

Identification and characterisation of the $P2Y_{14}$ purine receptor in the porcine coronary artery

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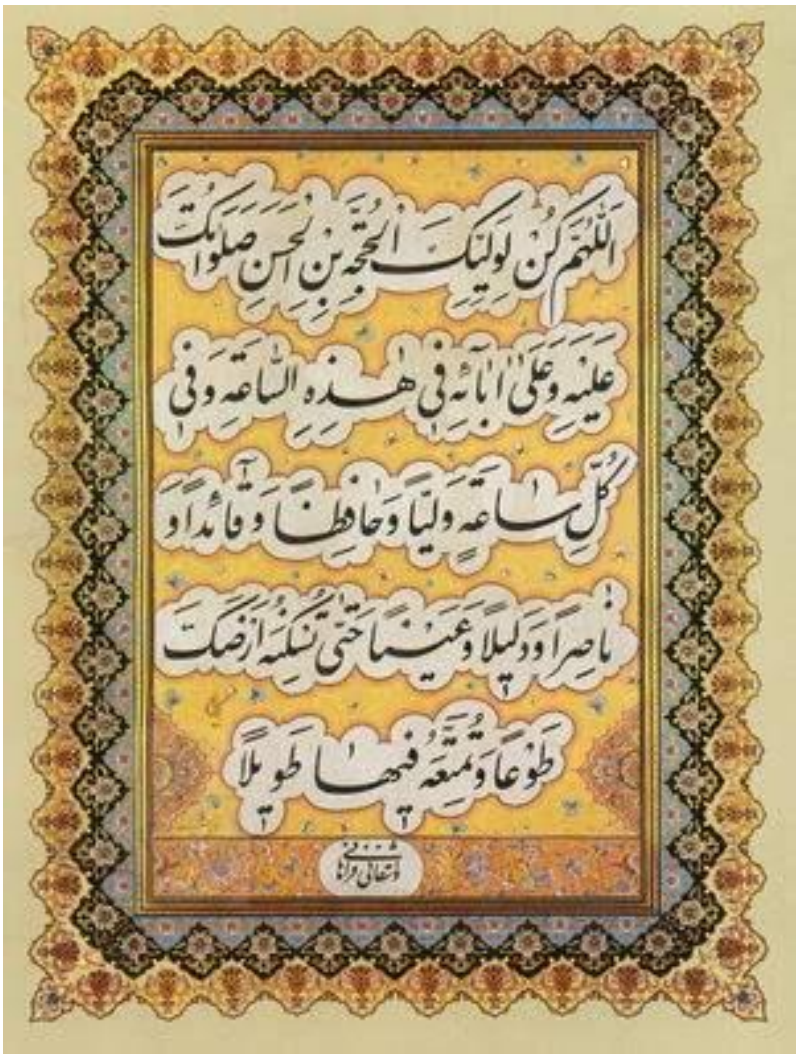
School of Life Sciences

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy in the area of Cardiovascular Pharmacology

April 2017

In the Name of Allah, the Most Gracious the Most Merciful

The Blessings of Allah are upon Prophet Muhammad and His Holy Progeny



Abstract

Introduction: The $P2Y_{14}$ receptor, with its unique pharmacologic profile, is the only member of its P2Y family that is activated by UDP-sugar nucleotides as well as MRS2690, the synthetic analogue of UDP-glucose. Its role in the immune and inflammatory systems is well documented; however its role in the cardiovascular system and, particularly, the vasculature is beginning to emerge.

Aims and Objectives: The aim was to provide evidence for the expression and the role of the $P2Y_{14}$ receptor in porcine coronary arteries. The objectives of the study were to characterize the $P2Y_{14}$ receptor response profile in the presence of a $P2Y_{14}$ receptor antagonist, PPTN, in the porcine coronary artery using organ bath pharmacology. The effects of non-selective P2 receptor antagonists, suramin and PPADS and the $P2Y_6$ receptor antagonist MRS2578, on responses to UDP-glucose and MRS2690 were investigated. $P2Y_{14}$ receptor protein expression and receptor localization was explored using Western blot and immunohistochemistry. $P2Y_{14}$ receptor coupling to the inhibition of adenylyl cyclase/cAMP activity, using a vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) bead assay, in porcine coronary arteries was determined. Human isolated red blood cells (RBCs) and platelets as sources of UDP-glucose were investigated. Hypoxia-induced release of UDP-glucose from porcine coronary arteries was also investigated. The function of the $P2Y_{14}$ receptor as an anti/pro-inflammatory stimulus in porcine coronary arteries was also explored.

Results: UDP-glucose, UDP-*N*-acetylglucosamine and UDP-glucuronic acid elicited concentration-dependent contractions in the porcine coronary artery;

MRS2690-induced contractions were 10-fold more potent than those of the UDP-sugars. The contractile responses to MRS2690, but not UDP-glucose, were significantly inhibited by PPTN (3 μM) (in presence of U46619 and forskolin to precontract and raise cAMP, respectively). The contractile responses to UDP-glucose, but not MRS2690, were altered (either inhibited or potentiated) in the presence of suramin and PPADS. MRS2578, a $P2Y_6$ receptor antagonist, did not change the contractile responses to UDP-glucose or MRS2690 in U46619 preconstricted coronary arteries. MRS2578 did not change the contractile responses to MRS2690, but did to UDP-glucose (in the presence of forskolin plus U46619). Immunoblotting showed that the $P2Y_{14}$ receptor protein exists in two isoforms, sized 41 kDa and 61 kDa in porcine coronary arteries. Immunohistochemistry revealed localization of $P2Y_{14}$ receptors in the vascular smooth muscle cells and endothelium of porcine coronary arteries. UDP-glucose and MRS2690 significantly decreased VASP-P in coronary arteries, an effect which was partially reversed in the presence of PPTN. Red blood cells induced contractions in preconstricted porcine coronary arteries; the response remained unchanged in the presence of PPTN. Platelets induced relaxations and contractions in the absence and presence of L-NAME (inhibitor of nitric oxide synthase), respectively, in porcine coronary arteries; the response remained unchanged in the presence of PPTN. PPTN does not change hypoxia induced relaxation in preconstricted coronary arteries. UDP-glucose and MRS2690 had no significant effect on the release of cytokines/chemokines from segments of coronary arteries.

Conclusion: The action of UDP-sugars and MRS2690, $P2Y_{14}$ receptor agonists, as inducers of contraction is consistent with an involvement of

functional $P2Y_{14}$ receptors in porcine coronary arteries. Inhibition by PPTN, a selective $P2Y_{14}$ receptor antagonist, of contractile responses to a selective $P2Y_{14}$ receptor agonist, MRS2690, further provides evidence of $P2Y_{14}$ receptors in porcine coronary arteries. Release of purine and pyrimidine nucleotides is known to be exaggerated in pathophysiological conditions, such as shear stress, endothelial cell damage and hypoxia. This may elicit chronic activation of P2 receptors including $P2Y_{14}$ receptors, consequently causing increased vasoconstriction of porcine coronary arteries. The patho/physiological sources and stimuli for local UDP/UDP-glucose release remain to be determined since PPTN did not block responses to RBCs, platelets and hypoxia under the conditions of the present study. The $P2Y_{14}$ receptor may be a novel target to control abnormal vascular contraction under pathophysiological conditions.

Conference presentations

1. Z Abbas, L Latif, V Ralevic. Effects of P2Y14 Receptor Agonists, UDP-glucose and MRS2690, on Porcine Coronary Artery Contractility. BPS Winter Meeting, London, 2011 Queen Elizabeth II Conference Centre, poster presentation http://www.pa2online.org/abstracts/vol9issue3abst051p.pdf

2. Z Abbas, W Dunn, V Ralevic.Effect of forskolin on contractile responses to the P2Y14 receptor agonists UDP-glucose and MRS2690 in porcine isolated coronary arteries. BPS Winter Meeting, London, 2012 Queen Elizabeth II Conference Centre, poster presentation http://www.pa2online.org/abstracts/1vol10issue4abst057p.pdf

3. ZSB Abbas, N Dovlatova, SC Fox, WR Dunn, V Ralevic. Effect of a novel P2Y14 receptor antagonist on the porcine coronary artery. BPS Winter Meeting, London, 2012 Queen Elizabeth II Conference Centre, poster presentation http://www.pa2online.org/abstracts/vol11issue3abst192p.pdf

Acknowledgements

My sincerest appreciation goes to Dr Vera Ralevic, my supervisor; I have known Vera to be a very kind and loving Lady who continuously gave her help and support and motivating words of encouragement throughout. My words are not enough to say how remarkable she is, I will not forget her. Secondly, to Dr William Dunn, my co-supervisor whose words and advice always got me thinking for the betterment of my work, his support is very much appreciated and I thank him truly. My thanks to Dr Michael Garle and Mr Liaque Latif, two extremely helpful and cheery laboratory technicians, their advice and guidance has been appreciated greatly. I would like to thank the academics of E34 Medical School, Dr Steve Alexandar, Dr Richard Roberts, Dr Sue Chan and Dr Vince Wilson and to all the staff and students whose names are too many too list, for making my time at the University of Nottingham a truly memorable one. I will miss them all.

I would like to acknowledge the British Heart Foundation who funded this PhD studentship and all staff and members of the University of Nottingham.

My love and appreciation to my Father and my Mother, their prayers and support, alongside my three brothers, has always been there. My love goes to my dear Husband Syed Asif Zaidi and my dear Mother in law, who continuously prayed for my studies and success. To my beautiful Princess Syeda Masooma Raza, I thank her for keeping me up at night so I could finish my thesis. My prayers are with my dear family always.

Contents

Chapter 1: Introduction 1
1.1.1 Historical introduction 2
1.1.3 P2X receptors 3
1.1.4 P2Y receptors 5
1.1.5 Metabolism of nucleotides 7
1.2.1 P2X receptor expression in the cardiovascular system 9
1.2.2 P2X1 receptors 10
1.2.3 P2X4 receptors 12
1.2.4 Other P2X receptors 14
1.3.1 P2Y receptor expression in the cardiovascular system 14
1.3.2 $P2Y_1$ receptors 15
1.3.3 $P2Y_2$ receptors and $P2Y_4$ receptors 16
1.3.4 $P2Y_6$ receptors 18
1.3.5 Trophic functions of P2Y receptors in the cardiovascular system 19
1.3.6 P2 receptors in the coronary artery 21
1.4.1 The $P2Y_{14}$ receptor 23
1.4.2 $P2Y_{14}$ receptor agonists 24
1.4.3 $P2Y_{14}$ receptor antagonist 25
1.5.1 GPR17 26
1.6.1 Release and regulation of ATP and UDP-glucose 27
1.6.2 Stress- and damage-induced UDP-glucose release 31
1.7.1 P2Y14 receptor signalling 34
1.7.2 $P2Y_{14}$ receptors in the heart 37
1.7.3 A vasocontractile role for the $P2Y_{14}$ receptor 37
1.8 Aims 38
Chapter 2: Methods 40
2.1 *In Vitro* pharmacology 41
2.1.1 $P2Y_{14}$ receptor agonist response curves 42
2.1.1.1 Basal tone 42
2.1.1.2 Preconstricted vessels 42
2.1.1.3 Forskolin conditions 42

2.1.1.4 Sodium nitroprusside 43
2.1.1.5 Coronary distal artery 43
2.1.2 P2 receptor antagonists 44
2.1.3 Endothelium-removal studies 44
2.1.5 Hypoxia studies 45
2.1.6 Blood cells 46
2.1.6.1 Preparation of blood cells 46
2.1.7 Lipopolysaccharide (LPS) studies 47
2.2 cAMP/VASP-phosphorylation measurement 48
2.3 Western Blotting 49
2.4 Cell culture 50
2.4.1 Preparation of coverslips 50
2.4.2 Isolation of PCA SMCs – using collagenase 51
2.4.3 Isolation of PCA SMCs – using tissue segments 51
2.5 Immunohistochemistry 52
2.5.1 Porcine coronary arteries 52
2.5.2 PCA smooth muscle cells (PCA SMCs) 53
2.6 Cytokine/Chemokine measurement 55
2.7 Materials 56
2.8 Statistical analysis 58
3.1 Introduction 60
3.2 Aims 62
3.3 Method 62
3.3.1 *In vitro* pharmacology 62
3.3.1.1 $P2Y_{14}$ receptor agonists 62
3.3.1.2 Effect of PPTN, a $P2Y_{14}$ receptor antagonist, on agonist responses 62
3.3.1.3 Effect of U46619 and forskolin on responses to acetylcholine and histamine 63
3.3.1.4 Effect of U46619 and sodium nitroprusside on responses to UDP-glucose 63
3.3.1.5 Endothelium removal studies 63
3.3.2 Western blot 64
3.3.3 Immunohistochemistry 64

3.4.1 $P2Y_{14}$ receptor protein is expressed in porcine coronary arteries 66

3.4.2 $P2Y_{14}$ receptors are expressed in the smooth muscle and endothelial cells of the porcine coronary artery..... 71

3.4.3 $P2Y_{14}$ receptor agonists constrict porcine coronary arteries 78

3.4.4 PPTN, a selective $P2Y_{14}$ receptor antagonist, blocks responses to MRS2690 and partially UDP-glucose, in porcine coronary arteries..... 87

3.4.5 UTP and UTPγS contractions are increased in the presence of forskolin and U46619..... 90

3.4.6 Contractile responses to histamine and acetylcholine are not affected by elevated cAMP levels..... 92

3.5 Discussion..... 96

3.5.1 $P2Y_{14}$ receptors are present the porcine coronary artery in two isoforms..... 96

3.5.3 $P2Y_{14}$ receptor agonists induce contraction of porcine coronary arteries 100

3.5.4 UDP-glucose contractions do not involve endothelial cells..... 101

3.5.5 PPTN antagonizes contractile responses to the selective $P2Y_{14}$ receptor agonist, MRS2690..... 103

3.5.6 Forskolin, an elevator of intracellular cAMP, enhances contractions to $P2Y_{14}$ receptor agonists..... 106

3.5.7 Investigating effects of UTP and UTPγS in the porcine coronary artery..... 107

3.5.8 PPTN does not block muscarinic receptors in PCA..... 111

3.5.9 Concluding comments..... 112

Summary..... 113

Chapter 4: Further characterization of $P2Y_{14}$ agonist responses: effect of P2 receptor antagonists, suramin and PPADS and involvement of cAMP production 114

4.1 Introduction..... 115

4.1.1 P2 receptor antagonists..... 115

4.1.2 $P2Y_{14}$ receptor signalling 117

4.2 Aims..... 120

4.3 Methods..... 120

4.3.1 Antagonist studies 120

4.3.2 Measurement of cAMP: see Chapter 2 Section 2.1.1 for full details. 121

4.4.1 Effect of suramin and PPADS on responses to MRS2690 and UDP-glucose in porcine coronary arteries 122

4.4.2 Effect of MRS2578, a $P2Y_6$ receptor antagonist, on responses to $P2Y_{14}$ receptor agonists in porcine coronary arteries 125

4.4.3 $P2Y_{14}$ receptor agonists decrease VASP-phosphorylation in porcine coronary arteries 127

4.5 Discussion 130

4.5.1 Effects of suramin and PPADS on responses to $P2Y_{14}$ receptor agonists 130

4.5.2 Effect of $P2Y_6$ receptor antagonist on UDP-glucose and MRS2690 133

4.5.3 The $P2Y_{14}$ receptor is coupled to the inhibition of cAMP and VASP-phosphorylation 134

Summary 137

Chapter 5: Pathophysiological roles of the $P2Y_{14}$ receptor in porcine coronary arteries: investigating potential sources of UDP-glucose release from blood cells and the coronary vasculature 138

5.1 Introduction 139

5.2 Methods 140

5.3 Results 142

5.3.1 Effect of isolated blood fractions on porcine coronary arteries 142

5.3.2 Responses to isolated red blood cells in coronary arteries in the presence of PPTN 146

5.3.3 Investigating coronary arteries as a source of UDP-glucose release during hypoxia 149

5.4.1 Investigation of red blood cells as possible sources of UDP-glucose release 150

5.4.2 Investigation of platelets as possible sources of UDP-glucose release 151

5.4.3 Effect of PPTN on platelets in endothelium denuded coronary arteries 153

5.4.4 Effect of PPTN on the hypoxic response of the porcine coronary arteries 155

Summary 157

Chapter 6: Effect of $P2Y_{14}$ receptor activation on release of inflammatory cytokines in porcine coronary artery 158

6.1 Introduction 159

6.1.1 $P2Y_{14}$ receptors control immune and inflammatory responses 159
6.1.2 Lipopolysaccharide induces changes in $P2Y_{14}$ receptor expression 161
6.2 Methods 162
6.2.1 Multiplex magnetic cytokine chemokine bead assay 162
6.2.2 Lipopolysaccharide incubation 163
6.2.3 Statistics 163
6.3.1 Effect of UDP-glucose and MRS2690 on the release of cytokines/chemokines from porcine coronary arteries 164
6.3.2 Effect of LPS on release of cytokines and chemokines from porcine coronary arteries 166
6.3.3 Effects of lipopolysaccharide incubation on porcine coronary arteries responses to $P2Y_{14}$ receptor agonist MRS2690 171
6.4 Discussion 174
6.4.1 $P2Y_{14}$ receptor activation does not significantly alter the release of cytokines and chemokines from porcine coronary arteries 174
6.4.2 Potential involvement of P2 receptor-mediated suppression of cytokines from porcine coronary arteries 175
6.4.4 Future studies: investigating signalling proteins involved in inflammatory pathways in the porcine coronary artery through $P2Y_{14}$ receptor activation 178
6.4.5 MRS2690-induced contractions are augmented in tissues exposed to the immunogenic agent, LPS 179
Summary 183
Chapter 7: General Discussion 184
7.1 Overview of General Discussion 185
7.1.1 $P2Y_{14}$ receptor in the porcine coronary artery 185
7.1.2 $P2Y_{14}$ receptor pharmacological response profile 187
7.1.3 PPTN blocks contractile responses to MRS2690 189
7.1.4 Suramin and PPADS 189
7.1.5 $P2Y_{14}$ receptors couple to the inhibition of $G_{i/o}$ proteins in porcine coronary arteries 191
7.1.6 Red blood cells and platelets do not induce coronary artery contractility through UDP-glucose release 192
7.1.7 Effect of $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690, on the release of cytokine/chemokine from porcine coronary arteries 193

7.2.2 Quantification of UDP-glucose levels 196
7.2.3 Investigating effects of PPTN on responses to UDP-sugar nucleotides 197
7.2.4 Plasma and white blood cells as source of UDP-glucose 198
7.2.5 Measurement of UDP-glucose in myocardial infarction patients 199
7.2.6 Effect of Montelukast on UDP-glucose induced contraction 199
7.2.7 $P2Y_{14}$ receptor knockouts and knockdowns 200
7.2.8 $P2Y_{14}$ receptors as inducers/inhibitors of apoptosis 203
7.2.9 Mitogenic and migratory effects of the $P2Y_{14}$ receptor in coronary arteries 204
7.2.10 Barrier function 205
7.3 Concluding comments 205
7.3.1 Clinical outcomes 206
8.1 Appendices 208
References 210

Abbreviations

5-HT	5-hydroxytrptamine
AC	Adenylyl cyclase
ADP	Adenosine-5'-diphosphate
ADPβS	Adenosine-5'-O-thiodiphosphate
AMP	Adenosine-5'-monophosphate
ANOVA	Analysis of variance
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
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
EDRFs	Endothelium-derived relaxing factors
EDTA	Ethylenediaminetetraacetic acid
ENPPs	Ecto-nucleotide pyrophosphatases
ENTPDases	Ecto-nucleoside-5'-triphosphate diphosphohydrolases
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK	Human embryonic kidney
HL-60	Human promyelocytic leukemia cells
HPLC	High performance liquid chromatography
IL-1α	Interleukin-1-alpha
IL-1β	Interleukin-1-beta
IL-1RA	Interleukin-1-receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
INFγ	Interferon-gamma
KCl	Potassium chloride
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase

NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PCA	Porcine coronary arteries
PLC	Phospholipase C
PPA	Porcine pancreatic arteries
PTX	Pertussis toxin
RBCs	Red blood cells
Rho	Ras homolog gene family member
SDS	Sodium dodecyl sulphate
SNP	Sodium nitroprusside
TCA	Tricarboxylic acid cycle
TNF-α	Tumour necrosis factor
UDP	Uridine-5'-diphosphate
UDP-glucose	Uridine-5'-diphosphate-glucose
UDPβS	Uridine-5'-O-thiodiphosphate
UMP	Uridine-5'-monophosphate
UTP	Uridine-5'-triphosphate
VASP/VASP-P	Vasodilator stimulated phosphoprotein/-phosphorylation
VSMC	Vascular smooth muscle cell
WBCs	White blood cells

Chemical names

α,β-meATP	α,β-methylene-adenosine 5’-triphosphate
2-meSATP	2-Methylthioadenosine 5'-triphosphate tetrasodium salt
ARL67156	6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt
MRS2690	2-thiouridine-5′-diphosphoglucose
MRS2802	α,β-difluoromethylene-uridine diphosphate
MRS2905	α,β-methylene-2-thio analogue
MRS2578	*N*,*N*''-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt
PPTN	4-((piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid
U46619	(5*Z*)-7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-Hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid
UK14304	5-Bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine

Chapter 1: Introduction

1.1.1 Historical introduction

In 1929, Drury and Szent-Gydrgyi documented the physiological actions of adenine nucleotides in the mammalian heart (Drury et al., 1929). Numerous reports followed, looking at the effects of purine nucleosides and nucleotides in the cardiovascular system (see review by Burnstock, 1997). Purinoceptor signalling was initially proposed by Geoffrey Burnstock in 1972 in which he stated that autonomic nerve stimulation caused smooth muscle changes that were not blocked by adrenoceptor antagonists. These nerves, now termed non-adrenergic non-cholinerginc (NANC) nerves, released a neurotransmitter that was neither noradrenaline nor acetylcholine. A surprising discovery was made that the high energy nucleotide purine adenosine 5'-triphosphate (ATP) or a similar acting nucleotide was the candidate identified that caused these changes in the gut and bladder. Hence, the term 'purinergic' signalling was created (Burnstock et al., 1970; Burnstock, 1972).

The hypothesis that purine and pyrimidine nucleotides act as signalling molecules was rejected for almost two decades. The concept of purinergic signalling is now well established and documented (Burnstock, 1997; Burnstock, 2006).

1.1.2 Purinoceptor classification

Purinoceptors are a class of membrane-bound cell surface receptors, present on all mammalian tissues, which are activated by purine and pyrimidine

nucleosides, nucleotides and UDP-sugars (Ralevic et al., 1998; Abbracchio et al., 2003; Burnstock et al., 2014). Purinoceptors are divided into two main classes, namely P1 and P2 receptors. There are four subtypes of P1 receptors, namely A_1, A_{2A}, A_{2B} and A_3 (Ralevic et al., 1998). P1 receptors will not be discussed in this thesis (see Ralevic and Burnstock, 1998 for a review).

1.1.3 P2X receptors

P2 receptors are divided into two subclasses, the inotropic P2X receptors and metabotropic P2Y receptors. P2X receptors are ligand gated ion channels and consist of seven subtypes: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7. The P2X receptors open following the binding of ATP and analogues, such as α,β-methylene ATP (α,β-meATP) and 2-methtlthio-ATP (2-meSATP) the receptor pore opens allowing the movement of small ions and larger organic cations, consequently membrane depolarisation and vasoconstriction in blood vessels (von Kugelgen, 2006; Ralevic, 2012).

P2X receptor desensitization, internalization and recycling

The P2X receptor subunits share 40-50% amino acid sequence similarity, with each subunit having two transmembrane domains and an extracellular domain. The P2X receptor subunits combine together in various configurations forming multimer channels. These can be homomeric channels which are of the same P2X receptor protein. All P2X receptors except P2X6 receptors form homomultimers (Burnstock, 2006; Coddou et al., 2011). The specificity of P2X

receptors has been outlined in extensive detail (Volonte et al., 2006). P2X receptors also form heteromeric channels which are a combination of P2X receptors. There are seven functional heteromultimers with pharmacological properties that vary from the individual homomultimers. These include P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 and P2X4/7, however others have been identified (Burnstock, 2006; Coddou et al., 2011). The activity of native P2X1, P2X2, P2X3, P2X4, P2X5, P2X7 and P2X2/3 has been reviewed (Khakh et al., 2001). The tissue-specificity of the P2X receptors has been well documented (Volonte et al., 2006). Here are some reviews that discuss P2X receptors in detail (Burnstock, 2007; Coddou et al., 2011).

Following activation of P2X1 (and P2X3) receptors by α,β-meATP, the receptors undergo rapid desensitization followed by long recovery periods until the receptors can be activated after subsequent exposure (Roberts et al., 2006; Kaczmarek-Hajek et al., 2012). P2X1 and P2X3 desensitization occurs within milliseconds in comparison to P2X2, P2X4, P2X5 and P2X7 which slowly desensitize, occurring 100 to 1000 times more slower (Ralevic et al., 1998; North, 2002); the time of signal transduction of P2X receptors is determined by receptor desensitization (Hausmann et al., 2015). The ability of P2X (and P2Y) receptors to be able to form homomers and heteromers adds to the biological complexity and heterogeneity and the response of these receptors (Torres et al., 1999; Nakata et al., 2004). The combinations of homomers and heteromers yields various characteristics such as ligand selectivity and binding strength, signalling, desensitization, receptor internalization and recycling;

given this alongside the receptor tissue selectivity leads to a diverse array of functions across the organism (Volonte et al., 2006).

1.1.4 P2Y receptors

The P2Y receptors are G protein-coupled receptors (GPCRs), consisting of eight subtypes (Ralevic et al., 1998; Knight et al., 2003; Burnstock et al., 2014). These are divided into two groups, the first include the $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ $P2Y_{6,}$ $P2Y_{11}$ receptors which couple to $G\alpha_q$ proteins to stimulate phospholipase C (PLC), this generates second messengers inositol 1,4,5-triphosphosphate (IP_3) and diacylglycerol (DAG), which subsequently cause mobilization of intracellular calcium stores and protein kinase C (PKC), activation respectively. The $P2Y_{11}$ receptor also couples to $G\alpha_s$ as well as $G\alpha_q$ proteins and the activation of adenylyl cyclase is followed by an increase in intracellular cAMP levels. The second group consists of $P2Y_{12,}$ $P2Y_{13}$ and $P2Y_{14}$ receptors which couple to $G_{i/o}$ proteins and their activation leads to the inhibition of adenylyl cyclase activity followed by decreases in intracellular cAMP levels (Abbracchio et al., 2003; von Kugelgen, 2006; Harden et al., 2010) (See Figure 1.1 for an illustration). Unlike P2X receptors, the cellular response of P2Y receptors is less than 100 milliseconds following receptor activation (Volonte et al., 2006).

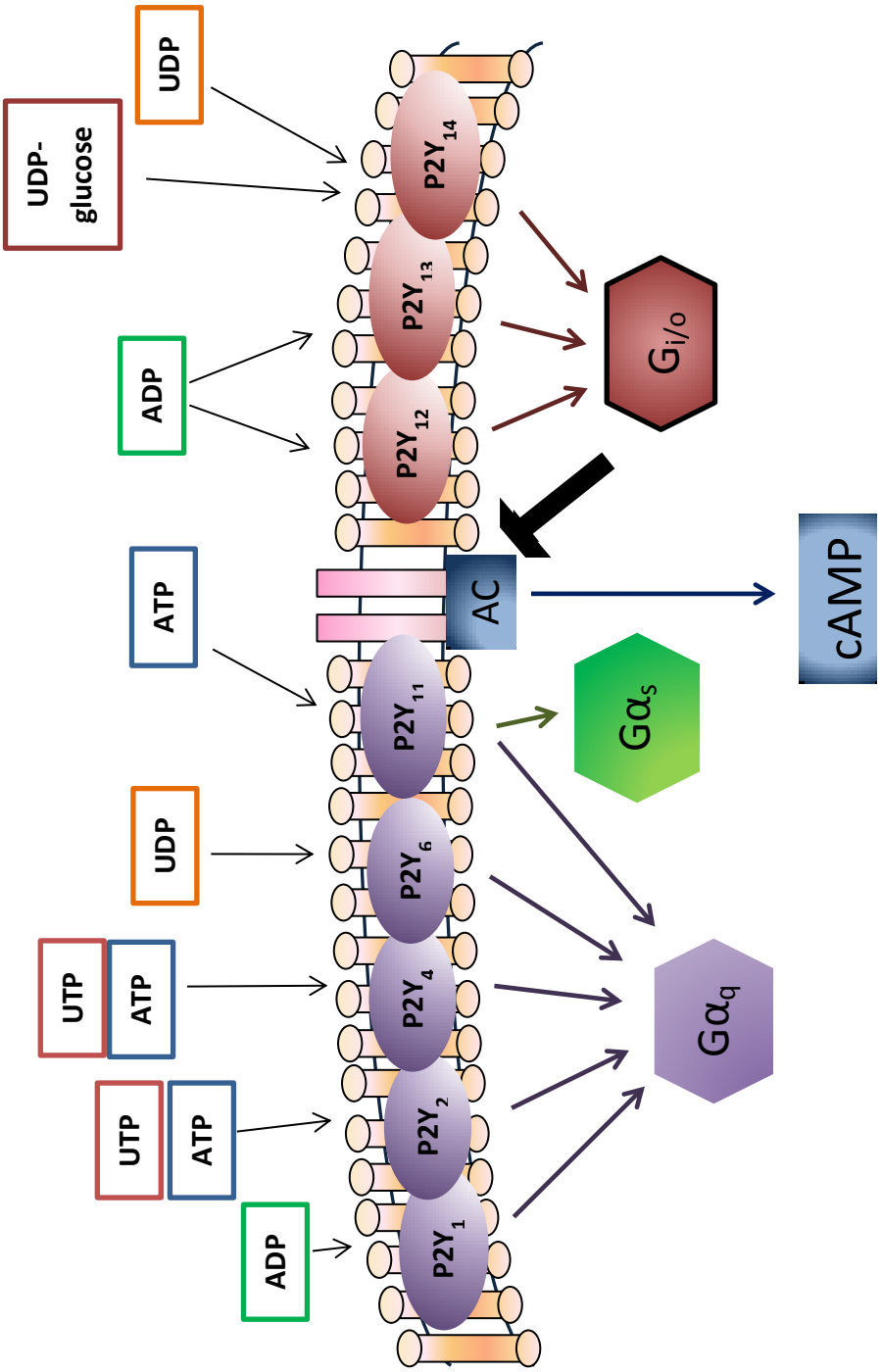


Figure 1.1: The P2Y receptor family showing their nucleotide ligands and protein coupling (figure modified from Harden et al., 2010). AC – adenylyl cyclase.

The factors that regulate the purinoceptor system include release of extracellular nucleotides, the rate at which they are degraded by ectonucleotidases, expression of purinoceptor subtypes, their degree of desensitization and their activation of signalling proteins (Erlinge et al., 2008). Endogenous agonist release occurs from a variety of sources including perivascular nerves, epithelial cells, endothelium and smooth muscle cells, activated platelets and erythrocytes (Burnstock et al., 2014). Purine receptor activation leads to many effects including alteration of vascular tone by inducing a contraction or relaxation, regulation of cardiac function and other effects such as mitogenesis, proliferation, migration and platelet aggregation (Burnstock, 2002; Ding et al., 2011; Burnstock et al., 2014). Homeostatic changes and abnormal environmental stimuli, including shear stress, cell damage and hypoxia can cause the release of abnormal levels of nucleotides (Burnstock et al., 2014).

1.1.5 Metabolism of nucleotides

Nucleotide metabolism results in the formation of the respective nucleoside and phosphate; the phosphate is subsequently reused for nucleotide formation (Pastor-Anglada et al., 1998). Enzymes for nucleotide metabolism were originally defined as ecto-ATPase, required for ATP metabolism, ecto-ADPase (ADP metabolism) or ecto-ADP diphosphohydrolase, ecto-apyrase (ADP/ATP metabolism). This has been changed since there are many enzymes that have multiple substrate specificities and tissue distributions (Zimmermann, 2000). The ectonucleotidases families have been discovered and functionally

characterised; these include the ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase family (E-NPP), alkaline phosphatases (APs) and ecto-5'-nucleotidase (ecto-5'-NT/CD73) (Zimmermann, 2000; Robson et al., 2006; Baqi, 2015). Ectonucleotidases can exist in membrane-bound form, but also when cleaved to become soluble extracellular isoforms known as exonucleotidases (Zimmermann, 2000). For a detailed review on the ectonucleotidase families, the reader is referred to Zimmerman (2000) and Baqi (2015).

There are eight members of the ENTPDase family. Some of the ectonucleotidases including NTPDase1, NTPDase2, NTPDase3 and NTPDase8 as well as E-NPP1 and ENPP3 are located on the cell surface (Kukulski et al., 2005; Stefan et al., 2005). NTPdase4, NTPDase5, NTPDase6 and NTPDase7 are located intracellularly; NTPDase5 and NTPDase6 may be cleaved to be released as soluble enzymes (Baqi, 2015). NTPDase-1 and NTPDase-2 are expressed on endothelial and smooth muscle cells of blood vessels (Pearson et al., 1980; Ralevic et al., 2015). NTPDase 1,2,3, and 8 hydrolyze ATP to ADP and ADP to AMP yielding inorganic phosphate (P_i). ENPPs 1,2 and 3, however, break down ATP in a single step to AMP yielding pyrophosphate (PP_i). AMP is hydrolyzed to adenosine and P_i by either ecto-5'-NT/CD73 or from the different ecto-alkaline phosphatase enzymes (Zimmermann, 2000; Lazarowski et al., 2003a). The action of these ectonucleotidases can be terminated by inhibitor enzymes. There are currently no known selective and potent inhibitors of NTPDases, however, the main one known is ARL67156,

originally named FPL67156 (Crack et al., 1995; Baqi, 2015). ARL67156 is also an inhibitor of APs, however, it is hydrolyzed by ENPPs (Baqi, 2015). Other ectonucleotidase inhibitors include PSB-6436, selective against NTPDase1, 2, 3 and 8 and polyoxometalates (Muller et al., 2006; Brunschweiger et al., 2008), as well as non-nucleotide inhibitors such as suramin, reactive blue and PPADS (Crack et al., 1994; Bultmann et al., 1996; Heine et al., 1999). For a detailed review of inhibitors of ectonucleotidases, see Baqi (2015). These ectonucleotidases are important homeostatic regulators of neurotransmission, platelet aggregation and regulators of vasomotor tone, as they control P2 receptor activation by terminating the action of nucleotides or yielding new nucleotides which activate other P2 receptors (Pearson et al., 1980).

This thesis will now discuss some of the P2X (P2X1 and P2X4) and P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) receptors in detail, particularly outlining their role and function in the cardiovascular system and emphasis on the coronary artery will be given. Information about the $P2Y_{14}$ receptor, including the relatively limited recent evidence for its expression in the cardiovascular system, will also be reported.

1.2.1 P2X receptor expression in the cardiovascular system

P2X receptors have a high binding affinity for ATP in comparison to adenine nucleotides and nucleosides. Activation by ATP leads to a conformational change in the receptors, opening a pore between the subunits which causes a

diffusion of extracellular cations, K^+, Na^+ and Ca^{2+}, to cross the cell membrane, causing cellular depolarisation. This subsequently leads to activation of voltage-gated calcium channels to assist a further influx of Ca^{2+} thereby amplifying the depolarisation, and in vascular tissues leads to vasoconstriction (Valera et al., 1994; Li et al., 2010). In the vasculature, P2X1 and P2X4 receptors are the predominant P2X receptors expressed in smooth muscle cells, although P2X7 receptors are also expressed (Lewis et al., 2001; Harhun et al., 2010). There is limited or no expression of the P2X2, P2X3, P2X5 and P2X6 receptors in vascular smooth muscle cells. Transcript and protein of all seven P2X receptors have been identified in vascular endothelial cells, however apart from P2X4 receptors, their functional roles remain undefined (Ralevic et al., 2015).

1.2.2 P2X1 receptors

ATP and αβ-methylene ATP (αβ-meATP) have been shown to excite the rat atrium and ventricle through P2X receptors expressed on the heart (Burnstock et al., 1983). P2X1 receptors are expressed in the cardiac myocytes (Vulchanova et al., 1996). P2X1 receptors are particularly localized in the gap junctions at intercalated discs in the human myocardium (Jiang et al., 2005) and also co-localized in patches with nerve terminal varicosities in the rat atrium and ventricle (Hansen et al., 1999). P2X1 receptor mRNA is increased in the left ventricle in a congestive heart failure rat model (Hou et al., 1999), Western blotting shows increased P2X1 receptors in atria of human failing hearts (Berry et al., 1998; Berry et al., 1999).

P2X1 receptors are highly expressed in most arterial smooth muscle cells (Bo et al., 1998) and its expression is considerably higher in smaller than larger arteries (Lewis et al., 2001). In line with P2X1 receptor activation by ATP released as a sympathetic cotransmitter, small arteries tend to be more densely innervated than conduit arteries (Nilsson et al., 1986). In general, P2X1 receptor activation elicits vasoconstriction in most vascular beds including the rat pulmonary, basilar, renal, coronary artery (Knight et al., 2003; Mitchell et al., 2012b) human cerebral arteries (Bo et al., 1998) and human saphenous vein (Borna et al., 2003). P2X1 receptor activation also elicits vasoconstriction in the porcine mesenteric artery (Shatarat et al., 2014), mouse mesenteric, femoral, uterine and tail arteries (Vial et al., 2002). In P2X1 receptor-deficient mice, the nerve evoked vasoconstriction and responses to αβ-meATP and ATP are abolished in these arteries (Vial et al., 2002). In P2X1 receptor-deficient mice, blood pressure is normal or slightly elevated (Mulryan et al., 2000). All of these blood vessels are densely innervated by sympathetic nerves which utilize noradrenaline and ATP as cotransmitters (Burnstock, 1990b; von Kugelgen et al., 1994). Thus, the role of the P2X1 receptors on smooth muscle is contraction due to ATP released as sympathetic cotransmitter. P2X1 can also be activated by ATP released from platelets or injured cells (Vial et al., 2002).

P2X1 receptors are expressed in the human coronary artery (Malmsjo et al., 2000b) but not in the rat coronary artery (Lewis et al., 2001), there is also no literature to molecular or functional presence of the receptor in porcine coronary arteries, or other larger mammals such as the dog, cat or rabbit. The proximal region of human coronary arteries has relatively little sympathetic

innervation (Gulbenkian et al., 1993). Interestingly, the P2X1 receptor appears to differ in its contribution to contractility in coronary arteries (compared to other vasocontractile P2 receptors) (see section on P2 receptors in the coronary artery 1.3.6).

In renal vascular smooth muscle cells, monomeric P2X1 receptors and heteromeric P2X1/4 receptors have been functionally characterized. In these vessels, ATP and αβ-meATP elicit prolonged membrane depolarization, thus controlling renal vascular tone (Harhun et al., 2010).

1.2.3 P2X4 receptors

P2X4 receptors are highly expressed in cells and tissues (Ralevic et al., 2015). P2X4 receptors have been found to be localized in murine cardiac ventricular myocytes (Musa et al., 2009). Murine lines overexpressing cardiac-specific P2X4 receptors are protected from heart failure and show enhanced cardiac function, which are also improved in models overexpressing the calsequestrin (CSQ) proteins (Yang et al., 2004). P2X4 receptor knockout mice have worse symptoms in heart failure after left anterior descending coronary artery ligation or pressure overload by transverse aorta constriction, compared to wild type mice (Yang et al., 2014).

Overexpression of human P2X4 receptors in transgenic mice leads to an enhanced cardiac output and contractility compared to non-transgenic mice, after activation of P2X4 receptors with 2-meSATP (Hu et al., 2001). In

Langendorff assessed heart from a mouse model overexpressing P2X4 receptors, there is increased left ventricular developed pressure and increased rate of contraction compared to non-transgenic mice. Crossing of a mouse that has a P2X4 receptor overexpressed gene with a mouse that has an overexpressed CSQ gene, increases the lifespan of the offspring compared to overexpressed CSQ mice alone, thus P2X4 receptor overexpression prolongs life in CSQ mice (Yang et al., 2004).

P2X4 receptors immunoreactivity has been shown in smooth muscle cells of the rat mesenteric artery however a functional role has not yet been demonstrated (Lewis et al., 2000b). The P2X4 receptor is also expressed on the endothelium and its activation leads to an increased production of endothelial derived nitric oxide (NO) as well as activation of guanylyl cyclase and formation of cGMP (Yang et al., 2004). ATP release from endothelial cells, triggered by shear stress, activates P2X4 receptors present on neighbouring endothelial cells, in proximity to the site of release consequently producing a vasodilatation (Yamamoto et al., 2000). In human umbilical cord endothelial cells (HUVECs), antisense oligonucleotides to downregulate P2X4 mRNA and protein levels, diminished ATP-induced and stress-induced responses (Yamamoto et al., 2000). In P2X4 receptor knockout mice, there is decreased production of NO, subsequent to diminished calcium mobilization, causing impairment in blood flow due to reduced vasodilatation, consequently causing hypertension (Yamamoto et al., 2006).

1.2.4 Other P2X receptors

High levels of P2X5 and P2X7 mRNA are abundantly expressed in the rat heart, and it may have an important role in modulation of pacemaking (Musa et al., 2009). However, there is limited information regarding a complete functional role of these P2X receptors in the heart and blood vessels. This thesis will not explore the roles of the other P2X receptors in the cardiovascular system; see Ralevic and Dunn (2015) for a detailed review.

1.3.1 P2Y receptor expression in the cardiovascular system

P2Y receptors have been identified in many vascular beds across several species (Ralevic et al., 1998). Nucleotides induce vasorelaxation through activation of P2Y receptors located on endothelial cells which stimulates release of factors including NO, endothelium-derived hyperpolarizing factor and prostaglandins. Activation of P2Y receptors located on smooth muscle cells typically elicits vasoconstriction (Ralevic et al., 1998; Kauffenstein et al., 2010).

As well as control of vascular tone, P2Y receptors control vascular permeability and vascular cell remodelling by proliferation and migration (Burnstock, 2008). Thus, nucleotides are important vasoactive mediators and modulate vascular tone of blood vessels and may be involved in control of blood pressure (Lewis et al., 2000a).

1.3.2 $P2Y_1$ receptors

$P2Y_1$ receptors are activated by ADP, ADPβS and 2-MeSADP (Burnstock, 1990a). In general, $P2Y_1$ receptor activation mediates vasodilatation in many vascular beds across several species, through the release of endothelium-derived NO (Burnstock, 1990a). Prostacyclin (PGI_2) is also produced from the endothelium in response to activation of P2Y receptors, however, it plays a smaller role in mediating vasodilation under physiological conditions (Ralevic et al., 1998). Both NO and PGI_2 are involved in the inhibition of platelet aggregation (Burnstock et al., 2014). $P2Y_1$ receptor activation elicits vasodilation in rat cerebral arteries (Lewis et al., 2000a), rat mesenteric arteries (Malmsjo et al., 2000b), mouse aorta (Guns et al., 2006; Kauffenstein et al., 2010), human left internal mammary arteries (Wihlborg et al., 2003) and porcine coronary arteries (Rayment et al., 2007b; Zhou et al., 2013).

Blood flow restriction in the heart causes an increase in the release of vasodilator factors, including adenosine and ADP, which decrease vascular resistance during periods of arterial occlusion (Olivecrona et al., 2004). Reperfusion causes a dramatic rise in blood flow which can cause severe inflammation and damage to arteries. The $P2Y_1$ receptors may play a major role in coronary artery blood flow during peak reactive hyperaemia which occurs following reperfusion. The targeting and inhibiting of $P2Y_1$ receptors has been proposed to reduce these effects during angioplasty (unblocking of a vessel) in acute myocardial infarction (Olivecrona et al., 2004).

1.3.3 $P2Y_2$ receptors and $P2Y_4$ receptors

$P2Y_2$ and $P2Y_4$ receptors will be discussed together since they are both activated by UTP and ATP and work together, in some tissues, to maintain homeostasis. $P2Y_2$ receptors are activated by UTP and ATP. Activation of endothelial $P2Y_2$ receptors mediates vasodilation through release of prostaglandins and NO (Burnstock, 1990a; Marrelli et al., 1999; Wang et al., 2002). UTP also activates $P2Y_4$ receptors; the receptors are partially responsive to ATP but not to ADP (Burnstock, 1990a). UTP and ATP induce endothelium-dependent relaxation in the murine aorta through activation of $P2Y_2$ receptors (Guns et al., 2006). In $P2Y_2$ receptor-deficient mice, ATP- and ATPγS- (a stable analogue of ATP) mediated relaxation in the aorta is diminished (Guns et al., 2006). $P2Y_2$ receptors and $P2Y_4$ receptors are also localised on vascular smooth muscle and mediate vasoconstriction after activation by UTP and ATP, in rat pulmonary and renal arteries and bovine middle cerebral artery (Ralevic et al., 1998; Mitchell et al., 2012a). Knockout of $P2Y_2$ receptors in mice results in an increase in blood pressure, however, $P2Y_4$ receptor knockout does not change blood pressure which appears to be normal (Guns et al., 2006; Rieg et al., 2011). These findings suggest that knockdown of $P2Y_2$ receptors results in an exaggerated $P2Y_4$ receptor-induced vasoconstriction, suggesting that under physiological conditions $P2Y_2$ receptors maintain stable blood pressure. These findings are consistent with reports which suggest that $P2Y_2$ and $P2Y_4$ receptor activation mediate vasodilatation and vasoconstriction, respectively, and thus may play a role together to regulate blood pressure (Guns et al., 2006; Rieg et al., 2011). UTP

also induces a vasodilatation through $P2Y_4$ receptor activation expressed on guinea pig aortic endothelial cells (Kaiser et al., 2002).

Pathophysiological conditions which cause the release of purine and pyrimidine nucleotides include ischaemia, stress and injury. These nucleotides act as stress stimuli to induce elevated levels of P2Y receptor expression (Yitzhaki et al., 2005; Blackburn, 2011). In rat aortic intimal lesion and aortic smooth muscle cells of human coronary atherosclerotic lesions, the $P2Y_2$ receptors are highly overexpressed suggesting a role of this receptor in diseased states (Pillois et al., 2002).

Myocardial ischaemia triggers the release of ATP, ADP and UTP in the interstitial fluid of mouse hearts (Wee et al., 2007). In a study that utilized the Langendorff heart, it was shown that after ischemic-reperfusion, UTP treatment improved left ventricular developed pressure, reducing the diastolic phase. A reduction in the necrotic factor lactate dehydrogenase was also seen. These results indicate that UTP attenuates myocardial dysfunction following ischaemia reperfusion injury through $P2Y_2$ receptors (and possibly $P2Y_4$ receptors) (Wee et al., 2007), thus UTP has protective effects on cardiomyocytes during hypoxia. These cardioprotective effects are reduced in the presence of the non-selective P2 receptor antagonist, suramin (Yitzhaki et al., 2005; Cohen et al., 2011; Golan et al., 2011).

MRS2768, a stable agonist at the $P2Y_2$ receptor, diminished hypoxia induced injury in mouse myocytes, as well as reducing infarct size and improving

cardiac function compared to untreated cells (Hochhauser et al., 2013). Moreover, MRS2768 reduced the tumor necrosis factor-alpha (TNF-α) levels which are a major sign of heart failure. $P2Y_2$ receptor blockade antagonized the cardioprotective effects of MRS2768 in hypoxic myocytes. Accordingly, $P2Y_2$ receptors are potential targets for treatment of ischaemia in the heart (Hochhauser et al., 2013).

1.3.4 $P2Y_6$ receptors

The $P2Y_6$ receptor is activated by UDP and also weakly by UTP (Nicholas et al., 1996). In general, $P2Y_6$ receptor activation elicits vasoconstriction in many vascular beds including human saphenous vein (Borna et al., 2003), rat cerebral parenchymal arterioles (Brayden et al., 2013), rat intrapulmonary arteries (Mitchell et al., 2012a), mouse mesenteric arteries (Vial et al., 2002) and mouse renal arteries (Ralevic et al., 1998; Vonend et al., 2005), and porcine coronary arteries (Rayment et al., 2007a). UDP also mediates a contraction in mouse aorta through activation of $P2Y_6$ receptors, and this effect is eliminated in $P2Y_6$ receptor knockout mice (Bar et al., 2008). Although initially proposed to be a contractile receptor (Malmsjo et al., 2000a), the $P2Y_6$ receptor mediates an endothelium-dependent relaxation in human pial arteries (Hardebo et al., 1987) and human left internal mammary artery segments (Wihlborg et al., 2003).

Uracil activation of P2Y receptors has been identified in human cerebral arteries which may play a role in initiating a vasospasm which occurs after a

subarachnoid haemorrhage (Malmsjo et al., 2003). UTP and UDP released from blood clots during haemorrhage cause chronic activation of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors which causes sustained constriction of cerebral arteries (Lewis et al., 2000a). These effects consequently cause tissue ischaemia and necrosis and have been proposed as targets for treatment of cerebral vasospasm (Malmsjo et al., 2003).

1.3.5 Trophic functions of P2Y receptors in the cardiovascular system

There are two different types of smooth muscle phenotypes, the contractile phenotype and the synthetic/proliferative phenotype (Campbell et al., 1985). The change from a contractile phenotype into a proliferative, matrix-producing synthetic phenotype is a condition that occurs in the development of cardiovascular disease (Erlinge et al., 2008). P2X1 receptors are highly expressed in contractile phenotypes, however, are downregulated in synthetic phenotypes, since their main role is mediating contractile effects (Erlinge et al., 1998). $P2Y_2$ receptor mRNA has been found to be significantly upregulated upon change to a synthetic phenotype from a contractile phenotype. This upregulation is said to occur because $P2Y_2$ receptor activation may contribute to trophic functions such as cellular growth, stimulation of matrix proteins or release of growth factors (Erlinge et al., 1998; Erlinge et al., 2008). The increase in $P2Y_2$ receptors mRNA has been found to occur after balloon angioplasty, thus, resulting in UTP- and ATP-mediated proliferation of vascular smooth muscle cells, implying the receptors play a key role in the

development of atherosclerosis and neointimal formation (Seye et al., 1997; Shen et al., 2004).

Adenine nucleotides, ADP and ATP have been shown to induce cell proliferation of porcine aortic vascular smooth muscle cells (Wang et al., 1992). Similarly, UTP and UDP act through $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors to stimulate proliferation and migration of rat aortic vascular smooth muscle cells and intimal hyperplasia; UTP is more potent as $P2Y_2$ receptors are more highly expressed (Pillois et al., 2002). As well as proliferation, activation of $P2Y_{1,}$ $P2Y_2$ and $P2Y_4$ receptors stimulates mitogenesis in smooth muscle cells (Erlinge et al., 1998). Similarly, UDP-induced $P2Y_6$ receptor activation mediates mitogenesis in synthetic vascular smooth muscle cells (Erlinge et al., 1998). Endothelial $P2Y_2$ and $P2Y_4$ receptor activation stimulates cytoskeletal changes, cellular adhesion and motility in the human umbilical cord (Kaczmarek et al., 2005).

$P2Y_4$ receptors are functionally present in cardiac endothelial cells in micro vessels where, after activation by UTP, endothelial cells undergo proliferation, migration and secrete platelet-derived growth factor beta polypeptide which is essential for cardiac development. These effects are all abolished in $P2Y_4$ receptor knockout mice where, after birth, mice appear to have reduced heart growth (Horckmans et al., 2012). $P2Y_4$ receptor knockout mice had a reduced left ventricular mass and decreased septal and posterior wall thickness. Thus, $P2Y_4$ receptors have a role in regulation of cardiac endothelial cell function which is essential for postnatal heart growth. As such, $P2Y_4$ receptor targeted

inhibition may be used for treatment of excessive angiogenesis whilst targeted activation can be used for regulating cardiac remodelling and restoring perfusion to micro vessels after ischaemia (Horckmans et al., 2012).

1.3.6 P2 receptors in the coronary artery

Interestingly the efficacy of the P2X1 receptor agonist α,β-meATP was less than half of that of the $P2Y_2$ and $P2Y_4$ receptor agonists in the human coronary artery, suggesting a primary role for the contractile $P2Y_2$ and $P2Y_4$ receptors versus the P2X1 receptor (Malmsjo et al., 2000b). P2X1 receptors are not expressed in the rat coronary artery (Lewis et al., 2001). Coronary arteries have limited sympathetic innervation (Gulbenkian et al., 1993; Yasuda et al., 1994), thus P2X1 receptor activation through ATP released as a cotransmitter from perivascular nerves may not play a significant role in inducing a change in coronary artery contractility. This raises the question of what are the physiological roles of the $P2Y_2$ and $P2Y_4$ receptors in the coronary artery and are there any other important P2Y receptors in the coronary artery.

A number of P2Y receptors have been identified in the coronary arteries of several species at the molecular level; however, not all receptors are functionally expressed. In endothelium denuded human coronary arteries mRNA expression is apparent for a number of P2 receptors including $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Malmsjo et al., 2000b). RT-PCR analysis of all eight P2Y receptors in human coronary arteries endothelial cells showed mRNA expression for $P2Y_2$ and $P2Y_{11}$ subtypes; with no detection of the other

P2Y subtypes (Ding et al., 2011). In porcine coronary arteries RT-PCR has revealed mRNA expression for $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and A_{2a} receptors (Rayment et al., 2007a; Zhou et al., 2013); in these studies by Rayment et al. (2007a) and Zhou et al. (2013) $P2Y_{14}$ mRNA expression was not investigated.

The $P2Y_2$ receptor has been detected in porcine coronary smooth muscle cells (Rayment et al., 2007a). Diadenosine tetraphosphate, UTP, ATP and stable analogues of UTP and ATP (UTPγS and ATPγS) which are agonists at $P2Y_2$ and $P2Y_4$ receptors, elicit vasoconstriction in human coronary arteries (Malmsjo et al., 2000b) and porcine coronary arteries (Rayment et al., 2007a).

$P2Y_2$ receptor mRNA was shown to increase in organ-cultured arteries compared to tissues that were freshly harvested or from cold-stored coronary arteries, however there were no changes in mRNA levels for $P2Y_4$ or $P2Y_6$ receptors (Hill et al., 2002; Shen et al., 2004). UTP evoked increases in calcium responses were higher in dedifferentiated smooth muscles compared with those from healthy coronary arteries and therefore the $P2Y_2$ or $P2Y_4$ receptors may have a significant role in vascular disease (Hill et al., 2002). UTP evoked increases in calcium in coronary arteries from diabetic dyslipdaemic pigs are also higher in organ-cultured coronary smooth muscle and these responses were attenuated by pertussis toxin, thus, these responses are mediated partially by $G_{i/o}$ proteins (Hill et al., 2001; Hill et al., 2002). UTP and UDP also mediate vasoconstriction in canine epicardial coronary arteries (Matsumoto et al., 1997).

Platelets contain high concentrations of nucleotides, including ATP, ADP and UTP which induce changes in vascular tone of canine coronary arteries (Cohen et al., 1983; Houston et al., 1986). These nucleotides may act through $P2Y_2$ and $P2Y_4$ receptors, causing increased vascular contractility. Thus, these P2Y receptors are proposed targets for treatment of coronary vasospasm and unstable angina (Malmsjo et al., 2000b; Rayment et al., 2007a).

Relatively little is known about the $P2Y_{14}$ receptor, so this thesis will now focus on the $P2Y_{14}$ receptor. The thesis will discuss the agonists and antagonist at the $P2Y_{14}$ receptor, the release of endogenous $P2Y_{14}$ receptor agonists, and the signalling proteins activated downstream to $P2Y_{14}$ receptor activation. Finally, the function of the $P2Y_{14}$ receptor in the cardiovascular system and blood vessels will be discussed.

1.4.1 The $P2Y_{14}$ receptor

The $P2Y_{14}$ receptor, previously known as KIAA001 or GPR105 (Freeman et al., 2001; Abbracchio et al., 2003), was initially cloned from the human immature human myeloid cell line and is the most recently discovered P2Y receptor (Chambers et al., 2000). It is most similar to the $P2Y_{12}$ and $P2Y_{13}$ receptors than to any other of its family members, having 47% sequence homology. The $P2Y_{14}$ receptor gene is located at position 3q24-3q25 of the human chromosome where several other GPCRs are present including other P2Y receptors as well as orphan receptors GPR87, GPR91 and H963 (Abbracchio et al., 2003).

High levels of $P2Y_{14}$ receptor mRNA have been detected in the placenta, adipose tissue, stomach and intestine, and moderately low levels have been detected in the brain, spleen, lung, heart, bone marrow and thymus (Chambers et al., 2000; Freeman et al., 2001). The $P2Y_{14}$ receptor has also been detected in bone marrow hematopoetic stem cells and several immune cells including neutrophils, lymphocytes and dendritic cells (Moore et al., 2003; Sesma et al., 2009). This thesis will not discuss the function of the $P2Y_{14}$ receptor in these tissues and cells.

1.4.2 $P2Y_{14}$ receptor agonists

The $P2Y_{14}$ receptor is the only member of its family to be activated by UDP 5'-diphosphoglucose (UDP-glucose) (Abbracchio et al., 2003). Other agonists which have been identified include the naturally occurring sugars, UDP-galactose, UDP-*N*-acetylglucosamine and UDP-glucuronic acid (Freeman et al., 2001). UDP, previously suggested to have no agonist activity at the $P2Y_{14}$ receptor (Chambers et al., 2000) and then later shown to act as a competitive antagonist in certain cell lines (Fricks et al., 2008), is now proposed to display agonist activity at the $P2Y_{14}$ receptor (Carter et al., 2009). Other naturally occurring uridine-5'-di/triphosphates or adenine nucleotides do not cause $P2Y_{14}$ receptor activation (Ko et al., 2007).

UDP and UDP-glucose are also ligands for the cysteinyl leukotriene receptor, GPR17 (see section on GPR17 1.5.1) which is structurally related to the P2Y receptor family. The GPR17 receptor has selectivity for both nucleotides and

cysteinyl leukotrienes (von Kugelgen, 2006; Daniele et al., 2011). The difference between the GPR17 receptor and the $P2Y_{14}$ receptor is that the GPR17 receptors bind to both cysteinyl leukotrienes and UDP-sugars, whilst $P2Y_{14}$ receptors are only activated by UDP-sugars and not cysteinyl leukotrienes, a review of the literature suggests there are no reports that examine effects of cysteinyl leukotrienes on the $P2Y_{14}$ receptor.

MRS2690 (2-thiouridine-5′-diphosphoglucose), is a synthetic analogue of UDP-glucose and has a potency 7-fold higher for the $P2Y_{14}$ receptor than UDP-glucose (Felix et al., 2012). The potency of MRS2690 (EC_{50} of 49 ± 2 nM) was identified in COS-7 cells which expressed the $P2Y_{14}$ receptor which coupled to the production of inositol phosphate (Ko et al., 2007). In addition, α,β-difluoromethylene-UDP, MRS2802 and α,β-methylene-2-thio analogue MRS2905 are reported to activate the human $P2Y_{14}$ receptor (Jacobson et al., 2012).

1.4.3 $P2Y_{14}$ receptor antagonist

A novel non-nucleotide compound, 4-((piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid (PPTN), was reported as a high-affinity competitive antagonist at the $P2Y_{14}$ receptor (Gauthier et al., 2011; Robichaud et al., 2011). PPTN showed a good binding affinity for the $P2Y_{14}$ receptor with a K_i of 1.9 nM (Robichaud et al., 2011). At concentrations as low as 1 μM, PPTN does not display any interaction at the other seven P2Y receptor family members (Barrett et al., 2013). At concentrations of 1 μM and

10 μM, PPTN has been shown to inhibit UDP-glucose mediated chemotaxis of neutrophils (Barrett et al., 2013), and UDP-glucose- and MRS2690-mediated mast cell degranulation (Gao et al., 2013). Thus, both of these immune cell types functionally express the $P2Y_{14}$ receptor (Scrivens et al., 2006; Gao et al., 2010; Barrett et al., 2013).

$P2Y_{14}$ receptors have recently been identified in intercalated cells in the kidney where UDP-glucose activates the receptor to stimulate the synthesis and release of pro-inflammatory chemokines as well as activating the MEK1/2 and ERK1/2 pathways; these effects were blocked by PPTN (10 μM) (Azroyan et al., 2015). Furthermore, in porcine pancreatic vessels, where $P2Y_{14}$ receptor activation by UDP-glucose and MRS2690 induced a vasoconstriction, PPTN (10 μM) has been shown to inhibit this vasoconstriction (Alsaqati et al., 2014).

1.5.1 GPR17

As well as activating the $P2Y_{14}$ receptor, UDP-glucose is also a potent agonist of the nucleotide and cysteinyl-leukotriene (CysLT) GPR17 receptor (Daniele et al., 2011), which has been detected at high levels in organs susceptible to ischaemic damage such as the brain, heart and kidney (Ciana et al., 2006). GPR17 is a G_i coupled receptor that is structurally related to the P2Y family (Daniele et al., 2011). GPR17 is also activated by cysteinyl-leukotrienes such as LTC_4 and LTD_4, which are inflammatory mediators known to be involved in stroke and cardiovascular diseases (Ciana et al., 2006). Both nucleotides and

CysLTs act as danger signals to alert cells and induce tissue repair (Lecca et al., 2008), suggesting they are important contributors to inflammation.

1.6.1 Release and regulation of ATP and UDP-glucose

Regulated release of nucleotides, and consequent activation of P2 receptor signalling pathways, controls many physiological processes (Ohman et al., 2012a). These pathways can be altered or inhibited by the action of ectonucleotidases which regulate processes such as inflammation, vascular tone and neurotransmission (Pearson et al., 1980). ATP is synthesized and stored as a co-transmitter with noradrenaline in sympathetic nerves (Burnstock, 1990b) and ATP in the vasculature, alongside noradrenaline and neuropeptide Y (NPY) (Burnstock, 1999). Levels of ATP within neuronal cytoplasm are approximately 2-5mM whilst vesicular-stored ATP levels are as high as 100mM (Burnstock, 2007). Other nucleotides including ADP, AMP, GTP and adenosine polyphosphates are stored within vesicles but at lower concentrations. Both ATP and noradrenaline are released from vesicles to act on P2X and alpha$_1$-adrenoceptors, respectively, that are expressed on smooth muscle cells. P2X receptor activation produces excitatory junction potentials, intracellular calcium influx subsequently causing fast acting and brief vasoconstriction. Alpha$_1$-adrenoceptor activation leads to inositol triphosphate production, calcium influx and slow and prolonged contraction. NPY also stored and released from vesicles acts as a prejunctional inhibitory regulator to prevent release of ATP and NA; alternatively, NPY acts as a postjunctional modulatory potentiator to the actions of ATP and NA. Inhibitory enzymes in

the form of soluble nucleotidases are released from nerve varicosities that terminate the action of nerve transmitters (Kennedy et al., 1997). ATP release transport mechanisms from neuronal cells include vesicular exocytosis and from non-neuronal cells, the various mechanisms include the ATP-binding cassette (ABC) transporters, connexon or pannexin hemichannels or plasma membrane F_1/F_0-ATP synthase. Pannexins will be discussed later (page 30). ATP release can occur from a variety of cell types in a controlled manner. These cells include nerve terminals, chromaffin cells, pancreatic acinar cells and also from epithelial cells, endothelial cells, astrocytes, glial cells, fibroblasts, keratinocytes, cardiomyocytes and blood cells (Yegutkin, 2008). For a comprehensive review on mechanisms of ATP release, see Lohman et al., (2012).

The release of ATP is usually complemented by the release of uracil nucleotides including UTP and UDP sugars (Lazarowski et al., 2003b; Lazarowski, 2012). The release of UDP-sugar nucleotides occurs through regulated and non-regulated mechanisms. The regulated release is thought to occur through two main mechanisms, involving exocytosis from vesicles or release from the cytosol through plasma membrane or channel transporters. UDP-sugars are synthesized in the cytosol and transported to the endoplasmic reticulum (ER) and Golgi apparatus through luminal UDP-sugar/UMP antiporters or SLC35 nucleotide sugar transporters expressed in these organelles (Figure 1.1) (Sesma et al., 2009; Lazarowski, 2012). These sugar nucleotides reach concentrations up to 20-fold higher in the ER and Golgi than cytosolic levels (Sesma et al., 2009; Harden et al., 2010). In the ER and Golgi

apparatus, UDP-sugars act as sugar nucleotide glycosyl donors for the synthesis of carbohydrates or to carry out protein glycosylation (Abbracchio et al., 2003). UDP-sugars do not return back into the cytosol, but are released with glycoconjugates and other cargo molecules in vesicles from non-excitatory cells including epithelial, endothelial and astrocytoma cells (Sesma et al., 2009).

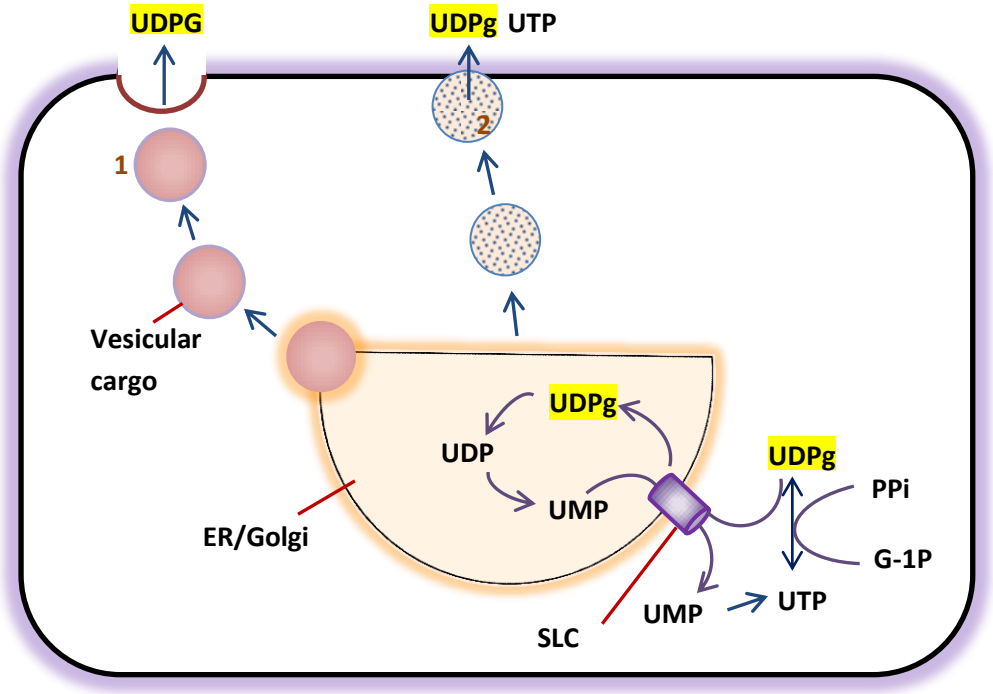


Figure 1.2: Release of UDP-glucose (UDPg). UDPg is synthesized in the cytosol and transported to the endoplasmic reticulum (ER) and Golgi apparatus through luminal UMP/UDPg anitporters, SLC35 nucleotide sugar transporters (1) UDP-sugar nucleotides are released with glycoconjugates and other cargo molecules through the constitutive secretory pathway. (2) Vesicles from Golgi apparatus contain UDP-sugars in granules which are subject to calcium regulated exocytosis. PPi: pyrophosphate; G-1P: glucose-1 phosphate. (Figure modified from Lazarowski and Harden, 2015).

Impairment of nucleotide release due to disruption of vesicular trafficking has been associated with Golgi-disrupting agents. A fungus metabolite, brefeldin A_1, blocks the trafficking between the ER and the Golgi and has been shown to disrupt UDP-sugar release from astrocytoma cells, yeast and also from airway epithelial cells (Esther et al., 2008; Sesma et al., 2009). Similarly, overexpression of a Golgi UDP-*N*-acetylglucosamine transporter gene in airway epithelial cells causes increased release of UDP-*N*-acetylglucosamine and UDP-glucose although the latter less robustly (Sesma et al., 2009). Deletion of the gene, Yea4, which encodes the UDP-*N*-acetylglucosamine transporter in yeast, causes impaired release of this sugar nucleotide and also diminished production of chitin, an acetylglucosamine-abundant constituent of the yeast cell wall (Lazarowski et al., 2015).

Pannexins consist of three types, pannexin 1, 2 and 3. They form transmembrane channel proteins that allow the passage of ATP as well as other nucleotides (Scemes et al., 2009). ATP release through pannexin 1 channels has been documented in several cell types including erythrocytes, T-lymphocytes, neutrophils, epithelial cells and skeletal muscle and blood vessels (Lazarowski, 2012). Activation of pannexin 1 channels in apoptotic lymphocytes induces the release of several nucleosides and nucleotides including ATP, ADP, AMP and UTP (Boyd-Tressler et al., 2014), thus pannexin 1 channels are not nucleotide specific and it is plausible that UDP-glucose could be released through pannexin 1 channels.

1.6.2 Stress- and damage-induced UDP-glucose release

Non-regulatory mechanisms causing UDP-glucose release can occur as a result of damaged or dying cells. This release occurs in conjunction with other nucleotides, including ATP. Apoptosis, stress and hypoxia can cause the unregulated release of nucleotides (Urban et al., 2012).

Constitutive ATP release is often higher than UDP-glucose release, however, over a period of time, UDP-glucose levels become significantly higher than ATP (Lazarowski et al., 2015). Unlike ATP, which is rapidly hydrolysed, UDP-glucose is highly stable and not easily degraded by specific ectonucleotidases, such as ecto-nucleoside triphospho-diphosphohydrolase (NTPDase), which are highly expressed on the surface of many cells. On the other hand, ecto-nucleotide pyrophosphatases (ENPPs) hydrolyse diadenosine polyphosphates including UDP-glucose (Sesma et al., 2012; Lazarowski et al., 2015).

UDP-glucose release has been shown to occur alongside ATP release from 1321N1 human astrocytoma cells upon mechanical stimulation. Levels of UDP-glucose remained elevated for up to 3 hours. These levels were almost three to five times higher than resting ATP levels. ATP levels peaked within 10 minutes and reverted back to basal (Lazarowski et al., 2003b). Furthermore, when neutrophils were incubated with 100 µM of ATP and UDP-glucose, ATP was rapidly hydrolysed whilst the concentration of UDP-glucose remained unchanged after 1 hour incubation (Sesma et al., 2012).

Intracellular UDP-glucose levels are believed be in nanomolar concentrations (Lazarowski et al., 2003b). In resting cells, UDP-glucose levels are much higher than ATP levels in several cell lines, including Calu-3 airway epithelial cells (Kreda et al., 2007) COS-7, CHO-K1 and C6 glioma cells (Lazarowski et al., 2003b). In mice, the plasma levels of UDP-glucose are approximately 5 nM, these levels are significantly higher (3-fold higher) in mice with diet induced obesity (Xu et al., 2012). Table 1.1 shows the intracellular levels of UDP-glucose and its release from a variety of cell types.

Cell type	Intracellular UDP-glucose levels	Mechanism of release	Reference
White blood cells	32 ± 10 µM/100g white blood cells	-	(Gibson et al., 1994)
Platelets	1231 pmol/mg	-	(Wandall et al., 2012)
Red cells	7-10 µM/ 100g haemoglobin	-	(Palmieri et al., 1991; Gibson et al., 1995)
	Extracellular release of UDP-glucose		
C6 glioma cells	1 ± 1 nM	constitutive	(Lazarowski et al., 2003b)
CHO-K1 cells	6 ± 3 nM	constitutive	(Lazarowski et al., 2003b)
COS-7 cells	22 ± 4 nM	constitutive	(Lazarowski et al., 2003b)
1321N1 Human astrocytoma cells	10 ± 4 nM	constitutive	(Lazarowski et al., 2003b)
Normal bronchial cells	12 ± 5 nM	constitutive	(Lazarowski et al., 2003b)
Cystic Fibrosis bronchial cells	14 ± 3 nM	constitutive	(Lazarowski et al., 2003b)
Calu-3 Human airway epithelial cells	16 ± 4 nM* 18 nM*	*constitutive **ionomycin induced (10 µM)	*(Lazarowski et al., 2003b; Kreda et al., 2007) **Kreda et al., 2007)
Human airway goblet cells (SPOC1 cells)	10 nM* 35 nM**	*constitutive **stimulated by ATP	(Kreda et al., 2007)
1321N1 Human astrocytoma cells	8 nM* 14 nM*	*stimulated by thrombin *stimulated by thrombin and β,γ-metATP	(Kreda et al., 2008)

Table 1.1: The intracellular levels of UDP-glucose in blood cells and constitutive/stimulated release of UDP-glucose from various cell types. Measurements of constitutive release of UDP-glucose from C6 glioma, CHO-

K1, COS-7, 1321N1, normal/cystic bronchial cells and Calu-3 cells were taken from cells incubated in 0.3 ml DMEM for 2 h (Lazarowski et al., 2003b). Calu-3 cells were incubated for 90 min in Hank's balanced salt solution followed by addition of 10 µM ionomycin with a further 5 min incubation, UDP-glucose was measured in the extracellular solution from 100 µl aliquots (Kreda et al., 2007). SPOC1 cells were incubated in 0.5 ml of minimal essential media treated with and without 300 µM ATP for 30 min, UDP-glucose was measured in the extracellular solution from 100 µl aliquots (Kreda et al., 2007). 1321N1 cells were incubated in serum-free minimum media for 2 h followed by incubation of 20 nM thrombin for 5 min, measurements were taken from the 0.3ml medium (Kreda et al., 2008).

1.7.1 P2Y14 receptor signalling

The $P2Y_{14}$ receptor couples to the inhibition of adenylyl cyclase through $G_{i/o}$ proteins which are inhibited by pertussis toxin (Chambers et al., 2000; Abbracchio et al., 2003; Scrivens et al., 2005b). Activation of Gi-coupled receptors causes inhibition of adenylyl cyclase, which is involved in the conversion of ATP to cyclic adenosine monophosphate (cAMP) (Scrivens et al., 2005b; Scrivens et al., 2006), cAMP activates protein kinase A which then phosphorylates a number of proteins including transcription factor cAMP response element binding protein (CREB). In microglial cells, the intracellular levels of CREB phosphorylation have been shown to be reduced upon stimulation with UDP-glucose and UDP-galactose. These results suggests that the $P2Y_{14}$ receptor couples to $G_{i/o}$ proteins (Brautigam et al., 2008).

In SH-SY5Y neuroblastoma cells, UDP-glucose has been found to inhibit forskolin (elevator of intracellular cAMP) induced cAMP elevation (Scrivens

et al., 2005b). These inhibitory effects of UDP-glucose were abolished in cells that had been pre-treated with pertussis toxin. Although UDP-glucose inhibited cAMP accumulation, the study suggested that the effects of UDP-glucose are independent of the $P2Y_{14}$ receptor as the mRNA for this protein was not detected in either SH-SY5Y or U373 MG cells (Scrivens et al., 2005b). Therefore the effects could have been through the GPR17 receptor, for which is known to be activated by UDP-glucose (Daniele et al., 2011).

UDP-glucose has also been found to induce a significant inhibition of forskolin stimulated cAMP accumulation in human neutrophils and T-lymphocytes, however, other related sugar nucleotides including UDP-galactose and UDP-glucuronic acid (100 μM) did not have any significant inhibitory effect (Scrivens et al., 2006). Pertussis toxin treatment of $P2Y_{14}$ receptor transfected HEK293 cells caused a block in UDP-glucose-stimulated GTPγS binding to cell membranes. Furthermore, UDP-glucose-stimulated Ca^{2+} increases in rat cortical astrocytes and human immature monocyte derived dendritic cells, and UDP-glucose-stimulated cAMP accumulation in U373-cells (glioblastoma astrocytoma cells), are all abolished with pertussis toxin treatment (Scrivens et al., 2005a; Scrivens et al., 2006).

In human embryonic kidney 293 cells (HEK-293), CHO cells, and C6 glioma cells, all stably expressing the $P2Y_{14}$ receptor, UDP-glucose and related sugar nucleotides inhibited forskolin-stimulated cAMP production, an effect eliminated after pre-treatment of cells with pertussis toxin (Carter et al., 2009). UDP-glucose does not have any effect in wild-type C6 glioma or CHO cells

(Carter et al., 2009; Fricks et al., 2009). The $P2Y_{14}$ receptor antagonist, PPTN, elevated basal and forskolin-stimulated cAMP accumulation in C6-glioma cells expressing $P2Y_{14}$ receptors (Barrett et al., 2013) which is consistent with previous studies which show that UDP-glucose is constitutively released from these cells (Lazarowski et al., 2003b). Thus $P2Y_{14}$ receptors in C6 glioma cells are coupled to the inhibition of adenylyl cyclase.

UDP-glucose activates MAPK/ERK1/2 proteins in HEK-293 cells expressing $P2Y_{14}$ receptors and in differentiated HL60 human myeloid leukaemia cells (Fricks et al., 2009). Increases in ERK1/2 phosphorylation were also evident after $P2Y_{14}$ receptor activation via UDP-glucose in neutrophils (Skelton et al., 2003).

In MDCK-C11 cells, an epithelial cell line, UDP-glucose induces phosphorylation of the MAPK related protein, ERK 1/2 but not p38 or JNK/SAPK. This effect was attenuated by the $P2Y_{14}$ receptor antagonist, PPTN (Azroyan et al., 2015). Nuclear factor kappa-β ligand (RANKL) activates the RANKL receptor expressed on osteoclasts leading to osteoclast differentiation. This RANKL mediates the activation of the p38 MAP kinase signalling pathway and also contributes to the induction of $P2Y_{14}$ receptor expression on osteoclasts. p38 MAP kinase pathway blockade in bone marrow-derived monocytes/macrophages (BMMs), results in an attenuation of the $P2Y_{14}$ receptor protein expression (Lee et al., 2013).

1.7.2 $P2Y_{14}$ receptors in the heart

Qualitative RT-PCR analysis has revealed that the $P2Y_{14}$ receptor is the most abundantly expressed P2Y receptor in the left ventricle of the rat heart. The $P2Y_{14}$ receptor is also highly expressed in the right atrium and in the sinoarterial node (SAN) of the rat heart (Musa et al., 2009). The expression of $P2Y_{14}$ receptor mRNA is localized in the cardiac myocytes and also SAN nodal myocytes. In the human SAN and the atrium, the $P2Y_{14}$ receptor transcript is the most highly expressed in the former (Musa et al., 2009). Although $P2Y_{14}$ receptor transcripts are expressed abundantly in the rat and human heart (Musa et al., 2009), as yet, there is no evidence to suggest a functional role for this receptor in these tissues. In mouse isolated, electrically-stimulated cardiomyocytes, the $P2Y_{14}$ receptor agonist, UDP-glucose, does not appear to have any effect on cardiomyocyte contractility (Wihlborg et al., 2006).

1.7.3 A vasocontractile role for the $P2Y_{14}$ receptor

My studies were the first to show a contractile role for the $P2Y_{14}$ receptor in the vasculature, in studies carried in porcine coronary arteries (Abbas et al., 2011, 2012a, 2012b). Recent reports have started to establish a role for the $P2Y_{14}$ receptor in the vasculature. UDP, UDP-glucose and MRS2690 (a more selective agonist at the $P2Y_{14}$ receptor) elicit contractions in porcine pancreatic arteries which are reduced in the presence of the selective $P2Y_{14}$ receptor antagonist, PPTN (Alsaqati et al., 2014). PPTN does not have any effect upon UTP mediated contraction in these vessels (Alsaqati et al., 2014). Similarly, a study which utilized $P2Y_2$ receptor knockout mice showed that UDP-glucose mediates contraction of mouse coronary and basilar arteries through activation

of $P2Y_{14}$ receptors (Haanes et al., 2014a). The $P2Y_{14}$ receptor has also been found to be expressed in the mouse uterus, salivary glands, bronchioles and pancreas) (Meister et al., 2014) which may signify increased levels of the $P2Y_{14}$ receptor in the innate immune system or in areas susceptible to pro-inflammatory reactions. Meister et al. (2014) also have shown increased levels of $P2Y_{14}$ receptors in blood vessels (mainly in venous blood vessels, increases in blood pressure and heart rate were observed in $P2Y_{14}$ receptor deficient mice (Meister et al., 2014).

1.8 Aims

Since there are recent reports to show contractile $P2Y_{14}$ receptors in the cardiovascular system, the aims were to identify and characterise a functional role of the $P2Y_{14}$ receptor in the porcine coronary artery, as there is evidence to show it has one of the highest mRNA expression levels in the heart.

The effects of $P2Y_{14}$ receptor agonists, UDP-glucose, UDP-sugar nucleotides and MRS2690 in the porcine coronary artery were characterised in the absence and presence of the novel $P2Y_{14}$ receptor antagonist, PPTN, using organ bath pharmacology. $P2Y_{14}$ receptor protein expression and localization in porcine coronary arteries was determined using Western blotting and immunohistochemical staining. Effects of non-selective P2 receptor antagonists, suramin, PPADS and selective $P2Y_6$ receptor antagonist, MRS2578, on responses to $P2Y_{14}$ receptor agonists were investigated. To

determine whether $P2Y_{14}$ receptors couple to the inhibition of adenylyl cyclase, an assay to measure vasodilator-stimulated phosphoprotein (activated downstream to cAMP activation), was employed using a bead assay and flow cytometry. Potential endogenous sources of UDP-glucose were employed by investigating the effects of human platelets and red blood cells, in the presence of PPTN in porcine coronary arteries. The role of $P2Y_{14}$ receptor in inflammation was investigated by examining the effects of $P2Y_{14}$ receptor agonist on release of cytokines/inflammatory markers from porcine coronary arteries.

Chapter 2: Methods

2.1 *In Vitro* pharmacology

Hearts were obtained from pigs (of either sex, around 6 months old and weighing approximately 50 kg) and were transported on ice from a local abattoir. Porcine left circumflex coronary arteries were dissected from hearts and stored in oxygenated Krebs-Henseleit buffer overnight at 4^0C. The Krebs-Henseleit buffer was composed of (mM): sodium chloride (NaCl) 118; potassium chloride (KCl) 4.8; magnesium sulphate ($MgSO_47H_2O$) 1.1; sodium hydrogen carbonate ($NaHCO_3$) 25.0; potassium hydrogen phosphate (KH_2PO_4) 1.2; glucose 12; calcium chloride ($CaCl_2H_2O$) 1.25. A fine dissection was carried out the following day. Two adjacent, approximately 5 mm, ring segments were cut from the proximal end of each vessel. Porcine coronary artery (PCA) segments were mounted for isometric tension recording (using isometric force transducers, Grass FT03) in warmed (37^0C) and gassed (95% O_2, 5% CO_2) Krebs-Henseleit buffer in organ baths (Rayment et al., 2007). Rings were placed under an initial tension of 10g. Arteries were left for approximately 45 min to equilibrate and reach a stable tone. Tissue viability was then assessed by eliciting contractions with 60mM KCl. A KCl response under 3 g in coronary artery segments was excluded from experiments. An initial contractile response to KCl was established then the KCl was washed out and after a further 15 min a second addition was added. After washout of KCl, arteries were left for 30-45 min to equilibrate and reach a stable tone. This was the standard procedure, carried out at the start of all organ bath experiments. The following procedures were subsequently carried out.

2.1.1 $P2Y_{14}$ receptor agonist response curves

2.1.1.1 Basal tone

After the second KCl had been washed out, vessels were left for approximately 45 min to relax completely to a stable baseline (basal tone). Cumulative concentration response curves to UDP-glucose (0.1 µM -1 mM), MRS2690 (1 nM -10 µM), UDP-glucuronic acid (0.1 µM -1 mM) and UDP-*N*-acetylglucosamine (0.1 µM -1 mM) were constructed at basal tone.

2.1.1.2 Preconstricted vessels

In some experiments, arteries were preconstricted with U46619 (5-10nM), a thromboxane A_2 mimetic, to 35-75% of the KCl-induced contraction and cumulative concentration-response curves to some of the $P2Y_{14}$ receptor agonists were carried out.

2.1.1.3 Forskolin conditions

In other experiments, forskolin (0.1 µM), an activator of adenylyl cyclase, was used to reverse the U46619-mediated contraction (by inducing a vasorelaxation) to restore the tone of the vessels to their original basal tone. When vessels had reached a stable tone (after about 30-45 min), cumulative concentration-response curves to UDP-glucose, MRS2690, UDP-glucuronic acid and UDP-*N*-acetylglucosamine were constructed. Cumulative response

curves to acetylcholine (10 nM-30 μM) and histamine (10 nM–100 μM) were also constructed as a negative control (to determine forskolin effects on responses to non-G_i coupled receptor agonists). Cumulative concentration response curve to UTP (0.1 μM -1 mM) and UTPgammaS (UTPγS) (0.1 μM - 100 μM), agonists at $P2Y_2$ and $P2Y_4$ receptors, were also constructed in forskolin conditions.

2.1.1.4 Sodium nitroprusside

In separate experiments, after preconstricting arteries with U46619 (5-7 nM), sodium nitroprusside (SNP 2.5–25 μM), an activator of guanylyl cyclase, was added to induce a relaxation and reverse the U46619-induced contraction. In control experiments, vessels were preconstricted with U46619 which was then washed out to reverse the U46619 induced contraction. Cumulative concentration-response curves to UDP-glucose and MRS2690 were constructed.

2.1.1.5 Coronary distal artery

Small coronary arteries from the distal ends (2 mm in length, approximate diameter of 0.8 mm) were dissected and were set up for isometric tension recording in a myograph. After eliciting two contractions with KCl (60 mM), preconstricting with U46619, and adding forskolin to reverse U46619-induced contraction, cumulative concentration-response curves to UDP-glucose and MRS2690 were generated.

2.1.2 P2 receptor antagonists

In separate experiments, PPTN (1 μM) was added 10 min prior to addition of U46619 and forskolin. Cumulative concentration-response curves to UDP-glucose, MRS269, UTP, UTPγS and acetylcholine were constructed in the absence and presence of PPTN (1 μM).

In separate experiments, suramin (100 μM) and PPADS (10 μM), non-selective P2 receptor antagonists, and MRS2578 (10 μM), a $P2Y_6$ receptor antagonist, were added 10 min before U46619 addition. After approximately 30 min, during which time a stable contraction to U46619 was produced, concentration-response curves to UDP-glucose and MRS2690 were constructed. Thus, the antagonists were in contact with the tissues at least 30 min prior to addition of agonists. In separate experiments, UDP-glucose concentration-response curves were generated in forskolin conditions (see 2.1.1.3) in the presence of MRS278, a $P2Y_6$ receptor antagonist.

2.1.3 Endothelium-removal studies

The endothelium of the PCA was removed by gently rubbing the lumen of the vessel Krebs-Henseleit-dampened tissue, using fine forceps (Rayment et al., 2007a). In other vessel segments, the endothelium remained intact. A cumulative concentration-response curve to UDP-glucose was generated as previously described under forskolin and U46619 conditions. Substance P, an endothelium-dependent vasorelaxant (10nM), was added at the end of the

experiment to assess whether the endothelium was intact (induced relaxation) or denuded (did not cause relaxation). The endothelium was considered to have been removed if substance P caused a relaxation that was less than 10% of the U46619-induced tone.

2.1.4 Porcine coronary veins

Porcine coronary veins were carefully dissected out similarly to coronary arteries. Vessels were set up in organ baths and were tensioned to 2g. Contractility was assessed with KCl (60mM) and vessels were preconstricted with U46619 as described for the arteries (see Section 2.1.0). Concentration response curves to UDP-glucose were generated.

2.1.5 Hypoxia studies

PCA were preconstricted with U46619, in which PPTN (1 μM) was added prior to preconstriction. In control vessels, the vehicle control, DMSO, was added (as PPTN is water insoluble). After vessels had achieved a stable preconstricted tone, the gassing of the Krebs-Henseleit solution was switched to 95% Nitrogen; 5% $C0_2$ to induce hypoxia, and the response was monitored over time (a biphasic response was seen).

2.1.6 Blood cells

2.1.6.1 Preparation of blood cells

Venous blood was obtained from healthy volunteers (see appendix, Table A) (from School of Life Sciences, University of Nottingham). Ethics approval was obtained by Thrombosis and Haemostasis Research Group, Division of Neuroscience, University of Nottingham. Blood was immediately put into a collecting tube containing EDTA (4mM), to prevent blood clotting. For platelet preparation, platelet rich plasma was obtained by centrifugation at 1,100 RPM (15 min) followed by a 2,000 RPM (20 min) centrifugation step to obtain a platelet pellet. Platelets were resuspended in sterile saline solution (0.9% sodium chloride) before centrifuging at 1,600 RMP (15 min). The pellet was resuspended in 2ml volume of saline. Cells were counted on Sysmex KX-21 Haematology analyser (Kobe, Japan). Cells were between 78.3% - 92.9% pure (contamination with red blood cells and white blood cells).

For red blood cells (RBCs), plasma and leukocytes were removed by centrifuging at 3,000 RPM (15 min). RBCs were resuspended in 5ml of sterile saline and centrifuged at 3,000 RPM (10 min), the supernatant was removed and the previous step was repeated. RBCs were analysed on Sysmex KX-21 Haematology analyser (Kobe, Japan). Cells were 99.5 - 99.8% pure.

2.1.6.2 Blood cells addition to PCA

PCA were prepared as previously described (contractile studies Section 2.1.0). In some vessels, the $P2Y_{14}$ receptor antagonist PPTN (1 μM) was added 10 min prior to U46619 addition. After a stable tone had been established, isolated platelets were added to the vessels; the final platelet concentration in the bath was approximately 24,000-30,000/μL. In other experiments, L-NAME (100 μM) was added to PCA and left for 10 min before adding PPTN (1 μM). After inducing precontraction with U46619, the isolated platelets were added. Platelets were also added in similar conditions but in endothelium removed PCA preparations. Volumes of platelets added to organ baths were approximately 10-25μl/ml; controls were not needed since addition of 10-30μl/ml of 0.9% sodium chloride in 118mM of Krebs-Henseleit buffer would not have a significant effect.

In other experiments, after preconstricting vessels with U46619, RBCs (final haematocrit of 0.4% (0.4 ml (w/v) of RBCs in 10 ml Krebs-Henseleit bath volume) were added in the vessels in the presence and absence of PPTN (1 μM). The volume of RBCs added was approximately 7-13 μl/ml (4,800,000 - 45,000,000) RBCs/ml of Krebs-Henseleit buffer, controls were not required.

2.1.7 Lipopolysaccharide (LPS) studies

Coronary arteries were dissected and cleaned of surrounding muscle on the same day of arrival from the abattoir. Connective tissue and fat remained on the same arteries. Four segments from each artery was cut and each were incubated with either no LPS, LPS 1 μg/ml, 10 μg/ml or 100 μg/ml (isolated

from *E.coli* strains) in Krebs-Henseleit buffer overnight at 4 ºC. The following day, tissues were set up in organ baths as described in Section 2.1.0. Cumulative concentration-response curves to MRS2690 were constructed in conditions of forskolin plus U46619 (see Section 2.1.1.3). To determine effect of LPS on endothelium, substance P (10 nM) was added at the end of the MRS2690 response curve.

2.2 cAMP/VASP-phosphorylation measurement

Porcine coronary arteries were set up in organ baths as described above (4 segments from the same artery). After addition of U46619 (5-10 nM) and forskolin (0.1 μM), vessels were left to relax to basal tone. These were control vessels. In some vessels, either 1mM UDP-glucose or 10 μM MRS2690 was added (in addition to the U46619 and forskolin). After the contractions had reached a maximum, vessels were immediately removed, put into lysis buffer, and frozen in dry ice. The VASP-phosphorylation assay was carried out due to the effectiveness in generated a larger amount of results over a shorter time period. The assay was carried out as follows. Tissues were homogenized and the supernatant was separated by centrifugation (3000 g, 10 min). Vasodilator-stimulated phosphoprotein (VASP) phosphorylation (VASP-P) was measured in tissues exposed to forskolin in the absence and presence of UDP-glucose or MRS2690, and in the absence and presence of PPTN. VASP-phosphorylation assay and flow cytometry was carried out according to Iyu et al. (2012).

2.3 Western Blotting

PCA were dissected as described above. Control tissues were brains and hearts obtained from male Wistar rats, which were put into lysis buffer (20 mM Tris, 1 mM EGTA, 0.1% (v/v) Triton X100, 1 mM NaF, 10 mM beta glycerophosphate, pH 7.6) (containing protease inhibitor enzyme) and frozen at -20 ^{0}C. Tissues were homogenized using a borosilicate glass homogeniser in lysis buffer before being centrifuged (3000 RPM, for 5 min, 4 ^{0}C). The supernatant (liquid containing proteins) above the pellet (insoluble tissue/fat of porcine coronary artery) was separated out for subsequent assaying whilst the pellet was discarded. A Lowry protein assay was carried out to measure the amount of proteins within the sample and to normalize samples to 1 mg/ml (Lowry et al., 1951). For the Western blotting procedure samples were diluted 1:6 in solubilisation buffer (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) in lysis buffer containing protease inhibitor before being heated to 95^{0}C for 5 min, vortexed and centrifuged (13,000 RPM, 1 min). Separation of proteins was performed using 4-20% Bio-Rad precast polyacrylamide SDS/PAGE gels for 45 min at 200 volts. Proteins were transferred onto nitrocellulose membranes. This was carried out using a cassette in which an absorbent sponge, filter paper, gel, nitrocellulose membrane, filter paper, absorbent sponge (bottom-up) were prepared. Transfer of proteins was carried out for 60 min at 100 volts Ponceau S stain was applied to membranes to visualize proteins bands, blots were cut depending on size-of-interest of bands required. Ponceau solution was removed within minutes by washing with Tris-buffered saline (TBS). Membranes were blocked for 1 hour

in 5% (w/v) milk in TBS containing 0.1% (w/v) Tween 20. Blots were incubated overnight at 4^0C with either anti-$P2Y_{14}$ antibody (two different antibodies were used; $P2Y_{14}$ receptor antibody (1:500) intracellular epitope, (LS-C120603, LifeSpan BioSciences, Seattle, Washington, USA) plus $P2Y_{14}$ receptor antibody (1:500) extracellular epitope: APR-018 (Alomone, Jerusalem) or anti-GPR17 antibody (1:500) (ab106781, Abcam, Cambridge, UK) GAPDH antibody (1:20,000) (G8795, Sigma-Aldrich, UK) was used as a house-keeping control antibody. All antibodies were diluted in the blocking solution. The following day, blots were washed 3 times briefly with TBST. This was followed by 3 times 10-min washes with TBS. Blots were incubated with secondary antibodies for anti-$P2Y_{14}$ (IRDye 800CW Goat anti-Rabbit IgG (H + L), 0.5 mg, LI-COR Biosciences, Lincoln, Nebraska, USA) for 1 hour at 37ºC on a shaker. Three quick washes were carried out using TBST, followed by three times 5-min then three times 15 min washes with TBST. A final wash with Millipore water was carried out. Immunocomplexes were detected using an Odyssey Infrared imaging system (Biosciences, Biotechnology) and analysed with image studio V3 software (Licor).

2.4 Cell culture

2.4.1 Preparation of coverslips

19 mm coverslips were left in decontamination solution (2% (w/v) distal surface disinfectant in distilled water) for 1 hour. Coverslips were washed in distilled water (dH_20) five times before immersion in Hydrochloric acid (1M) for 1 hour. Coverslips were washed again in dH_20 five times before being left to air dry and then autoclaved. Coverslips were put into 12 well plates and

coated with 0.01% (w/v) Poly-l-lysine for 30 min at room temperature; coverslips were washed twice with phosphate buffered saline (PBS) containing (w/v) 10% antibiotics and antimycotic (1000 units penicillin, 1mg streptomycin and 2.5 amphotericin B per ml). Collagen was diluted to 50 µg/ml using 0.01 M HCl before being applied to coverslips for 1 hour at room temperature. The solution was aspirated off and rinsed thrice using PBS containing antibiotics to remove any remaining acid. Coverslips were air dried, or stored at 2-8^{0}C for up to one week under sterile conditions for later use.

2.4.2 Isolation of PCA SMCs – using collagenase

PCA were dissected out fresh and cleaned of adventitia and connective tissue. Tissue was washed thrice in PBS containing (w/v) 10% antibiotics and antimycotic. Before the final wash, tissues were cut into 5mm segments and put into collagenase P with a ratio of 5:1 of collagenase (ml) to tissue (mg). 5mg/ml collagenase was made in Krebs buffer which was filter (0.45 micron syringe filters) sterilized. Tissues were incubated for 1 hour at 37 ^{0}C. Tissues were thoroughly mixed with using a cut-ended pipette (0.5mm cut made on 1000µl tip) until the solution became cloudy, the residual tissues were discarded and the solution was transferred into 5ml of serum-free media. The content was centrifuged for 5 min at 100 g (1200 RPM). The cell pellet was resuspended into warmed (37 ^{0}C) serum-free media (containing antibiotics) and used for assaying. Cells were plated onto coverslips (prepared as described above)

2.4.3 Isolation of PCA SMCs – using tissue segments

PCA were dissected as described above. 5mm segments were opened out and endothelium was removed using fine forceps. Segments were washed thrice in PBS containing antibiotics. Segments were carefully put onto coverslips (prepared as described above) lumen facing down. Serum-free media was added to segments, enough to submerge the tissue to allow a full covering of the tissue. A second coverslip was carefully placed onto the top of the segment in order to weigh the tissue down to prevent it from floating. Growth of the cells was checked over a number of days; once cells started appearing from the outside of the tissue segments, the segments were carefully removed leaving behind smooth muscle cells attached to the coverslips.

2.5 Immunohistochemistry

2.5.1 Porcine coronary arteries

Porcine coronary arteries were dissected and fixed in 4% (w/v) paraformaldehyde overnight at 4 ^{0}C. Segments were placed onto a cork and were frozen in OCT mounting solution using dry ice. 20 µM slices of tissue were cut using a microtome and transferred onto cold slides before storing overnight at -80 ^{0}C. Slides were removed and left to equilibrate at room temperature. Tissues were permeabilized using phosphate buffered saline (PBS) + 1% (w/v) bovine serum albumin (BSA) + 0.2% (w/v) Triton X-100 for 30 min at room temperature. Tissues were incubated with a blocking solution made up of human serum 1:25 in PBS + 1% (w/v) BSA for 30 min at room temperature. Tissues were incubated with a 1:50 dilution of $P2Y_{14}$ receptor antibody (rabbit anti-human polyclonal antibody-LS-A1486, intracellular C-terminal $P2Y_{14}$ receptor epitope, LifeSpan BioSciences, Seattle, Washington,

USA) and a 1:800 dilution of smooth muscle β-actin antibody, made in the blocking solution, overnight at 4 ^{0}C. The following day, slides were briefly washed with PBS + 0.2% (w/v) Triton X-100 this was followed by 3 times 15-min washes with PBS + 0.2% (w/v) Triton X-100. A 1:500 dilution of secondary antibody, Alexaflour 488 goat anti-Rabbit IgG (whole molecule), FITC conjugate and a 1:500 dilution of smooth muscle β-actin goat anti mouse 568 (Sigma-Aldrich, UK) made in PBS + 1% (w/v) BSA was added to slides which were incubated for 1 hour in the dark at room temperature. Control slides contained only secondary antibody. 5 µg/ml of DAPI (nuclei stain) made in PBS + 1% (w/v) BSA was added to slides for 5 min at room temperature. Three quick PBS + 0.2% (w/v) Triton X-100 washes were carried out, followed by three times 15-min washes PBS + 0.2% (w/v) Triton X-100 before a final quick wash with Millipore water was carried out. DABCO fluorescence mounting media and glass coverslips were carefully placed onto the slides. Samples were visualised using fluorescence microscopy using an objective magnification of x40 and x100.

2.5.2 PCA smooth muscle cells (PCA SMCs)

PCA were prepared as described in Section 2.4.2. Immunohistochemistry was carried out as follows. PCA SMCs were grown on 19 mm coverslips until confluent in a 24 well plate. Cells were removed from the incubator, serum-free media was aspirated off before ice cold PBS was added to cells which was then washed off immediately. Cells were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. This was aspirated off after

which cells were permeabilized with PBS +1% (w/v) bovine serum albumin (BSA), +0.15% (w/v) Triton X-100 for 30 min at room temperature. After aspirating, cells were then blocked in 0.5% milk (made in PBS) for 1 hour at room temperature. Coverslips with cells were carefully lifted out using a needle and fine forceps from the 24-well plate and placed onto a immunohistochemistry slide. A ring was drawn around the coverslip using an oil based pen (to prevent antibody from leaking out). Slides were placed into an immunohistochemistry chamber in which dampened tissue paper was placed beneath to prevent drying out of antibody. Four slides with coverslips were prepared. Antibodies made in PBS + (w/v) 0.5% milk were added to cells as follows: 1. Control- contained no antibody, 2. alpha-actin antibody (1:400 dilution), 3. $P2Y_{14}$ receptor antibody (1:250 dilution), 4. alpha-actin antibody (1:400) + $P2Y_{14}$ receptor antibody (1:250). Slides were left incubated for 24 hours at -4 ºC. The following day, the antibody was carefully removed by using an absorbent tissue paper. Three times PBS + 0.1% BSA washes were carried out, using absorbent tissue paper to remove residual liquid. Secondary antibodies made in PBS + 5% milk were added as follows. Fluorescein isothiocyanate (FITC) antibodies (1:300 dilution) and tetramethylrhodamine isothiocyanate (TRITC) antibodies (1:500 dilution), were added to control slides containing no antibody and to slides containing $P2Y_{14}$ primary antibody and alpha-actin primary antibody. FITC antibodies (1:300) were added to $P2Y_{14}$ containing primary antibody. TRITC antibodies (1:500) were added to alpha-actin containing primary antibody. Slides were incubated for 1 hour at room temperature. Two quick washes were carried out using PBS +0.1% (w/v) BSA, followed by three times 5-min washes. DAPI (1 µg/ml) was added to

slides for 1 min at room temperature. Three quick washes using PBS +0.1% (w/v) BSA were carried out before a final quick wash using Millipore water was carried out. Coverslips containing cells were removed and mounted onto new slides (cells facing downwards) using mounting media. Nail varnish was put around the coverslips to seal. Samples were visualised using fluorescence microscopy.

2.6 Cytokine/Chemokine measurement

PCA were dissected as described above, approximately 3-4 mm segments were cut from each vessel, and blotted weights were recorded. Segments were put into a 48-well plate containing Krebs-Henseleit. Serum free media RPMI media was prepared containing 5% (w/v) antibiotics antimycotic solution (100x) and L-glutamine (2µM). Krebs-Henseleit was aspirated off from the plate containing segments which were transferred to a new 48-well plate. Media containing antibiotics and antimycotic was added to segments which were left for approximately 10 min before being aspirated off. Fresh media containing single concentrations of either UDP-glucose 100 µM, UDP-glucose 1 mM, MRS2690 1 µM or MRS2690 10 µM was added to segments and control wells contained normal media. In other cell culture plates, media containing various concentrations of lipopolysaccharide (LPS) (1 ng/ml, 10 ng/ml, 1 µg/ml and 10 µg/ml) were added to vessels either alone or in combination with 1 µM MRS2690. The plates were incubated at 37 ºC for 24 hours. The following day, the plate was removed and put onto ice; media was taken off and put into Eppendorf tubes on ice. Segments were removed and put into Eppendorf tubes into dry ice. Media was centrifuged for 10 min at 3000 g

and aliquots of supernatant were taken before being stored at -80 ºC. Profiling of cytokines released from porcine coronary arteries was conducted using a Porcine Cytokine/Chemokine Magnetic bead panel (PCYTMG-23K-13PX) according to the manufacturer's instructions (Merck Millipore, USA). The plate was washed (and primed) with assay buffer for 10 min at room temperature. Standards, quality controls and neat samples were added according to the protocol. Magnetic beads were sonicated and vortexed and added to wells before leaving overnight at 4 ºC. The following day, the plate was fixed onto a magnetic plate holder and three washes were carried out using wash buffer. Detection antibodies were added to wells and the plate was incubated for 2 hours at room temperature. Streptavidin-phycoerythrin was added to wells containing antibodies which were left for a further 30 min. The plate was washed as carried out previously. Sheath fluid was added to plates which were incubated for 10 min on a plate shaker at room temperature. Plate reading was carried out using a Bioplex 200 System.

2.7 Materials

Krebs-Henseleit buffer ingredients were all purchased from Sigma-Aldrich, UK. Uridine 5′-diphosphoglucose disodium salt (UDP-glucose), uridine 5′-diphosphoglucuronic acid trisodium salt (UDP-glucuronic acid), uridine 5′-diphospho-N-acetylglucosamine sodium salt (UDP-*N*-acetylglucosamine), uridine 5′-triphosphate trisodium salt hydrate (UTP), 7-beta-acetoxy-8,13-epoxy-1-alpha, 6-beta, 9-alpha-trihydroxylabd-14-en-11-one; coleonol (adenylyl cyclase activator: forskolin), U46619 acetylcholine chloride, histamine dihydrochloride colforsin; suramin sodium salt (non-universal, non-

selective P2 receptor antagonist), Sodium nitroprusside (SNP) dihydratre, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were all obtained from Sigma-Aldrich, UK. Diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(4'-methylthio)uridin-5''-yl] ester disodium salt (MRS2690) *N*,*N*''-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea (selective $P2Y_6$ antagonist, MRS2578), uridine-5'-(γ-thio)-triphosphate trisodium (UTPγS) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (non-universal, non-selective P2 receptor antagonist PPADS), substance P were all obtained from Tocris Bioscience, Bristol, UK. UDP-sugar nucleotides, UTP, UTPγS, MRS2690, MRS2578, PPADS, suramin and substance P were all dissolved in distilled water and subsequent dilutions were made in distilled water. U46619 was dissolved in ethanol to a final stock concentration of 10Mm, subsequent dilutions were made using distilled water. PPTN (10mM made in DMSO) (selective $P2Y_{14}$-R antagonist) was received as a gift from Merck Frosst Centre for Therapeutic Research, USA. PPTN dilutions were made in DMSO. LPS was obtained from Sigma-Aldrich, UK. Paraformaldehyde, Triton X-100, Albumin; from bovine serum (BSA), Dulbecco's phosphate buffered saline (PBS), Human serum, lysis buffer ingredients and Ponceau S stain were all obtained from Sigma-Aldrich, UK. Tween 20 was purchased from Fisher Scientific, UK. Protease inhibitor cocktail tablet (EDTA-free) was obtained from Roche, Sigma-Aldrich, UK. Antibiotic Antimycotic Solution (100×), Stabilized (with 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL), collagenase P, serum-free media were all purchased from Sigma-Aldrich, UK

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 software. EC_{50} values (concentration of agonist required to produce 50% of the maximal responses) and R_{max} were obtained for $P2Y_{14}$ receptor agonist concentration response curves and comparisons of EC_{50} and R_{max} values were made using unpaired Student's *t*-test. One-way ANOVA, followed by Sidak's multiple comparisons test was used as a statistical analysis to make individual comparisons of single agonist concentrations in comparison to the same agonist concentration in the presence of an antagonist. Comparisons of overall responses between $P2Y_{14}$ receptor agonists were made using two-way ANOVA followed by Sidak's multiple comparisons test. All subsequent data (PPTN, UTP, UTPγS, Suramin, PPADS, MRS2578, blood experiments) was analysed using two-way ANOVA followed by Sidak's multiple comparisons test. Data from VASP-P experiments were analysed using one-way ANOVA, followed by Sidak's multiple comparisons test. Paired Student's *t*-test was used to make a comparison between VASP-P in vessels exposed to forskolin alone *versus* forskolin plus PPTN. $P<0.05$ was taken as significant. Experimental data are expressed as mean ± S.E.M. The "n" in the results signifies the number of animals. Chapter 5 gives results of blood cell responses from individual human donors, blood cells from the same donor were added to vessels from different animals where "n" signifies the number of animals. Results were analysed using unpaired Student's *t*-test, GraphPad Prism 6.0. $P <0.05$ was taken as statistically significant.

Chapter 3: Effects of $P2Y_{14}$ receptor agonists in porcine coronary in the absence and presence of a novel $P2Y_{14}$ receptor antagonist, PPTN

3.1 Introduction

P2Y receptors are G protein-coupled receptors activated by purine, pyrimidine and sugar nucleotides. There are eight members of the P2Y family, namely $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors with the latter being the most recently described (Chambers et al., 2000; Abbracchio et al., 2003). Quantitative analysis has revealed that $P2Y_{14}$ protein is expressed in many regions including the brain, adipose tissue, lung, spleen, heart and certain immune and inflammatory cells (Chambers et al., 2000); however, there is limited evidence of $P2Y_{14}$ receptor expression in the vascular system. The $P2Y_{14}$ receptor is a $G_{i/o}$ protein-coupled receptor (Scrivens and Dickenson, 2005a,b, 2006; Alsaqati et al., 2013) with a unique pharmacological profile as it is the only member of this family to be activated by UDP-sugars, including UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine (Abbracchio et al., 2003; Harden et al., 2010). The $P2Y_{14}$ receptor is also activated by UDP, and by MRS2690, a more selective agonist that has a 7-fold greater potency at the $P2Y_{14}$ receptor than UDP-glucose (Ko et al., 2007).

It is known that UDP-glucose can be released from a variety of cell types through mechanical, stressful or hypoxic stimuli (Lazarowski et al., 2003b; Kook et al., 2013). It has been postulated that after cell content leakage caused by exocytosis or cell damage, extracellular concentrations of UDP-glucose can range between 10 nM to 1 mM (Arase et al., 2009). There is emerging evidence for the expression of $P2Y_{14}$ receptor transcript and protein in endothelial and smooth muscle cells (Govindan et al., 2010; Alsaqati et al.,

2014), but relatively little is known about the functional expression of this receptor in blood vessels. It has recently been shown that UDP-glucose mediates vasoconstriction through activation of $P2Y_{14}$ receptors in porcine pancreatic arteries (Alsaqati et al., 2014) and also in mouse coronary arteries (Haanes et al., 2014a). However the function of the $P2Y_{14}$ receptor has not been characterized in porcine coronary arteries. Both the size of the porcine heart and the distribution of blood supply by the coronary artery system are very similar to that of humans (Swindle et al., 2012). Thus, the hypothesis of this study was that the $P2Y_{14}$ receptor is expressed in and mediates the vasomotor role of UDP-sugars (in the porcine coronary vasculature) and, therefore, it can provide valuable information about the $P2Y_{14}$ receptor as a potential target for control of vascular tone in coronary arteries in humans.

Until recently, the lack of availability of $P2Y_{14}$ receptor antagonists has made it difficult to determine a precise role for the $P2Y_{14}$ receptor. A novel non-nucleotide compound, 4-((piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid (PPTN), was recently reported as a high-affinity competitive antagonist at the $P2Y_{14}$ receptor (Robichaud et al., 2011). PPTN has been shown to inhibit UDP-glucose- and MRS2690-mediated mast cell degranulation (Gao et al., 2013) and UDP-glucose mediated chemotaxis of neutrophils (Barrett et al., 2013), thus both of these cell types functionally express the $P2Y_{14}$ receptor (Scrivens and Dickenson, 2006; Gao et al., 2010; Barrett et al., 2013).

In this chapter, the functional expression of a contractile $P2Y_{14}$ receptor, sensitive to UDP-sugars in the porcine coronary artery are described. The contractile response was mimicked by the selective $P2Y_{14}$ agonist MRS2690.

3.2 Aims

To visualise and identify the site-specific location of the $P2Y_{14}$ receptors in porcine coronary arteries, Western blotting and immunohistochemical techniques were used.

To functionally characterise the $P2Y_{14}$ receptor in porcine coronary arteries using $P2Y_{14}$ receptor agonists and the selective $P2Y_{14}$ receptor antagonist PPTN.

3.3 Method

3.3.1 *In vitro* pharmacology

A detailed description of the Methods is outlined in Chapter 2.

3.3.1.1 $P2Y_{14}$ receptor agonists

Cumulative concentration-response curves to $P2Y_{14}$ receptor agonists: UDP-glucose (0.1 µM-1 mM), MRS2690 (1 nM-10 µM), UDP-glucuronic acid (0.1 µM-1 mM), and UDP-*N*-acetylglucosamine (0.1 µM-1 mM), were constructed at basal tone, in U46619 induced preconstricted arteries, and also in vessels exposed to both U46619 and forskolin (see Chapter 2 Section 2.1.1.3).

3.3.1.2 Effect of PPTN, a $P2Y_{14}$ receptor antagonist, on agonist responses

Cumulative concentration response curves to UDP-glucose, MRS2690, UTP and UTPγS (agonists at $P2Y_2$ and $P2Y_4$ receptors) were constructed in the presence of PPTN (1 µM and 3 µM), a selective $P2Y_{14}$ receptor antagonist.

PPTN stock concentration was made in DMSO, DMSO was added to control tissues.

3.3.1.3 Effect of U46619 and forskolin on responses to acetylcholine and histamine

At basal tone, preconstricted tone (U46619 induced) and forskolin plus U46619 conditions, responses to acetylcholine (10 nM –10 µM) and histamine (10 nM-100 µM) were constructed. This was done to show the effects of non-Gi coupled-receptor agonists in conditions of elevated cAMP. ACh responses were examined in the presence of 3 µM PPTN. This was done to show the effect of PPTN on responses to the other, non-purinergic, receptors in porcine coronary arteries.

3.3.1.4 Effect of U46619 and sodium nitroprusside on responses to UDP-glucose

To compare the effect of elevated cGMP on agonist responses versus responses of agonists to elevated cAMP (forskolin); after preconstricting arteries with U46619 (5-7 nM), sodium nitroprusside (SNP, 2.5–25µM) was added to induce a relaxation and reverse the U46619-induced contraction; in control arteries, vessels were not preconstricted with U46619 and were at basal tone. Cumulative concentration-response curves to UDP-glucose were constructed.

3.3.1.5 Endothelium removal studies

Cumulative concentration-response curves to UDP-glucose were generated as previously described under forskolin and U46619 conditions (see Section

2.1.3). The endothelium was considered to have been removed if substance P (10nM) caused a relaxation that was less than 10% of the U46619-induced tone (see Chapter 2 Section 2.1.3).

3.3.2 Western blot

For full methods, refer to Chapter 2 Section 2.3.1. PCA, rat brains, rat hearts, porcine hearts and porcine pancreatic arteries were homogenized in lysis buffer. The supernatant was separated by centrifugation and a Lowry protein assay was carried out to normalize samples to 1 mg/ml. Samples were heated to 95 ^{0}C for 5 min, vortexed and centrifuged. Following, polyacrylamide SDS/PAGE gels, proteins were transferred onto nitrocellulose membranes and blocked for hour. Blots were incubated overnight at 4^{0}C with either anti-P2Y$_{14}$ antibody or anti-GPR17 antibody. The following day, blots were incubated with secondary antibodies for anti-P2Y$_{14}$ receptor. Immunocomplexes were detected using an Odyssey scanner with image studio V3 software.

3.3.3 Immunohistochemistry

For full methods, refer to Chapter 2 Section 2.5.1. PCA were fixed in paraformaldehyde and segments were cut to 20 µM and transferred onto slides. Tissues were permeabilized with PBS +1% (w/v) BSA +0.2% (w/v) Triton X-100 for 30 min at room temperature. Tissues were blocked with human serum +1% (w/v) BSA for 30 min at room temperature. Tissues were incubated with a P2Y$_{14}$ receptor antibody and smooth muscle β-actin antibody, overnight at 4 ^{0}C. The following day slides washed with PBS + 0.2% (w/v) Triton X-100. Slides were incubated with secondary antibodies for 1 hour at room

temperature. Control slides contained only secondary antibody. DAPI was added to slides for 5 min at room temperature. Slides were washed with PBS + 0.2% (w/v) Triton X-100 and a quick wash with Millipore water. DABCO fluorescence mounting media and glass coverslips were carefully placed onto the slides. Samples were visualised using fluorescence microscopy. Immunohistochemisty was also carried out on porcine coronary artery smooth muscle cells (PCA SMCs). A detailed description of the methods is outlined in Chapter 2 Section 2.5.2.

3.4 Results

3.4.1 $P2Y_{14}$ receptor protein is expressed in porcine coronary arteries

Western blotting using a $P2Y_{14}$ receptor antibody targeted against the extracellular $P2Y_{14}$ receptor epitope (Alomone Labs) showed that the $P2Y_{14}$ receptor protein is present in porcine coronary artery and is present as two bands around 54 kDa (Figure 3.1a). These bands are similar to the larger band in a report which has suggested that the $P2Y_{14}$ receptor is present in two isoforms, a glycosylated and non-glycosylated form in C6 glioma cells (with the glycosylated form predominating); band sizes were around 56 kDa and 34 kDa (Krzeminski et al., 2008). $P2Y_{14}$ receptor expression was also investigated in the porcine heart. In the current study in both the rat heart and porcine heart, bands appeared at different sizes to the PCA, $P2Y_{14}$ receptors have been shown to be present in rat hearts (Musa et al., 2009) but not in coronary arteries. These were approximately 47 kDa and 67 kDa (Figure 3.1a), the former band being similar sized to those reported (Azroyan et al., 2015). In order to show that these proteins were $P2Y_{14}$ receptor proteins, a second blot was incubated with a $P2Y_{14}$ receptor blocking peptide (Figure 3.1b). In the presence of the blocking peptide the bands in PCA, porcine heart and rat heart were absent suggesting that they represent $P2Y_{14}$ receptor proteins.

As there were inconsistencies between band sizes from PCA and control tissues using the Alomone antibody, a second antibody, this time targeted against the intracellular $P2Y_{14}$ receptor epitope (Lifespan), was used. Use of the Lifespan antibody revealed that immunoreactive bands in the PCA in two

forms sized around 41 kDa and 61 kDa; these same sized bands appeared in porcine pancreatic artery, rat heart and porcine heart (Figure 3.1c and 3.2). These bands are consistent with the manufactures who showed (not stated) single sized bands approximately 41 kDa in Human embryonic kidney 293 cells, Human colon adenocarcinoma cells, and double sized bands, approximately 41 kDa and 61 kDa in Human liver cancer cells. Recent findings show the $P2Y_{14}$ receptor is present in the porcine pancreatic artery (Alsaqati et al., 2014); thus, this was used as a positive control tissue along with porcine heart and rat heart tissues. This data is consistent with a report which showed $P2Y_{14}$ receptor expression in the mouse coronary and basilar artery with a band size of 60 kDa (Haanes et al., 2014a). The bands were absent in a second blot that was incubated with the omission of primary $P2Y_{14}$ receptor antibody (Figure 3.1d).

UDP-glucose has been shown to act at the GPR17 receptor, a cysteinyl/purinoceptor (Daniele et al., 2011), as well as at $P2Y_{14}$ receptors. Hence, western blotting was carried out to determine the expression of the GPR17 receptor in the porcine coronary artery. RT-PCR analysis has shown that the GPR17 receptor mRNA is present highly in the rat brain and also the rat heart (Ciana et al., 2006), thus both these tissues were used as positive control tissue. Western blotting showed a band of approximately 44 kDa in rat brain, slightly higher than their reported molecular weight according to the supplier's guidelines (Abcam), and a second band at 38 kDa was seen (Figure 3.3). In the current results, these bands were not seen in the rat heart (Figure 3.3), and since protein expression of GPR17 has not been looked at in the rat heart (Ciana et al., 2006), it can only be suggested that GPR17 protein is not

present in this tissue. There is also a lack of reports using this antibody. Further investigation into the lack of expression in rat heart was not carried out. No GPR17 protein was detected in the porcine coronary artery (Figure 3.3). Due to time restrains, only one Western blot experiment was conducted using samples obtained from two separate porcine coronary artery, thus, further experiments are required to show consistency between vessels from different hearts.

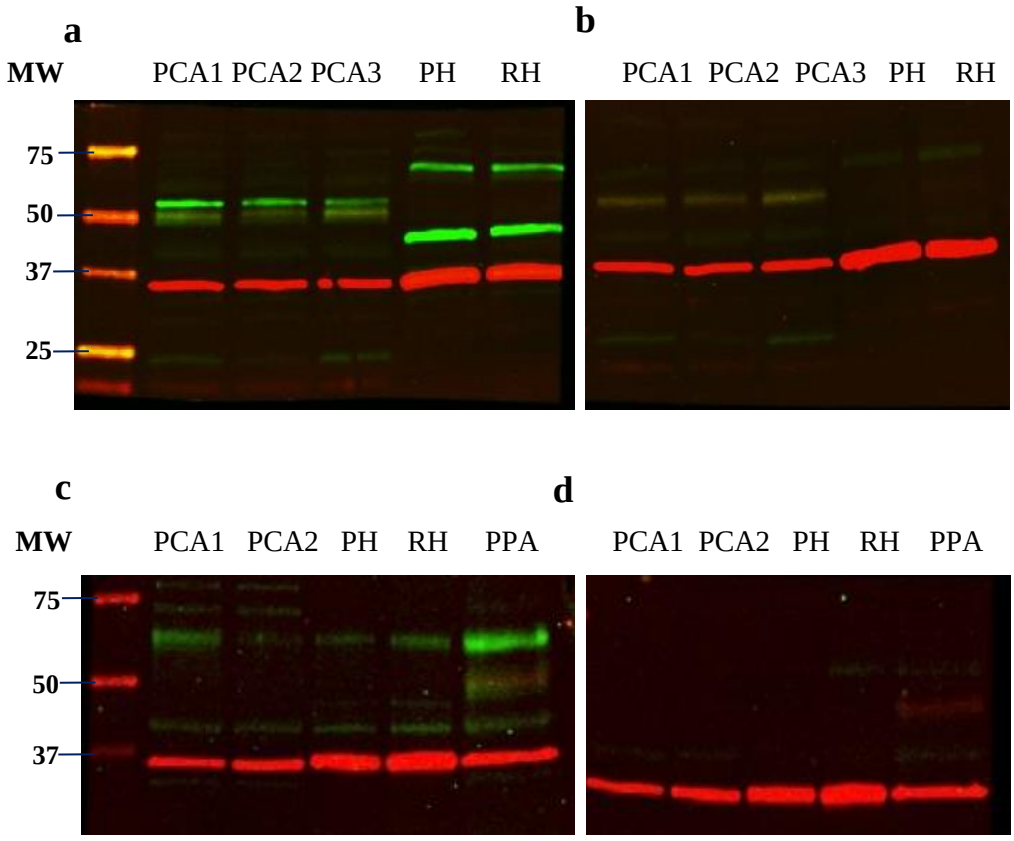


Figure 3.1: Western blot of $P2Y_{14}$ receptor protein expression in porcine coronary arteries using two different antibodies. (a) $P2Y_{14}$ receptors are present as a doublet at around 54 kDa in PCA and two isoforms at 47 kDa and 67 kDa in PH and RH, (Alomone, extracellular epitope). (b) blot incubated with $P2Y_{14}$ receptor antibody + blocking peptide (Alomone), results in omission of bands seen in blot a. (c) $P2Y_{14}$ receptors are in two isoforms at 41 and 61 kDa in PCA, RH and PPA, (LifeSpan, internal epitope). (d) blot was incubated in the absence of the primary $P2Y_{14}$ receptor antibody and presence of secondary antibody resulting in omission of bands seen in blot c. Red bands indicate housekeeping protein, GAPDH at 36 kDa. PCA, porcine coronary artery (5 µg) (from 3 different hearts), Porcine heart (PH) (5 µg), rat heart (RH) (5 µg), porcine pancreatic artery (PPA) (10 µg).

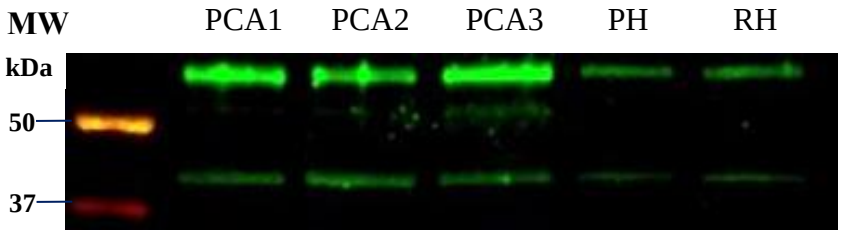


Figure 3.2: Western blot of $P2Y_{14}$ receptor expression in porcine coronary arteries. Bands of immunoreactivity for $P2Y_{14}$ receptors were observed at 41 kDa and 61 kDa in porcine coronary arteries (PCA) from two different hearts, porcine heart (PH) and Rat heart (RH), (using Lifespan $P2Y_{14}$ receptor antibody intracellular epitope).

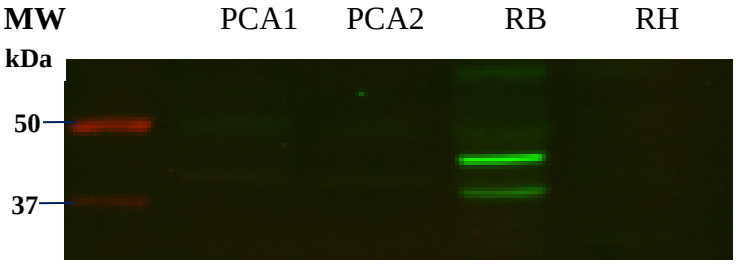


Figure 3.3 Lack of GPR17 receptor protein expression in porcine coronary artery (PCA); rat brain (RB) was used as a positive control to show GPR17 positive bands at 38 kDa and 44 kDa. Rat heart (RH) shows absence of GPR17 receptor protein. Blot repetitive of 1 experiment conducted.

3.4.2 $P2Y_{14}$ receptors are expressed in the smooth muscle and endothelial cells of the porcine coronary artery

Immunohistochemical analysis showed that $P2Y_{14}$ receptors are expressed in the smooth muscle and the endothelium of the PCA from sections of the whole intact vessels (Figures 3.4, 3.5 and 3.6). In order to show staining on the smooth muscle, an alpha-actin antibody was utilized which selectively stained smooth muscle cells (red) (Figures 3.4B, 3.4F, 3.5B and 3.5F). DAPI staining was done to stain nuclei (blue) (Figures 3.4C, 3.4G, 3.5C and 3.5G). A brighter band (yellow/green) appeared on endothelial cells (3.4H and 3.6H) indicating $P2Y_{14}$ receptors are also present on endothelial cells. However a positive marker was not used for endothelial cells and therefore the yellow/green band could have been the internal elastic lamina of smooth muscle cells. There was little or no staining in control sections of coronary artery in which primary antibody had been omitted.

Staining for the $P2Y_{14}$ receptor was also seen in isolated cultured coronary artery smooth muscle cells (Figures 3.7 and 3.8). These were isolated from whole intact vessels that were incubated with collagenase (detailed description of the methods is given in Chapter 2, Section 2.4.2). The antibody used for western blotting was the same one used here for immunohistochemistry. Figure 3.7a shows staining for the $P2Y_{14}$ receptor to be denser and more dispersed across the cells appearing in both the smooth muscle and the nucleus, which have a more elongated morphology, in comparison to Figure 3.7b where staining is restricted to the nucleus and cells have a rhomboid-like morphology. This apparent discrepancy between cells, from the same vessel could correspond to contractile and synthetic phenotypes, Figure 3.7a and 3.7b

respectively. On the other hand, cells (observed under a higher magnification) in Figures 3.8a and 3.8b have similar morphology to those seen in Figure 3.7b, cells are further apart from one another and staining of the $P2Y_{14}$ receptor appears restricted to the nucleus.

Evidence from western blotting and the immunohistochemical staining suggests that the $P2Y_{14}$ receptor is expressed in the PCA, but does not provide information about its function. Thus, the pharmacological profile of the $P2Y_{14}$ receptor was characterized using organ bath pharmacology, to investigate specifically whether this receptor has a role in regulation of blood vessel contractility.

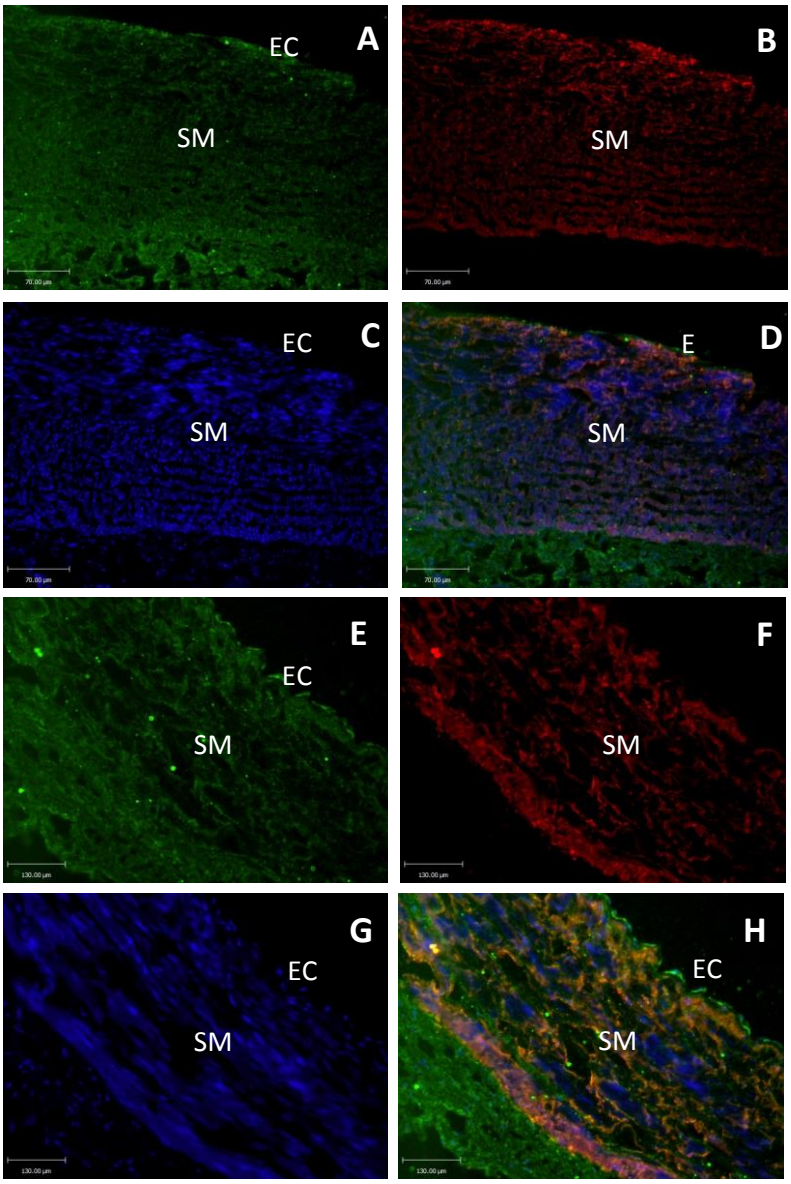


Figure 3.4: Immunohistochemical staining showing expression of $P2Y_{14}$ receptors in porcine coronary artery in longitudinal sections (A,B,C,D) and cross sections (E,F,G,H). $P2Y_{14}$ receptor expression was shown using C-terminal primary antibody (LifeSpan) and secondary anti-$P2Y_{14}$ goat anti rabbit 488 (green) (A,E), Smooth muscle beta actin goat anti mouse 568 (red) (B,F), DAPI nuceli staining (blue) (C,G) and a combined image of all three (D,H); showing yellow/orange staining of $P2Y_{14}$ protein predominantly in the vascular smooth muscle. $P2Y_{14}$ receptors are localized in smooth muscle (SM) and endothelial cells (EC). Scale bar 70µM (Figures A-D), 130µM (Figures E-H).

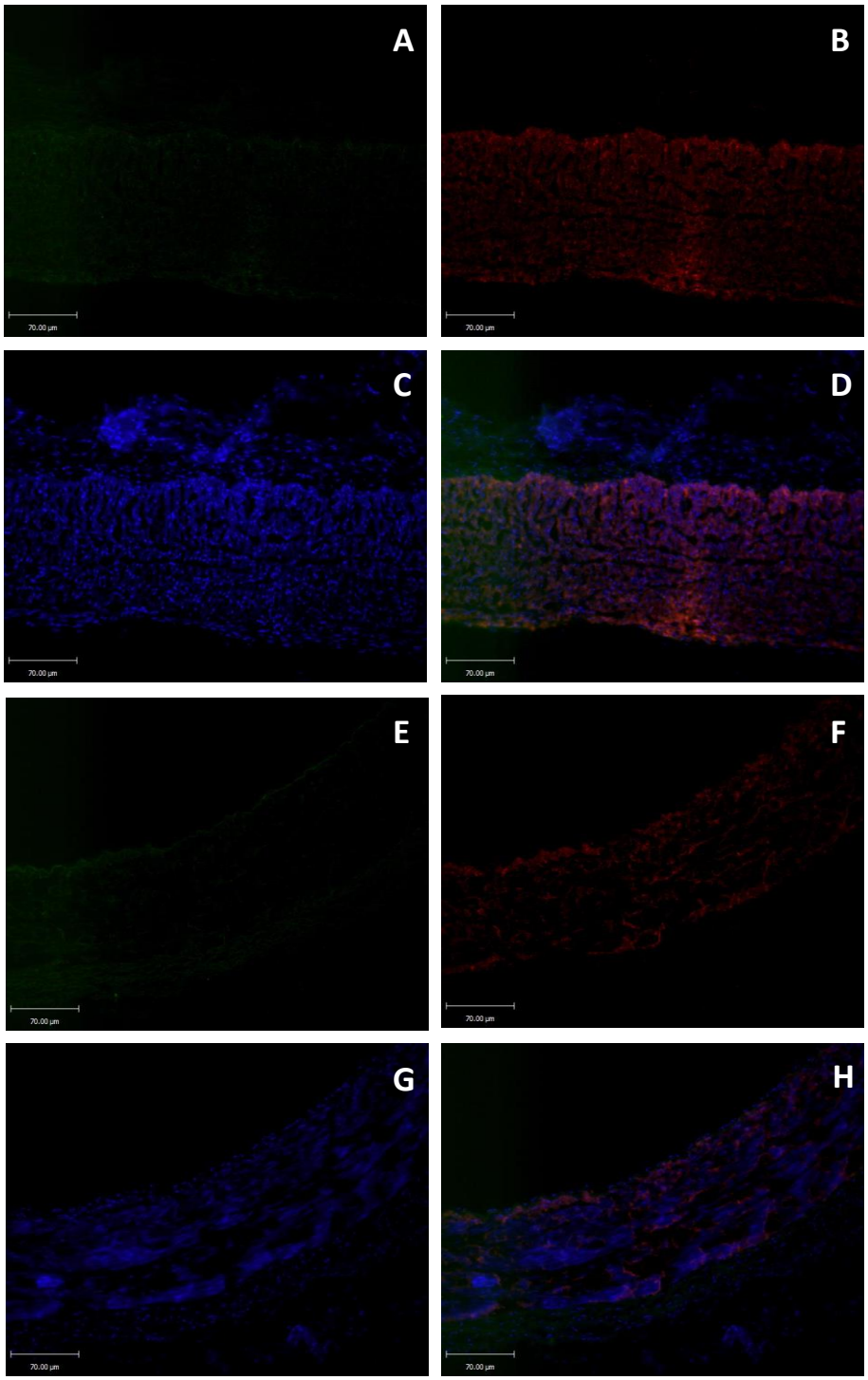


Figure 3.5. Cross-sections and longitudinal sections of porcine coronary arteries as shown in Figure 3.4, but with omission of primary antibody ($P2Y_{14}$ receptor C-terminal, LifeSpan). Anti-$P2Y_{14}$ goat anti rabbit 488 (A,E), Smooth muscle beta actin goat anti mouse 568 (B,F) DAPI nuclei staining (C,G) and Combined image of all three (D, H). Scale bar 70μM.

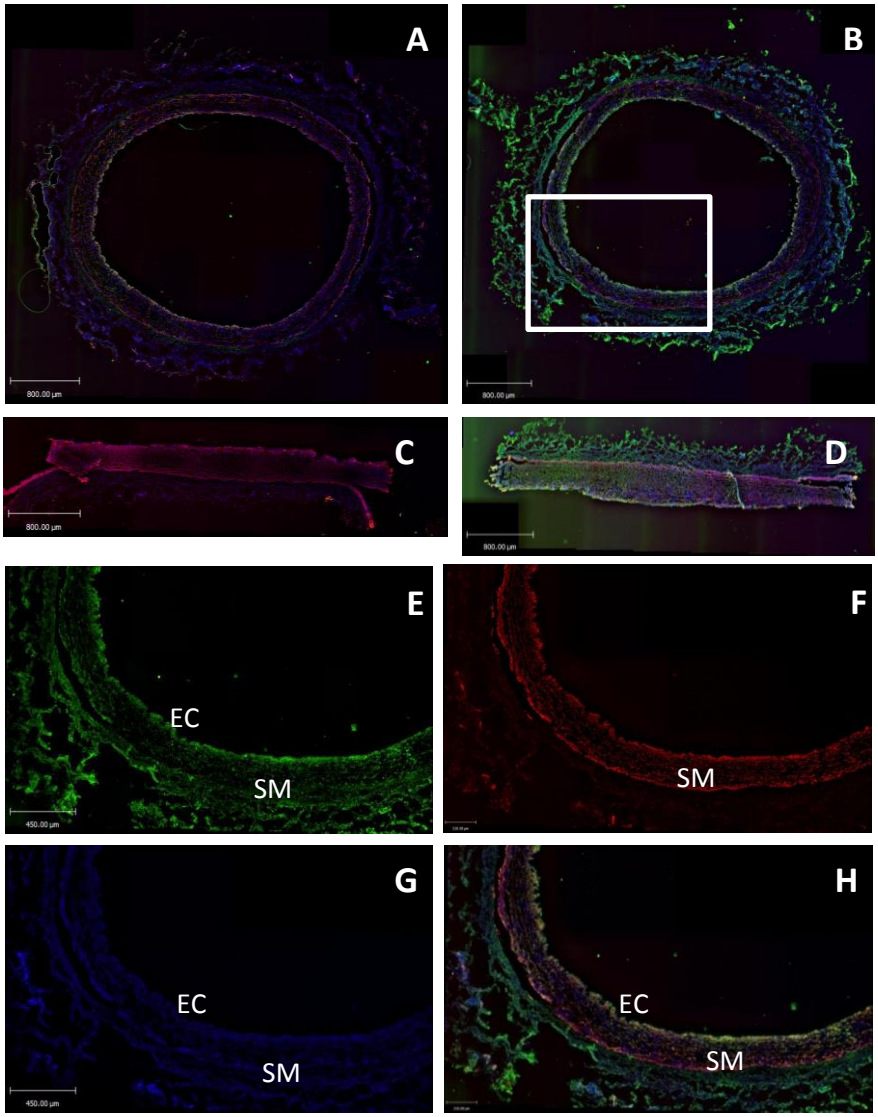


Figure 3.6: Immunohistochemical staining of $P2Y_{14}$ receptor in whole cross sections (A, B, E, F, G, H) and whole longitudinal sections of porcine coronary artery (C, D). Control sections, where primary $P2Y_{14}$ receptor antibody was omitted (A, C), shows no staining of the $P2Y_{14}$ receptor (indicated by lack of green colour) in smooth muscle (SM) and endothelial cells (EC), but shows SM staining (red) and nuclei staining (blue). White box (B) indicates the region which is magnified to produce images E, F, G and H. $P2Y_{14}$ receptor staining (E), smooth muscle alpha actin (F), DAPI nuclei staining (G) and merge of all three (H).

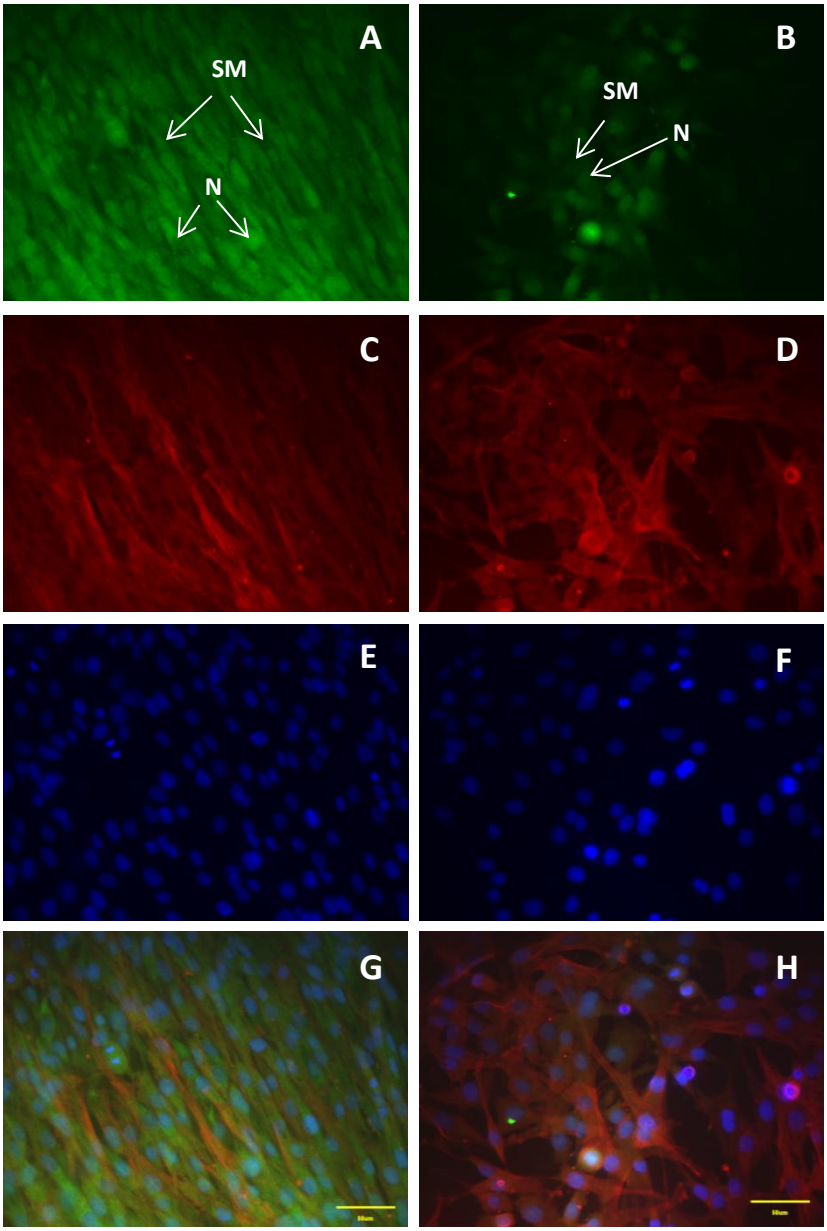


Figure 3.7: Immunohistochemical staining of the $P2Y_{14}$ receptor in cultured porcine coronary artery smooth muscle cells (PCA SMCs). Anti-$P2Y_{14}$ directed antibody (LifeSpan) was used to show $P2Y_{14}$ receptors expressed in the smooth muscle (SM) and nuclei (N) as shown in green (A, B). α-actin smooth muscle represented in red (C, D), DAPI nuclei staining shown in blue (E, F) and a merged image of all three (top to bottom) (G, H). Images are representative of studies carried out in 3 number of animals (cells from animals were pooled together). Scale bar – 50 µM as seen in bottom right of merged images.

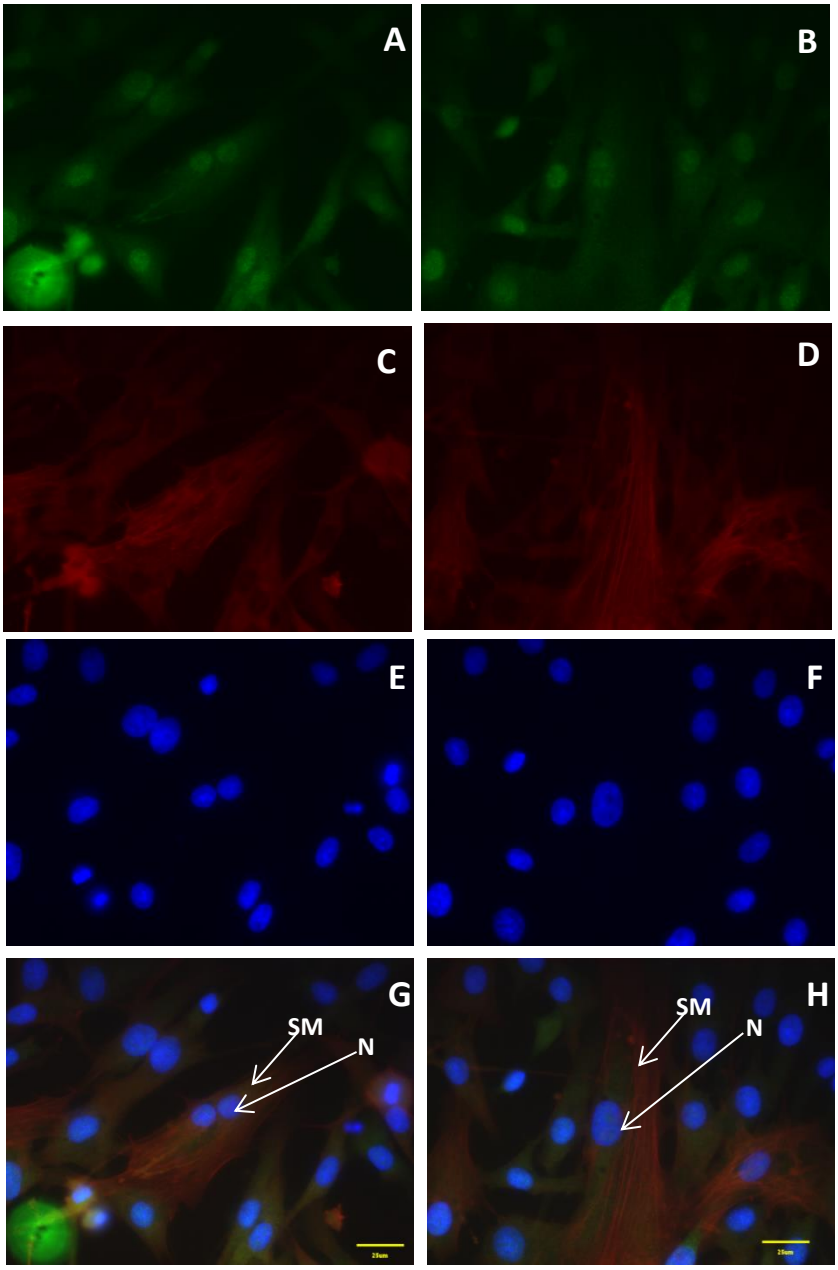


Figure 3.8: Immunohistochemical staining of the $P2Y_{14}$ receptor in cultured porcine coronary artery smooth muscle cells (PCA SMCs) viewed under a higher magnification. $P2Y_{14}$ receptors are expressed in the smooth muscle (SM) and nuclei (N) as shown in green (A,B). α-actin smooth muscle represented in red (C, D), DAPI nuclei staining shown in blue (E, F) and a merged image of all three (top to bottom) (G, H). Images are representative of studies carried out in 3 number of animals (cells from animals were pooled together). Scale bar – 25 µM as seen in bottom right of merged images.

3.4.3 $P2Y_{14}$ receptor agonists constrict porcine coronary arteries

Having established that the $P2Y_{14}$ receptor is expressed in the porcine coronary artery, the following experiments aimed to characterize its functional response and pharmacological profile. The $P2Y_{14}$ receptor is activated by various sugar nucleotides, including UDP-glucose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine (Abbracchio et al., 2003) and, more potently, by the synthetic analogue MRS2690 (Ko et al., 2007). To identify and characterize the $P2Y_{14}$ receptor in the porcine coronary artery, cumulative concentration-response curves to $P2Y_{14}$ receptor agonists were constructed at basal tone. Representative traces of responses to UDP-glucose and MRS2690 are under basal tone conditions are shown in Figure 3.9a and 3.9c. Under basal tone conditions, UDP-glucose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine (0.1µM - 1mM) produced concentration-dependent contractions of similar potency (Figure 3.11a, Table 3.1). MRS2690 (1 nM-10 µM) also caused concentration-dependent contraction, and was approximately 10-fold more potent than the UDP-sugars (Figure 3.11a, Table 3.1).

Since the $P2Y_{14}$ receptor is coupled to the inhibition of adenylyl cyclase, hence reducing cAMP production (Scrivens and Dickenson, 2005a,b, 2006), arteries were also pre-exposed to forskolin, an agent used to enhance intracellular cAMP levels, prior to conducting concentration-response curves to $P2Y_{14}$ receptor agonists. U46619 (5-10 nM), a thromboxane A_2 mimetic, was additionally present in these experiments to maintain a stable basal tone (since forskolin induces vasorelaxation). Representative traces of responses to UDP-

glucose and MRS2690 are under forskolin conditions are shown in Figure 3.9b and 3.9d.

All agonists produced greater contractions in tissues exposed to forskolin and U46619 than in tissues at basal tone, (Figure 3.11). The agonist R_{max} values in the presence of forskolin and U46619 were significantly greater than those at basal tone (Table 3.1). The log EC_{50} values were similar to those at basal tone (Table 3.1).

UDP-glucose and MRS2690 produced a contraction which was significantly greater ($P<0.0001$, ANOVA with Sidak's multiple comparisons) after treatment of forskolin and U46619 compared to tissues that were not exposed to forskolin (Figure 3.10). To determine whether U46619 was playing a part in potentiation of responses, the effect of sodium nitroprusside (SNP), a potent vasorelaxant and activator of cGMP (Arnold et al., 1977) was used in place of forskolin. Vessels were preconstricted with U46619, SNP was then added causing a relaxation bringing the tone of the vessels back to baseline. UDP-glucose produced a concentration-dependent contraction which was increased in the presence of SNP plus U46619 however this was not significant ($P>0.05$, ANOVA) (Figure 3.10a). Uridine but not UMP (0.1 µM -1 mM) caused a contraction in porcine coronary arteries, tested in the presence of forskolin and U46619 (Figure 3.12). Glucose alone was not used in these studies (Figure 3.12).

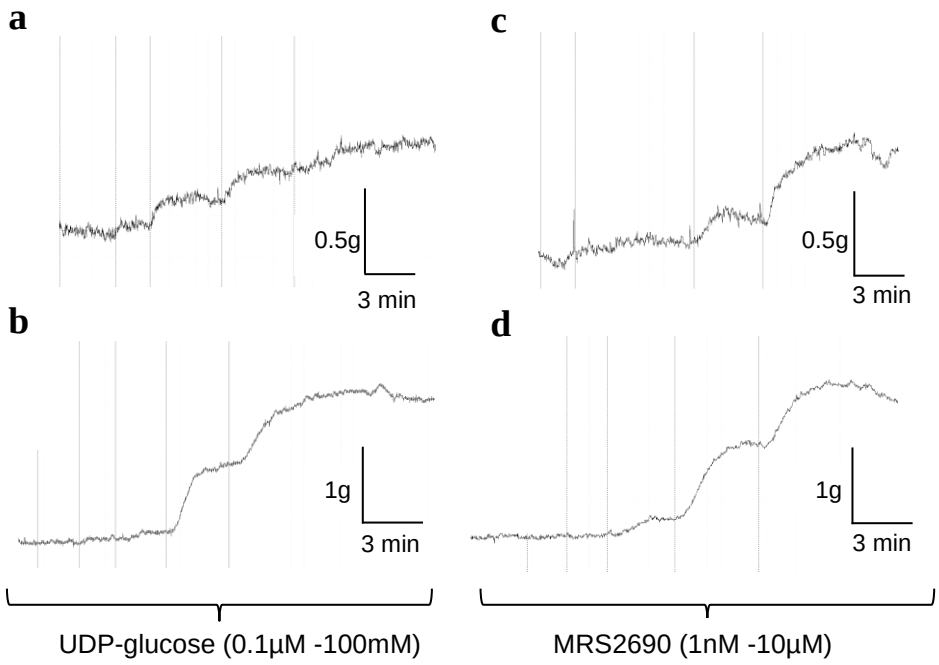


Figure 3.9: Representative traces showing concentration-dependent contractile responses of porcine coronary artery to UDP-glucose (a, b) and MRS2690 (selective $P2Y_{14}$ receptor agonist) (c, d) at basal tone (a,c) and at basal tone under forskolin conditions (presence of U46619 and forskolin) (b, d). Dashed vertical lines on traces represent addition of cumulative concentrations of drugs.

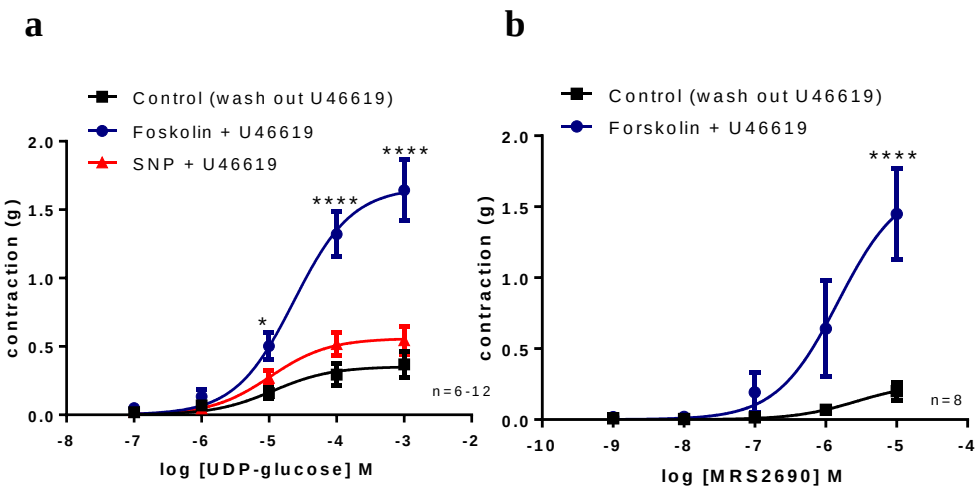


Figure 3.10: Contractile responses to UDP-glucose in the presence of forskolin plus U46619 and sodium nitroprusside (SNP) plus U46619 (a) and responses to MRS2690 (selective $P2Y_{14}$ receptor agonist) in the presence of forskolin plus U46619 (b), in porcine coronary arteries. Vessels were preconstricted with U46619 and then relaxed back to baseline by the addition of either forskolin or SNP. Data presented are mean ± S.E.M. (*P<0.05, Forskolin + U46619 compared to control, ****P<0.0001, Forskolin + U46619 compared to control and SNP + U46619, ANOVA with Sidak's multiple comparisons test).

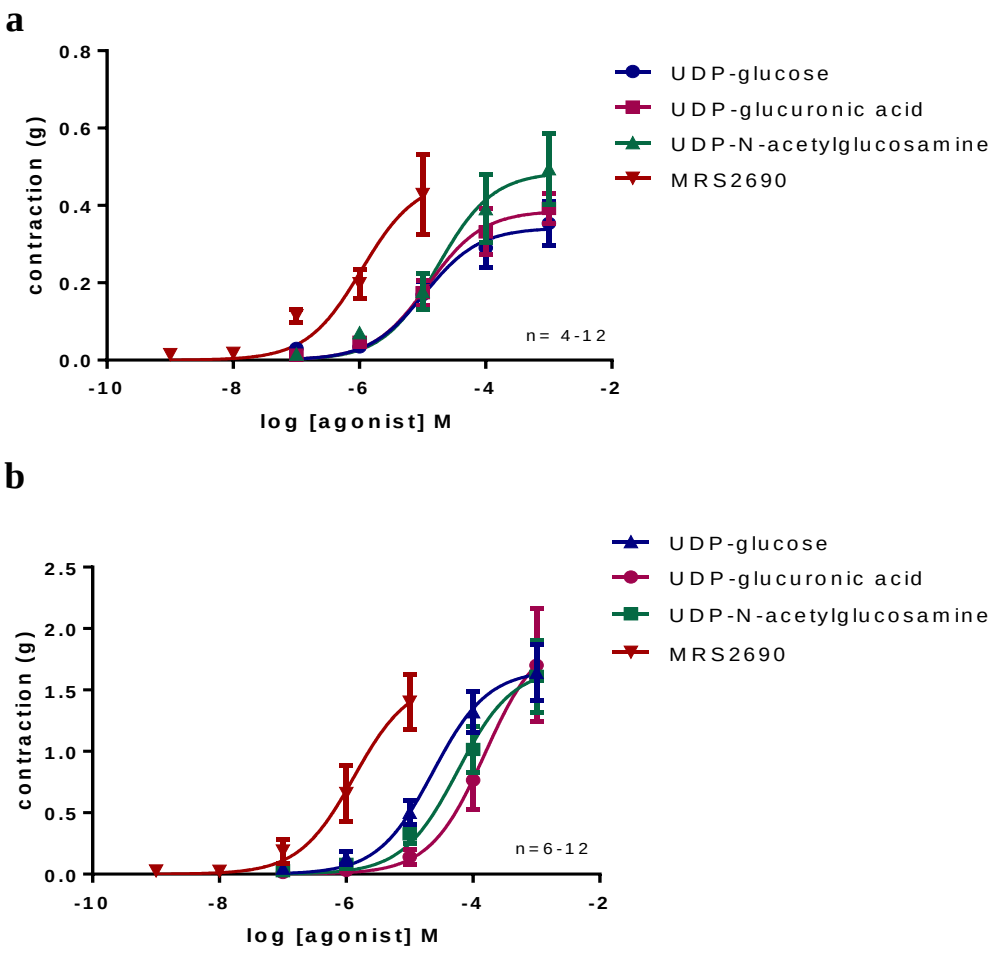


Figure 3.11: Cumulative concentration-response curves to $P2Y_{14}$ receptor agonists in the porcine coronary artery. UDP-glucose, UDP-glucuronic acid, UDP-*N*-acetylglucosamine and MRS2690 were investigated in isolated porcine coronary artery segments under basal tone conditions (a) and in arteries, also at basal tone, to which forskolin and U46619 had been added (b). Data presented are mean ± S.E.M.

Agonist	EC_{50} ± S.E.M (-log M)		R_{max} ± S.E.M (contraction in g)	
	Basal	**Forskolin**	**Basal**	**Forskolin**
UDP-glucose	4.97 ± 0.21 n=12	4.63 ± 0.16 n=6	0.34 ± 0.31	1.66 ± 0.12*
UDP-glucuronic acid	4.92 ± 0.17 n=7	3.81 ± 0.28$^{\#\#}$ n=7	0.39 ± 0.03	1.96 ± 0.36***
UDP-*N*-acetylglucosamine	4.75 ± 0.24 n=7	4.24 ± 0.21 n=7	0.49 ± 0.05	1.68 ± 0.19****
MRS2690	5.97 ± 0.25 n=4	5.88 ± 0.22 n=12	0.47 ± 0.07	1.58 ± 0.22*

Table 3.1: Contractile responses of the porcine coronary artery to UDP-sugars. Shown are the EC_{50} (-log M) and Rmax values. Contractile response curves were investigated in arteries at basal tone (Basal) and in arteries to which forskolin had been added to stimulate adenylyl cyclase (with U46619 present to maintain a basal tone; see Methods) (Forskolin). EC_{50} of UDP-glucuronic acid in forskolin conditions was significantly different to that of basal conditions ($^{\#\#}P<0.01$, unpaired Student's *t*-test). Agonist R_{max} values under basal tone conditions were significantly greater than their respective R_{max} values under forskolin plus U46619 conditions (*P<0.5, ***P<0.001 and ****P<0.0001 unpaired Student's *t*-test)

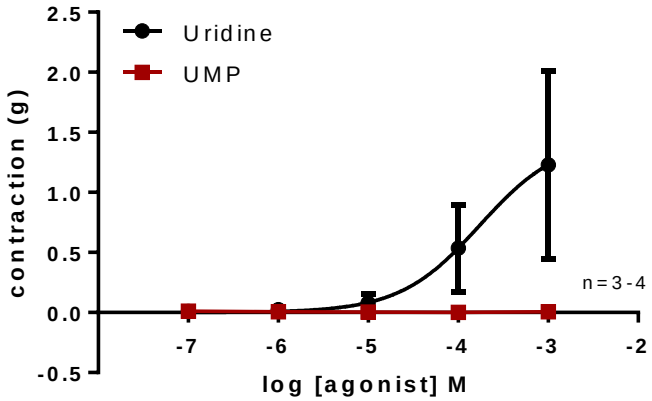


Figure 3.12: Contractile responses to Uridine and UMP in the presence of forskolin plus U46619 in porcine coronary arteries. Data presented are mean ± S.E.M.

To characterize whether the contractile responses to UDP-glucose was modulated by the endothelium, the endothelium was removed in some preparations of the PCA. The effect of UDP-glucose was compared between endothelium removal and endothelium intact vessels in the presence of forskolin plus U46619. Substance P (10nM) was used to confirm the presence of the endothelium in the porcine coronary arteries. UDP-glucose responses were not significantly different in endothelium intact or denuded vessels ($P>0.05$, ANOVA, Figure 3.13). In endothelium-intact arteries, relaxation to substance P (10nM) was 41 ± 6.4 % of the total U46619-induced contraction. In endothelium-denuded arteries, substance P did not cause a relaxation.

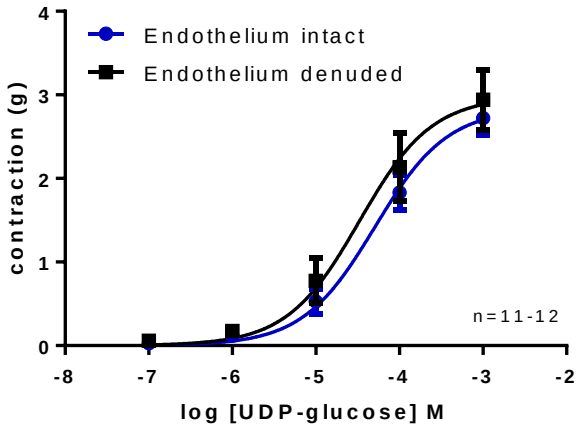


Figure 3.13: Concentration-response curves to UDP-glucose in endothelium-intact and endothelium-denuded porcine coronary arteries in the presence of forskolin plus U46619. Data presented are mean ± S.E.M. (P>0.05, ANOVA).

To determine whether $P2Y_{14}$ receptors are variably expressed in different regions of the coronary artery, contractions induced by UDP-glucose and MRS2690 were examined in distal segments after exposure to U46619 and forskolin (as all of the studies described above were carried out using segments of the proximal artery). The contractions to both UDP-glucose and MRS2690 were not significantly different between proximal and distal segments (2 mm in length, approximate diameter of 0.8 mm) (contractions were expressed as a percentage of KCl for both the proximal and distal segments in these experiments, to allow for any inherent differences in vascular contractility) (Figure 3.14).

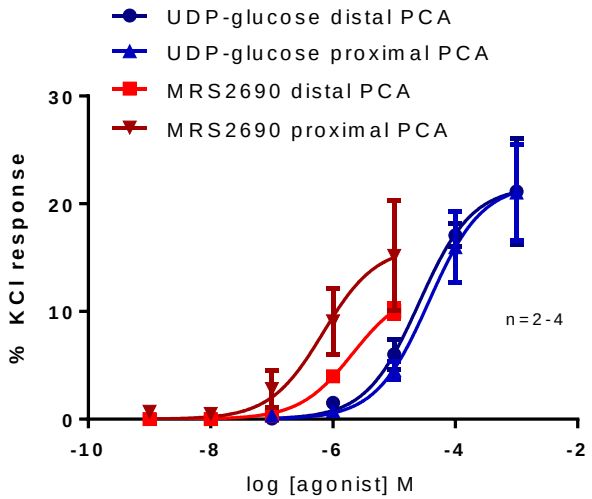


Figure 3.14: Concentration-response curves to UDP-glucose and MRS2690 in distal and proximal segments of porcine coronary arteries in the presence of U46619 and forskolin. Responses to agonists are not significant between distal and proximal segments. Data presented are mean ± S.E.M. (P>0.05, ANOVA)

Additionally, responses to UDP-glucose were measured in coronary veins in forskolin conditions and were compared to those seen in coronary arteries. The contractions induced by UDP-glucose were significantly smaller (expressed as a percentage of the KCl induced contractions) compared to contractions seen in coronary arteries (Figure 3.15); (P <0.001, ANOVA with Sidak's multiple comparisons test).

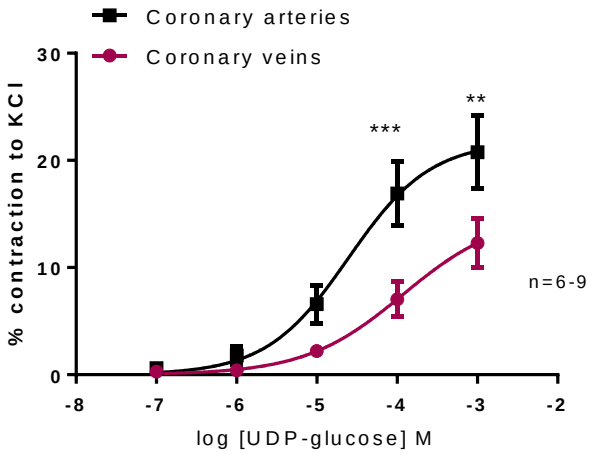


Figure 3.15: Concentration response curves to UDP-glucose in porcine coronary arteries and porcine coronary veins at basal tone in forskolin (plus U46619) conditions. UDP-glucose induced contractions are significantly different between arteries and veins, particularly at 100µM and 1mM UDP-glucose concentrations. Data presented are mean ± S.E.M (**P<0.01, ***P<0.001, ANOVA with Sidak's multiple comparisons test).

3.4.4 PPTN, a selective $P2Y_{14}$ receptor antagonist, blocks responses to MRS2690 and partially UDP-glucose, in porcine coronary arteries

In order to further pharmacologically characterize the $P2Y_{14}$ receptor in the porcine coronary artery, PPTN, a selective antagonist at the $P2Y_{14}$ receptor (Gauthier et al., 2011; Barrett et al., 2013), was used to examine the contractile response to the $P2Y_{14}$ receptor agonists UDP-glucose and MRS2690. PPTN at 1µM acts selectively to inhibit the $P2Y_{14}$ receptor with no effect at the other P2Y receptor subtypes (Barrett et al., 2013). The contraction to MRS290 (selective agonist at the $P2Y_{14}$ receptor) was significantly attenuated by PPTN (1µM), which produced a 10-fold rightward displacement in the concentration-

response curve (P <0.0001, ANOVA, with Sidak's multiple comparisons test). At 3μM PPTN, the responses to MRS2690 were not significantly different to the response in the presence of 1μM PPTN (Figure 3.16b, Table 3.2). In the presence of PPTN, there was an approximately 11-fold rightward shift in the UDP-glucose response curve, with 1μM PPTN. (P <0.01, ANOVA with Sidak's multiple comparisons test), (Figure 3.16a, Table 3.2). 1μM and 3μM PPTN significantly inhibited the contraction to 10μM UDP-glucose (P <0.01, ANOVA) but had no significant effect to the other UDP-glucose concentrations (P >0.05, ANOVA).

UTP is an agonist at $P2Y_2$ and $P2Y_4$ receptors, and elicits contraction of porcine coronary arteries through activation of these receptors (Rayment et al., 2007). UTP, up to concentrations of 1μM, does not have any effect at the $P2Y_{14}$ receptor (Chambers et al., 2000**).** To determine the selectivity of action of PPTN versus other P2Y receptors in the PCA, namely $P2Y_2$ and $P2Y_4$ receptors, the effect of PPTN on contractile responses to UTP was investigated. 1μM PPTN had no significant effect on the contractile responses to UTP (P > 0.05, ANOVA). Although 3μM PPTN, did not statistically change the responses to UTP, there was an approximate 5 fold shift in the concentration response curve (Figure 3.16c, Table 3.2).

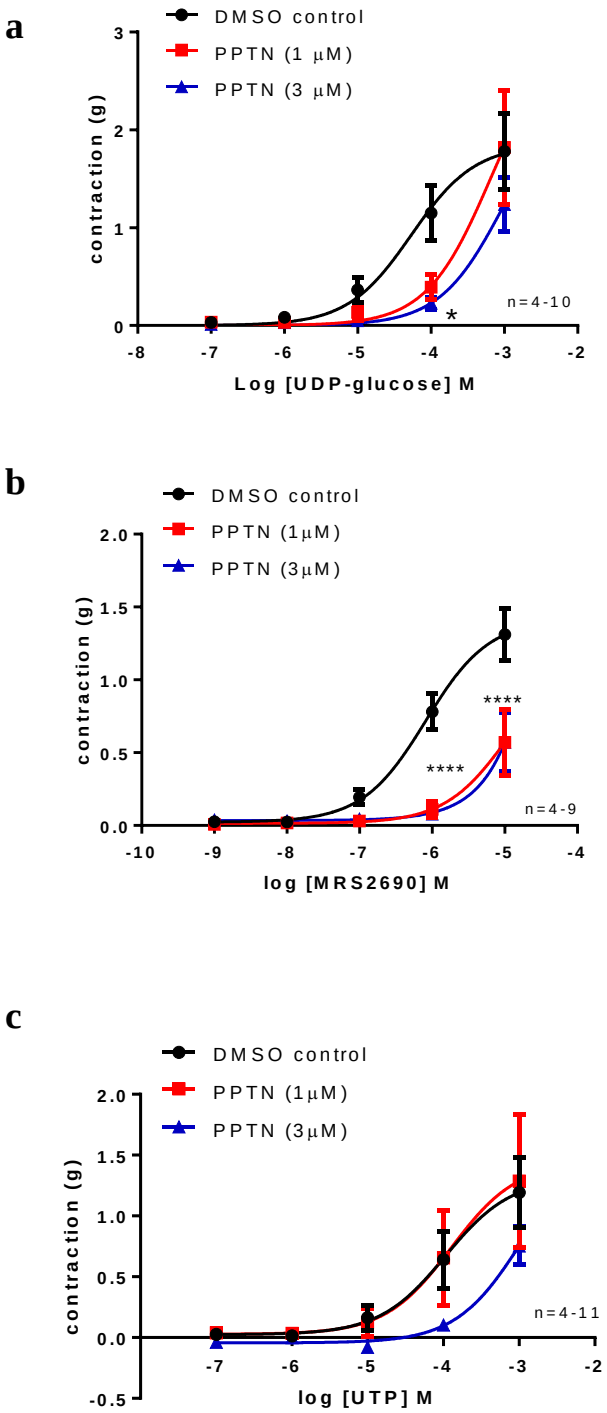


Figure 3.16: The effect of a selective $P2Y_{14}$ receptor antagonist, PPTN (1 µM and 3 µM), on contractile responses to UDP-glucose (a), MRS2690 (selective $P2Y_{14}$ agonist) (b), and UTP (c), in porcine coronary arteries in the presence of forskolin (and U46619). Data are means ± S.E.M. (*P<0.05, Control compared to 1 µM 3 µM PPTN ****P<0.0001, control compared to 1 µM and 3 µM PPTN, two-way ANOVA followed by Sidak's multiple comparisons test).

Agonist	Control EC_{50} (-log M)	PPTN (1 μM) EC_{50} (-log M)
UDP-glucose	4.27 ± 0.26 n=10	3.20 ± 0.58 n=6
MRS2690	6.12 ± 0.82 n=9	5.13 ± 0.82 n=7
UTP	3.91 ± 0.68 n=11	3.89 ± 0.95 n=7

Table 3.2: EC_{50} values (-log M) for contractile responses to $P2Y_{14}$ receptor agonists in porcine coronary arteries (PCA) in the absence (Control) and presence of PPTN, a selective $P2Y_{14}$ receptor antagonist. The responses were obtained in PCA in the presence of forskolin and U46619. Data are means ± S.E.M.

3.4.5 UTP and UTPγS contractions are increased in the presence of forskolin and U46619

$P2Y_2$ and $P2Y_4$ receptors couple to $G\alpha_q$ proteins (Harden et al., 2010) and therefore do not inhibit the production of cAMP. Responses to UTP (an agonist at $P2Y_2$ and $P2Y_4$ receptors) in the presence of forskolin (as a stimulant of cAMP production) plus U46619 were investigated in porcine coronary arteries.

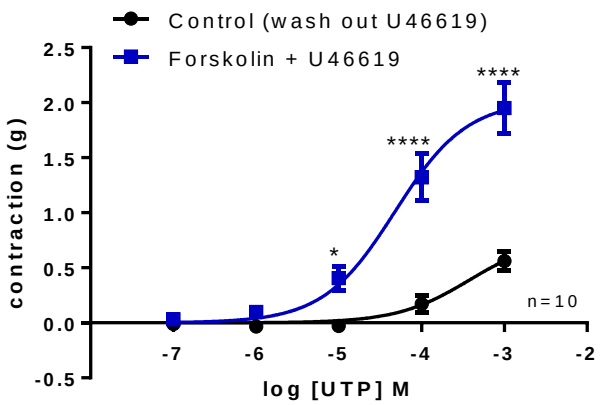


Figure 3.17: Concentration response curves to UTP in porcine coronary artery segments in the absence and presence of forskolin plus U46619. Data presented are mean ± S.E.M. (*P<0.05, ****P<0.0001, ANOVA with Sidak's multiple comparisons test).

UTP produced a concentration-dependent contraction which was significantly augmented in the presence of forskolin plus U46619 (P<0.0001, ANOVA) (Figure 3.17). To determine whether forskolin was causing direct potentiation of responses to UTP or following breakdown of UTP to UDP (an agonist at G_i-coupled $P2Y_{14}$ receptors), the effect of UTPγS, a more stable and potent analogue of UTP (Malmsjo et al., 2000b) in forskolin plus U46619 treated vessels was investigated. The effect of the PPTN (3μM) on responses to UTPγS in similar conditions was also investigated.

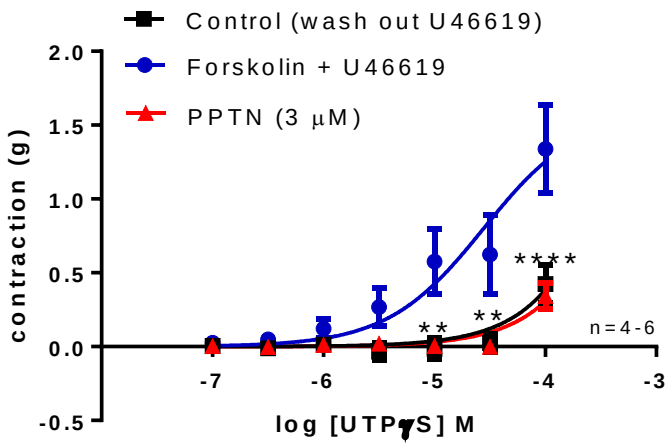


Figure 3.18: Cumulative concentration-response curve to UTPγS in porcine coronary artery segments in the presence of forskolin plus U46619 and in the presence of 3μM PPTN. Control conditions involved preconstricting with U46619 and subsequently washing out of the U46619 (vessels relaxed to a stable basal tone). Data presented are mean ± S.E.M. (**P<0.01, ****P<0.0001, Forskolin + U46619 compared to control and PPTN, ANOVA with Sidak's multiple comparisons test).

UTPγS produced a concentration dependent contraction which was significantly potentiated in the presence of forskolin plus U46619 (P<0.0001, ANOVA). This contraction was significantly inhibited in the presence of the 3μM PPTN (P<0.0001, ANOVA) (Figure 3.18).

3.4.6 Contractile responses to histamine and acetylcholine are not affected by elevated cAMP levels

Histamine and acetylcholine (ACh) causes vasoconstriction in coronary arteries by activating H_1 receptors ($G_{\alpha q}$-coupled receptor) (Chrusch et al., 1999) and M_3 receptors ($G_{\alpha q}$-coupled receptor), respectively (Weirich et al., 2005). As both receptors are non-G_i-coupled receptors, they do not inhibit cAMP formation.

Thus, the effect of forskolin plus U46619 on responses to both histamine and ACh was investigated. The effects of ACh on basal tone and raised tone (U46619 preconstricted vessels) compared to forskolin treatment were also examined.

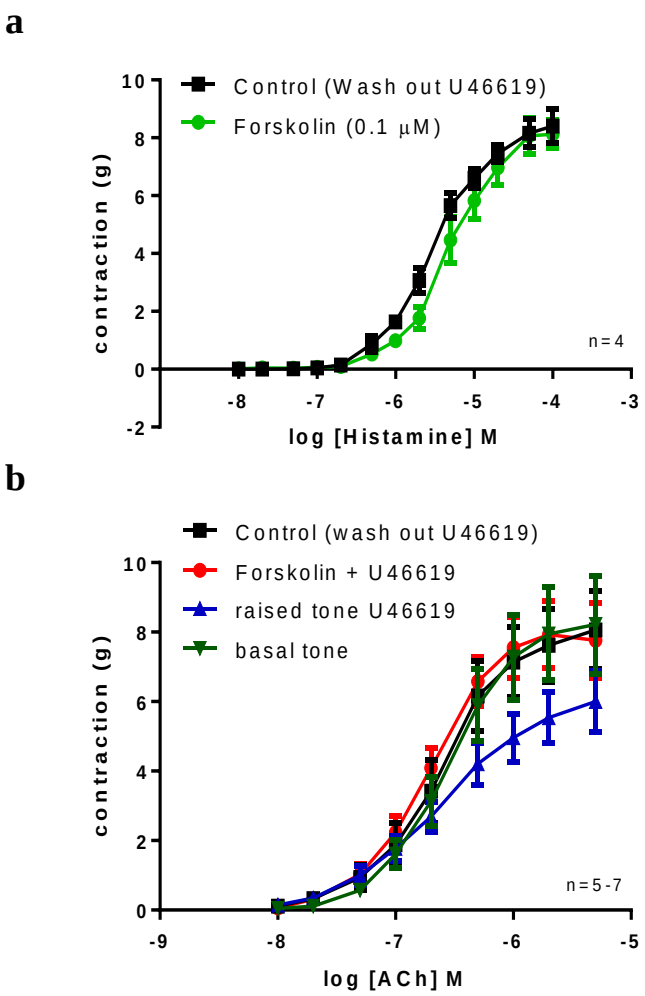


Figure 3.19: Porcine coronary artery cumulative concentration-response curves to histamine in the absence and presence of forskolin plus U46619 (a), and acetylcholine (ACh) in control conditions (vessels exposed to U46619 and subsequently washed out), in the presence of forskolin plus U46619, in U46619 preconstricted vessels (raised tone U46619) and at basal tone. Data presented are mean ± S.E.M.

Histamine produced a concentration dependent contraction which was not significantly different in the presence of forskolin plus U46619. The -log EC_{50} values for histamine in control vessels and forskolin treated vessels were 5.51 ± 0.05 and 5.31 ± 0.06 (n=4), respectively (Figure 3.19a).

ACh produced a concentration-dependent contraction. This contraction was not significantly different in the presence of forskolin plus U46619 or at basal tone. At raised tone, vessels were preconstricted to roughly 40-60% of the maximal KCl response causing a reduction in responses at the higher concentrations of ACh, however this reduction was not significant (Figure 3.19b). The –log EC_{50} values for ACh are in the various conditions are: control: 6.62 ± 0.11; forskolin: 6.73 ± 0.07; raised tone: 6.60 ± 0.15 and basal tone: 6.56 ± 0.12 (n=5-7).

ACh was used to investigate the selectivity of PPTN, in PCA in the presence of forskolin plus U46619.

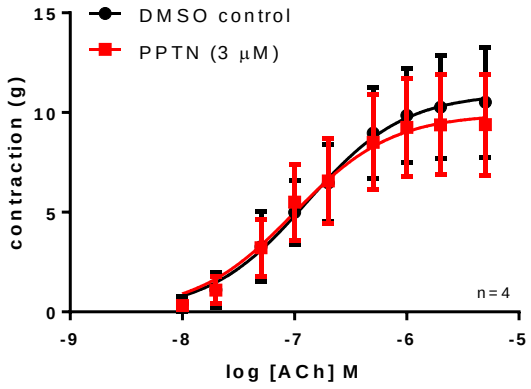


Figure 3.20: Effect of PPTN, a $P2Y_{14}$ receptor antagonist, on acetylcholine responses in the presence of forskolin plus U46619 in porcine coronary arteries. Data presented are mean ± S.E.M.

In the presence of 3μM PPTN there was no significant difference in the ACh concentration-response curve compared to control vessels ($P>0.05$, ANOVA) (Figure 3.20).

3.5 Discussion

In the present study it was shown for the first time that UDP-glucose and MRS2690 (a selective $P2Y_{14}$ receptor agonist) (Ko et al., 2007) induced vasoconstriction in PCA. This provides a functional correlate for Western blotting and immunohistochemical evidence for the expression of $P2Y_{14}$ receptors in porcine coronary arteries.

3.5.1 $P2Y_{14}$ receptors are present the porcine coronary artery in two isoforms

Western blotting using an antibody targeted against the extracellular $P2Y_{14}$ receptor epitope (Alomone antibody) and intracellular epitope (LifeSpan antibody) was carried out. A double band sized approximately 57 kDa was shown using the extracellular derived antibody against the $P2Y_{14}$ receptor. These bands are slightly higher in molecular weight than in a previous report, (which utilized the same antibody against the $P2Y_{14}$ receptor), in mice kidney cells, where a predominant band at 50 kDa and a weaker band at 40 kDa were identified (Azroyan et al., 2015). Furthermore, according to the manufacturers (Alomone Labs), applications of the antibody using rat heart membranes, blots showed bands sized between approximately 40 kDa and 47 kDa. $P2Y_{14}$ receptor mRNA is present in the rat heart (Musa et al., 2009) and thus this was used as a positive control tissue (although there are no reports of the $P2Y_{14}$ receptor protein being present in rat hearts).

The antibody derived against the intracellular $P2Y_{14}$ receptor epitope, showed that the $P2Y_{14}$ receptor protein is present in porcine coronary arteries as two isoforms, sized 41 and 61 kDa. These could correspond to glycosylated and non-glycosylated forms of the $P2Y_{14}$ receptor respectively, as shown in C6 glioma cells (Krzeminski et al., 2008). N-glycosylation and receptor oligomerization are processes that enable the release of functional GPCRs from the ER to the plasma membrane (Bulenger et al., 2005; Dong et al., 2007). Zhong et al. (2004) have demonstrated that N-linked glycosylated $P2Y_{12}$ receptors are essential for signal transduction in platelets whilst glycosylated forms retain cell surface expression and ligand binding activity but have no effect on signal transfer following ligand binding (Zhong et al., 2004). Thus glycosylated, or non-glycosylated forms of the $P2Y_{14}$ receptor, artery may modulate intracellular signalling whilst the other may retain its ability to bind agonists whilst having no further effect; however this remains to be established. A future study to identify the effect of glycosylated $P2Y_{14}$ receptors in the porcine coronary artery would be to utilize tunicamycin; this has been shown to inhibit N-linked glycosylation of the glucagon-like peptide 1 receptor thereby abolishing the binding of glucagon-like peptide 1 to the receptor as well as inhibiting the biological activity of the receptor

GPR17, a non-P2Y receptor is activated by UDP-glucose and cysteinyl leukotrienes (Daniele et al., 2011), does not appear be expressed in porcine coronary arteries since Western blot studies failed to detect GPR17 receptor protein expression in the porcine coronary artery. Two human isoforms of the GPR17 receptor, however, have been detected in human brain, heart and kidney (Benned-Jensen and Rosenkilde, 2010). RT-PCR has shown that the

GPR17 receptor is present in the rat brain, heart and kidney (Ciana et al., 2006). In the current study, two isoforms of the GPR17 receptor protein, sized 38 kDa and 44 kDa were seen in the rat brain however not the rat heart. It is possible that GPR17 receptors are expressed in these tissues at the molecular levels, however not functionally expressed in rat heart tissue. Human and pig hearts are similar (Swindle et al., 2012); GPR17 receptors are expressed in the human heart but not in porcine coronary arteries.

3.5.2 $P2Y_{14}$ receptors are present in high levels in porcine coronary artery smooth muscle cell contractile phenotypes?

Immunohistochemical analysis showed staining for the $P2Y_{14}$ receptor in both the vascular smooth muscle cells and endothelial cells of the porcine coronary artery, which, upon omission of the primary antibody (LifeSpan $P2Y_{14}$ receptor antibody, intracellular $P2Y_{14}$ receptor epitope), did not appear to be present. However, there are doubts about antibody reliability and specificity and also there is a lack of information regarding the $P2Y_{14}$ receptor antibodies within the literature. Recent reports that looked at three different antibodies directed against the $P2Y_6$ receptor in bladder smooth muscle, found that the antibodies appeared to bind to similar proteins in $P2Y_6$ receptor deficient knockout mice (Yu et al., 2013). Moreover, there have also been reports to show cross-reactivity of $P2X_1$ receptor-derived antibodies which stain both sites ($P2X_1$ receptors) in the central nervous system and also in $P2X_1$ receptor deficient mice (Ashour et al., 2006).

Staining of $P2Y_{14}$ receptors was looked at in isolated porcine coronary artery smooth muscle cells. It is well know that smooth muscle cells undergo

phenotypic modulation and range from contractile to synthetic phenotypes; both phenotypes can be present within the same vessel (Rensen et al., 2007). The Introduction Chapter, Section 1.3.5 has described how changes in P2 receptor levels in various phenotypes can occur depending on function required. The morphology of the cells isolated from porcine coronary arteries appears to be elongated and rhomboid-like for Figures 3.8g and 3.8h, respectively. The characteristics of smooth muscle cells have been described to correlate to contractile (elongated, spindle-shaped cells) and synthetic (epithelioid or rhomboid) phenotypes which have proliferative and migratory functional aspects (Chamley-Campbell et al., 1979; Hao et al., 2003), thus $P2Y_{14}$ receptors could be present in contractile and synthetic phenotypes as identified in the present study. Following from this speculation, the staining of $P2Y_{14}$ receptors appears to be more dispersed across the cytoplasm to the nucleus of cells in contractile phenotypes as opposed to synthetic phenotypes where staining of the receptors appears to be restrained to the nucleus. P2 receptors are present across the plasma membrane, the intracellular cytosolic compartments, ER, Golgi apparatus nuclear envelope, endosomes and neurosecretory granules. Depending upon the function required, P2 receptors can dynamically move between these structures (Atkinson et al., 2002; Volonte et al., 2006). It can be speculated that the staining seen in the nuclei of porcine coronary artery smooth muscle cells of synthetic phenotypes may be as a result of receptor internalization following phenotypic modulation or the lack of functional activity required in these phenotypes as opposed to the contractile phenotypes where staining is denser and there is an increased cell-cell contact. Thus $P2Y_{14}$ receptors may be required for communication signalling between

cells in contractile phenotypes. On the other hand the staining for $P2Y_{14}$ receptor expression may be as a result of a methodological artefact, however the above proposed suggestions warrant further investigation.

Smooth muscle cells can be manipulated in culture to adopt a particular phenotype, porcine coronary artery smooth muscle cells have been shown to display phenotypic modulation following fibroblast growth factor-2 treatment (Hao et al., 2002). Future experiments could be conducted to detect whether changes in $P2Y_{14}$ receptor expression occur following treatment using fibroblast growth factor-2. An increase, or decrease, in $P2Y_{14}$ receptor staining in coronary artery smooth muscle cells could suggest a regulatory function in contractile, synthetic or both phenotypes. Additional experiments could be done to confirm the presence of either contractile or synthetic phenotypes; smooth muscle myosin heavy chain and smoothelin are specific markers for mature contractile phenotypes which are absent in synthetic phenotypes (Rensen et al., 2007).

3.5.3 $P2Y_{14}$ receptor agonists induce contraction of porcine coronary arteries

In the present study, a functional $P2Y_{14}$ receptor was identified and characterized using pharmacological techniques. In the porcine coronary vasculature, we showed that this receptor mediates vasoconstriction to UDP-glucose, UDP-glucuronic acid, UDP-*N*-acetylglucosamine and MRS2690, all of which are known $P2Y_{14}$ receptor agonists (Chambers et al., 2000; Abbracchio et al., 2003). UDP-glucose also elicits contractions in mouse coronary arteries, where the approximate EC_{50} value was 100μM (Haanes et

al., 2014a). In the present study, UDP-glucose induced contractions of the porcine coronary artery were more potent, with an EC_{50} value of approximately 10μM (-log M is 4.97). A higher concentration of UDP-glucose above 1mM was not used since these would not have been physiologically relevant (see Introduction, Table 1.1,). It is possible that the greater potency in the porcine coronary artery is due to the overnight storage conditions (see Methods), since Haanes and Edvinsson found that the potency of UDP-glucose increased in organ cultured mouse coronary arteries to 2.1 μM (compared to 100 μM in fresh vessels). UDP-glucose also elicits weak contractions in the mouse mesenteric artery (Haanes et al., 2014b). To determine whether the potency of UDP-glucose changes in fresh porcine coronary arteries, a possible further experiment would be to look at the effects of UDP-glucose induced-contractions in freshly isolated arteries.

The contractile response induced by MRS2690 was more potent than those produced by the UDP-sugars, consistent with previous reports (Jacobson et al., 2009; Gao et al., 2010), that MRS2690 is a more potent and selective $P2Y_{14}$ receptor agonist (Ko et al., 2007). Contractions to MRS2690 were observed at concentrations equal to or below 10μM, and at 10μM MRS2690 is inactive at $P2Y_2$ receptors (Ko et al., 2009). Higher concentrations of MRS2690 were not used due to drug availability/expense.

3.5.4 UDP-glucose contractions do not involve endothelial cells

P2Y receptors located on vascular endothelial cells elicit vasodilatation through release of NO, endothelium-derived hyperpolarising factor and

prostaglandins (Burnstock and Ralevic, 2014). P2Y receptors expressed on vascular smooth muscle cells usually mediate vasoconstriction (Lewis et al., 2000a; Burnstock et al., 2014). Vasocontractile $P2Y_{14}$ receptors are expressed on the vascular smooth muscle cells, since endothelium removal had no effect on contractions to UDP-glucose, suggesting an absence of vasocontractile $P2Y_{14}$ receptors on endothelial cells. This is similar to $P2Y_{14}$ receptor expression in the mouse coronary and cerebral arteries, where it was shown that there was no difference in UDP-glucose induced contraction in vessels where endothelium was present or absent (Haanes et al., 2014a). However, this is different to the porcine pancreatic artery, in which the $P2Y_{14}$ receptor is located on both the smooth muscle and endothelium; removal of endothelium inhibits UDP-glucose-induced contractions (Alsaqati et al., 2014). RT-PCR analysis of all eight P2Y receptors in human coronary artery endothelial cells showed mRNA expression for $P2Y_2$ and $P2Y_{11}$ subtypes with no detection for the other subtypes including the $P2Y_{14}$ receptor (Ding et al., 2011). However this may vary for porcine vessels. Based on the results from immunohistochemical analysis, it is possible that endothelial $P2Y_{14}$ receptors (with no obvious role in regulation of contractility) may have a role in long-term signalling mechanisms. ATP and adenosine cause proliferation of endothelial cells via activation of $P2Y_1$ and A_2, receptors respectively (Burnstock, 2002). ATP and UTP induce migration of human umbilical vein endothelial cells by activation of $P2Y_2$ receptors (Gendaszewska-Darmach et al., 2011). These proliferative and migratory effects mediated by activation of P2Y receptors occur when there is an increase in nucleotide release caused by ischaemia, hypoxia and cell damage. The high concentration of nucleotides

stimulate tissue and endothelium repair and may also interact with growth factors to increase mitogenesis and angiogenesis (Abbracchio et al., 1998; Burnstock et al., 2014). Thus, it is possible that UDP-glucose may initiate proliferation and/or migration in coronary endothelial cells by activation of $P2Y_{14}$ receptors.

3.5.5 PPTN antagonizes contractile responses to the selective $P2Y_{14}$ receptor agonist, MRS2690

In a recent report, a novel compound, PPTN, was shown to be a selective high-affinity competitive antagonist at the $P2Y_{14}$ receptor (Gauthier et al., 2011; Barrett et al., 2013). PPTN was shown to antagonize the $P2Y_{14}$ receptor at low nanomolar levels, where chimpanzee $P2Y_{14}$ receptor binding assays revealed it had a K_i of 1.9nM (Robichaud et al., 2011). Another $P2Y_{14}$ receptor antagonist has been identified where this nonnucleotide compound referred to as 'compound A' has been shown to inhibit UDP-glucose mediated calcium signalling in a HEK cell line expressing the $P2Y_{14}$ receptors. In UDP binding assays, it was shown to have a K_i of 1280 nM (Hamel et al., 2011). In the present study, responses to MRS2690 were selectively antagonized by PPTN (3 μM) versus contractile responses to acetylcholine. These functional studies are supported by evidence of the expression of $P2Y_{14}$ receptor protein in the porcine coronary artery. The porcine coronary artery is known to express other uracil-activated P2Y receptors including $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, which mediate vasocontraction (Rayment et al., 2007). These are receptors for UTP and ATP ($P2Y_2$), UTP ($P2Y_4$) and UDP ($P2Y_6$) (Burnstock and Ralevic, 2014).

PPTN is highly selective antagonist of the $P2Y_{14}$ receptor, which does not act at the other P2Y receptor subtypes (Gauthier et al., 2011; Barrett et al., 2013). In other reports, higher concentrations of PPTN (10μM) have been utilized, where it has been shown that PPTN blocks UDP-glucose induced increases in ERK1/2 phosphorylation and also UDP-glucose increase in IL-8 and CC12 mRNA expression in renal intercalated cells (Azroyan et al., 2015). The effects of this high concentration of PPTN on responses to other P2Y receptor nucleotides were not investigated (Azroyan et al., 2015).

PPTN inhibited the contractile responses to MRS2690 and the responses were virtually abolished at 1 μM MRS2690. The UDP-glucose response curve was shifted in the presence of the $P2Y_{14}$ receptor antagonist PPTN which significantly inhibited the responses to 10 μM UDP-glucose but not to 100 μM UDP-glucose. It is possible that, in addition to its actions at $P2Y_{14}$ receptors, UDP-glucose may act at other contractile P2Y receptors. Indeed, actions at multiple P2Y receptor targets are common among purine/pyrimidine nucleotides (Burnstock and Ralevic, 2014). A likely target is the $P2Y_2$ receptor, at which UDP-glucose is known to act, albeit weakly (Ko et al., 2009). The lack of availability of a $P2Y_2$ receptor antagonist makes it difficult to determine whether UDP-glucose is acting at $P2Y_2$ receptors, which are known to be present in the porcine coronary artery (Rayment et al., 2007). However, a recent report showed that in $P2Y_2$ receptor knockout mice, the contractile responses to UDP-glucose induced in freshly isolated basilar arteries and organ cultured coronary arteries is slightly smaller, suggesting some involvement of UDP-glucose induced contraction through $P2Y_2$ receptors. However, these

UDP-glucose contractions are not significantly different in $P2Y_2$ receptor knockouts compared to wild type mice (Haanes et al., 2014a).

UMP and or uridine (formed as a result of UDP-glucose metabolism) are known not to contribute to $P2Y_{14}$ receptor activation (Chambers et al., 2000) and it was shown that UMP, (concentrations ranging between 0.1µM to 1mM) does not have a contractile effect on porcine coronary arteries. In vascular smooth muscle cells and endothelial cells, the ectonucleotidase, nucleoside triphosphate diphosphohydrolase-1 (NTPDase1) is expressed whilst NTPDase2 is expressed in the adventitia (Sevigny et al., 2002). NTPDase1 exerts its catalytic activity on ATP, ADP, UTP and UDP, as in a study looking at Entpd1 deficient mice, hydrolysis of these nucleotides is insignificant (Kauffenstein et al., 2010). UDP-glucose is not metabolized by NTPDase but by ectonucleotide pyrophasphatases (ENPP) leading to the formation of UMP (Lazarowski et al., 2015). In the rat coronary circulation, there is a high expression levels of ectonucleotidases that hydrolyse ATP, as seen in a study that utilized the Langendorff model, in which injection of ATP resulted in almost complete metabolism and formation of ADP, AMP, adenosine, inosine and hypoxanthine (Takahashi-Sato et al., 2013). There is limited evidence of the effects of ENPP in the coronary vasculature and, in the current study the actions of UDP-glucose on the coronary artery are likely to be direct rather than due to the actions of its metabolites. However, further research into the effects of ENPPs in porcine coronary artery is needed. Interestingly, constitutive release of UDP-glucose occurs from several cell types, including human astrocytoma cells, Calu-3 airway epithelial cells, COS-7, CHO-K1 and C6 glioma and, unlike ATP levels, the levels of UDP-glucose remain elevated for up to 3 hours

(Lazarowski et al., 2003), suggesting that UDP-glucose is not readily metabolized. This has important implications for $P2Y_{14}$ receptor signalling under conditions, such as ischaemia, where UDP-glucose may be released in the coronary vasculature.

3.5.6 Forskolin, an elevator of intracellular cAMP, enhances contractions to $P2Y_{14}$ receptor agonists

The $P2Y_{14}$ receptor couples to $G_{i/o}$ proteins since pertussis toxin treatment (which blocks $G_{i/o}$ proteins-coupling) inhibits UDP-glucose-induced activation of the $P2Y_{14}$ receptor (Chambers et al., 2000; Abbracchio et al., 2003). $G_{i/o}$ proteins couple to the inhibition of adenylyl cylase (AC)/cAMP pathway. This pathway can be activated by forskolin, which activates the AC activity by binding to the catalytic subunit of smooth muscle receptors (Muller et al., 1983; Wagh et al., 2012). UDP-glucose inhibits forskolin-stimulated cAMP accumulation in T-lymphocytes, neutrophils in human astrocytoma cells, suggesting $P2Y_{14}$ receptors couple to $G_{i/o}$ proteins (Scrivens et al., 2005b; Scrivens et al., 2005a; Scrivens et al., 2006).

The present study investigated the effect of forskolin as a stimulant of cAMP and effect of sodium nitroprusside (SNP), as a stimulant of cGMP (through activation of guanylate cyclase) (Arnold et al., 1977), on responses to UDP-glucose. U46619 was present in both cases to maintain basal tone since forskolin and SNP are vasodilators. UDP-glucose mediated contraction, which was significantly enhanced in the presence of U46619 and forskolin compared to vessels that had not been exposed to forskolin. These observations are

consistent with the effects of forskolin in the porcine isolated ear artery, where contractile responses to a $G_{i/o}$ coupled α_2-adrenoceptor agonist (UK 14304) were enhanced in the presence of U46619 and forskolin compared to tissues not exposed to either (Roberts et al., 1998). Responses to the more selective $P2Y_{14}$ agonist, MRS2690, were also significantly enhanced in the presence of U46619 plus forskolin.

Responses to UDP-glucose were increased by approximately 25% in the presence of SNP plus U46619 than vessels not exposed to SNP. However, in the presence of forskolin (plus U46619), the maximum contraction induced by UDP-glucose was enhanced by approximately 200%. From these results, it can be concluded that U46619 does not play a major role in the potentiation of responses to UDP-glucose observed in the presence of forskolin plus U46619. The potentiation of UDP-glucose and MRS2690 responses in conditions of elevated cAMP levels are consistent with the fact that the $P2Y_{14}$ receptor is a $G_{i/o}$ coupled receptor. Further investigation of the $P2Y_{14}$ receptor coupling to the inhibition of cAMP is given in Chapter 5.

3.5.7 Investigating effects of UTP and UTPγS in the porcine coronary artery

In previous studies, UTP has been shown to elicit vasodilation in the isolated rat aorta (Garcia-Velasco et al., 1995), rat heart (Godecke et al., 1996) and also the isolated guinea-pig heart (Vials et al., 1993). However, in rat coronary arteries, UTP does not elicit a vasodilation (Korchazhkina et al., 1999) and in porcine coronary arteries, UTP has been shown to mediate vasoconstriction

(Rayment et al., 2007a). The actions of UTP in the different systems are likely to be mediated through $P2Y_2$ receptors and $P2Y_4$ receptors which couple to $G\alpha_q$ proteins (Harden et al., 2010). Thus, the actions of UTP activating $P2Y_2$ and $P2Y_4$ receptors vary across species and vascular beds within the same species. UTP is also an agonist at $P2Y_6$ receptors; however, it is less potent than UDP. $P2Y_6$ receptors are indirectly activated by UTP which is metabolically degraded to UDP (Nicholas et al., 1996). The present study found that contractile responses to UTP were significantly increased in vessels treated with forskolin plus U46619 compared to vessels that had no exposure to forskolin. As UTP is not an agonist at the $P2Y_{14}$ receptor (Abbracchio et al., 2003) and does not activate G_i coupled receptors and hence does not inhibit cAMP, it is possible that the augmentation caused by forskolin plus U46619 was due to responses to UDP (acting as a $P2Y_{14}$ agonist) formed following metabolic degradation of UTP, which is rapidly degraded in aqueous solutions (Welford et al., 1987). Furthermore, ectonucleotidases and/or phosphatases are a cause of high UDP production as a by-product of extensive UTP breakdown (as shown in mucosal cells) (Lazarowski et al., 1997). In porcine coronary smooth muscle cells, UDP has been shown to inhibit forskolin-induced cAMP accumulation; the effect was abolished by PTX treatment suggesting the presence of a G_i coupled receptor. It was concluded that $P2Y_6$ receptors present in PCA are coupled to G_i proteins (Shen et al., 2006). Therefore, the present results suggest that, as a consequence of UTP breakdown, $P2Y_6$ and/or $P2Y_{14}$ receptors may have been activated by UDP causing the significant potentiation in responses in the presence of forskolin plus U46619.

To test the selectivity of PPTN, its effects were examined on UTP responses. In the porcine coronary artery, contractile response to UTP (agonist at $P2Y_2$ and $P2Y_4$ receptors) were unaffected by PPTN at a concentration (1 μM) that inhibited contractile responses to MRS2690. However, at a higher antagonist concentration (3 μM), responses to UTP were shifted to the right. These results may be because, as previously mentioned, UTP was being metabolized to UDP (UDP being a $P2Y_{14}$ and $P2Y_6$ agonist). Indeed, it has been shown that in binding assays, UTP is rapidly degraded to UDP in which the latter remains stable for longer (Hamel et al., 2011). In the current results, this is what may have accounted for PPTN shifting the responses to UTP to the right. Alternatively, PPTN may be acting non-selectively at other P2Y receptors, although PPTN has been shown to be selective at up to 10μM as there was no non-$P2Y_{14}$ receptor agonist induced activation of inositol phosphate accumulation (Barrett et al., 2013).

To examine which of the above is involved, the effect of uridine 5'-O-3-thiotriphoshate (UTPγS), a stable analogue of UTP was examined. UTPγS is a selective agonist at $P2Y_2$ and $P2Y_4$ receptors (Lazarowski et al., 1996) and is 1000-fold more potent than endogenous UTP (Malmsjo et al., 2000a). However, in human airway epithelial cells, where the ectonucleotidase activity has been reduced, UTP and UTPγS are equally potent (Lazarowski et al., 1996; Malmsjo et al., 2000a). UTPγS produced a contractile response which was significantly enhanced in the presence of forskolin plus U46619. These results suggest that as UTPγS is not susceptible to enzymatic breakdown, potentiation in responses is possibly because UTPγS is activating $G_{i/o}$ coupled receptors. Reports have shown that at concentrations above 1μM, UTPγS strongly

inhibits AC in S49 lymphoma cell membranes and at 100μM completely inhibits the AC activity (Gille et al., 2004). These reports are consistent with the current findings as concentrations of UTPγS reported to inhibit AC activity were also augmented during forskolin-induced activation of cAMP in PCA.

These results are also consistent with some reports that suggest although the $P2Y_2$ receptors have been shown to couple to the $G\alpha_{q/11}$ proteins, $P2Y_2$ receptors are partially inhibited by the G_i/G_o inhibitor pertussis toxin (PTX) (Abbracchio et al., 2006). PTX partially inhibits responses to UTP mediated activation of the MAP kinase pathway in CHO-A_1 cells (Dickenson et al., 1998) and also reduces UTP-mediated calcium influx in cultured coronary artery SMCs suggesting $P2Y_2$ receptors couple to $G_{i/o}$ proteins (Hill et al., 2002). Furthermore $P2Y_2$ receptor activation by UTP and ATP mediates an inhibition of AC and cAMP accumulation in NCB-20 cells (Ralevic et al., 1998). Thus, UTP/UTPγS may be inhibiting the formation of cAMP through activation of $G_{i/o}$ coupled receptors.

Enzymatic breakdown of UTP to UDP (agonist at the $P2Y_{14}$ receptor) was unlikely to be the cause of contractile responses to UTP being shifted to the right by PPTN, as responses to UTPγS were inhibited by 3μM PPTN. Whether UTPγS acts at $P2Y_{14}$ receptors is not known as there are no reports that examine the effects of UTPγS on $P2Y_{14}$ receptor expressing cells. These results suggest that, since UTPγS is a selective agonist at $P2Y_{2/4}$ receptors, PPTN may be acting non-selectively at other P2Y receptor subtypes when used at 3 μM and under the conditions of the present study. However, the possibility of UDP (agonist at $P2Y_{14}$ receptor) contamination within the UTPγS stock (resulting in PPTN blockade of UTPγS induced contractions) is yet to be investigated.

Another possibility is that activation of $P2Y_2$ and/or $P2Y_4$ receptors on the vascular smooth muscle causes a release of UDP and UDP-glucose from the smooth muscle cells. Thrombin was shown to cause a release of UDP-glucose from airway epithelial cells (Kreda et al., 2008) Moreover, P2 receptor induced ATP release from smooth muscle, concomitant with contraction, has been shown (Matsuo et al., 1997; Westfall et al., 2000).

3.5.8 PPTN does not block muscarinic receptors in PCA

Histamine mediates a vasoconstriction in PCA through activation of H_1 receptors (Hagen et al., 1979) and acetylcholine (ACh) causes vasoconstriction of PCA through activation of M_3 muscarinic receptors (Yamanoue et al., 1993). Both are coupled to $G_{q/11}$ proteins (Notcovich et al., 2010; Kruse et al., 2012) and therefore do not inhibit adenylyl cyclase activity. In the present study, it was revealed that histamine and ACh contractile responses were not significantly different in forskolin plus U46619 treated vessels suggesting that forskolin treatment was not augmenting responses to vasoconstrictors non-selectively. Furthermore, potentiation in agonist responses in the presence of forskolin occurs through activation of $G_{i/o}$ proteins, as seen with UDP-glucose and UTP/UTPγS.

It was important to determine the selectivity of PPTN versus other receptors in the porcine coronary artery which induce a vasoconstriction. It was shown that PPTN (3μM) did not have any significant effect on contractile responses to ACh.

3.5.9 Concluding comments

Nucleotides, such as UTP, are released from many cell types in response to stress and hypoxia, including activated platelets and blood cells (Ralevic et al., 1998; Erlinge et al., 2005). These nucleotides initiate an inflammatory and immune response and in blood vessels may contribute to the formation of atherosclerotic plaques (Martinez et al., 2012). UDP-glucose is also released from cells in response to stress and levels remain elevated for up to 3 hours *in vitro* (Lazarowski et al., 2003b). It can be hypothesized that UDP-glucose released from damaged cells may contribute to constriction of coronary arteries during myocardial ischaemia. These findings provide a novel link between the $P2Y_{14}$ receptor and induction of vasoconstriction of porcine coronary arteries and therefore a possible target of treatment for cardiovascular diseases. Furthermore, the $P2Y_{14}$ receptor is expressed highly in the human placenta, adipose tissue, stomach and intestine and plays a fundamental role in these tissues in both health and disease.

In summary, the $P2Y_{14}$ receptor has functionally been characterized in the porcine coronary artery, where it has been shown to mediate vasoconstriction following activation by UDP-sugar nucleotides and the selective agonist MRS2690. Responses to MRS2690 were selectively inhibited by PPTN, a novel selective $P2Y_{14}$ receptor antagonist. The $P2Y_{14}$ receptor may be involved in the control of coronary arterial blood flow and may, therefore, be a possible novel target for the treatment of diseases involving abnormal coronary artery contractility.

Summary

- $P2Y_{14}$ receptors are present in porcine coronary artery smooth muscle cells and endothelial cells and are present in two isoforms, 41 kDa and 61 kDa

- $P2Y_{14}$ receptor agonists, UDP-glucose, UDP-glucuronic acid and UDP-N-acetylglucosamine elicit contraction of porcine coronary arteries. MRS2690, a more selective $P2Y_{14}$ receptor agonist, elicits more potent contraction than UDP-sugars

- Forskolin (an agent used to increase intracellular cAMP levels) enhances the contraction induced by $P2Y_{14}$ receptor agonists, consistent with Gi coupling of the $P2Y_{14}$ receptor

- UTP (agonist at non-Gi coupled $P2Y_2$ and $P2Y_4$ receptors) contractile responses are elevated in the presence of forskolin (to increase cAMP levels) and, thus, it may be acting through Gi coupled P2Y receptors

- PPTN, a selective $P2Y_{14}$ receptor antagonist antagonizes MRS2690 responses

- PPTN (1 μM) does not inhibit contractile responses to UTP (an agonist at $P2Y_2$ and $P2Y_4$ receptors), but antagonizes responses to UTP and UTPγS at 3 μM. PPTN (1 μM) does not antagonize responses to acetylcholine in PCA

Chapter 4: Further characterization of $P2Y_{14}$ agonist responses: effect of P2 receptor antagonists, suramin and PPADS and involvement of cAMP production

4.1 Introduction

4.1.1 P2 receptor antagonists

Suramin and PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate) are non-selective P2 receptor antagonists that have been widely used to study P2 receptor physiology and pharmacology because of the general lack of subtype-selective antagonists for P2 receptors. Although a selective $P2Y_{14}$ receptor antagonist exists (PPTN, see Chapter 3), understanding of the activity of suramin and PPADS at the $P2Y_{14}$ receptor is useful because it may offer additional pharmacological tools for receptor characterisation. To the best of my knowledge, there are no published reports on the effects of suramin and PPADS at $P2Y_{14}$ receptors. Suramin and PPADS also have the ability to inhibit ectonucleotidases, various intracellular enzymes and a number of ligand-gated ion channels (Friedle et al., 2010).

Suramin is an antiparasitic polysufonated drug (Kim et al., 2001) that has antagonistic properties towards the P2X receptor family at high concentrations (30-100 μM) and it blocks most of the P2Y receptors (von Kugelgen, 2006). Studies looking at the antagonist effects of suramin have been carried out using recombinant cells expressing human and rat P2 receptors (von Kugelgen, 2006). Suramin has an IC_{50} of 1 to 15 μM at the P2X1, P2X2 P2X3 and P2X5 receptors and an IC_{50} of 78 μM at the P2X7 receptor (Evans et al., 1995; Garcia-Guzman et al., 1997b; Rassendren et al., 1997). Suramin is reported to antagonize the human but not the rat P2X4 receptor (Buell et al., 1996; Garcia-Guzman et al., 1997a). Suramin blocks the heteromeric P2X2/3 and the

homomeric P2X3 receptor (Burgard et al., 2000; Spelta et al., 2002). Suramin also blocks members of the P2Y receptor family. At native human and rat UTP-sensitive $P2Y_2$-like receptors, suramin has an approximate IC_{50} and IC_{75} of 50 µM and 100 µM respectively (Charlton et al., 1996a; 1996b; Wildman et al., 2003), but at the porcine $P2Y_2$ receptors, its potency is increased with an IC_{50} of 10 µM (Shen et al., 2004). Suramin is a weak antagonist at the rat recombinant $P2Y_4$ receptor with an IC_{50} of 1 mM (Wildman et al., 2003). It also antagonizes human and mouse $P2Y_4$ receptors with IC_{50} of 100µM (Communi et al., 1996; Suarez-Huerta et al., 2001). Suramin antagonizes native/endogenous human and rat $P2Y_6$ receptors (Rayment et al., 2007a). Suramin blocks the $P2Y_{11}$ receptor having an IC_{50} of 1 µM (Communi et al., 1999). At the human recombinant $P2Y_{12}$ receptor and $P2Y_{13}$ receptor, suramin has an IC_{50} of 50 µM and approximately 2 µM, respectively (Zhang et al., 2001; Marteau et al., 2003).

PPADS blocks P2X1 receptors with an IC_{50} of approximately 0.1 µM (Kim et al., 2001). It is less potent at the other P2X receptors with an IC_{50} between 1 µM to 1.7 µM at P2X2, P2X3 and P2X5 receptors, and an IC_{50} of 51 µM at P2X7 receptors. PPADS does not affect P2X4 and P2X6 receptors (Evans et al., 1995; Garcia-Guzman et al., 1997b; Rassendren et al., 1997). Many of the P2Y receptors are inhibited by PPADS (von Kugelgen, 2006). PPADS inhibits the native $P2Y_1$ receptor (IC_{50} approximately 10 µM) (Kim et al., 2001). It also inhibits the other P2Y receptors which have been stably expressed in cells: $P2Y_2$ receptor (IC_{50} of 30 µM) (Charlton et al., 1996b; Charlton et al., 1996a); the $P2Y_4$ receptor (IC_{50} of 100 µM) (Communi et al., 1996); and the $P2Y_6$ receptor (IC_{50} of 100 µM) (Robaye et al., 1997). PPADS inhibits both the

$P2Y_{11}$ and $P2Y_{12}$ receptors with an IC_{50} of 100 µM (Communi et al., 1999) and the $P2Y_{13}$ receptor with an IC_{50} of 10 µM (Marteau et al., 2003).

There are relatively few selective antagonists at P2 receptors: NF449 at P2X1, MRS2179 at $P2Y_1$, MRS2578 at $P2Y_6$ and PPTN at $P2Y_{14}$ receptors (Burnstock et al., 2014; Ralevic, 2015). Characterisation of native P2 receptors is further complicated by actions of the endogenous agonists at multiple subtypes and by nucleotide interconversion and metabolism (see Introduction Section 1.1.5).

4.1.2 $P2Y_{14}$ receptor signalling

Vasodilator-stimulated phosphoprotein (VASP) was originally identified in human platelets as a substrate for cAMP- and also cyclic guanosine monophosphate (cGMP)-dependent kinases (Halbrugge et al., 1990). Both cAMP and cGMP production in smooth muscle is stimulated by an elevation in endothelial NO and prostacyclin which causes smooth muscle relaxation (Aszodi et al., 1999). cGMP/cAMP-dependent protein kinase phosphorylate human VASP on three common sites including Ser-157, Ser-239 and Thr-278 (Gertler et al., 1996). VASP associates with actin filaments and is localized along the dynamic filamentous membrane structure, focal adhesions and stress fibres (Reinhard et al., 1992; Aszodi et al., 1999). The proline-rich region of VASP proteins binds to profilin an actin-binding protein which is involved in the actin polymerization and microfilament assembly (Wentworth et al., 2006). (Meinecke et al., 1994).

VASP phosphorylation (VASP-P) has been used as a measure of changes in intracellular cAMP levels in a number of cells (Eigenthaler et al., 1992; Halbrugge et al., 1992; Reinhard et al., 1992; Butt et al., 1994). There are many procedures that can measure levels of intracellular cAMP, however these procedures can be lengthy and time-consuming. The measurement of VASP-P is suggested to be more stable over time and is a reliable and robust application to measure the levels of cyclic nucleotide levels (Geiger et al., 2010). Preliminary studies conducted utilized ELISA-based assays to measure changes in cAMP in porcine coronary arteries following UDP-glucose and MRS2690 treatment, however this assay resulted in inconsistencies between results following lengthy sample preparation (results not shown) Thus, the flow cytometric VASP-P assay was used in the current study which provides a quicker and more effective means of testing intracellular levels of cAMP in porcine coronary arteries in the current study. Figure 4.1 illustrates the mechanism of action of the VASP-P assay.

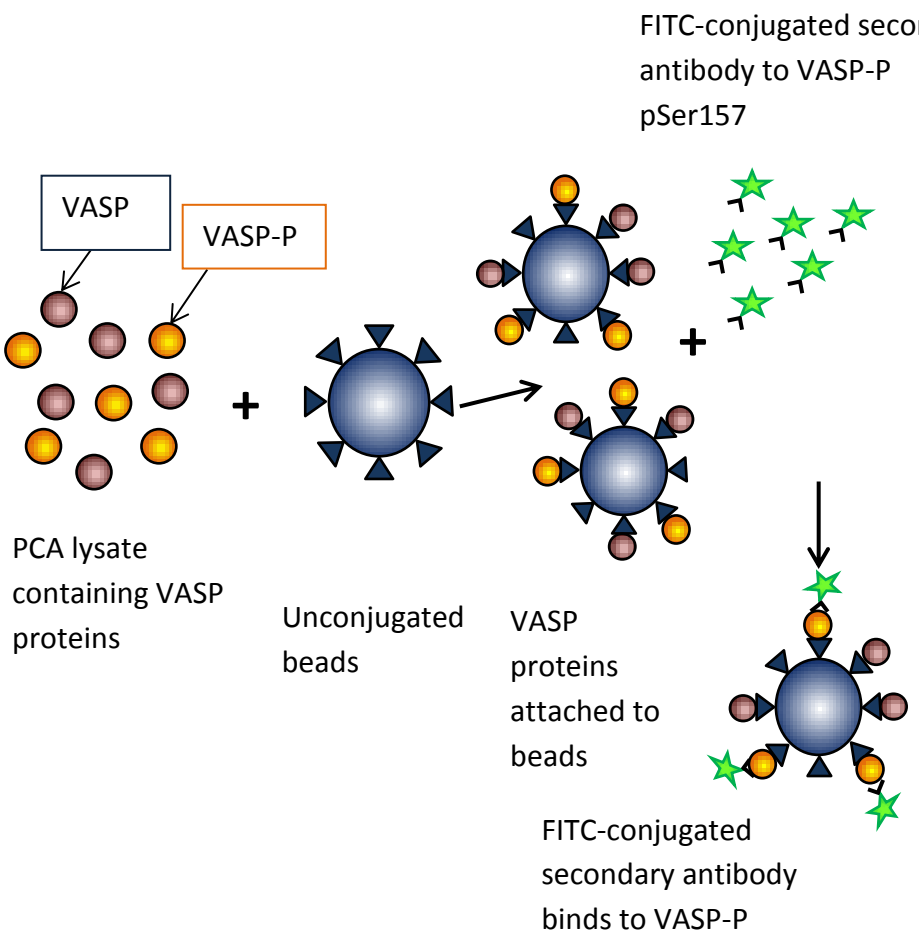


Figure 4.1: an illustration of the detection of vasodilator stimulated phosphoprotein (VASP) phosphorylated (VASP-P) proteins in porcine coronary arteries (PCA). PCA lysate (obtained from PCA segments stimulated with $P2Y_{14}$ receptor agonists; UDP-glucose and MRS2690) containing VASP proteins either unphosphorylated (red) or phosphorylated (yellow) are mixed with unconjugated beads that bind to VASP proteins. Conjugated beads-proteins are incubated with a fluorescence FITC-conjugated secondary antibody that binds to phosphorylated serine 157 residues. The mix is analysed by flow cytometry which quantitatively measures the levels of VASP-P in samples.

4.2 Aims

The first aim of the study was to evaluate the responses of non-selective P2 receptor antagonists, PPADS and suramin, on the responses to $P2Y_{14}$ receptor agonists UDP-glucose and MRS2690, a selective $P2Y_{14}$ receptor agonist, in porcine coronary arteries.

The possible involvement of $P2Y_6$ receptors, activated by UDP present as a contaminant or breakdown product of UDP-glucose or MRS2690, or present due to P2 receptor-mediated release from the smooth muscle cells, was also investigated using a selective $P2Y_6$ receptor antagonist, MRS2578.

The second aim of the study was to determine whether the $P2Y_{14}$ receptor couples to the inhibition of $G_{i/o}$ proteins. The objective was to determine the effects of UDP-glucose and MRS2690 on forskolin-induced VASP-P in porcine coronary arteries and to determine whether a selective $P2Y_{14}$ receptor antagonist, PPTN, inhibits UDP-glucose and MRS2690 induced inhibition of VASP-P.

4.3 Methods

4.3.1 Antagonist studies

See Chapter 2 Section 2.1.2 for full details.

Cumulative concentration-response curve to UDP-glucose and MRS2690 were constructed in the presence of the P2 receptor antagonists suramin and PPADS, and a $P2Y_6$ receptor antagonist, MRS2578. The studies were carried out in U46619 (5-10 nM) preconstricted coronary arteries and also in conditions of

forskolin plus U46619; (forskolin (0.1 μM) was added to reverse U46619-induced contraction and to elevate intracellular cAMP levels). The two conditions were carried out to be consistent with previous experiments (see Chapter 3) where these were found to optimize contractions to UDP-glucose and MRS2690.

4.3.2 Measurement of cAMP: see Chapter 2 Section 2.1.1 for full details.

PCA were set up in organ baths as described in Chapter 2 Section 2.1.0. Forskolin was added to reverse the U46619-induced contraction and to stimulate cAMP accumulation. Single concentrations of either UDP-glucose or MRS2690 were added to some vessels. After contractions reached a maximum (approximately 1-3 min), vessels were placed into lysis buffer in Eppendorf tubes which were immediately frozen in dry ice. VASP-P was measured using flow cytometry.

Statistical analysis was performed using GraphPad Prism 6.0 software. VASP-phosphorylation assay results were calculated using one-way ANOVA, followed by Sidak's multiple comparisons test. $P < 0.05$ was taken as statistically significant.

4.4 Results

4.4.1 Effect of suramin and PPADS on responses to MRS2690 and UDP-glucose in porcine coronary arteries

The effect of suramin and PPADS on responses to MRS2690 and UDP-glucose were investigated. This was carried out in either porcine coronary arteries that had been preconstricted with U46619 or in vessels that were exposed to forskolin plus U46619 (see Chapter 2 Sections 2.1.1.2 and 2.1.l.3).

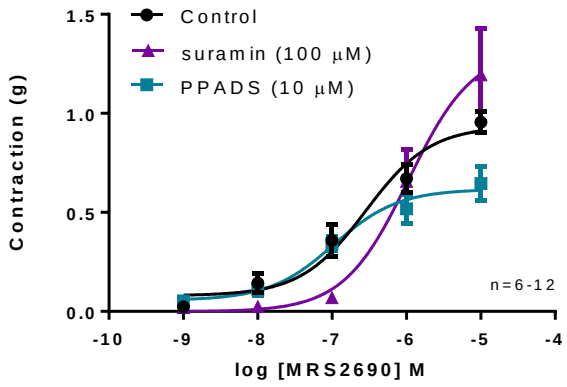


Figure 4.2: Effect of suramin (100 µM) and PPADS (10 µM) on responses to MRS2690, a selective $P2Y_{14}$ receptor agonist, in U46619-preconstricted porcine coronary arteries. Data presented are mean ± S.E.M

In the presence of suramin, contractions induced by MRS2690, a selective $P2Y_{14}$ receptor agonist, were not significantly different from those in the absence of suramin ($P > 0.05$, ANOVA) (Figure 4.2). Contractile responses to MRS2690 were also unaffected by PPADS ($P > 0.05$, ANOVA), (Figure 4.2). The EC_{50} values (-log M) for responses to MRS2690 in control vessels, in the

presence of suramin, or in the presence of PPADS are 6.55 ± 0.18 (n=6), 5.97 ± 0.24 (n=12) and 6.98 ± 0.21 (n=12), respectively.

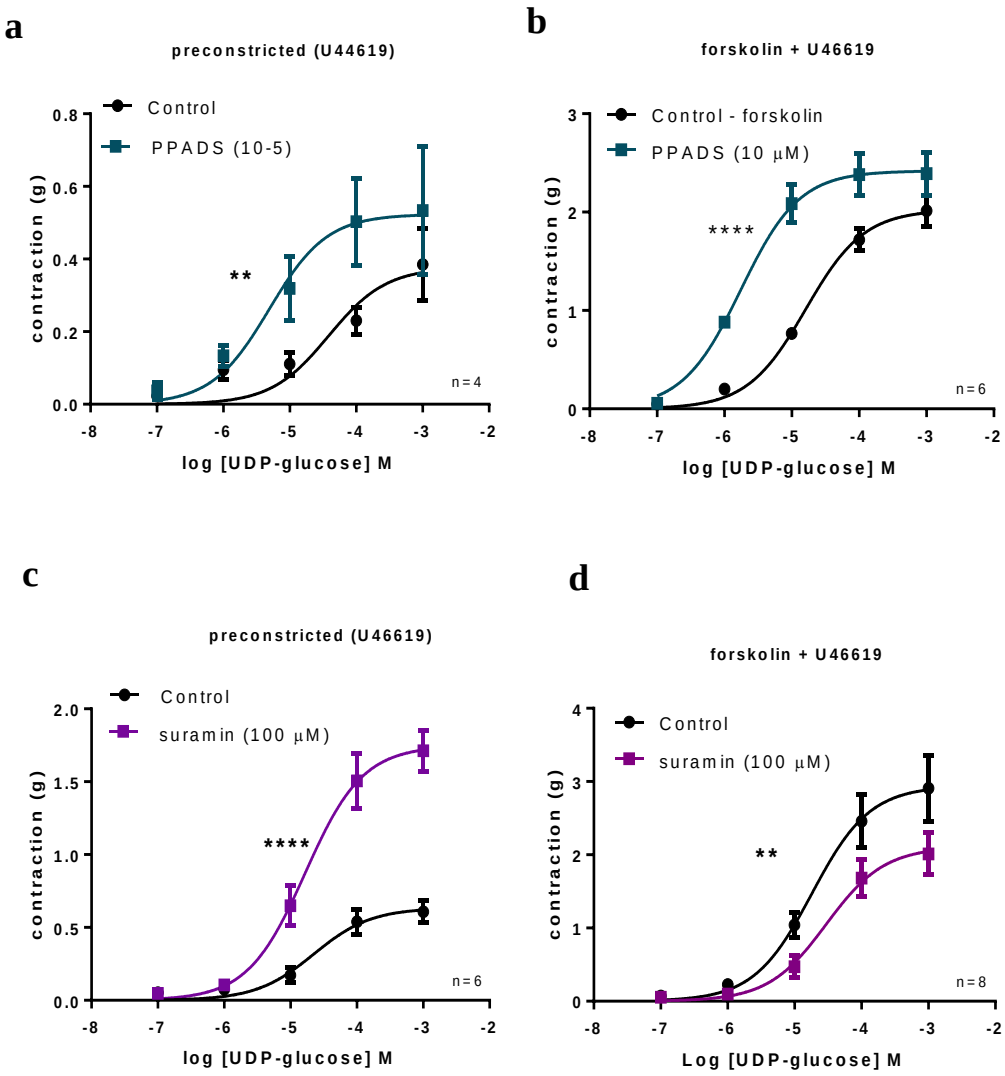


Figure 4.3: Concentration-response curves to UDP-glucose in the presence of PPADS (10 µM) (a, b) and suramin (100 µM) (c, d) in U46619 preconstricted porcine coronary arteries (a, c) and after exposure to forskolin plus U46619 (b, d). Data presented are mean ± S.E.M. **P<0.01; ****P<0.0001, two-way ANOVA.

Responses to UDP-glucose were significantly potentiated in the presence of PPADS (10 μM) in both preconstricted vessels and vessels exposed to forskolin plus U46619 ($P<0.05$, ANOVA) (Figure 4.3a and 4.3b). In both experimental conditions, there was no significant difference in the maximal contractions induced by UDP-glucose in the presence of PPADS. Responses to UDP-glucose were also significantly increased in the presence of suramin in preconstricted vessels ($P < 0.0001$, ANOVA) and unlike PPADS, the maximal contractions induced by UDP-glucose were significantly increased (Figure 3.3c). In conditions of forskolin plus U46619, responses to UDP-glucose were attenuated in the presence of suramin ($P <0.01$, ANOVA), (Figure 4.3d). Table 4.1 gives the EC_{50} values of UDP-glucose in the presence of PPADS and suramin in porcine coronary arteries.

UDP-glucose	**EC_{50} (-log M)**			
	control	**PPADS**	**control**	**suramin**
Preconstricted (U46619)	4.42 ± 0.31 n=4	5.31 ± 0.31 n=4	4.65 ± 0.19 n=6	4.78 ± 0.12 n=6
Forskolin + U46619	4.79 ± 0.08 n=6	5.76 ± 0.12 n=6	4.72 ± 0.18 n=8	4.52 ± 0.19 n=8

Table 4.1: EC_{50} (-log M) values of UDP-glucose in the presence of either PPADS (10 μM) or suramin (100 μM) in U46619-preconstricted arteries porcine coronary arteries or arteries exposed to forskolin plus U46619.

4.4.2 Effect of MRS2578, a $P2Y_6$ receptor antagonist, on responses to $P2Y_{14}$ receptor agonists in porcine coronary arteries

$P2Y_6$ receptors are present in the porcine coronary artery and, upon activation by their endogenous ligand UDP, elicit vasoconstriction (Rayment et al., 2007). The effect of a selective $P2Y_6$ receptor antagonist, MRS2578, was investigated to determine the involvement of $P2Y_6$ receptors in responses to UDP-glucose and MRS2690. The effect of MRS2578 on responses to $P2Y_{14}$ receptor agonists was examined in both U46619-preconstricted arteries (see Chapter 2, Section 2.1.1.2) and in conditions of forskolin plus U46619 (forskolin present to elevate cAMP levels and U46619 present to maintain vasomotor tone) (see Chapter 2, Section 2.1.1.3).

MRS2578 (10 μM) had no significant effect on contractile responses induced by UDP-glucose and MRS2690 in porcine coronary arteries preconstricted with U46619 (Figure 4.4a and 4.4c). In conditions of forskolin plus U46619, the response to UDP-glucose was reduced in the presence of MRS2578. At 1 mM UDP-glucose, the contractile induced response was significantly inhibited by MRS2578, from 2.45 ± 0.23 g in controls to 1.25 ± 0.32 g in the presence of MRS2578 suggesting that $P2Y_6$ receptor activation accounts for approximately 50% of the contractile response to UDP-glucose in porcine coronary arteries in conditions of forskolin plus U46619 ($P < 0.05$, ANOVA) (Figure 4.4b). In conditions of forskolin plus U46619, responses to MRS2690 were unaffected in the presence of MRS2578 ($P > 0.05$, ANOVA) (Figure 4.4d).

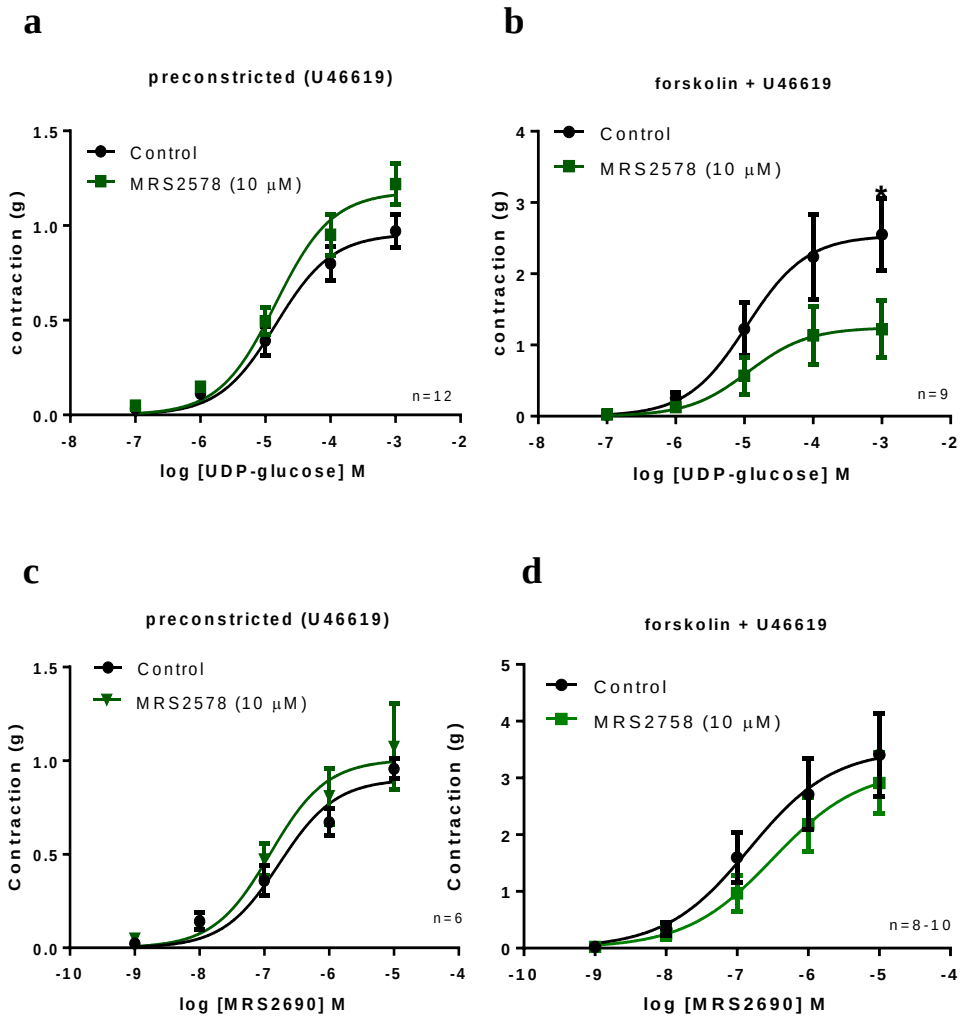


Figure 4.4: Effect of a $P2Y_6$ receptor antagonist, MRS2578, on concentration-response curves to UDP-glucose (a, b) and MRS2690 (c, d) in porcine coronary arteries in U46619 induced preconstricted vessels (a, c) and in the presence of forskolin plus U46619 (b, d). UDP-glucose and MRS260 caused concentration-dependent contractions in U46619-preconstricted porcine coronary arteries. These contractions were unaffected in the presence of MRS2578 ($P < 0.05$, ANOVA). MRS2768 antagonized responses to UDP-glucose (1 mM) in forskolin plus U46619 conditions (*$P<0.05$, ANOVA, followed by Sidak's multiple comparisons test). Data presented are mean ± S.E.M

4.4.3 $P2Y_{14}$ receptor agonists decrease VASP-phosphorylation in porcine coronary arteries

Increases in intracellular cAMP levels lead to the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) via cAMP-dependent protein kinase A (PKA) and other kinases (Wentworth et al., 2006). Thus VASP-phosphorylation (VASP-P) can be used as a tool to measure levels of cAMP. In the present study, forskolin, an activator of adenylate cyclase, was used to increase cAMP levels in porcine coronary arteries. Forskolin (0.1 μM) significantly increased VASP-P levels ($P<0.001$, ANOVA with Sidak's multiple comparisons test) (Figure 4.5). UDP-glucose (100 μM) and MRS2690 (10 μM) significantly decreased the forskolin-induced elevation of VASP-P, to levels that were similar to those measured in control tissues ($P<0.05$, ANOVA with Sidak's multiple comparisons test) (Figure 4.5a). PPTN (1 μM) was able to partially restore levels of VASP-P that were decreased by UDP-glucose and MRS2690 in porcine coronary arteries (Figure 4.5b). PPTN alone (1 μM) had no significant effect on VASP-P levels in forskolin exposed porcine coronary arteries ($P >0.05$, paired Student's *t*-test) (Figure 4.5c).

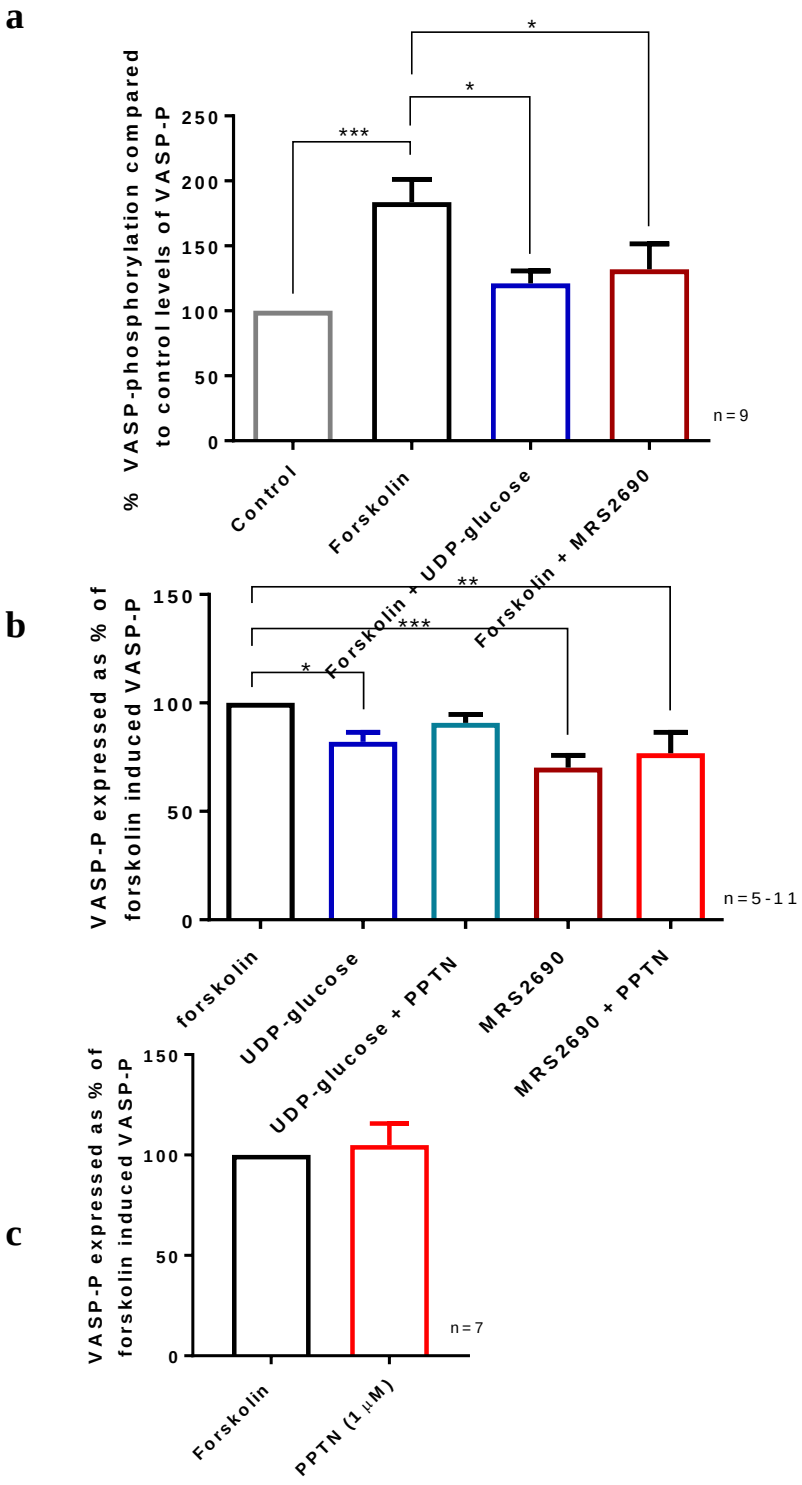


Figure 4.5: Levels of VASP phosphorylation (VASP-P) in porcine coronary arteries at basal tone, in the presence of forskolin and the effect of UDP-glucose, MRS2690 ($P2Y_{14}$ receptor agonist) and PPTN ($P2Y_{14}$ receptor antagonist). (a) Levels of VASP-P, in the presence of forskolin /+UDP-glucose (100 μM)/+MRS2690 (10 μM). (b) Effect of UDP-glucose (1 mM) and

MRS2690 (10 μM) on forskolin-induced VASP phosphorylation in porcine coronary arteries in the absence and presence of PPTN (1 μM). VASP-phosphorylation was measured using flow cytometry. (c) Effect of PPTN (1 μM) on forskolin-induced VASP phosphorylation in porcine coronary arteries. Results are presented as a percentage change of VASP-P compared to controls. Significant changes in comparison to forskolin (*P<0.05, **P<0.01; ***P<0.001, one-way ANOVA followed by Sidak's multiple comparisons test). Data presented are mean ± S.E.M

4.5 Discussion

Since there are no reports that have examined the effect of the non-selective P2 receptor antagonists, suramin and PPADS, on the $P2Y_{14}$ receptor, the present study examined the effects of these antagonists on responses to some of the $P2Y_{14}$ receptor agonists, namely UDP-glucose and MRS2690, in porcine coronary arteries. To determine the involvement of $P2Y_6$ receptors in the UDP-glucose and MRS2690 induced contractions, the effect of a selective $P2Y_6$ receptor antagonist, MRS2578, on contractile responses was also investigated. The involvement of cAMP in responses to $P2Y_{14}$ receptor agonists was also investigated, as an indirect means for investigation of G_{io} protein coupling.

4.5.1 Effects of suramin and PPADS on responses to $P2Y_{14}$ receptor agonists

In the presence of suramin, contractile responses to UDP-glucose were potentiated in preconstricted arteries, with a potentiation of the maximum response. One explanation may be that suramin is blocking the actions of UDP-glucose at vasorelaxant receptors in the porcine coronary artery. Since UDP-glucose has been reported to act weakly at the $P2Y_2$ receptor, with an EC_{50} of 10 μM (Ko et al., 2009), versus 0.24 μM at $P2Y_{14}$ receptors (Ko et al., 2009), the results suggest that suramin may have been inhibiting vasorelaxant $P2Y_2$ receptors, thus amplifying the actions of UDP-glucose at vasocontractile $P2Y_{14}$ receptors. In preliminary studies, I have shown that UTP induces a biphasic response consisting of a vasorelaxation (between 1-10μM) followed by a vasocontraction in porcine coronary arteries, suggesting the presence of vasorelaxant $P2Y_2$ and/or $P2Y_4$ receptors (data not shown). Since endothelium

removal of porcine coronary arteries does not change the UDP-glucose induced contractions (see Chapter 3), the location of these vasorelaxant receptors may be the smooth muscle rather than the endothelium. Direct smooth muscle, endothelium-independent, relaxations to ATP and UTP have been reported in human epicardial coronary arteries (Saetrum Opgaard and Edvinsson, 1997). The lack of effect of suramin on contractile responses to MRS2690 is consistent with this suggestion, since MRS2690 is a selective $P2Y_{14}$ receptor agonist without effect at other P2Y receptor subtypes (Ko et al., 2007). Alternatively, suramin may be acting as an ectonucleotidase inhibitor, preventing the breakdown of UDP-glucose thus increasing its contractile responses in the porcine coronary arteries. There is no information available about the stability of MRS2690.

In conditions of elevated cAMP (induced by forskolin in the presence of U46619), it was shown that suramin inhibited contractile responses to UDP-glucose, in contrast to what was seen in U46619-induced preconstricted vessels. The most likely explanation is that as contractile responses of the coronary arteries to UDP-glucose alone were much greater under these experimental conditions (R_{max} approximately 3 g in the presence of U46619 plus forskolin *versus* R_{max} approximately 0.6 g in the presence of U46619 alone), no further augmentation of the contractile machinery could take place. Under these conditions, the inhibitory effect of suramin which was observed could have involved antagonism of contractile $P2Y_2$ receptors expressed on porcine coronary arteries (Rayment et al., 2007).

UDP-glucose mediated contractions were also significantly potentiated in the presence of PPADS, both in coronary arteries that had been precontracted with

U46619 alone and in arteries in the presence of forskolin and U46619. Interestingly, in this case, the concentration response curves were leftward shifted, with a significant decrease in the-logEC_{50} and no change in the maximum response. It is possible that, as suggested for suramin earlier, PPADS acts to inhibit vasorelaxant $P2Y_2$ receptors activated by UDP-glucose, to remove their opposing effects on UDP-glucose contractile actions at $P2Y_{14}$ receptors. The lack of effect of PPADS on contractile response to MRS2690 is again consistent with this suggestion, because MRS2690 is selective for $P2Y_{14}$ receptors and, thus, would not be expected to have any actions at vasorelaxant $P2Y_2$ receptors. Alternatively, being a weak ectonucleotidase inhibitor (Windscheif et al., 1995), PPADS may have prevented the breakdown of UDP-glucose causing potentiation of the vasocontractile responses. Both suramin and PPADS act to inhibit ecto-nucleotide triphosphate diphosphohydrolases (eNTPDases) and ecto-nucleotide pyrophosphatases (Chen et al., 1996; Vollmayer et al., 2003), the latter of which breaks down UDP-glucose (Lazarowski et al., 2003b). ARL67156, which is an inhibitor of these enzymes (Levesque et al., 2007), could be used for potential future studies; the effect of ARL67156 on responses to UDP-glucose could be investigated. An increase in UDP-glucose induced contractions in the porcine coronary artery in the presence of ARL67156 would suggest that UDP-glucose breakdown occurs, hence, resulting in increased responses to UDP-glucose in the presence of PPADS and suramin.

In summary, both suramin and PPADS altered the contractile responses to UDP-glucose, but not to a selective $P2Y_{14}$ receptor agonist MRS2690. There was a fundamental difference in the shape change of the UDP-glucose

concentration response curve produced by suramin *versus* PPADS: PPADS produced a leftward shift in the concentration response curve to UDP-glucose (in both preconstricted arteries and conditions of elevated cAMP) without a change in the maximum response; in contrast, suramin altered the maximum response to UDP-glucose. Both of the above are consistent with the known actions of PPADS and suramin as competitive and non-competitive antagonists, respectively (Lambrecht et al., 2002; Rayment et al., 2007a). These data suggest that potentiation of UDP-glucose contractile responses by suramin and PPADS occurs through antagonism of its actions at opposing vasorelaxant $P2Y_2$ receptors, rather than through inhibition of ectonucleotidase activity.

The effects of suramin and PPADS on the contractile responses to UDP-glucose has provided an insight on the effects of these antagonists at the $P2Y_{14}$ receptor, however, it has been difficult to determine the mechanism of action. Thus, suramin and PPADS have not proved to be reliable antagonists for the characterisation of the $P2Y_{14}$ receptor.

4.5.2 Effect of $P2Y_6$ receptor antagonist on UDP-glucose and MRS2690

UDP is an agonist of the $P2Y_6$ receptor (Harden et al., 2010). Although initially reported to be an antagonist at the human (but not rat) $P2Y_{14}$ receptor (Fricks et al., 2008), UDP was subsequently shown to be a potent agonist of the $P2Y_{14}$ receptor (Carter et al., 2009). Radioligand binding assays have revealed that UDP has pEC_{50} values of 6.80 and 6.52 at the human $P2Y_{14}$ and $P2Y_6$ receptors, respectively (Harden et al., 2010; Jacobson et al., 2012). It is known that porcine coronary arteries express UDP-sensitive vasocontractile $P2Y_6$-like

receptors on the smooth muscle (Rayment et al., 2007a). There are no reports that show the effects of UDP-glucose at $P2Y_6$ receptors. Hence, the present study aimed to test whether $P2Y_6$ receptors are involved in UDP-glucose induced contractions in the porcine coronary artery. In the presence of MRS2578, a selective $P2Y_6$ receptor antagonist (Mamedova et al., 2004), contractile responses to UDP-glucose were unaffected in preconstricted coronary arteries suggesting UDP-glucose does not interact with $P2Y_6$ receptors in porcine coronary arteries. Responses to the selective $P2Y_{14}$ receptor agonist, MRS2690, were also unaffected by MRS2578. However, in conditions which arteries were exposed to forskolin plus U46619, responses to UDP-glucose were attenuated in the presence of MRS2578 suggesting an involvement of $P2Y_6$ receptors. Since there are no reports to suggest that UDP-glucose acts at $P2Y_6$ receptors, the results suggest that perhaps breakdown of UDP-glucose to UDP (or potential UDP contamination within the UDP-glucose stock), or possibly UDP-glucose mediated release of UDP from vascular smooth muscle, resulted in activation of $P2Y_6$ receptors. It is unclear why this was observed only in the presence of forskolin and not during U46619 contraction alone.

4.5.3 The $P2Y_{14}$ receptor is coupled to the inhibition of cAMP and VASP-phosphorylation

In the current study, VASP-P was measured as an indicator of cAMP accumulation. Forskolin significantly enhanced VASP-P in the porcine coronary artery, while UDP-glucose and MRS2690 inhibited the forskolin-induced increase in VASP-P. Moreover PPTN, was able to partially reverse the

UDP-glucose and MRS2690 mediated inhibition of forskolin-induced VASP-P. PPTN was shown to antagonise UDP-glucose-mediated inhibition of adenylyl cyclase in C6 glioma cells expressing $P2Y_{14}$ receptors (Barrett et al., 2013). UDP-glucose also inhibits forskolin-stimulated cAMP accumulation in T-lymphocytes, neutrophils and human astrocytoma cells (Scrivens and Dickenson, 2005a,b, 2006). Overall, the data suggest that, as in other tissues, the $P2Y_{14}$ receptor in the porcine coronary artery is $G_{i/o}$ protein-coupled and thus its activation inhibits adenylyl cyclase activity, hence, reducing levels of cAMP and VASP-P in porcine coronary arteries *in vitro*. Ideally, pertussis toxin would have been used to uncouple G_{io} proteins, but it is notoriously difficult to use this successfully other than in isolated cells, and previous attempts in our lab using porcine arteries were unsuccessful.

In the VASP-P assay, forskolin was used to elevate cAMP levels since my hypothesis was that the $P2Y_{14}$ receptor agonists would decrease cAMP levels (and thus I needed an elevated background of cAMP against which to test this). A similar approach is widely used by others in cell-based assays looking for evidence of agonist-induced inhibition of adenylyl cyclase (Scrivens et al., 2006). This approach was extended to the isolated artery segments studies, again using forskolin to raise cAMP levels, but adding U46619, a thromboxane A_2 agonist, to maintain vascular tone (since the increase in cAMP produced by forskolin would lead to vasodilatation and thus functional antagonism of vasocontractile responses). In the presence of forskolin plus U46619, contractile responses to the $P2Y_{14}$ receptor agonists were augmented (as discussed in Chapter 3). Under *in vivo* conditions, basal levels of cAMP in the cardiovascular system may normally be low, but the level may be increased

during pathophysiological conditions; for example, during hypoxia there is evidence that adenosine (Berne, 1980; Mubagwa et al., 1996) and calcitonin gene-related peptide (Hasbak et al., 2005) are released to cause coronary vasodilatation through mechanisms involving an increase in cAMP. If there is concomitant release of UDP-glucose during hypoxia, it may attenuate the hypoxic relaxation through actions at contractile $P2Y_{14}$ receptors. The use of an antagonist of $P2Y_{14}$ receptors may, therefore, be beneficial in promoting vasodilatation and protecting the heart from damage due to ischaemia or hypoxia.

Summary

- PPADS augments UDP-glucose-induced contractions, with a leftward shift in the concentration-response curves (in both U46619-preconstricted porcine coronary arteries and in the presence of forskolin plus U46619)
- Suramin augments UDP-glucose induced contractions in U46619-preconstricted vessels, significantly increasing the maximum response
- Contractile responses to MRS2690, a selective $P2Y_{14}$ receptor agonist, are unaffected by PPADS and suramin (in U46619-preconstricted vessels)
- A $P2Y_6$ receptor antagonist, MRS2578, has no effect on UDP-glucose- and MRS2690-induced contractions in U46619-preconstricted coronary arteries
- UDP-glucose-induced contractions, but not MRS2690 induced contractions, are partially mediated through $P2Y_6$ receptors in arteries in the presence of forskolin plus U46619
- UDP-glucose and MRS2690 inhibit the forskolin-induced elevation in VASP-phosphorylation in porcine coronary arteries
- PPTN, a $P2Y_{14}$ receptor antagonist, partially restores UDP-glucose- and MRS2690-induced inhibition of forskolin-induced increase in VASP-phosphorylation

Chapter 5: Pathophysiological roles of the $P2Y_{14}$ receptor in porcine coronary arteries: investigating potential sources of UDP-glucose release from blood cells and the coronary vasculature

5.1 Introduction

Cell damage results in the release of UDP-glucose which have been measured ranging from 10 nM to 1 mM (Lazarowski et al., 2003b). Constitutive release of UDP-glucose, accompanying release of ATP, occurs from many cell types including primary airway epithelial cells, 1321N1 human astrocytoma, COS-7, CHO-K1 and C6 glioma cells (Lazarowski et al., 2003b; Kreda et al., 2008). Table 1.1 in Chapter 1 outlines in more detail the levels of UDP-glucose that have been detected and released from various cell types. UDP-glucose levels have also been quantified in platelets (Wandall et al., 2012) and red blood cells (Palmieri et al., 1991; Gibson et al., 1995).

Houston et al. (1985) have shown that aggregating human platelets release adenine nucleotides, ATP and ADP which induce canine coronary artery relaxation (Houston et al., 1985). Red blood cells (RBCs) release ATP in response to hypoxia and hypercapnia (Bergfeld et al., 1992), as well as in conditions of acidic pH, stress and mechanical deformation (Sprague et al., 1996; Sprague et al., 1998). The release of these adenine nucleotides induces vasodilation in several tissues (Burnstock et al., 1986; Burnstock, 1987). Similarly, ectonucleotidases present on smooth muscle cells, endothelial cells and leukocytes break down ATP to adenosine which activates several physiological responses including vasodilatation, release of endothelial factors and anti-inflammatory effects (Cusack et al., 1983; Ohman et al., 2012b).

UDP-sugars, including UDP-glucose, have been detected in platelets and UDP-glucose is present at 1213 pmol/mg (Wandall et al., 2012). The intracellular level of UDP-glucose in RBCs is approximately 8-10 µM/100 g haemoglobin

(Palmieri et al., 1991) and in white blood cells is approximately 32 µM/100g protein (Palmieri et al., 1993). The findings from these studies led to the hypothesis that, since adenosine nucleotides are released from blood cells and have an effect on vascular contractility, UDP-glucose release from platelets and RBCs could also occur and contribute to vascular tone regulation. Thus, the effect of human isolated platelets and RBCs on porcine coronary artery contractility in the presence of PPTN, a selective $P2Y_{14}$ receptor antagonist was investigated.

Purine and pyrimidines nucleotides can be released from endothelial cells (Erlinge et al., 2008). The release of ATP during shear stress and hypoxia from endothelial cells is well documented (Bodin et al., 1991; Bodin et al., 1995). ATP can also be released from contracting smooth muscle (Matsuo et al., 1997) and during mechanical stress (Takahara et al., 2014). Thus, the aims of the study were to investigate whether porcine coronary arteries are potential sources of UDP-glucose release under hypoxic conditions and contribute to the hypoxic response. The effect of hypoxia was investigated in the presence of PPTN.

5.2 Methods

See Chapter 2, Section 2.1.6 for full details.

Red blood cells (7-13µl/ml) and platelets (10-25µl/ml) isolated from healthy donors (see Appendix, Table A) were added to U46619 preconstricted porcine coronary arteries in the presence and absence of PPTN (1 µM), in the absence and presence of L-NAME (100 µM). L-NAME was added to inhibit NO synthase, hence inhibiting platelet induced vasorelaxation; the platelet-induced

contractile response could then be investigated. Platelets were also added to endothelium denuded coronary arteries in the presence and absence of PPTN (1 µM), DMSO was added to control tissues at similar times to when PPTN was added. Controls were not needed since addition of 10-30µl/ml of 0.9% sodium chloride in 118mM of Krebs-Henseleit buffer would not have a significant effect.

Results were analysed using unpaired Student's *t*-test, GraphPad Prism 6.0. P <0.05 was taken as statistically significant.

5.3 Results

5.3.1 Effect of isolated blood fractions on porcine coronary arteries

To examine whether UDP-glucose release from RBCs and platelets has any effect on porcine coronary artery contractility, RBCs and platelet responses, in the absence and presence of a $P2Y_{14}$ receptor antagonist, PPTN (1 µM) were investigated. Isolated platelets (24,700-30,000 cells/µl) were added to vessels that had been preconstricted with U46619.

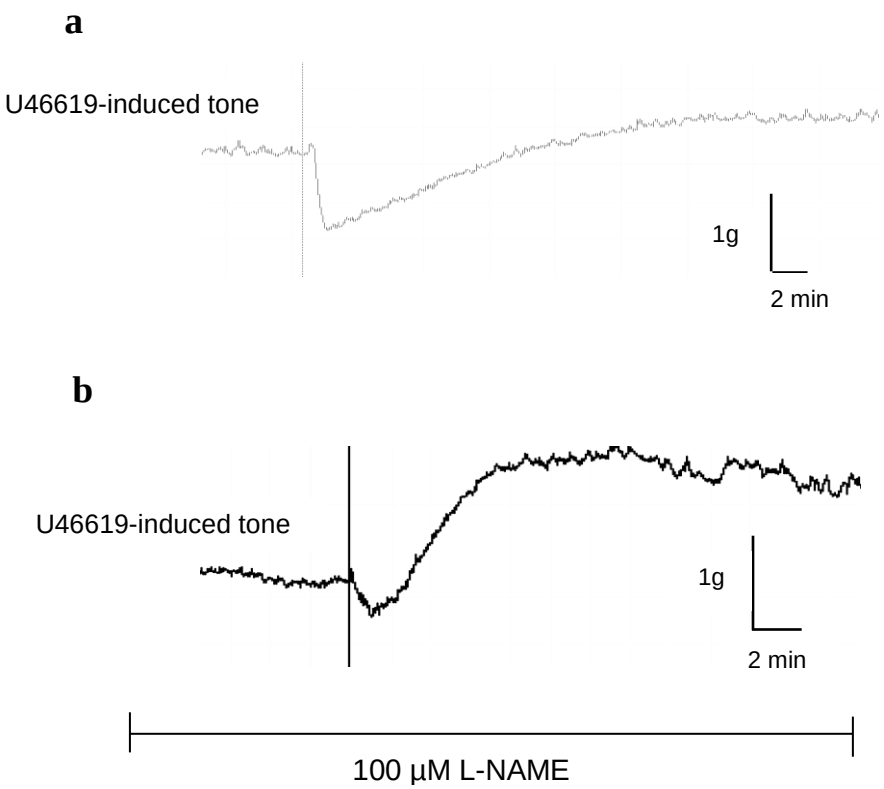


Figure 5.1: Representative traces of contractile responses to human isolated platelet addition in U46619-preconstricted porcine coronary arteries in a) the absence of L-NAME and b) presence of L-NAME (100 µM). Dashed lines indicate addition of platelets. Scale bar and time indicated on bottom right of images.

Platelets that were added to coronary arteries in the absence of L-NAME caused an instant relaxation followed by a slow and prolonged contraction which did not exceed the U46619 induced tone (Figure 5.1a). In the presence of L-NAME, platelets caused a minor relaxation followed by a contraction that exceeded the U46619 induced tone (Figure 5.1b). The effect of platelets on preconstricted coronary arteries in the presence of a $P2Y_{14}$ receptor antagonist, PPTN, was then investigated in the absence and presence of L-NAME.

PPTN (1 μM) did not significantly change the relaxation response induced by platelets isolated from donor 1 in porcine coronary arteries in the absence of L-NAME ($P > 0.05$, unpaired t-test) (Figure 5.2). However, platelets isolated from donors 2 and 3 induced relaxations that were significantly attenuated in the presence of PPTN ($P < 0.01$, unpaired Student's *t*-test, comparing overall platelet responses in control vessels *versus* PPTN treated vessels). The volumes of platelets added to vessels were variable between donors; however approximate volumes ranged from 10-20μl/ml. See Table A, Appendix for details of ages and genders of volunteers.

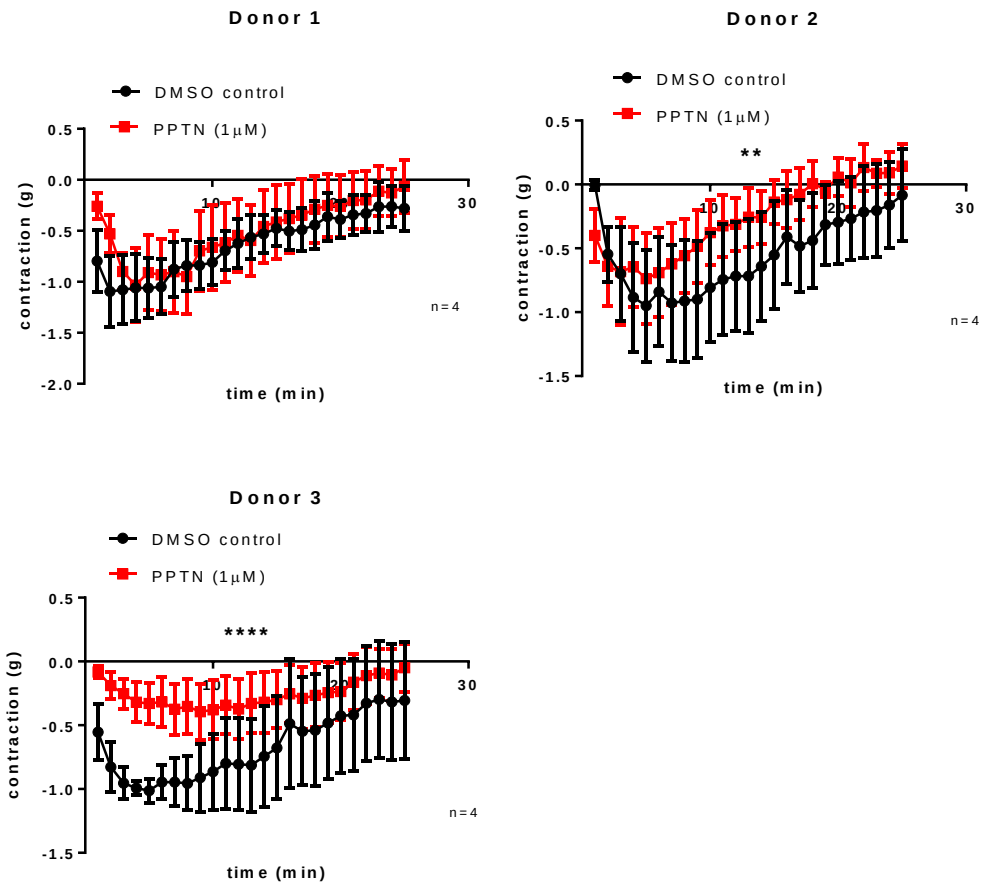


Figure 5.2: Effect of human isolated platelets in U46619-preconstricted porcine coronary arteries in the presence of PPTN (1 µM). **P<0.01, ****P<0.0001. Unpaired Student's *t*-test. Data are means ± standard error mean (S.E.M). n=number of different porcine vessels

In the presence of L-NAME platelets caused minor relaxations followed by a predominant contractile response (Figure 5.1b). In the presence of L-NAME, PPTN augmented the contractile response induced by platelets isolated from donor 4 (P <0.0001, unpaired t-test) compared to vehicle control vessels (Figure 5.3). However PPTN did not affect the contractile response induced by platelets isolated from donors 5, 6 and 7 compared to control vessels (Figure 5.3) (P >0.05, unpaired t-test).

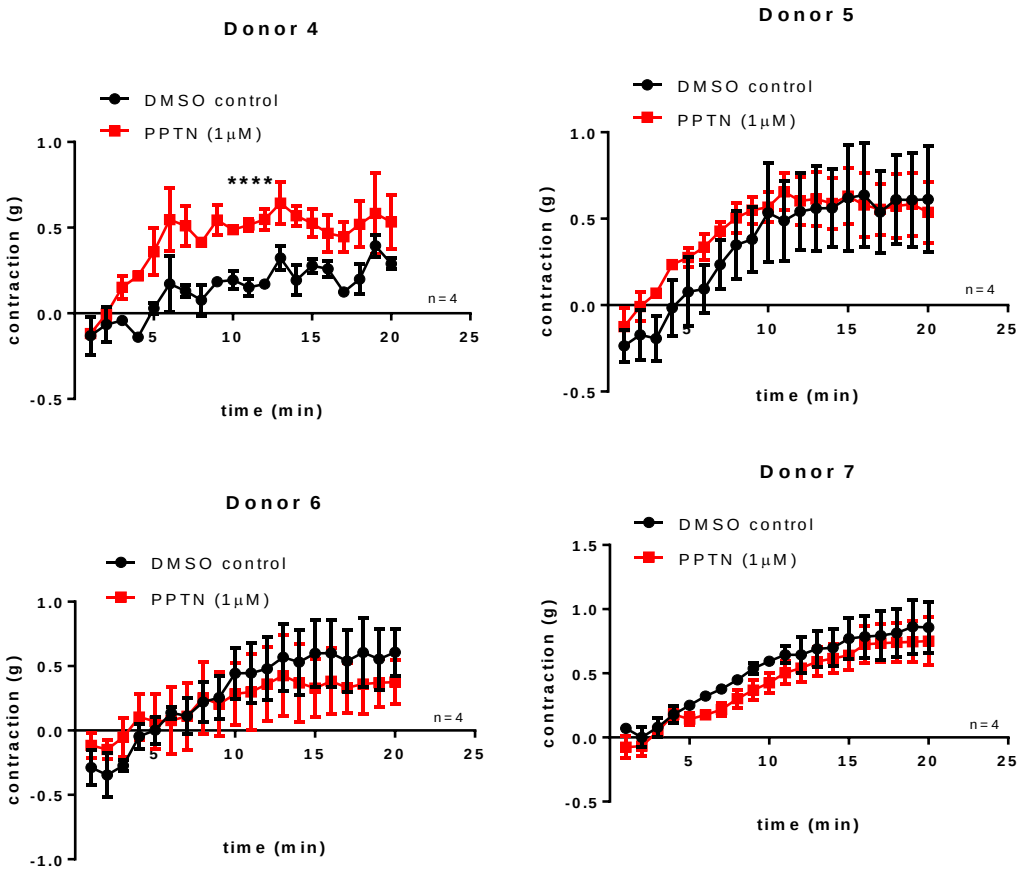


Figure 5.3: Effect of human isolated platelets in preconstricted porcine coronary arteries in the presence of PPTN (1 µM) and L-NAME (100 µM). Data are means ± S.E.M, ****P<0.0001, ANOVA. n=number of different porcine vessels

The endothelium was removed from some vessel segments as an alternative approach to using L-NAME to attenuate the endothelium-dependent relaxation to the platelets. The results from my previous studies showed that contractile $P2Y_{14}$ receptors are present on smooth muscle cells of porcine coronary arteries, since endothelium removal did not affect the UDP-glucose contractions (see Chapter 3, Figure 3.13). The endothelium removal would therefore be expected to expose $P2Y_{14}$ receptors and to mimic endothelium damage. The effect of platelet responses in these conditions was investigated in the absence and presence of PPTN.

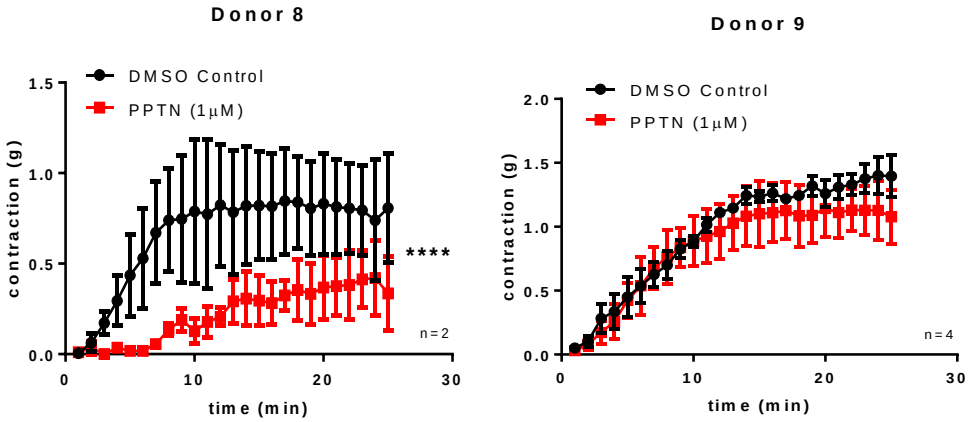


Figure 5.4: Effect of isolated human platelets in U46619-preconstricted endothelium denuded porcine coronary arteries in the presence of PPTN (1 µM) and L-NAME (100 µM). Data are means ± S.E.M, ****P<0.0001, ANOVA. n=number of different porcine vessels

PPTN significantly antagonized the contractile response to platelets isolated from donor 8 (P <0.0001, unpaired t-test) but not from donor 9 (P >0.05, unpaired t-test) (Figure 5.4). Further similar studies were performed, however in all experiments (additional 3 different donors), the platelets failed to produce a response.

5.3.2 Responses to isolated red blood cells in coronary arteries in the presence of PPTN

Isolated RBCs were added to U46619 induced preconstricted porcine coronary arteries to a final bath volume of 0.4% haematocrit as carried out by Diesen et al. (2008). RBCs caused a contraction in porcine coronary arteries. PPTN significantly inhibited the contractile response to RBCs from donor 10 (P <0.01, unpaired t-test), however it augmented the contractile response to RBCs from donors 11 (P <0.01 unpaired t-test) and 12 (P <0.05, unpaired t-test).

Responses to RBCs from donors 12 and 14 were unaffected in the presence of PPTN ($P > 0.05$, unpaired t-test) (Figure 5.5). Overall, there was no significant effect of PPTN on the responses to RBCs from all donors. See Table A, Appendix for details of ages and genders of volunteers.

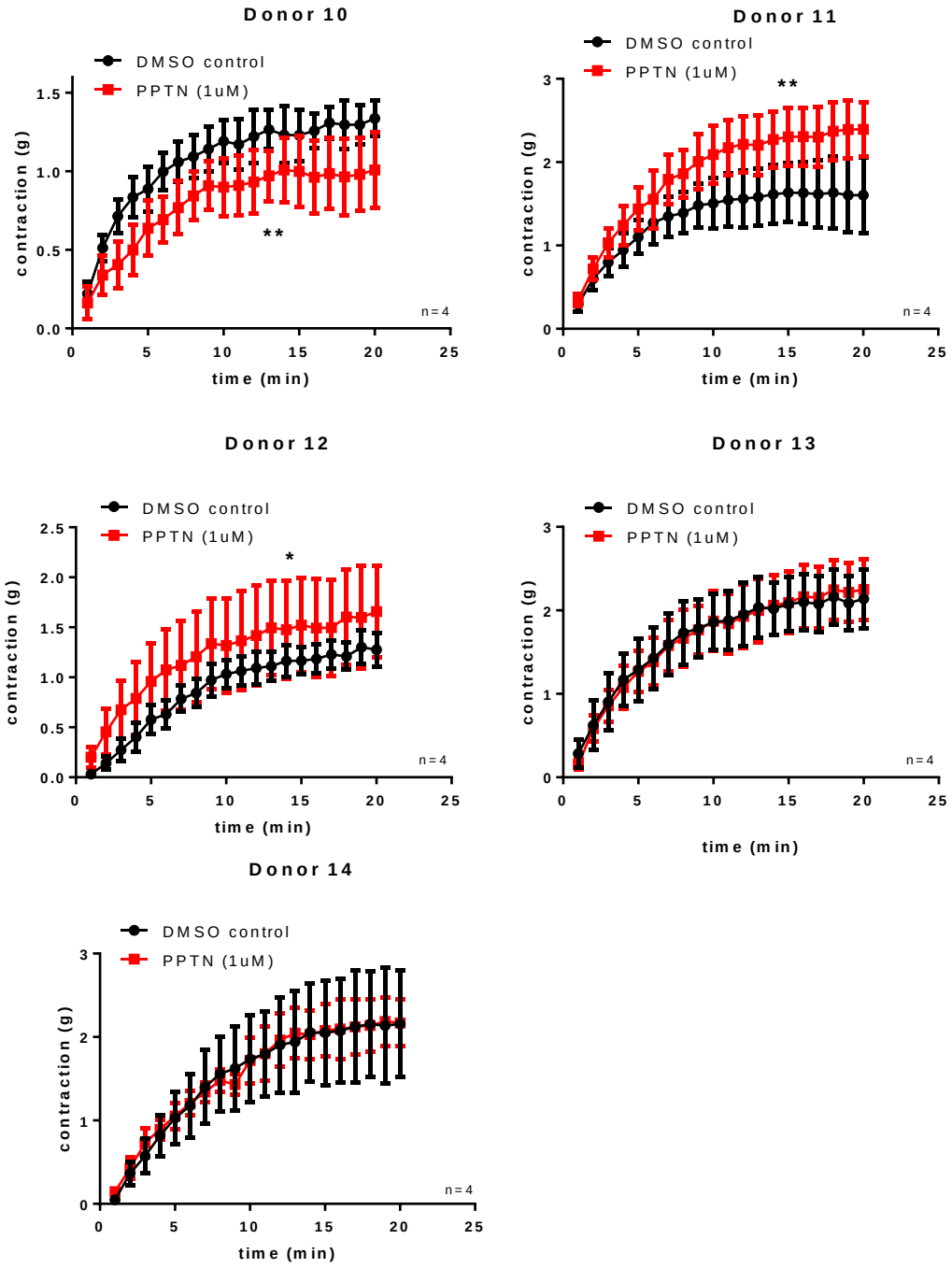


Figure 5.5: Effect of human isolated red blood cells in U46619-preconstricted porcine coronary arteries in the presence of PPTN (1 µM). *P<0.05, **P<0.01, Unpaired t-test. Data are means ± S.E.M

5.3.3 Investigating coronary arteries as a source of UDP-glucose release during hypoxia

It was determined whether UDP-glucose could potentially be released from porcine coronary arteries in conditions of hypoxia, stimulated by introducing 95% nitrogen, 5% CO_2 (see Methods Chapter 2, Section 2.1.5 for full details). Porcine coronary arteries were preconstricted with U46619 to induce a stable level of tone before hypoxia was introduced and the response was measured over 25 min in the absence and presence of PPTN (1 µM).

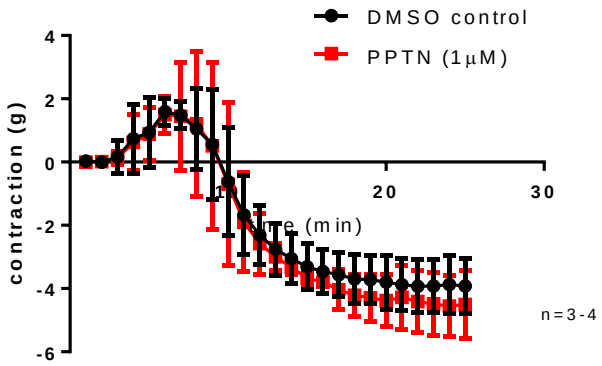


Figure 5.6: Effect of hypoxia on U46619-preconstricted porcine coronary arteries in the presence and absence of PPTN (1 µM). Data are means ± S.E.M

Porcine coronary arteries contracted within minutes after hypoxia was introduced, contractions peaked after approximately 10 minutes before a relaxation occurred that went below the U46619-induced tone. There was no change in this hypoxic response in the presence of PPTN (1 µM) compared to control vessels ($P > 0.05$, unpaired t-test), (Figure 5.6).

5.4 Discussion

The purpose of the current study was to determine whether UDP-glucose can be released from RBCs and platelets and whether these levels are sufficient to activate the $P2Y_{14}$ receptor in the coronary vasculature to produce a vasocontractile response. This was assessed using a pharmacological approach and a selective $P2Y_{14}$ receptor antagonist PPTN. The possible release of UDP-glucose from the porcine hypoxic coronary vasculature *in vitro* was also indirectly investigated using PPTN.

5.4.1 Investigation of red blood cells as possible sources of UDP-glucose release

The current findings showed that addition of isolated RBCs caused a contraction in coronary arteries. These findings are similar with a previous report which has shown RBCs-induced contractions in rabbit and mouse thoracic aortic segments (Diesen et al., 2008). The contraction induced by RBCs is likely to occur mainly through release of haemoglobin which inhibits endothelium-dependent vasorelaxation, through inhibition of EDRF/NO (Houston et al., 1990). In the current study, it can be seen that RBC-mediated contraction in porcine coronary arteries is unaffected in the presence of the PPTN suggesting that RBC-mediated contraction does not involve $P2Y_{14}$ receptors. Alternatively, UDP-glucose release from RBCs (and contraction of porcine coronary arteries) may have been very small, resulting in the absence of a visible effect of PPTN on red blood cell-induced contractions.

Previous studies have shown that RBCs act as oxygen sensors and release ATP under hypoxic conditions, low partial pressure (Bergfeld et al., 1992; Ellsworth

et al., 1995), mechanical deformation (Sprague et al., 1996; Sprague et al., 1998), high levels of potassium and hypotonic stress (Locovei et al., 2006). ATP release from RBCs is dependent on the extent and duration of stress upon them, and also the deformation of these cells is a necessary factor contributing to ATP release (Wan et al., 2008). ATP induces endothelium-dependent vasorelaxation in many vascular tissues and species (Burnstock, 1987) and RBCs induce vasorelaxation through release of ATP during stress and hypoxic conditions (Dietrich et al., 2000). The findings from these studies are different to what has been shown in the current study, since RBCs were not added in conditions of hypoxia and stress. As UDP-glucose is released simultaneously with ATP (Lazarowski et al., 2003b) and is present in RBCs (Palmieri et al., 1991), it is important to determine whether these stimuli are occurring (upon addition into organ baths) in order for these nucleotides to be released. These results suggest that PPTN does not have any effect on RBCs mediated contraction (through release of nucleotides) as insufficient levels of UDP-glucose are being released.

5.4.2 Investigation of platelets as possible sources of UDP-glucose release

In canine coronary arteries, aggregating platelets cause an endothelium-dependent relaxation which is largely due to the actions of ADP and ATP released from the platelets (Houston et al., 1986). This relaxation is reduced in vessels treated with apyrase, which hydrolyzes ATP and ADP (Houston et al., 1985), suggesting that nucleotides can be released from platelets which have an effect on the contractility of blood vessels. These findings led to the hypothesis that uracil nucleotides, including UDP-glucose may also be released from

platelets, in conjunction with ATP, and have an effect on porcine coronary artery contractility. The findings show that in endothelium intact porcine coronary artery, platelets caused a relaxation which was not seen in vessels treated with L-NAME (an inhibitor of nitric oxide). Previous studies by Houston et al. (1986) and Shimokawa et al. (1991) have shown that in endothelium denuded coronary arteries, platelets elicit vasoconstrictions that does not occur in endothelium intact coronary arteries (Houston et al., 1986; Shimokawa et al., 1991). A review of the literature has shown that this is the first study that has used L-NAME to block platelet-induced relaxations in vascular tissue. In the absence of L-NAME, the relaxations seen in the current experiment were biphasic responses, in which there was a rapid relaxation after addition of platelets, followed by a slower contraction that restored the U46619 induced tone. There were no significant changes to the platelet induced relaxations in vessels that had been treated with PPTN compared to control vessels. However, in 2 out of the 3 donors, PPTN reduced the relaxation induced by the platelets. This suggests that PPTN is perhaps blocking the actions of a vasorelaxant released from platelets. PPTN does not block any of the P2Y receptors, other than $P2Y_{14}$, at concentrations of up to 1 μM (Barrett et al., 2013) and, therefore, could not be blocking endothelial $P2Y_1$ receptors that are activated by ADP and ATP and cause vasodilatation in many vascular beds (Ralevic et al., 1998). It is possible that under these experimental conditions, a vasorelaxant effect of UDP-glucose at endothelial $P2Y_{14}$ receptors (shown by immunohistochemistry in Chapter 3) has been uncovered (although endothelium removal had no effect on UDP-glucose contractions under forskolin plus U46619 conditions of basal tone).

In endothelium-intact vessels, in the presence of L-NAME, the platelet induced relaxation was almost abolished and a more profound contraction was seen that exceeded the U46619 induced tone. In the presence of PPTN, there was no change in the responses induced by platelets. There was a rightward shift in the UDP-glucose induced contraction in the presence of PPTN in preconstricted vessels (see appendices Figure A.1), however the change was not significant. The results from the current study also show a change in the responses to platelet induced-relaxations in the presence of PPTN in some donors. The results suggest that PPTN may be non-selectively antagonizing the effects of a vasodilator component released from platelets, however further investigation is required to identify which component could elicit this effect.

5.4.3 Effect of PPTN on platelets in endothelium denuded coronary arteries

Endothelium removal was used as an alternative to L-NAME and to remove the platelet induced endothelium-dependent relaxation. However, endothelium removal has effects on platelets which will be discussed. Endothelial cells release NO and PGI_2 which prevent platelet activation. Consequent damage to the endothelial layer exposes the subendothelial matrix; platelets interact with collagen and von Willebrand Factor initiating signalling events and subsequent platelet activation (Golebiewska et al., 2015). Platelet activation induces release of several platelet derived components including thrombin, thromboxane A_2 and adenine nucleotides (Kunapuli, 2002), which aid in the coagulation cascade at the damaged site of vascular blood vessels, eventually forming a haemostatic plug (Dangelmaier et al., 2001). Damage to endothelial cells may also expose smooth muscle purinoceptors, consequently causing P2Y

activated-induced vasospasm (Malmsjo et al., 2000b). Hence, these studies of the effect of platelets on endothelium denuded coronary arteries mimic a local cellular damage and determine the effect of PPTN, and hence possible involvement of endogenous UDP-glucose, in these responses. The responses were variable, where PPTN blocked contraction to platelets from donor 1; however had no effect on contractile responses to platelets from donor 2. In subsequent experiments, to increase the number of experiments, the responses induced by platelets were very variable; the biphasic responses did not occur and there was no change in the U46619 tone after addition of platelets (three experiments – results not shown). Therefore, it is difficult to determine the effect of PPTN on platelet responses in these conditions.

The effects of UDP-glucose may be small in comparison to those of the other components released from platelets. These components may be adenosine, ATP, ADP and 5- hydroxy-tryptamine (5HT) which induce coronary artery vasorelaxation (Cohen et al., 1983; Houston et al., 1985; Houston et al., 1986) or sphingosine-1-phosphate (S1P) or thromboxane A_2 (TXA_2) which induce coronary artery vasoconstriction (Yanagisawa-Miwa et al., 1990; Sugiyama et al., 2000). Moreover, a limitation of this study has been a lack of quantifying the level of UDP-glucose released from platelets and RBCs, although reports have shown that UDP-glucose, and other UDP-sugars, are present in both cell types (Palmieri et al., 1991; Wandall et al., 2012). It is difficult, therefore, to determine whether there are sufficient concentrations of UDP-glucose being released from platelets, enough to see a partial change in the responses in the presence of PPTN.

High levels of $P2Y_{14}$ receptor mRNA have been detected in megakaryocytic cells and platelets (Moore et al., 2003). Although $P2Y_{14}$ receptors have been characterised in platelets using immunoblotting analysis, UDP-glucose and MRS2690 do not have any effect on human platelets; they did not induce platelet aggregation, inhibit adenylyl cyclase activity, induce changes in calcium mobilization or promote granule release (Dovlatova et al., 2008). The findings from Dovlatova et al. (2008) suggest that since $P2Y_{14}$ receptors expressed on platelets do not appear to have any apparent function, in the current study, PPTN may not be having a direct effect on platelet function.

Overall the variability of the responses to RBCs and platelets in isolated porcine coronary artery preparations could have been due to the shearing/cell damage during blood cell extraction and preparation, without conducting further experiments, using a high number of donors, it is difficult to determine whether the significant change of PPTN on responses to blood cell responses was due to the lack of UDP-glucose release from cells or donor-specific.

5.4.4 Effect of PPTN on the hypoxic response of the porcine coronary arteries

Adenosine is the classical metabolite identified as being released during hypoxia to increase coronary blood flow (Fox et al., 1974; Hori et al., 1991). A release of ATP has been shown to occur from endothelial cells that line the vessel lumen in response to hypoxia, ischaemia and shear stress (Bodin et al., 1991; Bodin et al., 1995). A number of other potent vasoactive mediators are also released from the endothelium in response to hypoxia (Aaronson et al., 2002; Stenmark et al., 2006).With regard to the porcine coronary artery hypoxic response, the initial contraction is endothelium-dependent and the

prolonged relaxation is endothelium-independent (Mellemkjaer et al., 1994). Hypoxia could cause a release of compounds from the endothelium and/or smooth muscle. Some sources of UDP-glucose release have been outlined in Table 1.1, Chapter 1. There is no report to suggest that UDP-glucose is released from endothelial cells, but a co-release with ATP during the stimuli listed above seems feasible. In the present study, PPTN did not antagonize the vasorelaxation induced through hypoxic stimulation of porcine coronary arteries suggesting that UDP-glucose is not involved in the vasomotor response to hypoxia under the conditions of the present study.

Due to the varying responses produced by platelet and RBCs in the presence of PPTN, it has been difficult to establish whether or not these cells are potential sources of extracellular UDP-glucose. There was certainly no strong indication that UDP-glucose contributes significantly to the porcine coronary artery vasomotor response produced by platelets and RBCs under the conditions of the present study. Moreover, endogenous UDP-glucose does not appear to be involved in the porcine coronary artery vasomotor response to hypoxia under the conditions of the present study.

Summary

- Human isolated platelets induce profound vasorelaxation in preconstricted porcine coronary arteries in the absence of L-NAME; these effects are unaffected in the presence of PPTN
- Human isolated platelets induce small vasorelaxation followed by vasoconstriction in preconstricted porcine coronary arteries in the presence of L-NAME; these effects are unaffected in the presence of PPTN
- Human isolated RBCs induce vasoconstriction in preconstricted porcine coronary arteries; these effects are unaffected in the presence of L-NAME
- Responses to platelets and RBCs were variable between donors and more n number are required in future studies
- PPTN does not change the hypoxic-induced response in porcine coronary arteries

Chapter 6: Effect of $P2Y_{14}$ receptor activation on release of inflammatory cytokines in porcine coronary artery

6.1 Introduction

6.1.1 $P2Y_{14}$ receptors control immune and inflammatory responses

Since the $P2Y_{14}$ receptor has been found to be expressed on immune and inflammatory cells (Lee et al., 2003; Skelton et al., 2003; Muller et al., 2005; Scrivens et al., 2005a; Scrivens et al., 2006), this may imply a role of this receptor in the regulation of these systems.

Cytokines can be released by non-immune cells, usually during inflammation. In the human lung airway epithelial cell lines, alveolar epithelial type II cells (A549) and bronchial epithelial cells (BEAS-2B), $P2Y_{14}$ receptor activation by UDP-glucose stimulates the mobilization of calcium from intracellular stores through activation of phospholipase C (PLC) (Muller et al., 2005). UDP-glucose also promoted vigorous release of the neutrophil chemokine, CXCL8/IL-8, from both A549 and BEAS-2B cells (Muller et al., 2005). In metaplastic goblet cell culture (an airway epithelial cell type), UDP-glucose is released alongside mucins into the airway surface liquid at significantly higher levels compared to non-metaplastic goblet cell cultures (Okada et al., 2011). Since $P2Y_{14}$ receptors are expressed on neutrophils, lymphocytes and macrophages (Scrivens et al., 2005a; Scrivens et al., 2006; Myrtek et al., 2008), the high concentrations of UDP-glucose from metaplastic cell cultures may suggests $P2Y_{14}$ receptors are involved in initiating airway immune responses. RT-PCR studies have shown that UDP-glucose contributes to upregulation of IL-8 and does not have an effect on the secretion of other cytokines including

CXCL9, CXCL10 and CXCL11 (Muller et al., 2005). The release of IL-8 caused an increase in concentration gradient to stimulate chemotaxis of inflammatory cells to the site of infection, suggesting an inflammatory role of the $P2Y_{14}$ receptor. UDP-glucose does not cause alveolar or bronchial cell proliferation (Muller et al., 2005; Sesma et al., 2012).

In kidney intercalated cells, UDP-glucose-induced activation of $P2Y_{14}$ receptors leads to increases in IL-8 and CCL-2 mRNA expression and does not increase other pro-inflammatory mediators such as CCL4, CCL5, IL1β or TNFα (Azroyan et al., 2015). The $P2Y_{14}$ receptor antagonist, PPTN, has been shown to inhibit UDP-glucose mediated increases in IL-8 and CCL-2 mRNA expression. The study demonstrated that UDP-glucose released from damaged intercalated cells acts as a damage-associated molecular pattern molecule to induce sterile inflammation in kidneys where $P2Y_{14}$ receptors plays a functional role in the pro-inflammatory mediated response in these cells (Azroyan et al., 2015).

In neutrophils, $P2Y_{14}$ receptor activation by UDP-glucose leads to cytoskeleton remodelling, changes in cell shape leading to increased motility and migration (Sesma et al., 2012); this UDP-glucose-promoted neutrophil migration is inhibited by PPTN (Barrett et al., 2013). Although UDP-glucose does not promote degranulation in neutrophils and does not induce release of the pro-inflammatory mediator elastase (Scrivens et al., 2005a; Scrivens et al., 2006; Sesma et al., 2012). UDP-glucose has been shown to induce significant increases in neutrophil chemoattractants CXCL1, CXCL2 and also monocyte

chemo-attractants CCL2 and CCL3. Thus UDP-glucose is a pro-inflammatory agonist in these cells (Azroyan et al., 2015).

6.1.2 Lipopolysaccharide induces changes in $P2Y_{14}$ receptor expression

$P2Y_{14}$ receptor expression levels and, indeed, UDP-glucose levels are significantly elevated in certain diseases and inflammatory disorders (Sesma et al., 2009). $P2Y_{14}$ receptors are upregulated by the immunogenic agent lipopolysaccharide (LPS) in the rat brain and spleen (Charlton et al., 1997; Moore et al., 2003). $P2Y_{14}$ receptors are significantly upregulated in N9 microglial cells after exposure to overnight incubation with LPS, without affecting other P2Y receptor subtypes ($P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors), signifying an inflammatory role of the $P2Y_{14}$ receptor in these cells (Bianco et al., 2005). In an endometrial epithelial cell line, LPS significantly increased $P2Y_{14}$ mRNA expression (Arase et al., 2009). In the mouse uterus, the LPS mediated increase in white blood cells was abolished with $P2Y_{14}$ receptor knockdown (Arase et al., 2009).

The effects of LPS on blood vessel contractility are well documented. For example, LPS has been shown to increase 5-HT (serotonin)-induced contractions in rat middle cerebral arteries (MCA) (Hernanz et al., 2003) and in rat aorta (Wylam et al., 2001). However, LPS has been found to reduce prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) contraction in MCA (Briones et al., 2002) and also reduces the U46619 induced contractions in porcine basilar arteries (Ueno et al., 1993). The increased contractility to 5-HT was due to an increase in COX-2 expression, enhanced release of thromboxane and superoxide anion and

hydrogen peroxide, subsequently resulting in increased vasocontractile responses to 5-HT (Hernanz et al., 2003). Conversely, increased COX-2 levels through LPS stimulation leads to COX-2-dependent vasodilatation in cerebral arterioles (Brian et al., 1998).

There is limited research into a role of the $P2Y_{14}$ receptor in regulation of immunity and inflammation in vascular tissues, thus, the current study investigated the effects of the immunogenic agent LPS on contractile responses to MRS2690 in porcine coronary arteries, as well as investigating the effects of UDP-glucose and MRS2690 on the release of 13 cytokines and chemokines from porcine coronary arteries.

6.2 Methods

6.2.1 Multiplex magnetic cytokine chemokine bead assay

See Chapter 2 Section 2.6 for full details.

Coronary artery segments were incubated with UDP-glucose (100 µM or 1mM) or MRS2690 (1 µM or 10 µM) in serum-free RPMI media for 24 hours at 37 ºC. In separate experiments, vessels were incubated with various concentrations of LPS (1 ng/ml 10 ng/ml 1 µg/ml or 10 µg/ml) or LPS (10 µg/ml) + MRS290 (10 µM). The following day, medium (containing cytokines) was removed and centrifuged. Supernatant was taken and was used to conduct an assay to measure cytokine release using the Porcine Cytokine/Chemokine Magnetic Bead Panel (PCYTMG-23K-13PX) (Merck,

Millipore). These cytokines/chemokines included GM-CSF, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL6, IL-8, IL-10, IL-12, IL18, IFNγ and TNFα.

6.2.2 Lipopolysaccharide incubation

See Chapter 2 Section 2.1.7 for full details.

Coronary artery segments were incubated with various concentrations of LPS (1 µg/ml 10 µg/ml or 100 µg/ml) or without LPS (control) overnight at 4ºC. The following day, response curves to MRS2690 were constructed in forskolin plus U46619 conditions. Substance P (10 nM) was used to assess endothelium integrity.

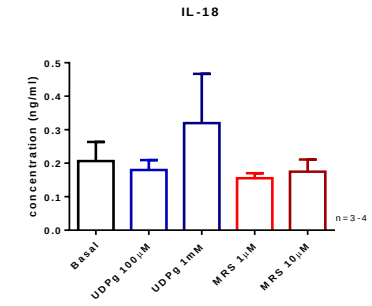
6.2.3 Statistics

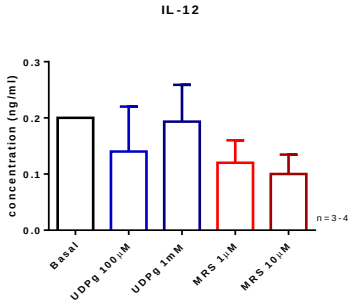
Multiplex cytokine/chemokine assay results were analysed using one-way ANOVA followed by Sidak's multiple comparisons test. MRS2690 contractile responses in LPS-incubated vessels were analysed using two-way ANOVA with Sidak's multiple comparisons test. $P < 0.05$ was taken as statistically significant.

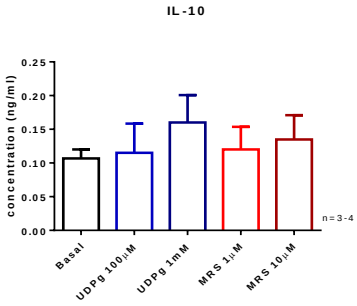
6.3 Results

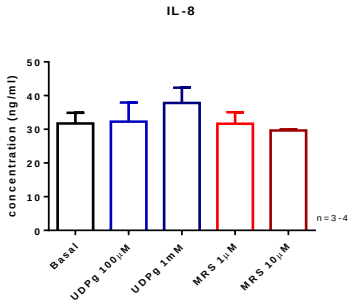
6.3.1 Effect of UDP-glucose and MRS2690 on the release of cytokines/chemokines from porcine coronary arteries

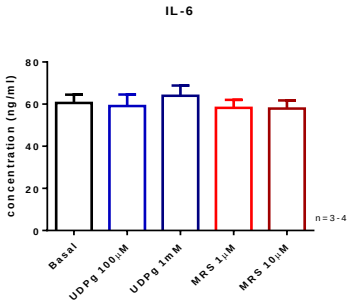
Levels of various cytokines and chemokines were measured in the media from porcine coronary arteries incubated with the $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690 for 24h.

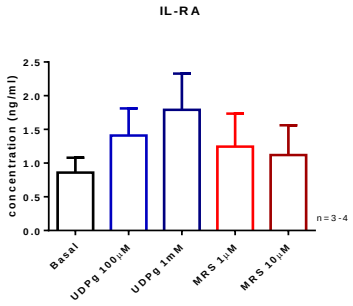

IL-18
concentration (ng/ml)
0.5
0.4
0.3
0.2
0.1
0.0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM

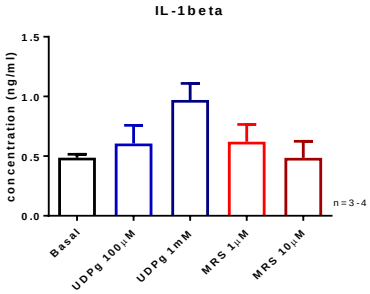

IL-12
concentration (ng/ml)
0.3
0.2
0.1
0.0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM

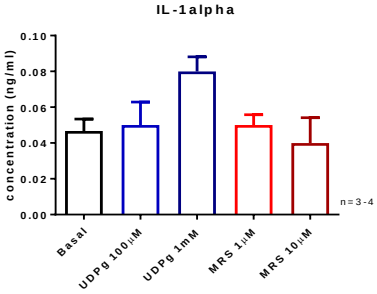

IL-10
concentration (ng/ml)
0.25
0.20
0.15
0.10
0.05
0.00
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM


IL-8
concentration (ng/ml)
50
40
30
20
10
0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM


IL-6
concentration (ng/ml)
80
60
40
20
0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM


IL-RA
concentration (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM


IL-1beta
concentration (ng/ml)
1.5
1.0
0.5
0.0
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM


IL-1alpha
concentration (ng/ml)
0.10
0.08
0.06
0.04
0.02
0.00
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM

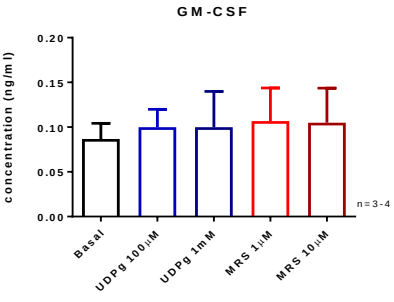

GM-CSF
concentration (ng/ml)
0.20
0.15
0.10
0.05
0.00
n=3-4
Basal
UDPg 100µM
UDPg 1mM
MRS 1µM
MRS 10µM

Figure 6.0: Levels of cytokine/chemokine present in the culture media containing porcine coronary artery segments incubated for 24h with $P2Y_{14}$ receptor agonists, UDP-glucose (UDPg) or MRS2690 (MRS). Data are means ± S.E.M.

The levels of cytokines/chemokines were not significantly different in vessels that were exposed to UDP-glucose or MRS2690 compared to control vessels ($P > 0.05$, ANOVA, Figure 6.1). The weights of the vessels between groups were not significantly different compared to each other ($P > 0.05$, ANOVA) (Table 6.1). IL-2, IL-4 and TNFα and IFNγ release from coronary arteries was not detected with the multiplex assay, since readings were below the range of minimum detection.

	Basal	**UDP-glucose (100µM)**	**UDP-glucose (1mM)**	**MRS2690 (10µM)**	**MRS2690 (1µM)**
Weight (g)	37.7 (n=3)	38.1 (n=4)	39.2 (n=4)	38.9 (n=4)	30.8 (n=4)

Table 6.1: The average weights of the vessel segments (g) used in Figure 6.1

6.3.2 Effect of LPS on release of cytokines and chemokines from porcine coronary arteries

In order to investigate the involvement of the $P2Y_{14}$ receptor in modulation of inflammation, the effect of the immunogenic agent LPS, in the absence and presence of the $P2Y_{14}$ receptor agonist MRS2690, on the release of cytokines/chemokines vessels was investigated. Initial experiments were carried out which involved using various concentrations of LPS (1, 10 or 100

ng/ml) to stimulate the release of inflammatory cytokines/chemokines. Subsequent experiments were carried out (using the LPS concentration that established a response), to see whether the effects were changed in the presence of MRS2690.

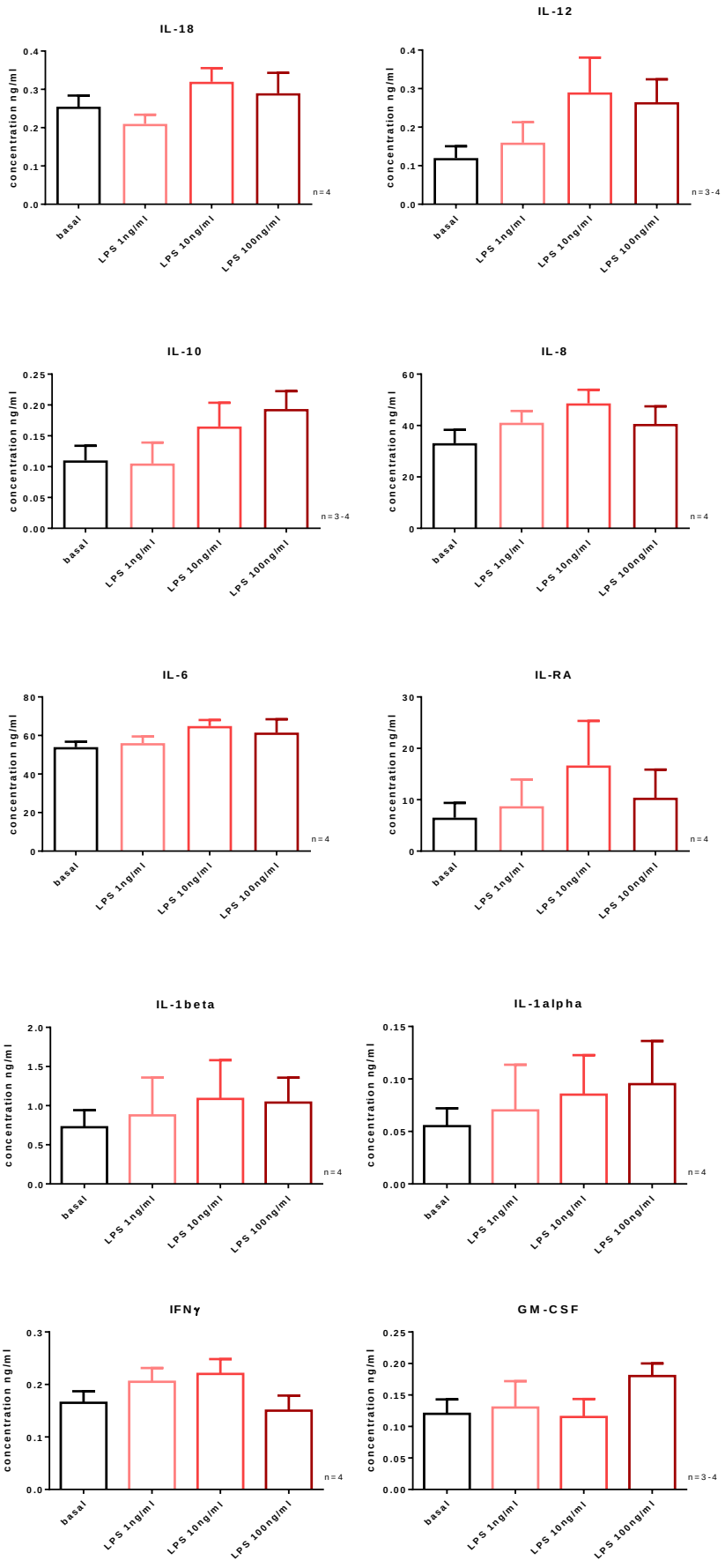

IL-18
concentration ng/ml
0.0
0.1
0.2
0.3
0.4
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-12
concentration ng/ml
0.0
0.1
0.2
0.3
0.4
n=3-4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-10
concentration ng/ml
0.00
0.05
0.10
0.15
0.20
0.25
n=3-4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-8
concentration ng/ml
0
20
40
60
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-6
concentration ng/ml
0
20
40
60
80
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-RA
concentration ng/ml
0
10
20
30
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-1beta
concentration ng/ml
0.0
0.5
1.0
1.5
2.0
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IL-1alpha
concentration ng/ml
0.00
0.05
0.10
0.15
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
IFNγ
concentration ng/ml
0.0
0.1
0.2
0.3
n=4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml
GM-CSF
concentration ng/ml
0.00
0.05
0.10
0.15
0.20
0.25
n=3-4
basal
LPS 1ng/ml
LPS 10ng/ml
LPS 100ng/ml

Figure 6.2: Effect of various concentrations of LPS (1, 10 or 100 ng/ml) on cytokine/chemokine release from porcine coronary arteries. Data are means ± S.E.M. Vessels were stored overnight at 37 ºC for 24 hours.

	Basal	**LPS (1ng/ml)**	**LPS (10ng/ml)**	**LPS (100ng/ml)**
Weight (g)	32.1 (n=4)	34.8 (n=4)	38.3 (n=4)	48.4 (n=4)

Table 6.2: The average weights of the vessel segments (g) according to Figure 6.2

Overall, there was no significant difference in the levels of cytokines/chemokines from porcine coronary arteries in vessels exposed to LPS compared to basal levels (P >0.05, ANOVA), (Figure 6.1). Similarly to the previous experiments, release of IL-2, IL-4 and TNFα was not detected as levels were below the minimum range detected. Weights of tissues are given in Table 6.2; these weights were not significantly different between groups (P >0.05, ANOVA).

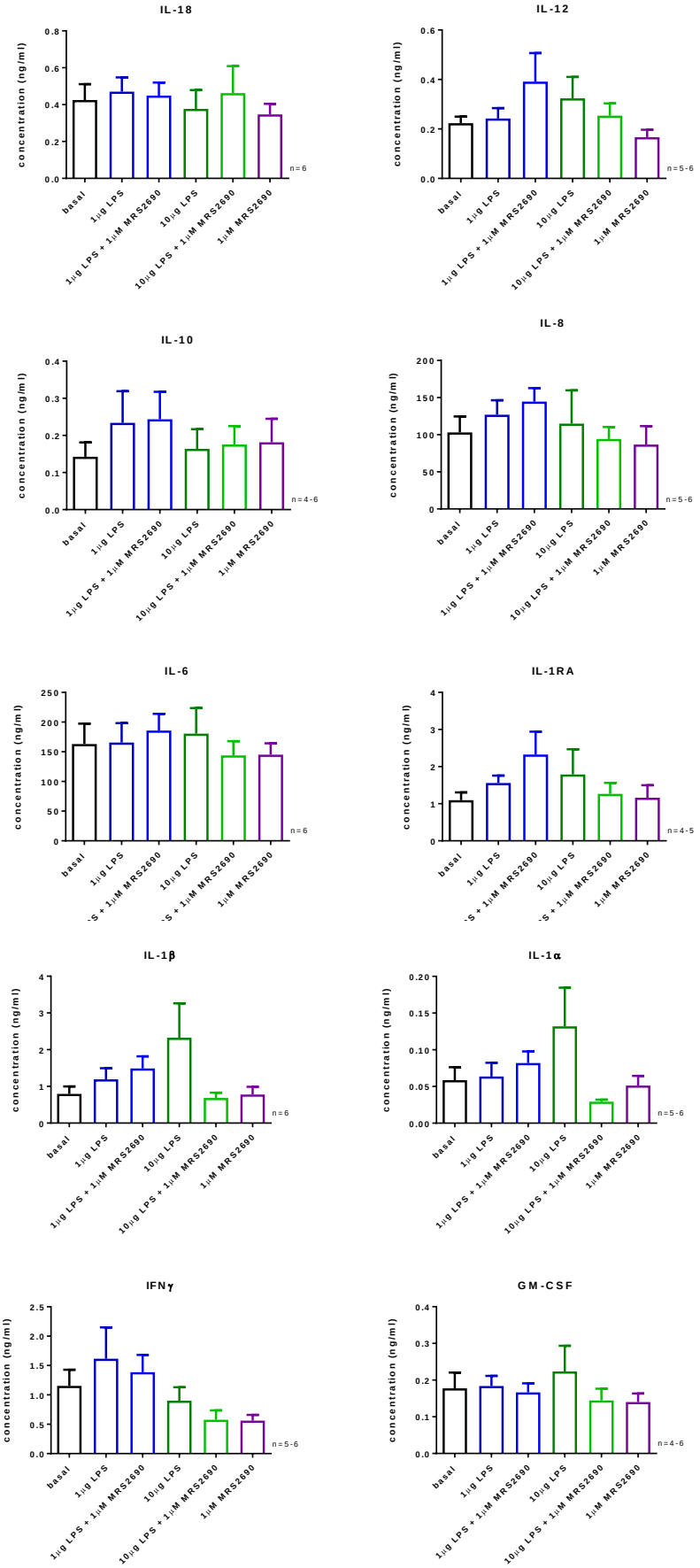

IL-18
IL-12
IL-10
IL-8
IL-6
IL-1RA
IL-1β
IL-1α
IFNγ
GM-CSF
concentration (ng/ml)
basal
1μg LPS
1μg LPS + 1μM MRS2690
10μg LPS
10μg LPS + 1μM MRS2690
1μM MRS2690
n=6
n=5-6
n=4-6
n=4-5

Figure 6.3: Levels of cytokines/chemokines present in the culture media containing porcine coronary artery segments incubated for in the presence of LPS (1µg/ml) +/- MRS2690 (1µM), LPS (10µg/ml) +/- MRS2690 (1µM) or MRS2690 (1µM). Data are means ± S.E.M.

LPS	-	1µg/ml	1µg/ml	10µg/ml	10µg/ml	-
MRS2690	-	-	1µM	-	1µM	1µM
Weight (g)	60.8 (n=6)	56.9 (n=6)	56.2 (n=6)	55.7 (n=6)	56.3 (n=6)	50.9 (n=6)

Table 6.3: The average weights of the vessel segments (g) according to Figure 6.3. Control vessel indicates the absence of both LPS and MRS2690.

Since 100 ng/ml LPS showed modest, but not significant, increases overall in cytokine/chemokine induced release in (Figure 6.1), subsequent experiments looked to see whether higher concentrations of LPS (1 or 10 µg/ml) could change the cytokine/chemokine induced release compared to control vessels and to see whether these changes could be altered in the presence of 1 µM MRS2690. There was no significant effect of the higher concentrations of LPS on cytokine/chemokine release (Figure 6.3). Moreover, MRS2690 had no significant effect on cytokine/chemokine levels in the presence of LPS ($P>0.05$, ANOVA), (Figure 6.3).

6.3.3 Effects of lipopolysaccharide incubation on porcine coronary arteries responses to $P2Y_{14}$ receptor agonist MRS2690

Since LPS can alter vascular contractility, the effect of LPS incubation on contractile responses to the selective $P2Y_{14}$ receptor agonist, MRS2690, was investigated in porcine coronary arteries.

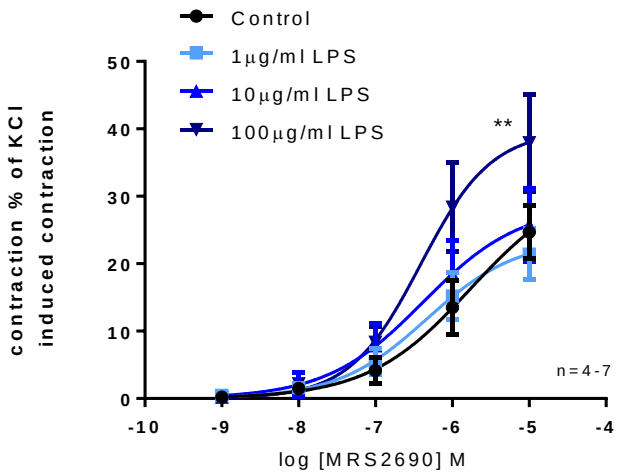


Figure 6.4: Concentration response curves to MRS2690 in porcine coronary arteries in forskolin plus U46619 conditions, after incubation with various concentrations of LPS (1 µg/ml, 10 µg/ml and 100 µg/ml). Porcine coronary artery segments were stored overnight for 18h with or without LPS. Contractions are expressed as a percentage of the KCl induced contraction. Data are mean ± S.E.M. **P<0.01, two-way ANOVA, followed by Sidak's multiple comparisons test.

With increasing concentrations of LPS, there appeared to be a trend towards an increase in the responses to MRS2690 compared to control vessels (Figure 6.4). There was a significant change in the MRS2690 induced contractions in vessels that were exposed to 100 µg/ml LPS in comparison to control vessels (P<0.01, two-way ANOVA, followed by Sidak's multiple comparisons test),

however there was no significant change in the MRS2690-induced contractions exposed to 1 µg/ml and 10 µg/ml compared to control vessels ($P>0.05$, two-way ANOVA, followed by Sidak's multiple comparisons test), (Figure 6.4).

Substance P (10 nM) was used to assess the endothelial integrity. After vessels reached a maximum contractile response and began to relax following addition of the final concentration of MRS2690 (1µM), substance P was added to vessels and a relaxation was seen (100% of the MRS2690-induced contraction) back to baseline; the basal tone achieved prior to conducting the contractile response curve to MRS2690.

6.4 Discussion

6.4.1 $P2Y_{14}$ receptor activation does not significantly alter the release of cytokines and chemokines from porcine coronary arteries

The current study demonstrated that the $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690, had no significant effect on the release of cytokines from porcine coronary arteries compared to basal release. It did appear that UDP-glucose, particularly at the higher concentration (1 mM), had some minor effect (not significant) on the release of several cytokines. Since UDP-glucose has some effect at $P2Y_2$ receptors (Ko et al., 2009) which are expressed in porcine coronary arteries (Rayment et al., 2007a), it is possible that $P2Y_2$ receptor activation is resulting in the release of inflammatory markers from coronary arteries. However, it is difficult to demonstrate this since there are currently no known selective antagonists available at $P2Y_2$ receptors. However, there is potential to discover an involvement using selective and stable $P2Y_2$ receptor agonists such as UTPγS (Malmsjo et al., 2000a).

The effect of LPS as an immunogenic stimulus on the release of these cytokines and chemokines was investigated to see whether release of inflammatory markers was augmented and determine whether MRS2690 could affect the LPS-induced increases in release of these inflammatory markers. LPS (1ng/ml - 10μg/ml) did not have any significant changes on the release of cytokines from porcine coronary arteries and MRS2690 had no further effect. Overall the results from these experiments did not show an involvement of the $P2Y_{14}$ receptor in release of inflammatory markers from porcine coronary arteries and there is a possibility that the $P2Y_{14}$ receptor does not have an

involvement in inflammation in these vessels under the conditions examined. This discussion will focus on possible effects occurring as a result of LPS treatment and also changes that could be carried out for future studies.

6.4.2 Potential involvement of P2 receptor-mediated suppression of cytokines from porcine coronary arteries

Nucleotides can be released in response to LPS which consequently activate P2X and P2Y receptors, leading to autocrine activation and release of inflammatory cytokines. In the current study, there is perhaps a possibility that LPS induced release of nucleotides from porcine coronary arteries which may have activated several purinoceptors in an autocrine mechanism resulting in the release or inhibition of cytokines. Therefore, the effects of LPS activating release of cytokines in the presence of MRS2690 (and subsequent $P2Y_{14}$ receptor activation) may have been counteracted by simultaneous release of opposing nucleotides/purinoceptor activation and hence suppression of the release of cytokines. LPS has been shown to secrete IL-1β from mouse microglial cells and human macrophages through ATP release and autocrine/paracrine signalling. Selective blockade of the P2X7 receptor and addition of apyrase inhibited the LPS-dependent IL-1β release (Ferrari et al., 1997). Addition of hexokinase, which generates glucose 6-phosphate and ADP from ATP, leads to ADP subsequently releasing IL-1β through P2X7 receptor activation (Ferrari et al., 1997). Similarly, LPS triggers the release of ATP from human endothelial cells which consequently mediates IL-1α release after P2X7 receptor activation (Imai et al., 2000). Furthermore, LPS stimulates the release of IL-8 secretion from monocytes through UDP-mediated activation of

$P2Y_6$ receptors (Warny et al., 2001). The results from these studies suggest that ADP, ATP, UTP or UDP could be released from porcine coronary arteries as a result of LPS stimulation, thereby activating multiple purinoceptors. On the other hand, ATP and ADP have been shown to decrease LPS induced IL-12p40 and TNF-α production in dendritic cells likely through $P2Y_1$ receptors (Myrtek et al., 2008), whilst UTP and UDP supress the production of IL-12 and TNF-α from macrophages (Hasko et al., 2000). Moreover, MRS2758 a selective $P2Y_2$ receptor agonist, reduces TNF-α in hypoxic myocytes (Hochhauser et al., 2013). These findings, therefore, present a possibility that firstly, LPS may trigger the release of nucleotides from porcine coronary arteries; secondly, these nucleotides may supress the activation and release of cytokines from porcine coronary arteries, and thus the release of cytokines through $P2Y_{14}$ receptor activation may be counteracted by activation of P2 receptors which supress the release of cytokines. Thus, it is important to determine the effects of LPS on the release of cytokines in porcine coronary arteries in the presence of PPTN, and possibly other selective P2Y receptor antagonists, in future studies.

6.4.3 Stability of $P2Y_{14}$ receptor agonists

Lazarowski et al. (2003) have shown that extracellular release of UDP-glucose has remained at a steady rate up to 3 hours, without being degraded, in comparison to ATP levels which peak within 10 minutes and return to resting levels (Lazarowski et al., 2003b). The selectivity of MRS2690 has been reported (Ko et al., 2007), however there are no reports of its stability. There are also no reports to show the stability of UDP-glucose longer than 3 hours. In the current study, porcine coronary arteries were incubated with UDP-glucose

and MRS2690 over a 24 hour time period, to induce the release of cytokines. There is a possibility that these agonists may have degraded over this time or lost efficacy resulting in a lack of change in cytokine levels compared to tissues not exposed to these agonists, however this warrants further investigation. LPS (10 µg/ml) has been used as a inducer for cytokine release in cells incubated with the immunogenic agent for as little as 2 hours (Ferrari et al., 1997). There is perhaps a chance that LPS may have induced the release of cytokines earlier than 24 hours (the time used in the current study). LPS (20µg/ml) stimulates the release of TNF-α from porcine coronary arteries which peaks at 6 hours and remains elevated up 24 hours (Newman et al., 1998). Further to this, future studies could be conducted in which cytokines could be measured from segments treated with LPS, in combination with/out MRS2690 at earlier time points similar to those reported.

In the current study, LPS treatment of porcine coronary arteries did not cause a significant change in inflammatory mediators compared to untreated vessels. There is a possibility that the LPS used from the E.coli strain was not significantly potent to induce a major change in response. The concentrations of LPS used in the current study (1-100ng/ml) were perhaps too low to show a significant change. Similar concentrations of LPS (0.1-10ng/ml) have been shown to stimulate the release from isolated human coronary artery endothelial cells and smooth muscle cells (Stoll et al., 2004). Much higher concentrations of LPS have been used to stimulate the release of cytokines from intact segments. These concentrations include 10 µg/ml (Wei et al., 2008) and 20 µg/ml in porcine coronary arteries (Newman et al., 1998), and as high as 10

μg/ml in human temporal arteries (Blain et al., 2002). Future studies conducted should aim to use higher concentration of LPS.

It has been documented that several membrane receptors can translocate into or move away from lipid rafts, specialized regions of the plasma membrane which have high levels of cholesterol and glycosphingolipids, following ligand binding. The dynamic properties of such receptors are linked to receptor signalling, modulation, desensitization or internalization (Zajchowski et al., 2002; Simons et al., 2011). Furthermore, in the presence of continuous agonist stimulation of GPCRs, leads to phosphorylation of GPCRs by specific kinases, recruitment of β-arrestins, termination of G protein signalling followed by receptor desensitization, inactivation and internalization (Pierce et al., 2002). It is plausible that $P2Y_{14}$ receptors may have been desensitized to UDP-glucose and MRS2690, leading to receptor internalization prior to their being any effect on signal-induced cytokine upregulation. However, further evidence is required to show whether desensitizing $P2Y_{14}$ receptors is the cause the significantly low levels of cytokines measured in the current study.

6.4.4 Future studies: investigating signalling proteins involved in inflammatory pathways in the porcine coronary artery through $P2Y_{14}$ receptor activation

Future studies could be conducted to determine whether $P2Y_{14}$ receptor activation supresses or enhances inflammatory-linked signalling proteins. The activation of the JAK-STAT (Janus kinase-signal transducer and activation of transcription) signalling pathway plays a key role in the transduction of cytokines and growth factors (Heinrich et al., 2003). The suppressor of cytokine signalling (SOCS) proteins, including SOCS-3 negatively regulates

the JAK-STAT signalling pathway by inhibiting the JAK tyrosine kinase activity (Ehlting et al., 2007); SOCS-3 upregulation supresses the effect of inflammatory cytokines (Rakesh et al., 2005). The effect of MRS2690 on the expression pattern of SOCS-3/JAK-STAT proteins can be carried out. It can be hypothesized that UDP-glucose mediated activation of $P2Y_{14}$ receptors could stimulate or inhibit the release of inflammatory cytokines from porcine coronary arteries; the resulting release of cytokines could act as autocrine signalling molecules that subsequently activate the JAK-STAT pathway. Consequent upregulation of SOCS-3 proteins as a result of $P2Y_{14}$ receptor activation could suggest that this receptor is required as a marker for anti-inflammatory signalling; alternatively down-regulation of SOCS-3 proteins, and therefore production of further inflammatory cytokines would suggest that the $P2Y_{14}$ receptor serves as a marker for pro-inflammatory regulation in porcine coronary arteries. This future study can be conducted based on the methodology outlined by Gupta et al., (2011); porcine coronary arteries or smooth muscle cells could be incubated in the absence and presence of $P2Y_{14}$ receptor agonist/antagonist for 24 hours and subsequent Western blots to identify SOCS-3/JAK-STAT expression can be carried out from cell lysate obtained.

6.4.5 MRS2690-induced contractions are augmented in tissues exposed to the immunogenic agent, LPS

The results demonstrated that overnight storage of coronary arteries with the immunogenic agent LPS caused changes in the contractions elicited by the selective $P2Y_{14}$ receptor agonist, MRS2690. There was a significant increase in the MRS2690 induced contractions in vessels incubated with 100 µg/ml LPS.

This is similar to what others have shown where the endothelin receptor agonist, Sarafotoxin 6c, induced increased contraction in rat coronary arteries incubated with LPS for 24 hours (Ghorbani et al., 2010), and also there is increased contraction to 5-HT in rat middle cerebral arteries after exposure to LPS (Hernanz et al., 2003).

Endothelial removal or damage can cause increases in contractions (Brutsaert et al., 1988; Li et al., 1991). Reports have shown that LPS impairs endothelium-dependent relaxations induced by substance P (Qi et al., 2007; Al-Shalmani et al., 2011). In the current study, LPS treatment did not damage the endothelium of coronary arteries, since vessels relaxed in response to substance P. However only a single concentration of substance P was used (10 nM) thus there remains the possibility that the endothelium may have been damaged resulting in an increase in contractile responses to MRS2690.

Overnight treatment of coronary arteries with LPS (100 µg/ml), may increase $P2Y_{14}$ receptor mRNA levels. Since induction of receptor expression requires at least 7 hours (Hasbak et al., 2005), and vessels were incubated with LPS at least 18 hours, thus increases in $P2Y_{14}$ receptor mRNA in coronary arteries is plausible. The $P2Y_{14}$ receptor mRNA is upregulated in response to inflammation and stress and injury (Malin et al., 2010; O'Keeffe et al., 2010a; O'Keeffe et al., 2010b). The present study investigated whether the inflammatory agent, LPS could stimulate an increase in $P2Y_{14}$ receptor by altering its pharmacological response profile.

Since LPS treatment (intraperitoneal injection of 250 µg/kg, 200-300g rats) LPS has been associated with increases in both mRNA and protein levels of

other inflammatory receptors; the interleukin-1 receptor antagonist (IL-1RA) receptor (Palin et al., 2001) and the CCR2 chemokine receptor in rat brain (100 µg/ml injected into 250-300g rats) (Banisadr et al., 2002), it is possible that $P2Y_{14}$ receptor protein levels in coronary arteries are increased resulting in an increase in MRS2690 induced contractions in LPS (100 µg/ml) treated vessels. Moore et al., (2003), have shown that LPS induces release of cytokines in the rat brain (intraperitoneal injection of 2.5 mg/kg LPS into 180-200g rats) which initiates astrocytes to activate and proliferate, a process termed reactive astrogliosis. This subsequently leads to an increase in $P2Y_{14}$ receptor mRNA expression on astrocytes and microglia (Moore et al., 2003). Thus $P2Y_{14}$ receptors may have a role in reactive astrogliosis and respond to the microbial insult in the brain and also respond to the release of UDP-glucose from damaged or distressed cells.

In rat coronary arteries, following 24 hour organ culture with LPS (250ng/ml), there is increased endothelin receptor type B (ETB) protein which causes an increased contractile response to the endothelin receptor agonist Sarafotoxin 6c (Ghorbani et al., 2010). These results suggest that LPS may stimulate increased in $P2Y_{14}$ receptor mRNA/protein levels resulting in the increased vasocontractile response to MRS2690, however further studies to identify whether molecular changes in $P2Y_{14}$ receptors are occurring needs to be explored.

Temperature decrease under physiological conditions has been associated with a decrease in membrane fluidity, stabilization of nucleic acids leading to a reduced cellular ability to undergo RNA transcription, translation and

degradation (Phadtare, 2004). In the current study, vessels were incubated overnight with LPS at 4ºC, this may have resulted in altered levels of $P2Y_{14}$ receptor expression. Kirkley et al. (2003) have showed that temperature effected the translation and release of cytokines from macrophages however had no effect on mRNA expression (Kirkley et al., 2003). Future studies conducted could look at the expression of $P2Y_{14}$ receptors (by observing effect of agonist induced contraction), in coronary arteries following incubation of LPS at various temperatures.

Monocytes express $P2Y_{14}$ receptors (Skelton et al., 2003) RT-PCR studies to look at CD68 or CD14 positive cells (as an indication of monocytes and macrophage levels in smooth muscle cells) (Bruun et al., 2007) could be carried out to determine the level of these cells in coronary arteries, as well as looking at potential infiltration of T-lymphocytes and neutrophils, which both express $P2Y_{14}$ receptors (Scrivens et al., 2005a; Scrivens et al., 2006), within the coronary arterial wall. These studies could suggest $P2Y_{14}$ receptor agonists induced release of cytokines/chemokines, however small, from immune cells embedded within the coronary arteries.

Summary

- $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690 have no significant effect on the release of cytokines from porcine coronary arteries

- LPS (at concentrations of up to 10µg/ml for 24h) does not stimulate a change in cytokine levels in porcine coronary arteries; future studies conducted could investigate the effect of higher concentrations of LPS and incubating for shorter time periods (2 h) including

- MRS2690 induced contraction is increased in vessels exposed to overnight (18h) LPS treatment suggesting LPS may be damaging the endothelium or changing $P2Y_{14}$ receptors protein expression or stimulating release of substances from coronary arteries to alter levels of MRS2690-induced contraction

Chapter 7: General Discussion

7.1 Overview of General Discussion

This thesis investigated the functional expression of the $P2Y_{14}$ purine receptor in the porcine coronary artery; the preliminary investigation was to establish the pharmacological profile of the $P2Y_{14}$ receptor. It was found that $P2Y_{14}$ receptor agonists, UDP-sugar nucleotides and a more selective agonist, MRS2690, induced contractile responses in the porcine coronary artery; MRS2690 more potently than UDP-sugar nucleotides. The responses to MRS2690, but not UDP-glucose, were blocked by a novel and selective $P2Y_{14}$ receptor antagonist, PPTN (Chapter 3). The study also aimed to determine the effect of the non-selective P2 receptor antagonists suramin and PPADS on responses to $P2Y_{14}$ receptor agonists, the coupling of $P2Y_{14}$ receptors to Gi-proteins using a vasodilator stimulated phosphoprotein-phosphorylation assay (Chapter 4), the potential sources of UDP-glucose in blood cells (Chapter 5) and the role of $P2Y_{14}$ receptors in inflammation in the porcine coronary artery (Chapter 6). The discussion will present the main findings of the study before potential future proposals are outlined. Some sections of the discussion of results have been integrated with discussion of future experiments.

7.1.1 $P2Y_{14}$ receptor in the porcine coronary artery

The current study aimed to identify and characterise the $P2Y_{14}$ receptor in the porcine coronary artery. The collective evidence from this study suggests that $P2Y_{14}$ receptors are expressed in the porcine coronary artery; immunohistochemical findings showed $P2Y_{14}$ receptors localized on smooth muscle and endothelial cells and organ bath pharmacology showed that $P2Y_{14}$ receptor agonists induce contractility of porcine coronary arteries. A

cAMP/VASP-phosphorylation assay provided evidence to show $P2Y_{14}$ receptor coupling to the inhibition of Gi-proteins.

Since there have been doubts raised against the P2 receptor antibodies, including $P2Y_6$ antibodies (Yu et al., 2013) and P2X7 receptor antibodies (Anderson et al., 2006), several studies have measured $P2Y_{14}$ receptor DNA and RNA using PCR and RT-PCR (Bassil et al., 2009). Although, these molecular techniques do not provide evidence for functional receptor expression, PCR may be used to confirm molecular presence of receptors in porcine coronary arteries whilst pharmacological and biochemical studies have provided evidence for functional expression.

The expression of the $P2Y_{14}$ receptor in other vascular beds has been relatively little investigated and is variable; in mouse thoracic aorta and isolated vascular smooth muscle cells $P2Y_{14}$ receptor transcripts are barely detected (Kauffenstein et al., 2010). PCR analysis from both freshly isolated and cultured rat aortic smooth muscle cells (ASMCs) has shown that the $P2Y_{14}$ receptor is one of the predominating P2Y receptor subtypes present in these cells (Govindan et al., 2010). However, functional receptors are not present since both MRS2690 and UDP-glucose fail to induce changes in calcium signals in ASMCs (Govindan et al., 2010). The apparent difference in functional expression may be a result of species variance, since contractile $P2Y_{14}$ receptors have been characterized in porcine pancreatic arteries, where both UDP-glucose and MRS2690 induced contractile responses (Alsaqati et al., 2014). Other studies have shown contractile $P2Y_{14}$ receptors in mouse coronary and cerebral arteries (Haanes et al., 2014a). The biggest difference

between these studies is that functional $P2Y_{14}$ receptors are not present in cultured cells, however they are present in fresh tissue.

7.1.2 $P2Y_{14}$ receptor pharmacological response profile

Overall, clear evidence has been provided for functional $P2Y_{14}$ receptor expression in porcine coronary arteries. The pharmacological response profile of the $P2Y_{14}$ receptor in these vessels shows that MRS2690 is a more potent agonist, inducing contractions that are approximately 10-fold greater than UDP-sugars; UDP-glucose, UDP-glucuronic acid and UDP-N-acetylglucosamine, which are all equally potent. These $P2Y_{14}$ receptor agonists, including UDP-galactose, are agonists at cloned human, rat and mouse receptors expressed in HEK 293 cells (Chambers et al., 2000; Freeman et al., 2001). Scrivens and Dickenson (2005) have shown that $P2Y_{14}$ receptors are expressed in murine T-lymphocytes, where UDP-glucose inhibits forskolin induced elevation of cAMP. They have also shown that UDP-galactose, UDP-glucuronic acid and UDP-N-acetylglucosamine block UDP-glucose mediated inhibition of forskolin-induced cAMP in these cells and act as partial agonists in this respect, indicating $G_{i/o}$ protein coupling (Scrivens et al., 2005a).

The results from the current study are the first to show that UDP-sugar nucleotides, UDP-glucuronic acid and UDP-N-acetylglucosamine, behave as full agonists in the porcine coronary vasculature through activation of the $P2Y_{14}$ receptor. Previous studies have not investigated the effects of these sugar nucleotides in vascular beds or, indeed, in similar cell systems.

Vasocontractile responses to $P2Y_{14}$ receptor agonists vary in different tones; in U46619-preconstricted tone, agonists induced more profound contractions than

those that were seen in basal tone (relaxed) coronary arteries. In conditions of elevated cAMP, using forskolin as an agent to increase cAMP levels, these contractions exceeded those that were produced in basal and U46619 conditions. These findings are consistent with reports which have shown that the actions of the α_2-adrenoceptor agonist UK14304 are higher in preconstricted arteries in the porcine ear artery compared to basal level; contractions to UK14304 are further increased in conditions of U46619 and forskolin compared to U46619 alone (Roberts et al., 1998). Similarly contractions to the P2X receptor agonist, α,β-methylene ATP are enhanced in U46619-induced preconstricted porcine splenic arteries (Roberts, 2012). The physiological relevance of inducing U46619-induced preconstriction is to mimic physiological conditions found *in vivo*; others have shown that using U46619 or endothelin-1 to induce preconstriction in the rat mesenteric artery, reveals a purinergic response that was not found under basal conditions (Pakdeechote et al., 2007). Hence, the contractions to UDP-glucose in preconstricted porcine coronary arteries may resemble more closely what may occur under physiological conditions.

Other $P2Y_{14}$ receptor agonists have been identified, including α,β-difluoromethylene-UDP (MRS2802) which has an EC_{50} of 50 nM (Carter et al., 2009), similar to the EC_{50} of MRS2690 (Ko et al., 2007). α,β-methylene-2-thio-UDP has an EC_{50} of 0.92 nM (Das et al., 2010), and so is a more potent agonist than MRS2690. These $P2Y_{14}$ receptor agonists were not used in the current study because they are not commercially available; it is worth investigating whether these agonists, particularly α,β-methylene-2-thio-UDP is more potent than MRS2690 to induce porcine coronary artery contractility.

7.1.3 PPTN blocks contractile responses to MRS2690

PPTN, a $P2Y_{14}$ receptor antagonist, has been shown to act selectively at the $P2Y_{14}$ receptor at concentrations as low as 1 μM, and reported to have no antagonist activity at the other P2Y receptor subtypes at concentrations as high as 10 μM (Barrett et al., 2013). PPTN did not significantly block responses to UDP-glucose; however a rightward shift in the contractile responses was seen. Conversely, PPTN significantly blocked responses mediated by the more selective $P2Y_{14}$ receptor agonist, MRS2690. In control experiments, PPTN did not block vasocontractile responses to UTP, an agonist at $P2Y_2$ and $P2Y_4$ receptors (Ralevic et al., 1998), consistent with evidence that it is a selective antagonist (Barrett et al., 2013). The results present novel findings of vasocontractile $P2Y_{14}$ receptors that are selectively antagonized by PPTN.

7.1.4 Suramin and PPADS

There are no reports that have provided evidence to show the effects of suramin and PPADS, both non-selective P2 receptor antagonists (Charlton et al., 1996b) at $P2Y_{14}$ receptors. To the best of my knowledge, this is the first study to report the effects of suramin and PPADS on $P2Y_{14}$ receptors.

Suramin is an antagonist at the $P2Y_2$ receptor (Wildman et al., 2003) and other P2Y receptors (listed in Chapter 4). In U46619 induced preconstricted porcine coronary arteries, suramin amplified UDP-glucose induced contractions suggesting UDP-glucose may be acting at vasorelaxant $P2Y_2$ receptors present in the porcine coronary arteries. However, in conditions of elevated cAMP, using forskolin, contractile responses were significantly blocked. Suramin has non-specific effects where it has been shown to inhibit U46619- and 5-HT-

induced platelet aggregation. Suramin is suggested to bind to plasma proteins at high concentrations, thus it may bind to fibrinogen resulting in inhibition of platelet aggregation (Hourani et al., 1992). In a neuroblastoma x glioma hybrid cell line (NG 108-15) and a glioma cell line (C6-BU1), suramin has been shown to interact with a pertussis-toxin-sensitive G protein receptor. Suramin also non-competitively inhibited the basal rate of hydrolysis of GTP by high-affinity GTPases in both cell lines (Butler et al., 1988)

From these observations, it is possible that suramin may be having non-specific effects on the porcine coronary artery, in the conditions of U46619 and forskolin or possibly be blocking contractile $P2Y_2$ receptors resulting in significantly inhibited UDP-glucose responses.

PPADS has been shown to competitively block the rat $P2Y_1$ receptor in both native cells and cells that have been transfected with the rat $P2Y_1$ receptor (Ralevic et al., 1996; Schachter et al., 1997). Reports have shown that PPADS inhibits $P2Y_2$ and $P2Y_4$ receptors that have been stably expressed in recombinant cells (Charlton et al., 1996a; Charlton et al., 1996b). There are no reports to show the effects of PPADS on $P2Y_{14}$ receptors; the present results suggest that UDP-glucose-induced contractions, but not MRS2690-induced contractions, are amplified in the presence of PPADS. The ectonucleotidases, specifically ectonucleotidase pyrophosphatases (ENPPs) are responsible for the degradation of UDP-sugars (Lazarowski et al., 2015). Since PPADS is an inhibitor of ENPP1 (Farahbakhsh, 2003), the augmented responses are possibly due to a diminished breakdown of UDP-glucose. In order to determine whether UDP-glucose metabolism is occurring under these conditions, ARL67156, an inhibitor of these ectonucleotidases (Levesque et al., 2007) could be used for

future studies. This could involve looking at the effects of ARL67156 on responses to UDP-glucose; increased contraction to UDP-glucose in porcine coronary arteries in the presence of ARL67156 would suggest that UDP-glucose breakdown is occurring. The results from this study suggest that suramin and PPADS cannot usefully be used to characterise the $P2Y_{14}$ receptor.

7.1.5 $P2Y_{14}$ receptors couple to the inhibition of $G_{i/o}$ proteins in porcine coronary arteries

The coupling of $P2Y_{14}$ receptors to the inhibition of adenylyl cyclase is well documented. $P2Y_{14}$ receptor agonists UDP and MRS2906 (β-ethyl ester of 2-thio-UDP) reduce forskolin stimulated cAMP accumulation in C6-glioma cells, which functionally expresses the $P2Y_{14}$ receptor, and PPTN inhibits the effect produced by UDP and MRS2906 (Carter et al., 2009; Das et al., 2010). UDP-glucose inhibits forskolin stimulated cAMP accumulation in SH-SY5Y neuroblastoma cells and U373 MG glioblastoma cells (Scrivens et al., 2005b) and in T-lymphocytes (Scrivens et al., 2005a).

Vasodilator stimulated phosphoprotein-phosphorylation (VASP) is a substrate of both cAMP-and cGMP-dependent protein kinases (Aszodi et al., 1999). The originality of this study has been that the application used for measurement of VASP-phosphorylation (VASP-P), used as an indirect measurement of intracellular cAMP levels in platelets, has been used in the porcine coronary artery vasculature. The VASP-P assay was used to determine $P2Y_{14}$ receptor coupling to the inhibition of $G_{i/o}$ proteins in the porcine coronary vasculature. Both UDP-glucose and MRS2690 were shown to inhibit forskolin-mediated elevation of VASP-P compared to basal levels. PPTN antagonized and partially

reversed the responses induced by UDP-glucose and MRS2690. The results suggest that the $P2Y_{14}$ receptor is coupled to $G_{i/o}$ proteins in the porcine coronary artery.

7.1.6 Red blood cells and platelets do not induce coronary artery contractility through UDP-glucose release

Human isolated red blood cells and platelets were added to coronary arteries in the presence of PPTN to determine whether UDP-glucose release constitutes a part of the contractile responses induced by these blood cells. Platelets induced immediate relaxations in preconstricted coronary arteries. This was followed by a slow but prolonged contraction. The results were similar to previous findings in which platelets induced relaxations and contractions in endothelium-intact and endothelium-denuded coronary arteries, respectively (Houston et al., 1986). These relaxations correspond to the release of ATP and ADP from platelets (Houston et al., 1986). These adenosine nucleotides activate vasorelaxant endothelial $P2Y_1$ receptors present in porcine coronary arteries (Olivecrona et al., 2004). In the presence of L-NAME, platelet induced relaxations were abolished; a small relaxation was seen followed by a much more profound contraction. Thus, it was hypothesized that platelet induced adenine nucleotide release is accompanied by release of uridine nucleotides, including UDP-glucose, which would activate vasocontractile $P2Y_{14}$ receptors in the porcine coronary artery. UDP-sugar levels have been quantified within platelets (Wandall et al., 2012). The release of UDP-glucose from cells is suggested to occur through regulated release; either exocytosis or release from channel membranes as well as pannexins or non-regulated release such as cellular damage or stress (see Introduction Section 1.6.2).

Thus, PPTN was used to determine whether vasocontractile responses, induced by platelets, could be partially inhibited signifying the presence of UDP-glucose release from platelets. The effect of PPTN on responses to platelets in endothelium-denuded coronary arteries was also investigated. In both conditions, endothelium-intact (presence of L-NAME) and endothelium-denuded (absence of L-NAME), platelet responses were not significantly changed in the presence of PPTN.

UDP-sugar levels have also been quantified in red blood cells (Gibson et al., 1995). In the present study, red blood cells elicited contractions in preconstricted coronary arteries; these effects were not affected by PPTN. Hence, it is concluded that UDP-glucose may not account for the responses induced by both platelets and red blood cell in porcine coronary arteries.

Because of limited antagonist availability (PPTN had been provided as a gift by Merck Biosciences and is not commercially available), a limitation of this study has been the ability to carry out a full investigation. Since there was variability between donors platelets/RBCs responses in the presence of PPTN, there is a need increase the number of subject to reduce the inconsistent responses.

7.1.7 Effect of $P2Y_{14}$ receptor agonists, UDP-glucose and MRS2690, on the release of cytokine/chemokine from porcine coronary arteries

$P2Y_{14}$ receptor expression is upregulated in response to inflammatory injury (Malin et al., 2010). In rat brain microvascular endothelial cells (BMECs), oxygen-glucose deprivation (OGD) increases $P2Y_{14}$ receptor expression.

Geniposide, a constituent of Gardenia and an anti-inflammatory herb, significantly down-regulated $P2Y_{14}$ receptor expression in OGD-induced BMECs (Li et al., 2016). UDP-glucose has been shown to act as a pro-inflammatory stimulant; injection of UDP-glucose into the tail vein of mice significantly increases neutrophil chemoattractants CXCL1, CXCL2 and monocyte chemoattractants CCL2 and CCL3 (Azroyan et al., 2015). The current study showed that $P2Y_{14}$ receptor activation had no significant effect on the release of cytokines from coronary arteries (Chapter 6). The role of the $P2Y_{14}$ receptor as an anti/pro-inflammatory mediator in porcine coronary arteries remains to be determined under physiological as well as pathophysiological conditions.

Zymosan, a component of the yeast Saccharomyces cerevisiae induces inflammatory effects through activation of macrophages (monocytes and dendritic cells (Underhill, 2003). Zymosan has been shown to significantly upregulate the $P2Y_{14}$ receptor putative rat orthologue, VTR 15-20, in microglial and astrocytes (Charlton et al., 1997). Macrophages embedded within coronary arteries could have responded to the UDP-glucose and MRS2690 treatment by releasing inflammatory mediators that were detected in initial experiments. In order to show an involvement of phagocytic cells, zymosan in the absence and presence of PPTN, could be used to investigate the contribution of macrophages as a source of release of inflammatory cytokines and chemokines that were measured.

There could be potential co-activation of P2X7 and $P2Y_{14}$ receptors in the coronary vasculature during inflammation. Damage-induced release of ATP accumulates at the site of inflammation (Bodin et al., 1991; Di Virgilio, 2007).

Increased ATP levels in the external milieu acts as a danger signal initiating innate immune responses. UDP-glucose is usually co-released with ATP under physiological conditions (Lazarowski et al., 2003b), and under pathophysiological conditions, from damaged or injured cells (Azroyan et al., 2015). The P2X7 receptor and other P2 receptors are classical inducers of inflammation. ATP-induced activation of P2X7 receptors expressed on immune cells and endothelial cells leads to the production of several cytokines including IL-1β, IL-18, IL-6 and TNF-α. Chronic exposure to low-dose ATP activates dendritic cells and macrophages to secrete anti-inflammatory markers such as IL-10 and IL-1RA, thus suppressing the effects of pro-inflammatory cytokines (la Sala et al., 2003; Sathanoori et al., 2015). Dendritic cells expressing P2X7 receptors migrate towards high concentrations of ATP (Liu et al., 1999). UDP-glucose induces neutrophil migration and stimulates release of pro-inflammatory mediators from these cells (Azroyan et al., 2015). Whereas ATP activation of P2X7 receptors may be required to induce immediate immune responses, prevention of long-term activation is prevented by metabolism of ATP to resting levels (Lazarowski et al., 2003b). In the current study, it was difficult to establish a definitive role of the $P2Y_{14}$ receptor agonists on cytokine secretion from porcine coronary arteries under the experimental conditions. UDP-glucose is not readily metabolized and remains elevated for up to 3 hours (Lazarowski et al., 2003b), it may act to inhibit or facilitate the release of cytokines under pathophysiological conditions. There may also be potential interactions between P2X7 and $P2Y_{14}$ receptors under pathophysiological conditions; this would be investigated by using selective P2X7 receptor antagonists, such as AZ11645373 (Stokes et al., 2006) to

determine the involvement of ATP acting to induce release of pro-inflammatory cytokines through activation of P2X7 receptors, and investigating these responses in the presence of the $P2Y_{14}$ receptor antagonist.

7.2 Future proposals

7.2.1 Measurement of UDP/UDP-glucose release from smooth muscle cells

Future experiments conducted could measure the potential release of UDP-glucose from porcine coronary arteries and/or smooth muscle and endothelial cells. Thrombin and similar serine proteases activate protease-activated receptors (PAR1-PAR4), a class of GPCRs (Coughlin, 2000). Thrombin has been shown to induce the release of ATP from aortic endothelial and smooth muscle cells (Pearson et al., 1979) and the release of UTP from a number cells including astrocytes, epithelial cells and human platelets (Lazarowski et al., 2003a). Thrombin has also been shown to induce the release of UDP-glucose from airway smooth muscle cells (Kreda et al., 2008). Since porcine coronary arteries express PAR1 and PAR2 receptors, future studies could involve identifying whether thrombin stimulates the release of UDP-glucose and/or UDP from porcine coronary arteries and/or porcine coronary artery smooth muscle cells (Hwa et al., 1996) and whether these levels are sufficient to induce vasoconstriction. There also may be a possibility that UDP/UDP-glucose are released from vascular smooth muscle cells following the activation of P2 receptors, however this remains to be investigated. The levels of UDP/UDP-glucose can be measured using HPLC (see section below).

7.2.2 Quantification of UDP-glucose levels

In preliminary studies in collaboration with Professor Stan Heptinstall and colleagues, (School of Medicine, University of Nottingham), HPLC was performed to measure UDP-glucose levels present in human homogenized blood cells; however a peak in the chromatograph was found which was not UDP-glucose and which interfered with the measurement of UDP-glucose. Therefore, the exact levels of UDP-glucose present within platelets and red blood cells were not identified. For this reason, levels of UDP-glucose proposedly released from platelets and red blood cells were unknown in the current study in which these cells were added to coronary arteries. It is possible that there were not sufficient levels of UDP-glucose released to activate $P2Y_{14}$ receptors. Lazarowski et al. (2003) have established a HPLC assay in which UDP-glucose levels as low as 1nM can be quantified. This is perhaps the only robust assay which can be carried out for future studies that can quantify UDP-glucose levels (Lazarowski et al., 2003b).

7.2.3 Investigating effects of PPTN on responses to UDP-sugar nucleotides

The effects of PPTN on responses to the other UDP-sugar nucleotides acting at $P2Y_{14}$ receptors were not investigated in the current study due to limited antagonist availability, (and indeed no other research has investigated this). As well as platelets being a source of UDP-glucose, there is evidence that has quantified levels of UDP-N-acetylgalactosamine (418 pmol/mg), UDP-N-acetylglucosamine (1195 pmol/mg), and UDP-galactose (320 pmol/mg) in platelets (Wandall et al., 2012). These sugar nucleotides may also be released from platelets and could contribute to porcine coronary artery contractility

through activation of $P2Y_{14}$ receptors. Thus, it would be useful to investigate the effects of PPTN on the responses to the other sugar nucleotides.

7.2.4 Plasma and white blood cells as source of UDP-glucose

Platelets express adhesion receptors on their cell surfaces that participate in the interactions with endothelial cells and white blood cells. Neutrophil-activating peptide-2 is released from the alpha-dense granules of platelets inside the thrombus formed which induces white blood cell migration (Ghasemzadeh et al., 2013; Golebiewska et al., 2015). As discussed in Chapter 5, neither human plasma nor white blood cells were looked at as potential sources of UDP-glucose. Intracellular levels of UDP-glucose measured in white blood cells have been recorded as approximately 32 µM/100g protein (Gibson et al., 1994). Arterial stress or a plaque rupture may subsequently release nucleotides including UDP-glucose from white blood cells causing abnormal coronary artery activation through $P2Y_{14}$ receptors. UTP has been quantitatively measured from human plasma (devoid of both platelets and ectonucleotidases using trichloroacetic acid) (Wihlborg et al., 2006), hence there may be also a pool of UDP-glucose within plasma. Furthermore, UDP-glucose measured in mouse plasma was significantly elevated in obesity compared to healthy controls (Xu et al., 2012), and was also elevated in some tissues from diabetic rats (Spiro, 1984). From these studies, further investigation could be to determine whether white blood cells and plasma are potential sources of UDP-glucose, and are these levels increased in obese individuals. This would perhaps show an involvement of UDP-glucose release from white blood cells and plasma, and consequent activation of $P2Y_{14}$ receptors, in the involvement

of coronary artery contractility during periods of ischaemia or damage, which may be worse in obese and/or diabetic individuals.

7.2.5 Measurement of UDP-glucose in myocardial infarction patients

Wihlborg et al. (2006) have shown certain diseases alter the levels of nucleotide release from man. UTP and UDP levels increase in patients during myocardial infarction and are proposed to contribute to the development of cardiac disease (Wihlborg et al., 2006). In patients diagnosed with symptoms of chest pain and with signs of cardiac disease, the levels of UTP are approximately 14 nM in human plasma (devoid of platelets and ectonucleotidases). Conversely patients diagnosed with ST-segment elevation myocardial infarction, the levels of UTP are significantly increased to approximately 22 nM. Overall, the levels of UTP are approximately 10% of that of ATP (Wihlborg et al., 2006). It can be speculated that since UDP-glucose release can occur in concurrence with ATP (Lazarowski et al., 2003b), it is plausible that UDP-glucose may also be released significantly higher in cardiac diseases (Wihlborg et al., 2006). Certainly as well as exploring other potential sources of UDP-glucose release, there is potential to investigate release of UDP-glucose in myocardial-related diseases. There was no difference in levels of UTP release from males and females, or patients with no cardiac disease and with non-ST elevation myocardial infarction (Wihlborg et al., 2006).

7.2.6 Effect of Montelukast on UDP-glucose induced contraction

$P2Y_{14}$ receptor agonists UDP-glucose, UDP and UDP-galactose are also agonists at the GPR17 receptor (see Chapter 1 for details). UDP-glucose has

been shown to increase the potency of CysLTs binding to the GPR17 receptor in 1321N1 astrocytoma cells (Daniele et al., 2011). Similarly, CysLTs increased the potency of UDP-glucose binding to this receptor. This was possibly due to a change in conformational structure of the receptor that increased the response of each ligand to the other (Daniele et al., 2011). Furthermore the dual functionality of the receptor is perhaps due to two different binding regions and the two ligands interact through allosteric modulation. The effects of UDP-glucose have been shown to be partially inhibited by a CysLT receptor antagonist, montelukast, as phospholipase C and increases in calcium were halted in 1321N1 astocytoma cells. The same effects were also seen when a P2Y receptor antagonist, cangrelor, inhibited the signalling pathways mediated via CysLT receptor agonists (Daniele et al., 2011). The results from these findings pose a potential future study to observe the effects of montelukast on responses to UDP-glucose in porcine coronary arteries.

7.2.7 $P2Y_{14}$ receptor knockouts and knockdowns

A number of $P2Y_{14}$ receptor mouse knockout studies have been carried out (Bassil et al., 2009; Cho et al., 2014; Meister et al., 2014). Bassil et al. (2009) showed that UDP-glucose and UDP-galactose promote rat stomach contractility during electrical field stimulation; however this effect is abolished in $P2Y_{14}$ receptor knockout mice (Bassil et al., 2009). Meister et al. (2014) showed $P2Y_{14}$ receptors are expressed in smooth muscle cells from the ileum to the colon since knockout mice showed that orally administered dextran blue in faeces was delayed compared to wild type mice, suggesting the $P2Y_{14}$

receptor has a role in control of meal passage way throughout the gastrointestinal tract (Meister et al., 2014). Meister et al. (2014) also created a LacZ reporter gene construct to create $P2Y_{14}$ receptor knockout mice; they showed positive staining of beta-galactosidase in several tissues; the uterus, bronchioles, blood vessels, pancreas, salivary glands and parts of the gastrointestinal tract. Expression of $P2Y_{14}$ receptors was found in mainly venous blood vessels, and not arteries, and it was shown that $P2Y_{14}$ receptor knockout mice have increased blood pressure compared to wild type mice (Meister et al., 2014). These findings of Meister et al. (2014) are obviously not in line with the present study since knockout of $P2Y_{14}$ receptors will abolish vasocontractile responses, resulting in a decreased blood pressure, if there is constitutive release of UDP/UDP-glucose which contributes to vascular tone regulation. Vasocontractile $P2Y_{14}$ receptors have been characterized in porcine pancreatic blood vessels (Alsaqati et al., 2014), mouse coronary and mouse mesenteric arteries (Haanes et al., 2014a; Haanes et al., 2014b). However, $P2Y_{14}$ receptors are also expressed in the brain (Bianco et al., 2005; Brautigam et al., 2008; Carter et al., 2009) and central changes in blood pressure control may override local changes produced with a lack of $P2Y_{14}$ receptors in the knockouts. Also, compensatory changes in the expression of other P2Y receptors may occur, in the cardiovascular system and/or CNS. The study carried out by Meister et al. (2014) utilized mouse arteries and, thus, there may be differences in $P2Y_{14}$ receptor responses across species. A future proposal of this study would be to investigate whether there is altered contractility to UDP-glucose/MRS2690 in blood vessels from $P2Y_{14}$ receptor knockout mice. The results from the studies by Bassil et al. (2009) and Meister et al. (2014)

certainly suggest a role of the $P2Y_{14}$ receptor in maintaining homeostasis in regards to vascular systems and gastrointestinal functions.

$P2Y_{14}$ receptors are expressed in haematopoietic stem cells (Cho et al., 2014). $P2Y_{14}$ receptor knockout does not affect haematopoiesis in mice which appear to have normal development. However, under pathophysiological conditions involving total body irradiation and stress, $P2Y_{14}$ knockout mice have significantly lower number of nucleated cells since they are more inclined to undergo ionizing irradiation-inducedsenescence compared to wild types (Cho et al., 2014).

Pathophysiological conditions including stress, injury and ischaemia induce the release of purine and pyrimidine nucleotides (Burnstock et al., 2014). UTP is released from pig hearts under ischaemic conditions, perhaps from the cardiac vein, endothelial cells and red blood cells (Wihlborg et al., 2006). These findings suggest it would be reasonable to investigate whether UDP-glucose could potentially act at other P2Y receptors, including $P2Y_2$ receptors (where it has been shown to act) and thus UDP-glucose could have cardioprotective effects in $P2Y_{14}$ receptor knockouts compared to wild type mice. This could be explored further using $P2Y_2$ knockout mice and double $P2Y_{14}/P2Y_2$ knockouts.

A review of the literature shows that $P2Y_{14}$ receptor knockdown studies have not been carried out in vascular tissues. RBL-2H3 mast cells express $P2Y_{14}$ - receptors, where UDP-glucose and MRS2690 induce degranulation resulting in release of β-hexosaminidase release. $P2Y_{14}$ receptor knockdown by small interfering RNA diminishes this response in mast cells (Gao et al., 2010). Studies have been carried out where siRNA approaches successfully

knockdown signalling proteins in intact arteries (Corteling et al., 2007; Smolock et al., 2007) and $P2Y_6$ receptors have been knocked down in THP-1 monocytic cells (Warny et al., 2001). Since PPTN is not commercially available and throughout my studies there was limited antagonist availability, knockdown studies could perhaps be carried out to determine the effects of $P2Y_{14}$ receptor agonists in intact coronary arteries or smooth muscle cells.

7.2.8 $P2Y_{14}$ receptors as inducers/inhibitors of apoptosis

ATP, UTP and a stable analogue of ATP, ATPγS, all agonists at $P2Y_2$ receptors, reduce serum starvation-induced DNA fragmentation in pheochromocytoma 12 cells (Arthur et al., 2006), a sign indicative of apoptosis (Batistatou et al., 1993). UDP, an agonist at $P2Y_6$ receptors,inhibited cell growth and induced apoptosis in SHSY5Y neuroblastoma cells overexpressed $P2Y_6$ cells (Apolloni et al., 2010). High concentrations of ATP have been shown to be cytotoxic and to induce apoptosis in dendritic cell that highly express P2X7 receptors (Coutinho-Silva et al., 1999). These studies provide an avenue to explore the effects of UDP-glucose, or MRS2690 as inducers or inhibitors of apoptosis in coronary artery smooth muscle or endothelial cells. Methodology outlined by Arthur et al. (2006) could be used to measured apoptosis; isolated coronary artery smooth muscle cells or endothelial cells could be serum starved for 12 hours, in the presence of $P2Y_{14}$ receptor agonists/antagonist, and DNA fragmentation could consequently be measured. Thus, the effect of the $P2Y_{14}$ receptor in regulation of cell survival in vasculature could be investigated.

7.2.9 Mitogenic and migratory effects of the $P2Y_{14}$ receptor in coronary arteries

As well as stimulators of vascular tone, P2Y receptor activation have been proposed to act as mitogenic stimulator in vascular smooth muscle cells (see Introduction, Section 1.3.5). Thus, $P2Y_{14}$ receptors function as stimulators of mitogenesis in porcine coronary artery smooth muscle cells could be explored. The methodology outlined by Erlinge et al. (1998) could be adopted as an approach to investigate this; in order to determine whether $P2Y_{14}$ receptor agonists stimulate mitogenesis, DNA synthesis can be measured using a tritiated (^{3}H) thymidine-incorporation assay. This would involve culturing coronary artery smooth muscle cells (from pigs and humans) and serum starving for 24 hours to induce quiescence, and after addition of $P2Y_{14}$ receptor agonists (ideally known to be stable) for approximately 24 hours, ^{3}H-thymidine could be added. The incorporated ^{3}H-thymidine in the DNA of coronary artery smooth muscle cells could then be measured using a scintillation counter. Increased levels of ^{3}H-thymidine incorporation in the presence of UDP-glucose or MRS2690 would suggest that $P2Y_{14}$ receptors acts to serve as a stimulator for mitogenesis in coronary artery smooth muscle cells.

$P2Y_{14}$ receptor activation in neutrophils leads to reorganization of actin and myosin filaments, cytoskeleton remodelling, changes in cell shape all leading to increased motility and migration (Sesma et al., 2012). $P2Y_{14}$ receptor activation in cardiac endothelial cells in microvessels stimulates proliferation, migration and secretion of growth factors (Horckmans et al., 2012). From these studies, a potential future experiment could be to investigate the effects of

$P2Y_{14}$ receptor activation as inhibitors of migration and thus a protective role of the $P2Y_{14}$ receptor in conditions of abnormal cell proliferation.

7.2.10 Barrier function

There is a report that shows an absence of $P2Y_{14}$ receptor mRNA in human cultured coronary artery endothelial cells (HCAECs) (Ding et al., 2011), thus experiments to investigate $P2Y_{14}$ receptor in barrier function were not carried out in this thesis. However, endothelial $P2Y_2$ receptors can affect vascular permeability and could be activated by UDP-glucose.

7.3 Concluding comments

Under physiological conditions, purine and pyrimidine nucleosides and nucleotides function as signalling molecules that target purinoceptors to maintain homeostasis. UDP-glucose and other UDP-sugars are released alongside other nucleotides including ATP. Therefore nucleotide effects in the cardiovascular system and blood vessels will be a combination of multiple P2 receptor subtypes including $P2Y_{14}$ receptors and the P1 and P2 receptors. Under pathophysiological conditions such as hypoxia, shear stress or endothelial damage due to an inflammatory response, there is a release of nucleotides, as well as other compounds (such as serotonin, endothelin, thromboxane) which adds complexity to the responses seen in the cardiovascular system. For example, results in Chapter 3 showed how UDP-glucose vasocontractile responses are augmented by raised tone due to U46619. Therefore, although characterisation of the $P2Y_{14}$ receptor, in coronary arteries, is potentially useful there is a much bigger and complex pathophysiological response that occurs.

The thesis investigated two potential roles of the $P2Y_{14}$ receptor in the cardiovascular system, namely agonist-induced contraction in coronary arteries and release of inflammatory markers from coronary arteries. Other trophic functions of the $P2Y_{14}$ receptor were not investigated; these may include a role of the $P2Y_{14}$ receptor in smooth muscle growth, proliferation, migration and apoptosis. Experiments conducted in this thesis only looked at coronary arteries from pigs, however, there may be species variation, therefore function of $P2Y_{14}$ receptors in human tissue could be investigated.

7.3.1 Clinical outcomes

Chronic activation of P2Y receptors, particularly $P2Y_1$ (Zerr et al., 2011) and $P2Y_2$ receptors (Seye et al., 2004), plays a pivotal role in the development of atherogenesis, ischaemia and stroke. Severe damage to endothelial cells caused by abnormal blood flow, platelet aggregation and cell lysis, releases a high concentration of nucleotides that causes endothelial dysfunction and damage (Di Virgilio et al., 2002). The high concentration of nucleotides causes sustained P2Y receptor activation which may increase the expression of adhesion molecules, stimulate the recruitment of immune cells leading to the development of an inflammatory environment which progresses the formation of atherosclerotic plaques (Martinez et al., 2012). Furthermore, a rupture in atherosclerotic plaques can cause further damage to the endothelial layer causing excessive nucleotide release; this may expose purinoceptors, including $P2Y_{14}$ receptors, located on the smooth muscle which are consequently activated causing vasospasm (Malmsjo et al., 2000b). The $P2Y_{14}$ receptor plays a role in regulation of vascular tone (Alsaqati et al., 2013; Alsaqati et al., 2014;

Haanes et al., 2014a). Thus, the $P2Y_{14}$ receptor may be a potential target for the treatment of cardiovascular diseases, for example in the event of a pathophysiological response such as stress and hypoxia.

8.1 Appendices

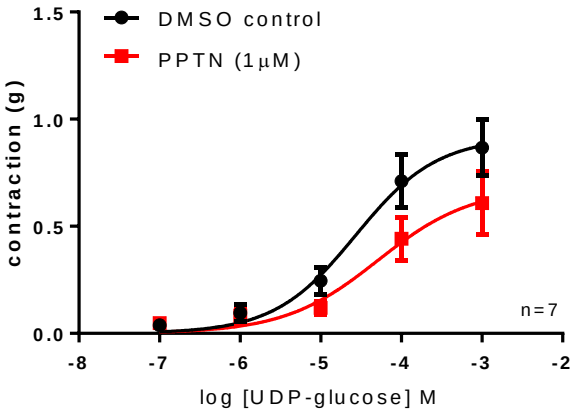


Figure A.1: The effect of the selective $P2Y_{14}$ receptor antagonist, PPTN (1 μM), on contractile responses to UDP-glucose in U46619-induced preconstricted porcine coronary arteries. There was no significant change in the responses to UDP-glucose in the presence of PPTN ($P > 0.05$, ANOVA followed by Sidak's multiple comparisons test).

Donor	Gender	Age	Blood cell taken
1	Female	35	platelets
2	Male	28	platelets
3	Male	30	platelets
4	Male	42	platelets
5	Female	30	platelets
6	Male	32	platelets
7	Male	32	platelets
8	Female	30	platelets
9	Female	27	platelets
10	Male	35	RBCs
11	Male	29	RBCs
2	Male	30	RBCs
13	Male	42	RBCs
14	Male	28	RBCs

Table A: The gender and ages of the blood donors and types of blood cell taken.

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