Characterisation of the $P2Y_{14}$ receptor in the pancreas: control of vascular tone and insulin secretion

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Abstract

The $P2Y_{14}$ receptor is the most recently identified member of the P2Y family of receptors for adenine and uridine nucleotides and nucleotide sugars. It is activated by UDP, UDP-glucose and its analogues, as well as the synthetic analogue MRS2690, which exhibits greater potency and selectivity at the $P2Y_{14}$ receptor. The principle aim of this study was to investigate the functional expression of the $P2Y_{14}$ receptor in porcine pancreatic arteries, and the signalling pathways underlying the vasoconstriction evoked by $P2Y_{14}$ receptor agonists, together with an examination of the effects of UDP-glucose and MRS2690 on insulin secretion from the rat INS-1 823/13 β-cell line.

Segments of porcine pancreatic arteries were prepared for isometric tension recordings in warmed oxygenated Krebs'-Henseleit buffer. Agonists were applied after preconstriction with U46619, a thromboxane A_2 mimetic. ATP induced vasoconstriction followed by a vasorelaxation in pancreatic arteries; the contraction was blocked by NF449 (a P2X1 receptor selective antagonist), while the relaxation to ATP was blocked by an adenosine receptor antagonist. Neither the contraction, nor the relaxation to ATP were affected by removal of the endothelium. ADP evoked vasorelaxation, which was inhibited in the presence of SCH58261 (a selective adenosine A_{2A} receptor antagonist). UTP-induced vasoconstriction was attenuated significantly in endothelium-denuded arteries. UDP, UDP-glucose and MRS2690 induced concentration-dependent contractions in porcine pancreatic arteries with a rank order of potency of MRS2690 (10-fold) > UDP-glucose = UDP. The contractions evoked by UDP-glucose and MRS2690 were significantly attenuated in the presence of PPTN (a selective $P2Y_{14}$ receptor antagonist), indicating actions at $P2Y_{14}$ receptors. The

expression of $P2Y_{14}$-like receptor was shown by immunohistochemical and contractile studies to be on the endothelium of the pancreatic arteries. UDP-glucose and MRS2690 inhibited forskolin-stimulated cAMP production. UDP-glucose and MRS2690 increased the level of MLC2 phosphorylation; this effect was blocked by PPTN, indicating the involvement of $P2Y_{14}$ receptors. UDP-glucose increased the level of ERK1/2 phosphorylation. UDP-glucose and MRS2690 inhibited glucose-induced insulin release from the rat INS-1 823/13 β-cell line; this effect was blocked by PPTN, indicating actions through $P2Y_{14}$ receptors. PPTN itself was able to elevate significantly basal insulin secretion from INS-1 823/13 β-cells, which may suggest a constitutive release of UDP-glucose from these cells.

These results suggest that, in porcine pancreatic arteries, ATP induces a vasoconstriction mediated by P2X1 receptors followed by a vasorelaxation evoked by adenosine receptors present on the vascular smooth muscle. ADP induced a relaxation mediated by adenosine A_{2A} receptor. Moreover, my data indicate for the first time, an endothelium-dependent contraction evoked by UTP. A novel vasocontractile role of $P2Y_{14}$ receptors in porcine pancreatic arteries was also documented. The contractile response was mediated largely by the endothelium. $P2Y_{14}$-mediated contraction involves a cAMP-dependent mechanism, which is consistent with $P2Y_{14}$ receptor coupling to G_i protein, and an elevation in phosphorylated MLC2 and ERK1/2. Activation of the $P2Y_{14}$ receptor evoked a decrease in the level of insulin secreted from the rat pancreas. The current data have identified novel roles of the $P2Y_{14}$ receptor as a mediator of pancreatic artery contractility and in regulation of insulin secretion. If its role within the vasculature is shown to be more widespread, the $P2Y_{14}$ receptor may be a novel target for the treatment of cardiovascular disease.

Publications

1. Alsaqati M, Chan SL, Ralevic V (2013). Investigation of the functional expression of purine and pyrimidine receptors in porcine isolated pancreatic arteries. *Purinergic signalling***:** 1-9.

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Conference presentations

1. Alsaqati M, Latif ML, Chan SLF, Ralevic V (2010). Identification of the novel $P2Y_{14}$ receptor in porcine isolated pancreatic arteries. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre, poster presentation http://www.pa2online.org/abstracts/vol8issue1abst028p.pdf.

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Table of Contents

Abbreviations

AC	Adenylyl cyclase
Ac-CoA	Acetyl-coenzyme A
Ado	Adenosine
ADP	Adenosine-5′-diphosphate
ADPβS	Adenosine-5′-O-thiodiphosphate
AMP	Adenosine-5′-monophosphate
ANOVA	Analysis of variance
APs	Alkaline phosphatases
ATP	Adenosine-5′-triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5′-monophosphate
cGMP	Cyclic guanosine-5′-monophosphate
CHO	Chinese hamster ovary
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
EDCFs	Endothelium-derived contractile factors
EDRFs	Endothelium-derived relaxing factors
EDTA	Ethylenediaminetetraacetic acid
ENDPKs	Ecto-nucleotide diphosphokinases
ENPPs	Ecto-nucleotide pyrophosphatases
ENTPDases	Ecto-nucleoside-5′-triphosphate diphosphohydrolases
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
G&G	Gey & Gey
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporters
GSIS	Glucose stimulated insulin secretion
HEK	Human embryonic kidney
HL-60	Human promyelocytic leukemia cells
IAB	Insulin assay buffer

IP_3	Inositol 1,4,5-trisphosphate
IUPHAR	International Union of basic and clinical Pharmacology
KCl	Potassium chloride
KRBH	Krebs-Ringer bicarbonate HEPES buffer
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PKC	protein kinase C
PLC	phospholipase C
PTX	Pertussis toxin
Rho	Ras homolog gene family member
RIA	Radioimmunoassay
ROCK	Rho-associated protein kinase
SAR	Structure activity relationship
SB	Solubilisation buffer
SNP	Sodium nitroprusside
TCA	Tricarboxylic acid cycle
TM	Transmembrane
TP	Thromboxane receptors
UDP	Uridine-5'-diphosphate
UDP-glucose	Uridine-5'-diphosphate-glucose
UDPβS	Uridine-5'-O-thiodiphosphate
UMP	Uridine-5'-monophosphate
UTP	Uridine-5'-triphosphate
VSMC	Vascular smooth muscle cell

Chemical Names

αβ-meATP	αβ-methylene-adenosine-5′-triphosphate
βγ-meATP	βγ-methylene-adenosine-5′-triphosphate
2-MeSADP	2-methylthio-adenosine-5′-diphosphate
2-MeSATP	2-methylthio-adenosine-5′-triphosphate
A317491	5-({[3-phenoxybenzyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetricarboxylic acid
A804598	(S)-1-(1-(4-bromophenyl)ethyl)-2-cyano-3-(quinoline-5-yl)guanidine; A839977,1-(2,3-dichlorophenyl)-*N*-[2-(pyridin-2-yloxy)benzyl]-1H-tetrazol-5-amine
AF353	(5-(5-iodo-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine
ARC67085	2-propylthio-βγ-dichloromethylene-ATP
ARL66096	2-propylthio-βγ-difluoromethylene ATP
ARL67156	6-N,N-diethyl-D-βγ-dibromomethyleneATP
ATL-146e	4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester
Bay60-6583	2-(6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl) pyridin-2-ylthio)acetamide
BBG	Brilliant blue green
BQ123	(*cyc*(DTrp-DAsp-Pro-D-Val-Leu))
BQ788	(*N*-*cis*-2,6-dimethylpiperidinocarbonyl-L-gmethylleucyl-D-1-methoxycarboyl-D-norleucine)
BzATP	2′-&3′-*O*-(4-benzoyl-benzoyl)-ATP
CCPA	2-chloro-*N*6-cyclopentyladenosine
CGS21680	2-(4-[2-carboxyethyl]-phenethylamino)adenosine-5′-N-ethyluronamide
Cl-IB-MECA	2-chloro-*N*6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide

CP-532,903	(2S,3S,4R,5R)-3-amino-5-[6-(2,5-dichlorobenzylamino)purin-9-yl]-4-hydroxytetrahydrofuran-2-carboxylic acid
CPA	*N*6-cyclopentyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DUP 697	(5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene)
GR79236	*N*-[(1s,2s)-2-hydroxycyclopentyl adenosine; HENECA, 2-(1-(*E*)-hexenyl)adenosine-5′-*N*-ethyluronamide; KN62, 1-(*N,O-bis*[5-isoquinolinesulphonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine
KW3902	8-(Hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-1,3-dipropyl-1*H*-purine-2,6-di one
L-655,240	1-[(4-Chlorophenyl)methyl]-5-fluoro-α,α,3-trimethyl-1H-indole-2-propanoic acid
MRS1191	1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester
MRS1220	9-chloro-2-(2-furyl)5-phenylacetylamino[1,2,4]triazolo[1,5*c*]quinazol ine
MRS1523	2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate
MRS1706	N-(4-acetylphenyl)-2-(4-[2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl]phenoxy)acetamide
MRS1754	8-(4-[{(4-cyanophenyl)carbamoylmethyl}oxy]phenyl)-1,3-di(n-propyl)xanthine
MRS2179	N6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate
MRS2211	Pyridoxal-5′- phosphate-6-azo (2-chloro-5-nitrophenyl)-2,4-disulfonate
MRS2279	2-chloro-N6-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
MRS2365	(N)-methanocarba-2-MeSADP
MRS2500	*N*6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5-bisphosphate

MRS2578	*N*,*N*″-1,4-butanediyl *bis*(*N*′-[3-isothiocynatophenyl)] thiourea
MRS2690	2-thiouridine-5′-diphosphoglucose
MRS2698	[[(2R,3S,4R,5R)-4-amino-3-hydroxy-5-(4-oxo-2-sulfanylidenepyrimidin-1-yl)oxolan-2-yl]methoxy-hydroxyphosphoryl] phosphono hydrogen phosphate
MRS2768	Uridine-5′-tetraphosphate δ-phenyl ester
MRS2802	[({[(2R,3R,4S)-5-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)difluoromethyl]phosphonic acid
MRS4062	N4-phenylpropoxycytidine-5′-triphosphate
NDGA	Nordihydroguiaretic acid
NF 340	4,4'-(Carbonyl*bis*(imino-3,1-(4-methyl-phenylene)carbonylimino))*bis*(naphthalene-2,6-disulfonic acid) tetrasodium salt
NF023	8,8′-(carbonyl*bis*[imino-3,1-phenylene carbonylimino])bis-1,3,5-naphthalenetrisulfonic acid
NF279	8,8'-[Carbonyl*bis*(imino-4,1-phenylen ecarbonylimino-4,1-phenylenecarbonylimino)]*bis*-1,3,5-naphthalenetrisulfonic acid hexasodium salt
NF449	4,4′,4″,4′″-(carbonylbis[imino-5,1,3-benzenetriyl-bis{carbonylimino}]) tetrakisbenzene-1,3-disulfonic acid octasodium salt
NF546	4,4′-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-a,a′-diphosphonic acid); PPTN 4-(4-(piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid
Nifedipine	1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester
PD98059	(2-(2-amino-3-methoxyphenyl)-4H-1-be-nzopyran-4-one)
PPADS	pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate

PSB 0739	1-Amino-9,10-dihydro-9,10-dioxo-4-[[4-(phenylamino)-3-sulfophenyl]amino]-2-anthracenesulfonic acid sodium salt
PSB1115	4-[2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)benzenesulphonic acid
PSB-36	1-butyl-8-(3-noradamantanyl)-3-(3-hydroxypropyl)xanthine
PSB603	8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine
PSB-716	1-amino-4-(2-methoxyphenyl)-2-sulfoanthraquinone
SCH4421416	2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo [4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine
SCH58261	5-amino-2-(2-furyl)-7-phenylethylpyrazolo[4,3-*e*]-1,2,4-triazolo[1,5*c*]pyrimidine
S-ENBA	(2S)-*N*6-(2-endonorbanyl)adenosine; SLV320, *trans*-4-[(2-phenyl-7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclohexanol
Thapsigargin	(3*S*,3a*R*,4*S*,6*S*,6A*R*,7*S*,8*S*,9b*S*)-6- (Acetyloxy)-2,3,3a,4,5,6,6a,7,8,9b-decahydro-3,3a-dihydroxy-3,6,9-trimethyl-8-[[(2*Z*)-2-methyl-1-oxo-2-butenyl]oxy]-2-oxo-4-(1-oxobutoxy)azuleno[4,5-*b*]furan-7-yl octanoate
TNP-ATP	2’,3’-O-(2,4,6-trinitrophenyl)-ATP
UK14304	5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline)
VUF5574	*N*-(2-methoxyphenyl)-*N*-(2-[3-pyridyl]quinazolin-4-yl)urea
XAC	Xanthine amine congener
Y-27632	Trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride
Zafirlukast	N-[3-[[2-Methoxy-4-[[[(2-methylphenyl) sulfonyl]amino]carbonyl]phenyl]methyl]-1-methyl-1H-indol-5-yl]carbamic acid cyclopentyl ester
ZM241385	4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl amino]ethyl)phenol

Chapter One

General introduction

This chapter will begin with a description of the structure of blood vessels and their layers, as well as the ways of controlling the blood flow into these vessels. This will then be followed by a review about purine receptors and their classification. This review will focus mainly on the most recently discovered member of the P2Y receptor family, the $P2Y_{14}$ receptor and its ligands. Subsequently, the chapter will provide an overview about the pancreas; its structure, the arterial supply to the pancreas, as well as the hormones and enzymes released from it. Finally, the structure, biosynthesis, secretion and role of insulin will be considered. The general introduction will conclude with a description of the aims and the objectives of the project.

1.1. Vascular system

1.1.1. Blood vessel structure

The structure of blood vessels varies according to the function, but in general they comprise of three distinctive layers (Figure 1.1). The innermost layer (tunica intima) which contains the endothelial cell layer. It consists of a single layer of endothelial cells and thus appears flattened and smooth. This layer is surrounded by the middle layer (tunica media) which is a thick wall containing the smooth muscle cells, this layer is innervated by motor nerves, most of which are part of the sympathetic nervous system (Rutishauser, 1994; Clancy & McVicar, 2009). The outer layer (tunica adventitia) contains an abundance of two types of connective tissues; collagen fibers and elastin fibers (Figure 1.1), which provide the

arterial walls with strength and flexibility. This layer is innervated by sensory neurones (Rutishauser, 1994; Sherwood, 1997). The main role of the vessels is to drive the blood from the heart to the tissues and then back to the heart, as well as passing the deoxygenated blood, by the pulmonary artery, to the lung to remove the carbon dioxide (CO_2) and to reoxygenate it (Rutishauser, 1994). The capillaries are the simplest among the blood vessels, and they just contain a single layer of endothelial cells surrounded by a basement membrane (Rutishauser, 1994).

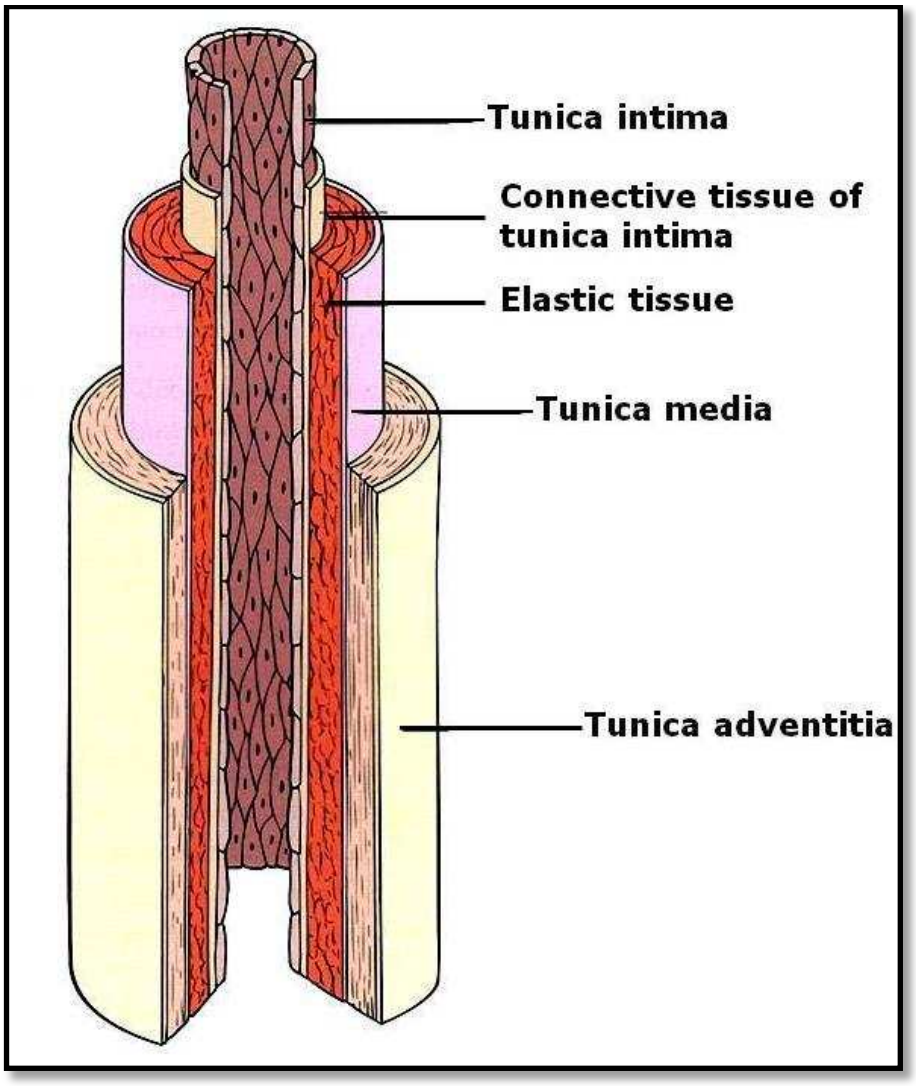


Figure 1.1. A schematic representation of the structure of the blood vessels. Adapted from Clancy & McVicar (2009).

1.1.2. Control of arterial blood pressure

Arterial blood pressure acts as the driving force for blood flow through the tissues of the body. The arterial blood pressure depends on two major factors, the cardiac output and the peripheral resistance (Rutishauser, 1994; McGeown, 2002). The cardiac output is the amount of blood ejected from the heart per minute, and it is determined by the heart rate and the stroke volume. The peripheral resistance is regulated by the diameter of the blood vessels. The diameter of the blood vessels depends on the balance of a variety of factors (neural, hormonal and local) that exert an influence on the smooth muscles at any given time (Rutishauser, 1994). It has been shown that a change in the diameter of the arterioles has a greater effect on the blood flow than that in large arteries. Consequently, the arterioles offer the greatest peripheral resistance. The veins drive the flow of the blood back to the heart, this is significant in determining the cardiac output. The resistance to the blood flow through the veins is low comparing with that through the arteries. However, a change in diameter of the veins would alter the venous return of blood back to the heart, and that will affect the stroke volume.

1.2. Purinergic signalling

Purinergic signalling is one of the most ancient biological systems, and it is believed to be the most ubiquitous intracellular signalling system in living tissues (see review by Burnstock *et al.*, 2010). Purine and pyrimidine receptors play a major role as signal transduction proteins in eukaryotes. These receptors consist of several subtypes with extracellular nucleotides

and nucleotide sugars as potent ligands. These receptors are found on the surface of almost all cells with widespread expression and effect.

1.3. A brief historical perspective

The role of purinergic signalling was first identified in 1929 by Drury & Szent-Gyorgyi, through investigation of the effects of sheep heart, brain and kidney extracts on guinea-pig, cat and dog heart. These extracts, when were injected intravenously, resulted in the slowing down of the heart rate and vasodilatation of the coronary arteries, hence causing hypotensive actions. The active component in the extracts were found to be the adenosine-5'-monophosphate (AMP) (Drury & Szent-Gyorgyi, 1929). These findings were confirmed by further experiments showing the ability of purine nucleotides to induce a heart block and to act as vasodilators of coronary arteries (Bennett & Drury, 1931; Lindner & Rigler, 1931; Wedd, 1931; Wedd & Drury, 1934; Ralevic & Burnstock, 1998; Winbury *et al.*, 1953; Wolf & Berne, 1956), and other arteries (Gaddum & Holtz, 1933; Houck *et al.*, 1948). Meanwhile, some reports showed that adenosine-5'-triphosphate (ATP) was more effective than adenosine (ado) at producing a heart block (Drury, 1936; Green & Stoner, 1950). It was also shown that extracellular nucleotides evoked non-cardiovascular responses, including ATP-induced contraction of the intestine (Gillespie, 1934) and uterus (Deuticke, 1932; Watts, 1953). In addition, the role of ATP in the nervous system has been investigated since 1947 (see review by Burnstock *et al.*, 2010).

In the 1960s, the term '' non-adrenergic, non-cholinergic nerves'' (NANC) was suggested to describe the nerves which were other than adrenergic and cholinergic (Burnstock *et al.*, 1963). These nerves were determined

following the inhibition of the activities of adrenergic and cholinergic neurotransmission in the guinea-pig taenia coli using blocking agents. It was found that the electrical stimulation of guinea-pig taenia coli was still able to induce a hyperpolarisation and relaxation (Burnstock *et al.*, 1963). The previous effect was blocked in the presence of a neurotoxin, indicating the involvement of some nerves which were distinct from adrenergic and cholinergic in that response (Bulbring & Tomita, 1967). Subsequently, in the 1970s, studies were conducted to identify the neurotransmitter utilised by the NANC nerves of the gastrointestinal tract and urinary bladder (Eccles, 1964; Burnstock, 1971). After investigating several substances as potential neurotransmitters, ATP was found to be the substance which acted as a co-transmitter with catecholamines in adrenergic nerves and with acetylcholine in a number of cholinergic nerves (see review by Burnstock *et al.*, 2010). In 1972, the word '' purinergic'' was introduced to describe the nerves which utilise ATP as a neurotransmitter (Burnstock, 1972).

In 1978, Burnstock introduced the first classification of the purinergic receptors (Burnstock, 1978). The nomenclature used by Burnstock divided these receptors into P1 and P2 purinoceptors. P1 receptors were reported to be much more responsive to the adenosine and AMP than to ATP and adenosine-5'-diphosphate (ADP), while P2 receptors were proposed to be much more responsive to ATP and ADP than to AMP and adenosine (Burnstock, 1978). In 1985, a report suggested a pharmacological basis for discriminating two types of P2 receptors (P2X and P2Y receptors) (Burnstock & Kennedy, 1985). They showed that P2X receptors were most potently activated by $\alpha\beta$-methylene-adenosine-5'-triphosphate ($\alpha\beta$-meATP), while 2-methylthio-adenosine-5'-triphosphate (2-MeSATP) was at that time the most potent agonist at P2Y receptors. Later in 1989, it was

observed that some P2Y receptors responded to pyrimidine nucleotides as well as to purine nucleotides (Seifert & Schultz, 1989).

The current classification was first defined in 1994 on the basis of molecular structure and transduction mechanisms (Abbracchio & Burnstock, 1994; Abbracchio *et al.*, 2003). It was proposed that purinergic receptors may be sub-divided into two major families: a P2X receptor family of ligand-gated ion channel receptors, which are activated by ATP and its analogues, in addition to a P2Y receptor family of G protein-coupled receptors (Abbracchio & Burnstock, 1994; Burnstock, 2007). P2Y receptors are activated by ATP, ADP, uridine-5'-triphosphate (UTP), uridine-5'-diphosphate (UDP) and UDP-sugars and their analogues. In fact, both P1 and P2 receptors may be responsive to ATP and ADP since adenosine can be produced from ATP or ADP in metabolic breakdown (section 1.7).

It is believed that P1 and P2 receptors and their signalling are involved in many non-neuronal and neuronal mechanisms, including immune responses, inflammation, platelet aggregation, pain, modulation of cardiac functions, exocrine and endocrine secretion (Burnstock & Knight, 2004; Burnstock, 2006a). Purinergic signalling can mediate cell proliferation, cell death and cell differentiation (Abbracchio & Burnstock, 1998; Burnstock, 2002). In addition, extracellular purine and pyrimidine nucleotides play a significant role in regulating the blood flow in a variety of tissues, via inducing vasoconstriction or vasorelaxation by activating purine or pyrimidine receptors (Burnstock *et al.*, 2010).

1.4. Purine and pyrimidine receptors

The diagram below shows the classification of the main families of adenosine, purine and pyrimidine receptors (P1 and P2 receptors), and their respective subtypes along with their signal transduction mechanisms (Figure 1.2).

1.4.1. P1 receptors

P1 receptors (adenosine receptors) can be sub-divided into four distinct subtypes; A_1, A_{2A}, A_{2B} and A_3 receptors (Figure 1.2, Table 1.1) (Olah & Stiles, 2000; Fredholm *et al.*, 2001; Cobb & Clancy, 2003; Yaar *et al.*, 2005). Adenosine receptors are G protein-coupled receptors with a structure common with other G protein-coupled receptors. They consist of seven putative transmembrane (TM) domains of hydrophobic amino acids, each domain has an α-helix with approximately 21-28 amino acids, with an extracellular N-terminus and an intracellular C-terminus (see review by Ralevic & Burnstock, 1998) (Figure 1.3). The seven transmembrane domains are linked via three extracellular and three intracellular hydrophilic loops (Figure 1.3) (Ralevic & Burnstock, 1998). P1 receptors primarily couple to adenylyl cyclase, where A_1 and A_3 are negatively coupled to adenylyl cyclase through $G_{i/o}$ protein, A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase through G_s protein (Reshkin *et al.*, 2000). Adenosine receptors have been reported to play a significant role in the cardiovascular system especially by regulating blood pressure and cell function, and mediating cardioprotection (see reviews by Ledent *et al.*, 1997; Peart & Headrick, 2007; Headrick *et al.*, 2011). The main distribution and the signalling transduction mechanisms of adenosine

receptors, together with their selective agonists and antagonists are listed in Table 1.1.

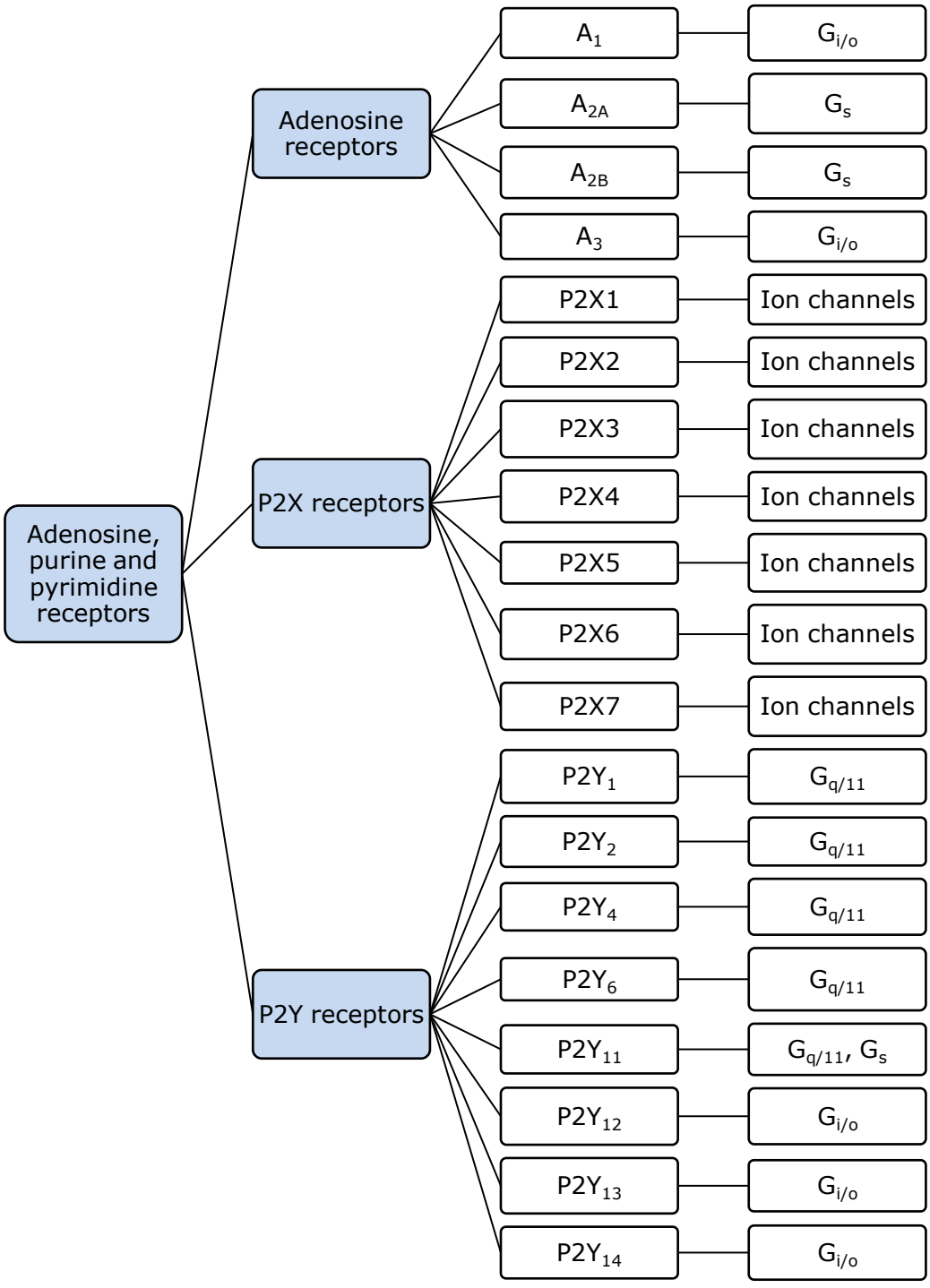


Figure 1.2. Diagrammatic representation of the subtypes of adenosine, purine and pyrimidine receptors along with their transduction mechanisms.

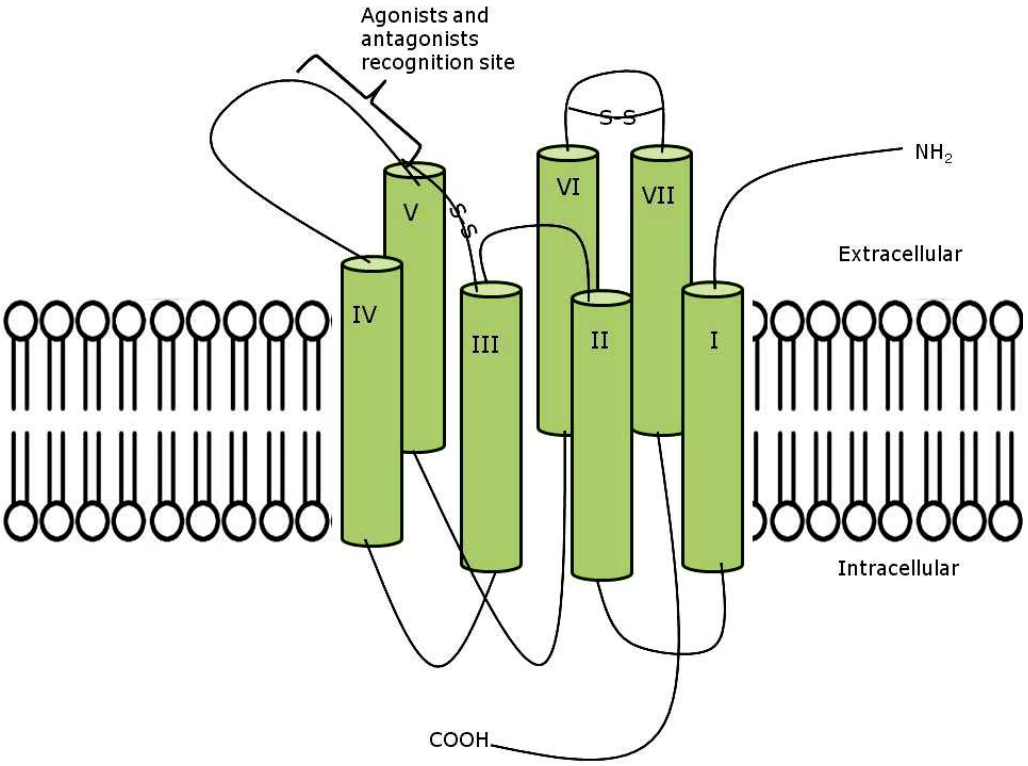


Figure 1.3. A schematic representation of the adenosine receptor. In common with other G protein-coupled receptors, adenosine receptors have seven putative transmembrane domains (I-VII), each domain contains an α-helix. These domains are linked via three extracellular and three intracellular hydrophilic loops. The arrangement of the transmembrane regions forms a pocket for the ligand binding site. S-S denotes the presence of hypothetical disulfide bridges. Figure is based on data from Jacobson *et al.* 1993; Ralevic & Burnstock, 1998.

1.4.2. P2 receptors

P2 receptors are membrane bound receptors responsive to the extracellular nucleotides and nucleotide sugars. These receptors are divided into two distinct families: P2X receptors which are ligand-gated ion channels and P2Y receptors, which are metabotropic G protein-coupled receptors.

To date, the nomenclature subcommittee of the International Union of Basic and Clinical Pharmacology (IUPHAR) has recognised seven mammalian P2X receptor subunits: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7 receptors (Figure 1.2) (Khakh *et al.*, 2001), while IUPHAR has recognised eight mammalian subtypes of P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (Figure 1.2) (Abbracchio *et al.*, 2006). The missing numbers among P2Y receptors (p2y3, p2y5, p2y7, p2y8, p2y9, p2y10 and p2y15) represent either receptors that have some sequence homology to the P2Y receptors but they are not responsive to nucleotides, or they are non-mammalian orthologues, or that are mammalian orphan receptors; in these cases a lower case (p2y) has been suggested to be used (Abbracchio *et al.*, 2006).

The ligands for P2 receptors are ATP, ADP, UTP, UDP and UDP-sugars (Kügelgen, 2008). A number of selective agonists and antagonists at P2 receptors are shown in Table 1.1. Several compounds can be used as non-selective antagonists at P2 receptors, including suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulphonate (PPADS), Reactive blue-2 and some other antagonists (see reviews by Ralevic & Burnstock, 1998; von Kügelgen, 2006). However, some P2 receptors are resistant to these antagonists. For example, it was shown that suramin and PPADS are weak/inactive at rat recombinant $P2Y_4$ receptors (Bogdanov *et al.*, 1998b; Wildman *et al.*, 2003). To date, there is no report of the antagonist

sensitivity of $P2Y_{14}$ receptors for suramin and PPADS. It should be noted that some P2 receptor antagonists, including suramin and PPADS, can inhibit the ecto-nucleotidase enzymes (section 1.7). That may reduce their efficacy as P2 receptor antagonists, which results in an increase in the responses to P2 receptor ligands via a decrease in their breakdown (Chen *et al.*, 1996; Ralevic & Burnstock, 1998; Grobben *et al.*, 1999; Vollmayer *et al.*, 2003; Munkonda *et al.*, 2007).

1.4.2.1. P2X receptors

P2X receptors are ligand-gated ion channels. They comprise seven distinct homomeric subtypes P2X1-7 (Khakh *et al.*, 2001). P2X receptor identities range between 26-47% and the length of each subtype is between 379 and 595 amino acids (Khakh *et al.*, 2001). The native ligand at all P2X receptors is ATP (Coddou *et al.*, 2011). $\alpha\beta$-meATP was initially shown to be an agonist only at homomeric P2X1 and P2X3 receptors. Subsequently, it was shown to be also an active agonist at P2X5, P2X6 and P2X4 receptors (Kennedy *et al.*, 2013). The structure of P2X receptors is shown in Figure 1.4. P2X subunits have two hydrophobic, transmembrane spanning regions which cross the plasma membrane, they are connected by an extracellular loop, with approximately 280–300 amino acids, the latter contains the ATP binding site (Jiang *et al.*, 2000; Kawate *et al.*, 2009; Kennedy *et al.*, 2013), in addition to a further site where the antagonist can bind (Garcia-Guzman *et al.*, 1997; Kawate *et al.*, 2009). Since P2X subunits have only two transmembrane regions, a single subunit on its own is not able to form a functional receptor. Reports using a variety of experimental techniques showed that three subunits are required to form a functional P2X receptor,

and hence three agonist molecules are required to bind to a single receptor in order to activate it (see review by Burnstock & Kennedy, 2011).

P2X receptors are multisubunit, as they may be homomeric (contain identical subunits) or heteromeric (contain different subunits). For example, it was reported that the heteromeric P2X2/3 receptor can be formed via the combination of P2X2 and P2X3 receptors (Lewis *et al.*, 1995). Similarly, the heteromeric P2X1/2, P2X1/4, P2X1/5, P2X2/6, P2X4/7 and P2X4/6 receptors have been characterised (see reviews by Coddou *et al.*, 2011; Kennedy *et al.*, 2013). P2X receptors are non-selective cation-conducting, which mediate initially a rapid non-selective passage of cations (Na^+, K^+, Ca^{2+}) through the cell membrane, resulting in depolarisation and thus Ca^{2+} influx via L-type voltage-dependent calcium channels (Bean, 1992; Dubyak & El-Moatassim, 1993; Kennedy *et al.*, 2013). P2X receptors have approximately equal permeability to Na^+ and K^+ cations with significant permeability to Ca^{2+} (Evans & Kennedy, 1994). Cations can also regulate ATP-activated currents in P2X receptors, since Ca^{2+} could inhibit P2X receptor currents via decreasing the affinity of P2X receptor ATP binding sites (Honore *et al.*, 1989; Khakh *et al.*, 2001).

P2X receptors can be divided into two groups depending on whether they desensitise rapidly (within 100-300 ms) or not. P2X1 and P2X3 receptors were shown to be rapidly desensitised, while other P2X receptors (namely, P2X2, P2X4, P2X5, P2X6 and P2X7) are not desensitised rapidly or do not desensitise at all (Ralevic & Burnstock, 1998; Coddou *et al.*, 2011). The mechanism by which the desensitisation is induced, requires an interaction between the two hydrophobic channels at P2X1 or P2X3 receptors, as well as an involvement of a number of amino acids of the intracellular terminus,

which are located just before the first transmembrane (TM1) domain of P2X receptors (Werner *et al.*, 1996; Allsopp & Evans, 2011).

P2X receptors are found throughout the body, and they are abundantly present in the nervous system, where they are involved in rapid purinergic synaptic transmission. Thus P2X receptors are involved in physiological regulation of the periphery and the central nervous system (CNS). Some of P2X receptors, including P2X1, P2X4 and P2X7, are functionally expressed in the cardiovascular system, and they have been proposed to be potential targets for treatment of some cardiovascular disorders (see review by Kennedy *et al.*, 2013). A number of P2X receptor selective agonists and antagonists, in addition to the receptors main distribution and their signal transduction mechanisms, are listed in Table 1.1.

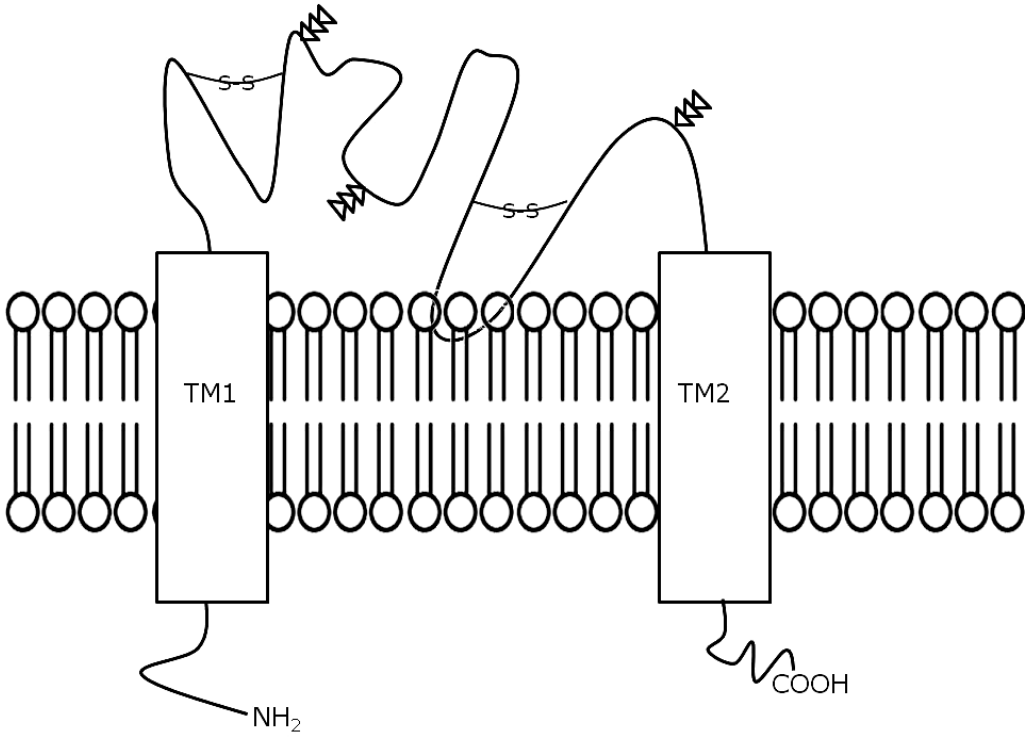


Figure 1.4. A schematic representation of P2X receptor, showing two hydrophobic transmembrane domains (TM1 and TM2) crossing the lipid bilayer of the plasma membrane, with intracellular N- and C-termini. The putative extracellular domain contains two disulfide-bonded loops (S-S) and three N-linked glycosyl chains (triangles). Figure is adapted from Ralevic & Burnstock, 1998.

1.4.2.2. P2Y receptors

P2Y receptors are G protein coupled receptors which are responsive to purine and pyrimidine nucleotides and nucleotide sugars (Ralevic & Burnstock, 1998; Abbracchio *et al*., 2006). P2Y receptors contain 308-377 amino acids with a mass of 41-53 kDa after glycosylation (Ralevic & Burnstock, 1998). Their structure is common to that of other G protein-coupled receptors (Figure 1.3). They contain seven α-helical transmembrane domains of hydrophobic amino acids, these domains are

connected via three extracellular and three intracellular loops, with an extracellular N-terminus and an intracellular C-terminus (Ralevic & Burnstock, 1998; Jacobson *et al.*, 2012). It has been shown that four amino acid residues of TM6 and TM7 might be essential for agonist binding, potency and specificity (Erb *et al.*, 1995; Jiang *et al.*, 1997; Barnard & Simon, 2001).

P2Y receptors can be divided on the basis of their endogenous ligands into adenine nucleotide-preferring ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors) and uracil nucleotide or UDP-sugar-preferring ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors) (von Kugelgen, 2006). Alternatively, P2Y receptors can be distinguished as $P2Y_1$-like family and $P2Y_{12}$-like family based on their sequence alignments and effector coupling. The $P2Y_1$-like family couples to G_q protein and involves an activation of the phospholipase C (PLC) signalling pathway (Costanzi *et al.*, 2004). This sub-family contains $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, although $P2Y_{11}$ receptor can couple to G_s protein too, leading to an activation of adenylyl cyclase (Communi *et al.*, 1997). The $P2Y_{12}$-like family can couple to G_i protein leading to an inhibition of adenylyl cyclase (Jacobson *et al.*, 2012). The sequence homology between the two sub-families is low, for instance, the sequence identity between $P2Y_1$ and $P2Y_{12}$ receptors is only 20%. While the sequence identity between the members within the same sub-family is higher, for instance, the sequence identity between $P2Y_{12}$ and $P2Y_{14}$ receptors is 45% (Jacobson *et al.*, 2012).

P2Y receptors have a wide distribution throughout the body and they mediate various responses in a variety of tissues (see reviews by Burnstock, 2007; Erlinge & Burnstock, 2008; Burnstock *et al.*, 2010). They have been found to be present in the central nervous system (Ralevic *et*

al., 1999; Moore *et al.*, 2000; Brown & Dale, 2002; Vasiljev *et al.*, 2003), cardiovascular system (Patel *et al.*, 1996; Bogdanov *et al.*, 1998a; Muraki *et al.*, 1998), stomach (Otsuguro *et al.*, 1996), liver (Dixon *et al.*, 2000, 2003), placenta (Somers *et al.*, 1999) and kidney (Takeda *et al.*, 1996). It has been reported that P2Y receptors play significant roles in the exocrine and endocrine pancreas (Luo *et al.*, 1999; Coutinho-Silva *et al.*, 2001) and adipose tissue (Yegutkin & Burnstock, 1999). A number of P2Y receptor selective agonists and antagonists, in addition to the receptors main distribution and their signal transduction mechanisms, are listed in Table 1.1.

Table 1.1. Characteristics of adenosine, purine and pyrimidine receptors

Receptor	Main distribution	Selective agonists	Selective antagonists	Transduction mechanism
P1 (adenosine) receptors				
A_1	brain, spinal cord, heart, endothelial cells, adipose tissue	CPA, CCPA, S-ENBA, GR79236	PSB-36, DPCPX, SLV320, KW3902	$G_{i/o}$
A_{2A}	brain, heart, lungs, spleen,	CGS21680, HE-NECA, ATL-146e	SCH442416, ZM241385, SCH58261	G_s
A_{2B}	large intestine, bladder, endothelial cells	Bay60-6583	PSB-603, MRS1754, MRS1706, PSB1115	G_s
A_3	lung, liver, brain testis heart	Cl-IB-MECA, CP532,903	MRS1220, VUF5574, MRS1523, MRS1191	$G_{i/o}$
P2X receptors				
P2X1	smooth muscle, platelets,	L-βγ-meATP, αβ-meATP, BzATP	TNP-ATP, NF449, NF023	Intrinsic cation channel (Ca^{2+}, Na^+)
P2X2	smooth muscle, CNS, autonomic and sensory ganglia	-	NF279	Intrinsic cation channel (particularly Ca^{2+})
P2X3	sensory and some sympathetic neurons	αβ-meATP, BzATP, A317491	TNP-ATP, A317491, AF353	Intrinsic cation channel
P2X4	CNS, colon, smooth muscle	-	BBG, 5-BDBD	Intrinsic cation channel

P2X5	proliferating cells in skin, gut, bladder	αβ-meATP	-	Intrinsic cation channel
P2X6	CNS, motor neuron in spinal cord	αβ-meATP	-	Intrinsic cation channel
P2X7	stomach, kidney, bladder	BzATP	KN62, A804598, A839977	Intrinsic cation channel
P2Y receptors				
$P2Y_1$	epithelial and endothelial cells, platelets	2-MeSADP, MRS2365	MRS2500, MRS2279, MRS2179	$G_{q/11}$
$P2Y_2$	epithelial and endothelial cells, immune cells, smooth muscle	MRS2698, MRS2768, PSB1114	PSB-716	$G_{q/11}$
$P2Y_4$	epithelial cells	MRS4062, UTPγS	-	$G_{q/11}$
$P2Y_6$	epithelial cells, placenta	5-iodoUDP, PSB-0474	MRS2578	$G_{q/11}$
$P2Y_{11}$	spleen, intestine	ARC67085, NF546	NF340	$G_{q/11}$, G_s
$P2Y_{12}$	platelets, glial cells	2-MeSADP	ARL66096, AZ11931285	$G_{i/o}$
$P2Y_{13}$	spleen, brain, bone marrow	-	MRS2211	$G_{i/o}$
$P2Y_{14}$	placenta, adipose tissue, stomach	MRS2690, MRS2802	PPTN	$G_{i/o}$

Adapted from Burnstock, (2007); Burnstock *et al.* (2010); Jacobson *et al.* (2012); Barrett *et al.* (2013); Kennedy *et al.* (2013); Burnstock & Ralevic (2014).

1.4.2.2.1. $P2Y_{14}$ receptor

The $P2Y_{14}$ receptor (also known as GPR105), which was identified in 2000 as the eighth member of the P2Y receptor family, is responsive to uridine-5'-diphosphate-glucose (UDP-glucose) and other sugar nucleotides (Chambers *et al.*, 2000; Abbracchio *et al.*, 2003). It was originally cloned from immature human myeloid cells (KIAA0001) (GenBank™ accession number D13626) (Nomura *et al.*, 1994). The rat and mouse orthologues of $P2Y_{14}$ receptor showed 80% and 83% amino acid identities respectively, to the human GPR105 protein. In addition, the human $P2Y_{14}$ receptor shares 45% amino acid identity with human $P2Y_{12}$ and $P2Y_{13}$ receptors and 22% with the $P2Y_1$ receptor (Freeman *et al.*, 2001; Abbracchio *et al.*, 2003; Moore *et al.*, 2003).

$P2Y_{14}$ receptor is activated by UDP-glucose (Figure 1.5B) and other nucleotide sugars, with a rank order of potency of $P2Y_{14}$ receptor ligands as follows: UDP-glucose ≥ UDP-glucuronic acid > UDP-galactose > UDP-N-acetylglucosamine (Chambers *et al.*, 2000; Ko *et al.*, 2007). The structure activity relationship (SAR) of synthetic nucleotides for activation of the human $P2Y_{14}$ receptor resulted in development of a compound with a 2-thiouracil modification, MRS2690 (2-thiouridine-5'-diphosphoglucose) (Figure 1.5C), with 7-fold greater potency than UDP-glucose at $P2Y_{14}$ receptors (Ko *et al.*, 2009). $P2Y_{14}$ receptor-dependent inhibition of cyclic adenosine-5'-monophosphate (cAMP) accumulation in C6 glioma cells expressing $P2Y_{14}$ receptor, human embryonic kidney 293 (HEK-293) cells and Chinese hamster ovary (CHO) cells was shown in the presence of UDP-glucose, as well as in the presence of UDP with similar efficacies, indicating that UDP (Figure 1.5A) is a potent agonist at $P2Y_{14}$ receptor (Carter *et al.*, 2009). In contrast, other nucleotides (CDP, GDP and ADP) exhibited 100-

fold less potency than that of UDP or UDP-glucose in activating the human $P2Y_{14}$ receptors (Carter *et al.*, 2009).

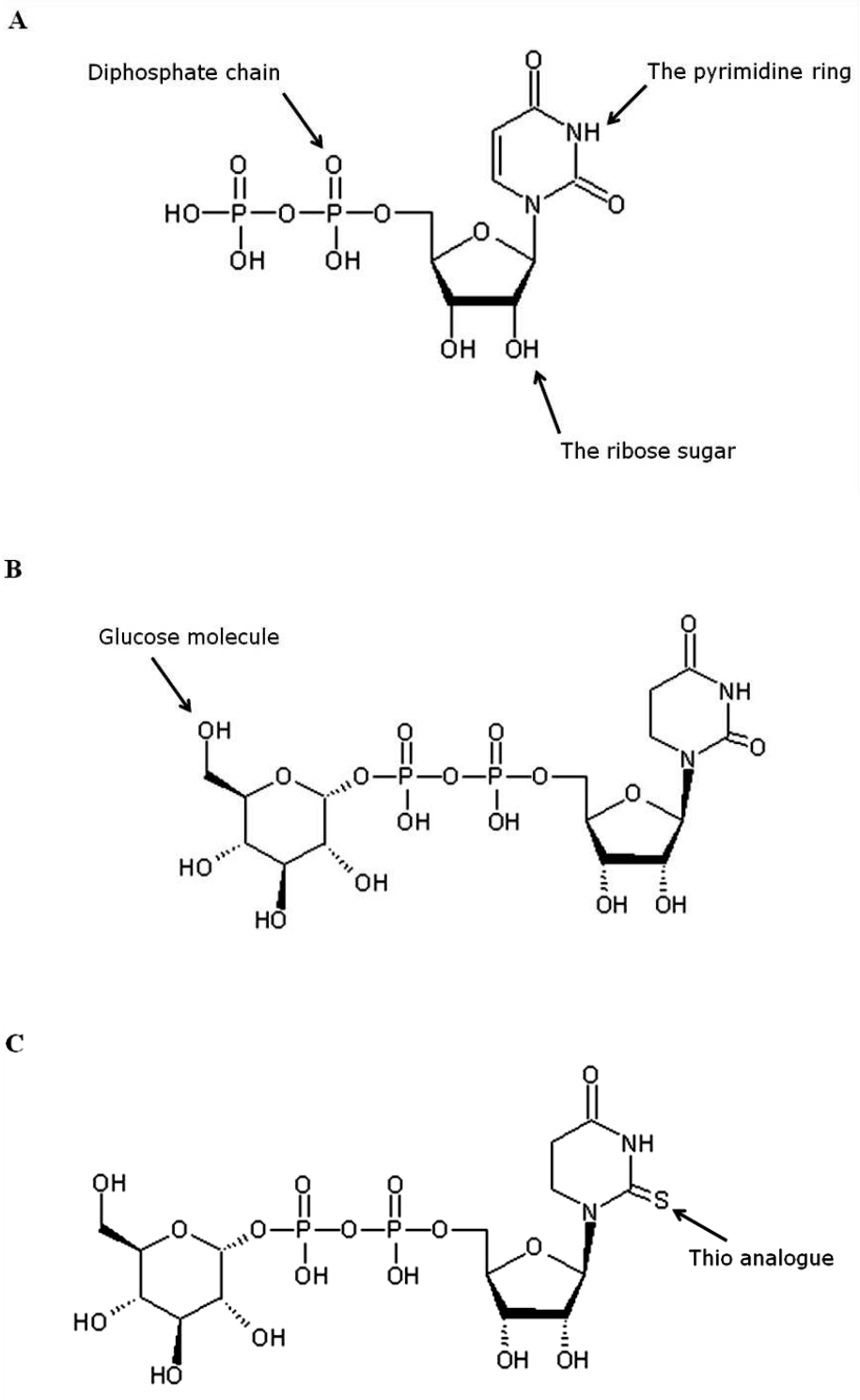


Figure 1.5. Chemical structure of UDP (A), UDP-glucose (B) and MRS2690 (C), the agonists at $P2Y_{14}$ receptor.

Recently, a novel high affinity competitive antagonist at $P2Y_{14}$ receptors was identified, this antagonist, named 4-(4-(piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid (PPTN) (Figure 1.6). It was synthesised, as a naphthoic acid derivative, as described by Gauthier *et al.* (2011) and Barrett *et al.* (2013). PPTN was initially characterised in $P2Y_{14}$ receptor-expressing HEK cells through its ability to inhibit UDP-glucose-stimulated Ca^{2+} mobilisation (Robichaud *et al.*, 2011). It showed good affinity for the $P2Y_{14}$ receptor (K_i = 1.9 nM in a chimpanzee $P2Y_{14}$ binding assay) (Robichaud *et al.*, 2011). When it was examined in human C6 glioma cells, PPTN at a concentration as low as 1 nM abolished the ability of UDP-glucose to inhibit forskolin-stimulated cAMP accumulation (Barrett *et al.*, 2013). Similarly, the activity of UDP at $P2Y_{14}$ receptors were blocked by PPTN (Barrett *et al.*, 2013). PPTN showed selectivity for $P2Y_{14}$ receptors since it did not exhibit agonist or antagonist affinity at $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ or $P2Y_{13}$ receptors with a range of concentrations up to 10 μM (Barrett *et al.*, 2013).

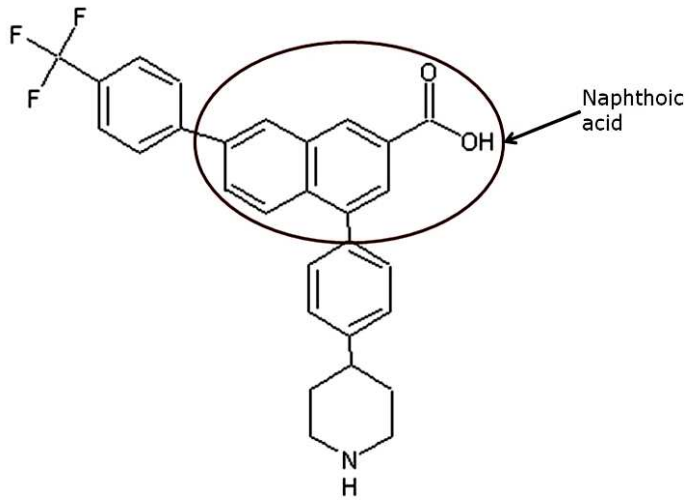


Figure 1.6. Chemical structure of 4-(4-(piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid (PPTN), a selective antagonist at the $P2Y_{14}$ receptor.

The $P2Y_{14}$ receptor is involved in heterotrimeric G_i protein mediated signalling, which induces an interaction of the $G_{i\alpha}$ subunit with adenylyl cyclase and subsequent inhibition of cAMP, and hence pertussis toxin (PTX) was able to completely abolish the $P2Y_{14}$ receptor agonist responses in C6 glioma cells and HEK-293 cells expressing $P2Y_{14}$ receptors (Carter *et al.*, 2009; Fricks *et al.*, 2009). In addition, G_i protein derived $G_{\beta\gamma}$-dimers are also involved in the signalling pathways of $P2Y_{14}$ receptor leading to the activation of phospholipase Cβ signalling pathway which results in activation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Rebecchi & Pentyala, 2000; Schulte & Fredholm, 2003). The $P2Y_{14}$ receptor was reported to activate ras homolog gene family member (Rho) Rho-associated protein kinase (ROCK) signalling in human neutrophils, this effect was abolished following incubation with PTX (Sesma *et al.*, 2012). UDP-glucose-evoked extracellular signal-regulated kinase (ERK1/2) phosphorylation was also shown in HEK-293 cells (Fricks *et al.*, 2009).

The widespread distribution of $P2Y_{14}$ receptor was observed in human and mouse tissues including adipose tissue, placenta, liver, kidney, spleen, stomach and intestine, in which the $P2Y_{14}$ receptor is expressed at high level (Charlton *et al.*, 1997; Chambers *et al.*, 2000; Freeman *et al.*, 2001). In addition $P2Y_{14}$ receptor is expressed in the immune system including primary neutrophils and primary lymphocytes (Charlton *et al.*, 1997; Moore *et al.*, 2003). The $P2Y_{14}$ receptor is present at a moderate level in brain, heart and lung (Chambers *et al.*, 2000; Freeman *et al.*, 2001; Moore *et al.*, 2003). A moderate level of $P2Y_{14}$ receptor was also detected in the HEK-293 cell line, while $P2Y_{14}$ mRNA was not detected in cell lines derived from human brain including glial and neuronal origin (Moore *et al.*, 2003).

P2Y receptor desensitisation is a regulatory phenomenon that terminates receptor signalling following prolonged exposure of the receptor to the agonist, leading to down-regulation of surface receptors (Galas & Harden, 1995). It was shown previously that $P2Y_2$ receptors desensitised following constant application of UTP (Sanabria *et al.*, 2008; Rodriguez-Rodriguez *et al.*, 2009). Studies on UTP and UDP activated human $P2Y_4$ and $P2Y_6$ receptors revealed that UTP induced a rapid desensitisation of the IP_3 response and a 50% loss of cell surface receptors. Subsequently, the removal of the agonist (UTP) results in a rapid recovery of the surface receptors. On the other hand, UDP did not induce a rapid desensitisation but it also did not result in a rapid recovery after removal of the agonist (Brinson & Harden, 2001). Regarding the $P2Y_1$ and $P2Y_{12}$ receptors, it has been shown that $P2Y_1$ and $P2Y_{12}$-mediated platelet responses desensitise quickly (Hardy *et al.*, 2005). There appears to be no report which has investigated whether $P2Y_{14}$ receptor may exhibit desensitisation.

1.5. Mechanism of nucleotides release

The existence of ATP in the extracellular milieu has been identified a long time ago (see reviews by Ralevic & Burnstock, 1998; Burnstock *et al.*, 2010). The release of ATP to the extracellular milieu may occur from excitatory/secretory tissues or from nonexcitatory tissues (see review by Lazarowski *et al.*, 2003a). The exocytotic release of ATP by excitatory cells (neurons, platelets and secretory cells) features the release of ATP as a co-transmitter from terminal nerves. The storage and the release of ATP from these cells are controlled by electrochemical force generated by the activity of ATPase (Evans *et al.*, 1992). Furthermore, ATP can be released from nonexcitatory cells, including endothelial and epithelial cells, smooth

muscle, circulating lymphocytes, erythrocytes, monocytes and hepatocytes (see reviews by Lazarowski *et al.*, 2003a; Lazarowski *et al.*, 2011). The nonexocytotic release of ATP is believed to occur in three different contexts: 1) ATP may be released due to the mechanical stimulation of epithelial, endothelial cells and some other cells; 2) A number of agonists may promote the release of ATP; 3) ATP could be released by resting cells in the absence of any external stimulus (Lazarowski *et al.*, 2003a).

1) The mechanism by which ATP can be mechanically released from the nonexcitatory cells (endothelial cells) is by subjecting these cells to shear stress, following an increase in flow rate (Bodin & Burnstock, 2001). Likewise, ATP can be generated from 1321N1 cell line during a medium change (Lazarowski *et al.*, 2003a). Both cases resulted in approximately 50 times elevation of the level of ATP released from these cells (Lazarowski *et al.*, 2003a).

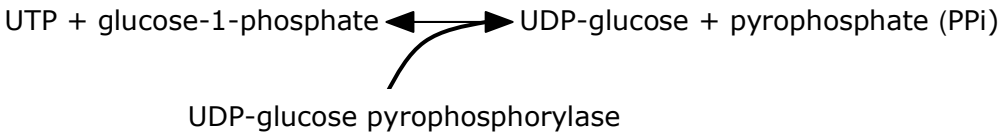
2) The release of ATP to extracellular side can also be triggered in the presence of some pharmacological stimuli (Lazarowski *et al.*, 2003a), including thrombin which has been shown to promote the release of ATP from aortic endothelial and smooth muscle cells (Yang *et al.*, 1994). ADP and UTP induced an increase within the level of extracellular ATP in endothelial cell culture, COS-7 and HEK-293 cells (Yang *et al.*, 1994; Ostrom *et al.*, 2000). 3) ATP was found to be continuously released from many resting (nonsecretory) cells, including 1321N1, C6 rat glioma and airway epithelial cells (Lazarowski *et al.*, 2000), since the level of ATP in the extracellular milieu persists at low nanomolar (1-10 nM) concentrations in the presence of ecto-ATPase enzymes (Lazarowski *et al.*, 2000).

Following release of ATP to the extracellular medium, it may be broken down to ADP with an inorganic phosphate (Pi) as well as to AMP with

inorganic pyrophosphate (PPi), and subsequently to adenosine by activities of ecto-nucleotidase enzymes (Joseph *et al.*, 2004) (section 1.7). Other nucleotides namely UTP, ADP and UDP-glucose are also released by resting and mechanically stimulated tissues with similar mechanisms as described for ATP (see reviews by Lazarowski *et al.*, 1997; Lazarowski *et al.*, 2003b; Simon *et al.*, 2008).

1.6. UDP-glucose

UDP-glucose is a nucleotide sugar which is biosynthesised in the cytosol and transported into the lumen of the endoplasmic reticulum (ER) and Golgi apparatus (ER/Golgi), and serves as a sugar donor involve in glycosyltransferase-catalysed reactions (Sesma *et al.*, 2009). This nucleotide sugar can be biosynthesised from UTP by the activity of a UDP-glucose pyrophosphorylase enzyme in the presence of glucose-1-phosphate. The latter results from glucose by the activity of glucokinase, before being converted to glycogen as the activity of glycogen synthase (Seoane *et al.*, 1996). UDP-glucose pyrophosphorylase catalyses a reversible production of UDP-glucose and pyrophosphate (PPi) (Kleczkowski *et al.*, 2004).



The transport of UDP-glucose from the cytosol into the lumen of the endoplasmic reticulum and Golgi apparatus occurs by means of ER/Golgi nucleotide sugar transporters (identified as SLC35 nucleotide sugar transporters). These transport the cytosolic UDP-sugars to ER/Golgi apparatus using uridine-5'-monophosphate (UMP) as an antiporter substrate (Hirschberg *et al.*, 1998; Ishida & Kawakita, 2004). It has been shown that the nucleotide sugar concentrations in ER/Golgi are 20-fold higher than those in the cytosol (Hirschberg *et al.*, 1998). In addition, brefeldin A which blocks the traffic between ER and the Golgi, has been shown to decrease UDP-sugars release from astrocytoma cells and yeast (Esther *et al.*, 2008; Kreda *et al.*, 2008). Overexpression of SLC35 transporters in mucosal and basolateral compartments resulted in an enhancement of the cellular release of UDP-glucose, and displayed a 3.3-fold increase in the rate of UDP-glucose release compared with that in cells that do not express SLC35 (Sesma *et al.*, 2009).

The concentration of intracellular cardiac UDP-glucose was estimated at 100 mM (Laughlin *et al.*, 1988). UDP-glucose has been shown to be released constitutively from a number of cells, including differentiated human airway epithelial cells, COS-7, CHO-K1, yeast, 1321N1 and C6 glioma cells, to the extracellular milieu in two different ways: exocytosis from vesicles or cytosolic release through plasma membrane transporters or channels. Subsequently, UDP-glucose can act as an extracellular signalling molecule (Abbracchio *et al.*, 2003; Lazarowski *et al.*, 2003a; Lazarowski *et al.*, 2003b; Sabirov & Okada, 2005; Sesma *et al.*, 2009), as the concentration of extracellular UDP-glucose may reach effective levels ranging between 1-20 nM (Arase *et al.*, 2009). On the other hand, the concentration of extracellular UDP-glucose may reach effective levels

ranging between 10 nM- 1 mM during cell damage and/or injury (Arase *et al.*, 2009).

Mechanical stimulation, induced by medium change, promoted ATP and UDP-glucose release from 1321N1 cells, the extracellular UDP-glucose concentration ranged within 10- 20 nM and was constant over 2-3 h, whereas the extracellular concentration of ATP reached a maximum concentration of 100 nM within 10-20 min, but it was followed by a substantial decrease of the extracellular concentration to a resting steady-state level (3 nM). These findings suggest that the UDP-glucose rate of metabolism was much lower than that of ATP or the hydrolysis of UDP-glucose induced by ecto-nucleotidase enzymes is balanced by its constitutive release (Lazarowski *et al.*, 2003b).

1.7. Ecto-nucleotidases

Following released to the extracellular compartments (section 1.5), the biological activities of ATP, ADP, UDP, UTP and UDP-glucose are controlled by the activity of membrane-bound enzymes regulating nucleotide hydrolysis and phosphorylation (Zimmermann, 2000). Four major families of ecto-nucleotidases have been described by Zimmermann (2000): the ecto-nucleotide triphosphate diphosphohydrolase family (eNTPDase), the ecto-nucleotide pyrophosphatases (eNNPs), the glycosylphosphatidylinositol (GPI)-anchored ecto-5'-nucleotidase, and GPI-anchored alkaline phosphatases (APs) (Zimmermann, 2000). In addition, an ecto-nucleotide diphosphokinases (eNDPKs) family was identified in 2001 (Yegutkin *et al.*, 2001).

The ENTPDase family can hydrolyse nucleoside-5'-triphosphates (NTP) and nucleoside-5'-diphosphate (NDP). This family contains eight members: eNTPDase1-8 (Zimmermann, 2000; Kukulski *et al.*, 2005). They can degrade NTP to NDP with Pi as well as NDP to NMP with PPi (Joseph *et al.*, 2004). The, eNPP family contains three subtypes (eNPP1-3), and they can hydrolyse ATP directly to AMP, as well as hydrolysing the pyrophosphate linkages of UDP-glucose resulting in glucose-1-phosphate + UMP (Figure 1.7) (Zimmermann, 2000; Lazarowski *et al.*, 2003b). GPI-anchored ecto-5'-nucleotidase and APs are the enzymes which degrade extracellular AMP to adenosine with Pi (Lazarowski *et al.*, 2003a,b; Joseph *et al.*, 2004). The eNDPKs facilitate the synthesis of ATP via catalysing the phosphorylation of extracellular ADP to ATP using nucleotide triphosphates, such as UTP or guanosine-5'-triphosphate (GTP), as substrates (Figure 1.7) (Yegutkin *et al.*, 2001, 2002).

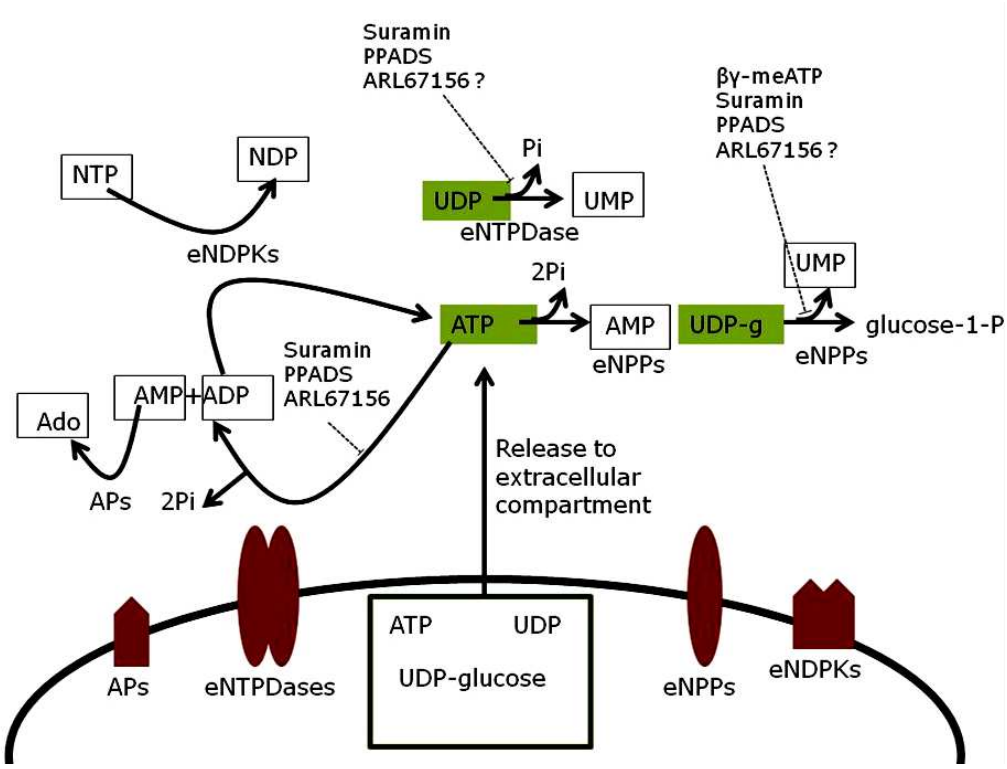


Figure 1.7. A schematic representation of the effect of ecto-nucleotidase enzymes on the extracellular nucleotides and nucleotide sugars (ATP, UDP and ADP, UDP-glucose). Following extracellular release ATP, UDP and UDP-glucose are broken down by ecto-nucleotide triphosphate diphosphohydrolase family (eNTPDase) or ecto-nucleotide pyrophosphatases (eNNPs). AMP is broken down to adenosine (Ado) by GPI-anchored alkaline phosphatases (APs). ATP is re-synthesised by the activity of ecto-nucleotide diphosphokinases (eNDPKs) mediated by *de novo* ATP synthesis. βγ-meATP, suramin and PPADS are inhibitors of eNTPDase and eNNPs. Arrows represent positive influences, while dotted arrows represent the negative influence. Figure is based on data from Zimmermann (2000) and Joseph *et al.* (2004).

1.8. Functional expression of P1 and P2 receptors in the vasculature

The expression of $P2Y_1$, $P2Y_2$, $P2Y_6$ and P2X4 receptors on the vascular endothelial cells and $P2Y_2$, $P2Y_4$, $P2Y_{12}$, P2X1 and P2X7 receptors on vascular smooth muscle has been documented (Figure 1.8) (see review by Erlinge & Burnstock, 2008). ATP can induce vasoconstriction or vasorelaxation, following activating purine or pyrimidine receptors expressed on the vascular smooth muscle or on the vascular endothelial cells (Burnstock, 1988). The vasoconstriction induced by ATP was shown to be mediated mainly by P2X1 receptors as seen in mesenteric arteries (Vial & Evans, 2002), rabbit saphenous arteries (Ramme *et al.*, 1987) and renal vasculature (Chan *et al.*, 1998; Inscho *et al.*, 2004). Likewise, other nucleotides are able to elicit a vasoconstriction in a variety of arteries, including UTP and UDP activating $P2Y_2$ and $P2Y_6$ receptors respectively, expressed on the vascular smooth muscle cells (VSMCs) (Figure 1.8) (Malmsjo *et al.*, 2000). Besides, ADP was reported to induce a contraction of human mammary arteries via activating $P2Y_{12}$ receptors present on the VSMCs (Wihlborg *et al.*, 2004).

The vasorelaxation induced by ATP was shown to be mediated by endothelial $P2Y_1$, $P2Y_2$ and P2X4 receptors (Figure 1.8) (Wang *et al.*, 2002). In addition, other nucleotides were able to induce a relaxation, including UTP, UDP and ADP via acting at endothelial $P2Y_2$, $P2Y_6$, $P2Y_4$ or $P2Y_1$ receptors (Figure 1.8) (Ralevic *et al.*, 1991b; Wang *et al.*, 2002; Wihlborg *et al.*, 2003). The mechanism by which the nucleotides induce vasorelaxation involve the release of endothelial derived relaxing factors (EDRFs) or prostacyclin (PGI_2). Alternatively, vasorelaxation can be triggered by the release of endothelium-derived hyperpolarising factors

(EDHFs), which relax the smooth muscles by activating potassium channels followed by a hyperpolarisation (Malmsjo *et al.*, 1998; Mistry *et al.*, 2003).

P1 receptors are expressed throughout the vasculature with the adenosine as an endogenous ligand (Burnstock, 1978). A_{2A} or A_{2B} receptors can be found on the vascular endothelial cells or smooth muscle (Schulte & Fredholm, 2003), the activation of these receptors results in vasodilatation in a number of vessels (Figure 1.8) (Belardinelli *et al.*, 1996; Ongini *et al.*, 1996; Conti *et al.*, 1997; Feoktistov & Biaggioni, 1997; Kemp *et al.*, 1999; Olanrewaju *et al.*, 2000). Activation of A_1 adenosine receptors leads to an elevation in intracellular calcium in airway smooth muscle cells resulting in airway constriction (Figure 1.8) (Zhou *et al.*, 2013). Activation of A_3 receptors in mice aorta induced an endothelium-dependent contraction involving an increase in the level of cyclooxygenase (Ansari *et al.*, 2007).

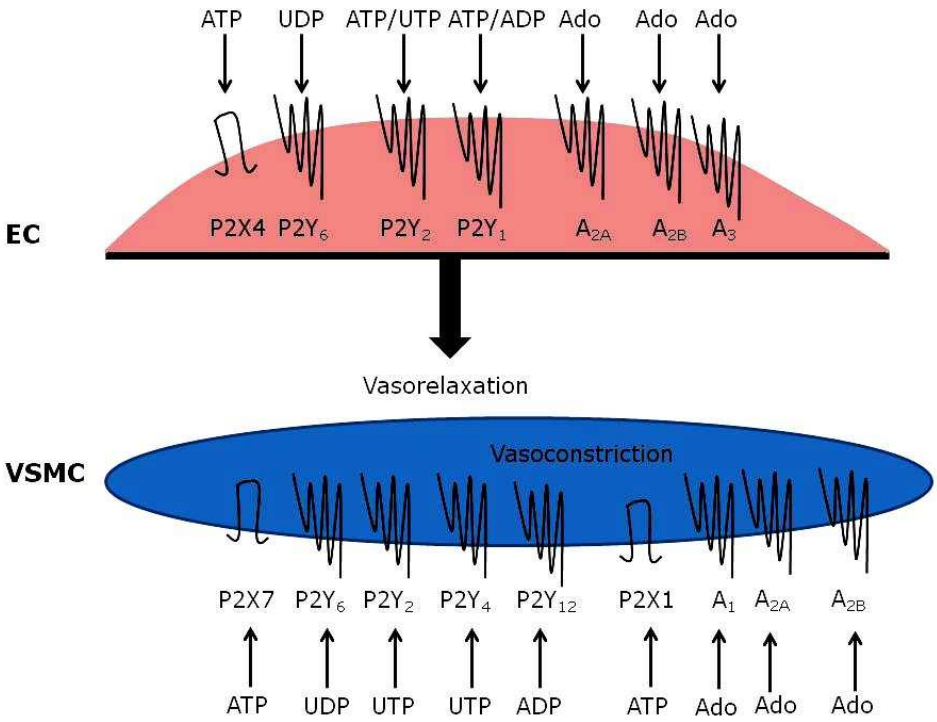


Figure 1.8. A schematic representation of P1 and P2 receptor-mediated regulation of the circulation. Extracellular ATP, UDP, ADP and UTP can act at P2 receptors expressed on the endothelial cells (ECs) or vascular smooth muscle cells (VSMCs) to elicit a vasorelaxation or a vasoconstriction respectively. Similarly, adenosine (Ado) can act on P1 receptors expressed on the EC or VSMC to elicit a vasorelaxation or a vasoconstriction respectively. Arrows represent positive influences. Figure is based on data from Erlinge & Burnstock, 2008.

1.9. The pancreas (overview)

The pancreas is a glandular organ (endocrine and exocrine gland) which lies below the stomach, above the first loop of the duodenum, between the spleen on the left and the duodenum on the right (Sherwood, 1997). The length of the human pancreas is approximately 12-15 cm and its weight is approximately 60-100 g (Schaefer, 1926), while the porcine pancreas weight is 190-698 g (Ferrer *et al.*, 2008). The pancreas consists of four parts: the head, the uncinate process, the neck and the tail (Figure 1.6, inset) (see review by Woodburne & Olsen, 1951). The pancreas is composed of exocrine and endocrine cells; the exocrine cells surround the endocrine cells. Its bulk varies depending on the species, and in rodents, it comprises 70-90% of acinar cells plus 5-25% of duct cells (Naya *et al.*, 1997). Exocrine cells secrete a pancreatic juice consisting of the enzymatic secretion which includes lipase, α-amylase, colipase, carboxyl ester lipase, DNAse and RNAse and some other proteins including trypsin inhibitor, which play a significant role in digestion. These enzymes are secreted from the acinar cells. In addition, exocrine cells secrete an aqueous alkaline solution, which is secreted by the pancreatic ducts, and allows optimal functioning of the pancreatic enzymes (Sherwood, 1997).

The islet endocrine cells constitute only 1-2% of the pancreas bulk (Naya *et al.*, 1997; Novak, 2008), these cells consist of isolated islands of endocrine tissues which are called the islets of Langerhans. These tissues are clusters of cells scattered throughout the pancreas and composed of α, β, δ and F cells. In rat and mouse, β-cells constitute 60-80% of the bulk of the islet and they are located in the islet core, while the remaining endocrine cells are dispersed at the periphery of the islet (Naya *et al.*, 1997). In human, the α- and δ-islets are not confined to the islet

periphery, rather they are dispersed throughout the islets (Dubois *et al.*, 2000). Islet cells secrete several hormones: glucagon is secreted from α-cells, and insulin and amylin are secreted from β-cells (more details about insulin are provided in section 1.12). The effects of insulin and glucagon are opposing, while an elevation in glucose concentration suppresses glucagon secretion, it promotes insulin secretion (Ohneda *et al.*, 1969). Somatostatin is released from δ-cells; its role is to regulate the secretion of both insulin and glucagon, since δ-cell somatostatin was shown to evoke a tonic inhibitory effect on glucagon and insulin secretion (Hauge-Evans *et al.*, 2009). In addition, somatostatin exerts its effect by inhibiting the digestion of nutrients and decreasing nutrient absorption (Sherwood, 1997). Pancreatic polypeptide is secreted from endocrine F-cells (Ekblad & Sundler, 2002), it has been reported to have an influence on hunger, and energy balance, since it slows down the gastrointestinal motion of chyme and reduces further food intake, hence pancreatic polypeptide can modify metabolic and energy homeostasis (Holzer *et al.*, 2012). In addition, there is some evidence for the expression of ε-cells in the islet which are ghrelin-producing (see review by Gittes, 2009).

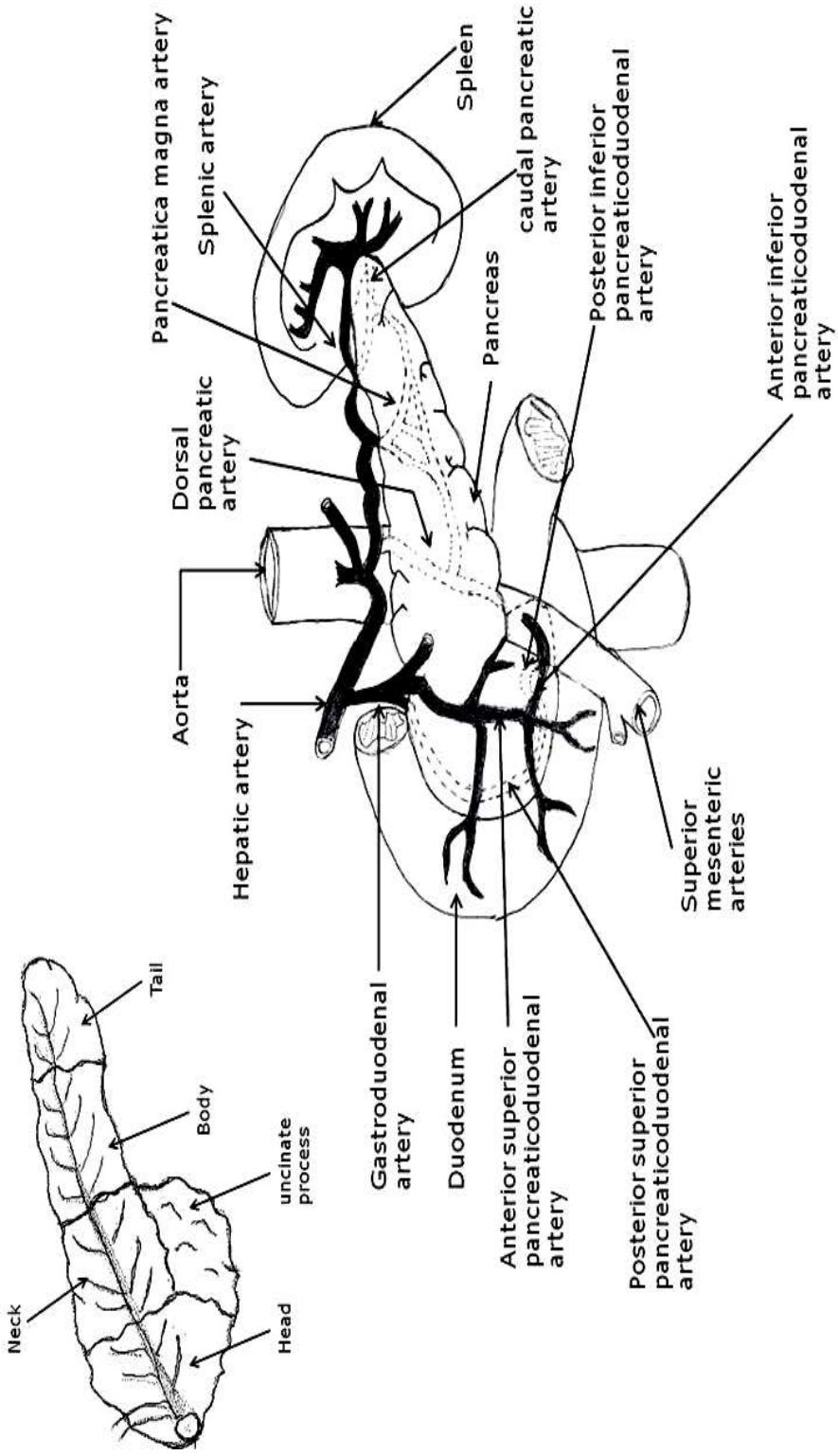


Figure 1.9: Gross structure of the pancreas (inset), General pattern of arterial supply of the pancreas (main), hand drawn figure, information is based on data from Woodburne & Olsen, 1951.

1.10. The arterial blood supply of the pancreas

The pancreas is supplied by the blood mainly via three sources: the splenic, hepatic and superior mesenteric arteries (Figure 1.9, main), and drains into the portal vein (see reviews by Bonner-Weir, 1993; Bertelli *et al.,* 1998). Two double arterial branches (posterior superior and anterior superior pancreaticoduodenal arteries) provide the head of the pancreas and the uncinate process with the blood supply (see review by Woodburne & Olsen, 1951), in addition to two inferior pancreaticoduodenal arteries (posterior and anterior) (Figure 1.9, main). The superior and inferior pancreaticoduodenal arteries branch originally from the gastroduodenal artery and the superior mesenteric artery respectively (Figure 1.9, main) (Woodburne & Olsen, 1951).

One of the major arteries among the pancreatic arteries designated as the dorsal pancreatic artery, is also called the superior pancreatic artery (Bertelli *et al.*, 1998). The dorsal pancreatic artery supplies the neck and the body of the pancreas with blood. It branches originally from the splenic artery and it is considered as one of the largest vessels leaving the splenic artery (Figure 1.9, main) (Vandamme & Bonte, 1986). It descends to the lower border of the pancreas and it is divided into two branches (right and left branches). The right branch of the dorsal artery appears on the surface of the pancreas close to the neck and to the uncinate process; this branch provides the head of the pancreas with blood (Figure 1.9, main), as well as forming an anastomosis with the little left branch of the anterior superior pancreaticoduodenal artery. The left branch of the dorsal pancreatic artery forms the inferior pancreatic artery which runs through the inferior border of the body of the pancreas and anastomoses with the caudal pancreatic artery and with the pancreatica magna artery (Figure 1.9, main)

(Woodburne & Olsen, 1951). This latter artery (pancreatica magna artery) is a branch of splenic artery, and it accesses the pancreas at approximately the junction of the middle and left thirds of the gland. Then it sends branches to right and left sides of the pancreas which are oriented along the main pancreatic duct (Figure 1.9, main) (Woodburne & Olsen, 1951). The tail of the pancreas is provided with its blood supply by means of the caudal pancreatic artery which is also a branch of the splenic artery (Figure 1.9, main). It anastomoses with the dorsal pancreatic artery and pancreatica magna artery (Bertelli *et al.*, 1998).

The microvasculature of the pancreas is supplied from anastomosing arteries derived from the hepatic and superior mesenteric arteries. These arteries form arcades within the glands from which smaller branches are produced as interlobular arteries (Bockman, 1992). Then the interlobular arteries branch further to form the intralobular arterioles, which supply the lobules of exocrine and endocrine tissues with blood. It has been found that the proportion of blood going to islets is approximately 10% of the pancreatic blood (Jansson & Hellerstrom, 1983) indicating that the blood flow to the endocrine tissues (expressed as flow per unit weight) is 10 times greater than that of the exocrine tissues (Bockman, 1992). The islet blood flow is regulated by hormonal, nervous and nutrient signals which are produced in the islets or in distal tissues (Ballian & Brunicardi, 2007). The islets of Langerhans are embedded in a dense capillary network, and one to five arterioles per islet supply the endocrine pancreas, these arterioles branch into capillaries and form a spherical framework with structural similarities to a glomerulus (Jansson *et al.*, 2002; Zanone *et al.*, 2005), and then these arterioles exit the islets as veins (Ballian & Brunicardi, 2007).

1.11. The role of the blood supply on pancreatic function

Pancreatic blood vessels deliver proper gas exchange and nutrients to the pancreas. Besides, most secretory stimuli, which are carried by the blood, should diffuse rapidly into the endocrine tissues to reach their targets (Schaeffer *et al.*, 2011). In addition, the blood takes up the secreted islet hormones to deliver them to target tissues and they are also involved in secretion of these hormones into the blood stream and in elimination of wastes from pancreatic cells (Cabrera *et al.*, 2006; Eberhard *et al.*, 2010). The relationship between the pancreatic exocrine and endocrine secretion and blood flow has been well documented (Goodhead *et al.*, 1970; Sendur & Pawlik, 1994; Schaeffer *et al.*, 2011; Cleaver & Dor, 2012). For example, the pancreatic islets are embedded in a very dense microvascular network; this network has been shown to play a significant role in endocrine cells during secretory episodes (Eberhard *et al.*, 2010). Therefore, the release of vasoactive substances most likely play a pivotal role to compensate for the deficit of the oxygen level due to the higher metabolic demand (Schaeffer *et al.*, 2011). In pancreatic islets, both endothelial cells and β-cells express nitric oxide synthase (NOS) (Corbett *et al.*, 1992), and nitric oxide (NO) influences the regulation of the islet blood flow and the hormone secretion (Salehi *et al.*, 1996). It was shown that administration of glucose doubled the islet perfusion to promote the insulin influx into the circulation, as well as many abnormalities of islet blood flow regulation have been identified in animal models of diabetes mellitus and impaired glucose tolerance, indicating that the deregulation of islet perfusion could contribute to islet dysfunction (Ballian & Brunicardi, 2007). Although the microvascular network in exocrine tissues was shown to be almost two- to three-fold less dense than that in endocrine tissues, it was reported that drugs which

decreased the pancreatic tissue blood flow were able to inhibit exocrine pancreatic secretion, this indicates the importance of the blood flow in regulating the pancreatic exocrine functions as well as the endocrine functions (Sumi *et al.*, 1991; Schaeffer *et al.*, 2011).

1.12. Insulin

Insulin plays a major role in glucose homeostasis. Human insulin is a peptide hormone which has molecular weight of approximately 6,000 Daltons and contains 51 amino acids. The mature insulin molecule consists of two polypeptide chains designated as A and B which are linked by two pairs of disulfide bonds (Melloul *et al.*, 2002; Steiner *et al.*, 2009). Insulin is stored in the secretory granules with proinsulin which has an additional polypeptide (C-peptide) (Figure 1.10). Proinsulin is converted to insulin in the Golgi complex via specific proteases (Steiner *et al.*, 2009). The pathway in which the insulin is biosynthesised is shown in Figure 1.10. Proinsulin is produced from preproinsulin by cleaving the signal peptide (green bar) in the endoplasmic reticulum. Proinsulin, similar to preproinsulin, contains a connecting domain (the C-domain) (red bar) which connects A and B chains (black and blue respectively). Proinsulin undergoes folding in the ER, with the formation of three pairs of disulfide bridges, which are necessary for stability and bioactivity (Narhi *et al.*, 1993). Proinsulin is an inactive hormone which reacts weakly with insulin receptors. In order to obtain the active hormone (insulin), the C-peptide domain in proinsulin needs to be cleaved away by employing the proteolytic enzymes in the Golgi apparatus (Figure 1.10). The bioactive hormone is stored as Zn^{2+}-stabilised hexamers (6× insulin: 2× Zn^{2+}) in the secretory granules. When insulin hexamers are secreted into the

circulation, they dissociate to yield insulin monomers which are bioactive and bind strongly to the insulin receptors (Weiss, 2009).

Insulin exerts its effect by binding to insulin receptors. These receptors are expressed on many cells including those of the liver, fat and skeletal muscles (see review by Fritsche *et al.*, 2008). The primary effect of insulin is to regulate the clearance of glucose from the circulation which occurs by insulin-induced increase of glucose uptake by skeletal muscle (Gerich *et al.*, 1974). In addition, insulin promotes glycogenesis by acting on the liver (Aronoff *et al.*, 2004; Fritsche *et al.*, 2008). Insulin also inhibits glucagon secretion from α-cells which results in glycogenolysis inhibition (Ohneda *et al.*, 1969; Aronoff *et al.*, 2004). Insulin is involved in other actions including activation of fat synthesis, simulation of triglyceride storage in fat cells, stimulation of the synthesis of the protein in the liver or skeletal muscles and inducing cell proliferation and differentiation (Cryer, 1992; Fritsche *et al.*, 2008).

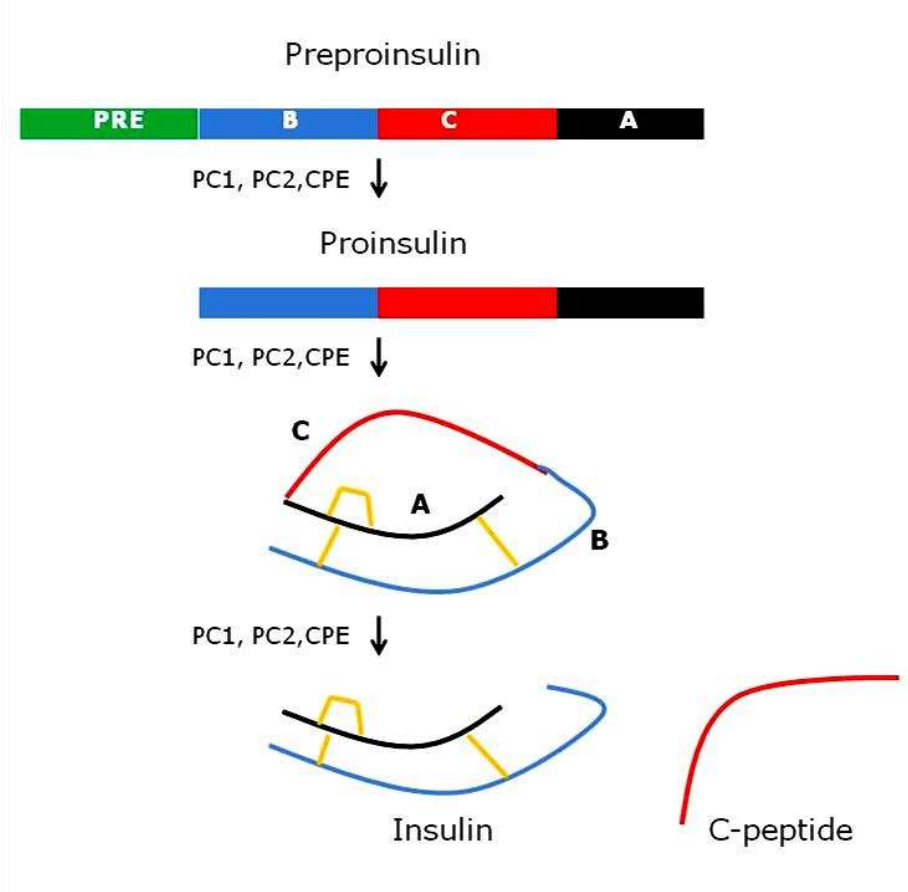


Figure 1.10: A schematic representation of the conversion of preproinsulin to proinsulin and then to insulin and C-peptide by prohormone convertases 1 and 2 (PC1, PC2) and carboxypeptidase E (CPE). The conversion of preproinsulin to folded proinsulin occurs in the endoplasmic reticulum (ER), while production of insulin by the cleavage of C-peptide domain occurs in the Golgi apparatus via the activity of proteolytic enzymes.

1.13. Glucose-stimulated insulin secretion (GSIS)

Numerous nutrients can act as insulin secretagogues, including glucose, some amino acid and free fatty acids. However, glucose is the most potent secretagogue, since it promotes robust insulin release within a few minutes

following its administration, and its stimulatory effect lasts as long as the glucose level in the plasma is elevated. The current section will investigate briefly the pathways in which glucose stimulates insulin secretion from islet β-cells.

Following administration of glucose, insulin secretion from pancreatic β-cell is elevated, involving mainly two signalling pathways: the K_{ATP} channel-dependent and K_{ATP} channel-independent pathways. It has been reported that GSIS is biphasic (Curry *et al.*, 1968). The first peak obtained after 4-8 min is followed by a nadir in release. Following the nadir in secretion, the second phase occurs in which there is a gradual elevation of the rate of the insulin release reaching its peak after a further 25-30 min (see reviews by Straub & Sharp, 2002; Henquin *et al.*, 2006a,b).

1.13.1. ATP-sensitive potassium channel-dependent (K_{ATP}) insulin secretion

The β-cell K_{ATP} channels play an essential role in GSIS. In the absence of substimulatory glucose concentrations (<8 mM), the membrane potential of the β-cell is -60 to -70 mV (Rorsman, 1997). When plasma glucose concentration increases above the threshold (2-4 mM) (Henquin *et al.*, 2006a), glucose enters the β-cells via the glucose transporters (GLUTs) GLUT2, GLUT1 or GLUT3 (De Vos *et al.*, 1995) (Figure 1.11). Glucose may then be phosphorylated to glucose-6-phosphate which is then catabolised to pyruvate. The latter is subsequently decarboxylated to acetyl-Coenzyme A (Ac-CoA). Ac-CoA then may be metabolised within the mitochondria to generate ATP which elevates the ratio of cytosolic ATP/ADP (MacDonald *et al.*, 1991; Srivastava & Goren, 2003). High levels of ATP will result in an inhibition of K_{ATP} channels which increases the intracellular $[K^+]$. The high

level of K^+ will result in depolarisation of β-cell membrane potential and subsequently opening of the voltage-dependent Ca^{2+} channels, which induce Ca^{2+} influx, thus triggering insulin granule exocytosis (Smith *et al.*, 1990) (Figure 1.11). Subsequently, the high intracellular Ca^{2+} activates the voltage-dependent K^+ channel, which will result in repolarisation of the membrane potential of β-cells, subsequently the insulin secretion may be reduced (Smith *et al.*, 1990; Rorsman, 1997) (Figure 1.11).

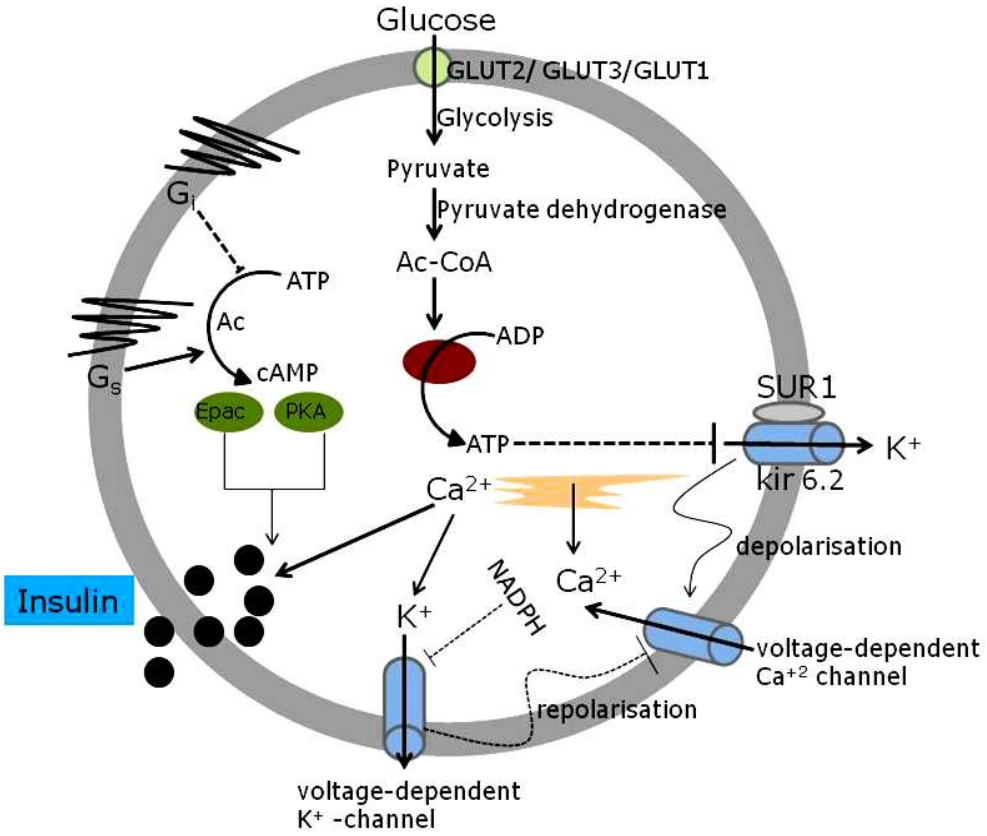


Figure 1.11. A schematic representation of K_{ATP} channel-dependent signalling. The facilitative glucose transporters 1/2/3 (GLUT1/2/3) mediate glucose entry. The metabolism of glucose induces production of ATP, following a production of Acetyl-coenzyme A (Ac-CoA). ATP inhibits the K_{ATP} channel. Closure of which results in depolarisation of the β-cells membrane potential and then opening of the voltage-dependent Ca^{2+} channel, which induces Ca^{2+} influx. The high level of intracellular Ca^{2+} triggers insulin granule exocytosis. Subsequently, Ca^{2+} will activate voltage-dependent K^+ channel which induces repolarisation of the membranes potential of β-cells and inhibits further insulin release. Some P2Y receptors can activate the cAMP pathway, which triggers the insulin secretion. Arrows represent positive influences, while dotted arrows represent the negative influence. Figure is based on data from Tipparaju *et al.* 2007; Novak, 2008; Burnstock & Novak, 2012.

1.13.2. ATP-sensitive potassium channel-independent (K_{ATP}) insulin secretion

It has been suggested since 1992 that glucose is able to stimulate insulin secretion in both mouse and rat via a second pathway other than K_{ATP} channel-dependent signalling (Best *et al.*, 1992; Gembal *et al.*, 1992). This effect was observed when the K_{ATP} channel was clamped open in the presence of an activator of K_{ATP} channels, diazoxide. It was found that glucose was still able to induce an insulin secretion. The mechanisms underlying the K_{ATP} channel-independent signalling are discussed in detail in reviews by Straub & Sharp, 2002; Jitrapakdee *et al.* 2010.

In summary, glucose-induced insulin secretion through the K_{ATP} channel-dependent pathways was shown to be involved in the mechanisms underlying the first phase of insulin secretion (section 1.13) (see review by Straub & Sharp, 2002). Whereas, the second phase of insulin secretion is induced by the K_{ATP} channel-independent pathways (Taguchi *et al.*, 1995), which produces an amplifying signal maintaining a long-lasting second phase of insulin release (Gembal *et al.*, 1992).

1.14. Aims and objectives

The aims of the current study were to investigate the functional expression of the P1 and P2 receptors in porcine isolated pancreatic artery, since the roles of these receptors are not well characterised in these arteries. The present study focused on the last member of P2Y receptor family, namely the $P2Y_{14}$ receptor, since the role of this receptor in the cardiovascular system is novel, and it has not been addressed previously, although, it was shown previously that $P2Y_{14}$ mRNA and protein are present in the heart and the blood vessels. Therefore, the primary aim of this study was to examine the functional expression of this receptor in porcine pancreatic artery as well as identifying the signalling pathways underlying the responses to $P2Y_{14}$ receptor agonists in these arteries.

The study aimed also to examine the effects of $P2Y_{14}$ receptor agonists on insulin secretion from the pancreas. Therefore, the effects of UDP-glucose and MRS2690 on insulin secretion from rat INS-1 832/13 β-cells and from rat isolated islets of Langerhans were indentified in the current study, to obtain a comprehensive view about the roles of the $P2Y_{14}$ receptor in the pig pancreas, since it is more closely related to that of man.

Chapter Two

Investigation of the effects of ATP, αβ-meATP, UTP, MRS2768 and ADP on vascular tone in porcine isolated pancreatic arteries

2.1. Introduction

There are two main families of purine and pyrimidine receptors: ionotropic P2X and G protein-coupled P2Y receptors (section 1.4.2). Molecular cloning has identified seven mammalian P2X-receptor subunits: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7 receptors (Khakh *et al.*, 2001), while eight mammalian P2Y receptors have been identified: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (Abbracchio *et al.*, 2006). P2X receptors are activated by ATP and its stable analogue, αβ-meATP (Kasakov & Burnstock, 1982; Burnstock, 2006b). P2Y receptors can be subdivided on the basis of their endogenous agonists into adenine nucleotide-preferring ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors) and uracil nucleotide or UDP-sugar-preferring ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors) (section 1.4.2.2) (Kügelgen, 2008). Among the adenine nucleotide group, the human $P2Y_{11}$ receptor is activated by ATP and αβ-meATP but it fails to respond to ADP (Communi *et al.*, 1997), although the dog orthologue responds to both ADP and ATP (Qi *et al.*, 2001; Kennedy *et al.*, 2013). $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors are activated by ADP and, with lower potency, by ATP (Léon *et al.*, 1997; Bodor *et al.*, 2003; Marteau *et al.*, 2003; Waldo & Harden, 2004). Among the uracil nucleotide or UDP-sugar

receptors, the $P2Y_2$ receptor is equally activated by ATP and UTP, while the $P2Y_4$ receptor is highly selective for UTP over ATP (Nicholas *et al.*, 1996). The $P2Y_6$ receptor is activated by UDP and UTP, while the $P2Y_{14}$ receptor is activated by UDP and UDP-sugars (Burnstock, 2006b; Carter *et al.*, 2009).

Within the pancreatic vasculature, P2X1, P2X2, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were detected by immunohistochemistry (Coutinho-Silva *et al.*, 2001; Coutinho-Silva *et al.*, 2003). More than two decades ago, it was shown that P2X receptors mediate pancreatic artery vasoconstriction and P2Y receptors mediate vasodilatation in response to ATP in perfused rat pancreas, this effect was shown by using P2X receptor desensitising agent or P2Y receptor antagonist respectively (Hillaire-Buys *et al.*, 1991), and subsequent studies showed an additional involvement of contractile receptors sensitive to UTP (named P2U receptors) (Hillaire-Buys *et al.*, 1999). Purine and pyrimidine receptor sub-classification has advanced significantly since that time. A re-evaluation of purine receptors in the pancreatic vasculature is clearly warranted. In the current study, I characterised pharmacologically $P2Y_1$ and A_{2A} receptor-mediated relaxatory responses, in addition to P2X1, $P2Y_2$ and/or $P2Y_4$ receptor-mediated contractile responses, of porcine isolated pancreatic artery preparations. $P2Y_2$ and/or $P2Y_4$ receptors appear to be expressed mainly on the vascular endothelial cells, while P2X1 and A_{2A} receptors appear to be expressed on the smooth muscle cells of the pancreatic arteries.

2.2. Materials and methods

2.2.1. Tissue preparation

Pancreata from pigs (either sex, age less than 6 months, weight ~50 kg) were obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield). A crude dissection was conducted to isolate the pancreatic artery (the dorsal pancreatic artery) which was located in the body of the pancreas (Figure 1.9, main). The vessels were dissected out and placed in Krebs'-Henseleit buffer (section 2.2.3) containing 2% (w/v) Ficoll (type 70) and refrigerated overnight at 4ºC. The next day, a fine dissection was performed, and the arteries were cut into rings of 0.5 cm in length, ~1.5-2 mm inner diameter and ~0.5 mm thickness, and suspended between two supports (wires), in organ baths containing Krebs'-Henseleit buffer maintained at 37°C (gassed constantly, 95% O_2, 5% CO_2). The lower wire was inserted through the lumen of the arterial ring and attached to a glass rod and placed in the organ bath, with the upper wire which was also inserted through the lumen and connected to the transducer for isometric recording (Figure 2.1). The whole setup was linked to a Maclab data acquisition system (AD Instruments Ltd., Hastings, UK) via an amplifier.

The endothelium of some arteries was removed by gently rubbing the lumen with forceps before attaching the vessels to the setup (Rayment *et al.*, 2007b). Successful removal of endothelium was tested using substance P (10 nM). Endothelium-denuded arteries relaxed in response to substance P to less than 10% of the U46619 (a thromboxane A_2 mimetic)-induced contraction, while in endothelium-intact arteries the relaxation to substance P was 36% ± 8 (n=7).

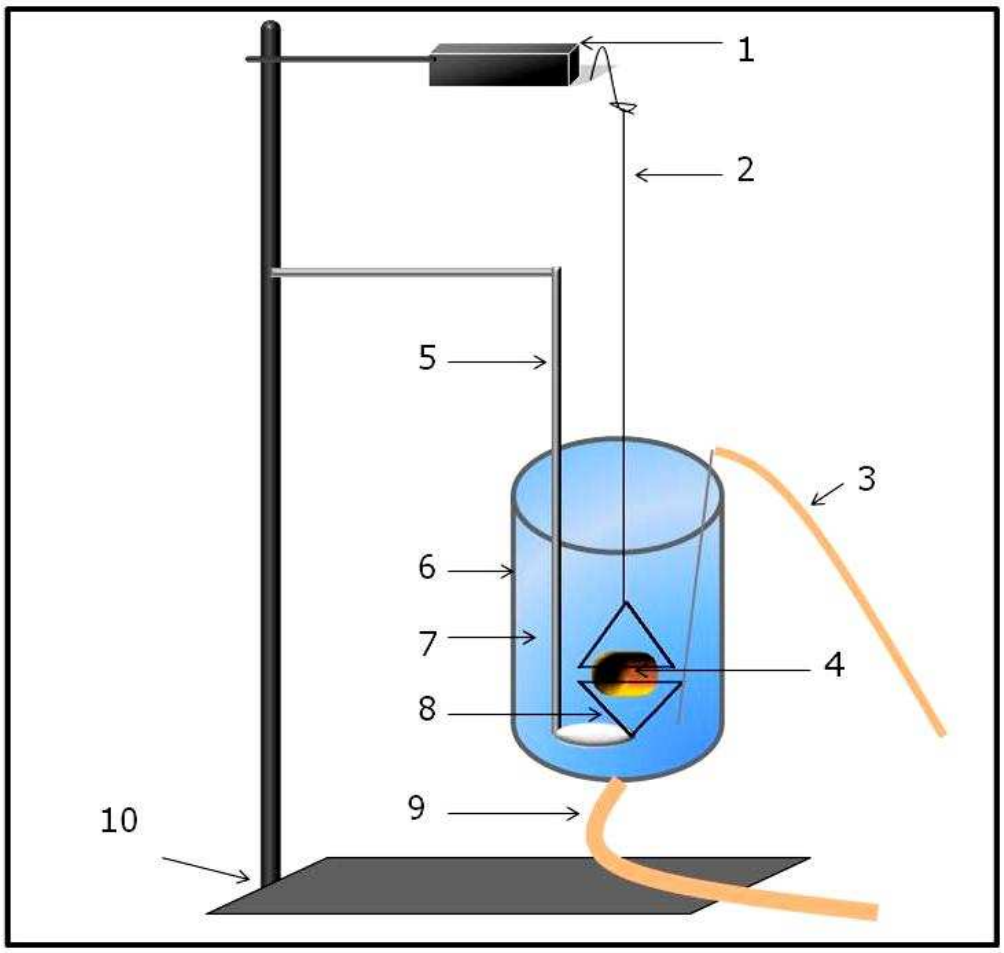


Figure 2.1. Diagrammatic representation of an isometric set up including (1) transducer, (2) a thin thread attached to a thin wire (upper support), (3) gas tube supplying 95% O_2: 5% CO_2, (4) blood vessel mounted between upper and lower wire supports, (5) glass tissue holder, (6) organ bath, (7) Krebs'-Henseleit buffer, (8) thin wire (lower support) attached to a tissue holder, (9) drain, and (10) base.

2.2.2. Responses in porcine isolated pancreatic arteries

Arterial rings were mounted onto wires in tissue baths (20 ml) containing warmed (37°C) oxygenated Krebs'-Henseleit solution and were connected via isometric force transducers (ADInstruments, Sydney, Australia) to a PC running the computer program LabChart (ADInstruments, Sydney, Australia). Rings were put under tension (15 g) and allowed to equilibrate for 60 min, before assessing viability with two challenges of 75 mM potassium chloride (KCl). The tissues were then allowed to equilibrate for 60 min, after which U46619 (10-100 nM) was used to contract the tissues to 40-80% of the second KCl response. This ensured that if there was a vasodilator component to the response, for example, due to activation of multiple P2 receptor subtypes, this could be detected. Once an appropriate level of U46619 response had been achieved, ATP, αβ-meATP, UTP, ADP or MRS2768 were applied. Antagonists, inhibitors or desensitisation agents [suramin (100 μM), PPADS (10 μM), αβ-meATP (1 μM), UTP (1 μM), ATP (1 μM), NF449 (10 μM), xanthine amine congener (XAC) (10 μM), MRS2578 (10 μM), DUP 697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene) (3 μM), MRS2179 (10 μM), SCH58261 (1 μM)] were applied 10 min prior to the addition of U46619, allowing incubation with the tissues for a minimum of 30 min prior to the application of agonists. In experiments using dimethyl sulfoxide (DMSO) as the solvent (see reagents and drugs section 2.2.3), DMSO 0.1 % (v/v) was added to the arteries as vehicle controls.

2.2.3. Reagents and drugs

Krebs'-Henseleit buffer was composed of the following (mM): NaCl 118, KCl 4.8, $CaCl_2.H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2 and glucose 11.1. Suramin, UTP, ATP, αβ-meATP, ADP, U46619, XAC, and SCH58261 were purchased from Sigma (Poole, Dorset, UK), while DUP 697, PPADS, MRS2578, MRS2179, MRS2768 and substance P were purchased from Tocris Biosciences Ltd. (Bristol, UK). NF449 was purchased from Calbiochem-Merck Biosciences (Darmstadt, Germany). U46619 was dissolved in ethanol at 10 mM stock concentration. PPADS, suramin, αβ-meATP, ATP, ADP, UTP, NF449, MRS2179, MRS2768 and substance P were dissolved in distilled water. DUP 697, XAC, MRS2578 and SCH58261 were dissolved in DMSO at 10 mM stock concentration.

2.3. Statistical analysis

The contractions to ATP, αβ-meATP and UTP were measured from the stabilised U46619-induced response and were expressed in g, while the relaxations to ATP and ADP were expressed as a percentage of the U46619-induced contraction. Data were expressed as log concentration-response plots. Values for all figures refer to mean ± S.E.M with 95% confidence. Results were compared by two-way analysis of variance (ANOVA) or one-way ANOVA with Bonferroni's post hoc test or unpaired Student's *t*-test (Prism, GraphPad, San Diego, CA, USA). Differences were considered to be significant when the P value was < 0.05. The "n" in the results expresses the number of animals.

2.4. Results

2.4.1. Effect of purine and pyrimidine nucleotides on vascular tone in porcine isolated pancreatic arteries

To investigate the effects of purine and pyrimidine receptor agonists on porcine pancreatic arteries, αβ-meATP (10 nM to 100 μM), ATP (1 μM to 10 mM), UTP (10 μM to 1 mM), ADP (1 μM to 1 mM) and MRS2768 (100 nM to 30 μM) were applied after pre-constriction with U46619. The responses to ATP and αβ-meATP were found to desensitise rapidly. Therefore, they were applied at single concentrations (one concentration per tissue segment). The responses to UTP, ADP and MRS2768 did not desensitise rapidly, thus cumulative concentration-response curves were generated. ATP, αβ-meATP, UTP and MRS2768 induced concentration-dependent contractions with a potency order of αβ-meATP > MRS2768 > UTP ≥ ATP (two-way ANOVA; Figure 2.2A), [(mean EC_{50} value for αβ-meATP was 1.6 μM (95% confidence interval (CI): 1.05 to 2.53 μM; n=8; it was 0.5 mM (95% confidence interval (CI): 0.1 to 1.8 mM; n=7 for ATP; Figure 2.2A). The response to ATP was biphasic, since its contraction was followed by a relaxation (Figure 2.2B) which was equipotent to the concentration-dependent relaxation induced by ADP (Figure 2.2A). The R_{max} value of αβ-meATP in inducing contraction of pancreatic artery was 9.3 ± 0.2g. The efficacy of ATP in inducing contraction was similar to that of αβ-meATP, and it was greater than those of, of UTP or MRS2768. The relaxations to ADP and ATP at the highest concentration of the agonists used (1 mM) were similar at 4.5 ± 0.5g (n=10) and 5.5 ± 0.2g (n=7) respectively; there was no significant difference between these responses (Figure 2.2A). UTP, MRS2768 and αβ-meATP (Figure 2.2B) did not elicit vasorelaxation.

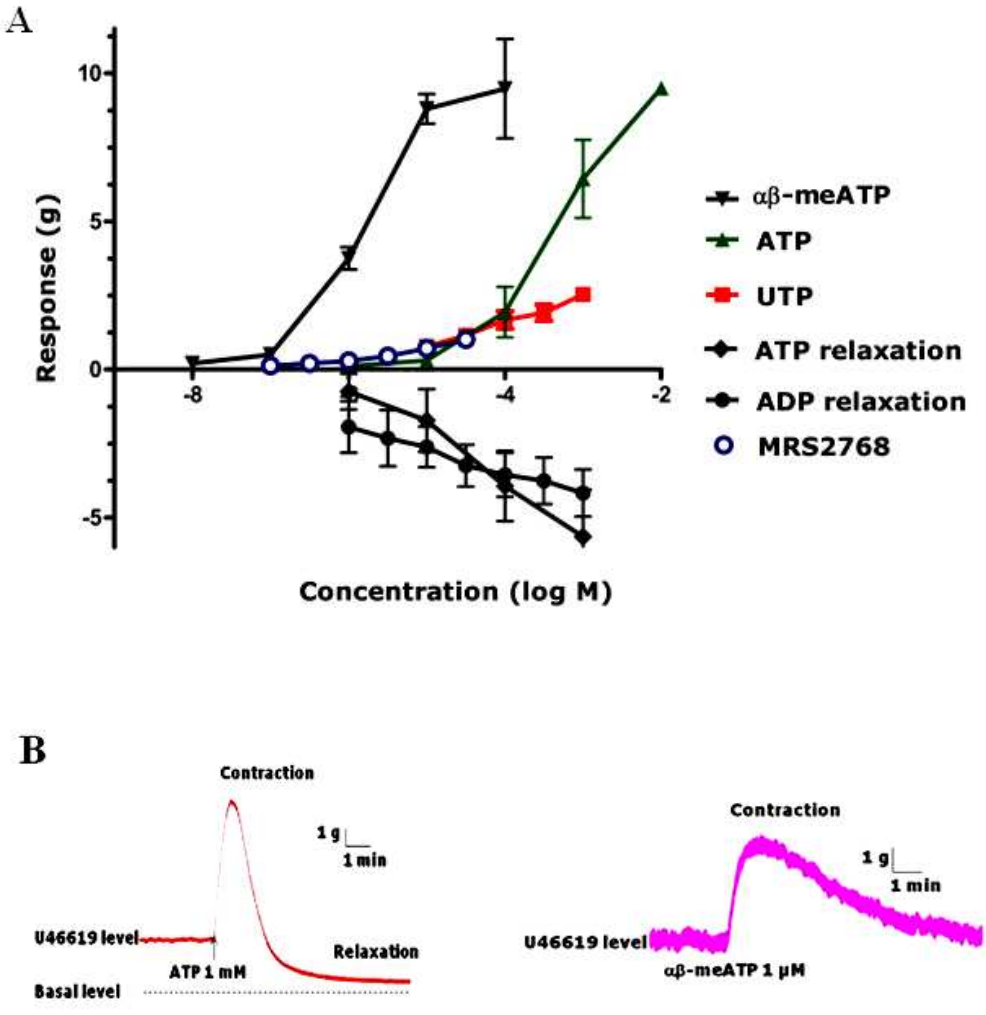


Figure 2.2. (A) Concentration-dependent contractions of ATP, αβ-meATP, UTP and MRS2768, a selective $P2Y_2$ receptor agonist, and concentration-dependent relaxation of ADP and ATP in U46619-preconstricted porcine pancreatic arteries (n=7-12). (B) Typical traces showing the biphasic response to ATP (contraction followed by relaxation) on the left hand side, as well as the monophasic response to αβ-meATP (just contraction) on the right hand side.

2.4.2. Characterisation of responses to ATP and αβ-meATP in U46619-preconstricted porcine isolated pancreatic arteries

2.4.2.1. Effect of suramin, PPADS and αβ-meATP

Responses to ATP and αβ-meATP were characterised using the non-selective P2 receptor antagonists, suramin (100 μM) and PPADS (10 μM). Both suramin and PPADS significantly attenuated the contractions-evoked by ATP (1 mM) and αβ-meATP (1 μM) ($P < 0.01$, one-way ANOVA, Figure 2.3A, B). These concentrations of ATP and αβ-meATP were chosen since they produced robust, submaximal responses and were close to the EC_{50}s (section 2.4.1). The relaxation to ATP was not affected in the presence of suramin or PPADS (Figure 2.3C). Since αβ-meATP induces desensitisation of P2X1 and P2X3 receptors more readily than ATP because it is broken down more slowly than ATP (Kasakov & Burnstock, 1982; Ralevic & Burnstock, 1998; Coddou *et al.*, 2011), the responses to ATP and αβ-meATP were studied in the presence of αβ-meATP, in which αβ-meATP (1 μM) was added 10 min prior to the addition of U46619 (section 2.2.2). As seen in Figure 2.3A, and 2.3B, the contractions to ATP and αβ-meATP were reduced significantly in the presence of the desensitising agent ($P < 0.001$, one-way ANOVA, Figure 2.3A, B), while the relaxation to ATP was not affected (Figure 2.3C), indicating involvement of P2X1 or P2X3 in the contractions to ATP and αβ-meATP in the porcine pancreatic arteries.

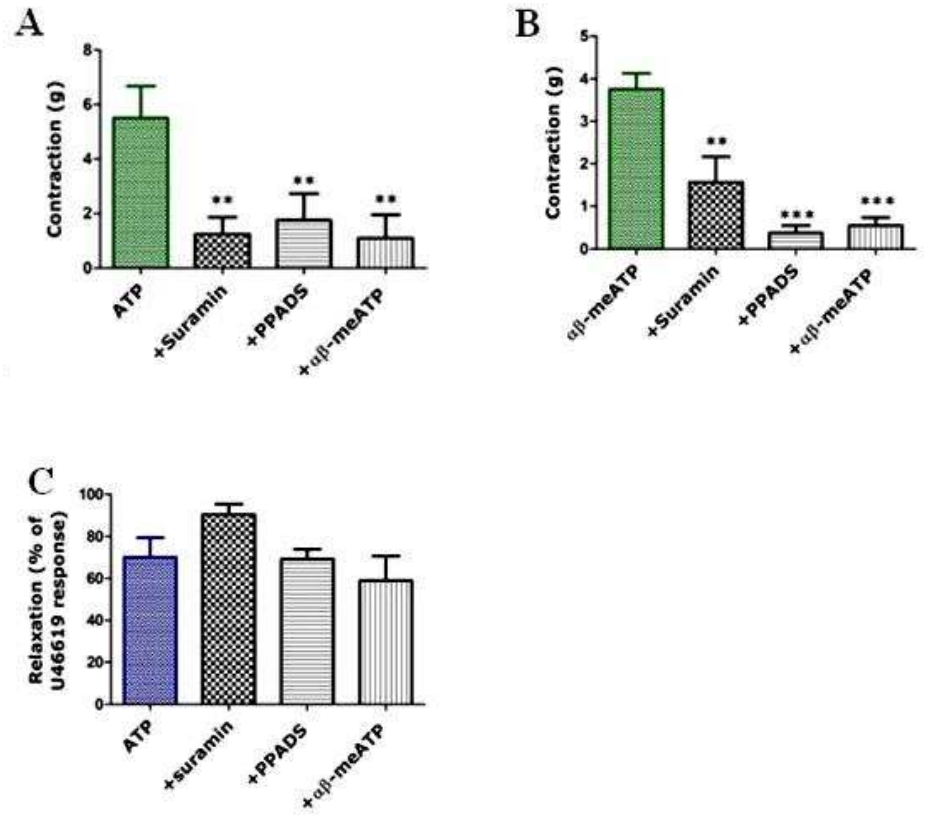


Figure 2.3. Effects of suramin (100 μM), PPADS (10 μM) and desensitisation by αβ-meATP (1 μM) on contractions to (A) ATP (1 mM), (B) αβ-meATP (1 μM), (C) on the relaxation to ATP in U46619-preconstricted porcine pancreatic arteries. PPADS, suramin and αβ-meATP reduced the contractions of (A) ATP and (B) αβ-meATP (**P < 0.01, ***P < 0.001, one-way ANOVA with Bonferroni's post hoc test, responses of ATP or αβ-meATP vs their responses in the presence of PPADS, suramin or αβ-meATP, n=6-9). (C) The relaxations to ATP were not significantly different in the absence or in the presence of PPADS, suramin or αβ-meATP (n=7).

2.4.2.2. Effect of NF449, a selective P2X1 receptor antagonist

Contractile response to αβ-meATP suggests an expression of P2X1 receptors in pancreatic arteries (Figure 2.3B). In turn, the involvement of P2X1 receptors in the contraction to ATP seems likely because contraction was significantly blocked by αβ-meATP (Figure 2.3A), since P2X3 receptor is not expressed in the VSMCs of the blood vessels (see review by Burnstock *et al.*, 2010). The responses to ATP and αβ-meATP were examined further in the presence of NF449 (10 μM), a P2X1 receptor selective antagonist (IC_{50} value of 1.6 μM in large rat pulmonary arteries) (Rettinger *et al.*, 2005; Syed *et al.*, 2010). The contractions to ATP and αβ-meATP were significantly inhibited in the presence of NF449 ($P < 0.001$, unpaired Student's *t*-test, Figure 2.4).

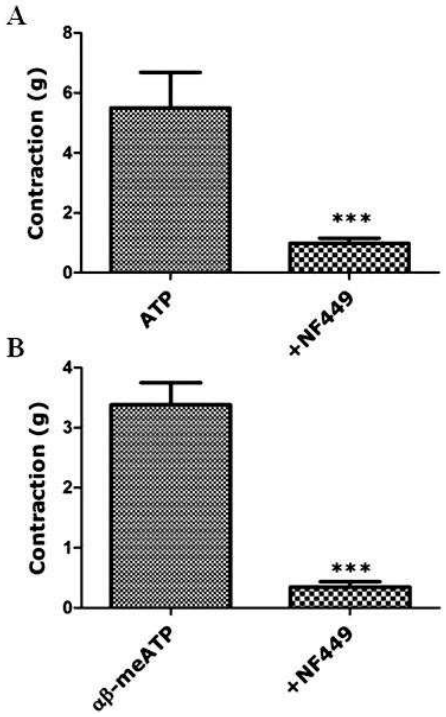


Figure 2.4. Effect of NF449 (10 μM), a selective P2X1 receptor antagonist, on contractions to (A) ATP (1 mM), (B) αβ-meATP (1 μM) in U46619-preconstricted porcine pancreatic arteries. NF449 reduced the effects of (A) ATP, (B) αβ-meATP (***P < 0.001, unpaired Student's *t*-test, n=10-13).

2.4.2.3. Effect of endothelium removal

The response to ATP was examined after the endothelium had been removed. The contraction and the relaxation induced by 1 mM ATP (Figure 2.5A, B) and the contraction to 1 μM αβ-meATP (Figure 2.5C) were not significantly different in the absence or presence of the endothelium (P > 0.05, unpaired Student's *t*-test). Similarly, removal of the endothelium had no effects on the contractions to KCl or U46619; for example, the contraction to 75 mM KCl was 9.5 ± 0.5g in endothelium-intact arteries, while it was 9 ± 0.5g in endothelium-denuded arteries (n=7-9). The contraction to 10-100 nM U46619 was 5.5 ± 0.5g in endothelium-intact

arteries, while it was 5.8 ± 0.6g in endothelium-denuded arteries (n=12-14); there was no significant difference between these responses. On the other hand, as can be seen in Figure 2.5A, the contraction to ATP in endothelium-denuded pancreatic arteries was reduced slightly (but not significantly) relative to its contraction in endothelium-intact arteries (Figure 2.5A), indicating involvement of other receptors (P2Y receptors).

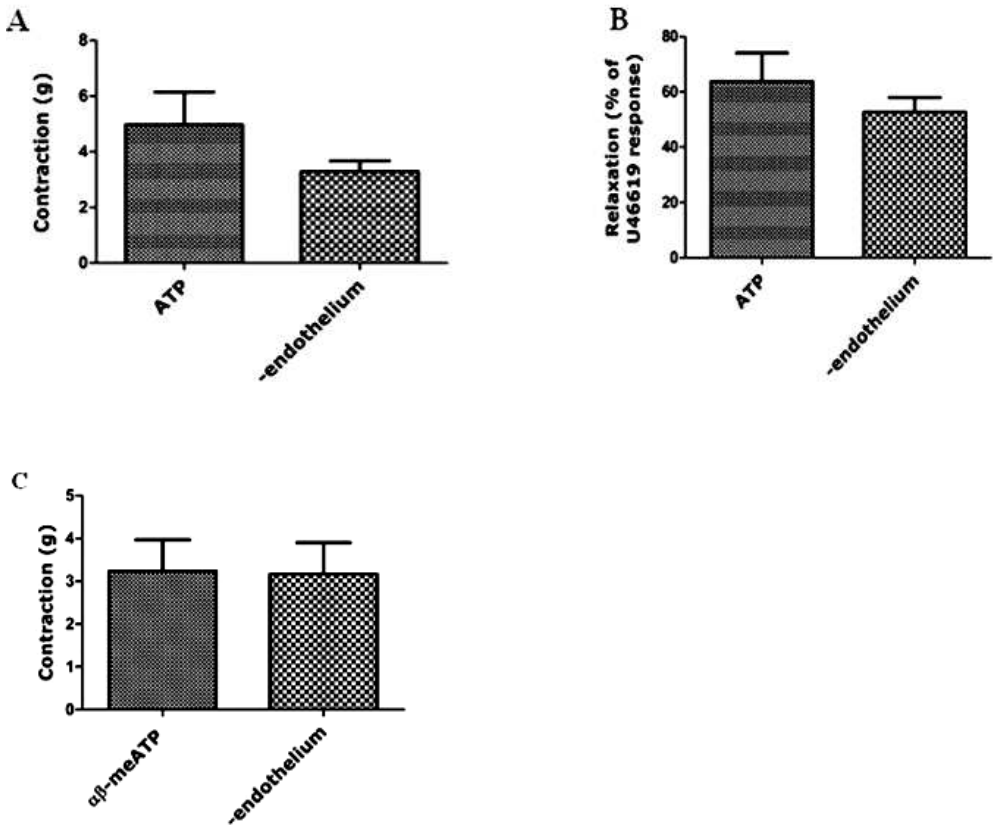


Figure 2.5. Effect of removal of the endothelium on (A) contraction, (B) relaxation to ATP (1 mM), (C) contraction to αβ-meATP (1 μM) in U46619-preconstricted porcine pancreatic arteries. (A), (B), (C) The removal of the endothelium had no significant effect on the contraction or relaxation of ATP or on the contraction to αβ-meATP (n=9-11).

2.4.2.4. Effect of XAC, an adenosine receptor antagonist

Since P2 receptor antagonists (suramin and PPADS) had no effect on the relaxation to ATP, the latter was investigated in the presence of a non-selective adenosine receptor antagonist. XAC (10 μM) had no effect on the contraction evoked by ATP ($P > 0.05$, unpaired Student's *t*-test, Figure 2.6A), while it reduced significantly the relaxation to ATP ($P < 0.001$, unpaired Student's *t*-test, Figure 2.6B).

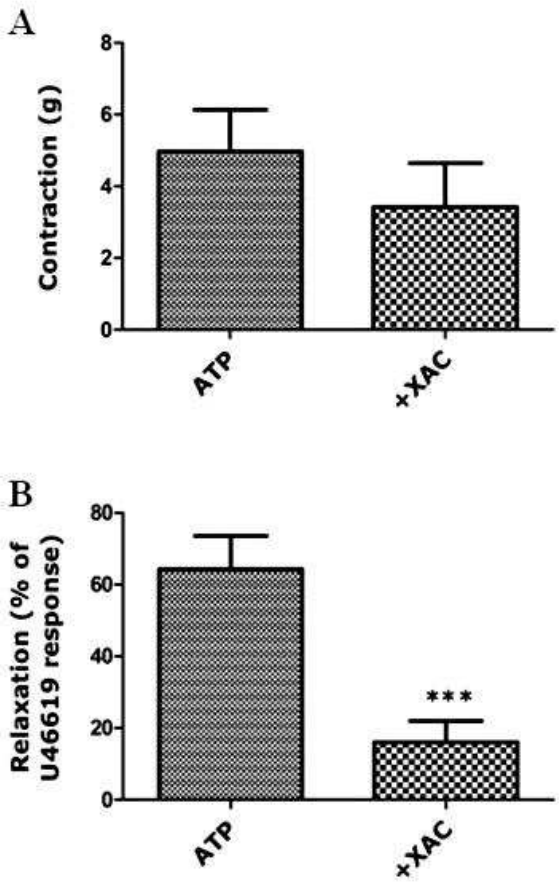


Figure 2.6. Effect of XAC (10 μM) on (A) contraction, (B) relaxation to ATP (1 mM) in U46619-preconstricted porcine pancreatic arteries. (A) XAC had no effect on the contraction to ATP (n=8-10), (B) XAC reduced the relaxation to ATP (***$P < 0.001$, unpaired Student's *t*-test, n=8-10).

2.4.3. Characterisation of response to UTP in U46619-preconstricted porcine isolated pancreatic arteries

2.4.3.1. Effect of suramin, PPADS, αβ-meATP and MRS2578, a selective $P2Y_6$ receptor antagonist

The contractions to UTP were examined in the presence of suramin (100 μM), PPADS (10 μM), αβ-meATP (1 μM) and MRS2578 (10 μM). Suramin and PPADS significantly reduced the contractions to UTP ($P < 0.001$, two-way ANOVA, Figure 2.7); for example, the contraction to 1 mM UTP was 2.5 ± 0.2g in the absence of suramin, PPADS, while it was 1.8 ± 0.3g, 1.9 ± 0.2g in the presence of suramin, PPADS respectively ($P < 0.001$, n=9-12, Figure 2.7). These findings were consistent with those of Shen *et al.* (2004), as 10 μM of suramin and PPADS was able to block the UTP-induced increase in peak of $[Ca^{2+}]$ in porcine $P2Y_2$-1321N1 cells. While the UTP responses were not affected in the presence of MRS2578 (Figure 2.8A), which selectively blocks $P2Y_6$ receptor with an IC_{50} of 98 nM at rat $P2Y_6$ receptor (Mamedova *et al.*, 2004). In addition, the contraction to UTP was unaltered in the presence of P2X receptor desensitising agent, αβ-meATP (1 μM) (Kasakov & Burnstock, 1982) (Figure 2.8B); for example, the contraction to 1 mM UTP was 1.8 ± 0.2g in the absence of MRS2578, while it was 2.1 ± 0.2g in the presence of MRS2578 (n=6-7). The contraction to 1 mM UTP was 2.2 ± 0.2g in the absence of αβ-meATP, while it was 2.5 ± 0.4g in the presence of αβ-meATP (n=7); there was no significant difference between these responses.

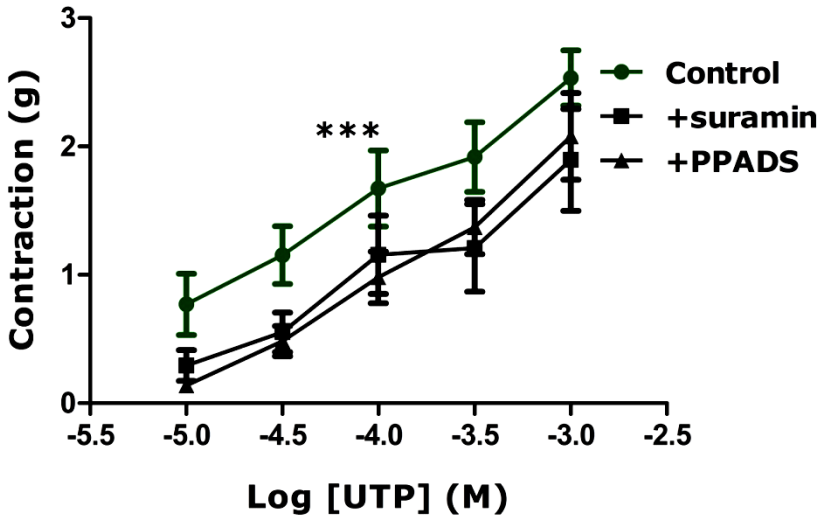


Figure 2.7. Effect of suramin (100 μM) and PPADS (10 μM) on contraction to UTP in U46619-preconstricted porcine pancreatic arteries. Suramin and PPADS significantly reduced the contraction evoked by UTP (***P < 0.001, two-way ANOVA, F=14.47, 12.48 respectively; n=9-12).

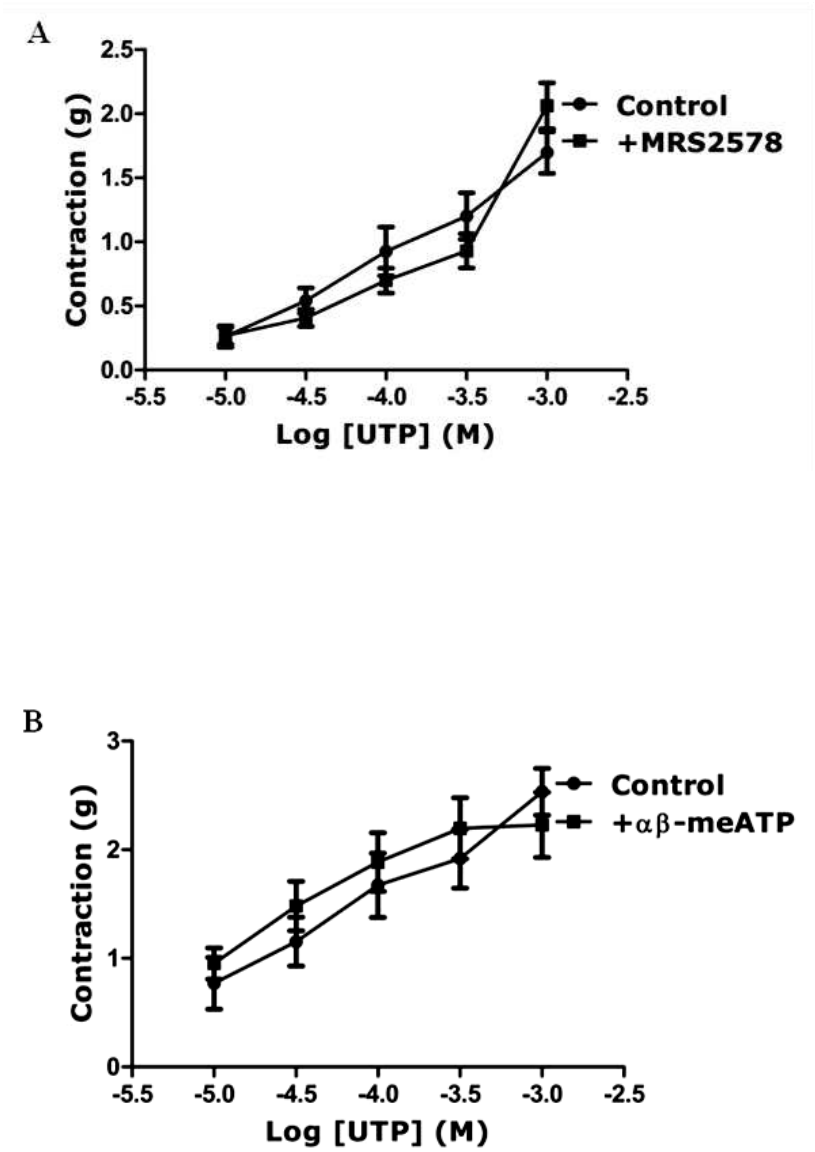


Figure 2.8. Effect of (A) MRS2578 (10 μM), and (B) αβ-meATP (1 μM) on the contractions to UTP in U46619-preconstricted porcine pancreatic arteries. (A), (B) Both MRS2578 and αβ-meATP had no significant effect on the contraction to UTP (6-7).

2.4.3.2. Effect of endothelium removal

To investigate the involvement of the endothelium in the contraction to UTP, the response of UTP was studied after the endothelium had been removed. The contraction induced by UTP was significantly attenuated in the endothelium-denuded arteries ($P < 0.001$, two-way ANOVA, Figure 2.9).

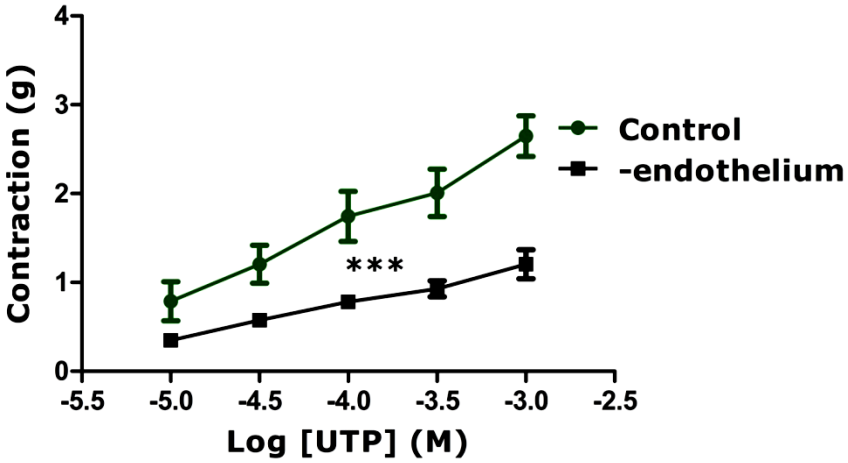


Figure 2.9. Effect of removal of the endothelium on the contraction to UTP in U46619-preconstricted porcine pancreatic arteries. Removal of endothelium reduced significantly the contraction evoked by UTP (***$P < 0.001$, two-way ANOVA, $F=43$; $n=10$-12).

2.4.3.3. Effect of DUP 697, a cyclooxygenase-2 inhibitor

Because the contraction to UTP was largely endothelium-dependent, the contraction was studied in the presence of DUP 697, a cyclooxygenase-2 (COX-2) inhibitor (IC_{50} value of 10 nM for COX-2), since cyclooxygenase facilitates the release of agents which are responsible for endothelium-dependent contraction (Mombouli & Vanhoutte, 1993; Gierse *et al.*, 1995; Wong *et al.*, 2009). DUP 697 (3 μM) diminished the contraction to UTP ($P < 0.001$, two-way ANOVA, Figure 2.10) to a similar extent as removal of the endothelium (Figure 2.9). While DUP 697 had no effect on the contractions to U46619 (the pre-constriction agent) or ATP. The contraction to 1 mM ATP was 1.1 ± 0.3g in the absence of DUP 697, while it was 0.9 ± 0.2g in the presence of it (n=8-9); there was no significant difference between these responses.

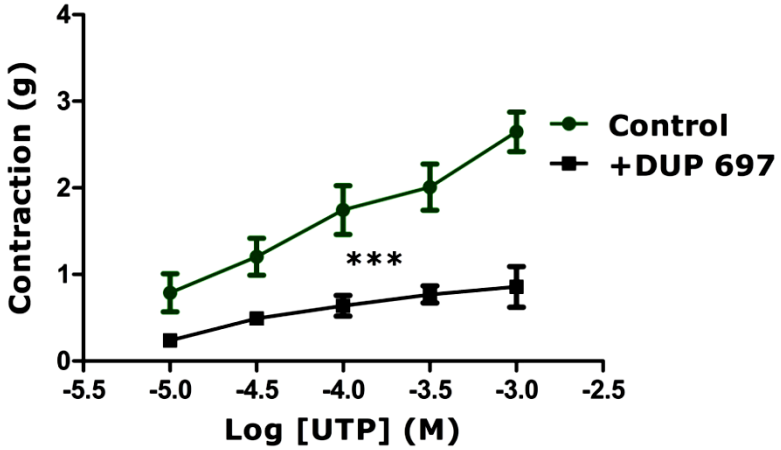


Figure 2.10. Effect of DUP 679 (3 μM), a cyclooxygenase-2 inhibitor, on the contraction to UTP in U46619-preconstricted porcine pancreatic arteries. DUP 679 reduced significantly the contraction evoked by UTP (***$P < 0.001$, two-way ANOVA, $F=50.8$; n=8-12).

2.4.3.4. Desensitisation of UTP-induced contraction in the presence of ATP or UTP

Both ATP and UTP separately induced significant attenuation of the response to UTP, when the pancreatic arteries were exposed to these ligands 30 min prior to the addition of UTP, for example, the response to 300 μM UTP was 0.7 ± 0.2g in the presence of 1 μM ATP, and it was 0.8 ± 0.2 in the presence of 1 μM UTP, while the contraction to UTP was 1.2 ± 0.2 in the absence of these ligands ($P < 0.01$, n=10-13, Figure 2.11).

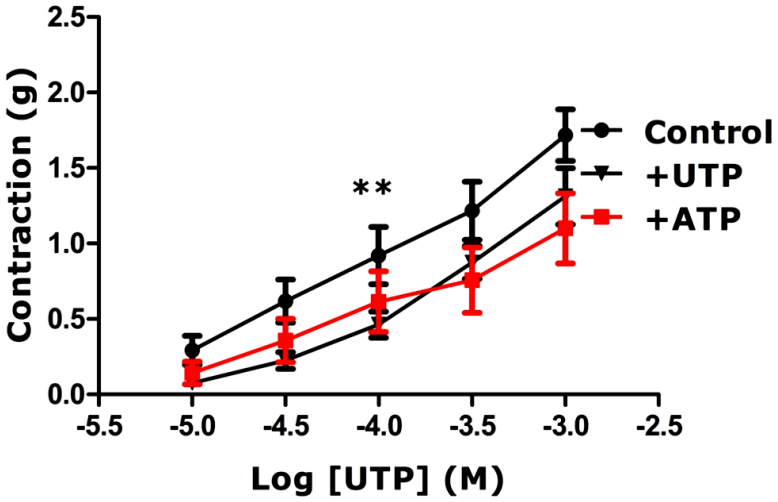


Figure 2.11. Attenuation of UTP-induced contraction (control) in the presence of ATP (1 μM) or UTP (1 μM) in U46619-preconstricted porcine pancreatic arteries. Both UTP and ATP significantly attenuated the contraction evoked by UTP (**$P < 0.01$, two-way ANOVA, UTP contraction in the absence or in the presence of UTP or ATP, F=18.21, 14.04 respectively; n=10-13).

2.4.4. Characterisation of responses to ADP in U46619-preconstricted porcine isolated pancreatic arteries

2.4.4.1. Effect of MRS2179, a $P2Y_1$ receptor selective antagonist, and endothelium removal

The relaxation to ADP in pancreatic arteries was studied in the presence of MRS2179 (10 μM), and after the endothelium had been removed. The relaxation to ADP was reduced slightly but significantly in the presence of MRS2179 ($P < 0.01$, two-way ANOVA, Figure 2.12A) and in the endothelium-denuded arteries ($P < 0.001$, two-way ANOVA, Figure 2.12B); for example, the relaxation to 100 μM ADP was 28 ± 5% in the absence of MRS2179 and in endothelium-intact arteries, while it was 16 ± 7% in the presence of MRS2179 ($P < 0.01$, n=8-12), and it was 13 ± 3% ($P < 0.001$, n=10-12) in endothelium-denuded arteries, which indicates the involvement of $P2Y_1$ receptors and the endothelium in ADP-mediated relaxation of porcine pancreatic arteries.

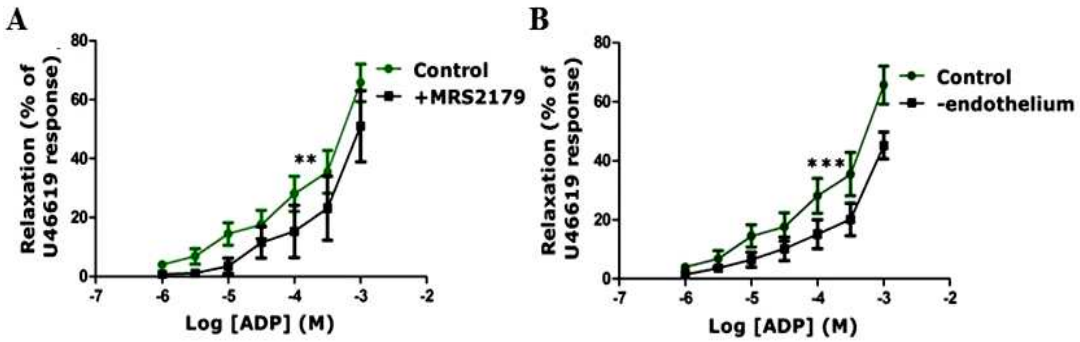


Figure 2.12. Effect of (A) MRS2179 (10 μM), (B) the removal of the endothelium on the relaxation to ADP in U46619-preconstricted porcine pancreatic arteries. MRS2179 and removal of endothelium reduced the relaxation-evoked by ADP (**P < 0.01, ***P < 0.001, two-way ANOVA, F=21.42, F=32.04 respectively; n=10-12).

2.4.4.2. Effect of XAC, an adenosine receptor antagonist, and SCH58261, a selective adenosine A_{2A} receptor antagonist

The relaxation to ADP was investigated in the presence of XAC (10 μM). The relaxation to ADP was largely reduced in the presence of this inhibitor which indicates the involvement of adenosine receptors ($P < 0.001$, two-way ANOVA, Figure 2.13). To identify the adenosine subtype involved in the relaxation to ADP, the response to ADP was investigated in the presence of SCH58261, a selective adenosine A_{2A} receptor antagonist with K_i of 1.3 nM (Zocchi *et al.*, 1996). This antagonist significantly inhibited the relaxation to ADP, to a similar extent as seen with XAC ($P < 0.001$, two-way ANOVA, Figure 2.13). This showed that the relaxation to ADP is mainly mediated by A_{2A} adenosine receptors.

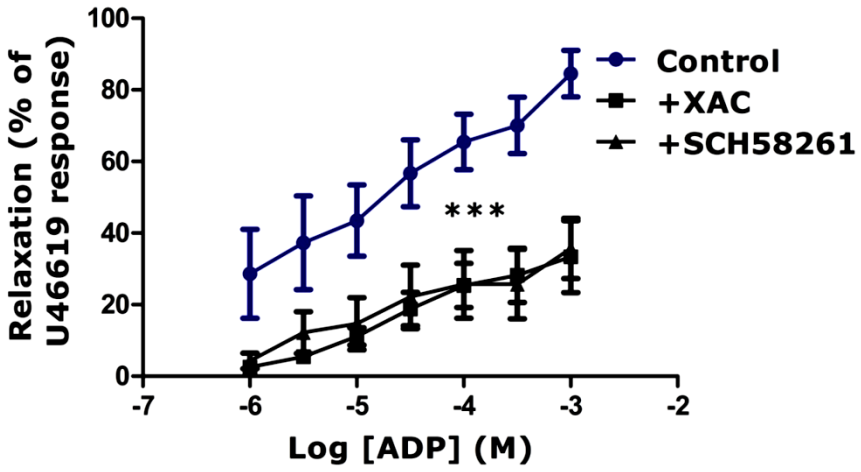


Figure 2.13. Effect of XAC (10 μM), a non-selective adenosine receptor antagonist, and SCH58261 (1 μM), a selective adenosine A_{2A} receptor antagonist, on the relaxation to ADP in U46619-preconstricted porcine pancreatic arteries. XAC and SCH58261 inhibited significantly the relaxation-evoked by ADP (***$P < 0.001$, two-way ANOVA, $F=71.19$, 58.16 respectively; $n=9$-14).

2.4.4.3. Effect of αβ-meATP, a P2X receptors desensitisation agent

To find out whether ADP acts at P2X receptors, the relaxation to ADP was investigated in the presence of αβ-meATP, since the latter induces desensitisation of P2X receptors (Kasakov & Burnstock, 1982; Ralevic & Burnstock, 1998; Coddou *et al.*, 2011). αβ-meATP (1 μM) was applied 10 min prior to the addition of U46619 (section 2.2.2). As can be seen in Figure 2.14, the relaxation to ADP was not significantly altered in the presence of the desensitising agent, which rules out the involvement of P2X receptors in the relaxation-evoked by ADP.

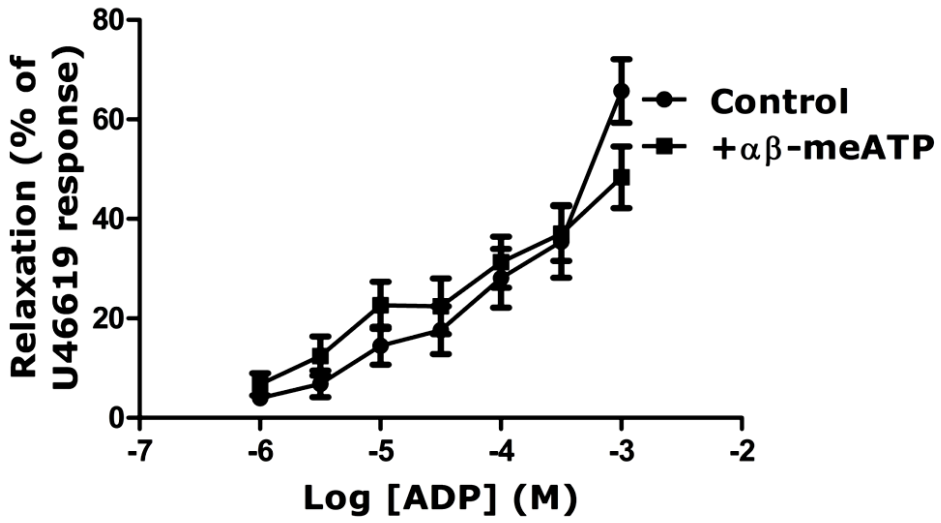


Figure 2.14. Effect of αβ-meATP (1 μM) on the relaxation to ADP in U46619-preconstricted porcine pancreatic arteries. αβ-meATP had no significant effect on the relaxation to ADP (n=14).

2.5. Discussion

The current report has provided evidence for the functional expression of contractile P2X1, $P2Y_2$ and/or $P2Y_4$ receptors, and vasorelaxant $P2Y_1$ and A_{2A} adenosine receptors in porcine isolated pancreatic arteries. These receptors are sensitive to the extracellular nucleotides ATP (P2X1), UTP ($P2Y_2$ and $P2Y_4$) and ADP/ado ($P2Y_1$ and A_{2A}). The contraction to ATP was endothelium-independent, while UTP induced an endothelium-dependent contraction which may involve $P2Y_2$ and/or $P2Y_4$ receptors. The relaxation to ADP involved the endothelium, $P2Y_1$ receptors and mainly A_{2A} adenosine receptors present on the VSMCs.

2.5.1. Characterisation of the response-evoked by ATP and αβ-meATP in porcine pancreatic arteries

A vasoconstrictor response elicited by ATP has been reported in a number of different arteries, including rabbit ear arteries, rat mesenteric arterial bed and rat pulmonary vascular bed (Kügelgen *et al.*, 1987; Ralevic & Burnstock, 1991a; Rubino & Burnstock, 1996). ATP may also induce vasorelaxation depending on the experimental conditions (level of pre-tone) and relative expression of relevant vasocontractile or vasorelaxant receptors (Ralevic & Burnstock 1996; Korchazhkina *et al.*, 1999). In porcine pancreatic arteries, ATP induced a biphasic response consisting of a contraction followed by a relaxation (Figure 2.2B). In addition, αβ-meATP elicited a monophasic response consisting of only a contraction in porcine pancreatic arteries (Figure 2.2B). Since the contractions to ATP and αβ-meATP were rapidly desensitising, non-cumulative concentration response curves were investigated. The contractions to ATP and αβ-meATP were significantly reduced in the presence of suramin, PPADS, αβ-meATP (a

desensitiser of P2X1 receptor) and NF449 (a P2X1 selective antagonist) (Figure 2.3A, 2.3B and 2.4), which indicates that a large part of the contraction to ATP could be attributed to the activation of P2X1 receptors, since P2X3 receptor, which is also desensitised in the presence of αβ-meATP, is not likely to be expressed in the VSMCs of the blood vessels (see review by Burnstock *et al.*, 2010). Moreover, the contractile effect of αβ-meATP is consistent with the expression of P2X1 receptors in porcine pancreatic arteries. In the current study, αβ-meATP was shown to be approximately 300-fold more potent than ATP in eliciting a vasoconstriction, most likely due to its greater stability. This finding was consistent with previous reports which showed that αβ-meATP was 10-fold more potent than ATP in eliciting the contraction of the rat vas deferens, and this effect was attributed to the greater stability of αβ-meATP, since it was shown in that report that αβ-meATP was resistant to breakdown by the ecto-nucleotidases for 2h, while ATP was metabolised rapidly (Khakh *et al.*, 1995).

Since the contractions to ATP and αβ-meATP were not significantly changed after the endothelium had been removed (Figure 2.5A, C) thus, the expression of P2X1 receptors was proposed to be on the vascular smooth muscle cells of the pancreatic arteries. This is consistent with the abundant expression of P2X1 receptors on VSMCs of most tissues (Kügelgen, 2008). On the other hand, the contraction to ATP in endothelium-denuded pancreatic arteries was reduced slightly relative to its contraction in endothelium-intact arteries (Figure 2.5A). Although this reduction was not significant, it is likely that other receptors (P2Y receptors) may be involved. These receptors may be expressed, at least partly, on the endothelium, and they were responsible for eliciting part of the observed contraction. ATP is a potent agonist at $P2Y_2$ receptors which can be expressed on the

endothelium (Erlinge & Burnstock, 2008). Likewise, the current study showed (as it is discussed later on in this section) that UTP may act at $P2Y_2$ receptors expressed on the endothelium of the pancreatic arteries to induce a contraction, which was also reduced in endothelium-denuded vessels. Taken together, it can be concluded that ATP acted at P2X1 receptors expressed on the VSMCs to induce the major part of the observed contraction, as well as acting partly at the endothelial-$P2Y_2$ receptors to induce the rest of that contraction, which was reduced slightly (not significantly) after the endothelium had been removed. Therefore, it is also required to examine the contraction to ATP in the presence of a $P2Y_2$ selective antagonist for that hypothesis to be confirmed.

ATP-induced vasorelaxation was not affected after the endothelium had been removed, or in the presence of suramin or PPADS, which suggests that the relaxation to ATP was not due to its action at $P2Y_1$ or $P2Y_2$ receptors. However, the relaxation to ATP was significantly inhibited in the presence of XAC, which suggested an involvement of adenosine receptors expressed on VSMCs of the pancreatic arteries. It is likely that this is due to the activity of adenosine derived from ATP metabolism by ecto-nucleotidase-5'-triphosphate diphosphohydrolase (ENTPDases) enzymes followed by the activity of (GPI)-anchored ecto-5'-nucleotidase or APs enzymes (section 1.7) (Zimmermann, 2000). Similarly, in rat coronary arteries, the relaxation to ATP involved P1 receptors, although there was an additional involvement of P2Y receptors (Korchazhkina *et al.*, 1999). The relaxation evoked by ATP in mouse thoracic aorta was mainly mediated by $P2Y_2$ receptors (Guns *et al.*, 2006). In the current study, further investigation of the adenosine receptor subtypes involved in the relaxation to ATP is required. Although, it is possible that the adenosine A_{2A} receptor is the subtype responsible of the observed relaxation, since the relaxation

to ADP in porcine pancreatic arteries (as it is discussed later on in this section) was shown to be mediated by this subtype (Figure 2.13). Other reports have shown previously a slow relaxation in response to αβ-meATP in rat mesenteric arteries, subsequent to the contraction (Stanford & Mitchell, 1998; Ralevic, 2001, 2002), but this was not observed in the present study in porcine pancreatic arteries.

2.5.2. Characterisation of the response-evoked by UTP in porcine pancreatic arteries

The vasoconstriction to UTP did not desensitise quickly, therefore, cumulative concentration response curves were used to study the effect of UTP on pancreatic arteries. This contraction was significantly inhibited in the presence of suramin and PPADS (Figure 2.7), and there was a significant reduction of the response after the endothelium had been removed (Figure 2.9). That would indicate, for the first time, an endothelium-dependent vasoconstriction-evoked by UTP, since UTP-mediated vasoconstrictions, in the previous reports, were mainly mediated by P2Y receptors present on the vascular smooth muscle. Moreover, when the endothelium of the rat, rabbit and bovine cerebral arteries had been removed, the contractions to UTP were not significantly altered (von Kugelgen & Starke, 1990; Miyagi *et al.*, 1996; Lopez *et al.*, 2000; Lacza *et al.*, 2001; Miyagi *et al.*, 2004).

UTP is known to be active at $P2Y_2$, $P2Y_4$ receptors and less potently at $P2Y_6$ receptors (Burnstock & Williams, 2000). The expression of these receptors in the endothelium and the smooth muscle of vessels has been reported previously (Burnstock, 2007). In the current study, since MRS2578 was not able to alter the contraction to UTP (Figure 2.8A), this indicates that the

contraction induced by UTP was not due to its action at $P2Y_6$ receptors. There are currently no commercially available selective antagonists for either $P2Y_2$ or $P2Y_4$ receptors. However, it is believed that UTP acted mainly at $P2Y_4$ receptors since the contraction to UTP was significantly inhibited by both the removal of the endothelium and in the presence of DUP 697, but contraction to ATP (a potent agonist at $P2Y_2$ receptor) did not change significantly in endothelium-denuded arteries (section 2.4.2.3) or in the presence of DUP 697 (section 2.4.3.3), indicating actions at distinct receptors. UTP induced-contraction may also be mediated by $P2Y_2$ receptors, since MRS2768, which is a selective agonist at $P2Y_2$ receptors and displays no affinity for $P2Y_4$ or $P2Y_6$ receptors (Ko *et al.*, 2008), was able to evoke a contraction in porcine pancreatic arteries (Figure 2.2A).

UTP-induced vasoconstriction has been documented in a number of arteries, including rat pulmonary arteries in which the contraction was attributed to $P2Y_2$ receptors, and in rabbit basilar arteries in which the contraction to UTP was due to its action at $P2Y_4$ receptors (Hartley *et al.*, 1998; Miyagi & Zhang, 2004). UTP produced an endothelium-dependent relaxation in rabbit pulmonary arteries and in rat mesenteric arterial bed, but the receptor subtypes were undefined (Ralevic & Burnstock, 1991a; Qasabian *et al.*, 1997). In bovine middle cerebral arterial strips, UTP had a dual response, it induced a contraction in endothelium-denuded arteries but a relaxation in endothelium-intact arteries (Miyagi *et al.*, 1996). The absence of endothelium-dependent or -independent relaxation to UTP and some other nucleotides in rat renal arteries was reported (Knight *et al.*, 2003), which is consistent with the current study since there was no evidence of a UTP-mediated relaxation in porcine pancreatic arteries. Hence, porcine pancreatic arteries appear not to express relaxant $P2Y_2$ and/or $P2Y_4$ receptors.

To investigate the mechanism underlying the contraction mediated by UTP in porcine pancreatic arteries, the response to UTP was examined in the presence of DUP 697. As seen in Figure 2.10, the endothelium-dependent contraction was attenuated in the presence of the selective COX-2 inhibitor. Endothelial cells can release endothelium-derived contractile factors, which may include thromboxane A_2, prostaglandin $F_2\alpha$/ prostaglandin H_2, leukotrienes and endothelin-1. Thromboxane A_2 and prostaglandin $F_2\alpha$/ prostaglandin H_2 are released from the endothelium due to the activity of cyclooxygenase (Mombouli & Vanhoutte, 1993; Wong *et al.*, 2009). The reduction of the contraction to UTP in the presence of DUP 697 indicated that thromboxane A_2 and prostaglandins, the prominent vasoconstrictors arachidonic acid derivatives, can be suspected of mediating the UTP-induced contraction. These agents, after being released from the endothelium, may act on their receptors on VSMCs to elicit a contraction (Wong *et al.*, 2009). Similarly, UTP-induced contraction in rat cerebral arteries was associated with an elevation of thromboxane A_2 release (Lacza *et al.*, 2001). To test whether UTP interacts with P2X receptors, expressed in porcine pancreatic arteries (section 2.4.2.2), αβ-meATP, which desensitises P2X1 and P2X3 receptors (Kasakov & Burnstock, 1982; Ralevic & Burnstock, 1998; Coddou *et al.*, 2011), was employed. However, as can be seen in Figure 2.8B, αβ-meATP had no significant effect on the response to UTP, which ruled out the involvement of P2X receptors in the contraction to UTP.

2.5.3. Characterisation of the response-evoked by ADP in porcine pancreatic arteries

The relaxation to ADP did not desensitise rapidly, therefore, cumulative concentration response curves were used to study the effect of ADP on pancreatic arteries. The relaxation was significantly attenuated in the presence of MRS2179, a selective $P2Y_1$ receptor antagonist (Figure 2.12A). In addition, the relaxation to ADP was significantly reduced after the endothelium had been removed, by a similar extent as observed in the presence of MRS2179 (Figure 2.12B). This may suggest that $P2Y_1$ receptors are expressed on the endothelium. Indeed, a number of reports show that $P2Y_1$ receptors are expressed on the endothelium and are responsible for relaxation of the arteries, including rat thoracic aortic and porcine mesenteric arteries (Dol-Gleizes *et al*., 1999; Alefishat *et al*., 2010). The relaxation to ADP in our study was largely reduced in the presence of XAC (an adenosine receptor antagonist) and SCH58261 (a selective adenosine A_{2A} receptor antagonist). Adenosine receptors may be expressed on the endothelium or the vascular smooth muscle (Schulte & Fredholm, 2003). Since XAC and SCH58261 produced a greater reduction in the relaxation to ADP than the inhibition induced by removal of the endothelium (Figure 2.13). This suggested that relaxation to ADP involves A_{2A} adenosine receptors expressed, at least in part, on VSMCs. The mechanism by which ADP would produce adenosine to act at the adenosine receptors is still to be elucidated. The simplest explanation is that ADP may be broken down by ENTPDases followed by the activity of (GPI)-anchored ecto-5'-nucleotidase or APs enzymes (section 1.7) (Zimmermann, 2000). Alternatively, as suggested in porcine coronary arteries, ADP mediates a relaxation via a mechanism that involves ADP-evoked adenosine release and the subsequent activation of A_{2A} receptors (Rayment *et al.*, 2007b). In

contrast to the porcine pancreatic vessels, ADP in rat pancreatic arteries induced a contraction at a high concentration (1 mM), this contraction was similar to that produced by ATP and was much lower than the contraction induced by αβ-meATP (Chapal & Loubatieres-Mariani, 1983). Further investigation is required to determine the involvement of endothelium-derived relaxing factors or endothelium-derived hyperpolarising factors released from the endothelium in the ADP-induced relaxation. To test whether ADP binds to P2X receptors expressed in porcine pancreatic arteries (section 2.4.2.2), αβ-meATP was employed. However, as seen in Figure 2.14, αβ-meATP had no effect on the response to ADP which ruled out the involvement of P2X receptors in the response to ADP in porcine pancreatic arteries.

2.6. Conclusion

The data presented in this chapter determined the functional expression of P2X1 and A_{2A} adenosine receptors expressed on VSMCs, and $P2Y_2$ and/or $P2Y_4$ receptors expressed on the vascular endothelial cells of porcine pancreatic arteries. Activation of P2X1 receptors by ATP or αβ-meATP induces a vasoconstriction, and UTP acts at $P2Y_2$ and/or $P2Y_4$ receptors to induce a contraction. The slight reduction of the contraction to ATP, observed in endothelium-denuded arteries, suggests an involvement of $P2Y_2$ receptors in the contraction to ATP. ADP and ATP activate A_{2A} adenosine receptors on the VSMCs to induce relaxation, together with an action of ADP at $P2Y_1$ receptors expressed on the endothelial cells. Pancreatic arteries appear to lack vasorelaxant $P2Y_2$ and/or $P2Y_4$ receptors. The data rule out the involvement of P2X receptors in the contraction or the relaxation to UTP and ADP respectively.

Chapter Three

Investigation of the effects of UDP-glucose, UDP and MRS2690 on vascular tone in porcine isolated pancreatic arteries

3.1. Introduction

The $P2Y_{14}$ receptor is a recently described member of the P2Y receptor family, and was identified in 2000 (Chambers *et al.*, 2000). In contrast to other P2Y receptors, $P2Y_{14}$ receptors can be activated by nucleotide sugars such as UDP-glucose (Figure 1.5B), in addition to UDP-galactose and UDP-glucuronic acid which are less potent than UDP-glucose at $P2Y_{14}$ receptors (Abbracchio *et al.*, 2003; Fricks *et al.*, 2008; Harden *et al.*, 2010). $P2Y_{14}$ receptors are also activated by UDP (Figure 1.5A) and by MRS2690 (Figure 1.5C) which is more selective at $P2Y_{14}$ receptors and was shown to be 7-10 fold more potent than UDP-glucose, as well as $\alpha\beta$-difluoromethylene-UDP which is also more potent and selective at $P2Y_{14}$ receptors (Carter *et al.*, 2009; Jacobson *et al.*, 2009; Gao *et al.*, 2010).

PPADS and suramin are non-selective antagonists at most of the P2Y receptors, but some P2Y receptors are insensitive to these antagonists (Chootip *et al.*, 2005). There is currently no report of antagonist sensitivity of $P2Y_{14}$ receptors for suramin and PPADS. Recently, a novel selective antagonist at $P2Y_{14}$ receptors was identified, namely PPTN (Figure 1.6) (Barrett *et al.*, 2013). It was characterised in human embryonic kidney cells expressing $P2Y_{14}$ receptor through its ability to inhibit UDP-glucose-

stimulated Ca^{2+} mobilisation (Robichaud *et al.*, 2011). In addition, it showed high affinity for the $P2Y_{14}$ receptor (K_i = 1.9 nM in a chimpanzee $P2Y_{14}$ binding assay) (Robichaud *et al.*, 2011). When it was studied in human C6 glioma cells, PPTN showed selectivity for $P2Y_{14}$ receptors, with no agonist or antagonist affinity at other P2Y receptors family (Barrett *et al.*, 2013).

$P2Y_{14}$ receptor mRNA and protein have a varied expression in the body; they have been found in spleen, placenta, lung, heart, adipose tissue, gastrointestinal smooth muscle, endothelial cells, and immune cells (section 1.4.2.2.1) (Chambers *et al.*, 2000; Scrivens & Dickenson, 2005; Umapathy et al., 2010).

Relatively little is known, however, about the functional expression of the $P2Y_{14}$ receptor in cardiovascular system, best characterised is its role in regulation of the immune system, as a number of studies have described an involvement of $P2Y_{14}$ receptors in modulation of the function of human neutrophils and T-lymphocytes. Activation of $P2Y_{14}$ receptors inhibited T-lymphocyte proliferation and increased secretion of the pro-inflammatory cytokine interleukin 8 from airway epithelial cells (Scrivens & Dickenson, 2005, 2006; Muller *et al.*, 2005). Therefore, the aim of this chapter was to investigate the functional expression of $P2Y_{14}$ receptor-mediated contractile response of porcine isolated pancreatic artery preparations.

3.2. Materials and methods

3.2.1. Tissue preparation

Pancreata from pigs were obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield). A crude dissection was conducted to isolate the pancreatic arteries (dorsal pancreatic artery) (Figure 1.9, main), followed by a fine dissection to obtained rings of 0.5 cm in length, which were suspended between two wires in Krebs'-Henseleit buffer (gassed, 95% O_2, 5% CO_2), as described in (section 2.2.1).

Endothelium denudation (by gentle rubbing) was performed using the same protocol described in (section 2.2.1).

3.2.2. Responses in the porcine isolated pancreatic artery

Arterial rings were mounted onto wires in tissue baths containing warm (37°C) oxygenated Krebs'-Henseleit solution and were connected via isometric force transducers (ADInstruments, Sydney, Australia), to a PC running the computer program LabChart (ADInstruments, Sydney, Australia). Rings were put under tension (15 g) and allowed to equilibrate for 60 min before being treated as described in (section 2.2.2). U46619 (10-100 nM) was used to contract the tissues to 40-80% of the second KCl response. Once an appropriate level of U46619 response had been achieved, cumulative addition of UDP-glucose, UDP or MRS2690 was applied. Antagonists or inhibitors [suramin (100 μM), PPADS (10 μM), PPTN (1 μM), αβ-meATP (1 μM), ARL67156 (6-N,N-diethyl-D-βγ-dibromomethyleneATP) (10 μM), MRS2578 (1 μM) or (10 μM) and drug X

(1 μM)] were applied 10 min prior to the addition of U46619, allowing incubation with the tissues for a minimum of 30 min prior to the application of agonists. In some experiments with PPTN, the contraction to UDP-glucose was constructed at basal tone, in which UDP-glucose was added an hour following the second KCl addition in the presence or in the absence of PPTN, which was pre-incubated with the tissues for 30 min.

Desensitisation of the contraction to UDP-glucose in the presence of $P2Y_{14}$ receptor ligands was generated by exposing the arteries to UDP (100 μM) or UDP-glucose (100 μM) ($P2Y_{14}$ receptor ligands) 10 min prior to the addition of U46619, followed by cumulative addition of UDP-glucose at stabilised U46619 level. In experiments using DMSO as the solvent (see reagents and drugs section 3.2.4), DMSO 0.1 % (v/v) was added to the arteries as vehicle controls.

3.2.3. Immunohistochemical staining

Segments of porcine pancreatic arteries were collected and fixed in 4% (w/v) paraformaldehyde overnight at 4°C. Specimens were then washed in phosphate-buffered saline (PBS). Slices of vessels were created by freezing the tissues with optimal cutting temperature (OCT) mounting solution, then cutting to size (14 μm thick) using a microtome. The slices were then transferred onto slides and stored at -80°C.

Whole-mount segments of porcine pancreatic arteries were stained using the standard indirect immunofluorescence technique. The tissues were permeablised using PBS + 1% (w/v) bovine serum albumin (BSA) + 0.15% Triton X-100 at room temperature for 30 min. Non-specific binding was blocked with human serum 1:25 in PBS at room temperature for 30 min.

The slides were incubated overnight at 4ºC with antibody against G protein-coupled receptor GPR105/$P2Y_{14}$, diluted 1:100 in the blocking solution, while control slides were incubated overnight with the blocking solution. Subsequently, the samples were washed with PBS + 0.1% (w/v) bovine serum albumin (BSA), the secondary antibody (anti-rabbit IgG FITC) 1:100, diluted in PBS + 0.1% (w/v) BSA, was incubated with the samples at 37^{o}C for 30 min followed by further washes. The slides were covered using Vector shield mounting solution and glass cover slips. Samples were visualised using fluorescence microscopy using an objective magnification of x40.

3.2.4. Western blotting

Segments of porcine pancreatic arteries (PPA), rat heart (RH) and porcine heart (PH) were collected and stored in -80^{o}C freezer. They were then homogenised with a borosilicate glass homogeniser in lysis buffer (see reagents and drugs section 3.2.6), containing protease inhibitor cocktail tablets, EDTA-free. After removal of a sample for a protein assay (section 3.2.5), samples were diluted 1:6 into solubilisation buffer 6×SB: (see reagents and drugs section 3.2.6), and were heated at 95°C for 5 min. Subsequently, electrophoresis was carried out on 4-20% Tris-Glycine (PAGE) Gold Precast Gels (Bio-Rad, Hercules, CA, U.S.A.), 20 µg protein per lane was loaded for PPA, 10 µg protein per lane was loaded for RH and PH.

Samples were transferred to nitrocellulose membranes. Next, blots were incubated in blocking solution (5% (w/v) powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (Fisher Scientific UK Ltd., Loughborough, UK)) for 60 min, at room temperature. Blots were incubated overnight at 4°C with primary antibodies against $P2Y_{14}$ protein

(1:500) and against GAPDH (1:10000) diluted in the blocking solution. After washing in Tris-buffered saline containing 0.1% (v/v) Tween 20, the blots were incubated with an appropriate IRDye® secondary antibody (Li-Cor Biosciences, Biotechnology, Lincoln, NE, USA). Proteins were visualised using the Licor/Odyssey infrared imaging system (Biosciences, Biotechnology).

Antibody	**Cat No.**	**Host**	**MW (kDa)**	**Sequence**
GAPDH	G8795	mouse	37	
anti-$P2Y_{14}$ receptor (IHC)	LS-A1486	rabbit		synthetic 20 amino acid peptide from C-terminus of human $P2RY_{14}$
anti-$P2Y_{14}$ receptor (WB)	LS-C120603	rabbit	41	amino acids; 146-195

3.2.5. Determination of the protein level

The total level of the protein in porcine pancreatic samples was determined using a Lowry test, which is based on measuring the amount of proteins with Folin phenol reagent in the presence of alkaline copper conditions (Lowry *et al.*, 1951). A stock of bovine serum albumin (BSA) 1 mg/ml was made in distilled water, a serial dilution (from 0.05-0.45 mg/ml) was prepared from that stock to produce the standard curve.

Samples and standard were mixed with Lowry A and Lowry B (section 3.2.6), followed by 10 min incubation at room temperature. Subsequently, Folin phenol reagent, diluted 1:1 in distilled water, was added to the samples and standards followed by 45 min incubation in the room temperature. After mixing, samples and standards were transferred onto 96-well plates and the resultant absorbance was determined using spectra MAX 340pc plate reader at 750 nm.

3.2.6. Reagents and drugs

Lowry A: 0.4% (w/v) NaoH, 0.2% (w/v) sodium dodecyl sulphate, 2% (w/v) Na_2CO_3. Lowry B: 1% (w/v) $CuSO_4$, 2% (w/v) NaK Tartrate. Human serum, UDP and UDP-glucose was purchased from Sigma (Poole, Dorset, UK), while MRS2690 and ARL67156 were purchased from Tocris Biosciences Ltd. (Bristol, UK). PPTN, a selective high affinity antagonist of $P2Y_{14}$ receptor, was kindly gifted from Merck Frosst Centre for Therapeutic Research. Drug X was kindly gifted from Dr. Sue C. Fox, Thrombosis and Haemostasis Research Group, School of Medicine, University of Nottingham, company name withheld. Lysis buffer (20 mM Tris, 1 mM EGTA, 0.1% (v/v) Triton X100, 1 mM NaF, 10 mM beta glycerophosphate, pH 7.6). Solubilisation buffer, 6×SB: (24% (w/v) sodium dodecyl sulphate, 30% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5M Tris HCl, pH 6.8). The antiserum against $P2Y_{14}$ receptor protein, used for western blotting study, was purchased from Lifespan Biosciences, Inc (cat No. LS-C120603). The mouse monoclonal GAPDH antibody was purchased from Sigma (Poole, Dorset, UK) (cat No. G8795). The primary antibody (anti-$P2Y_{14}$ receptor) used for immunohistochemical staining study was purchased from Lifespan

Biosciences (cat No. LS-A1486). UDP, UDP-glucose, MRS2690, Drug X and ARL67156 were dissolved in water, while PPTN were dissolved in DMSO. For information about the sources and the solvents of other reagents and drugs, see section 2.2.3.

3.3. Statistical analysis

Data were expressed as log concentration-response plots. The contraction to all agonists was expressed in g, and measured from the stabilised U46619 response. Values for all figures refer to mean ± S.E.M with 95% confidence. Results were compared by two-way analysis of variance (Prism version 5, GraphPad, San Diego, CA, USA). Differences were considered to be significant when the P value was < 0.05. The "n" in the results expresses the number of animals.

3.4. Results

3.4.1. Effect of UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

To investigate the possible functional expression of $P2Y_{14}$ receptors and their role in porcine pancreatic arteries, agonists for these receptors were applied as cumulative concentrations. MRS2690, a selective $P2Y_{14}$ receptor agonist (10 nM to 30 μM), UDP-glucose (1 μM to 1 mM) and UDP (1 μM to 1 mM) were added after pre-constriction with U46619. All of the agonists induced a concentration-dependent contraction with a rank order of potency of MRS2690 > UDP-glucose = UDP. MRS2690 was significantly more potent, by approximately 10-fold, than UDP-glucose ($P < 0.01$, two-

way ANOVA, Figure 3.1), while UDP-glucose and UDP responses were equipotent (Figure 3.1). The contraction to 30 μM MRS2690 was 1.9 ± 0.4g, while it was 0.97 ± 0.2 and 1.0 ± 0.2 for 30 μM UDP and 30 μM UDP-glucose respectively ($P < 0.01$, n=9-12, Figure 3.1). There was no significant difference between the contractions to UDP and UDP-glucose.

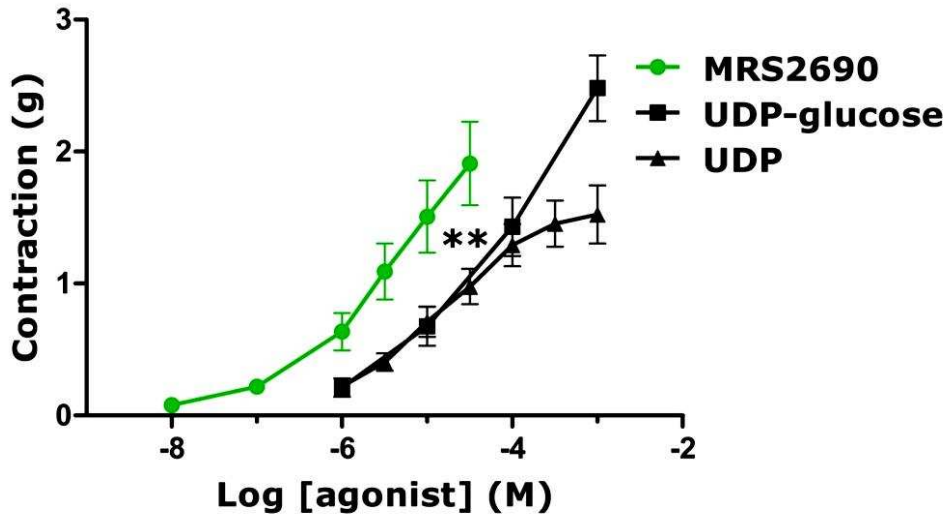


Figure 3.1. Concentration-dependent contractions evoked by MRS2690, UDP and UDP-glucose in U46619-preconstricted porcine pancreatic arteries (**P < 0.01, two-way ANOVA, MRS2690 response vs UDP-glucose and UDP responses, F=13.74, 16.03; n=9-12).

3.4.2. Effect of PPTN on responses to UDP-glucose and MRS2690 in porcine isolated pancreatic arteries

The responses to UDP-glucose and MRS2690 were examined in the presence of PPTN (1 μM), a selective high affinity antagonist of $P2Y_{14}$ receptors (Robichaud *et al.*, 2011; Barrett *et al.*, 2013). This compound significantly reduced the contractions evoked by UDP-glucose and MRS2690 in U46619-preconstricted pancreatic arteries (Figure 3.2A, B).

PPTN decreased the contractions to 100 μM UDP-glucose and to 10 μM MRS2690 by 0.5 ± 0.1g ($P < 0.05$, n=7) and by 0.7 ± 0.2g ($P < 0.01$, n=9) respectively (Figure 3.2A, B). To find out about the selectivity of PPTN at $P2Y_{14}$ receptors over $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors, the contraction to UTP, which is known to be a ligand at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Burnstock & Williams, 2000), was examined in the presence of PPTN (1 μM) (the same concentration that induced inhibition of UDP-glucose and MRS2690 contractions). As seen in Figure 3.2C, the contraction to UTP was not altered in the presence of PPTN, indicating some selectivity of PPTN at $P2Y_{14}$ receptor over some other P2Y receptors in porcine pancreatic arteries. Typical traces, showing the effect of MRS2690 in the absence and in the presence of PPTN, are shown in Figure 3.3. Similarly, at basal tone (section 3.3.2), the concentration-dependent vasoconstriction elicited by UDP-glucose was statistically decreased in the presence of PPTN (Figure 3.4).

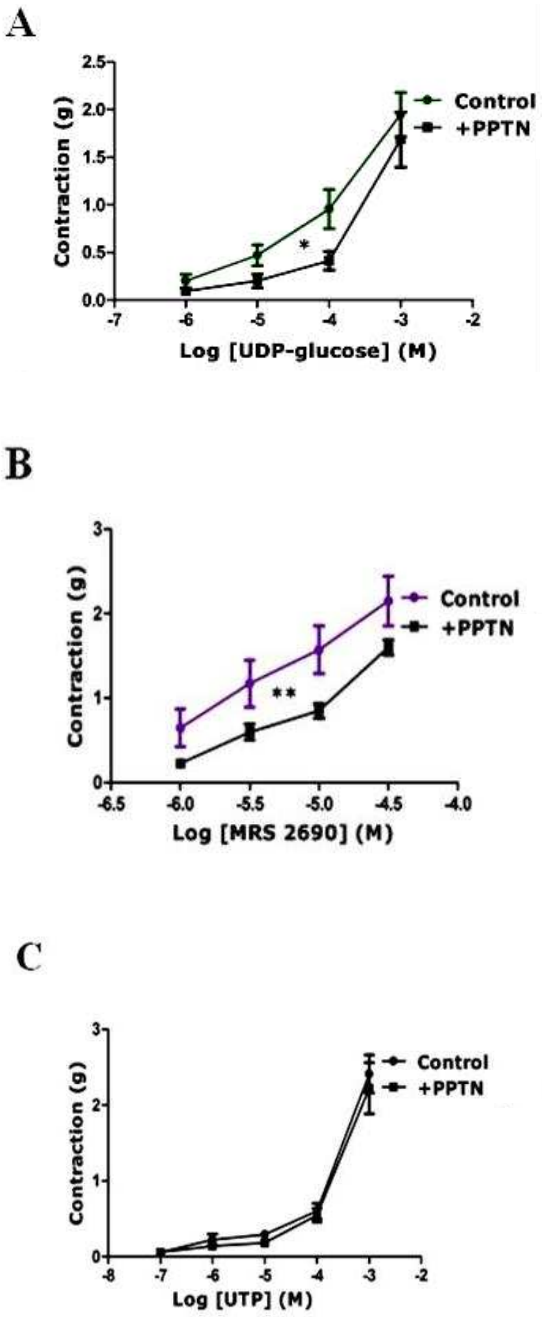


Figure 3.2. Effect of PPTN (1 μM), a $P2Y_{14}$ receptor selective antagonist, on responses to (A) UDP-glucose, (B) MRS2690, (C) UTP in U46619-preconstricted porcine pancreatic arteries. (A), (B) PPTN inhibited the effects of UDP-glucose and MRS2690 (*P < 0.05, **P < 0.01, two-way ANOVA, F=6.56, F=12.85 respectively; n=7-9). (C) PPTN had no effect on the response to UTP (n=8-10).

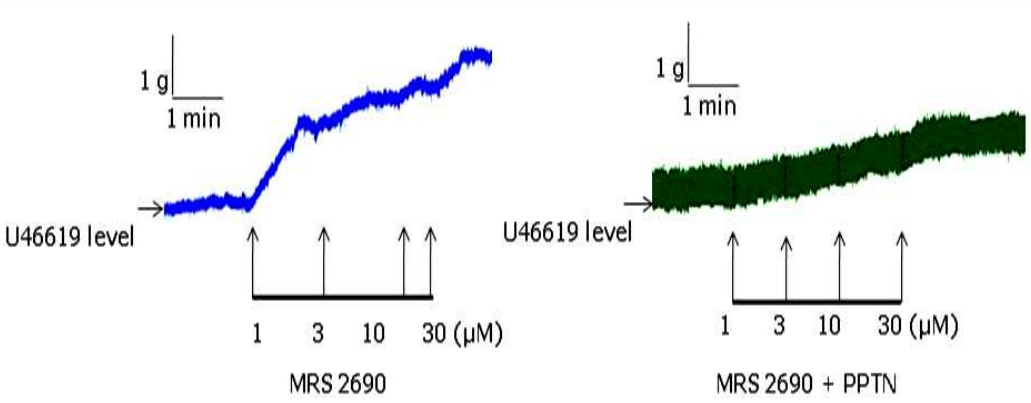


Figure 3.3. Typical traces showing the effect of MRS2690 in the absence and in the presence of PPTN (1 μM).

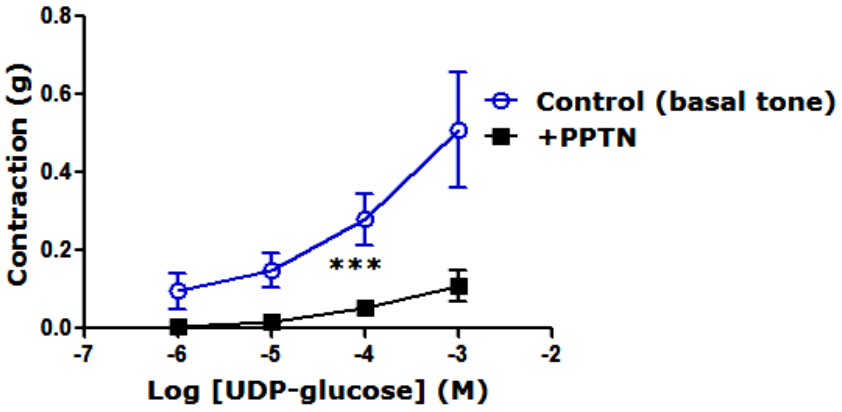


Figure 3.4. Effect of PPTN (1 μM), a $P2Y_{14}$ receptor selective antagonist, on contraction to UDP-glucose at basal tone in porcine pancreatic arteries. PPTN inhibited the contraction induced by UDP-glucose (***P < 0.001, two-way ANOVA, F=24.35; n=12).

3.4.3. Effect of PPADS and suramin on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Responses to UDP-glucose, UDP and MRS2690 were characterised using the non-selective P2 receptor antagonists PPADS (10 μM) and suramin (100 μM) (Rayment *et al.*, 2007a). Both PPADS and suramin significantly enhanced the contractions evoked by UDP-glucose and UDP (Figure 3.5A, B). The contraction to 100 μM UDP-glucose was enhanced by 1.9 ± 0.2g ($P < 0.001$, n=7) and by 1.7 ± 0.2g ($P < 0.001$, n=10) in the presence of PPADS and suramin respectively (Figure 3.5A). The contraction to 1 mM UDP was enhanced by 2 ± 0.3g ($P < 0.001$, n=8), and by 2.3 ± 0.3g ($P < 0.001$, n=10) in the presence of PPADS and suramin respectively (Figure 3.5B). Suramin and PPADS failed to alter the contraction to MRS2690 (Figure 3.5C).

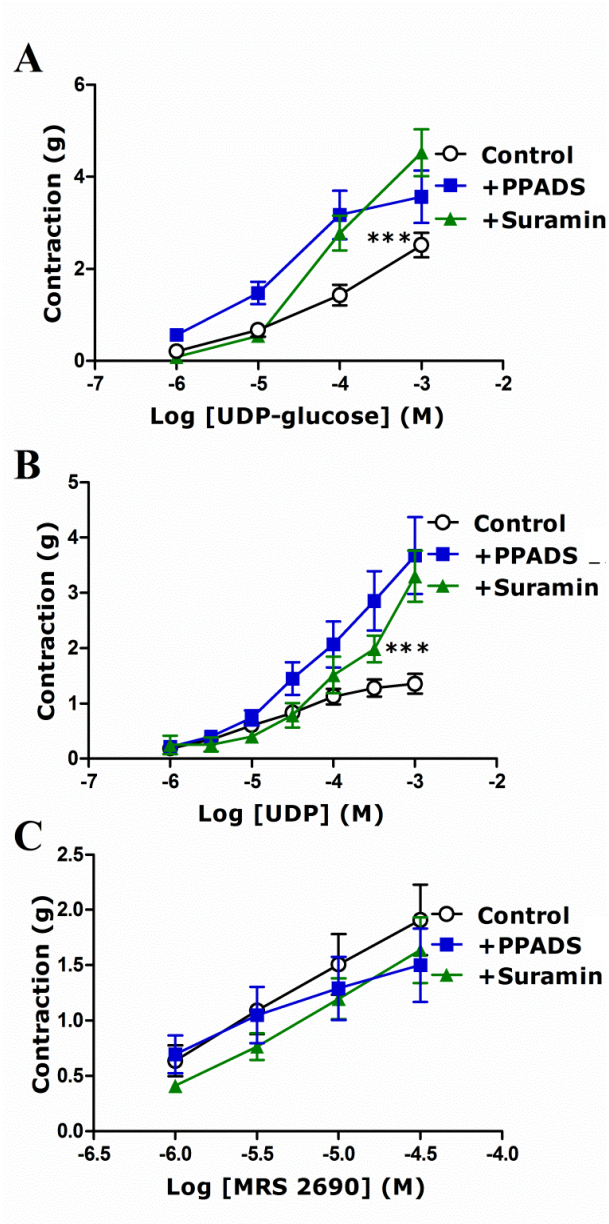


Figure 3.5. Effect of PPADS (10 μM) and suramin (100 μM) on responses to (A) UDP-glucose, (B) UDP, and (C) MRS2690 in U46619-preconstricted porcine pancreatic arteries. (A) Suramin, PPADS enhanced the effects of UDP-glucose (***P < 0.001, two-way ANOVA, UDP-glucose with suramin or PPADS vs UDP-glucose alone, F=19.85, 23.07; n=7-10). (B) Suramin and PPADS enhanced the effects of UDP (***P < 0.001, two-way ANOVA, UDP with suramin or PPADS vs UDP alone, F=16.83, 45.24; n=8-10). (C) Suramin and PPADS had no significant effect on the contraction to MRS2690 (n=5-9).

3.4.4. Effect of MRS2578 on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

UDP is a ligand at $P2Y_6$ receptors (Mamedova *et al.*, 2004) as well as being a ligand at $P2Y_{14}$ receptors (Carter *et al.*, 2009). Therefore, the effects of UDP, UDP-glucose and MRS2690 were examined in the presence of MRS2578 (1 μM) and (10 μM), a $P2Y_6$ receptor selective antagonist (Mamedova *et al.*, 2004). The contraction evoked by UDP was unaffected at lower concentrations but was augmented at higher concentrations of UDP (Figure 3.6A). The contraction to 1 mM UDP was enhanced by 0.7 ± 0.3g ($P < 0.001$, n=8-16) in the presence of MRS2578 (Figure 3.6A), while MRS2578 did not alter the responses to UDP-glucose or MRS2690 (Figure 3.6B, C).

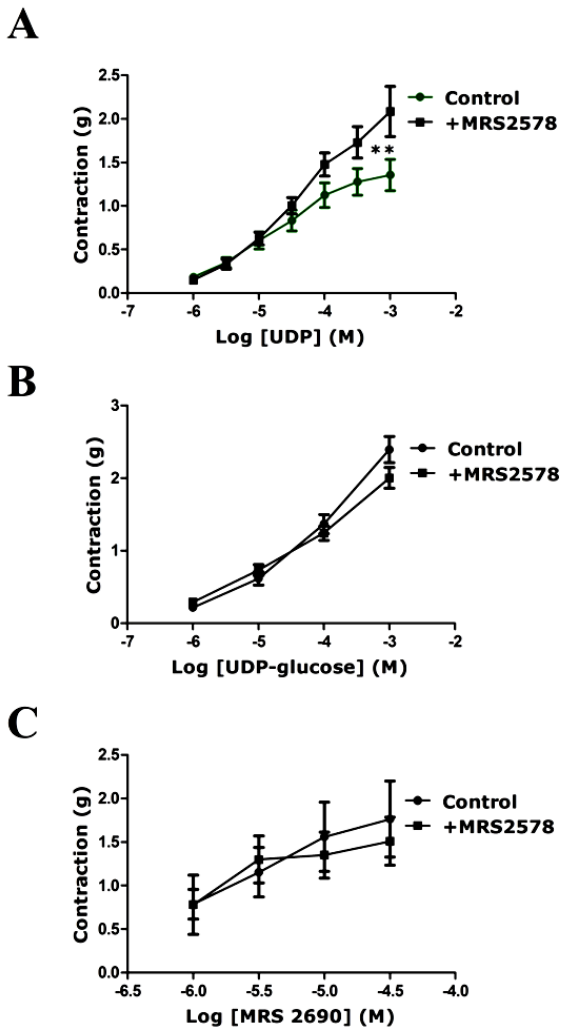


Figure 3.6. Effect of MRS2578 (10 μM) on responses to UDP, UDP-glucose and MRS2690 in U46619-preconstricted porcine pancreatic arteries. (A) MRS2578 enhanced significantly the contraction evoked by UDP (**P < 0.01, F= 9.953; n=8-16). (B), (C) MRS2578 failed to alter the contractions to UDP-glucose and MRS2690 (n=6-13).

3.4.5. Effect of ARL67156 on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Suramin and PPADS can act as ecto-nucleotide pyrophosphatases (eNPPs) inhibitors (Grobben *et al.*, 1999; Vollmayer *et al.*, 2003). In addition, they act as inhibitors for the ecto-nucleoside-5′-triphosphate diphosphohydrolases (eNTPDases) (Chen *et al.*, 1996; Munkonda *et al.*, 2007). It has been shown that nucleotide sugars are resistant to hydrolysis by the nucleotide-hydrolyzing eNTPDases (Zimmermann, 2000), but they could be broken down by eNPPs, as was shown for UDP-glucose (Lazarowski *et al.*, 2003b). While, UDP is broken down by eNTPDases (section 1.7) (Murphy-Piedmonte *et al.*, 2005; Levesque *et al.*, 2007). Since suramin and PPADS were able to potentiate the contractions to UDP-glucose and UDP, that might raise the possibility that they may act as ecto-nucleotidase inhibitors (Chen *et al.*, 1996). Therefore, the responses to UDP-glucose, UDP and MRS2690 were studied following the exposure to ARL67156 (10 μM), which is an ecto-nucleotidase inhibitor with pIC_{50} of 4.62 in human blood (Crack *et al.*, 1995; Liu *et al.*, 2004; Levesque *et al.*, 2007). As shown in Figure 3.8, ARL67156 failed to significantly alter the responses to UDP-glucose, UDP and MRS2690.

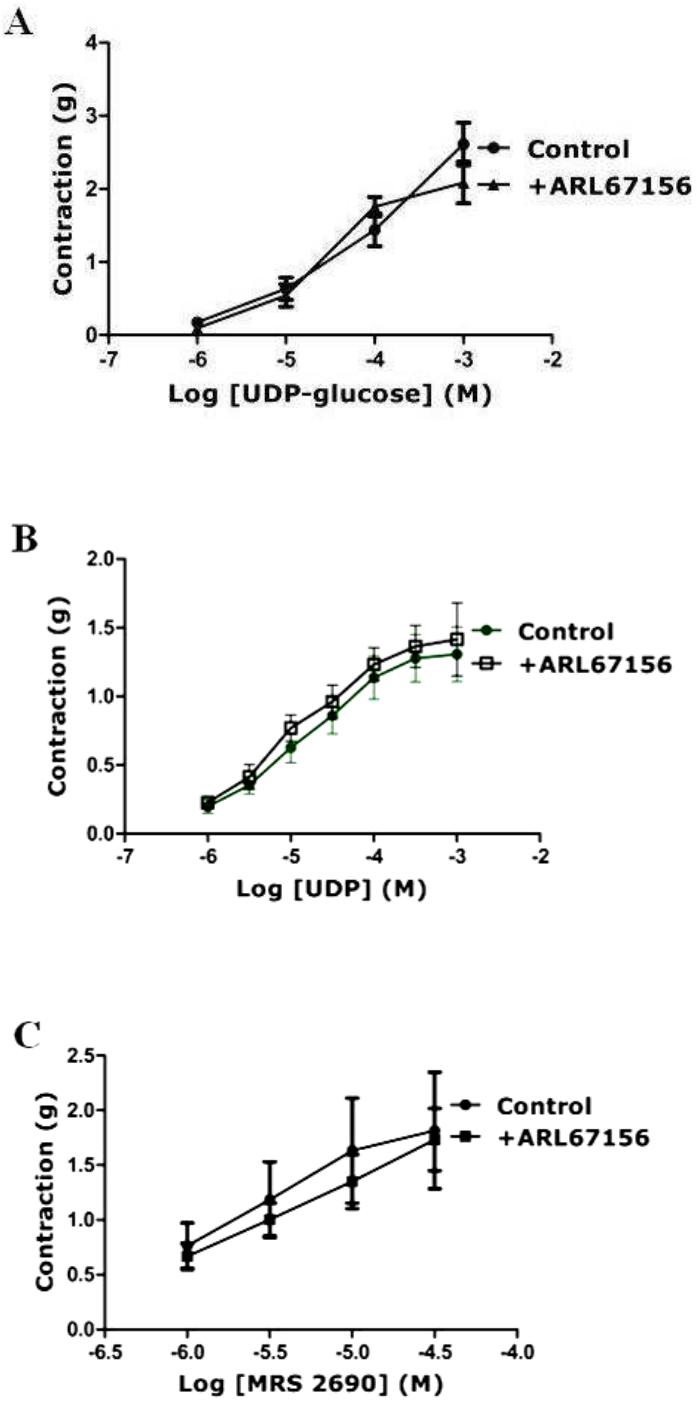


Figure 3.7. Effect of ARL67156 (10 μM), an ecto-nucleotidase inhibitor, on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690 in U46619-preconstricted porcine pancreatic arteries. ARL67156 had no significant effect on responses to (A) UDP-glucose (n=5-8), (B) UDP (n=6-14) and (C) MRS2690 (n=4-5).

3.4.6. Effect of endothelium removal on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

The responses of UDP-glucose, UDP and MRS2690 were studied after the endothelium had been removed (section 2.2.1). The contractions induced by UDP-glucose, UDP and MRS2690 were significantly attenuated in the endothelium-denuded arteries (Figure 3.8). Removal of endothelium reduced the contractions to 1 mM UDP-glucose by 1.3 ± 0.2g ($P < 0.001$, n=12, Figure 3.8A), that to 1 mM UDP by 0.5 ± 0.2g ($P < 0.001$, n=15, Figure 3.8B) and that to 30 μM MRS2690 by 0.5 ± 0.2g ($P < 0.01$, n=5, Figure 3.8C).

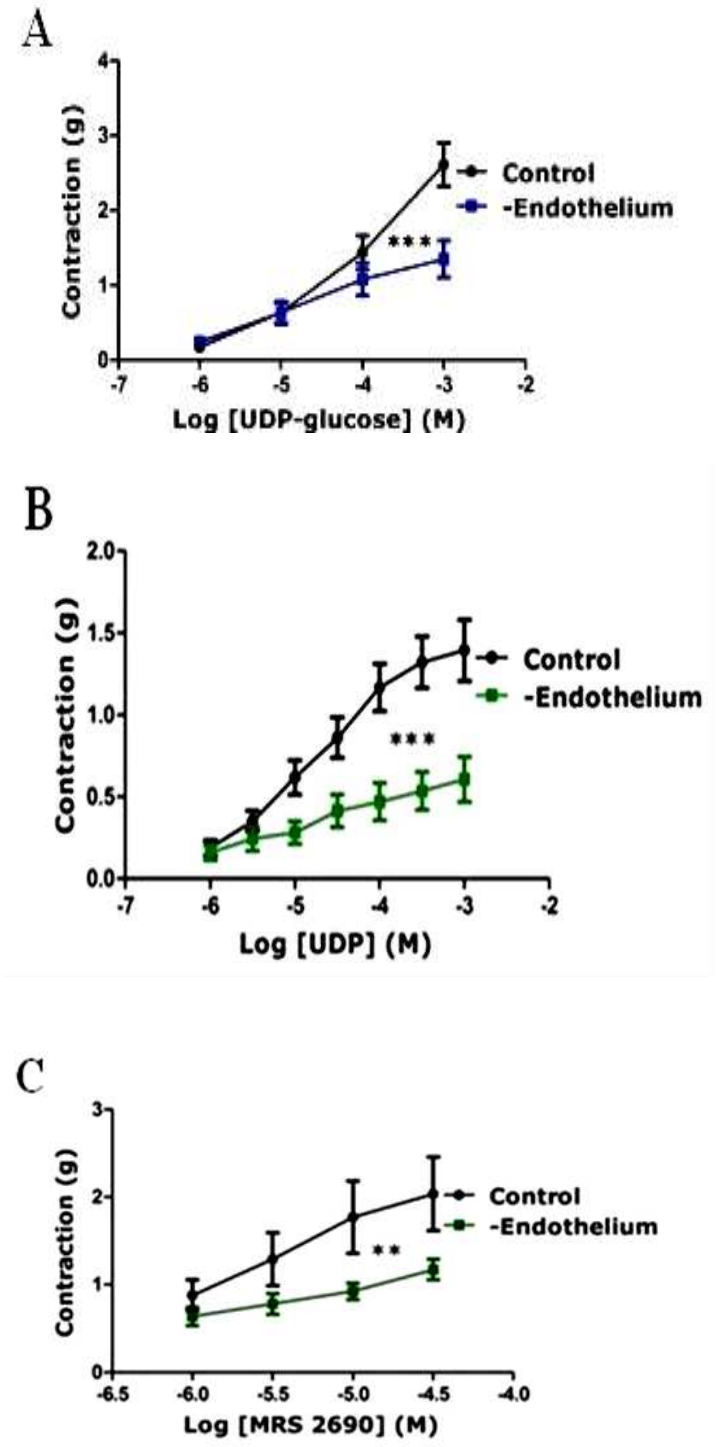


Figure 3.8. Effect of removal of the endothelium on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690, in U46619-preconstricted porcine pancreatic arteries. The removal of endothelium significantly reduced the contraction evoked by (A) UDP-glucose, (B) UDP, (C) MRS2690 (**P < 0.01, ***P < 0.001, two-way ANOVA, F=8.15, 51.24, 9.48; n=5-15).

3.4.7. Desensitisation of the contraction to UDP-glucose induced by UDP-glucose or UDP

Both UDP-glucose and UDP ($P2Y_{14}$ receptor ligands), added 10 min prior to U46619 addition, separately induced significant attenuation of the response to subsequent administration of UDP-glucose. When the pancreatic arteries were exposed to these ligands half an hour prior to the addition of UDP-glucose. The contraction to UDP-glucose was significantly attenuated in the presence of prior exposure to UDP-glucose (100 μM) and (1 mM), UDP (100 μM) and (1 mM) (Figure 3.9), while the lower concentrations of UDP (10 μM) or (1 μM) had no significant effect on the subsequent contraction to UDP-glucose (data not shown). The response to 100 μM UDP-glucose was decreased by 2.5 ± 0.1g in the presence of 100 μM UDP-glucose, and by 2.4 ± 0.1g in the presence of 100 μM UDP ($P < 0.001$, n=10-13, Figure 3.9).

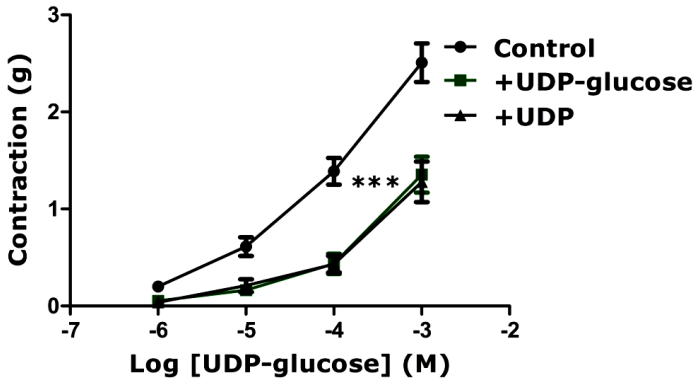


Figure 3.9. Effect of UDP-glucose (100 μM) and UDP (100 μM) on the contraction to UDP-glucose (the control) in U46619-preconstricted porcine pancreatic arteries. Both UDP-glucose and UDP significantly attenuated the contraction evoked by UDP-glucose (***$P < 0.001$, two-way ANOVA, UDP-glucose contraction in the absence or presence of UDP-glucose or UDP, F=63.11, 56.48; n=10-13).

3.4.8. Effect of αβ-meATP on the contractions to UDP-glucose and UDP in porcine isolated pancreatic arteries

To find out whether UDP-glucose or UDP act at P2X receptors, the contractions to UDP-glucose and UDP were investigated in the presence of αβ-meATP (1 μM). As seen in Figure 3.10, the contractions to UDP-glucose or UDP were not altered in the presence of the desensitising agent, which rule out the involvement of P2X receptors in the contraction to $P2Y_{14}$ ligands.

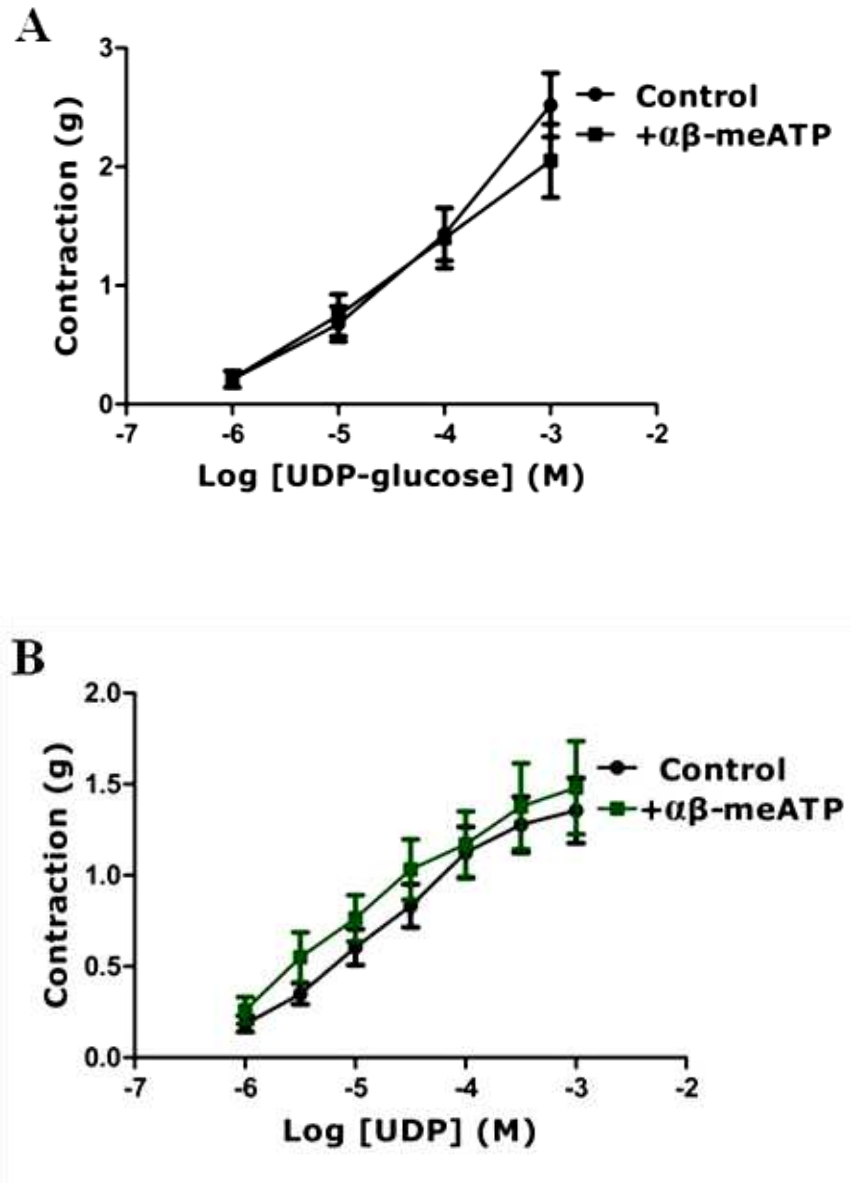


Figure 3.10. Effect of αβ-meATP (1 μM), a P2X receptors desensitising agent, on contraction to (A) UDP-glucose and (B) UDP in U46619-preconstricted porcine pancreatic arteries. αβ-meATP had no significant effect on the responses to (A) UDP-glucose (n=8-10) or (B) UDP (n=10-16).

3.4.9. Investigation of the expression of $P2Y_{14}$ receptors in porcine pancreatic arteries

The expression of $P2Y_{14}$ receptor was investigated in porcine pancreatic arteries using western blotting in the presence of a rabbit polyclonal antiserum against C-terminal tail of the $P2Y_{14}$ receptor (green bars). This was performed in the presence of mouse GAPDH monoclonal antibody (red bars), which determines the total amount of the protein in the sample, and showed an immunoreactive band at around 37 kDa. The $P2Y_{14}$ receptor mRNA has been identified previously in the rat heart (Musa *et al.*, 2009), and thus it has been used as a positive control in the current study.

In the absence of the $P2Y_{14}$ primary antibody, no bands were evident (Figure 3.11A), similarly, no bands were evident following pre-incubation with the neutralizing antigen (Lifespan Biosciences, Inc, cat No. LS-C120603) (data not shown). While two immunoreactive bands were obtained in the presence of the $P2Y_{14}$ antibody, one around 59 kDa with second band at around 41 kDa in rat heart (RH) and porcine heart (PH), which are used as positive controls in the current study (Figure 3.11B). These findings were consistent with other reports which showed the presence of $P2Y_{14}$ receptors in HEK-293 cells and liver hepatocellular cells (HepG2) with an immunoreactive band of approximately 41 kDa (Lifespan Biosciences, cat No. LS-C120603), and in human brain membranes and human $P2Y_{14}$ receptor-transfected HEK-293 cells with multiple immunoreactive bands of around 40-65 kDa (Moore *et al.*, 2003; Krzemiński *et al.,* 2008). In contrast, the previous immunoreactive bands were also apparent in undifferentiated HL-60, in differentiated SH-SY-5Y (neuronal cells) and in mouse thoracic aorta (Moore *et al.*, 2003; Fricks *et al.*, 2009; Kauffenstein *et al.*, 2010) (Figure 3.12), which were reported not to express $P2Y_{14}$ mRNA, and used as negative controls in my study. The

previous observations suggested some lack of specificity of the $P2Y_{14}$ receptor antibody, used in the current study. In porcine pancreatic arteries (PPA), two immunoreactive bands were also apparent at 59 kDa and 41 kDa, indicating the expression of $P2Y_{14}$ receptor in my arteries.

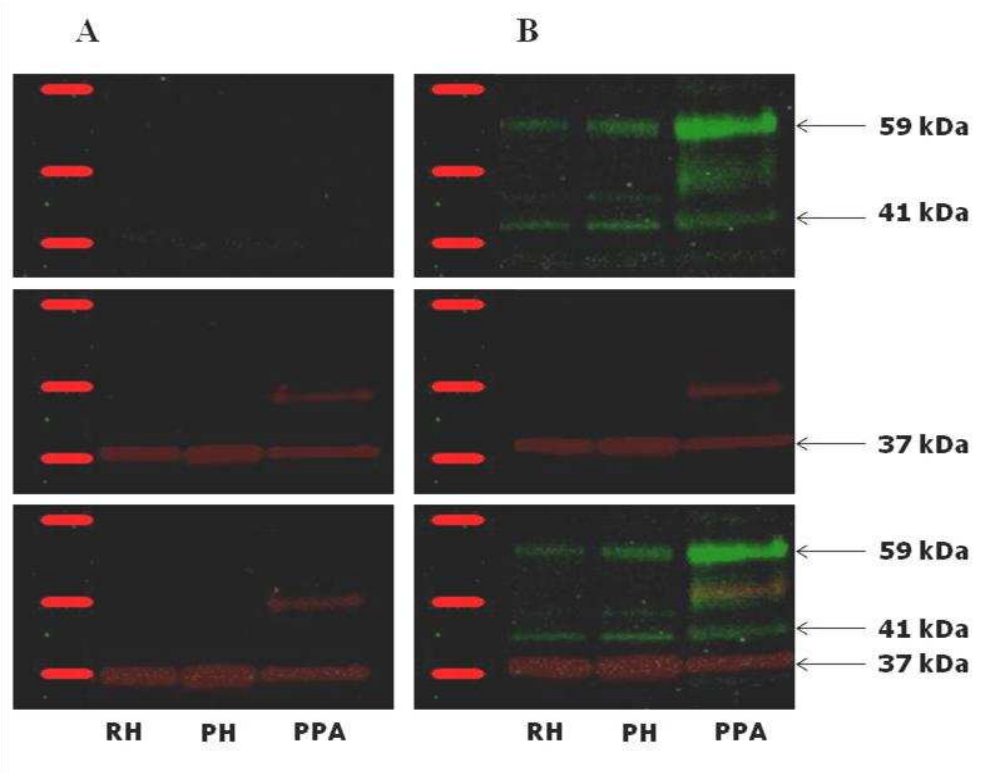


Figure 3.11. The $P2Y_{14}$ protein expression detected using western blotting, in the absence (A) or in the presence (B) of $P2Y_{14}$ antiserum (green bars). (A), (B) The total protein was determined using GAPDH antibody (red bars). Two immunoreactive bands were evident in rat heart (RH) (10 µg/lane), in porcine heart (PH) (10 µg/lane) and in porcine pancreatic arteries (PPA) (20 µg/lane) at around 59 and 41 kDa. The band sizes of the molecular weight markers from top to bottom are 75, 50, 36 kDa respectively. Blots are representative of staining from six separate experiments.

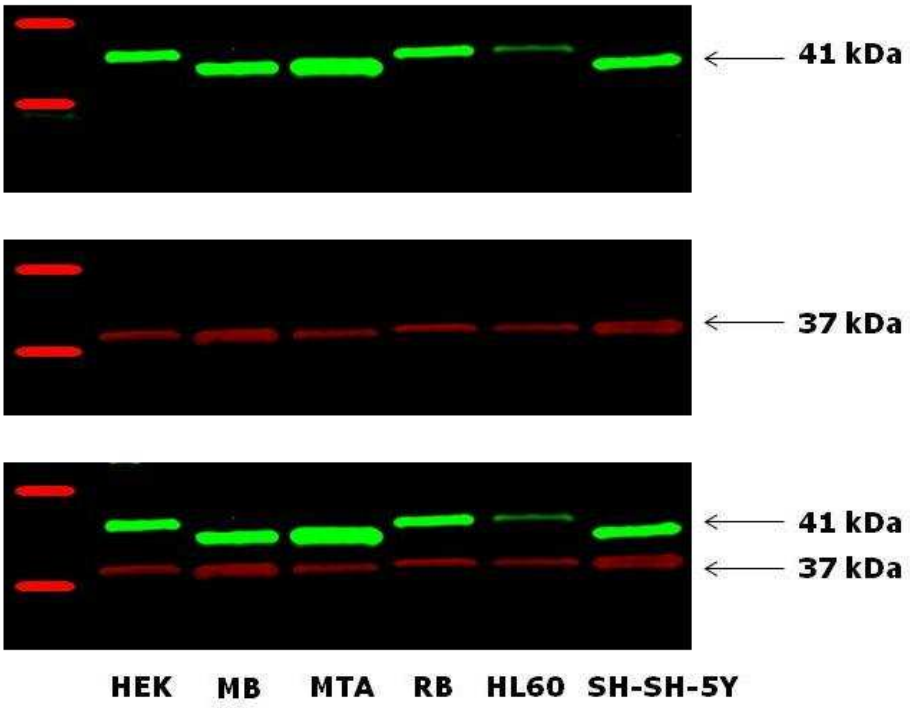


Figure 3.12. The $P2Y_{14}$ protein expression detected using western blotting, in presence of $P2Y_{14}$ antiserum (green bars). The total protein was determined using GAPDH antibody (red bars). Samples were loaded at 10 µg/lane. An immunoreactive band was evident in HEK-293 cells (HEK), mouse brain (MB), mouse thoracic aorta (MTA), rat brain (RB), undifferentiated HL-60 cells and differentiated SH-SY-5Y at 41 kDa. The band sizes of the molecular weight markers from top to bottom are 50, 36 kDa respectively. Blots are representative of staining from three separate experiments.

3.4.10. $P2Y_{14}$-like receptor immunostaining in porcine isolated pancreatic arteries

Since removal of the endothelium significantly attenuated the contraction evoked by $P2Y_{14}$ receptor agonists (by almost half), the expression of $P2Y_{14}$ receptors in porcine pancreatic arteries was investigated using immunohistochemistry. An intense $P2Y_{14}$-like receptor immunoreactivity was observed in the endothelial cells of the pancreatic arteries (Figure 3.13A, C). Staining was evident, but was less intense, in the smooth muscle of the pancreatic arteries (Figure 3.13A, C). In the absence of anti GPR105/$P2Y_{14}$ receptors, no staining was obtained (Figure 3.13D).

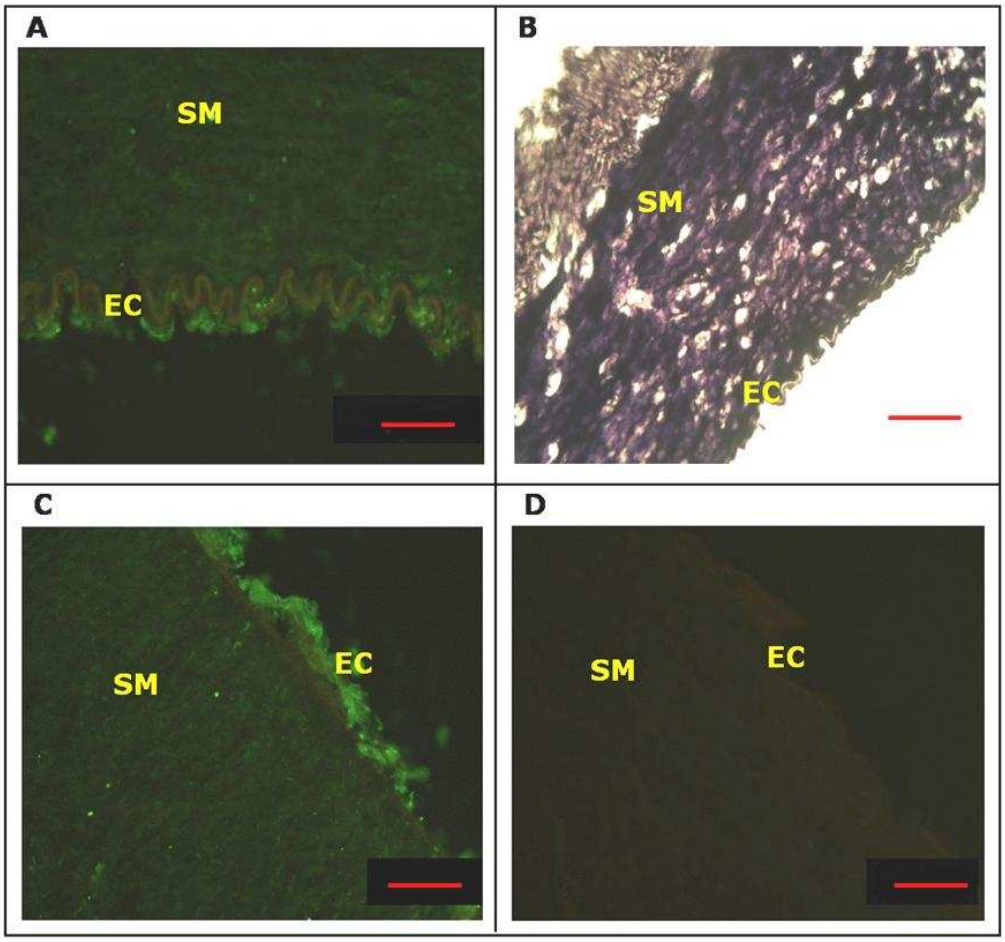


Figure 3.13. $P2Y_{14}$–like receptor immunoreactivity in porcine pancreatic arteries. (A), (B) Cross-sections of the arteries showing staining in the presence of (A) $P2Y_{14}$ receptor antiserum, (B) 1% (w/v) toluidine blue, in smooth muscle (SM) and endothelial cells (EC). (C), (D) Longitudinal sections of the arteries showing immunostaining in the presence (C) and absence (D) of $P2Y_{14}$ receptor antiserum in smooth muscle (SM) and endothelial cells (EC). Scale bar = 2 μm. Images are representative of staining from three separate experiments.

3.4.11. Effect of GPR17 receptor antagonist on the contraction to UDP-glucose in porcine isolated pancreatic arteries

Recently, a new G protein-coupled receptor, GPR17 was identified as a P2Y-like receptor, which is activated by UDP-glucose, UDP and cysteinyl leukotriene, and is primarily coupled to G_i protein leading to an inhibition of cAMP (Fumagalli *et al.*, 2011). This receptor was mainly found in neurons and it plays a significant role in some physiological and pathological processes, including brain injury, spinal cord injury (Zhang *et al.*, 2013), but little is known about its influence in the cardiovascular system. To investigate the possible functional expression of GPR17 receptor in pancreatic arteries (Fumagalli *et al.,* 2011), the contraction to UDP-glucose was studied in the presence of Drug X (1 μM), an antagonist of the purine binding site on GPR17 receptor. Drug X failed to inhibit the contraction to UDP-glucose (Figure 3.14), indicating that GPR17 receptor is not involved in the contraction to UDP-glucose.

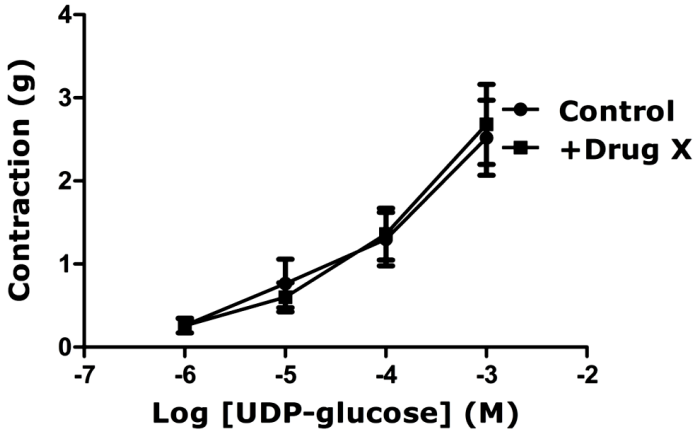


Figure 3.14. Effect of Drug X (1 μM), an antagonist of the purine binding site on GPR17 receptor, on contraction to UDP-glucose in U46619-preconstricted porcine pancreatic arteries. Drug X had no significant effect on the response to UDP-glucose (n=5).

3.5. Discussion

The current study presents evidence for the functional expression of contractile $P2Y_{14}$ receptors, sensitive to the endogenous nucleotides, UDP-glucose and UDP, in arteries of pig pancreas. Evidence from the immunostaining along with the results obtained in endothelium-denuded arteries strongly suggested that the expression of $P2Y_{14}$ receptors is mainly on the endothelium of the porcine pancreatic arteries.

3.5.1. Functional expression of $P2Y_{14}$ receptor in porcine pancreatic arteries

The contractile studies (Figure 3.1) together with the immunoblotting studies (Figure 3.11) suggested the expression of the $P2Y_{14}$ receptor in porcine pancreatic arteries. The contractions to UDP-glucose and UDP were almost equipotent, whereas the contraction to MRS2690, a selective $P2Y_{14}$ receptor agonist, was approximately 10-fold more potent than those of UDP-glucose and UDP at the $P2Y_{14}$ receptor (Figure 3.1). This is consistent with previous reports which suggested a 7-10 fold greater potency of MRS2690 over UDP-glucose (Jacobson *et al.*, 2009; Gao *et al.*, 2010). In the current study, MRS2690 activity was observed at ≤ 10 μM; at 10 μM, MRS2690 has been reported to be inactive at $P2Y_2$ receptors, indicating that the contraction observed in the presence of MRS2690 is mediated by acting at $P2Y_{14}$ receptor in pancreatic arteries (Ko *et al.*, 2009).

PPTN is a non-nucleotide, high affinity competitive antagonist at $P2Y_{14}$ receptors. When it was assessed in HEK-293 cells using a calcium mobilisation assay, PPTN inhibited UDP-glucose mediated signalling (Robichaud *et al.*, 2011). In addition, PPTN showed no effect on other P2Y

receptors at concentrations up to 10 μM (Barrett *et al.*, 2013). In the current study, when the responses to $P2Y_{14}$ receptor agonists were examined in the presence of this antagonist, PPTN blocked the contractions induced by UDP-glucose and MRS2690 which was consistent with the involvement of $P2Y_{14}$ receptors in pancreatic arteries (Figure 3.2A, B). The selectivity of PPTN for $P2Y_{14}$ receptor over $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors has been assessed in porcine pancreatic arteries, by examining the contraction induced by UTP (an agonist for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, Burnstock & Williams, 2000) in the presence of PPTN. The data suggested some selectivity of PPTN at $P2Y_{14}$ receptor because the response to UTP in the presence of PPTN was different from that of UDP-glucose (Figure 3.2C),. UDP showed a high selectivity at $P2Y_{14}$ receptors in the previous reports (Carter *et al.*, 2009), besides, in the current study UDP induced a contraction of porcine pancreatic arteries (Figure 3.1), which can be mediated by $P2Y_{14}$ receptor.. Therefore, further studies are still required to investigate the effect of PPTN on the contraction induced by UDP in pancreatic arteries to confirm whether it is mediated by action at $P2Y_{14}$ receptors. Unfortunately, due to the limited availability of PPTN, these experiments were not performed.

3.5.2. Investigation of the effect of non-selective P2 receptor antagonists on the contraction to $P2Y_{14}$ receptor agonists

The responses to $P2Y_{14}$ receptor agonists were examined in the presence of the non-selective P2 receptor antagonists, suramin and PPADS, as there is no information available on the sensitivity of $P2Y_{14}$ receptor to suramin and PPADS. The non-selective antagonists induced an increase in the contractions to UDP and UDP-glucose (Figure 3.5A, B). However, they

failed to alter the response to MRS2690 (Figure 3.5C). The lack of effect of suramin and PPADS, as inhibitors of the contractions-evoked by UDP-glucose and UDP, appears to rule out an involvement of $P2Y_2$ and/or $P2Y_4$ receptors. Since it has been shown, in the current study, that they blocked the responses to UTP in porcine pancreatic arteries (section 2.4.3.1) and other tissues, including porcine coronary arteries and porcine ear arteries (Rayment *et al.*, 2007a). It has been reported that UDP-glucose and UDP can be broken down by eNPPs and eNTPDases respectively, resulting in decreased nucleotide activities (Murphy-Piedmonte *et al.*, 2005; Kauffenstein *et al.*, 2010; O'Keeffe *et al.*, 2010). Some reports indicate that both suramin and PPADS act as inhibitors for eNPPs as well as for eNTPDases (Chen *et al.*, 1996; Grobben *et al.*, 1999; Vollmayer *et al.*, 2003; Munkonda *et al.*, 2007). Therefore, one explanation of the enhancement to the contractions to UDP-glucose and UDP, occurred in the presence of suramin and PPADS is that when the responses to UDP and UDP-glucose were tested in the presence of suramin and PPADS, the vasoconstrictions induced by these agonists were enhanced due to the enhancement with their availability (Figure 3.5A, B). Whereas, there was no change to the response to MRS2690 in the presence of suramin and PPADS (Figure 3.5C), since the MRS2690 could be more stable and it is not affected by ecto-nucleotidase enzymes.

To test this hypothesis, the responses to UDP-glucose, UDP and MRS2690, were examined in the presence of ARL67156. In contrast, ARL67156 failed to alter the responses to all of these agonists (Figure 3.7). However, ARL67156 at concentrations used in the literature 50-100 μM has been shown to be a weak inhibitor of some human and mouse ecto-nucleotidase isoforms (NTPDase1, NTPDase3 and NPP1) (Levesque *et al.,* 2007). In the current study, the concentration used for ARL67156 was 10 μM. Therefore,

insufficient blockade of ecto-nucleotidases by 10 μM may explain the lack of effect of ARL67156 on the contractions to $P2Y_{14}$ receptor agonists. We could not investigate the effects of higher concentrations due to cost considerations. Therefore, investigating the contractions to $P2Y_{14}$ receptor agonists in the presence of higher concentrations of ARL67156 is required. It would be also useful to use βγ-meATP as an ecto-nucleotidases inhibitor, since it was shown in the report of Joseph *et al.* (2004) that it acts as eNPPs inhibitor as well as eNTPDases inhibitor, which was also found to significantly inhibit the hydrolysis of UDP-glucose in 1321N1 cell line (Kreda *et al.*, 2008).

Alternatively, the enhancement of the responses to UDP and UDP-glucose may involve other P2Y receptor subtypes. UDP and UDP-glucose are not selective agonists at $P2Y_{14}$ receptors. UDP-glucose was found to be a weak full agonist, with an EC_{50} of 10 μM, at $P2Y_2$ receptors and its effect could be antagonised by suramin and PPADS (Shen *et al.*, 2004; Ko *et al.*, 2009). Additionally, UDP was reported to be a ligand at $P2Y_6$ receptors, which may be present on the endothelial cells and account for relaxation to UDP observed in some arteries, such as mouse thoracic aorta (Guns *et al.*, 2005; Bar *et al.*, 2008). Similarly, endothelial $P2Y_2$ receptor was reported to induce a vasorelaxation in human vascular endothelial cells and in mouse thoracic aorta mediated by ATP and UTP (Guns *et al.*, 2006; Raqeeb *et al.*, 2011). In my preparations, UDP-glucose and UDP induced contractions which were significantly increased in the presence of suramin, PPADS and MRS2578 (in the case of UDP) (Figure 3.5A, 3.5B, 3.6A). It is possible that UDP-glucose acted at $P2Y_{14}$ receptors to induce a contraction, as well as acting partly at $P2Y_2$ receptors on endothelial cells to induce a relaxation. Meanwhile, UDP acted at $P2Y_{14}$ receptors to induce a contraction, as well as acting partly at $P2Y_6$ receptors on endothelial cells to

induce a relaxation. The relaxation induced by UDP and UDP-glucose was blocked by suramin, PPADS and MRS2578 (in the case of UDP), and accordingly that would increase the contractile response to UDP and UDP-glucose. MRS2690 is a selective agonist at $P2Y_{14}$ receptors (Jacobson *et al.,* 2009), and consequently its response was not altered in the presence of these antagonists. Indeed, it is not fully clear why these antagonists enhanced the effects of $P2Y_{14}$ receptor agonists. However, it is apparent that suramin and PPADS had different effects on UTP responses (section 2.4.3.1) versus responses of MRS2690, UDP and UDP-glucose indicating actions at distinct receptors.

3.5.3. Effect of the removal of the endothelium, and the involvement of P2X or GPR17 receptors

Contractile responses to UDP-glucose, UDP and MRS2690 were significantly inhibited after the endothelium was removed (Figure 3.8). In addition, immunostaining showed that the expression of $P2Y_{14}$-like receptors is mainly on the endothelium of the pancreatic arteries, with slight expression on the smooth muscle (Figure 3.13). This indicated an involvement of endothelial $P2Y_{14}$ receptor-mediated contraction in pancreatic arteries. Similarly, the protein for $P2Y_{14}$ receptor has been reported to be expressed in the endothelial cells of the porcine coronary artery, the portion and mRNA were also evidenced in human lung microvascular endothelial cells, and mRNA for $P2Y_{14}$ receptor was shown in pulmonary artery vasa vasorum endothelial cells (Umapathy *et al.*, 2010; Abbas *et al.*, 2011; Lyubchenko *et al.*, 2011). While mRNA expression for $P2Y_{14}$ receptor was barely detectable in mouse thoracic aorta and in human coronary artery endothelial cells (Kauffenstein *et al.*, 2010; Ding *et al.*, 2011). In contrast, rat aortic smooth muscle cells showed robust expression of mRNA for the

receptor, as observed in freshly isolated and cultured cells (Govindan *et al.*, 2010).

It has been reported that pyrimidine nucleotides might interact with P2X receptors, which would influence their responses (Froldi *et al.*, 1997; Froldi *et al.*, 2001; Mo *et al.*, 2013). To test whether UDP-glucose or UDP bind to P2X receptors expressed in porcine pancreatic arteries (section 2.4.2.2), αβ-meATP, which desensitises P2X1 and P2X3 receptors (Kasakov & Burnstock, 1982), was employed. However, as seen in Figure 3.10, αβ-meATP had no effect on the responses to UDP-glucose or UDP, which ruled out the involvement of P2X receptors in the responses to UDP-glucose and UDP. P2Y-like GPR17 receptor is reported to play a significant role in the central nervous system (Fumagalli *et al.*, 2011). A recent study showed that inhibition of the activity of GPR17 receptor is effective for the modulation of bronchoconstriction in humans with bronchial asthma (Maekawa *et al.*, 2010). In addition, this receptor can be activated by UDP-glucose and UDP (Fumagalli *et al.*, 2011). Therefore, a study was conducted to identify whether GPR17 receptor plays a role in the contraction to UDP-glucose in porcine pancreatic arteries. The data in Figure 3.14 indicated that GPR17 receptor is not involved in the contraction-mediated by UDP-glucose, which indicated that the contraction to UDP and UDP-glucose were solely mediated by acting at $P2Y_{14}$ receptor.

3.6. Conclusion

The data presented in this chapter described novel vasocontractile actions of UDP-glucose, UDP and MRS2690 which are mediated by the $P2Y_{14}$ receptor in porcine isolated pancreatic arteries. The functional expression of $P2Y_{14}$ receptor was shown, by the contractile studies and by immunostaining for $P2Y_{14}$ receptors, to be mainly on the endothelium, with slight expression on the smooth muscle of the pancreatic arteries. The data rule out the involvement of P2X receptors or P2Y-like GPR17 receptors in the responses to UDP or UDP-glucose, which indicate that $P2Y_{14}$ receptor is the most influential receptor regarding the contraction obtained in responses to UDP, UDP-glucose or MRS2690. Since alterations in blood flow can influence physiological and pathological functions of the pancreas, a drug which decreases the activity of $P2Y_{14}$ receptor as a vasoconstrictor of porcine pancreatic arteries, such as PPTN, may serve as a potential therapeutic approach for the treatment of some pancreatic diseases.

Chapter Four

Investigation of the signalling pathways underlying the responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

4.1. Introduction

$P2Y_{14}$ receptor activation involves G_i protein-mediated signalling, leading to an inhibition of adenylyl cyclase activity (hence decreases in cAMP level) and, accordingly, it is pertussis toxin-sensitive (Jacobson *et al.*, 2009). G_i protein-derived $G_{\beta\gamma}$-dimers can initiate phospholipase Cβ signalling pathways, which lead to the stimulation of diacylglycerol and inositol 1,4,5-trisphosphate and subsequent activation of RhoA/ROCK signalling, protein kinase C (PKC) and myosin light chain kinase (MLCK) (Amano *et al.*, 1996; Hartshorne & Gorecka, 2011; Sesma *et al.*, 2012).

It has been indicated in previous reports that $P2Y_{14}$ receptors were involved in the regulation of intracellular Ca^{2+} (Skelton *et al.*, 2003). This effect was also shown by further studies which demonstrated the ability of UDP-glucose and UDP to increase the intracellular calcium in RBL-2H3 mast cells, as well as showing the ability of UDP-glucose to induced a rapid and concentration-dependent Ca^{2+} elevation in epithelial cell lines, A549 and BEAS-2B (Muller *et al.*, 2005; Gao *et al.*, 2010). The effects of UDP-glucose and UDP on Ca^{2+} elevation in the previous cells was abolished following pre-treatment with PTX, which catalyses ADP-ribosylation of the αi subunits, resulting in inactivation of G_i protein. The inhibition in the effect

of UDP-glucose following pre-treatment with PTX confirmed an involvement of $P2Y_{14}$ receptor coupled to G_i proteins (Muller *et al.*, 2005; Gao *et al.*, 2010).

Activation of G_i protein coupled receptors may also involve an activation of mitogen-activated protein kinases (MAPK), thus, when the effect of UDP-glucose on (ERK1/2) was tested in HEK-293 cells, UDP-glucose evoked a concentration-dependent elevation in phosphorylated ERK (Fricks *et al.*, 2009; Harden *et al.*, 2010). The ability of UDP-glucose to induce elevation in phosphorylated ERK1/2 in HEK-293 cell line was abolished when the cells were pre-incubated with pertussis toxin indicating signalling through G_i proteins (Fricks *et al.*, 2009; Harden *et al.*, 2010). Although many attempts have been carried out to investigate the signalling pathways involving the $P2Y_{14}$ receptor activation, there is still a lack of knowledge in that respect in the cardiovascular system. Therefore, the aim of this chapter is to investigate the signalling pathways underlying the contraction evoked by UDP-glucose, MRS2690 and UDP through activating $P2Y_{14}$ receptors in porcine isolated pancreatic arteries.

4.2. Materials and methods

4.2.1.Tissue preparation

Pancreata from pigs were obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield). A crude dissection was conducted to isolate the pancreatic arteries (dorsal pancreatic artery) (Figure 1.9, main), followed by a fine dissection to obtained rings of 0.5 cm in length, which were

suspended between two wires in Krebs'-Henseleit buffer (gassed, 95% O_2, 5% CO_2), as described in section 2.2.1.

Endothelium denudation (by gentle rubbing) was conducted using the same protocol described in section 2.2.1.

4.2.2. Responses in the porcine isolated pancreatic artery

Arterial rings were mounted onto wires in tissue baths containing warm (37°C) oxygenated Krebs'-Henseleit solution and were connected via isometric force transducers (ADInstruments, Sydney, Australia) to a PC running the computer program LabChart (ADInstruments, Sydney, Australia). Rings were put under tension (15 g) and allowed to equilibrate for 60 min before being treated as described in section 2.2.2. Then U46619 (10-100 nM) was used to contract the tissues to 40-80% of the second KCl response. Once an appropriate level of U46619 response had been achieved, cumulative addition of UDP-glucose, UDP or MRS2690 were applied. Antagonists or inhibitors [nordihydroguiaretic acid (NDGA) (10 μM), nifedipine (10 μM), thapsigargin (100 nM), zafirlukast (10 μM), BQ788 (*N*-*cis*-2,6-dimethylpiperidinocarbonyl-L-gmethylleucyl-D-1-methoxycarboyl-D-norleucine) (1 μM), PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-be-nzopyran-4-one) (10 μM), DUP 697 (3 μM), BQ123 (*cyc*(DTrp-DAsp-Pro-D-Val-Leu)) (1 μM), Y-27632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinyl-cyclohexane carboxamide) (10 μM), (5 μM)] were applied 10 min prior to the addition of U46619, allowing incubation with the tissues for a minimum of 30 min prior to the application of the agonists. An exception to the pre-constriction with U46619, were experiments with L-655,240 (1-[(4-Chlorophenyl)methyl]-5-fluoro-α,α,3-

trimethyl-1H-indole-2-propanoic acid) (1 μM), in which arteries were pre-constricted with endothelin-1 (1–10 nM). In experiments using DMSO as the solvent (see reagents and drugs section 4.2.6), DMSO 0.1 % (v/v) was added to the arteries as vehicle controls.

4.2.3. Effect of forskolin on subsequent UDP-glucose or UTP responses

Tissues were exposed to U46619 (10-100 nM) and relaxed with forskolin (1 μM, to induce an elevation of cAMP level), or with sodium nitroprusside (SNP) (100 μM, to induce an elevation of cyclic guanosine-5'-monophosphate (cGMP) level), back to the baseline. Cumulative concentration-response curves were then constructed for UDP-glucose (1 μM–1 mM) or UTP (10 μM–1 mM). Responses to UDP-glucose or UTP obtained under these conditions were compared to the control responses in which drugs were added at basal tone without exposing to either U46619 or forskolin. The tissues were allowed to recover for 20 min before concentration-response curves to UDP-glucose or UTP were constructed.

4.2.4. Western blotting

Segments of porcine pancreatic arteries were set up in the organ baths (20 ml) and tensioned to 15 g, then left for approximately 60 min to reach a new baseline of resting tension. Then tissues were incubated with 100 μM UDP-glucose for 30, 60, 120, 180, 240 and 300 s for MLC, isoform 2 phosphorylation study and for 30, 60, 120 and 300 s for ERK1/2, JNK and p38 phosphorylation studies. Segments were rapidly removed from the organ baths and immediately frozen on dry ice. Control tissues were not

exposed to any compound (basal conditions). Segments were then homogenised with a borosilicate glass homogeniser in lysis buffer (section 3.2.5), containing protease inhibitor cocktail tablets, EDTA-free. After removal of a sample for a protein assay (section 3.2.5), samples were diluted 1:6 into solubilisation buffer 6×SB: (section 3.2.6), and were heated at 95°C for 5 min. Subsequently, electrophoresis was carried out on 4-20% Tris-Glycine (PAGE) Gold Precast Gels (Bio-Rad, Hercules, CA, U.S.A.), 15 µg protein per lane was loaded for MLC2 study, 10 µg protein was loaded for JNK and p38 studies, while 0.5 µg protein per lane was loaded for ERK1/2 study.

Samples were transferred to nitrocellulose membranes. Next, blots were incubated in blocking solution (5% (w/v) powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (Fisher Scientific UK Ltd., Loughborough, UK)) for 60 min, at room temperature. Blots were incubated overnight at 4°C with primary antibodies against phosphorylated MLC2 (1:500) and total MLC2 (1:1000) or against phosphorylated p38 MAPK (1:1000) and total p38 MAPK (1:1000) or against phosphorylated SAPK/JNK (1:1000) and total SAPK/JNK (1:1000) or against phosphorylated ERK1/2 (1:1000) and total ERK1/2 (1:1000) diluted in the blocking solution. After washing in Tris-buffered saline containing 0.1% (v/v) Tween 20, the blots were incubated with an appropriate IRDye® secondary antibody (Li-Cor Biosciences, Biotechnology, Lincoln, NE, USA). Proteins were visualised using the Licor/Odyssey infrared imaging system (Biosciences, Biotechnology). Bands were analyzed by densitometry using the Odyssey application software, and expressed as phosphorylated MLC2 or ERK1/2 normalised to total MLC2 or ERK1/2 respectively.

Antibody	Cat No.	Host	MW	Sequence
PMLC	3674	rabbit	18	threonine 18/serine 19
MLC	3672	mouse	18	threonine 18/serine 19
PERK1/2	4370	rabbit	44/42	threonine 202/Tyrosine 204, threonine 185/tyrosine 187
ERK1/2	9102	mouse	44/42	threonine 202/Tyrosine 204, threonine 185/tyrosine 187

4.2.5. cAMP measurement in porcine pancreatic arteries

Pancreatic artery rings were stimulated with 75 mM KCl and then challenged with UDP-glucose (1 mM), MRS2690 (1 μM), UTP (1 mM) or distilled water (control group), preceded by U46619 (10-100 nM) plus forskolin (1 μM). When the contractions to UDP-glucose or MRS2690 reached a plateau (~3 min after addition of agonists), the segments were quickly removed from the tissue baths, and immediately frozen on dry ice and stored in vials at -80°C, for later use. For cAMP measurement, the tissue samples were homogenised in 5% (w/v) trichloroacetic acid (TCA) in distilled water with a borosilicate glass homogeniser, and then centrifuged for 10 min at 1500 g. TCA was extracted from the supernatant samples using water-saturated ether at room temperature, and then evaporated for 5 min at 70°C to remove the residual ether from the aqueous fractions. Samples were diluted (1:2) in ether-extracted 5% (w/v) TCA. cAMP concentration was measured using a competitive enzyme immunoassay kit (EIA kit) (see reagents and drugs section 4.2.6). The working range of the

cAMP assay was 0.1–1000 pmol/ml. cAMP concentration was expressed as a percentage of forskolin-induced cAMP elevation.

4.2.6. Reagents and drugs

NDGA, nifedipine, thapsigargin, SNP, zafirlukast, Tween 20, UK14304 (5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline), BQ788, UDP-glucose and UDP were purchased from Sigma (Poole, Dorset, UK), while DUP 697, MRS2578, PD98059, L-655,240, endothelin-1, BQ123, pertussis toxin, Y-27632 and forskolin were purchased from Tocris Biosciences Ltd. (Bristol, UK). Primary antibodies for western blotting were purchased from Cell Signalling Technology (Danvers, MA, USA) for phosphorylated MLC2 antibody (cat No. 3674), for total MLC2 antibody (cat No. 3672), for phosphorylated p44/42 MAPK (ERK1/2) (cat No. 4370), for total p44/42 MAPK (ERK1/2) (cat No. 9102), for phosphorylated p38 MAPK (cat No. 9216), for total p38 MAPK (cat No. 8690), for phosphorylated SAPK/JNK (cat No. 9255) and for total SAPK/JNK (cat No. 9258). Cyclic AMP EIA kit (cat No. 581001) was purchased from Cayman Chemical Company, (Ann Arbor, MI). DUP 697, BQ788, L-655,240, nifedipine, thapsigargin, zafirlukast, UK14304, PD98059 and forskolin were dissolved in DMSO. All other drugs were dissolved in distilled water. For information about the sources and the solvents of other reagents and drugs, see section 2.2.3 and 3.2.5.

4.3. Statistical analysis

Data were expressed as log concentration-response plots. The contraction to all agonists was expressed in g, and measured from the stabilised U46619 response. Values for all figures refer to mean ± S.E.M with 95% confidence. Results were compared by two-way ANOVA or Student's unpaired *t*-test (Prism, GraphPad, San Diego, CA, USA). Differences were considered to be significant when the P value was < 0.05. The "n" in the results expresses the number of animals.

4.4. Results

4.4.1. Effect of DUP 697 on responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Because the contractions to $P2Y_{14}$ receptor agonists were mainly endothelium-dependent (section 3.4.6), the effect of DUP 697, a cyclooxygenase-2 inhibitor, on the contraction to $P2Y_{14}$ receptor agonists was investigated, since COX-2 facilitates the release of some agents which are responsible for the endothelium-dependent contraction (Mombouli & Vanhoutte, 1993; Wong *et al.*, 2009). DUP 697 (3 μM) diminished the contractions to UDP-glucose, UDP and MRS2690 to a similar extent as removal of the endothelium (Figure 3.8), while DUP 697 did not alter the contraction to U46619 (the pre-constriction agent) or the contraction to ATP (section 2.4.3.3). DUP 697 reduced the contraction to 1 mM UDP-glucose by 1 ± 0.2g ($P < 0.001$, n=11), that to 1 mM UDP by 0.55 ± 0.2g ($P < 0.001$, n=15) and that to 30 μM MRS2690 by 0.5 ± 0.4g ($P < 0.01$, n=4), (Figure 4.1).

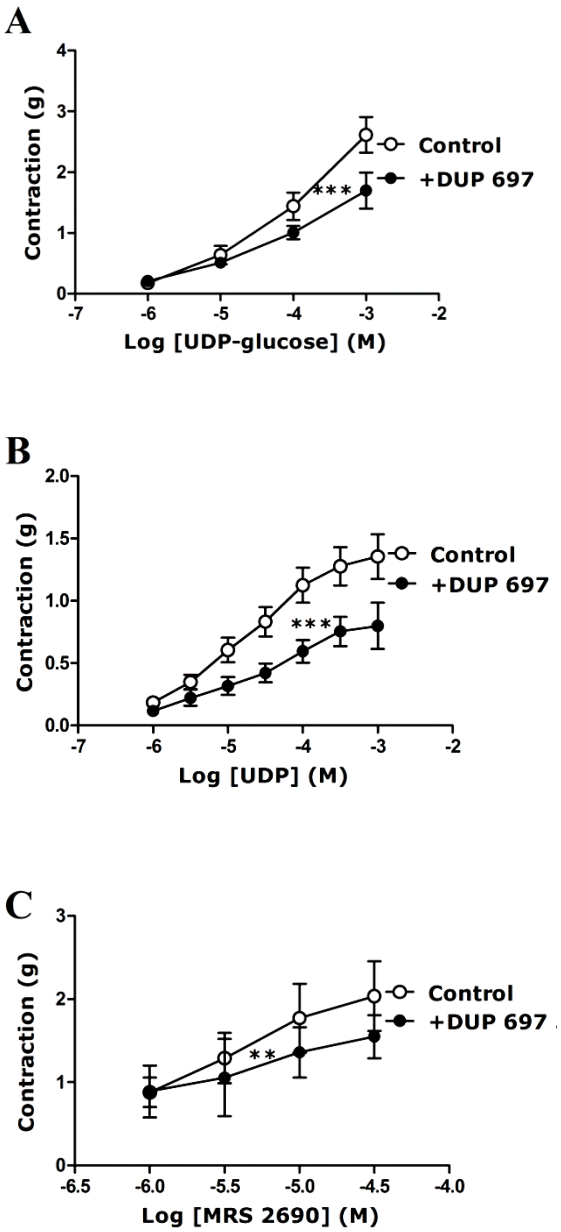


Figure 4.1. Effect of DUP 697 (3 μM) on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690 in U46619-preconstricted porcine pancreatic arteries. DUP 697 inhibited the contractions-evoked by (A) UDP-glucose, (B) UDP, (C) MRS2690 (**P < 0.01, ***P < 0.001, two-way ANOVA, F=7.85, 35.31, 4.95 respectively; n=4-15).

4.4.2. The role of endothelium-derived contractile factors in the response to UDP-glucose in porcine isolated pancreatic arteries

The following experiments were carried out using mainly UDP-glucose or UDP due to the cost considerations involved with use of MRS2690. The possible involvement of thromboxane A_2, leukotrienes, endothelin-1 and prostaglandins, which can be released from endothelial cells (Mombouli & Vanhoutte, 1993; Kurahashi *et al.*, 2003; Wong *et al.*, 2009), in endothelially-mediated contraction to UDP-glucose was investigated. The contraction to UDP-glucose was not altered in the presence of NDGA (10 μM), a lipoxygenase inhibitor (Figure 4.2A) or zafirlukast (10 μM), a selective cysteinyl leukotriene type 1 receptor antagonist (Figure 4.2B). The contraction to UDP-glucose was reduced in the presence of BQ123 (1 μM), a selective endothelin ET_A receptor antagonist (Ki value is 1.4 nM at ET_A receptor (Sakamoto *et al.*, 1993)); for example, the response to 100 μM UDP-glucose was attenuated by 0.2g ($P < 0.05$, n=14) in the presence of BQ123 (Figure 4.2C), which was only effective in the arteries with intact endothelium (Figure 4.2D). The contraction to UDP-glucose was unaltered in the presence of BQ788 (1 μM), a selective endothelin ET_B receptor antagonist (IC_{50} of 1.2 nM for inhibition of endothelin-1 in human girardi heart cells (Okada *et al.*, 2002)) (Figure 4.2E). UDP-glucose induced-contraction was reduced in the presence of L-655,240 (1 μM), a selective thromboxane A_2/prostaglandin receptor antagonist (IC_{50} of 7 nM for inhibition of endothelin-1 in human platelet aggregation) (Hall *et al.*, 1987; Wong *et al*., 2009); for example, the response to 1 mM UDP-glucose was inhibited by 0.6 ± 0.1g ($P < 0.001$, n=9) in the presence of L-655,240 (Figure 4.2F). These data indicated that UDP-glucose mediated-contraction

occurs mainly via thromboxane and prostaglandins, with a lesser involvement of endothelin-1.

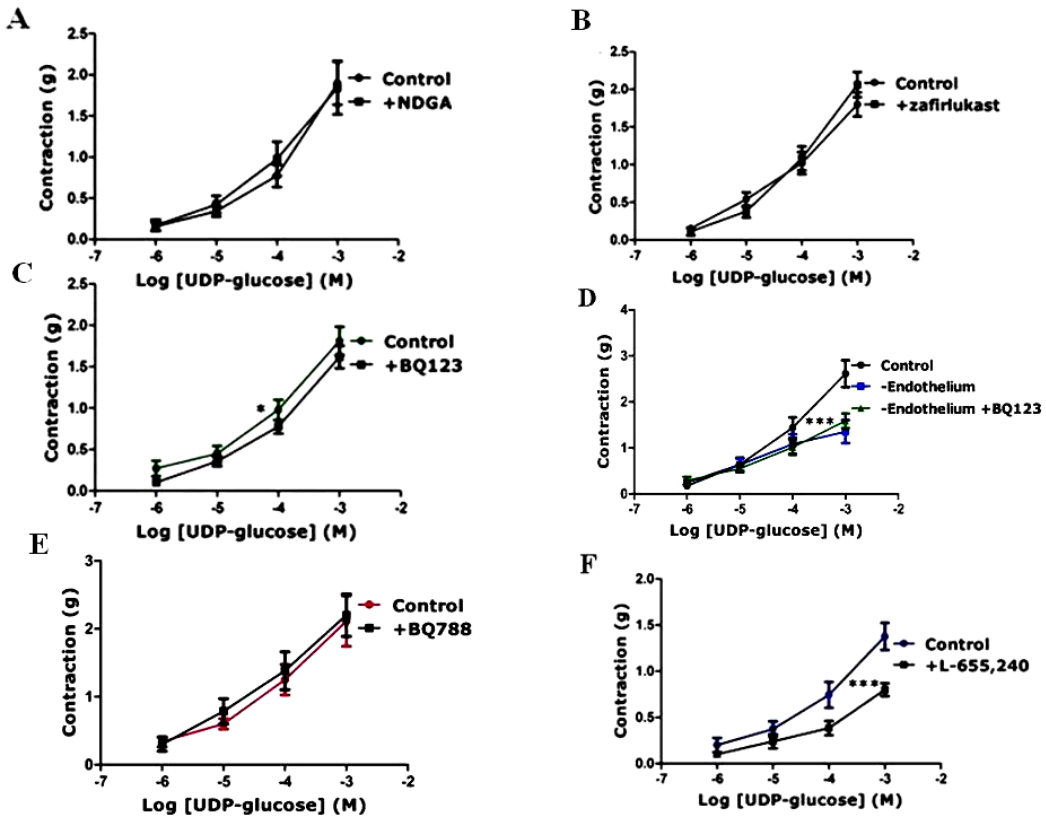


Figure 4.2. The contraction evoked by UDP-glucose in the presence of (A) NDGA (10 μM), (B) zafirlukast (10 μM), (C) BQ123 (1 μM), (D) in endothelium-denuded arteries in the presence or absence of BQ123 (1 μM) (E) BQ788 (1 μM) in U46619-preconstricted porcine pancreatic arteries, (F) L-655,240 (1 μM) in endothelin-1-preconstricted porcine pancreatic arteries. (A), (B), (E) NDGA, zafirlukast and BQ788 did not alter the responses to UDP-glucose (n=7-10). (C), (F) BQ123 and L-655,240 inhibited the responses evoked by UDP-glucose (*$P < 0.05$, ***$P < 0.001$, two-way ANOVA, $F=4.97$, 19.03 respectively; n=9-14). (D) BQ123 had no effect on the contraction evoked by UDP-glucose in endothelium-denuded pancreatic arteries.

4.4.3. Effect of BQ123 and BQ788 on contraction to endothelin-1 in porcine isolated pancreatic arteries

Since the contraction to UDP-glucose was decreased in the presence of a selective ET_A receptor antagonist (Figure 4.2C), that may suggest the involvement of endogenous endothelin-1, released as a consequence of $P2Y_{14}$ receptor activation via UDP-glucose. Endothelin-1 may act at endothelin ET_A receptor in porcine pancreatic arteries to induce a contraction. To investigate this further, the contraction to exogenous endothelin-1 (0.1–10 nM) was studied in U46619-preconstricted pancreatic arteries. As seen in Figure 4.3, endothelin-1 induced a vasoconstriction which was inhibited in the presence of BQ123 ($P < 0.0001$, two-way ANOVA), while it was not affected in the presence of BQ788. These findings were in agreement with the data in Figure 4.2C, E which indicated the involvement of endothelin ET_A receptor only in the contraction evoked by UDP-glucose, without an involvement of endothelin ET_B receptor.

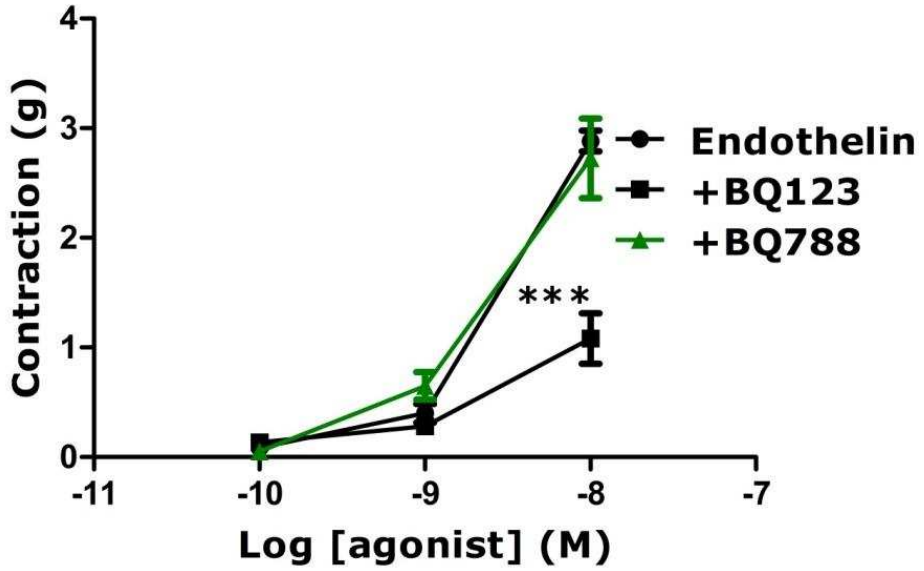


Figure 4.3. Effects of BQ123 (1 μM) and BQ788 (1 μM) on contraction to endothelin-1 in U46619-preconstricted porcine pancreatic arteries. BQ123 decreased significantly the contraction to endothelin-1 (***P < 0.001, F= 51.77; n=4-6), while BQ788 had no effect on the contraction to endothelin-1.

4.4.4. Effect of pre-constriction with U46619, and relaxation with forskolin or incubation with pertussis toxin on the response to UDP-glucose in porcine isolated pancreatic arteries

Agonist-promoted activation of G_i and subsequent inhibition of adenylyl cyclase is one of the signalling responses of $P2Y_{14}$ receptors. Therefore, the response to UDP-glucose was tested after the exposure to U46619 and subsequent relaxation by forskolin (back to the baseline), involving an elevation of intracellular cyclic AMP. UDP-glucose induced a greater contraction compared with the control, in which UDP-glucose was applied at basal tone, without the tissues being exposed to U46619 or forskolin (Figure 4.4A). The contraction to 1 mM UDP-glucose was enhanced by 2.7 ± 0.2g ($P < 0.001$, n=11) after exposure to U46619 and forskolin (Figure 4.4A). In agreement with the data obtained in endothelium-denuded pancreatic arteries (section 3.4.6). When the endothelium has been removed, exposing the tissue to U46619 and subsequently to forskolin enhanced the contraction to UDP-glucose relative to the control (UDP-glucose was applied at basal tone). However, this elevation was significantly less than that in endothelium-intact pancreatic arteries; Removal of endothelium reduced the contractions to 1 mM UDP-glucose by 1 ± 0.3g ($P < 0.01$, n=15).

In contrast, when responses to UTP, an agonist at $P2Y_2$ and $P2Y_4$ receptors ($G_{q/11}$ protein coupled receptors) were investigated in the presence and absence of U46619 and forskolin, there was no change within its contractions (Figure 4.4B). In addition, the response to UDP-glucose was not altered after the arteries were pre-contracted with U46619 and relaxed with SNP (100 μM), which elevates intracellular cyclic GMP (Figure 4.5) (Roberts *et al.,* 1999), which ruled out the involvement of cGMP in the

contraction to UDP-glucose. Typical traces showing the effect of UDP-glucose on basal tone (inset) and after the tissues had been contracted by U46619 and then relaxed back with forskolin (main) are shown in Figure 4.4C.

Since $P2Y_{14}$ receptor is a G_i protein-coupled receptor (Jacobson et al., 2009), the contraction to $P2Y_{14}$ receptor agonist (UDP-glucose) was examined following overnight incubation of pancreatic arteries with pertussis toxin (100 ng/ml or 1 μg/ml), which inhibits αi subunit of G_i protein. The study was conducted in the presence of UK14304 (30 μM) which is an adrenergic α_2 agonist, and it was used in the current study as a positive control. By contrast, overnight incubation with PTX had no effect on the contractions to UK14304 or UDP-glucose (Figure 4.6).

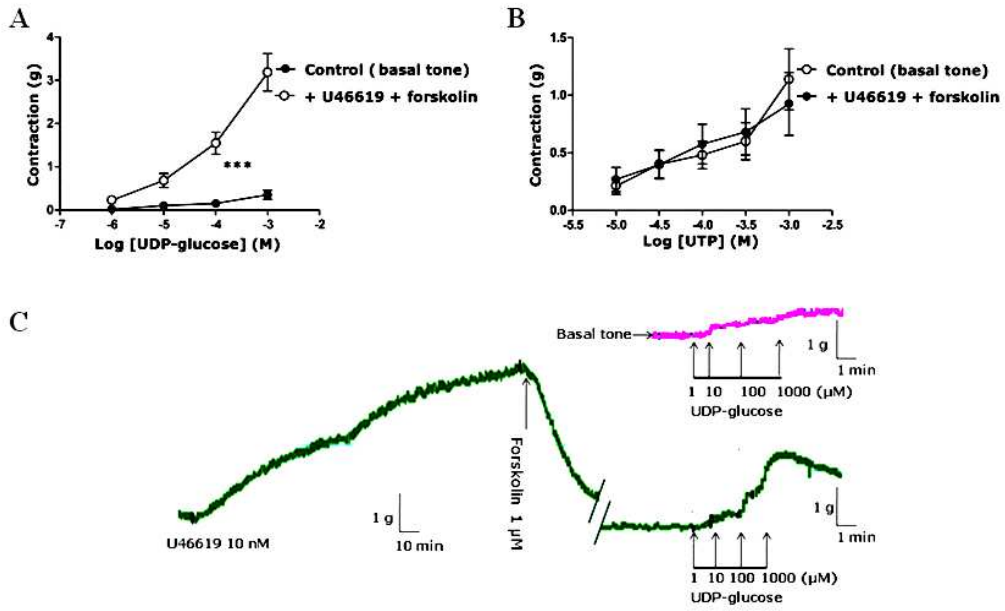


Figure 4.4. Effect of pre-constriction with U46619 followed by relaxation with forskolin (1 μM) on the responses to (A) UDP-glucose, (B) UTP in porcine pancreatic arteries. (A) Exposing the tissues to U46619 followed by forskolin significantly enhanced the contraction evoked by UDP-glucose (***P < 0.001, two-way ANOVA, F=54.34; n=8-13). (B) Exposing the tissues to U46619 followed by forskolin failed to alter the response to UTP. (C) Typical traces showing the effect of UDP-glucose on basal tone (inset) and after the tissues were pre-constricted with U46619 and then relaxed with forskolin (main).

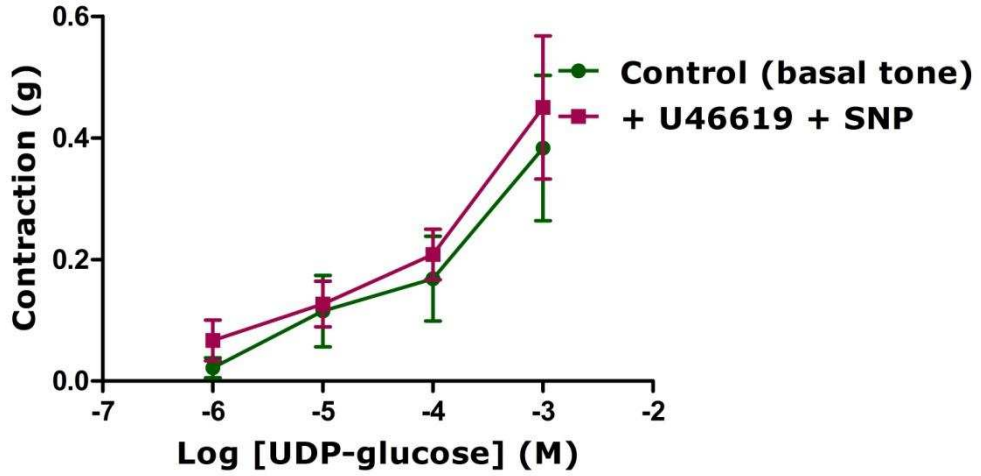


Figure 4.5. Effect of pre-constriction with U46619 followed by relaxation with SNP (100 μM) on the response to UDP-glucose in porcine pancreatic arteries. Exposing the tissues to U46619 followed by SNP had no significant effect on the contraction evoked by UDP-glucose (n=6).

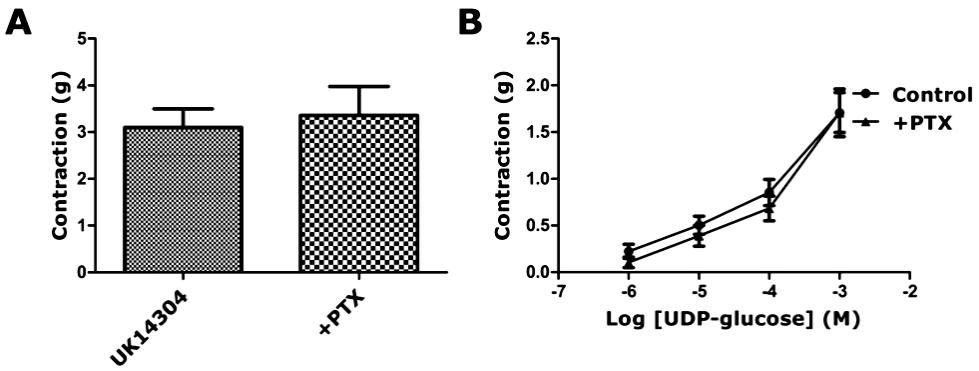


Figure 4.6. Effect of pertussis toxin (PTX; 1 μg/ml) on contraction responses to (A) UK14304 (30 μM), (B) UDP-glucose in U46619-preconstricted porcine pancreatic arteries. (A), (B) overnight incubation with PTX had no effect on the contractions to UK14304 or UDP-glucose (n=5-9).

4.4.5. Effect of UTP, UDP-glucose and MRS2690 on the cAMP level in porcine isolated pancreatic arteries

On the basis that cAMP is involved in the contraction to UDP-glucose (section 4.4.4), the cellular levels of this second messenger were measured in pancreatic arterial rings. We investigated the effects of UDP-glucose, MRS2690 and UTP (as a negative control, since it is coupled to $G_{q/11}$ protein) on cAMP level in the presence of U46619 plus forskolin (to mimic the raised tone condition of the pharmacology experiments). UDP-glucose (1 mM) and MRS2690 (10 μM) induced a significant decrease within the level of cAMP relative to the control, in which the tissues were exposed just to U46619 and forskolin; UDP-glucose (1 mM) decreased the level of cAMP by 0.19 ± 0.06 pmol\mg tissue, while MRS2690 (10 μM) decreased it by 0.13 ± 0.02 pmol\mg tissue (*$P < 0.05$, n=4, Figure 4.7). By contrast, UTP had no significant effect on cAMP level (Figure 4.7).

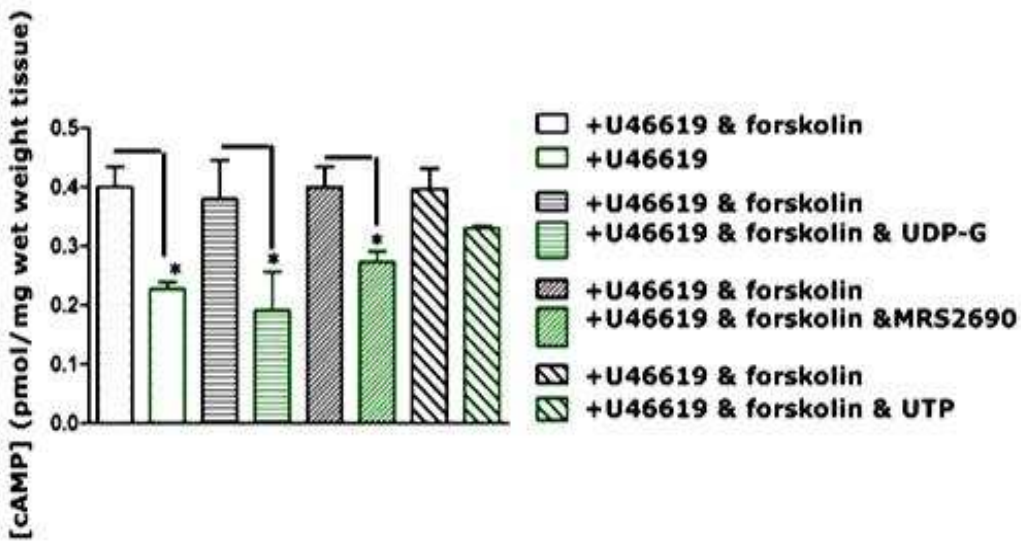


Figure 4.7. Effect of UDP-glucose (UDP-G) (1 mM), MRS2690 (10 μM)and UTP (1 mM) on the cAMP concentrations in porcine pancreatic arteries exposed to U46619 (10-100 nM) followed by forskolin (1 μM). UTP had no significant effect on cAMP levels, while UDP-glucose and MRS2690 reduced significantly the cAMP level (*P < 0.05, Student's unpaired *t*-test, the responses to UTP or UDP-glucose or MRS2690 vs their respective controls, n=4). Basal cyclic AMP level represents the level of cAMP in the absence of forskolin. Distilled water was added to the arteries as vehicle controls.

4.4.6. Effect of inhibition of calcium release and calcium entry on the responses to UDP-glucose and UDP in porcine isolated pancreatic arteries

Binding of agonists to the $P2Y_{14}$ receptor leads to increased Ca^{2+} mobilisation in some cells, including A549, BEAS-2B and RBL-2H3 cell lines. This elevation could result from triggering PLCβ signalling pathways following activation G_i protein-derived $G_{\beta\gamma}$-dimers of $P2Y_{14}$ receptor. Activation of PLCβ signalling pathways results in an elevation of the level of IP_3 and DAG, which induce an elevation of the level of intracellular Ca^{2+}

(Skelton *et al.*, 2003; Gao *et al.*, 2010; Verin *et al*., 2011). To test this mechanism in porcine pancreatic arteries, responses to UDP-glucose and UDP were examined in the presence and absence of nifedipine (1 μM), a L-type voltage-gated calcium channel blocker, and thapsigargin (100 nM), a potent inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases which leads to depletion of intracellular calcium. Both of these inhibitors reduced significantly the contraction- evoked by UDP-glucose; for example, the contraction to 1 mM UDP-glucose was reduced by 1 ± 0.1g ($P < 0.001$, n=15) in the presence of nifedipine, and by 0.6 ± 0.2g ($P < 0.05$, n=15) in the presence of thapsigargin (Figure 4.8). Similarly, they attenuated the contraction to UDP; for example, contraction to 100 μM UDP was inhibited by 0.6 ± 0.2g ($P < 0.001$, n=9) in the presence of nifedipine, and by 0.4 ± 0.1g ($P < 0.001$, n=9) in the presence of thapsigargin. Typical traces showing the effect of UDP-glucose in the absence and in the presence of nifedipine are shown in Figure 4.8C.

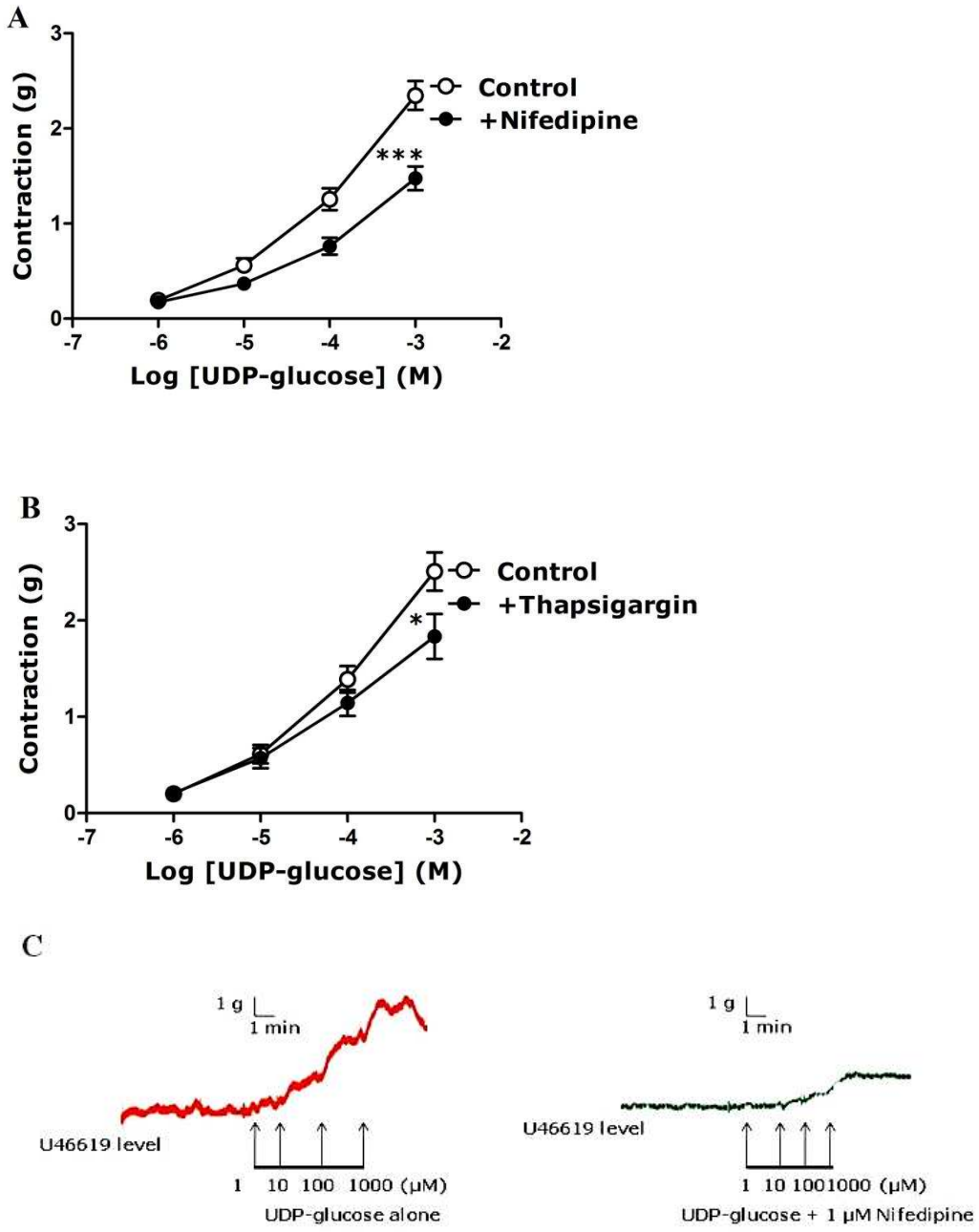


Figure 4.8. Effect of (A) nifedipine (1 μM) and (B) thapsigargin (100 nM) on the response to UDP-glucose in U46619-preconstricted porcine pancreatic arteries. Both inhibitors, nifedipine and thapsigargin, inhibited the contraction evoked by UDP-glucose (*P < 0.05, ***P < 0.001, two-way ANOVA, F=32.5, 5.84; n= 9-15). (C) Typical traces showing the responses of UDP-glucose in the absence and in the presence of nifedipine.

4.4.7. Effect of inhibition of the Rho-kinase pathway on the responses to UDP-glucose, UDP and MRS2690 in porcine isolated pancreatic arteries

Activation of $P2Y_{14}$ receptors may cause stimulation of RhoA/ROCK signalling (Sesma *et al.*, 2012). To test the possible involvement of this pathway, experiments were conducted to study the contractions to UDP-glucose, UDP and MRS2690 in the presence of Y-27632 (5 10 μM), a selective inhibitor of the Rho-associated protein kinase (K_i value is 0.14 μM for ROCK) (Uehata *et al.*, 1997; Narumiya *et al.*, 2000). Y-27632 significantly inhibited the contraction evoked by UDP-glucose, UDP and MRS2690. For instance, the response to 1 mM UDP-glucose was reduced by 1.3 ± 0.2g ($P < 0.001$, n=9, Figure 4.9A), that to 300 μM UDP by 0.7 ± 0.1g ($P < 0.001$, n=15, Figure 4.9B) and that to 30 μM MRS2690 by 1 ± 0.4g ($P < 0.001$, n=9, Figure 4.9C).

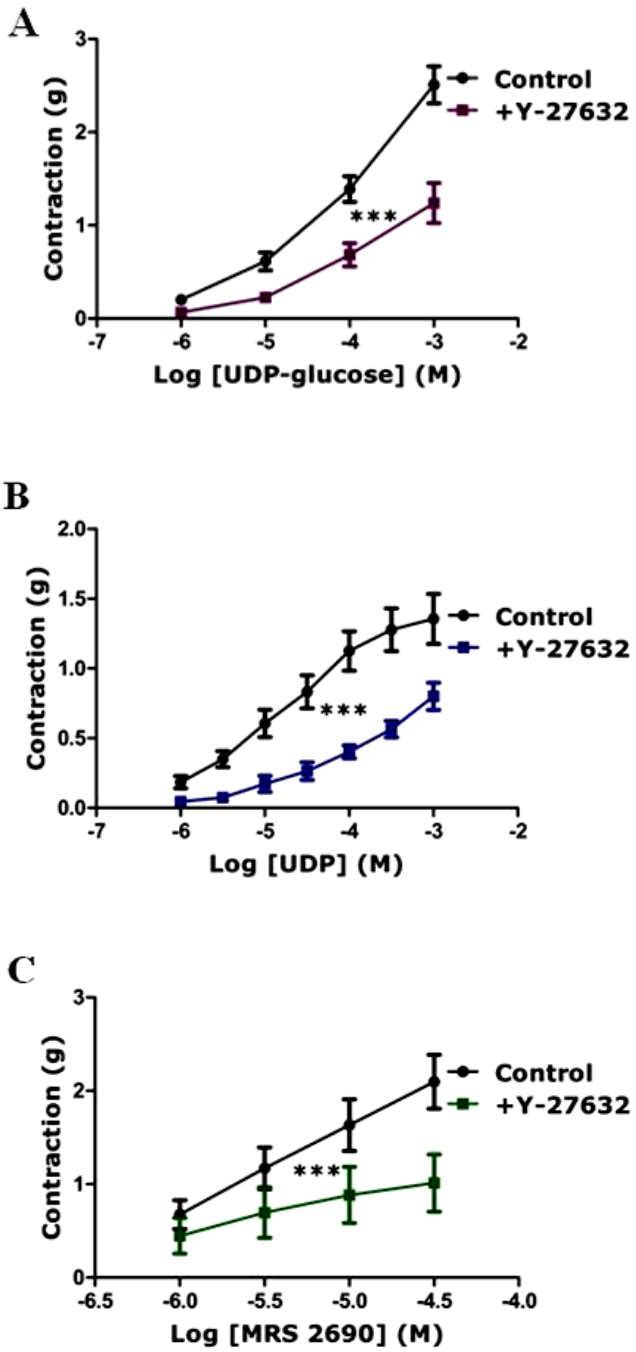


Figure 4.9. Effect of Y-27632 (5 μM), a selective inhibitor of the Rho-associated protein kinase on responses to (A) UDP-glucose, (B) UDP, (C) MRS2690 in U46619-preconstricted porcine pancreatic arteries. Y-27632 reduced significantly the contractions to (A) UDP-glucose, (B) UDP (C) MRS2690 (***P < 0.001, two-way ANOVA, F=42.68, 53.07, 10.32; n=9-15).

4.4.8. Effect of UDP-glucose on the level of MLC phosphorylation in porcine isolated pancreatic arteries

The response to UDP-glucose was associated with an elevation of the level of MLC2 phosphorylation at 18 kDa, which was consistent with the expected band size reported previously (Cell Signalling Technology, cat No. 3674). The elevation of the level of phosphorylated MLC2 occurred within 30-60 s of treatment with 100 μM UDP-glucose, and returned to the basal level of phosphorylated MLC within 120-300s, (Figure 4.10), which suggested an involvement of MLC activation in the signalling pathways underlying the response to $P2Y_{14}$ receptor agonist. The ability of UDP-glucose (100 μM) or MRS2690 (10 μM) to elevate the MLC phosphorylation (within 30 s of treatment) was decreased by 0.22 ± 0.03 normalised intensity (NI) (the ratio of phospho protein to total protein) and 0.12 ± 0.03 NI respectively (*$P < 0.05$, n=3, Figure 4.11) in the presence of PPTN (1 μM), which indicated that the ability of UDP-glucose or MRS2690 to elevate MLC phosphorylation in porcine pancreatic arteries is mediated by the activation of $P2Y_{14}$ receptors.

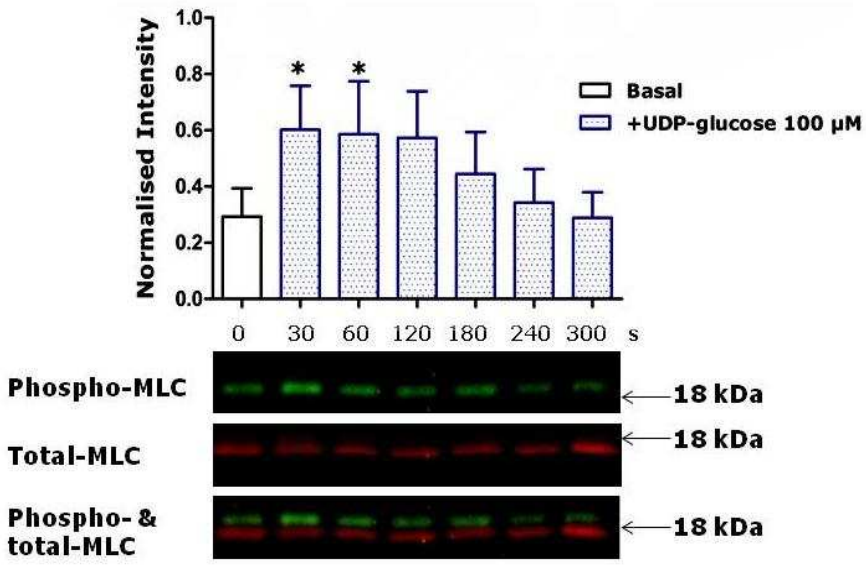
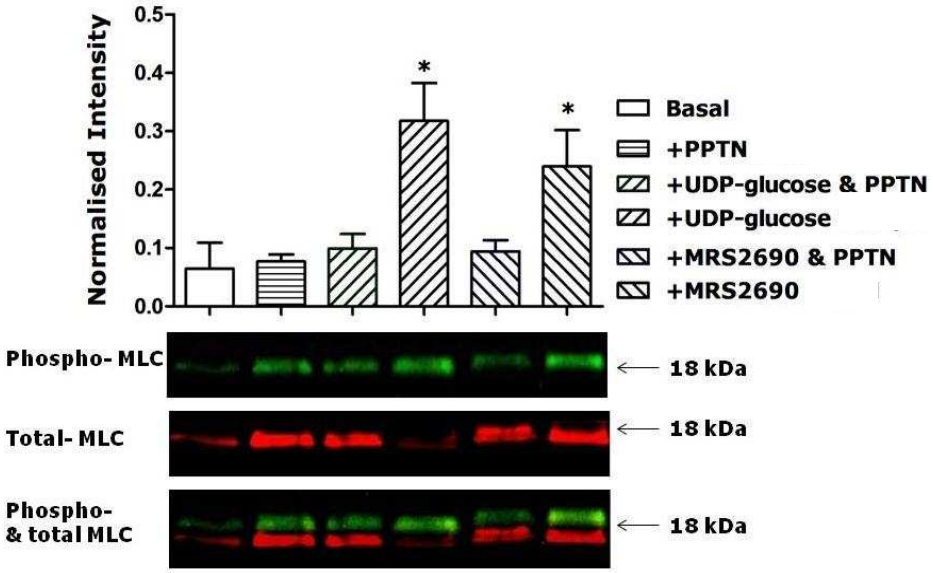


Figure 4.10. Effect of UDP-glucose (100 μM) on MLC phosphorylation in porcine pancreatic arteries. UDP-glucose induced changes in MLC phosphorylation in a time-dependent manner, the level of MLC phosphorylation was elevated within 30-60 s (*P < 0.05, one-way ANOVA with Bonferroni's post hoc test, n=4). Representative immunoblots of a time course of UDP-glucose-induced change within the level of phosphorylated MLC2 from four separate experiments.



\Figure 4.11. PPTN (1 μM) abolished the ability of UDP-glucose (100 μM) and MRS2690 (10 μM) to elevate the level of phosphorylated MLC within 30 s of treatment (*P < 0.05, one-way ANOVA with Bonferroni's post hoc test, n=3). Representative immunoblots of MLC2 phosphorylation from three separate experiments in the absence or presence of PPTN.

4.4.9. Effect of UDP-glucose and UDP on extracellular signal-regulated kinase ERK1/2 phosphorylation

It is previously reported that MAP kinase signalling pathways are stimulated via activation of receptors that couple to G_i protein (Harden *et al.*, 2010). This effect occurs following triggering PLCβ signalling pathways following activation $G_{\beta\gamma}$ subunits of $P2Y_{14}$ receptor. Activation of PLCβ signalling pathways results in an elevation of the level of DAG, which activates PKC, the latter is associated with phosphorylation of ERK in many cell types (Langan *et al.*, 1994; Marquardt *et al.*, 1994; Zhao *et al.*, 2005). That was also shown after activation of $P2Y_{14}$ receptors in HEK-293 cells via UDP-glucose or UDP (Carter *et al.*, 2009). To investigate the involvement

of ERK1/2 in the responses to UDP-glucose or UDP in pancreatic arteries, PD98059 (10 μM), a MEK inhibitor with an IC_{50} of 4 μM (Alessi *et al.*, 1995), was employed. PD98059 was not able to alter the contractions to UDP-glucose or UDP (n=9-16) (Figure 4.12A, B). On the other hand, when the level of phosphorylated ERK1/2 was determined by western blotting using 0.5 μg protein per lane (section 4.2.4), the response to UDP-glucose (100 μM) was associated with an increase with the level of phosphorylated ERK1/2 at 44, 42 kDa respectively. These were consistent with the expected bands size reported previously (Cell Signalling Technology, cat No. 4370). The elevation in the level of phosphorylated ERK1/2 was apparent within 60 s of the treatment, and peaked within 300 s (*P < 0.05, **P < 0.01, Student's unpaired *t*-test, n=6) (Figure 4.12C), before it returned back to the basal level of phosphorylated ERK1/2 within 10 min (data not shown). In contrast, UDP-glucose was not able to elevate phosphorylated JNK or p38 level up to 5 min of treatment with 100 μM UDP-glucose (data not shown).

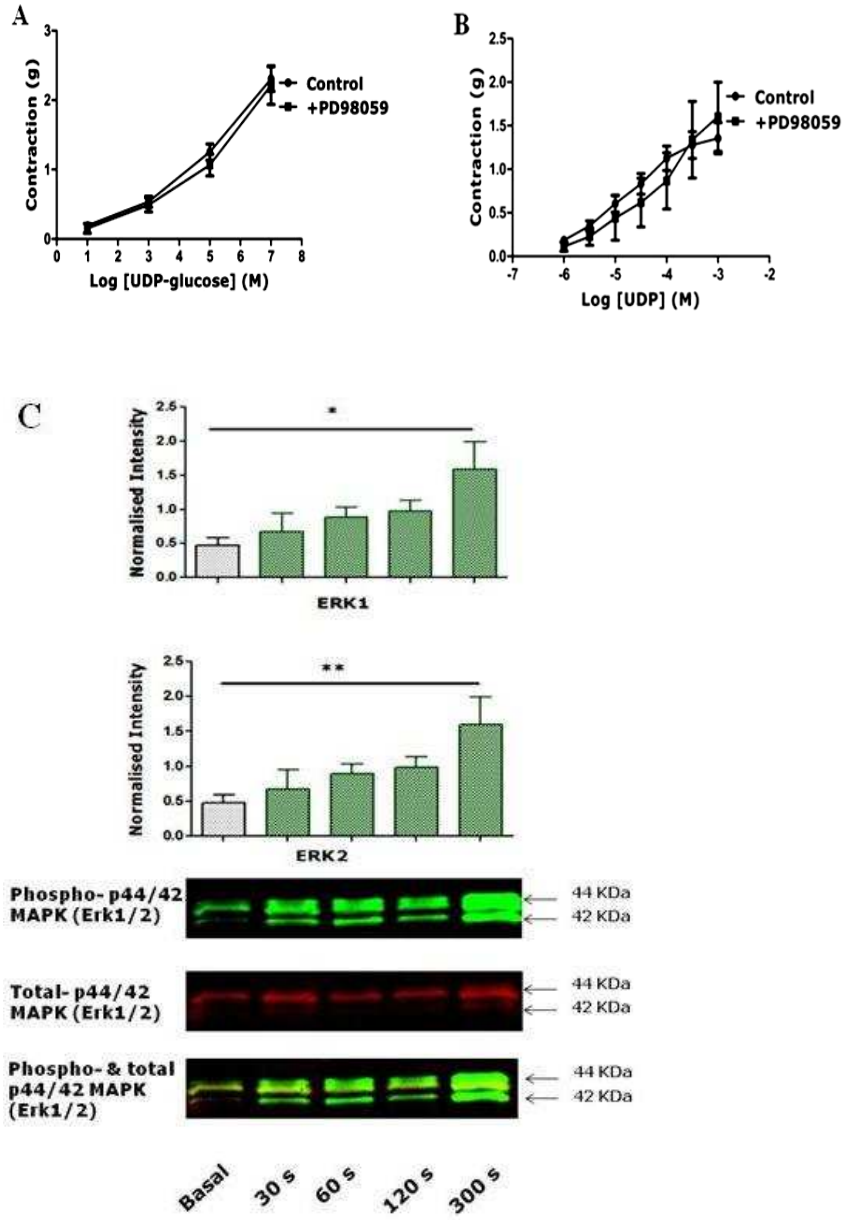


Figure 4.12. Effect of PD98059 (10 μM) on the contractions to (A) UDP-glucose, (B) UDP in U46619-preconstricted porcine pancreatic arteries. PD98059 had no significant effect on the responses to UDP-glucose or UDP (n=9-16). (C) UDP-glucose (100 μM) induced elevation with the level of ERK1/2 phosphorylation in a time-dependent manner. The level of ERK1/2 phosphorylation was apparent at 60 s and peaked within 300 s (*P < 0.05, **P < 0.01, one-way ANOVA with Bonferroni's post hoc test, n=6). Representative immunoblots of ERK1/2 phosphorylation from six separate experiments.

4.5. Discussion

The current study describes the signalling pathways underlying the responses to $P2Y_{14}$ receptor agonists in arteries of pig pancreas. The contractile response was mediated largely by the endothelium with an involvement of endothelin-1, thromboxane A_2 and $PGF_2\alpha/PGH_2$. Evidence from the contractile studies and the cAMP immunoassay indicates that $P2Y_{14}$ receptor couples to G_i protein, and hence the activation of this receptor can lead to an inhibition of the level of adenylyl cyclase. While immunoblotting study for MLC2 and ERK1/2 indicates the downstream involvement of phosphorylated MLC2 and ERK1/2.

4.5.1. The involvement of Endothelium-derived contractile factors (EDCFs) in the vasoconstriction-evoked by UDP-glucose

To investigate the mechanism underlying the contraction to $P2Y_{14}$ receptor agonists in pancreatic arteries, responses to UDP-glucose, UDP and MRS2690 were examined in the presence of DUP 697. As seen in Figure 4.1, the endothelium-dependent contractions were significantly attenuated in the presence of the selective COX-2 inhibitor. Endothelial cells can release endothelium-derived contractile factors in addition to endothelium-derived relaxing factors. EDCFs may include thromboxane A_2, $PGF_2\alpha/PGH_2$, leukotrienes and endothelin-1. thromboxane A_2 and $PGF_2\alpha/PGH_2$ are released from the endothelium due to the activity of cyclooxygenase (Mombouli & Vanhoutte, 1993; Wong *et al.,* 2009). To characterise the EDCFs involved in the contraction to UDP-glucose, experiments were conducted to study the responses to UDP-glucose in the presence of NDGA, zafirlukast, BQ123, BQ788 and L-655,240. The results showed that only

BQ123 and L-655,240 were able to decrease significantly the contraction evoked by UDP-glucose, which indicated an involvement of thromboxane A_2, prostaglandins and endothelin-1 (mainly via ET_A receptor). These agents, after being released from the endothelium, may act on their receptors on the vascular smooth muscle cells to elicit a contraction (Wong et al., 2009). Since L-655,240 is a selective thromboxane A_2/prostaglandin receptor antagonist (Wong *et al.*, 2009), the contraction of U46619 may be inhibited in the presence of that antagonist, as I found also in the current study, since U46619 was unable to induce a contraction to the pancreatic arteries, with concentrations up to 10 μM. Therefore, U46619, as a pre-constriction agent, was substituted by endothelin-1, since endothelin-1 induces contraction via acting at endothelin receptors expressed on the VSMCs, which are distinct from thromboxane receptors. In turn, a control (in the absence of L-655,240) of concentration-dependent contraction of UDP-glucose in endothelin-1-preconstricted arteries was generated (Figure 4.2F).

To confirm the involvement of endothelin receptors in the contraction to UDP-glucose, since BQ123 induced little inhibition of the contraction to UDP-glucose (Figure 4.2C), the contraction to exogenous endothelin-1 was investigated in U46619-preconstricted pancreatic arteries. Endothelin-1-evoked contraction was significantly inhibited in the presence of BQ123 (Figure 4.3), while BQ788 had no effect on the endothelin-1-evoked contraction (Figure 4.3), which was in accordance with the results obtained in the presence of UDP-glucose. Taken together, these findings indicated an involvement of ET_A receptors in the contraction to exogenous endothelin-1, as well as to the endogenous endothelin-1, released as a result of $P2Y_{14}$ receptor activation by UDP-glucose. The results obtained in (Figure 4.2D) showed that BQ123 had no effect on the contraction to UDP-

glucose in endothelium-denuded arteries which indicates that the contribution of endothelin in the UDP-glucose-evoked contraction is solely endothelium-dependent.

The involvement of extracellular calcium influx and calcium released from sarcoplasmic reticulum as a part of the response to $P2Y_{14}$ receptor activation has been reported previously (Verin *et al*., 2011). In addition, calcium-induced release of calcium from SR and influx of external Ca^{2+} in response to some activated receptors have been also reported (Fabiato, 1983; Li *et al.*, 2003). In porcine pancreatic arteries, contractions to UDP-glucose (Figure 4.8A, B) and UDP (section 4.4.6) were significantly decreased in the presence of nifedipine or thapsigargin. This indicated an involvement of elevated intracellular Ca^{2+} level, mainly via Ca^{2+} influx from extracellular milieu, in the contraction to UDP and UDP-glucose in porcine pancreatic arteries. In contrast, activation of $P2Y_{14}$ receptors in A549 and BEAS-2B cells regulated mobilisation of Ca^{2+} from intracellular stores rather than from extracellular milieu, since the ability of UDP-glucose to elevate Ca^{2+} level in these cells was not influenced by the absence of external Ca^{2+} (Muller *et al.*, 2005). The dependency on the extracellular calcium could be also determined by examining the contractions to UDP-glucose or MRS2690 following removal of the extracellular calcium ions using a calcium-free Krebs' buffer, and then observing any change within these contractions following reintroduction of calcium to the bathing solution.

Collectively, our results suggest that when UDP-glucose, UDP and MRS2690 activate $P2Y_{14}$ receptors, which are expressed mainly on the endothelium, the intracellular Ca^{2+} levels may be elevated. This may then activate COX-2 and the release of the endothelin (Luscher *et al.*, 1992; Vanhoutte *et al.*, 2005; Wong et al., 2009). COX-2 facilitates the

production of the contractile agents; thromboxane A_2 and prostaglandins, which bind to thromboxane receptors (TP) on the vascular smooth muscle cells to induce a contraction. Whereas, endothelin binds to endothelin ET_A receptors on the vascular smooth muscle cells, which contributes to that contraction. The ability of the nucleotides to stimulate the biosynthesis and release of prostanoids has been reported previously (Bruner & Murphy, 1990; Bowden *et al.*, 1995). In addition, it was shown in isolated rat middle cerebral arteries that the vasoconstriction-evoked by UTP was mediated by thromboxane A_2 release, since the contraction to UTP was significantly attenuated in the presence of a thromboxane receptor antagonist (Lacza *et al.*, 2001), which was in agreement with the current findings.

Thromboxane receptors and endothelin ET_A receptors are $G_{q/11}$ protein-coupled receptors (Wilson *et al.*, 2005). Thus, activation of these receptors leads to an activation of PLC, the latter hydrolyses plasma membrane phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG, that will result in activation of PKC and calcium release from endoplasmic reticulum (Falkenburger *et al.*, 2013). Consequently, $P2Y_{14}$ receptor activation can indirectly initiate $G_{q/11}$ signalling pathway via a downstream regulation of the level of endothelin, thromboxane and prostaglandins. This finding was in agreement with the report of Skelton *et al.* (2003), where they found that PTX was only able to block part of the response-evoked by UDP-glucose activates $P2Y_{14}$ receptors, while the rest of the response was attributed to an activation of $G_{q/11}$ signalling pathways. Activation of intracellular Ca^{2+} release following the stimulation of $G_{q/11}$ protein-coupled receptors by endothelin, thromboxane and prostaglandins may serve to amplify the signalling induced by $P2Y_{14}$ receptor agonists, resulting in

greater elevation in the level of intracellular Ca^{2+} following activation of $P2Y_{14}$ receptors.

4.5.2. The involvement of cAMP, ERK1/2, MLC and RhoA in the vasoconstriction to $P2Y_{14}$ receptor agonists

It is well established that $P2Y_{14}$ receptor is coupled to G_i protein, leading to an inhibition of forskolin-stimulated adenylyl cyclase activity, and hence inhibition of the cyclic AMP level. Accordingly, $P2Y_{14}$ receptor is pertussis toxin-sensitive (Jacobson *et al.*, 2009). It is notoriously difficult to successfully block G_i protein-coupled receptors with PTX in isolated blood vessels, as it was advised by Dr. Vincent Wilson and his group (The University of Nottingham, School of Life Sciences), and as I have also found in my preliminary studies. PTX (0.1–2 μg/ml) incubated with the tissues overnight, in Krebs'-Henseleit buffer or in Dulbecco's modified Eagle's medium, with 5% FCS (fetal calf serum) or without it, failed to block the responses to either UDP-glucose or UK14304, an agonist of α_2 adrenoceptors, classical G_i protein-coupled receptors (Figure 4.6) (Bylund *et al.*, 1994). This showed that our attempts to inhibit the responses to G_i protein-coupled receptors agonists (UDP-glucose or UK14304) with PTX was unsuccessful. However, in tissues which had been pre-constricted with U46619 and relaxed with forskolin (to elevate the cAMP levels), subsequent addition of UDP-glucose produced a significantly greater contraction compared with the control (UDP-glucose added at basal tone) (Figure 4.4A). The contraction to UTP ($P2Y_2$, $P2Y_4$ and $P2Y_6$ agonist, $G_{q/11}$ protein-coupled receptor), applied after pre-constriction with U46619 and relaxation with forskolin, was not different from its respective control (UTP added at basal tone) (Figure 4.4B). This was consistent with $P2Y_{14}$ receptor coupled to G_i proteins, since other P2Y receptors, namely $P2Y_1$, $P2Y_2$, $P2Y_4$,

$P2Y_6$ receptors, are generally $G_{q/11}$ protein-coupled receptors. The significant decrease within the contraction of UDP-glucose occurred in endothelium-denuded pancreatic arteries (section 4.4.4), confirmed the findings in the Chapter 3, since it was indicated that $P2Y_{14}$ receptor was mainly present on the endothelial cells of the porcine pancreatic arteries. On the other hand, relaxation with SNP (to elevate cGMP levels) after pre-constriction with U46619 had no significant effect on the contractile response to UDP-glucose (Figure 4.5), which indicates that cGMP elevation is not a part of the signalling pathways involved in the contraction evoked by UDP-glucose.

These findings together with the data obtained from cAMP assay (Figure 4.7), which showed the ability of UDP-glucose (1 mM) and MRS2690 (10 μM), but not UTP (1 mM), to diminish the cAMP level, indicated that the enhanced contraction to UDP-glucose seen in Figure 4.4A is mainly dependent on the agonist's ability to lower the cAMP levels. In Figure 4.7, UDP-glucose (1 mM) decreased cAMP level by 0.19 ± 0.06 pmol\mg tissue, which was slightly greater than the reduction induced by MRS2690 (10 μM) (0.13 ± 0.02 pmol\mg tissue). It was shown using pharmacological experiment that MRS2690 was more potent than UDP-glucose (section 3.4.1), in the current experiment the concentration used for MRS2690 was 100-fold lower than that of UDP-glucose, which may justify the greater inhibition occurred in the presence of UDP-glucose. . Further experiments seem vital to investigate the effect of UDP, a $P2Y_{14}$ receptor agonist (Carter *et al.*, 2009) on the cAMP level. In addition to examining the effects of $P2Y_{14}$ receptor agonists (UDP-glucose, MRS2690 and UDP) on cAMP level in the presence of PPTN to confirm that the decrease within the level of cAMP in the presence of UDP-glucose, MRS2690 and UDP occurs mainly through acting at $P2Y_{14}$ receptors.

A number of studies have considered the signalling mechanisms underlying the response of $P2Y_{14}$ receptor agonists (Harden *et al.,* 2010; Sesma *et al.,* 2012). Recent reports claimed that UDP-glucose promotes concentration-dependent activation of RhoA in isolated human neutrophils (Sesma *et al.,* 2012). This action was examined in porcine pancreatic arteries where contractions to UDP-glucose, UDP and MRS2690 were investigated in the presence of Y-27632. This compound inhibited the contraction evoked by these agonists (Figure 4.9A, B, C), which indicated an involvement of RhoA in the response to $P2Y_{14}$ receptor agonists. The level of activated RhoA could be determined using immunoblotting technique, in which it can be examined in a time-dependent manner, as the report of Sesma *et al.* (2012) showed that RhoA/ROCK signalling was activated within 1 min in the presence of 100 μM UDP-glucose in isolated human neutrophils. Therefore, further experiments could be performed to quantify the level of activated RhoA resulting from $P2Y_{14}$ receptors activation in porcine pancreatic arteries.

Activation of RhoA can lead to an inhibition of myosin light-chain phosphatase (MLCP), and thus phosphorylation of MLC and subsequently contraction of the blood vessels in a calcium-independent manner (Amano *et al.,* 1996; Hartshorne & Gorecka, 2011). Accordingly, when UDP-glucose was incubated with the tissues, the level of phosphorylated MLC2 was elevated within 30–60 s of incubation (Figure 4.10). This observation was confirmed by employing PPTN which was able to abolish the ability of UDP-glucose or MRS2690 to elevate the MLC2 phosphorylation (Figure 4.11). This finding was consistent with the involvement of MLC2 phosphorylation in the signalling pathways involved in $P2Y_{14}$ receptor activation. Moreover, these data were in accordance with the ability of UDP-glucose and MRS2690 to diminish the level of cAMP, since it was reported that an

increase in the level of cAMP in blood vessels leads to an inhibition of myosin light chain kinase, which is responsible for the phosphorylation of the MLC and subsequently inducing a contraction of the smooth muscles (Raina *et al.*, 2009).

It has been reported that activation of $P2Y_{14}$ receptors via UDP-glucose or UDP in some cells, including HEK-293 or differentiated human promyelocytic leukemia cells (HL-60) leads to stimulation of phosphorylated ERK1/2 (Carter *et al.*, 2009; Fricks *et al.*, 2009; Harden *et al.*, 2010). In the current study, the involvement of MAP kinase pathways in the contractions to UDP-glucose and UDP was examined by employing the MEK inhibitor, PD98059. The latter had no effect on the contractions-evoked by UDP-glucose or UDP. Likewise, when the level of phosphorylated ERK1/2 was quantified using immunoblotting study, following loading 10 µg protein per lane, there was no difference between the samples (treated or untreated samples) (data not shown). However, when the blots were analysed in more detail, I found that the level of ERK1/2 in untreated samples (the basal level of ERK1/2) was too high already, which may be because of some factors in the Krebs'-Henseleit buffer (used throughout the experiment), which activate ERK1/2. The previous observation may be the reason behind the high level of the activated ERK1/2 obtained. To overcome that issue, 0.5 µg of the samples (treated and untreated) were loaded per lane (section 4.2.4), which allowed obtaining low level of the basal ERK1/2 phosphorylation. As can be seen in Figure 4.12C, the response to UDP-glucose was associated with an increase within the level of ERK1/2 phosphorylation, which was apparent within 60 s of the treatment, and peaked within 300 s, before it returned back to the basal level of phosphorylated ERK1/2 within 600 s. On the other hand, UDP-glucose-induced phosphorylated ERK1/2, in HEK-293 cells, did not peak

until at least 15 min of incubation, and was retained for approximately 30 min (Fricks *et al.*, 2009; Harden *et al.,* 2010).

Thus, the lack of effect of PD98059 on the contractions to UDP-glucose or UDP may be because of the elevated levels of activated ERK1/2 in the tissues, that kept in the Krebs'-Henseleit buffer throughout the experiments. Furthermore, in agreement with other reports of HEK-293 and differentiated HL-60 cells, UDP-glucose was not able to elevate the levels of phosphorylated JNK or p38 in porcine pancreatic arteries too (Fricks *et al.*, 2009). Further experimentation seems vital to investigate the contraction to UDP-glucose or UDP in the presence of higher concentrations of PD98059, to find out whether PD98059 might be able to decrease the responses to UDP-glucose or UDP and overcome the high level of the phosphorylated ERK1/2 within the solution. In the current study, PD98059 was used at 10 μM, while other report suggested to use it within 10-50 μM (Roberts, 2001).

The contraction evoked by 100 μM UDP-glucose plateaued within 60-180 s (Figure 4.8C), in addition, 100 μM UDP-glucose induced an elevation with the level of phosphorylated MLC2 which peaked within 30-60 s (Figure 4.10). This indicates an involvement of the phosphorylated MLC2 in the signalling pathways of UDP-glucose-induced contraction. In contrast, UDP-glucose 100 μM elevated ERK1/2 phosphorylation, but this elevation was not significant until a later time of 300 s, while UDP-glucose failed to enhance significantly the level of phosphorylated ERK1/2 at lower time points (Figure 4.12C), where UDP-glucose induced its contraction. This is indicating that phosphorylated ERK1/2 may be not a major part of the signalling pathways involved in UDP-glucose-induced contraction. However, ERK signalling can function in the VSMC since it is associated with the cell

growth and differentiation (Howe *et al.*, 2002; Roy *et al.*, 2002), with phenotypic modulation in VSMC and regulating of contractile responses (Moses *et al.*, 2001; Schauwienold *et al.*, 2003). Therefore, further experiments could be conducted to investigate the functions of phosphorylated ERK1/2 in the smooth muscle of the pancreatic arteries. It is also important to examine the ability of UDP-glucose and MRS2690 to elevate the level of phosphorylated ERK1/2 in the presence of PPTN, to investigate whether this effect was mediated by the actions at $P2Y_{14}$ receptors, since other receptors could be involved in that effect namely, $P2Y_2$ receptor. In the current study, these experiments were not performed due to the limited availability of PPTN.

In summary, when $P2Y_{14}$ receptor (expressed mainly on the endothelium of the porcine pancreatic arteries) is activated by its agonists, this leads to dissociation of $G_{i\alpha}$ subunit from $G_{\beta\gamma}$ subunits and the receptor. Free $G_{i\alpha}$ subunits interact with adenylyl cyclase leading to an inhibition of the level of cAMP. The latter effect induces a calcium-independent contraction of the porcine pancreatic arteries through an elevation of the level of MLCK which phosphorylates MLC. G_i protein-derived $G_{\beta\gamma}$ subunits trigger PLCβ signalling pathways, resulting in an elevation of the level of IP_3 and DAG. IP_3 induces an elevation of the level of intracellular Ca^{2+}. The high level of Ca^{2+} may activate the release of endothelin, thromboxane and $PGF_2\alpha/PGH_2$, which then bind to their receptors, endothelin receptors and TP respectively. Endothelin receptors and TP receptors are $G_{q/11}$ protein-coupled receptors which involves an activation of PLC and hence a further elevation of the levels of IP_3 and DAG. Elevation of the level of IP_3 may increase the release of Ca^{2+} from ER, which will result in further increase within the level of intracellular Ca^{2+}. DAG induces an activation of PKC and Rho/ROCK. PKC activation is associated with phosphorylation of ERK in many cell types

(Langan *et al.*, 1994; Marquardt *et al.*, 1994; Zhao *et al.*, 2005). In addition, PKC together with Rho/ROCK signalling inhibit MLCP, increases Ca^{2+} sensitivity and promotes phosphorylation of MLC and contraction (Zhao *et al.*, 2005). The high level of Ca^{2+}, following binding to calmodulin, may activate MLCK, which leads to a phosphorylation of MLC, resulting in initiating the crossbridge between the actin and myosin leading to inducing a contraction of the porcine pancreatic arteries.

4.6. Conclusion

This chapter has demonstrated the signalling pathways underlying the responses to UDP-glucose, MRS2690 or UDP activating $P2Y_{14}$ receptor in porcine isolated pancreatic arteries. $P2Y_{14}$ receptor agonists induce vasoconstriction with an involvement of endothelin-1, thromboxane A_2 and prostaglandins, released from the endothelium, and then act at their respective receptors on the smooth muscles of the pancreatic arteries. In addition, $P2Y_{14}$ receptor activation by UDP-glucose involves an activation of Rho/ROCK signalling pathways, and the subsequent phosphorylation of MLC2, as well as the phosphorylation of ERK1/2. The ability of UDP-glucose and MRS2690 to inhibit cAMP levels indicates that $P2Y_{14}$ receptor is involved in G_i protein-coupled receptor mediated signalling. This study has provided evidence supporting the functional expression of $P2Y_{14}$ receptor and the signalling pathways involving in the contraction to the receptor agonists in porcine isolated pancreatic arteries.

Chapter Five

Investigation of the effects of UDP-glucose and MRS2690 on insulin secreted from the rat INS-1 823/13 β-cell line and rat isolated islets of Langerhans

5.1. Introduction

The activities of both endocrine and exocrine cells are regulated by parasympathetic and sympathetic nerves, as well as by hormones, autocrine and paracrine mediators. The pancreatic β-cell behaves as a fuel sensor that maintains blood glucose concentration within a narrow range by secreting insulin. The modulation of glucose stimulated insulin secretion occurs by various pathways (section 1.13). The purinergic system has been described for a long time as a significant pathway involved in the regulation of GSIS (Rodriguez Candela *et al.*, 1963; Loubatieres-Mariani *et al.*, 1979; Chapal & Loubatieres-Mariani 1981; Burnstock & Novak, 2013). Previously, studies using immunohistochemistry have shown the expression of P2X1, P2X4, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor subtypes in rat pancreas (Coutinho-Silva *et al.*, 2001; Coutinho-Silva *et al.*, 2003). In addition $P2Y_2$ receptor expression was shown in human pancreas, as well as $P2Y_{11}$ and $P2Y_{12}$ receptors which were found in the human pancreatic islets (Stam *et al.*, 1996; Lugo-Garcia *et al.*, 2008). Recently, reports showed the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptors in the INS-1 β-cell line, both at the mRNA and protein levels (Lugo-Garcia *et al.*, 2007).

Regarding the effect of nucleotides on insulin release from β-cells, ATP was the first nucleotide studied in 1963, and it induced an increase in insulin release in both rabbit pancreas and in isolated rat and hamster pancreas (Rodrigue-Candela *et al.*, 1963; Loubatieres *et al.*, 1972; Feldman & Jackson, 1974; Loubatieres-Mariani *et al.*, 1976). The ability of ATP to induce an elevation in the insulin release is mediated by two different types of receptors: P2X and P2Y receptors (Petit *et al.*, 1998). The mechanism by which ATP induces insulin secretion via P2X or P2Y receptors involves $[Ca^{2+}]$ elevation (Squires *et al.*, 1994; Jacques-Silva *et al.*, 2010). Other P2 receptor agonists have also the ability to regulate insulin release from β-cells, including αβ-meATP which mimics the effect of ATP on insulin release, suggesting the involvement of P2X1 or P2X3 receptors (Petit *et al.*, 1987).

P2Y receptors may mediate biphasic insulin secretion from β-cells. UDPβS, a selective $P2Y_6$ agonist, induced concentration-dependent stimulation of insulin and glucagon secretion in isolated mouse pancreatic islets and purified β-cells during short-term incubation (1h), while the activation of $P2Y_6$ receptors, via UDPβS, during a longer period of 24h, enhanced insulin release only, especially at high glucose levels at 20 mM. This effect was abolished in the presence of MRS2578, a selective antagonist of $P2Y_6$ receptor (Parandeh *et al.*, 2008). UTP had no effect on insulin release in isolated mouse pancreatic islets and purified β-cells, but it increased insulin secretion in INS-1 cell line very weakly, indicating the involvement of $P2Y_2$ or $P2Y_4$ receptors in insulin secretion is not likely to be of major physiological importance (Verspohl *et al.*, 2002; Parandeh *et al.*, 2008).

Studies on both intact mouse pancreatic islets and isolated β-cells using quantitative real-time PCR revealed an expression of $P2Y_1$ and $P2Y_{13}$ receptors. The role of these receptors has been investigated using ADP, a

ligand of the $P2Y_1$ and $P2Y_{13}$ receptors. ADP had a dual effect on mouse pancreatic islets: promoting insulin secretion via $P2Y_1$ receptors and inhibiting the secretion via $P2Y_{13}$ receptors (Amisten *et al.*, 2010). This was shown by using the selective $P2Y_1$ antagonist, MRS2179, and the selective $P2Y_{13}$ antagonist, MRS2211, where the effects mediated by ADP at the respective receptors were confirmed (Amisten *et al.*, 2010). Regarding effects mediated by adenosine receptors on GSIS, several studies suggested that A_1 and A_{2B} receptors mediate inhibition of insulin secretion (see review by Rusing *et al.*, 2006; Burnstock & Novak, 2013). A_{2A} receptors mediated augmentation of GSIS in mouse pancreatic arteries (Ohtani *et al.*, 2013), which indicated that activation of P1 and P2 receptors can exert synchronised effects on insulin secretion which contributes to the regulation of glucose homeostasis.

There is increasing evidence to suggest that UDP-glucose can act as extracellular signalling molecules via cell surface $P2Y_{14}$ receptors (Xu *et al.*, 2012; Meister *et al.,* 2013). However, no report has indicated the direct effect of activation of $P2Y_{14}$ receptor on insulin or any other hormones released from the islet of Langerhans. Therefore, the aim of this chapter is to investigate the functional expression of $P2Y_{14}$ receptors in both rat INS-1 823/13 β-cell line and freshly isolated rat islet of Langerhans.

5.2. Materials and methods

5.2.1. Insulin secretion studies

Insulin secretion can be studied using several models, it can be studied in vivo or from perfused pancreas, isolated islets, β-cell line (monolayers or pseudoislets) (Howell & Taylor, 1968; Park *et al.*, 1999; Hohmeier *et al.*, 2000; Guo-Parke *et al.*, 2012; Lee *et al.*, 2013). Among these models, the use of freshly isolated exocrine-free islets of Langerhans in this study has some advantages: the effects of treatments on insulin release directly exhibit variations in islet cell activities. In addition, the isolated islets architecture is maintained, as well as the elimination of the influences of exocrine and acinar tissues, blood and immune system which would normally interfere with the islet responses to the agents. However, since this method utilises the collagenase enzyme (section 5.2.1.3) to digest the pancreas and liberates the islets, one limitation would be the potential damage to cell surface proteins during the isolation procedure which may affect the functions of the islets. In addition, proteolytic enzymes (e.g. protease, phospholipase) are released from the exocrine tissues during the tissue dissociation (autodigestion) which may hydrolyse the proteins and reduce the islet yields (Wolters *et al.*, 1989). This loss can be overcome by culturing the islets to allow recovery (Gingerich *et al.*, 1979; Marchetti *et al.*, 1995). In addition, it has also been suggested that addition of a high concentration (10%) of bovine serum albumin (BSA) to the digestion medium may be beneficial, as it suppresses the release of proteolytic enzymes from the exocrine tissues in the pancreas during the collagenase digestion, which would result in an increase of the islet yield (Wolters *et al.*, 1989; van Suylichem *et al.*, 1992).

The other model used in this chapter to study the insulin secretion is the rat β-cell line. The pancreatic β-cell line has some advantages, including as an abundant source of β-cells, in addition, the study could be performed in the absence of other cell types. Moreover, these cells display a lot of essential characteristics of the pancreatic β-cells, including the high level of insulin contents and the responsiveness to glucose (Asfari *et al.*, 1992). Nevertheless, it is been reported that the magnitude of the insulin response of these cell lines is less than that detected in freshly isolated rat islets (Asfari *et al.*, 1992). However, among β-cell line derivatives, the INS-1 823/13 β-cell line has been shown to be one of the highly responsive clones and was able to secrete relatively large amounts of insulin in response to glucose concentrations in a physiological range (Hohmeier *et al.*, 2000). In addition, the amount of insulin released from this cell line compares well with the response exhibited by freshly isolated rat islets (Zawalich & Zawalich 1996; Hohmeier *et al.*, 2000).

5.2.1.1. INS-1 823/13 β-cell secretion studies

Cell culture, The rat INS-1 832/13 β-cells were grown in T75 flasks at 37°C and 95% O_2, 5% CO_2 in a humidified atmosphere. The cells were passaged every 5 days by using 1 ml 0.05% (w/v) trypsin-EDTA. The culture medium was RPMI-1640 with 11.1 mM glucose supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, with 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells from passage 86-91 were used for all experiments.

Secretion assays, INS-1 823/13 β-cells were seeded in 24-well plates at a density of ~1 × 10^5 cells/well in 1 ml culture medium, and were grown to 100% confluence before assay. At 18 h before secretion experiments, the standard tissue culture medium containing 11.1 mM glucose was switched to fresh RPMI-1640 media with 5 mM glucose. Cells were washed in 1 ml Krebs-Ringer bicarbonate HEPES buffer (KRBH) (section 5.2.3), followed by 1 h incubation in 1 ml of the same buffer. Subsequently, the cells were incubated for 1 h at 37^{o}C in 0.5 ml of the incubation buffer with two different concentrations of glucose: basal glucose (2 mM) and maximal stimulatory glucose (20 mM), with or without UDP-glucose (100 μM, 1mM) or MRS2690 (10 μM), which was tested in the absence and presence of PPTN (1 μM), a $P2Y_{14}$ receptor selective antagonist. For determination of secreted insulin during glucose stimulation of INS-1 823/13 β-cells, samples were diluted (1:2) in assay buffer then analyzed using rat insulin radioimmunoassay (RIA) kit with ^{125}I-labelled insulin (section 5.2.3). The total amount of the protein per well was determined using the protocol in section 3.2.5, followed by determination of the amount of insulin secreted per well. The data were presented as a percentage of basal glucose (2 mM) induced insulin secretion.

5.2.1.2. Rat isolated islets of Langerhans

Islet Isolation, As the islets are dispersed throughout the pancreas, they first need to be isolated from the surrounding exocrine tissue. A two-step method was used to isolate the islets, utilising collagenase enzyme to digest the pancreas and to liberate the islets. Collagenase enzymes are usually applied by injection via the pancreatic bile duct or by chopping of the pancreas into small pieces followed by an incubation of the pancreatic tissues with collagenase at 37^{o}C (van Suylichem *et al.*, 1992). This procedure will specifically hydrolyse the collagen fibres which connect the endocrine and exocrine tissues together. Separation of the exocrine tissue from islets is then achieved by picking the islets or by density gradient purification techniques (Noguchi *et al.*, 2012).

5.2.1.3. Isolation of rat islets of Langerhans (performed by Dr. S.L.F. Chan)

The method that we utilised for the isolation of rat islets described below is based upon the protocols described by Howell & Taylor (1968); Chan *et al.* (2001) and Anderson *et al.* (2013) which used bicarbonate-buffered physiological saline solution (Gey & Gey, 1936) (G&G) (section 5.2.3) supplemented with glucose 4 mM.

Male Wistar rats 200-250g in weight were obtained from Charles River (England, UK), and were used in this study. After stunning, they were killed by cervical dislocation. Rats were killed using the Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals (Scientific Procedures) Act 1986. The pancreas was isolated and separated from the spleen, fat and other tissues (intestine, lymph nodes, large blood vessels). The pancreas was distended with G&G bicarbonate-buffered medium using

a syringe fitted with a needle (a total volume of 7-l0 ml). Excess fat and blood vessels, which were clearly visible, were then dissected. Subsequently, the pancreas was placed in a small beaker and chopped vigorously as finely as possible with scissors to obtain uniformly small pieces (~1 mm^3 pieces). The pieces were then transferred to a 15 ml centrifuge tube and were spun down gently (2000 rpm, 3 s) and the supernatant was pipetted off completely. The segments were then poured into a 25 ml conical flask and 10 ml of a solution of collagenase P (0.5 mg/ml) in bicarbonate buffer was applied. The flask was vigorously shaken (~200 shakes/minute) for 5-6 min at 37^{o}C using Griffin flask shaker (Griffin and George Ltd., UK) until a fine, smooth digest, free of exocrine fragments was obtained. The incubation time was checked by the size and appearance of the fragments in the flask. The reaction was ended by pouring the solution into a 20 ml conical flask, and adding the bicarbonate buffer followed by spinning down gently. Islets and fragments of exocrine tissue separated out at the bottom of the tube and the supernatant was poured off. The fragments were resuspended in a further 10 ml of G&G buffer. Exocrine-free islets were manually isolated from the digest using finely drawn-out Pasteur pipettes under a dissecting binocular SZ4045 microscope (Olympus, Essex, UK). The total time taken from the death of the animal to the isolation of islets, ready for incubation, was 45-60 min. Yields of 150-300 islets were obtained from a single rat pancreas.

5.2.1.4. Islet static incubation studies

The buffer used for these experiments was G&G bicarbonate-buffered saline with 1 mg/ml BSA. The following protocol describes the use of 96-well plates for the islets studies. 200 µl buffer (at the required glucose

concentrations and reagents) were first added to the wells. Batches of 3 islets were carefully added to each well using a finely drawn-out Pasteur pipette. The islets were incubated at 37°C for 1 h. At the end of the incubation period, the 96-well plate was agitated to allow mixing and then transferred on ice for 5 min to stop further insulin secretion. Samples of the incubation media were then taken from each well and were analysed using rat insulin radioimmunoassay (RIA) kit with ^{125}I-labelled insulin (section 5.2.3) or samples were stored at -20°C for later use. The data were presented as ng of insulin secreted/islet/hour. The working range of the insulin assay was from 0.156-5 ng/ml.

5.2.1.5. Radioimmunoassay (RIA)

RIA is a very sensitive, competition-based assay which is utilised to quantify the antigens (e.g. insulin) using specific antibodies (e.g. anti-insulin) to form an antigen-antibody complex. To perform a RIA, a known quantity of an antigen is made radioactive, by labelling it with gamma-radioactive isotopes of iodine (Iodine-125 (^{125}I)). This radiolabelled antigen is then mixed with a known amount of antibody for that antigen, and as a result, a complex of antigen-antibody would be constructed. A sample with unknown quantity of that same antigen (non-radiolabelled) is added. This causes the unlabelled (or "cold") antigen from the unknown sample to compete with the radiolabelled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabelled variant, and reducing the ratio of antibody-bound radiolabelled antigen to free radiolabelled antigen. At the end of the reaction, a separation step is performed to isolate antigen-antibody complex from unbound reagents, “hot” and “cold” antigen. Thus,

level of radioactivity of antigen-antibody complex will be inversely proportional to the concentration of "cold" antigen. Using standard samples of known amounts of "cold" antigen, a binding curve can then be generated where concentration of the "cold" antigen (x-axis) is plotted against radioactivity (y-axis). That will allow the amount of antigen to be estimated by the amount of radioactivity in the antigen-antibody complex.

5.2.1.6. Insulin RIA

All solutions used in the experiment were made using insulin assay buffer (IAB) (section 5.3.3). The primary antibody was raised in guinea-pig against highly purified rat insulin. Purified rat insulin was used as a standard and at a stock of 10 ng/ml concentration. The standard curve of insulin standards of concentration range (0.156-5 ng/ml) was constructed by two-fold serial dilution of the 10 ng/ml standard in IAB. The assay also contained non-specific binding and total radioactivity controls.

All samples and standards were assayed in duplicate, where 50 µl samples or standards were incubated with 50 µl of guinea pig anti-rat insulin serum and 50 µl of ^{125}I-insulin tracer. Subsequently, the samples and the standards were incubated overnight at 4^{o}C. The next day, the primary antibody was separated from solution using 0.5 ml of the precipitating reagent, followed by mixing and incubating of the tubes at 4^{o}C for 20 min. The role of precipitating reagent is to precipitate the secondary-primary antibody complex, which would otherwise normally need an additional overnight incubation period to get precipitated (Peterson & Swerdloff, 1979). The samples were then centrifuged at 3000 rpm (Eppendorf Centrifuge 5810R) for 20 min at 4^{o}C and the supernatant was then carefully aspirated off, making sure not to disturb the pellets. The amount

of radiation being emitted by the pellets was counted using a Packard Cobra II γ-counter (Perkin-Elmer, MA, USA). The insulin content within each sample was automatically determined, as ng of insulin/ml, by interpolation of the RIA standard curve by the Packard Cobra II γ-counter.

5.2.2. Western blotting

Segments of porcine heart (PH) and rat brain (RB) were collected and homogenised with a borosilicate glass homogeniser in lysis buffer (see reagents and drugs section 3.2.6), containing protease inhibitor cocktail tablets, EDTA-free. INS-1 823/13 cells and rat isolated islets were collected in lysis buffer. After removal of a sample for a protein assay (section 3.2.5), samples were diluted 1:6 into solubilisation buffer 6×SB: (see reagents and drugs section 3.2.6), and were heated at 95°C for 5 min. Subsequently, electrophoresis was carried out on 4-20% Tris-Glycine (PAGE) Gold Precast Gels (Bio-Rad, Hercules, CA, U.S.A.), 10 µg protein per lane was loaded for all of these samples.

Samples were transferred to nitrocellulose membranes. Next, blots were incubated in blocking solution (5% (w/v) powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (Fisher Scientific UK Ltd., Loughborough, UK)) for 60 min, at room temperature. Blots were incubated overnight at 4°C with primary antibodies against $P2Y_{14}$ protein (1:500) and against GAPDH (1:10000), diluted in the blocking solution. After washing in Tris-buffered saline containing 0.1% (v/v) Tween 20, the blots were incubated with an appropriate IRDye® secondary antibody (Li-Cor Biosciences, Biotechnology, Lincoln, NE, USA). Proteins were visualised

using the Licor/Odyssey infrared imaging system (Biosciences, Biotechnology).

5.2.3. Reagents and drugs and cell culture media

1x G & G bicarbonate-buffered saline was composed of the following (mM): NaCl 111, $NaHCO_3$ 25.2, KCl 5, $MgCl_2.6H_2O$ 1, $MgSO_4.7H_2O$ 0.3, Na_2HPO_4 0.4, KH_2PO_4 0.3 and $CaCl_2$ 1. This was gassed for 10 min with 95% O_2, 5% CO_2 prior to the experiment to oxygenate the buffer and adjust the pH of the buffer to 7.4. During the experiment, the buffer was periodically re-gassed to maintain the pH at 7.4. KRBH was composed of the following (mM): NaCl 135, KCL 3.6, NaH_2PO_4 5, $MgCl_2$ 0.5, $CaCl_2$ 1.5, HEPES 10, and 1 mg/ml BSA pH 7.4. Precipitating reagent and IAB were part of the rat insulin RIA Kit (cat No. #RI-13K) which was purchased from LINCO Research (St. Charles, MI, USA). BSA was purchased from Wilfred Smith, Edgeware, U.K. For information about the sources and the solvents of other reagents and drugs, see section 3.2.6.

5.3. Statistical analysis

The mean of insulin secretion rate was determined for each group (both control and test groups). Outliers were removed based on objective judgement to reduce variation (standard error of the mean > 2 SDs). For rat INS-1 832/13 β-cells experiments, the mean of insulin secretion rates within the experiment were then standardised against the 2 mM glucose control group, as the mean of insulin secretion rates were expressed as a percentage of insulin released in the presence of the 2 mM glucose control (which was always 100%). Variation was expressed as the mean ± SEM. Non-parametric Kruskal-Wallis test was used followed by non-parametric Mann-Whitney unpaired test (Prism, GraphPad, San Diego, CA, USA). The non-parametric tests were used since the values were standardised to a control. Therefore, normal distribution of data cannot be assumed. For all experiments, P value < 0.05 was considered significant.

With regards to the secretion experiments with rat isolated islets, variation was expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used followed by Student's unpaired *t*-test (Prism, GraphPad, San Diego, CA, USA). For all experiments, P value < 0.05 was considered significant.

5.4. Results

5.4.1. Effect of $P2Y_{14}$ receptor activation on insulin released from rat INS-1 823/13 β-cells

To determine the effect of $P2Y_{14}$ receptor activation on the insulin released from β-cell, INS-1 823/13 β-cells were grown in appropriate medium (section 5.2.1.1) until they were 100% confluent. On the day of the experiment the cells were treated with two different concentrations of glucose: one is a basal (2 mM) and the other is the maximal stimulatory concentration (20 mM) of glucose. As seen in Figure 5.1, the insulin secretion was stimulated five-fold as glucose concentration was increased from 2-20 mM ($P < 0.01$, n=9). This level of elevation was similar to the range described by Hohmeier *et al.* (2000), Yang *et al.* (2004), Winzell *et al.* (2006) and Youl *et al.* (2010).

In the presence of UDP-glucose (100 μM, 1 mM) or MRS2690 (10 μM), the level of insulin secreted from INS-1 β-cells was significantly attenuated ($P < 0.05$, n=4-9) (Figure 5.1). In contrast, MRS2690 was unable to alter the amount of insulin secreted from INS-1 823/13 β-cells in the presence of PPTN, a selective high affinity antagonist of $P2Y_{14}$ receptor (Figure 5.1), indicating that its effect was mediated by acting at $P2Y_{14}$ receptor. In the presence of basal glucose concentration (2 mM), PPTN itself was able to elevate significantly the level of insulin secreted from INS-1 823/13 β-cells ($P < 0.05$, n=4-9). In contrast, it had no significant effect on the insulin secretion in the presence of stimulatory glucose concentration (20 mM) (Figure 5.1).

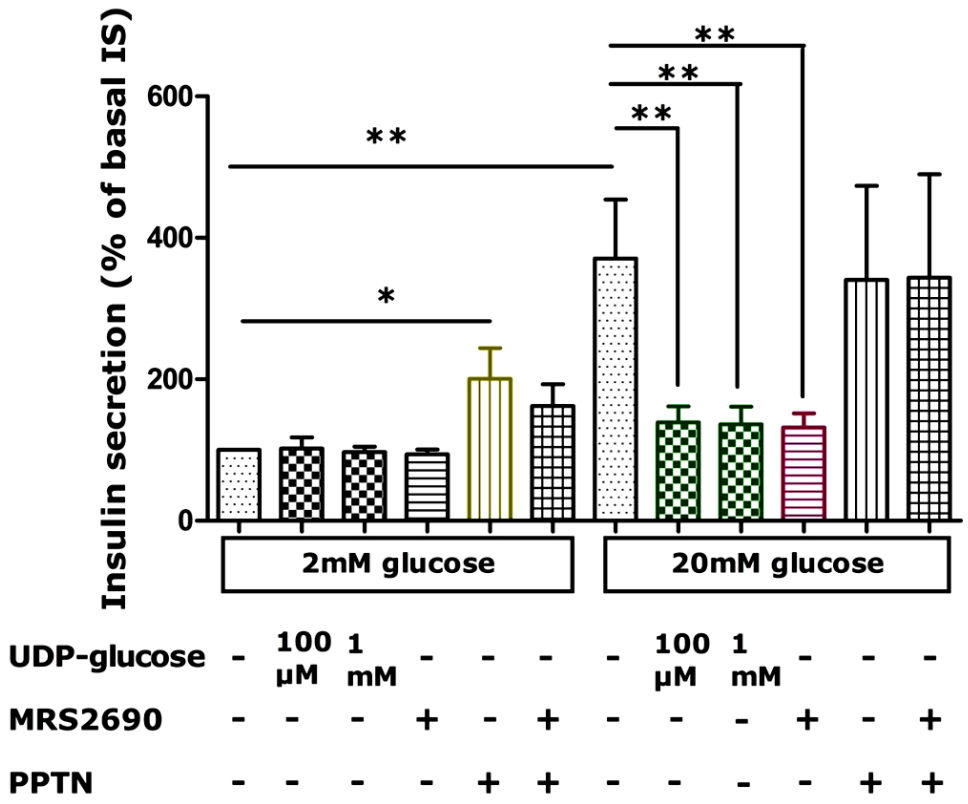


Figure 5.1. The effect of $P2Y_{14}$ receptor agonists, UDP-glucose (100 µM, 1 mM) or MRS2690 (10 µM), in the presence or absence of PPTN (1 µM) on insulin released from rat INS-1 832/13 β-cells. Cells were incubated with low (2 mM) or high (20 mM) concentrations of glucose. Data were presented as a percentage of basal insulin secretion. (*P < 0.05, **P < 0.01, non-parametric Mann-Whitney unpaired test, n=4-9).

5.4.2. Effect of UDP-glucose on GSIS in rat isolated islets of Langerhans

Islets were incubated at 3 mM, 10 mM and 20 mM glucose in order to assess for islet viability and glucose responsiveness. These concentrations represent basal, intermediate and maximal levels of GSIS (Figure 5.2). The insulin secretion was stimulated by two-fold as glucose concentration was increased from 3-10 mM, while the stimulation was three-fold as the glucose concentration was increased to 20 mM (Figure 5.2). These findings were similar to reports by Chan *et al.* (2001) and Anderson *et al.* (2013) in which the stimulation of insulin secreted by rat isolated islets was four-fold when the glucose concentration was increased from 4-20 mM.

To determine the effect of $P2Y_{14}$ receptors activation on the insulin released from the islet of Langerhans, islets were incubated with UDP-glucose (100 µM or 1 mM) in the presence of glucose 10 mM. As seen in Figure 5.2, in the presence of UDP-glucose (100 µM or 1 mM), the insulin secreted from the islets were significantly attenuated to the basal level relative to the amount of insulin secreted at 10 mM glucose ($P < 0.05$, $n=5$) (Figure 5.2), indicating the ability of the $P2Y_{14}$ receptor ligand to regulate the insulin secretion by rat isolated islets.

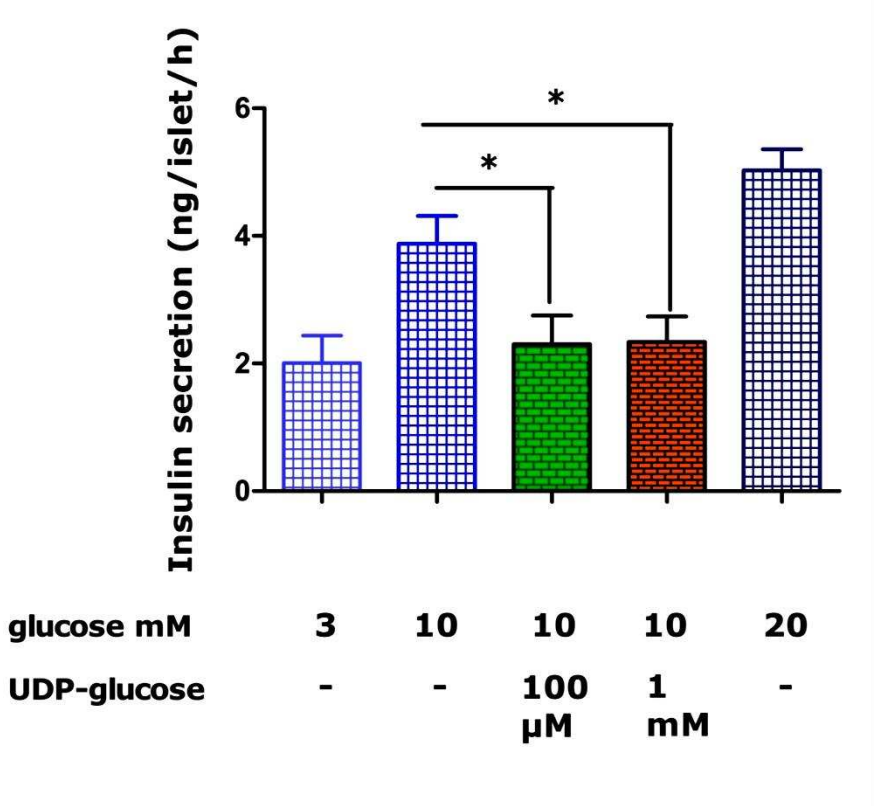


Figure 5.2. UDP-glucose inhibited GSIS in rat isolated islets of Langerhans. Islets were incubated at the indicated glucose concentrations for 1 h at 37°C. The treatments ($P2Y_{14}$ receptor ligand, UDP-glucose 100 µM or 1 mM) were performed in the presence of glucose 10 mM. Data were presented as changes in quantity of insulin released from the islet cells per hour. UDP-glucose at both concentrations significantly attenuated the insulin secreted from the islets (*P < 0.05, one-way ANOVA with Bonferroni's post hoc test, n=5).

5.4.3. Investigation of the expression of $P2Y_{14}$ receptor in rat isolated islets of Langerhans and in rat INS-1 823/13 β-cells

The expression of $P2Y_{14}$ receptor was investigated using a rabbit polyclonal antiserum against C-terminal tail of the $P2Y_{14}$ receptor (green bars), while the total amount of the protein in each sample was determined in the presence of mouse GAPDH monoclonal antibody (red bars).. GAPDH monoclonal antibody showed an immunoreactive band at around 37 kDa, while $P2Y_{14}$ receptor antibody showed an immunoreactive band at around 41 kDa. As seen in Figure 5.3, $P2Y_{14}$ protein was expressed in rat brain (RB), used as a positive control, since $P2Y_{14}$ receptor protein is widely expressed throughout the rat and human brain (Moore *et al.*, 2003). In addition, $P2Y_{14}$ protein was also present in porcine heart (PH), which was also used as a positive control in the current study (section 3.4.9).

An immunoreactive band was obtained in the presence of the $P2Y_{14}$ antibody at around 41 kDa in rat brain and in porcine heart (Figure 5.3). These findings were consistent with other reports which showed the presence of $P2Y_{14}$ receptors in HEK-293 cells and liver hepatocellular cells (HepG2) with an immunoreactive band of approximately 41 kDa (Lifespan Biosciences, cat No. LS-C120603), and in human brain membranes and human $P2Y_{14}$ receptor-transfected HEK-293 cells with multiple immunoreactive bands of around 40-65 kDa (Moore *et al.*, 2003; Krzemiński *et al.,* 2008). Similarly, an immunoreactive band with the same size (41 kDa) was observed in rat islet (RI), rat INS-1 823/13 cells indicating the presence of $P2Y_{14}$ receptor in these cells. The band size observed in this section (41 kDa) was in accordance with that observed in rat heart and porcine pancreatic arteries in section 3.4.9.

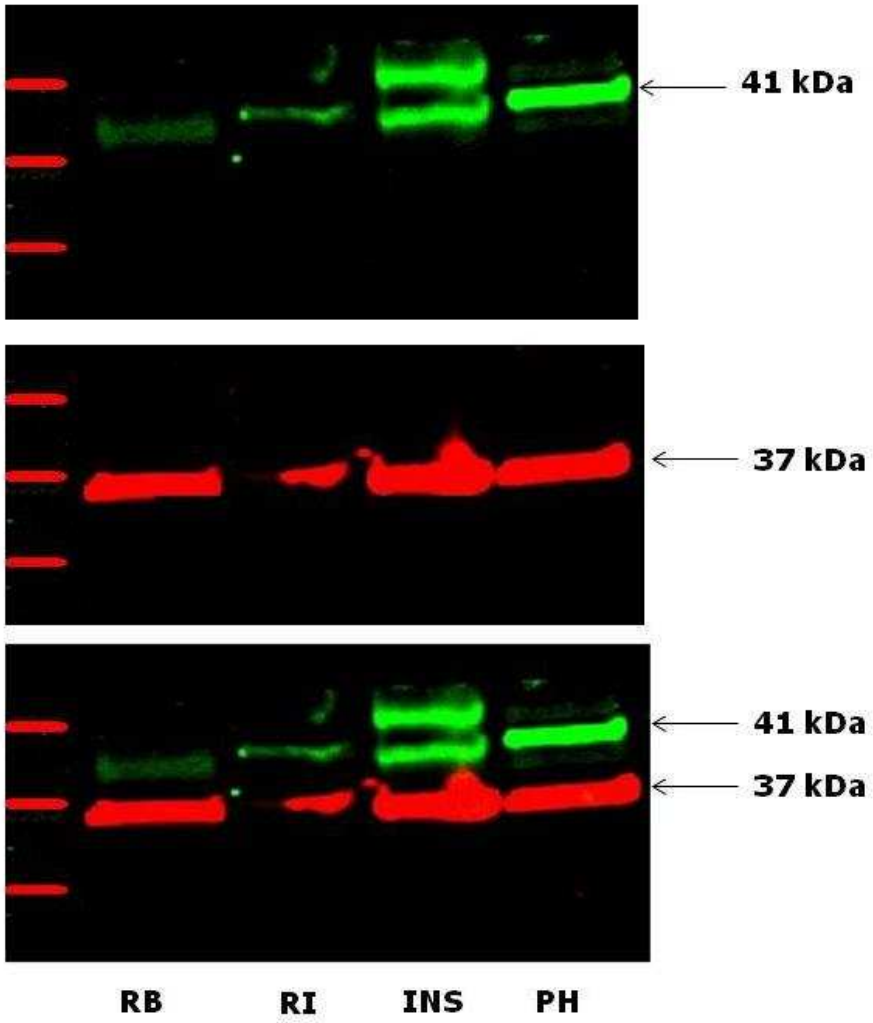


Figure 5.3. $P2Y_{14}$ protein expression detected by western blotting, in the presence of $P2Y_{14}$ antiserum (green bars). The total protein was determined using GAPDH antibody (red bars). An immunoreactive band was evident in rat brain (RB) (10 µg/lane), rat islet (RI) (10 µg/lane), rat INS-1 823/13 (10 µg/lane) and porcine heart (PH) (10 µg/lane) at around 41 kDa. The band sizes of the molecular weight marker from top to bottom are 50, 36, 25 kDa respectively. Blots are representative of staining from three separate experiments.

5.5. Discussion

The role of purine and pyrimidine receptors in modulation of insulin secretion has been described for a long time (Loubatieres-Mariani *et al.*, 1979; Chapal & Loubatieres-Mariani 1981; Burnstock & Novak, 2013). The expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, P2X1, P2X4 and P2X7 receptors was detected by immunohistochemical studies in the rat and mouse pancreas (Coutinho-Silva *et al.*, 2001; Coutinho-Silva *et al.*, 2003). In the current study, the expression of $P2Y_{14}$ receptor protein in rat INS-1 823/13 β-cell line and in rat isolated islets was determined using a specific antiserum against $P2Y_{14}$ protein. Samples taken from rat brain and porcine heart were used as positive controls, since the expression of $P2Y_{14}$ receptors in rat brain was shown elsewhere (Moore *et al.*, 2003; Krzemiński *et al.,* 2008). The expression of $P2Y_{14}$ protein in porcine heart was demonstrated in the current study in section 3.4.9. GSIS from rat INS-1 823/13 β-cell line, in the presence of 20 mM glucose, was significantly deceased following activation of $P2Y_{14}$ receptor with its selective agonists, UDP-glucose or MRS2690. While these agonists were not able to alter the insulin released from these cells under basal conditions (in the presence of 2 mM glucose) (Figure 5.1). In Figure 5.1, 10 μM MRS2690 decreased the insulin secretion to the same extent as 100 μM and 1 mM UDP-glucose although the concentration used for MRS2690 was 10-100-fold less than that of UDP-glucose, which suggests some greater potency of MRS2690 over UDP-glucose in the current experiment. The attenuation induced by the activation of $P2Y_{14}$ receptors by MRS2690 in rat INS-1 823/13 β-cell line was abolished in the presence of PPTN, a selective antagonist at $P2Y_{14}$ receptors (Figure 5.1), which indicated that the ability of MRS2690 to

decrease the level of insulin released from β-cells occurred through acting at $P2Y_{14}$ receptors.

Moreover, PPTN itself was able to elevate the basal insulin secretion from rat INS-1 823/13 β-cells (Figure 5.1). This suggests that there may be a constitutive release of UDP-glucose occurring by rat INS-1 823/13 β-cells. This release may attenuate the insulin secretion rate. The tonic inhibition of insulin release was then blocked in the presence of $P2Y_{14}$ receptor antagonist. Constitutive release of UDP-glucose from various cell lines including Calu-3 airway epithelial, COS-7, CHO-K1, and C6 glioma cells has been reported elsewhere (Lazarowski *et al.*, 2003b). Similarly PPTN induced concentration-dependent elevation of the basal forskolin-stimulated cAMP accumulation in C6 glioma expressed $P2Y_{14}$ receptor, with EC_{50} of 3-10 nM, while it exhibited no effect in wild-type cells. The previous findings were justified by the idea that the effect of PPTN on cAMP levels (in the absence of added agonist) occurs due to the blockade of the effect of UDP-glucose, released from these cells into the medium (Barrett *et al.*, 2013). Alternatively, PPTN could act as an inverse agonist since $P2Y_{14}$ receptor may be constitutively active that is, its activity does not depend on binding of ligand (Kenakin, 2004).

A large number of reports have indicated the ability of purine and pyrimidine nucleotides to regulate the level of insulin secreted from pancreatic islets (Loubatieres-Mariani *et al.*, 1979; Fernandez-Alvarez *et al.*, 2001; Parandeh *et al.*, 2008; Meister et al., 2013). In general, the role of purine and pyrimidine nucleotides as modulators of insulin secretion involves stimulation of insulin secretion (see reviews by Burnstock & Novak 2012, Novak, 2008). In contrast, few reports described the ability of some nucleotides to inhibit the insulin secretion by pancreatic islets. One such is

the report of Amisten *et al.* (2010) where ADP was able to decrease the level of insulin secreted from mouse pancreatic islets following activation of $P2Y_{13}$ receptors, and this effect was blocked by a competitive antagonist at $P2Y_{13}$ receptor. In the current study, following activation of $P2Y_{14}$ receptors in isolated rat islets by the selective ligand, UDP-glucose, the level of insulin secreted from the cells was significantly decreased in the presence of glucose 10 mM (Figure 5.2), which showed the ability of UDP-glucose to inhibit the insulin released from freshly isolated rat islets. Similarly, $P2Y_{14}$ receptor was described to play a significant role in glucose homeostasis in the report of Xu *et al.* (2012), which was shown via using a GPR105 knocked-out model. Alternatively, it is recommended to investigate the concentration-response curves for multiple concentrations of UDP-glucose and MRS2690 which would allow calculation of the pharmacological values for these agonists, including EC_{50}, R_{max}. By calculating these values, a comparison between their efficacies and potencies would be conducted. In addition, this would be useful for characterisation the effects of MRS2690 and UDP-glucose in the presence of PPTN. It is also recommended to determine whether there is a constitutive release of UDP-glucose in rat isolated islets, which could be addressed by examining the effect of PPTN in the absence of exogenous UDP-glucose. If PPTN is able to elevate the basal insulin secretion from rat isolated islets that may suggest a physiological role of this receptor in rat islets. Alternatively, the level of UDP-glucose can be measured at basal, intermediate and maximal levels of glucose. This measurement will determine whether there is any constitutive release of UDP-glucose in rat islets. The level of UDP-glucose can be assayed based on the protocol used by Lazarowski *et al.* (2003b); UDP-glucose pyrophosphorylase catalysed the conversion of UDP-glucose to [^{32}P]UTP in the presence of [^{32}P]pyrophosphate. Subsequently, HPLC separates

[^{32}P]UTP from [^{32}P]PPi, then the determination of [^{32}P]UTP would allow an accurate quantification of the extracellular UDP-glucose concentrations (Lazarowski *et al.,* 2003b).

The ability of $P2Y_{14}$ receptor agonists to lower the level of insulin released from INS-1 823/13 β-cell line and from isolated rat islets is consistent with their coupling to $G_{i/o}$ protein, since the ability of cAMP to increase the GSIS from pancreatic β-cells is well-established (see review by Furman *et al.*, 2010). The mechanism by which cAMP modulates insulin release has been studied in several reports, including the review of Furman *et al.* 2010. Some reports suggested that cAMP/PKA inhibits β-cell K_{ATP} channels, resulting in depolarisation of the β-cell membrane potential, and hence Ca^{2+} influx, resulting in insulin granule exocytosis (Brisson & Malaisse 1973; Suga *et al.*, 1997; Kang *et al.*, 2006). Other studies showed that the ability of cAMP/PKA to promote insulin release involves a mechanism at a site distal to the elevation of intracellular Ca^{2+}, with increased effectiveness of the K_{ATP} channel-independent action of glucose (section 1.10.2) (Yajima *et al.*, 1999). The ability of $P2Y_{14}$ receptor agonists to lower cAMP level was shown earlier in section 4.4.5, which is consistent with their ability to decrease the level of insulin released from pancreatic β-cells in the current study. Further studies are essential to investigate the effect of UDP-glucose or MRS2690 on cAMP level in rat INS-1 823/13 β-cell line or in isolated rat islets, which can be addressed by a direct measurement of cellular cAMP levels in these cells (using the protocol in section 4.2.6) in the presence of UDP-glucose or MRS2690 in the absence or presence of PPTN. In addition, the effect of $P2Y_{14}$ receptor activation in rat INS-1 823/13 β-cell line or in isolated rat islets, needs to be investigated in the presence of PTX (G_i inhibitor), to confirm the involvement of $P2Y_{14}$ receptor coupling to G_i protein in the UDP-glucose and MRS2690 mediated inhibition of the insulin

secretion. Other signalling pathways might be also involved in the $P2Y_{14}$ receptors mediated inhibition of insulin secretion.

Further studies are required to investigate the effect of UDP on insulin secretion from INS-1 823/13 β-cell line and from isolated rat islets, since UDP was reported to be a potent agonist at $P2Y_{14}$ receptors (Carter *et al.*, 2009; Harden *et al.*, 2010). Additionally, UDP also acts as an agonist at $P2Y_6$ receptors (Mamedova *et al.*, 2004). The data presented by Parandeh *et al.* (2008) and Ohtani *et al.* (2008) showed that UDP induced a dose-dependent stimulation of insulin in isolated mouse pancreatic islets at lower concentrations up to 10 μM, which was mediated by $P2Y_6$ receptors, while UDP at higher concentration (100 μM) decreased the insulin release from isolated mouse pancreatic islets, which was suggested to be mediated via acting at other receptors. Therefore, investigation of the effect of UDP on insulin release from rat INS-1 823/13 β-cell line and from isolated rat islets, with a range of concentrations, seems essential. This determines whether UDP would induce insulin secretion elevation or inhibition mediated by $P2Y_6$ or $P2Y_{14}$ receptors respectively. In addition, investigation of the effect of $P2Y_{14}$ receptor activation on insulin secretion by human islets would indicate whether species differences exist in the role of $P2Y_{14}$ receptor controlling the insulin secretion. Moreover, if PPTN is able to abolish the inhibitory effect evoked by UDP-glucose on insulin release from human islets that may suggest a therapeutic value of PPTN for patients with impaired glucose tolerance or type 2 diabetes. In addition, as part of characterisation of the role of $P2Y_{14}$ receptor in the pancreas, the effects of $P2Y_{14}$ receptor agonists on other hormones secreted from the pancreas including glucagon should be examined, to identify completely the role of this receptor in the endocrine pancreas, and that might suggest a physiological role of $P2Y_{14}$ receptor in the pancreas. Additionally, it is

necessary to examine the effect of UDP-glucose on the exocrine secretion to get a comprehensive view about the role of $P2Y_{14}$ receptors in the pancreas.

5.6. Conclusion

The data presented in this chapter provides evidence for the functional expression of $P2Y_{14}$ receptors in rat isolated islets and in rat INS-1 823/13 β–cell line. The expression of $P2Y_{14}$ protein in rat isolated islets and in rat INS-1 823/13 β-cell line was shown by western blotting. Activation of $P2Y_{14}$ receptors by the selective agonists, UDP-glucose and MRS2690 induced an inhibition of the glucose-stimulated insulin release from rat isolated islets and from rat INS-1 823/13 β–cell line. The mechanism by which $P2Y_{14}$ receptors induced the attenuation of the insulin released from β-cells is still to be elucidated. However, it is believed that cAMP inhibition by $P2Y_{14}$ receptor agonists (section 4.4.5) is at least part of the signalling pathways underlying $P2Y_{14}$ receptor-induced inhibition of the insulin release, although other signalling may be involved. When $P2Y_{14}$ receptors were antagonised by PPTN, MRS2690 failed to decrease the insulin secreted from β-cells. In addition, PPTN was able to elevate basal insulin secreted from INS-1 823/13 β-cells, suggesting that $P2Y_{14}$ receptor may play a role as an autocrine regulator of insulin secretion and may serve as a target for treatment of diabetes mellitus. Modulation of the effect of $P2Y_{14}$ receptors on insulin released from islets of Langerhans may be of importance in patients with impaired glucose tolerance or with insulin resistance.

Chapter Six

General discussion

This study investigated the functional expression of P2X1, $P2Y_1$, $P2Y_2$ and/or $P2Y_4$, $P2Y_{14}$ and A_{2A} adenosine receptors in porcine isolated pancreatic arteries (Chapter 2,3,4), as well as characterisation of $P2Y_{14}$ receptors in rat INS-1 832/13 β-cells and in rat isolated islets of Langerhans, including examining the effects of UDP-glucose and MRS2690 on insulin secretion (Chapter 5). The study focused primarily on the functional expression of $P2Y_{14}$ receptors and the signalling pathways underlying the vasoconstriction-evoked by $P2Y_{14}$ receptor agonists in porcine pancreatic arteries. There is currently a lack of information about the role of $P2Y_{14}$ receptors in the cardiovascular system, although both $P2Y_{14}$ mRNA and protein are present in the heart and blood vessels (Musa *et al.*, 2009; Umapathy *et al.*, 2010; Abbas *et al.*, 2011).

6.1. Characterisation of P1 and P2 receptors in porcine pancreatic arteries

The expression of P2X1, P2X2, $P2Y_1$ and $P2Y_2$ receptors has been detected by immunohistochemistry earlier in rat pancreatic vasculature (Coutinho-Silva *et al.*, 2001; Coutinho-Silva *et al.*, 2003). Although a few reports investigated the functional expression of P1 and P2 receptors in rat pancreatic arteries, there is no report investigating P1 and P2 receptors in porcine pancreatic arteries. In the current study, P1 and P2 receptors and their signalling are described in arteries of the pig pancreas. The expression of P1 and P2 receptors on the VSMCs or ECs of the porcine pancreatic arteries, which has been observed in the current study, is shown in figure 6.1.

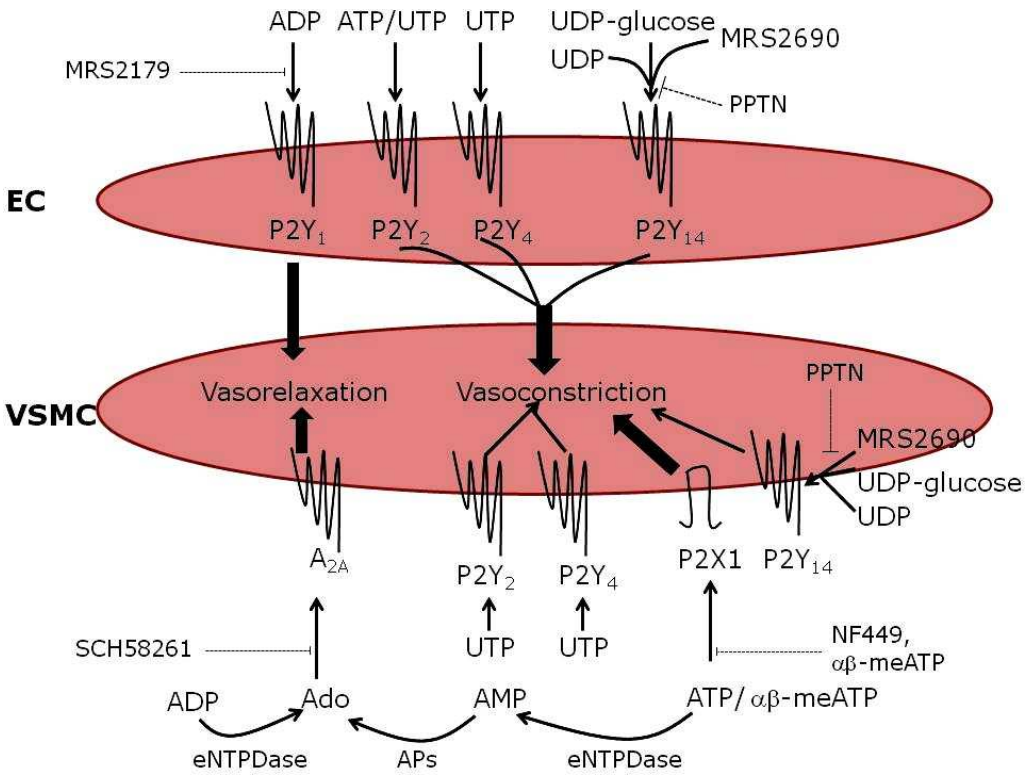


Figure 6.1. A schematic representation of P2X1, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{14}$ and A_{2A} adenosine receptors expressed on the endothelial cells (ECs) or vascular smooth muscle cells (VSMCs) of the porcine pancreatic arteries, together with their selective agonists and antagonists. ATP and ADP are broken down by ecto-nucleoside-5′-triphosphate diphosphohydrolases (eNTPDases) to AMP, which is broken down to adenosine (Ado) by the activity of GPI-anchored alkaline phosphatases (APs). Arrows represent positive influences, while dotted arrows represent the negative influence.

Porcine pancreatic arteries express some P1 and P2 relaxatory receptors, since ADP and ATP induced vasorelaxation in these arteries (Chapter 2). The relaxation to ADP was shown to be mainly mediated by A_{2A} receptors, as that relaxation was inhibited in the presence of SCH58261, a selective adenosine A_{2A} receptor antagonist, and XAC, an adenosine receptor antagonist. The expression of A_{2A} receptors was shown to be on the VSMCs

of the pancreatic arteries, since the removal of the endothelium had less effect on the ADP-induced relaxation than that of SCH58261 or XAC. Similarly, the expression of A_{2A} receptors was shown to be on the VSMCs of rat smooth muscle cells and porcine coronary arteries (Schulte & Fredholm, 2003; Rayment *et al.*, 2007b). ATP also induced a relaxation in porcine pancreatic arteries following its contraction, and this relaxation was also evoked by adenosine receptors expressed on the VSMCs of the pancreatic arteries, without an involvement of the endothelium, since the relaxation was significantly inhibited in the presence of XAC, but not in endothelium-denuded arteries. The adenosine receptor subtypes involved in the relaxation to ATP were not identified in the current study, due to the time limit. Nevertheless, the findings obtained with ADP identify relaxatory adenosine A_{2A} receptors on the VSMCs of the pancreatic arteries, and these may be the subtype responsible for the relaxation to ATP, since the relaxations to ADP and ATP were significantly inhibited in the presence of XAC (10 μM), but this relaxation was not abolished in endothelium-denuded arteries. The relaxation to ADP was also inhibited in the presence of a selective adenosine A_{2A} receptor antagonist, SCH58261 (1 μM) to the same extent observed in the presence of XAC. However, further studies are still needed to identify the adenosine receptor subtype involved in ATP-induced relaxation in porcine pancreatic arteries, which could be addressed using SCH58261.

6.2. Characterisation of $P2Y_{14}$ receptor in porcine pancreatic arteries

The contractions to $P2Y_{14}$ receptor agonists were investigated following raising the vascular tone with U46619. The main reason for pre-constricting with U46619 was to allow investigation of a possible

vasodilator component to the response. Additionally, the presence of a vasoconstrictor such as U46619 represents the physiological state since the vascular tone, which is defined as the degree of vessel constriction relative to their maximally dilated state under basal tone conditions in vivo, is controlled by a number of mechanisms (Kur & Newman, 2013). These mechanisms include extrinsic or intrinsic innervation by the autonomic nervous system, and the local release of vasoactive agents from vascular cells (Hamel, 2006). In the current study, the contractions to $P2Y_{14}$ receptor agonists were also observed on basal tone, although the amplitude of that contraction was less than that in the presence of U46619, but that contraction was significantly attenuated in the presence of PPTN (section 3.4.3). Moreover, in the arteries which were pre-constricted with endothelin-1 (instead of U46619) (section 4.4.2), the contraction to UDP-glucose was apparent with a similar amplitude seen in the presence of U46619. Taken together, the previous observations indicate that the contractions to $P2Y_{14}$ receptor agonists are not restricted to contractions evoked by U46619, which is consistent with the functionally expressed $P2Y_{14}$ receptor in the arteries of the pig pancreas.

In Chapter 3, the functional expression of $P2Y_{14}$ receptor has been shown by using the selective agonists (UDP-glucose and MRS2690) at this receptor. The rank order of potencies of MRS2690 (10-fold) > UDP-glucose in eliciting vasoconstrictions in the pancreatic arteries was consistent with that reported previously in the literature, which showed that MRS2690 was 7-10-fold more potent than UDP-glucose (Jacobson *et al.*, 2009; Gao *et al.*, 2010), suggesting the involvement of $P2Y_{14}$ receptor in pancreatic arteries. Moreover, the inhibition of the contractions to UDP-glucose and MRS2690 which occurred in the presence of PPTN, a selective antagonist at $P2Y_{14}$ receptors (Robichaud *et al.*, 2011; Barrett *et al.*, 2013) indicated that the

contractions to UDP-glucose and MRS2690 occurred via actions at $P2Y_{14}$ receptors expressed in porcine pancreatic arteries. It is still required to investigate the concentration-response curves of UDP-glucose or MRS2690 in the presence of multiple concentrations of PPTN to allow us to create Schild plots, and determine slope, which would indicate whether PPTN is a competitive antagonist at $P2Y_{14}$ receptor in porcine pancreatic arteries. In addition, the IC_{50} value for PPTN needs to be determined, which would indicate the potency of that antagonist in the inhibition of the contractions to $P2Y_{14}$ receptor agonists. These experiments were not performed here due to the limited availability of PPTN.

A number of selective antagonists at $P2Y_{14}$ receptor have been developed as naphthoic acid derivatives by Gauthier *et al.* (2011). However, some of these antagonists were either non-competitive or bound with high affinity to serum albumin. PPTN was identified as a naphthoic acid derivative which bound with high affinity to $P2Y_{14}$ receptor but exhibited less human serum albumin binding (Robichaud *et al.*, 2011). Moreover, a prodrug derivative of PPTN (an ester of carboxylic acid) was also prepared which enhanced its bioavailability (Robichaud *et al.*, 2011). In the current study, PPTN showed some selectivity at the $P2Y_{14}$ receptor over $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in porcine pancreatic arteries (Chapter 3). In addition, Barrett *et al.* (2013) showed that PPTN at concentrations as high as 1 μM exhibited no effect on the ability of agonists to activate their respective P2Y receptors in cell lines. It can be anticipated from the previous observations that PPTN may exhibit selectivity at $P2Y_{14}$ receptor over other P2Y receptors. Although, it is still recommended to investigate the specificity of PPTN in in vivo studies, which may be of important for any clinical use in future. Subsequently, I believe that PPTN is a promising compound which may be used to develop

a drug with specificity at $P2Y_{14}$ receptors and with high bioavailability. This compound may be used for managing cardiovascular disease.

6.3. Characterisation of $P2Y_{14}$ receptor in rat β-cells; therapeutic approach of PPTN

The expression of purine and pyrimidine receptors in rat and mouse pancreas has been shown previously using immunohistochemical studies (Coutinho-Silva *et al.*, 2001; Coutinho-Silva *et al.*, 2003). However, the involvement of $P2Y_{14}$ receptor in endocrine or exocrine tissues has not been addressed previously. The $P2Y_{14}$ receptor could play an important role in these tissues, as its endogenous ligand (UDP-glucose) was shown to be released constitutively from different types of cells (but not endocrine or exocrine cells). Therefore, in Chapter 5, the effects of $P2Y_{14}$ receptor activation on insulin secretion were investigated. MRS2690 and UDP-glucose were able to inhibit glucose-induced insulin secretion, back to the basal level, in INS-1 832/13 β-cells. In addition, UDP-glucose attenuated the level of insulin secreted from rat isolated islets. The ability of MRS2690 to inhibit the insulin secretion from INS-1 823/13 β-cell line was abolished in the presence of PPTN. The findings in the current study are consistent with the ability of $P2Y_{14}$ receptor agonists to decrease the level of insulin secreted from these cells. However, it is still necessary to examine the ability of UDP-glucose and MRS2690 to inhibit the secretion of insulin from rat isolated islets in the presence of PPTN, to confirm that inhibition is mediated by an action at $P2Y_{14}$ receptors. Moreover, PPTN itself was able to increase the level of insulin secreted from the rat INS-1 823/13 β-cell line in the presence of basal glucose concentration, which may suggest a constitutive release of UDP-glucose from this cell. To confirm the

constitutive release of UDP-glucose occurred in rat INS-1 823/13 β-cell line, the basal level of UDP-glucose can be measured in this cell based on the protocol used by Lazarowski *et al.* (2003b), as described in Chapter 5.

Although the exact mechanisms remain to be established, an increase in pancreatic endocrine cell activity during hormone secretion corresponds with an increase in blood flow, to meet metabolic demand (Schaeffer *et al.*, 2011). Thus, alterations in blood flow can influence pancreatic function, as a reduction in pancreatic blood flow has been observed in acute and chronic pancreatitis and some other pancreatic diseases such as diabetes mellitus and impaired glucose tolerance (Satoh *et al.*, 2000; Ballian & Brunicardi, 2007; Nguyen *et al.*, 2010), implicating pancreatic tissue perfusion as an important factor in pathogenesis of pancreatic diseases and symptoms. There is increasing evidence for the role of purinergic signalling in the pathophysiology of the pancreas, as well as the involvement of purine and pyrimidine receptors in hormone secretion (see reviews by Burnstock & Knight 2004; Burnstock & Novak, 2012). Drugs designed to target specific component of the purinergic system may be of relevance to the management of pancreatic disorders including pancreatitis, cystic fibrosis, pancreatic cancer and diabetes.

In the current study, it has been shown that activation of $P2Y_{14}$ receptor by UDP-glucose in porcine pancreatic arteries induced a vasoconstriction, and hence that would result in decrease in blood flow to the pancreatic cells. The effect of UDP-glucose was abolished in the presence of PPTN. Moreover, UDP-glucose has been shown to be released constitutively from different type of cells, with levels ranging within 1-20 nM (Arase *et al.*, 2009). The level of the extracellular UDP-glucose may reach effective levels ranging within 10 nM- 1 mM during damage and/or injury to cells or when

the cells are subjected to mechanical forces (e.g., shear, cell swelling, hydrostatic pressure) (Lazarowski *et al.*, 2003b; Arase *et al.*, 2009). Furthermore, the level of intracellular UDP-glucose may be regulated by the glucose level, as the level of UDP-glucose would be elevated following an increase in the level of glucose, since glucose is converted to glucose-1-phosphate before being converted to glycogen by the activity of glycogen synthase (Seoane *et al.*, 1996). Glucose-1-phosphate is the precursor for UDP-glucose synthesis (Chapter 1), which suggests a relationship between the glucose level and the production of UDP-glucose. Therefore, it can be proposed that when the level of UDP-glucose is elevated (during some disorders, such as diabetes), that would induce a decrease in blood flow to the pancreas, which may result in a decrease of the hormone secreted from the islets, including the secretion of the insulin. In Chapter 5, UDP-glucose had also a direct inhibitory effect on insulin secretion from β-cells, an effect which was abolished in the presence of PPTN. Consequently, that would raise the therapeutic value of PPTN, since the usage of PPTN would abolish the tonic inhibitory activity induced by endogenous UDP-glucose, which may result in an attenuation of the complications accompanied with some diseases including diabetes. It is recommended to measure the level of UDP-glucose in tissue cultured with increasing glucose concentrations, to indentify the relationship between the level of UDP-glucose and different concentrations of glucose.

It is still required to examine the effect of UDP-glucose on the blood flow in small porcine pancreatic arteries, using the wire myograph system, since it is well known that smaller arteries are more involved in the control of blood flow than larger arteries (Rutishauser, 1994). Thus, if we can control the blood flow in smaller pancreatic arteries, in the presence of PPTN, this would have a greater impact on patients with exocrine/endocrine

pancreatic dysfunctions than that in larger arteries. Alternatively, the blood flow to the exocrine/endocrine pancreatic cells can be determined (in vivo) in the pancreas (such as rodent) by using nonradioactive microspheres, injected into the left ventricle, according to the protocol of Lifson *et al.* (1980). This approach involves locating and counting microscopically the spheres in fixed and stained portions of the pancreas, following the animal sacrifice (Lifson *et al.*, 1980). By applying this approach the flow rate per bead can be determined in the presence of various compounds (including UDP-glucose in the presence and absence of PPTN) or in animal models of diabetes.

6.4. Functional expression of $P2Y_{14}$ receptor in other vessels; validation of the specificity of the $P2Y_{14}$ receptor antibody used in the study

Pharmacological studies demonstrated the functional expression of the $P2Y_{14}$ receptor in porcine coronary arteries, which was shown to be expressed on the VSMCs and the ECs (Abbas *et al.*, 2011). Activation of $P2Y_{14}$ receptor by its selective agonists, UDP-glucose and MRS2690, induced concentration-dependent contractions in coronary arteries (Abbas *et al.*, 2011). The previous observations were in agreement with the findings in the current studies. Similarly, Umapathy *et al.* (2010) showed that activation of $P2Y_{14}$ receptors with UDP-glucose induced inhibition in the forskolin-induced cAMP production, with an involvement of ERK1/2, in human lung microvascular endothelial cells. These findings were also in accordance with my findings in the current study. Moreover, the expression of $P2Y_{14}$ mRNA and protein was shown using RT-PCR, western blot, and immunofluorescence studies in these cells (Umapathy *et al.*, 2010) as well

as in pulmonary artery vasa vasorum endothelial cells (Lyubchenko *et al.*, 2011). On the other hand, contractile $P2Y_{14}$ receptor was not present in endothelium-denuded rat intrapulmonary arteries, as UDP-glucose had no effect on these arteries (Mitchell *et al.*, 2012). In addition, the absence of $P2Y_{14}$ mRNA, demonstrated by using RT-PCR studies, has been documented in human coronary artery endothelial cells and in mouse thoracic aorta (Kauffenstein *et al.*, 2010; Ding *et al.*, 2011). Taken together, the expression of contractile $P2Y_{14}$ receptor is not limited to the porcine pancreatic arteries, although some arteries lack the contractile $P2Y_{14}$ receptor. Therefore, it is recommended to investigate the functional expression of the contractile $P2Y_{14}$ receptor in various arteries, and if the role of this receptor is shown to be widespread, then it can be a novel target for the treatment of cardiovascular diseases.

In Chapter three and five, the expression of $P2Y_{14}$ receptor protein was shown by using western blotting, which indicated the presence of this receptor in porcine pancreatic arteries, rat INS-1 823/13 β-cell line and in isolated rat islet of Langerhans. Although the immunoreactive bands were completely eliminated in the absence of the $P2Y_{14}$ primary antibody (Figure 3.11) or following pre-incubation with the neutralizing antigen (section 3.4.9), attempts to validate the specificity of this antibody, by performing the same assay on undifferentiated HL-60 cells and on mouse thoracic aorta as negative controls, since they were shown to not express $P2Y_{14}$ mRNA (Fricks *et al.*, 2009; Kauffenstein *et al.*, 2010) were unsuccessful. In the current study, $P2Y_{14}$ receptor immunoreactive bands were observed in these tissues (Chapter 3). This observation suggested that there may be another cellular antigen identified by this antibody. In fact, there are a growing number of GPCRs which have been described as having poorly validated detection reagents. Some of these receptors are dopamine

receptors, α and β-adrenoceptors as well as $P2Y_6$ receptors (Yu *et al.*, 2013). Therefore, $P2Y_{14}$ receptor might be added to these collections of GPCRs, which may have deficient antibodies. Nevertheless, it is still required to look for rigorous controls to allow us to validate the specificity of $P2Y_{14}$ antibodies. Alternatively, the generation of GPCR-specific nanobodies (functional antibodies devoid of heavy chains) may become a useful approach to obtain specific GPCR antibodies (Harmsen *et al.*, 2007).

6.5. Future directions in identifying the role of $P2Y_{14}$ receptor

Inflammation has been implicated as a key initial trigger for type 2 diabetes, and as $P2Y_{14}$ receptor participates in inflammation reactions (Xu *et al.*, 2012). Thus the effect of PPTN on the insulin secretion from islet β-cells needs to be examined appropriate in in vivo animal models of type 2 diabetes, and then if PPTN were able to abolish the inhibitory effect of endogenous UDP-glucose on insulin secretion, that may suggest the use of PPTN as a lead compound for the design of drugs which can be used as a potential treatment for patients with type 2 diabetes. That may confirm the possible therapeutic benefit of this antagonist in prevention of the decrease of the insulin secretion, induced by elevated level of endogenous UDP-glucose, since it was discussed earlier that the level of UDP-glucose may be elevated during diabetes, as the level of blood sugar is increased.

In the current study, the physiological role of $P2Y_{14}$ receptor has not been investigated. Therefore, it is necessary to identify the role of this receptor in cardiovascular physiology to find out whether $P2Y_{14}$ receptor plays a major role in blood pressure regulation. Since the $P2Y_{14}$ receptor knockout mice is available (Bassil *et al.*, 2009), as well as $P2Y_{14}$ specific siRNA (Gao

et al., 2010), these effects could be addressed. However, $P2Y_{14}$ receptor knockout mice showed only an improvement in insulin sensitivity by inhibiting macrophage recruitment and tissue inflammation (Xu *et al.*, 2012), as well as exhibiting a marked resistance to tissue injury induced by infra red in utero (Kook *et al.*, 2013). In addition, UDP-glucose did not induce contractions in the stomach of $P2Y_{14}$ receptor knockout mice (Bassil *et al.*, 2009). Thus, it would seem from these reports that $P2Y_{14}$ receptor does not have a major role in blood pressure regulation. Further studies of this kind are still of relevance to investigate the effect of $P2Y_{14}$ receptors and its role in blood pressure regulation, since compensatory mechanisms may occur after receptor knockout.

In summary, this project characterised the $P2Y_{14}$ receptor in the pancreas, and identified a novel vasocontractile role of $P2Y_{14}$ receptor in porcine pancreatic arteries, antagonised by PPTN. This study confirmed that $P2Y_{14}$ receptor is coupled to G_i protein, which was consistent with previous reports. $P2Y_{14}$ receptor evoked an inhibitory effect on the insulin secreted from the pancreas (Chapter 5), this effect was abolished by PPTN. This would give a hint that PPTN may be used as a lead for compounds that may be used in the management of some pancreatic disorders. It is hoped that the information presented in this thesis will aid future investigations in the cardiovascular field to obtain a more complete understanding of the roles of purine and pyrimidine receptors in cardiovascular diseases.

References

Abbas Z, Latif L, Ralevic V (2011). Effects of $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690, on porcine coronary artery contractility, http://www.pA2online.org/abstracts/Vol9Issue3abst051P.pdf.

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, et al. (2003). Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24**(2): 52-55.

Abbracchio MP, Burnstock G (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacology & therapeutics* **64**(3)**:** 445-475.

Abbracchio MP, Burnstock G (1998). Purinergic signalling: pathophysiological roles. *Japanese journal of pharmacology* **78**(2)**:** 113-145.

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C*, et al.* (2006). International Union of Pharmacology LVIII: Update on the P2Y G Protein-Coupled Nucleotide Receptors: From Molecular Mechanisms and Pathophysiology to Therapy. *Pharmacol Rev* **58**(3)**:** 281-341.

Alefishat E, Alexander S, Ralevic V, (2010). Effect of palmitoyl CoA on ADP-evoked vasorelaxations in porcine isolated coronary and mesenteric arteries. http://www.fasebj.org/cgi/content/meeting_abstract/24/1_MeetingAbstracts/lb426.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995). PD 098059 Is a Specific Inhibitor of the Activation of Mitogen-activated Protein Kinase Kinase in Vitro and in Vivo. *Journal of Biological Chemistry* **270**(46)**:** 27489-27494.

Allsopp RC, Evans RJ (2011). The intracellular amino terminus plays a dominant role in desensitization of ATP-gated P2X receptor ion channels. *J Biol Chem* **286**(52)**:** 44691-44701.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T*, et al.* (1996). Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho-kinase). *Journal of Biological Chemistry***271**(34)**:** 20246-20249.

Amisten S, Meidute-Abaraviciene S, Tan C, Olde B, Lundquist I, Salehi A*, et al.* (2010). ADP mediates inhibition of insulin secretion by activation of $P2Y_{13}$ receptors in mice. *Diabetologia* **53**(9): 1927-1934.

Anderson RL, Randall MD, Chan SL (2013). The complex effects of cannabinoids on insulin secretion from rat isolated islets of Langerhans. *Eur J Pharmacol* **706**(1-3)**:** 56-62.

Ansari HR, Nadeem A, Tilley SL, Mustafa SJ (2007). Involvement of COX-1 in A_3 adenosine receptor-mediated contraction through endothelium in mice aorta. *Am J Physiol Heart Circ Physiol* **293**(6)**:** H3448-3455.

Arase T, Uchida H, Kajitani T, Ono M, Tamaki K, Oda H*, et al.* (2009). The UDP-glucose receptor $P2RY_{14}$ triggers innate mucosal immunity in the female reproductive tract by inducing IL-8. *Journal of immunology* **182**(11)**:** 7074-7084.

Aronoff SL, Berkowitz K, Shreiner B, Want L (2004). Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. *Diabetes Spectrum* **17**(3)**:** 183-190.

Asfari M, Janjic D, Meda P, Li GD, Halban PA, Wollheim CB (1992). Establishment of 2-Mercaptoethanol-Dependent Differentiated Insulin-Secreting Cell-Lines. *Endocrinology* **130**(1)**:** 167-178.

Ballian N, Brunicardi FC (2007). Islet vasculature as a regulator of endocrine pancreas function. *World J Surg* **31**(4)**:** 705-714.

Bar I, Guns PJ, Metallo J, Cammarata D, Wilkin F, Boeynams JM*, et al.* (2008). Knockout mice reveal a role for $P2Y_6$ receptor in macrophages, endothelial cells, and vascular smooth muscle cells. *Molecular pharmacology* **74**(3)**:** 777-784.

Barnard EA, Simon J (2001). An elusive receptor is finally caught: $P2Y_{12}$, an important drug target in platelets. *Trends Pharmacol Sci* **22**(8)**:** 388-391.

Barrett MO, Sesma JI, Ball CB, Jayasekara PS, Jacobson KA, Lazarowski ER*, et al.* (2013). A selective high-affinity antagonist of the $P2Y_{14}$ receptor inhibits UDP-glucose-stimulated chemotaxis of human neutrophils. *Mol Pharmacol* **84**(1)**:** 41-49.

Bassil AK, Bourdu S, Townson KA, Wheeldon A, Jarvie EM, Zebda N*, et al.* (2009). UDP-glucose modulates gastric function through $P2Y_{14}$ receptor-dependent and -independent mechanisms. *American journal of physiology. Gastrointestinal and liver physiology* **296**(4)**:** G923-930.

Bean BP (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol Sci* **13**(3)**:** 87-90.

Belardinelli L, Shryock JC, Ruble J, Monopoli A, Dionisotti S, Ongini E*, et al.* (1996). Binding of the novel nonxanthine A_{2A} adenosine receptor antagonist [3H]SCH58261 to coronary artery membranes. *Circ Res* **79**(6)**:** 1153-1160.

Bennett DW, Drury AN (1931). Further observations relating to the physiological activity of adenine compounds. *J Physiol* **72**, 28–320.

Bertelli E, Di Gregorio F, Mosca S, Bastianini A (1998). The arterial blood supply of the pancreas: a review. V. The dorsal pancreatic artery. An anatomic review and a radiologic study. *Surgical and radiologic anatomy* : SRA **20**(6): 445-452.

Best L, Yates AP, Tomlinson S (1992). Stimulation of insulin secretion by glucose in the absence of diminished potassium (86Rb+) permeability. *Biochemical pharmacology* **43**(11): 2483-2485.

Bockman DE (1992). Microvasculature of the pancreas. Relation to pancreatitis. *Int J Pancreatol* **12**(1)**:** 11-21.

Bodin P, Burnstock G (2001). Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol* **38**(6)**:** 900-908.

Bodor ET, Waldo GL, Hooks SB, Corbitt J, Boyer JL, Harden TK (2003). Purification and Functional Reconstitution of the Human $P2Y_{12}$ Receptor. *Molecular pharmacology* **64**(5)**:** 1210-1216.

Bogdanov Y, Rubino A, Burnstock G (1998a). Characterisation of subtypes of the P2X and P2Y families of ATP receptors in the foetal human heart. *Life sciences* **62**(8)**:** 697-703.

Bogdanov YD, Wildman SS, Clements MP, King BF, Burnstock G (1998b). Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br J Pharmacol* **124**(3)**:** 428-430.

Bonner-Weir S (1993). The pancreas: Biology, pathobiology, and Disease. In: *The microvasculature of the pancreas, with emphasis on that of the islet of langerhans: anatomy and functional implications*, Vay Liang W. Go ea (ed), 2nd edn, pp 759-768. New York: Raven Press.

Bourgeois C, Robert B, Rebourcet R, Mondon F, Mignot TM, Duc-Goiran P*, et al.* (1997). Endothelin-1 and ETA receptor expression in vascular smooth muscle cells from human placenta: a new ETA receptor messenger ribonucleic acid is generated by alternative splicing of exon 3. *J Clin Endocrinol Metab* **82**(9)**:** 3116-3123.

Bowden A, Patel V, Brown C, Boarder MR (1995). Evidence for requirement of tyrosine phosphorylation in endothelial P2Y- and P2U- purinoceptor stimulation of prostacyclin release. *Br J Pharmacol* **116**(6)**:** 2563-2568.

Brinson AE, Harden TK (2001). Differential regulation of the uridine nucleotide-activated $P2Y_4$ and $P2Y_6$ receptors. SER-333 and SER-334 in the carboxyl terminus are involved in agonist-dependent phosphorylation desensitization and internalization of the $P2Y_4$ receptor. *The Journal of biological chemistry* **276**(15)**:** 11939-11948.

Brisson GR, Malaisse WJ (1973). The stimulus-secretion coupling of glucose-induced insulin release. XI. Effects of theophylline and epinephrine on Ca^{2+} efflux from perifused islets. *Metabolism: clinical and experimental* **22**(3)**:** 455-465.

Brown P, Dale N (2002). Modulation of K^+ currents in Xenopus spinal neurons by P2Y receptors: a role for ATP and ADP in motor pattern generation. *The Journal of physiology* **540**(Pt 3)**:** 843-850.

Bruner G, Murphy S (1990). ATP-evoked arachidonic acid mobilization in astrocytes is via a P2Y-purinergic receptor. *J Neurochem* **55**(5)**:** 1569-1575.

Bulbring, E. & Tomita, T. (1967). Properties of the inhibitory potential of smooth muscle as observed in the response to field stimulation of the guinea-pig taenia coli. *J Physiol* **189**, 299–315.

Burnstock G (1971). Neural nomenclature. *Nature* **229**, 282–283.

Burnstock G (1972). Purinergic nerves. *Pharmacol Rev* **24**(3)**:** 509-581.

Burnstock G (1978). A basis for distinguishing two types of purinergic receptor. In: R.W. Straub & L. Boils (eds) *Cell Membrane Receptors for Drugs and Hormones: A multidisciplinary Approach*, pp. 107–118. Raven Press, New York.

Burnstock G (1988). Sympathetic purinergic transmission in small blood vessels. *Trends Pharmacol Sci* **9**(4)**:** 116-117.

Burnstock G (2002). Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* **22**(3)**:** 364-373.

Burnstock G (2006a). Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* **58**(1)**:** 58-86.

Burnstock G (2006b). Purinergic signalling. *Br J Pharmacol* **147 Suppl 1:** S172-181.

Burnstock G (2007). Purine and pyrimidine receptors. *Cellular and molecular life sciences : CMLS* **64**(12)**:** 1471-1483.

Burnstock G, Campbell G, Bennett M, Holman ME (1963). The effects of drugs on the transmission of inhibition from autonomic nerves to the smooth muscle of the guinea pig taenia coli. *Biochem Pharmacol* **12**(Suppl.), 134–135.

Burnstock G, Fredholm BB, North RA, Verkhratsky A (2010). The birth and postnatal development of purinergic signalling. *Acta Physiol (Oxf)* **199**(2)**:** 93-147.

Burnstock G, Kennedy C (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *General pharmacology* **16**(5)**:** 433-440.

Burnstock G, Kennedy C (2011). P2X receptors in health and disease. *Advances in pharmacology* **61:** 333-372.

Burnstock G, Knight GE (2004). Cellular distribution and functions of P2 receptor subtypes in different systems. *International review of cytology* **240:** 31-304.

Burnstock G, Novak I (2012). Purinergic signalling in the pancreas in health and disease. *The Journal of endocrinology* **213**(2): 123-141.

Burnstock G, Novak I (2013). Purinergic signalling and diabetes. *Purinergic signalling*.

Burnstock G, Ralevic V (2014). Purinergic signaling and blood vessels in health and disease. *Pharmacological reviews* **66**(1)**:** 102-192.

Burnstock G, Williams M (2000). P2 purinergic receptors: modulation of cell function and therapeutic potential. *The Journal of pharmacology and experimental therapeutics* **295**(3): 862-869.

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP*, et al.* (1994). International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev* **46**(2)**:** 121-136.

Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc Natl Acad Sci U S A* **103**(7)**:** 2334-2339.

Carter RL, Fricks IP, Barrett MO, Burianek LE, Zhou Y, Ko H, et al. (2009). Quantification of G_i-mediated inhibition of adenylyl cyclase activity reveals that UDP is a potent agonist of the human $P2Y_{14}$ receptor. *Molecular pharmacology* **76**(6): 1341-1348.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, et al. (2000). A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**(15): 10767-10771.

Chan CM, Unwin RJ, Bardini M, Oglesby IB, Ford AP, Townsend-Nicholson A*, et al.* (1998). Localization of P2X1 purinoceptors by autoradiography and immunohistochemistry in rat kidneys. *Am J Physiol* **274**(4 Pt 2)**:** F799-804.

Chan SL, Mourtada M, Morgan NG (2001). Characterization of a K^+_{ATP} channel-independent pathway involved in potentiation of insulin secretion by efaroxan. *Diabetes* **50**(2)**:** 340-347.

Chapal J, Loubatieres-Mariani MM (1981). Effects of phosphate-modified adenine nucleotide analogues on insulin secretion from perfused rat pancreas. *Br J Pharmacol* **73**(1): 105-110.

Chapal J, Loubatieres-Mariani MM (1983). Evidence for purinergic receptors on vascular smooth muscle in rat pancreas. *Eur J Pharmacol* **87**(4)**:** 423-430.

Charlton ME, Williams AS, Fogliano M, Sweetnam PM, Duman RS (1997). The isolation and characterization of a novel G protein-coupled receptor regulated by immunologic challenge. *Brain Res* **764**(1-2)**:** 141-148.

Chen, BC, Lee, CM, Lin, WW (1996) Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C6 glioma cells and RAW 264.7 macrophages. *Br J Pharmacol* **119**(8): 1628-1634.

Chootip K, Gurney AM, Kennedy C (2005). Multiple P2Y receptors couple to calcium-dependent, chloride channels in smooth muscle cells of the rat pulmonary artery. *Respir Res* **6**(1): 124.

Clancy J, McVicar A (2009). *Physiology and Anatomy for Nurses and Healthcare Practitioners: A homeostatic approach*. 3rd edn: London, Uk.

Cleaver O, Dor Y (2012). Vascular instruction of pancreas development. *Development* **139**(16)**:** 2833-2843.

Cobb BR, Clancy JP (2003). Molecular and Cell Biology of Adenosine Receptors. In: (ed)^(eds). *Current Topics in Membranes*, edn, Vol. Volume 54: Academic Press. p^pp 151-181.

Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, Stojilkovic SS (2011). Activation and regulation of purinergic P2X receptor channels. *Pharmacol Rev* **63**(3)**:** 641-683.

Communi D, Govaerts C, Parmentier M, Boeynaems JM (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *The Journal of biological chemistry* **272**(51)**:** 31969-31973.

Conti A, Lozza G, Monopoli A (1997). Prolonged exposure to 5'-N-ethylcarboxamidoadenosine (NECA) does not affect the adenosine A2A-mediated vasodilation in porcine coronary arteries. *Pharmacol Res* **35**(2)**:** 123-128.

Corbett JA, Wang JL, Hughes JH, Wolf BA, Sweetland MA, Lancaster JR, Jr.*, et al.* (1992). Nitric oxide and cyclic GMP formation induced by interleukin 1 beta in islets of Langerhans. Evidence for an effector role of nitric oxide in islet dysfunction. *Biochem J* **287 (Pt 1):** 229-235.

Costanzi S, Mamedova L, Gao ZG, Jacobson KA (2004). Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *Journal of medicinal chemistry* **47**(22)**:** 5393-5404.

Coutinho-Silva R, Parsons M, Robson T, Burnstock G (2001). Changes in expression of P2 receptors in rat and mouse pancreas during development and ageing. *Cell and tissue research* **306**(3): 373-383.

Coutinho-Silva R, Parsons M, Robson T, Lincoln J, Burnstock G (2003). P2X and P2Y purinoceptor expression in pancreas from streptozotocin-

diabetic rats. *Molecular and cellular endocrinology* **204**(1-2): 141-154.

Crack B, Pollard C, Beukers M, Roberts S, Hunt S, Ingall A*, et al.* (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *British journal of pharmacology* **114**(2)**:** 475-481.

Cryer P (1992). Glucose homeostasis and hypoglycaemia, Wilson J, Foster D (eds), pp p. 1223–1253. Philadelphia, Pa., W.B. Saunders Company: In William's Textbook of Endocrinology.

Curry DL, Bennett LL, Grodsky GM (1968). Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* **83**(3): 572-584.

De Vos A, Heimberg H, Quartier E, Huypens P, Bouwens L, Pipeleers D, et al. (1995). Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. *The Journal of clinical investigation* **96**(5): 2489-2495.

Deuticke HJ (1932). Über den einfluss von adenosin und ädenosinphosphorauren auf den isolierten meerschweinchenuterus. *Pflugers Arch* **230**: 555.

Ding L, Ma W, Littmann T, Camp R, Shen J (2011). The $P2Y_2$ nucleotide receptor mediates tissue factor expression in human coronary artery endothelial cells. *The Journal of biological chemistry* **286**(30)**:** 27027-27038.

Dixon CJ, Hall JF, Boarder MR (2003). ADP stimulation of inositol phosphates in hepatocytes: role of conversion to ATP and stimulation of P2Y2 receptors. *Br J Pharmacol* **138**(1)**:** 272-278.

Dixon CJ, Woods NM, Webb TE, Green AK (2000). Evidence that rat hepatocytes co-express functional $P2Y_1$ and $P2Y_2$ receptors. *Br J Pharmacol* **129**(4): 764-770.

Dol-Gleizes F, Mares AM, Savi P, Herbert JM (1999). Relaxant effect of 2-methyl-thio-adenosine diphosphate on rat thoracic aorta: effect of clopidogrel. *Eur J Pharmacol* **367**(2-3)**:** 247-253.

Drury AN (1936). The physiological activity of nucleic acid and its derivatives. *Physiol Rev* **16**: 292–325.

Drury AN, Szent-Gyorgyi A (1929). The physiological activity of adenine compounds with special reference to their action upon mammalian heart. *J Physiol* **68**: 213–237.

Dubois M, Pattou F, Kerr-Conte J, Gmyr V, Vandewalle B, Desreumaux P*, et al.* (2000). Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in normal human pancreatic islet cells. *Diabetologia* **43**(9)**:** 1165-1169.

Dubyak GR, El-Moatassim C (1993). Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *The American journal of physiology* **265**(3 Pt 1): C577-606.

Eberhard D, Kragl M, Lammert E (2010). 'Giving and taking': endothelial and beta-cells in the islets of Langerhans. *Trends Endocrinol Metab* **21**(8)**:** 457-463.

Eccles JC (1964). The Physiology of Synapses. Springer Verlag, Heidelberg.

Ekblad E, Sundler F (2002). Distribution of pancreatic polypeptide and peptide YY. *Peptides* **23**(2): 251-261.

Erb L, Garrad R, Wang Y, Quinn T, Turner JT, Weisman GA (1995). Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. *The Journal of biological chemistry* **270**(9)**:** 4185-4188.

Erlinge D, Burnstock G (2008). P2 receptors in cardiovascular regulation and disease. *Purinergic signalling* **4**(1)**:** 1-20.

Esther CR, Jr., Sesma JI, Dohlman HG, Ault AD, Clas ML, Lazarowski ER*, et al.* (2008). Similarities between UDP-glucose and adenine nucleotide release in yeast: involvement of the secretory pathway. *Biochemistry* **47**(35)**:** 9269-9278.

Evans RJ, Derkach V, Surprenant A (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature* **357**(6378)**:** 503-505.

Evans RJ, Kennedy C (1994). Characterization of P2-purinoceptors in the smooth muscle of the rat tail artery: a comparison between contractile and electrophysiological responses. *British Journal of Pharmacology* **113**(3)**:** 853-860.

Fabiato A (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *The American journal of physiology* **245**(1)**:** C1-14.

Falkenburger BH, Dickson EJ, Hille B (2013). Quantitative properties and receptor reserve of the DAG and PKC branch of G_q-coupled receptor signaling. *J Gen Physiol* **141**(5)**:** 537-555.

Feldman JM, Jackson TB (1974). Specificity of nucleotide-induced insulin secretion. *Endocrinology* **94**(2): 388-394.

Feoktistov I, Biaggioni I (1997). Adenosine A_{2B} receptors. *Pharmacol Rev* **49**(4)**:** 381-402.

Fernandez-Alvarez J, Hillaire-Buys D, Loubatieres-Mariani MM, Gomis R, Petit P (2001). P2 receptor agonists stimulate insulin release from human pancreatic islets. *Pancreas* **22**(1): 69-71.

Ferrer J, Scott WE, 3rd, Weegman BP, Suszynski TM, Sutherland DE, Hering BJ, et al. (2008). Pig pancreas anatomy: implications for pancreas procurement, preservation, and islet isolation. *Transplantation* **86**(11): 1503-1510.

Fredholm BB, AP IJ, Jacobson KA, Klotz KN, Linden J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**(4)**:** 527-552.

Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S*, et al.* (2001). Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78**(3)**:** 124-128.

Fricks IP, Carter RL, Lazarowski ER, Harden TK (2009). G_i-dependent cell signaling responses of the human $P2Y_{14}$ receptor in model cell systems. *The Journal of pharmacology and experimental therapeutics* **330**(1)**:** 162-168.

Fricks IP, Maddileti S, Carter RL, Lazarowski ER, Nicholas RA, Jacobson KA, et al. (2008). UDP is a competitive antagonist at the human $P2Y_{14}$ receptor. *J Pharmacol Exp Ther* **325**(2): 588-594.

Fritsche L, Weigert C, Haring HU, Lehmann R (2008). How insulin receptor substrate proteins regulate the metabolic capacity of the liver--implications for health and disease. *Current medicinal chemistry* **15**(13): 1316-1329.

Froldi G, Ragazzi E, Caparrotta L (2001). Do ATP and UTP involve cGMP in positive inotropism on rat atria? *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* **128**(2)**:** 265-274.

Froldi G, Varani K, Chinellato A, Ragazzi E, Caparrotta L, Borea PA (1997). P2X-purinoceptors in the heart: actions of ATP and UTP. *Life sciences* **60**(17)**:** 1419-1430.

Fumagalli M, Daniele S, Lecca D, Lee PR, Parravicini C, Fields RD, et al. (2011). Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation. *The Journal of biological chemistry* **286**(12): 10593-10604.

Furman B, Ong WK, Pyne NJ (2010). Cyclic AMP signaling in pancreatic islets. *Advances in experimental medicine and biology* **654:** 281-304.

Gaddum JH, Holtz P (1933). The localization of the action of drugs on the pulmonary vessels of dogs and cats. *The Journal of physiology* **77**(2)**:** 139-158.

Galas MC, Harden TK (1995). Receptor-induced heterologous desensitization of receptor-regulated phospholipase C. *Eur J Pharmacol* **291**(2)**:** 175-182.

Gao ZG, Ding Y, Jacobson KA (2010). UDP-glucose acting at $P2Y_{14}$ receptors is a mediator of mast cell degranulation. *Biochemical pharmacology* **79**(6)**:** 873-879.

Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund PE, Stuhmer W (1997). Characterization of recombinant human P2X4 receptor reveals pharmacological differences to the rat homologue. *Molecular pharmacology* **51**(1)**:** 109-118.

Gauthier JY, Belley M, Deschenes D, Fournier JF, Gagne S, Gareau Y*, et al.* (2011). The identification of 4,7-disubstituted naphthoic acid derivatives as UDP-competitive antagonists of $P2Y_{14}$. *Bioorganic & medicinal chemistry letters* **21**(10)**:** 2836-2839.

Gembal M, Gilon P, Henquin JC (1992). Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B cells. *The Journal of clinical investigation* **89**(4): 1288-1295.

Gerich JE, Schneider V, Dippe SE, Langlois M, Noacco C, Karam JH*, et al.* (1974). Characterization of the glucagon response to hypoglycemia in man. *J Clin Endocrinol Metab* **38**(1)**:** 77-82.

Gierse JK, Hauser SD, Creely DP, Koboldt C, Rangwala SH, Isakson PC*, et al.* (1995). Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. *The Biochemical journal* **305 (Pt 2):** 479-484.

Gillespie JH (1934). The biological significance of the linkages in adenosine triphosphoric acid. *The Journal of physiology* **80**(4)**:** 345-359.

Gingerich RL, Aronoff SL, Kipnis DM, Lacy PE (1979). Insulin and Glucagon-Secretion from Rat Islets Maintained in a Tissue Culture Perifusion System. *Diabetes* **28**(4)**:** 276-281.

Gittes GK (2009). Developmental biology of the pancreas: a comprehensive review. *Developmental biology* **326**(1): 4-35.

Goodhead B, Himal HS, Zanbilowicz J (1970). Relationship between pancreatic secretion and pancreatic blood flow. *Gut* **11**(1)**:** 62-68.

Govindan S, Taylor EJ, Taylor CW (2010). Ca^{2+} signalling by P2Y receptors in cultured rat aortic smooth muscle cells. *Br J Pharmacol* **160**: 1953–1962.

Green HN, Stoner HB, (1950). Biological Actions of Adenine Nucleotides. H.K. Lewis & Co, London.

Grobben B, Anciaux K, Roymans D, Stefan C, Bollen M, Esmans EL, et al. (1999). An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. *Journal of neurochemistry* **72**(2): 826-834.

Guns PJ, Van Assche T, Fransen P, Robaye B, Boeynaems JM, Bult H (2006). Endothelium-dependent relaxation evoked by ATP and UTP in the aorta of $P2Y_2$-deficient mice. *Br J Pharmacol* **147**(5)**:** 569-574.

Guns P-JDF, Korda A, Crauwels HM, Van Assche T, Robaye B, Boeynaems J-M*, et al.* (2005). Pharmacological characterization of nucleotide P2Y receptors on endothelial cells of the mouse aorta. *British journal of pharmacology* **146**(2)**:** 288-295.

Guo-Parke H, McCluskey JT, Kelly C, Hamid M, McClenaghan NH, Flatt PR (2012). Configuration of electrofusion-derived human insulin-secreting cell line as pseudoislets enhances functionality and therapeutic utility. *The Journal of endocrinology* **214**(3)**:** 257-265.

Hall RA, Gillard J, Guindon Y, Letts G, Champion E, Ethier D*, et al.* (1987). Pharmacology of L-655,240 (3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethylpropanoic acid); a potent, selective thromboxane/prostaglandin endoperoxide antagonist. *European Journal of Pharmacology* **135**(2)**:** 193-201.

Hamel E (2006). Perivascular nerves and the regulation of cerebrovascular tone. *Journal of applied physiology* **100**(3)**:** 1059-1064.

Harden TK, Sesma JI, Fricks IP, Lazarowski ER (2010). Signalling and pharmacological properties of the P2Y receptor. *Acta Physiol (Oxf)* **199**(2): 149-160.

Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ (2005). $P2Y_1$ and $P2Y_{12}$ receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* **105**(9)**:** 3552-3560.

Harmsen MM, De Haard HJ (2007). Properties, production, and applications of camelid single-domain antibody fragments. *Applied microbiology and biotechnology* **77**(1)**:** 13-22.

Hartley SA, Kato K, Salter KJ, Kozlowski RZ (1998). Functional evidence for a novel suramin-insensitive pyrimidine receptor in rat small pulmonary arteries. *Circ Res* **83**(9)**:** 940-946.

Hartshorne DJ, Gorecka A (2011). Biochemistry of the Contractile Proteins of Smooth Muscle. *Comprehensive Physiology*, edn: John Wiley & Sons, chapter **4**, 93-120.

Hauge-Evans AC, King AJ, Carmignac D, Richardson CC, Robinson IC, Low MJ, et al. (2009). Somatostatin secreted by islet delta-cells fulfills

multiple roles as a paracrine regulator of islet function. *Diabetes* **58**(2): 403-411.

Headrick JP, Peart JN, Reichelt ME, Haseler LJ (2011). Adenosine and its receptors in the heart: regulation, retaliation and adaptation. *Biochimica et biophysica acta* **1808**(5)**:** 1413-1428.

Henquin JC, Dufrane D, Nenquin M (2006a). Nutrient control of insulin secretion in isolated normal human islets. *Diabetes* **55**(12): 3470-3477.

Henquin JC, Nenquin M, Stiernet P, Ahren B (2006b). In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca^{2+} and amplification signals in beta-cells. *Diabetes* **55**(2): 441-451.

Hillaire-Buys D, Chapal J, Petit P, Loubatières-Mariani M-M (1991). Dual regulation of pancreatic vascular tone by P2X and P2Y purinoceptor subtypes. *Eur J Pharmacol* **199**(3): 309-314.

Hillaire-Buys D, Dietz S, Chapal J, Petit P, Loubatieres-Mariani MM (1999). [Involvement of P2X and P2U receptors in the constrictor effect of ATP on the pancreatic vascular bed]. *Journal de la Societe de biologie* **193**(1): 57-61.

Hirschberg CB, Robbins PW, Abeijon C (1998). Transporters of nucleotide sugars, ATP, and nucleotide sulfate in the endoplasmic reticulum and Golgi apparatus. *Annual review of biochemistry* **67:** 49-69.

Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB (2000). Isolation of INS-1-derived cell lines with robust ATP-sensitive K^+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**(3)**:** 424-430.

Holzer P, Reichmann F, Farzi A (2012). Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. *Neuropeptides* **46**(6): 261-274.

Honore E, Martin C, Mironneau C, Mironneau J (1989). An ATP-sensitive conductance in cultured smooth muscle cells from pregnant rat myometrium. *The American journal of physiology* **257**(2 Pt 1)**:** C297-305.

Houck CR, Bing RJ, et al. (1948). Renal hyperemia after the intravenous infusion of adenylic acid adenosine, or adenosine-triphosphate in the dog. *The American journal of physiology* **153**(1)**:** 159-168.

Howe AK, Aplin AE, Juliano RL (2002). Anchorage-dependent ERK signaling mechanisms and consequences. *Curr Opin Genet Dev* **12**(1)**:** 30-35.

Howell SL, Taylor KW (1968). Potassium ions and the secretion of insulin by islets of Langerhans incubated in vitro. *The Biochemical journal* **108**(1)**:** 17-24.

Inscho EW, Cook AK, Imig JD, Vial C, Evans RJ (2004). Renal autoregulation in P2X1 knockout mice. *Acta Physiol Scand* **181**(4)**:** 445-453.

Ishida N, Kawakita M (2004). Molecular physiology and pathology of the nucleotide sugar transporter family (SLC35). *Pflugers Archiv : European journal of physiology* **447**(5)**:** 768-775.

Jacobson KA, Balasubramanian R, Deflorian F, Gao ZG (2012). G protein-coupled adenosine (P1) and P2Y receptors: ligand design and receptor interactions. *Purinergic signalling* **8**(3)**:** 419-436.

Jacobson KA, Ivanov AA, de Castro S, Harden TK, Ko H (2009). Development of selective agonists and antagonists of P2Y receptors. *Purinergic Signal* **5**(1): 75-89.

Jacobson KA, van Galen PJM, Ji X-D, Ramkumar V, Olah ME, Stiles GL (1993). Molecular characterization of A_1 and A_{2A} adenosine receptors. *Drug Development Research* **28**(3)**:** 226-231.

Jacques-Silva MC, Correa-Medina M, Cabrera O, Rodriguez-Diaz R, Makeeva N, Fachado A, et al. (2010). ATP-gated P2X3 receptors constitute a positive autocrine signal for insulin release in the human pancreatic beta cell. *Proceedings of the National Academy of Sciences of the United States of America* **107**(14): 6465-6470.

Jansson L, Carlsson PO (2002). Graft vascular function after transplantation of pancreatic islets. *Diabetologia* **45**(6)**:** 749-763.

Jansson L, Hellerstrom C (1983). Stimulation by glucose of the blood flow to the pancreatic islets of the rat. *Diabetologia* **25**(1)**:** 45-50.

Jiang LH, Rassendren F, Surprenant A, North RA (2000). Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. *The Journal of biological chemistry* **275**(44)**:** 34190-34196.

Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA*, et al.* (1997). A mutational analysis of residues essential for ligand recognition at the human $P2Y_1$ receptor. *Molecular pharmacology* **52**(3)**:** 499-507.

Jitrapakdee S, Wutthisathapornchai A, Wallace JC, MacDonald MJ (2010). Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia* **53**(6): 1019-1032.

Joseph SM, Pifer MA, Przybylski RJ, Dubyak GR (2004). Methylene ATP analogs as modulators of extracellular ATP metabolism and accumulation. *Br J Pharmacol* **142**(6): 1002-1014.

Kang G, Chepurny OG, Malester B, Rindler MJ, Rehmann H, Bos JL*, et al.* (2006). cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic beta cells and rat INS-1 cells. *The Journal of physiology* **573**(Pt 3)**:** 595-609.

Kasakov L, Burnstock G (1982). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitisation of the P2-purinoceptor: Effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *European Journal of Pharmacology* **86**(2)**:** 291-294.

Kauffenstein G, Drouin A, Thorin-Trescases N, Bachelard H, Robaye B, D'Orléans-Juste P*, et al.* (2010). NTPDase1 (CD39) controls nucleotide-dependent vasoconstriction in mouse. *Cardiovasc Res* **85**(1)**:** 204-213.

Kawate T, Michel JC, Birdsong WT, Gouaux E (2009). Crystal structure of the ATP-gated P2X4 ion channel in the closed state. *Nature* **460**(7255)**:** 592-598.

Kemp BK, Cocks TM (1999). Adenosine mediates relaxation of human small resistance-like coronary arteries via A_{2B} receptors. *Br J Pharmacol* **126**(8)**:** 1796-1800.

Kenakin T (2004). Principles: receptor theory in pharmacology. *Trends Pharmacol Sci* **25**(4)**:** 186-192.

Kennedy C, Chootip K, Mitchell C, Syed NI, Tengah A (2013). P2X and P2Y nucleotide receptors as targets in cardiovascular disease. *Future medicinal chemistry* **5**(4)**:** 431-449.

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Séguéla P*, et al.* (2001). International Union of Pharmacology. XXIV. Current Status of the Nomenclature and Properties of P2X Receptors and Their Subunits. *Pharmacological Reviews* **53**(1)**:** 107-118.

Khakh BS, Surprenant A, Humphrey PP (1995). A study on P2X purinoceptors mediating the electrophysiological and contractile effects of purine nucleotides in rat vas deferens. *Br J Pharmacol* **115**(1)**:** 177-185.

Kleczkowski LA, Geisler M, Ciereszko I, Johansson H (2004). UDP-glucose pyrophosphorylase. An old protein with new tricks. *Plant physiology* **134**(3)**:** 912-918.

Knight GE, Oliver-Redgate R, Burnstock G (2003). Unusual absence of endothelium-dependent or -independent vasodilatation to purines or pyrimidines in the rat renal artery. *Kidney international* **64**(4)**:** 1389-1397.

Ko H, Carter RL, Cosyn L, Petrelli R, de Castro S, Besada P*, et al.* (2008). Synthesis and potency of novel uracil nucleotides and derivatives as $P2Y_2$ and $P2Y_6$ receptor agonists. *Bioorganic & medicinal chemistry* **16**(12)**:** 6319-6332.

Ko H, Das A, Carter RL, Fricks IP, Zhou Y, Ivanov AA, et al. (2009). Molecular recognition in the $P2Y_{14}$ receptor: Probing the structurally permissive terminal sugar moiety of uridine-5'-diphosphoglucose. Bioorganic & medicinal chemistry **17**(14): 5298-5311.

Ko H, Fricks I, Ivanov AA, Harden TK, Jacobson KA (2007). Structure-activity relationship of uridine-5'-diphosphoglucose analogues as agonists of the human $P2Y_{14}$ receptor. *Journal of medicinal chemistry* **50**(9)**:** 2030-2039.

Kook SH, Cho JS, Morrison A, Wiener E, Lee SB, Scadden D*, et al.* (2013). The purinergic $P2Y_{14}$ receptor axis is a molecular determinant for organism survival under in utero radiation toxicity. *Cell death & disease* **4:** e703.

Korchazhkina O, Wright G, Exley C (1999). Intravascular ATP and coronary vasodilation in the isolated working rat heart. *British Journal of Pharmacology* **127**(3)**:** 701-708.

Kreda SM, Seminario-Vidal L, Heusden C, Lazarowski ER (2008). Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol* **153**(7): 1528-1537.

Krzemiński P, Pomorski P, Barańska J (2008). The $P2Y_{14}$ receptor activity in glioma C6 cells. *European Journal of Pharmacology* **594**(1–3)**:** 49-54.

Kügelgen I (2008). Pharmacology of mammalian P2X and P2Y receptors. Biotrend Reviews, No. 3.

Kügelgen I, Häussinger D, Starker K (1987). Evidence for a vasoconstriction-mediating receptor for UTP, distinct from the P2 purinoceptor, in rabbit ear artery. *Naunyn-Schmiedeberg's Archives of Pharmacology* **336**(5)**:** 556-560.

Kukulski F, Levesque SA, Lavoie EG, Lecka J, Bigonnesse F, Knowles AF*, et al.* (2005). Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic signalling* **1**(2)**:** 193-204.

Kur J, Newman EA (2013). Purinergic control of vascular tone in the retina. *The Journal of physiology*.

Kurahashi K, Nishihashi T, Trandafir CC, Wang AM, Murakami S, Ji X (2003). Diversity of endothelium-derived vasocontracting factors--arachidonic acid metabolites. *Acta Pharmacol Sin* **24**(11)**:** 1065-1069.

Lacza Z, Kaldi K, Kovecs K, Gorlach C, Nagy Z, Sandor P*, et al.* (2001). Involvement of prostanoid release in the mediation of UTP-induced cerebrovascular contraction in the rat. *Brain Res* **896**(1-2)**:** 169-174.

Langan EM, 3rd, Youkey JR, Elmore JR, Franklin DP, Singer HA (1994). Regulation of MAP kinase activity by growth stimuli in vascular smooth muscle. *The Journal of surgical research* **57**(1)**:** 215-220.

Laughlin MR, Petit WA, Jr., Dizon JM, Shulman RG, Barrett EJ (1988). NMR measurements of in vivo myocardial glycogen metabolism. *The Journal of biological chemistry* **263**(5)**:** 2285-2291.

Lavan BE, Lane WS, Lienhard GE (1997). The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *The Journal of biological chemistry* **272**(17): 11439-11443.

Lazarowski ER, Boucher RC, Harden TK (2000). Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *The Journal of biological chemistry* **275**(40)**:** 31061-31068.

Lazarowski ER, Boucher RC, Harden TK (2003a). Mechanisms of Release of Nucleotides and Integration of Their Action as P2X- and P2Y-Receptor Activating Molecules. *Molecular pharmacology* **64**(4)**:** 785-795.

Lazarowski ER, Homolya L, Boucher RC, Harden TK (1997). Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *The Journal of biological chemistry* **272**(39)**:** 24348-24354.

Lazarowski ER, Sesma JI, Seminario-Vidal L, Kreda SM (2011). Molecular mechanisms of purine and pyrimidine nucleotide release. *Adv Pharmacol* **61:** 221-261.

Lazarowski ER, Shea DA, Boucher RC, Harden TK (2003b). Release of cellular UDP-glucose as a potential extracellular signaling molecule. *Molecular pharmacology* **63**(5)**:** 1190-1197.

Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ*, et al.* (1997). Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388**(6643)**:** 674-678.

Lee YS, Morinaga H, Kim JJ, Lagakos W, Taylor S, Keshwani M*, et al.* (2013). The fractalkine/CX3CR1 system regulates beta cell function and insulin secretion. *Cell* **153**(2)**:** 413-425.

Léon C, Hechler B, Vial C, Leray C, Cazenave J-P, Gachet C (1997). The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS letters* **403**(1)**:** 26-30.

Levesque SA, Lavoie EG, Lecka J, Bigonnesse F, Sevigny J (2007). Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. *Br J Pharmacol* **152**(1): 141-150.

Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995). Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* **377**(6548)**:** 432-435.

Li N, Sul JY, Haydon PG (2003). A calcium-induced calcium influx factor, nitric oxide, modulates the refilling of calcium stores in astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**(32)**:** 10302-10310.

Lifson N, Kramlinger KG, Mayrand RR, Lender EJ (1980). Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. *Gastroenterology* **79**(3)**:** 466-473.

Lindner F, Rigler R (1931). U¨ber die Beeinflussung der Weite der Herzkranzgefa¨ sse durch Produkte des Zellkernstoffwechsels. *Pflugers Arch* **226**: 697–708.

Liu C, Mather S, Huang Y, Garland CJ, Yao X (2004). Extracellular ATP facilitates flow-induced vasodilatation in rat small mesenteric arteries. *American journal of physiology. Heart and circulatory physiology* **286**(5)**:** H1688-1695.

Lopez C, Sanchez M, Hidalgo A, Garcia de Boto MJ (2000). Mechanisms involved in UTP-induced contraction in isolated rat aorta. *Eur J Pharmacol* **391**(3)**:** 299-303.

Loubatieres AL, Loubatieres-Mariani MM, Chapal J (1972). [Adenosine triphosphate (ATP), cyclic adenosine 3'5' monophosphate (cycl 3'5' AMP) and insulin secretion]. *Comptes rendus des seances de la Societe de biologie et de ses filiales* **166**(12): 1742-1746.

Loubatieres-Mariani MM, Chapal J, Lignon F, Valette G (1979). Structural specificity of nucleotides for insulin secretory action from the isolated perfused rat pancreas. *Eur J Pharmacol* **59**(3-4): 277-286.

Loubatieres-Mariani MM, Loubatieres AL, Chapal J, Valette G (1976). [Adenosine triphosphate (ATP) and glucose. Action on insulin and glucagon secretion]. *Comptes rendus des seances de la Societe de biologie et de ses filiales* **170**(4): 833-836.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**(1)**:** 265-275.

Lugo-Garcia L, Filhol R, Lajoix AD, Gross R, Petit P, Vignon J (2007). Expression of purinergic P2Y receptor subtypes by INS-1 insulinoma beta-cells: a molecular and binding characterization. *Eur J Pharmacol* **568**(1-3): 54-60.

Lugo-Garcia L, Nadal B, Gomis R, Petit P, Gross R, Lajoix AD (2008). Human pancreatic islets express the purinergic $P2Y_{11}$ and $P2Y_{12}$ receptors. *Hormone and metabolic research* **40**(11): 827-830.

Luo X, Zheng W, Yan M, Lee MG, Muallem S (1999). Multiple functional P2X and P2Y receptors in the luminal and basolateral membranes of pancreatic duct cells. *The American journal of physiology* **277**(2 Pt 1)**:** C205-215.

Luscher TF, Boulanger CM, Dohi Y, Yang ZH (1992). Endothelium-derived contracting factors. *Hypertension* **19**(2)**:** 117-130.

Lyubchenko T, Woodward H, Veo KD, Burns N, Nijmeh H, Liubchenko GA*, et al.* (2011). $P2Y_1$ and $P2Y_{13}$ purinergic receptors mediate Ca^{2+} signaling and proliferative responses in pulmonary artery vasa vasorum endothelial cells. *American Journal of Physiology - Cell Physiology* **300**(2)**:** C266-C275.

MacDonald MJ, Kaysen JH, Moran SM, Pomije CE (1991). Pyruvate dehydrogenase and pyruvate carboxylase. Sites of pretranslational regulation by glucose of glucose-induced insulin release in pancreatic islets. *The Journal of biological chemistry* **266**(33): 22392-22397.

Maekawa A, Xing W, Austen KF, Kanaoka Y (2010). GPR17 regulates immune pulmonary inflammation induced by house dust mites. *J Immunol* **185**(3)**:** 1846-1854.

Malmsjo M, Adner M, Harden TK, Pendergast W, Edvinsson L, Erlinge D (2000). The stable pyrimidines UDPbetaS and UTPgammaS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. *Br J Pharmacol* **131**(1)**:** 51-56.

Malmsjo M, Edvinsson L, Erlinge D (1998). P2U-receptor mediated endothelium-dependent but nitric oxide-independent vascular relaxation. *Br J Pharmacol* **123**(4)**:** 719-729.

Mamedova LK, Joshi BV, Gao ZG, von Kugelgen I, Jacobson KA (2004). Diisothiocyanate derivatives as potent, insurmountable antagonists of $P2Y_6$ nucleotide receptors. *Biochemical pharmacology* **67**(9): 1763-1770.

Marchetti P, Giannarelli R, Cosimi S, Masiello P, Coppelli A, Viacava P*, et al.* (1995). Massive Isolation, Morphological and Functional-Characterization, and Xenotransplantation of Bovine Pancreatic-Islets. *Diabetes* **44**(4)**:** 375-381.

Marquardt B, Frith D, Stabel S (1994). Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway in vitro. *Oncogene* **9**(11)**:** 3213-3218.

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P*, et al.* (2003). Pharmacological Characterization of the Human $P2Y_{13}$ Receptor. *Molecular pharmacology* **64**(1)**:** 104-112.

McGeown JG (2002). *Physiology: a clinical core text of human physiology with self-assessment*. Churchill Livingstone: UK.

Meister J RA, Schöneberg T, Schulz A, (2013). The metabolic relevance of the UDP-glucose receptor $P2Y_{14}$. *Diabetologie und Stoffwechsel.*

Melloul D, Marshak S, Cerasi E (2002). Regulation of insulin gene transcription. *Diabetologia* **45**(3): 309-326.

Mistry H, Gitlin JM, Mitchell JA, Hiley CR (2003). Endothelium-dependent relaxation and endothelial hyperpolarization by P2Y receptor agonists in rat-isolated mesenteric artery. *Br J Pharmacol* **139**(3)**:** 661-671.

Mitchell C, Syed NI, Tengah A, Gurney AM, Kennedy C (2012). Identification of contractile $P2Y_1$, $P2Y_6$, and $P2Y_{12}$ receptors in rat intrapulmonary artery using selective ligands. *The Journal of pharmacology and experimental therapeutics* **343**(3)**:** 755-762.

Miyagi Y, Kimura H, Carpenter RC, Parent AD, Zhang J (2004). alpha,beta-MeATP augments the UTP contraction of rabbit basilar artery. *Eur J Pharmacol* **488**(1-3)**:** 117-125.

Miyagi Y, Kobayashi S, Nishimura J, Fukui M, Kanaide H (1996). Dual regulation of cerebrovascular tone by UTP: P2U receptor-mediated contraction and endothelium-dependent relaxation. *Br J Pharmacol* **118**(4)**:** 847-856.

Miyagi Y, Zhang JH (2004). αβ-Methylene ATP enhances $P2Y_4$ contraction of rabbit basilar artery. *American Journal of Physiology - Heart and Circulatory Physiology* **286**(4)**:** H1546-H1551.

Mo G, Peleshok JC, Cao CQ, Ribeiro-da-Silva A, Seguela P (2013). Control of P2X3 channel function by metabotropic $P2Y_2$ UTP receptors in primary sensory neurons. *Molecular pharmacology* **83**(3)**:** 640-647.

Mombouli JV, Vanhoutte PM (1993). Purinergic endothelium-dependent and -independent contractions in rat aorta. *Hypertension* **22**(4)**:** 577-583.

Moore D, Chambers J, Waldvogel H, Faull R, Emson P (2000). Regional and cellular distribution of the $P2Y_1$ purinergic receptor in the human brain: striking neuronal localisation. *The Journal of comparative neurology* **421**(3)**:** 374-384.

Moore DJ, Murdock PR, Watson JM, Faull RL, Waldvogel HJ, Szekeres PG*, et al.* (2003). GPR105, a novel $G_{i/o}$-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain research. Molecular brain research* **118**(1-2)**:** 10-23.

Moses S, Franzen A, Lovdahl C, Hultgardh-Nilsson A (2001). Injury-induced osteopontin gene expression in rat arterial smooth muscle cells is dependent on mitogen-activated protein kinases ERK1/ERK2. *Arch Biochem Biophys* **396**(1)**:** 133-137.

Muller T, Bayer H, Myrtek D, Ferrari D, Sorichter S, Ziegenhagen MW*, et al.* (2005). The $P2Y_{14}$ receptor of airway epithelial cells: coupling to intracellular Ca^{2+} and IL-8 secretion. *American journal of respiratory cell and molecular biology* **33**(6)**:** 601-609.

Munkonda MN, Kauffenstein G, Kukulski F, Levesque SA, Legendre C, Pelletier J, et al. (2007). Inhibition of human and mouse plasma membrane bound NTPDases by P2 receptor antagonists. *Biochemical pharmacology* **74**(10): 1524-1534.

Muraki K, Imaizumi Y, Watanabe M (1998). Effects of UTP on membrane current and potential in rat aortic myocytes. *Eur J Pharmacol* **360**(2-3)**:** 239-247.

Murphy-Piedmonte, DM, Crawford, PA, Kirley, TL (2005) Bacterial expression, folding, purification and characterization of soluble NTPDase5 (CD39L4) ecto-nucleotidase. *Biochim Biophys Acta* **1747**(2): 251-259.

Musa H, Tellez JO, Chandler NJ, Greener ID, Maczewski M, Mackiewicz U*, et al.* (2009). P2 purinergic receptor mRNA in rat and human sinoatrial node and other heart regions. *Naunyn Schmiedebergs Arch Pharmacol* **379**(6)**:** 541-549.

Narhi LO, Hua QX, Arakawa T, Fox GM, Tsai L, Rosenfeld R, et al. (1993). Role of native disulfide bonds in the structure and activity of insulin-like growth factor 1: genetic models of protein-folding intermediates. *Biochemistry* **32**(19): 5214-5221.

Narumiya S, Ishizaki T, Ufhata M (2000). Use and properties of ROCK-specific inhibitor Y-27632. In: W.E. Balch CJD, Alan H (ed)^(eds).

Methods Enzymol, edn, Vol. Volume 325: Academic Press. p^pp 273-284.

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB*, et al.* (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* **11**(18)**:** 2323-2334.

Nguyen NC, Taalab K, Osman MM (2010). Decreased blood flow with increased metabolic activity: a novel sign of pancreatic tumor aggressiveness. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**(1)**:** 367; author reply 567.

Nicholas RA, Lazarowski ER, Watt WC, Li Q, Boyer J, Harden TK (1996). Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. *Journal of autonomic pharmacology* **16**(6)**:** 319-323.

Noguchi H, Naziruddin B, Shimoda M, Fujita Y, Chujo D, Takita M*, et al.* (2012). Evaluation of osmolality of density gradient for human islet purification. *Cell Transplant* **21**(2-3)**:** 493-500.

Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S*, et al.* (1994). Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1 (supplement). *DNA research : an international journal for rapid publication of reports on genes and genomes* **1**(1)**:** 47-56.

Novak I (2008). Purinergic receptors in the endocrine and exocrine pancreas. *Purinergic signalling* **4**(3)**:** 237-253.

Ohneda A, Aguilar-Parada E, Eisentraut AM, Unger RH (1969). Control of pancreatic glucagon secretion by glucose. *Diabetes* **18**(1): 1-10.

Ohtani M, Oka T, Ohura K (2013). Possible involvement of A_{2A} and A_3 receptors in modulation of insulin secretion and beta-cell survival in mouse pancreatic islets. *General and comparative endocrinology* **15**(187): 86-94.

Ohtani M, Suzuki J, Jacobson KA, Oka T (2008). Evidence for the possible involvement of the $P2Y_6$ receptor in Ca^{2+} mobilization and insulin secretion in mouse pancreatic islets. *Purinergic signalling* **4**(4)**:** 365-375.

Okada M, Nishikibe M (2002). BQ-788, A Selective Endothelin ETB Receptor Antagonist. *Cardiovascular drug reviews* **20**(1)**:** 53-66.

O'Keeffe MG, Thorne PR, Housley GD, Robson SC, Vlajkovic SM (2010). Distribution of NTPDase5 and NTPDase6 and the regulation of P2Y receptor signalling in the rat cochlea. *Purinergic signalling* **6**(2): 249-261.

Olah ME, Stiles GL (2000). The role of receptor structure in determining adenosine receptor activity. *Pharmacology & therapeutics* **85**(2)**:** 55-75.

Olanrewaju HA, Qin W, Feoktistov I, Scemama JL, Mustafa SJ (2000). Adenosine A_{2A} and A_{2B} receptors in cultured human and porcine coronary artery endothelial cells. *Am J Physiol Heart Circ Physiol* **279**(2)**:** H650-656.

Ongini E, Fredholm BB (1996). Pharmacology of adenosine A_{2A} receptors. *Trends Pharmacol Sci* **17**(10)**:** 364-372.

Ostrom RS, Gregorian C, Insel PA (2000). Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. *The Journal of biological chemistry* **275**(16)**:** 11735-11739.

Otsuguro K, Ito S, Ohta T, Nakazato Y (1996). Influence of purines and pyrimidines on circular muscle of the rat proximal stomach. *Eur J Pharmacol* **317**(1)**:** 97-105.

Parandeh F, Abaraviciene SM, Amisten S, Erlinge D, Salehi A (2008). Uridine diphosphate (UDP) stimulates insulin secretion by activation of $P2Y_6$ receptors. *Biochemical and biophysical research communications* **370**(3): 499-503.

Park HS, Park IS, Lee YL, Kwon HY, Park HJ (1999). Effects of intrapancreatic neuronal activation on cholecystokinin-induced exocrine secretion of isolated perfused rat pancreas. *Pflugers Archiv : European journal of physiology* **437**(4)**:** 511-516.

Patel V, Brown C, Boarder MR (1996). Protein kinase C isoforms in bovine aortic endothelial cells: role in regulation of P2Y- and P2U-purinoceptor-stimulated prostacyclin release. *Br J Pharmacol* **118**(1)**:** 123-130.

Peart JN, Headrick JP (2007). Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacology & therapeutics* **114**(2)**:** 208-221.

Peterson MA, Swerdloff RS (1979). Separation of bound from free hormone in radioimmunoassay of lutropin and follitropin. *Clinical chemistry* **25**(7): 1239-1241.

Petit P, Hillaire-Buys D, Manteghetti M, Debrus S, Chapal J, Loubatieres-Mariani MM (1998). Evidence for two different types of P2 receptors stimulating insulin secretion from pancreatic B cell. *Br J Pharmacol* **125**(6): 1368-1374.

Petit P, Manteghetti M, Puech R, Loubatieres-Mariani MM (1987). ATP and phosphate-modified adenine nucleotide analogues. Effects on insulin secretion and calcium uptake. *Biochemical pharmacology* **36**(3): 377-380.

Qasabian RA, Schyvens C, Owe-Young R, Killen JP, Macdonald PS, Conigrave AD*, et al.* (1997). Characterization of the P2 receptors in rabbit pulmonary artery. *Br J Pharmacol* **120**(4)**:** 553-558.

Qi A-D, Zambon AC, Insel PA, Nicholas RA (2001). An Arginine/Glutamine Difference at the Juxtaposition of Transmembrane Domain 6 and the Third Extracellular Loop Contributes to the Markedly Different Nucleotide Selectivities of Human and Canine $P2Y_{11}$ Receptors. *Molecular pharmacology* **60**(6)**:** 1375-1382.

Raina H, Zacharia J, Li M, Wier WG (2009). Activation by Ca^{2+}/calmodulin of an exogenous myosin light chain kinase in mouse arteries. *J Physiol* **587**(Pt 11)**:** 2599-2612.

Ralevic V (2001). Mechanism of prolonged vasorelaxation to ATP in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **132**(3)**:** 685-692.

Ralevic V (2002). The involvement of smooth muscle P2X receptors in the prolonged vasorelaxation response to purine nucleotides in the rat mesenteric arterial bed. *Br J Pharmacol* **135**(8)**:** 1988-1994.

Ralevic V, Burnstock G (1991a). Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Circulation Research* **69**(6)**:** 1583-1590.

Ralevic V, Burnstock G (1991b). Roles of P2-purinoceptors in the cardiovascular system. *Circulation* **84**(1)**:** 1-14.

Ralevic V, Burnstock G (1996). Relative contribution of P2U- and P2Y-purinoceptors to endothelium-dependent vasodilatation in the golden hamster isolated mesenteric arterial bed. Br J Pharmacol 117(8)**:** 1797-1802.

Ralevic V, Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**(3)**:** 413-492.

Ralevic V, Thomas T, Burnstock G, Spyer KM (1999). Characterization of P2 receptors modulating neural activity in rat rostral ventrolateral medulla. *Neuroscience* **94**(3)**:** 867-878.

Ramme D, Regenold JT, Starke K, Busse R, Illes P (1987). Identification of the neuroeffector transmitter in jejunal branches of the rabbit mesenteric artery. *Naunyn Schmiedebergs Arch Pharmacol* **336**(3)**:** 267-273.

Raqeeb A, Sheng J, Ao N, Braun AP (2011). Purinergic $P2Y_2$ receptors mediate rapid Ca^{2+} mobilization, membrane hyperpolarization and nitric oxide production in human vascular endothelial cells. *Cell calcium* **49**(4)**:** 240-248.

Rayment SJ, Latif ML, Ralevic V, Alexander SP (2007a). Evidence for the expression of multiple uracil nucleotide-stimulated P2 receptors coupled to smooth muscle contraction in porcine isolated arteries. *Br J Pharmacol* **150**(5): 604-612.

Rayment, SJ, Ralevic, V, Barrett, DA, Cordell, R, Alexander, SP (2007b) A novel mechanism of vasoregulation: ADP-induced relaxation of the porcine isolated coronary artery is mediated via adenosine release. *FASEB J* **21**(2)**:** 577-585.

Rebecchi MJ, Pentyala SN (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiological reviews* **80**(4)**:** 1291-1335.

Reshkin SJ, Guerra L, Bagorda A, Debellis L, Cardone R, Li AH*, et al.* (2000). Activation of A_3 adenosine receptor induces calcium entry and chloride secretion in A_6 cells. *The Journal of membrane biology* **178**(2)**:** 103-113.

Rettinger J, Braun K, Hochmann H, Kassack MU, Ullmann H, Nickel P*, et al.* (2005). Profiling at recombinant homomeric and heteromeric rat P2X receptors identifies the suramin analogue NF449 as a highly potent P2X1 receptor antagonist. *Neuropharmacology* **48**(3)**:** 461-468.

Roberts RE (2001). Role of the extracellular signal-regulated kinase (Erk) signal transduction cascade in alpha(2) adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein. *Br J Pharmacol* **133**(6)**:** 859-866.

Roberts RE, Kendall DA, Wilson VG (1999). α_2-Adrenoceptor and NPY receptor-mediated contractions of porcine isolated blood vessels:

evidence for involvement of the vascular endothelium. *Br J Pharmacol* **128**(8)**:** 1705-1712.

Robichaud J, Fournier JF, Gagne S, Gauthier JY, Hamel M, Han Y, et al. (2011). Applying the pro-drug approach to afford highly bioavailable antagonists of $P2Y_{14}$. *Bioorganic & medicinal chemistry letters* **21**(14): 4366-4368.

Rodrigue-Candela JL, Martin-Hernandez D, Castilla-Cortazar T (1963). Stimulation of insulin secretion in vitro by adenosine triphosphate. *Nature* **197**: 1304.

Rodriguez Candela JL, Garcia-Fernandez MC (1963). Stimulation of secretion of insulin by adenosine-triphosphate. *Nature* **197**: 1210.

Rodriguez-Rodriguez R, Yarova P, Winter P, Dora KA (2009). Desensitization of endothelial $P2Y_1$ receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries. *Br J Pharmacol* **158**(6)**:** 1609-1620.

Rorsman P (1997). The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. *Diabetologia* **40**(5): 487-495.

Roy J, Tran PK, Religa P, Kazi M, Henderson B, Lundmark K*, et al.* (2002). Fibronectin promotes cell cycle entry in smooth muscle cells in primary culture. *Exp Cell Res* **273**(2)**:** 169-177.

Rubino A, Burnstock G (1996) Evidence for a P2-purinoceptor mediating vasoconstriction by UTP, ATP and related nucleotides in the isolated pulmonary vascular bed of the rat. *British Journal of Pharmacology* **118**(6):1415-20.

Rusing D, Muller CE, Verspohl EJ (2006). The impact of adenosine and A(2B) receptors on glucose homoeostasis. *The Journal of pharmacy and pharmacology* **58**(12): 1639-1645.

Rutishauser S (1994). *Physiology and anatomy: a basis of nursing and health care*. Churchill Livingstone: UK.

Sabirov RZ, Okada Y (2005). ATP release via anion channels. *Purinergic signalling* **1**(4)**:** 311-328.

Sakamoto A, Yanagisawa M, Sawamura T, Enoki T, Ohtani T, Sakurai T*, et al.* (1993). Distinct subdomains of human endothelin receptors determine their selectivity to endothelinA-selective antagonist and endothelinB-selective agonists. *Journal of Biological Chemistry* **268**(12)**:** 8547-8553.

Salehi A, Carlberg M, Henningson R, Lundquist I (1996). Islet constitutive nitric oxide synthase: biochemical determination and regulatory function. *Am J Physiol* **270**(6 Pt 1)**:** C1634-1641.

Sanabria P, Ross E, Ramirez E, Colon K, Hernandez M, Maldonado HM*, et al.* (2008). P2Y2 receptor desensitization on single endothelial cells. *Endothelium : journal of endothelial cell research* **15**(1)**:** 43-51.

Satoh A, Shimosegawa T, Satoh K, Ito H, Kohno Y, Masamune A*, et al.* (2000). Activation of adenosine A_1-receptor pathway induces edema formation in the pancreas of rats. *Gastroenterology* **119**(3)**:** 829-836.

Schaefer JH (1926). The normal weight of the pancreas in the adult human being: A biometric study. *The Anatomical Record* **32**(2): 119-132.

Schaeffer M, Hodson DJ, Lafont C, Mollard P (2011). Endocrine cells and blood vessels work in tandem to generate hormone pulses. *J Mol Endocrinol* **47**(2)**:** R59-66.

Schauwienold D, Plum C, Helbing T, Voigt P, Bobbert T, Hoffmann D*, et al.* (2003). ERK1/2-dependent contractile protein expression in vascular smooth muscle cells. *Hypertension* **41**(3)**:** 546-552.

Schulte G, Fredholm BB (2003). Signalling from adenosine receptors to mitogen-activated protein kinases. *Cellular signalling* **15**(9)**:** 813-827.

Scrivens M, Dickenson JM (2005). Functional expression of the $P2Y_{14}$ receptor in murine T-lymphocytes. *Br J Pharmacol* **146**(3): 435-444.

Scrivens M, Dickenson JM (2006). Functional expression of the $P2Y_{14}$ receptor in human neutrophils. *Eur J Pharmacol* **543**(1-3): 166-173.

Seifert R, Schultz G (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. *Trends Pharmacol Sci* **10**(9)**:** 365-369.

Sendur R, Pawlik WW (1994). [Pancreatic blood flow and its regulation]. *Przegl Lek* **51**(5)**:** 224-228.

Seoane J, Gomez-Foix AM, O'Doherty RM, Gomez-Ara C, Newgard CB, Guinovart JJ (1996). Glucose 6-phosphate produced by glucokinase, but not hexokinase I, promotes the activation of hepatic glycogen synthase. *The Journal of biological chemistry* **271**(39)**:** 23756-23760.

Sesma JI, Esther CR, Jr., Kreda SM, Jones L, O'Neal W, Nishihara S*, et al.* (2009). Endoplasmic reticulum/golgi nucleotide sugar transporters contribute to the cellular release of UDP-sugar signaling molecules. *The Journal of biological chemistry* **284**(18)**:** 12572-12583.

Sesma JI, Kreda SM, Steinckwich-Besancon N, Dang H, García-Mata R, Harden TK*, et al.* (2012). The UDP-sugar-sensing $P2Y_{14}$ receptor promotes Rho-mediated signalling and chemotaxis in human neutrophils. *American Journal of Physiology - Cell Physiology* **303**(5)**:** C490-C498.

Shen J, Seye CI, Wang M, Weisman GA, Wilden PA, Sturek M (2004). Cloning, up-regulation, and mitogenic role of porcine $P2Y_2$ receptor in coronary artery smooth muscle cells. *Molecular pharmacology* **66**(5)**:** 1265-1274.

Sherwood L (1997). *Human physiology: from cells to systems*. 3rd edn. Belmont, CA: Wadsworth Publishing Company.

Simon D, Kunicki T, Nugent D (2008). Platelet function defects. *Haemophilia* **14**(6)**:** 1240-1249.

Skelton L, Cooper M, Murphy M, Platt A (2003). Human immature monocyte-derived dendritic cells express the G protein-coupled receptor GPR105 (KIAA0001, $P2Y_{14}$) and increase intracellular calcium in response to its agonist, uridine diphosphoglucose. *J Immunol* **171**(4)**:** 1941-1949.

Smith PA, Bokvist K, Arkhammar P, Berggren PO, Rorsman P (1990). Delayed rectifying and calcium-activated K^+ channels and their significance for action potential repolarization in mouse pancreatic beta-cells. *The Journal of general physiology* **95**(6): 1041-1059.

Somers GR, Bradbury R, Trute L, Conigrave A, Venter DJ (1999). Expression of the human $P2Y_6$ nucleotide receptor in normal placenta

and gestational trophoblastic disease. *Laboratory investigation; a journal of technical methods and pathology* **79**(2)**:** 131-139.

Squires PE, James RF, London NJ, Dunne MJ (1994). ATP-induced intracellular Ca^{2+} signals in isolated human insulin-secreting cells. Pflugers Archiv : European journal of physiology **427**(1-2): 181-183.

Srivastava S, Goren HJ (2003). Insulin constitutively secreted by beta-cells is necessary for glucose-stimulated insulin secretion. *Diabetes* **52**(8): 2049-2056.

Stam NJ, Klomp J, Van de Heuvel N, Olijve W (1996). Molecular cloning and characterization of a novel orphan receptor (P2P) expressed in human pancreas that shows high structural homology to the P2U purinoceptor. *FEBS letters* **384**(3): 260-264.

Stanford, S.J. & Mitchell, J.A. (1998). ATP-induced vasodilatation in the rat isolated mesenteric bed exhibits two apparent phases. Br. J. Pharmacol., **125**, 94P.

Steiner DF, Park SY, Stoy J, Philipson LH, Bell GI (2009). A brief perspective on insulin production. *Diabetes, obesity & metabolism 11 Suppl* **4**: 189-196.

Straub SG, Sharp GW (2002). Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes/metabolism research and reviews* **18**(6): 451-463.

Suga S, Kanno T, Nakano K, Takeo T, Dobashi Y, Wakui M (1997). GLP-I(7-36) amide augments Ba^{2+} current through L-type Ca^{2+} channel of rat pancreatic beta-cell in a cAMP-dependent manner. *Diabetes* **46**(11)**:** 1755-1760.

Sumi S, Inoue K, Kogire M, Doi R, Yun M, Kaji H*, et al.* (1991). Effect of synthetic porcine neuropeptide Y (NPY) on splanchnic blood flows and exocrine pancreatic secretion in dogs. *Dig Dis Sci* **36**(11)**:** 1523-1528.

Syed N-i-H, Tengah A, Paul A, Kennedy C (2010). Characterisation of P2X receptors expressed in rat pulmonary arteries. *European Journal of Pharmacology* **649**(1–3)**:** 342-348.

Taguchi N, Aizawa T, Sato Y, Ishihara F, Hashizume K (1995). Mechanism of glucose-induced biphasic insulin release: physiological role of adenosine triphosphate-sensitive K^+ channel-independent glucose action. *Endocrinology* **136**(9): 3942-3948.

Takeda M, Kawamura T, Kobayashi M, Endou H (1996). ATP-induced calcium mobilization in glomerular mesangial cells is mediated by P2U purinoceptor. *Biochemistry and molecular biology international* **39**(6)**:** 1193-1200.

Tipparaju SM, Liu SQ, Barski OA, Bhatnagar A (2007). NADPH binding to beta-subunit regulates inactivation of voltage-gated K^+ channels. *Biochemical and biophysical research communications* **359**(2): 269-276.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T*, et al.* (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**(6654)**:** 990-994.

Umapathy NS, Hick RT, Yu Y, Verin AD, (2010). Functional Expression of the Purinergic Receptor $P2Y_{14}$ in the Human Lung Microvascular

Endothelial Cells (HLMVEC). FASEB J. (Meeting Abstract Supplement) 111.9.

van Suylichem PT, Wolters GH, van Schilfgaarde R (1992). Peri-insular presence of collagenase during islet isolation procedures. *The Journal of surgical research* **53**(5)**:** 502-509.

Vandamme JP, Bonte J (1986). Systematisation of the arteries in the splenic hilus. *Acta anatomica* **125**(4): 217-224.

Vanhoutte PM, Feletou M, Taddei S (2005). Endothelium-dependent contractions in hypertension. *Br J Pharmacol* **144**(4)**:** 449-458.

Vasiljev KS, Uri A, Laitinen JT (2003). 2-Alkylthio-substituted platelet $P2Y_{12}$ receptor antagonists reveal pharmacological identity between the rat brain G_i-linked ADP receptors and $P2Y_{12}$. *Neuropharmacology* **45**(1)**:** 145-154.

Verin A, Zemskov E, Umapathy N, Lucas R (2011). P2Y receptors as regulators of lung endothelial barrier integrity. *J Cardiovasc Dis Res* **2**(1)**:** 14-22.

Verspohl EJ, Johannwille B, Waheed A, Neye H (2002). Effect of purinergic agonists and antagonists on insulin secretion from INS-1 cells (insulinoma cell line) and rat pancreatic islets. *Canadian journal of physiology and pharmacology* **80**(6): 562-568.

Vial C, Evans RJ (2002). P2X1 receptor-deficient mice establish the native P2X receptor and a $P2Y_6$-like receptor in arteries. *Mol Pharmacol* **62**(6)**:** 1438-1445.

Vollmayer P, Clair T, Goding JW, Sano K, Servos J, Zimmermann H (2003). Hydrolysis of diadenosine polyphosphates by nucleotide pyrophosphatases/phosphodiesterases. *European journal of biochemistry / FEBS* **270**(14): 2971-2978.

von Kugelgen I (2006). Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacology & therapeutics* **110**(3): 415-432.

von Kugelgen I, Starke K (1990). Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P2X-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **341**(6)**:** 538-546.

Waldo GL, Harden TK (2004). Agonist Binding and G_q-Stimulating Activities of the Purified Human $P2Y_1$ Receptor. *Molecular pharmacology* **65**(2)**:** 426-436.

Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C*, et al.* (2002). P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* **40**(6)**:** 841-853.

Watts DT (1953). Stimulation of uterine muscle by adenosine triphosphate. *Am J Physiol* **173**: 291–296.

Wedd AM (1931). The action of adenosine and certain related compounds on coronary flow of the perfused heart of the rabbit. *J Pharmacol Exp Ther* **41**: 355–366.

Wedd AM, Drury AN (1934). The action of certain nuclei acid derivatives on the coronary flow in the dog. *J Pharmacol Exp Ther* **50**: 157–164.

Weiss MA (2009). Proinsulin and the genetics of diabetes mellitus. *The Journal of biological chemistry* **284**(29): 19159-19163.

Werner P, Seward EP, Buell GN, North RA (1996). Domains of P2X receptors involved in desensitization. *Proceedings of the National Academy of Sciences of the United States of America* **93**(26)**:** 15485-15490.

Wihlborg AK, Malmsjo M, Eyjolfsson A, Gustafsson R, Jacobson K, Erlinge D (2003). Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. *Br J Pharmacol* **138**(8)**:** 1451-1458.

Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T*, et al.* (2004). ADP receptor $P2Y_{12}$ is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol* **24**(10)**:** 1810-1815.

Wildman SS, Unwin RJ, King BF (2003). Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. *Br J Pharmacol* **140**(7)**:** 1177-1186.

Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP (2005). Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J* **389**(Pt 3)**:** 763-774.

Winbury MM, Papierski DH, Hemmer ML, Hambourger WE (1953). Coronary dilator action of the adenine-ATP series. *The Journal of pharmacology and experimental therapeutics* **109**(3)**:** 255-260.

Winzell MS, Strom K, Holm C, Ahren B (2006). Glucose-stimulated insulin secretion correlates with beta-cell lipolysis. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **16 Suppl 1:** S11-16.

Wolf MM, Berne RM (1956). Coronary vasodilator properties of purine and pyrimidine derivatives. *Circ Res* **4**(3)**:** 343-348.

Wolters GH, van Suylichem PT, van Deijnen JH, van Schilfgaarde R (1989). Increased islet yield by improved pancreatic tissue dissociation: the effects of bovine serum albumin and calcium. *Transplantation proceedings* **21**(1 Pt 3)**:** 2626-2627.

Wong SL, Leung FP, Lau CW, Au CL, Yung LM, Yao X*, et al.* (2009). Cyclooxygenase-2-derived prostaglandin $F_{2\alpha}$ mediates endothelium-dependent contractions in the aortae of hamsters with increased impact during aging. *Circ Res* **104**(2)**:** 228-235.

Woodburne RT, Olsen LL (1951). The arteries of the pancreas. *The Anatomical Record* **111**(2): 255-270.

Xu J, Morinaga H, Oh D, Li P, Chen A, Talukdar S*, et al.* (2012). GPR105 ablation prevents inflammation and improves insulin sensitivity in mice with diet-induced obesity. *J Immunol* **189**(4)**:** 1992-1999.

Yaar R, Jones MR, Chen JF, Ravid K (2005). Animal models for the study of adenosine receptor function. *Journal of cellular physiology* **202**(1)**:** 9-20.

Yajima H, Komatsu M, Schermerhorn T, Aizawa T, Kaneko T, Nagai M*, et al.* (1999). cAMP enhances insulin secretion by an action on the ATP-sensitive K^+ channel-independent pathway of glucose signaling in rat pancreatic islets. *Diabetes* **48**(5)**:** 1006-1012.

Yang S, Cheek DJ, Westfall DP, Buxton IL (1994). Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ Res* **74**(3)**:** 401-407.

Yang S, Fransson U, Fagerhus L, Holst LS, Hohmeier HE, Renstrom E*, et al.* (2004). Enhanced cAMP protein kinase A signaling determines improved insulin secretion in a clonal insulin-producing beta-cell line (INS-1 832/13). *Mol Endocrinol* **18**(9)**:** 2312-2320.

Yegutkin GG, Burnstock G (1999). Steady-state binding of adenine nucleotides ATP, ADP and AMP to rat liver and adipose plasma membranes. *Journal of receptor and signal transduction research* **19**(1-4)**:** 437-448.

Yegutkin GG, Henttinen T, Jalkanen S (2001). Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **15**(1)**:** 251-260.

Yegutkin GG, Henttinen T, Samburski SS, Spychala J, Jalkanen S (2002). The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *The Biochemical journal* **367**(Pt 1)**:** 121-128.

Youl E, Bardy G, Magous R, Cros G, Sejalon F, Virsolvy A*, et al.* (2010). Quercetin potentiates insulin secretion and protects INS-1 pancreatic beta-cells against oxidative damage via the ERK1/2 pathway. *Br J Pharmacol* **161**(4)**:** 799-814.

Yu W, Hill WG (2013). Lack of specificity shown by $P2Y_6$ receptor antibodies. *Naunyn-Schmiedeberg's archives of pharmacology* **386**(10)**:** 885-891.

Zanone MM, Favaro E, Doublier S, Lozanoska-Ochser B, Deregibus MC, Greening J*, et al.* (2005). Expression of nephrin by human pancreatic islet endothelial cells. *Diabetologia* **48**(9)**:** 1789-1797.

Zawalich WS, Zawalich KC (1996). Regulation of insulin secretion by phospholipase C. *Am J Physiol-Endoc M* **271**(3)**:** E409-E416.

Zhang Z, Wei EQ, Lu YB (2013). [Role of G protein-coupled receptor 17 in central nervous system injury]. Zhejiang da xue xue bao. Yi xue ban = Journal of Zhejiang University. *Medical sciences* **42**(3): 355-359.

Zhao Y, Zhang L, Longo LD (2005). PKC-induced ERK1/2 interactions and downstream effectors in ovine cerebral arteries. *American journal of physiology. Regulatory, integrative and comparative physiology* **289**(1)**:** R164-171.

Zhou J, Alvarez-Elizondo MB, Botvinick E, George SC (2013). Adenosine A_1 and prostaglandin E receptor 3 receptors mediate global airway contraction after local epithelial injury. *Am J Respir Cell Mol Biol* **48**(3)**:** 299-305.

Zimmermann H (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362**(4-5)**:** 299-309.

Zocchi C, Ongini E, Conti A, Monopoli A, Negretti A, Baraldi PG*, et al.* (1996). The non-xanthine heterocyclic compound SCH 58261 is a new potent and selective A2a adenosine receptor antagonist. *Journal of Pharmacology and Experimental Therapeutics* **276**(2)**:** 398-404.