Characterisation of responses to PGE_2-G in the porcine coronary artery, rat aorta and murine microglial cell line BV-2.

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Abstract

Prostaglandin glyceryl esters are derived from cyclooxygenase-2 oxygenation of the endocannabinoid 2-arachidonoylglycerol. Prostaglandin E_2 glyceryl ester (PGE_2G) may be involved in inflammation but very little else is known about its patho/physiological roles. A recent study identified interaction of PGE_2G with $P2Y_6$ receptors in isolated cells. $P2Y_6$ receptors are involved in cardiovascular regulation and UDP is the agonist. In this study, it was investigated whether PGE_2G can influence vascular contractility through $P2Y_6$ receptors.

Isometric tension recordings were used to evaluate responses of segments of porcine coronary artery (PCA) (stored at 4ºC overnight) and rat thoracic aorta (freshly isolated). In PCA, UDP (1.3×10^{-6}-1.3×10^{-3} M) and PGE_2-G (1.3×10^{-9}-4×10^{-6} M) produced concentration-dependent contractions which were unaffected by $P2Y_6$ receptor antagonist, MRS 2578. UDP contractions were blocked by P2 receptor antagonist suramin ($p<0.001$; two-way ANOVA). UDP also acts at $P2Y_{14}$ and $P2Y_2$ receptors; antagonists PPTN ($P2Y_{14}$) and AR-C118925XX ($P2Y_2$) failed to alter UDP responses. UDP responses were also characterised in U46619-precontracted PCAs in presence of AR-C 118925XX in which there was no evidence for the presence of relaxant $P2Y_6$ receptors and enhanced PGE_2-G responses were produced in endothelium-denuded vessels, pointing out involvement of a smooth muscle coupled receptor in mediating responses. PGE_2-G activates EP receptors; nonselective EP antagonist AH-6809 significantly blocked contractile responses evoked by both PGE_2-G ($p<0.05$ at 4×10^{-6} M) and UDP ($p<0.001$ at 10^{-3} M).

In rat aorta, UDP (1×10^{-8}-1×10^{-5}M) produced concentration-dependent relaxations (pEC_{50} 6.4 ± 0.1, R_{max} 77 ± 8%) which were unaffected by MRS 2578 (pEC_{50} 6.4 ± 0.1, R_{max} 80 ± 10% (n=7). PGE_2-G (1×10^{-10}-3×10^{-6}M) produced concentration-dependent relaxations (pEC_{50} value of 8.6 ± 0.2 and R_{max} 31 ± 5%) which were also unaffected by MRS 2578 (pEC_{50} value of 8.6 ± 0.3; R_{max} 25 ± 5%, n = 7). UDP also acts at $P2Y_2$ receptors; responses characterised in presence of AR-C 118925XX demonstrated enhanced responses, showing involvement of another P2Y receptor. Endothelium-denuded

experiments presented evidence for attenuated UDP responses demonstrating involvement of a receptor coupled to endothelium. Application of the nonselective EP antagonist AH-6809 did not alter PGE_2-G evoked relaxations but concentration-dependent contractions evoked at 0.3 μM were abolished ($p<0.0001$ at 3×10^{-6} M) and UDP responses (pEC_{50} value 5.2 ± 1.6 and R_{max} of 77 ± 8%) were also enhanced (pEC_{50} value of 6.7 ± 0.1 and R_{max} of 89 ± 13%, $p<0.05$, n=6). Calcium release evoked by UDP (1×10^{-4}-6×10^{-9} M) in presence of MRS 2578 was significantly attenuated in $P2Y_6$ expressing BV-2 cells. PGE_2-G (1×10^{-4}-6×10^{-9} M) responses remained unchanged.

In summary, UDP and PGE_2-G responses appear to behave independently to the $P2Y_6$ receptor in both blood vessels however, UDP mediated calcium release demonstrated involvement of $P2Y_6$ receptors unlike PGE_2-G. Evidence points out that PGE_2-G responses do not involve activation of $P2Y_6$ receptors but is more potent than UDP at evoking responses. Inhibition by AH-6809 suggests that UDP may be stimulating a P2Y receptor mediated prostanoid release. Overall, the identities of UDP and PGE_2-G-sensitive receptors in the blood vessels is yet unclear but it does not involve $P2Y_2$, $P2Y_6$ or $P2Y_{14}$ receptors. Thus, further molecular targets are being investigated.

Keywords: $P2Y_6$ receptor, UDP, PGE_2-G, prostanoid, smooth muscle, endothelium, porcine coronary artery, rat aorta

Abbreviations: U46619, 9,11-dideoxy-11α,9α - epoxy-methano-prostaglandin F2α; MRS 2578, N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)thiourea; PPTN, 4-((piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid; AR-C 118925XX, 5-[[5-(2,8-Dimethyl-5*H*-dibenzo[*a*,*d*]cyclohepten-5-yl)-3,4-dihydro-2-oxo-4-thioxo-1(2*H*)-pyrimidinyl]methyl]-*N*-2*H*-tetrazol-5-yl-2-furancarboxamide; AH-6809, 6-Isopropoxy-9-xanthone-2-carboxylic acid.

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Abbreviations

2-AG	2-arachidonoylglycerol
AC	Adenylate cyclase
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BK	Bradykinin
$[Ca^{2+}]i$	Intracellular calcium
cAMP	Cyclic adenosine-5'-monophosphate
CCh	Carbachol
COX	Cyclooxygenase
CNS	Central Nervous System
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
EDCFs	Endothelium-derived contractile factors
EDHF	Endothelium-derived hyperpolarising factor
EDRFs	Endothelium-derived relaxing factors
EGFR	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GPCRs	G-protein-coupled receptors
IP_3	Inositol 1.4.5-triphosphate
HEK	Human embryonic kidney
KO	Knockout
KCl	Potassium chloride
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NA	Noradrenaline

NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Phenylephrine
PCA	Porcine coronary artery
PG-Gs	Prostaglandin glyceryl esters
PGE_2	Prostaglandin E_2
PGE_2-G	Prostaglandin E_2 glyceryl ester
PKC	Protein kinase C
PLC	Phospholipase C
RFU	Relative fluorescence units
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
TXA_2	Thromboxane
UDP	Uridine-5'-diphosphate
UTP	Uridine-5'-triphosphate
VSMC	Vascular smooth muscle cell

Chemical Names

AH-6809 6-Isopropoxy-9-xanthone-2-carboxylic acid

AR-C 118925XX 5-[[5-(2,8-Dimethyl-5*H*-dibenzo[*a*,*d*]cyclohepten-5-yl)-3,4-dihydro-2-oxo-4-thioxo-1(2*H*)-pyrimidinyl]methyl]-*N*-2*H*-tetrazol-5-yl-2-furancarboxamide

MRS 2578 N,N″-1,4-butanediyl bis(N′-[3- isothiocynatophenyl)] thiourea

PPTN 4-[4-(4-Piperidinyl)phenyl]-7-[4-(trifluoromethyl)phenyl]-2-naphthalenecarboxylic acid hydrochloride

PSB 0474 3-(2-Oxo-2-phenylethyl)-uridine-5'-diphosphate disodium salt

U46619 9,11-dideoxy-11α,9α - epoxy-methano-prostaglandin F2α

Chapter One

General Introduction

This chapter will initially focus on the description of blood vessel structure followed by mechanisms controlling blood flow. Thereafter, P2 receptors and their classification will be reviewed, with a primary focus on the $P2Y_6$ receptor and its ligands. Finally, the introduction will conclude with a description of the aims and objectives of the project.

1.1. The Cardiovascular System and The Vasculature

1.1.1. Blood vessel structure

The cardiovascular system (CV) is a complex network consisting of essential components; the heart, blood and vasculature (arteries, veins and capillaries). All the blood vessels with the exception of capillaries, have the same basic structure: an outer layer also known as *tunica externa* (adventitia), a middle layer also known as *tunica media* (media) and an inner layer also known as *tunica intima* (intima). The adventitia provides structural support to the vessel and is comprised of perivascular nerves, connective tissue, elastic and collagen fibres. The medial layer is the thickest layer and has characteristics to provide muscular support and ability for the vessel to stretch. This layer comprises of multiple layers of circumferentially oriented vascular smooth muscle cells (VSMCs) and elastic fibres. The vascular tone is determined on the basis of the contractile state of the media, essential in the control and regulation of blood pressure. The intimal layer is the thinnest, innermost layer comprising of endothelial cells which line the entire vasculature. This endothelial layer forms an interface between blood and the vessel wall and is vital for the control of vascular tone. The endothelial cells (ECs) line the entire vasculature and display different morphological and phenotypic features depending on the type of vessel. In arteries and veins, ECs are thicker and continuous as opposed to a thinner and

fenestrated appearance in capillaries, to enable exchange of gases and metabolites (Ghitescu & Robert, 2002; dela Paz & D'Amore, 2008).

The endothelium is known as the largest regulatory organ that is essential for cardiovascular homeostasis based on its numerous physiological and pathological functions and key localisation within the vasculature. Under normal physiological conditions, the endothelium contributes to maintaining vascular smooth-muscle tone, cellular growth and proliferation, angiogenesis immune and inflammatory mechanisms in the vessel wall. Control of the vascular tone is mediated by the endothelial cells which release various constricting and relaxing factors in response to physiological and pathological conditions (Rubanyi, 1993; Sandoo et al., 2010).

In addition, the endothelium has antithrombotic, anticoagulant and fibrinolytic regulatory properties and is involved in maintaining a nonadhesive luminal surface which is disrupted when the endothelium is damaged during pathological conditions. External stimuli (hemodynamic forces, vasoactive substances and mediators released from cells), trigger synthesis and release of biologically active substances which control the calibre of the blood vessels and structure of vascular smooth muscle layer to control blood flow (Rubanyi, 1993; Sandoo et. al 2010). Stimuli-induced vascular tone changes are mediated via intercellular junctions which mediate both the intracellular adhesion and communication between the endothelial cells and VSMCs (dela Paz & D'Amore, 2008).

1.1.2. **Mechanism of control of arterial blood pressure**

Regulation of the vascular tone plays an essential role in maintenance of blood flow and pressure. Increases in blood pressure is associated with various cardiovascular diseases such as atherosclerosis, heart failure, ischaemic heart

disease, stroke and renal damage. The control of blood vessel tone is regulated by the endothelium which releases various vasoactive factors. These include vasodilatory factors such as prostacyclin (PGI_2), nitric oxide (NO) and the endothelium derived hyperpolarizing factor (EDHF) and vasoconstrictive factors such as endothelin-1 (ET-1) and thromboxane (TXA_2). Nitric oxide is known as an endothelium-dependent vasodilator associated with maintaining a basal vasodilator tone and is synthesised under the influence of a nitric oxide synthase enzyme (NOS). Three isoforms of NOS exist namely: neuronal isoform (nNOS) which produces NO which behaves as a neuronal messenger to regulate neurotransmitter release at the synapse; inducible isoform (iNOS) which is expressed in cells exposed to injurious/inflammatory stimuli, enabling activation of macrophages and endothelial NOS (eNOS) which produces NO in the vasculature. In the blood vessels, NO diffuses from the endothelium into the smooth muscle adjacently where it binds to the enzyme soluble guanylyl cyclase (sGC). The activated enzyme converts the inactive guanosine triphosphate (GTP) to active cyclic guanosine monophosphate (cGMP) which is associated to decrease smooth muscle tension. Calcium release from the sarcoplasmic reticulum is reduced in the smooth muscle cells and is also associated to restore calcium levels in the sarcoplasmic reticulum, reducing the contraction of smooth muscle cells. During pathological conditions, inflammatory mediators such as chemokines stimulate release of iNOS which induces release of NO to prevent platelet and leukocyte activation and adhesion to the vessel wall (Sandoo et al., 2010)

Prostacyclin (PGI_2) and thromboxane (TXA_2) are products of the cyclooxygenase (COX) enzymes. Prostacyclin binds and activates prostacyclin receptors (IP) expressed on both vascular smooth muscle cells (VSMCs) and platelets. On platelets, activation of IP receptors results in platelet aggregation whereas activation of IP receptors on the VSMCs, activates adenylate cyclase (AC), inducing synthesis of cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A, relaxing the smooth muscle. It is

associated to play a role as a compensatory vasodilator when the bioavailability of NO is reduced. TXA_2 on the other hand, binds to thromboxane-prostanoid (TP) receptors located on platelets to induce platelet aggregation and activation of TP receptors on the VSMCs, evokes intracellular calcium release, leading to vasoconstriction. The balance in both activities of TXA_2 and PGI_2 is essential in maintaining the homeostasis of the vessel (Sandoo et al., 2010).

Endothelin-1 (ET-1) binds and activates ET-1 receptors on the SMCs and ECs. Activation of receptors on the SMCs, promotes opening of calcium channels to allow extracellular calcium into the cell, inducing vasoconstriction in the same mechanism as TXA_2. Activation of ET-1 receptors on the endothelium induces vasodilation by promoting release of NO and PGI_2. ET-1 also contributes to inflammation and smooth muscle cell proliferation in the vessel. Finally, EDHF hyperpolarises the underlying smooth muscle by making the membrane potential of the cell more negative. The change in the membrane potential of the VSMCs results in reduced intracellular calcium levels, mediating relaxation (Sandoo et al., 2010). It has been known that NO is the predominant relaxing factor in larger arteries and EDHF in smaller arteries/arterioles.

1.1.3. Nucleotide control of vascular tone

Stimuli such as shear stress, hypoxia and injury alter the morphology, function and gene expression of the ECs (Jacobson et.al., 2012; Ralevic & Dunn, 2015). This functional alteration results in release of nucleotides such as ATP, ADP, UTP and UDP from endothelial cells and various other cells (including, erythrocytes, circulating cells and platelets). These ubiquitous intracellular messengers are released in the extracellular space and act in an autocrine or paracrine manner. These nucleotides and nucleosides (such as adenosine) activate ligand-specific, membrane-bound receptors (P1, P2X and P2Y) which activate various intracellular signalling cascades to regulate cellular processes

such as proliferation, nociception, cell adhesion and migration. These receptors are present in the perivascular nerves, endothelial cells and smooth muscle cells in the blood vessels and contribute to functions such as vascular tone and remodelling, growth and immune function. (Ralevic & Dunn, 2015). To activate these receptors, nucleotides are released from cells to the extracellular space via various mechanisms such as vesicular transport, exocytosis and membrane channels (ABC transporters, connexins and pannexins) (Lazarowski., 2012). For example, ATP is released by exocytosis, co-packaged with other molecules from excitatory and non-excitatory cells. Once released, nucleotides mediate pleiotropic vascular effects, including vessel contraction.

1.2. The Role of Purinergic Signalling in the Cardiovascular System

1.2.1. Types of purine and pyrimidine receptors

Purinergic signalling plays an essential role in various physiological and pathological processes including neurotransmission, cardiovascular function, post-natal development and aging (Burnstock, 2016). The various subtypes of purinoceptors are widely distributed throughout tissues and living cells. A total of 19 receptors are present for adenine, uridine, adenosine and nucleotide sugars. Based on their pharmacological properties, molecular cloning and signal transduction mechanisms, these receptors are classified into 2 subtypes namely: P1 receptors also known as ‘adenosine receptors’ and P2 receptors. P1 receptors comprise of 4 subtypes namely: A_1, A_{2A}, A_{2B} and A_3 which are all G-protein-coupled receptors (GPCRs) activated by adenosine. These heterogenous GPCRs consist of a typical GPCR structure involving 7 hydrophobic transmembrane regions connected by intracellular and extracellular loops. They couple with heterotrimeric G proteins which consist of an α subunit (Gα) tightly bound to a βγ subunit at the inner surface of the plasma membrane. These G proteins are divided into subfamilies namely: Gs, Gq, Gi/o and G12/13, each coupling to different downstream effectors (Nishimura et al., 2017).

A_1 and A_3 receptors couple to Gi/o proteins, supressing adenylyl cyclase activity and increase cAMP, whereas A_{2A} and A_{2B} receptors couple to Gs proteins, activating AC, reducing cAMP levels. All four subtypes are expressed on ECs and VSMCs of blood vessels with distinct roles in regulating the vascular tone (Erb & Weisman, 2012). A_{2A} receptors have higher affinity to adenosine in comparison to A_{2B} and are therefore activated based on levels of adenosine concentrations. Both involve nitric oxide (NO) production in coronary artery ECs, activation of ATP-sensitive K^+ channel (K_{ATP}) channels to mediate coronary vasodilation and possibly activation of p38 mitogen activated protein kinase (MAPK) (Teng et. al., 2005; Sanjani et al., 2011).

1.2.2 P2X and P2Y receptors

P2 receptors are divided into 2 major families: Fast P2X ion channels and slow metabotropic P2Y receptors. To date there are 7 ligand-gated P2X (P2X1-7) and 8 transmembrane P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) receptors (Jacobson et al., 2012). P2X receptors are activated by ATP, concomitantly released with noradrenaline (NA) as a sympathetic neurotransmitter or released during cell damage from either necrotic/apoptotic cells to mediate smooth muscle cell depolarization and thereby inducing vascular contraction via actions on the P2X receptors on the vascular smooth muscle. Via activation of smooth muscle and endothelial P2Y receptors, ATP is also associated to mediate vasorelaxation. In addition, ATP is also associated to play a role as a neurotransmitter in non-adrenergic non-cholinergic (NANC) perivascular nerves. ATP and NA relative contribution as functional cotransmitters is associated to vary with size and type of the blood vessel, tone/pressure of the vessel, species type, neuronal firing pattern and physio/pathological condition. (Ralevic & Dunn 2015).

The P2X receptors are more structurally restrictive than P2Y in agonist selectivity and are associated with contributing to short-term purinergic signalling (Burnstock, 2016). P2X receptors are widely expressed in the cardiovascular system with P2X1 receptor being a highly expressed receptor in the heart, platelets, smooth muscle (SM) and majority of blood vessels. Additionally, P2X receptors also have pivotal roles in immune functions, regulation of contractility, platelet aggregation, cell proliferation and macrophage activation (Kaczmarek-Hájek, Lörinczi et al., 2012; Erb & Weisman, 2012). Detection techniques such as immunostaining and RT-PCR have reported localisation of all seven P2X receptor proteins and transcripts on the endothelium and the smooth muscle (Ralevic & Dunn, 2015).

P2Y receptors belong to class A or Family 1 GPCRs and are coupled to induce long-term purinergic signalling (Burnstock, 2016). They are grouped further into subfamilies based on their G-protein coupling and sequence similarities. P2Y1-like receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) all share 28-52% sequence homology and couple mainly with Gq, activating phospholipase Cβ and mobilizing calcium. Gi/o coupled P2Y12-like receptors ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) share 45-50% sequence homology and are coupled to inhibit AC activity. P2Y11R is able to couple with Gs, activating AC activity. In addition, most Gq coupled receptors are known to couple with other G protein subfamilies especially Gi/o and G12/13 (Erb & Weisman, 2012). $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ show sensitivity to adenosine diphosphate (ADP); $P2Y_2$, rodent $P2Y_4$ and $P2Y_{11}$ show sensitivity to ATP; $P2Y_2$ and $P2Y_4$ show sensitivity to uridine triphosphate (UTP); $P2Y_6$ and $P2Y_{14}$ demonstrate sensitivity to uridine diphosphate (UDP) and $P2Y_{14}$ shows sensitivity to UDP, UDP-glucose and other UDP-sugars (Jacobson et al., 2012). P2Y receptor expression is widespread in terms of both species and vessels examined and cell types (i.e.: those involved in inflammation, innate immunity and thrombosis all express multiple subtypes of P2 receptor) (Hechler$_i$ & Gachet, 2015). P1 and P2 receptors for purines and pyrimidines are known to be expressed in various biological systems including

the cardiovascular systems, peripheral and central nervous systems, gastrointestinal, respiratory and immune systems and mediate a variety of diverse effects including neurotransmission, immune function, regulation of contractility, cell migration and proliferation.

In the vasculature, extracellular nucleotides control blood flow via activation of P2 receptors. This can be a complex interplay considering the widespread nature of the nucleotide release, influence of site of expression (endothelium vs smooth muscle) and the number of P2 receptors (Rayment et. al., 2009). The complexity also arises when ectonucleotidases are in play; they belong to a family of ectoenzymes that control the concentration of extracellular nucleotides by catalysing the breakdown of nucleotides to nucleosides; leading to interconversion between ligands enzymatically (e.g.: metabolism of ATP to adenosine) and therefore, initiating complex integrated responses via activation of different purine receptor subtypes. These enzymes are present on smooth muscle and ECs surface and may be coreleased with neurotransmitters from perivascular nerves (Kauffenstein et al., 2010; Ralevic & Dunn, 2015).

In the vasculature, ectoenzymes such as ectonucleotide triphosphate diphosphohydrolase 1 (E-NTPDase also known as CD39 or ecto-apyrase) is present which is associated with breaking down ATP and ADP to produce AMP, which can be further metabolized into adenosine by the enzyme ecto-5'-nucleotidase (CD73). During conditions such as shear stress, there is enhanced release of nucleotides as well as an accompanied increase in the activity of ectonucleotidases (Yegutkin et al., 2000). E-NTPDase1/CD39 is expressed on the endothelium, leukocytes and platelets and by metabolising ATP and ADP, inhibits platelet accumulation, vascular occlusion and thrombus formation (Sévigny et al., 2002; Drosopoulos et al., 2010). CD39 localized to arterial smooth muscle cells is associated with reducing number of nucleotides (ATP, ADP, UTP, UDP) to limit the activation of P2 receptors and therefore, the extent and duration of constriction. Ectonucleotidases therefore, play an essential role

in regulating levels of nucleotides such as ATP in the lumen of the vessel as well as in the extracellular milieu, therefore reinforcing the dual effect of purines on both the blood pressure and peripheral resistance.

In general, activation of P2X and P2Y receptors on the VSMCs generally mediates vasoconstriction of the vessel whereas endothelial activation of P2 receptors is generally associated with promoting a vasorelaxation. Vascular purinergic system is therefore considered a vital signalling process involved in regulating both physiological (vasoconstriction and vasorelaxation) and pathological (vascular inflammation) processes and studies have shown their implications in a number of cardiovascular diseases (Ralevic & Dunn, 2015; Burnstock & Ralevic, 2013) and therefore, are potential targets for therapeutic interventions. However, with the exception of the use of adenosine via A1 for the treatment of supraventricular tachycardia and $P2Y_{12}R$ inhibitors such as clopidogrel as antithrombotic agents, therapeutic interventions targeting other P2 receptors are not yet resolved (Nishimura et al., 2017).

1.2.3 $P2Y_6$ receptor

The nucleotide $P2Y_6$ receptor is ubiquitously expressed in various organs, tissues and blood vessels (Bar et al., 2008). The receptor is classified as a GPCR coupled to Gq protein associated with activating phospholipase Cβ and therefore an increase in intracellular calcium levels through inositol 1,4,5-triphosphate stores and activity of protein kinase C (PKC) (Erb & Weisman, 2012). Uridine 5' diphosphate (UDP) is a potent agonist at the $P2Y_6$ receptor but also acts at $P2Y_2$ (Burnstock & Williams, 2000) and $P2Y_{14}$ receptors (Carter et al., 2009; Harden et al., 2010). Molecular modifications for UDP have been carried out to increase selectivity at the $P2Y_6$ receptor and this has resulted in formation of stable analogues, UDPβS, 3-phenacyl UDP (PSB 0474), 5-iodo-UDP (MRS 2693) and 5-methoxy-UDP which are all coupled to increase cytosolic calcium

levels via Gq coupling. (Besada et al., 2006; Jacobson et al., 2010; Jacobson et al., 2012).

$P2Y_6$ receptor profile has been well established in terms of their functions e.g.: Regulation of blood pressure (Bar et al., 2008), potentiating secretion of insulin for treatment of diabetes (Balasubramanian et al., 2013; Ohtani et al., 2008); treating ocular hypertension and glaucoma (Jacobson & Civan., 2016; Shinozaki et al., 2017), beneficial in skeletal muscle injury treatments (Mamedova et al., 2008), promoting cellular repair responses (Ide et al., 2014) and more clinical implications.

In addition to the known $P2Y_6$ agonists, a recent study has identified that prostaglandin glyceryl ester (PGE_2-G) couples to the $P2Y_6$ receptor with an EC_{50} value of ~1pM. Since most of the endogenous agonists for GPCRs have EC_{50} values of >1nM, it suggests that PGE_2-G has an extraordinarily high affinity for the receptor $P2Y_6$ (Brüser et al., 2017). In contrast to prostaglandins which have been well known, the function and signalling pathways for prostaglandin glyceryl esters (PG-Gs) are still being studied. Cyclooxygenase-2 (COX-2) isoenzyme is associated with oxygenating 2-arachidonoylglycerol (2-AG) leading to the formation of PG-G endoperoxides which are further converted to PGE_2-G, PGD_2-G, $PGF_{2\alpha}$-G and PGI_2-G (Kozak et al., 2002). PGE_2-G has shown independent properties to prostaglandin PGE_2 and does not effectively activate the known prostanoid receptors $EP_{1\text{-}4}$, DP, FP, TP or IP and possesses at least 2 orders of magnitude less potency than the prostaglandin E_2 at EP receptors (Nirodi et al., 2004).

The PGE2-G/$P2Y_6$ signalling by Brüser et. al (2017) was established using $P2Y_6$ transfected HEK 293 cells. Reduction of intracellular cAMP levels, IP formation, ERK1/2 phosphorylation and intracellular calcium release associated with activation of this signalling pathway was similar to cells highly expressing $P2Y_6$ receptor endogenously (RAW264.7 and H1819 cells). Furthermore, based

on a mutagenesis study and $P2Y_6$ homology model, it was found that UDP and PGE_2-G shared the predicted binding pocket but also had individual binding pockets/determinants specific to each of the agonists at the $P2Y_6$ receptor (Brüser et. al 2017) and therefore, each agonist mediated effects in distinct ways. Very little information is known about this signalling mechanism, especially since it has not been studied extensively and is therefore of interest to understand this mechanism.

1.3. $P2Y_6$ receptor expression in microglia

Microglia are also known commonly as resident macrophages in the central nervous system (CNS). Under normal conditions, CNS is exposed to extracellular nucleotides under various conditions such as the synaptic cleft where they behave as neurotransmitters (Ralevic and Burnstock 1998). During pathological conditions such as tissue injury, extracellular nucleotides such as ATP are released from neurons/damaged cells which function as warning molecules to mediate functions such as astrogliosis, chemotaxis, phagocytosis, activation of the inflammasome or release of cytokines to sustain neuroinflammation (Dubyak and El-Moatassim 1993). The activation of microglia is classified as a neuroinflammatory response. There is an enhanced nucleotide release and therefore, an increased activation of P2 purinoceptors (P2X ligand-gated ion channels and P2Y G-protein-coupled receptors) on the microglial cells. Microglial cells express both P2X and P2Y receptors and activation of either, is associated with increasing intracellular calcium levels, either by increasing calcium permeability of the sarcolemma (P2X-mediated) or phospholipase (PLC) mediated release from internal stores (P2Y-mediated). Activation of P2 receptors on the microglia leads to multiple intracellular signal transduction pathways and regulation of both pro- and anti-inflammatory microglial cell functions including activating mitogen-activated protein kinase (MAPK), transcription factors (NF-kB, NFAT, AP-1 and CREB), promotes

release of pro-inflammatory cytokines and is largely involved in controlling the inflammatory gene expression (James and Butt, 2002).

$P2Y_6$ is present in the microglia and has an inflammatory phenotype associated with it. The murine microglial cell line, BV-2 is of particular interest. It is an immortalized microglial cell line that exhibits functional and morphological features to that of primary microglial and are therefore, most commonly used as a reliable alternative model for primary microglia cultures (Henn et al., 2009). This cell line is also associated with possessing complex cell-cell interaction. $P2X_4$ and $P2X_7$ are the predominant P2X purinoceptors expressed on the cells (Raouf et al., 2007). Detection techniques such as RT-PCR was implemented to study the expressional levels of various purinoceptors in BV-2 cells. Using immunofluorescence and western-blot assay, $P2Y_{12}$ was shown to be predominantly expressed followed by significant levels of $P2Y_{14}$, $P2Y_{13}$, $P2Y_6$ and then $P2Y_2$ receptors. This is also supported by Gendron et al (2003) suggesting that mRNA for $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ as well as $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ are present with the exception of $P2Y_4$ and $P2X_2$ receptors in BV-2 cells and the less detected, $P2Y_{11}$ receptors.

In the cellular system, extracellular nucleotides are associated to mediate a range of physiological functions. For example: $P2Y_6$ receptor was coupled to promote microglial phagocytosis resulting from calcium mobilisation (Jiang et al., 2017). Hyperactivation of the microglia is a consequence that may arise during conditions such as brain injury, during which nucleotide release is abundant in the extracellular milieu. The microglia release cytokines, nitrogen species and reactive oxygen species (ROS) which play a role in exacerbating neuronal injury Purinoceptors are also increasingly activated and may therefore become desensitized in BV-2 cells, delaying the response time (Stoll et al., 2000).

With the understanding that PGE_2-G is not widely studied, the PGE_2-G/$P2Y_6$ signalling was investigated based on their calcium responses in endogenously

$P2Y_6$ receptor expressing- BV-2 cells in which this signalling mechanism has not yet been established.

1.4. The role of $P2Y_6$ receptor in physiological and pathophysiological processes involving the cardiovascular system

Studies have pointed out the roles of $P2Y_6$ in cardiovascular function with many supporting that inhibition of the receptor is beneficial. MRS 2578 (1,4-di-(phenylthioureido) butane is a potent, insurmountable selective antagonist for the $P2Y_6$ receptor (human and rat $P2Y_6$), associated with inhibiting phospholipase C activity. The mode of inhibition is non-competitive and is predicted to irreversibly react with the receptor (concentration-dependently) defined by the presence of two isothiocyanate groups. The low solubility and limited aqueous solubility of MRS 2578 hinders the use as a pharmacological probe (Mamedova et.al., 2004; Jacobson et al., 2012).

Inhibiting the receptor/knocking out the $P2Y_6$ receptor gene is associated with attenuating systemic inflammatory responses in vivo and in vitro. Human microvascular endothelia and mice knockout (KO) model (P2Y6-/-) were challenged with tumor necrosis factor α (TNF-α) and lipopolysaccharide (LPS) in vitro and in vivo respectively. $P2Y_6$ receptor induction was abolished in vitro using a $P2Y_6$ receptor antagonist, MRS 2578 which abolished the nuclear factor κB (NF-kB) reporter activity and proinflammatory gene expression. In vivo, $P2Y_6$ knockout mice showed attenuated LPS-induced vascular inflammation, implicating $P2Y_6$ receptor as a therapeutic target during systemic inflammation (Riegel et al., 2010).

P2 receptor activation has shown to enhance atherosclerosis. $P2Y_6$ is associated with driving vascular inflammation by inducing expression of chemokines and contributing to leukocyte recruitment. The use of $P2Y_6$ knockout mouse

macrophages in a study by (Bar et al., 2008) depicted attenuated release of interleukin-6 and macrophage inflammatory protein-2 upon LPS stimulation in presence of UDP. $P2Y_6$ (-/-) KO mice model showed that deficiency of $P2Y_6$

limited atherosclerosis and plaque formation. This points out how strategies that dampen $P2Y_6$ signalling may be essential in treatment of vascular inflammatory conditions such as sepsis (Stachon et al., 2014). Furthermore, in conditions such as hypertension, pathogenesis is associated with excessive proliferation of vascular smooth muscle cells. Silencing the vascular $P2Y_6$ receptor by siRNA, downregulated angiotensin-II induced human aortic vascular smooth muscle cells (HAVSMC) proliferation suggesting that inhibition of $P2Y_6$ may represent a therapeutic strategy against hypertension and vascular pathologies (Wang et al., 2017). $P2Y_6$ receptor has also been linked to age-related hypertension. In a mouse model, $P2Y_6$R was depicted to age-dependently promote vascular remodelling, an effect that was inhibited by $P2Y_6$ receptor antagonist, MRS 2578 (Sunggip et al., 2017).

In cardiomyocytes, $P2Y_6$ receptors are also coupled to Gα12/13 proteins (Rho-activation). During mechanical stretch, release of nucleotides such as UDP via actions on $P2Y_6$ results in secretion of fibrogenic factors and triggered pressure overload-induced cardiac fibrosis; one of the inflammatory responses of the heart. Inhibition of $P2Y_6$ was associated with suppressing the expression of fibrogenic genes and cardiac fibrosis. The study also supported that $P2Y_6$ receptor couples to Gα12/13 and Rho-activation in cardiomyocytes unlike Gq coupling in blood vessels (Nishida et al., 2008).

On the other hand, $P2Y_6$ receptor mediated effects have been implicated in cardiac hypertrophy where loss of the receptor was associated with amplified pathological cardiac hypertrophy and macrocardia phenotype. UDP appeared to inhibit physiological and pathological cardiac hypertrophy and therefore, $P2Y_6$

receptors could play a therapeutic role in regulating cardiac hypertrophy (Clouet et al., 2016). There is growing evidence for the role of $P2Y_6$ in regulating cardiovascular homeostasis and a pivotal role in the control of vascular tone and remodelling. Dampening $P2Y_6$ signalling is considered therapeutic by studies which have pointed out the role of the receptor in contributing to the atherogenic/inflammatory conditions.

1.5. Aims and Objectives

The aims of the current study were therefore, to characterize PGE_2-G as a potential $P2Y_6$ receptor agonist using porcine isolated coronary arteries and freshly isolated rat thoracic aortic rings. Since PGE_2-G/$P2Y_6$ signalling was suggested in isolated cells by Brüser et al (2017), it was of interest to confirm whether this signalling phenomenon was also achieved using blood vessels. The functional expression of the $P2Y_6$ receptor has been previously shown in these blood vessels (Rayment et al., 2007; Guns et al., 2005) and BV-2 cell line (Gendron et al., 2003; Jiang et al., 2017) The PGE_2-G/$P2Y_6$ signalling will also be investigated in BV-2 cells, in which this mechanism is yet to be established.

Chapter Two

2.1. Materials and Methods

2.1.1 Tissue preparation

Hearts from pigs (either sex, about 4-6 months old, weighing ~ 50kg) obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield) and thoracic aorta from male Sprague Dawley rats (225-250g) obtained from Charles River (England, UK) were used in this study. A crude dissection was performed to isolate the porcine coronary arteries, which were then stored overnight at 4 °C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (see section 2.1.4). Rats were killed by cervical dislocation, a procedure carried out in accordance with the Code of Practice for the Humane Killing of Animals as specified under Schedule 1 of the Animals (Scientific Procedures) Act 1986. Rat thoracic aorta was freshly isolated for use on the same day. Tissues were finely dissected on a wax plate on the day of use, to remove all the adhering connective tissue and fat. Porcine coronary arteries (4-5 mm) and rat aortic rings (3 mm) were cut into ring segments and mounted onto wires which passed through the lumen of the vessels; the lower hook was connected to a glass support rod and the upper hook connected to the transducer via a thread (Figure 1). The rings were then suspended in the organ bath containing warmed (37°C) and gassed (95% O_2, 5% CO_2) Krebs Henseleit buffer. The transducer was connected to a computer system (LabChart, AD Instruments Ltd) via an amplifier for isometric tension recording. For some rat aortic ring preparations, the endothelium was removed by inserting fine forceps into the artery lumen and then gently rolling the segment backwards and forwards 5-7 times on Krebs-moistened absorbent paper.

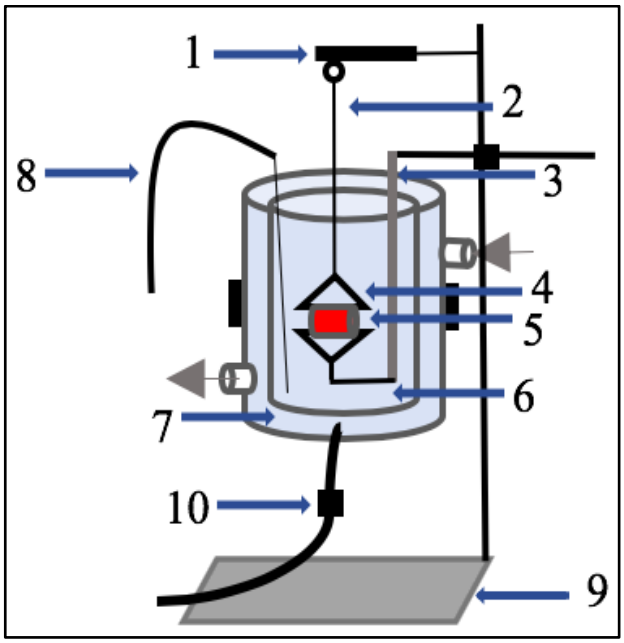


Figure 1. Diagrammatic representation of an organ bath tissue set-up including (1) transducer, (2) thread attaching the top hook to the transducer, (3) glass rod attached to the bottom hook, (4) hook support, (5) blood vessel mounted between hooks, (6) organ bath filled with Krebs-Henseleit buffer, (7) thermostatic jacket, (8) gas tube supplying 95% O_2 :5% CO_2, (9) base, (10) drain.

2.1.2 Responses in porcine isolated coronary artery and rat aorta

Porcine coronary arteries (PCAs) were suspended in 15 ml organ baths, tensioned to 10 g and left to equilibrate for ~ 45 min, after which viability was assessed with two challenges of 80 mM potassium chloride (KCl) with washout after each addition, followed by an equilibration period of ~30 min. Rat thoracic aortic rings were suspended in 10 ml organ baths, initially set at 1g tension and allowed to equilibrate for 30 min during which the tension fell below 1g, after which the tension was increased by a further 1g, giving a final resting tension of

below 2 g. The rings were left to equilibrate for ~30 min before assessing the viability with 60 mM KCl, followed by washout (Alefishat et al., 2014).

In PCAs, cumulative concentration response curves to PSB 0474 (3-phenacyl UDP) (1 x 10^{-7}-3×10^{-5} M), UDP (uridine diphosphate) (10^{-6}-10^{-3} M) and PGE2-G (prostaglandin E_2 glyceryl ester) (10^{-9}-3×10^{-6} M) were produced at basal tone in presence and absence of antagonists. In some experiments, PCAs were preconstricted between 40-60% of the second KCl response by cumulative addition of U46619 (1 x 10^{-9} - 1×10^{-7} M), a thromboxane A_2 mimetic. This was to assess if there was any vasodilator component. Rat thoracic aortic rings were preconstricted between 60-80% of the KCl response by cumulative addition of phenylephrine (1 x 10^{-9} - 1×10^{-6} M) and once a stable tone was achieved, UDP (1 x 10^{-8} - 10^{-5} M) and PGE2-G (1 x 10^{-10} - 3×10^{-6} M) were cumulatively added to the bath in the presence and absence of the antagonists.

Antagonists were added after washout of KCl and return of tone to baseline and before precontraction with U46619/phenylephrine, for a period of at least 30 minutes. In PCAs antagonists used were: suramin (133 μM; a P2 receptor antagonist), MRS 2578 (13 μM; a $P2Y_6$ receptor antagonist), PPTN (1.4 μM; $P2Y_{14}$ receptor antagonist), AR-C 118925XX (13 μM; $P2Y_2$ receptor antagonist) and AH-6809 (35 μM; EP_1, EP_2, EP_3 and DP receptor antagonist). In rat aortic rings, antagonists used were: MRS 2578 (10 μM; a $P2Y_6$ receptor antagonist), AH-6809 (35 μM; EP_1, EP_2, EP_3 and DP receptor antagonist) and AR-C 118925XX (10 μM; $P2Y_2$ receptor antagonist). Some arteries were incubated with DMSO/distilled water for a vehicle control (Rayment et al., 2007; Abbas et al., 2018; Abramovitz et al., 2000)

2.1.3 Maintenance of the murine microglial cell line BV-2.

BV-2 cells were received originally as a gift from Dr Nephi Stella, University of Washington. The cells were cultured in 175 cm^2 culture flasks at 37 °C in a 5% CO_2 humidified incubator containing the Dulbecco's Modified Eagle Medium (DMEM) – Nutrient Mixture high glucose supplemented with 10% foetal bovine serum, 400 µg/ml G 418 and 2mM L-glutamine. The BV-2 cells were passaged when reached ~90% confluency and were cultured every 2-3 days. Cells were cultured in a sterile condition, in a class II laminar airflow cabinet.

The cells were washed once with phosphate buffered saline (PBS) and thereafter incubated with trypsin-EDTA solution for ~3 min until majority of the cells were detached from the culture flask. The cells were loosened from the flask bottom by gently tapping. To prevent further action of trypsin, the cell suspension was diluted in the culture medium and thereafter, cells were collected by centrifugation at 1000 rpm for 5 min. The cell pellet was resuspended in fresh culture medium.

2.1.4 Measurement of intracellular calcium $[Ca^{2+}]_i$ changes using FlexStation III

BV-2 cells were collected and seeded in the 96-well black-walled plates. To measure changes in intracellular calcium $[Ca^{2+}]_i$, the Fluo-4 AM (Invitrogen) dye was used. The old culture media of the plated cells was aspirated and replaced with Fluo-4 AM solution (consisting of probenecid and high glucose DMEM media). Using a Multichannel pipette, 100 µl of this Fluo-4 AM solution was transferred to the 96-well plate (wrapped in aluminium foil) and incubated for 50 min. After the incubation period, plated cells were washed once with 100 µl of loading buffer (consisting of 500 µl of probenecid in 50 ml HEPES buffer). The next step involved incubating with the antagonist, where 100 µl of the $P2Y_6$ antagonist, MRS 2578 or loading buffer was added to alternate well columns on

the plate and incubated with cells for at least 30 min (wrapped in aluminium foil) in a CO_2 free incubator, after which the intracellular calcium responses were recorded using a FlexStation III plate reader. During the incubation period, agonist dilutions (UDP/PGE_2-G) were prepared 6 times their desired concentrations (stocks) and transferred onto the compound plate, ranging from the highest concentration to the lowest (down the column), with the first two wells of the columns for the control, ATP (10 µM) to allow normalization of the results.

Agonist and antagonist dilutions were prepared in loading buffer solution. The cell plate, compound plate and the tip rack were incubated for at least 15 min at 37 °C in the FlexStation device before performing analysis. Calcium responses were measured at 37 °C for 2 min with 1.52 second interval between successive readings along the row, at 525 nm (samples were excited at 488 nm and emission spectra was recorded at 525 nm). Data were transferred to an Excel sheet and then analysed using GraphPad Prism 8 software (GraphPad, San Diego).

2.1.5 Reagents and Drugs

Krebs-Henseleit buffer was composed of: sodium chloride (NaCl) 118 mM, potassium chloride (KCl) 4.8 mM, magnesium sulphate ($MgSO_4.7H_2O$) 1.1 mM, sodium hydrogen carbonate ($NaHCO_3$) 25 mM; potassium hydrogen phosphate (KH_2PO_4) 1.2 mM, glucose 12 mM and calcium chloride ($CaCl_2.H_2O$) 1.3 mM. The HEPES Buffer was composed of: sodium pyruvate 2 mM, sodium chloride (NaCl) 137 mM, glucose 10 mM, potassium chloride (KCl) 5 mM, magnesium sulphate heptahydrate ($MgSO_4.7H_20$) 1 mM, HEPES 10 mM, calcium chloride ($CaCl_2$) 1.3 mM, sodium hydrogen carbonate ($NaHCO_3$) 1.5 mM and probenecid, 2.5 mM. The pH of the HEPES buffer was adjusted with NaOH. Probenecid was obtained from Sigma Aldrich. Pluronic acid (F-127) and Fluo-4 AM dye were obtained from Thermo Fisher Scientific.

Suramin and UDP were purchased from Sigma Aldrich. PSB 0474, MRS 2578, AH-6809, PPTN and AR-C 118925XX were obtained from Tocris Bioscience Ltd (Bristol, UK). PGE_2-G was purchased from Cayman Chemicals (Ann Arbor, MI, USA). U46619, UDP, suramin, bradykinin (BK), carbachol (CCh) and phenylephrine (PE) were dissolved in distilled water. PGE_2-G stock was made up in ethanol and subsequent serial dilutions made in distilled water, with any remaining amounts discarded at the end of the experiment. AR-C 118925XX, MRS 2578, PPTN and AH-6809 were dissolved in DMSO at their respective stock concentrations. MRS 2578 was made up in DMSO, aliquoted and stored at -80 °C, PPTN and suramin were stored at -4 °C, AH-6809 and AR-C 118925XX were stored at -20 °C. All stock concentrations of agonists were stored at -4 °C.

2.2. Statistical analysis

Contractile responses to PSB 0474, UDP and PGE_2-G were expressed as a percentage of KCl contraction in PCAs. In experiments which involved pre-contracting the PCAs with U46619, the responses were expressed as a percentage of the contraction to U46619. Relaxations observed in the rat aortic segments were expressed as a percentage of the PE-induced contraction. Data were fitted using non-linear regression and statistical analyses were performed using GraphPad Prism 8.0 software. R_{max} is the maximal response and log EC_{50} is the log of the molar concentration of agonist required to generate 50% of the R_{max}. Results for the organ bath experiments were compared by two-way analysis of variance (2-way ANOVA) followed by *post hoc* Sidak's multiple comparisons test (Prism, GraphPad). After analysis with 2-way ANOVA, Sidak's post hoc was only conducted if the necessary level of significance ($p<0.05$) was achieved. Results for the calcium mobilisation experiments using FlexStation III were compared by students paired t-test. Calcium measurements are expressed in peak values (delta) and relative fluorescence units (RFU).

Differences against control were considered significant if the p value was <0.05. Data for organ bath experiments are expressed as log concentration-responses and are presented as mean ± S.E.M. The 'n' represents number of animals.

Chapter Three

3.1. Effect of PSB 0474, UDP and PGE_2-G on vascular tone in porcine isolated coronary arteries

In endothelial-intact porcine coronary arteries, PSB 0474 (1.3×10^{-7}-4×10^{-5} M) ($P2Y_6$ receptor agonist), UDP (1.3×10^{-6}-1.3×10^{-3} M) and PGE_2-G (1.3×10^{-9} - 4×10^{-6} M) were cumulatively applied at basal tone in the presence and absence of various antagonists and log concentration responses were generated. Each of the agonists evoked concentration-dependent contractions with a potency order of PGE_2-G > PSB 0474 > UDP (pEC_{50} value for PGE_2-G was 6.7 ± 0.1 (n = 6); 4.9 ± 0.3 (n = 6) for PSB 0474; 3.7 ± 0.3 (n = 6) for UDP) (Figure 2). PSB 0474 is a structural analog of UDP and is associated to have a greater potency than UDP (Jacobson et al., 2012). The difference in potencies between PGE_2-G and PSB 0474 was almost two orders of magnitude and three orders of magnitude against UDP. Contractile responses evoked by PGE_2-G produced an R_{max} of 11 ± 2% in comparison to responses evoked by PSB 0474 (11 ± 4%) and UDP (7 ± 1%) (all n = 6).

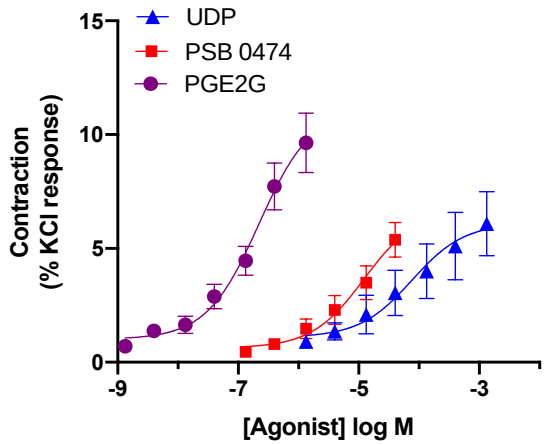


Figure 2. Concentration-dependent contractions of UDP, PSB 0474 ($P2Y_6$ receptor agonists) and PGE_2-G in porcine coronary arteries. Data presented are mean ± SEM (n=6).

3.2. Characterisation of responses to UDP in porcine isolated coronary arteries

3.2.1. Effect of a non-selective P2 receptor antagonist suramin

UDP is an agonist at the $P2Y_6$ receptor and the functional expression of this receptor has been previously shown in porcine coronary arteries (PCAs) (Rayment et al., 2007). The aim was to therefore, confirm the $P2Y_6$ expression in the PCAs using UDP, a $P2Y_6$ receptor agonist before, investigating the actions of PGE_2-G. A non-selective P2 receptor antagonist, suramin (133 μM) was first applied to investigate involvement of P2 receptors in UDP responses. Concentration-dependent contractions evoked by UDP (1.3×10^{-6}-1.3×10^{-3} M) were significantly attenuated by suramin ($p<0.001$; two-way ANOVA; figure 3).

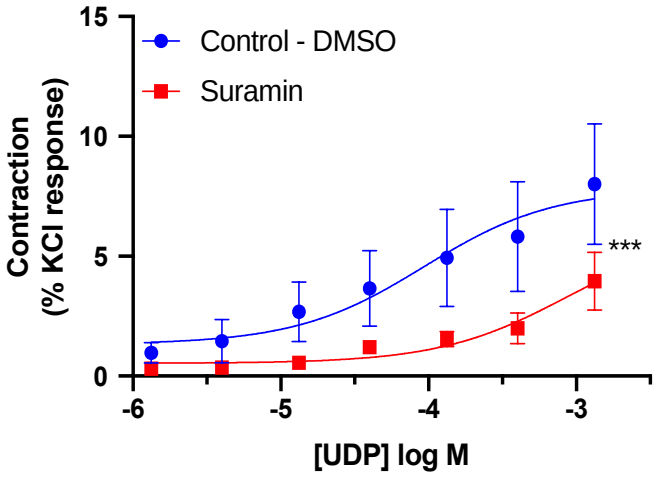
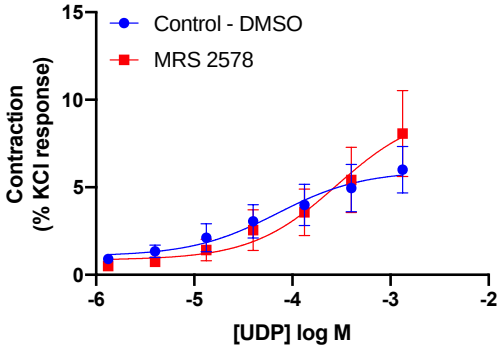


Figure 3. Effect of suramin, a P2 receptor antagonist (133 μM) on concentration-dependent contractions of UDP in PCAs. UDP responses were attenuated in presence of suramin. Data presented are mean ± SEM (n=6). ****P<0.001*.

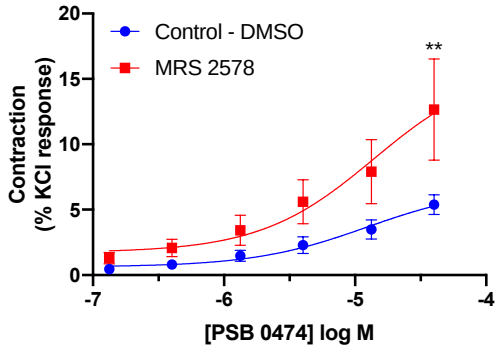
3.2.2. Effect of a P2Y$_6$ receptor antagonist MRS 2578 on UDP, PSB 0474 and PGE$_2$-G responses

MRS 2578 (13 μM), a P2Y$_6$ receptor antagonist was applied next to investigate the presence of P2Y$_6$ receptors in the PCA rings. UDP (1.3×10^{-6}-1.3×10^{-3} M) elicited concentration-dependent contractions which remained unchanged in presence of MRS 2578 (Figure 4A). PSB 0474 is a P2Y$_6$ receptor agonist and PGE$_2$-G has recently been suggested to mediate effects via the P2Y$_6$ receptor (Brüser., et al 2017). Concentration-dependent contractions evoked by PSB 0474 (1.3×10^{-7}-4×10^{-5} M) were significantly enhanced by MRS 2578 ($p<0.01$ at the last concentration (4×10^{-5} M); two-way ANOVA; (n=6); figure 4B). In the presence of MRS 2578, the PGE$_2$-G (1.3×10^{-9}-4×10^{-6} M) mediated responses were unaffected (Figure 4C).

A.



B.



C.

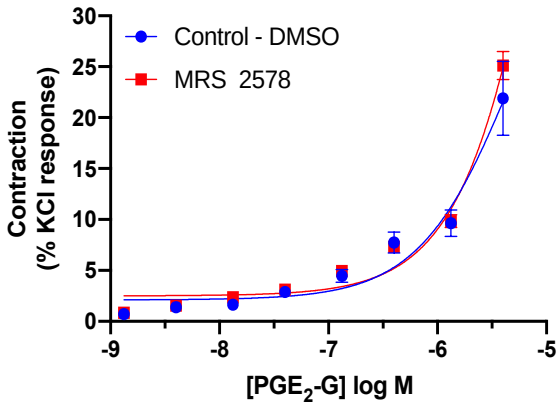


Figure 4. Effect of MRS 2578, a $P2Y_6$ receptor antagonist (13 μM) on concentration-dependent contractions mediated by (A) UDP (B) PSB 0474 and (C) PGE_2-G in PCAs. MRS 2578 significantly enhanced contractile responses of PSB 0474 but responses of UDP and PGE_2-G remained unaffected (n=6). Data presented are mean ± SEM. ***P<0.01*

3.2.3. Effect of the $P2Y_2$ receptor antagonist, AR-C 118925XX and $P2Y_{14}$ receptor antagonist, PPTN

UDP is also an agonist at $P2Y_2$ and $P2Y_{14}$ receptors. The next step involved applying selective $P2Y_2$ and $P2Y_{14}$ receptor antagonists; AR-C 118925XX (13 μM) and PPTN (1.4 μM). Concentration-dependent contractions evoked by UDP (1.3×10^{-6}-1.3×10^{-3} M) remained unchanged in the presence of AR-C 118925XX (Figure 5A) and PPTN (Figure 5B). Both antagonists failed to attenuate UDP mediated contractile responses in PCAs.

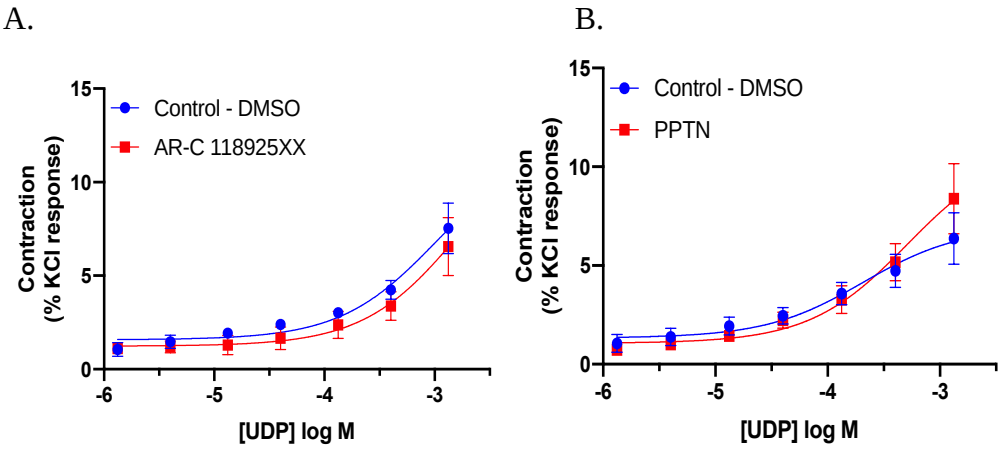


Figure 5. Effect of (A) AR-C 118925XX, a $P2Y_2$ receptor antagonist (13 μM) and (B) PPTN, a $P2Y_{14}$ receptor antagonist (1.4 μM) on concentration-dependent contractions of UDP in PCAs. UDP responses remained unaffected in presence of both antagonists. Data presented are mean ± SEM (n=6)

3.3. Investigation of the expression of vasorelaxant P2Y$_6$ receptors in porcine coronary arteries

Since UDP had only generated contractile responses in the porcine coronary arteries, the next step involved investigating the functional presence of any relaxant P2Y$_6$ receptors. This involved incubating the U46619-precontracted PCA rings with a P2Y$_2$ receptor antagonist, AR-C 118925XX (13 μM). The purpose was to firstly determine whether UDP evoked any relaxations in precontracted arteries and the purpose of using the P2Y$_2$ antagonist was to determine whether any relaxations produced were P2Y$_2$ receptor-dependent as P2Y$_2$ receptors are expressed predominantly on the endothelium of coronary vessels coupled to NO/cGMP and primarily associated to mediate vasodilation (Gödecke et al., 1996). Unattenuated relaxations, if produced, would suggest possible involvement of vasorelaxant P2Y$_6$ receptors as UDP is an agonist at the P2Y$_6$ receptors. Cumulative addition of UDP (1.3×10^{-6}- 4×10^{-4} M) to the U46619-precontracted PCA rings, produced no relaxations. Only concentration-dependent contractions were generated by UDP in the precontracted PCA rings (Figure 6). UDP responses were unchanged in presence and absence of the antagonist, AR-C 118925XX (Figure 6).

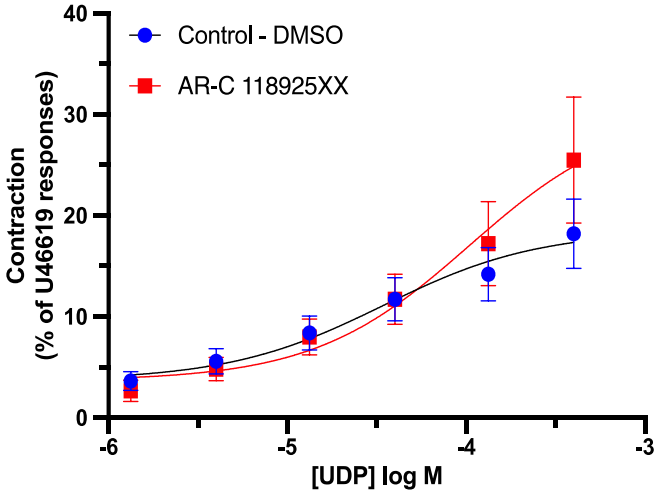


Figure 6. Concentration-dependent contractions evoked by UDP in U46619-preconstricted porcine coronary in presence and absence of the $P2Y_2$ receptor antagonist, AR-C 118925XX (13 μM). Data presented are mean ± SEM (n=5).

3.4. Effect of EP receptor antagonist, AH-6809 on PGE_2-G and UDP responses in porcine coronary arteries

The next step involved investigating the effects of AH-6809 on PGE_2-G and UDP responses. AH-6809 is a nonselective EP receptor antagonist with affinities for each of the EP receptors; EP1, EP2, EP3 and DP receptors with minimal/no effect on TP, IP and FP receptors (Abramovitz et al., 2000). PGE_2-G is known to have some actions on EP receptors although with a lower potency than PGE_2 (Turcotte et al., 2017; Nirodi et al., 2004). Responses to UDP were also investigated to determine whether EP receptors may be involved in mediating its contractile responses. The responses of UDP and PGE_2-G were characterised in presence and absence of AH-6809 (35 μM; Ki = 350 nM at EP2 receptors). Concentration-dependent contractions evoked by PGE_2-G (1.3×10^{-9}-4×10^{-6} M) were significantly attenuated in the presence of AH-6809 ($p<0.05$ at the last concentration (4×10^{-6} M); two-way ANOVA; n=8 Figure 7A). Concentration-dependent contractions of UDP (1.3×10^{-6}-1.3×10^{-3} M) were also significantly attenuated in presence of AH-6809 ($p<0.001$ at 1×10^{-3} M; n=7 Figure 7B).

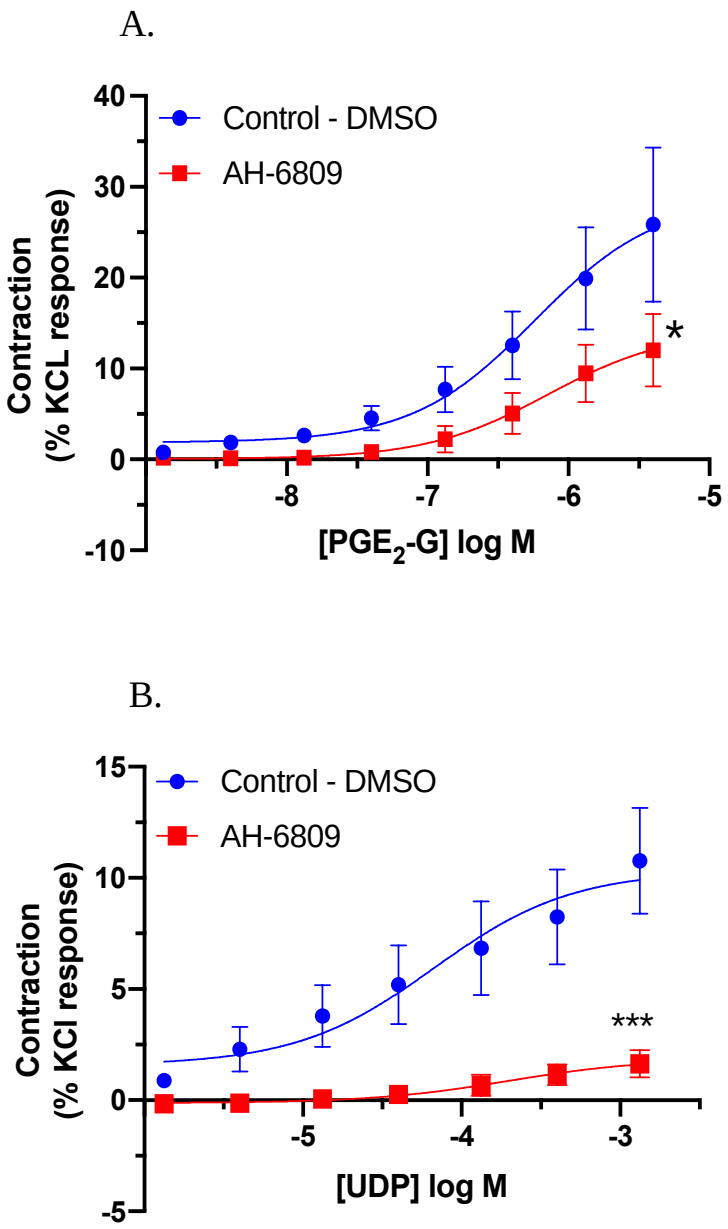


Figure 7. Effect of a nonselective EP receptor antagonist, AH-6809 (35 μM) on concentration-dependent contractions of (A) PGE_2-G and (B) UDP in PCAs. AH-6809 significantly attenuated responses of both PGE_2-G and UDP (n=7-8). Data are represented as mean ± SEM. **P<0.05, *** P<0.001*.

3.5. Characterisation of responses to PGE_2-G on vascular tone in endothelium denuded porcine coronary arteries

To investigate the involvement of the endothelium in the contractile responses to PGE_2-G, concentration-response curves were generated to PGE_2-G (1.3×10^{-9}-4×10^{-6} M) in endothelium-denuded PCAs. Coronary artery segments were denuded of the endothelium using the procedure mentioned in the methods section (section 2.1.1). PGE_2-G evoked concentration-dependent contractions with a pEC50 value of 6.7 ± 0.2 and R_{max} value of 19 ± 6% (n=6) in endothelium-intact PCA rings in comparison to an enhanced contractile response evoked by PGE_2-G in endothelium-denuded PCA rings (R_{max} value of 61 ± 17%; $p<0.001$ at 4×10^{-6} M, n=6; Figure 8) with an unchanged potency (pEC50 value of 6.7 ± 0.1; n=6).

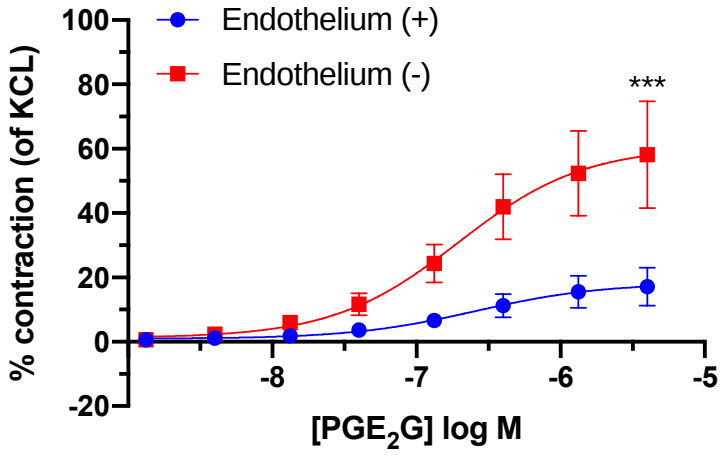


Figure 8. Effect of endothelial removal on responses produced by PGE_2-G in PCA rings. Endothelium-denuded PCA rings produced enhanced contractile responses to PGE_2-G (n=6). Data are represented as mean ± SEM. *** *P<0.001*.

3.6. Characterisation of responses to UDP and PGE2-G on vascular tone in rat thoracic aorta

In porcine coronary arteries, characterisation of the $P2Y_6$ receptor using a range of agonists and antagonists questioned the presence of the functional $P2Y_6$ receptor. The next step therefore, involved using the rat thoracic aorta to investigate possible actions of PGE_2-G at the $P2Y_6$ receptor as studies have previously demonstrated expression of the $P2Y_6$ receptor in rodent (mouse) aorta (Guns et al., 2005). In endothelium-intact aortic rings, concentration-response curves were generated to UDP (1×10^{-8}-1×10^{-5} M) and PGE_2-G (1×10^{-10}-3×10^{-6} M) which were added cumulatively to the bath to phenylephrine (PE) precontracted rings. PGE_2-G evoked concentration-dependent relaxations with a pEC_{50} value of 8.5 ± 0.2 and R_{max} value of 36 ± 6% (n = 9). A biphasic response curve was observed where concentrations greater than 0.3 μM produced concentration-dependent contractions. UDP evoked concentration-dependent relaxations with a pEC_{50} value of 6.6 ± 0.2 and R_{max} value of 80 ± 12% (n = 9). Both agonists evoked concentration-dependent relaxations with a potency order of PGE_2-G > UDP (Figure 9).

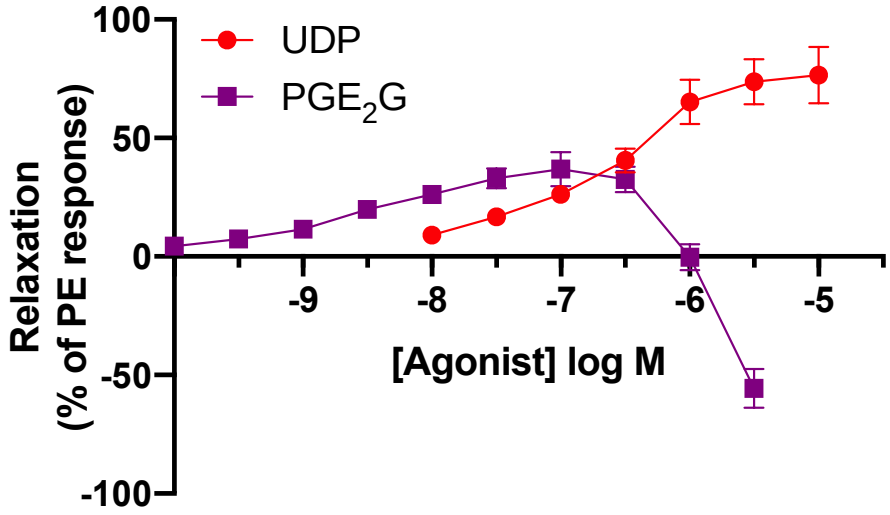


Figure 9. Concentration-dependent responses of UDP and PGE_2-G in rat thoracic aorta precontracted with phenylephrine (PE). Data presented are mean ± SEM (n=9).

3.6.1. Effect of $P2Y_6$ receptor antagonist, MRS 2578 on UDP, PGE_2-G and carbachol responses in rat thoracic aorta.

The next step involved characterising the responses of UDP (1×10^{-8}-1×10^{-5} M) and PGE_2-G (1×10^{-10}-3×10^{-6} M) in the presence of the $P2Y_6$ receptor antagonist, MRS 2578 (10 μM). Concentration-dependent relaxations were evoked by UDP with a pEC_{50} value of 6.4 ± 0.1 and R_{max} of 77 ± 8% (n = 7) which remained unchanged in presence of MRS 2578 (pEC_{50} value of 6.4 ± 0.1 and R_{max} of 80 ± 10%, n = 7; Figure 10A). Similarly, concentration-dependent relaxations evoked by PGE_2-G (pEC_{50} value of 8.6 ± 0.2 and R_{max} 31 ± 5%, n = 7) also remained unaffected in presence of the $P2Y_6$ antagonist, MRS 2578 (pEC_{50} value of 8.6 ± 0.3; R_{max} 25 ± 5%, n = 7). Concentrations of PGE_2-G greater than 0.3 μM evoked

concentration dependent contractions (Figure 10B). Carbachol (1×10^{-10}-1×10^{-7} M), a muscarinic receptor agonist, was used as a control to test whether MRS 2578 influenced relaxation responses of other agents other than UDP and PGE_2-G. Concentration-dependent relaxations were evoked by carbachol with a pEC_{50} value of (8.9 ± 0.1) and R_{max} of 113 ± 6% (n = 6) which remained unchanged in presence of MRS 2578 (pEC50 value of 8.9 ± 0.1 and R_{max} of 110 ± 4%, n = 6; Figure 10C).

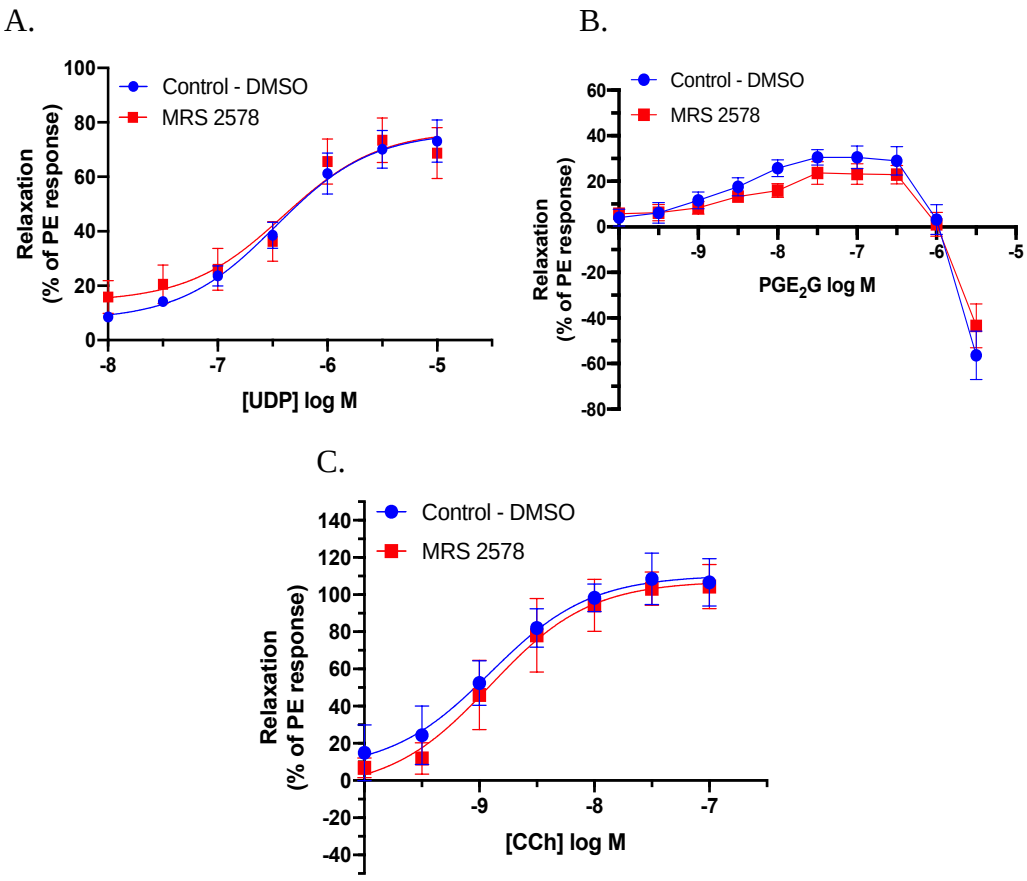


Figure 10. Effect of MRS 2578 (10 μM), a $P2Y_6$ receptor antagonist on concentration-dependent relaxations of (A) UDP (B) PGE_2-G and (C) carbachol in rat thoracic aortic rings. MRS 2578 had no effect on the responses of either agonists (n=6-7). Data are represented as mean ± SEM.

3.6.2. Effect of P2Y2 receptor antagonist, AR-C 118925XX on UDP responses in rat thoracic aorta

Since UDP is associated to mediate actions at the $P2Y_2$ receptors, the contribution of relaxation or contraction was investigated in presence of the $P2Y_2$ receptor antagonist, AR-C 118925XX (10 μM). Concentration-dependent relaxations were evoked by UDP (1×10^{-8}-1×10^{-5} M) with a pEC50 value of 6.6 ± 0.1 and R_{max} of 51 ± 6% (n=5) which were significantly enhanced in presence of AR-C 118925XX (pEC_{50} value of 6.5 ± 0.1 and R_{max} of 80 ± 11%, n = 5; $p<0.01$ at 1 μM) (Figure 11).

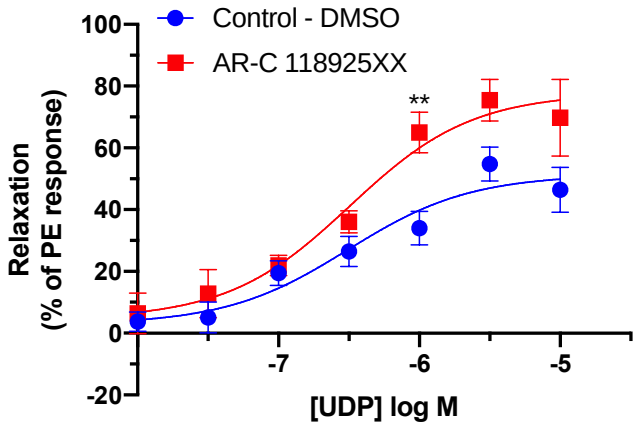
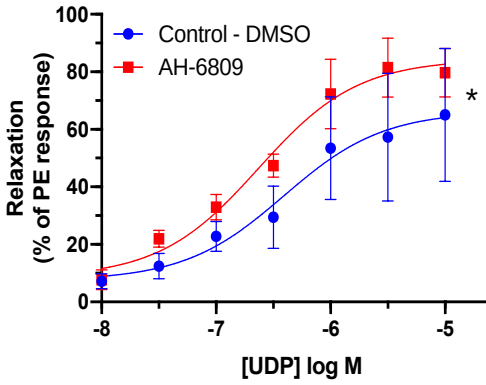


Figure 11. Effect of AR-C 118925XX (10 μM), a $P2Y_2$ receptor antagonist on concentration-dependent relaxations of UDP in rat thoracic aortic rings. AR-C 118925XX significantly enhanced responses of UDP (n=5). Data are represented as mean ± SEM. **P<0.01*

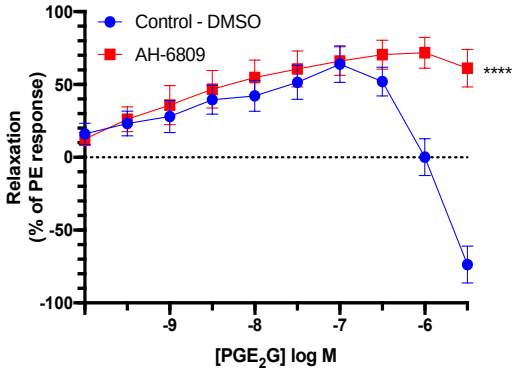
3.6.3. Effect of EP receptor antagonist, AH-6809 on UDP, PGE_2-G and carbachol responses in rat thoracic aorta

The next step involved characterising the responses of UDP (1×10^{-8}-1×10^{-5} M) and PGE_2-G (1×10^{-10}-3×10^{-6} M) in presence of the non-selective EP antagonist, AH-6809 (35 μM). UDP evoked concentration-dependent relaxations with a pEC_{50} value of 5.2 ± 1.6 and R_{max} of 77 ± 8% (n = 6) which were significantly enhanced in presence of AH-6809 (pEC_{50} value of 6.7 ± 0.1 and R_{max} of 89 ± 13%, n = 6; $p<0.05$; two-way ANOVA) (Figure 12A). Concentration-dependent relaxations evoked by PGE_2-G remained unchanged in the presence (pEC_{50} value of 8.7 ± 0.3 and R_{max} of 67 ± 10%, n = 5) and absence (pEC_{50} value of 8.5 ± 0.2 and R_{max} 57 ± 10%, n = 5) of AH-6809. However, concentration-dependent contractions obtained by PGE_2-G starting at 0.3 μM were significantly blocked by AH-6809 ($p<0.0001$ at 3×10^{-6} M; Figure 12B). Carbachol (1×10^{-10}-1×10^{-7}) was used as a control to test whether AH-6809 influenced relaxation responses of other agents other than UDP and PGE_2-G. Concentration-dependent relaxations were evoked by carbachol with a pEC_{50} value of 8.3 ± 0.3 and R_{max} of 112 ± 7% (n = 7) which remained unchanged in the presence of AH-6809 (pEC_{50} value of 8.3 ± 0.2 and R_{max} of 113 ± 11% (n = 7); Figure 12C).

A.



B.



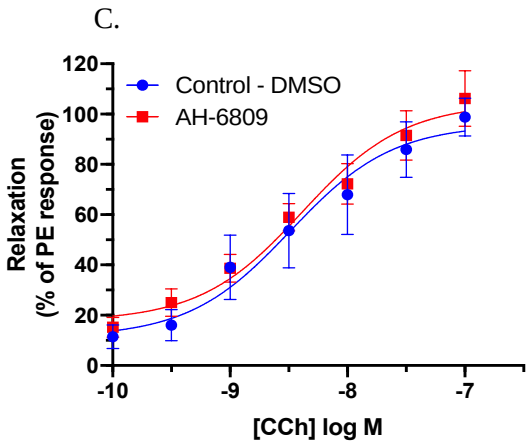


Figure 12. Effect of a nonselective EP receptor antagonist, AH-6809 (35 μM) on responses of (A) UDP (B) PGE_2-G and (C) carbachol in rat thoracic aortic rings. AH-6809 had a significant effect on both UDP and PGE_2-G (n=5-7). Data are represented as mean ± SEM. **P<0.05*, **** *P<0.0001*.

3.7. Characterisation of responses to UDP and PGE_2-G on vascular tone in endothelium denuded rat thoracic aorta

To investigate the involvement of the endothelium in the relaxation to UDP and PGE_2-G, concentration-response curves were generated to UDP (1×10^{-8}-1×10^{-5} M) and PGE_2-G (1×10^{-10}-3×10^{-6} M) in endothelium-denuded aortic rings. Aortic rings were denuded of the endothelium using the procedure mentioned in the methods section (section 2.1.1). Rings were precontracted with phenylephrine followed by cumulative addition of the agonists. UDP evoked concentration-dependent relaxations with a pEC_{50} value of 6.5 ± 0.1 and R_{max} value of 61 ± 8% (n = 8) in endothelium-intact aortic rings in comparison to UDP responses obtained in endothelium-denuded aortic rings (pEC_{50} value of 4.8 ± 1.1) which were significantly attenuated ($p<0.001$; two-way ANOVA; n=8; Figure 13A). PGE_2-G evoked concentration-dependent relaxations with a

pEC_{50} value of 8.5 ± 0.2 and R_{max} value of 32 ± 4%, n=8; Figure 13B. A biphasic response curve was observed where concentrations greater than 0.3 μM produced concentration-dependent contractions. Due to minimal relaxation responses produced by PGE_2-G in endothelium-intact rings, the responses in endothelium-denuded aortic rings showed unchanged responses as well as the contractile responses which remained unaffected (n=8; Figure 13B).

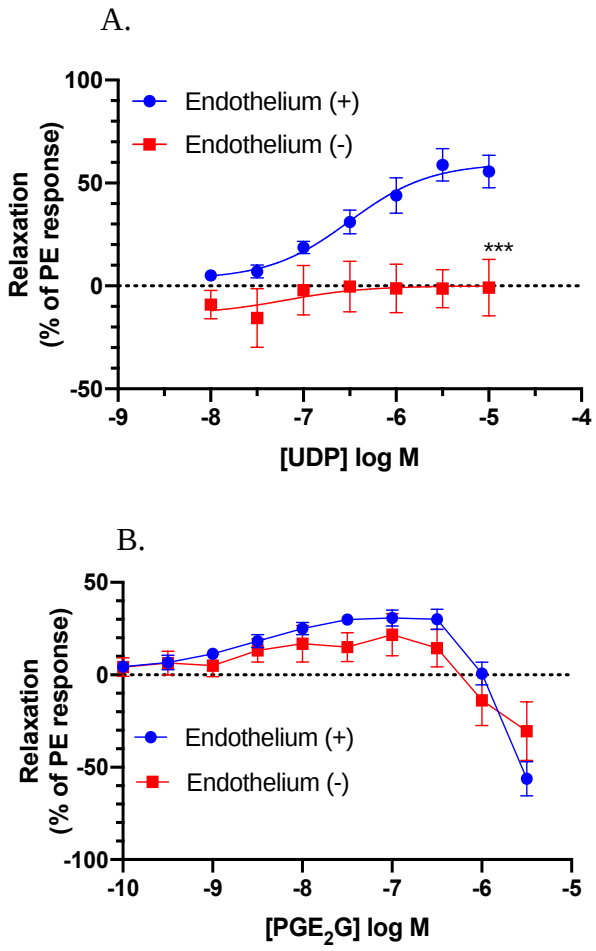


Figure 13. Effect of endothelial removal on responses produced by (A) UDP and (B) PGE_2-G in phenylephrine-precontracted rat thoracic aortic rings. Data are represented as mean ± SEM (n=8). *** *P<0.001*.

3.7 Effect of $P2Y_6$ antagonist, MRS 2578 on UDP and PGE_2-G- evoked intracellular calcium release in BV-2 cells

Since $P2Y_6$ receptors have an inflammatory phenotype associated with them and its expression has been previously characterised using RT-PCR in the BV-2 cell line (Gendron et al., 2003; Jiang et al., 2017), the study involved characterising UDP and PGE_2-G responses in presence and absence of the antagonist, MRS 2578. Upon activation of the endogenous receptor, intracellular calcium levels were measured and compared. UDP (1×10^{-4}-6×10^{-9} M) and PGE_2-G (1×10^{-4}-6×10^{-9} M) evoked concentration-dependent calcium responses in BV-2 cells. MRS 2578 (10 μM) failed to attenuate the intracellular calcium levels evoked by PGE_2-G (pEC_{50} value of 4.1 ± 0.1 and R_{max} of 607 ± 61) (Figure 14A). Responses of PGE_2-G were unaffected in presence of the $P2Y_6$ antagonist, MRS 2578 (Figure 14A & Figure 14C). UDP (1×10^{-4}-6×10^{-9} M) evoked concentration-dependent calcium responses in BV-2 cells with a pEC_{50} value of 5.6 ± 0.2 and R_{max} value of 114 ± 17. The potency remained unchanged in presence of the antagonist, but responses were attenuated, non-competitively (pEC_{50} value of 5.6 ± 0.3 and Rmax value of 86 ± 14%; $p<0.05$; paired t-test; n=4; Figure 14B). Concentration-dependent calcium responses (expressed in delta) evoked by UDP (pEC_{50} value of 5.6 ± 0.2 and R_{max} value of 76 ± 11) were significantly blocked, but in a competitive mechanism, by the antagonist (pEC_{50} value of 4.7 ± 0.3 and R_{max} value of 79 ± 7%; $p<0.05$; paired t-test; n=4; Figure 14D).

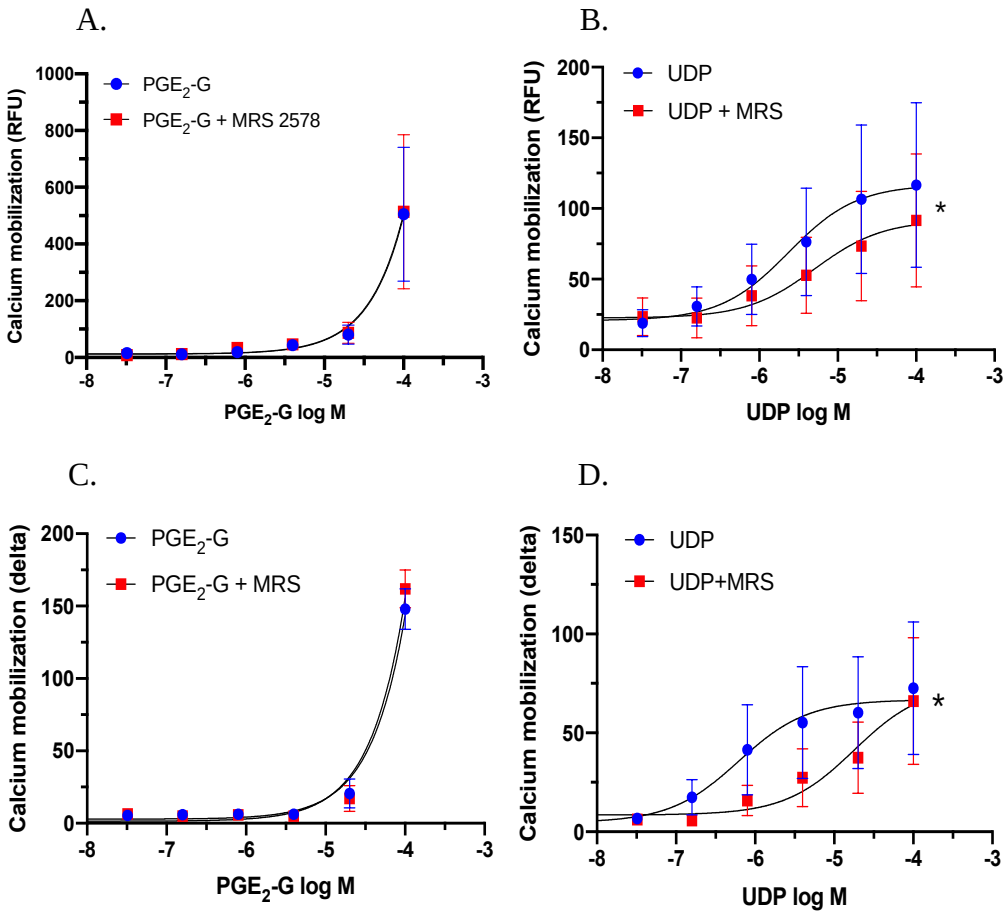


Figure 14. Effect of the P2Y6 receptor antagonist, MRS 2578 (10 μM) on calcium responses of BV-2 cells to (A) PGE2-G and (B) UDP expressed in relative fluorescence units (RFU) and (C) PGE2-G and (D) UDP expressed in peak (delta values). Responses to UDP were significantly attenuated but the responses to PGE2-G remained unchanged. Calcium measurements are expressed as both RFU and delta values. Data are represented as mean ± SEM (n=4) *$P<0.05$*

Chapter Four

3.1. Discussion

In this study, the aim of the study was to characterise PGE_2-G responses in porcine coronary arteries and rat thoracic aorta and further investigate its possible involvement with the $P2Y_6$ receptor. UDP and PSB 0474 are known agonists at the $P2Y_6$ receptor and will therefore be implemented for characterisation of the receptor using a range of selective and nonselective antagonists. Results obtained so far questioned the functional presence of $P2Y_6$ receptors in these arteries, however, a P2Y and prostaglandin signalling crosstalk was discovered. Furthermore, UDP and PGE_2-G evoked calcium responses were also characterised in BV-2 cells, in the presence and absence of the $P2Y_6$ receptor antagonist.

3.1.1. Characterisation of responses evoked by UDP in porcine coronary arteries and rat thoracic aorta

In this study, UDP evoked vasoconstriction in the porcine coronary artery at basal tone and vasorelaxation in the phenylephrine precontracted rat thoracic aorta which is consistent with previous findings. UDP has been previously shown to cause vasoconstriction in porcine (Rayment et al., 2007), human (Malmsjo et al., 2003) and mouse coronary arteries (Haanes et al., 2016). Other vessels in which UDP causes vasoconstriction include porcine pancreatic arteries (Alsaqati et al., 2013) and guinea pig mesenteric vein (Mutafova-Yambolieva et al., 2000). Bar et al. (2008) and Guns et al. (2005) observed endothelium-dependent relaxations to UDP in mouse thoracic aorta. UDP mediated relaxant responses have also been characterised in rat mesenteric arteries (Malmsjö et al., 2000), rat pulmonary vasculature, intrapulmonary artery (Rubino and Burnstock, 1996; Rubino et al., 1999; Chootip et al., 2002; Mitchell et al., 2012), rat basilar artery (von Kügelgen and Starke, 1990) and mouse mesenteric (Vial and Evans, 2002) arteries.

Bar et al (2008) observed that UDP evoked endothelial-dependent relaxations in mouse aorta which were abolished in the isolated mouse aorta from $P2Y_6$ null mice thus, pointing out the functional expression of $P2Y_6$ receptors predominantly on the endothelium in the mouse aorta and coupled primarily to mediate vasorelaxant responses. Guns et al (2009) also confirmed functional presence of $P2Y_6$ receptors in rodent (mouse) aorta. Chang et al (1995) also provided evidence for the presence of the $P2Y_6$ receptor mRNA in freshly isolated rat aorta and rat aortic smooth muscle cells in primary culture.

Species, vessel type, region/size of blood vessel, level of contractile tone and the involvement of multiple receptors can all influence the functional response to UDP and other nucleotides leading to considerable diversity in responses. There are multiple contractile and relaxant purine receptors expressed on blood vessels which are activated by UDP. Studies have shown that nucleotide activation of endothelial P2 receptors is associated with inducing a local vasorelaxation involving mediators such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) which are released from the endothelium (see Introduction). Direct activation of P2 receptors on the vascular smooth muscle promotes vasoconstriction (Wihlborg et al., 2003).

Regional based changes in purinergic function was also identified from previous studies which have reported between the thoracic aorta and the aortic root, where the root was suggested to be more sensitive to nucleotide actions. ATP produced only a partial relaxation in the thoracic aorta as opposed to the root, where there was a complete relaxation achieved (Guns et al., 2005). In physiological conditions, ATP is also released from the sympathetic nerves. The balance between the ATP release and purine release in the endothelium determines the blood flow and vessel tone control. ATP is coreleased along with noradrenaline

(NA) which acts at smooth muscle receptors to induce vasoconstriction. (Burnstock & Ralevic, 2013).

Regional differences in contractility to $P2Y_6$ in mouse descending coronary arteries was shown by Haanes et al (2016) whereby large diameter segments with a lumen size of ~ 150 μm had $P2Y_6$ receptor as the predominant receptor for UTP and UDP-evoked contraction and $P2Y_2$ receptor coupled to producing endothelial-dependent relaxations whereas in smaller diameter arterial segments with a lumen size of ~ 50 μm, functions were reversed with the $P2Y_2$ receptor mediating contractions and $P2Y_6$ coupled to promoting relaxation (Haanes et al., 2016).

Responses to UDP observed in both the porcine coronary arteries and rat thoracic aorta could be mediated by one or more of the $P2Y_2$, $P2Y_6$ and $P2Y_{14}$ receptors since UDP is an agonist at all of these (Burnstock & Williams, 2000; Carter et al., 2009; Harden et al., 2010). The expression of the $P2Y_{14}$ and $P2Y_2$ receptors have been previously characterised in porcine coronary arteries (Abbas et al., 2013; Abbas et al., 2018; Rayment et al., 2007) and rat thoracic aorta (rodent) (for P2Y2 receptors- Guns et al., 2009). Studies using mouse thoracic aorta and human coronary artery endothelial cells had previously shown a barely detectable mRNA for $P2Y_{14}$ receptor (Kauffenstein et al., 2010; Ding et al., 2011) but a rather robust mRNA expression for the receptor was produced in freshly isolated and cultured rat aortic smooth muscle cells, providing evidence for the expression of $P2Y_{14}$ in aortic smooth muscle (Govindan et al., 2010) and the endothelium (Alsaqati et al., 2014), associated to mediate vasocontractile and vasorelaxant responses respectively.

The functional presence of $P2Y_2$ and $P2Y_6$ receptors coupled to mediate vasocontractile responses in porcine isolated coronary arteries was also confirmed by Rayment et al (2007). It is of note that contractile responses produced by UDP in the PCAs were relatively small (less than 10% contraction

to KCl), possibly suggesting a low expression of the target receptors (Figure 2). Phenylephrine (PE) precontracted aortic rings produced endothelial-dependent relaxations in response to UDP (Figure 13A), consistent with the findings of Kobayashi et al (2017).

UDP contractile responses in PCAs are likely to be mediated via the smooth muscle, since Rayment et al (2007) demonstrated UDP contractions in endothelium-denuded PCAs. In rat aortic rings, relaxations evoked by UDP (1×10^{-8}-1×10^{-5} M) were abolished ($p<0.001$; Figure 13A) in endothelium denuded vessels suggesting that UDP mediated endothelial-dependent relaxant responses in rat thoracic aortic rings. The receptors involved were predominantly expressed on the endothelium to mediate UDP responses. The component of relaxation produced is dependent on the endothelial integrity of the vessels.

The main purpose of denuding the endothelium was to determine whether UDP responses were endothelium-dependent however, physiologically, this condition would mimic an endothelium dysfunction situation in which there is an imbalance in the protective and destructing factors. EDRF (NO) synthesis and release is limited which results in nitric oxide (oxygen radical scavenger) remaining into a superoxide anion radical and therefore contributing to cellular dysfunction and manifestation of an atherogenic condition since low levels of EDRF contribute to events such as intimal proliferation and atheroma formation. Thus, endothelial dysfunction is the underlying cause of several cardiovascular diseases such as atherosclerosis, congestive heart failure, peripheral artery disease, diabetic angiopathies and reperfusion injury (Rubanyi, 1993).

Thus, the expression of uracil nucleotide sensitive P2 receptors is widespread both in terms of the type of species and vessels examined, which coupled with the metabolism/interconversion of nucleotides, and the fact that they can act on more than one P2 receptor, makes it complex in understanding the interplay of P2 receptors in vascular tone regulation (Rayment et al., 2007).

3.1.2. Investigation for functional P2 receptors in porcine coronary arteries and rat thoracic aorta using the non-selective antagonist, suramin

Based on the recently identified PGE_2-G-$P2Y_6$ signalling reported by Brüser et al. (2017) in isolated cells, the aim of the study was to investigate whether PGE2-G activated the $P2Y_6$ receptor with the same potency (~1pM) in blood vessels, specifically porcine coronary arteries and rat thoracic aorta. The first step was to therefore, characterise the $P2Y_6$ receptor in these blood vessels. UDP is a known agonist at the $P2Y_6$ receptor (Communi et al., 1996; Ralevic & Dunn, 2015) therefore, concentration response curves to UDP were generated in the presence and absence of selective antagonists. The first step involved implementing a non-selective P2 receptor antagonist, suramin. Suramin (von Kügelgen, 2006) has been previously shown to block most of the P2Y receptors, in a non-selective manner. However, some P2Y receptors have shown resistance to suramin, such as rat $P2Y_4$ receptors which have shown weak sensitivity to suramin (Wildman et al., 2003; Chootip et al., 2005). In porcine coronary arteries, UDP mediated concentration-dependent contractions which were significantly attenuated in presence of suramin (Figure 3). The simplest interpretation of the significant attenuation of UDP responses by suramin is that, they demonstrated the involvement of P2 receptors in mediating the contractile responses to UDP but no clear identification of which P2 receptors were involved. However, suramin is known to have a range of other actions at targets other than P2 receptors. It has been previously suggested that concentrations of suramin >100 μmol/L inhibited UDP evoked responses however, with nonspecific actions on other functions (Hartley, Kato, Salter & Kozlowski, 1998) such as inhibition of growth factors from binding to their receptors (Neufeld & Gospodarowicz, 1988; Williams et al., 1984) and influence on ATP release by endothelial cells in blood vessels (Yang et al., 1994**).**

3.1.3. Investigation for functional $P2Y_6$ receptors in porcine coronary arteries and rat thoracic aorta using a $P2Y_6$ antagonist, MRS 2578

The next step involved using a $P2Y_6$ receptor antagonist, MRS 2578 to confirm the functional presence of $P2Y_6$ receptors in the porcine coronary arteries and rat thoracic aorta. UDP was employed to determine the functional presence of the $P2Y_6$ receptors in the porcine coronary arteries and rat thoracic aorta.

N,N'-1,4-butanediylbis-N'-(3-isothiocyanatophenyl)thiourea also known as MRS 2578 is a potent, insurmountable antagonist at the $P2Y_6$ receptors associated with blocking UDP effects at both human and rat $P2Y_6$ receptors and is able to mediate blockade at concentrations of less than 1 μM of UDP (for human $P2Y_6$ at IC_{50} values 37 ± 16 nM, for rat $P2Y_6$ at IC_{50} values 98 ± 11 nM) (Mamedova et al., 2004) with little/no effect on $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors. With the presence of two isothiocyanate groups, this aryl diisothiocyanate derivative is classified as an insurmountable $P2Y_6$ antagonist as observed by the inability of the antagonist to produce a parallel rightward shift of the concentration-response curves (Vauquelin et al., 2002) and is therefore characterised as a non-competitive antagonist, predicted to react irreversibly with the $P2Y_6$ receptor but has disadvantages as it exhibits low solubility and limited aqueous stability (Jacobson et al., 2012).

UDP mediated concentration dependent contractile responses in PCAs were unaffected in presence of MRS 2578, evident by the unchanged potency and efficacy (Figure 4A). The same was observed by UDP mediated concentration-dependent relaxant responses in the rat thoracic aorta which were also unaffected in presence of MRS 2578 (Figure 10A). This finding was unexpected because $P2Y_6$ mRNA and protein have been detected in both the porcine coronary artery and rat thoracic aorta and because there exists some evidence for their functional expression in porcine coronary artery and rodent thoracic aorta.

The evidence for functional expression of $P2Y_6$ receptors in rodent thoracic aorta is more robust. Bar et al. (2008) studied isolated mouse aorta from $P2Y_6$ KO mice in which endothelium-dependent relaxation of the aorta evoked by UDP was abolished and the contractile effects evoked by UDP was abolished when endothelial nitric oxide synthase was blocked in the $P2Y_6$ KO mice, both evidence pointing out involvement of the $P2Y_6$ receptors in regulating vascular tone and blood flow.

In the porcine coronary artery, the functional evidence provided by Rayment et al. (2007) relied on non-selective antagonists (suramin, PPADS) as a selective antagonist for the $P2Y_6$ receptor was not commercially available at the time. The paper also suggested how the $P2Y_6$ receptors appear silent in terms of their contractile responses, which may be the reason for the small contractile responses produced by UDP in the PCAs (Rayment et al., 2007).

Because the lack of inhibition of UDP responses by MRS 2578 was unexpected, I investigated the possibility that there were issues with its stability in solution leading to a decrease in its activity with repeated freezing and thawing of the same stock solution. Freshly ordered MRS 2578 was dissolved (in DMSO), it was aliquoted and stored at -80 °C, defrosted immediately prior to use and any residual antagonist discarded. MRS 2578 handled in this way (rather than the same stock solution, kept at -20°C, being used) was still unable to block responses to UDP in both the porcine coronary artery and rat thoracic aorta. (Data reported in the results section are a combination of MRS 2578 handled in both of the ways described here).

Furthermore, a study by Kobayashi et al. (2017) in rat thoracic aorta showed that in the presence of MRS 2578, the magnitude of relaxant responses produced by UDP was inhibited however, no change was observed in UDP-mediated contractile responses in endothelial-denuded aortic segments in presence of MRS 2578, raising the question of whether MRS 2578 blocked only endothelial-

based $P2Y_6$ receptors or whether there may be "MRS 2578-sensitive" or "MRS-insensitive" $P2Y_6$ receptors, implying that UDP may be acting on other P2 receptors that are insensitive to blockade by the $P2Y_6$ inhibitor.

In addition, a study by Mitchell et al. (2012) has provided evidence for the non-selective actions of MRS 2578 suggesting that concentrations higher than 100 nM was associated with depressing the UDP-evoked contractions in rat intrapulmonary arteries and how the contribution of $P2Y_6$ receptors to the contractile responses mediated by UDP would not be determined. This is because, the antagonist was suggested to possess nonspecific actions to other sites, depressing the smooth muscle contractility. It was demonstrated that, at concentrations of 1 and 10 μM, MRS 2578 was associated with inhibiting contractions evoked by KCl. This mechanism was linked to the fact that isothiocyanate groups in the compound has potential to react chemically with nucleophilic groups on proteins.

In the present study, the evidence obtained using MRS 2578 suggested that the $P2Y_6$ receptor is not involved in mediating UDP responses in both porcine coronary arteries and rat thoracic aorta. The next step involved employing another $P2Y_6$ agonist, to confirm the findings obtained so far. PSB 0474 (3-(2-oxo-2-phenylethyl)-UDP, also known as 3-phenacyl UDP, was added cumulatively to porcine coronary arteries at basal levels. PSB 0474 is a structural analog of UDP and has >500-fold selectivity at the $P2Y_6$ over the $P2Y_2$ receptors with inactivity at $P2Y_4$ and $P2Y_{14}$ receptors (El-Tayeb et al., 2006; Gao et al., 2010). PSB 0474 evoked concentration dependent contractions in porcine coronary arteries (Figure 2) with a pEC_{50} value of 4.9 ± 0.3. The responses were more potent than UDP-mediated contractile responses by one order of magnitude. In the presence of the $P2Y_6$ antagonist, MRS 2578, the responses were significantly enhanced (for which the pEC50 was not determined as a plateau was not achieved). It is possible that MRS 2578 is blocking vasorelaxant $P2Y_6$ receptors activated by UDP, thereby enhancing contraction mediated by

UDP at non-$P2Y_6$ receptors. However, investigation of the effects of UDP in precontracted porcine coronary arteries showed that it did not mediate relaxation (Figure 6).

3.1.4. Characterisation of responses to PGE_2-G in porcine coronary arteries and rat thoracic aorta

Prostaglandin E_2 glyceryl ester (PGE_2-G) was recently demonstrated to activate $P2Y_6$ receptors with sub-picomolar potency in isolated cells (Brüser et al., 2017). The idea was to investigate whether PGE_2-G also demonstrated the same phenomenon in blood vessels. Being derived from cyclooxygenase 2 (COX-2) mediated oxygenation of the endocannabinoid, 2-arachidonoylglycerol (Nirodi et al., 2004), these underappreciated COX-2 products have not been widely studied and therefore, their biological functions are not fully understood. Since $P2Y_6$ receptor is associated with beneficial clinical implications, it was interesting to understand whether PGE_2-G could mediate any such effects by activating the $P2Y_6$ receptor. However, the results in the study so far have not shown the potential presence of the $P2Y_6$ receptor in the PCAs and rat thoracic aorta, despite previous studies showing their expression in these vessels.

PGE_2-G produced concentration-dependent contractile responses (pEC50 value of 6.7 ± 0.1 and R_{max} 11 ± 2%) in porcine coronary arteries with a potency at least two orders of magnitude greater than PSB 0474 (pEC_{50} value of 4.9 ± 0.3) and at least three orders of magnitude greater than UDP (pEC_{50} value of 3.8 ± 0.3) (Figure 2). In rat thoracic aortic rings, PGE_2-G produced concentration-dependent relaxations (pEC_{50} value of 8.5 ± 0.2 and R_{max} 36 ± 6%). A biphasic response was observed whereby, concentrations greater than 0.3 μM produced concentration dependent contractions. (Figure 9) Vasorelaxations evoked by PGE_2-G were more potent than UDP (pEC_{50} value of 6.6 ± 0.2 and R_{max} 80 ± 12%) relaxations but a smaller magnitude in comparison to UDP (Figure 9). The

relative potency of PGE_2-G is much lower than the suggested potency of PGE_2-G (~1 pM) by Brüser et al (2017) in isolated cells.

In porcine coronary arteries, PGE_2-G evoked concentration-dependent contractions were significantly inhibited in presence of AH-6809 (Figure 7A). Since no evidence for functional $P2Y_6$ was obtained in presence of the $P2Y_6$ receptor antagonist MRS 2578, it ruled out the possibility of its involvement in both contractile and relaxant responses produced by PGE_2-G and therefore, raising the possibility of it acting at a distinct receptor to mediate these responses (Figure 4C; Figure 10B).

Since EP (EP_1, EP_3 and EP_4) receptors have shown sensitivity to PGE_2-G, their involvement in mediating PGE_2-G evoked vascular actions was investigated. Unlike PGE_2-G, its metabolite, PGE_2 binds to all the EP receptors. The nonselective EP receptor antagonist, AH-6809 has significantly blocked PGE_2-G responses in PCAs, which suggests that the contractile responses may have been mediated by actions at EP_2 receptors but since EP_2 receptors are coupled to Gs, associated with elevating cAMP levels, it rules out its involvement (St-Jacques and Ma, 2016).

The biphasic responses produced by PGE_2-G in this study may either be a result of PGE_2-G hydrolysis, in which its metabolites may be activating their putative receptor and mediating the contractile responses. For example, PGE_2-G can hydrolyse into PGE_2, which can play a role in triggering calcium release via actions at EP receptors in the vascular smooth muscle. Hydrolysis mechanism was observed in neutrophils incubated with 300 nM of PGE_2-G which resulted in a time-dependent decrease in its levels (half-life of about 60 mins) and a concomitant PGE_2 (metabolite) build-up (Turcotte et al., 2017; Nirodi et al., 2004). According to the results obtained in the rat aorta, concentration-dependent contractions are produced after 35 min suggesting that PGE_2-G levels

are decreasing time-dependently as relaxations are no longer evoked. The same concentration (300 nM) elicited concentration-dependent contractions in PE-precontracted rat aortic rings, which may point out the involvement of PGE_2-G metabolites in mediating responses.

The other possibility is that at higher concentrations, PGE_2-G may have affinity to activate another receptor. Since PGE_2-G also activates EP receptors, although at lower potency than PGE_2 (its metabolite), the involvement of these receptors was investigated by employing a non-selective EP antagonist, AH-6809 (Ki = 350 nM for EP_2 receptors) (Figure 12B) whereby no effect was observed on the concentration-dependent relaxations mediated by PGE_2-G but concentration-dependent contractions observed after 0.1 μM were significantly inhibited. This also ruled out the possibility that PGE_2-G may have activated EP_2 receptors to mediate the relaxation responses, in which case they would also be inhibited by AH-6809 since the concentration used for AH-6809 is targeted to block EP_2 receptors in this study (35 μM), a concentration at which maximal occupancy for EP_2 receptors is achieved. No change was observed in relaxation responses mediated by PGE_2-G up to 0.1 μM (Figure 12B). Generally, EP_1 is characterised as a Gq coupled receptor; EP_2 and EP_4 as Gs coupled receptors and EP_3 as a Gi coupled receptor (St-Jacques and Ma, 2016).

PGE_2-G acts on EP_1 receptors which are coupled to increase cytosolic calcium levels, which may have been the activated receptor instead. Abramovitz et al. (2000) has reported pA_2 values of 6.4-7.0 for EP_1 which correlates with the binding affinity for the EP_1 receptor, although reported as a weak EP_1 antagonist. (Abramovitz et al., 2000). It is classified as a nonselective antagonist as it displays similar affinities for EP_2, EP_3 and DP receptors. Inhibition constants (K_i) for AH-6809 at recombinant prostanoid receptors were also suggested by Abramovitz et al. (2000) in which AH-6809 displayed Ki value of 1 217±98 nM (EP_1), 1 150±36 nM (EP_2), 1 597±140 nM (EP_3), >100 000 nM (EP_4), 1 415±104 nM (DP), >100 000 (FP) and >100 000 nM (IP) receptors.

On the other hand, vascular tone is maintained by the local release of endothelium-derived relaxing and contracting factors. The endothelial derived vasoconstrictor prostanoids may play a role in regulating vascular tone and the release of prostanoids such as thromboxane need to be considered. The ability of AH-6809 to inhibit endothelium-dependent contractions is attributed to its partial antagonism at thromboxane-prostanoid (TP) receptors (Tang et al., 2008). It is unclear from the present study whether the actions are mediated via receptors on the smooth muscle and/or endothelium.

Therefore, to determine whether contractions involved the endothelium/smooth muscle, PGE_2-G responses were characterised in endothelium-denuded PCAs and thoracic aortic rings. Due to minimal relaxation responses produced by PGE_2-G in endothelium-intact aortic rings, the responses in endothelium-denuded aortic rings showed almost unaffected responses. The contractile responses observed at 0.3 μM onwards, also remained unchanged (Figure 13B). In PCAs, PGE_2-G evoked an enhanced contractile response in comparison to the control (endothelium-intact vessels) (Figure 8). Since PGE_2-G did not cause relaxation in U46619-precontracted PCAs it seems unlikely that the removal of an endothelium-dependent vasorelaxant response to PGE_2-G led to the augmentation of PGE_2-G-mediated smooth muscle contraction. It is possible that the enhanced PGE_2-G contractile response is due to the removal of the endothelium barrier function, such that in its absence there is improved access of PGE_2-G to the vascular smooth muscle. Removal of the endothelium mimics an endothelial dysfunction situation during which production of nucleotides and contractile factors such as thromboxane are enhanced resulting in intimal proliferation of smooth muscle cells and therefore, a reduction in the vessel lumen. The present results suggest that during inflammation any increased production of PGE_2-G will be accompanied by an increase in its vasocontractile effects, exacerbating vascular dysfunction (Rubanyi, 1993).

3.1.5. Investigation for functional $P2Y_{14}$ receptors in porcine coronary arteries

Considering UDP is a potent agonist at $P2Y_{14}$ receptors, a selective $P2Y_{14}$ antagonist PPTN (4-(4-(piperidin-4-yl)phenyl)-7-(4-(trifluoromethyl)phenyl)-2-naphthoic acid) was employed to investigate whether contractile responses mediated by UDP in porcine coronary arteries involved $P2Y_{14}$ receptors. This non-nucleotide, high affinity competitive antagonist was initially characterised in the $P2Y_{14}$ receptor-expressing HEK 293 cells through its inability to inhibit UDP- glucose stimulated calcium mobilisation (Barett et al., 2013). In human C6 glioma cells, PPTN (1 nM), low concentration abolished activity of UDP at $P2Y_{14}$ receptors. The mode of antagonism is selective as no affinity for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ or $P2Y_{13}$ is exhibited up to a concentration of 10 μM (Barett et al., 2013). According to Fricks et al. (2008), UDP was also reported to behave as a competitive antagonist at hP2Y14 receptors.

In porcine coronary arteries, concentration dependent contractions mediated by UDP were unchanged in presence of the $P2Y_{14}$ antagonist PPTN, suggesting that $P2Y_{14}$ receptors are not involved in mediating contractile responses of UDP. Vasoconstrictor responses to UDP-glucose that were sensitive to PPTN have been previously reported in porcine coronary arteries under different experimental conditions (Abbas et al., 2018).

3.1.6. Investigation for functional $P2Y_2$ receptors in porcine coronary arteries and rat thoracic aorta

The next step involved determining whether $P2Y_2$ receptors contributed to the contractile responses mediated by UDP in porcine coronary arteries. Since UDP is associated with mediating actions at the $P2Y_2$ receptors, the contribution of $P2Y_2$ receptors in the contractile and relaxant responses in porcine coronary

arteries and rat thoracic aorta respectively, were investigated under the influence of a $P2Y_2$ receptor antagonist, AR-C 118925XX.

In porcine coronary arteries, concentration dependent contractions mediated by UDP were unchanged in presence of the $P2Y_2$ receptor antagonist, suggesting that $P2Y_2$ receptors are not involved in mediating contractile responses of UDP. Furthermore, the study also involved precontracting porcine coronary arteries with U46619 in presence of AR-C 118925XX. This is because $P2Y_2$ receptors have been suggested to be present primarily in the endothelium of coronary vessels, where a vasodilatory role is served (Gödecke et al., 1996) however, no relaxant responses were observed. Blocking the $P2Y_2$ receptors was conducted to understand whether $P2Y_6$ receptors may have a vasodilatory component however, concentration-dependent contractions were produced which rules out the possible involvement of $P2Y_6$ receptors in mediating relaxant responses in PCAs.

3.1.7. Investigation of a P2Y and PGE_2 signalling crosstalk in porcine coronary arteries and rat thoracic aorta

During physiological or pathological conditions, nucleotides and prostaglandins coexist in the extracellular milieu. The effects produced, whether beneficial or detrimental, depends on nucleotide release and duration of other prostaglandin production. Studies have previously shown that PGE_2 can regulate nucleotide levels and nucleotides can influence production of PGE_2 (Chen and Lin, 2000; Ito and Matsuoka, 2015). In this study, the non-selective EP receptor antagonist AH-6809 significantly attenuated the contractile responses mediated by UDP in porcine coronary arteries (Figure 7B) but enhanced the relaxant responses produced by UDP in rat thoracic aorta (Figure 12A) demonstrated by the change in R_{max}. The simplest explanation for the attenuation of UDP contractile responses in the porcine coronary artery is that UDP acts at a P2Y receptor which

stimulates the production of a contractile prostanoid. In the porcine pancreatic artery UDP-glucose contraction, which appears to involve the $P2Y_{14}$ receptor, is mediated by TXA_2/PGs (Alsaqati et al., 2014). Similarly, the enhanced UDP relaxation in rat aorta could be due to AH-6809 mediated inhibition of contractile responses mediated by contractile P2 receptors coupled to contractile prostanoid release. As discussed above, the identities of these UDP-sensitive P2 receptors in porcine coronary artery and rat thoracic aorta is not clear, but they do not appear to be any of the $P2Y_2$, $P2Y_6$ or $P2Y_{14}$ receptors.

Cyclooxygenase (COX) enzyme is responsible for the production of prostanoids by catalysing the conversion of arachidonic acid (AA). The potential involvement of the prostanoids in UDP evoked responses can be investigated by implementing COX inhibitors such as indomethacin which non-selectively blocks the COX enzyme. COX inhibitors may suppress the nucleotide mediated release of the prostanoid. This may allow identifying and confirming the potential involvement of the prostanoids. In addition, techniques such as immunoassay and employing liquid chromatography tandem mass spectrometry (LC-MS/MS) will allow to assess levels of prostanoid release from isolated vessels (Kirkby et al., 2013)

Previous study involving the use of rat cerebral astrocytes demonstrated that PGE_2 interfered with the calcium responses elicited by UTP and inhibiting this P2Y signalling resulted in impaired astrocyte migration. This was suggested to be mediated via EP_3 receptors. PGE_2 activation of EP_3 receptors not only affected levels of calcium but also UTP induced extracellular regulated kinases (ERK) and Akt phosphorylation (Paniagua-Herranz et al., 2017). Furthermore, the involvement of EGFR (epidermal growth factor receptor) transactivation by PGE_2 was suggested to be involved in dampening P2Y signalling. The receptor has been characterised in rat thoracic aorta, where it is predominantly present in smooth muscle. Phenylephrine is also associated to mediate contractions via EGFR transactivation along with alpha-1 receptors (Ulu et al., 2010). This may

be one of the possibilities to understand the crosstalk between the P2Y signalling and PGE_2 responses. Another PGE_2 and P2Y signalling crosstalk was observed in macrophages, whereby PGE_2 showed an inhibition of responses mediated by $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. In this scenario, the selective impairment of P2Y signalling was associated to be independent on EP receptors but rather involving activation of nPKC and PKD protein kinases, with functional implications in their metabolism and migration (Traves et al., 2013).

3.1.8. Calcium responses in murine microglial cell line - BV-2

$P2Y_6$ receptors are expressed in microglia and their expression has been previously characterised using reverse transcriptase polymerase chain reaction (RT-PCR) in the BV-2 cell line (Gendron et al., 2003; Jiang et al., 2017). The $P2Y_6$ receptors are coupled to elevation of intracellular calcium, which is readily quantified and therefore, the effects of UDP and PGE_2-G were tested in this established model. Concentration-dependent increase in calcium responses were observed in response to both PGE_2-G and UDP as both agonists were presumably associated to mediate actions via a Gq-coupled receptor. UDP is a known canonical receptor at the $P2Y_6$ receptors and characterisation of the responses in presence of the $P2Y_6$ antagonist, demonstrated a non-competitive inhibition of the calcium responses (Figure 14B & 14D). Responses obtained in presence of PGE_2-G, portrayed an increase in intracellular calcium, with a higher magnitude than UDP however, responses remained unattenuated in presence of the $P2Y_6$ antagonist. Actions of PGE_2-G failed to demonstrate actions at the $P2Y_6$ receptors (Figure 14A & 14C). The inhibition of UDP-evoked calcium responses by MRS 2578 further demonstrated that the antagonist was functional and performed its native actions of blocking the $P2Y_6$ receptors in the BV-2 cells. In contrast to the responses produced by Brüser et al (2017) in $P2Y_6$ transfected HEK-293 cells, unaffected calcium responses achieved in BV-2 cells by PGE_2-G in the presence of MRS 2578 demonstrated a lack of PGE_2-G/$P2Y_6$ signalling.

Overall, results obtained so far demonstrate that further work will have to be implemented to determine the receptor at which UDP and PGE_2G acts. In this study, both PCA and rat aorta have not demonstrated functional presence of the $P2Y_6$. This study can be a useful contribution to the current knowledge regarding functional presence of the $P2Y_6$. receptors and therefore, also provides an awareness of how currently established information may need revisiting since identification of functional P2 receptors is essential for therapeutic targeting. Techniques such as western blotting and RT-PCR may be implemented to identify receptors involved in the blood vessels. Furthermore, the concept of nucleotide and prostanoid signalling may require wider research. Prostanoid release evoked in presence of nucleotides in both physiological and pathological conditions can be measured in both cell and tissue-based experiments, which will enable better understanding of the nucleotide/prostanoid signalling mechanism.

3.3. Conclusion

In the current study, PGE_2-G/$P2Y_6$ signalling mechanism was assessed in porcine coronary arteries, rat thoracic aortic rings and the murine microglial cell line (BV-2). Overall, the results have not established the functional presence of the $P2Y_6$ receptor in blood vessels as characterisation of responses to UDP and PGE_2-G using various antagonists have failed to demonstrate $P2Y_6$ involvement.

In porcine coronary arteries, UDP and PGE_2-G evoked concentration-dependent contractions whereas in the rat aorta, concentration-dependent relaxations were produced. Endothelium-denuded vessel preparations were implemented to confirm vascular responses in either vessel. UDP responses were independent of the $P2Y_2$, $P2Y_6$ and $P2Y_{14}$ receptors and therefore, suggesting potential involvement of a different P2 receptor coupled to promote vasoconstriction in PCAs and vasodilation in rat aorta. PGE_2G evoked potent responses in

comparison to UDP but failed to activate $P2Y_6$ receptors in the PCAs and rat aorta, contradicting the theory suggested by Brüser et al (2017).

In BV-2 cells however, intracellular calcium responses evoked by UDP has shown attenuation of responses in presence of the $P2Y_6$ antagonist MRS 2578, demonstrating the presence of functional $P2Y_6$ receptors. However, PGE_2-G, failed to demonstrate any $P2Y_6$ receptor signalling mechanism but the robust intracellular calcium release, points out involvement of a G-protein coupled receptor.

In PCAs and rat thoracic aorta, $P2Y_6$ may not be coupled to induce vascular contractility changes but may be coupled to perform other functions such as cell migration and proliferation. The interesting phenomenon that was observed in these blood vessels, was the potential involvement of EP receptors in influencing UDP responses, which will require further experimental work to establish the signalling mechanism.

APPENDICES

Characterisation of responses to Bradykinin to determine endothelial integrity in porcine coronary arteries.

Endothelial integrity was assessed by precontracting the porcine coronary arteries, up to 40-60% of the second KCL response by cumulative addition of U46619, a thromboxane A_2 mimetic and once a stable precontraction was achieved, Bradykinin (1×10^{-10}-3×10^{-7} M) was added cumulatively. Concentration-dependent relaxations were produced by Bradykinin with a pEC_{50} value of 8.5 ± 0.1 and R_{max} of 83 ± 4% (Figure 15).

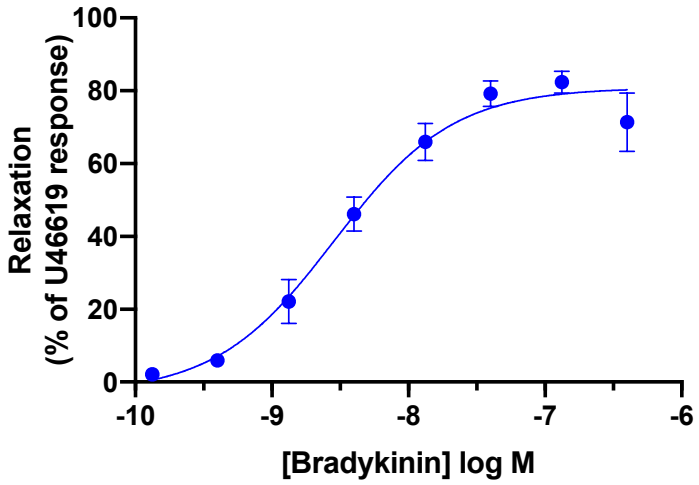


Figure 15. Concentration-dependent relaxations of Bradykinin in U46619-preconstricted porcine coronary arteries. Data presented are mean ± SEM (n=8).

Effect of AH-6809, a nonselective EP receptor antagonist in the porcine coronary arteries and rat thoracic aorta

In the porcine coronary arteries and rat aorta, AH-6809 (35 μM) was implemented to characterise the responses of UDP and PGE_2-G. During the incubation period of 30 minutes, there was a noticeable decline in the baseline observed in the PCAs unlike the rat aorta, in which the baseline remained very

stable. This may be one of the reasons why contractions evoked by UDP and PGE_2-G demonstrated a larger inhibition of responses in presence of the antagonist (Figure 7A & 7B). Representative trace below, demonstrate that the drop in the baseline has augmented the contractile responses produced by PGE_2-G. The same was achieved in presence of UDP.

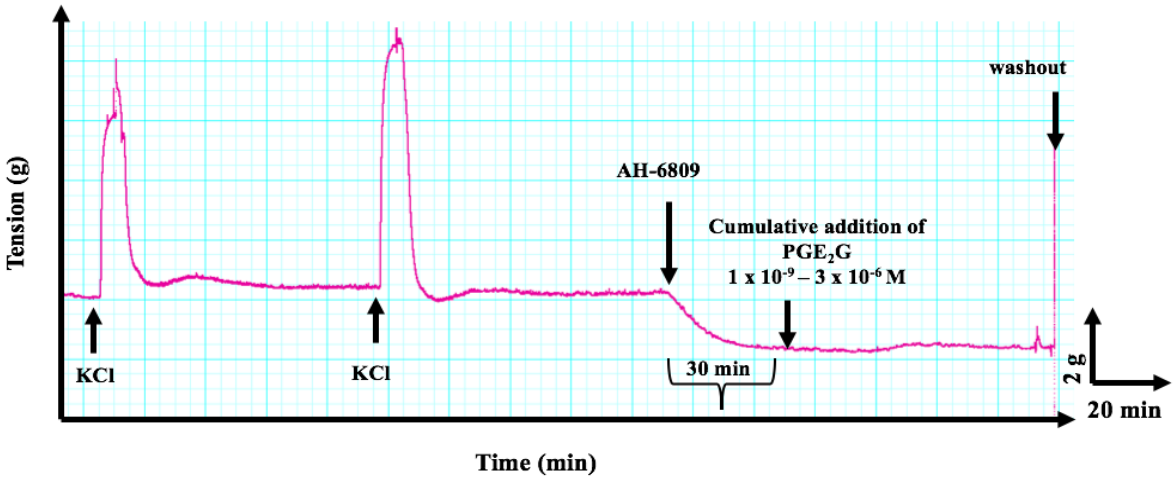


Figure 16. Representative trace showing the decline in baseline achieved by AH-6809 (non-selective EP receptor antagonist) in porcine coronary arteries.

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