strong affinity similar to that observed on platelets to ³³P-2MeS-ADP, a stable analogue of ADP (Savi *et al.*, 2001). MRS2279, a selective high-affinity non-nucleotide antagonist for the P2Y₁ receptor radiolabeled with tritium ([³H] MRS2279) has been reported to be a reliable radioactive probe able to bind with high selectivity to the P2Y₁ receptor (Waldo *et al.*, 2002). However, high affinity, subtype specific agents still remains a desideratum.

3.1.1 Human bladder cancer epithelial cell line ECV304

Previous studies of ECV304 cells showed an increase in intracellular calcium in response to UTP, ATP and 2MeSATP suggesting the presence of at least two P2Y receptors (Conant *et al.*, 1998). Recent studies have further evaluated these cells for the functional expression of P2Y receptors. The stimulation of ECV304 cells with nucleotides suggested that UTP and ATP were equipotent agonists at the P2Y₂ receptors. The desensitisation caused by UTP and ATP for the subsequent action of ATP and UTP respectively strongly suggested the expression of P2Y₂ receptor (Brown *et al.*, 2000). Furthermore, stimulation of cAMP by ADP β S indicated the existence of

P2Y₁₁ receptors coupled to adenylate cyclase (AC) (Brown *et al.*, 2000; Brown, 2001). The aim of current study was to further evaluate these cells with P2Y₁₁ subtype specific nucleotides to confirm the expression of this receptor subtype and use the functional expression data in conjunction with the RT-PCR results for mRNA expression to set the basis for western blot analysis with P2Y receptor subtype specific antibodies.

3.1.2 Endothelial EAhy926 cells

Previous studies in EAhy926 cells reported that nucleotides stimulate activation of MAP kinase in a protein kinase C-dependent manner through interaction with a P2U-purinoceptor (Graham *et al.*, 1996a; Graham *et al.*, 1996b). This finding was further supported by the fact that the inhibitory effect of UTP on tumor necrosis factor α (TNF α) - stimulated stress activated protein kinases in EAhy926 cells involved Ca²⁺ independent PKC isoforms and mediation of the P2Y₂ receptor (Paul *et al.*, 2000). Thus, it is necessary to fully characterize the EAhy926 cells for functional expression of P2Y receptor subtypes based on their responses to natural agonists and also to confirm previously published P2Y receptor pharmacology in these cells. This will provide a basic expression profile of P2Y receptors and a platform to carry out RT-PCR studies to determine the mRNA expression and western blot analysis using P2Y receptor subtype specific antibodies.

3.1.3 Bovine aortic endothelial (BAE) cells

In addition to the above cells, Bovine aortic endothelial cells have been studied for P2Y receptor expression. The rank order of agonist potency of responses to natural agonists were consistent with action at receptors of P2Y₁ and P2Y₂ subtype in bovine aortic endothelial cells (Allsup and Boarder, 1990). The additive and desensitizing responses for the nucleotides further indicated the expression of P2Y₁ and P2Y₂ receptors in these cells (Piroton *et al.*, 1996). These receptors were thought to stimulate endothelial nitric oxide synthase (NOS) in a manner that is dependent on protein kinase C (PKC) activity (Brown *et al.*, 1996). Investigation of increases in intracellular calcium ([Ca²⁺]_i) in single BAE cells in response to agonists further supported the hypothesis that different intracellular signaling pathways could be involved in increasing [Ca²⁺]_i (Duchene and Takeda, 1997). More recently, studies have reported the accumulation of inositol

phosphates by 2MeSATP, 2MeSADP and UTP suggesting the native expression of P2Y₁ and P2Y₂ receptors in these cells (Brown and Brown, 2002). Therefore, because of the native expression of P2Y₁ and P2Y₂ receptors, BAE cells will be used to analyse whether human P2Y receptor specific primers could be used to detect the presence of mRNA transcripts and to study the cross species reactivity of P2Y₂ specific antibodies.

3.2 Materials and Methods

3.2.1 Tissue culture

Human ECV304, EAhy926 and bovine aortic endothelial (BAE) cells were cultured as described in section 2.2.2

3.2.2 Measurement of total inositol (poly) phosphates

The protocol for the measurement of total inositol (poly) phosphates was described in section 2.3.1. Cells were stimulated with agonists for 15 minutes.

3.2.3 Measurement of cAMP production

The protocol for the measurement of cAMP production was described in section 2.3.2

3.2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism analysis program (San Diego, USA). The equation built-in to Prism is defined in terms of the $\log(\text{EC}_{50})$, so Prism finds the best-fit value of the $\log(\text{EC}_{50})$ along with its SE and 95% CI. Prism reports the EC_{50} and its 95% CI by taking the antilog of the $\log(\text{EC}_{50})$ and both ends of the 95% CI. Therefore, caution need to be taken when interpreting data as values for EC_{50} will still be calculated for curves that do not plateau. Data are expressed as mean values \pm SEM.

3.3 Results

3.3.1 Measurement of inositol phosphate accumulation in ECV304 cells in response to BzATP and ATP γ S

The method of measuring inositol phosphates was modified from a previously published method (Brown *et al.*, 2000). A dose-dependent accumulation of [3 H] InsP $_{1-4}$ was also induced by BzATP and ATP γ S in ECV304 cells (Figure 3.1) with mean EC $_{50}$ values of $1.51 \pm 0.09 \times 10^{-6}$ M for BzATP and $1.39 \pm 0.08 \times 10^{-6}$ M for ATP γ S

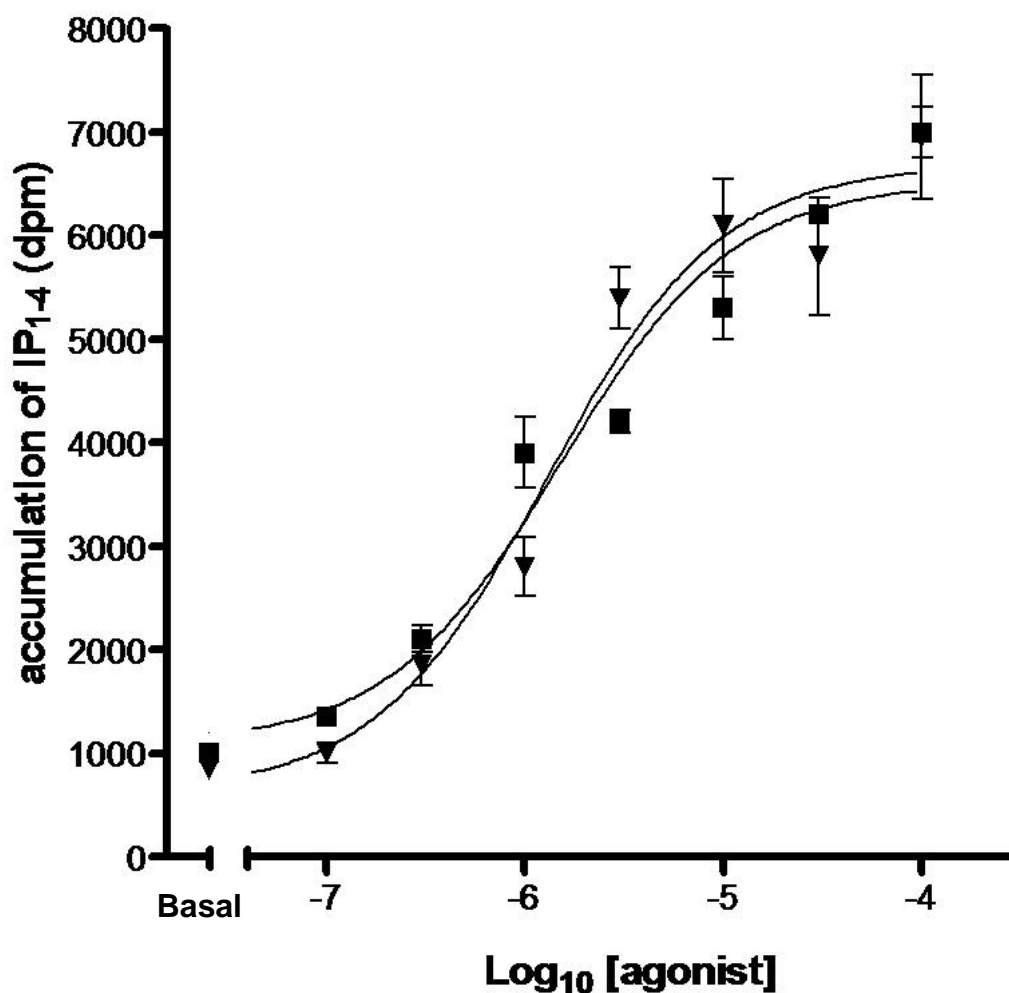


Figure 3.1. Dose-dependent accumulation of [3 H] IP $_{1-4}$ in ECV304 cells in response to BzATP (■) and ATP γ S (▼). Data are \pm SEM from three experiments performed in triplicate.

3.3.2 Measurement of cAMP production in ECV304 cells in response to ADP β S and ATP γ S

ECV304 cells produced a dose-dependent accumulation of cAMP in response to ADP β S and ATP γ S (Figure 3.2) with mean EC₅₀ values of $2.5 \pm 0.02 \times 10^{-3}$ M for ADP β S and $2.2 \pm 0.01 \times 10^{-4}$ M for ATP γ S. (EC₅₀ values calculated by GraphPad Prism, these values are estimates only since neither response reaches plateau and so cannot be taken as absolute values and highlights a limitation of this software).

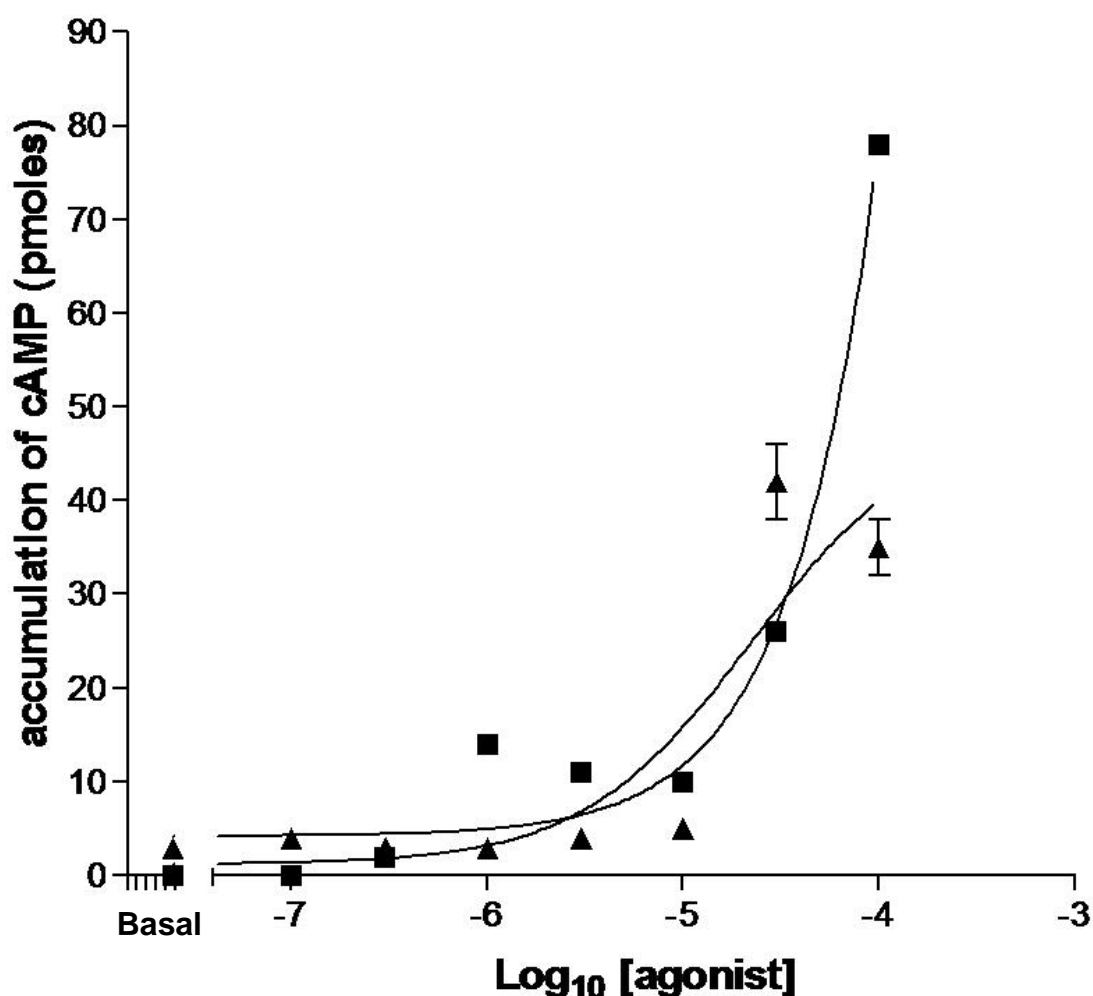


Figure 3.2. Dose-dependent accumulation of cAMP in ECV304 cells in response to ADP β S (■) and ATP γ S (▲). Data are \pm SEM from three experiments performed in triplicate.

3.3.3 Measurement of total inositol (poly) phosphates in ECV304 cells in response to UTP, ATP and ADP

Figure 3.3 shows that ECV304 cells accumulated inositol phosphates ($[^3\text{H}]$ InsP₁₋₄) in response to UTP, ATP and hexokinase-treated ADP with mean EC₅₀ values of 3.04×10^{-6} M, 6.85×10^{-6} M and 0.0001667 respectively. This figure is reproduced with kind permission from the author (Brown, 2001).

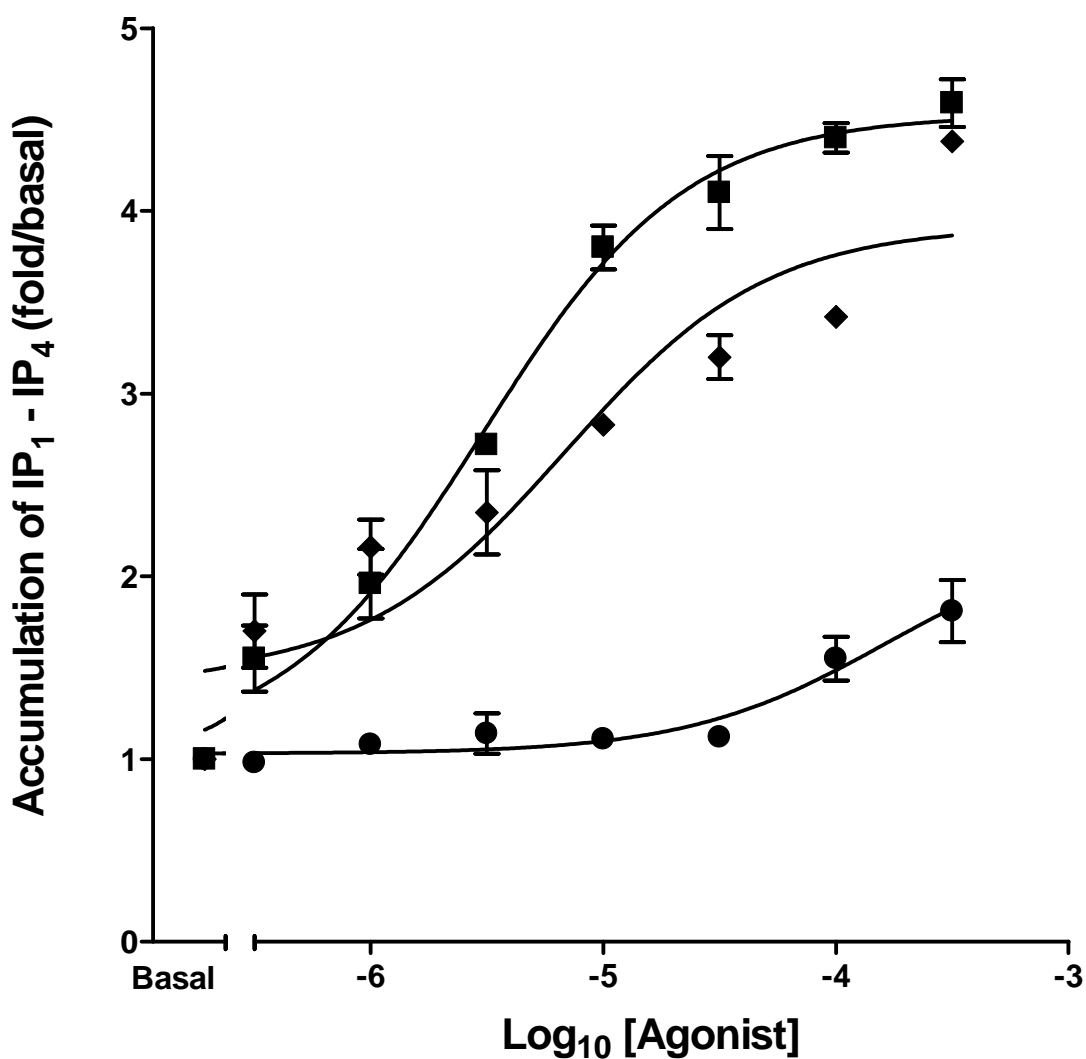


Figure 3.3 Dose-dependent accumulation of $[^3\text{H}]$ Inositol phosphates in response to UTP (■), ATP (◆) and ADP (●) in ECV304 cells. Data are mean results \pm SEM from a representative experiment performed in triplicate.

3.3.4 Measurement of total inositol (poly) phosphates in EAhy926 cells in response to BzATP

The method of measuring inositol phosphates was carried out as per the previously published method (Brown *et al.*, 2000). BzATP did not induce a dose-dependent accumulation of [³H] InsP1-4 in EAhy926 cells (Figure 3.4).

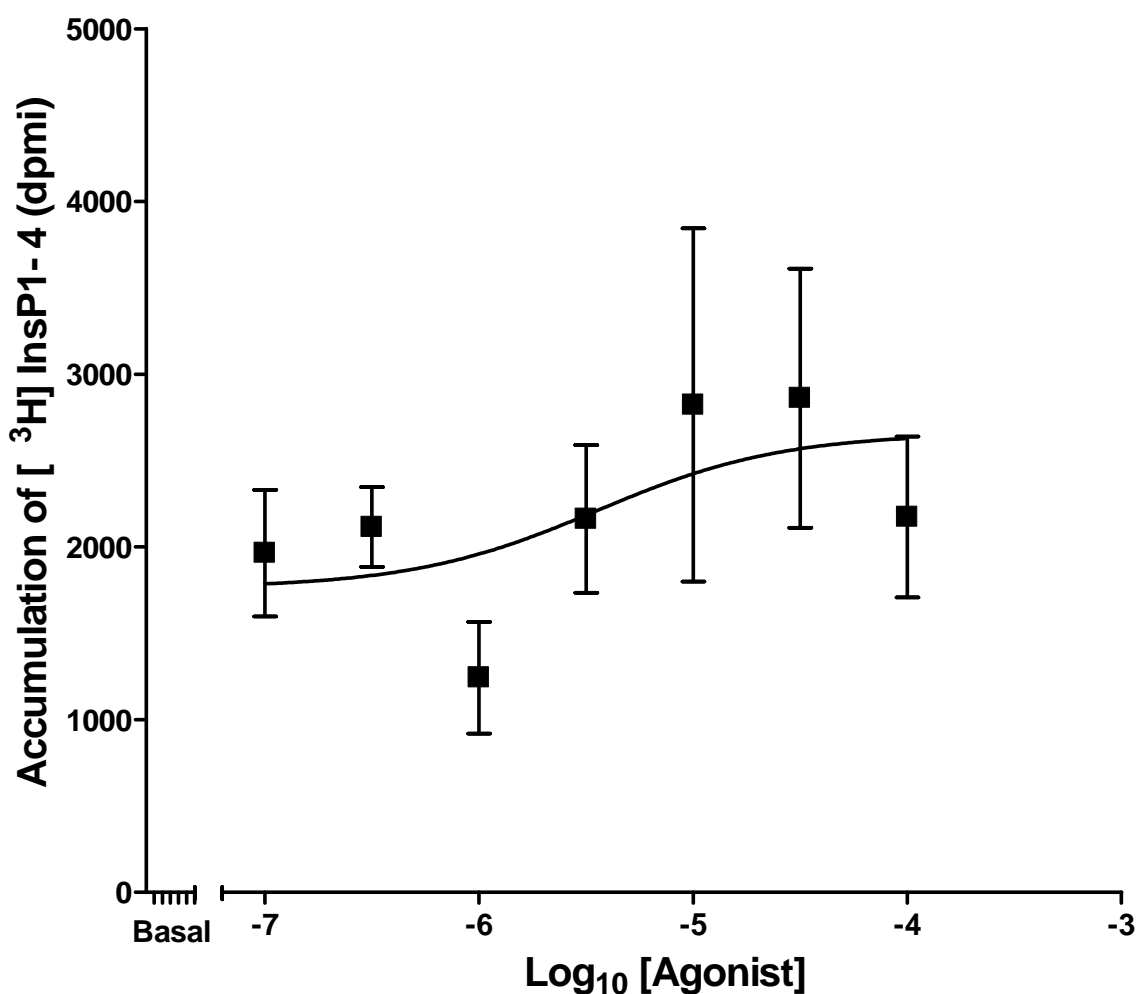


Figure 3.4 Dose dependent accumulations of [³H] InsP 1-4 in EAhy926 cells in response to BzATP. Data are \pm SEM from three experiments performed in triplicate.

3.3.5 Measurement of total inositol (poly) phosphates in EAhy926 cells in response to UTP and ATP

Figure 3.5 shows that EAhy926 cells accumulated inositol phosphates ($[^3\text{H}]$ InsP1-4) in response to UTP and ATP with mean EC_{50} values of 2.46×10^{-5} M and 4.01×10^{-6} M respectively.

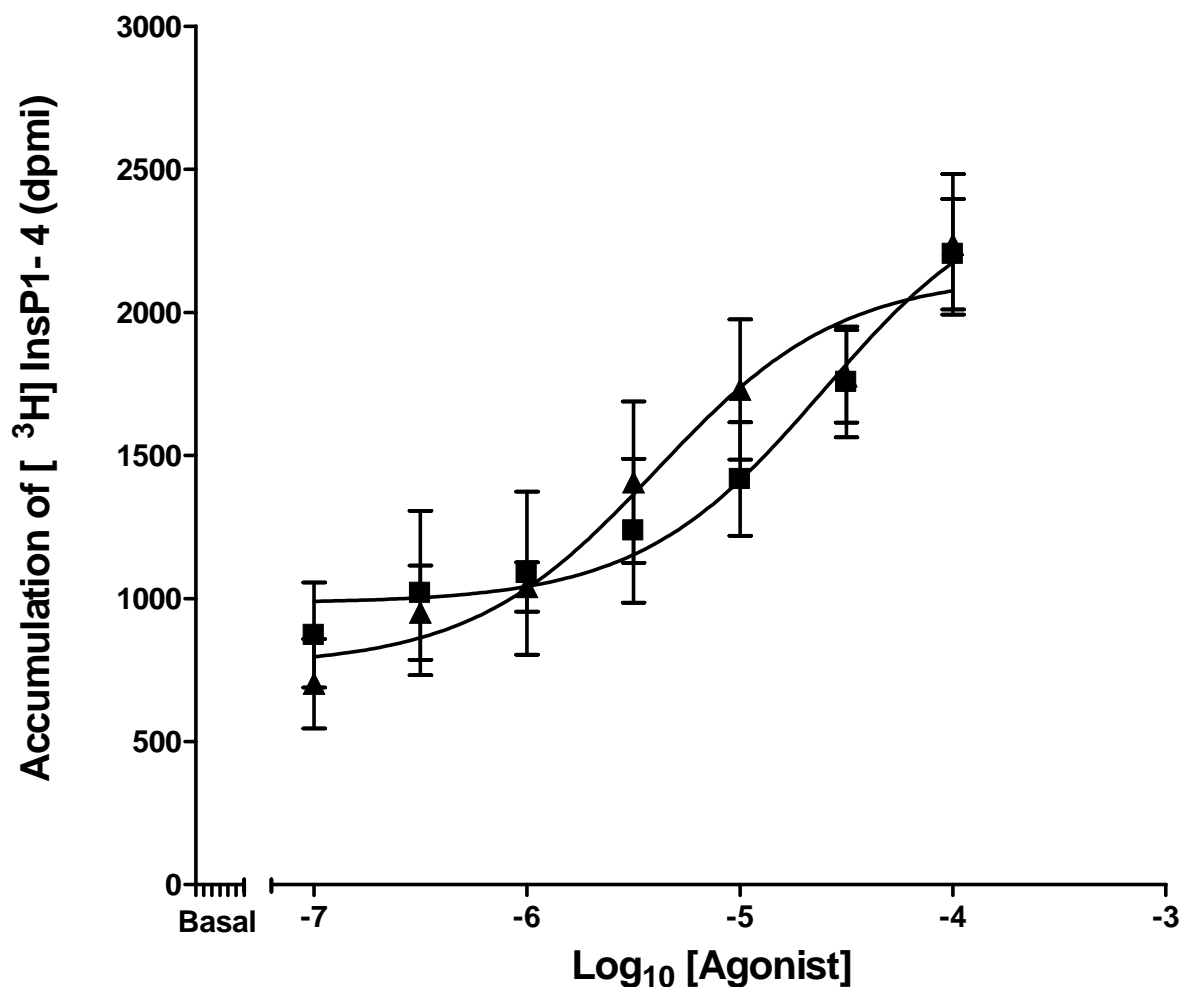


Figure 3.5 Dose dependent accumulations of $[^3\text{H}]$ InsP 1-4 in EAhy926 cells in response to UTP (■) and ATP (▲). Data are \pm SEM from three experiments performed in triplicate.

3.3.6 Measurement of total inositol (poly) phosphates in EAhy926 cells in response to 2MeSATP

2MeSATP did not result in a dose-dependent accumulation of [³H] InsP1-4 in EAhy926 cells (Figure 3.6). However, small increase was observed at the highest concentration of 2MeSATP. It was not possible to calculate EC₅₀, which is >10⁻⁴M.

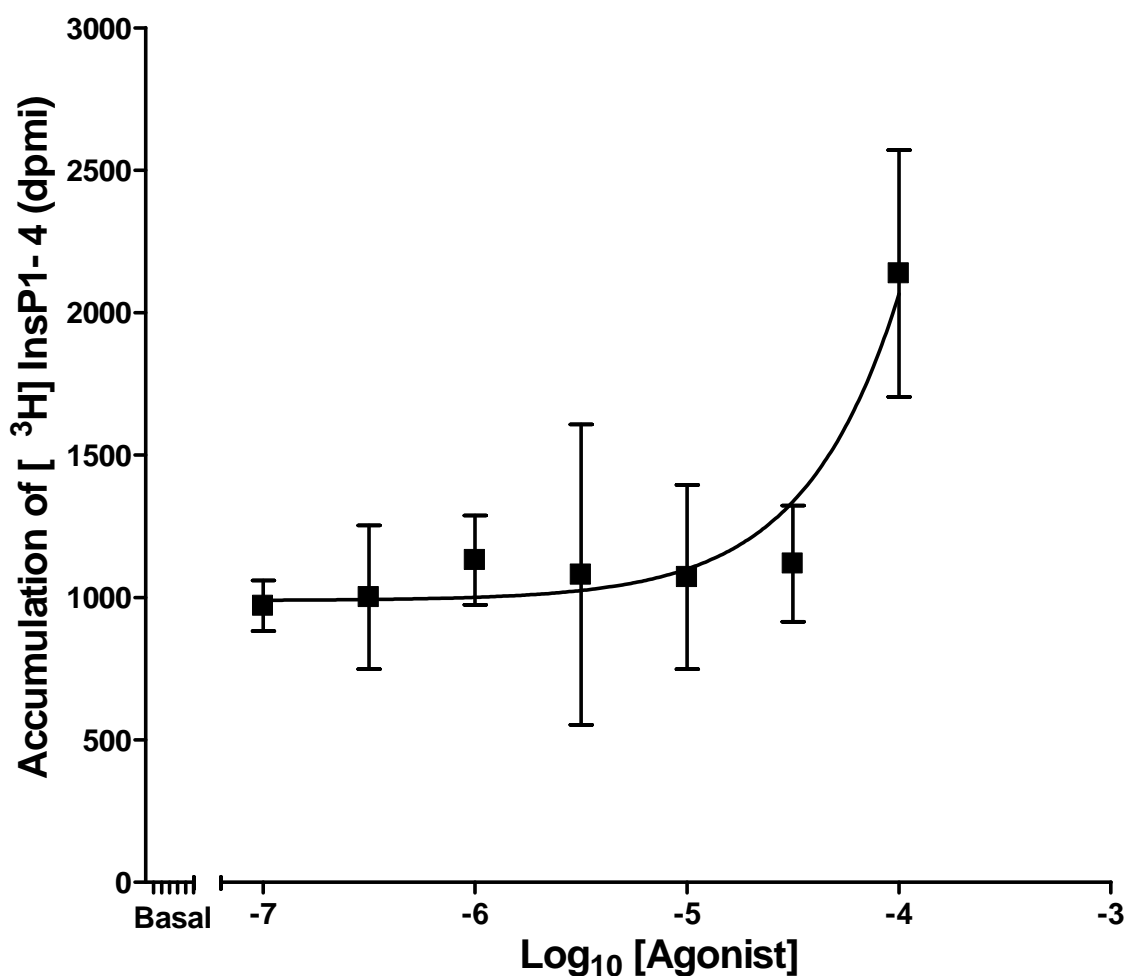


Figure 3.6 Dose dependent accumulations of [³H] InsP 1-4 in EAhy926 cells in response to 2MeSATP. Data are ± SEM from three experiments performed in triplicate.

3.3.7 Measurement of total inositol (poly) phosphates in EAhy926 cells in response to ADP

ADP did not result in a dose-dependent accumulation of [³H] InsP1-4 in EAhy926 cells (Figure 3.7).

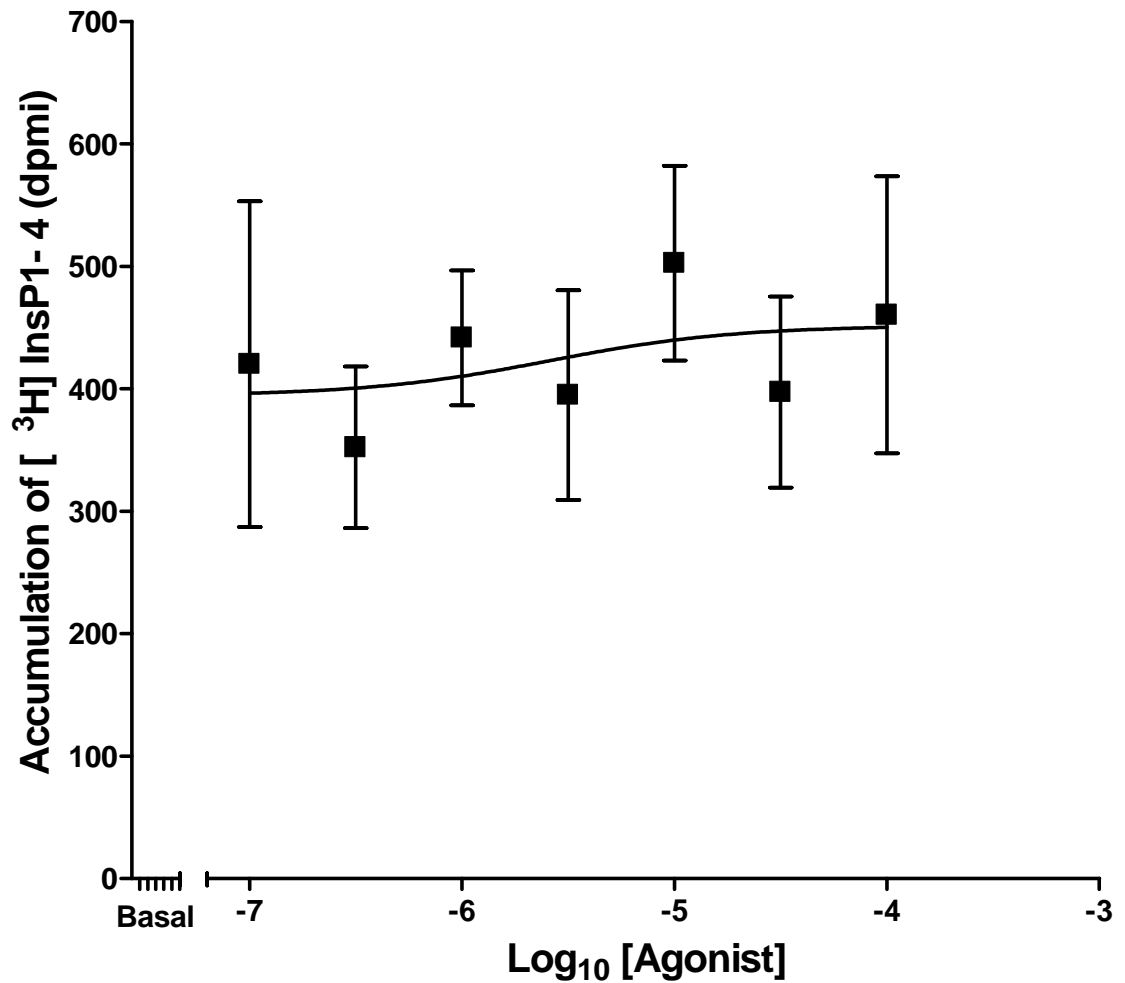


Figure 3.7 Dose dependent accumulations of [³H] InsP 1-4 in EAhy926 cells in response to ADP. Data are \pm SEM from three experiments performed in triplicate.

3.3.8 Measurement of total inositol (poly) phosphates in EAhy926 cells in response to UDP

Figure 3.8 shows that EAhy926 cells accumulated inositol phosphates ($[^3\text{H}]$ InsP1-4) in response to UDP with mean EC_{50} values of $1.62 \times 10^{-5}\text{M}$

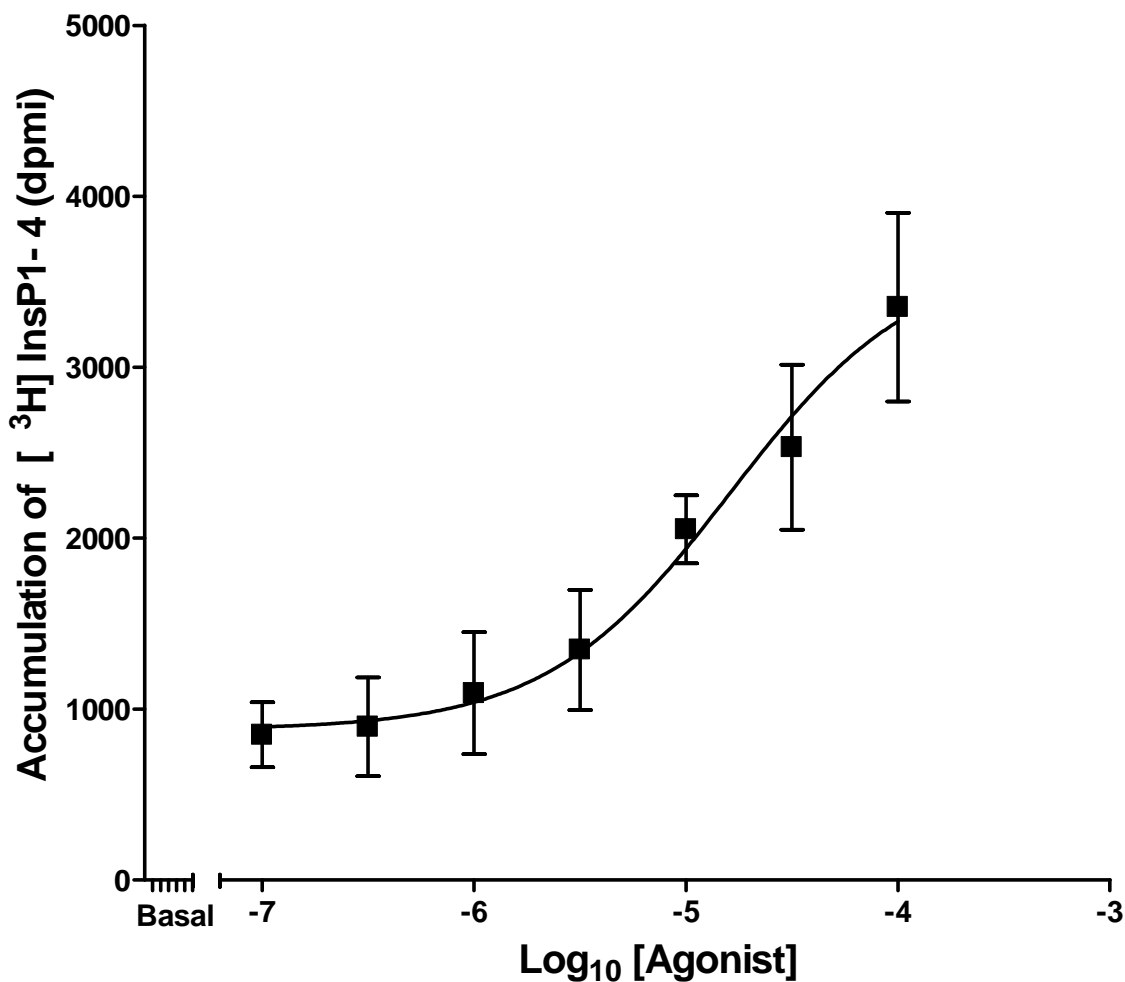


Figure 3.8 Dose dependent accumulations of $[^3\text{H}]$ InsP 1-4 in EAhy926 cells in response to UDP. Data are \pm SEM from three experiments performed in triplicate.

3.3.9 Measurement of total inositol (poly) phosphates in BAE cells in response to UTP

Figure 3.9 shows that BAE cells accumulated inositol phosphates ($[^3\text{H}]$ InsP₁₋₄) in response to UTP with mean EC₅₀ values of 2.06×10^{-5} M. This figure is reproduced with kind permission from the author (Brown and Brown, 2002).

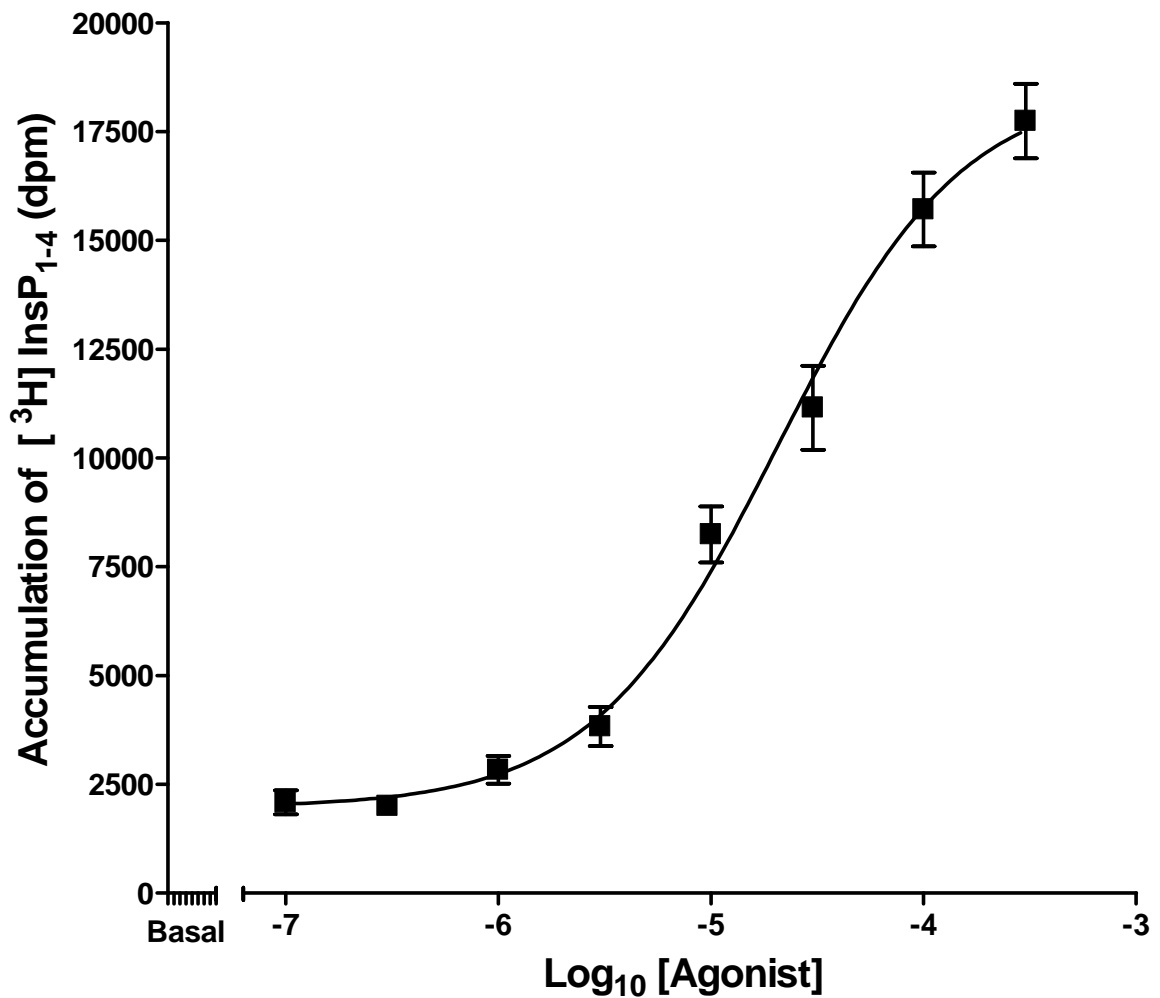


Figure 3.9 Dose-dependent accumulation of inositol phosphates in response to UTP (■) in bovine aortic endothelial cells. Data are mean results \pm SEM from three experiments.

3.3.10 Measurement of total inositol (poly) phosphates in BAE cells in response to 2MeSADP and 2MeSATP

Figure 3.10 shows that BAE cells accumulated inositol phosphates ($[^3\text{H}]$ InsP₁₋₄) in response to 2MeSADP and 2MeSATP with mean EC₅₀ values of 2.51×10^{-6} M and 2.77×10^{-6} M respectively. This figure is reproduced with kind permission from the author (Brown and Brown, 2002).

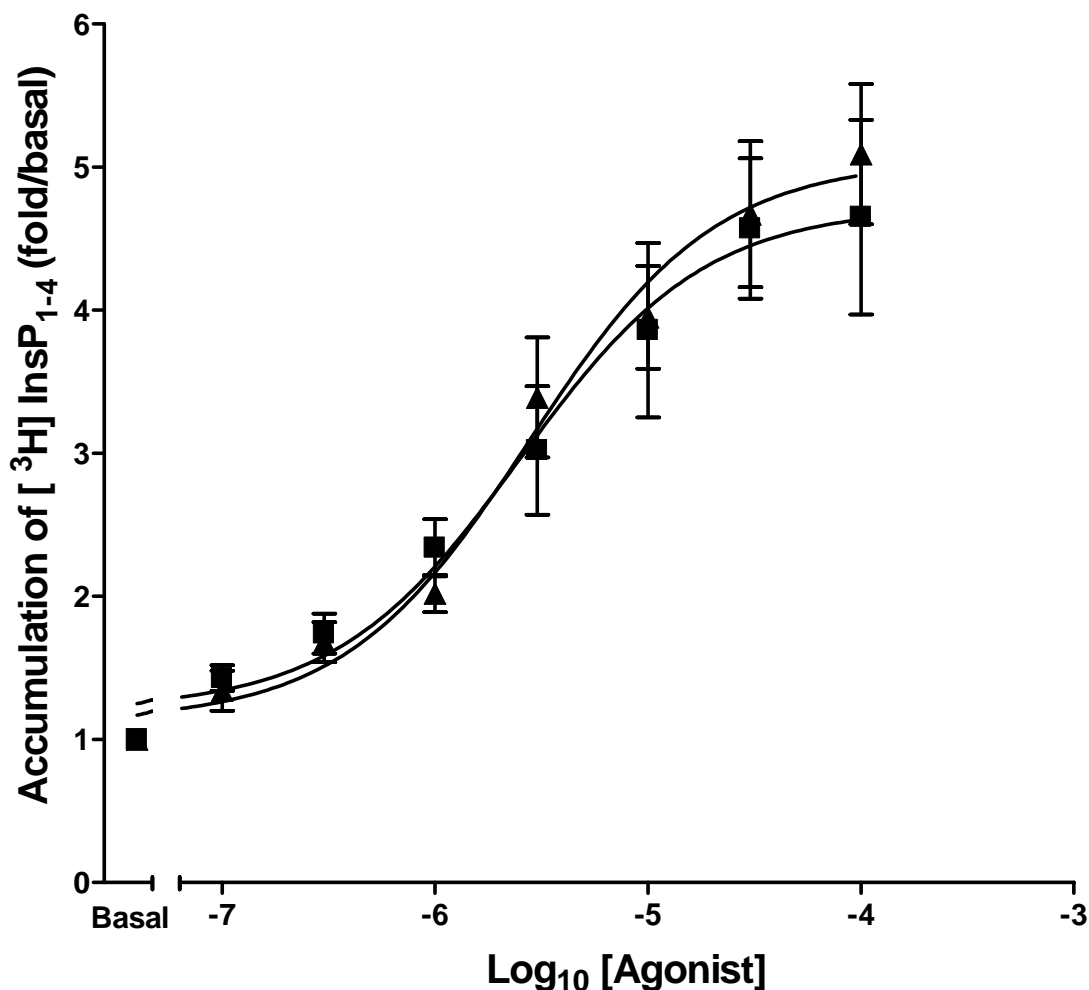


Figure 3.10 Dose-dependent accumulation of inositol phosphates in response to 2MeSADP (■) and 2MeSATP (▲) in bovine aortic endothelial cells. Data are mean results \pm SEM from three experiments.

Table 3.1 Summary of functional responses induced by P2Y receptor agonists in ECV304, EAhy926 and BAE cells

Cell Type	Agonists induced Response									
		ADP	ADP β S	2MeSADP	ATP	ATP γ S	2MeSATP	BzATP	UDP	UTP
ECV304	IP3 Assay	+	+		+	+	-	+	-	+
	[Ca ²⁺] _i Release	+	+	+	+		-		-	+
	cAMP Assay		+			+				
	NOS Activity				+					+
EAhy926	IP3 Assay	-			+		-	-	+	+
BAE	IP3 Assay	+		+			+			+
	NOS Activity				+		+			+

+: Positive stimulation; -: No stimulation

The data for agonists induced [Ca²⁺]_i release and NOS activity is obtained with kind permission from the author (Brown, 2001).

3.4 Discussion

The accumulation of inositol phosphates in response to BzATP and ATP γ S, the potent agonists of P2Y₁₁ receptors, strongly suggests the presence of P2Y₁₁ receptors in ECV304 cells. In addition, the dose-dependent accumulation of cAMP in ECV304 cells in response to ADP β S and ATP γ S further supports the presence of functional human P2Y₁₁ receptors. High concentrations of ADP β S, the partial agonist at human P2Y₁₁ receptor has also been reported to evoke inositol phosphate accumulation and intracellular calcium release (Brown, 2001). Previous studies have reported an identical rank order potency of ATP γ S and BzATP for two pathways. That is the potency of ATP γ S and BzATP were almost identical in inositol trisphosphate measurements and cyclic AMP assays respectively in two different cell lines expressing human P2Y₁₁ receptor (Communi *et al.*, 1999b).

Previous studies in ECV304 cells have shown evidence for the expression of P2Y₁ receptors (Conant *et al.*, 1998). Further studies with the potent P2Y₁ agonist 2MeSATP produced cytosolic calcium responses but not inositol phosphate accumulation (Conant *et al.*, 1998; Brown *et al.*, 2000). The P2Y₁ selective agonist 2MeSADP was without effect suggesting the possibility that either 2MeSATP is acting on the same receptor to induce intracellular Ca²⁺ by different mechanisms or is having effects on different receptors altogether (Brown, 2001). Similar responses have been seen with human P2Y₁₁ receptors expressed in 1321N1 cells where UTP acted as a Ca²⁺-mobilizing agonist with a potency and maximal response similar to ATP but was without effect on inositol phosphate accumulation, whilst ATP was a potent agonist (White *et al.*, 2003). Furthermore, recent studies have reported a biphasic stimulation of [Ca²⁺]_i increase produced by activation of the P2Y₁ receptor; the initial transient phase was IP₃ dependent and the second sustained phase was dependent on pertussis toxin-sensitive kinases such as calcium calmodulin kinase II (CaMKII) and protein kinase C (PKC) (Paredes-Gamero *et al.*, 2006). However, the analysis of agonist specific signaling pathways is not the objective of this study. Furthermore, given the inconclusive agonist mediated response with P2Y₁ agonists and for the purpose of establishing the functional expression, these results should be analyzed together with results obtained with RT-

PCR studies and western blot analysis to detect the presence of mRNA and expression of P2Y₁ receptors in these cells.

Previous reports showed the presence of P2Y₂ receptors in ECV304 cells as both UTP and ATP, the equipotent agonists at P2Y₂ receptors induced inositol phosphate accumulation and calcium mobilisation (Brown *et al.*, 2000). In experiments measuring intracellular calcium release the maximal concentration of ATP desensitized the receptor for subsequent UTP mediated action; and the maximal concentration of UTP desensitized the receptor for ATP mediated action suggesting both ATP and UTP acted at the same receptor thus excluding the possibility of the presence of P2Y₄ receptor in ECV304 cells (Brown, 2001).

Hexokinase – treated UDP failed to elicit accumulation inositol phosphates and cytosolic calcium response in ECV304 cells suggesting the absence of P2Y₆ receptors (Brown *et al.*, 2000). These results will be confirmed by RT-PCR studies for the presence of mRNA and western blot studies with anti-P2Y₆ antibodies.

Previous studies with ADP, the potent agonist of P2Y₁₂ and P2Y₁₃ receptors has shown weak agonistic potency in ECV304 cells (Brown *et al.*, 2000). In addition, ADPβS, an agonist of lower potency at P2Y₁₃ receptor evoked the accumulation of inositol phosphates and a rise in intracellular calcium release in ECV304 cells. However, 2MeSADP, the potent agonist of P2Y₁, P2Y₁₂ and P2Y₁₃ receptors was without effect, and 2MeSATP induced a small increase in the intracellular calcium release in ECV304 cells (Brown, 2001). Thus, it is not possible to establish the functional expression of P2Y₁, P2Y₁₂ and P2Y₁₃ receptors in ECV304 cells from these data. Moreover, it is possible that the responses obtained by ADP, ADPβS and 2MeSATP may be mediated by P2Y₁ or P2Y₁₁ or both. These results highlight the difficulty in studying pharmacology of P2Y receptors, and in the absence of receptor specific agonists or antagonists, the need for other approaches such as RT-PCR and western blot analysis to determine their presence and expression. However, based on these results it could be hypothesized that P2Y₂ and P2Y₁₁ receptors are expressed in ECV304 cells. Nevertheless, RT-PCR studies and western blot studies with receptor specific antibodies

will be explored in order to reach a definitive profile of P2Y receptors expressed in these cells.

The results obtained with EAhy926 cells are consistent with previous reports suggesting the expression of P2Y₂ receptors in these cells (Graham *et al.*, 1996a; Graham *et al.*, 1996b; Paul *et al.*, 2000). In this study the P2Y₂ receptor selective agonist, UTP showed a dose dependent accumulation of inositol phosphates in EAhy926 cells. The non selective agonist, ATP also showed a similar response in these studies. However these results may also suggest that these cells express P2Y₄ receptor, for which UTP is a full agonist. Previous studies involving these agonists reported that UTP as being 50-fold more potent than ATP at P2Y₄ receptor (Nicholas *et al.*, 1996). Measurement of intracellular calcium with ATP and UTP and studies to determine if the maximal concentration of ATP or UTP desensitizes the receptor for subsequent action of UTP or ATP might determine the functional expression of P2Y₄ in EAhy926 cells. At the same time, the detection of mRNA transcripts for P2Y₂ and P2Y₄ receptor using RT-PCR studies should determine the existence of either P2Y₂/P2Y₄ or both. Furthermore, results obtained from inositol phosphate assays and RT-PCR studies would be compared to the results of the western blot analysis using anti-P2Y₂ and anti-P2Y₄ antibodies.

The dose-dependent accumulation of inositol phosphates in response to UDP, a potent agonist of P2Y₆ suggests the presence of P2Y₆ receptor in EAhy926 cells. 2MeSATP, a potent agonist of both P2Y₁ and P2Y₁₁ did not show any significant dose-dependent accumulation of inositol phosphates. Furthermore, BzATP, which is a full agonist of P2Y₁₁, also did not result in significant agonist response. The lack of response to either 2MeSATP or the potent agonist BzATP suggests the absence of P2Y₁₁ receptors in these cells. However, these results will be confirmed with RT-PCR studies using primers specific for human P2Y₁₁ primers to investigate the presence of P2Y₁₁ mRNA transcripts in these cells. In addition, western blot analysis of these cell extracts using anti-P2Y₁₁ antibodies will give further evidence in determining the functional expression of P2Y₁₁ receptor. The lack of any significant stimulation of inositol phosphates to ADP, a potent agonist of P2Y₁₂ and P2Y₁₃ receptors seem to suggest their absence in these cells. However RT-PCR and western blot analysis will be used to further determine whether these receptors are functionally expressed in EAhy926 cells.

This study suggests that EAhy926 cells express P2Y₂/P2Y₄ and P2Y₆ receptors. To confirm these findings, future work will involve RT-PCR studies to determine the mRNA expression of P2Y receptors in these cells. Immunodetection techniques such as immunohistochemistry and immunofluorescence could be used to study the binding and cellular localisation of anti-P2Y₂ and anti-P2Y₆ antibodies in EAhy926 cells. Functional studies may also be extended to determine the ability of receptor specific antibodies to inhibit/augment responses of natural agonists. These, together with other commercially available polyclonal antibodies to P2Y receptor subtypes will be investigated in western blot analysis.

In single bovine aortic endothelial cells ATP, 2MeSATP and UTP produced inositol accumulation suggesting the expression of P2Y₁ and P2Y₂ receptors (Duchene and Takeda, 1997). Further studies reported that 2MeSADP and 2MeSATP were equipotent in evoking the accumulation of inositol phosphates suggesting the expression of P2Y₁ receptor (Brown and Brown, 2002). In the same study 2MeSATP, the agonist of P2Y₁₁ failed to elicit a cAMP response suggesting that the inositol phosphate accumulation in response to 2MeSATP was mediated by P2Y₁. Furthermore, UTP also stimulated a strong dose dependent inositol phosphate response suggesting the expression of P2Y₂ receptors (Brown and Brown, 2002).

Results from these studies and previous studies have provided an expression profile of functional P2Y receptor subtypes in ECV304, EAhy926 and BAE cells. These preliminary results have provided the basis for further investigation using RT-PCR to determine the presence of mRNA transcripts and western blot analysis to verify the functional expression of P2Y receptor subtypes.

CHAPTER 4

ESTABLISHING THE MOLECULAR IDENTITY OF P2Y RECEPTORS IN ECV304 AND EAhy926 CELLS

4.1 Introduction

The extracellular nucleotides regulate cellular function and resultant physiological responses through the activation of P2Y receptors. The P2Y receptor belonging to the super family of G-protein-coupled receptors family consists of eight cloned and functionally defined receptor subtypes namely P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ based on their specific pharmacological selectivities for different nucleotides (Ralevic and Burnstock, 1998; von K ugelgen and Wetter, 2000; Jacobson *et al.*, 2002; Abbracchio *et al.*, 2003). The pharmacological and functional studies of P2Y receptor subtypes in both endothelial and epithelial cells are of great physiological importance. Studies of these cells derived from human and animals have shown a potential regulatory role for the P2Y receptor subtypes in various physiological processes (see Section 1.8).

P2Y receptor subtypes have been shown to be expressed in endothelial cells of human myocardium, human coronary artery graft vessels including internal mammary artery, radial artery and saphenous vein and in human umbilical veins at the mRNA and protein levels (Jin *et al.*, 1998b; Hou *et al.*, 1999; Moore *et al.*, 2001; Ray *et al.*, 2002; Wang *et al.*, 2002). In addition, the P2Y₂ receptor has been found to be up-regulated in CHF (congestive heart failure) heart (Hou *et al.*, 1999). Furthermore, stimulation of P2Y₁, P2Y_{2/4} and P2Y₆ receptors have been shown to cause vasodilation in human vessels (Wihlborg *et al.*, 2003).

Activation of P2Y receptors has been shown to regulate ion transport in human airway epithelial tissues (Leipziger, 2003), gastrointestinal epithelium including human cystic fibrosis pancreatic adenocarcinoma cell line (CFPAC-1) (Chan *et al.*, 1996), pancreatic duct epithelial cells of dogs (Nguyen *et al.*, 1998), rat distal colonic mucosa (Kerstan *et al.*, 1998), secretory epithelium of the equine sweat gland (Wilson *et al.*, 1998), trachea and gall bladder of mice (Cressman *et al.*, 1999; Clarke *et al.*, 2000) rat bile duct epithelia (Dranoff *et al.*, 2001) and rabbit conjunctival epithelium (Li *et al.*, 2001). Furthermore, P2Y receptors have been implicated in the process of wound healing in human corneal epithelial cells (Klepeis *et al.*, 2004). Recent studies have reported the polarised expression of human P2Y receptor subtypes in epithelial cells from kidney, lung, and colon (Qi *et al.*, 2005; Wolff *et al.*, 2005). Therefore, it is desirable to be able

to determine the molecular identity of P2Y receptors in endothelial and epithelial cell types used in this study.

Functional studies have revealed that human bladder cancer epithelial cell line, ECV304 express a P2Y₂-like receptor (Brown *et al.*, 2000). Further studies on this cell line have shown that P2Y₂ receptors, through activation of PLC (phospholipase C) and mobilisation of intracellular calcium, stimulate production of nitric oxide. In addition, these studies reported evidence of the presence of a second P2Y receptor coupled to adenylate cyclase (AC) stimulation (Brown, 2001).

Studies of the human endothelial cell line EAhy926 reported that nucleotides stimulate activation of MAP kinase in a protein kinase C-dependent manner through interaction with a P2Y₂ receptor (Graham *et al.*, 1996a). Furthermore, the P2Y₂ agonist UTP stimulated inhibitory effect on tumor necrosis factor α (TNF α) - stimulated stress activated protein kinases such as JNK and p38 MAP kinase activity in EAhy926 cells was thought to involve Ca²⁺ independent PKC isoforms and the mediation of the P2Y₂ receptor (Paul *et al.*, 2000). Furthermore, ATP, UTP and UDP showed a dose dependent accumulation of inositol phosphates in EAhy926 cells suggesting the presence of P2Y₂/P2Y₄ or both and P2Y₆ receptor in these studies (see Chapter 3).

The expression of receptors of the P2Y subtype in bovine aortic endothelial (BAE) cells is well established with reported effects being mediated via P2Y₁ and P2Y₂ receptors (see Chapter 3). Although the functional responses ascribed to receptor agonists indicate the type of receptor expressed, the pharmacology is confusing as a single agonist activates more than one receptor. Thus, in order to characterise the endothelial and epithelial cells for their P2Y receptor subtype expression and also to confirm or verify previously published pharmacology a complete expression profile is necessary.

Therefore, RT-PCR studies were used to determine the expression of mRNA transcripts of P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors in ECV304, EAhy926 and BAE cells. These studies also included 1321N1, human astrocytoma cells as a null cell line and BRIN-BD11, insulin-secreting cell line. Currently, there are no reports to indicate that BRIN-BD11 cells express any functional P2Y receptors.

4.2 Materials and Methods

4.2.1 Tissue culture of 1321N1, ECV304, EAhy926, BAE and BRIN-BD11 cells

1321N1, ECV304, EAhy926, BAE and BRIN-BD11 cells were maintained as described in section 2.2.2.

4.2.2 Primer design

The primer sequences for P2Y₁-P2Y₁₁ and GAPDH (Table 4.1) were taken from previously published work (Adrian *et al.*, 2000) and the sequences for P2Y₁₂- P2Y₁₄ were designed using the program Lasergene DNA Star as described in section 2.4.1.

Table 4.1 P2Y₁₋₁₁ receptor specific primers (Adrian *et al.*, 2000)

Primer	Strand	Sequence	Corresponds to nt	GeneBank accession no.
GAPDH	S	5'- cac cac cat gga gaa ggc tgg-3'	366-386	M33197
	AS	5'- gaa gtc aga gga gac cac cag-3'	918-898	
P2Y ₁	S	5'- cgg tcc ggg ttc gtc c-3'	194-209	U42030
	AS	5'- cgg acc cgg gta cct-3'	721-707	
P2Y ₂	S	5'- ctc tac ttt gtc acc acc agc g-3'	750-777	S74902 (U07225)
	AS	5'- ttc tgc tcc tac agc cga atg tcc-3'	1387-1364	
P2Y ₄	S	5'- cca cct ggc att gtc aga cac c-3'	405-426	X91852
	AS	5'- gag tga cca ggc agg gca cgc-3'	829-809	
P2Y ₆	S	5'- cgc ttc ctc ttc tat gcc aac-3'	510-531	U52464
	AS	5'- cca tcc tgg cgg cac agg cgg c-3'	874-853	
P2Y ₁₁	S	5'- cag cgt cat ctt cat cac c-3'	336-354	AF030335
	AS	5'- gct ata cgc tct gta ggc -3'	608-593	

S: sense oligonucleotide; AS: antisense oligonucleotide

Table 4.2 P2Y₁₂₋₁₄ receptor specific primers

Primer	Strand	Sequence	Corresponds to nt	GeneBank accession no.
P2Y ₁₂	S	5'-ctg ggc att cat gtt ctt act ctc-3'	688-711	BC017898
	AS	5'-att ggg gca ctt cag cat act ta-3'	1195-1173	
P2Y ₁₃	S	5'-caa gcc aag aaa atc ata gca gtc-3'	1126-1149	NM_023914
	AS	5'-tta agg ggc aca aaa gca gta gt-3'	1567-1545	
P2Y ₁₄	S	5'-gct cac tgg gca aaa cac ct-3'	125-144	NM_014879
	AS	5'-gat gag ccc aaa gaa cac aat g-3'	557-536	

S: sense oligonucleotide; AS: antisense oligonucleotide

4.2.3 RT-PCR

The protocol for RT-PCR studies for P2Y (P2Y₁₋₁₄) receptor mRNA transcripts is described in section 2.4.2.

4.2.4 Agarose gel electrophoresis

RT-PCR products were analysed by ethidium bromide stained 2% 'Hi-Pure' Low EEO agarose gels (Bio/Gene) as described in section 2.4.2.

4.2.5 PCR product purification

The PCR products were purified using GenElute™ PCR Clean-Up Kit as described in section 2.4.3.

4.2.6 DNA sequencing

DNA sequencing of the purified PCR products was performed by the Sequencing Service at the School of Life Sciences of University of Dundee as described in section 2.4.4.

4.2.7 Database search

The DNA sequence of the PCR products for P2Y receptor subtypes were used to run a database search. They were aligned with the reference mRNA sequence used for primer designing using BLAST bl2seq sequence alignment program.

4.3 Results

4.3.1 RT-PCR studies on 1321N1, ECV304, EAhy926, BAEC and BRIN-BD11 cells

Expression of cloned human P2Y receptors were analysed in RNA samples from 1321N1, ECV304, EAhy926, BAEC and BRIN-BD11 by a semi quantitative RT-PCR assay using P2Y receptor subtype specific primers. The mRNA expression was also determined for GAPDH, a housekeeping gene in these cells. The resultant RT-PCR products were eluted from the ethidium bromide stained 2% 'Hi-Pure' Low EEO agarose and analysed by sequencing. All the experiments were repeated three times using independent batches of cells.

4.3.2 Determining the presence of P2Y₁ transcripts

Evidence of P2Y₁ mRNA transcripts with a strong band of the expected size of 527bp was detected only in ECV304 cells (Figure 4.1). However, 1321N1 (Figure 4.1), EAhy926, BAEC and BRIN-BD11 cells did not show evidence of P2Y₁ transcripts (Figure 4.2). Both the images represent one run of the experiment.

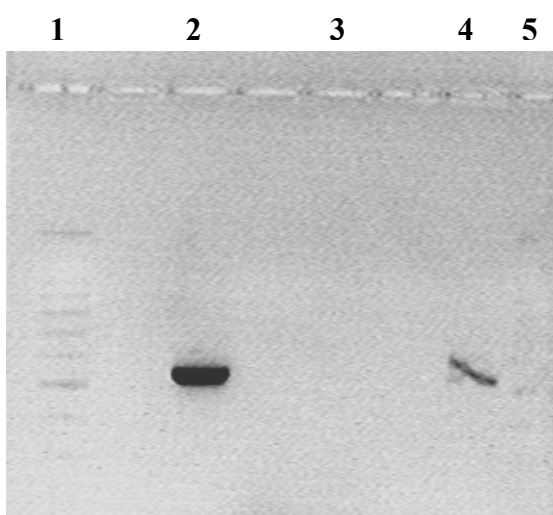


Figure 4.1 RT-PCR analysis of P2Y₁ (527bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (527bp); Lane 5. 100bp markers.

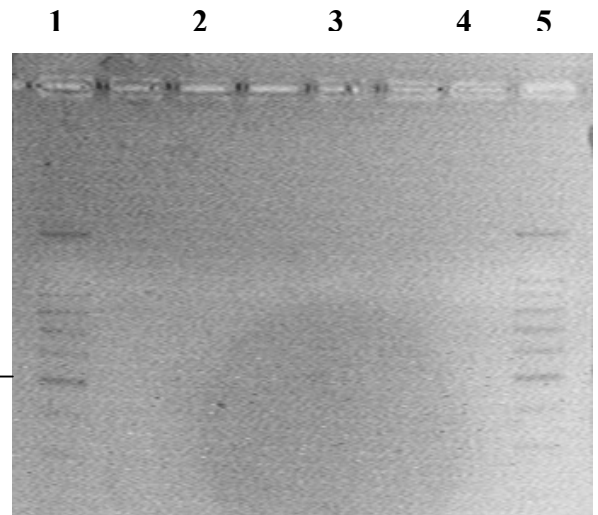


Figure 4.2 RT-PCR analysis of P2Y₁ (527bp) mRNA transcripts. Lane 1. 100bp markers; Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.3 Determining the presence of P2Y₂ transcripts

P2Y₂ mRNA transcripts with strong bands of 637bp size were detected in ECV304 (Lane 4. Figure 4.3) and EAhy926 cells (Lane 2. Figure 4.4). Evidence of P2Y₂ mRNA transcripts was not detected in 1321N1 (Figure 4.3), BAEC and BRIN-BD11 cells (Figure 4.4). Both the images represent one run of the experiment.

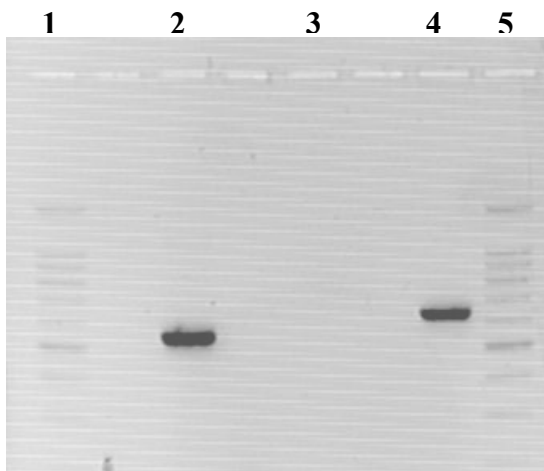


Figure 4.3 RT-PCR analysis of P2Y₂ (637bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (637bp); Lane 5. 100bp markers.

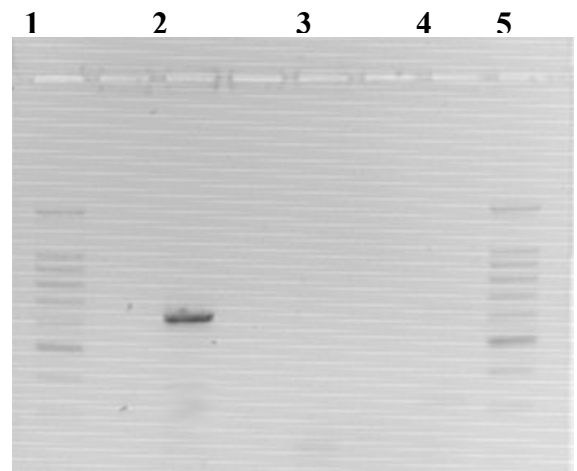


Figure 4.4 -PCR analysis of P2Y₂ (637bp) mRNA transcripts. Lane 1. 100bp markers; Lane 2. EAhy926 sample (637bp); Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.4 Determining the presence of P2Y₄ transcripts

P2Y₄ mRNA transcripts with a strong band of 424bp were observed in ECV304 cells (Lane 4. Figure 4.5). However, 1321N1 (Figure 4.5), EAhy926, BAEC and BRIN-BD11 cells did not show evidence of P2Y₄ mRNA transcripts (Figure 4.6). Both the images represent one run of the experiment.

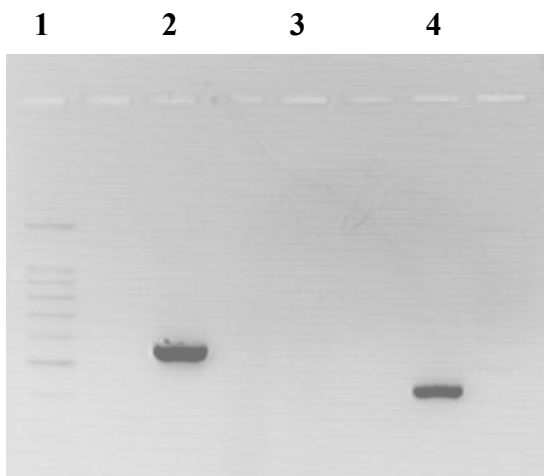


Figure 4.5 RT-PCR analysis of P2Y₄ (424bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (424bp).

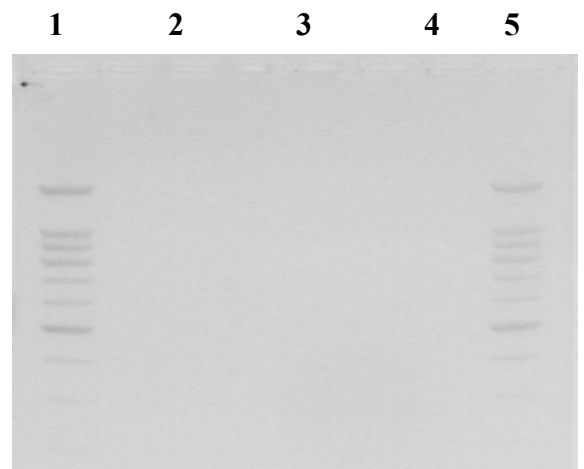


Figure 4.6 RT-PCR analysis of P2Y₄ (424bp) mRNA transcripts. Lane 1. 100bp markers; Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.5 Determining the presence of P2Y₆ transcripts

P2Y₆ mRNA transcripts with bands in expected size of 365bp were detected in ECV304 (Lane 4. Figure 4.7) and EAhy926 cells (Lane 2. Figure 4.8). However, 1321N1 (Figure 4.7), BAEC and BRIN-BD11 cells did not show evidence of P2Y₆ mRNA transcripts (Figure 4.8). Both the images represent one run of the experiment.

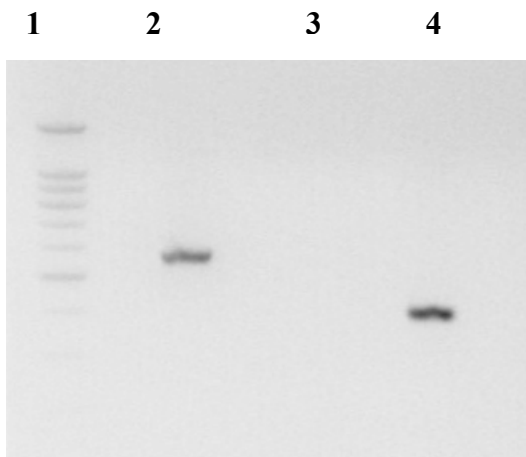


Figure 4.7 RT-PCR analysis of P2Y₆ (365bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (365bp).

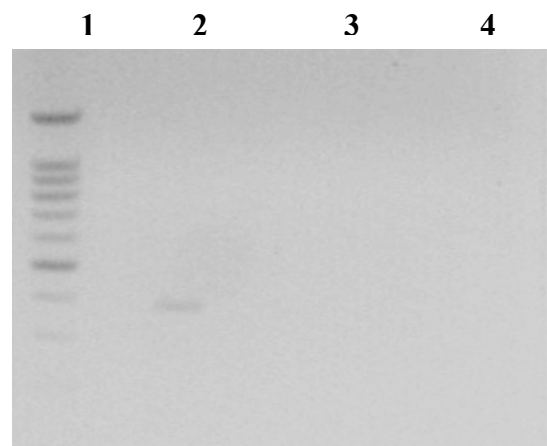


Figure 4.8 RT-PCR analysis of P2Y₆ (365bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. EAhy926 sample (365bp); Lane 3. BAE sample; Lane 4. BRIN-BD11 sample.

4.3.6 Determining the presence of P2Y₁₁ transcripts

Evidence for P2Y₁₁ mRNA transcripts with a weak band of 272bp was observed in ECV304 cells (Lane 3. Figure 4.9). There was no evidence for P2Y₁₁ mRNA transcripts in 1321N1 (Figure 4.9), EAhy926, BAEC and BRIN-BD11 cells (Figure 4.10). Both the images represent one run of the experiment.

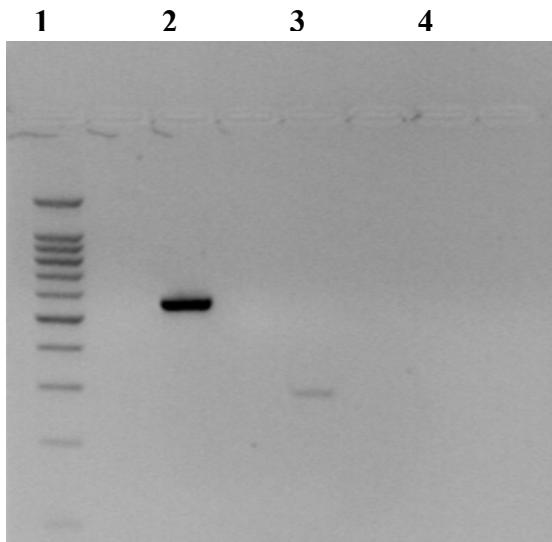


Figure 4.9 RT-PCR analysis of P2Y₁₁ (272bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. ECV304 sample (272bp); Lane 4. 1321N1 sample.

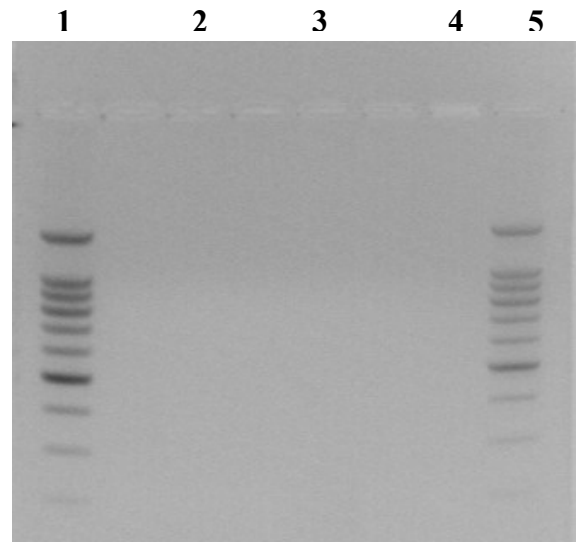


Figure 4.10 RT-PCR analysis of P2Y₁₁ (272bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.7 Determining the presence of P2Y₁₂ transcripts

P2Y₁₂ mRNA transcripts with band of 507bp were detected in ECV304 cells (Lane 4. Figure 4.11). However, 1321N1 (Figure 4.11), EAhy926, BAEC and BRIN-BD11 cells did not show evidence of P2Y₁₂ mRNA transcripts (Figure 4.12). Both the images represent one run of the experiment.

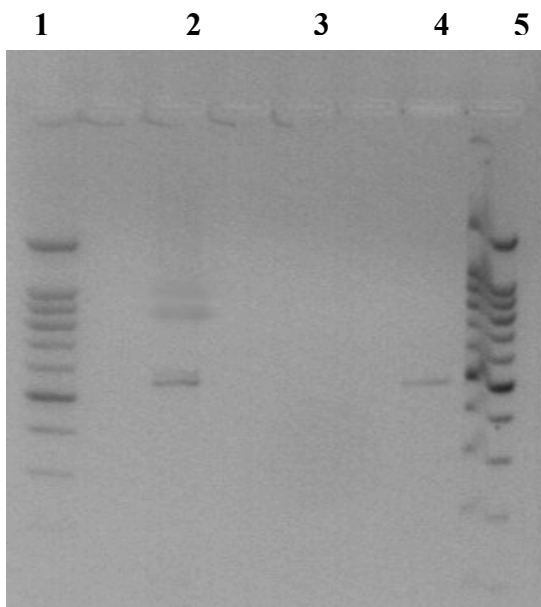


Figure 4.11 RT-PCR analysis of P2Y₁₂ (507bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (507bp); Lane 5. 100bp markers.

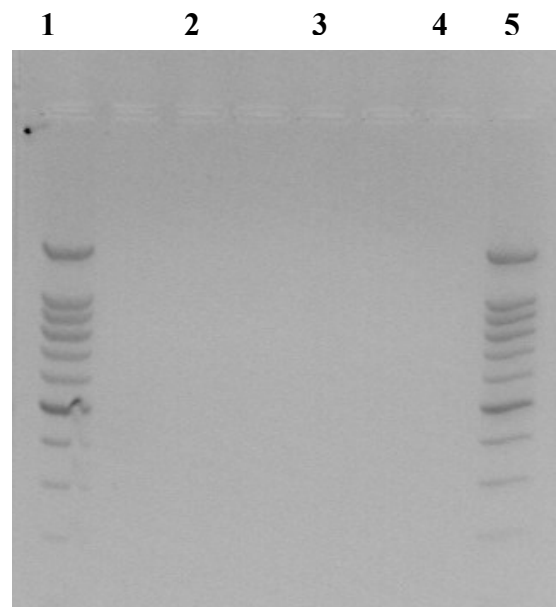


Figure 4.12 RT-PCR analysis of P2Y₁₂ (507bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.8 Determining the presence of P2Y₁₃ transcripts

P2Y₁₃ mRNA transcripts with a strong band of 441bp were detected in ECV304 cells (Lane 4. Figure 4.13). However, 1321N1 (Figure 4.13), EAhy926, BAEC and BRIN-BD11 cells did not show evidence of P2Y₁₃ mRNA transcripts (Figure 4.14). Both the images represent one run of the experiment.

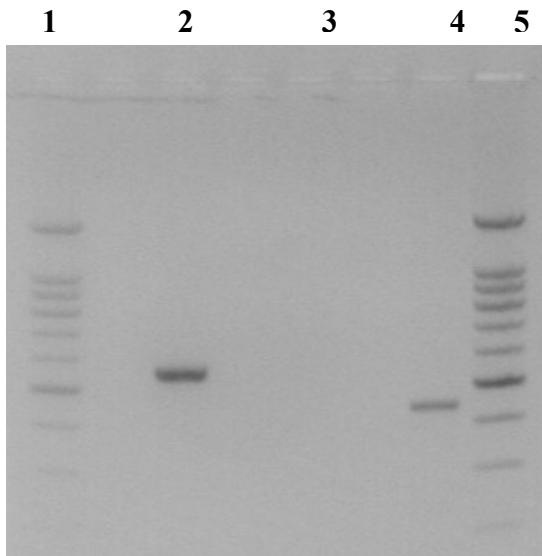


Figure 4.13 RT-PCR analysis of P2Y₁₃ (441bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample (441bp); Lane 5. 100bp markers.

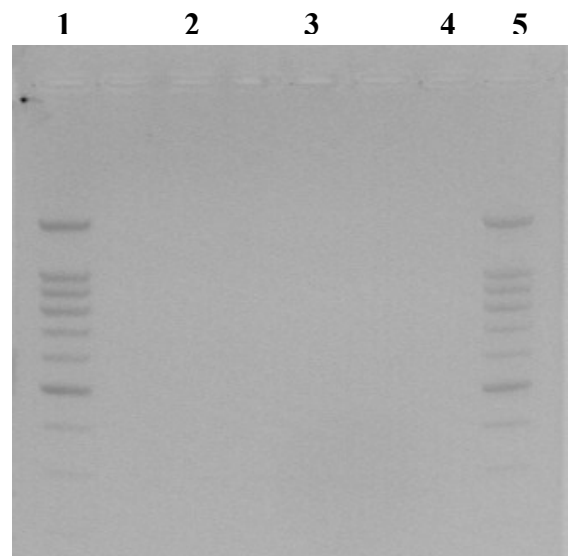


Figure 4.14 RT-PCR analysis of P2Y₁₃ (441bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

4.3.9 Determining the presence of P2Y₁₄ transcripts

P2Y₁₄ mRNA transcripts (the PCR product size of 432bp) were not detected in 1321N1, ECV304, EAhy926, BAEC and BRIN-BD11 cells after amplification with P2Y₁₄ specific primers (Figure 4.15 and Figure 4.16). Both the images represent one run of the experiment.

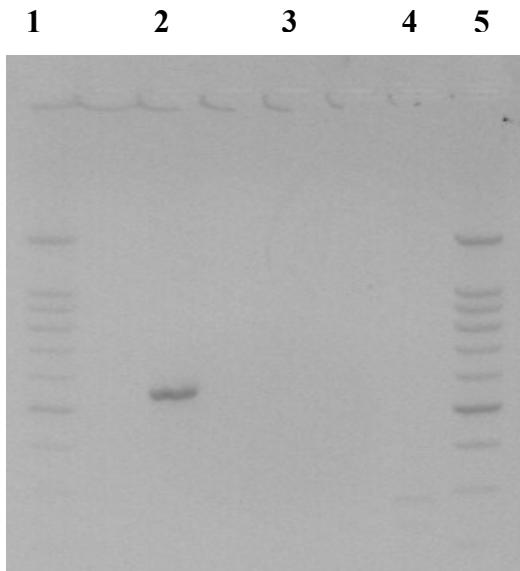


Figure 4.15 RT-PCR analysis of P2Y₁₄ (432bp) mRNA transcripts. Lane 1. 100bp markers; Lane 2. GAPDH (552bp); Lane 3. 1321N1 sample; Lane 4. ECV304 sample; Lane 5. 100bp markers.

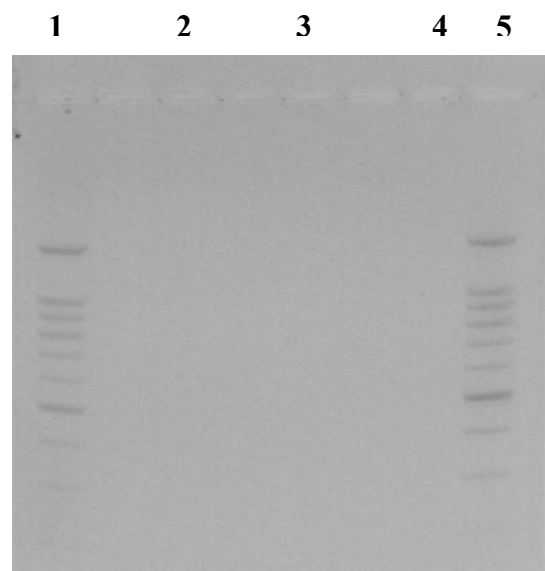


Figure 4.16 RT-PCR analysis of P2Y₁₄ (433bp) mRNA transcripts. Lane 1. 100bp markers. Lane 2. EAhy926 sample; Lane 3. BAE sample; Lane 4. BRIN-BD11 sample. Lane 5. 100bp markers.

Table 4.3 Summary of RT-PCR results of P2Y receptor subtypes in 1321N1, ECV304, EAhy926, BAEC and BRIN-BD11 cells

Receptor Type	Cell Type				
	1321N1	ECV304	EAhy926	BAEC	BRIN-BD11
P2Y ₁	-	+	-	-	-
P2Y ₂	-	+	+	-	-
P2Y ₄	-	+	-	-	-
P2Y ₆	-	+	+	-	-
P2Y ₁₁	-	+	-	-	-
P2Y ₁₂	-	+	-	-	-
P2Y ₁₃	-	+	-	-	-
P2Y ₁₄	-	-	-	-	-

**+: Presence of P2Y receptor subtype transcripts;
- : Absence of P2Y receptor subtype transcripts**

4.3.10 Database search

The DNA sequence of the RT-PCR products for P2Y receptor subtypes aligned with the reference mRNA sequence used for primer designing using BLAST bl2seq sequence alignment program shows the sequence similarity as below.

Table 4.4 Summary of RT-PCR-DNA sequence alignment of P2Y receptor subtypes with reference sequence in ECV304 cells

RT-PCR Product	Primer Strand	bl2seq Alignment: Percent Sequence Identities
P2Y ₁	S	404/463 (87%)
	AS	474/480 (98%)
P2Y ₂	S	593/597 (99%)
	AS	574/579 (99%), Gaps = 1/579 (0%)
P2Y ₄	S	352/364 (96%), Gaps = 4/364 (1%)
	AS	371/374 (99%), Gaps = 3/374 (0%)
P2Y ₆	S	232/238 (97%), Gaps = 2/238 (0%)
	AS	275/278 (98%), Gaps = 2/278 (0%)
P2Y ₁₁	S	223/227 (98%), Gaps = 2/227 (0%)
	AS	224/228 (98%), Gaps = 1/228 (0%)
P2Y ₁₂	S	455/458 (99%)
	AS	456/457 (99%), Gaps = 1/457 (0%)
P2Y ₁₃	S	324/396 (81%), Gaps = 1/396 (0%)
	AS	331/374 (88%)

S: sense oligonucleotide; AS: antisense oligonucleotide

Table 4.5 Summary of RT-PCR-DNA sequence alignment of P2Y receptor subtypes with reference sequence in EAhy926 cells

RT-PCR Product	Primer Strand	bl2seq Alignment: Percent Sequence Identities
P2Y ₂	S	416/487 (85%)
	AS	439/515 (85%)
P2Y ₆	S	214/249 (85%)
	AS	258/296 (87%)

S: sense oligonucleotide; AS: antisense oligonucleotide

4.4 Discussion

These results establish the presence of mRNA transcripts for P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃ in ECV304 cells. The presence of P2Y₁ mRNA in ECV304 cells could be supported by previous published results (Conant *et al.*, 1998). However, these receptor subtypes were also shown to be absent in ECV304 cells based on functional responses to 2MeSATP, a potent agonist of P2Y₁ (Conant *et al.*, 1998). This inconsistency in the ability of the agonist to produce cytosolic calcium responses but not to induce inositol phosphate accumulation had been attributed to a sub – population or passage specific effect on these cells (Conant *et al.*, 1998; Brown *et al.*, 2000). Similarly, 2MeSATP has been reported to induce a small increase in the intracellular calcium release whereas the P2Y₁ selective agonist 2MeSADP was without effect (Brown, 2001).

However, in this study the presence of P2Y₁ mRNA was consistent in cells taken from different batches. In addition, the DNA sequence results for RT-PCR product of the P2Y₁ receptor in ECV304 cells when aligned with the primer specific mRNA sequence using the BLAST bl2seq sequence alignment program, showed a high percentage (>85%) of sequence similarity. Western blot studies with an anti-P2Y₁ antibody could clarify whether the transcribed receptor has been translated into functional receptor. It may be that the transcribed P2Y₁ receptor has a low receptor reserve. The receptor reserve is reported to affect the degree of agonist/antagonist activity that has been reported for P2Y₁ receptor (Palmer *et al.*, 1998).

The detection of P2Y₂ mRNA and the subsequent DNA sequence results in ECV304 cells is consistent with previous reports which showed UTP and ATP as equipotent agonists at P2Y₂ receptors which induced inositol phosphate accumulation and calcium mobilisation (Brown *et al.*, 2000). In this study P2Y₄ and P2Y₆ mRNA were observed in ECV304 cells and RT-PCR-DNA sequence alignment with reference sequence showed >95% similarity. However, previous studies with the potent P2Y₆ agonist, UDP failed to elicit accumulation inositol phosphates and cytosolic calcium response in ECV304 cells (Brown *et al.*, 2000). This needs to be clarified with immunoblotting studies with anti-P2Y₆ antibodies.

The detection of P2Y₁₁ mRNA in ECV304 cells in these studies is consistent with previous pharmacological studies of dose-dependent accumulation of inositol phosphates in these cells in response to ADPβS, a P2Y₁ and P2Y₁₁ agonist (Communi *et al.*, 1999b; Brown, 2001). Previous studies on ECV304 cells reported that these cells express receptors coupled to adenylyl cyclase (Brown *et al.*, 2000). Later studies have reported the accumulation of cAMP in response to ADPβS in ECV304 cells (Brown, 2001). Furthermore, the small increase in the intracellular calcium release by 2MeSATP reported in ECV304 cells may be mediated by P2Y₁₁ receptors (Brown, 2001). Finally, DNA sequence results for RT-PCR product of P2Y₁₁ receptor showed >98% similarity with primer specific mRNA sequence.

Previous studies with human P2Y₁₁ receptors stably expressed in Chinese hamster ovary cells (CHO-K1) and 1321N1 human astrocytoma cells showed the potency order ATPγS ≥ 2MeSATP ≥ ATP ≈ ADPβS > 2MeSADP > ADP in promoting both inositol phosphate and cyclic AMP accumulation (Qi *et al.*, 2001). These studies suggested that human P2Y₁₁ receptor was potently activated by adenosine triphosphate nucleotides rather than their corresponding diphosphates, which acted as partial agonists. This further supports the idea that increases in the intracellular calcium release observed in response to 2MeSATP reported in ECV304 cells was mediated by P2Y₁₁ receptors (Brown, 2001). Furthermore, the dose dependent functional responses observed in these studies with more selective P2Y₁₁ agonists like BzATP and ATPγS provide evidence for the functional expression of this receptor (see Chapter 3).

The DNA sequence results for RT-PCR product showed >99% (P2Y₁₂) and >81% (P2Y₁₃) similarity with primer specific mRNA sequences. So far, there are no published reports which suggest that P2Y₁₂ and P2Y₁₃ receptors are expressed in ECV304 cells and/or these cells express receptors that are coupled to the inhibition of adenylyl cyclase. Previous studies did not find evidence for expression of P2Y receptors linked to the inhibition of adenylyl cyclase in these cells (Brown *et al.*, 2000). Nonetheless, the potent agonist of these receptors, ADP, has shown weak agonistic potency in ECV304 cells (Brown *et al.*, 2000; Zhang *et al.*, 2001). In addition, accumulation of inositol phosphates and a rise in intracellular calcium release is evoked by ADPβS, a common agonist of P2Y₁₂ and P2Y₁₃ receptors. However, previous studies on ECV304

cells also reported that 2MeSADP, the potent agonist of P2Y₁, P2Y₁₂ and P2Y₁₃ receptors was without effect. Furthermore, 2MeSATP induced only a small increase in the intracellular calcium release, thus highlighting the inability of nucleotides to produce conclusive pharmacological evidence to determine the existence of the functional P2Y receptors (Brown, 2001). It would be interesting to see if stimulation of ECV304 cells with 2MeSADP and 2MeSATP reduce the levels of cAMP in order to determine whether ECV304 cells express functional receptors that are coupled to the inhibition of adenylyl cyclase.

According to this study EAhy926 cells show the presence of mRNA for P2Y₂ and P2Y₆ receptors. In addition, the DNA sequence of the RT-PCR products for P2Y₂ and P2Y₆ receptor subtypes when aligned with the reference mRNA sequence used for primer designing using BLAST bl2seq sequence alignment program has shown a high percentage (>85%) of similarity. The presence of P2Y₂ in EAhy926 supports previous reports that nucleotides stimulate activation of MAP kinase in a protein kinase C-dependent manner through interaction with a P2Y receptor (Graham *et al.*, 1996a). Further studies have also reported that P2Y₂ receptor mediated inhibitory effect on tumor necrosis factor α (TNF α) - stimulated stress activated protein kinases such as JNK and p38 MAP kinase activity in EAhy926 cells involving Ca²⁺ independent PKC isoforms. The P2Y₆ receptor agonist UDP, however, was ineffective in this cell line (Paul *et al.*, 2000). However, studies have shown a dose-dependent accumulation of inositol phosphates in response to UDP in EAhy926 cells (see Chapter 3).

Bovine aortic endothelial cells have been shown to express of both P2Y₁ and P2Y₂ receptors coupled to Gq/G₁₁ and G₁ proteins (Wilkinson *et al.*, 1993; Piroton *et al.*, 1996). However, P2Y transcripts were not observed in these cells under the conditions which showed presence of mRNA for P2Y receptors in ECV304 and EAhy926 cells when human P2Y receptor sequence specific primers were used. Further RT-PCR studies with bovine P2Y receptor specific primers in these cells are needed to investigate the expression of P2Y receptor mRNA transcripts. Bovine corneal endothelial cells (BCECs) showed expression of mRNAs for P2Y₁ and P2Y₂ receptors (Gomes *et al.*, 2005).

Expression for P2Y receptor transcripts in 1321N1 and BRIN-BD11 cells were not observed. However, previous reports of the presence mRNA for P2Y₆ and P2Y₁₁ in 1321N1 cells using TaqMan – quantitative RT–PCR analysis is said to be due to the clonal variations in the cells used in that study (Moore *et al.*, 2001). The P2Y primer sequences were compared with the Genbank using Basic Local Alignment Search Tool (BLAST). No significant similarities were found between human P2Y primer pair sequence with bovine P2Y receptors. Similarly, the human P2Y primer pair sequences showed no homology to the rat P2Y receptor gene suggesting that these primers are not suitable for amplifying bovine and rat P2Y PCR products.

Overall, these RT-PCR studies have given a thorough profile of the P2Y receptor transcripts in 1321NI, ECV304 and EAhy926 cells. It has been observed that the studies to determine the presence of mRNA transcripts should only be used to indicate the presence or absence of receptor transcripts and not to prove the functional level of protein expression (Moore *et al.*, 2001). That may be one of the reasons why P2Y₁, P2Y₆, P2Y₁₂ and P2Y₁₃ receptor selective agonists were unable to stimulate second messengers in EV304 cells. Further studies with selective ligands or western blot analysis using P2Y receptor specific antibodies would help to determine the functional level of protein expression. Moreover, RT-PCR studies with species specific P2Y primers could elucidate the expression of P2Y receptor subtypes in BAE and BRIN-BD11 cells.

CHAPTER 5

PURIFICATION OF POLYCLONAL ANTIBODIES

5.1 Introduction

Peptides designed to mimic the defined regions of extracellular loops of peptide receptor (rat liver V1a vasopressin receptor), which share the sequence homology with P2Y receptor, have been shown to act like functional agonists, suggesting a role for extracellular loops in ligand binding (Van Rhee *et al.*, 1995; Howl and Wheatley, 1996). Further studies indicated that for peptide receptors the neuropeptide binding involves residues on the top of several transmembrane domains and in extracellular loops of the receptors while the non peptide type ligands to the same receptors tend to bind deeper in the plane of the membrane, between several transmembrane domains suggesting the importance of the extracellular loops (ELs) for ligand recognition (Berthold and Bartfai, 1997).

For human P2Y₁ receptors, it has been shown that positively charged and other conserved residues in transmembrane (TM) regions 3, TM6, and TM7 e.g. Arg¹²⁸, Arg³¹⁰, and Ser³¹⁴, are critical for the activation by nucleotides (Moro *et al.*, 1998). Mutational analysis of P2Y₁ receptors suggested that positively charged residues on the exofacial side of TM3 and TM7 are involved in binding the negatively charged phosphate groups of nucleotides (Jiang *et al.*, 1997; Jacobson *et al.*, 1999). The mutation of the basic amino acid lysine (Lys²⁸⁰) on TM6 of the human P2Y₁ receptor has also shown to affect the potency and affinity of receptor antagonists (Guo *et al.*, 2002). Site-directed mutagenesis of P2Y₂ receptors suggested that positively charged amino acids histidine²⁶² and arginine²⁶⁵ on TM6 and TM7 are involved in nucleotide binding (Erb *et al.*, 1995). Additionally, it was also reported that the presence of two critical disulfide bridges (first between the EL2 and TM3 and the second between EL3 and the N-terminal domain) and charged residues in the extracellular domains are involved in ligand recognition and activation of P2Y₁ receptor (Hoffmann *et al.*, 1999).

Based on this evidence, previous work in the lab had focussed on design, synthesis and subsequent evaluation of peptides specifically mimicking the extracellular regions of the human P2Y₂ receptor (these peptides were designed to be specific to the extracellular loops of the human P2Y₂ receptor subtype). In particular, a peptide (L247) mimicking the third extra cellular loop has been shown to stimulate nitric oxide synthase activity in BAE cells (Brown, 2001). In addition, L247 was reported to act like a weak agonist of

inositol phosphate production when added alone, but when added simultaneously with ATP has shown synergistic effects on inositol phosphate production and intracellular calcium release in ECV304 cells. This synergistic effect was specific to purinergic and pyrimidinergic agonists with no such effects being observed upon the simultaneous addition of L247 and histamine. L247 was equipotent to 100 μ M ATP in activating phospholipase D in porcine aortic endothelial cells (Brown, 2001).

These results prompted the commissioning of polyclonal antisera from a commercial source (Severn Biotech) which were raised in rabbit using L247 peptide as an immunogen. This study presents purification of this antisera by affinity chromatography and evaluation by indirect ELISA. Affinity purification has been used as the method of choice for antibody purification; this relies on the structure of immunoglobulin and its specificity for the antigen (Huse *et al.*, 2002). However, ligands other than those used for immunization have been used effectively for affinity purification. For example, thiophilic-adsorption chromatography, in which the presence of high concentrations of lyotropic salts facilitate selective adsorption of proteins on to thiophilic adsorbents. This has been used for the purification of murine monoclonal antibodies from hybridoma cell culture (Finger *et al.*, 1996) and in the separation of immunoglobulins from bovine colostrum and milk whey (Hutchens *et al.*, 1990; Konecny *et al.*, 1994). Thiophilic chromatography has also been used to separate antibodies from cell-culture supernatants and ascites fluids (Belew *et al.*, 1987; Sulk *et al.*, 1992; Bog-Hansen, 1997).

PROSEP-Thiosorb, a thiophilic affinity adsorbent is prepared by the covalent attachment of a nonbiological thiophilic ligand to a porous glass (glass particles permeated by interconnecting pores of uniform and precisely controlled size) of 75 -125 micron. PROSEP-Thiosorb selectively binds protein regions common to all immunoglobulins and has a static binding capacity of 8 mg/ml for polyclonal Rabbit IgG. This matrix binds antibodies in the presence of high concentrations of lyotropic salts such as ammonium sulphate or potassium sulphate which influences the strength of the interaction. Due to the nonbiological nature of ligand, the possible contamination by leachable protein is eliminated. Furthermore, synthetic adsorbent ligands have been shown to retain binding capacity even after subjecting them to thorough cleaning after the run (Bak and Thomas, 2007). Furthermore, mouse MAbs (Monoclonal antibodies)

from cell culture supernatants have been successfully affinity purified using PROSEP-Thiosorb M and has been optimized for the purification of both monoclonal and polyclonal immunoglobulin M (IgM) (Waldron *et al.*, 2002; Nelson *et al.*, 2005). Therefore, PROSEP-Thiosorb was used in this study to purify rabbit polyclonal antibody by employing thiophilic-adsorption chromatography technique.

5.2 Materials and Methods

5.2.1 Buffer preparation

Wash Buffer: 20 mM HEPES, 7.5% $(\text{NH}_4)_2\text{SO}_4$ and 0.5M NaCl, pH 7.5

Elution Buffer 1(A): 20mM HEPES, 1M NaCl, pH 7.5

Elution Buffer 2(B): 20mM HEPES, 2M NaCl, pH 7.5

Elution Buffer 3: 60% Ethylene glycol)

Regeneration Solution: 6M Urea

5.2.2 Sample preparation

Sufficient reagent was added to the starting material (serum or cell culture supernatant) so that it contained 20 mM HEPES, 7.5% $(\text{NH}_4)_2\text{SO}_4$ and 0.5M NaCl. The pH of the solutions were adjusted to 7.5 using 5M NaOH and the solution was filtered through a 0.45 or 0.2 μm filter

5.2.3 Affinity chromatography

The polyclonal antisera was purified by affinity chromatography as described in section 2.5.2.

5.2.4 Dialysis

The purified fractions were then dialysed as described in section 2.5.3.

5.2.5 Bicinchoninic acid protein assay

The concentration of purified antibody fractions was quantified by Bicinchoninic Acid Protein Assay as described in section 2.5.4.

5.2.6 Indirect ELISA

Indirect ELISA was performed as described in section 2.5.5.

5.3 Results

5.3.1 Thiophilic-adsorption chromatography

The terminal bleed of L247 polyclonal antisera was affinity purified using a PROSEP-Thosorb (Millipore) affinity adsorbent column in BioLogic HR Chromatography System (Bio-Rad). Table 5.1 elucidates the gradual plan of sample injection and buffer loading on BioLogic HR Chromatography System. Various fractions were collected during the elution stages and were analysed for protein contents and assayed for antibody immunoreactivity by indirect Elisa.

Table 5.1 Biologic HR run report

Step Number	Start (h:m)	Step
1	0:00.0	Collect Fractions of size 1.00 m above 0.1000 threshold during entire run
2	0:00.0	Turn UV lamp on
3	0:00.0	Set UV baseline to 0.0
4	0:00.0	Turn Chart Recorder ON
5	0:00.0	Set UV baseline to 0.0
6	0:00.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 15.0 min
7	0:15.0	Static loop: inject 5.0 ml sample at 1.00 ml/min for 5.0 min
8	0:20.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 5.0 min
9	0:25.0	Static loop: inject 5.0 ml sample at 1.00 ml/min for 5.0 min
10	0:30.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 5.0 min
11	0:35.0	Static loop: inject 5.0 ml sample at 1.00 ml/min for 5.0 min
12	0:40.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 5.0 min
13	0:45.0	Static loop: inject 5.0 ml sample at 1.00 ml/min for 5.0 min
14	0:50.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 5.0 min
15	0:55.0	Static loop: inject 5.0 ml sample at 1.00 ml/min for 5.0 min
16	1:00.0	Isocratic Flow with Wash Buffer at 1.50 ml/min for 45.0 min
17	1:45.0	Linear Gradient with 0% to 100% Elution Buffer 2 (100% to 0% Elution Buffer 1) at 1.50 ml/min for 30.0 min
18	2:15.0	Linear Gradient with 100% to 0% Elution Buffer 2 (0% to 100% Elution Buffer 1) at 1.50 ml/min for 30.0 min
19	2:45.0	Isocratic Flow with Elution Buffer 3 at 1.50 ml/min for 30.0 min
20	3:15.0	Turn Chart Recorder ON
21	3:15.0	Sound Alarm
22	3:15.0	End of Protocol
23	3:15.0	Isocratic Flow with Regeneration Solution at 1.50 ml/min for 30.0 min

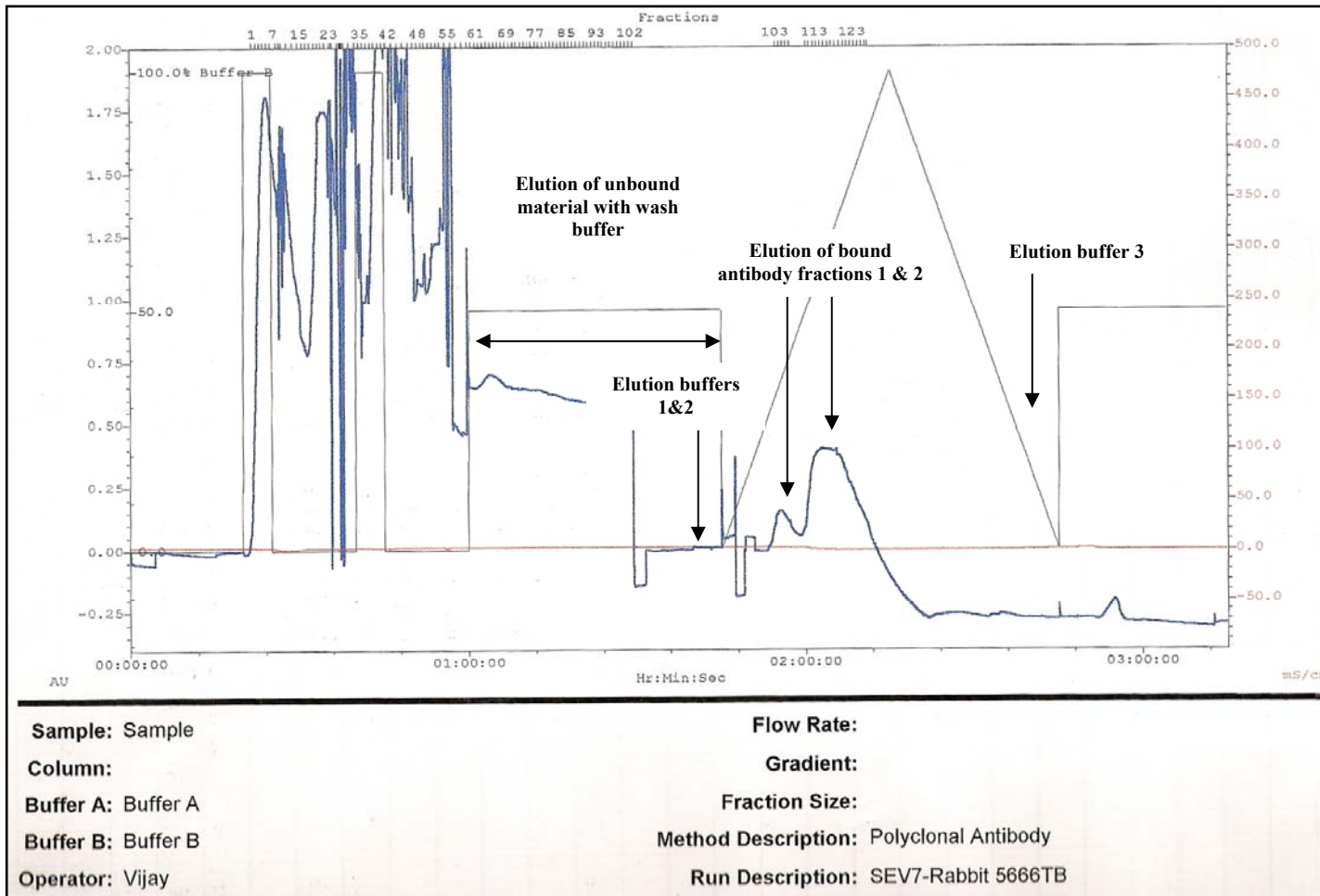


Figure 5.1 A profile of thiophilic-adsorption chromatography run performed on L247 polyclonal antisera using a BioLogic HR Chromatography System (Bio-Rad) and PROSEP- Thosorb (Millipore) affinity adsorbent column.

5.3.2 Protein quantification

Eluted bound fractions of 1ml each were collected corresponding to the positive UV value peak in the profile run (Fig 5.1) and quantified for the protein/antibody concentration using a Bicinchoninic Acid Protein Assay Kit (BCA1-Sigma). The concentration of the unknown sample was determined by comparing its absorbance at 562 nm to the standard curve prepared using BSA (Bovine Serum Albumin) protein standards. The concentration of fraction I was 1.6mg/ml and that of fraction II was 2.3mg/ml.

5.3.3 Indirect ELISA

Fraction I and fraction II were analyzed for their binding efficiency to L247 peptide by the indirect ELISA method. As expected the antibody fractions at serially diluted concentrations bound to 30µg/ml of L247 peptide in a dose-related manner (Figures 5.2 and 5.3). Significantly, the binding observed for fraction II was four fold higher than that observed for fraction I (Figure 5.4). Control experiments using an unrelated peptide designed to Human endogenous retrovirus K *env* (HERV-K *env*: GKTCPKETPKGKNI) confirmed the specificity of binding to L247 peptide (at 30µg/ml peptide: absorbance 0.036 units for HERV-K *env* peptide versus 0.586 for L247 peptide; and absorbance 1.065 for HERV-K *env* peptide versus 2.670 for L247 peptide). Moreover, there were no significant similarities when L247 peptide sequence (RSLDLSCHTLNAIN) was aligned with HERV-K *env* protein suggesting that the binding of antibody with HERV-K *env* peptide may have been due to non-specific immunoglobulins.

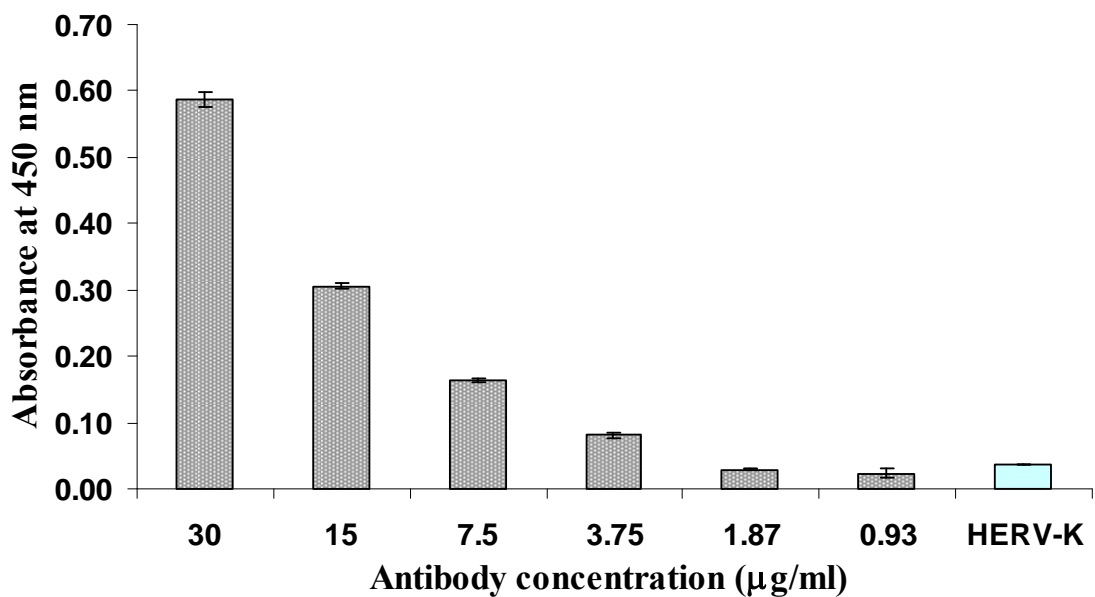


Figure 5.2 Reactivity of serially diluted L247 antibody fraction I against 30µg/ml of L247 peptide and 30µg/ml of L247 antibody fraction I against 30µg/ml of HERV-K *env* peptide. Data are mean results \pm SEM performed in triplicate and one representation of three experiments.

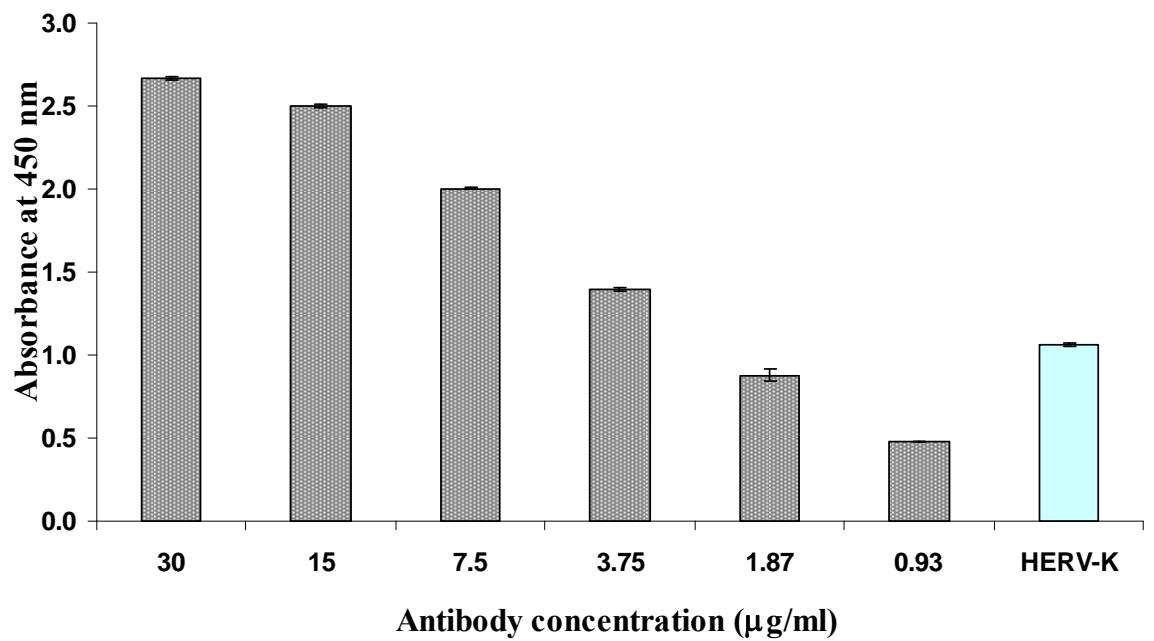


Figure 5.3 Reactivity of serially diluted L247 antibody fraction II against 30µg/ml of L247 peptide and 30ug/ml of L247 antibody fraction II against 30µg/ml of HERV-K *env* peptide. Data are mean results ± SEM performed in triplicate and one representation of three experiments.

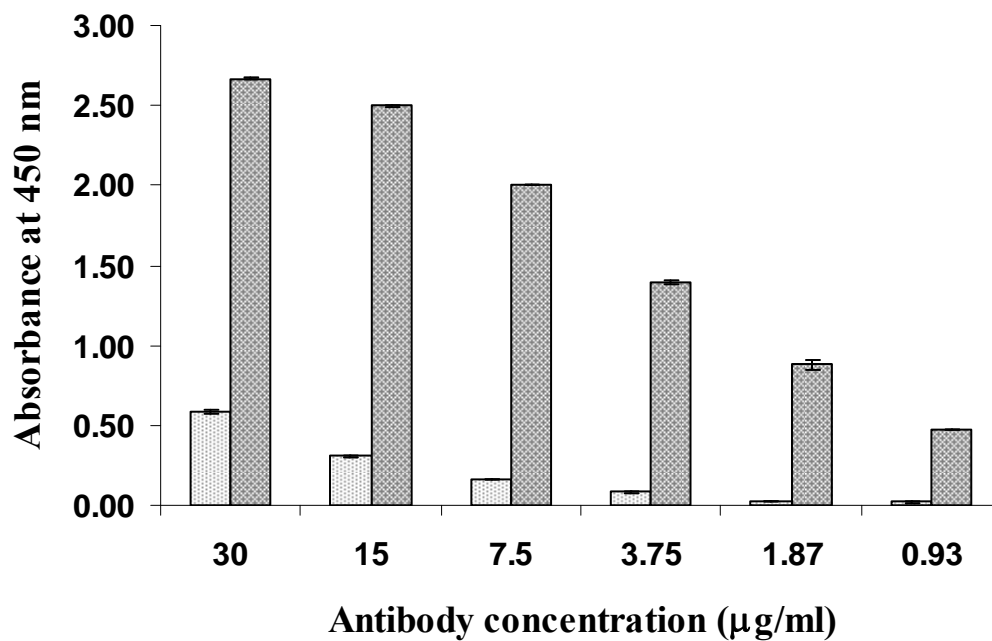


Figure 5.4 Comparison of the relative reactivity of the serially diluted L247 Antibody fraction I (▨) & II (▩) against 30µg/ml of L247 peptide. Data are mean results ± SEM performed in triplicate and one representation of three experiments.

5.4 Discussion

The rabbit polyclonal antiserum was affinity purified using a BioLogic HR Chromatography System (Bio-Rad) and PROSEP- Thiosorb affinity adsorbent columns. Prior to elution of adsorbed protein, the affinity adsorbent columns were washed to remove all the traces of albumin and other contaminants present as non-adsorbed or unbound content. After elution of the unbound material the adsorbed material was eluted to yield two fractions, corresponding to the two elution buffers suggested by adsorbent column manufacturers for the elution of IgG. The incorporation of different elution buffers will have eluted these antibody fractions with differing affinity to the adsorbent column (Muronetz and Korpela, 2003). The purified fractions were then dialysed against multiple buffer changes for the required time before storing them in aliquots at -20°C .

The protein substance of the adsorbed fractions showed that the concentration of fraction II was twice that of fraction I. This may be due to the degree of severity of the elution buffer on bound protein to the columns. To check whether the immunoreactivity of the antibody fractions with the antigen remained unscathed after affinity purification was ascertained with indirect ELISA using immobilized antigen (L247 peptide). The antibody fraction I and fraction II at serially diluted concentrations bound strongly to L247 peptide in a dose-related manner suggests the reaction specificity of the antibody to antigenic immunogen. Significantly, the binding observed for fraction II was four fold higher than that observed for fraction I. Control experiments using an unrelated peptide (designed to HERV-K *env*) confirmed the specificity of binding to L247 peptide. The minimal binding that occurred with HERV-K peptide may be due to cross reactivity of antibody fractions. The presence of non specific immunoglobulins in the fraction II may have accounted for this minimal binding. Isolation of these antibodies with different specificities using chromatography on immobilized synthetic antigen would provide the antibody with desired specificity (Muronetz and Korpela, 2003).

The results from indirect ELISA suggest the successful generation of sequence specific antibody to L247 peptide designed to mimic specific region in the extracellular domain of the human P2Y₂ receptor (Brown, 2001). The ability of the antibody fraction to bind strongly with the antigen (immunogen) suggests the reliability of the purification

system. However, SDS-PAGE, western blot analysis and immunohistochemistry or immunofluorescence experiments should be able to determine whether the purified fractions detect the immunogenic peptide in denatured as well as in their native environment in cells expressing human P2Y₂ receptors.

CHAPTER 6

WESTERN BLOT ANALYSIS OF P2Y RECEPTOR SUBTYPES

6.1 Introduction

RT-PCR studies have shown the presence of mRNA transcripts for P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃ in ECV304 cells. Likewise, RT-PCR studies of EAhy926 cells have indicated the presence of mRNA transcripts for P2Y₂ and P2Y₆ which seems to confirm the predicted profile from the functional studies (Chapter 3). These studies provide a detailed profile of the P2Y receptor transcripts in 1321N1, ECV304 and EAhy926 cells. However, RT-PCR studies do not conclusively prove the functional level of protein expression (Moore *et al.*, 2001). This has been confirmed by both the pharmacological studies and western blot analysis. Therefore, western blot analysis using P2Y receptor specific antibodies will be used to determine the functional protein expression. A point to note is that there were no antibodies available at the beginning of this project; which have subsequently become available during the course of the study.

Thus western blot analysis will be carried out using polyclonal anti-P2Y antibodies for P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃ obtained from commercial sources to establish which P2Y subtypes are expressed and to confirm the pharmacological data and expression of P2Y receptor mRNA transcripts. The novel antibody being used in this investigation is L247 anti-P2Y₂ antibody, which is a rabbit polyclonal antibody, raised to immunogenic peptide L247, which is designed to mimic a specific region of third extracellular loop of human P2Y₂ receptor. Therefore, the main aim of this study is the analysis and characterization of the anti-P2Y₂ antibody by western blot analysis of epithelial ECV304 and endothelial cells EAhy926 and BAE cells, which known to express P2Y₂ receptor. Thus western blot analysis will use immunogenic peptide in the native protein i.e in cells expressing human P2Y₂ receptor to determine the specificity of the antibody fractions. In addition, L247 anti-P2Y₂ antibody will be compared with two other polyclonal anti-P2Y₂ antibodies obtained commercially. These studies have also included 1321N1 cell line as negative control. BRIN-BD11 cells will also be used in addition to BAE cells to study the cross species reactivity of the antibodies.

Moreover, this study is aimed to categorically establish the immunospecificity of L247 anti-P2Y₂ antibody with respect to others.

It is well documented that the consensus pattern for a potential N-glycosylation is Asn-Xaa-Ser/Thr or N - [P] - [ST] - [P], where - Asn (Asparagine) or (N) is the glycosylation site and -Xaa- represents a variable amino acid. However, the presence of the consensus tripeptide is not sufficient to conclude that an Asparagine residue is glycosylated, due to the fact that the folding of the protein plays an important role in the regulation of N-glycosylation (Pless and Lennarz, 1977). The presence of Proline between Asparagine (Asn) and Serine/ Threonine (Ser/Thr) has been shown to inhibit N-glycosylation (Bause, 1983; Gavel and Heijne, 1990). Three potential N-glycosylation sites have been identified in P2Y₂ receptors. These sites are situated at amino acid residues, 9 – 12 (NDTI), 13 – 16 (NGTW), 66 – 69 (NAST). Moreover, the importance of phosphorylation sites in the third intracellular loop and C-terminal tail of the P2Y₂ receptor for agonist induced desensitisation has been well documented (Flores *et al.*, 2005). Therefore it is possible for anti-P2Y₂ antibodies to detect the receptor protein at higher molecular mass. Increased molecular mass has been attributed to N-linked glycosylations in the extracellular domains in P2Y₁ and P2Y₂ receptors (Wang *et al.*, 2002). Likewise, a non-uniform distribution of P2Y₂ receptor at higher molecular mass than that predicted has been reported in HA-tagged P2Y₂ receptors in 1321N1 cells, again ascribed to heterogeneous complex glycosylation (Flores *et al.*, 2005). Moreover, recent studies have shown that N-linked glycosylation of the P2Y₁₂ receptor is essential for signal transduction but not for ligand binding or cell surface expression (Zhong *et al.*, 2004).

Hence this study will incorporate potential deglycosylation analysis and subsequent protein sequencing of protein blots stained by L247 anti-P2Y₂ and other anti-P2Y₂ antibodies to see if the resultant sequence matches that with human P2Y₂ receptor. Alternatively, agonist induced desensitisation will be used to determine the immunospecificity of anti-P2Y₂ antibodies. Additionally immunofluorescence analysis will be used to compare and analyse P2Y₂ receptor localisation.

6.2 Materials and Methods

6.2.1 Tissue culture

ECV304, EAhy926, BAEC, 1321N1 and BRIN-BD11 cells were maintained as described in section 2.2.2.

6.2.2 Protein extraction

The extraction of membrane protein from ECV304, EAhy926, BAEC, 1321N1 and BRIN-BD11 cells was carried out as described in section 2.6.1.

6.2.3 Protein quantification

Extracted membrane protein fractions were quantified for the protein concentration using a DC Protein Assay Kit (BIO-RAD). The concentration of the unknown sample was determined by comparing its absorbance at 690 nm to the standard curve prepared using BSA (Bovine Serum Albumin) protein standards as described in section 2.6.2.

6.2.4 SDS-PAGE and western blot analysis

The protein fractions were analysed by loading 40µg of total protein in each well and reducing them in 10% Sodium dodecyl sulphate-polyacrylamide gel. The protein was then transferred electrophoretically onto nitrocellulose membranes and western blot analysis was performed on these membranes to determine the specificity of antibodies to P2Y receptors and to verify their expression in both human and bovine endothelial cells as described in section 2.6.3.

6.2.5 Antibodies

For details of antibodies used in this investigation see section 2.6.4.

6.2.6 Enzymatic N- deglycosylation

ECV304 cells were deglycosylated using an N-Glycosidase F Deglycosylation Kit as described in section 2.6.5.

6.2.7 Protein sequencing

The protein sequencing was achieved by the established technique of Edman degradation as described in section 2.6.6 and was carried out by Alta Biosciences, University of Birmingham.

6.2.8 Receptor desensitisation

ECV304 cells were grown to 90% confluency in 75cm² flasks under conditions described in section 2.2.2. The cells were incubated with 300µM of UTP and histamine for various time periods. Control cells were incubated without UTP to allow the normal binding of the antibody to the P2Y₂ receptor. Cells were removed from the flasks and the protein was extracted and quantified as described in sections 2.6.1 and 2.6.2 respectively.

6.2.9 Immunofluorescence analysis

The immunofluorescence analysis was performed as described in section 2.7.

6.3 Results

6.3.1 Protein quantification

The membrane protein fractions from ECV304, EAhy926, BAEC, 1321N1 and BRIN-BD1 cells were quantified for their protein concentration using the Bio-Rad *DC* (detergent compatible) protein assay kit. The concentration of the unknown sample was determined by comparing its absorbance at 690 nm to the standard curve prepared using BSA (Bovine Serum Albumin) protein standards, measured with a standard laboratory spectrophotometer or microplate reader.

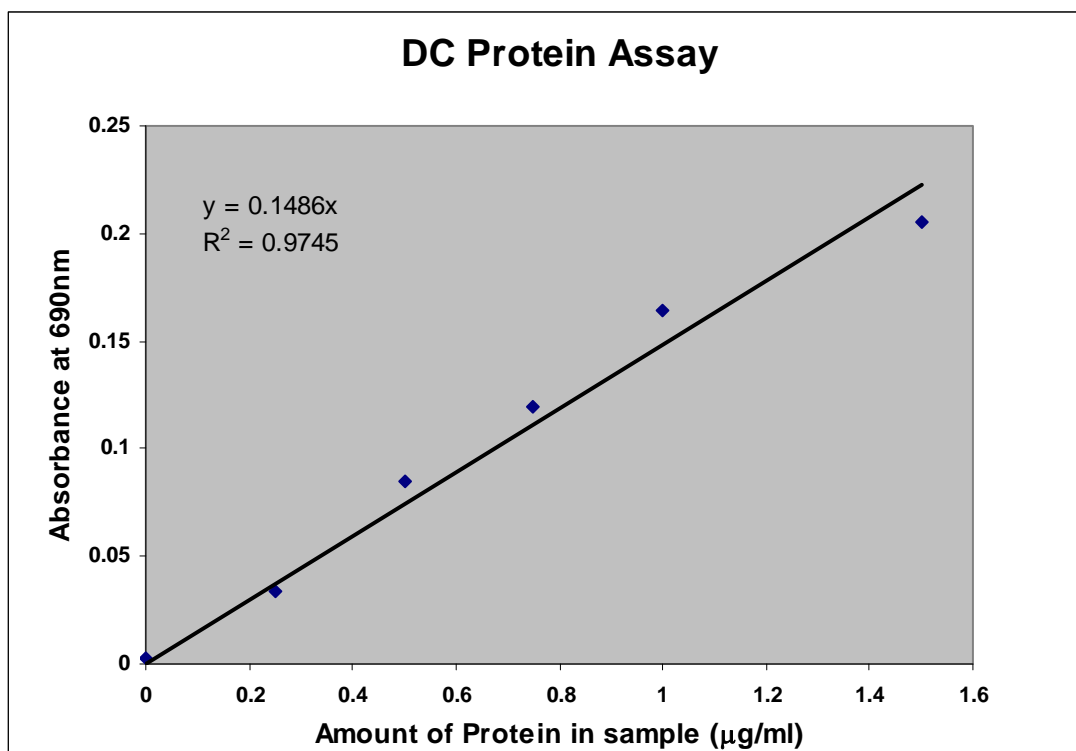


Figure 6.1 A representative BSA (Bovine Serum Albumin) protein standards curve generated to determine the concentration of the unknown protein sample by comparing its absorbance at 690 nm.

6.3.2 Western blot analysis for protein loading control with α -Tubulin

All the Western blotting experiments were performed on at least three different protein extractions from each cell type. As a protein loading control the western blot was performed for α -Tubulin and a strong single band at 50-64kDa (Fig 6.2) was observed in all the cell types.

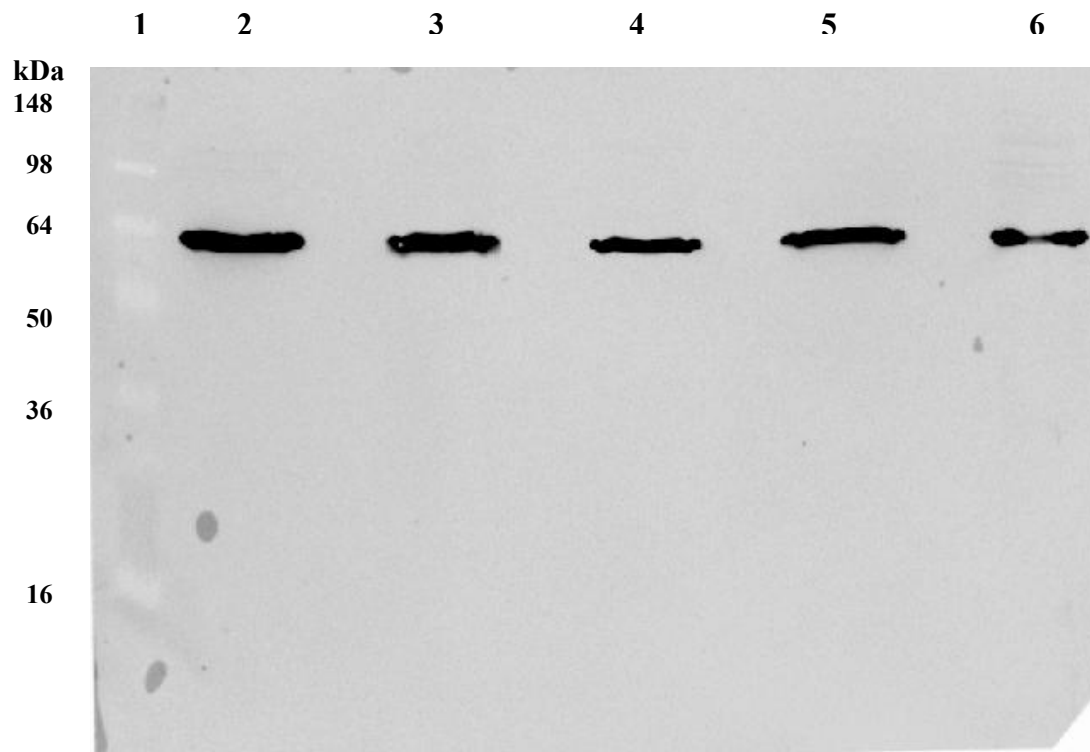


Figure 6.2 Western blot analysis of cell extracts using Mouse Monoclonal Anti- α - Tubulin Clone B-5-1-2 AT 1:2500 dilution as a protein loading control. Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.3 Western blot analysis using anti-P2Y₂ antibodies

Western blot analysis was performed using L247 anti-P2Y₂ antibody, the novel antibody used in this study which was raised in rabbit to a peptide (RSLDLSCHTLNAIN) designed to the third extracellular loop of the human P2Y₂ receptor. ImageQuantTm TL software was used to detect and to calibrate the molecular weights of the bands in western blot analysis using anti-P2Y antibodies. Once the values are assigned to the bands of the standard lane (lane with known values of molecular weight for its bands), the software interpolate and extrapolate contour lines of known molecular weight horizontally across the gel image. After the horizontal contours have been determined, the software uses the selected curve-fitting algorithm to determine the relationship of molecular size to a position along the lane for all the points in all the lanes to determine the molecular size of any given band in any of the lanes.

Results show that no band was observed in the extracts of cell line, 1321N1. A single band was observed at 84 kDa in ECV304 and EAhy926 cell extracts and a single band was observed at 92 kDa in BAEC and BRIN-BD11 cell extracts (Figure 6.3). In western blots where membranes had been preincubated with L247 peptide, bands were observed in BAEC and BRIN-BD11 cell extracts at 92 kDa (Figure 6.4).

Western blot analysis using rabbit anti-P2Y₂ receptor antibody from Santacruz Biotechnology (Sc-20124) showed a strong band in addition to multiple bands in all cell extracts including 1321N1 cells, where these cell extracts showed a strong band at 52 kDa with other weak bands at 57 kDa and 76 kDa. A strong band was detected at 53 kDa and a weaker band detected at 77 kDa in ECV304 cell extracts. A strong band was detected at 52 kDa and weaker bands detected at 47 kDa and 78 kDa in EAhy926 cell extract. A strong band was detected at 54 kDa in BAE cell extracts. Two strong bands were detected at 49 kDa and 54 kDa in BRIN-BD11 cell extracts (Figure 6.5). Western blot analysis with rabbit anti-P2Y₂ receptor antibody obtained from Zymed Laboratories (34-7600) detected a strong band at 148 kDa in ECV304 cell extracts. However, a weak band was also detected at 58 kDa in EAhy926 cell extracts. A weak band at 210 kDa was also detected in BAE cell extracts, whilst weak bands were observed at 57 kDa, 65 kDa,

94 kDa, 150 kDa and 199 kDa in BRIN-BD11 cell extracts (Figure 6.6). Western blot analysis using swine anti-rabbit HRP as a negative control for all rabbit P2Y receptor subtype polyclonal antibodies did not produce any bands (data not shown).

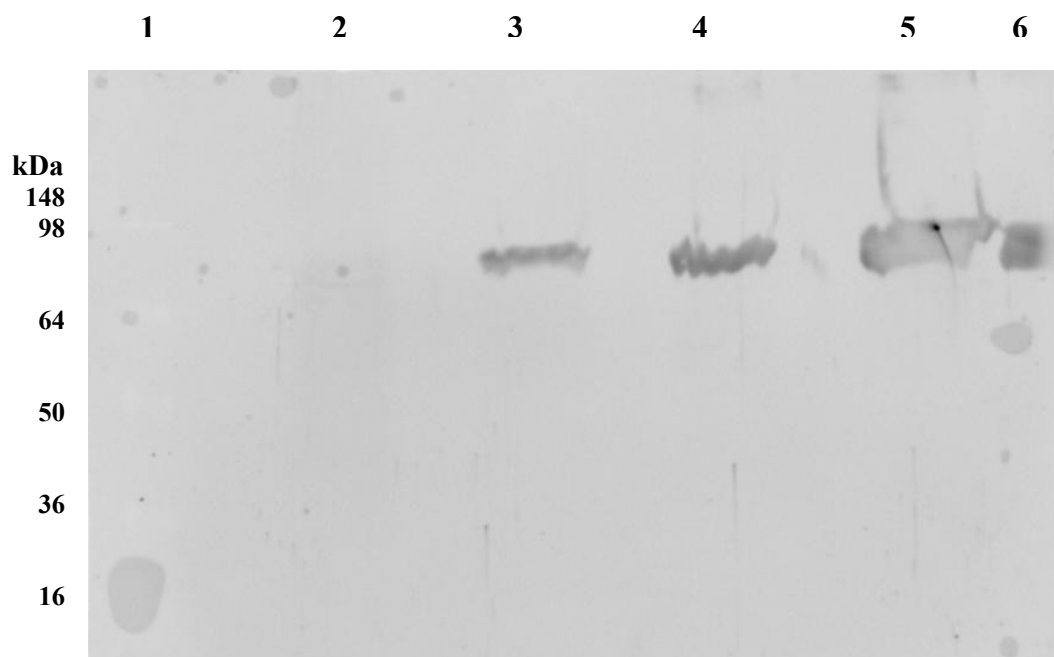


Figure 6.3 Western blot analysis of cell extracts using 5 $\mu\text{g}/\text{ml}$ of anti-L247 antibody to the P2Y₂ receptor. Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

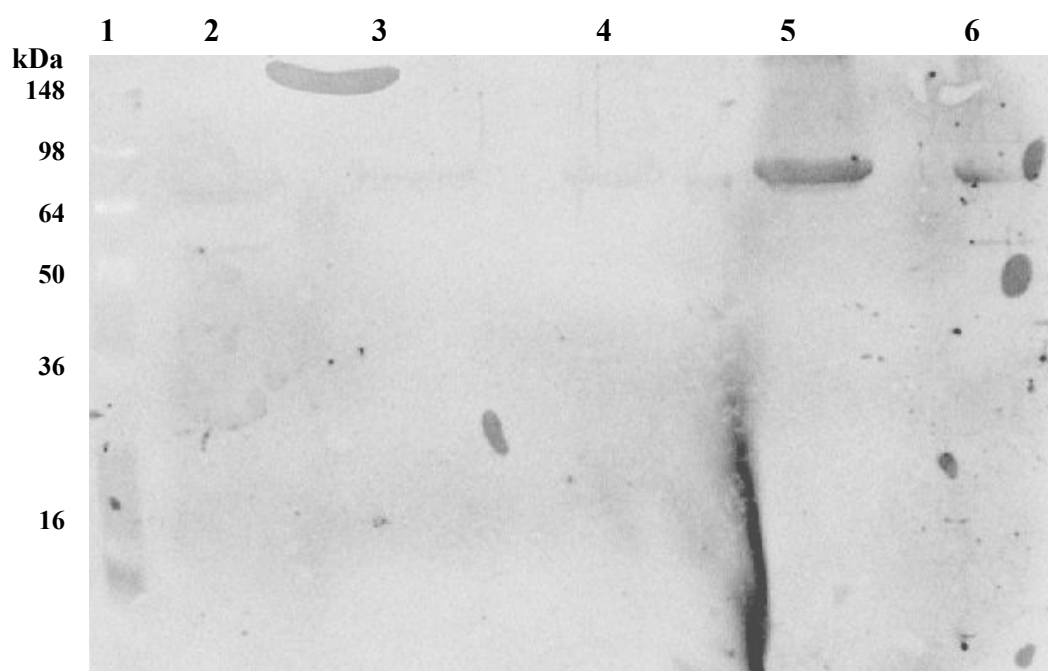


Figure 6.4 Western blot analysis of cell extracts using 5 $\mu\text{g}/\text{ml}$ of anti-L247 antibody to the P2Y_2 receptor with peptide control (Anti- P2Y_2 antibody preabsorbed with peptide antigen). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

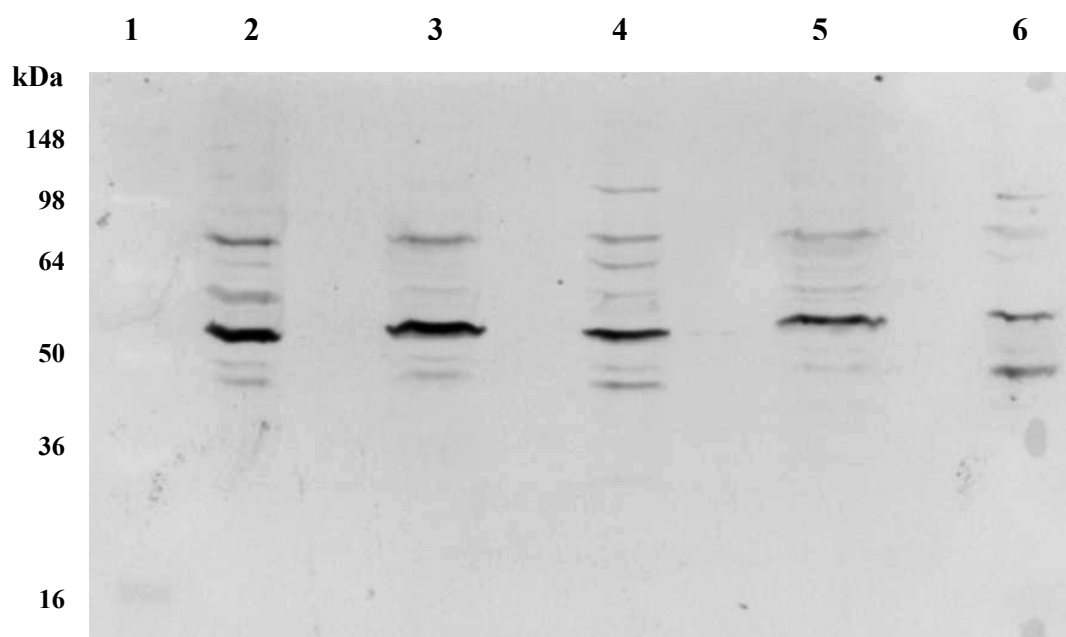


Figure 6.5 Western blot analysis of cell extracts using 2 $\mu\text{g/ml}$ of anti-P2Y₂ antibody (Santa Cruz Biotechnology). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

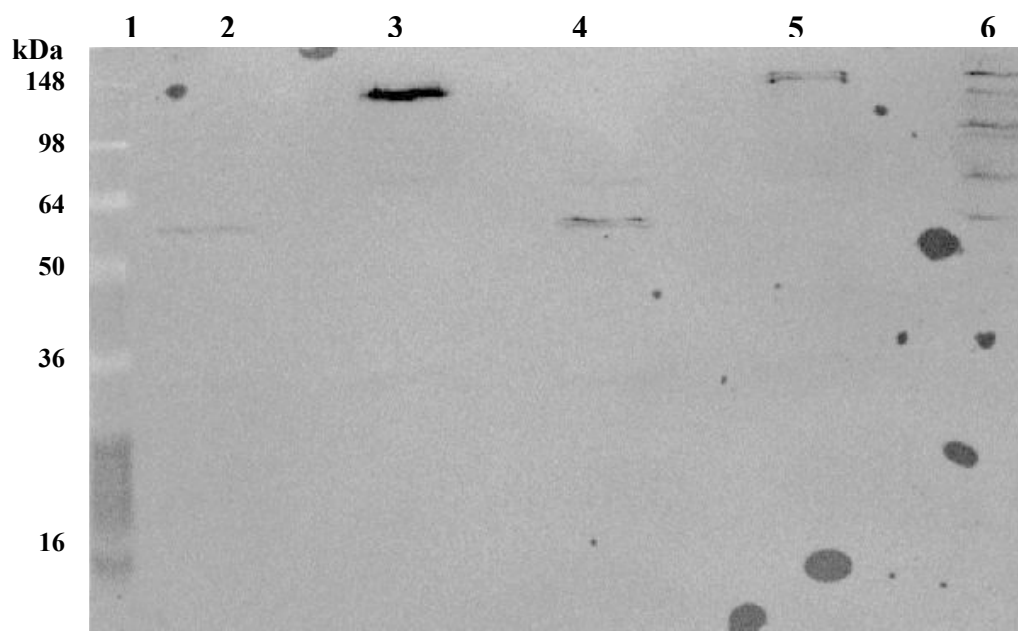


Figure 6.6 Western blot analysis of cell extracts using 10 $\mu\text{g/ml}$ of anti-P2Y₂ antibody (Zymed). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.4 Western blot analysis using anti-P2Y₂ antibodies for protein sequencing

Western blot analysis was performed using L247 anti-P2Y₂ antibody and other anti-P2Y₂ receptor rabbit polyclonal antibodies on PVDF membrane for protein sequencing. All the anti-P2Y₂ antibodies including L247 anti-P2Y₂ antibody showed bands in the same region as reported in section 6.3.3. The anti-P2Y₂ antibody raised to L247 peptide mimicking the third extracellular loop of P2Y₂ receptor revealed a strong band of 84 kDa (Figure 6.7). Western blot analysis with rabbit anti-P2Y₂ receptor antibody from Santacruz Biotechnology (Sc-20124) yielded a strong band at 52 kDa and another very faint band at 76 kDa (Figure 6.8, Lane 3). Western blot analysis with a high concentration (10µg/ml) of rabbit anti-P2Y₂ receptor antibody obtained from Zymed Laboratories (34-7600) yielded a strong band at 148 kDa and a weaker band at 65 kDa (Figure 6.9, Lane 3). The protein blots from the unused sections of PVDF membranes corresponding to the region stained with anti-P2Y₂ antibodies were used for protein sequencing.

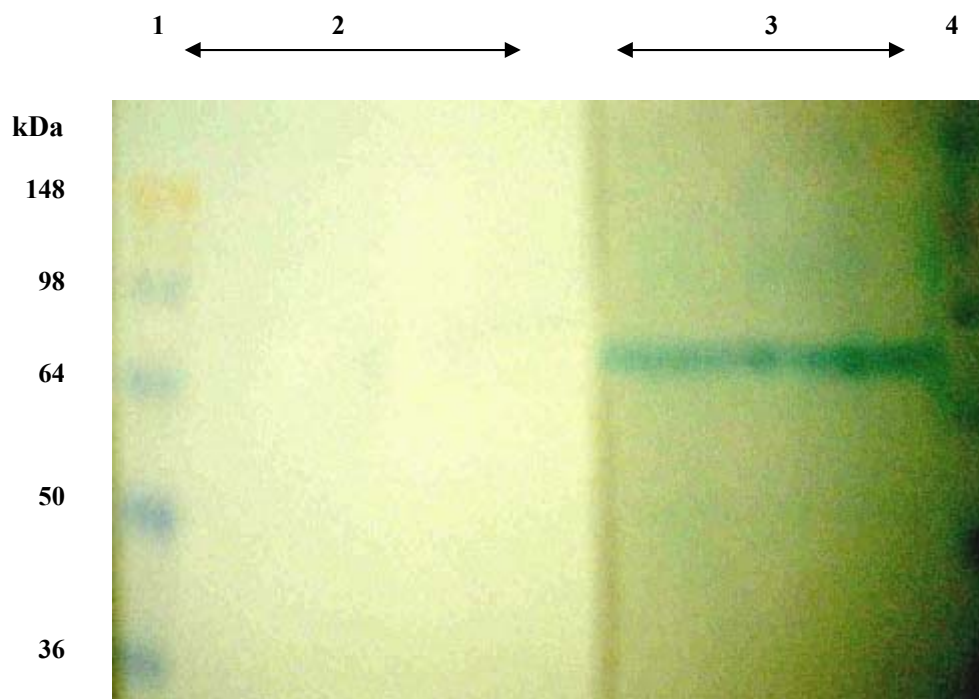


Figure 6.7 Western blot analysis of ECV304 cell extracts using L247 anti-P2Y₂ antibody. Lane 1. Pre Stained Standards; Lane 2. ECV304 cell extracts (non western blot membrane for Micro Sequencing); Lane 3. ECV304 cell extracts; Lane 4. Pre Stained Standards. Data are representative of three independent experiments.

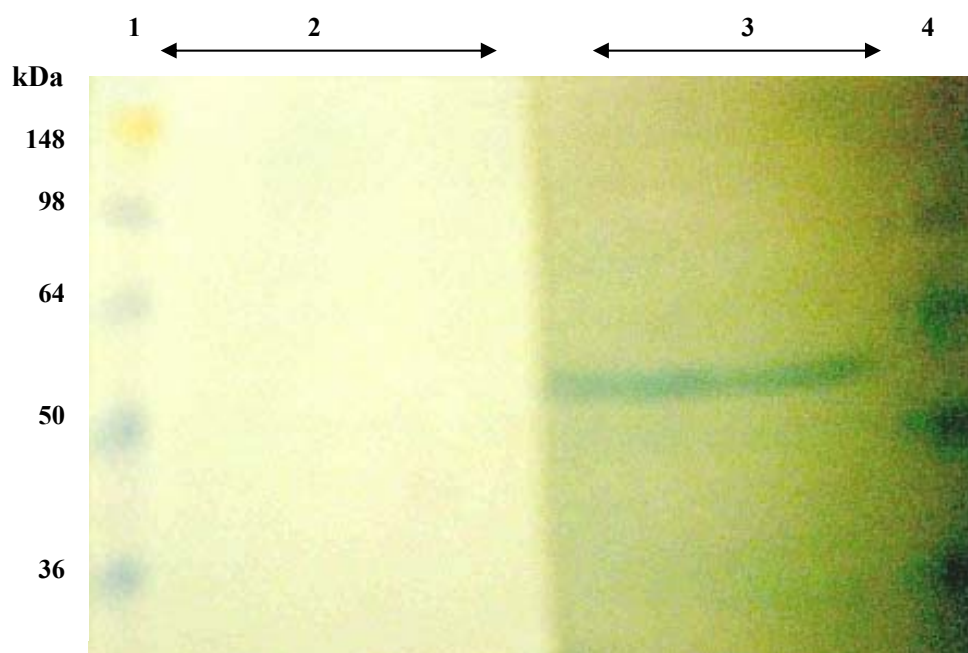


Figure 6.8 Western blot analysis of ECV304 cell extracts using anti-P2Y₂ antibody (Santa Cruz Biotechnology). Lane 1. Pre Stained Standards; Lane 2. ECV304 cell extracts (non western blot membrane for Micro Sequencing); Lane 3. ECV304 cell extracts; Lane 4. Pre Stained Standards. Data are representative of three independent experiments.

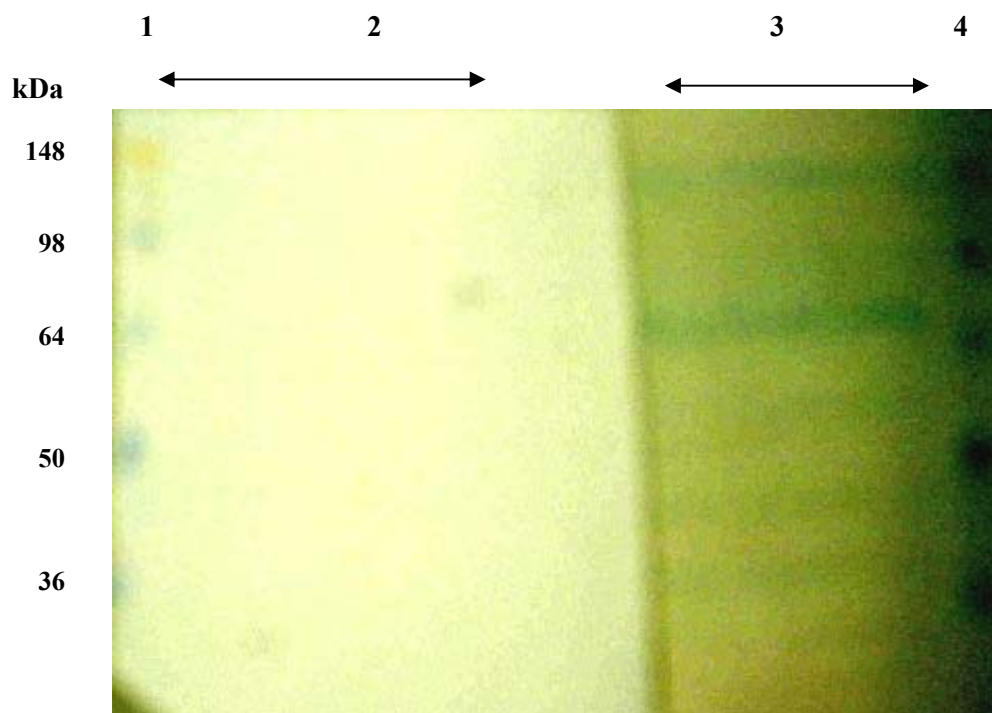


Figure 6.9 Western blot analysis of ECV304 cell extracts using anti-P2Y₂ antibody (Zymed). Lane 1. Pre Stained Standards; Lane 2. ECV304 cell extracts (non western blot membrane for Micro Sequencing); Lane 3. ECV304 cell extracts; Lane 4. Pre Stained Standards. Data are representative of three independent experiments.

6.3.5 Protein sequencing

The protein sequencing with the blot corresponding to the region stained with L247 anti-P2Y₂ antibody did not detect any amino acid residues. Large amounts of glycine were observed in the first few cycles. As the signals with other anti-P2Y₂ antibodies from Santacruz Biotechnology (Figure 6.8) and Zymed Laboratories (Figure 6.9) were weaker than those obtained for L247 anti-P2Y₂ antibody the blots corresponding those weaker regions were not sequenced.

6.3.6 Western blot analysis of digested cell extracts using L247 anti-P2Y₂ antibody

The western blot analysis for P2Y₂ receptor in ECV304 cell extracts with L247 Anti-P2Y₂ antibody revealed a strong protein band at 84 kDa (Figure 6.10). However, in the digested (upon digestion with N-glycosidase F Kit) ECV304 cell extracts (deglycosylated receptor protein), the band was weaker and was reduced to 80 kDa (Figure 6.10).

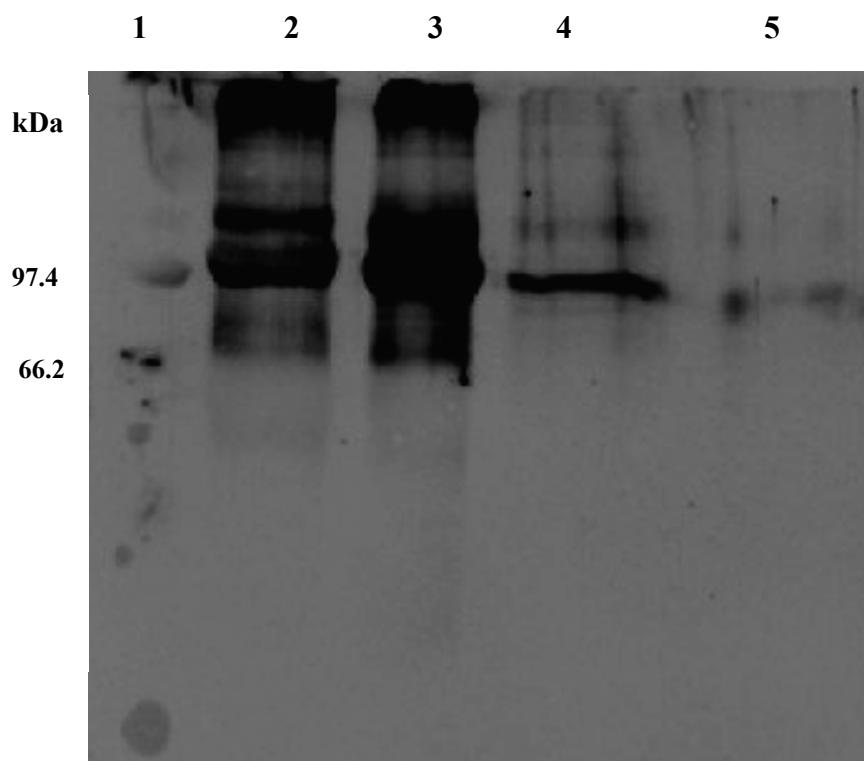


Figure 6.10 Western blot analysis of ECV304 cell extracts for deglycosylated P2Y₂ receptor using L247 anti-P2Y₂ antibody. Lane 1. Protein Molecular Weight Markers; Lane 2. Undigested control glycoproteins; Lane 3. Digested control glycoproteins; Lane 4. Undigested ECV304 cell extracts; Lane 5. Digested ECV304 cell extracts. Data are representative of three independent experiments.

6.3.7 Western blot analysis of digested cell extracts using anti-P2Y₂ antibodies for protein sequencing

The protein fractions from a sodium dodecyl sulphate-polyacrylamide gel were transferred onto Sequi-Blot PVDF membrane (BIO RAD) for protein sequencing. As reported in section 6.3.6 the digested ECV304 protein extracts showed a weaker band at 80 kDa with L247 anti-P2Y₂ antibody (Figure 6.11). The anti-P2Y₂ antibody obtained from Santacruz Biotechnology detected two bands in ECV304 cell extracts which, however, did not change from those observed in undigested ECV304 cell extracts (Figure 6.12). The results with anti-P2Y₂ antibody obtained from Zymed yielded two very weak bands at a concentration of 10µg/ml also did not change from those observed in undigested ECV304 cell extracts (Figure 6.13). For the purpose of microsequencing, pictures of the blots were taken immediately using digital camera and the blots were stored in -20⁰C. The poor quality of the pictures was due to the translucent nature of Sequi-Blot PVDF membrane resulting in the poor capture of the protein bands by digital photography. The protein blots from Sequi-Blot PVDF membranes (not used for western blot analysis) corresponding to the region stained with anti-P2Y₂ antibodies were used for protein sequencing.

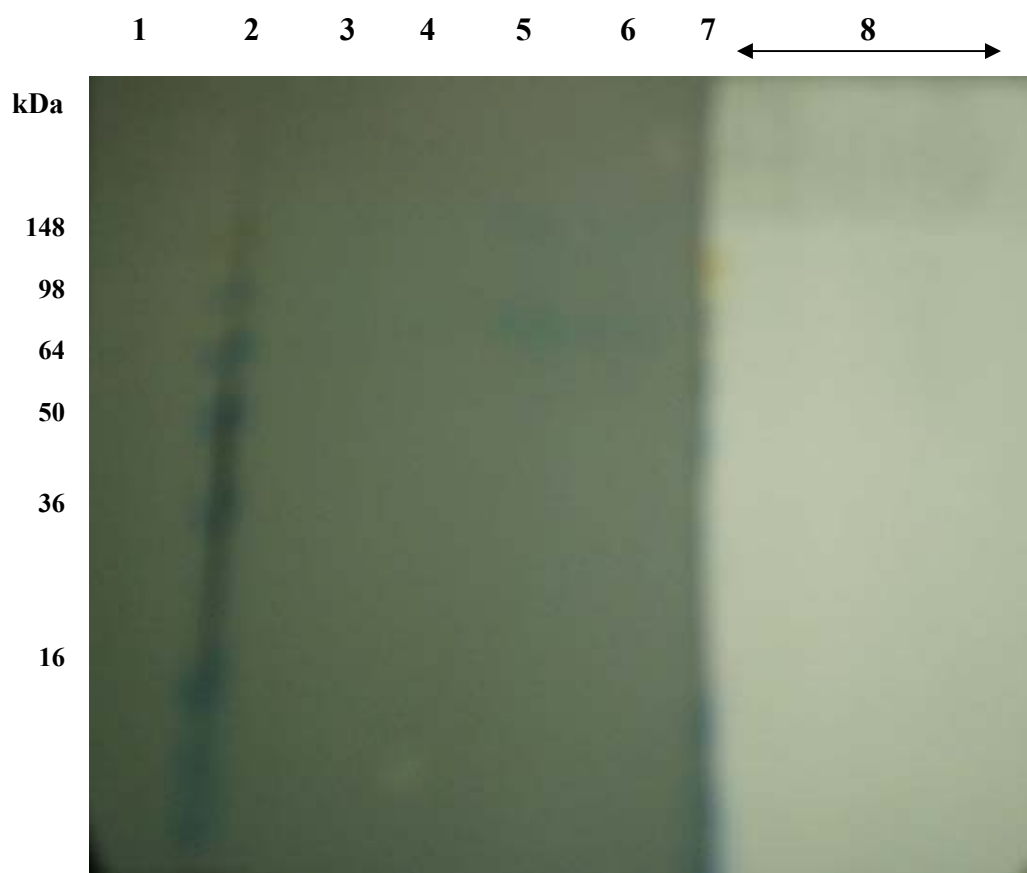


Figure 6.11 Western blot analysis of deglycosylated P2Y₂ receptor with L247 anti-P2Y₂ antibody in ECV304 cell extracts. Lane 1. Protein Molecular Weight Markers; Lane 2. See Blue Plus 2 Pre Stained Standards; Lane 3. Undigested control glycoproteins; Lane 4. Digested control glycoproteins; Lane 5. Undigested ECV304 cell extracts; Lane 6. Digested ECV304 cell extracts. Lane 7. See Blue Plus 2 Pre Stained Standards; Lane 8. Digested ECV304 cell extracts for Micro Sequencing. Data are representative of three independent experiments.

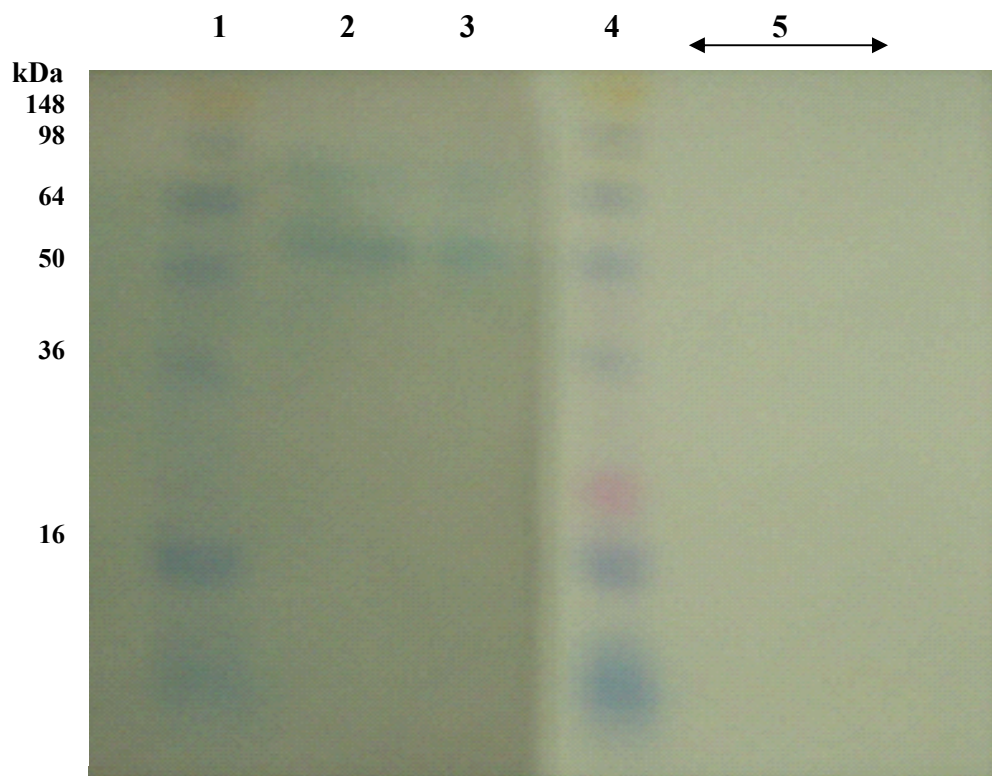


Figure 6.12 Western blot analysis of deglycosylated P2Y₂ with anti-P2Y₂ antibody (Santa Cruz Biotechnology) in ECV304 cell extracts. Lane 1. See Blue Plus 2 Pre Stained Standards; Lane 2. Undigested ECV304 cell extracts; Lane 3. Digested ECV304 cell extracts. Lane 4. See Blue Plus 2 Pre Stained Standards; Lane 5. Digested ECV304 cell extracts for Micro Sequencing. Data are representative of three independent experiments.

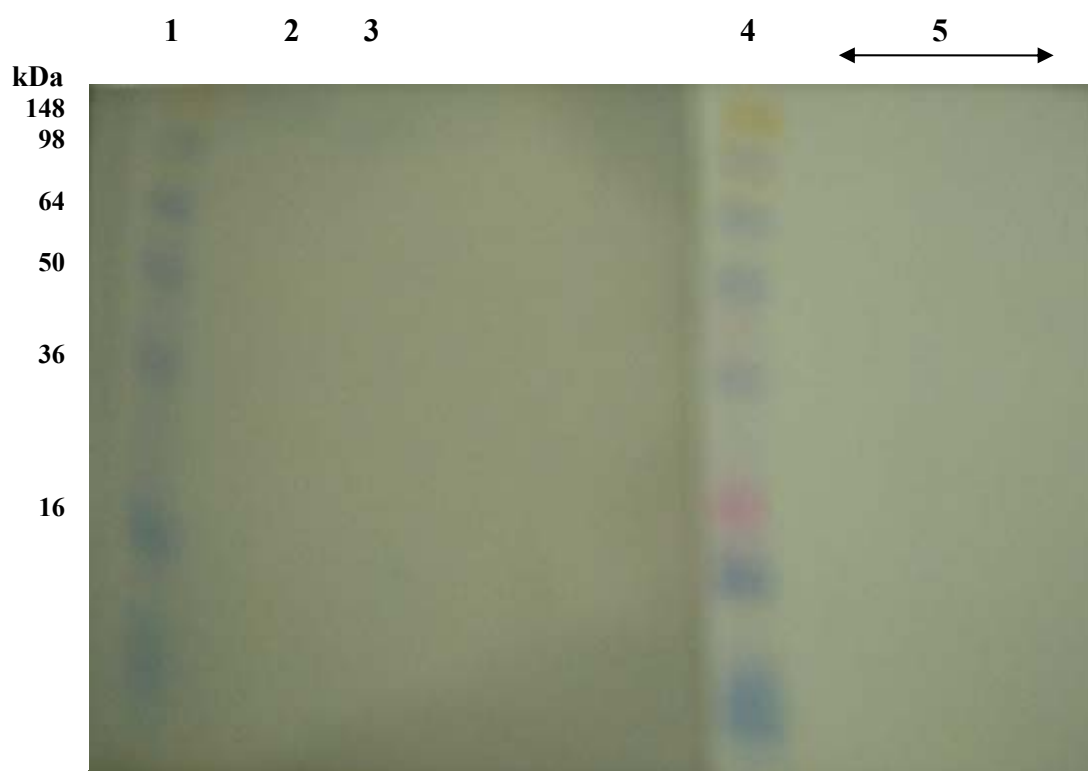


Figure 6.13 Western blot analysis of deglycosylated P2Y₂ with anti-P2Y₂ antibody (Zymed) in ECV304 cell extracts. Lane 1. See Blue Plus 2 Pre Stained Standards; Lane 2. Undigested ECV304 cell extracts; Lane 3. Digested ECV304 cell extracts. Lane 4. See Blue Plus 2 Pre Stained Standards; Lane 5. Digested ECV304 cell extracts for Micro Sequencing. Data are representative of three independent experiments.

6.3.8 Protein sequencing of deglycosylated P2Y₂ receptor

The results of protein sequencing of the protein blot corresponding to the region stained with L247 anti-P2Y₂ antibody (5µg/ml) in western blot analysis detected the following amino acid residues. However, the micro sequencing of protein blots corresponding to the region stained with anti-P2Y₂ antibody from Santa Cruz Biotechnology did not detect any amino acid residues. As the protein band produced by anti-P2Y₂ antibody from Zymed was very weak even at higher concentration of the antibody used (10µg/ml), the blot was not sequenced as advised by the service provider.

Table 6.1 Amino acid residues obtained after N-terminal protein sequencing by Alta Biosciences. The identity of Asp and Leu is not certain.

Residue	Amino Acid	Single Letter Code
1	---	---
2	Asp?	D
3	Arg	R
4	Ile	I
5	Lys	K
6	Asp	D
7	Glu	E
8	Phe	F
9	Gln	Q
10	Leu?	L

The above sequence was used to identify and find similarity between matching protein sequences using Basic Local Alignment Search Tool (BLAST). The results indicate that the sequence (DRIKDEFQL) matches the amino acid residues 94-102 of human amino-terminal enhancer of split isoform a (gi number: 39812019), 27-35 of human amino-terminal enhancer of split isoform c (gi number: 39812027), 27-35 of amino-terminal enhancer of split isoform b (gi number: 5706731), 26-34 of gp130 associated protein GAM (gi number: 3309589), 27-34 of transducin-like enhancer of split 3 splice variant 2 (gi number: 46391802), 27-34 of transducin-like enhancer of split 3 splice variant 1 (gi number: 46391800), 27-34 of TLE3 protein (gi number: 34783362), 27-34 of Transducin-like enhancer protein 3 (gi number: 20532417), 27-34 of transducin-like enhancer protein 3 (gi number: 4827030), 33-40 of TLE3 protein (gi number: 27469815), 130-137 of KIAA1547 protein (gi number: 10047159), 54-61 (87%) of transducin-like enhancer protein 1 variant (gi number: 62089294).

6.3.9 Receptor desensitisation

The P2Y₂ receptor was subjected to agonist induced desensitisation and the cell extracts expressing the P2Y₂ receptor were used in western blot analysis. The results of western blot analysis suggested that extracellular UTP caused P2Y₂ receptor desensitisation after a 1 hour incubation with 300µM UTP (Figure 6.14) as the protein staining bands in the cells incubated with UTP for an hour were almost abolished. However, the protein staining bands in control cells were unaffected (Figure 6.14). Notably, the cells incubated with UTP for 24 hours showed a prominent binding in response to anti-P2Y₂ antibody and appeared to be of higher intensity (Figure 6.14). However, incubation of the cells with histamine for similar time periods shows no such masking of protein staining bands both in cells incubated for 1 hour and 24 hours (Figure 6.15). Although the bands in lane 2 and 3 (Figure 6.15) are eclipsed by a blot, ImageQuant[™] TL software was used to detect and to calibrate the molecular weights of the bands.

This receptor desensitisation study was also used to investigate receptor specificity of anti-P2Y₂ antibody obtained from Santa Cruz Biotechnology. This anti-P2Y₂ receptor antibody detected a strong band at 52 kDa and a weaker band at 76 kDa (Figure 6.16). There was no observed desensitisation of the weak band as there was no difference in

the protein staining between control cells and cells incubated with UTP for 1 hour (Figure 6.16). However, the cells incubated with UTP for 24 hours showed prominent bands (Figure 6.16). Similarly, in the case of the strong band at 52 kDa there was no noticeable desensitisation effect. There appeared to be no difference in the band intensity between cells incubated with UTP for an hour and control cells (Figure 6.16). In this case too, the protein staining bands were prominent in cells incubated with UTP for 24 hours (Figure 6.16). Interestingly, in the cells incubated with histamine the bands produced at 52 kDa and 76 kDa were almost identical (Figure 6.17). Moreover, there was no discernible difference in protein staining bands between control cells and cells incubated for an hour or 24 hours (Figure 6.17).

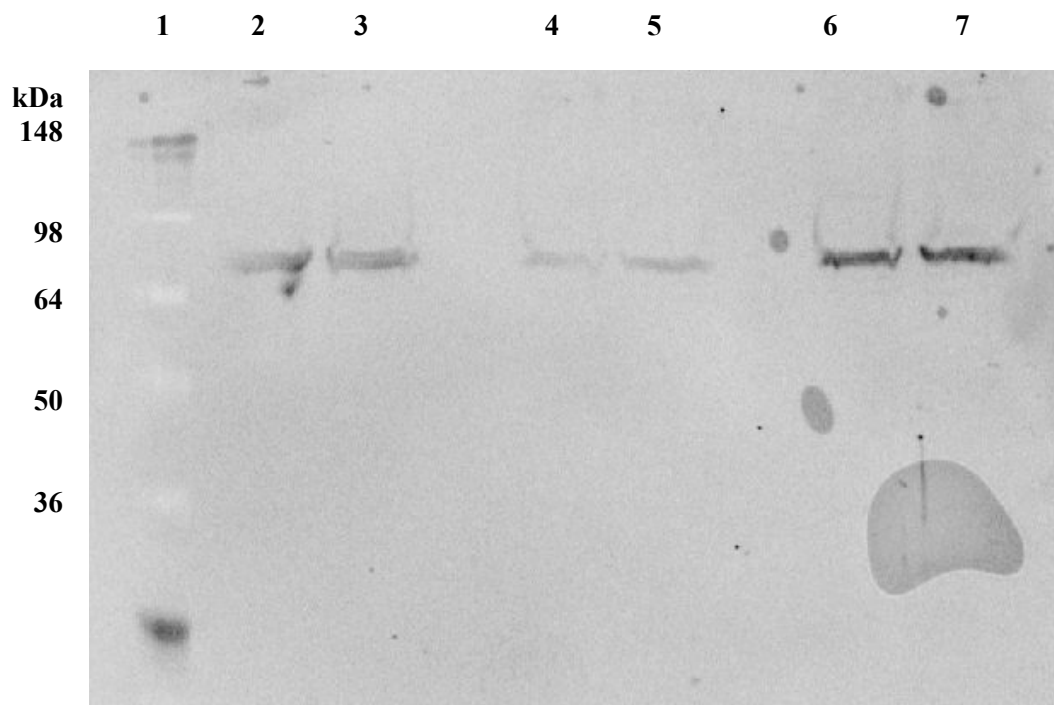


Figure 6.14 Western blot analysis of ECV304 cell extracts using 5 $\mu\text{g/ml}$ of L247 anti-P2Y₂ antibody. Lane 1. See Blue Plus 2 Pre Stained Standards; Lane 2&3. Untreated ECV304 cell extracts; Lane 4&5. ECV304 cell extracts incubated with 300 μM UTP for 1 hour; Lane 6&7. ECV304 cell extracts incubated with 300 μM UTP for 24 hours; Data are representative of three independent experiments.

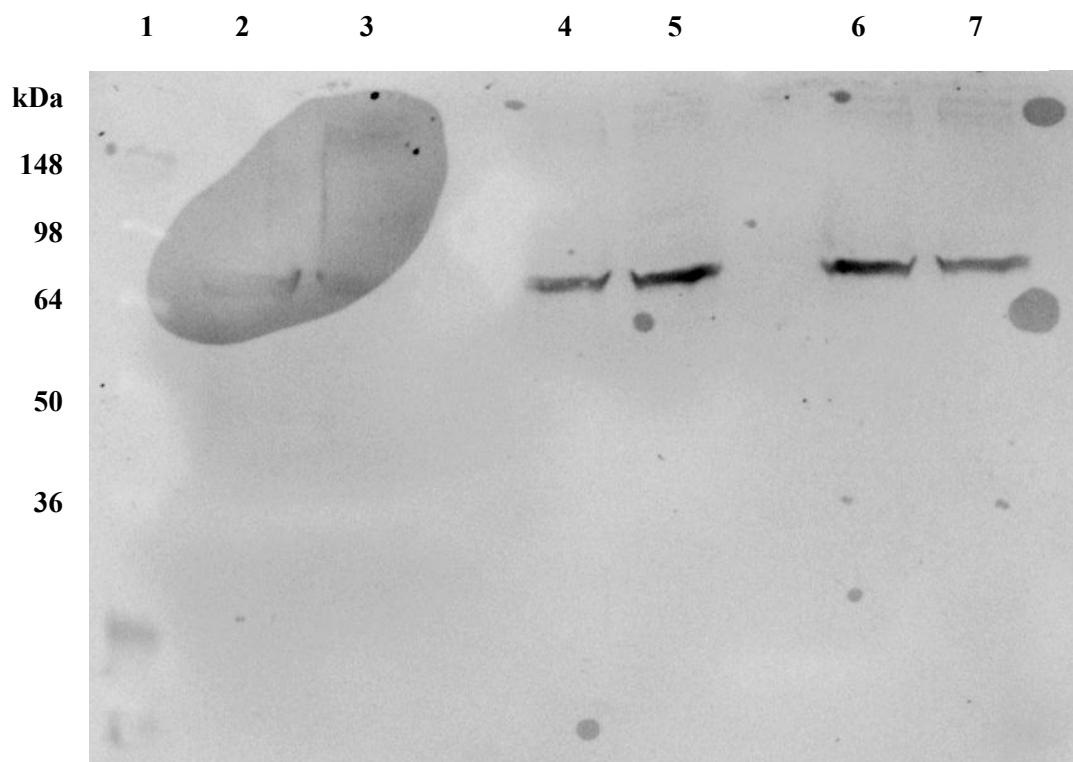


Figure 6.15 Western blot analysis of ECV304 cell extracts using 5 µg/ml of L247 anti-P2Y₂ antibody. 1. See Blue Plus 2 Pre Stained Standards; Lane 2&3. Untreated ECV304 cell extracts; Lane 4&5. ECV304 cell extracts incubated with 300µM Histamine for 1 hour; Lane 6&7. ECV304 cell extracts incubated with 300µM Histamine for 24 hours; Data are representative of three independent experiments.

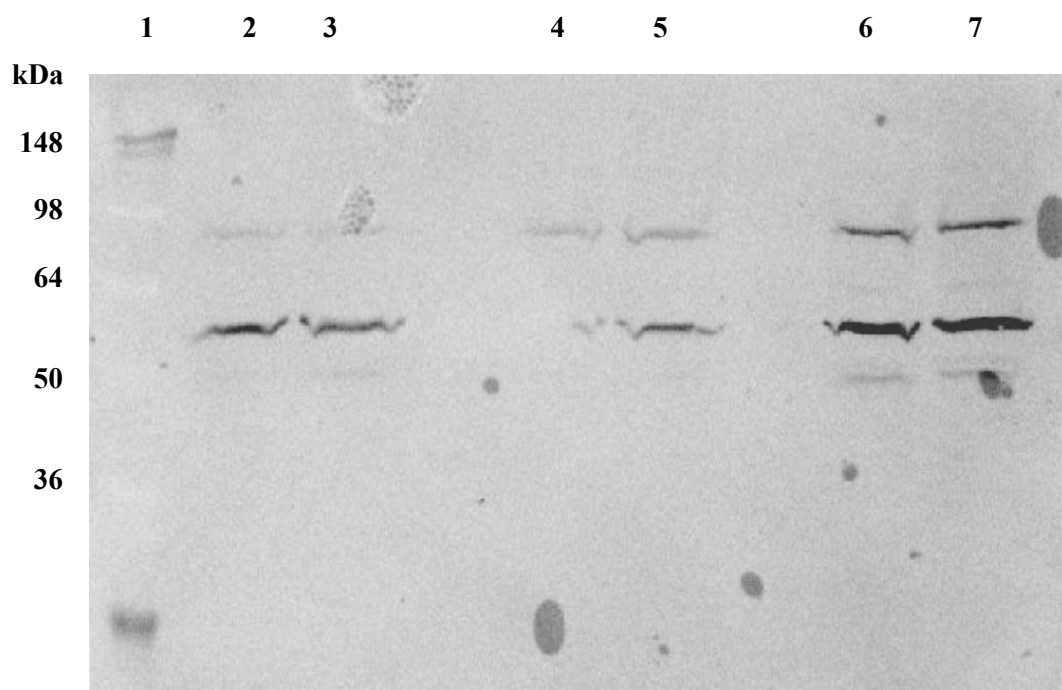


Figure 6.16 Western blotting analysis for P2Y₂ with anti-P2Y₂ antibody (Santa Cruz Biotechnology) in ECV304 cells. 1. See Blue Plus 2 Pre Stained Standards; Lane 2&3. Untreated ECV304 cells; Lane 4&5. ECV304 cells incubated with 300μM UTP for 1 hour; Lane 6&7. ECV304 cells incubated with 300μM UTP for 24 hours; Data are representative of three independent experiments.

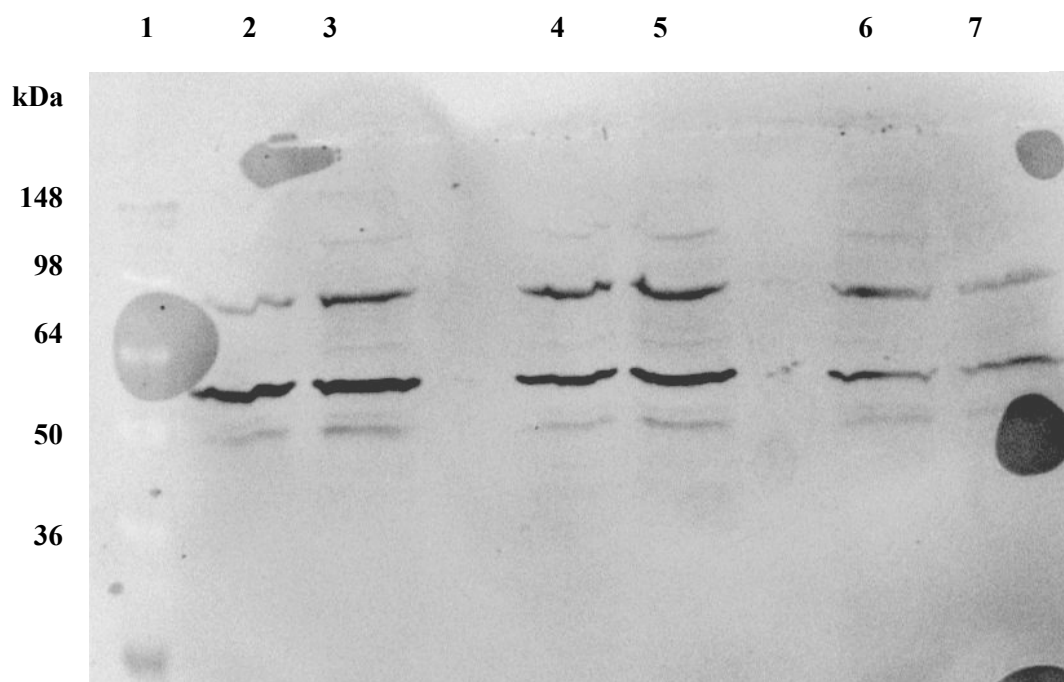


Figure 6.17 Western blotting analysis for P2Y₂ with anti-P2Y₂ antibody (Santa Cruz Biotechnology) in ECV304 cells. 1. See Blue Plus 2 Pre Stained Standards; Lane 2&3. Untreated ECV304 cells; Lane 4&5. ECV304 cells incubated with 300μM Histamine for 1 hour; Lane 6&7. ECV304 cells incubated with 300μM Histamine for 24 hours; Data are representative of three independent experiments.

6.3.10 Western blot analysis using anti-P2Y₁ antibody

Western blot analysis of the electrophoretically transferred protein extracts with anti-P2Y₁ antibody (P6487-Sigma) showed no band in 1321N1, ECV304 and BAE cells. However, strong bands were detected at 52 kDa in both EAhy926 and BRIN-BD11 cell extracts (Figure 6.18).

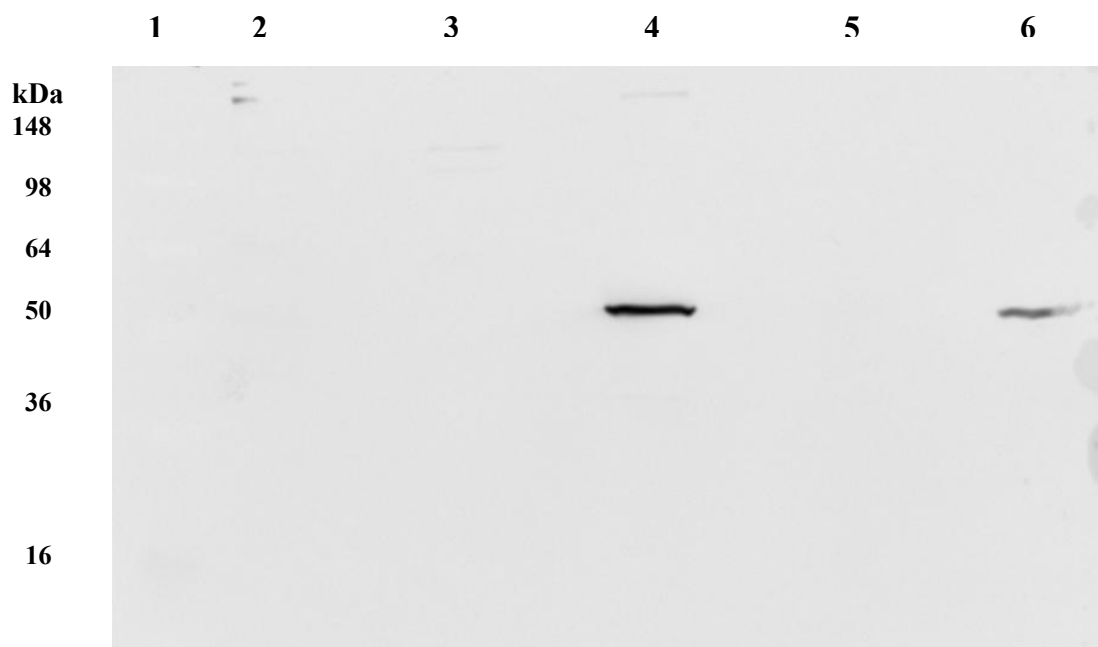


Figure 6.18 Western blot analysis of cell extracts using anti-P2Y₁ antibody (Sigma) at 1µg/ml. Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.11 Western blot analysis using anti-P2Y₄ antibody

Western blot analysis using rabbit anti-P2Y₄ receptor antibody from Santacruz Biotechnology showed strong multiple bands in all cell extracts including 1321N1 cells. Results show strong protein bands at 37 kDa, 51 kDa, 55 kDa, 64 kDa, 78 kDa in 1321N1 cell extracts (Figure 6.19). Strong bands were detected at 36 kDa, 50 kDa, 58 kDa and 70 kDa in ECV304 cell extracts while strong bands were detected at 51 kDa, 60 kDa, and 64 kDa in EAhy926 cell extracts (Figure 6.19). Strong bands were detected at 51 kDa, 53 kDa and 58 kDa in BAE cell extracts and at 35 kDa, 50 kDa and 63 kDa in BRIN-BD11 cell extracts (Figure 6.19).

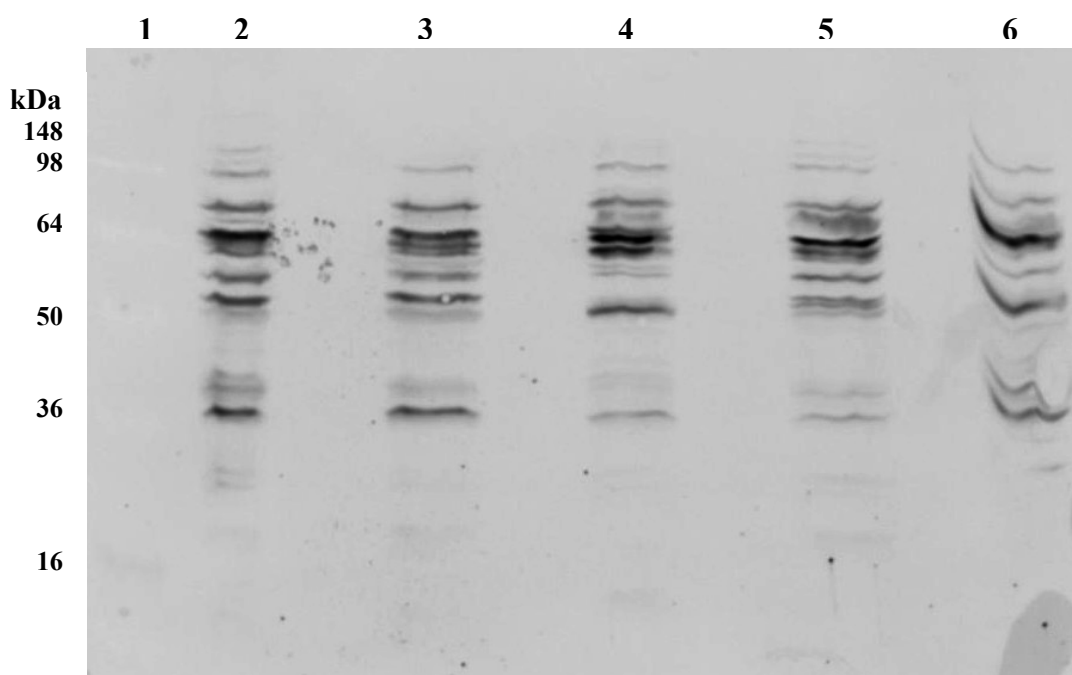


Figure 6.19 Western blot analysis of cell extracts using anti-P2Y₄ antibody (Santacruz Biotechnology) at 4 μ g/ml. Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.12 Western blot analysis using anti-P2Y₆ antibody

Results of the western blot studies using rabbit anti-P2Y₆ receptor antibody from Santacruz Biotechnology show strong protein bands in all cell extracts used including 1321N1. Strong bands were detected at 51 kDa, 62 kDa and 78 kDa in 1321N1 cells (Figure 6.20). Likewise, strong bands were detected at 39 kDa, 53 kDa, 65 kDa and 80 kDa in ECV304 cell extracts and at 52 kDa, 71 kDa and 83 kDa in EAhy926 cell extracts (Figure 6.20). Strong bands were detected at 53 kDa, 61 kDa, 69 kDa and 83 kDa in BAE cell extracts and at 52 kDa, 61 kDa, 72 kDa, 82 kDa and 122 kDa in BRIN-BD11 cell extracts (Figure 6.20).

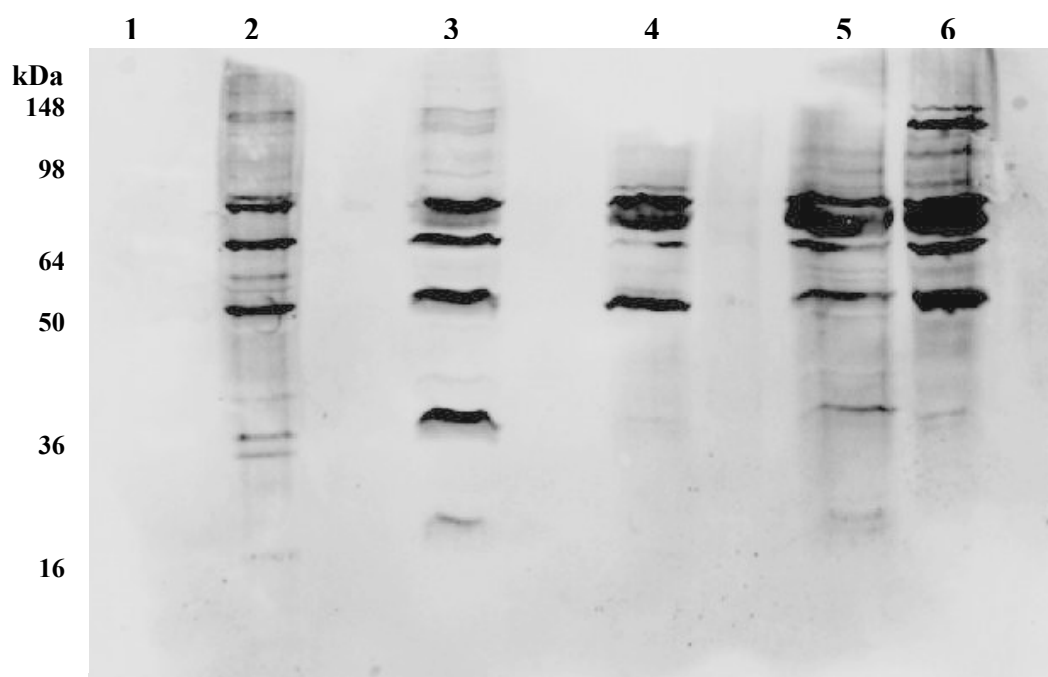


Figure 6.20 Western blot analysis of cell extracts using anti-P2Y₆ antibody (Santacruz Biotechnology) at 3.5µg/ml. Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.13 Western blot analysis using anti-P2Y₁₁ antibody

Western blot analysis using rabbit anti-P2Y₁₁ receptor antibody from Affinity Bioreagents did not yield any strong protein bands in any of the samples of cell extracts. However, some weak bands were observed at 101 kDa and 114 kDa in 1321N1 cell extracts, at 44 kDa and 60 kDa in ECV304 cell extracts, at 60 kDa, 88 kDa, 114 kDa and 132 kDa in EAhy926 cell extracts, at 65.12 kDa in BAE cell extracts and at 120 kDa in BRIN-BD11 cell extracts (Figure 6.21).

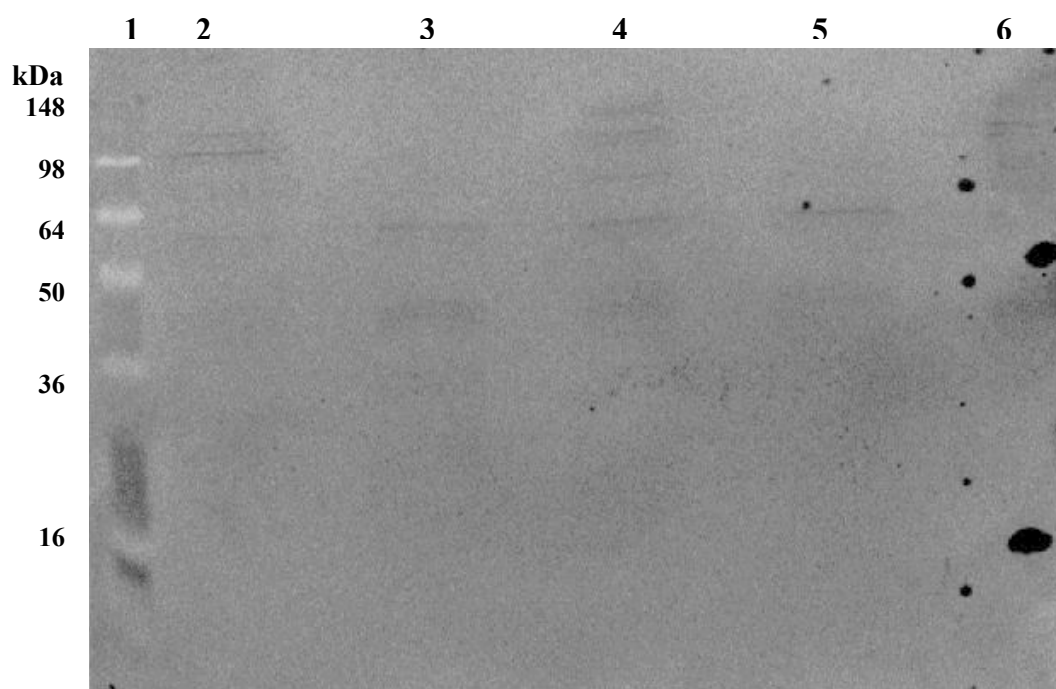


Figure 6.21 Western blot analysis of cell extracts using anti-P2Y₁₁ antibody (Affinity Bioreagents). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.14 Western blot analysis using anti-P2Y₁₂ antibody

Results of the western blot studies using rabbit anti-P2Y₁₂ receptor antibody from Sigma did not detect any bands in 1321N1, ECV304, EAhy926, BAEC or BRIN-BD11 cell extracts (Figure 6.22).

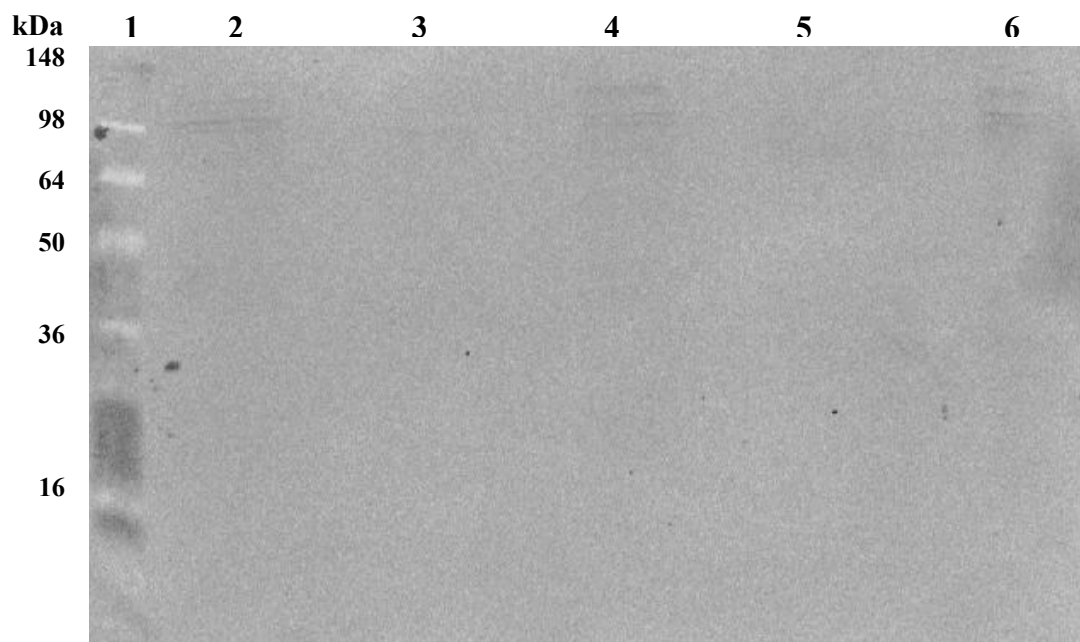


Figure 6.22 Western blot analysis of cell extracts using anti-P2Y₁₂ antibody (Sigma). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

6.3.15 Western blot analysis using anti-P2Y₁₃ antibody

Western blot analysis using rabbit anti-P2Y₁₃ receptor antibody from Acris Antibodies detected strong bands at 115 kDa in 1321N1 cell extracts. Strong bands were detected at 115 kDa and weaker bands at 47 kDa and 79 kDa in ECV304 cell extracts. Strong bands were detected at 74 kDa and 120 kDa in EAhy926 cell extracts. A strong band was detected at 123 kDa in BAE cell extracts and weaker bands were detected at 49 kDa and 118 kDa in BRIN-BD11 cell extracts (Figure 6.23).

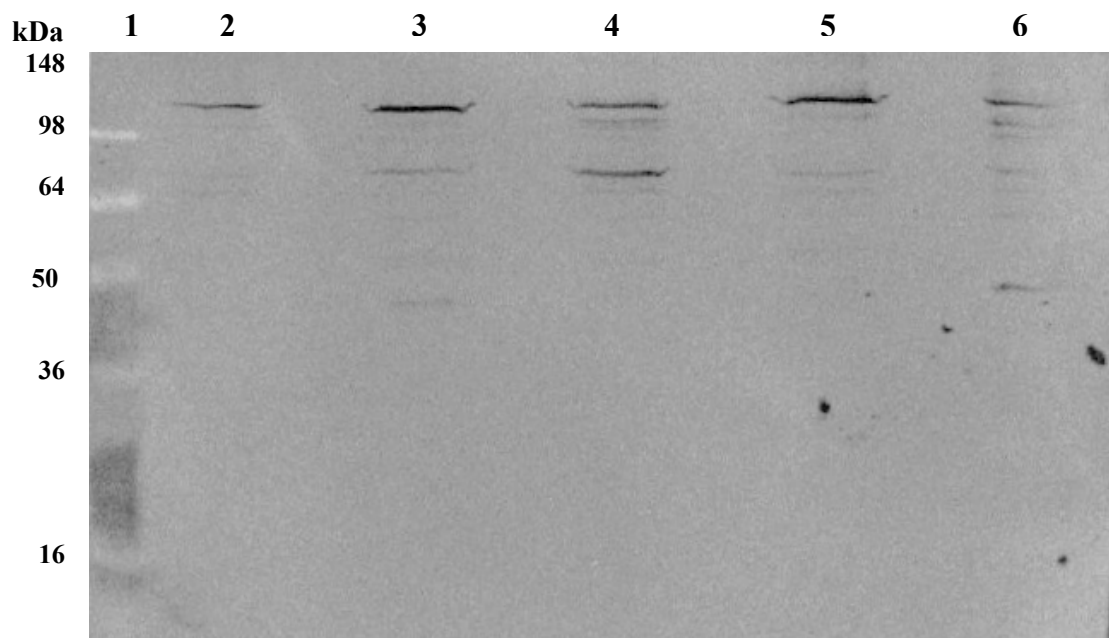


Figure 6.23 Western blot analysis of cell extracts using anti-P2Y₁₃ antibody (Acris). Lane 1. Pre Stained Standards; Lane 2. 1321N1 cell extracts; Lane 3. ECV304 cell extracts; Lane 4. EAhy926 cell extracts; Lane 5. BAE cell extracts; Lane 6. BRIN-BD11 cell extracts. Data are representative of three independent experiments.

Table 6.2 Summary of RT-PCR and western blot results of P2Y receptor subtypes in 1321N1, ECV304, EAhy926, BAE and BRIN-BD11 cells

Receptor Type	Cell Type									
	1321N1		ECV304		EAhy926		BAEC		BRIN-BD11	
	PCR	WB	PCR	WB	PCR	WB	PCR	WB	PCR	WB
P2Y ₁	-	-	+	-	-	+	-	-	-	+
P2Y ₂	-	-	+	+	+	+	-	+	-	+
P2Y ₄	-	+	+	+	-	+	-	+	-	+
P2Y ₆	-	+	+	+	+	+	-	+	-	+
P2Y ₁₁	-	+	+	+	-	+	-	+	-	+
P2Y ₁₂	-	-	+	-	-	-	-	-	-	-
P2Y ₁₃	-	+	+	+	-	+	-	+	-	+
P2Y ₁₄	-		-		-		-		-	

+: Positive result; -: Negative result

6.3.16 Immunofluorescence analysis of ECV304 cells using anti-P2Y₂ antibodies

Immunofluorescence analysis was performed in ECV304 cells using anti-P2Y₂ antibodies. The data obtained using L247 anti-P2Y₂ antibody suggests that this antibody detects proteins in the cell membrane and the nucleus (Figure 6.24). However, it is difficult to quantify the degree of binding with these results. These studies were also extended to other anti-P2Y₂ antibodies and the results suggest that the antibodies obtained from Santacruz Biotechnology and Zymed (Figure 6.26 & 6.27) stain the cells in a manner similar to L247 anti-P2Y₂ antibody. In the control experiments no signal was observed when only the secondary antibody was used (Figure 6.25).

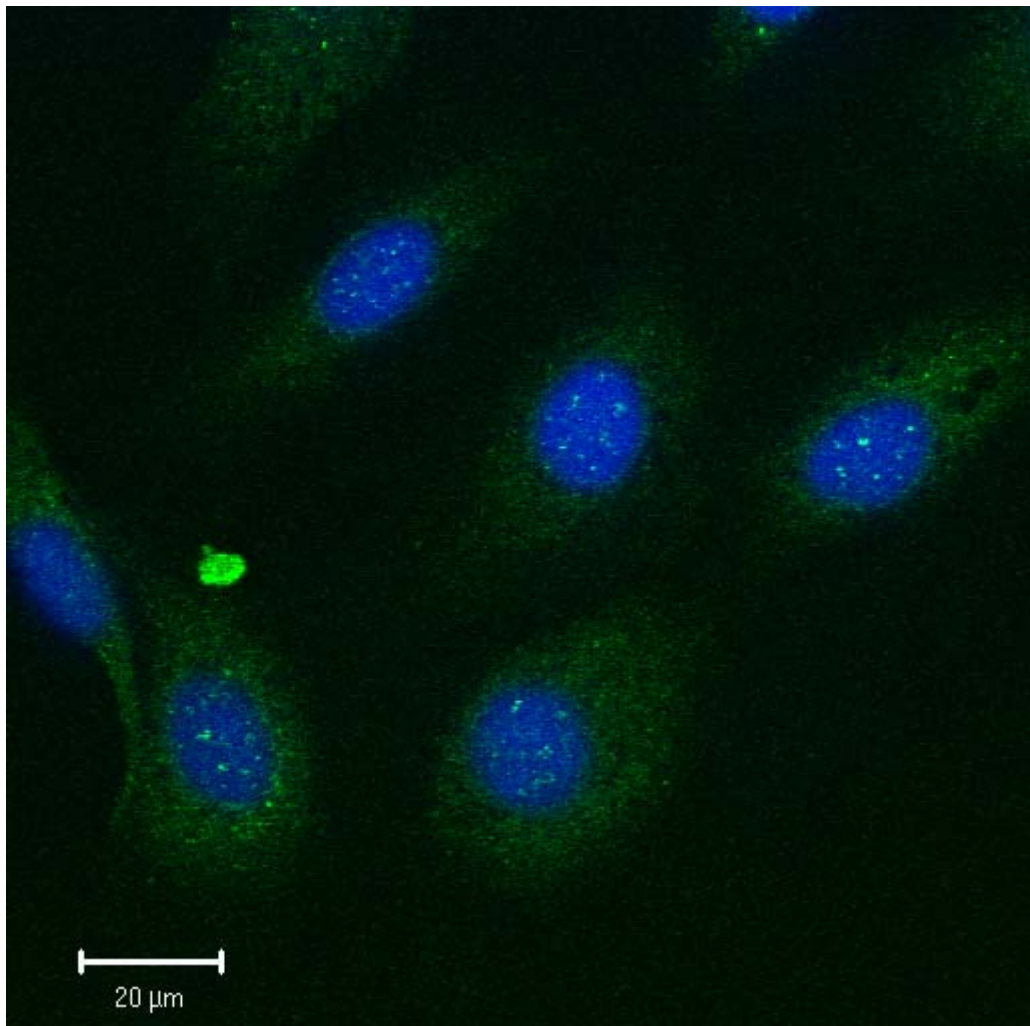


Figure 6.24 Immunofluorescence analysis of P2Y₂ receptor using L247 anti-P2Y₂ antibody at 1:200 dilution range in ECV304 cells. Data are representative of three independent experiments.

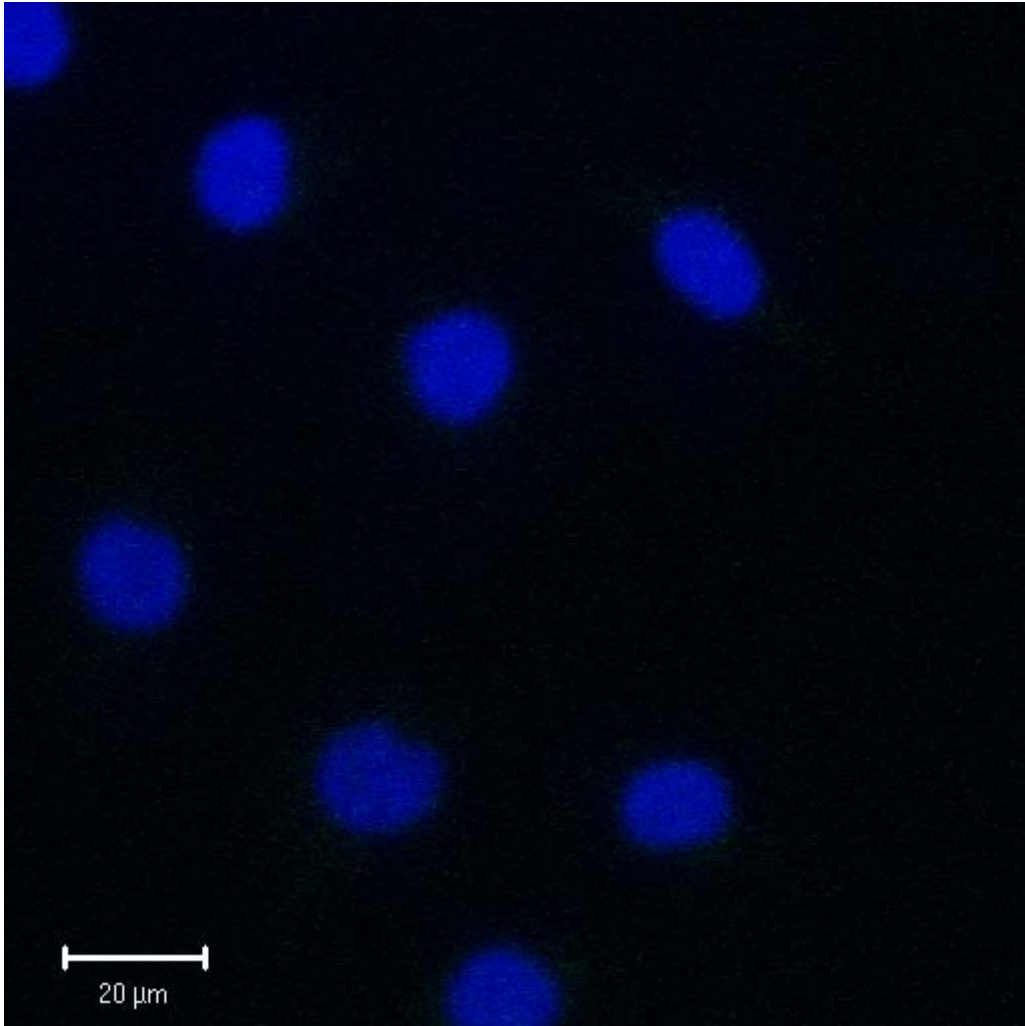


Figure 6.25 Negative control for immunofluorescence analysis of P2Y₂ receptor in ECV304 cells. Data are representative of three independent experiments.

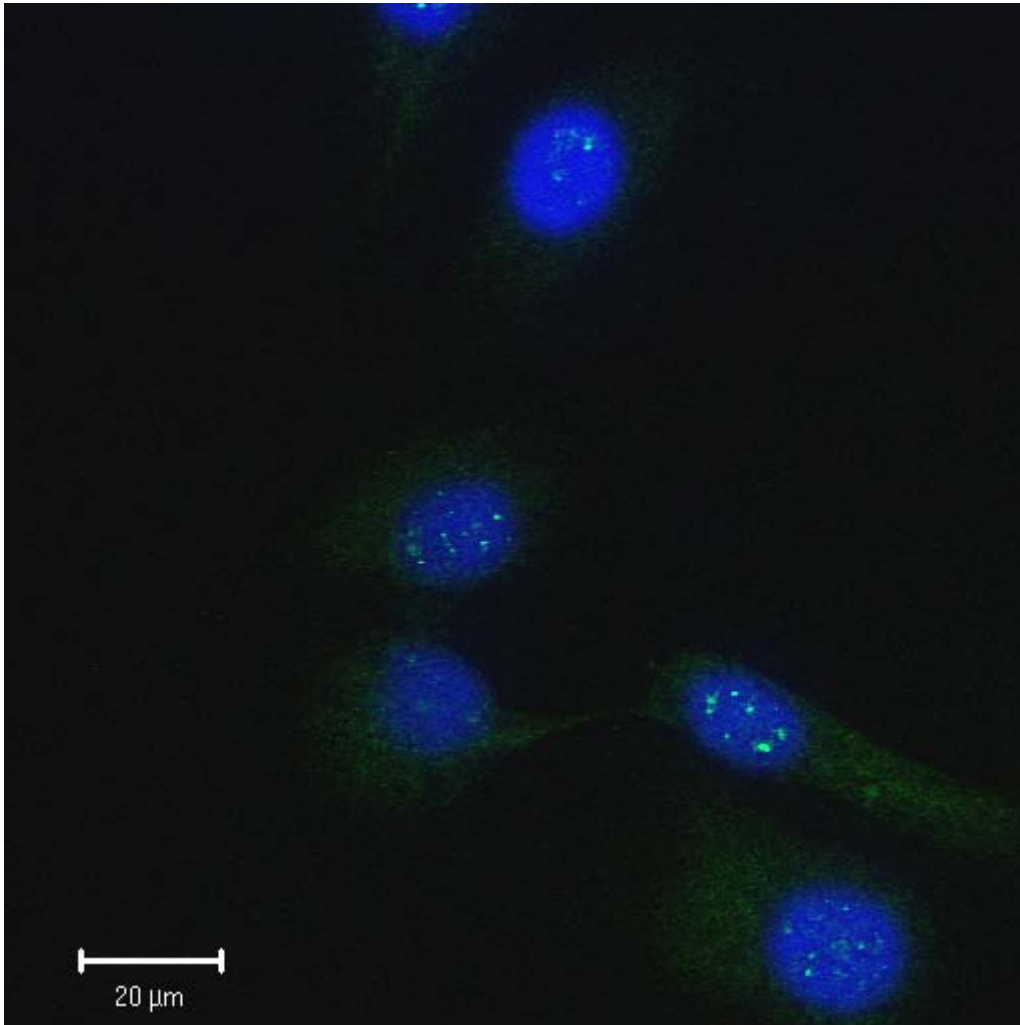


Figure 6.26 Immunofluorescence analysis of P2Y₂ receptor using anti-P2Y₂ antibody (Santa Cruz Biotechnology) at 1:400 dilution range in ECV304 cells. Data are representative of three independent experiments.

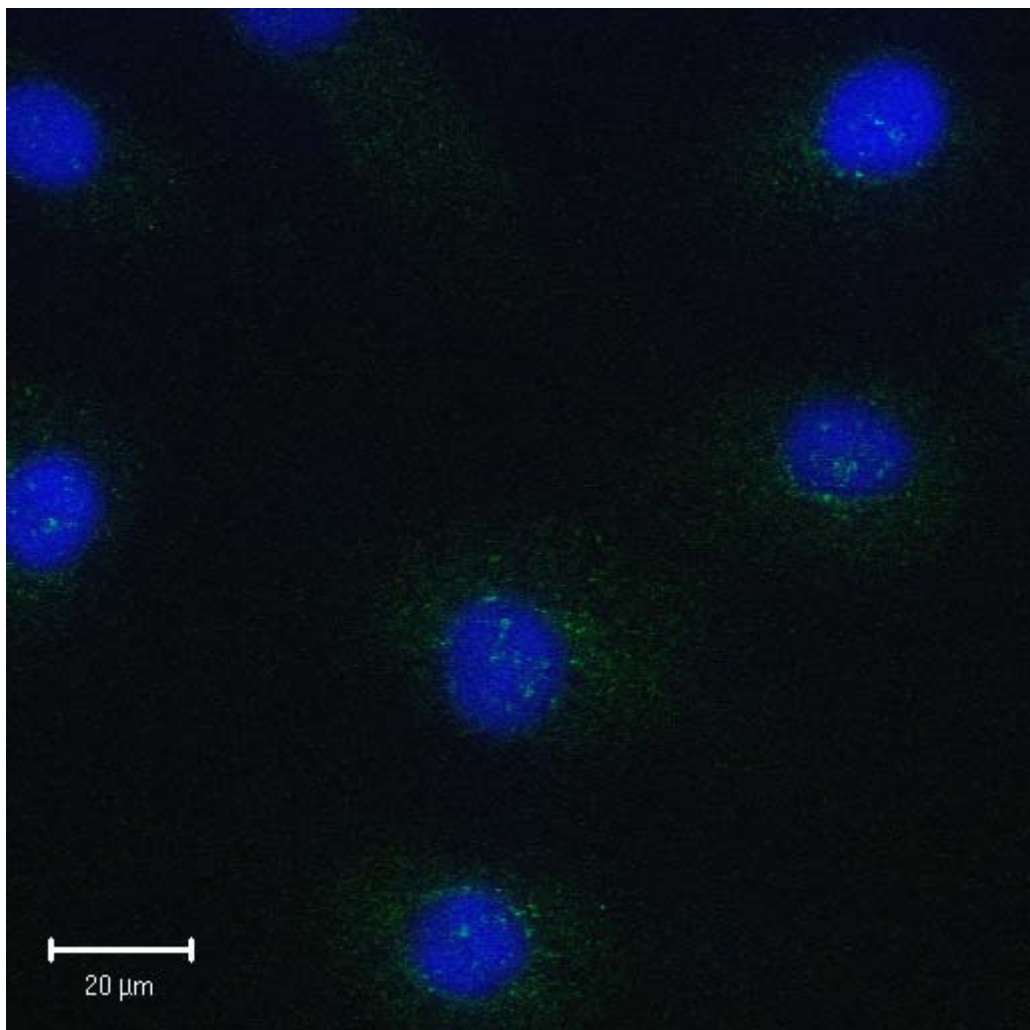


Figure 6.27 Immunofluorescence analysis of P2Y₂ receptor using anti-P2Y₂ antibody (Zymed) at 3µg/ml dilution range in ECV304 cells. Data are representative of three independent experiments.

6.4 Discussion

This study aimed to evaluate the expression of P2Y receptors in human epithelial and endothelial cells using western blot analysis. Rabbit anti-P2Y receptor subtype antibodies were used including a rabbit polyclonal anti-P2Y₂ antibody raised to L247 peptide (a peptide that mimics the third extracellular loop of the human P2Y₂ receptor).

Anti-P2Y₂ antibody raised against the peptide L247 revealed strong immunoreactivity in ECV304, EAhy926, BAEC and BRIN-BD11 cells. These responses were antigen specific in ECV304 and EAhy926 cell extracts as the pre-absorption with the immunogenic peptide abolished these responses. This suggests that the antibody is immunogen specific and that it recognises human P2Y₂ receptor reported to be expressed in ECV304 and EAhy926 cells (Graham *et al.*, 1996a; Graham *et al.*, 1996b; Brown *et al.*, 2000; Paul *et al.*, 2000; Brown, 2001). Furthermore, ECV304 and EAhy926 cells have also been shown to transcribe P2Y₂ mRNA (Chapter 4). The band observed for BAE cells, however, after pre-absorption with immunogenic peptide did not disappear. Pharmacological studies using BAE cell extracts have been reported that they express P2Y₂-like receptors. However, molecular biology studies with bovine P2Y receptor specific primers and antibodies are necessary to determine expression of P2Y receptors in these cells. The calculated molecular weight of the human P2Y₂ receptor is 42.14 kDa. Studies have reported a band size of 45-47 kDa (Santacruz Biotech). Immunoreactivity at higher molecular masses has been reported in response to anti-P2Y₂ antibodies in endothelial cells (36 and 50 kDa) and is possibly due to glycosylation (Wang *et al.*, 2002).

Immunoprecipitation of HA-tagged P2Y₂ receptor expressed in 1321N1 cells has shown a non-uniform distribution of the receptor (57-76 kDa and a single band of 45 kDa) because of heterogeneous complex glycosylation (Flores *et al.*, 2005). The bands observed in BRIN-BD11 cell extracts may be of non specific nature. The immunogenic peptide sequence also corresponds to amino acid residues 271-284 of P2Y₂ receptor of Norway rat (gi: 38197686), which may explain the cross species reactivity in BRIN-BD11 cells.

Rabbit anti-P2Y₂ receptor antibodies from commercial sources produced immunoreactive profiles with multiple bands (at different molecular mass) in ECV304 and EAhy926 cells. The immunoreactive profile was different than that yielded by L247 anti-P2Y₂ antibody and without peptide control it is not possible to characterise the specificity of these antibodies. These antibodies also showed immunoreactivity with 1321N1, BAEC and BRIN-BD11 cells. The results obtained with commercial antibodies show a different immunoreactivity profile and do not appear to be receptor specific. In conclusion, these results suggest that L247 anti-P2Y₂ antibody is receptor specific. However, western blot analysis with peptide control is necessary to characterise the immunoreactivity of commercial antibodies.

Immunofluorescence analysis using anti-P2Y₂ antibodies in ECV304 cells show that L247 anti-P2Y₂ antibody strongly binds to these cells. The widespread binding makes it difficult to localise cellular compartments and the degree of binding with these results. However, based on this result it appears that this antibody strongly stains the membrane and the nucleus. Immunofluorescence results obtained using other commercial anti-P2Y₂ antibodies suggest that they too bind to the cell membrane and nucleus in a manner similar to L247 anti-P2Y₂ antibody. Most of these commercially available antibodies are poorly characterised with only immunohistochemistry or immunofluorescence assays. These assays only confirm immunoreactivity of these antibodies with the non-denatured (intact) receptor. Therefore, even though these antibodies may be receptor specific their lack of immunoreactivity with denatured receptor in western blot analysis limit their usefulness as a diagnostic tool in receptor pharmacology.

Based on results of western blot analysis of ECV304 cell extracts, it appears that the P2Y₂ receptor protein is glycosylated in these cells. The reduced band size from 84 kDa to 80 kDa of the protein post-deglycosylation confirms this. Glycosylation of P2Y receptors has been reported in previous studies where immunoprecipitation of the HA-tagged P2Y₂ receptor expressed in 1321N1 cells revealed a non uniform distribution of the receptor protein typical of membrane glycoproteins with heterogenous complex glycosylation. Asparagine linked high mannose carbohydrates as well as hybrid and complex oligosaccharides were proposed to be the glycoproteins (Flores *et al.*, 2005).

The agonist induced P2Y₂ receptor sequestration/down-regulation studies were used to analyse the immunospecificity of anti-P2Y₂ antibody. The reduction in the ability of the anti-P2Y₂ antibody to stain protein bands as a result of sequestration/down-regulation in protein extract from ECV304 cells after 1 hour preincubation with 300µM UTP suggest that the L247 anti-P2Y₂ antibody is P2Y₂ receptor specific. However, L247 anti-P2Y₂ antibody produced strong immunoreactivity in cells after a prolonged (24 hour) agonist incubation which appears to be of higher intensity. Further investigations are required to determine why this happens. The pre-treatment of cells with agonist causes the rapid weakening of the stimulated response known as receptor desensitisation. This may also lead to receptor sequestration whereby the receptor selectively loses its capacity to bind hydrophilic (membrane-impermeable ligands) but not hydrophobic ligands due to rapid internalization of the surface receptor. Furthermore, the prolonged incubation (>1 h) with the agonist is shown to cause the down-regulation of the receptor, resulting in reduction in the receptor number (Yu *et al.*, 1993). Desensitisation and sequestration are thought to be reversible upon the removal of agonist (Yu *et al.*, 1993). However, the results from this study suggest that because of the nature of prolonged incubation, the agonist may have been degraded by ectonucleotidases and therefore not present at 24 hours. This may explain the strong immunoreactivity seen with cell extracts after prolonged (24 hour) agonist incubation.

Phosphorylation of β₂- adrenergic receptors has been reported in agonist induced desensitisation and sequestration of the receptors from the cell surface. These studies suggested that the desensitisation of β₂- adrenergic receptors was independent of sequestration (Hausdorff *et al.*, 1990; Campbell *et al.*, 1991). Sequestration is thought to be essential to facilitate resensitization of the receptors by a process involving dephosphorylation and subsequent recycling of the receptor back to the cell surface. Sequestration-defective mutant β₂- adrenergic receptors were unable to resensitise (Barak *et al.*, 1994). Agonist-induced desensitisation and internalization (sequestration) of P2Y₂ receptors has also been reported in HA-tagged P2Y₂ receptors expressed in 1321N1 cells. Removal of agonist from the medium resulted in recovery of cell surface immunoreactivity to control levels within approximately 1 hour. Desensitisation was more rapid and occurred to a greater extent than the agonist induced loss of surface receptors (Sromek and Harden, 1998).

Human P2Y₂ receptors have also been reported to undergo UTP induced homologous receptor desensitization in receptors expressed in 1321N1 astrocytoma cells as well as in human A431 cells which endogenously express P2Y₂ receptor (Velázquez *et al.*, 2000). Furthermore, potent P2Y₂ receptor desensitization observed in human 1321N1 astrocytoma cells expressing recombinant wild type and C-terminal truncation mutants of the P2Y₂ receptor was not related to depletion of calcium from intracellular stores. The same study also reported that the phosphorylation / dephosphorylation regulate receptor desensitization/resensitization; and phosphorylation of the C-terminus of the P2Y₂ receptor by protein kinases other than protein kinase C mediates agonist-induced receptor desensitization (Otero *et al.*, 2000). The involvement of protein kinase C (PKC) has also been ruled out in sequestration of P2Y₂ receptor after co-incubation with protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) and UTP produced sequestration similar to that obtained with UTP alone (Garrad *et al.*, 1998).

Wild-type P2Y₂ receptor expressed in 1321N1 cells have been shown to desensitise and sequester in a time-dependant manner when treated with UTP. These studies also reported resensitisation of a sequestered receptor truncated by 30 amino acids from C-terminal to resist desensitisation. Notably, this mutant P2Y₂ receptor was treated for more than 3 hours with agonist to bring about sequestration, which might have involved receptor down-regulation (Garrad *et al.*, 1998).

Later studies, however, reported that phosphorylation leads to receptor desensitisation and internalisation. Truncation of three potential GRK and PKC phosphorylation sites in C-terminus and the third intracellular loop (S243, T344, S356) to alanine reduced agonist-induced receptor phosphorylation as well as the efficacy of UTP to desensitize P2Y₂ receptor signalling to intracellular calcium mobilization, a finding suggestive of the importance of C-terminus for receptor desensitisation and sequestration (Garrad *et al.*, 1998). Agonist induced desensitisation involving an increase in receptor phosphorylation was reported in HA-tagged P2Y₂ receptor expressed in 1321N1 cells in which agonist induced internalisation of the P2Y₂ receptor was impaired after truncation of potential phosphorylation sites (Flores *et al.*, 2005).

Notably, in the above study an unphosphorylated 45 kDa protein band was produced in agonist stimulated, detergent solubilised, total cell extracts suggesting unglycosylated receptors from intracellular membranes are not accessible to phosphorylation (Flores *et al.*, 2005). However in this study no bands were observed at lower molecular mass which may be due to the fact that specially extracted membrane protein were used in this study. As reported by Garrad, *et al.*, 1998, where P2Y₂ receptors were resensitised even after 3 hours of agonist stimulation, in this study too P2Y₂ receptors which underwent prolonged agonist stimulation (24 hours) were resensitised. This may suggest that UTP stimulation for an hour just causes the cells to sequester/internalise so they are inaccessible for L247 anti-P2Y₂ antibody. However, over a period of 24 hours the receptors are recycled back to the surface and hence the binding with the antibody.

Irrespective of whether internalisation was due to sequestration or down-regulation or whether this process involves receptor phosphorylation, it is true that the antibody exhibits immunospecificity with the P2Y₂ receptor. In addition to the results obtained with antigen control, these studies strongly suggest that L247 anti-P2Y₂ antibody shows strong immunoreactivity with P2Y₂ receptors. Besides, the protein extract from the cells preincubated with histamine for similar time periods showed no such masking of protein staining as observed by their strong bands. However, the desensitisation effect was not observed in protein extracts using anti-P2Y₂ antibody from Santa Cruz Biotechnology. There was no visible difference between the bands produced by the antibody in control cells and cells challenged with agonist for 1 hour. Thus, suggesting that this antibody may not be binding to the P2Y₂ receptor.

In this study, the sequence obtained through Edman sequencing does not show homology with the P2Y₂ receptor sequence. This is probably as a result of sequencing of some other protein expressed in close proximity to P2Y₂ receptor molecular range and/or which may be present in higher concentration. However, based on this result it cannot be said if the rank order of protein quantity determines the sequence during Edman sequencing. Alternatively, based on the protein sequencing results it may also appear that P2Y₂ receptor protein is associated with some other protein as the resultant amino acid residues from protein sequencing do not match the sequence of P2Y₂ receptor.

Furthermore, the sequence DRIKDEFQL or RIKDEFQ - as the identity of D and L is not certain - aligned with the human amino-terminal enhancer of split isoform a, b and c (gi: 39812019; gi: 5706731; gi: 39812027). The protein encoded by this gene is similar in sequence to the amino terminus of *Drosophila* enhancer of split groucho, a protein involved in neurogenesis during embryonic development. This protein belonging to the groucho/TLE family of proteins can function as a homo-oligomer or as a hetero-oligomer with other family members to dominantly repress the expression of other family member genes (Chen and Courey, 2000). The predicted molecular mass of this protein is 28.96 kDa and in digested ECV304 cell extracts the protein band detected by L247 anti-P2Y₂ antibody had a molecular mass of 80 kDa. It is possible that this protein is associated with the P2Y₂ receptor. Post-translational modifications such as glycosylation, ubiquitination, and sumoylation are known to increase the mass of a protein (Ahmad *et al.*, 2005). The variability in the molecular weight of P2Y₂ receptor in ECV304 cells could be due to the fact that the P2Y₂ protein may have had post-translational modifications that are different for different tissues (Sage and Marcus, 2002).

The P2Y₂ receptor has been reported to exist as homo-oligomer in non-hematopoietic and hematopoietic cells (Kotevic *et al.*, 2005). Moreover, human amino-terminal enhancer of split isoform protein may be present as a homo or hetro-oligomer. However, under reducing conditions of SDS-PAGE it is unlikely for a homo or hetero-oligomeric P2Y₂ receptor complex to remain intact. Nevertheless, it is possible that the native quaternary structure of P2Y₂ receptor may not have been dissociated by SDS as resistance to dissociation by SDS has been reported by other membrane proteins such as tetrameric AqpZ aquaporin from *E. coli*, the prokaryotic K⁺ channel and the calcium regulatory protein phospholamban (Fujii *et al.*, 1989; Heginbotham *et al.*, 1997; Borgnia *et al.*, 1999). However, the dissociation has been achieved in membrane proteins, phospholamban and *E. coli* AmtB by boiling of the protein prior to SDS-PAGE (Fujii *et al.*, 1989; Blakey *et al.*, 2002). Dopamine D₁ receptor and dopamine D₂ receptors have been shown to be capable of forming homo-oligomers (Ng *et al.*, 1994a; Ng *et al.*, 1994b). Homodimers in G-protein coupled receptors such as β₂ adrenergic receptors have also been reported to resist SDS dissociation (Hebert *et al.*, 1996). Furthermore, the larger protein bands detecting the dopamine D₃ receptor oligomers expressed in brain tissues of rodent, primate and human appeared to be multiples of

monomers and were resistant to both SDS and heat (Nimchinsky *et al.*, 1997). In this study, the protein was heated for 3 minutes at 95°C before SDS-PAGE and results from both the peptide control and desensitisation studies suggest that the band detected in ECV304 cell extracts was that of P2Y₂ receptor. Furthermore, L247 anti-P2Y₂ antibody detected only a single band, thus suggesting that P2Y₂ receptor may exist as a homo-oligomer or hetero-oligomer in ECV304 cells. Therefore, it appears that either the P2Y₂ protein is partially deglycosylated in digested ECV304 cell extracts or that the receptor is part of a complex that is SDS-resistant and thermostable. Proteins like arrestins that promote the processes of desensitization, internalization, down-regulation, and resensitization of G protein-coupled receptors may be forming a complex with P2Y₂ receptors (Krupnick and Benovic, 1998). However, studies have reported that the desensitization of the P2Y₁ and P2Y₂ receptors was unaffected when arrestin levels were reduced in HEK293 cells (Mundell and Benovic, 2000). Moreover, A₁ adenosine receptor has been shown to form a heteromeric complex with P2Y₁ receptor in heterologously transfected cells and rat brain tissues generating an adenosine receptor with P2Y-like agonistic pharmacology (Yoshioka *et al.*, 2001; Yoshioka *et al.*, 2002a; Yoshioka *et al.*, 2002b; Nakata *et al.*, 2005). Therefore, it is possible that P2Y₂ receptor may form heteromeric association with different G protein-coupled receptors to regulate its functional activity.

Furthermore, the resultant sequence also aligned with gp130 associated protein GAM (gi: 3309589), transducin-like enhancer of split 3 splice variant 1, 2 (gi: 46391800; gi: 46391802), Transducin-like enhancer protein 3 (TLE3) (gi: 34783362; gi: 27469815; gi: 20532417; gi: 4827030), KIAA1547 protein (gi: 10047159). Transducin-like enhancer protein 3 is a transcriptional corepressor that binds to a number of transcription factors. Inhibits the transcriptional activation mediated by CTNNB1 and TCF (transcriptional co-activator of T cell factor) family members in Wnt signaling. The effects of full-length TLE family members may be modulated by association with dominant-negative AES (amino-terminal enhancer of split proteins). The predicted molecular mass of Transducin-like enhancer protein 3 is 83.41 kDa (swissprot: locus TLE3_HUMAN, accession Q04726). However, there is no basis for L247 anti-P2Y₂ antibody to cross react with this protein as the immunogenic peptide (RSLDLSCHTLNAIN) when aligned with above protein sequences no significant similarity was found suggesting the antibodies are not directed to these protein sequences.

The first extracellular loop of the P2Y₂ receptor has been reported to bind to integrins through the cell attachment site RGD (Arg-Gly-Asp) motif; and RGD along with Leu¹⁰⁸ is reported to be involved in apical targeting of the P2Y₂ receptor (Ruoslahti, 1996; Qi *et al.*, 2005). RGD sequence in the first extracellular loop has also been reported to facilitate the interaction of P2Y₂ nucleotide receptor with vitronectin receptors (Gonzalez *et al.*, 2005). This interaction has been shown to be essential for effective signal transduction to activate mitogen-activated protein kinases ERK1/2, to mobilize intracellular calcium stores via activation of phospholipase C, protein kinase C isoforms, and to activate focal adhesion kinase and other signaling events. Ligation of vitronectin receptors with specific antibodies caused an inhibition of P2Y₂ receptor-induced ERK1/2 and p38 phosphorylation and P2Y₂ receptor-induced cytoskeleton rearrangement and DNA synthesis (Gonzalez *et al.*, 2005). Activated P2Y₂ receptors through RGD have also been reported to bind α v β 3/ β 5 integrins and stimulate integrin signalling pathways that regulates cytoskeletal reorganization and cell motility (Weisman *et al.*, 2005).

However, human amino-terminal enhancer of split isoforms a, b and c, gp130 associated protein GAM, transducin-like enhancer of split 3 splice variants 1 and 2, TLE3 protein, Transducin-like enhancer protein 3 do not have a RGD motif in their structure. Moreover, the protein was sequenced from the unstained section of PVDF membrane area (not used in western blot analysis), which was corresponding to the band detected with anti-P2Y₂ antibody. Therefore, it is possible that Transducin-like enhancer protein 3 may have been sequenced instead of P2Y₂ receptor. Thus, it is not clear from these studies if P2Y₂ receptor protein is associated with some other protein or exists as a separate entity in ECV304 cells. Therefore, the identity of other proteins in the complex may provide information of any regulatory role played by these proteins on P2Y₂ receptors. Nevertheless, above results strongly support the immunospecificity of L247 anti-P2Y₂ antibody to human P2Y₂ receptor.

The western blot analysis using anti-P2Y₁ antibody showed immunoreactive bands in the cell extracts of EAhy926 and BRIN-BD11 cells. In EAhy926 cells the immunoreactive bands were of higher molecular mass than 42 kDa expected for wild-type P2Y₁ receptor (Hoffmann *et al.*, 1999). However, this antibody has been reported

to recognize P2Y₁ protein at 66 kDa from human platelets and rat brain membranes by immunoblotting (Sigma). These results obtained here are in contrast with those obtained with RT-PCR studies (Chapter 4) where the presence of P2Y₁ receptor mRNA transcripts has been reported in ECV304 cells. Moreover, previous studies have supported the functional expression of P2Y₁ receptor in ECV304 cells based on P2Y₁ receptor mediated agonist responses (Conant *et al.*, 1998; Brown *et al.*, 2000; Brown, 2001).

In EAhy926 this antibody produced a strong immunoreactive band at 52 kDa. However, it is to be noted that 2MeSATP, a potent agonist of P2Y₁ did not show (Chapter 3) any significant dose-dependent accumulation of inositol phosphates (Graham *et al.*, 1996a). Besides, under the experimental conditions RT-PCR studies did not provide evidence for mRNA expression of P2Y₁ receptors (Chapter 4). Therefore, this antibody may not be recognising the P2Y₁ receptor. No bands were observed in BAE cell extracts, which are known to express P2Y₁ receptors (Brown and Brown, 2002).

RT-PCR studies (Chapter 4) did not show evidence for the expression of P2Y receptor subtypes in 1321N1 cells and there are no published studies to relate the expression of P2Y receptors in BRIN-BD11 cells. The similar profile in EAhy926 and BRIN-BD11 cells, given the absence of mRNA data for P2Y₁ receptors suggest non-specific binding of this antibody. Moreover, the immunogenic peptide used to raise this antibody also corresponds to amino acid residues 242-258 of bovine and rat P2Y₁ receptor protein, which may explain the cross species immunoreactivity observed in BRIN-BD11 cells.

Polyclonal antibodies to human P2Y₁ receptors have also been reported to bind P2Y₁ protein in human and rat brain membranes at 63 kDa (Moore *et al.*, 2000).

Antibody to rat P2Y₁ receptor (330-345 amino acid residues of C-terminus) has been reported to show immunoreactivity in rat and bovine brain at 42 kDa (Moran-Jimenez and Matute, 2000). Sheep anti-P2Y₁ antibody raised to 36-51 amino acid residues of rat N-terminal segment (also corresponding to human P2Y₁ varied by 2 residues) bound rat brain at 65 kDa (Fong *et al.*, 2002). Furthermore, the higher bands (increased molecular mass) observed for anti-P2Y₁ antibody has been attributed to glycosylation of the receptor in its extracellular domain (Wang *et al.*, 2002). Therefore, further western blot

studies should include antigen control and analysis of the receptor protein for N-linked glycosylation to determine specificity and the immunoreactivity of this antibody.

The anti-P2Y₄ receptor antibody detected strong multiple bands in all the cell types including the control 1321N1 cells. The calculated molecular weight of human P2Y₄ receptor is 40.83 kDa (gi: 4505561). Most of the prominent bands observed in all cell types including ECV304 and EAhy926 cell extracts are of higher molecular mass, in the range of 50-70 kDa. However, unlike in EAhy926 cells RT-PCR studies (Chapter 4) in ECV304 cells have shown the presence of P2Y₄ mRNA transcripts. The detection of P2Y₄ mRNA and the subsequent DNA sequence result (Chapter 3) in ECV304 cells, however, is inconsistent with previous functional studies, which suggest that the P2Y₄ receptor is not present in ECV304 cells (Brown, 2001). There is no evidence to support the expression of P2Y₄ receptors by BAE cells (Brown and Brown, 2002) and there is no published evidence that BRIN-BD11 cells express P2Y₄ receptors. However, the immunogenic peptide sequence of this antibody also corresponds to amino acid residues (70%) 306-365 of bovine predicted uridine nucleotide receptor (gi:61868522) and amino acid residues (68%) of 302-361 of rat P2Y₄ receptor (gi:13928944). Nonetheless, the detection of multiple bands in all cells including 1321N1 cells suggests that this antibody may not be P2Y₄ receptor specific and not useful in P2Y₄ receptor research.

The anti-P2Y₆ receptor antibody stained multiple protein bands in all the cell extracts. The calculated molecular weight of human P2Y₆ receptor is 36.29 kDa (gi: 29029612) and in ECV304 cells this antibody produced a strong band at 39 kDa. Other bands of higher molecular range between 50-85 kDa appeared in both ECV304 and EAhy926 cells. The binding at higher molecular mass is also reported previously with other anti-P2Y₆ antibodies. The anti-P2Y₆ antibody (Glaxo Wellcome) stained one band at 45 kDa and 75 kDa in human umbilical vein endothelial cells (Wang *et al.*, 2002). RT-PCR studies have shown that both ECV304 and EAhy926 cells express P2Y₆ mRNA transcripts (Chapter 4). However, P2Y₆ agonist hexokinase – treated UDP failed to elicit accumulation inositol phosphates and cytosolic calcium response in ECV304 cells (Brown *et al.*, 2000) and EAhy926 cells have reported to show the lack of functional response to P2Y₆ receptor agonists (Paul *et al.*, 2000). However, studies have shown the dose-dependent accumulation of inositol phosphates in response to UDP (Chapter 3)

supporting expression of P2Y₆ mRNA in these cells. Even though the RT-PCR studies have indicated the presence of P2Y₆ mRNA in both ECV304 and EAhy926 cells; the contrasting pharmacological results obtained in response to receptor specific agonists highlight the difficulty of P2Y receptor pharmacology. Furthermore, anti-P2Y₆ antibody detects multiple bands in 1321N1 and ECV304 cell extracts in which P2Y₆ receptors are not expressed. Therefore, taking these results into consideration the next main objective is to analyse the immunospecificity of this antibody with antigen control to determine whether the strong bands showed by this antibody are P2Y₆ receptor specific.

The multiple strong bands produced by this antibody in 1321N1 are probably due to non specific binding as these cells do not respond to nucleotides. Furthermore, RT-PCR studies showed no sign of P2Y₆ mRNA in 1321N1 cells (Chapter 4). This anti- P2Y₆ rabbit polyclonal antibody corresponding to amino acids 1-70 mapping the N-terminus of human P2Y₆ also corresponds to amino acid residues (81%) 1-70 of rat P2Y₆ receptor (gi:47938969) and residues 1-89 (39%) of bovine G protein-coupled receptor 8 (gi:27806597). However, the binding shown in BAE and BRIN-BD11 cells by this antibody cannot be accounted for cross species reactivity as there is no published data for the expression of P2Y₆ receptor by BRIN-BD11 and BAE cells. Therefore, as with anti-P2Y₄ antibodies, the anti-P2Y₆ antibody appears to be of no value in P2Y receptor research.

The anti-P2Y₁₁ receptor antibody detected weak bands in both ECV304 (44 kDa and 60 kDa) and EAhy926 cells (60 kDa, 88 kDa, 114 kDa and 132 kDa). The calculated molecular weight of human P2Y₁₁ receptor is 40.21 kDa (gi: 23463303). In human umbilical vein endothelial cells, P2Y₁₁ antibody (Glaxo Wellcome) has been reported to detect a band at 60 kDa, which disappeared when the antibody was pre-absorbed with the immunogenic peptide (Wang *et al.*, 2002). Moreover, the RT-PCR studies have shown that ECV304 cells express P2Y₁₁ mRNA transcripts which matched with primer specific mRNA sequence with >98% similarity (Chapter 4). Besides, ECV304 cells has been shown to dose-dependently accumulate inositol phosphates in these cells in response to ADPβS, a P2Y₁ and P2Y₁₁ agonist (Brown, 2001). Furthermore, the expression of P2Y₁₁ receptor has been supported by studies showing the accumulation of cAMP and inositol phosphates and intracellular calcium release in response to

ADP β S in ECV304 cells (Brown, 2001). The accumulation of inositol phosphates in response to BzATP and ATP γ S, the potent agonists of P2Y₁₁ receptors suggest the presence of P2Y₁₁ receptors in ECV304 cells. In addition, the dose-dependent accumulation of cAMP in ECV304 cells in response to ATP γ S further supports the presence of functional human P2Y₁₁ receptors coupled to both the phosphoinositide and the cyclic AMP pathways (Chapter 3). Therefore, based on pharmacological and RT-PCR studies it appears that functional P2Y₁₁ receptors are expressed in ECV304 cells.

RT-PCR studies have shown that EAhy926 cells do not express P2Y₁₁ receptors and the functional studies with 2MeSATP, and BzATP a potent agonist of P2Y₁₁ did not show any significant dose-dependent accumulation of inositol phosphates (Chapter 3 and Chapter 4). These results suggest that EAhy926 cells do not express functional P2Y₁₁ receptors. There are no reports to support the expression of P2Y₁₁ receptors in BAE cells. However, similar pattern has been also seen with 1321N1 and BRIN-BD11 cells. Therefore, western blot analysis with anti-P2Y₁₁ antibody pre-absorbed with immunogenic peptide may prove to be vital in determining the immunospecificity of this antibody. Nevertheless, the response is very weak at the highest possible concentration used from the available stocks, suggesting that this antibody is not sensitive.

The anti-P2Y₁₂ receptor antibody did not show any immunoreactive bands in any of the cell extracts used in this study. The calculated molecular weight of the human P2Y₁₂ receptor is 39.30 kDa (gi: 12232483). RT-PCR studies have shown the presence of P2Y₁₂ mRNA transcripts in ECV304 cells (Chapter 4). ADP, an agonist of P2Y₁₂ receptors has shown weak agonistic potency in ECV304 cells (Brown *et al.*, 2000). Likewise ADP β S (agonist at P2Y₁, P2Y₁₁, P2Y₁₂) has caused the accumulation of inositol phosphates and a rise in intracellular calcium release. However, these responses cannot be attributed to the functional expression of P2Y₁₂ receptors because of their shared affinity to other P2Y subtypes. Therefore, the absence of pharmacological data and the evident western blot results suggest that ECV304 cells do not express P2Y₁₂ receptors.

The anti-P2Y₁₃ receptor antibody detected strong bands in 1321N1, ECV304, EAhy926, BAE and BRIN-BD11 cells. All these bands are of higher molecular mass than the calculated molecular weight of 38.27 kDa for human P2Y₁₃ receptor (gi: 29171721). RT-PCR studies have shown the presence of P2Y₁₃ mRNA transcripts in ECV304 cells (Chapter 4). ECV304 cells showed weak agonistic potency whereas EAhy926 cells (Chapter 3) did not produce significant stimulation of inositol phosphates in response to ADP, a potent agonist of P2Y₁₂ and P2Y₁₃ receptors (Brown *et al.*, 2000). Pharmacological data and RT-PCR studies suggest that both ECV304 and EAhy926 cells do not express P2Y₁₃ receptors. Future studies may include measurement of cAMP in response to 2MeSADP and 2MeSATP; and inositol phosphate assays with P2Y₁₂ and P2Y₁₃ antagonists to determine whether these cells express receptors that are coupled to the inhibition of adenylyl cyclase. Furthermore, without exact sequence of immunising peptide (obtained from second extracellular loop of the human P2Y₁₃ receptor) it is difficult to ascertain the cross species reactivity in BAE and BRIN-BD11 cells. Therefore, western blot analysis of anti-P2Y₁₃ antibody pre-absorbed with immunogenic peptide may determine the immunospecificity of this antibody. However, the immunoreactivity of anti-P2Y₁₃ antibody in 1321N1 cell extracts coupled with a lack of evidence from RT-PCR studies suggest that this antibody is not suitable to study P2Y receptors.

These results suggest that L247 anti-P2Y₂ antibody is P2Y₂ receptor specific and recognises denatured receptors in both ECV304 and EAhy926 cells. Furthermore, this antibody also recognises native non-denatured P2Y₂ receptors in ECV304 cells as shown by immunofluorescence studies. The commercially available anti-P2Y₂ antibodies show dissimilar and non specific reactivity in western blot studies and therefore may not be recognising the denatured P2Y₂ receptors. A weak band was detected in ECV304 cell extracts by anti-P2Y₁₁ antibody. Therefore, based on the established pharmacological and RT-PCR data it appears that ECV304 cells co-express P2Y₂ and P2Y₁₁ receptors. Furthermore, pharmacological and RT-PCR studies also confirm that the EAhy926 cells co-express P2Y₂ and P2Y₆ receptors.

CHAPTER 7

GENERAL DISCUSSION

7.1 Justification for this research project

Study of P2Y receptors has been compromised by the lack of selective ligands. Functional studies using nucleotide agonists provide inconclusive data for the expression of P2Y receptors. This is because P2Y receptors can be activated by different nucleotides and a single nucleotide activates more than one receptor. Moreover, the use of radiolabelled nucleotides has been constrained by their metabolic instability and their binding to non-P2 receptor proteins. In addition, anti-P2Y receptor antibodies were not available at the beginning of this study. Building on studies of peptide receptors, with which P2Y receptors share the sequence homology, peptides were designed and synthesised specifically mimicking the extracellular regions of the human P2Y₂ receptor. Peptide L247 from ECIV (extracellular region IV) and spanning amino acid residues 271-284, mimicking the third extra cellular loop of the human P2Y₂ receptor was shown to be a weak agonist of inositol phosphate (IP₃) production and shown to stimulate nitric oxide synthase activity in both epithelial and endothelial cells. In addition, when added simultaneously with ATP, L247 has been shown to have synergistic effects on inositol phosphate production and intracellular calcium release in epithelial cells suggesting that L247 acted as allosteric modulator of the P2Y₂ receptor (Brown, 2001). These results appeared to agree with reports that peptides designed to mimic the defined regions extracellular loops of peptide receptors act like a functional agonist (Van Rhee *et al.*, 1995; Howl and Wheatley, 1996). Furthermore, these results were in accord with the suggestion that for peptide receptors the neuropeptide binding involves residues on the top of several transmembrane domains and in extracellular loops of the receptors (Berthold and Bartfai, 1997). Based on these promising results, L247 peptide was used as an immunogen to raise P2Y₂ receptor sensitive and selective antibody in order to study endogenous P2Y₂ receptors in their native environment. Moreover, the immunogenic peptide sequence (RSLDLSCHTLNAIN) is unique for the P2Y₂ receptor as it did not show homology when aligned with other P2Y receptor sequences. Thus, peptide-specific antibody strategy was selected to raise the antibody and also to minimize or eliminate the cross-reactivity with other closely related P2Y receptor subtypes.

7.2 Purification of polyclonal antibodies

The rabbit polyclonal antiserum was purified using a PROSEP- Thosorb thiophilic affinity adsorbent column and the results suggest that this system can be reliably used for the purification of antibodies. The analysis of the purified antibody fractions using indirect ELISA suggests that the antibody fractions have a strong and specific immunoreactivity with L247 peptide, the immunising antigen (L247 peptide). The two antibody fractions that were tested bound to L247 peptide in a dose-related manner suggesting the reaction specificity of the antibody to antigenic immunogen, although fraction II bound four fold higher than fraction I. Furthermore, the binding observed for an unrelated peptide (designed to HERV) was minimal, confirming the specificity of antibody to L247 peptide. The minimal binding that occurred with HERV peptide may be due to cross reactivity of antibody fractions and the presence of non-specific immunoglobulins eluted during the purification process. Synthetic antigen linked to the affinity adsorbent column can eliminate the presence of non-specific immunoglobulins during the purification process to provide the antibody with desired specificity (Muronetz and Korpela, 2003). The results from indirect ELISA suggest the successful generation of sequence specific antibody to L247 peptide designed to mimic specific region in the extracellular domain of the human P2Y₂ receptor.

7.3 Evaluation of P2Y₂ receptors in epithelial and endothelial cells

The dose-dependent accumulation of inositol phosphate and intracellular calcium release with UTP and ATP, the equipotent agonists at P2Y₂ receptors, have already established the presence of functional human P2Y₂-like receptors in ECV304 cells (Brown *et al.*, 2000). Furthermore, receptor desensitisation studies carried out in tandem with intracellular calcium release suggests the absence of human P2Y₄ receptors in these cells (Brown, 2001). The detection of mRNA transcripts for P2Y₂ receptors is consistent with previous reports suggesting the expression of P2Y₂ receptors in ECV304 cells (Brown *et al.*, 2000).

The dose-dependent accumulation of inositol phosphates in response to UTP and ATP observed in this study are consistent with previous reports suggesting the expression of P2Y₂ receptors in EAhy926 cells (Graham *et al.*, 1996a; Graham *et al.*, 1996b; Paul *et*

al., 2000). As both UTP and ATP are full agonists at both P2Y₂ and P2Y₄ receptors, RT-PCR studies were used to determine which receptor is expressed in EAhy926 cells. Functional data (Chapter 3) and the presence of mRNA transcripts for P2Y₂ and not P2Y₄ receptors (Chapter 4) suggest that the P2Y₂ receptor is functionally expressed in EAhy926 cells.

7.4 Characterisation of a novel anti-P2Y₂ antibody

Evaluation of rabbit polyclonal anti-P2Y₂ antibodies raised to L247 peptide designed to mimic the third extracellular loop of human P2Y₂ receptor, suggests that this antibody is not only immunospecific but can also bind to the P2Y₂ receptor in its native environment. This anti-P2Y₂ antibody detected bands in ECV304, EAhy926, BAEC and BRIN-BD11 cell extracts. Furthermore, these responses were antigen specific as the pre-absorption of the antibody with the immunogenic peptide abolished the response seen for ECV304 and EAhy926 cells. This further confirms the specificity of this antibody to the peptide immunogen. These results are consistent with the pharmacological data and detection of mRNA transcripts supporting the expression of P2Y₂ receptors in ECV304 and EAhy926 cells (Graham *et al.*, 1996a; Graham *et al.*, 1996b; Brown *et al.*, 2000; Paul *et al.*, 2000; Brown, 2001). The bands observed for BAE cell extracts, known to express P2Y₁ and P2Y₂ receptors and BRIN-BD11 cells may be of non specific nature as they did not disappear after pre-absorption with immunogenic peptide.

The higher observed molecular weight of the P2Y₂ receptor in ECV304 and EAhy926 cells may have been due to post-translational modifications involving glycosylation, ubiquitination, and sumoylation that are known to increase mass of a protein (Sage and Marcus, 2002; Ahmad *et al.*, 2005). Receptor glycosylation has been shown to be a feature of P2Y₂ receptors (Flores *et al.*, 2005). The reduced band size of the protein post-deglycosylation confirmed this. Further evidence for the glycosylation of P2Y₂ receptor in ECV304 cells was provided by the detection of a short protein sequence which was not evident prior to deglycosylation of samples. However, the protein sequence did not match with the human P2Y₂ receptor. The protein was sequenced from the unstained section of PVDF membrane area, which was corresponding to the band detected with anti-P2Y₂ antibody. Therefore, it is most likely that some other protein

that migrates in close proximity to the P2Y₂ receptor on the SDS-PAGE gel may have been sequenced. Analysis of the sequence obtained suggests that it closely matches with the sequence of Transducin-like enhancer protein 3 with the predicted molecular mass of 83.41 kDa, which is in the range of the reduced band size of 80 kDa of the protein post-deglycosylation. Therefore it is possible to have sequenced transducin protein that is of similar molecular size and migrates at the same rate as P2Y₂ receptor protein.

On the other hand, P2Y₂ receptor protein in ECV304 cells may exist as a homo-oligomer or hetero-oligomer and may exist in the form of a complex with some unknown protein which is resistant to SDS dissociation and heat. However, complex formation is not likely to occur between the receptor and transducin, but perhaps may be between another, much smaller as yet unidentified protein. Activated P2Y₂ receptors through RGD motif in the first extracellular loop have been reported to bind $\alpha\beta3/\beta5$ integrins and to stimulate integrin signalling pathways that regulate cytoskeletal reorganization and cell motility (Weisman *et al.*, 2005). Interaction of P2Y₂ receptors with vitronectin receptors has been reported to be essential for P2Y₂ mediated signal transduction (Gonzalez *et al.*, 2005). Furthermore, heteromeric association between A₁ adenosine receptor and P2Y₁ receptor in heterologously transfected cells and rat brain tissues has been shown to alter the pharmacology of A₁ adenosine receptor and generate P2Y-like agonistic pharmacology (Yoshioka *et al.*, 2001; Yoshioka *et al.*, 2002a; Yoshioka *et al.*, 2002b; Nakata *et al.*, 2005). Therefore, it is possible that the close association of the unknown protein in P2Y₂ receptor complex is essential for P2Y₂ receptor mediated signaling. Furthermore, the detection of a protein band at high molecular mass in EAhy926 cells may suggest that the P2Y₂ receptor is forming a complex in cells that contain a tumour component.

Agonist induced receptor desensitisation studies further confirmed the specificity of L247 anti-P2Y₂ antibody. The fading or loss of bands after desensitisation suggests that the desensitised P2Y₂ receptor was not accessible for antibody binding. From these studies it appears that the receptor is sequestered (internalised) on agonist stimulation for more than 1 hour. Furthermore, the antibody could not detect the internalised receptor in the membrane enriched fractions used in this study suggesting that only an insignificant number of receptors may have remained on the membrane surface after

agonist induced sequestration. The prominent bands observed with L247 anti-P2Y₂ antibody after 24 hours of UTP stimulation suggest that the receptor is resensitised possibly as a result of agonist degradation over 24 hours by endogenous ectonucleotidases. The resensitisation of the receptor observed in this study after 24 hour incubation with the agonist is significant. Studies in the past have reported that the receptor is resensitised after 3 hour incubation with the agonist (Garrad *et al.*, 1998). Thus, this study supports the argument that the prolonged agonist incubation causes internalisation of the receptor, causing the reduction in the surface receptor number (Yu *et al.*, 1993). Furthermore, it also appears that prolonged agonist incubation causes its degradation resulting in resensitisation of the receptor and that the desensitisation and sequestration are reversible (Sromek and Harden, 1998). These results are also consistent with the argument that the sequestration is essential to facilitate resensitization of the receptors by a process involving dephosphorylation and subsequent recycling of the receptor as reported in β_2 -adrenergic receptors (Barak *et al.*, 1994). Therefore, agonist induced desensitisation/sequestration of P2Y₂ receptors seen here may involve an increase in receptor phosphorylation as reported previously (Flores *et al.*, 2005). Moreover, pre-incubation with histamine for similar time periods did not show any difference on antibody binding. Thus, agonist stimulation for an hour causes the desensitisation and sequestration of the P2Y₂ receptor, which is resensitised and recycled back to the surface after degradation of the agonist caused by prolonged incubation.

The commercially available rabbit anti-P2Y₂ receptor antibodies detected different immunoreactive profiles with multiple bands (of different molecular mass) both in ECV304 and EAhy926 cells. These antibodies also showed strong immunoreactivity with 1321N1 cells, which are not responsive to nucleotides. Furthermore, the desensitisation effect did not manifest when commercial rabbit anti-P2Y₂ receptor antibody was used to study the effect of receptor desensitisation on antibody binding. There was no noticeable difference between the bands in control cells and cells challenged with agonist for 1 hour. Immunofluorescence analysis using L247 anti-P2Y₂ antibody in ECV304 cells showed widespread binding including the membrane and the nucleus making it difficult to localise P2Y₂ receptors. Immunofluorescence results obtained using other commercial anti-P2Y₂ antibodies showed similar results to L247

anti-P2Y₂ antibody. However, these studies only suggest immunoreactivity of the antibodies with the non-denatured (intact) receptor. The lack of receptor specific immunoreactivity with denatured receptor shown by commercial antibodies suggest that results obtained from immunofluorescence studies may be due to the recognition epitopes of unrelated proteins. Therefore, even if the commercial antibodies are receptor specific their lack of immunoreactivity with denatured receptor in western blot analysis limit their usefulness as a diagnostic tool in receptor pharmacology. Therefore, these antibodies do not detect denatured P2Y₂ receptor and are marketed without full characterisation. In conclusion, these results suggest that L247 anti-P2Y₂ antibody is a highly sensitive and specific antibody to human P2Y₂ receptor.

7.5 Evaluation of P2Y₁ receptors in epithelial and endothelial cells

RT-PCR studies and subsequent DNA sequence results show that the P2Y₁ receptors are transcribed in ECV304 cells. The pharmacological data in ECV304 is not conclusive as potent P2Y₁ agonist 2MeSADP was without effect and increase in the intracellular calcium release observed in response to 2MeSATP may have been mediated by P2Y₁₁ receptors and are not due to sub – population or passage specific effect, suggested previously (Conant *et al.*, 1998; Brown *et al.*, 2000; Brown, 2001). Similarly, a small accumulation of inositol phosphates observed in ECV304 cells in response to ADPβS, may have been caused by its agonistic activity at P2Y₁₁ receptor (Communi *et al.*, 1999b; Brown, 2001). Moreover, adenosine diphosphates have been shown to act as partial agonists at P2Y₁₁ receptors (Qi *et al.*, 2001). In EAhy926 cells, P2Y₁ agonist 2MeSATP, did not generate significant dose-dependent accumulation of inositol phosphates (Graham *et al.*, 1996a). Furthermore, RT-PCR studies did not detect the presence of P2Y₁ mRNA transcripts to suggest that these receptors are not expressed in EAhy926 cells. Under these circumstances, the strong band detected by anti-P2Y₁ antibody in EAhy926 cells suggests that this antibody is not detecting P2Y₁ receptor and therefore not suitable for P2Y research.

7.6 Evaluation of P2Y₄ receptors in epithelial and endothelial cells

Receptor desensitisation studies carried out in ECV304 cells in tandem with intracellular calcium release have established that both UTP and ATP are both acting at the P2Y₂ receptor suggesting that P2Y₄ receptors are not expressed in ECV304 cells (Brown, 2001). Although RT-PCR studies show the presence of P2Y₄ mRNA transcripts in ECV304 cells, the lack of functional data suggest that P2Y₄ receptors are not functionally expressed in ECV304 cells. The dose dependent accumulation of inositol phosphates in response to UTP and ATP are consistent with previous reports suggesting the expression of P2Y_{2/4} receptors in EAhy926 cells (Graham *et al.*, 1996a; Graham *et al.*, 1996b; Paul *et al.*, 2000). However, RT-PCR and DNA sequence results do not show the presence of P2Y₄ mRNA transcripts suggesting that these receptors are not present in EAhy926 cells. The anti-P2Y₄ receptor antibody used in this study detected strong multiple bands in all the cell types including 1321N1 cells, which do not respond to nucleotides. Given the absence of P2Y₄ receptors in ECV304 and EAhy926 cells, the bands detected by this antibody are not specific to P2Y₄ receptors. Therefore, these results suggest that this anti-P2Y₄ antibody is not useful for P2Y research.

7.7 Evaluation of P2Y₆ receptors in epithelial and endothelial cells

Previous studies in ECV304 cells reported the absence of P2Y₆ receptors in these cells based on the lack of response to P2Y₆ agonist in assays to determine the accumulation of inositol phosphate and cytosolic calcium response (Brown *et al.*, 2000). Even though RT-PCR studies detected the mRNA transcripts for P2Y₆ receptors, the lack of P2Y₆ receptor mediated functional response suggests that these receptors are not functionally expressed in ECV304 cells. UDP has been shown to dose-dependently accumulate inositol phosphates in EAhy926 cells. The presence of P2Y₆ mRNA transcripts further support the functional responses elicited by P2Y₆ receptors in EAhy926 cells. The anti-P2Y₆ receptor antibody used in this study detected multiple bands in all the cell extracts including 1321N1 cells which do not express P2Y receptors. Therefore, even if this antibody detects the P2Y₆ receptor in EAhy926 cells, the strong immunoreactive profile of this antibody in cells which do not express P2Y₆ receptors makes it unreliable for P2Y research.

7.8 Evaluation of P2Y₁₁ receptors in epithelial and endothelial cells

Previous studies in ECV304 cells reported the expression of receptors that are coupled to adenylyl cyclase in ECV304 cells (Howl *et al.*, 1998; Brown *et al.*, 2000). High concentrations of ADPβS, the partial agonist at human P2Y₁₁ receptor has been shown to evoke inositol phosphate accumulation and intracellular calcium release (Brown, 2001). Furthermore, the accumulation of inositol phosphates in response to BzATP and ATPγS and the dose-dependent accumulation of cAMP in response to ADPβS and ATPγS suggest the expression of P2Y₁₁ receptors in ECV304 cells. RT-PCR studies and DNA sequence results showed that P2Y₁₁ mRNA is transcribed in ECV304 cells consistent with pharmacological data. Functional studies involving 2MeSATP and BzATP did not show any significant dose-dependent accumulation of inositol phosphates in EAhy926 cells suggesting that P2Y₁₁ receptors are not expressed in them. Furthermore, RT-PCR studies did not show the presence of P2Y₁₁ mRNA transcripts in EAhy926 cells. The anti-P2Y₁₁ receptor antibody used in this study detected weak bands in all cell types used in this study including ECV304 and EAhy926 cells. Even if this antibody detects P2Y₁₁ receptors in ECV304 cells, the response is very weak even at the highest possible concentration used from the available source. These results suggest that this antibody is not sensitive and may not be useful to study P2Y receptors.

7.9 Evaluation of P2Y₁₂ and P2Y₁₃ receptors in epithelial and endothelial cells

ADP the potent agonist of P2Y₁₂ and P2Y₁₃ receptors has shown mild agonistic potency in ECV304 cells (Brown *et al.*, 2000). However, ADP is also an agonist at P2Y₁ receptor and a mild agonist at P2Y₁₁ receptor. Besides, there are no reports to suggest that these cells express receptors that are coupled to the inhibition of adenylyl cyclase. Furthermore, ADPβS, an agonist at P2Y₁ and P2Y₁₃ receptors and a partial agonist at P2Y₁₁ receptors, has shown only weak agonistic potency in ECV304 cells (Brown, 2001). Given the absence of P2Y₁ receptors in ECV304 cells, it is possible that these weak responses may have been mediated by P2Y₁₁ receptors. Previous studies have reported that 2MeSADP, the potent agonist at P2Y₁₂ and P2Y₁₃ receptors, did not induce functional response in ECV304 cells (Brown, 2001). Although, RT-PCR studies have shown the presence of P2Y₁₂ and P2Y₁₃ mRNA transcripts in ECV304 cells, the lack of

functional responses mediated by these receptors suggests that they are not functionally expressed in ECV304 cells. Furthermore, RT-PCR studies did not show the presence P2Y₁₂ and P2Y₁₃ mRNA transcripts in EAhy926 cells confirming their absence. The anti-P2Y₁₂ antibody did not detect bands in any cell types used in this study including ECV304 and EAhy926 cells. However, given the absence of P2Y₁₂ receptors in ECV304 and EAhy926 cells, the receptor specificity of this antibody cannot be determined. The anti-P2Y₁₃ receptor antibody produced strong bands in all cell types used in this study including 1321N1, ECV304 and EAhy926 cells. As P2Y₁₃ receptors are not functionally expressed in ECV304 and EAhy926 cells, these responses by anti-P2Y₁₃ antibody seems non-specific. Therefore, these results suggest that this anti-P2Y₁₃ antibody do not detect P2Y₁₃ receptors and is not useful in P2Y research.

7.10 Antibodies for P2Y research

Metabolic instability and binding to non-P2 receptor proteins has restricted the use of radiolabelled nucleotides in P2Y research. Results from this study highlight the well-known problems associated with studying P2Y receptor pharmacology. It is evident from these studies that it is not possible to establish the functional expression of P2Y receptors using nucleotides alone, thus emphasising the need for a receptor subtype specific agonist or antibody. Highly sensitive and specific antibody is essential to study the localization and quantitation of the receptor in different human tissues. Antibodies directed to different receptor subtypes have been available recently, which were not available when this study was commenced. The commercial polyclonal antibodies used in this study appear as if they are not receptor specific. Manufacturers of these antibodies claim that they detect non-denatured receptor in immunofluorescence studies. However, when used in western blot studies they do not detect denatured receptors. These results raise questions about the immunoreactivity shown by these antibodies in immunofluorescence studies and whether it was a consequence of binding with seemingly unrelated proteins. This assumption is realistic as the immunogenic peptide chosen to raise the antibody may have homology with epitopes on other proteins from same organism. Therefore, it is necessary to determine if this reactivity is antigen specific or the result of non-specific reactivity. However, most of these antibodies are marketed without this data making the selection of antibodies extremely tricky.

C-terminus of the proteins is often used to generate anti-peptide antibodies as they are hydrophilic, exposed and flexible. However, if the C-terminus is part of the transmembrane segment, the sequence tends to be hydrophobic (Thornton and Sibanda, 1983; Van Regenmortel, 1986). Commercial anti-P2Y₂ antibodies and anti-P2Y₄ antibodies used in this study were raised against amino acids mapping the C-terminus of receptor proteins. Likewise, N-terminus is also frequently used as an ideal candidate for antibody generation as peptides derived from this region have been shown to be comparatively diverse between closely related receptors (Gupta and Devi, 2006). The third extracellular loop of diverse family A receptors has been shown to regulate transition to the active conformation and in ligand recognition (Lawson and Wheatley, 2004).

In this study, L247 anti-P2Y₂ antibody raised against peptide designed to mimic specific region in the third extracellular loop has been shown to be a good candidate for receptor specific antibody to study endogenously expressed P2Y₂ receptors. This highly specific and sensitive antibody provides an important tool to study tissue distribution and cellular expression levels of P2Y₂ receptor, with negligible cross-reactivity from the other members of the P2Y family. Therefore, because of its unique selectivity and the current state of lack of selective ligands to P2Y₂ receptors, L247 anti-P2Y₂ antibody can be used to distinguish P2Y₂ receptors from other P2Y receptor subtypes. Moreover, because polyclonal antibody interacts with the target molecule at multiple sites, this antibody will be expected to give stronger signals and more effective at immunoprecipitation studies.

7.11 Future work

These studies have highlighted the problems associated with studying P2Y receptor pharmacology because of lack of selective ligands and the generation of receptor specific antibody to P2Y₂ receptors. It is clear from these studies that carefully selected peptide sequence mapping the epitope of the extracellular region of P2Y receptor can be used as an immunogen for the generation of antibodies. Furthermore, this antibody successfully binds to P2Y₂ receptor protein in both native and denatured conditions. Nonetheless, this antibody should be further characterised to exploit its full potential.

Future studies will involve confocal microscopy using molecular markers to determine localisation and map distribution of P2Y₂ receptors in human cells and tissues. Furthermore, using an ELISA for cells or membranes coated onto 96 well-plates, a rapid screening assay will be generated to reliably measure the levels of P2Y₂ receptors in human endogenous tissues. This antibody will be used in conjunction with other P2Y₂ agonists in pharmacological assays such as measurement of inositol phosphates, nitric oxide synthase studies and intracellular calcium assays to determine the ability of this antibody to generate receptor specific activity and inhibit or augment responses of natural agonists. These studies will be extended to determine if the binding of the antibody changes P2Y₂ receptor conformation affecting its ligand binding. This study will involve capturing of extracellular region of P2Y₂ receptor and receptor agonists by anti-P2Y₂ antibody in a P2Y₂ receptor - capture ELISA or P2Y₂ ligand-capture ELISA (Schmidt *et al.*, 2001). Similar studies will be used to generate antibodies using the peptide sequences mimicking other extracellular regions of P2Y₂ receptors to establish the regions important for high affinity ligand binding.

Further studies will include stable homologous or heterologous expression of P2Y₂ receptors to determine binding affinity of the antibody and its effect on signal transduction. These studies will help to understand the oligomeric nature of P2Y₂ receptor in ECV304 cells and may lead to the development of superior therapeutic agents that target these receptors. Furthermore, these studies will also determine the effect of molecular density and/or the cellular environment of the target receptors on antibody binding. Finally, RT-PCR studies to establish the molecular identity of P2Y receptor subtypes in BAE cells with receptor specific primers will be able to confirm the

functional expression of P2Y₁ and P2Y₂ receptors arrived from the pharmacological studies (Gomes *et al.*, 2005).

CONCLUDING REMARKS

On the basis of the data outlined in this thesis the following conclusions can be made:

1. The accumulation of inositol phosphates in response to BzATP and ATP γ S, the potent agonists of P2Y₁₁ receptors, suggests the presence of P2Y₁₁ receptors in ECV304 cells. Furthermore, the dose-dependent accumulation of cAMP in ECV304 cells in response to ADP β S and ATP γ S further supports the presence of functional human P2Y₁₁ receptors in addition to already established P2Y₂ receptors in these cells.
2. The dose-dependent accumulation of inositol phosphates in response to ATP, UTP and UDP confirms previously published data in support of P2Y₂/P2Y₄ and P2Y₆ like receptors in EAhy926 cells.
3. RT-PCR and DNA sequence results established the presence of mRNA transcripts for P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃ in ECV304 cells. However, this data only support the functional expression of P2Y₂ and P2Y₁₁ receptors in ECV304 cells. The presence of mRNA transcripts for P2Y₁, P2Y₄, P2Y₆, P2Y₁₂ and P2Y₁₃ receptors seems to suggest that these receptors are not expressed at functional level to initiate receptor specific signalling pathways. This strengthens the concept that RT-PCR studies only indicate the presence or absence and not the functional expression of receptors.
4. RT-PCR and DNA sequence studies in EAhy926 cells show the presence of mRNA for P2Y₂ and P2Y₆ receptors thereby confirming the pharmacological data for functional expression of P2Y₂ and P2Y₆ receptors in these cells. P2Y receptor transcripts were not detected in 1321N1 cells, which confirm the functional absence of P2Y receptors in these cells.
5. L247 rabbit polyclonal antiserum was successfully affinity purified. The purified fractions showed strong immunoreactivity with immobilised immunogenic antigen (L247 peptide) in ELISA suggesting successful generation of sequence

specific antibody to L247 peptide designed to mimic specific region in the extracellular domain of the human P2Y₂ receptor. Control experiments using an unrelated peptide (designed to HERV) confirmed the specificity of binding to L247 peptide.

6. L247 rabbit polyclonal anti-P2Y₂ antibody showed strong bands in ECV304, EAhy926 cells and on pre-absorption with the immunogenic peptide these responses were abolished suggesting that the antibody is antigen specific. These results also confirmed the functional expression of P2Y₂ receptors in both ECV304 and EAhy926 cells as established by functional studies and RT-PCR results.
7. The deglycosylation studies revealed that the P2Y₂ receptors are glycosylated in ECV304 cells as shown by the higher molecular mass bands with L247 anti-P2Y₂ antibody prior to deglycosylation.
8. Post-deglycosylation it appears that the P2Y₂ receptor protein in ECV304 cells exists as a homo-oligomer or hetero-oligomer resistant to SDS dissociation and heat.
9. Agonist induced P2Y₂ receptor desensitisation in ECV304 cells further confirmed the specificity of L247 antibody as the prolonged agonist incubation caused the receptors to internalise and they were not available for interaction with the antibody. The receptor internalisation was further supported by the fact that this study used only membrane fractions and therefore there were not enough receptors in the extracted membrane fractions or a very small number of receptors remained on the membrane to interact with the antibody.
10. The polyclonal rabbit anti-P2Y₂ receptor antibodies from commercial sources produced completely different immunoreactive profiles with multiple bands even in 1321N1 cells. Furthermore, in comparison to L247 anti-P2Y₂ antibody the commercial antibody showed no difference between normal and agonist incubated cells suggesting that this antibody may not be recognising the P2Y₂ receptors in ECV304 cells.

11. Immunofluorescence analysis using L247 anti-P2Y₂ antibody and commercial anti-P2Y₂ antibodies in ECV304 cells showed that commercial antibodies bind cell membranes in a manner similar to L247 anti-P2Y₂ antibody suggesting that they may be only recognising non-denatured receptors.
12. Polyclonal rabbit antibodies to P2Y₁, P2Y₄, P2Y₆, P2Y₁₁ and P2Y₁₃ receptors either showed no response or showed strong immunoreactive profile with multiple bands in all cell types including 1321N1 cells suggesting that these antibodies are not receptor specific and may not have been extensively characterized. Given the absence of P2Y₁₂ receptors in ECV304 and EAhy926 cells and the lack of response from anti-P2Y₁₂ antibody, the receptor specificity of this antibody cannot be determined.
13. These studies suggest that the L247 anti-P2Y₂ antibody raised against peptide designed to mimic specific region in the third extracellular loop of human P2Y₂ receptor is highly specific and sensitive and provides an important tool to study P2Y₂ receptor pharmacology.

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