Characterisation of P2 receptors that modulate vascular tone in pulmonary arteries

A thesis presented in fulfilment of the requirement for the degree of Doctor of Philosophy by

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Date: 27/02/22

Published work

“Chapter 3 of this thesis includes previously published work for which I have been responsible. I was involved in the planning of the experiments with my supervisors, carried them out and analysed the data. I was also involved in writing the paper with my supervisors.

Signed: Markie Obukowho Dales

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Articles in preparation

Identification of endothelial $P2Y_2$ receptors in rat pulmonary arteries using the selective antagonist, AR-C118925XX.

How selective antagonists and receptor knockout and knockdown have helped identify vascular P2Y receptor functions.

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ABSTRACT

Purinergic P2Y receptors are a family of eight G protein-coupled receptors ($P2Y_{1,2,4,6,11,12,13,14}$) that are activated by nucleotides to elicit both vasoconstriction and vasodilation. The role of individual P2Y subtypes in these actions is unclear due to the poor selectivity of most antagonists available. The aim of this project was to determine the role of $P2Y_2$ receptors in nucleotide-evoked vascular responses using the novel $P2Y_2$ receptor antagonist, AR-C118925XX.

First, its pharmacological actions were characterised using intracellular Ca^{2+} imaging as a bioassay. Uridine 5'-triphosphate (UTP) acted at recombinant $P2Y_2$ receptors stably expressed in 1321N1 cells to evoked concentration-dependent rises in Ca^{2+}. AR-C118925XX had no effect *per se* on intracellular Ca^{2+}, but reversibly and competitively antagonised the actions of UTP (pA_2=8.43, K_B=3.7 nM). 1 μM AR-C118925XX had no effect at native or recombinant $hP2Y_1$, $hP2Y_4$, $rP2Y_6$ or $hP2Y_{11}$ receptors.

Next, the role of native $P2Y_2$ receptors in the vascular actions of nucleotides was investigated. AR-C118925XX inhibited UTP-evoked Ca^{2+} mobilisation in human EAhy926 vascular endothelial cells (K_B=3.0 nM). UTP, uridine 5'-diphosphate (UDP) and adenosine 5'-triphosphate (ATP) elicited contractions of rat isolated intrapulmonary and tail arteries at resting tone that were both unaffected by AR-C118925XX (1 μM). At raised tone, AR-C118925XX (1 μM) partially inhibited endothelium-dependent vasodilation of intrapulmonary arteries evoked by UTP and UDP, but had no effect on responses to ATP and ADP. At raised tone, these agonists caused vasoconstriction rather than vasodilation of tail arteries.

These data show that AR-C118925XX is a potent, selective and competitive $P2Y_2$ receptor antagonist. They also demonstrate that $P2Y_2$ receptors do not mediate contraction of intrapulmonary and systemic arteries, and that they are natively expressed in vascular endothelial cells, where they mediate Ca^{2+} mobilisation and vasodilation. As the only selective $P2Y_2$ antagonist available, AR-C118925XX will be invaluable in identifying the functions of $P2Y_2$ receptors function and their potential as a novel therapeutic target.

1 INTRODUCTION

1.1 Pulmonary Circulation

1.1.1 The Pulmonary Vascular System

Blood is delivered to tissues throughout the human body by the pulmonary and systemic circulatory systems. The pulmonary circulation is a high capacity, low-resistance and low-pressure circuit that serves the lungs from the right side of the heart; while the systemic circulation serves the rest of the body from the left side. The main function of the pulmonary circulation is to enable gas exchange across the alveoli of the lungs (Lammers *et al.*, 2012; Rizzo *et al.,* 2014). Deoxygenated blood from the systemic circulation is returned to the right atrium of the heart via the superior and inferior vena cava (figure 1.1). Blood flows through the tricuspid valve into the right ventricle, and is then pumped into the pulmonary artery via the pulmonary semi-lunar valve. The artery divides into two branches, which serve the right and left lung. These then branch into smaller arteries, arterioles and then capillaries. The capillaries surround the alveoli where the exchange of carbon dioxide (CO_2) and oxygen (O_2) takes place. The capillaries then come together to form venules that merge into veins, and eventually into the pulmonary veins that return blood to the left atrium. The oxygenated blood enters the left atrium and travels through the mitral valve into the left ventricle, where blood is pumped throughout the whole body in the systemic circulation. Once the blood has been transported through the systemic circulation, deoxygenated blood is recollected in the vena cava and the process is restarted.

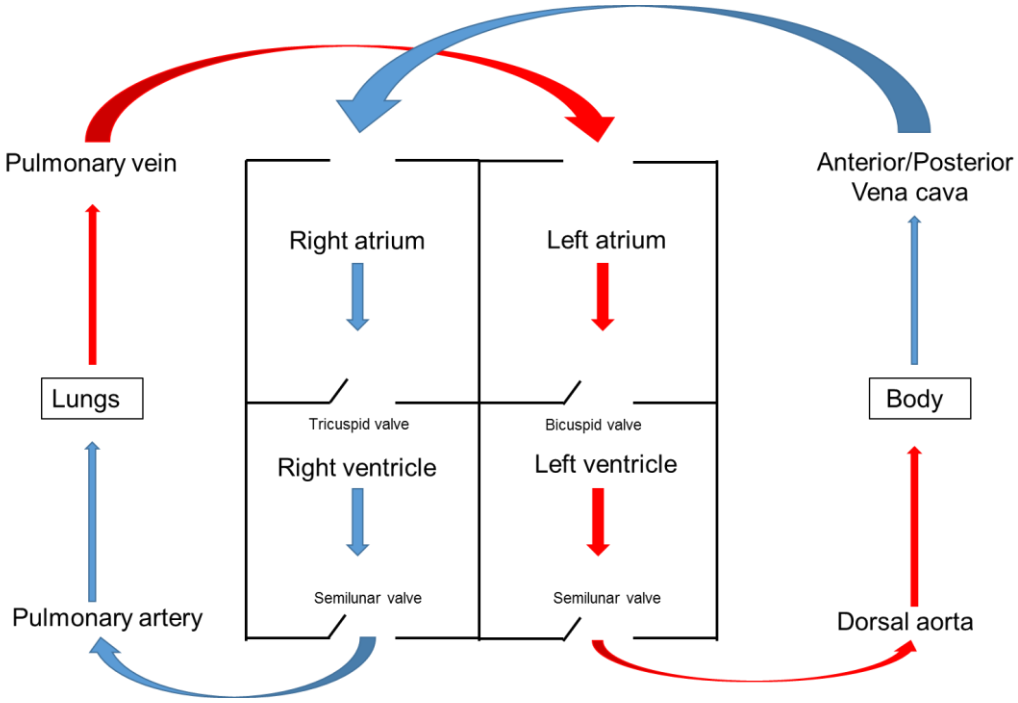


Figure 1.1: The exchange of blood between the pulmonary and systemic circulation. The pulmonary circulation carries blood to the lungs to be oxygenated, while the systemic circulation distributes oxygenated blood to the rest of the body.

1.1.2 Pulmonary Vasculature Structure

The vasculature of the pulmonary circulation is formed by extensive branching of arteries, veins and capillaries. The pulmonary arteries are elastic laminae and muscular coats characterised by their wall structure, size and branching system. To classify the pulmonary artery, several ordering systems have been used, including the divergent, convergent and the Strahler approaches. In the divergent approach, the main pulmonary artery is classified as “generation 1”, with the subsequent division becoming “generation 2, 3” and so on. An alternative is the convergent approach, in which the structure of the arteries are related to their function and in this approach the ordering starts with the end branches. The peripheral branch of the pulmonary artery (a precapillary artery with diameter of 13 µm) is numbered ‘order 1’, and this branching order/system increases until it reaches the main pulmonary artery (diameter of 3 cm; order 17). In the Strahler approach, the most distal branches are order 1, two of these braches merge to form an order 2 branch; two order 2 branches merge and form order 3 and so on up to single main vessel (Singhal *et al.,* 1973).

Structurally, the pulmonary artery can be sub-divided into 5 categories: *Elastic arteries*: Larger arteries with adventitial, muscular (media) and intimal layers. The muscular layer is bound by internal and external elastic laminae with three or more layers in the muscle coat. They are 3,200-2,000 µm in external diameter. According to the convergent approach, the elastic arteries have a branching order/system of 17-13. *Muscular arteries*: Arteries greater than 150 µm in external diameter, with internal and external laminae and a branching order of 13-3; there is an absence of an internal elastic laminae in the smallest arteries. *Partial muscular arteries*: These are 40-80 µm in external diameter and have a branching order of 5-3; the spiral arrangement of smooth muscle cells becomes apparent on removal of the surrounding muscle coat. *Non-muscular arteries*: These represent most of the vessel population of < 50 µm external diameter, they have a branching order of 5-1 and are characterised by the absence of an elastic laminae and replacement of smooth muscle cells by pericytes, which are involved in the production of basement membranes. *Supernumerary arteries*: Relatively small thin-walled arteries with their branching order between 11 and 12, act as a short cut to blood supplying the alveoli adjacent to conduit arteries (Singhal *et al.*, 1973; Hughes and Morrell, 2001; Jones and Capen, 2011).

The pulmonary veins branch in the same way as the pulmonary arteries, however they have a branching tree with 15 orders because the four pulmonary veins drain into the left atrium without forming an additional two orders. Also, venous system vessels have no elastic lamina and their walls contain more elastic tissue and less muscle than the pulmonary arteries (Hughes and Morrell, 2001).

1.1.3 Regulation of Vascular Tone

In the pulmonary circulation, active and passive factors regulate vascular tone (Barnes and Lui 1995; Barrett *et al.,* 2010). Passive factors include cardiac output, left atrial pressure, vascular obstruction, lung volume and gravitational force (Grover *et al.,* 2011). On the other hand, active regulation involves vasomotor activities regulated by non-neural and neural factors, which modulate the release of vasodilators and vasoconstrictors that lead to relaxation and contraction of vascular smooth muscle located within the medial layer of the arterial wall (Barrett *et al.,* 2010). The neural and non-neural factors include the autonomic nervous system, the endothelium, humoral agents, and respiratory gases. Active regulation has a greater influence on the pulmonary circulation than passive regulation and the majority of this control takes place in the small pulmonary arteries and arterioles (Chan and Joseph, 2011; Lumb, 2017).

1.1.3.1 Vascular smooth muscle regulation of vascular tone

The major determinant of blood flow resistance through the circulation is the contractile state of the vascular smooth muscle (VSM), which is dependent on the relationship between vasodilator and vasoconstrictor stimuli from endothelium-derived factors, circulating hormones and neurotransmitters. These signals are integrated by VSM to determine the diameter and hydraulic resistance of a blood vessel; ion channels play a role in this process. The VSM uses Ca^{2+} as the trigger for contraction, the influx of Ca^{2+} through ion channels in the plasma membrane and its release from intracellular stores are the major source of activator Ca^{2+} (Nelson *et al.,* 1990). In addition, the flow of ions through ion channels determines to a large extent the membrane potential, which regulates and modulates Ca^{2+} influx and release through ion channels (Ganitkevich and Isenberg, 1993; Kukuljan *et al.*, 1994). There are two general mechanisms involving membrane potential in the contraction of smooth muscle cells: 1) *Pharmacomechanical coupling*- contraction and relaxation that is independent of changes in the resting membrane potential (E_m) and dependent upon activation of receptors; and 2) *Electromechanical coupling*- contractions and relaxation generated through changes in the E_m. Regardless of the mechanism, an increase in cytosolic Ca^{2+} is the major trigger for smooth muscle contraction and pulmonary vasoconstriction in both (Firth and Yuan, 2011).

The VSM expresses various ion channels in their plasma membrane, all of which are involved in regulating vascular tone. They include: one to two types of voltage-gated Ca^{2+} channels (VGCC), store-operated Ca^{2+} channels, stretch-activated cation channels and several types of K^+ and Cl^- channels (Nelson *et al.,* 1990; Davis *et al.,* 1992; Nelson and Quayle, 1995; Large and Wang, 1996; Gibson *et al.,* 1998). VGCC are essential in regulating vascular tone. They are activated by membrane depolarisation, leading to Ca^{2+} influx; the increase in cytosolic Ca^{2+} concentration triggers vasoconstriction (figure 1.2a). In contrast, opening of K^+ channels results in the outflow of K^+, causing membrane hyperpolarisation that leads to the closure of VGCC and decrease in Ca^{2+} influx, initiating vasodilation (figure 1.2a; Jackson, 1998). Closure of K^+ channels, most especially large conductance K^+ channels (BK_{Ca}) and voltage-gated K^+ channels (K_V) promotes depolarisation, resulting in the opening of VGCC, Ca^{2+} influx and vasoconstriction (Jackson, 2017). Protein kinase C (PKC), commonly activated by vasoconstrictors that activate $G_{q/11}$ coupled receptors, may be involved in the closure of BK_{Ca} channels (Minami *et al.,* 1993; Lange *et al.,* 1997).

An increase in cytosolic Ca^{2+} concentration results not only from an influx of extracellular Ca^{2+} via VGCC, but also from 1) influx of extracellular Ca^{2+} via store- and receptor-operated Ca^{2+} channels (SOC, ROCs) and/or 2) release of intracellular Ca^{2+} from the sarcoplasmic reticulum (SR) through inositol 1,4,5-trisphosphate (IP_3) receptors and ryanodine receptors (Karaki *et al.,* 1997). When cytosolic Ca^{2+} increases, Ca^{2+} binds to calmodulin to form a Ca^{2+}-calmodulin complex that activates myosin light chain kinase (MLCK), which in turn phosphorylates myosin light chain (MLC) (figure 1.2b**;** Kamm and Stull, 1985). Phosphorylated MLC (MLC-P) increases the activity of myosin ATPase, which converts adenosine 5'-triphosphate (ATP) to adenosine 5'-diphosphate (ADP). It also combines with actin to form a cross bridge (actomyosin) that leads to contraction. Afterwards, MLC-P is dephosphorylated by myosin light chain phosphatase, causing the smooth muscle to relax (Kamm and Stull, 1985; Sylvester, 2004; Firth and Yuan, 2011).

MLC phosphorylation also occurs independently of changes in the intracellular Ca^{2+} level of pulmonary artery smooth muscle cells (PASMC), a phenomenon known as

myofilament Ca^{2+} sensitisation (Kitazawal *et al.,* 1989). This process occurs when Rho kinase, PKC or tyrosine kinases are stimulated in response to acute hypoxia, mechanical stimuli and G-protein coupled receptor (GPCR) ligands. Stimulation of these kinases initiates MLC phosphatase inactivation, leading to accumulation of MLC-P and contraction of the PASMC (Ming Cui Gong *et al.,* 1992; Nishimura *et al.,* 1992; Di Salvo *et al.,* 1993; Broughton, Walker and Resta, 2008). RhoA/Rho kinase is an important player in Ca^{2+} sensitisation and a key regulator of vascular tone. RhoA, a hydrolysing enzyme belonging to the Ras superfamily that binds and hydrolyses guanosine-5´-triphosphate (GTP), is activated when GPCR stimulation activates guanine nucleotide exchange factors (GEFs), which facilitates the exchange of guanosine-5´-diphosphate (GDP) bound RhoA (inactive) for GTP-bound RhoA (active). GTP-bound RhoA kinase interacts with Rho kinase, which phosphorylates (at threonine 696 and 853) and inhibits myosin phosphatase targeting subunit (MYPT1) of myosin light chain phosphatase. This leads to increased MLC phosphorylation and contraction of the PASMC (Somlyo and Somlyo, 2003; Grassie *et al.,* 2011).

Another Ca^{2+} sensitisation pathway is mediated by isoforms of PKC, a key regulatory enzyme that contributes to contractility and proliferation of PASMC (Dempsey et al., 2000). There are at least 13 isoforms divided into three subclasses: classical PKC isoforms (α, βI, βII, γ) regulated by both Ca^{2+} and diacylglycerol (DAG), novel PKC isoforms (δ, μ, η, θ, ε) requiring only DAG for activation and atypical PKC isoforms (ζ λ) activated by phosphatidyl serine and phosphorylation (Dempsey *et al.,* 2000). Existence of these isoforms has made it difficult to determine which of these individual PKC isoenzymes contribute to the regulation of pulmonary vascular reactivity. However, what is known is that PKC elicits PASMC contraction through Ca^{2+} -dependent and -independent mechanisms. An example of the role PKC plays in Ca^{2+} sensitisation is the inhibition of protein phosphatase 1 catalytic subunit (PP1c) of myosin light chain phosphatase (Chang *et al.,* 2018). This is an enzyme essential for smooth muscle contraction and its action is inhibited by phosphorylation of CPI-17 (17 kDa PKC-potentiated myosin phosphatase inhibitor protein), a phosphorylation-dependent inhibitory protein for smooth muscle cells (Hamaguchi *et al.,* 2000). The

inhibition of PP1c increases myofilament Ca^{2+} sensitivity that leads to PASMC contraction.

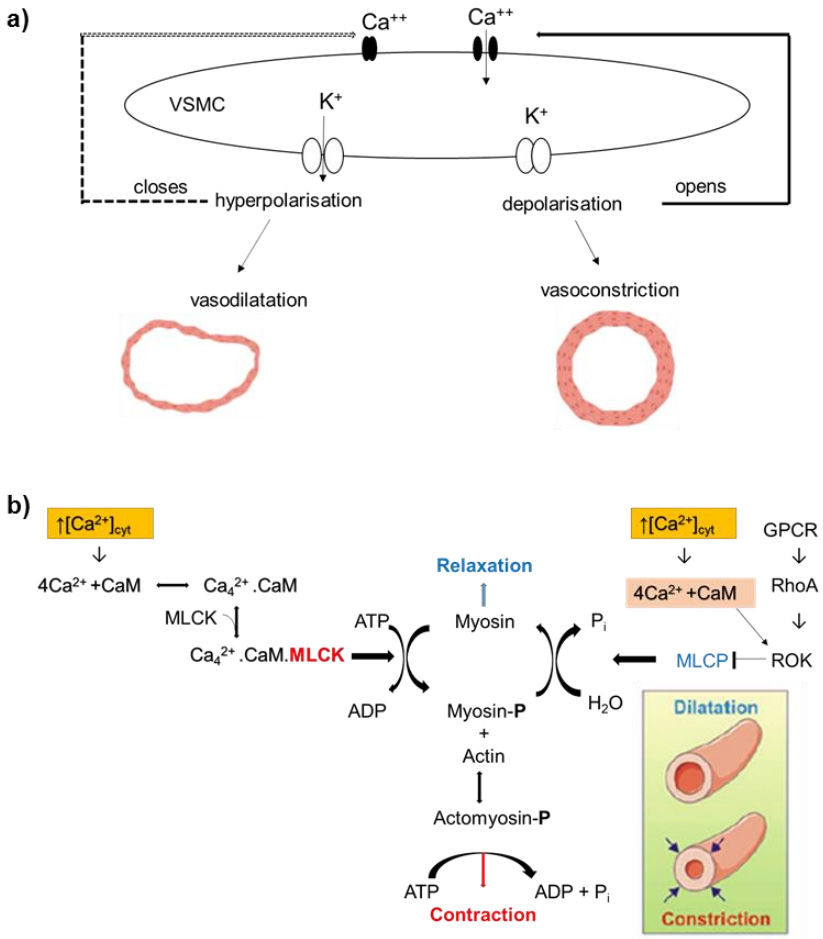


Figure 1.2*:* Regulation of vascular tone through Ca^{2+} influx. *a*) Vascular smooth muscle cell (top) showing the influx of Ca^{2+} and the outflow K^+. Cross section of arterioles (bottom) showing the opening of K^+ channels that leads to hyperpolarisation and decrease in intracellular Ca^{2+} concentration, resulting in vasodilatation. Closing of the K^+ channels leads to depolarisation and vasoconstriction. b) Influence of Ca^{2+} on dilatation and constriction. Cystolic Ca^{2+} concentration rises, Ca^{2+} binds to calmodulin (CaM) which activates MLCK to phosphorylate MLC. This increases myosin ATPase activity, hydrolyses ATP to release energy. Phosphorylated actomyosin produces displacement of myosin filament causing contraction. An increase in Ca^{2+} concentration (far right), and activation of RhoA leads to activation of Rho kinase (ROK) which subsequently inactivates MLC phosphatase causing contraction.

1.1.3.2 Endothelium and its regulation of vascular tone

The endothelium is composed of endothelial cells that line the internal surfaces of the vascular system. It is multifunctional and acts as a semi-permeable barrier that controls the movement of large and small molecules from the plasma into the tissue and *vice versa*. It is also important in maintaining the vessel wall and circulatory function. Other functions of the endothelium include: maintaining vascular tone by acting in an autocrine fashion to autoregulate endothelial function, acting in a paracrine fashion to influence neighbouring VSMCs and circulating blood elements (white blood cells, platelets), and regulating inflammatory responses by controlling lymphocytes, leukocyte interactions, monocyte interactions, vasculogenesis and angiogenesis (Sumpio *et al.*, 2002). Any disruption to the endothelial function or structure can lead to pathophysiological states such as: atherosclerosis, altered immune and inflammatory responses, haemostatic dysfunction and pulmonary arterial hypertension (PAH) (Sandoo *et al.,* 2010; Huertas *et al.,* 2018).

The endothelial cells releases specific paracrine and endocrine factors that control vascular tone, either by evoking contraction (endothelin-1 (ET-1), serotonin (5-HT) and thromboxane-2 (TXA_2)) or by causing relaxation (nitric oxide (NO), prostacyclin (PGI_2) and the endothelium-derived hyperpolarising factor (EDHF)). Endothelium-dependent vasodilation was first reported by Furchgott and Zawadzki (1980) and plays an important role in maintaining the basal vasodilator tone of the blood vessels. NO was subsequently identified as the endothelium-dependent vasodilator (Palmer *et al.,* 1987) and is formed by nitric oxide synthase (NOS), which exists in three isoforms: a neuronal isoform (nNOS) that acts as a neurotransmitter and a neuronal messenger regulating synaptic neurotransmitter release, an inducible isoform (iNOS) expressed in cells exposed to inflammatory mediators that activate macrophages and endothelial NOS (eNOS) that produces NO in the vasculature. Blood vessels are largely dependent on eNOS to dilate, so the focus will be on this particular isoform (Lamas *et al.,* 1992; Michel *et al.,* 1997; Prast and Philippu, 2001).

Inactive eNOS is bound to the protein caveolin and an increase in the intracellular Ca^{2+} concentration activates eNOS by promoting its detachment from caveolin (Bucci *et al.,* 2000). GPCR agonists, such as acetylcholine (ACh), bradykinin (BK), ATP and

ADP promote this dissociation by releasing Ca^{2+} from the endoplasmic reticulum (figure 1.3; Bucci *et al.*, 2000). Ca^{2+} then binds to calmodulin in the cell cytoplasm, which in turn undergoes structural changes that allows it to bind to eNOS; eNOS then converts L-arginine into NO (Fleming and Busse, 1999). A decrease in Ca^{2+} causes eNOS to dissociate from the Ca^{2+}-calmodulin complex and re-associate with caveolin, rendering eNOS inactive.

Ca^{2+}-independent mechanisms can also be activated to regulate the production of NO, including phosphorylation of eNOS, a process that occurs via protein kinases (protein kinase A (PKA), protein kinase B (PKB), adenosine 5' monophosphate (AMP) kinase (AMPK)) and cyclic guanosine-3',5-monophosphate (cGMP) protein kinase dependent II (Michel *et al.,* 1997; Butt *et al.,* 2000). The endothelium is constantly exposed to shear stress, a dragging force generated by blood flow. Shear stress triggers a range of biochemical changes in cell function, one of which is the production of NO by a mechanism that does not involve changes in the intracellular Ca^{2+} level (Boo *et al.,* 2002). Evidence suggests that shear stress initiates phosphorylation of eNOS via a PKA- and PKB-dependent mechanism (Boo *et al.,* 2002). Shear stress-dependent phosphorylation of eNOS also occurs by activation of Ca^{2+}-activated K^+ channels (K_{Ca}) on the endothelial cell surface. The effect of opening K^+ channels is to hyperpolarise the endothelial cell, which in turn increases the driving force for Ca^{2+} entry (via flow mediated vasodilation), thereby promoting the association of Ca^{2+}-calmodulin with eNOS to produce NO (Moens *et al.,* 2005; figure 1.3). Several additional mechanisms can also mediate shear stress-dependent activation of eNOS, including acylation and translocation of eNOS, as well as eNOS interaction with other molecules, such as heat shock protein 90 (Liu and Sessa, 1994; Prabhakar *et al.,* 1998; Dimmeler *et al.,* 1999; Fulton *et al.,* 1999; Gallis *et al.,* 1999; Su and Block, 2000). The mechanisms described above involving eNOS are continuously active and help to produce NO to maintain basal vasodilator tone. Consistent with this, the eNOS inhibitor, N^G monomethyl-L-arginine (L-NMMA), increases blood pressure *in vivo*, an effect that can be reversed by administering NO (Rees *et al.,* 1989).

Following NO synthesis, NO diffuses to and enters adjacent VSMC, where it binds to soluble guanylyl cyclase (sGC), which promotes the conversion of GTP to cGMP,

which triggers relaxation of VSMC by activating a series of intracellular pathways that results in a reduction of cytoplasmic Ca^{2+} levels (Morgado *et al.,* 2012). Four main pathways are thought to be involved in the vasodilator effect by cGMP: 1) A decrease in intracellular Ca^{2+} levels that can be achieved through inhibition of Ca^{2+} release from the SR, activation of Ca^{2+} uptake by the SR, increased intracellular Ca^{2+} efflux and decreased extracellular Ca^{2+} influx; 2) Hyperpolarisation of smooth muscle cell through activation of K^+ channels and inactivation of Ca^{2+} channels 3) Reduction in the sensitivity of the contractile mechanism by decreasing the cytosolic Ca^{2+} concentration sensitivity of MLC phosphorylation as a result of a decrease in MLCK activity and/or an increase in MLC phosphatase activity; and 4) Reduction in the sensitivity of the contractile machinery by uncoupling contraction from MLC phosphorylation via a thin-filament regulatory process (Morgado *et al.*, 2012).

An endothelium-derived hyperpolarising factor distinct from NO, is also involved in smooth muscle hyperpolarisation and relaxation (Feletou and Vanhoutte, 1988). There are three main suggestions as to the nature of EDHF, which are not mutually exclusive, but may represent differences between species and vascular beds. One suggestion is that EDHF represents direct charge transfer from the endothelial cells to the VSMCs. In this model, endothelial hyperpolarisation is generated by activation of Ca^{2+}-activated K^+ channels, $K_{Ca}3.1$ and $K_{Ca}2.3$ (small-conductance K^+ channel) that are expressed in most endothelial cells and the hyperpolarisation spreads passively via myoendothelial gap junctions (Adeagbo and Triggle, 1993; Eichler *et al.,* 2003) comprising of proteins, such as connexin 37 (Chaytor *et al.,* 2005; Figueroa and Duling, 2009; Figueroa *et al.,* 2013), resulting in hyperpolarisation of VSMC. This in turn reduces VSMC contractile activity by reducing Ca^{2+} influx via VGCC and by suppressing key enzymes involved in agonist-induced pathways (Coleman *et al,* 2001). Another hypotheses is that EDHF is a product of a cytochrome P450 (CYP450) pathway, such as epoxyeicosatrienoic acid (EET), an arachidonic acid-derived product of CYP450 epoxygenases. which evokes hyperpolarisation through the activation of BK_{Ca} in the VSMCs (Hecker *et al.,* 1994; Campbell and Harder, 1999). The final hypothesis is that K^+ efflux from endothelial cells via small- and intermediate-conductance Ca^{2+} -activated K^+ channels (SK_{Ca} and IK_{Ca}) activates inward rectifier K^+ channels (K_{IR}) and Na^+/K^+-ATPase on the VSMCs (Edwards *et al.,* 1998).

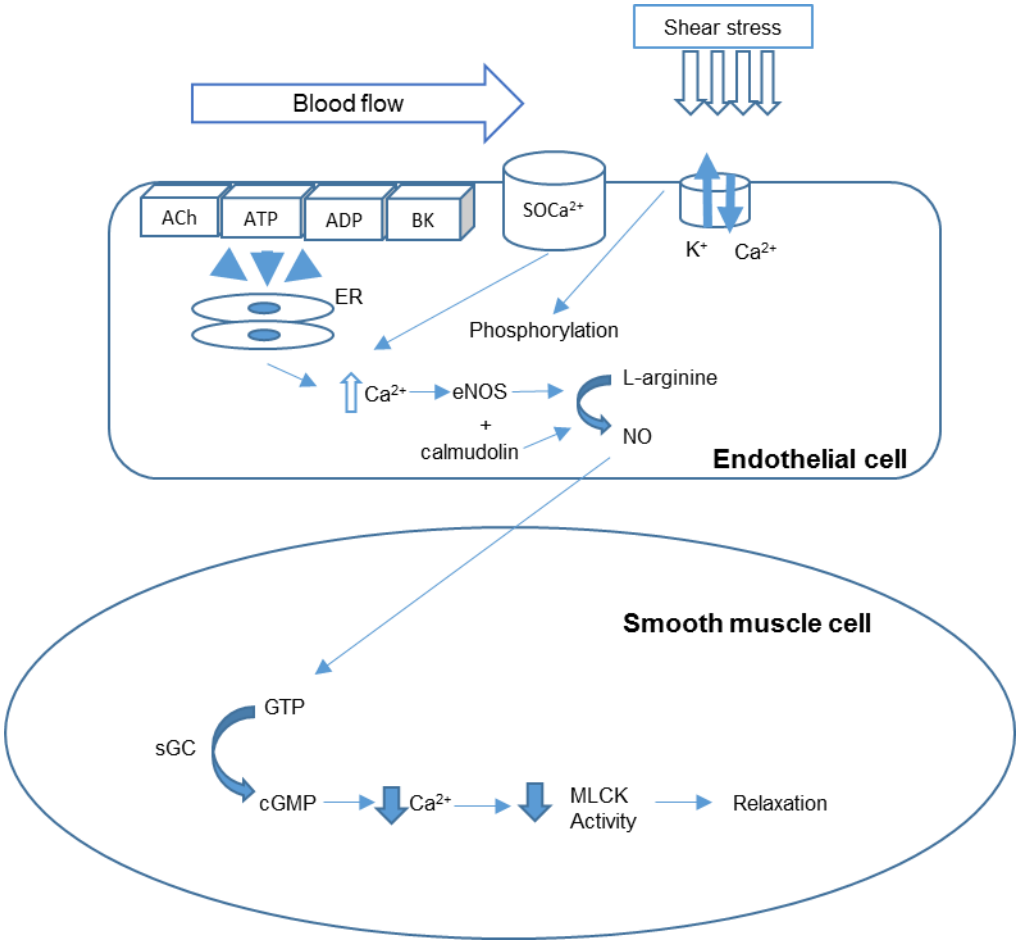


Figure 1.3: NO production and its effect on vascular tone. Agonists such as ACh, BK and ATP stimulate the release of Ca^{2+} from the endoplasmic reticulum (ER) in the endothelial cells; which enables the coupling of Ca^{2+} -calmodulin that in turn bind to eNOS to illicit the production of NO in the endothelial cell. Shear stress also initiates the phosphorylation of eNOS that also promotes the production of NO. NO then diffuses in to the smooth muscle cell to trigger the conversion of GTP to cGMP that leads to relaxation of the muscle.

1.1.3.3 Neural Regulation of Vascular Tone

Pulmonary vascular tone can be modulated by sympathetic, parasympathetic and non-adrenergic, non-cholinergic (NANC) components of the autonomic nervous system. The nerve supply of the autonomic system to the lungs arises from branches of the sympathetic neurones, vagal preganglionic and postganglionic neurones, which form the pulmonary plexus (Fisher, 1965; Kummer, 2011; Vaillancourt *et al.,* 2017).

1.1.3.3.1 Sympathetic control

The terms, sympathetic and '(nor)adrenergic' are sometimes used interchangeably to describe the fibres that originate from the first five thoracic ganglia and travel to the pulmonary vessels via the cervical ganglia. The sympathetic innervation acts mainly on the smooth muscle of the arteries and arterioles (Cech, 1969). In all species, the sympathetic innervation is highest in large extrapulmonary and hilar (the region where arteries, veins, bronchi and nerves enter and exit the lungs) blood vessels and then declines as the vessel diameter narrows. It varies from species to species how far sympathetic axons reach into the lungs. Small arteries down to 50 µm in diameter are innervated in humans, rabbit, sheep, dog, and cat, whereas in mouse, hedgehog, and rat the sympathetic innervation stops after the lung hilus (Fisher, 1965; Cech, 1969; Haberberger *et al.,* 1997).

The sympathetic control of the pulmonary circulation involves α_1 adrenoceptors, which mediate vasoconstriction in response to noradrenaline release, and β_2 adrenoceptors, which produce vasodilatation in response to circulating adrenaline (Lumb, 2017; Vaillancourt *et al.,* 2017). In the rat tail artery, both α_{1A} and α_{1B} adrenoceptors have been implicated in contractions induced by released noradrenaline (Jähnichen *et al.,* 2004). α_2 adrenoceptors have also been implicated in regulating pulmonary vascular tone (Magnenant *et al.,* 2003; Jantschak and Pertz, 2012). Magnenant *et al.,* (2003) showed that basal α_2 adrenoceptor activation is related to NO production and noradrenaline-mediated pulmonary vasodilation in ovine foetus. On the other hand, Jantschak and Pertz, (2012) showed that α_{2c} adrenoceptors mediate contraction in porcine pulmonary arteries.

1.1.3.3.2 Parasympathetic Control

The parasympathetic pathway to the lungs comprises of a chain of two groups of cholinergic neurones, vagal preganglionic and postganglionic neurones. The vagal preganglionic neurones arise in the nucleus ambiguus in the brainstem, while the postganglionic neurones are present in small ganglia located along the airways and hilar blood vessels (Hadziefendic and Haxhiu, 1999). Similar to the sympathetic fibres, the parasympathetic fibres have the highest density at the large extrapulmonary and hilar vessels. Their distribution also varies among species. For example, in rabbit the cholinesterase activity, a marker of parasympathetic nerves, was high compared to the guinea pig, where no nerve fibres where found (Čech, 1973; El-Bermani *et al.*, 1982; Haberberger *et al.,* 1997). The parasympathetic nerves mediate pulmonary vasodilation by releasing ACh and stimulation of M_3 muscarinic receptors, for example in precontracted feline pulmonary arteries (McMahon *et al.,* 1992). ACh-mediated vasodilation is endothelium- and NO-dependent, and in the absence of the endothelium, ACh is a vasoconstrictor (Buels and Fryer, 2012; Gao *et al,* 2016). The importance of cholinergic nerves in humans compared to the sympathetic system is unclear, although cholinergic nerve fibres have been shown histologically to be present in the human pulmonary vessels (Kummer, 2011; Lumb, 2017).

1.1.3.3.3 NANC Control

As the name implies, NANC mechanisms are not inhibited by blockade of cholinergic and adrenergic pathways. They are rather a manifestation of neurotransmission in the sensory, sympathetic and parasympathetic nerves that is mediated by neurotransmitters other than noradrenaline and ACh (Lumb, 2017). Barnes and Lui, (1995) showed that NANC responses can either be excitatory (e-NANC) or inhibitory (i-NANC) in rat small pulmonary artery. The frequency-dependent relaxations of pulmonary artery rings of cat were unaffected by adrenergic and cholinergic antagonists, but blocked by tetrodotoxin, indicating that they were neurogenic and not mediated by noradrenaline or ACh. Purines, substance P and vasoactive peptide (VIP) have all been suggested to be neurotransmitters within this system (Kummer, 2011; Ochs and O'Brodovich, 2019).

1.1.3.4 Humoral Regulation of Vascular Tone

Circulating mediators also modulate pulmonary vascular tone, and their effects vary depending upon the species, pre-existing tone and age (Barnes and Lui, 1995). Mediators such as angiotensin II, thrombin, and prostaglandins (D_2, E_2) are vasoconstrictors, whilst VIP and prostaglandins (E_1, I_2) are vasodilators (Barnes and Lui, 1995; Barrett *et al.,* 2010; Lumb, 2017). The role of many of these mediators remains unclear, but it is possible that some are involved in pulmonary vascular disease. Table 1.1 shows receptor-agonist systems demonstrated *in vitro* using pulmonary arteries of various species. The activities of some of the mechanisms listed below, but not all, are dependent on the endothelial lining of the pulmonary blood vessels. However, the basic mechanisms for these activities occur within the smooth muscle cells, while the endothelium acts as a modulator of the response. Take note that the substances listed below have mostly only been studied in animals and their physiological or pathological relevance in humans still remains unclear.

Table 1.1: Humoral mediators that modulate pulmonary vascular tone. Reconstructed using the information from (Yuta Kobayashi and Amenta, 1994; Barnes and Lui, 1995).

Mediators	Receptor Subtype	Action	Species	Endothelium-dependent?
Angiotensin II	A-II	Contraction	Human Rat	No
Atrial naturetic peptide (ANP)	ANP_A	Relaxation	Humans Pigs Rabbits	No
	ANP_B	Relaxation		No
Adenosine	A_1	Contraction	Humans Rats	No
	A_2	Relaxation		No
Endothelin	ET_A	Contraction	Adult sheep	No
	ET_B	Relaxation		Yes
Bradykinin	B_1	Relaxation	Pig Bovine	Yes
	B_2	Relaxation		
Serotonin (5-hydroxytryptamine)	$5\text{-}HT_1$	Contraction	Cats Sheep Bovine	No
	$5\text{-}HT_{1C}$	Relaxation		Yes
Histamine	H_1	Relaxation	Humans Rats Guinea Pig	Yes
	H_2	Relaxation		No
Vasopressin	V_1	Relaxation	Rat	Yes
Thromboxane	TP	Contraction	Dog Cats	No

1.1.3.5 Respiratory Gases

Pulmonary vascular tone is also affected by the respiratory gases O_2 and CO_2. An increase in CO_2 partial pressure (PCO_2) and decrease in O_2 partial pressure (PO_2) causes local vasoconstriction of pulmonary artery smooth muscle cells (PASMCs), otherwise known as hypoxic pulmonary vasoconstriction (HPV), a protective physiological response unique to pulmonary arterioles and arteries (Moudgil *et al.,* 2005). Humans experience hypoxia throughout life, for example in the uterus and at high altitude. HPV, a conserved homeostatic vasomotor response of resistance intrapulmonary arteries (IPA) to alveoli hypoxia, diverts blood away from hypoxic areas of the lungs to areas that are better oxygenated, thereby enhancing ventilation/perfusion matching and systemic oxygen delivery. (Houweling, 2007; Dunham-Snary *et al.,* 2017). Ventricular-perfusion (V/Q) matching is an important physiological determinant of gas exchange; the lungs attempt to pair oxygenated/ventilated regions with areas of sufficient blood supply (perfusion). In areas of low-oxygen state, pulmonary vessels constrict to shunt blood to better-ventilated regions of the lung. In an ideal lung, perfusion and ventilation are distributed identical with perfect matching (Tarry and Powell, 2017).

The mechanism of HPV has been subject to extensive research and has been proposed to be located within the mitochondria (Waypa *et al.,* 2001). HPV is triggered by a mitochondrial sensor that modifies reactive oxygen species (ROS) and redox couples in the PASMCs. This mitochondrial redox signal involves a coordinated response of voltage- and redox-sensitive K^+ and Ca^{2+} channels. Changes in the energy, ROS and redox states inhibit K^+ channels and increases the generation of superoxide and AMPK. These changes lead to the depolarisation of PASMCs, which in turn activates VGCC and SOC, leading to an increase in cytosolic Ca^{2+}, causing vasoconstriction. This sustained state of hypoxia activates Rho kinase, which reinforces vasoconstriction via Ca^{2+}-sensitisation, and hypoxia-inducible factor, leading to pulmonary hypertension and adverse pulmonary vascular remodelling (Sylvester *et al.,* 2012; Swenson, 2013; Dunham-Snary *et al.,* 2017).

It is important to note that although there are several modulatory influences on HPV, the effector mechanism of this physiological response resides solely in the PASMC,

where it is triggered by mitochondrial redox signals. Modulatory influences include the endothelium, erythrocytes and neurohumoral mediators. The pulmonary vascular endothelium produces vasodilators (NO and prostacyclin) and vasoconstrictors (ET-1 and TXA_1) that contribute to HPV modulation (Aaronson *et al.*, 2002). Also, pulmonary endothelial cells may be a source of ROS because when exposed to 3% O_2, isolated endothelial cells produce more hydrogen peroxide (Irwin *et al.,* 2009). Erythrocyte modulation involves red blood cell (RBC)-mediated changes in the pulmonary vascular resistance. NO scavenging by oxyheamoglobin and the generation of ROS by hypoxic RBC are some of the contributions of RBC that enhance HPV. These contributions are complicated and vary depending on the degree and duration of hypoxia (Deem *et al.,* 1998; Kiefmann *et al.,* 2008). In neurohumoral modulation, arterial PO_2 is monitored by peripheral chemoreceptors, which project afferents to the medullary cardiovascular control and the respiratory control areas of the brain stem and activate the sympathetic and the parasympathetic outflows to the lungs, causing vasoconstriction by the release of noradrenaline and NO-dependent vasodilation by the release of ACh, respectively. The loss of afferent input from peripheral chemoreceptors and denervation of the carotid bodies increases HPV (Naeije *et al.,* 1989).

There are also physiological factors that influence HPV, including temperature, age, iron status, extracellular pH, and PCO_2 (Gao and Raj, 2010; Tarry and Powell, 2017). HPV is potentiated and inhibited by hyperthermia and hypothermia respectively. Hypercapnia increases pulmonary vascular resistance (PVR) resulting in an increase in pulmonary arterial pressure (Kiely et al.,1996). Respiratory and metabolic alkalosis leads to attenuation of HPV and decreased PVR. It has been reported that Individuals with an iron deficiency have an enhanced HPV response (Smith et al., 2009). Age also has an influence on HPV. At birth, HPV is a key physiological process for the transition from foetal to an adult circulation (Gao and Raj, 2010). In the nonventilated foetal lung, HPV directs blood to the systemic vasculature. This process originates in the foetus and contributes significantly to the high PVR in the foetus; mainly due to the pulmonary artery being more muscularised and PO_2 lower (18mmHg) than they are after birth. The low PO_2 is a result of “streaming” in the right ventricle, whereby oxygen-poor blood from tissues enter via the superior vena cava into the pulmonary

circulation and oxygen-enriched blood through the placental vein enters from inferior vena cava and passes into the left ventricle via the foramen ovale. At first breath, the infants' lungs expands thereby increasing the PO_2, which reduces HPV and induces the synthesis and release of vasodilators such as NO. All of these factors reduce the PVR by five-fold. HPV is switched off at birth, but in adult life activated in response to external (high altitude) or internal (local hyperventilation) hypoxia (Gao and Raj 2010; Sylvester et al. 2012; Hughes 2016).

In addition to the above mechanisms that control the pulmonary vascular tone in a hypoxic environment, genetic background, environmental factors, such as cold, intensity of exercise activities, and a history of familial susceptibility to high altitude pulmonary oedema or pulmonary hypertension, also contribute to the occurrence of HPV (Stobdan *et al.,* 2008; Grünig *et al.,* 2009).

1.2 Pulmonary Vascular Disease

As discussed above, the pulmonary circulation is a high-flow, low-pressure system that is highly adapted for efficient gas exchange at the alveoli. The larger, more proximal arteries are conduit vessels; thus, they have thick medial walls to cope with high transmural pressures. However, as the pulmonary arteries divide, the vessel structure changes to accommodate efficient gas exchange. These vessels become greater in number, with no musculature in their walls, creating a high volume, low-pressure network to improve the efficiency of gas exchange (Davies and Howard, 2012). Several pulmonary vascular diseases affect the maintenance of gas exchange, including pulmonary embolism (PE), pulmonary hypertension (PH) and chronic obstructive pulmonary disease (COPD).

1.2.1 Pulmonary embolism

This disease is identified by the presence of thrombosis in the pulmonary circulation and is a major cause of mortality. In the United Kingdom, 57 out of 100,000 patients have been reported to develop PE in long-term care or in hospital (Davies and Howard, 2012; Morris and Fedullo, 2015). PE is categorised by the clinical presentation as high, intermediate or low risk. At *high risk*, there is a 15% in-hospital mortality rate and this requires immediate treatment. It is characterised by the presence of circulatory shock and systemic hypotension (< 90mm Hg systolic arterial pressure), with no identifiable cause; *Intermediate risk* patients are haemodynamically stable and have either a right ventricular dysfunction (RVD) or raised levels of cardiac biomarkers, such as N-terminal-pro-brain natriuretic peptides (NT-pro-BNP) and troponin I. A study showed that patients with RVD showed higher right/left ventricular ratios and NT-pro-BNP/troponin I levels compared to those without RVD (Henzler *et al.,* 2012). *Low risk* patients present with no systemic hypotension, but show acute symptoms, such as cough, chest pain and dyspnoea (Davies and Howard, 2012).

There are number of consequences of developing PE: a) obstruction to pulmonary blood flow diverts the flow to unobstructed areas of the lung, altering the ventilation-perfusion balance in obstructed and unobstructed areas; b) in areas where blood flow is severely obstructed, bronchoconstriction occurs in areas of the lungs distal to the obstruction as a result of alveolar hypocapnia (reduced CO_2 concentration in the

blood) and c) arterial hypoxemia (reduced O_2 concentration in the blood) is worsened, with an increase in right ventricular afterload. Another uncommon local consequence of PE is pulmonary infarction (PI), defined by the occlusion of pulmonary arteries, resulting in death due to inadequate supply of O_2-rich blood (Morris and Fedullo, 2015). It develops in 20-30% of patients with existing pulmonary or cardiac diseases and is rare in patients with no cardiopulmonary disease.

The haemodynamic effect of PE is related to three factors: pre-existing status of cardiopulmonary system, rate of reduction of cross-sectional area of the pulmonary vascular bed and the consequences of neurohumoral and hypoxic-mediated vasoconstriction (McIntyre and Sasahara, 1974; Elliott, 1992). The obstruction of the pulmonary vascular bed by an embolism accounts for most of the increase in pulmonary vascular resistance (Morris and Fedullo, 2015). Anticoagulants are given to those at immediate or low risk PE, whilst low molecular weight heparin is given if PE is not confirmed. When PE is confirmed, anticocoagulants, such as warfarin, rivaroxaban, apixaban, dabigatran and edoxaban, are given. The treatment course normally lasts three months (Davies and Howard, 2012; NICE-Guidelines, 2020).

1.2.2 Pulmonary hypertension

PH manifests as a complication of other conditions that are related to an increase in pulmonary arterial blood flow, pulmonary vascular resistance or left heart filling pressure (Bonow *et al.,* 2015). At the 6th world symposium PH was classified into five major groups: PAH, PH due to left heart disease, PH associated with lung disease and/or hypoxia, PH due to pulmonary artery obstructions (chronic thromboembolic pulmonary hypertension) and PH with unclear and/or multifactorial mechanisms, such as sarcoid lung disease, thyroid disorders or glycogen storage disease (Table 1.2; Galiè *et al.,* 2015; Simonneau *et al.,* 2019).

1.2.2.1 Pulmonary arterial hypertension

PAH is a disease of the small pulmonary arteries that is characterised by an increase in pulmonary vascular resistance, pulmonary artery pressure above 20 mmHg at rest and vascular remodelling of the distal pulmonary artery as a result of an increase in vascular tone and cell proliferation respectively. This eventually leads to right heart

failure (Rich *et al.,* 1987; Caruso *et al.,* 2012; Rose-Jones and Mclaughlin 2015; Docherty *et al.,* 2018; Simonneau *et al.,* 2019). The main pathological process in the development of PAH is vascular remodelling (formation of neointima) involving fibroblasts, pericytes, myofibroblasts, PASMCs, endothelial, progenitor and inflammatory cells.

Multiple processes contribute to the occurrence of PAH, including; endothelial dysfunction leading to an imbalanced release of endothelial factors (NO, endothelin, TXA_2 and prostacyclin), reduced anticoagulatory endothelial properties, endothelial release of different growth factors, chemokines and growth factors, and increased expression of adhesion molecules (vascular cell adhesion molecules, E-selectin, and intercellular adhesion molecule 1; Huertas *et al.,* 2018). PAH is characterised by disruption of the artery endothelial layer, incorporation of fibroblasts into the medial layer of the vessels and smooth muscle cell hypertrophy and hyperplasia. Disruption of the smooth muscle cells and hyperplasia leads to the obliteration of the lumen of distal vessels. As a result, an increase in vascular resistance of the pulmonary circulation ensues (Jeffery and Morrell, 2002; Chan and Loscalzo, 2008). The endothelial layer serves as a sensor for injurious stimuli, such as shear stress, inflammation and hypoxia. Injury or disruption to the endothelial cell wall is as a result of dysregulation of downstream vascular effectors (decrease in vasodilators and an increase in vasoconstrictors). Vasoconstriction is enhanced by the rapid hydrolysis of cGMP to inactive GMP, thereby impeding the vasodilatory endothelial response to NO (Davies and Howard, 2012; Rose-Jones and Mclaughlin, 2015).

Mutations leading to genetic susceptibility is another process that may contribute to PAH, including of voltage-gated K^+ channels, bone morphogenetic protein receptor 2 (BMPR2), K^+ channel subfamily K member 3 and activing receptor-like kinase (Rose-Jones and Mclaughlin, 2015). A common histological feature in all forms of PAH is the accumulation of cells expressing smooth muscle-specific α-actin (SMA) in the pulmonary arteries. This includes the appearance and extension of the SMA-positive cells in the neointima and precapillary arterioles devoid of smooth muscles respectively (Mandegar *et al.,* 2004). Mutations in the gene coding for the BMPR2, a receptor for the transforming growth factor superfamily (TGF)-β, have been identified

in 70% of patients with heritable PAH. In iPAH, the BMPR2 expression is markedly reduced (Morrell *et al.,* 2001). Mutations in BMPR2 in PASMCs are associated with an abnormal growth response to bone morphogenetic proteins and TGF-β (Yang *et al.,* 2005). In addition, these BMPR2 mutations increase the susceptibility of pulmonary artery endothelial cells to apoptosis (Morrell *et al.,* 2001; Teichert-Kuliszewska *et al.,* 2006).

In addition to the processes discussed above, other pathogenetic alterations involved in the PAH development include; i) Environmental trigger factors, such as hypoxia, smoke exposure, shear stress and air pollution. ii) Systemic and circulating factors, such as hormones, pro-coagulatory and inflammatory disposition. iii) Dysregulated cellular mechanism, altered expression or function of ion channels and growth factor receptors, and activation or deactivation of transcription factors, such as hypoxia-inducible factor 1 and nuclear factor of activated T-cells. iv) Lastly, disturbance of repair mechanisms counteracting cellular remodelling such as DNA and endothelial repair mechanism (Boucherat *et al.,* 2017; Humbert *et al.,* 2019; Morris *et al.,* 2019; Sofianopoulou *et al.,* 2019).

The multifactorial nature of PAH has made treatment of the disease very difficult and unsurprisingly, in the covid-19 pandemic, patients with PAH are on the high risk scale of the shielding group (Zheng *et al.,* 2020). Currently, treatment can be divided into three main steps. 1) The initial approach includes supportive therapy, such as birth control advice, diuretics, supervised rehabilitation and if needed, O_2 supplementation (Ulrich *et al.,* 2019). 2) The second step involves initial therapy with high dose Ca^{2+} channel blockers (nifedipine) in vasoreactive patients or therapies which target three pathways that regulate endothelial factors with vasoconstrictive, vasodilatory, proliferative or mitogenic properties including; a) Endothelin receptor antagonists that target both ET_A and ET_B non-selectively (bosentan) or ET_A receptors selectively (macitentan and ambrisentan). b) PDE5 inhibitors that target NO-cGMP signalling (tadalafil and sildenafil) and soluble guanylyl cyclase stimulator (riociguat) and c) Prostacyclin analogues (epoprostenol, iloprost and treprostinil) or an orally-available prostacyclin IP receptor agonist (selexipag) that increases prostacyclin signalling. 3) The third approach involves a combination of approved drugs and lung transplantation

for patients deteriorating despite maximal medical therapy (Galiè *et al.,* 2015; Sommer *et al.,* 2021).

Recent improvements in the treatment of PAH patients reflect the use of combining drug therapy that target multiple pathways. Presently, there are novel treatment options in pre-clinical stages, which target specific pathways in PAH, such as GPCR pathways (Rho-associated protein kinase, apelin) circulating hormones (sex hormones, dehydroepiandrosterone, renin-angiotensin-aldosterone system, neurohumoral regulation; Sommer *et al.,* 2021). There are also treatment options undergoing clinical trials including: selexipag, riociguat, sildenafil, bosentan, and tadalafil (Sommer *et al.,* 2021), Despite these treatment advances, PAH remains a fatal disease, and identification of new pathogenic mechanisms and development of new therapies still need to be identified. Hence, the focus of my studies was to investigate other possible factors that may influence pulmonary vascular tone, in particular the purinergic receptors (Jacobson *et al.,* 2020; Illes *et al.,* 2021).

Table 1.2: Clinical classification of Pulmonary hypertension (Simonneau *et al.,* 2004, 2019; Galiè *et al.,* 2015)

Group 1: PAH

- 1.1 Idiopathic PAH- PAH with no apparent cause
- 1.2 Heritable PAH- includes familial PAH (occurrence in ≥ 2 family members) and simplex PAH (single occurrence in family) when a pathogenic variant (eg BMPR2) in one of the known genes has been identified
- 1.3 Drug- and toxin- induced PAH (table 1.3)
- 1.4 PAH associated with:
  - 1.4.1 Connective tissue disease
  - 1.4.2 HIV infection
  - 1.4.3 Portal hypertension
  - 1.4.4 Congenital heart disease
  - 1.4.5 Schistosomiasis
- 1.5 PAH long term responders to Ca^{2+} channel blockers
- 1.6 PAH with overt features of venous/capillaries (pulmonary veno-occlusive disease/pulmonary capillary haemangiomatosis) involvement
- 1.7 Persistent PH of newborn syndrome

Group 2: PH due to left heart disease

- 2.1 PH due to heart failure with preserved left ventricular ejection fraction (LVEF)
- 2.2 PH due to heart failure with reduced LVEF
- 2.3 Valvular heart disease
- 2.4 Congenital/acquired cardiovascular conditions leading to post-capilary PH

Group 3: PH due to lung disease and/or hypoxia

- 3.1 Obstructive lung disease
- 3.2 Restrictive lung disease

3.3 Other lung disease with mixed restrictive/obstructive pattern 3.4 Hypoxia without lung disease 3.5 Developmental lung disorders
Group 4: PH due to pulmonary artery obstructions 4.1 Chronic thromboembolic PH 4.2 Other pulmonary artery obstructions
Group 5: PH with unclear and/or multifactorial mechanisms 5.1 Heamtological disorders 5.2 Systemic and metabolic disorders 5.3 Others 5.4 Complex congenital heart disease

Table 1.3**: Drugs and toxins associated with PAH (Simonneau *et al.,* 2019).**

Definite	**Possible**
Aminorex	Cocaine
Fenfluramine	Phenylpropanolamine
Dexfenfluramine	L- tryptophan
Benflourex	St John's wort
Methamphetamines	Amphetamines
Dasatinib	Interferon-α and –β
Toxic rapseed oil	Alkylating agents
	Bosutinib
	Direc-acting antiviral agents against hepatitis C virus
	Leflunomide
	Indirubin (Chinese herb Qing-Dai)

1.3 Purinergic Receptors and Signalling

In 1929, Drury and Szent-Gyorgyi first recognised the action of purine nucleotides and nucleosides as signalling molecules in the heart and blood vessels. Since then they have been shown to have pharmacological actions in most tissues and cell types (Jacobson *et al.,* 2020; Illes *et al.,* 2021). In 1978, the identification of purinergic receptors began when two purinergic receptor subtypes, P1 and P2, were proposed, based on four criteria 1) Relative potency of adenosine, ATP, ADP and adenosine 5'-monophosphate (AMP); 2) Selective actions of antagonists, especially methylxanthines; 3) Modulation of adenylyl cyclase activity by adenosine, but not by ATP; 4) Synthesis of prostaglandins by ATP and ADP (Burnstock, 1978, 1990). Thus, the following classification was proposed: The P1 purinergic receptors were defined as more responsive to AMP and adenosine than ADP and ATP; methylxanthines, such as theophylline and caffeine, are selective competitive P1 receptor antagonists. Activation of P1 receptors leads to activation or inhibition of an adenylate cyclase system, which results in an increase or decrease of intracellular cyclic AMP (cAMP) level. On the other hand, P2 receptors were defined as being more responsive to ATP and ADP than AMP and adenosine and their activation did not lead to changes in intracellular cAMP, but did induce synthesis of prostaglandins in certain tissues. Finally, methylxanthines do not antagonise P2 receptors (Burnstock, 1990).

The P1 purinergic receptors are GPCR and based on molecular, biochemical and pharmacological studies, they are subdivided into the A_1, A_{2a}, A_{2b}, and A_3 adenosine subtypes (Maenhaut *et al.,* 1990; Libert *et al.,* 1991; Meyerhof, Müller-Brechlin and Richter 1991; Stehle *et al.,* 1992). These subtypes have been cloned from numerous species and characterised following their functional expression in *Xenopus laevis* oocytes or mammalian cells. A_1 and A_3 receptors interact with members of the $G\alpha_{i/o}$ family of G proteins and the A_{2a}, and A_{2b} receptors interact with $G\alpha_s$ proteins. Adenosine is the endogenous agonist at all four receptors, but inosine can act as a partial agonist at A_1 and A_3 receptors (Fredholm *et al.,* 2011). The focus of this thesis is P2 receptors, which will now be discussed in detail.

1.3.1 P2 Receptors

In 1985, Burnstock and Kennedy subdivided P2 receptors into two subtypes based on pharmacological criteria, namely P2X purinoceptors that mediate contraction of smooth muscle, with α,β-methyleneATP as a potent agonist; and P2Y purinoceptors, which mediate relaxation of smooth muscle, with 2-methylthioATP (2-MeSATP) as a potent agonist (Burnstock and Kennedy, 1985). By the early 1990s, further subtypes had been proposed: P_{2T} purinoceptor, an ADP-selective receptor involved in platelet aggregation, P_{2Z} purinoceptor, a receptor activated by ATP and prominent in lymphocytes, macrophages and mast cells, P_{2U} purinoceptor, which is activated by both ATP and uridine 5'-triphosphate (UTP) and P_{2D} purinoceptor, which is activated by diadenosine polyphosphates (Abbracchio *et al.,* 2006). However, their properties, distribution and physiological roles were largely unclear due to the lack of selective antagonists and the complex pharmacological profiles of the receptors endogenous agonists. In the next few years, these uncertainties were clarified by the cloning of numerous adenine and/or uracil nucleotide-sensitive receptors (Abbracchio *et al.,* 2006, 2019; Jacobson and Müller, 2016). This led to the proposal that P2 receptors be sub-classified into two families, as follows: all ligand-gated ionotropic channels, including the P_{2Z} purinoceptor, involved in fast excitatory signalling, were named P2X receptors and all GPCR, including P_{2T} and P_{2U} purinoceptors, were called P2Y receptors. It is now known that these two receptors both comprise of multiple subtypes that are sensitive to purines and pyrimidines (figure 1.4), as will be discussed below (Jacobson *et al.,* 2020).

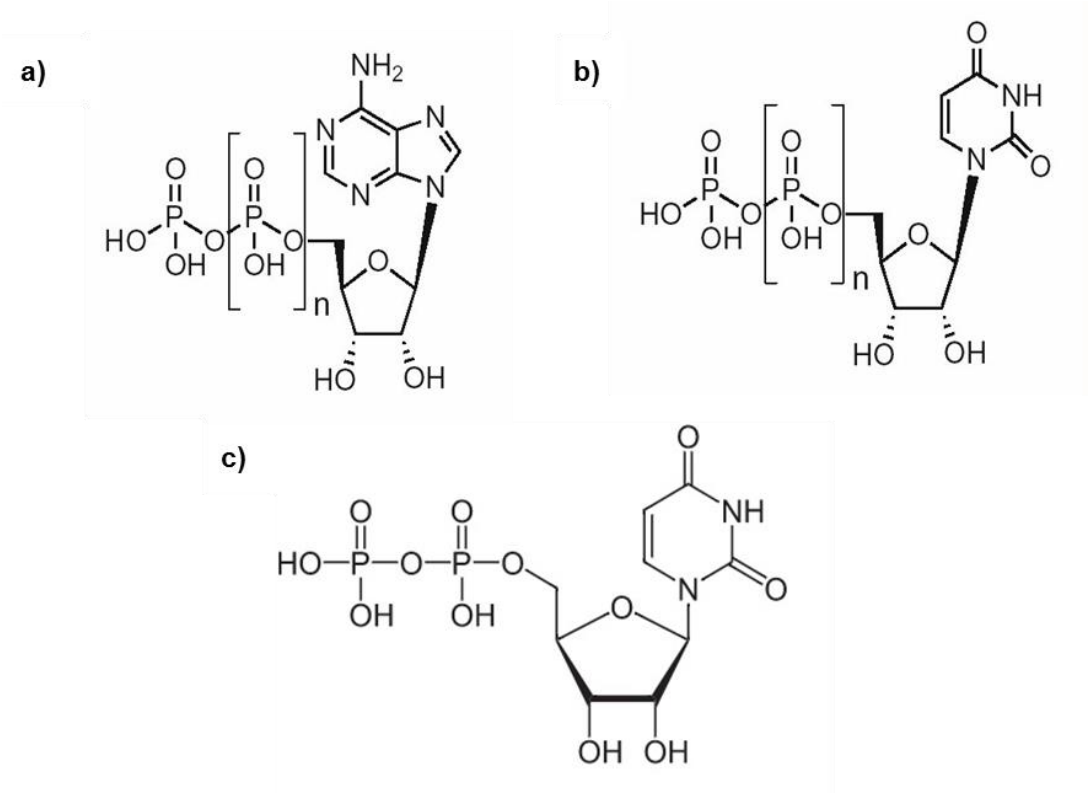


Figure 1.4: Chemical structure of purines and pyrimidines on purinergic receptors. a) Purines -ADP (n=1) and ATP (n=2); b) Pyrimidines- UDP (n=1) and UTP (n=2); and c) UDP-glucose

1.3.1.1 P2X Receptors

P2X receptors are ligand-gated cation channels that are permeable to Na^+, K^+ and Ca^{2+} and are activated by ATP (Burnstock and Kennedy 1985; Khakh *et al.,* 2001). There are seven P2X receptor subtypes, P2X1-P2X7, each of which has two transmembrane domains (TM) separated by an extracellular domain (Coddou *et al.,* 2011). All, except the P2X6 subtype, form functional homomeric receptors and further heteromeric assemblies are possible (Saul *et al.,* 2013). For example, $P2X_{2/3}$, $P2X_{1/5}$, $P2X_{2/6}$ and $P2X_{4/6}$ heteromeric channels have been characterised through heterologous expression (Egan *et al.,* 2006; Syed and Kennedy, 2012; Kennedy *et al.,* 2013). The crystal structure of the zebrafish P2X4 receptor in the closed and open state demonstrated that each functional receptor comprises of three subunits arranged in a head-to tail order, with each subunit having an intracellular N- and C-termini and a large extracellular loop with an ATP binding site (figure 1.5; Kawate *et al.,* 2009; Hattori and Gouaux, 2012). The ATP-bound P2X4 receptor revealed that ATP adopts a U-shaped conformation, where the phosphates are folded towards the adenosine group (Hattori and Gouaux, 2012). The elucidation of the crystal structures of P2X3 and P2X7 receptors supported these findings by showing that that each functional receptor comprises of three subunits arranged in a head-to tail order and that the binding of the U-shaped ATP is not specific to P2X4 receptors alone, but to all members of the P2X receptor family (Mansoor *et al.,* 2016; Kasuya *et al.,* 2017; Kawate 2017).

Activation of P2X receptors leads to membrane depolarisation (North, 2002) initiated by the influx of Na^+ and Ca^{2+}, which can trigger the opening of VGCC and further influx of Ca^{2+}, leading to cellular events, such as vasoconstriction of PASMCs. They are expressed widely in vascular and visceral smooth muscle (Collo *et al.,* 1996) and mediate the neurotransmitter actions of ATP when it is released as a cotransmitter from sympathetic and parasympathetic nerves (Syed and Kennedy, 2012). These receptors are implicated in a range of physiological processes, such as nociception, taste and inflammation; making them possible therapeutic target for inflammatory, neuronal and cardiovascular diseases (Jacobson and Müller, 2016).

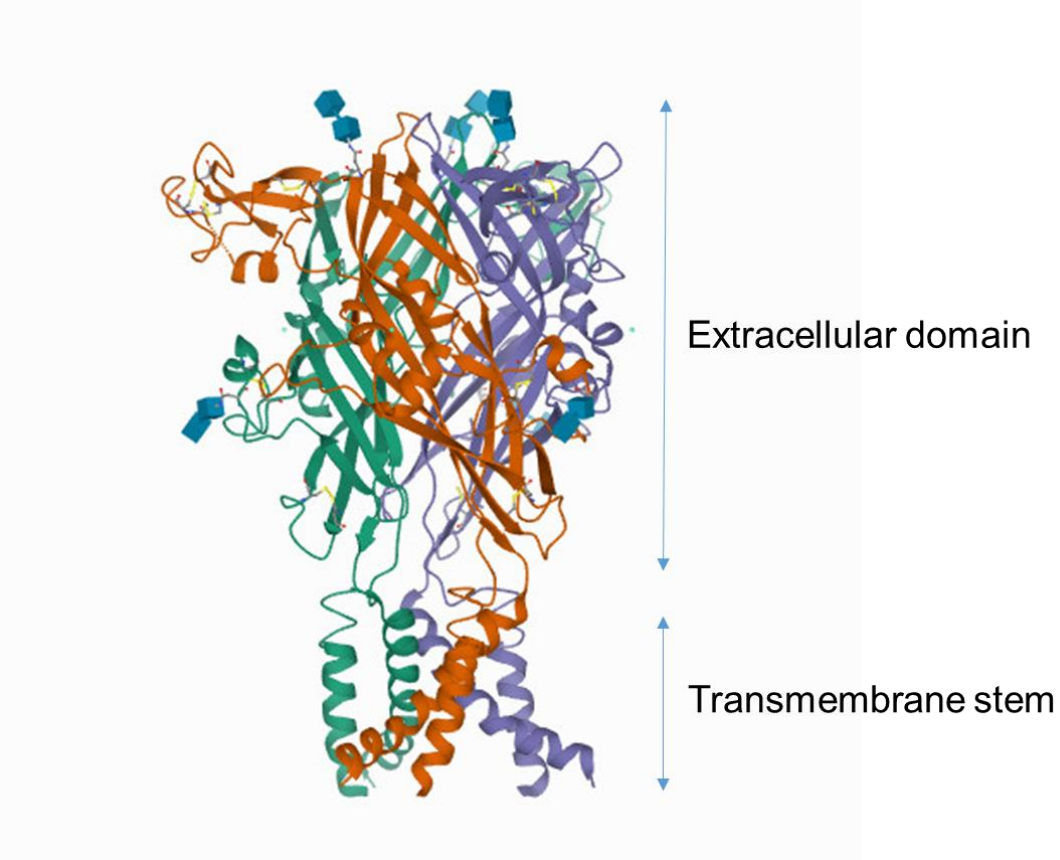


Figure 1.5: The crystal structure of P2X4 receptors in closed apo-state. The homotrimeric P2X4 structure, subunits are indicated in different colours. The zebrafish P2X4 receptor has a chalice-like shape with a small transmembrane stem extending through the membrane and a large extracellular domain protruding above the membrane. Glycosylated asparagine and *N*-acetyl-D-glucosamine resides are shown in stick representation (protein database (PDB): 3H9V).

1.3.1.2 P2Y Receptors

1.3.1.2.1 Introduction

P2Y receptors are a family of GPCR that comprise eight subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, and\ 14}$) in mammals and which have been cloned from many species (table 1.4). The missing numbers from these subtypes represent non-mammalian orthologs or orphan receptors that have amino acid sequences similar to P2Y receptors and were initially identified as P2Y receptors, but which were subsequently shown to not be activated by endogenous nucleotides (Abbracchio *et al.,* 2006; Jacobson *et al.,* 2020). P2Y receptors can be divided into two groups, A) $P2Y_{1, 2, 4, 6, 11}$ receptors and B) $P2Y_{12, 13, 14}$ receptors, based on their phylogenetic similarity, presence of amino acid motifs and selectivity of primary G protein coupling (Jacobson *et al.,* 2002; Abbracchio *et al.,* 2003). The amino acid sequences show that the two P2Y subgroups are 21-48% identical, with the highest degree of amino acid similarity found among group B ($P2Y_{12, 13, 14}$ receptors; table 1.5; Abbracchio *et al.,* 2003; Cattaneo *et al.*, 2003). Furthermore, these motifs have been shown to be important for nucleotide binding (Erb *et al.*, 1995; Jiang *et al.,* 1997; Jacobson *et al.,* 2020). The TM6 (H-X-X-R/K) motif present in all human P2Y receptors has been found to be important in agonist binding, while the TM7 motif (Q/K-X-X-R) has been proposed to be important in ligand binding in the $P2Y_{1, 2, 4, 6, and\ 11}$ receptors. The motif (K-E-X-X-L) is substituted in TM7 of $P2Y_{12, 13, 14}$ receptors and this may affect the ligand binding characteristics of these receptors (Boeynaems *et al.,* 2012).

Table 1.4: Distribution and function of P2Y receptors in mammalian tissues. (Burnstock and Knight, 2004; Boeynaems *et al.,* 2012; Von Kügelgen and Hoffmann, 2016; von Kügelgen, 2019; Jacobson *et al.,* 2020).

Receptors	Species in which receptor was first cloned	Tissue distribution	Tissue function	References
$P2Y_1$	Chick Human Cow Rat	Lung, brain, placenta, prostate, heart skeletal muscle, platelets, neuronal tissue and digestive tract	Smooth muscle relaxation and endothelium-dependent relaxation, platelet aggregation and shape change, mitogenic action in rat aorta	(Henderson *et al.,* 1995; Tokuyama *et al.,* 1995; Ayyanathan *et al.,* 1996; Leon *et al.,* 1996; Hechler *et al.,* 1998; Ralevic and Burnstock 1998; Communi *et al.,* 1999; Moore *et al.,* 2001)
$P2Y_2$	Human Rat Mouse Dog Pig	Heart, skeletal muscle, lung, bone marrow, macrophages, spleen, kidney, vascular smooth muscle and endothelial cells	Pulmonary surfactant secretion, smooth muscle vasoconstriction, endothelial cell-mediated NO release and vasodilatation, arterial cell wall growth.	(Lustig *et al.,* 1993; Parr *et al.,* 1994; Rice, Burton and Fieldeldey 1995; Chen *et al.,* 1996; Ralevic and Burnstock 1998; Zambon *et al.,* 2000; Seye *et al.,* 2002; Shen *et al.,* 2004)
$P2Y_4$	Human Rat Mouse	Lungs, vascular smooth muscle, brain, placenta, liver, intestine, pituitary, lymphocytes and monocytes	Endothelial cell-dependent NO release and vasodilatation, epithelial Cl^- transport in jejunum, mitogenicity of VSMCs	(Nguyen *et al.,* 1995; Bogdanov *et al.,* 1998; Jin *et al.,* 1998; Moore *et al.,* 2001; Burnstock 2002; Robaye *et al.,* 2003)
$P2Y_6$	Human Rat Mouse	Placenta, intestine, lung, vascular smooth muscle, spleen, thymus, heart, aorta, brain	Human cerebral and rat mesenteric arteries contraction, TNF-α related signals interaction, $NaCl^-$ secretion in colonic epithelial cells, proliferation of lung epithelial tumor cells, chemokine production	(Chang *et al.,* 1995; Communi, Parmentier and Boeynaems 1996; Lazarowski *et al.,* 2001; Warny *et al.,* 2001; Kim *et al.,* 2003; Malmsjö *et al.,* 2000, 2003 ; Schäfer *et al.,* 2003; Burnstock and Knight 2004)

			and monocytes release.	
$P2Y_{11}$	Human Dog	Spleen, lymphocytes, intestine, brain, liver,	Dendritic cells migration and maturation, pancreatic duct secretion, granulocytic differentiation	(Communi *et al.,* 1997, 2000; Moore *et al.,* 2001; Nguyen *et al.,* 2001; Qi *et al.,* 2001a; Wilkin *et al.,* 2001; Zambon *et al.,* 2001; Schnurr *et al.,* 2003)
$P2Y_{12}$	Human Rat Mouse Cow	Spinal cord, platelets, brain, neural tissues.	Platelet aggregation at injury sites, platelet activation via thrombin and thromboxane A_2, thrombus formation and fibrinogen activation.	(Jin, Daniel and Kunapuli 1998; Cattaneo *et al.,* 2000, 2003; Hollopeter *et al.,* 2001; Zhang *et al.,* 2001; Ennion, Powell and Seward 2004; Gachet 2005)
$P2Y_{13}$	Human Rat Mouse	Liver, bone marrow, heart, spleen, heart, peripheral leukocyte, brain	Balance of adipocyte and osteoblast in bone marrow, high density lipoprotein uptake, promotes reverse cholesterol transport, protection against stress-induced neuronal death	(Communi *et al.,* 2001; Zhang *et al.,* 2002; Fumagalli *et al.,* 2004; Jacquet *et al.,* 2005; Espada *et al.,* 2010; Fabre *et al.,* 2010; Biver *et al.,* 2013)
$P2Y_{14}$	Human Rat Mouse	Spleen, lung, bone marrow, brain glia, stomach, intestine, adipose tissue, placenta	Chemoatttractant receptor in bone marrow, dendritic cells activation and neuroimmune function.	(Charlton *et al.,* 1997; Chambers *et al.,* 2000a; Freeman *et al.,* 2001; Abbracchio *et al.,* 2003; Moore *et al.,* 2003; Skelton *et al.,* 2003)

Table 1.5: Characteristics of P2Y subgroups based on their amino acid sequence. (Abbracchio *et al.*, 2006)

Receptor	Percentage of Identity							
	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
$P2Y_1$	/	38	44	46	32	24	24	27
$P2Y_2$		/	41	41	29	25	26	26
$P2Y_4$			/	43	32	25	26	28
$P2Y_6$				/	34	24	24	23
$P2Y_{11}$					/	22	21	23
$P2Y_{12}$						/	48	47
$P2Y_{13}$							/	47
$P2Y_{14}$								/

P2Y receptor subtypes are extensively distributed in mammalian organs and have been proposed to regulate cellular processes, such as proliferation, phagocytosis, secretion, cell adhesion and migration, by activating intracellular signalling cascades (Abbracchio *et al.,* 2006). These cascades operate by sequential activation or deactivation of heterotrimeric and monomeric G proteins, adenylyl and guanylyl cyclases, protein kinases, phospholipases and phosphodiesterases (Erb and Weisman, 2012). Group A members of the P2Y receptors couple to the $G\alpha_{q/11}$ family of G proteins which, when activated, stimulate phospholipase C-β (PLCβ), which cleaves phosphatidylinositol- 4,5-bisphosphate in the plasma membrane to the second messengers IP_3 and DAG. IP_3 and DAG are involved in vasoconstriction by releasing Ca^{2+} from intracellular stores and activation of PKC respectively (Waldo and Harden, 2004; Abbracchio *et al.,* 2006). Uniquely, $P2Y_{11}$ receptors couple to both $G\alpha_{q/11}$ and $G\alpha_s$. On the other hand group B subtypes couple to the $G\alpha i_{/o}$ family of G proteins that inhibit adenylyl cyclase, resulting in a reduction of PKA activity. P2Y receptors can also be classified pharmacologically into four groups: i) adenine nucleotide-preferring receptors that respond to ATP and/or ADP ($P2Y_{1,\ 11,\ 12\ and\ 13}$); ii) uracil nucleotide-preferring receptors that respond to either UTP or uridine 5'-

diphosphate (UDP) ($P2Y_{4 \text{ and } 6}$): iii) adenine and uracil nucleotide-preferring receptors ($P2Y_{2, 4 \text{ and } 11}$); and iv) receptors that prefer UDP, UDP-glucose and UDP-galactose ($P2Y_{14}$) (Abbracchio *et al.,* 2006; Kennedy *et al.*, 2013).

1.3.1.2.2 P2Y Receptor Structure

The recently published crystal structures of $P2Y_1$ and $P2Y_{12}$ receptors confirm that P2Y receptors have the canonical seven hydrophobic transmembrane regions of GPCR that are connected by three intracellular loops and three extracellular loops (ELs) that are structurally distinct (Zhang *et al.,* 2014a,b; Zhang *et al.,* 2015). The elucidation of five conformations, 2 antagonist-bound $P2Y_1$ conformations and 2 agonist- and 1 antagonist-bound $P2Y_{12}$ conformations, has also provided insight into the ligand recognition sites and activation of P2Y receptors. $P2Y_1$ receptors and agonist-bound $P2Y_{12}$ receptors exhibited two disulphide bonds connecting the N-terminus to helix VII and helix III to EL2. This was not the case for antagonist-bound $P2Y_{12}$ receptors; only the EL3 and N-terminus helix VII disulphide bond could be determined, making the EL of the $P2Y_{12}$ receptors more labile (Zhang *et al.*, 2014a,b).These findings confirm previous mutational studies that suggested the presence of these disulphide bonds (Ding *et al.*, 2003). Another structural difference between these receptors is the conformation of their structure based on TM5. The TM5 of the $P2Y_1$ receptor contains a proline residue that introduces a bend into the helix, while the TM5 of the $P2Y_{12}$ receptor contains an asparagine residue that produces a straight elongated confirmation. In addition, $P2Y_{12}$ receptors contain a C-terminal helix VIII that is absent in the $P2Y_1$ receptor crystal structure.

Zhang *et al.,* (2015) reported the structure of the $P2Y_1$ receptor in complex with the nucleotide antagonist, 2-iodo- N^6 –methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-biphosphate (MRS2500),and non-nucleotide antagonist 1-(2-(2-tert-butyl)phenoxy)pyridine-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea (BPTU), and demonstrated two distinct ligand-binding sites. BPTU binds to the $P2Y_1$ receptor at the lipid interface of the TM domain in a ligand binding pocket formed by aromatic and hydrophobic residues in helices I, II, III and EL1 that interact with BPTU through hydrophobic interactions to prevent agonist induced receptor activation (figure 1.6a). On the other hand, MRS2500 binds in a pocket defined by residues from N-terminus, EL2, TM6 and TM7 (figure 1.6b). The adenine ring of MRS2500 interacts with amino acids within sub pockets formed by the N-terminus, TM6 and TM7 through hydrophobic interactions. The 2-iodo group of MRS2500 forms a hydrogen bond with cysteine-42 in the N-terminus; a substituent that is critical for high ligand binding

affinity to $P2Y_1$ receptor. In the $P2Y_1$ receptor-MRS2500 structure, each terminal oxygen of the two phosphate groups form hydrogen bonds and salt bridge interactions with polar residues in the N-terminus, TM2 and TM7.

The crystal structure of the $P2Y_{12}$ receptor has also been determined in the agonist- and antagonist-bound states using the full agonist 2-methylthioADP (2-MeSADP) and AZD1283, a non-nucleotide $P2Y_{12}$ receptor antagonist and anti-thrombotic agent (Zhang *et al.,* 2014a,b). It is the only P2Y receptor that has been solved in an agonist bound state. The adenine ring of the 2-MeSADP interacts with tyrosine-105 in TM3 of the $P2Y_{12}$ receptor, while its 2-thioether moiety attaches to the hydrophobic pockets formed by TM3 and TM4. AZD1283 also interacts with tyrosine-105 and other amino acid residues, such as phenylalanine-106 and lysine-155, however, it differs from 2-MeSADP in that it has a distinct polar and hydrophobic interactions with side chains from helices II-VII.

Recently, a homology model of human $P2Y_2$ receptor ($hP2Y_2$), based on the crystal structures of $P2Y_1$ receptors was published (Rafehi *et al.,* 2017). This updated homology model will be useful for screening and design of superior ligand and drug candidate.

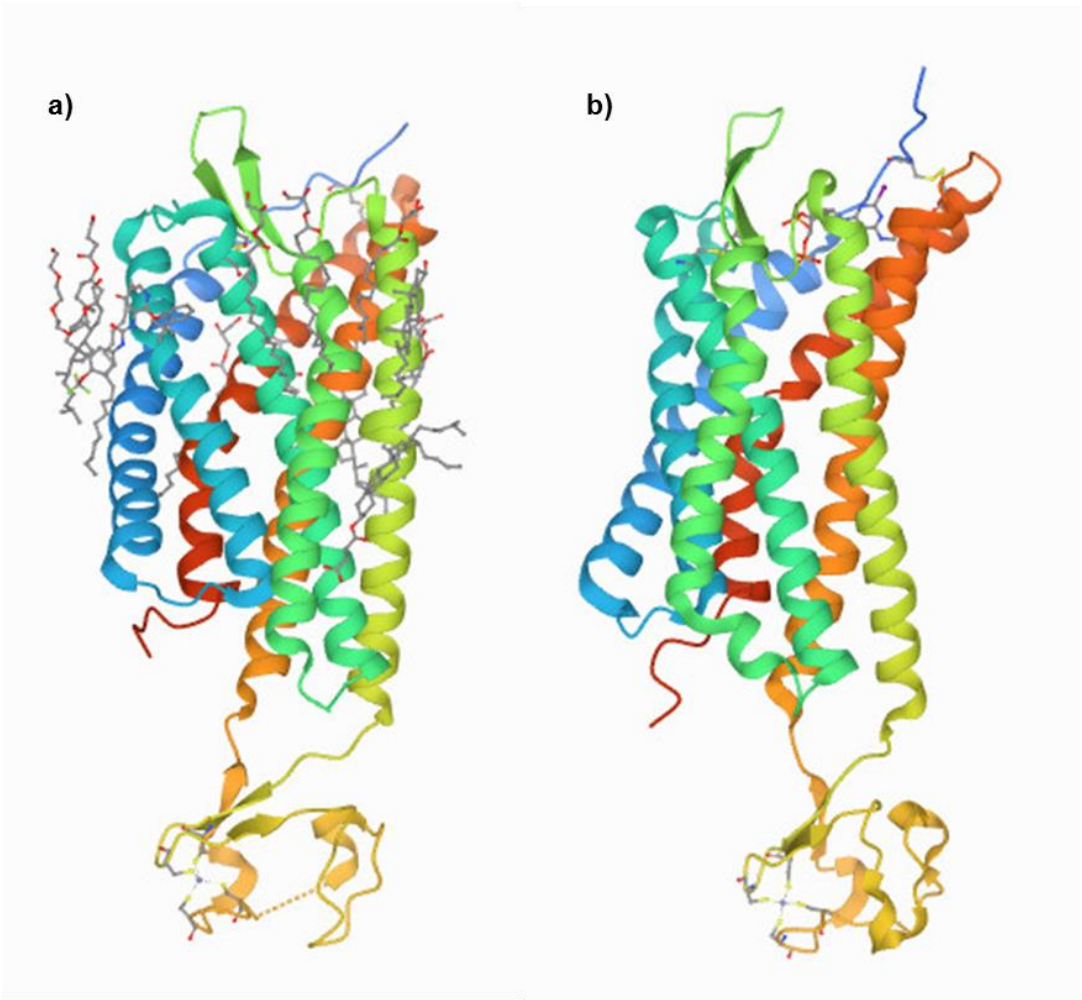


Figure 1.6: The crystal structure of $P2Y_1$ receptor. a) Structure of $P2Y_1$ receptor-BPTU complex. $P2Y_1$ receptor is shown as blue spirals while BPTU ligand are gray sticks with red carbons (PDB ID: 4XNV). b) Structure of $P2Y_1$ receptor-MRS2500 complex. $P2Y_1$ receptor is shown as orange spirals while MRS2500 ligand are blue sticks (PDB ID: 4XNW).

1.3.1.2.2.1 $P2Y_1$ Receptors

Cloning of the $P2Y_1$ receptor, cellular functions and signalling mechanism

The $P2Y_1$ receptor was first cloned from late embryonic chick brain by Webb *et al.*, (1993), who showed that expression of $P2Y_1$ receptors in *Xenopus laevis* oocytes resulted in Ca^{2+} activated Cl^- currents being generated in response to adenine nucleotides. Similar sequences were subsequently detected in mammals, including humans (table 1.2; Henderson *et al.,* 1995; Tokuyama *et al.,* 1995; Ayyanathan *et al.,* 1996). As of 2021, the $P2Y_1$ receptor has been cloned from 191 species according to the *Ensembl* gene database and is widely expressed in humans (von Kügelgen, 2019). Studies in $P2Y_1^{(-/-)}$ mice indicate roles for $P2Y_1$ receptors in platelet aggregation (Léon *et al.,* 1999), control of blood glucose levels and weight (Laplante *et al.,* 2010), control of bone mass (Orriss *et al.,* 2011a), vascular inflammation (Zerr *et al.,* 2011), purinergic neuromuscular transmission in the colon (Gallego *et al.,* 2012) and regulation of osmotic volume in glial cells (Grosche *et al.,* 2013). Studies using selective $P2Y_1$ receptor antagonist confirm the involvement of $P2Y_1$ receptors in pharmacological responses, such as adenine nucleotide-induced bone resorption, vasodilation, platelet aggregation and astroglial signalling and pain sensitisation (Abbracchio *et al.,* 2006; Ohlmann *et al.,* 2010; Franke and Illes, 2014; Jacob *et al.,* 2014; Barragán-Iglesias *et al.,* 2015).

$P2Y_1$ receptor activation of Gα-subunits of the G_q family was first indicated when stimulation of recombinant $P2Y_1$ receptors promoted changes in inositol, but not cAMP signalling (Filtz *et al.,* 1994; Schachter *et al.,* 1996). Activation of PLC occurs mainly via $G\alpha_q$, but some PLC-β isozymes are activated by the release of Gβγ-subunits from $G\alpha_i$ or $G\alpha_o$ (Harden and Sondek, 2006). However, the PLC-mediated signalling of $P2Y_1$ receptors is generally not sensitive to pertussis toxin, which inactivates $G\alpha_i$ and $G\alpha_0$ (Schachter *et al.*, 1996).

$P2Y_1$ receptors also mediate membrane-delimited, G protein-mediated regulation of ion channels, which doesn't involve cytosolic second messenger systems. It is thought that this involves the direct physical interaction between the active G protein subunit and the ion channel. For example, ADP-sensitive K^+ channels in rat cerebellar

neurons are activated directly by Gβγ-subunits, with no participation of intracellular components (Ikeuchi and Nishizaki, 1995a, b, 1996; Ikeuchi *et al.,* 1995; Wickman and Clapham, 1995).

Generally, $P2Y_1$ receptors do not readily desensitise following activation, but when they do the process involves phosphorylation of the receptor by protein kinases and the uncoupling from the associated G protein (Hardy *et al.,* 2005; Rodríguez-Rodríguez *et al.,* 2009; Erb and Weisman, 2012). However, studies carried out in turkey erythrocytes showed that $P2Y_1$-like receptor desensitisation does not involve PKC or intracellular Ca^{2+}, but is heterologous and involves multiple mechanisms (Galas and Harden, 1995).

$P2Y_1$ agonists and antagonists

ADP and ATP were reported initially to both be potent agonists at cloned $P2Y_1$ receptors (Henderson *et al.,* 1995; Tokuyama *et al.,* 1995). However, studies using highly purified nucleotides and conditions that limited the conversion of ATP to ADP, revealed that the $P2Y_1$ receptor is an ADP-preferring GPCR and that ATP is a partial agonist (Palmer *et al.,* 1998). Subsequent studies involving radioligand-binding assays (Waldo *et al.,* 2002) and purified recombinant human $P2Y_1$ receptors (Waldo and Harden, 2004) confirmed that the affinity of ADP (EC_{50} 150 nM) for this receptor is 20-fold greater than that of ATP (EC_{50} 3 µM). 2-MeSADP, an ADP analogue, has a higher potency at human $P2Y_1$ receptors than ADP and the N-methanocarba analog of 2-MeS-ADP, (1′S, 2′R, 3′S, 4′R, 5′S)-4-[(6-amino-2-methylthio-*9H*-purin-9-yl-1-diphosphoryl-oxymethyl] bicyclo[3.1.0] hexane-2,3-diol (MRS2365) is even more potent (EC_{50} 0.4 nM; Chhatriwala *et al.,* 2004). It is also more selective for the $P2Y_1$ receptor over $P2Y_{12}$ and $P2Y_{13}$ receptors. Adenosine 5'-O-(3-thio)triphosphate (ATPγS) and 2-MeSADP act as agonists at $P2Y_1$ receptors with similar potencies (Waldo and Harden, 2004). Thus the relative potency of agonists at $P2Y_1$ receptors is MRS2365 > 2-MeSADP > ADP = ADPγS (adenosine-5'-O-(2-thiodiphosphate); (Von Kügelgen and Hoffmann,2016).

Agonist stimulation of the $P2Y_1$ receptor is inhibited by the non-selective, non-competitive allosteric P2 antagonists, suramin, pyridoxal-5′-phosphate-6-azophenyl-

2′,4′-disulfonate (PPADS) and Reactive blue 2 (RB-2) (Boyer *et al.,* 1994). NF023 (8′-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulphonic acid), an analogue of suramin selective for P2X1 receptors (Lambrecht 1996) and MRS2210 (6-(2′-chloro-azophenyl)-pyridoxal-α5-phosphate), an analogue of PPADS (Kim *et al.,* 1998), are also $P2Y_1$ receptor antagonists. Competitive biphosphate antagonists with high affinity and selectivity for the $P2Y_1$ receptor include 2′-deoxy-N^6-methyl-adenosine-3′,5′-biphosphate (MRS2179 pK_B 6.99), 2-chloro-N^6–methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-biphosphate (MRS2279 pK_B 8.10) and MRS2500 (Boyer *et al.,* 1998, 2010; Hechler *et al.,* 2006). These biphosphate analogs have shown no interaction with other P2Y receptors (von Kügelgen, 2019).

These $P2Y_1$ receptor antagonists have a limitation of being polar structures that are poorly absorbed when administered orally. Several non-nucleotide $P2Y_1$ antagonists have been more recently developed to circumvent this limitation, including 1-(3,4-dichlorophenyl)-3-[4-[[(3,4-dimethyl-1,2-oxazol-5-yl)amino]-dimethylidene-λ^6–sulfanyl]phenyl]urea (MRS2950), which interacts with an orthosteric binding site (Costanzi *et al.,* 2012), and allosteric modulators 2,2′-pyridylisatogen tosylate and BPTU (Gao *et al.,* 2004; Zhang *et al.,* 2015; Gao and Jacobson, 2017). A diaryl urea analog of aryl-oxyprazole urea was shown to be orally bioavailable and inhibited $P2Y_1$ receptor-promoted platelet aggregation *in vitro* (Pfefferkorn *et al.,* 2008).

1.3.1.2.2.2 $P2Y_2$ receptors

Cloning of the $P2Y_2$ receptor, cellular function and signalling mechanism

The $P2Y_2$ receptor was first cloned from mouse NG108-15 neuroblastoma and glioma hybrid cells (Lustig *et al.,* 1993). The recombinant receptor was activated by ATP and UTP, a response that was similar to the native P2U-receptor. When the $hP2Y_2$ receptor was stably expressed in 1321N1 astrocytoma cells, ATP and UTP evoked a rise in intracellular Ca^{2+} levels, with similar potency, but had no effect on adenylyl cyclase activity (Parr *et al.,* 1994; Lazarowski *et al.,* 1995). The $P2Y_2$ receptor has been cloned from 200 species (table 1.2) according to the *Ensembl* gene database and is expressed in high levels in various tissues, including the lungs, skeletal muscle, macrophages, heart and vascular smooth muscle cells and vascular endothelial cells (Burnstock and Knight, 2004; von Kügelgen 2006, 2019).

Studies using mice in which the $P2Y_2$ gene was deleted indicate that these receptors are involved in the following physiological roles: regulation of airway epithelial ion transport; reduced Cl^- secretion in the trachea (Cressman *et al.,* 1999), aggravation of chronic kidney disease progression (Potthoff *et al.,* 2013), and resistance to the lithium-induced polyuria development (Zhang *et al.,* 2012, 2013). Studies using $P2Y_2$ receptor agonists confirm the involvement of $P2Y_2$ receptors in pharmacological responses, such as reduced vasodilatory responses to agonists such as ATP (Rieg *et al.,* 2011), regulation of inflammatory responses (Burnstock, 2007), bone formation regulation (Hoebertz *et al.,* 2002), chemotaxis of neutrophils and other cell types (Chen *et al.,* 2006), and a loss of responses to UTP after myocardial infarction (Cohen *et al.,* 2011). Clinical studies have supported $P2Y_2$ receptor to be an important therapeutic target in cystic fibrosis (CF) treatment (Yerxa 2001). A selective $P2Y_2$ receptor agonist, [P(1)-(uridine 5')-P(4)-(2'-deoxycytidine 5')tetraphosphate, tetrasodium salt] (INS37217; denufosol), was developed for the treatment of CF due to its long duration of action and enhanced metabolic stability. So far, *in vitro* studies have shown denufosol to stimulate $P2Y_2$ receptor dependent Ca^{2+} mobilisation, which thereby induces mucin and Cl^- secretion (Yerxa, 2001). This pharmacological action of denufosol provided the rationale that $P2Y_2$ receptors may be a therapeutic target for patients with CF. However, it has since been published that while denufosol was in its phase III trials, it has been discontinued (Verkman and Galietta 2009; Ratjen *et*

al., 2012; Almughem *et al.,* 2020). Diquafosol tetrasodium (UP_4U, INS365; Pendergast *et al.*, 2001), a selective $P2Y_2$ receptor agonist, has been found useful for the treatment of dry eyes disease. This dinucleoside polyphosphate promotes water secretion, secretory mucin secretion and membrane associated mucin expression on the ocular surface (Fujihara *et al.*, 2001, 2002; Nakamura *et al.*, 2012). In Japan and South Korea, 3% diquafosol ophthalmic solution has been approved for the treatment of dry eyes (Shigeyasu *et al.,* 2015; Mun *et al.,* 2018; Watanabe, 2018; Ohashi *et al.,* 2020).

Cloned $P2Y_2$ receptors couple to $G_{q/11}$ to mediate phospholipid breakdown and Ca^{2+} mobilisation via PLCβ. $P2Y_2$ receptors also appear to be able to couple to to G_i to induce the release of βγ subunits that then stimulate PLC-β2, IP_3 formation, Ca^{2+} mobilisation and various signalling components, such as PKC, phospholipase A_2, Ca^{2+}-dependent K^+ channels, and formation of NO and EDHF (Dubyak and el-Moatassim, 1993; Ralevic and Burnstock, 1998). The involvement of each given signalling pathway downstream depends on the cell type in which $P2Y_2$ receptor is expressed. Secondary to PLC activation and Ca^{2+} mobilisation, the $P2Y_2$ receptor mediates; the opening of Ca^{2+}- sensitive Cl^- channels in airway epithelia (Clarke and Boucher, 1992), intrahepatic epithelial cell lines (Wolkoff *et al.,* 1995) and avian exocrine salt glands (Martin and Shuttleworth 1995). Inhibition of N-type Ca^{2+} currents in sympathetic neurones by $P2Y_2$ receptors has also been reported (Filippov *et al.,* 1997).

$P2Y_2$ receptors do not desensitise readily (Ralevic and Burnstock, 1998). When they do it involves phosphorylation of intracellular regions of the receptor. The C terminal might be of importance, as the rate and magnitude of desensitisation decreased with progressive shortening of the C-termini (Garrad *et al.,* 1998; Flores *et al.,* 2005). In mouse epithelial cells, maximum desensitisation of $P2Y_2$ receptors was observed on exposure to UTP and receptor responsiveness was restored after removal of UTP (Garrad *et al.,* 1998). Up-regulation of $P2Y_2$ receptors has also been observed in rat submandibular glands, where $P2Y_2$ receptor mRNA levels were increased after ligation of the main excretory duct (Turner *et al.,* 1997; Hou *et al.,* 2000; Tak *et al.*, 2016).

$P2Y_2$ Agonists and Antagonists

The nucleotides, UTP, ATP, uridine-(O-3-thiotriphosphate) (UTPγS), and ATPγS are full agonists at $P2Y_2$ receptors (Lustig *et al.,* 1993; Lazarowski *et al.,* 1995, 1996); UTP and ATP are endogenous ligands that are approximately equipotent. At recombinant $P2Y_2$ receptors, UTPγS is equipotent with ATP and UTP, while ATPγS is less potent than ATP and UTP. UTPγS also has an advantage over the natural ligands by being resistant to enzymatic hydrolysis. 2-Thio-UTP, its 2´amino-2´-deoxy analog, (MRS2698) and 4-thio-β,γ-difluoromethylene-UTP (PSB-1114) are selective agonists at the $P2Y_2$ receptor; PSB-1114 having a 50-fold selectivity as compared to $P2Y_4$ and $P2Y_6$ receptors (El-Tayeb *et al.,* 2006, 2011; Ivanov *et al.,* 2007). In addition to triphosphate nucleotides, $P2Y_2$ receptors react to dinucleoside polyphosphates, such as diadenosine-tetraphosphate (AP_4A) (Lazarowski *et al.,* 1995; Patel *et al.,* 2001), diquafosol tetrasodium (UP_4U*,* INS365; Pendergast *et al.*, 2001), and P1-(uridine 5´)-P4-(2´-deoxycytidine-5´)tetraphosphate (INS37217; Yerxa, 2001).

Suramin, RB-2 and its analogue P2B416 acts as low affinity antagonists at $P2Y_2$ receptor (Brunschweiger and Muller, 2006; Weyler *et al.,* 2008; Hillmann *et al.,* 2009). Recently, a selective and potent $P2Y_2$ receptor antagonist, AR-C118925XX, (5-[[5-{2.8-dimethy;-5H-dibenzo[a,d]cyclohepten-5-yl}-3,4-dihydro-2-oxo-4-thioxo-1(2H)-pyrimidinyl]methyl]-N-[1H-tetrazol-5-yl]-2-furancarboxide) (figure 1.7) that was developed by AstraZeneca around 20 years ago, became commercially-available. Only a conference abstract was released at the time (Meghani, 2002). Kemp *et al.,* (2004) then reported that AR-C118925XX was inactive against 37 other receptors, but when human bronchial epithelial cells were pre-treated with AR-C118925XX for 30 min, it inhibited ATP-induced mucin section with an IC_{50} of 1 µM. Also, Onnheim *et al.,* (2014) found that AR-C118925XX antagonises ATP- and UTP-induced superoxide production in neutrophils. However, none of these studies reported the pharmacological properties of AR-C118925XX, such as its K_B and pA_2, or related the concentration used to these values. Hence there is a need to investigate its pharmacological properties, which will then enable the role of $P2Y_2$ receptors throughout the body to be studied.

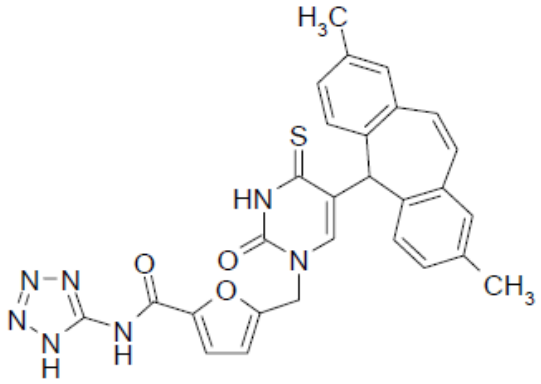


Figure 1.7*:* Chemical structure of $P2Y_2$ antagonist AR-C118925XX.

1.3.1.2.2.3 $P2Y_4$ receptors

Cloning of the $P2Y_4$ receptor, cellular function and signalling mechanism

The initial cloning of $P2Y_4$ receptors was carried out using human (Communi *et al.,* 1995; Nguyen *et al.,* 1995), rat (Bogdanov *et al.,* 1998) and mouse (Suarez-Huerta *et al.,* 2001) tissues. At present, the $P2Y_4$ receptor has been cloned from 167 species (table 1.2) according to the *Ensembl* gene database and is expressed in high levels in various tissues, including brain, intestine and pituitary gland (Abbracchio *et al.,* 2006, von Kügelgen, 2019). Studies in $P2Y_4^{(-/-)}$ mice indicate roles for $P2Y_4$ receptors in regulation of epithelial Cl^- secretion (Robaye *et al.,* 2003), control of endothelial function (Horckmans *et al.,* 2012b, 2012a) and protection against myocardial infarction through ET-1 down-regulation (Horckmans *et al.,* 2015).

$P2Y_4$ receptors signals through $G_{q/11}$ proteins and downstream signalling pathways, including PLC-β, PKC, ion channels (N-type Ca^{2+} channels and M-type K^+ channels) and mitogen activated protein (MAP) kinases (Communi *et al.*, 1996b; Filippov *et al.,* 2003; Scodelaro Bilbao *et al.,* 2010; von Kügelgen and Harden, 2011).

$P2Y_4$ agonists and antagonists

Agonist activity at recombinant $P2Y_4$ receptors has been shown to be species-dependent (Nicholas *et al.,* 1996; Bogdanov *et al.,* 1998; Kennedy *et al.,* 2000). Rat $P2Y_4$ receptors are activated by all common nucleoside triphosphates, including ATP, UTP, GTP, xanthosine 5′-triphosphate (XTP), inosine 5´-triphosphate (ITP), and cytidine 5′-triphosphate (CTP). The human $P2Y_4$ receptor is also activated by UTP. However there is a difference in activity between rat and human $P2Y_4$ receptors; while ATP is a full agonist at the rat $P2Y_4$ receptors, it acts as a competitive antagonist at the human $P2Y_4$ receptors. The N-terminal and three residues (asparagine-177, isoleucine-183 and leucine-190) located in the second EL of the $P2Y_4$ receptor may be the determining factor for the dual effects of ATP at $hP2Y_4$ and $rP2Y_4$ receptor; the second EL and NH_2-terminus form a functional motif involved in receptor activation (Herold *et al.,* 2004). In respect to rat $P2Y_4$ receptors, this profile is identical to that of the $P2Y_2$ receptor; the recombinant receptor is activated equivocally by ATP and UTP, indicating that in rat tissues $P2Y_2$-like responses may be mediated by $P2Y_4$ receptor (Bogdanov *et al.,* 1998). N(4)-(Phenylpropxy)-CTP (MRS4062), Up_4-[1]3'-deoxy-3'-fluoroglucose) (MRS2927), and N(4)-(phenylethoxy)-CTP are $P2Y_4$ receptor agonists

that have a ≥10-fold selectivity for human $P2Y_4$ over $P2Y_2$ and $P2Y_6$ receptors (Maruoka *et al.,* 2011).

The antagonistic effect of suramin, PPADS and RB-2 at the $P2Y_4$ receptor also differs in human and rat $P2Y_4$ receptors. Suramin (100 µM) had little (rat) or no (human) effect on UTP-evoked responses. PPADS (100 µM) reduced UTP response at human $P2Y_4$ receptors by 50%, however, it did not abolish the UTP response at rat $P2Y_4$ receptors. RB-2 abolished the UTP response at the rat $P2Y_4$ receptor, but only modestly inhibited the UTP-evoked response at the human $P2Y_4$ receptor (Communi *et al.,* 1996b; Bogdanov *et al.*, 1998; Wildman *et al.,* 2003). PSB16133, an allosteric modulator, has been reported to be a potent $P2Y_4$ receptor antagonist (Rafehi *et al.,* 2017).

1.3.1.2.2.4 $P2Y_6$ receptors

Cloning of the $P2Y_6$ receptor, cellular function and signalling mechanism

The $P2Y_6$ receptor is a uridine dinucleotide-preferring receptor and the smallest member of the P2Y receptor family with 328 amino acid residues (Communi *et al.,* 1996a). The receptor was first cloned from a rat aortic smooth muscle (Chang *et al.,* 1995). Since then, $P2Y_6$ receptor has been cloned from 185 species according to the *Ensembl* gene database and is widely expressed in VSMCs, placenta, thymus, lung and intestine (von Kügelgen, 2019).

Studies using $P2Y_6^{(-/-)}$ mice indicate a role for $P2Y_6$ receptors in regulation of bone cell function (Orriss *et al.,* 2011a, 2011b). Fewer roles for this receptor in wild-type tissues have been reported in physiological and pharmacological studies, which may be due to the difficulty in differentiating between the actions of UTP and UDP at $P2Y_6$ versus $P2Y_2$ and $P2Y_4$ receptors (von Kügelgen and Harden, 2011). The pharmacological responses that have been reported include, contraction of the vascular smooth muscle (Malmsjö *et al.,* 2000, 2003; Mitchell *et al.,* 2012), Cl^- secretion in epithelial cells (Lazarowski *et al.,* 2001; Leipziger, 2003), control of effector T cell activation (Giannattasio *et al.,* 2011), increased glucose uptake in adipocytes and skeletal muscle cells (Balasubramanian *et al.,* 2014), potentiation of pro-inflammatory responses in macrophages (Garcia *et al.,* 2014) and immune response, such as phagocytosis, NO release and astrocyte apoptosis in microglial (Koizumi *et al.,* 2007, 2013; Quintas *et al.,* 2014).

The $P2Y_6$ receptor selectively couples to G_q and induces inositol lipid signalling through PLC-β isozymes. Its stable expression in 1321N1 human astrocytoma cells promoted nucleotide activation of PLC (Chang *et al.,* 1995; Robaye *et al.*, 1997; von Kügelgen and Harden, 2011). This receptor displays slow desensitisation and internalisation, a unique feature that separates this receptor from other P2Y receptors. The process occurs due to the short intercellular C-terminal sequence of $P2Y_6$ receptor that lacks specific residues (serine-334, 334). These residues are important for agonist-induced phosphorylation, internalisation and desensitisation of $P2Y_4$ and $P2Y_6$ receptors (Brinson and Harden, 2001).

$P2Y_6$ agonists and antagonists

UDP is a more potent agonist than UTP at the $P2Y_6$ receptor, while adenine nucleotides are inactive (Nicholas *et al.,* 1996). Potent and selective agonists at the $P2Y_6$ receptor include 5-iodo UDP (MRS2693; Besada *et al.,* 2006), 3-phenacyl-UDP (PSB-0474; El-Tayeb *et al.,* 2006), diuridine 5´-triphosphate (Up3U; Shaver *et al.*, 2005), uridine 5´-O-thiodiphosphate (UDPβS; Hou *et al.,* 2002), α,β-methylene-UDP (MRS2782; Ko *et al.,* 2008), 5-O-methyl-UDP (Ginsburg-Shmuel *et al.,* 2010), INS48823 (Korcok *et al.,* 2005) and a boranophosphate analogue R(p) isomer of 5-O-methyl-UDP (a-B) (Ginsburg-Shmuel *et al.,* 2012). Thymidine 5´-O-monophosphorothioate is a partial agonist (Gendaszewska-Darmach and Szustak, 2016).

The $P2Y_6$ receptor is blocked by the non-selective antagonists RB-2, PPADS, and suramin, with the following order of potency; RB2 > PPADS > suramin (Robaye *et al.,* 1997). 4,4´-Diisothiocyanatostilbene-2,2´-disulfonate (DIDS; (von Kügelgen 2006) and its analogue N,N´´-1,4-buta-nediylbis[N´-(3-isothiocyanatophenyl)thiourea (MRS2578; Mamedova *et al.*, 2004) act as irreversible antagonists at human and rat $P2Y_6$ receptors, with no interaction with other P2Y receptors. Uridylyl phosphosulfate is also an antagonist at the $P2Y_6$ receptor; with a preference over $P2Y_2$ and $P2Y_4$ receptors (Meltzer *et al.,* 2015).

1.3.1.2.2.5 $P2Y_{11}$ receptors

Cloning of the $P2Y_{11}$ receptor, cellular function and signalling mechanism

$P2Y_{11}$ receptors were first cloned from a human placental library and showed 33% amino acid sequence similarity with $P2Y_1$ receptors (Communi *et al.,* 1997). The canine $P2Y_{11}$ receptor was isolated soon after and is 70% homologous to the human receptor (Qi *et al.,* 2001b; Zambon *et al.,* 2001). According to *Ensembl* gene, the $P2Y_{11}$ receptor has been cloned from 144 species, but interestingly, the $P2Y_{11}$ receptor is not present in the rat or mouse genomes. In humans, $P2Y_{11}$ receptors are expressed in the liver, brain, spleen, intestine and pituitary gland (von Kügelgen, 2019). The absence of the $P2Y_{11}$ receptor from the murine genome means that the consequences of its genetic deletion cannot be investigated, but studies using $P2Y_{11}$ receptor agonists and antagonists indicate the involvement of $P2Y_{11}$ receptors in pharmacological responses, such as dendritic cell maturation (Wilkin *et al.,* 2001), chemotactic responses of neutrophils and neuropathic pain (Alkayed *et al.,* 2012; Barragán-Iglesias *et al.,* 2014, 2015) and immunosuppressive responses (Chadet *et al.,* 2015). Variants of $P2Y_{11}$ receptor protein has also been shown to be associated with increased risks of myocardial infarctions (Amisten *et al.,* 2007), development of narcolepsy (Kornum *et al.,* 2011) and impaired receptor signalling (Haas *et al.,* 2014).

The human $P2Y_{11}$ ($hP2Y_{11}$) receptor couples to $G_{q/11}$ and activates inositol lipid hydrolysis (Communi *et al.,* 1997; Communi *et al.,* 1999; Qi *et al.,* 2001a). In contrast to other P2Y receptors, activating $P2Y_{11}$ receptor also results in an increase of adenylyl cyclase activity.

$P2Y_{11}$ agonists and antagonists

The nucleotide ATP is the primary physiological agonist at the $P2Y_{11}$ receptor (Communi *et al.*, 1999). For the recombinant human $P2Y_{11}$ receptor, the rank order of potency with which nucleotides increase cAMP or IP_3 levels are as follows; 2-propylthio-d-β,γ-dichloromethylene-ATP (AR-C67085MX) ≥ ATPγS ≈ 2′-3′-*O*-(4-benzoylbenzoyl)ATP > 2′-deoxyATP > ATP > ADP (Communi *et al.*, 1997, 1999; Qi *et al.*, 2001a). Thymidine 5'-triphosphate (TTP), UTP, GTP, ITP, CTP, and dinucleotides (AP_4A, AP_5A, AP_6A) are inactive. White *et al.,* (2003) suggested that UTP is a biased agonist at $hP2Y_{11}$ receptors, increasing intracellular Ca^{2+} levels

independently of inositol phosphates, but Morrow *et al.,* (2014) were unable to replicate their results, even though they used the same cell line. The canine $P2Y_{11}$ receptor has a different pharmacological profile, as it is characterised by greater potency of diphosphates compared to triphosphates and the rank order of agonist potency is 2-MeSADP > ADPγS > ADP > ATP (Zambon *et al.,* 2001). A mutational analysis suggested that this difference in profile may be due to the change in the arginine-265, located at the junction between TM6 and the third extracellular loop in the humans to glutamine in dogs (Qi *et al.*, 2001b). Nicotinamide adenine dinucleotide (NAD^+) and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) also activate the $hP2Y_{11}$ receptor; producing a two-step elevation of intracellular Ca^{2+} (Moreschi *et al.,* 2006, 2008). The suramin analogue, NF546, is also a potent and selective non-nucleotide $P2Y_{11}$ receptor agonist (Meis *et al.,* 2010) and adenosine-3´-phosphate-5´-phosphosulfate, a bisphosphate derivative, is a partial agonist (Communi *et al.,* 1999).

Suramin and its analogue NF157 have low affinity for the $P2Y_{11}$ receptor (Ullmann *et al.,* 2005; Moreschi *et al.,* 2006), but another analogue, NF340, is a potent antagonist ($pA_2 = 8.0$; Meis *et al.,* 2010). RB-2 blocks the $P2Y_{11}$ receptor, but PPADS is inactive. With medium potency, RB-2 produced full inhibition of IP_3 accumulation and partially inhibited the cAMP response to ATP in 1321N1 astrocytoma and CHO-K1 cells stably expressing $P2Y_{11}$ receptor respectively (Communi *et al.*, 1999).

1.3.1.2.2.6 $P2Y_{12}$ receptors

Cloning of the $P2Y_{12}$ receptor, cellular function and signalling mechanism

In 1979, Cooper and Rodwell discovered that ADP promotes inhibition of adenylyl cyclase in platelet membranes (Cooper and Rodbell, 1979). Subsequently, pharmacological studies and data from $P2Y_{12}^{(-/-)}$ mice showed that platelets express PLC-activating $P2Y_1$ receptors and $P2Y_{12}$ receptors that inhibits adenylyl cyclase and that both are required for platelet aggregation induced by ADP (Gachet, 2006; Hollopeter *et al.*, 2001). Since then, $P2Y_{12}$ receptors have been cloned from 180 species according to the *Ensembl* gene database, and it is highly expressed in platelets, neural tissues and megakaryocytes (von Kügelgen, 2019). Studies with $P2Y_{12}^{(-/-)}$ mice indicate that $P2Y_{12}$ receptors play roles in osteoclast activity (Jørgensen *et al.,* 2012; Syberg *et al.,* 2012), immune responses (Ben Addi *et al.,* 2010; Mansour *et al.,* 2020), regulation of microglial activation (Haynes *et al.*, 2006; Koizumi *et al.*, 2013), neuroinflammatory disease and neuropathic pain (Tozaki-Saitoh *et al.,* 2008; Webster *et al.,* 2013; Amadio *et al.,* 2014; Horváth *et al.,* 2014; Bekő *et al.,* 2017; Calovi *et al.,* 2019) and defense against neurotropic viruses (Fekete *et al.,* 2018). Studies using selective $P2Y_{12}$ receptor antagonists showed the involvement of $P2Y_{12}$ receptors in pharmacological responses, such as ADP-stimulated vasoconstriction (Wihlborg *et al.,* 2004), ADP-promoted platelet aggregation (Foster *et al.,* 2001; Andre *et al.,* 2003; Cattaneo, 2011) and an increase in renal urine concentrating ability (Zhang *et al.,* 2015c).

The $P2Y_{12}$ receptor couples to the $G\alpha_{i2}$ G protein (Jantzen *et al.,* 2001), causing inhibition of cAMP production (Bodor *et al.,* 2003; Chhatriwala *et al.,* 2004) and an associated decrease in cAMP-dependent PKA-mediated phosphorylation of vasodilator-stimulated phosphoprotein (Hechler and Gachet, 2015), as well as activation of phosphatidylinositol-3-kinase, PKC isoforms, K^+ channels and the small G protein, Rap1b (Gachet, 2006; von Kügelgen and Harden, 2011; Hechler and Gachet, 2015). In platelets, $P2Y_{12}$ receptors have been demonstrated to be re-localised at the level of the cell membrane by the involvement of arrestin (Baurand *et al.,* 2005; Mundell *et al.,* 2006, 2008).

$P2Y_{12}$ Agonists and Antagonists

ADP is the native agonist at the $P2Y_{12}$ receptor (von Kügelgen, 2019). 2-MeSADP and 2-MeSATP are more potent agonists, with nanomolar half-maximal concentrations (von Kügelgen, 2006, 2019; Zhang *et al.,* 2014a). The rank order of agonist potency is 2-MeSADP >> ADP ≥ ADPγS (Hollopeter *et al.,* 2001; Zhang *et al.,* 2001; Bodor *et al.,* 2003).

The $P2Y_{12}$ receptor is blocked with relatively high potency by RB-2 and suramin, while PPADS is inactive (Boyer *et al.,* 1994; Hoffmann *et al.,* 2008). PSB-0739, a RB-2 analogue, (Baqi *et al*. 2009; Baqi 2016) is a very potent, competitive and selective $P2Y_{12}$ receptor antagonist (pA_2 = 9.8; Hoffmann *et al.,* 2009). In contrast to 2-MeSADP, 2-methylthioAMP (2-MeSAMP) is a low-affinity antagonist (Hollopeter *et al.,* 2001). Also, ATP (Kauffenstein *et al.,* 2004) and its triphosphate derivatives (Ingall *et al.,* 1999; Vasiljev *et al.,* 2003) acts as antagonist at the human $P2Y_{12}$ ($hP2Y_{12}$) receptor. Where ATP is a low-affinity antagonist, its triphosphate derivatives such as cangrelor (AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP; pA_2 = 8.7 at native rat $P2Y_{12}$ receptor; Kubista *et al.,* 2003), and AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP; pA_2 value = 8.2 at native $hP2Y_{12}$ receptor; Vasiljev *et al.,* 2003), are highly potent and competitive antagonists. However, these compounds are not subtype selective, as AR-C67085 is an agonist at $hP2Y_{11}$ receptors and both AR-C67085 and cangrelor block human and rat $P2Y_{13}$ receptors (Marteau *et al.,* 2003; Vasiljev *et al.,* 2003; Fumagalli *et al.,* 2004).

In contrast, thienopyridine compounds acts as selective $P2Y_{12}$-selective antagonists. Clopidogrel (Herbert *et al.,* 1993), prasugrel (Sugidachi *et al.,* 2000) and ticlopidine (Maffrand *et al.,* 1988) are used clinically to inhibit platelet aggregation for the prevention of cardiovascular events. They interact in an irreversible manner with the cysteine-97 of the $hP2Y_{12}$ receptor (Savi *et al.,* 2006; Algaier *et al.,* 2008; Ding *et al.,* 2009). Off-target effects of metabolites of clopidogrel and prasugrel in immune function have been reported and receptor antagonism by these thienopyridines could directly alter immune response in inflammation (Liverani *et al.,* 2013, 2014, 2016). Ticragelor (AZD6140), an orally-active, competitive $P2Y_{12}$ receptor antagonist (pA_2 = 8.7) is also used to inhibit platelet aggregation (Springthorpe *et al.,* 2007; Hoffmann

et al., 2014). Off-targets effect have also been reported for ticagrelor, such as blockade of the $P2Y_{13}$ receptor (Björquist *et al.,* 2016) and nucleoside transporter 1 (Aungraheeta *et al.,* 2016).

Several novel compounds (Zetterberg and Svensson 2016; Islam *et al.,* 2018; Baqi and Müller 2019) that block $P2Y_{12}$ receptors have been developed. These include piperazinyl glutamates (Parlow *et al.,* 2010; Zech *et al.,* 2012), ACT-246475 (Caroff *et al.,* 2014, 2015), ethyl 6-aminonicotinate acyl sulphonamide compound (AZD1283, K_D 11 nM; (Bach *et al.,* 2013; Zhang *et al.,* 2014a; Zhou *et al.,* 2017), morpholine analogues (Ahn *et al.,* 2016), flavonolignans, salvianaolic acids (Bijak *et al.,* 2017, 2018), and 6-amino-2thio-3H-pyrimidin-4-one derivatives (Crepaldi *et al.,* 2009; Cacciari *et al.,* 2019).

1.3.1.2.2.7 $P2Y_{13}$ receptors

Cloning of the $P2Y_{13}$ receptor, cellular function and signalling mechanism

Identification of the $P2Y_{12}$ gene resulted in the discovery of the $P2Y_{13}$ receptor, a receptor that exhibited 50% amino acid sequence similarity with the $P2Y_{12}$ receptor (Communi *et al.,* 2001). The initial cloning of $P2Y_{13}$ receptors was carried out using human (Communi *et al.,* 2001; Zhang *et al.,* 2002), rat (Fumagalli *et al.,* 2004) and mouse (Zhang *et al.,* 2002) tissues. Since then, $P2Y_{13}$ receptors have been cloned from 276 species according to the *Ensembl* gene database, and is expressed in the brain, liver, heart and bone marrow (von Kügelgen, 2019). Studies using $P2Y_{13}^{(-/-)}$ mice indicate roles of $P2Y_{13}$ receptors in skeletal development and regulation of lipoprotein metabolism (Blom *et al.,* 2010; Wang *et al.,* 2014; Lichtenstein *et al.,* 2015). Studies using $P2Y_{13}$ receptor antagonists showed the involvement of $P2Y_{13}$ receptors in pharmacological responses, such as degranulation of rat mast cells (Gao *et al.,* 2010), neuroprotective effects on cerebellar astrocytes and neurons (Ortega *et al.,* 2011; Voss *et al.,* 2014; Pérez-Sen *et al.,* 2015), and reduced apoptosis in pancreatic beta cells (Tan *et al.,* 2013).

Similar to the $P2Y_{12}$ receptors, the $P2Y_{13}$ receptor signals through G_i to inhibit adenylyl cyclase, leading to inhibition of cAMP formation, and phosphorylation of extracellular signal-regulated kinases (ERK1/2) (Communi *et al.,* 2001; Marteau *et al.,* 2003). These processes were inhibited by pertussis toxin, consistent with the $P2Y_{13}$ receptor coupling with $G\alpha_{i/o}$.

$P2Y_{13}$ agonists and antagonists

ADP is a potent agonist, while ATP and 2-MeSATP are partial agonists with a low potency (Marteau *et al.,* 2003). RB-2, suramin and high concentrations of PPADS are low affinity $P2Y_{13}$ receptor antagonists (Marteau *et al.*, 2003). MRS2211, a 2-chloro-5-nitro analogue of PPADS, is a competitive antagonist at the human $P2Y_{13}$ receptor (pA_2 = 6.3; (Kim *et al.,* 2005). Cangrelor, as previously mentioned, blocks both the rat and human $P2Y_{13}$ receptor in a non-competitive mode of action (Marteau *et al.,* 2003; Fumagalli *et al.,* 2004), in contrast to its competitive mode of interaction at the $P2Y_{12}$ receptor (Ingall *et al.,* 1999; Van Giezen and Humphries, 2005).

1.3.1.2.2.8 $P2Y_{14}$ receptors

Cloning of the $P2Y_{14}$ receptor, cellular function and signalling mechanism

$P2Y_{14}$ receptors, formerly known as GPR105, KIAA0001 and the UDP-glucose receptor, was first cloned using human (Chambers *et al.,* 2000; Abbracchio *et al.*, 2003) and rat (Charlton *et al.,* 1997) tissues. Based on its amino acid sequence, $P2Y_{14}$ receptors are 18-45% identical to other human P2Y receptors, however it has the highest similarity (47% identical) to $P2Y_{12}$ and $P2Y_{13}$ receptors (Von Kügelgen and Hoffmann, 2016). According to the *Ensemble* gene database, $P2Y_{14}$ receptors have been cloned from 197 species and are expressed in brain, spleen, placenta, thymus, stomach, intestine and adipose tissue (Abbracchio *et al.,* 2006; von Kügelgen, 2019). $P2Y_{14}^{(-/-)}$ mice indicate roles of $P2Y_{14}$ receptors in control of stress induced stem cell senescence (Cho *et al*., 2014) and insulin levels (Meister *et al.,* 2014). Studies using $P2Y_{14}$ receptor antagonists indicated the involvement of $P2Y_{14}$ receptors in pharmacological responses such as mast cell degranulation (Gao *et al.,* 2013), renal inflammation (Azroyan *et al.,* 2015), pro-inflammatory roles in neutrophils (Harden *et al.,* 2010; Sesma *et al.,* 2012; Barrett *et al.,* 2013), release of tumour necrosis factor-alpha from astrocytes (Kinoshita *et al.,* 2013), proliferation of glioma cells (Curet and Watters, 2018) and vasoconstriction (Abbas *et al.,* 2018).

The $P2Y_{14}$ receptor couples to activation of $G_{i/o}$ G proteins (von Kügelgen, 2019) and a more detailed investigation revealed binding with $G\alpha_{i5,}$ $G\alpha_{o5,}$ $G\alpha_{16}$ and of the $G_{i/o}$ family, but not G_s or endogenous $G_{q/11}$ proteins (Moore *et al.,* 2003). Stimulation of native $P2Y_{14}$ receptor resulted in a transient increase in intracellular Ca^{2+} in rat cortical astrocytes and microglial cells, however, the mechanism of this effect is still unknown (Fumagalli *et al.,* 2003; Bianco *et al.,* 2005).

$P2Y_{14}$ agonists and antagonists

UDP and UDP-glucose are agonists at the $P2Y_{14}$ receptor, UDP being the most potent natural agonist (Chambers *et al.,* 2000). Initially, UDP was reported to be a competitive antagonist (Fricks *et al.,* 2008), but this study used Ca^{2+} as a bioassay in cells that coexpressed a G-protein mutant that couples $G_{i/o}$ receptors to release Ca^{2+}. When cAMP was subsequently measured, UDP was found to be a potent agonist (Carter *et al.,* 2009). Several analogues of UDP-glucose are potent agonists,

including: MRS2690 (2-thio analogue of UDP-glucose; (Ko *et al.,* 2007), MRS2802 (α,β-Difluoromethylene-UDP; Carter *et al.,* 2009), and α,β-methylene-2-thio-UDP (Das *et al.,* 2010). A fluorescent ligand, MRS4174 (Kiselev *et al.,* 2015) is also an agonist, with a biding constant of 21 nM.

PPTN, (4-(4-(piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid) is a potent and selective $P2Y_{14}$ receptor antagonist, with an affinity below 1 nM and no effect at any other P2Y receptor at a concentration of 1 µM (Barrett *et al.,* 2013). Other non-nucleoside antagonists include 3-aminopropyl congener (MRS4458) and phenyl p-carboxamide derivative (MRS4478) derived from 3-(-4-phenyl-1 H-1,2,3-triazol-1-yl)-5-(aryl) benzoic acid (Gauthier *et al.,* 2011; Guay *et al.,* 2011; Robichaud *et al.,* 2011; Yu *et al.,* 2018).

1.3.1.2.3 P2 Receptors in the cardiovascular system

P2X and P2Y purinergic receptors are expressed throughout the cardiovascular system in VSMCs, endothelial cells and cardiac muscle cells (figure 1.8; Kennedy *et al.,* 2013) and when activated, they produce many effects, including vasoconstriction, vasodilation, angiogenesis, growth of VSMCs and endothelial cells, vascular remodelling and platelet aggregation (Erlinge and Burnstock, 2008; Burnstock, 2009, 2017; Strassheim *et al.,* 2020). In addition, nucleotides are released by various types of cardiovascular cells, including perivascular nerves, endothelial cells and blood cells, both constitutively and in a regulated manner (Erlinge and Burnstock, 2008; Kennedy *et al.,* 2013). Consequently, P2X and P2Y receptors have been proposed to play a number of roles in the control of vascular tone and blood pressure under physiological and pathophysiological conditions and suggested to be viable therapeutic targets in cardiovascular disorders, such as vasospasm, hypertension, congestive heart failure and cardiac damage during ischemic episodes (Kennedy *et al.,* 2013; Burnstock, 2017; Strassheim *et al.,* 2020).

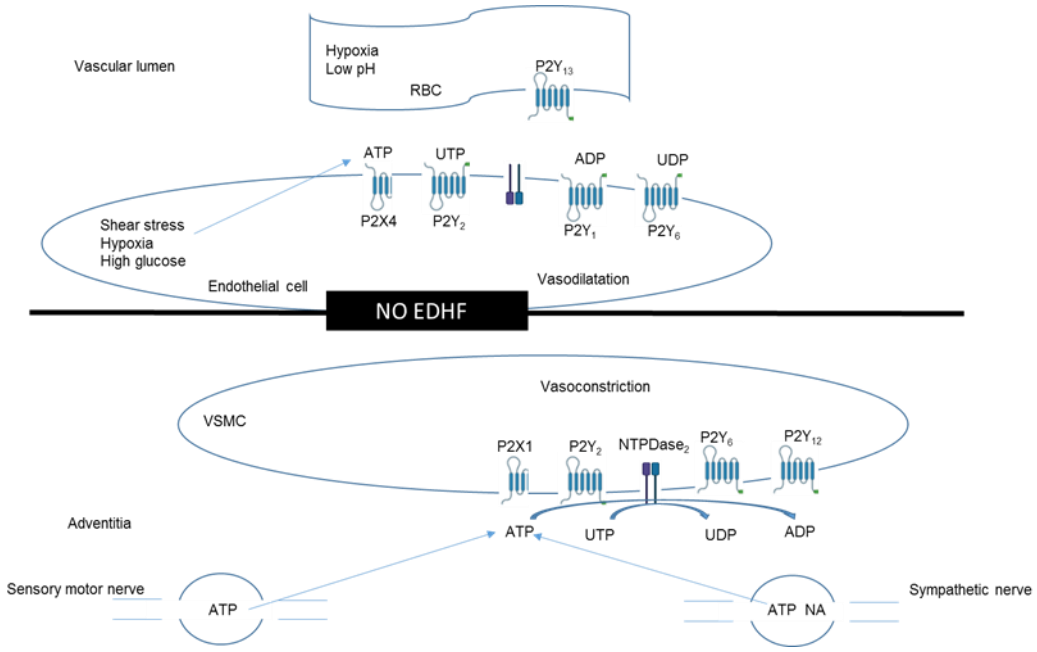


Figure 1.8: Action of endogenous agonists on purinergic receptors. These nucleotides are released from endothelial cells and red blood cells (RBC) in response to hypoxia, sheer stress and other stimuli. An intact endothelium cell layer enables vasodilatation by releasing NO and EDHF, while a damaged endothelium leads to platelet accumulation and release of ATP and ADP leading to vasoconstriction via P2 receptors present in the VSMC. NTPDase1I present in the endothelium rapidly degrade ATP to AMP and then adenosine by CD73 adventitial side, ATP is released from sympathetic and sensory nerves to mediate vasoconstriction.

P2 receptors in VSMCs

The first evidence of P2 receptors subtypes in the vasculature suggested that P2X receptors were present in the VSMC, where they mediated vasoconstriction, and P2Y receptors were expressed on the endothelial cells, where they mediated vasodilation (Kennedy *et al.,* 1985; Erlinge and Burnstock, 2008). Activation of P2X1 receptors by ATP in arterial SMCs to elicit vasoconstriction has since been widely studied and underlies the cotransmitter actions of ATP when co-released with noradrenaline and neuropeptide Y from perivascular sympathetic nerves (Erlinge and Burnstock, 2008; Kennedy *et al.,* 2013). However, subsequent studies showed that P2Y receptors are also present on VSMCs, where they mediate contraction. P2Y receptor protein and mRNA are expressed in VSMCs of a wide range on arteries, with $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ and $P2Y_{6}$ receptors being the most commonly seen (Burnstock and Knight, 2004). Furthermore, UDP and UTP also elicit vasoconstriction via P2Y receptors in many arteries, including the pulmonary artery (Rubino *et al.,* 1999; Chootip *et al.,* 2002; Mitchell *et al.,* 2012).

P2 receptors and endothelial regulation

Vasodilation can be induced by a variety of stimuli and is protective against high blood pressure, one of the pathological factors of cardiovascular disease (Wang *et al.,* 2015). Pharmacological studies have shown that in most blood vessels, one or more of ATP, UTP, ADP and UDP elicit vasodilation in an endothelium-dependent manner (Burnstock, 2017). mRNA of all P2X subunits and, in some cases protein, have been found in endothelial cells (Burnstock and Knight, 2004), but the function of endothelial P2X receptors is largely unclear. The only well-characterised effect is the vasodilation evoked by flow-dependent shear stress on the surface of endothelial cells, which is mediated by endothelial ATP release, and Ca^{2+} influx via P2X4 receptors (Bodin *et al.,* 1991; Yamamoto *et al.,* 2000). Interestingly, $P2X4^{(-/-)}$ mice have a higher blood pressure and display lower flow-dependent vasodilation than wild-type mice (Yamamoto *et al.,* 2006), indicating that this is a physiological function of ATP and P2X4 receptors.

mRNA of most P2Y subtypes and, in some cases protein, have been found in endothelial cells (Burnstock and Knight, 2004) and in contrast to P2X receptors, the

effects that they mediate have been much more widely studied and characterised. Based on the actions of selective agonists and antagonists, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors all mediate vasodilation, with the responses elicited by $P2Y_1$ and $P2Y_2$ receptors tending to be more pronounced (Wihlborg *et al.,* 2003; Burnstock and Knight, 2004; Erlinge and Burnstock, 2008; Kennedy *et al.*, 2013). Knockout mice have also been used to identify which subtypes mediate endothelium-dependent vasodilation in mouse aorta. In $P2Y_2^{(-/-)}$ mice the response to ATP was reduced, but responses to ADP, UTP and UDP were unaffected (Guns *et al.,* 2005, 2006) and these animals had salt-resistant hypertension (Rieg *et al.,* 2007; Pochynyuk *et al.,* 2010). Also, mice in which endothelium-specific $P2Y_2$ receptors or $G\alpha_q/G\alpha_{11}$ were knocked out, lacked flow-induced vasodilation, exhibited lower eNOS activation and were hypertensive (Wang *et al.,* 2015). Finally, studies on $P2Y_4^{(-/-)}$ animals showed that the $P2Y_4$ subtype does not mediate endothelium-dependent vasodilation (Guns *et al.,* 2005).

NO is the best characterised mediator of endothelium-dependent vasodilation (for example, in rat mesenteric arterial bed (Ralevic and Burnstock 1991)), but NO-independent mechanisms have also been identified, including endothelial-derived hyperpolarization (Malmsjö *et al.,* 1999, 2002; Wihlborg *et al.,* 2003) and prostacyclin (Hammer *et al.,* 2003; Wihlborg *et al.,* 2003). The $P2Y_2$ receptor mediated vasodilation and fall in blood pressure in mice are NO-independent (Rieg *et al.,* 2011) and the underlying mechanisms have been studied in some detail (Dominguez Rieg *et al.,* 2015). Knockout of $K_{Ca}3.1$, an intermediate conductance Ca^{2+}-activated K^+ channel, or connexin 37, which forms myoendothelial gap junctions between endothelial and smooth muscle cells, both reduced the fall in blood pressure induced by $P2Y_2$ receptor stimulation. Based on these and other studies, the proposed mechanism is that when activated, $P2Y_2$ receptors couple to $G\alpha_q/G\alpha_{11,}$ leading to release of Ca^{2+} from intracellular stores. Ca^{2+} then activates the $K_{Ca}3.1$ ion channels, leading to K^+ efflux and endothelial hyperpolarisation, which passes directly to the arterial smooth muscle cells by charge transfer through myoendothelial gap junctions composed of connexin 37. Note that in contrast to $P2Y_2$ receptor knockout, $K_{Ca}3.1$ and connexin 37 knockout did not abolish the $P2Y_2$ receptor mediated vasodilation, indicating that other signalling components are also involved.

Besides mediating vasodilation, the endothelium has secretory and adhesive properties, and it is an active part of the vasculature. It is also a semi-selective diffusion barrier between the plasma and the interstitial fluid (Aird, 2007). P2Y receptors are involved in the regulation of barrier function, activation of vascular inflammation and vascular growth processes, all of which are tightly linked with vascular diseases (Strassheim *et al.,* 2020). P2Y-mediated signalling is involved in crosstalk with other regulatory factors, such as cytokines, extracellular adenosine, toll-like receptor input and prostaglandins. An example is the $P2Y_2$ receptor-mediated stimulation of endothelial cell proliferation, which is potentiated by growth factors and cytokines (Li *et al.,* 2015). In addition, endothelial cell P2Y receptors are protective against the damaging effect of chemotherapies towards cardiovascular system (Aho *et al.,* 2016).

Release of endogenous nucleotides

Several reports discussed above indicate physiological roles for endothelial P2Y receptors, which implies that endogenous nucleotides must be released close to these receptors in order to activate them. Under normal conditions, the level of these nucleotides in the blood is low, but intracellular stores of ATP and UTP are released by shear stress and stimuli, such as hypoxia and thrombin, at levels that enable them to stimulate endothelial P2Y receptors and evoke vasodilation (Strassheim *et al.,* 2020). For example, fluid shear stress due to increased blood flow rate elicits a negative feedback response to initiate local vasodilation and reduce pressure (Wang *et al.,* 2016). PIEZO1 is an endothelial mechanosensitive cation channel, which when activated by shear stress, induces ATP release via pannexin channels, leading to activation of $P2Y_2$ receptors, downstream stimulation of the $G\alpha_q$-PLC-Ca^{2+} pathway in the endothelium and eNOS-NO-mediated vasodilation of mouse isolated mesenteric artery (Wang *et al.,* 2015, 2016). In addition, the shear-stress-induced fall in blood pressure in mice was almost completely lost following endothelium-specific knockout of PIEZO1 and the animals became hypertensive.

RBC acts as sensors for hypoxia and several reports have suggested that deoxygenated RBC stimulate vasodilation (figure 1.8). ATP has been shown to be released in millimolar amounts from RBC in response to a reduction in pH and O_2 tension and this is associated with a reduction in the Po_2 and O_2 saturation of haemoglobin (Ellsworth and Sprague, 2012; González-Alonso, 2012; Ellsworth *et al.,* 2016). During exercise, RBC are required to deliver O_2 to the skeletal muscle to maintain muscle activity and to ensure that skeletal muscle blood flow can be adjusted through local vasodilation to increase blood supply to O_2-deficient sites. On exposure to β-adrenergic stimulation and reduced O_2 tension, RBC release ATP, which, by stimulating endothelial P2Y receptors, triggers the release of local vasodilators that control vascular calibre (Ellsworth *et al.,* 1995; González-Alonso, 2012; Marginedas-Freixa *et al.,* 2018).

Termination of the actions of endogenous nucleotides

The extracellular actions of ATP and other nucleotides are terminated by a cascade of ecto-nucleotidases that catalyse their progressive dephosphorylation to produce

the respective nucleosides (Robson *et al.,* 2006; figure 1.8). These include ecto-nucleoside triphosphate diphosphohydrolases (CD39), nucleotide pyrophosphatase/phosphodiesterases, alkaline phosphatases and ecto-5'-nucleotidase (CD73). The most widely studied and best characterised of these is the ecto-nucleoside triphosphate diphosphohydrolase family, which has four members that functional extracellularly (eNTPDase1, 2, 3, 8). When, for example, ATP is released, one or more of these enzymes phosphohydrolyses it to ADP and AMP, which in turn is dephosphorylated to adenosine by ecto-5'-nucleotidase (Robson *et al.,* 2006). Adenosine is further degraded by adenosine deaminase to inosine and both adenosine and inosine are taken back up into cells by specific transporters. Thus these enzymes act to limit the action of extracellular nucleotides.

1.3.1.2.3.1 P2Y receptors in the pulmonary system

It has been known for some time that P2 receptor agonists modify the tone of pulmonary blood vessels of a variety of species, including humans. For example, in rat perfused lung at resting tone, ATP and other nucleotides evoked vasoconstriction, whereas if muscle tone was first raised, they evoked vasodilation (McCormack *et al.,* 1989; Hasséssian and Burnstock, 1995; Rubino and Burnstock, 1996). A similar pattern was seen in large diameter branches of rat isolated intrapulmonary arteries (rIPA; (Liu *et al.,* 1989; Rubino *et al.,* 1999b). However, the role of individual P2X and P2Y receptor subtypes in mediating these effects is unclear. Liu *et al.,* (1989b) suggested that P2X receptors, specifically P2X1 receptors, are expressed primarily in the smooth muscle and mediate vasoconstriction. Consistent with this, Syed *et al.,* (2010) showed that ATP acts via P2X1 receptors to induce contractions of rIPA. Liu *et al.,* (1989b) also suggested that P2Y receptors present in rIPA are restricted to the endothelium and mediate relaxation. Indeed, P2Y receptor mRNA has been reported in rabbit pulmonary artery endthothelial cells (Konduri *et al.,* 2004). However, mRNA of all seven P2Y subtypes has been detected in rat pulmonary artery SMCs (Hartley *et al.,* 1998; Gui *et al.,* 2008; Kennedy *et al.,* 2013). Furthermore, Rubino and Burnstock, (1996) found that the P2Y receptor agonists, UTP and UDP, also elicited vasoconstriction in the rat perfused pulmonary vascular bed. Subsequently, UTP and UDP were also shown to elicit vasoconstriction in isolated pulmonary vessels (Chootip *et al.,* 2002, 2005), UDP acting, in part, via $P2Y_6$ receptors (Mitchell *et al.,* 2012). Apart from this, the functional expression of P2Y subtypes in the PASMCs has not been properly classified, due to the lack of availability of selective P2Y receptor antagonists.

P2Y receptors and pulmonary vascular disease

A variety of experimental data suggests that contractile smooth muscle P2Y receptors are possible therapeutic targets for treatment of pulmonary vascular disease, especially PH (Cai *et al.,* 2020). As discussed above, in healthy pulmonary arteries, nucleotides promote vasodilation via the endothelial P2Y receptors by inducing release of NO and EDHF, thereby contributing to the maintenance of low pulmonary vascular resistance that is essential for the delivery of deoxygenated blood to the alveoli (Kennedy *et al.,* 2013; Dominguez Rieg *et al.,* 2015). However, vasodilation is

altered in diseases with impaired endothelial function, such as PAH and COPD (Adnot *et al.,* 1991; Dinh-Xuan *et al.,* 1991). For example, in COPD patients, ADP-induced pulmonary vasodilation is reduced (Dinh-Xuan *et al.,* 1991) and the level of extracellular ATP is increased (Lommatzsch *et al.,* 2010), possibly because CD39 expression is decreased (Aliagas *et al.,* 2018). This change in ATP results in an increase in its contractile activity, thereby lowering blood flow to the alveoli and O_2 passage into the blood. As discussed above, the release of ATP by RBC appears to regulate pulmonary resistance under certain conditions and this release is impaired in patients with PAH (Sprague *et al.,* 2001; Erlinge, 2011), though downregulation of CD39 has also been reported in the endothelium of the pulmonary small arteries in patients with PAH, again leading to increased levels of extracellular ATP (Helenius *et al.,* 2015; Visovatti *et al.,* 2016). Inducing chronic hypoxia in CD39 knockout mice produced more extracellular ATP, a greater rise in pulmonary arterial pressure (PAP) and greater right ventricular and arterial medial hypertrophy than did chronic hypoxia in wild-type animals (Visovatti *et al.*, 2016).

Little is known about the potential roles for nucleotides and P2Y receptors in the vascular remodelling seen in pulmonary vascular diseases, but ATP is a mitogen for pulmonary artery cells, which may be relevant for the media hypertrophy present in pulmonary arteries of PAH patients (Zhang *et al.,* 2004). Also, $P2Y_{11}$ receptor agonists have been reported to increase viability and survival response of healthy human pulmonary arterial endothelial cells, which was absent when $P2Y_{11}$ receptors were knocked down (Helenius *et al.,* 2015). This may be relevant, as the $P2Y_{11}$ receptor was the most markedly dysregulated purinergic receptor subtype in human pulmonary endothelial cells in which BMPR2 signalling was suppressed (Helenius *et al.,* 2015).

Evidence has also been reported that nucleotides contribute to acute HPV, as suramin, a broad spectrum P2X/P2Y receptor antagonist, reduced the rise in pulmonary blood pressure induced by hypoxia in perfused rabbit lungs (Baek *et al.,* 2008). Subsequently, P2Y receptors were shown to play a role in hypoxia-induced PH in pigs, as blocking $P2Y_1$ and $P2Y_{12}$ receptors with MRS2500 and cangrelor respectively, decreased PAP (Kylhammar *et al.,* 2014). Pulmonary vascular resistance (PVR) was also decreased by the $P2Y_1$, but not the $P2Y_{12}$ receptor antagonist. Consistent with these effects, infusing ADP into the pulmonary vascular system increased both PAP and PVR and these responses were abolished by

blocking $P2Y_1$ and $P2Y_{12}$ receptors. Thus ADP appears to have opposite effects on vascular tone in hypoxia-induced PH compared with PAH and COPD. Interestingly, ATP induced pulmonary vasodilation in hypoxic piglets (Paidas *et al.,* 1989) and lambs (Konduri and Woodard, 1991), as shown by a decrease in PAP and PVR. In addition, ATP-$MgCl_2$ infusions have been shown to be safe, effective and useful in treating children with PAH associated with congenital heart disease (Brook *et al.,* 1994), but at present, the possibility that its positive effect was produced by adenosine following breakdown by ecto-nucleotidases cannot be ruled out. Nonetheless, the balance between endothelial vs smooth muscle stimulation by ATP and other nucleotides to regulate blood pressure in different directions is still unclear, so it is important to investigate other therapeutic targets for the treatment of PAH and the role of the purinergic system, in this case $P2Y_2$ receptors, in the pulmonary artery.

1.4 Aims of this study

Nucleotides evoke both vasoconstriction and vasodilation via P2Y receptors located on vascular smooth muscle and endothelial cells. The role of individual subtypes of P2Y receptor in these actions is, however, unclear due to the poor selectivity of most agonists and antagonists available. Thus there is a clear need to develop selective ligands, particularly, antagonists. This will enhance our ability to identify the contribution of these subtypes to the effects of nucleotides and the roles they play in health and disease. The overall aim of this project, therefore, was to determine the role of $P2Y_2$ receptors in nucleotide-evoked vasoconstriction and vasodilation in systemic and pulmonary arteries by using the novel, selective $P2Y_2$ receptor antagonist, AR-C118925XX.

As little had been published at the start of the project on the pharmacological actions of AR-C118925XX, the first aim was to quantify the pA_2 of AR-C11895XX at recombinant $P2Y_2$ receptors stably expressed in a cell line, using Ca^{2+} imaging as a bioassay. Then the reversibility of AR-C118925XX action on $P2Y_2$ receptors was investigated. Thereafter, AR-C11895XX selectivity for $P2Y_2$ receptors was examined by studying its effect at other P2Y subtypes. Having characterised these basic pharmacological properties, AR-C118925XX was then used to study the functional expression of native $P2Y_2$ receptors in human vascular EAhy926 endothelial cells.

The next set of experiments employed AR-C118925XX to determine the role of $P2Y_2$ receptors in the vasoconstriction elicited by ATP, UTP, and UDP of rIPA and of a systemic artery, the rat isolated tail artery (rTA). Thereafter, AR-C118925XX was used to investigate the contribution of endothelial $P2Y_2$ receptors to the vasodilation of rIPA elicited by ATP, ADP, UTP, and UDP.

The final series of experiments were designed to investigate the contribution of other P2Y receptor subtypes to nucleotide-evoked vasodilation of rIPA. Specifically, the subtypes through which ATP and ADP elicit vasodilation were studied using MRS2179, a selective $P2Y_1$ antagonist, AR-C69913MX, a $P2Y_{12}$ and $P2Y_{13}$ antagonist, suramin and PPADS, which are non-selective, non-competitive allosteric P2 antagonists and the adenosine receptor antagonist, CGS1593. Finally, the role of

$P2Y_6$ receptors in UTP- and UDP-evoked vasodilation was studied using the selective $P2Y_6$ antagonist, MRS2578.

2 Methods and Materials

2.1 Pharmacological characteristics of the properties of AR-C118925XX

2.1.1 Cell Culture

The following cell lines were used: 1321N1 cells (ECACC Cat# 86030402, RRID:CVCL_0110) stably expressing recombinant human $P2Y_1$ (1321N1-h$P2Y_1$), h$P2Y_2$ (1321N1-h$P2Y_2$), $P2Y_4$ (1321N1-h$P2Y_4$), $P2Y_{11}$ (1321N1-h$P2Y_{11}$) or rat $P2Y_6$ (1321N1-r$P2Y_6$) receptors, tSA201 cells (ECACC Cat# 96121229, RRID:CVCL_2737) and EAhy926 cells (ATCC Cat# CRL-2922, RRID:CVCL_3901). 1321N1 is a human astrocytoma cell line that does not endogenously express any of the eight P2Y receptor subtypes or respond to the naturally-occurring nucleotide agonists, such as UTP and ATP (Filtz *et al*. 1994; Parr *et al*. 1994; Abbracchio *et al*. 2006) and so have been used widely for stable transfection and expression of recombinant P2Y receptors. tSA201 cells are a modified human embryonic kidney 293 cell line that expresses endogenous $P2Y_1$ receptors (tSA201-$P2Y_1$; Shrestha *et al.*, 2010). EAhy926 cells are a human vascular endothelial cell line from umbilical vein (Edgell *et al.,* 1983) that was previously shown to be responsive to UTP (Graham *et al*. 1996; Paul *et al*. 2000). The cells were grown in T25 flasks in media consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Paisley, UK) supplemented with 1% penicillin (10,000 units/ml) and streptomycin (10 mg/ml), 10% (v/v) foetal calf serum (FCS) and 1% non-essential amino acid and maintained at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% O_2.

2.1.2 Intracellular Ca^{2+} Measurements

Prior to recording intracellular Ca^{2+}, the cells were plated onto 13 mm diameter glass coverslips that were coated with poly-L-lysine (0.1 mg/ml) and experiments were performed once a confluent monolayer of cells had developed. The cells were then incubated in the dark at 37 °C for 60 min with a Ca^{2+}-sensitive fluorescent indicator, Cal-520 AM ester (5 µM), in a mixture of Pluronic™ F127 (0.05% w/v) and 1.5 ml of a buffer solution comprising: 122 mM NaCl, 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N´-[2-ethane-sulphonic acid]), 5 mM KCl, 1.8 mM $CaCl_2$, 11 mM glucose, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, titrated to pH 7.3 with NaOH. The coverslips were washed

with HEPES buffer and mounted vertically in the recording chamber of a Perkin Elmer LS50B Luminescence spectrometer and continuously superfused under gravity at room temperature with HEPES buffer at 4 ml/min. Cal-520 fluorescence intensity was recorded in a population of cells as arbitrary units (AU). It was sampled at 10 Hz following stimulation at 490±15 nm and light emitted was measured at 525±15 nm using FL Winlab software (V4.00.02). Resting Ca^{2+} levels were stable over the course of the experiment. Agonists were added in the superfusate for 60-90 sec at 10-15 min intervals to monitor changes in free intracellular Ca^{2+}. For each drug addition, the data were exported to GraphPad Prism v7.01 (GraphPad, San Diego, CA) for analysis. This experimental set-up enabled full agonist concentration-response curves (CRC) to be constructed on a single population of cells.

Experimental Protocols

The protocols described below followed previous studies on individual cell lines (Kennedy *et al*., 2000; Morrow *et al*., 2014; unpublished data from the Kennedy lab). Data were excluded if initial responses to the high concentration of agonists were too small for responses to lower concentrations to be measured accurately.

2.1.3 Effects of UTP and ATP on Intracellular Ca^{2+} in 1321N1-hP2Y$_2$ Cells

All coverslips of 1321N1-hP2Y$_2$ cells were first exposed to UTP (1 µM) twice to confirm cell viability. CRC were then generated by superfusing cells with increasing concentrations of UTP or ATP. The lower concentrations were added for 90 sec at 10 min intervals, while the higher concentrations were added for 60 sec at 15 min intervals. To compare the potencies of UTP and ATP, an initial CRC to UTP was constructed, followed by a CRC to ATP on the same cells. The data were measured by calculating responses in AU and normalised by calculating responses as a percentage of the response evoked by UTP (1 µM) during the construction of its CRC. To determine the reproducibility of nucleotide-evoked responses, an initial CRC to UTP was constructed, followed by a second CRC on the same cells. Again, all responses were normalised as a percentage of the response evoked by UTP (1 µM) during the construction of the first CRC. This second CRC also served as a time-matched control

when studying the effects of the antagonist AR-C11892XX, as described below. CRCs were constructed using non-linear regression analysis by fitting the Hill equation to the data to obtain EC_{50} values and maximum values. These values were then compared using Student's paired *t*-test.

2.1.4 Effect of AR-C118925XX at Recombinant hP2Y$_2$ Receptors

The effects of AR-C118925XX at hP2Y$_2$ receptors were determined by generating two UTP CRC for each coverslip of 1321N1-hP2Y$_2$ cells. First, a UTP CRC was constructed using the protocol described above in section 2.1.3. The cells were then superfused with a given concentration of AR-C118925XX for 5 min to establish if it had an effect on basal intracellular Ca^{2+} levels. Thereafter, the second UTP CRC was constructed in the continuous presence of that concentration of AR-C118925XX. The data were normalised as a percentage of the response evoked by UTP (1 µM) during the construction of the first CRC. The dose ratio (DR) for the rightwards shift induced by AR-C118925XX was calculated from the EC_{50} values of the two curves. Data generated using different concentrations of AR-C118925XX were collated and used to construct a Schild plot. To determine if AR-C11895XX reduced the maximum response to UTP, the maximum values calculated by fitting the Hill equation to the second CRC were compared with the maximum values of the time-matched controls obtained as described in section 2.1.3 using one-way ANOVA with Dunnett's comparison.

2.1.5 Reversibility of Inhibitory Effects of AR-C118925XX at Recombinant hP2Y$_2$ Receptors

The reversibility of the inhibitory actions of AR-C118925XX following its washout was studied using a single concentration of UTP (100 nM), which was chosen because it is just above the EC_{50} and it was the lowest concentration at which the response was abolished by AR-C118925XX (30 nM). UTP (100 nM) was added four times at 10 min intervals to obtain control responses. A given concentration of AR-C118925XX was then added for 5 min, and then UTP and AR-C118925XX were co-applied. Both drugs

were then washed out and UTP (100 nM) re-added at 10 min intervals until the inhibitory action of AR-C118925XX was fully reversed. The data were normalised by calculating the response in AU as a percentage of the control response to UTP (100 nM). No statistical tests were applied to these data since the point of this experiment was to determine if the responses fully recovered from the co-application with AR-C118925XX.

2.1.6 Selectivity of AR-C118925XX

To determine if AR-C118925XX is an antagonist at P2Y subtypes other than the $P2Y_2$ receptor, agonist CRCs were constructed in cells that expressed an individual P2Y receptor subtype. From these results two concentrations of agonist were chosen that were (a) 50-75% of the maximum response (test concentration) and (b) close to the top of the CRC (reference concentration), as follows; $hP2Y_1$-ADP (100 nM / 1 µM), $hP2Y_4$-UTP (1 µM / 10 µM), $rP2Y_6$-UDP (100 nM / 1 µM), $hP2Y_{11}$-ATP (2 µM / 10 µM), and tSA201-$P2Y_1$- ADP (300 nM / 10 µM). In all experiments, the reference concentration of agonist was first added twice to confirm the integrity of the cells. The second of these responses also served as a reference for statistical analysis, as described in the next paragraph. The test concentration of agonist was then added four times at 10 min intervals to obtain control responses. Next, AR-C118925XX (1 µM) was added for 5 min, then the test concentration of the agonist plus AR-C118925XX (1 µM) was added immediately after. Both drugs were then washed out and the test concentration of the agonist was re-added at 10 min intervals. A time-matched control was obtained by following the same protocol, but omitting addition of AR-C118925XX.

Since the aim of this experiment was to determine if ARC118925XX acts as an antagonist at the other P2Y subtypes, the data are shown as a percentage of the control response to the test concentration of the agonist. To allow parametric analysis, however, responses to the test concentration were also calculated as a percentage of the reference concentration in each cell line. The values thus obtained in the absence and presence of AR-C118925XX were then compared using Student's paired *t*-test.

2.1.7 Effects of UTP and AR-C118925XX on intracellular Ca^{2+} in EAhy926 Cells

To confirm cell viability, EAhy926 cells were first exposed to UTP (10 µM) twice. Two UTP CRC were then constructed consecutively, in the same manner as described above in section 2.1.3 for 1321N1-hP2Y_2 cells, to determine the reproducibility of the nucleotide responses. To compare the two CRC, the data were normalised by calculating each response in AU as a percentage of the response evoked by UTP (10 µM) in the first CRC. The concentration used is close to the top of the UTP CRC. CRCs were constructed using non-linear regression analysis by fitting the Hill equation to the data to obtain EC_{50} and maximum values. The effect of a single concentration of AR-C118925XX, 30 nM, on the UTP CRC was investigated using the same protocol described above in section 2.1.5 for 1321N1-hP2Y_2 cells.

2.2 Characterisation of P2Y Receptors in rat intrapulmonary artery.

2.2.1 Myography

Male Sprague-Dawley rats (200-450 g) were killed by cervical dislocation, according to the Schedule 1 guidelines. The lungs were cut out and placed in HEPES buffer. The rIPA (internal diameter 200-500 µm) was dissected out, cleaned of connective tissue and cut into rings approximately 5 mm long. Where appropriate, the endothelium was removed by carefully passing a thread or hair through the lumen. The rings were mounted horizontally in a 1 ml organ bath on a pair of intraluminal wires (figure 2.1). Tissues were equilibrated under a resting tension of 0.5 g at 37^{0}C for 60 min and bubbled with medical air (21% O_2, 5% CO_2 and 74% N_2). Tissue tension was recorded by Grass FT03 isometric force transducers (Grass Instruments, Quincy, MA) connected to a PowerLab/4e system using Chart 5 software (ADInstruments Ltd, UK). Drugs were added directly to the tissue bath and washed out by replacement with drug-free HEPES buffer solution.

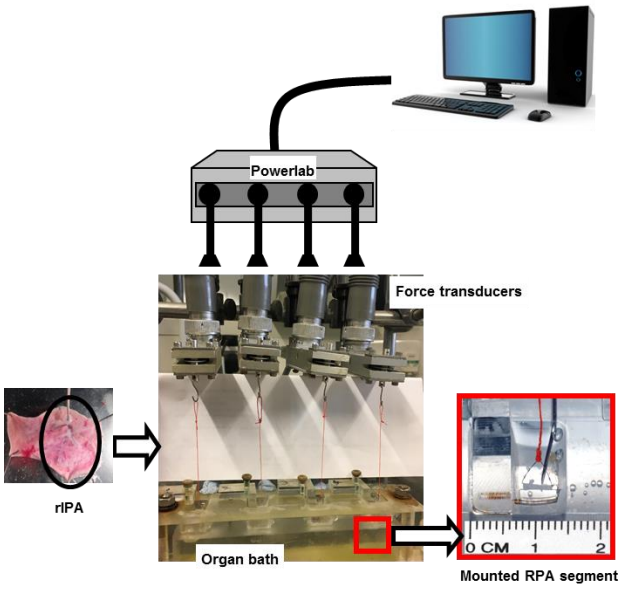


Figure 2.1: Organ bath set up for myography of rIPA. The rIPA (left hand panel) was dissected from the lung and mounted in a 1ml organ bath on a pair of intraluminal wires (highlighted red box). Muscle tension was recorded by Grass FT03 isometric force transducers (Grass Instruments, Quincy, MA) connected to a PowerLab/4e system.

2.2.2 Effects of KCl, UTP, UDP and ATP at Resting Tone

First, to verify the integrity of the tissue, tissues were exposed twice to isotonic 40 mM KCl solution (whole bath replacement) for 5 min at 30 min intervals. ACh (10 µM) was applied when the response had plateaued to assess the presence of the endothelium. Nucleotide contractile CRC in rIPA do not reach a maximum (Chootip *et al*., 2002; 2005); therefore, a single, equi-effective concentration, 300 µM, of UTP, UDP and ATP was used throughout these experiments. When studying responses evoked at basal tone, KCl, UTP, UDP, and ATP were added for 5 min six times at 30 min intervals with several washes in between; only one of these agonists were applied to an individual artery ring. Previous studies have shown this protocol to produce reproducible contractions (Mitchell *et al.,* 2012).

Contractions are presented as mg tension, which was calculated by subtracting the baseline of contractions from the peak. The responses evoked by repeated addition of any given agonist were compared statistically to the first response using one-way ANOVA, with Dunnett's comparison. The amplitudes of the first contraction evoked by an agonist in the presence and absence of the endothelium were analysed using Student's unpaired *t*-test. To compare responses obtained in endothelium-intact and endothelium-denuded tissues the mg values were normalized as a percentage of the first response of each individual agonist and responses from the second to sixth addition of both tissue types were then compared pairwise using Student's unpaired *t*-test.

2.2.3 Effects of AR-C118925XX on KCl- and Nucleotide-Evoked Contractions

To determine the role of $P2Y_2$ receptors in evoking vasoconstriction, three control responses were obtained for KCl (40 mM), UTP, ATP and UDP (300 µM). The tissue was then incubated with the $P2Y_2$ receptor antagonist, AR-C118925XX (1 µM) for 20 min before the readdition of the agonist. This concentration was used because it is 270 times greater than the K_B at $P2Y_2$ receptors (3.7 nM) calculated in our previous study. Both drugs were then washed out and a further response obtained to determine if any effect that the antagonist may have had was reversible. The responses are

shown as a percentage of the third control response, but to enable parametric analysis of the data, the peak amplitudes of the contractions (mg) were compared using one-way ANOVA with Dunnett's comparison.

2.2.4 Effects of UTP, UDP, ATP and ADP at Raised Tone

All tissues were first exposed twice to isotonic 40 mM KCl solution (whole bath replacement) for 5 min at 30 min intervals to verify their integrity. Following washout of KCl the rIPA was then pre-contracted with phenylephrine (PE) (0.1 μM) and ACh (10 μM) applied to confirm the presence of an intact endothelium. (Note that on a small number of occasions the contractions evoked by 0.1 μM PE were too small for vasodilation to be studied meaningfully and so the concentration of PE was increased to 0.3 μM. These instances are noted where appropriate in the results section of chapter 4). Both drugs were then washed out, the tissue was again pre-contracted with PE (0.1 μM) and the action of nucleotides, alone or in the presence of antagonists determined.

Relaxant CRC to UDP, UTP, ATP, ADP (100 nM - 30 μM) and the selective P2Y agonists, MRS2365 (100 nM-100 μM), P-UDP (100 nM-1 μM), and INS48823 (100 nM-3 μM), were then constructed. The peak amplitude of the relaxation evoked by each concentration of nucleotide was calculated as a percentage of the amplitude of the PE contraction. CRC were constructed using the non-linear regression analysis by fitting the Hill equation to the data to obtain EC_{50} values.

Single agonist concentrations that were above the EC_{50} were then selected to be used as the test concentrations in subsequent experiments (UTP and UDP - 3 μM, ATP and ADP - 10 μM). In each case, the nucleotide was added twice to PE-precontracted tissues, 30 min apart. The first response served as the control. Horizontal bars shown in the figures indicates where relaxation produced by these nucleotides were measured.

2.2.4.1 Reproducibility and the Effects of Antagonists on Nucleotide-Induced Vasodilation

In the first set of experiments both responses were obtained in the absence of antagonist to determine if the relaxations were reproducible. Thereafter, the roles of a variety receptor subtypes in evoking vasodilatation in endothelium-intact rIPA were investigated. Once a control relaxation had been obtained, the tissue was incubated with an antagonist for 20 min before readdition of the agonist. The peak relaxation amplitude was calculated as a percentage of the PE contraction amplitude and the two values were compared using Student's paired *t*-test.

2.3 Characterisation of P2 Receptors in rat tail artery.

2.3.1 Myography

Male Sprague-Dawley rats (200-450g) were killed by cervical dislocation according to the Schedule 1 guidelines. The tail was cut off and the rTA (internal diameter ~300 µm) was dissected out, cleaned of connective tissue and cut into rings approximately 5 mm long. Where appropriate, the endothelium was removed by carefully passing a thread or hair through the lumen. The rings were then mounted horizontally in a 1ml organ bath on a pair of intraluminal wires (figure 2.2) and equilibrated under a resting tension of 0.5g at 37 ^{0}C for 60 min and bubbled with medical air (21% O_2, 5% CO_2 and 74% N_2). Muscle tension was recorded thereafter as described above for the rIPA. Drugs were added directly to the tissue bath and washed out by replacement with drug-free HEPES buffer solution.

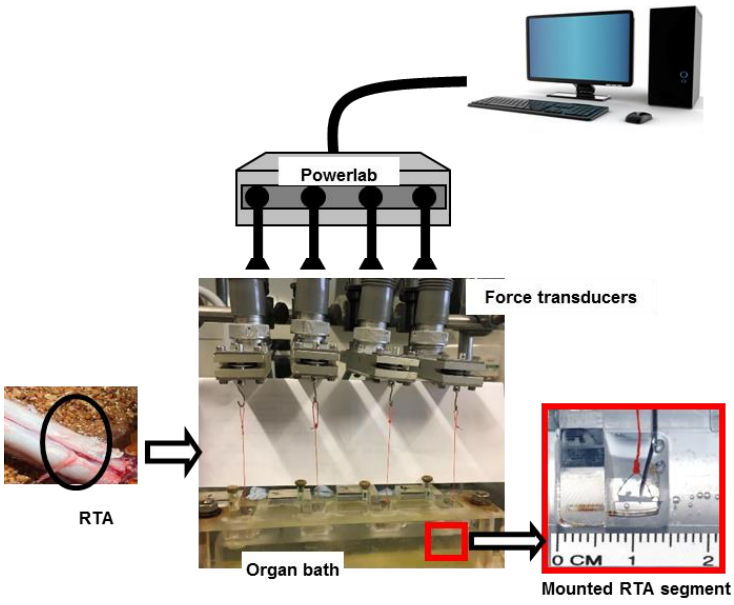


Figure 2.2: Organ bath set up for myography of rTA. The rTA (left hand panel) was dissected out and mounted in a 1ml organ bath on a pair of intraluminal wires (highlighted red box). Muscle tension was recorded by Grass FT03 isometric force transducers (Grass Instruments, Quincy, MA) connected to a PowerLab/4e system.

2.3.2 Effects of KCl, UTP, UDP and ATP at Resting Tone

First, the integrity of tissues was verified by exposing them twice to isotonic 40 mM KCl solution (whole bath replacement) for 5 min at 30 min intervals. ACh (10 µM) was applied when the response had plateaued to assess the presence of the endothelium. Previous experiments showed that nucleotide contractile CRC do not reach a maximum in rTA (Evans and Kennedy, 1994). In addition, nucleotide potency is reduced by breakdown by ecto-nucleotidases, which in turn limits the extent to which CRC can be right shifted by an antagonist (Evans and Kennedy, 1994; McLaren *et al*., 1998a). Consequently, a single, equi-effective concentration, 1 mM, of UTP, UDP and ATP was used throughout these experiments. When studying contractions at basal tone, KCl, UTP, UDP, and ATP were added for 5 min six times at 30 min intervals with several washes in between; only one of these agonists were applied to an individual artery ring.

Contractions are presented as mg tension, which was calculated by subtracting the baseline of contractions from the peak. The responses evoked by repeated addition of any given agonist were compared statistically to the first response using one-way ANOVA, with Dunnett's comparison.

2.3.3 Effects of AR-C118925XX on KCl- and Nucleotide-induced Contractions

To determine the role of $P2Y_2$ receptors in evoking vasoconstriction, three control responses were obtained for KCl (40 mM), UTP, ATP and UDP (1 mM). The tissue was then incubated with AR-C118925XX (10 µM), for 20 min before readdition of the agonist. Both drugs were then washed out and a further response obtained to determine if any effect that the antagonist may have had was reversible. The responses are shown as a percentage of the third control response, but to enable parametric analysis of the data, the peak amplitudes of the contractions (mg) were compared using one-way ANOVA with Dunnett's comparison.

2.3.4 Effects of UTP, UDP and ATP at Raised Tone

First, to verify the integrity of the tissue, tissues were exposed twice to isotonic 40 mM KCl solution (whole bath replacement) and NA (1 µM) for 5 min, 30 min apart. ACh (10 µM) was applied once the response had reached a peak in order to assess the presence of the endothelium. Thereafter, the rTA was pre-contracted with KCl (40 mM) or NA (1 µM), followed by the cumulative additions of UTP, UDP and ATP (10 µM - 1 mM). Contractions and relaxations are presented as mg tension, which was calculated by subtracting the tension just as the agonist was added from the peak change. The responses evoked by UTP, ATP and UDP at each concentration were compared statistically using one-way ANOVA, with Tukey's comparison.

2.4 Materials

UTP ($Na_3(H_2O)_2$ salt), ATP (Na_2 salt), UDP (Na salt), ADP (Na salt), P-UDP (Na_2 salt) (Sigma-Aldrich Co, Gillingham, Dorset, UK), suramin hexasodium, PPADS tetrasodium (Sigma/RBI, Natick, MA), MRS2179, MRS2365 (Na_2 salt) (Tocris Bioscience, Bristol, UK), INS48823 (a generous gift from Inspire Pharmaceuticals, Durham, NC, USA) and AR-C69913MX (a generous gift from The Medicines Company, Parsippany, NJ, USA) were dissolved in distilled water as 1, 10 or 100 mM stock solutions, as appropriate. AR-C118925XX, MRS2578, CGS15943 (Tocris Bioscience, Bristol, UK) and Cal-520-AM ester (Life Technologies, Paisley, UK) were dissolved in DMSO as a 1 or 10 mM stock solution. All were immediately frozen, and stored at -20ºC. Stock solutions were diluted to the appropriate concentrations using HEPES-based buffer on the day of use, before application to tissues. Pluronic™ F-127 (Life Technologies) was supplied as a 20% w/v solution in DMSO and stored at room temperature. The isotonic 40 mM K^+ solution was prepared by replacing NaCl in the HEPES solution with an equimolar amount of KCl to maintain osmolarity of the solution. Common chemicals were supplied by Sigma-Aldrich Co, Fisher Scientific UK (Loughborough, UK) and VWR International, (Lutterworth, UK) and were of the highest purity available.

2.5 Data and Statistical Analysis

Data are shown as the mean ± *SEM* or geometric mean with 95% confidence limits (95% cl) for EC_{50} values, with (n) being the number of coverslips of cells used in each experiment or the number of arteries taken from different rats (ie n=6, 6 arteries from 6 different rats). When appropriate, CRC were fitted to the data by logistic (Hill equation; R = Rmin + [(Rmax - Rmin)/1-(EC_{50}/D]$^{Hill\ slope}$], nonlinear regression analysis (GraphPad Prism v7.01, San Diego, CA) and EC_{50} and maximum values calculated. Hill equation parameters are: R - response; R_{max} - maximum response; D - concentration of the drug; EC_{50} - concentration of agonist that produces 50% of maximum response; Hill slope - slope of the curve. The Gaddum-Schild equation was used to calculate the dissociation constant (K_B) of AR-C118925XX (K_B = [AR-C118925XX]/DR-1).

Statistical analysis was carried out as indicated in each section above. Differences were considered significant when $P < 0.05$.

3 Characterisation of the Pharmacological Properties of AR-C118925XX

3.1 INTRODUCTION

P2Y receptors are expressed in cells and tissues throughout the body, but their physiological roles largely remain unclear. In part this is because the endogenous nucleotides, ATP, UTP, ADP and UDP, all act at multiple P2Y receptor subtypes and can be broken down by ecto-nucleotidases, which reduces their potency and produces metabolites that have effects of their own (Chootip *et al.,* 2005; Jacobson *et al.,* 2009). The biggest issue, however, is the low potency and selectivity of many of the available antagonists. Currently, highly selective and potent antagonists have only been developed for $P2Y_1$ (MRS2179, MRS2500) and $P2Y_{12}$ (clopidogrel, ticagrelor, cangrelor) receptors, and they have been used to identify physiological roles, such as that of $P2Y_1$ receptors in peristalsis in the gut (see Kennedy, 2015) and of $P2Y_1$ and $P2Y_{12}$ receptors in platelet aggregation (Abbracchio *et al.,* 2006; von Kügelgen, 2017). Clearly, the development of potent and selective antagonists at the other P2Y subtypes would greatly enhance our ability to determine their functions in health and disease.

Recently, the characterisation of $P2Y_2$ receptor function has been made possible due to the commercial availability of AR-C118925XX. This antagonist was developed by AstraZeneca around 20 years ago, but only a conference abstract was published at the time (Meghani, 2002). Several studies have since been published that used AR-C118925XX to investigate the role of native $P2Y_2$ receptors in the actions of P2Y agonists in various cell types (Kemp *et al.,* 2004; Cosentino *et al.,* 2012; Hochhauser *et al.,* 2013; Onnheim *et al.,* 2014; Magni *et al.,* 2015; Wang *et al.,* 2015; Gabl *et al.,* 2016; see also review by Rafehi and Müller, 2018). However, these studies did not report the K_B or pA_2 of AR-C118925XX, or relate the concentrations used (mostly 1 and 10 µM) to its potency or selectivity. Inhibition of other P2Y subtypes could not, therefore, be ruled out based on the data published at this time.

It is essential to have accurate values of antagonist potency for effective experimental use. Thus the aims of the experiments reported in this chapter were first to quantify the pA_2 of AR-C118925XX at recombinant $P2Y_2$ receptors stably expressed in a cell line, using a system that allowed full agonist CRC to be constructed in the absence and presence of AR-C118925XX on a single population of cells. The selectivity of AR-C118925XX for $P2Y_2$ receptors was then determined by studying its effects at other

recombinant P2Y receptor subtypes. Finally, AR-C118925XX was used to investigate the presence of functional native $P2Y_2$ receptors in human vascular endothelial cells.

3.2 RESULTS

3.2.1 Effects of UTP and ATP on Intracellular Ca^{2+}

UTP and ATP are both agonists at human $P2Y_2$ receptors (Abbracchio *et al*., 2006; Jacobson *et al*., 2020), so in the initial experiments UTP and ATP CRC were constructed to determine their potencies at the recombinant $hP2Y_2$ receptor when expressed in 1321N1 cells. Figure 3.1a shows that both produced a rapid, transient increase in intracellular Ca^{2+} in a concentration-dependent manner. There was no difference in the maximum responses produced, but UTP was 2.6 times more potent than ATP (EC_{50} values: UTP= 54 nM, 95% cl. 43-67 nM; ATP= 142 nM, 95% cl. 108-186 nM; n=4, $P<0.05$) (figure 3.1b). UTP was, therefore, used in all subsequent experiments.

The next objective was to determine if these responses were reproducible on repeated administration of agonist. When two consecutive UTP CRC were constructed on the same cells, there was no significant difference between the two EC_{50} values (1^{st} = 76 nM, 95% cl. 67-87 nM; 2^{nd} = 91 nM, 95% cl. 76-108 nM), but there was a small (~6%) decrease in the CRC maximum, which was statistically significant ($P<0.05$; figure 3.1c). This decrease may be due to slight run down of the $P2Y_2$ receptors.

These data show that UTP and ATP are both agonists at the $hP2Y_2$ receptor and that responses to UTP are reproducible on repeated administration. Thus, it was possible to quantify the actions of ARC118925XX at the recombinant $hP2Y_2$ receptor using UTP as the agonist.

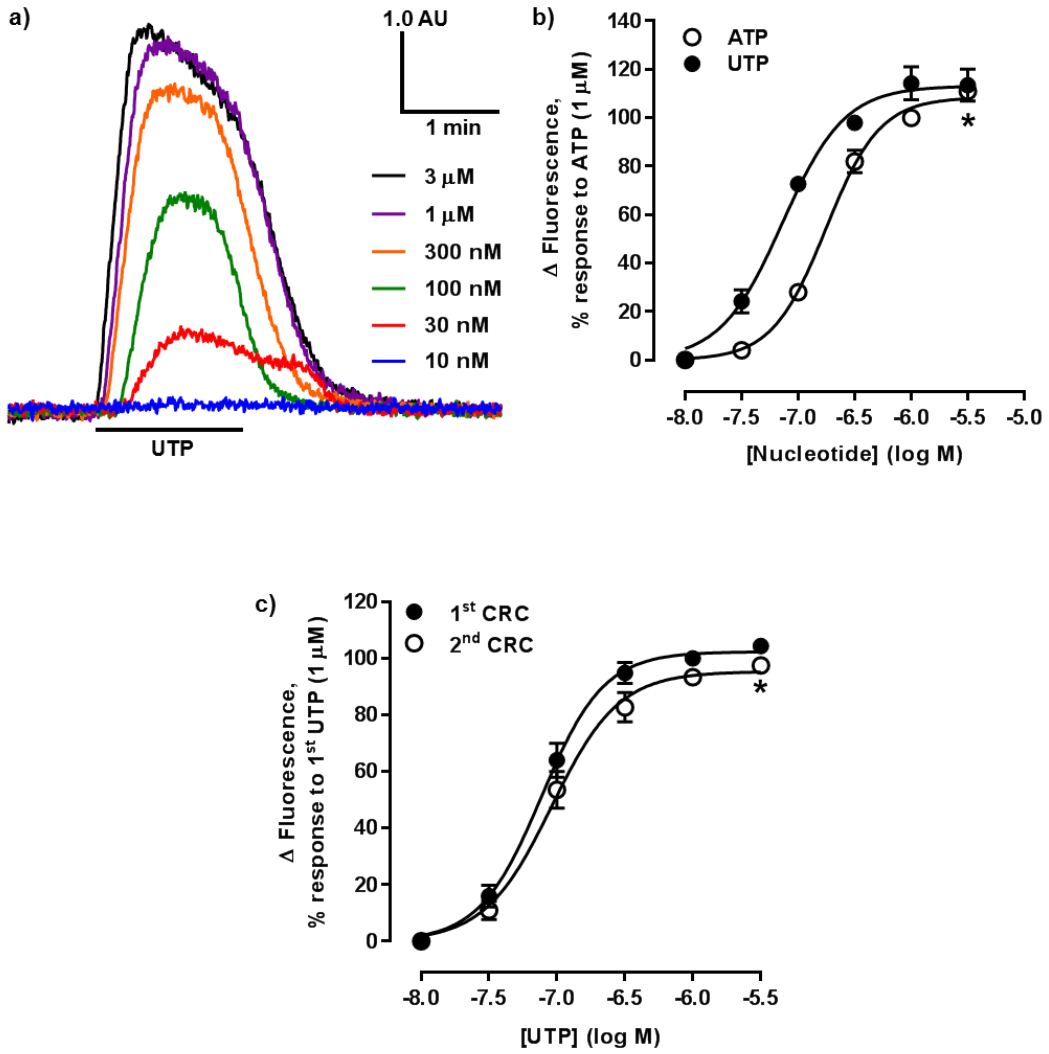


Figure 3.1: UTP and ATP increase intracellular Ca^{2+} in 1321N1-hP2Y$_2$ cells. a) The superimposed traces show changes in Cal-520 fluorescence evoked by superfusion of cells with UTP (10 nM - 3 µM) as indicated by the horizontal bar. b) CRC of the mean peak amplitude of responses evoked by UTP and ATP in the same population of cells are shown. The data are expressed as a percentage of the response to ATP (1 µM). n=4 each. * $P < 0.05$ for maximum responses produced by ATP. c) The mean amplitude of two consecutive UTP CRC in the same population of cells is shown. The data are expressed as a percentage of the response to UTP (1 µM) in the first CRC. n=6. * $P < 0.05$ for maximum responses produced by second UTP CRC. Vertical lines show SEM. For some points, the error bars are shorter than the height of the symbol. The curves represent the fit of the Hill equation to the data.

3.2.2 The Effect of AR-C118925XX at Recombinant hP2Y$_2$ Receptors

To investigate the antagonist effect of AR-C118925XX, two consecutive UTP CRC were generated on individual coverslips of cells, the first in the absence and the second in the presence of a single concentration of the antagonist. Because of the small decrease in the maximum response generated by UTP on repeated administration reported above, the 2nd control UTP CRC was used as a time-matched control for responses in the presence of the antagonist.

AR-C118925XX (10 nM - 1 µM) had no effects *per se* on intracellular Ca^{2+}, but progressively shifted the UTP CRC to the right in a parallel fashion, with no significant change in the maximum response (figure 3.2a). The X-intercept of the Schild plot of these data = 8.30 and the slope = 0.985±0.028 (figure 3.2b), giving a pA_2 = 8.43.

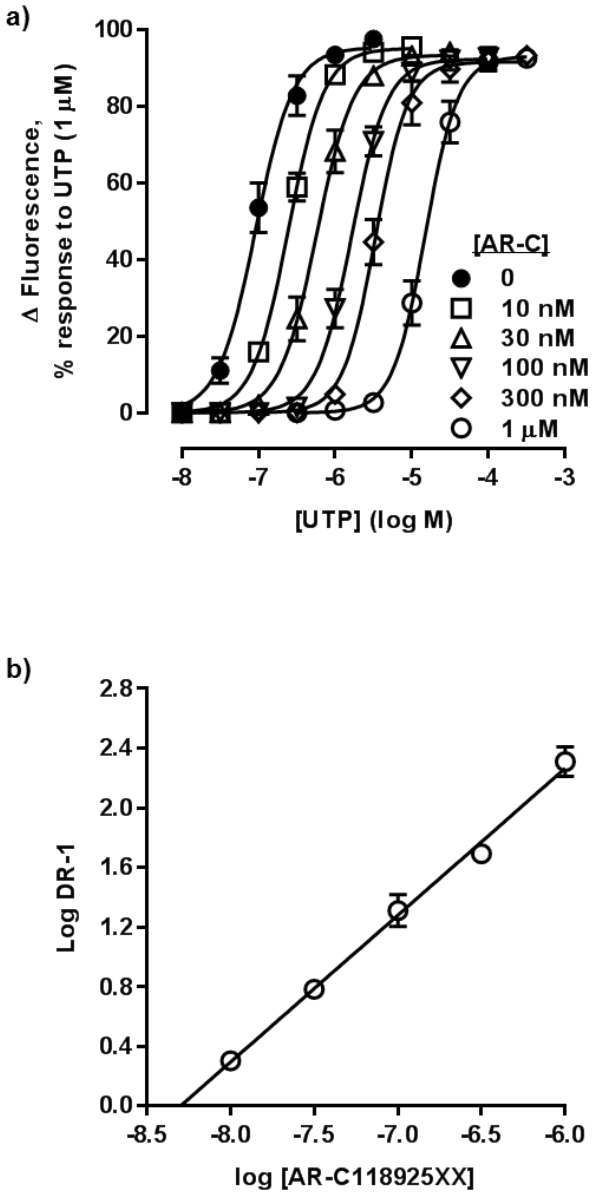


Figure 3.2: AR-C118925XX is a competitive antagonist at hP2Y$_2$ receptors. a) The mean peak amplitude of responses evoked by UTP (10 nM - 300 μM) in 1321N1-hP2Y$_2$ cells in the absence (control) and presence of AR-C118925XX (10 nM - 1 μM) is shown (n=6 each). The data are expressed as a percentage of the response to UTP (1 μM) in the control CRC in each coverslip of cells. The curves represent the fit of the Hill equation to the data. b) The Schild analysis of the data in panel (a) is shown. The straight line represents the fit of the data by linear regression (r^2= 0.996). Vertical lines show SEM. For some points, the error bars are shorter than the height of the symbol.

3.2.3 Reversibility of the Inhibitory Effects of AR-C118925XX

To investigate if the inhibitory effects of AR-C118925XX are reversible on washout, a single concentration of UTP (100 nM), which is just above its EC_{50}, was applied repeatedly at 10 min intervals. Under these conditions UTP evoked highly reproducible responses in the absence of AR-C118925XX (figure 3.3). Coapplication of each concentration of AR-C118925XX (30 nM - 1 µM) abolished the response to UTP (figure 3.3). Following washout of AR-C118925XX, the response to UTP quickly returned to time-matched control values. However, as the concentration of AR-C118925XX increased, so recovery of the UTP response was slower and it took longer for the UTP response to recover to control levels, with no difference between the washouts for each AR-C118925XX concentration.

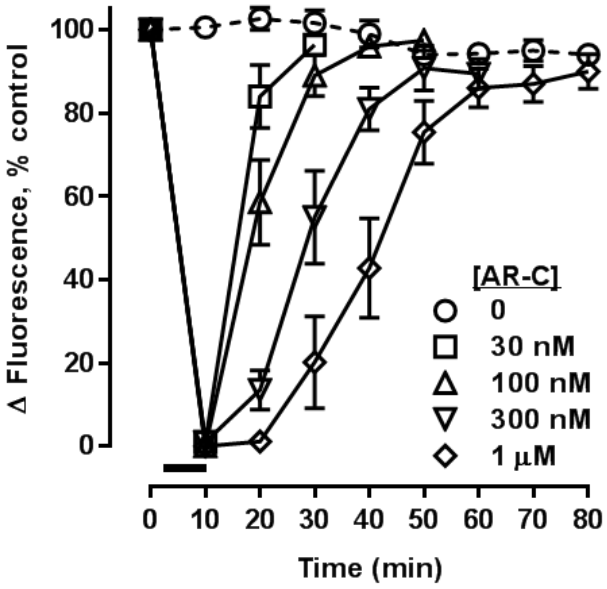


Figure 3.3: The inhibitory actions of AR-C118925XX are reversible. The figure shows the time-course of responses evoked by repeated addition of UTP (100 nM) to 131N1-hP2Y_2 cells at 10 min intervals in the absence (0 min), presence (10 min) and following washout of AR-C11895XX (30 nM - 1 µM; 20-80 min; n=5 each). AR-C118925XX was added for 5 min, as indicated by the horizontal bar. The data are expressed as the percentage of the control response evoked by UTP, prior to the addition of AR-C118925XX (0 min). The open circles and dashed line show time-matched control when AR-C118925XX was not applied to the cells. Vertical lines show SEM.

3.2.4 Selectivity of AR-C118925XX

The next series of experiments were designed to determine if AR-C118925XX also had inhibitory actions at other subtypes of P2Y receptor. In preliminary experiments, a single CRC for an appropriate agonist was constructed in cells expressing one of the other P2Y subtypes that couple to Ca^{2+} mobilisation (figures 3.4 - 3.8).

AR-C118925XX (1 µM), a concentration that is 270 times greater than its K_B at $hP2Y_2$ receptors (3.7 nM) had no effect on basal intracellular Ca^{2+} on its own in any of the cell lines (132N1-$hP2Y_1$, -$hP2Y_4$, -$rP2Y_6$, -$hP2Y_{11}$, and tSA201 cells) and had no significant effect on the responses mediated via recombinant (figure 3.4b, c) and native (figure 3.5b, c) $hP2Y_1$ receptors nor on recombinant $hP2Y_4$ (figure 3.6b,c), $rP2Y_6$ (figure 3.7b,c) or $hP2Y_{11}$ (figure 3.8b,c) receptors. Thus AR-C118925XX appears to be highly selective for $P2Y_2$ receptors over the other P2Y subtypes that couple to Ca^{2+} mobilisation.

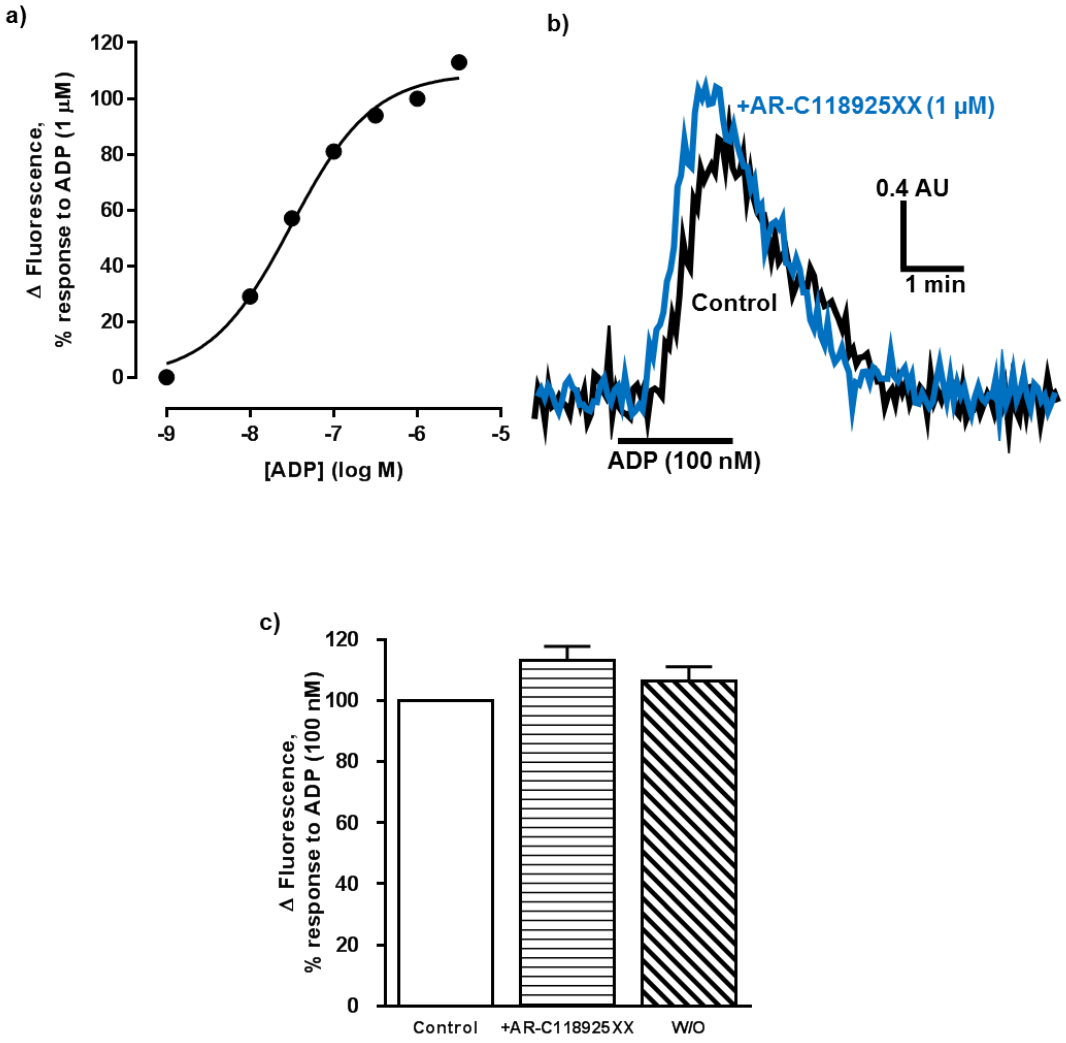


Figure 3.4: AR-C118925XX has no effect on 1321N1-hP2Y$_1$ cells. a) The peak amplitude of changes in Cal-520 fluorescence evoked by ADP (1 nM - 3 µM) is shown (n=1). Values were calculated as the percentage of the response to the reference concentration of ADP (1 µM). The curve represents the fit of the Hill equation to the data. b) The superimposed traces show responses evoked by addition of ADP (100 nM), as indicated by the horizontal bar, in the same population of cells in the absence and then presence of AR-C118925XX (1 µM). c) The mean peak amplitude of responses evoked by ADP (100 nM), first in the absence (control), then in the presence (+AR-C118925XX) and following washout (w/o) of AR-C118925XX (1 µM) are shown. Vertical lines indicate SEM, n=5.

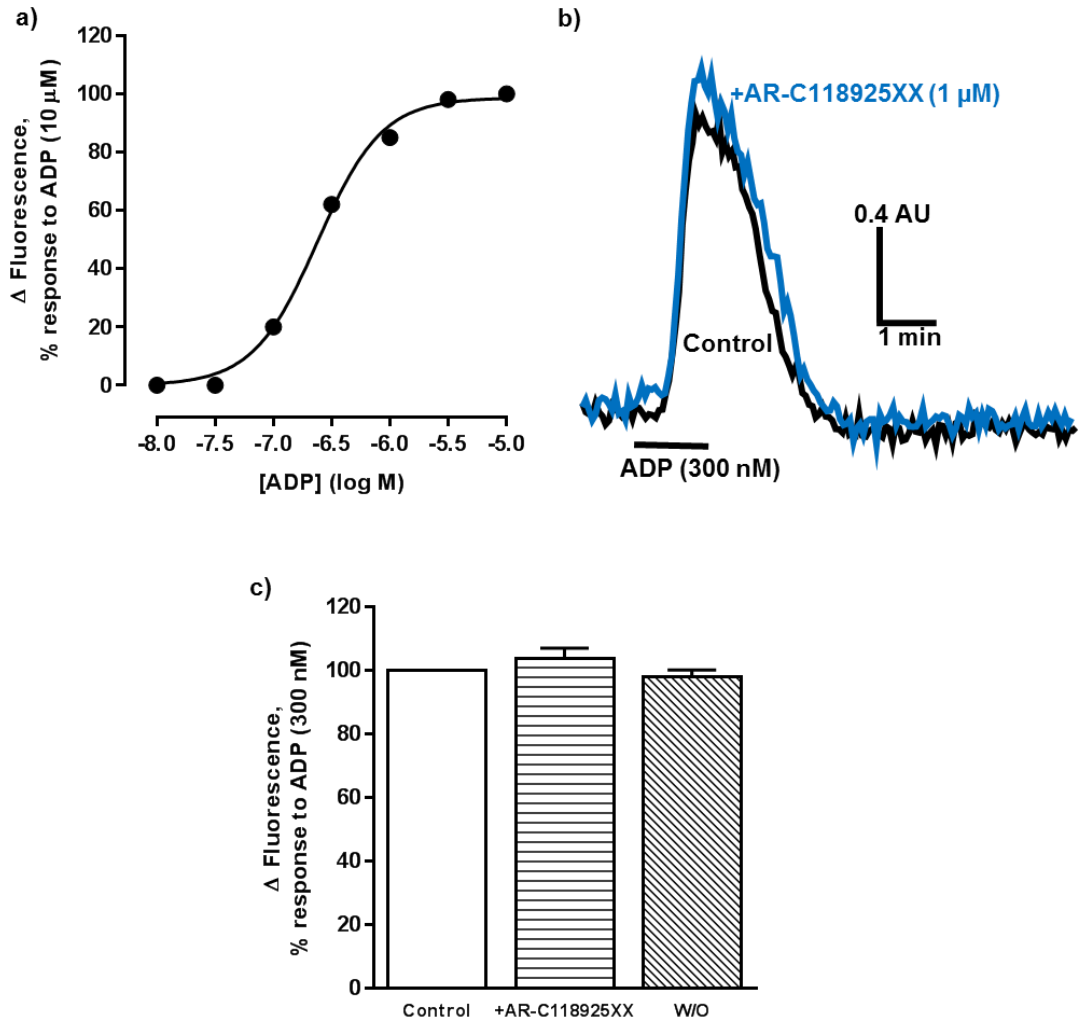


Figure 3.5: AR-C118925XX has no effect at hP2Y$_1$ receptors in tSA201 cells. a) The peak amplitude of changes in Cal-520 fluorescence evoked by ADP (10 nM - 3 µM) is shown (n=1). Values were calculated as the percentage of the response to the reference concentration of ADP, 10 µM. The curve represents the fit of the Hill equation to the data. b) The superimposed traces show responses evoked by addition of ADP (300 nM), as indicated by horizontal bar, in the same population of cells for in the absence and then presence of AR-C118925XX (1 µM). c) The mean peak amplitude of responses evoked by ADP (300 nM), first in the absence (control), then in the presence (+AR-C118925XX) and following washout (w/o) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response. Vertical lines indicate SEM, n=5.

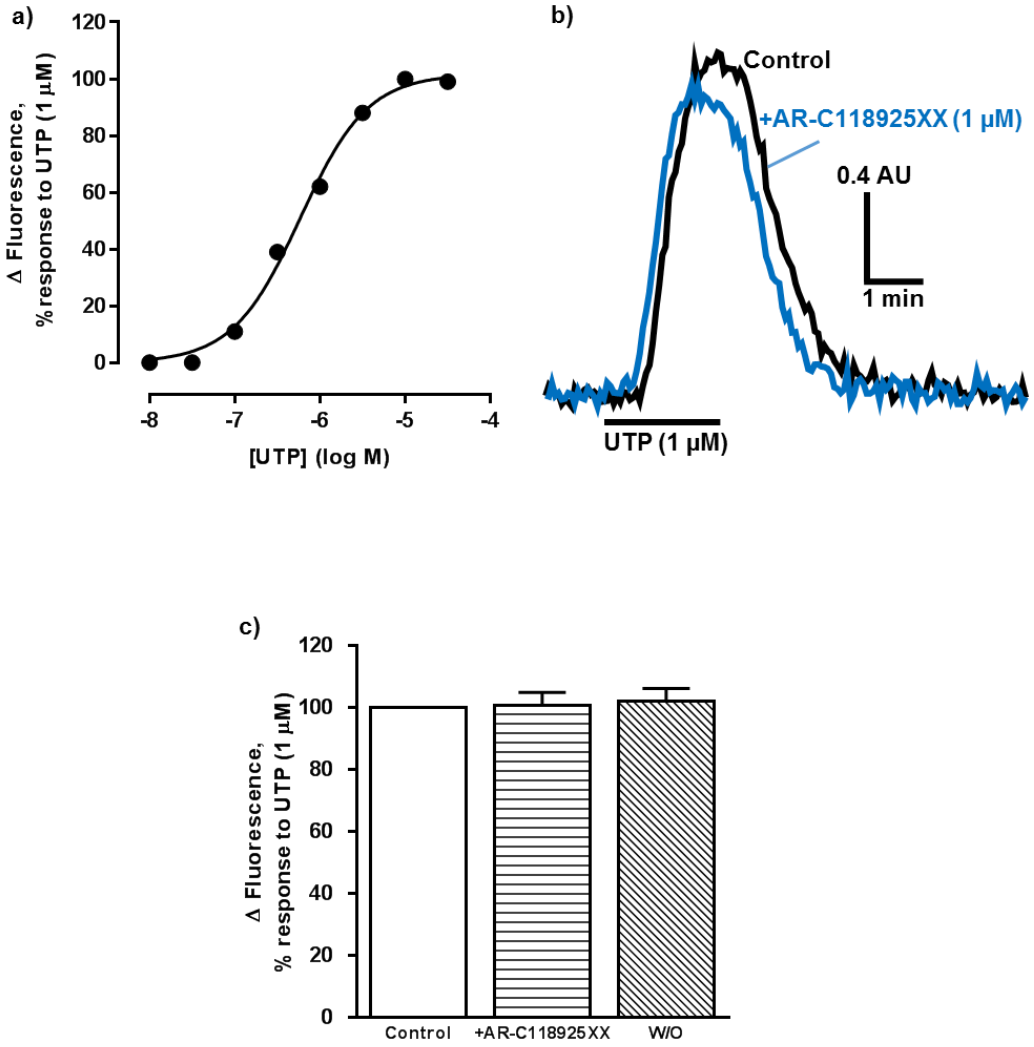


Figure 3.6: AR-C118925XX has no effect on 1321N1-hP2Y$_4$ cells. a) The peak amplitude of changes in Cal-520 fluorescence evoked by UTP (10 nM - 30 µM) is shown (n=1). Values were calculated as the percentage of the response to the reference concentration of UTP, 10 µM. The curve represents the fit of the Hill equation to the data. b) The superimposed traces show responses evoked by addition of UTP (1 µM), as indicated by horizontal bar, in the same population of cells in the absence and then presence of AR-C118925XX (1 µM). c) The mean peak amplitude of responses evoked by UTP (1 µM), first in the absence (control), then in the presence (+AR-C118925XX) and following washout (w/o) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response. Vertical lines indicate SEM, n=5.

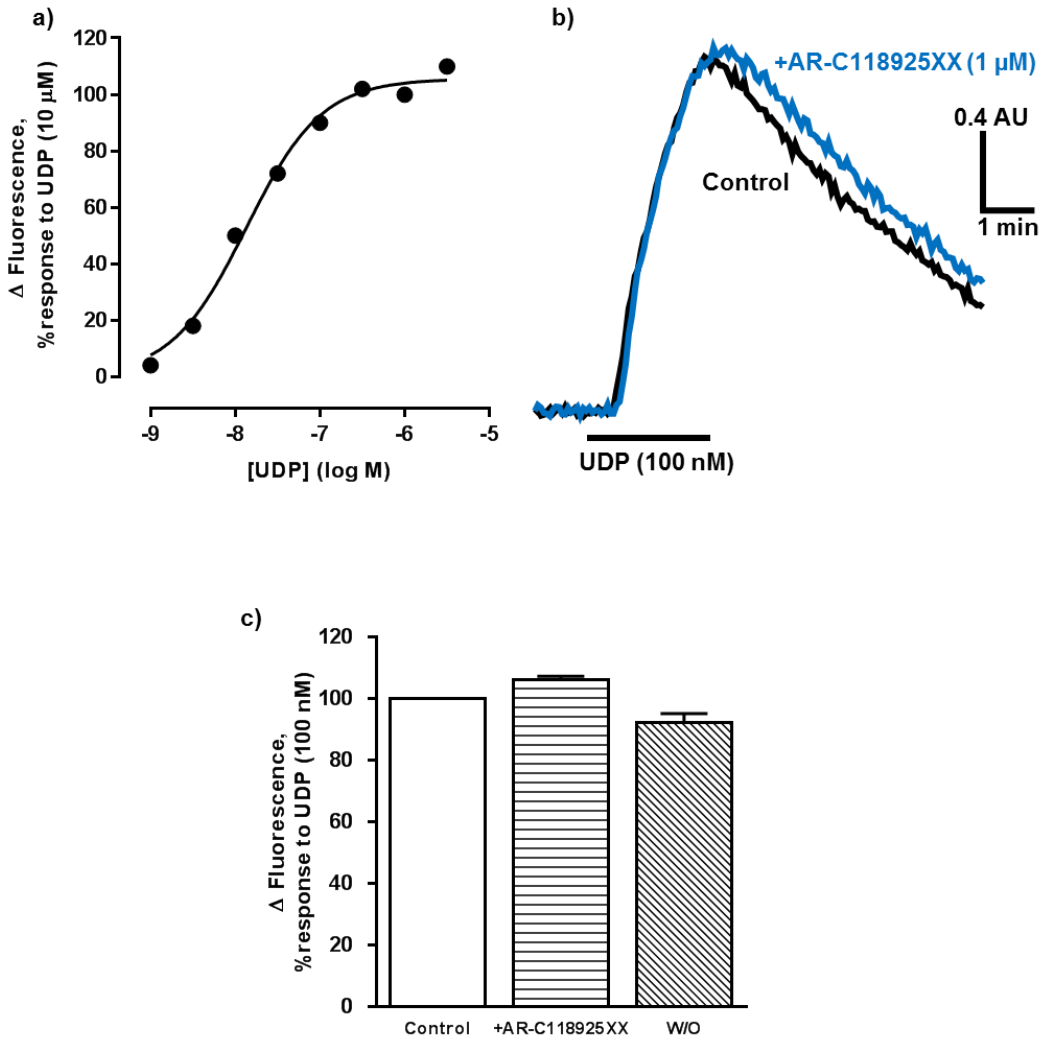


Figure 3.7: AR-C118925XX has no effect on 1321N1-rP2Y$_6$ cells. a) The peak amplitude of changes in Cal-520 fluorescence evoked by UDP (10 nM - 3 µM) is shown (n=1). Values were calculated as the percentage of the response to the reference concentration of UDP, 1 µM. The curve represents the fit of the Hill equation to the data. b) The superimposed traces show responses evoked by addition of UDP (100 nM), as indicated by horizontal bar, in the same population of cells in the absence and then presence of AR-C118925XX (1 µM). c) The mean peak amplitude of responses evoked by UDP (100 nM), first in the absence (control), then in the presence (+AR-C118925XX) and following washout (w/o) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response. Vertical lines indicate SEM, n=5.

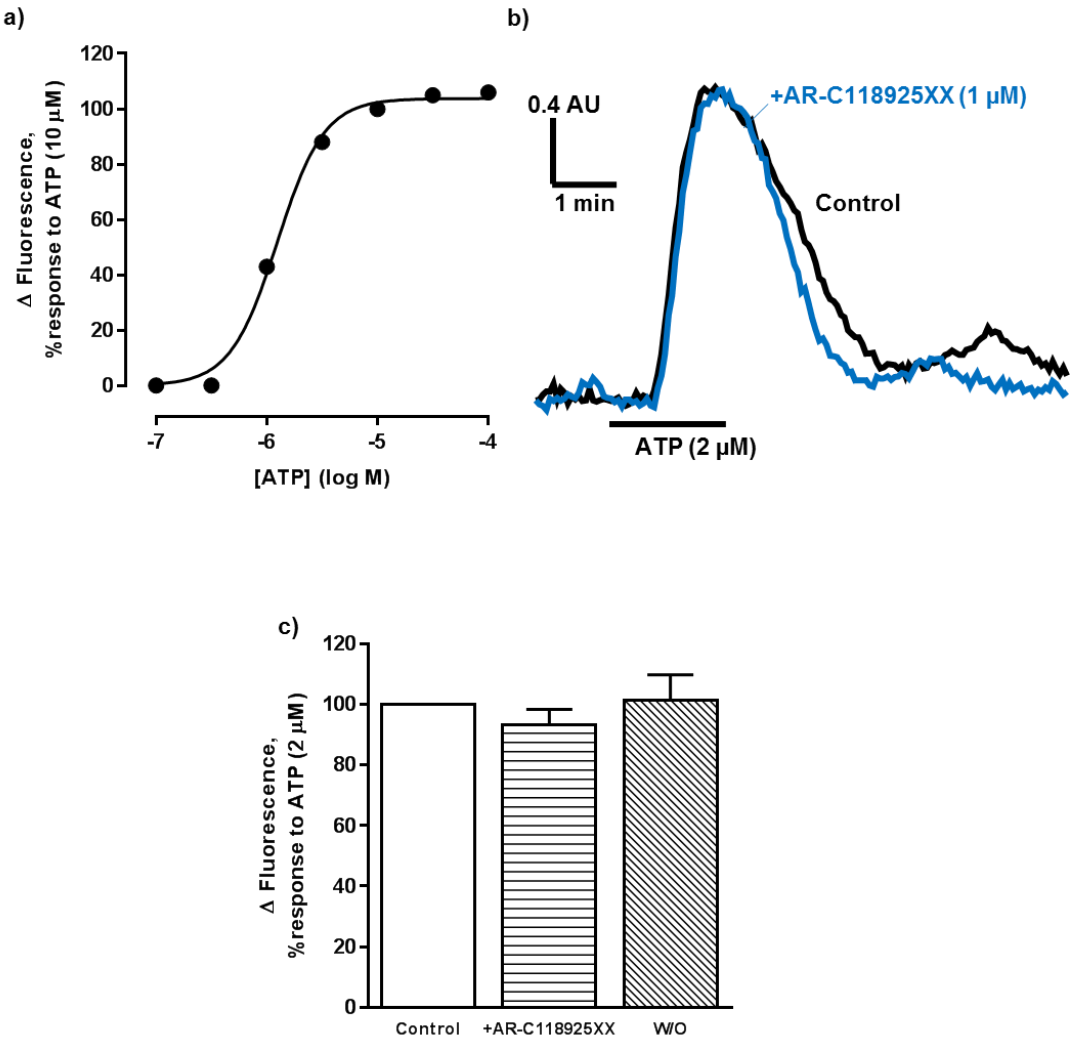


Figure 3.8: AR-C118925XX has no effect on 1321N1-hP2Y$_{11}$ cells. a) The peak amplitude of changes in Cal-520 fluorescence evoked by ATP (100 nM - 100 µM) is shown (n=1). Values were calculated as the percentage of the response to the reference concentration of ATP, 10 µM. The curve represents the fit of the Hill equation to the data. b) The superimposed traces show responses evoked by addition of ATP (2 µM), as indicated by horizontal bar, in the same population of cells, in the absence and then presence of AR-C118925XX (1 µM). c) The mean peak amplitude of responses evoked by ATP (2 µM), first in the absence (control), then in the presence (+AR-C118925XX) and following washout (w/o) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response. Vertical lines indicate SEM, n=5.

3.2.5 The Presence of Functional $P2Y_2$ receptors in EAhy926 Endothelial Cells

Having characterised the pharmacological properties of AR-C118925XX, it was then used to determine if $P2Y_2$ receptors are functionally expressed in the immortalised human vascular endothelial cell line, EAhy926 (Edgell *et al.,* 1983) that was previously shown to be responsive to UTP (Graham *et al.,* 1996; Paul *et al.,* 2000). Figure 3.9a shows that UTP produced a transient increase in intracellular Ca^{2+} in a concentration-dependent manner with an EC_{50} = 670 nM, 95% cl. 535-837 nM. There was no significant change in the EC_{50} when a second UTP CRC was constructed in the same population of cells (EC_{50} = 680 nM, 95% cl. 506-912 nM), but there was a small, significant decrease in the CRC maximum (1st UTP CRC= 108.3±3.1% of the response to UTP (10 µM) in the first CRC, 2nd UTP CRC= 95.5±3.7% ($P<0.05$); figure 3.9b). Thus UTP evokes Ca^{2+} mobilisation in EAhy926 cells and these responses are reproducible.

To determine if UTP acted via $P2Y_2$ receptors, a UTP CRC was constructed first in the absence and then in the presence of AR-C118925XX (30 nM). This concentration is almost 10-fold higher than its K_B at $P2Y_2$ receptors and substantially lower than the concentration that was shown above to be inactive at other P2Y-subtypes. When applied alone, AR-C118925XX did not affect the intracellular Ca^{2+}, but it produced a parallel rightward shift of the UTP CRC (EC_{50} = 7.6 µM, 95% cl. = 4.4–13.2 µM), with no change in the maximum response (92.4±8.2%) compared to the time-matched control (figure 3.10). Using the Gaddum-Schild equation gave the K_B for AR-C118925XX as 3.0 nM.

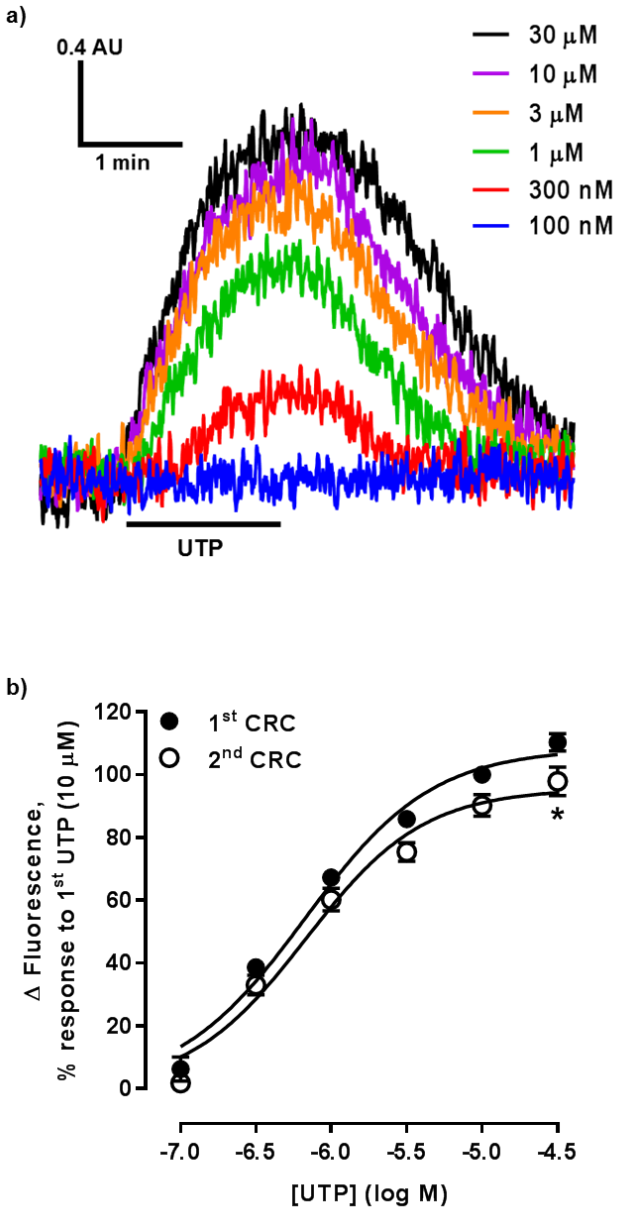


Figure 3.9: UTP mobilises Ca^{2+} in human EAhy926 endothelial cells. a) The superimposed traces show changes in Cal-520 fluorescence evoked by superfusion of cells with UTP (100 nM - 30 µM), as indicated by horizontal bar. b) The mean peak amplitude of two consecutive UTP CRC in the same population of cells is shown. The data are expressed as a percentage of the response to UTP (10 µM) in the first CRC. n=5. * $P < 0.05$ for maximum responses produced by second UTP CRC. Vertical lines indicate SEM. For some points, the error bars are shorter than the height of the symbol. The curves represent the fit of the Hill equation to the data.

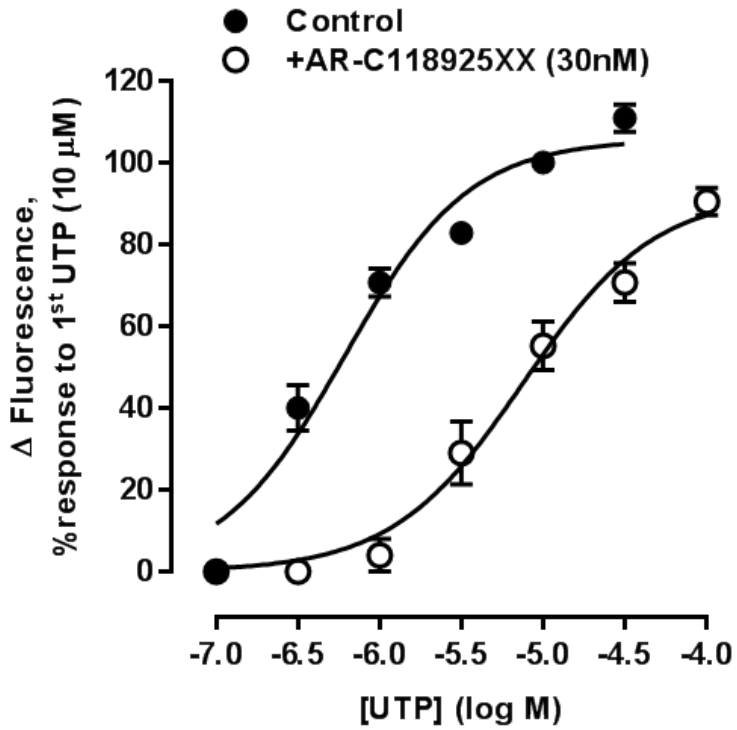


Figure 3.10: UTP acts at $P2Y_2$ receptors in human EAhy926 endothelial cells. The mean peak amplitude of changes in Cal-520 fluorescence evoked by superfusion of cells with UTP (100 nM - 30 µM) in the absence and then in the presence of AR-C118925XX (30 nM) in the same population of cells is shown. The data are expressed as a percentage of the response to UTP (10 µM) in the first CRC. Vertical lines indicate SEM, n=5. For some points, the error bars are shorter than the height of the symbol. The curves represent the fit of the Hill equation to the data.

3.3 DISCUSSION

3.3.1 Effects of Agonists on Intracellular Ca^{2+}

$P2Y_2$ receptor are G protein-coupled receptors**,** which when activated mediate the release of Ca^{2+} from the sarcoplasmic reticulum via IP_3 receptors. In this chapter I demonstrated that $hP2Y_2$ receptors are functionally expressed following stable transfection in 1321N1 cells, as UTP and ATP produced an increase in intracellular Ca^{2+}, with EC_{50} values of 54 nM and 142 nM respectively. This is consistent with a previous study from the Kennedy lab using 1321N1-$P2Y_2$ cells in which UTP and ATP evoked a rise in intracellular Ca^{2+} with similar EC_{50} values (73 nM and 176 nM respectively) (Morrow *et al*., 2014). The present data also showed that the responses elicited by UTP were reproducible on repeated application. Although there was a small decrease in the maximum response evoked, there was no difference in the EC_{50} values of two consecutive UTP CRC. $P2Y_2$ receptors are known to undergo desensitisation in a time- and concentration-dependent manner, but this requires longer exposure times to UTP than was used in my experiments (Garrad *et al.,* 1998; Flores *et al.,* 2005). Thus constantly superfusing 1321N1-$P2Y_2$ cells and applying UTP for short periods enabled me to quantify the actions of ARC118925XX at the $hP2Y_2$ receptor.

3.3.2 Mode of action of AR-C118925XX

The above data showed that increasing concentrations of AR-C118925XX had no effect on intracellular Ca^{2+} on its own but caused a progressive rightward shift in the UTP CRC. This confirmed that AR-C118925XX is an antagonist at the $P2Y_2$ receptor. A Schild plot was then constructed to analyse how AR-C11895XX acted and this showed that it is a competitive antagonist rather than a negative allosteric modulator, as the slope of the plot was very close to 1. Furthermore, it had a pA_2 of 8.43, equivalent to a K_B of 3.7 nM. An antagonist is competitive if Schild analysis produces a slope that is not significantly different from 1 (Wyllie and Chen, 2009). It is important to note that the antagonist properties of AR-C118925XX have only been determined using Ca^{2+} changes as a bioassay. The mode of antagonism of negative allosteric modulators varies depending on the agonist used, extent of signalling amplification

and the signalling pathway studied. An example is the $P2Y_1$ negative allosteric modulator, BPTU, which has two different effects on CRC generated by the agonist 2-MeSADP (Gao and Jacobson, 2017). A progressive rightward shift of the CRC was observed, with no decrease in maximum, when measuring IP_3 production or ERK1/2 stimulation. However, BPTU suppressed the maximum of β-arrestin2-mediated, $P2Y_1$ receptor internalisation CRC. This biased functional antagonism suggests that each signalling event may be mediated via a specific receptor conformation.

In my experiments, AR-C118925XX had no effect on resting Ca^{2+}, indicating that superfusion did not induce nucleotide release from the 1321N1-$P2Y_2$ cells, or that if it did, then the flow rate was fast enough to wash the nucleotides away from the cell surface, thereby preventing $P2Y_2$ receptor activation. This is an important observation, because superfusion-induced shear stress may cause UTP and ATP release from endothelial cells, which then act in an autocrine or paracrine manner to stimulate P2Y receptors in the same or neighbouring cells (Wang *et al.,* 2015; Burnstock, 2017). My data also showed that the inhibitory action of the antagonist was reversible following washout, but, a more prolonged perfusion of the cells with AR-C118925XX-free buffer was needed as the antagonist concentration increased.

After these experiments had been completed and while writing them up for publication, several other reports were published on the mode of action of AR-C118925XX. Rafehi *et al.,* (2017a) first published details of the pharmacological action of AR-C118925XX in 1321N1-h$P2Y_2$ cells, reporting a pA_2 of 7.43, K_B of 37.2 nM and a Schild plot slope of 0.816. This potency is approximately 10-fold lower than I found and the Schild plot slope is substantially less than 1. In a subsequent study, Rafehi *et al.,* (2017b) calculated the IC_{50} of AR-C118925XX as 62.9 nM, based on inhibition of responses evoked by the EC_{80} of UTP which is four times the EC_{50} (5.61 nM). When the K_i of AR-C118925XX was calculated using the Cheng-Prusoff equation ($K_i = IC_{50}/1+([UTP]/EC_{50})$, a value of 13 nM was generated; which is closer to the K_B calculated in my experiments. Kindon *et al.*, (2017) also described the original design and synthesis of AR-C118925XX and reported pA_2 and K_B values of 7.8 and 15.8 nM at h$P2Y_2$ receptors expressed in Jurkat cells. These values are closer to the ones that I found. The mode of antagonism could not be confirmed, however, because neither the Schild plot nor the slope of the plot was included.

The difference in values in this study and the ones generated by Rafehi *et al*., (2017 a, b) and Kindon *et al*., (2017) might be due to differences in the methods used to record cytoplasmic Ca^{2+} levels. Multi-well plates and a microplate reader were used in the other studies to generate a CRC by stimulating multiple population of cells with a single concentration of UTP. In contrast, I superfused a single population of cells to generate a control CRC by repeated addition of increasing concentration of UTP. The process was then repeated in the presence of AR-C118925XX. This system is equivalent to myography studies, where data can be generated on a single tissue. This system produces less variable data than microplate readers, which may be the reason why the Schild analysis produced a slope close to 1. Disadvantages of using this system are that more drug is used and it takes longer to generate data.

3.3.3 Selectivity of AR-C118925XX

In this study, ADP, ATP, UTP and UDP stimulated individual recombinant P2Y subtypes expressed in 1321N1 cells and ADP stimulated native $P2Y_1$ receptors in tSA201 cells to produce a concentration-dependent increase in intracellular Ca^{2+}. This is consistent with previous reports of the pharmacological profile of these agonists (Léon *et al.,* 1997; Harper *et al.,* 1998; Zhang *et al.,* 2011; Morrow *et al.,* 2014; Gafar *et al.,* 2016) and enabled me to study the selectivity of AR-C118925XX. At a concentration that is 270 times greater than the K_B at $P2Y_2$ receptors, 1 µM, AR-C118925XX had no effect on the rise in intracellular Ca^{2+} produced via these other P2Y subtypes, showing that it is highly selective for the $P2Y_2$ receptors. Consistent with this conclusion, Kemp *et al*., (2004) reported that 10 µM AR-C118925XX had no effect at 37 other GPCR and ion channels. The only clear indication of an off-target action of sub-µM concentrations is at P2X3 receptors, with an IC_{50} of 819 nM (Rafehi *et al.,* 2017a). The K_B was not calculated, however.

This high selectivity of AR-C118925XX means that it can be used as a tool to investigate native $P2Y_2$ receptors, their function in diseases, as well as identifying potential therapeutic targets. The endogenous nucleotides have complex pharmacological profiles, as they can all stimulate at least two of the eight P2Y subtypes. UTP is an agonist at $P2Y_2$ receptors, as shown here, and also an agonist at $P2Y_4$ receptors and possibly $P2Y_6$ receptors (Abbracchio *et al*., 2006; Guns *et al*.,

2006; Kennedy *et al*., 2013; Rafehi and Müller, 2018), so sensitivity of a cell or tissue to UTP is not proof of $P2Y_2$ receptor expression. No $P2Y_4$ receptor antagonists are currently commercially available and although an antagonist for $P2Y_6$ receptors, MRS2578, is available, its action is irreversible and insurmountable (Mamedova *et al*., 2004) and effects at sites other than $P2Y_6$ receptors have been noted (Mitchell *et al.,* 2012). Thus it has been difficult to determine which native receptors mediate the effects of UTP. Hence, AR-C118925XX being selective for the $P2Y_2$ receptors is a great advantage to the purinergic field of research.

3.3.4 Native $P2Y_2$ receptors in Human Vascular Endothelial cells.

In this study, UTP evoked a rise in intracellular Ca^{2+} in human EAhy926 vascular endothelial cells. This response was inhibited by AR-C118925XX in a surmountable manner, by a concentration (30 nM) that was 33.3 fold lower than the concentration reported above to have no effect at other P2Y subtypes. The K_B calculated (3.0 nM) was very close to that which I found at recombinant $hP2Y_2$ receptors (3.7 nM), suggesting that UTP acts via $P2Y_2$ receptors in EAhy926 cells to raise intracellular Ca^{2+} levels. This is consistent with the detection of $P2Y_2$ mRNA and protein (Raqeeb *et al.,* 2011) and the demonstration of $P2Y_2$-like immunoreactivity (Muoboghare *et al.,* 2019) in these cells. Another recent study involving the Kennedy lab used the same dual approach of AR-C118925XX and immunoreactivity to show that $P2Y_2$ receptors are present in rat carotid artery endothelial cells and couple to Ca^{2+} mobilisation (Lee *et al.,* 2018). Thus these data indicate that $P2Y_2$ receptors may be a major site of action of UTP in vascular endothelial cells in general. This will be investigated in rIPA in the next chapter.

In conclusion, in this chapter I showed that AR-C118925XX is a very potent, selective and reversible $P2Y_2$ receptor antagonist. Furthermore, it inhibited responses evoked by UTP in human vascular endothelial cells, indicating expression of endogenous $P2Y_2$ receptors. Thus AR-C118925XX is a powerful tool for identifying the functions of native $P2Y_2$ receptors and I, therefore, proceeded to investigate $P2Y_2$ receptor function in the rIPA, using UTP, UDP, ATP and ADP as agonists and AR-C118925XX as a $P2Y_2$ receptor antagonist.

4 Characterisation of P2Y Receptors in Rat Intrapulmonary Artery.

4.1 INTRODUCTION

It has been known for some time that P2 receptor agonists modify the tone of pulmonary blood vessels of a variety of species, including humans, but the role of individual P2X and P2Y receptor subtypes in mediating these effects is largely unclear. The P2X1 receptor appears to be the main P2X subtype expressed in the smooth muscle of the pulmonary vasculature (Syed *et al.,* 2010), but which P2Y receptor subtypes are present in pulmonary arterial smooth muscle and endothelial cells is unknown. In rats, UTP, UDP, ATP and ADP elicited vasoconstriction in isolated, perfused pulmonary vascular bed (Rubino and Burnstock, 1996) and isolated pulmonary arteries (Rubino *et al.,* 1999; Chootip *et al*., 2002, 2005), though ADP was much less potent. Subsequently, on the basis of experiments using the non-selective antagonists, suramin and PPADS, it was hypothesised that UTP and UDP act via the $P2Y_6$ and either the $P2Y_2$ or $P2Y_4$ receptors, while ATP acts via $P2Y_2$ receptors, as well as $P2X_1$ receptors, to elicit vasoconstriction (Chootip *et al*., 2002, 2005). ATP also acts via endothelial P2Y receptors to elicit pulmonary vasodilation in rats (Hasséssian *et al*., 1995; Rubino *et al*., 1999), but again the role of individual subtype is unclear.

It is important to know which P2X and P2Y receptor subtypes mediate pulmonary vasoconstriction and vasodilation because ATP, ADP, UTP and UDP, as endogenous nucleotides, may contribute to the regulation of pulmonary vascular tone under physiological and pathophysiological conditions. For example, ATP present in and released from red blood cells, induces an NO-dependent decrease in pulmonary vascular resistance via endothelial P2Y receptors (Sprague *et al.,* 1996, 2003). Also, extracellular ATP is elevated in COPD (Lommatzsch *et al.,* 2010), though ADP-induced pulmonary vasodilation is reduced in COPD patients (Dinh-Xuan *et al.,* 1991). In addition. P2X and P2Y receptors may play a role in HPV, as suramin inhibited the vasoconstriction in rabbit perfused lung (Baek *et al.,* 2008).

Our poor understanding of the receptors through which nucleotides evoke vasoconstriction and vasodilation of the rIPA is due to the absence of selective antagonists for most subtypes of P2X and P2Y receptor. As reported in the previous chapter, AR-C118925XX is a potent and selective, competitive antagonist at $P2Y_2$ receptors, therefore, the aim of this chapter was to use AR-C118925XX to investigate

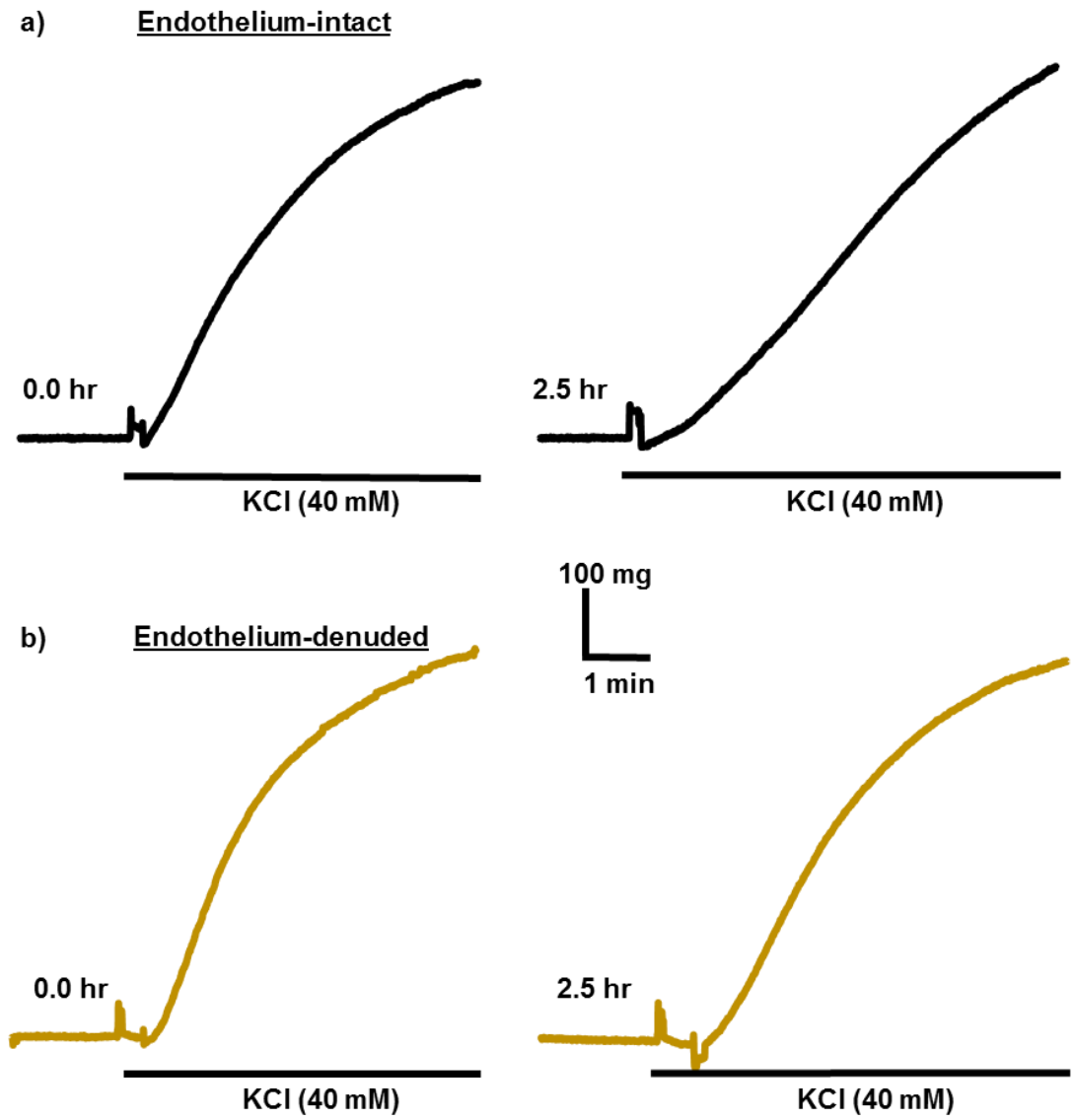
the role of $P2Y_2$ receptors in nucleotide-evoked vasoconstriction and vasodilation of the rIPA.

4.2 RESULTS

4.2.1 Reproducibility of Nucleotide-Induced Contractions

As discussed above, UTP, ATP, UDP and ADP evoke vasoconstriction of rIPA via smooth muscle P2X and/or P2Y receptors and ATP elicits vasodilation via endothelial receptors. The focus of the first part of this chapter was on the role of $P2Y_2$ receptors in vasoconstriction and the aims of the initial experiments were to determine if these contractions are reproducible and if they are affected by the physical removal of the endothelial cell layer. Previous studies in the Kennedy lab found that contractions evoked by ADP were small (Chootip *et al.,* 2002) and abolished by the $P2Y_{12/13}$ receptor antagonist, AR-C69931MX (Mitchell *et al.,* 2012), so these were not studied.

KCl, which evokes vasoconstriction independently of receptors and the endothelium, was used as a control. When added to endothelium-intact rIPA for 5 min, six times at 30 min intervals, KCl (40 mM) evoked contractions that initially increased in amplitude slightly and significantly, at 1.0 hr ($P < 0.001$) and 1.5 hr ($P < 0.05$) compared to the first response, but there was no significant difference thereafter (figures 4.1a, c). When this protocol was repeated in tissues from which the endothelium had been removed mechanically, there was no change in the amplitude of the first contraction evoked by KCl (40 mM) (399 ± 63 mg, n=6) compared to that evoked in endothelial-intact tissues (407 ± 30 mg, n=10; figures 4.1b, d). There was also no significant change in the amplitude of subsequent contractions relative to the first response. Comparing the contractions produced by KCl at each time point in endothelium-intact and endothelium-denuded rIPA showed no significant difference between each of the first to fourth additions, but responses in the absence of endothelium were significantly smaller than those in the presence at 2.0 hr ($P < 0.05$) and 2.5 hr ($P < 0.01$) (figure 4.1e).


a)
Endothelium-intact
0.0 hr
KCl (40 mM)
2.5 hr
KCl (40 mM)
100 mg
1 min
b)
Endothelium-denuded
0.0 hr
KCl (40 mM)
2.5 hr
KCl (40 mM)

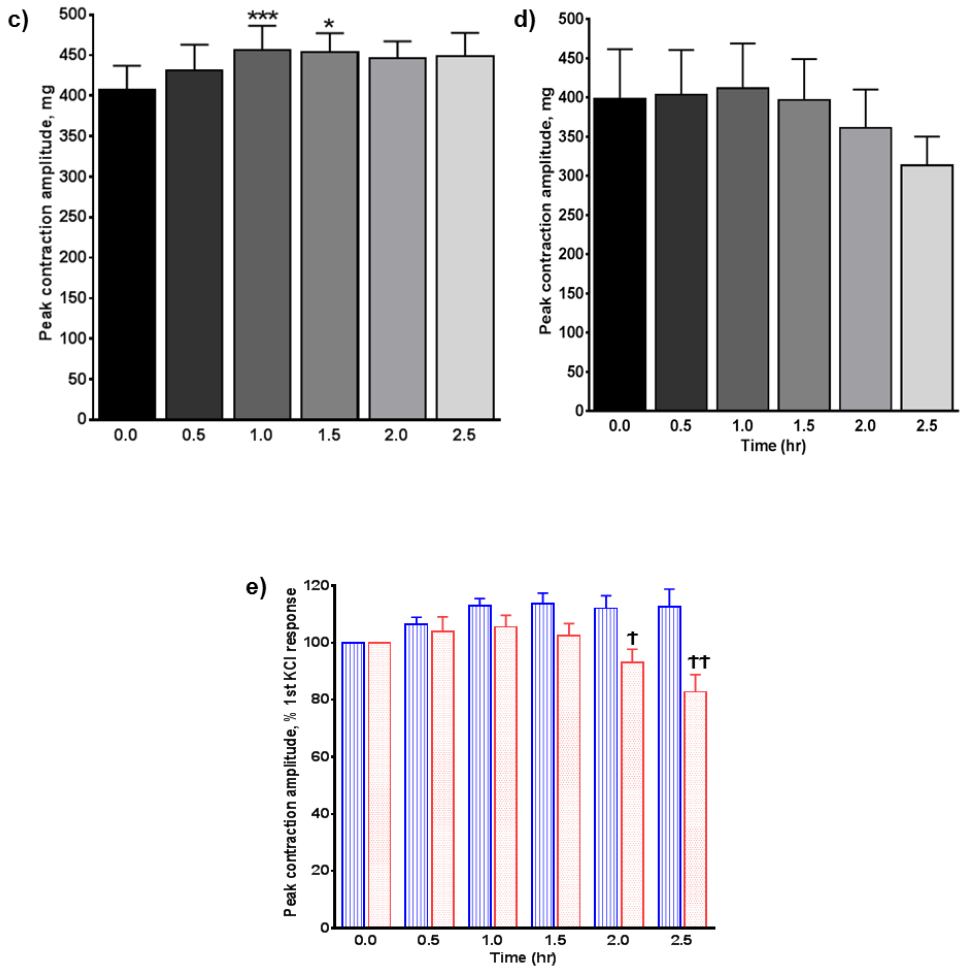
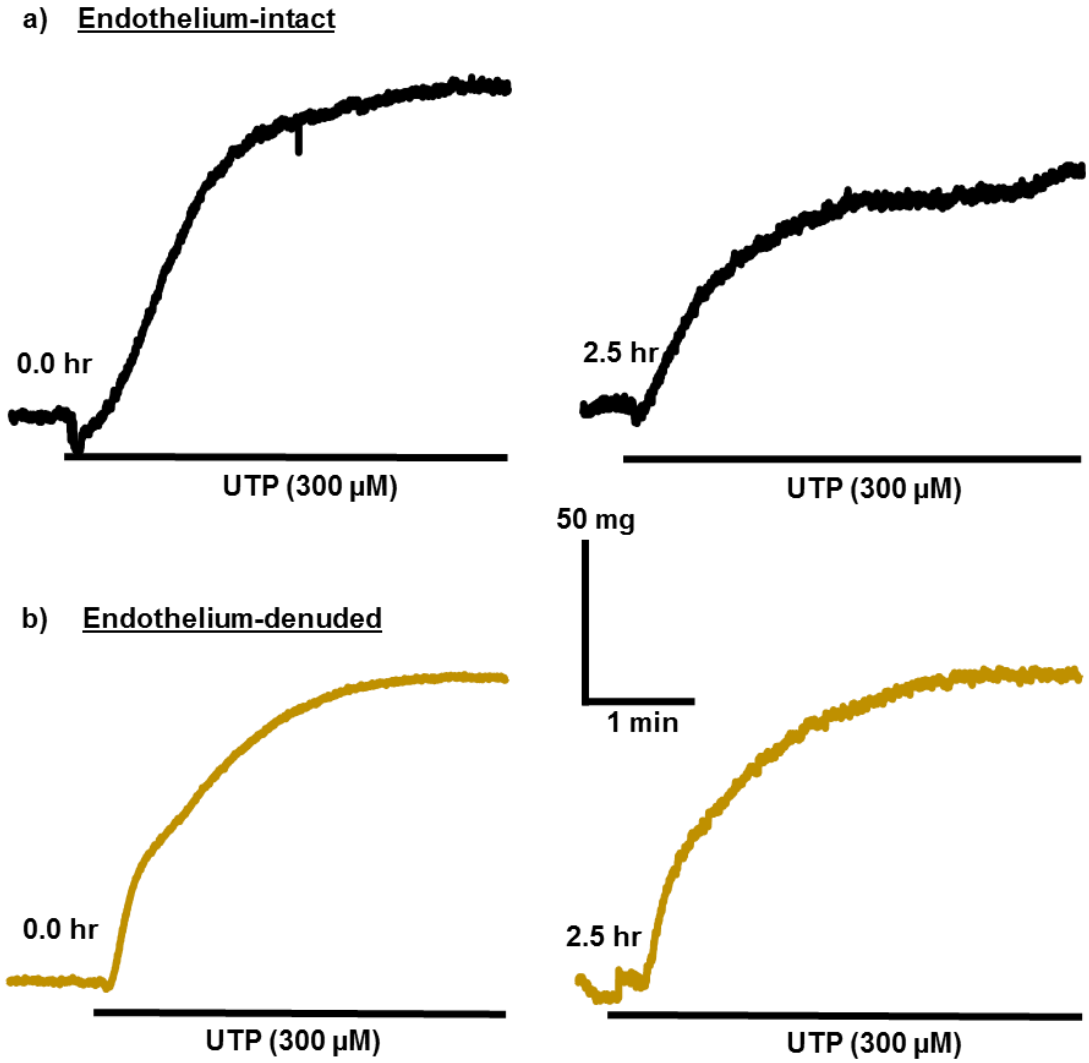


Figure 4.1: KCl-induced vasoconstriction in endothelium-intact and -denuded rIPA. The traces show typical contractions of a) endothelium-intact (black) and b) -denuded (gold) rIPA evoked by addition of KCl (40 mM), as indicated by the horizontal bars, at 0.0 hr and 2.5 hr. The mean peak amplitude of contractions induced by KCl (40 mM) in c) endothelium-intact and d) endothelium-denuded rIPA are shown and e) shows a comparison between endothelium-intact (blue) and endothelium-denuded (red) rIPA when the responses are expressed as a percentage of the first KCl response. Vertical lines indicate SEM. (n = 10 (endothelium-intact) and n=6 (endothelium-denuded)). * $P < 0.05$ for responses produced at 1.5 hr, and *** $P < 0.001$ for responses produced at 1 hr in endothelium-intact rIPA compared to those evoked at 0.0 hr. † $P < 0.05$ for responses produced at 2.0 hr, and †† $P < 0.01$ for responses produced at 2.5 hr in endothelium-denuded compared to endothelium-intact tissues.

Previous studies in the Kennedy lab found that UTP, ATP and UDP evoked concentration-dependent contractions with moderate potency (EC_{50} = 42, 47, 204 µM respectively) and that the CRC did not reach a maximum (Chootip *et al.,* 2002; 2005); therefore, the nucleotides were applied here at a single concentration that was greater than the EC_{50}. When UTP (300 µM) was added to the endothelium-intact rIPA for 5 min, six times at 30 min intervals, it produced vasoconstrictions that tended to progressively decrease in amplitude, but the mean contraction amplitudes at each time point were not significantly different from the first response (figures 4.2a,c). Using the same protocol in endothelium-denuded rIPA, there was no significant difference in amplitude of the first contraction evoked by UTP (277 ± 46 mg, n=6) compared to that evoked in endothelial-intact tissues (171 ± 39 mg, n=6) and in relation to the first response, there was no significant change in the amplitude of the second response, but a significant decrease in responses at 1.0 hr to 2.5 hr was seen ($P < 0.05$; figures 4.2b,d). Comparing the contractions produced by UTP at each time point in the endothelium-intact and endothelium-denuded rIPA showed there was no significant difference in the additions at 2.5 hr, but responses in the absence of endothelium were significantly smaller than those in the endothelium-intact rIPA at 0.5 hr, 1.5 hr, 2.0 hr ($P < 0.05$) and at 1.0 hr ($P < 0.01$; figure 4.2e).


a) Endothelium-intact
0.0 hr
UTP (300 µM)
2.5 hr
UTP (300 µM)
50 mg
1 min
b) Endothelium-denuded
0.0 hr
UTP (300 µM)
2.5 hr
UTP (300 µM)

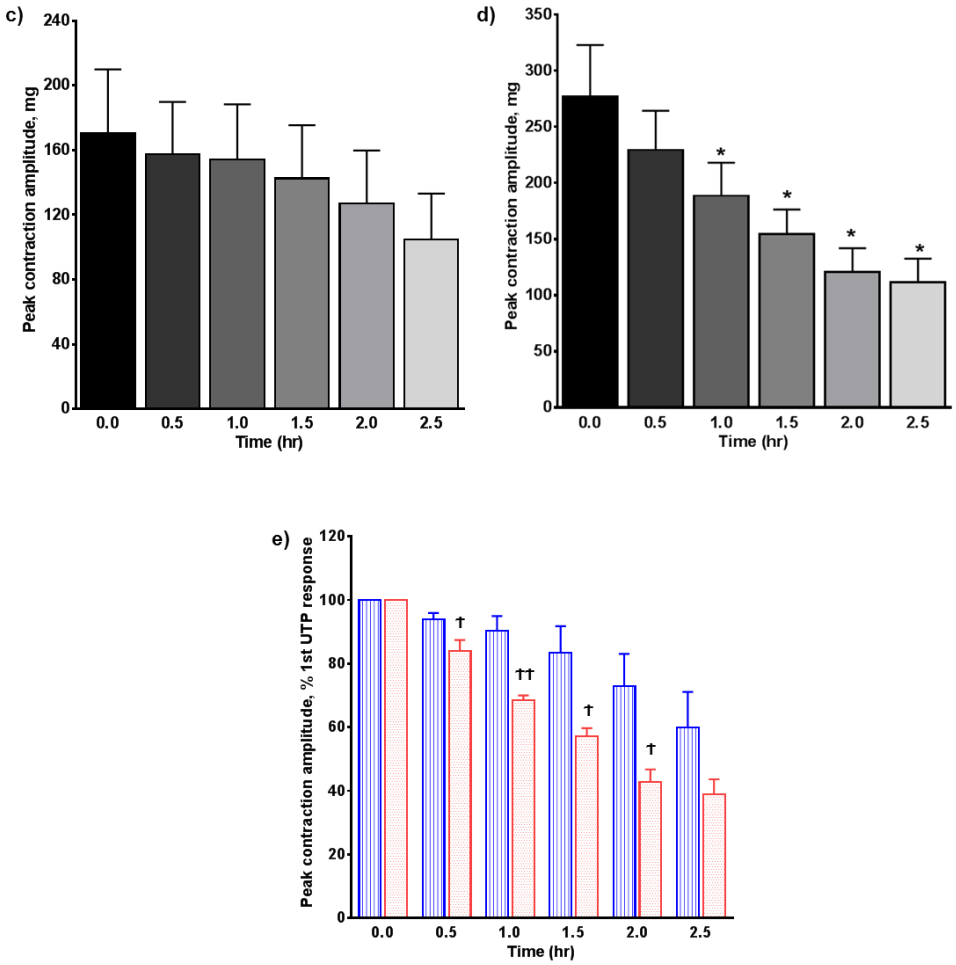
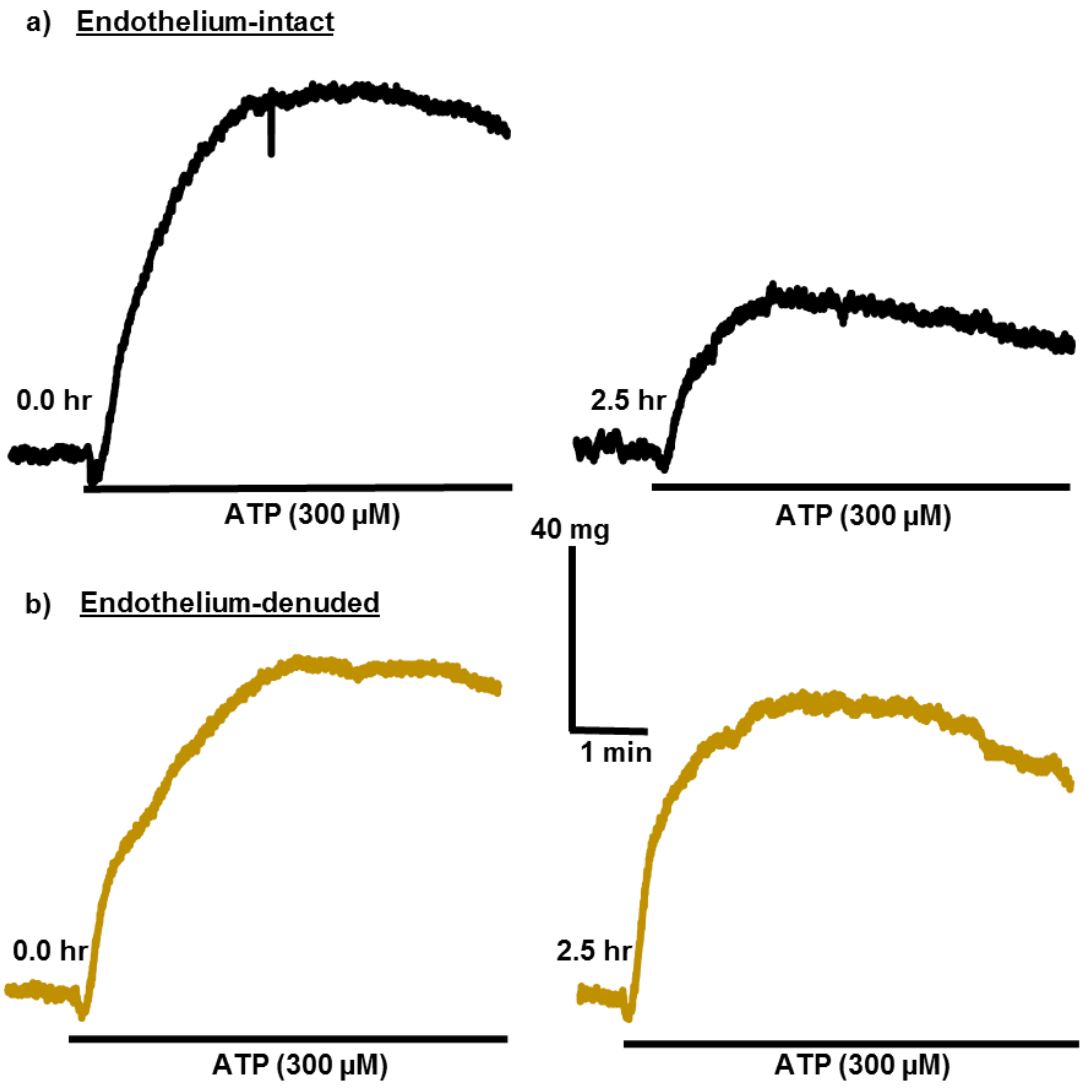


Figure 4.2: UTP-induced vasoconstriction in endothelium-intact and -denuded rIPA. The traces show typical contractions of a) endothelium-intact (black) and b) -denuded (gold) rIPA evoked by addition of UTP (300 µM), as indicated by the horizontal bars, at a) 0.0 hr and b) 2.5 hr. The mean peak amplitude of contractions induced by UTP (300 µM) in c) endothelium-intact and d) endothelium-denuded rIPA are shown and e) shows a comparison between endothelium-intact (blue) and endothelium-denuded (red) rIPA when the responses are expressed as a percentage of the first UTP response. Vertical lines indicate SEM. (n = 6 (endothelium-intact) and n=6 (endothelium-denuded)). * $P < 0.05$ for responses produced at 0.5 hr to 2.5 hr in endothelium-denuded rIPA compared to those evoked at 0.0 hr. † $P < 0.05$ for responses produced at 0.5 hr, 1.5 hr and 2.0 hr, and †† $P < 0.01$ for responses produced at 1.0 hr in endothelium-denuded compared to endothelium-intact tissues.

ATP (300 µM) produced vasoconstrictions that tended to progressively decrease in amplitude, with no significant difference from the first response, when added to endothelium-intact rIPA for 5 min, six times at 30 min intervals (figures 4.3a,c). When this protocol was repeated in tissues from which the endothelium had been removed mechanically, there was no difference in the amplitude of the first contraction evoked by ATP (300 µM; 139 ± 13 mg, n=6) compared to that evoked in endothelial-intact tissues (177 ± 38 mg, n=6) and there was also no significant change in the amplitude of contraction in the second addition relative to the first response (figures 4.3b, d). A significant decrease in amplitude of contractions was observed, however, in responses at 1.0 ($P < 0.05$) and at 1.5 hr to 2.5 hr ($P < 0.01$). Comparing the contractions produced by ATP at each time point in endothelium-intact and endothelium-denuded rIPA showed no significant difference between each of the first and second additions, but responses in the absence of endothelium were significantly smaller than those in the presence at 1.0 hr and 2.5 hr ($P < 0.05$), 1.5 hr and 2.0 hr ($P < 0.01$) (figure 4.3e).


a) Endothelium-intact
0.0 hr
ATP (300 μM)
2.5 hr
ATP (300 μM)
40 mg
1 min
b) Endothelium-denuded
0.0 hr
ATP (300 μM)
2.5 hr
ATP (300 μM)

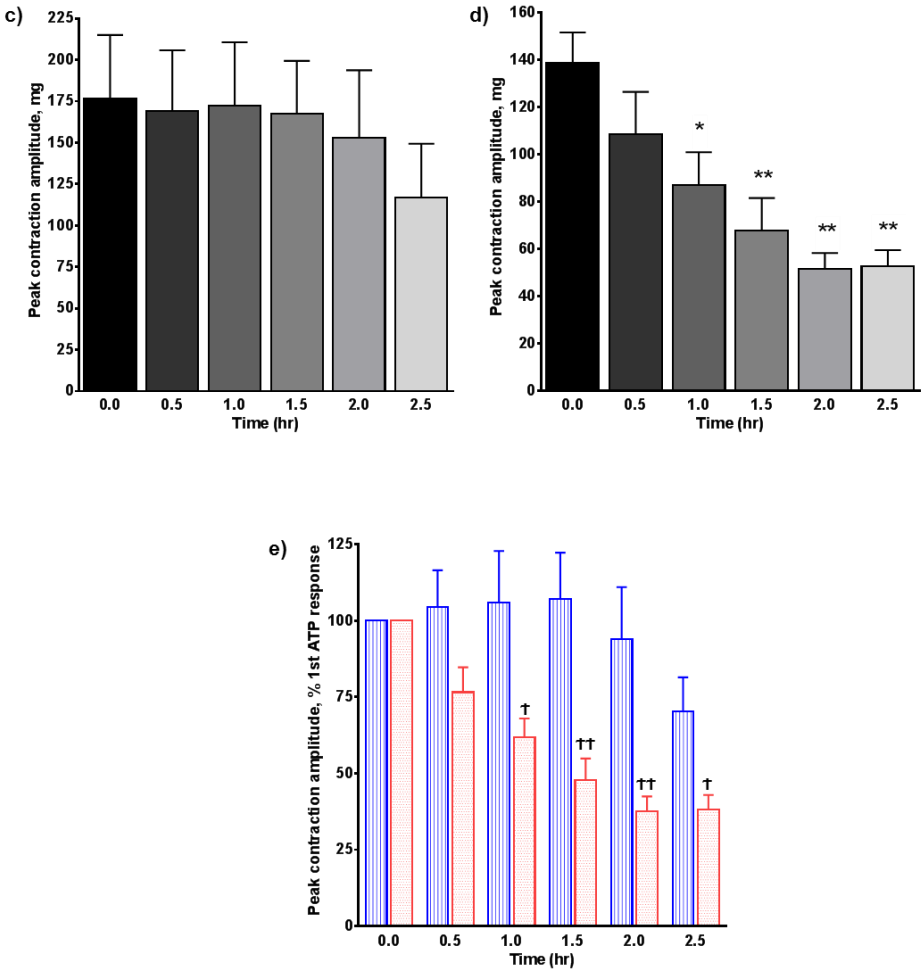
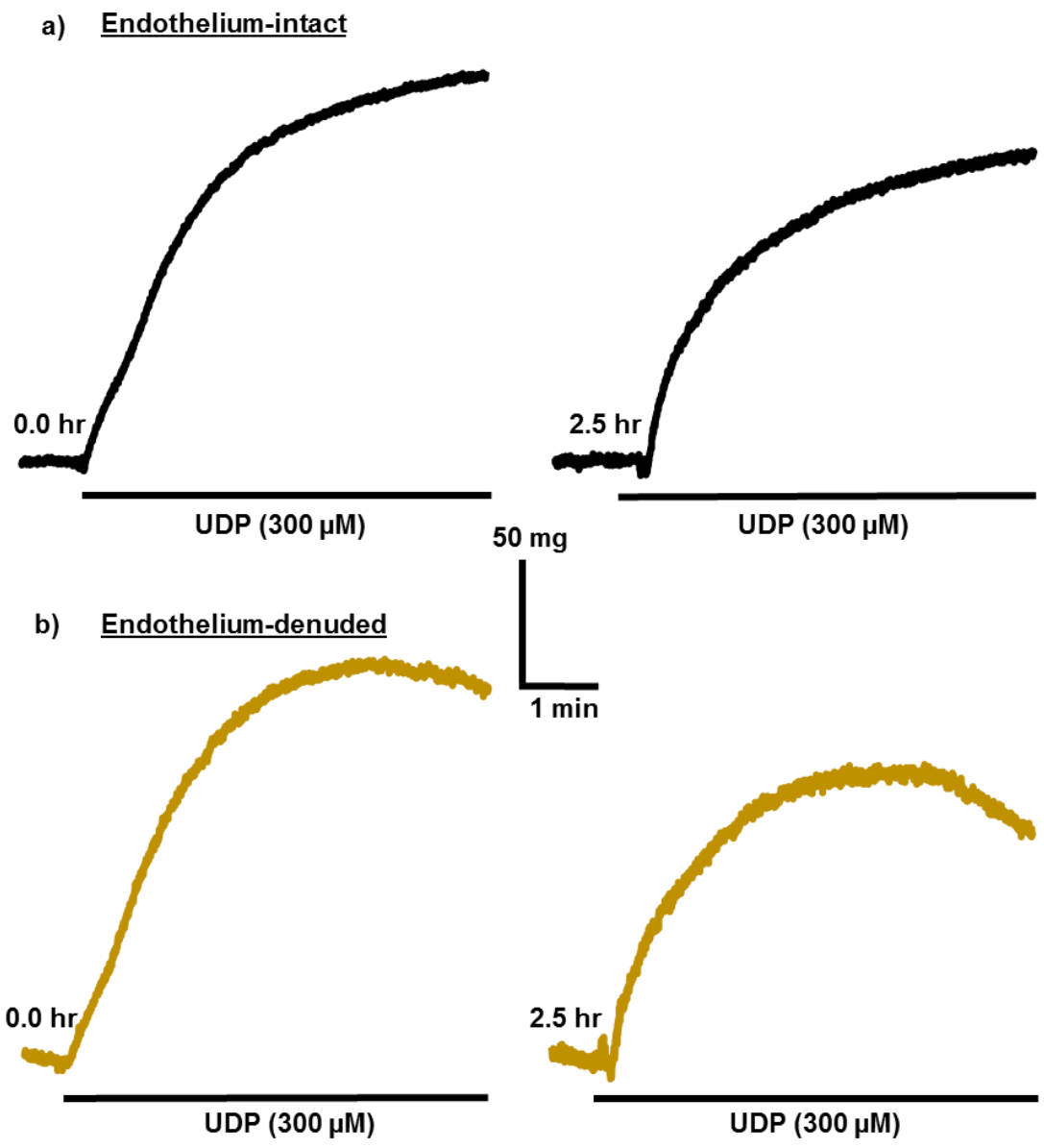


Figure 4.3: ATP-induced vasoconstriction in endothelium-intact and -denuded rIPA. The traces show typical contractions of a) endothelium-intact (black) and b) -denuded (gold) rIPA evoked by addition of ATP (300 µM), as indicated by the horizontal bars, at 0.0 hr and 2.5 hr. The mean peak amplitude of contractions induced by ATP (300 µM) in c) endothelium-intact and d) endothelium-denuded rIPA are shown and e) shows a comparison between endothelium-intact (blue) and -denuded (red) rIPA when responses are expressed as a percentage of the first ATP response. Vertical lines indicate SEM. (n = 6 (endothelium-intact) and n=6 (endothelium-denuded)). * $P < 0.05$ for responses produced at 1.0 hr, ** $P < 0.01$ for responses produced at 1.5, 2.0 and 2.5 hr in endothelium-denuded rIPA compared to those evoked at 0.0 hr. † $P < 0.05$ for responses produced at 1.0 hr and 2.5 hr, and †† $P < 0.01$ for responses produced at 1.5 hr and 2.0 hr in endothelium-denuded compared to endothelium-intact tissues.

When UDP (300 μM) was added to the endothelium-intact rIPA for 5 min, six times at 30 min intervals, it produced vasoconstrictions that tended to progressively decrease in amplitude, with no significant difference between the first to fifth additions, but the responses to the sixth ($P < 0.01$) additions were significantly smaller than the first response (figures 4.4a, c). Using the same protocol in endothelium-denuded rIPA, there was no difference in the amplitude of the first contraction evoked by UDP (212 ± 29 mg, n=6) compared to that evoked in endothelial-intact tissues (165 ± 20 mg, n=9), but the amplitude of subsequent contractions were significantly smaller than the first contractile response ($P < 0.05$ at 0.5 and $P < 0.01$ at 1.0 - 2.5 hr) (figures 4.4b, d). When each time point of endothelium-denuded IPA was compared to that of the endothelium-intact IPA, there was no significant difference in the first to third additions (figure 4.4e), but responses produced in 1.5 hr, 2.0 hr and 2.5 hr ($P < 0.05$) were significantly smaller.

These data show that UTP, ATP and UDP evoke reproducible contractions when the endothelial layer is left intact, but not when it is removed. The subsequent experiments were, therefore, performed on endothelium-intact tissues.


a) Endothelium-intact
0.0 hr
UDP (300 µM)
2.5 hr
UDP (300 µM)
50 mg
1 min
b) Endothelium-denuded
0.0 hr
UDP (300 µM)
2.5 hr
UDP (300 µM)

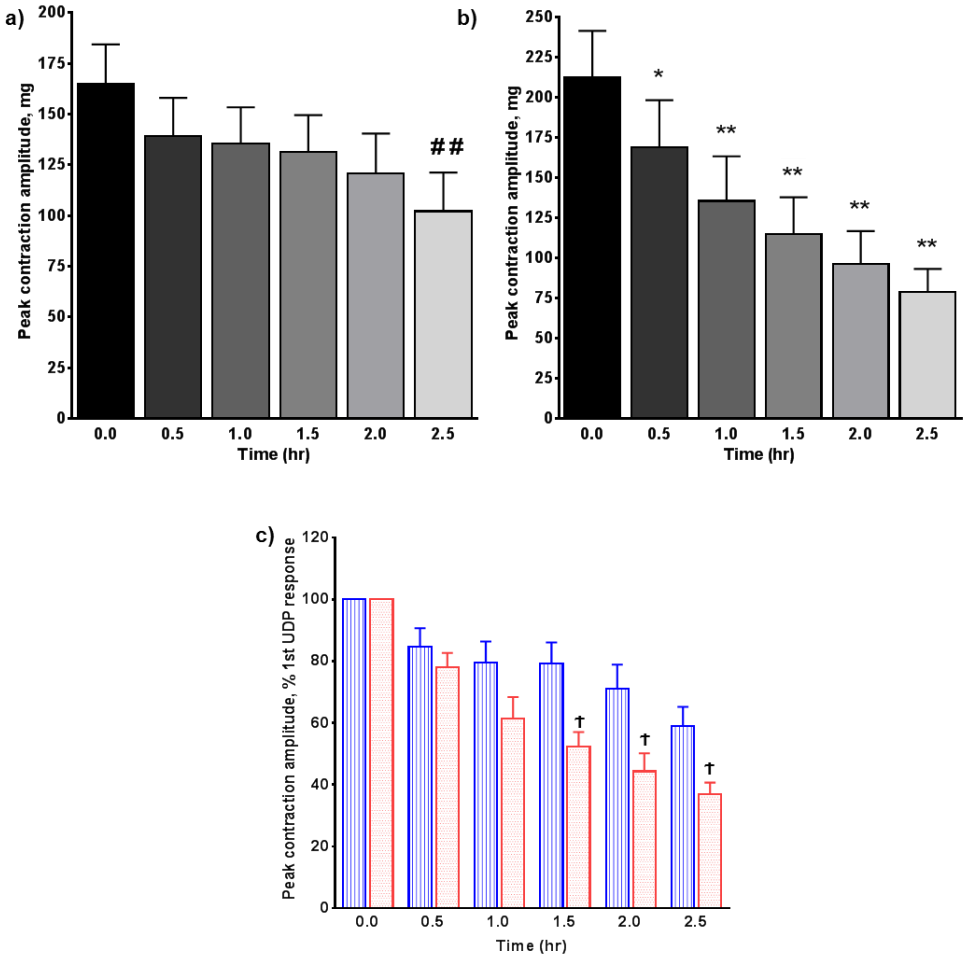


Figure 4.4: UDP-induced vasoconstriction in endothelium-intact and denuded rIPA. The traces show typical contractions of a) endothelium-intact (black) and b) -denuded (gold) rIPA evoked by addition of UDP (300 µM), as indicated by the horizontal bars, at 0.0 hr and 2.5 hr. The mean peak amplitude of contractions induced by UDP (300 µM) in c) endothelium-intact and d) endothelium-denuded rIPA are shown and e) shows a comparison between endothelium-intact (blue) and endothelium-denuded (red) rIPA when the responses are expressed as a percentage of the first UDP response. Vertical lines indicate SEM. (n = 9 (endothelium-intact) and n=6 (endothelium-denuded)). $^{\#\#}$ $P < 0.01$ for responses produced at 2.5 hr in endothelium-intact rIPA compared to those evoked at 0.0 hr. * $P < 0.05$ for responses produced at 0.5 hr, and ** $P < 0.01$ for responses produced at 1.0 hr, 1.5 hr, 2 hr and 2.5 hr in endothelium-denuded rIPA compared to those evoked at 0.0 hr. † $P < 0.05$ for responses produced at 1.0 hr, 1.5 hr and 2.5 hr in endothelium-denuded compared to endothelium-intact tissues.

4.2.2 Effects of AR-C118925XX on KCl- and Nucleotide-Induced Contractions

In the next set of experiments, the role of $P2Y_2$ receptors in the nucleotide-evoked contractions of rIPA were investigated by determining the effects of AR-C118925XX (1 μM), a concentration that is 270 times greater than the K_B at $P2Y_2$ receptors (3.7 nM) calculated in the previous chapter. When preincubated for 20 min, this high concentration of AR-C118925XX had no effect on basal tone of rIPA or on contractions evoked by KCl (40 mM) (figure 4.5). AR-C118925XX also had no effect on the contractions evoked by 300 μM UTP (figure 4.6a), ATP (figure 4.6b) or UDP (figure 4.6c). Thus $P2Y_2$ receptors do not appear to contribute to contractions of rIPA evoked by ATP, UTP or UDP.

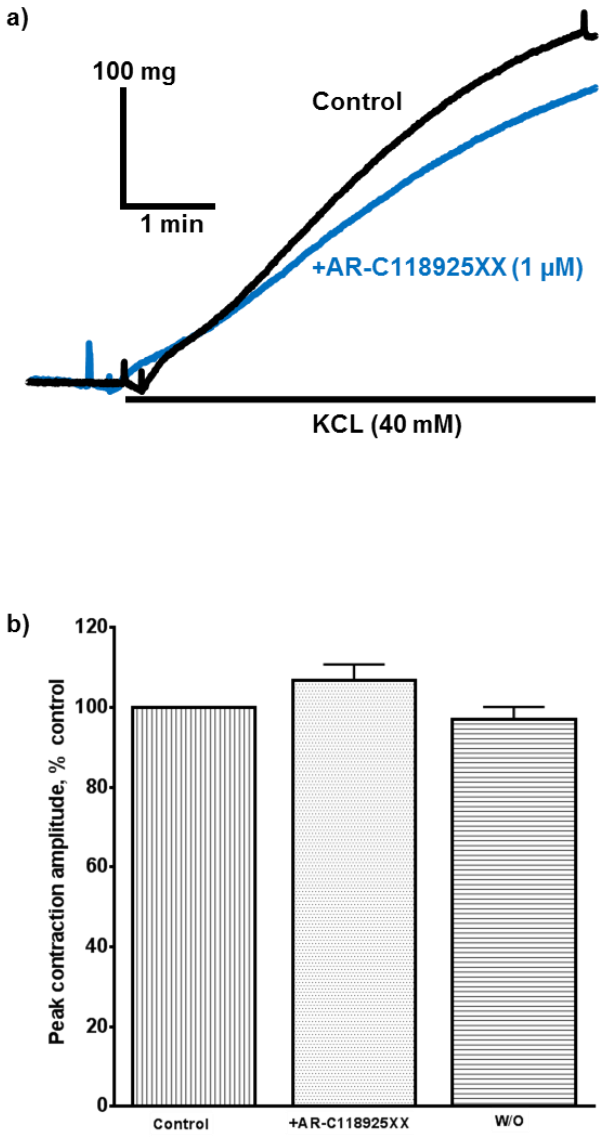
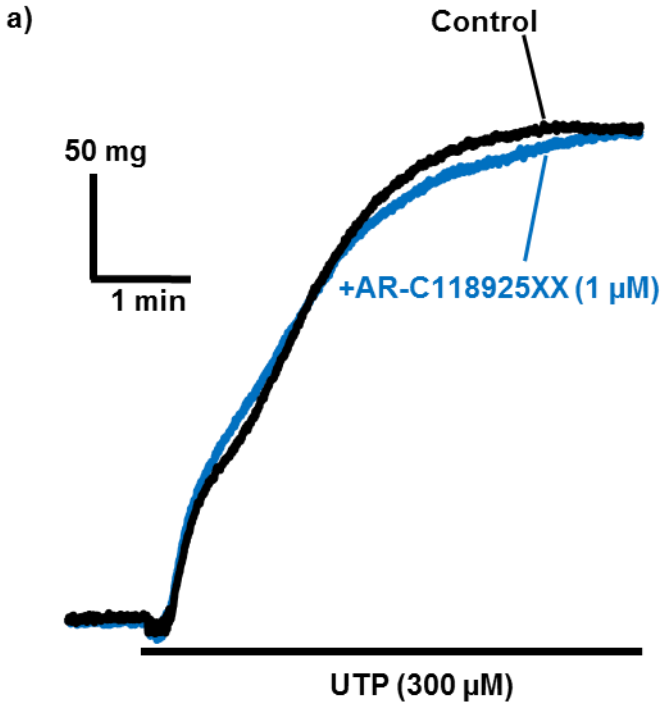
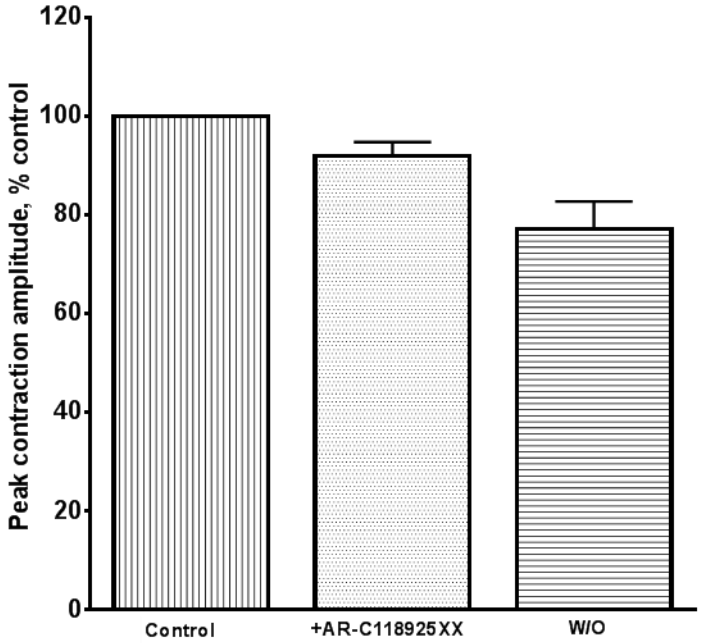
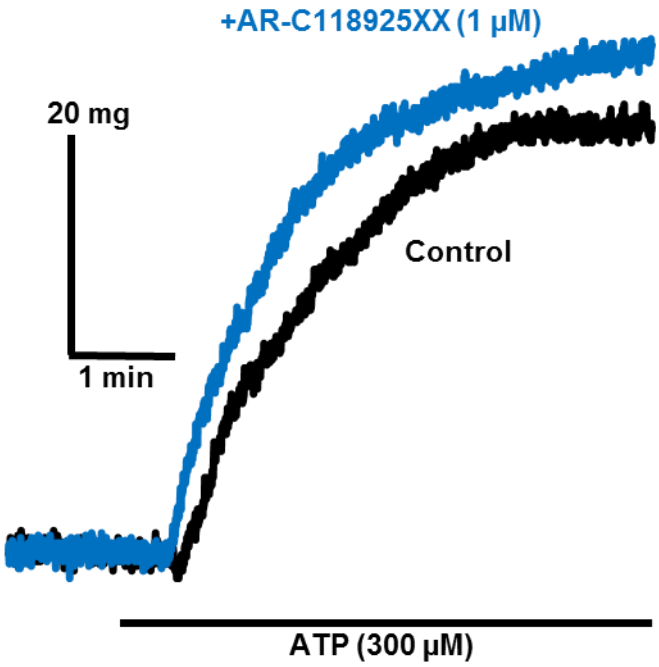


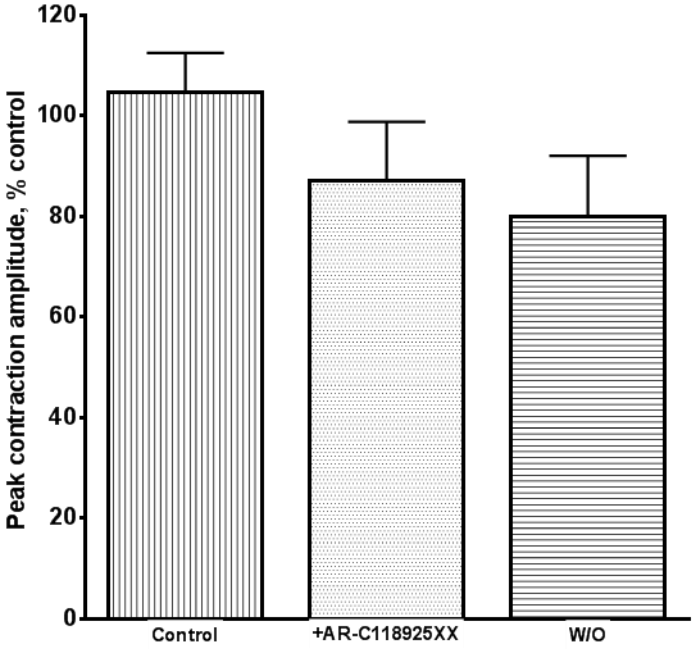
Figure 4.5: Effects of AR-C118925XX on contractions evoked by KCl. a) The superimposed traces show typical contractions of endothelium-intact rIPA evoked by addition of KCl (40 mM), as indicated by the horizontal bar, in the absence (black) and presence of AR-C118925XX (1 µM) (blue). b) The mean peak amplitude of contractions evoked by KCl (40 mM) alone (Control), in the presence (+AR-C11895XX) and after washout (W/O) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response to KCl (40 mM). Vertical lines indicate SEM., n=6.


a)
Control
50 mg
1 min
+AR-C118925XX (1 µM)
UTP (300 µM)


Peak contraction amplitude, % control
120
100
80
60
40
20
0
Control
+AR-C118925XX
W/O

b)


+AR-C118925XX (1 µM)
20 mg
Control
1 min
ATP (300 µM)


Peak contraction amplitude, % control
120
100
80
60
40
20
0
Control
+AR-C118925XX
W/O

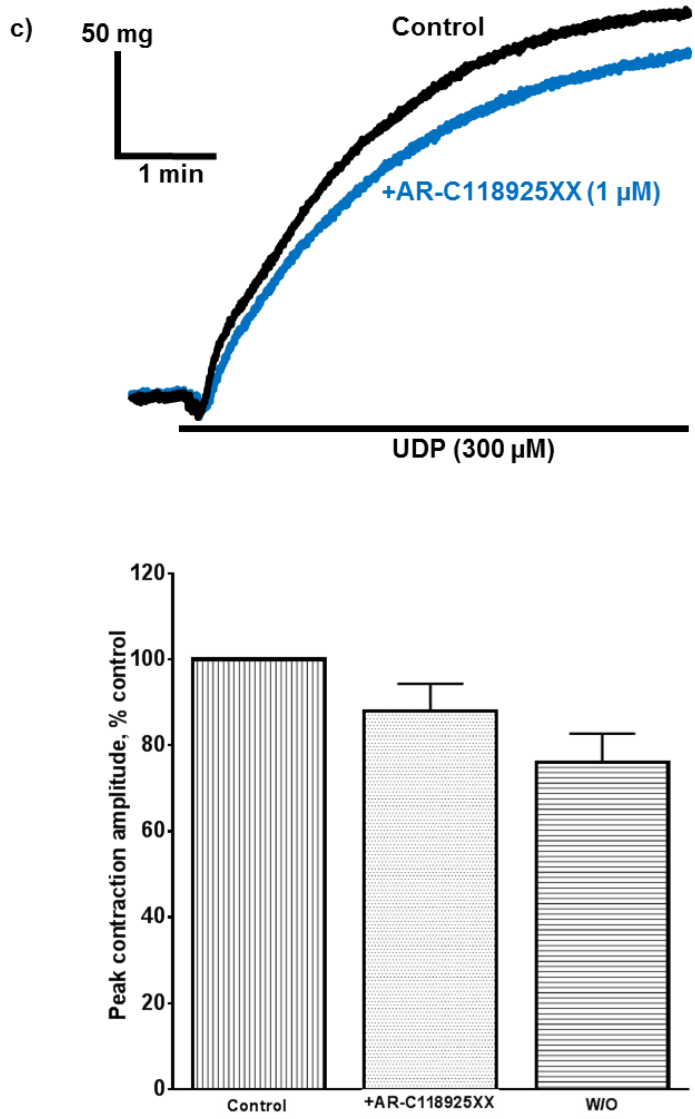


Figure 4.6: Effects of AR-C118925XX on contractions evoked by UTP, ATP and UDP. Upper panels: The superimposed traces show typical contractions of endothelium-intact rIPA evoked by addition of a) UTP, b) ATP and c) UDP (all 300 µM), as indicated by the horizontal bars, in the absence (black) and presence (blue) of AR-C118925XX (1 µM). Lower panels: The mean peak amplitude of contractions evoked by a) UTP, b) ATP and c) UDP (all 300 µM) alone (Control), in the presence (+AR-C118925XX) and on washout (W/O) of AR-C118925XX (1 µM) are shown. The data are expressed as a percentage of the control response to the nucleotide. Vertical lines indicate SEM. n=6 (UTP), n=5 (ATP) and n=7 (UDP).

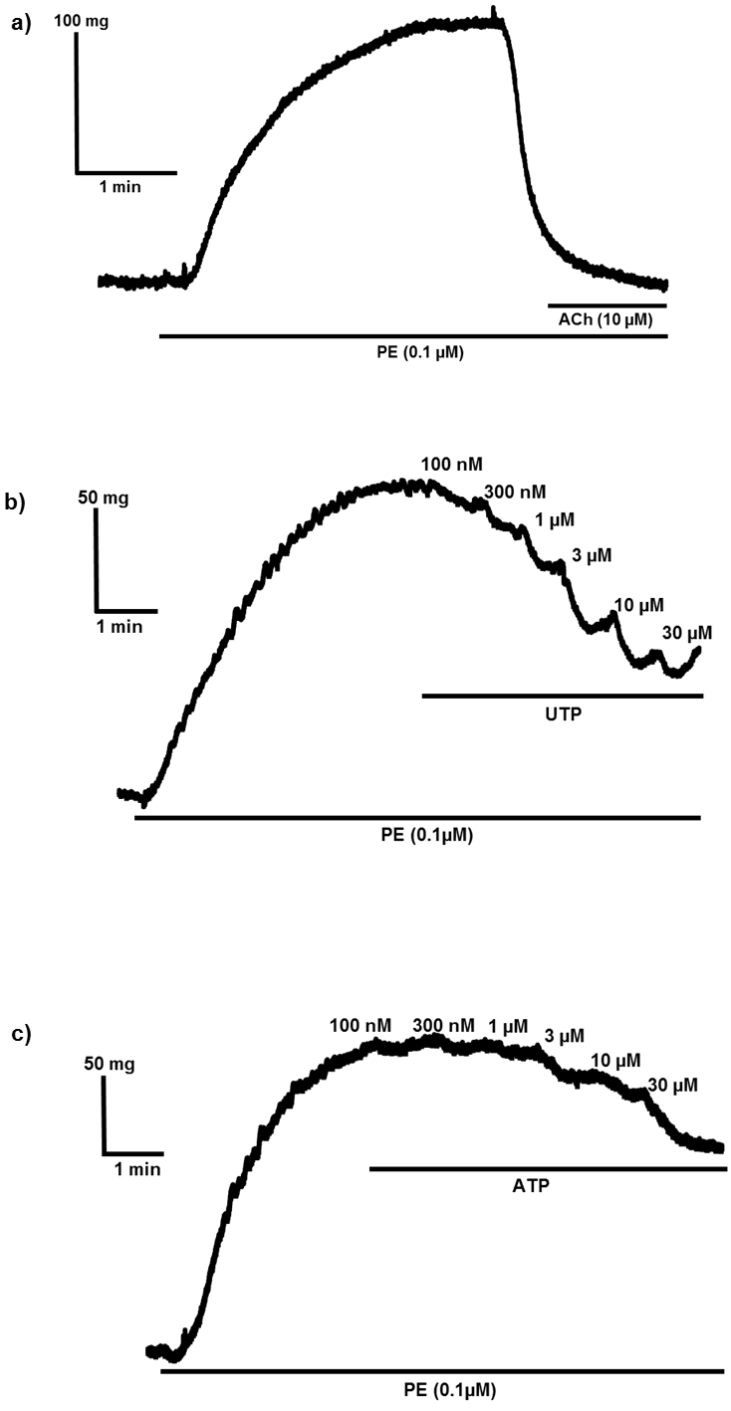
4.2.3 Effects of UTP, UDP, ATP and ADP at Raised Tone

UTP, ATP, ADP and UDP have all been reported previously to evoke endothelium-dependent vasodilation in rIPA (Rubino and Burnstock, 1996; Rubino *et al*., 1999) and the focus of the rest of this chapter is the contribution of $P2Y_2$ and other P2Y receptor subtypes to these responses. The first aim was to determine the potency of the nucleotides in endothelium-intact tissues by constructing CRC. First, tissues were pre-contracted with PE (0.1 or 0.3 µM) and the endothelium-dependent vasodilator, ACh, was applied to determine the integrity of the endothelium. PE-induced contractions reached peak amplitude within 1 min (209 ± 15 mg, n=32) and ACh (10 µM) relaxed the contractions by 78.8 ± 3.5% (n=32, figure 4.7a). UTP, ATP, UDP and ADP all produced concentration-dependent relaxation of the rIPA, with EC_{50} values in the low µM range (figures 4.7b-e, 4.8, table 4.1). ATP and ADP evoked larger maximum vasodilation than UTP and UDP, but were slightly less potent.

These data confirm that UTP, ATP, UDP and ADP evoke vasodilation of the rIPA. However, it was very noticeable that the slopes for all nucleotide CRC were substantially less than 1. In addition, relaxations were not always well-maintained and at the highest concentrations tested, UTP, UDP and ATP, but not ADP, evoked biphasic responses, with large contractions following the vasodilation, which meant that a plateau to the CRC could not be obtained. Finally, fitting the Hill equation to the data could not provide an upper limit for the EC_{50} values of ATP and UDP. Shallow CRC can arise when an agonist acts at more than one receptor and with different potencies to produce its effect, which might be the case here, or if agonist steady-state concentrations at the biophase next to the plasma membrane are not maintained due to agonist breakdown by ecto-enzymes or cellular uptake by transporters. Nucleotides are known to be progressively dephosphorylated by a family of ecto-nucleotidases, which can reduce their apparent potency and produce shallow CRC (Evans and Kennedy 1994; Kennedy and Leff, 1995; Kauffenstein *et al.,* 2010). An ecto-nucleotidase inhibitor, ARL67156, is available, but it has only moderate potency (pIC_{50} = 4.62 in human blood cells), is also a weak antagonist at P2X1 receptors (pA_2 = 3.3) and is expensive (Crack *et al.,* 1995; Kennedy and Leff, 1995).

The ideal protocol when characterizing the actions of an antagonist is to generate agonist CRC, first in the absence of the antagonist and then in its presence. The

issues described above, however, limit the usefulness of this approach. An alternative approach was, therefore, adopted in which the effects of AR-C118925XX were studied against single concentrations of nucleotides that were in the EC_{50} - EC_{70} range (UTP and UDP - 3 µM, ATP and ADP - 10 µM).



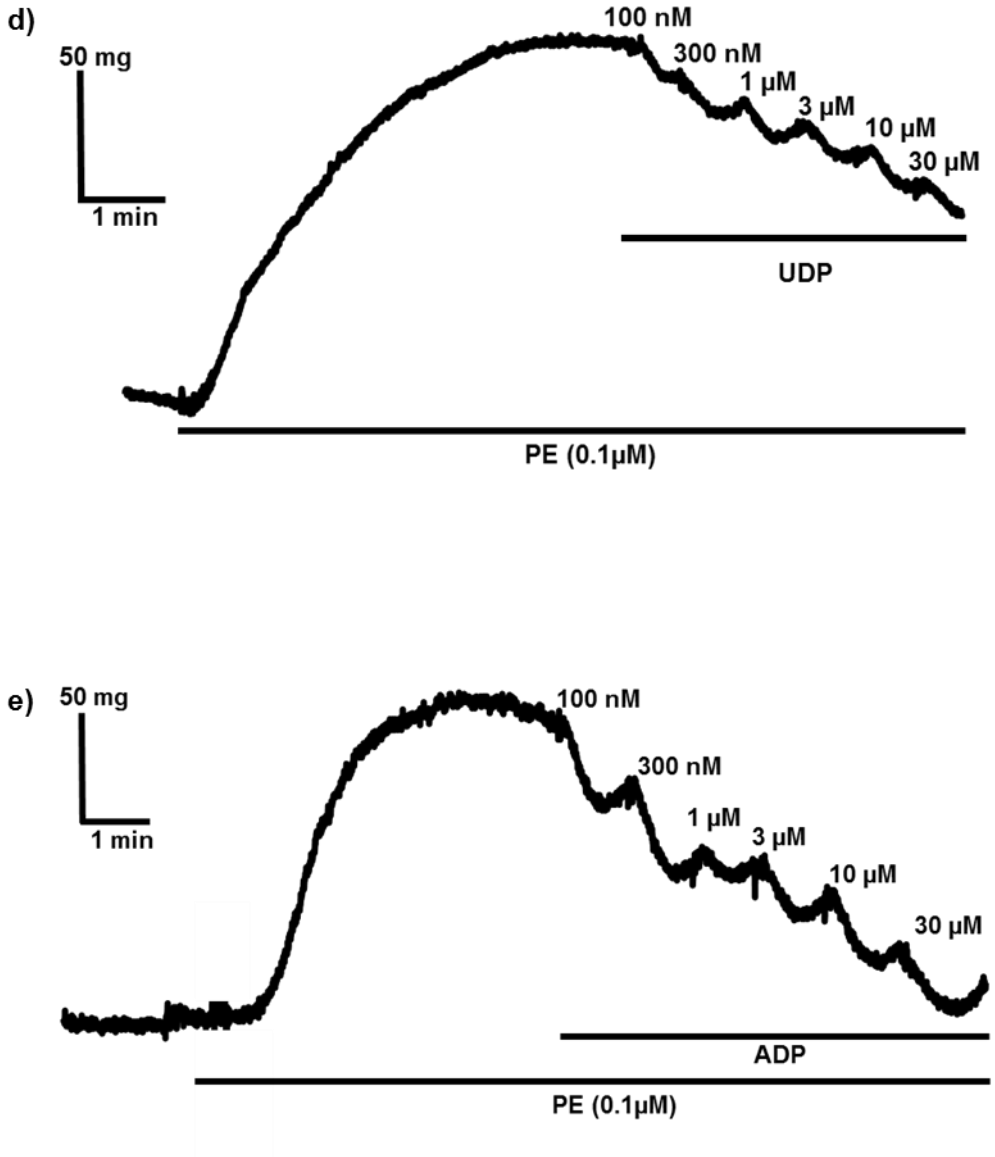


Figure 4.7: Nucleotides elicit vasodilation of rIPA at raised tone. The traces show typical relaxations of PE (0.1 µM)-precontracted, endothelium-intact rIPA induced by a) ACh (10 µM) and cumulative additions of b) UTP, c) ATP, d) UDP, and e) ADP (100 nM - 30 µM), as indicated by the horizontal bars.

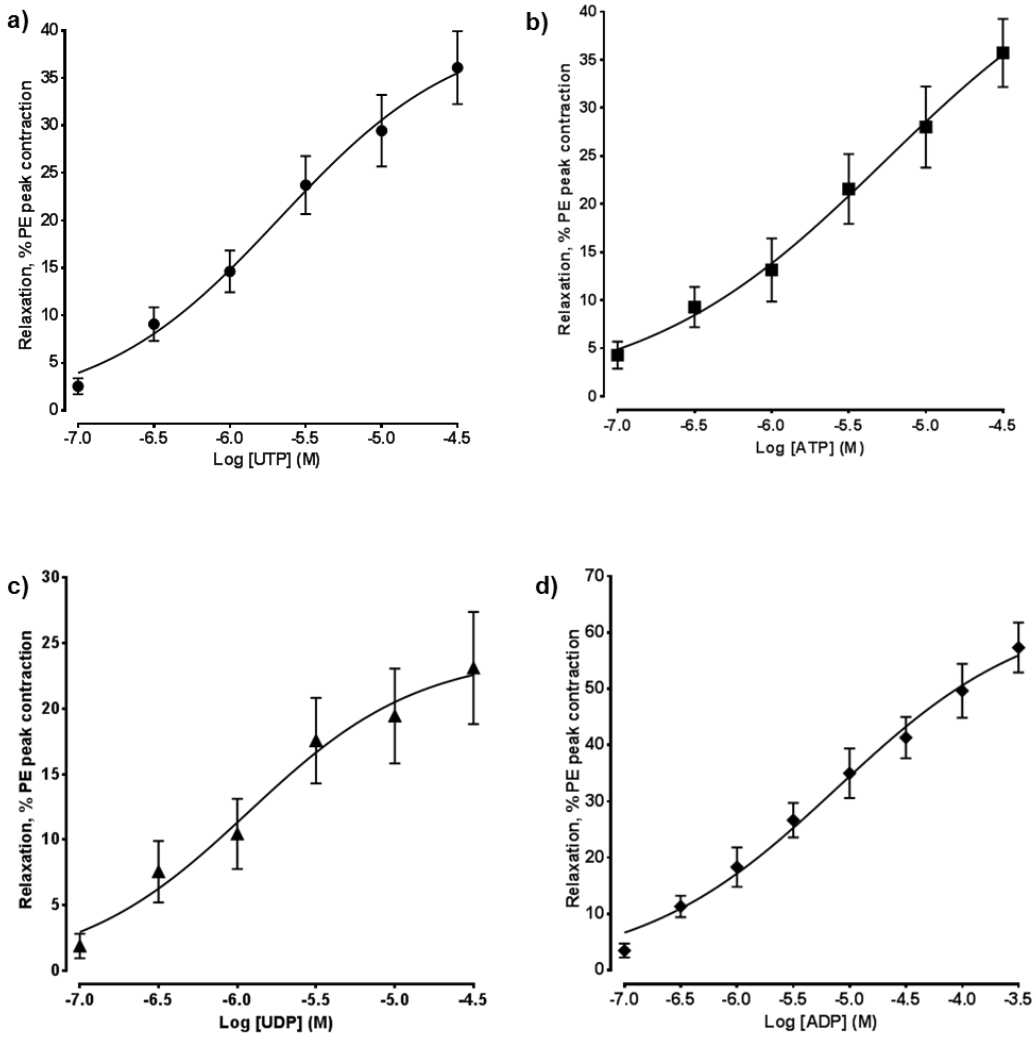


Figure 4.8: Nucleotides elicit concentration-dependent vasodilation of rIPA at raised tone. The mean peak amplitude of relaxations evoked by cumulative additions of a) UTP, b) ATP, c) UDP, and d) ADP (100 nM - 30 µM) on PE (0.1/0.3 µM) pre-contracted rIPA is shown. The data are expressed as a percentage of the contraction evoked by PE. Vertical lines indicate SEM and the curves represent the fit of the Hill equation to the data. n=11 (UTP), n=7 (ATP), n=9 (UDP) and n=6 (ADP).

Table 4.1: Properties of nucleotide relaxant CRC at raised tone. Values shown are the EC_{50} (μM) with 95% confidence intervals, Hill slope ± SEM and maximum response ± SEM for UTP, ATP, UDP and ADP relaxant CRC in endothelium-intact rIPA that were pre-contracted by PE (0.1/0.3 μM). * indicates the values were to infinity.**

Agonist	EC_{50} (μM) (95% confidence interval)	Max (%PE Vc)	Hill slope	n
UTP	2.1 (824 nM-262 μM)	40.5 ± 7.1	0.73 ± 0.21	11
ATP	5.5 (946 nM-***)	49.0 ± 20.1	0.55 ± 0.22	7
UDP	2.3 (420 nM-***)	29.7 ± 10.9	0.65 ± 0.33	9
ADP	7.7 (2.5-229 μM)	64.8 ± 9.0	0.50 ± 0.11	6

4.2.3.1 Reproducibility of nucleotide-evoked vasodilation and the role of the endothelium

The aims of the next set of experiments were first to confirm the effectiveness of physical removal of the endothelium and then to determine if the vasodilations evoked by nucleotides are reproducible and if they are endothelium-dependent. PE (0.1 μM) evoked contractions of endothelium-intact rIPA with a mean peak amplitude of 230 ± 7 mg (SD = 76 mg, range = 75-459 mg, n=127), equivalent to 58.6 ± 1.5% (SD = 16.5%, range = 27-95%) of the peak amplitude of the contractions produced by KCl (40 mM) (mean KCl contractions = 398 ± 9 mg, SD = 98 mg, range = 159-795 mg, n=127). The PE-elicited contractions were reproducible, as when PE was added twice, 30 min apart, the second response was 101.2 ± 2.2% of the first (n=24). This served as a time-matched control when later studying the effects of P2Y receptor antagonists and determined if they had any non-specific effects on tissue contraction.

ACh (10 μM) induced relaxations of endothelium-intact rIPA that were 86.1 ± 0.8% of the peak amplitude of the contractions evoked by PE (SD = 9.6%, range = 50-100%, n=127). This was significantly reduced to 1.7 ± 0.7% (range = 0-7.5%, n=12) in endothelium-denuded tissues ($P < 0.0001$), confirming that physical removal of the endothelial cell layer had been effective.

When UTP (3 μM) was added to the pre-contracted, endothelium-intact rIPA, it produced relaxations that tended to be fast and transient (figure 4.9a) and with a mean amplitude of 26 ± 2 mg (range = 7-42 mg, n=24), which was equivalent to 10.8 ± 1.0% of the PE-induced contraction (range = 3.0-21.1%). In tissues where UTP (3 μM) was added twice, 30 min apart, there was no significant difference in the mean amplitude of the responses, with the second being 106.4 ± 6.0% of that of the first (n=6; figure 4.9a). This served as a time-matched control when studying the actions of AR-C118925XX and other antagonists on vasodilation evoked by UTP. The same approach was also used with the other nucleotides. In endothelium-denuded tissues the relaxations evoked by UTP were substantially and significantly smaller ($P < 0.01$), with a mean amplitude of 8 ± 1 mg (n=6), equivalent to 2.2 ± 0.4% of the PE-induced contraction (figure 4.9b). Thus these experiments demonstrate that UTP evokes small, but reproducible, relaxations of rIPA that are largely dependent upon an intact endothelial layer.

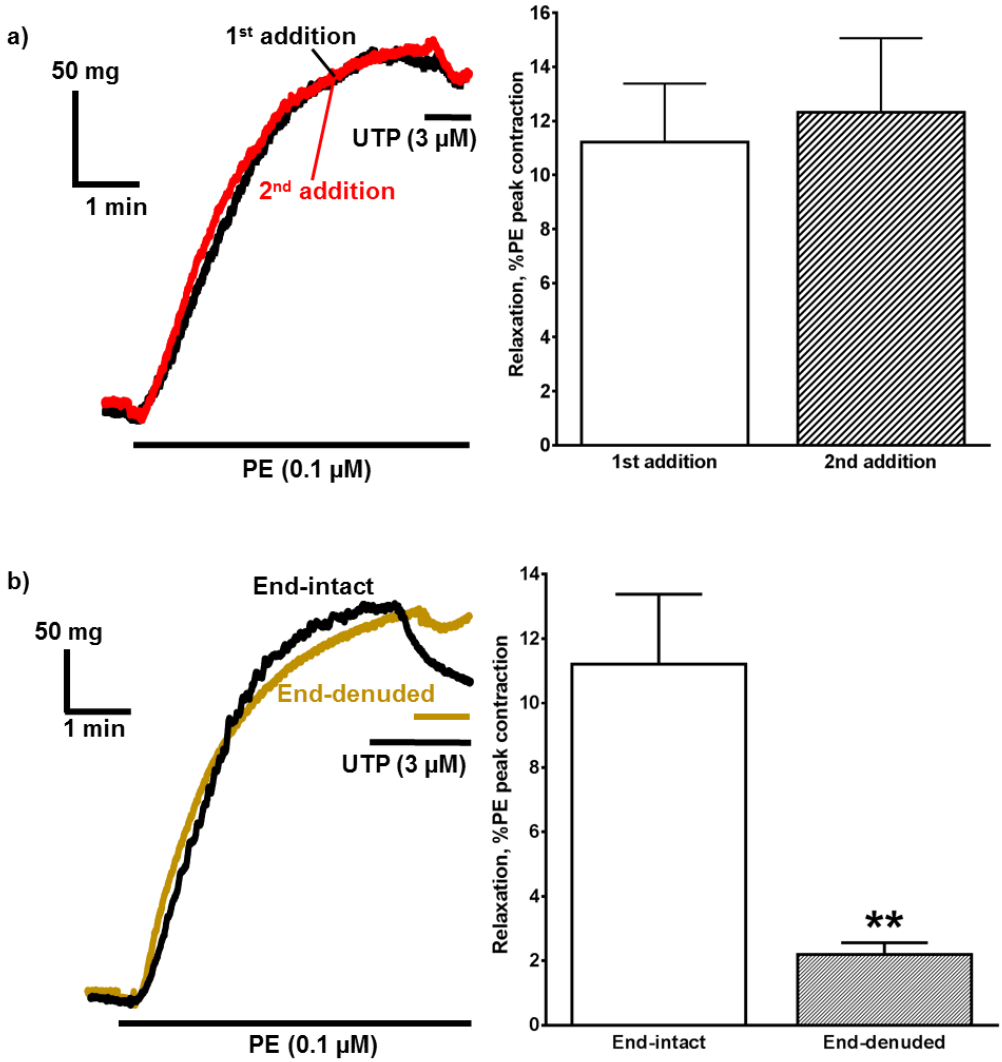


Figure 4.9: UTP-induced vasodilatation in endothelium-intact and -denuded rIPA. Left-hand panels: The superimposed traces show a) typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by two additions of UTP (3 µM), 30 min apart (1st addition - black, 2nd addition - red) and b) a comparison of relaxations seen in endothelium-intact (black) and -denuded (gold) rIPA. PE and UTP were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by UTP (3 µM) in a) endothelium-intact rIPA, and b) endothelium-intact and -denuded rIPA, expressed as a percentage of the contraction elicited by PE is shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by UTP in endothelium-denuded rIPA compared to responses in endothelium-intact rIPA.

ATP (10 µM), also produced rapid, transient relaxations of the endothelium-intact rIPA (figure 4.10a) that had a mean amplitude of 28 ± 2 mg (range = 10-63 mg, n=37), which was equivalent to 14.1 ± 0.8% of the PE-induced contraction (range = 3.4-28.3%). When ATP (10 µM) was added twice, 30 min apart, there was no significant difference in the mean amplitude of the responses (figure 4.10a), with the second being 103.9 ± 5.1% of that of the first (n=6). In endothelium-denuded tissues the relaxations were substantially and significantly smaller ($P < 0.0001$), with a mean amplitude of 9 ± 4 mg (n=6), equivalent to 1.6 ± 0.7% of the PE-induced contraction (figure 4.10b).

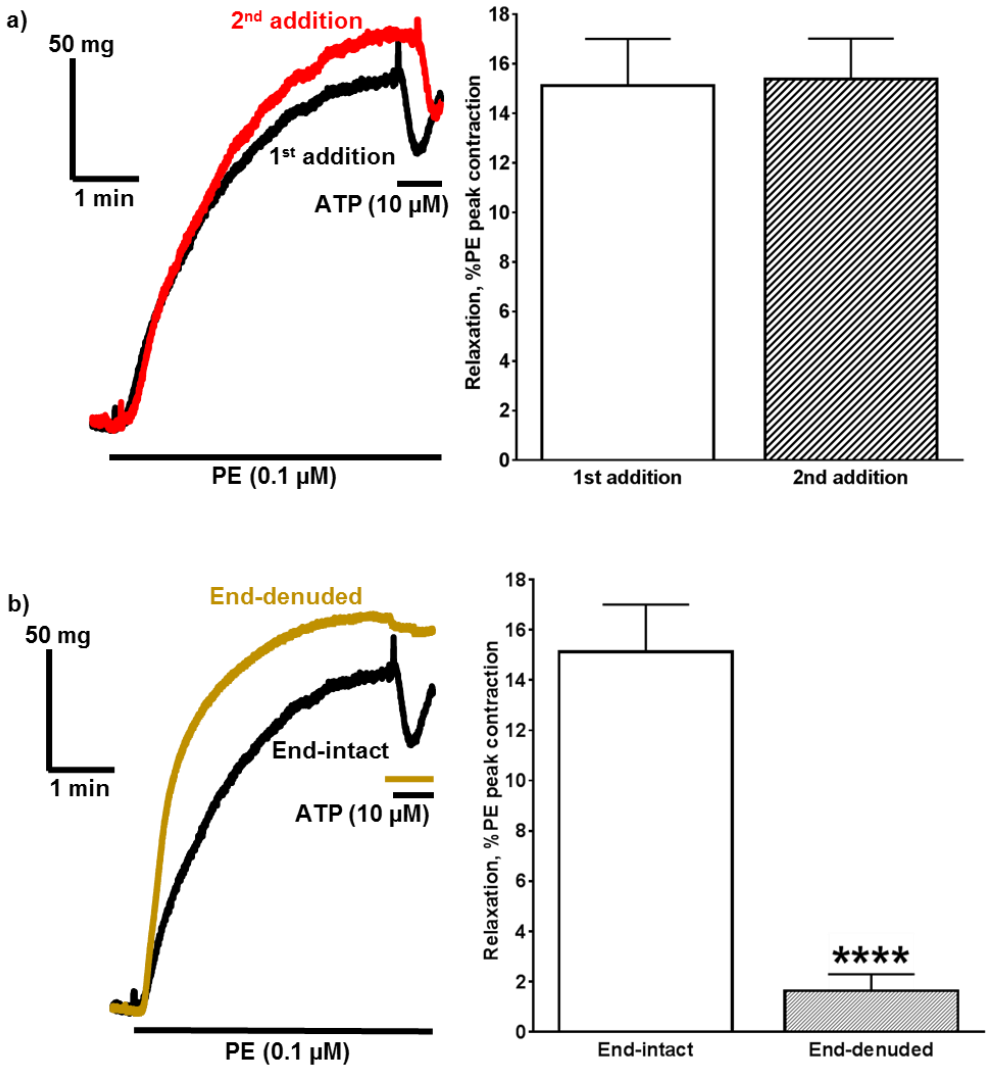


Figure 4.10: ATP-induced vasodilatation in endothelium-intact and -denuded rIPA. Left-hand panels: The superimposed traces show a) typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by two additions of ATP (10 µM), 30 min apart (1st addition - black, 2nd addition - red) and b) a comparison of relaxations seen in endothelium-intact (black) and -denuded (gold) rIPA. PE and ATP were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by ATP (10 µM) in a) endothelium-intact rIPA, and b) endothelium-intact and -denuded rIPA, expressed as a percentage of the contraction elicited by PE is shown. Vertical lines indicate SEM. n=6 each. **** $P < 0.0001$ for responses produced by ATP in endothelium-denuded rIPA compared to responses in endothelium-intact rIPA.

UDP (3 μM) also produced rapid, transient relaxations in the endothelium-intact rIPA (figure 4.11a) and with a mean amplitude of 17 ± 1 mg (range = 7-32 mg, n=24), which was equivalent to 7.2 ± 0.2% of the PE-induced contraction (range = 4.0-10.0%). In tissues where UDP (3 μM) was added twice, 30 min apart, there was no significant difference in the mean amplitude of the responses (figure 4.11a), with the second being 95.7 ± 2.8% of that of the first (n=6). When added to tissues from which the endothelium had been removed, the relaxations induced by UDP (3 μM) were significantly smaller ($P < 0.0001$), with a mean amplitude of 6 ± 1 mg (n=6), equivalent to 1.7 ± 0.4% of the PE-induced contraction (figure 4.11b).

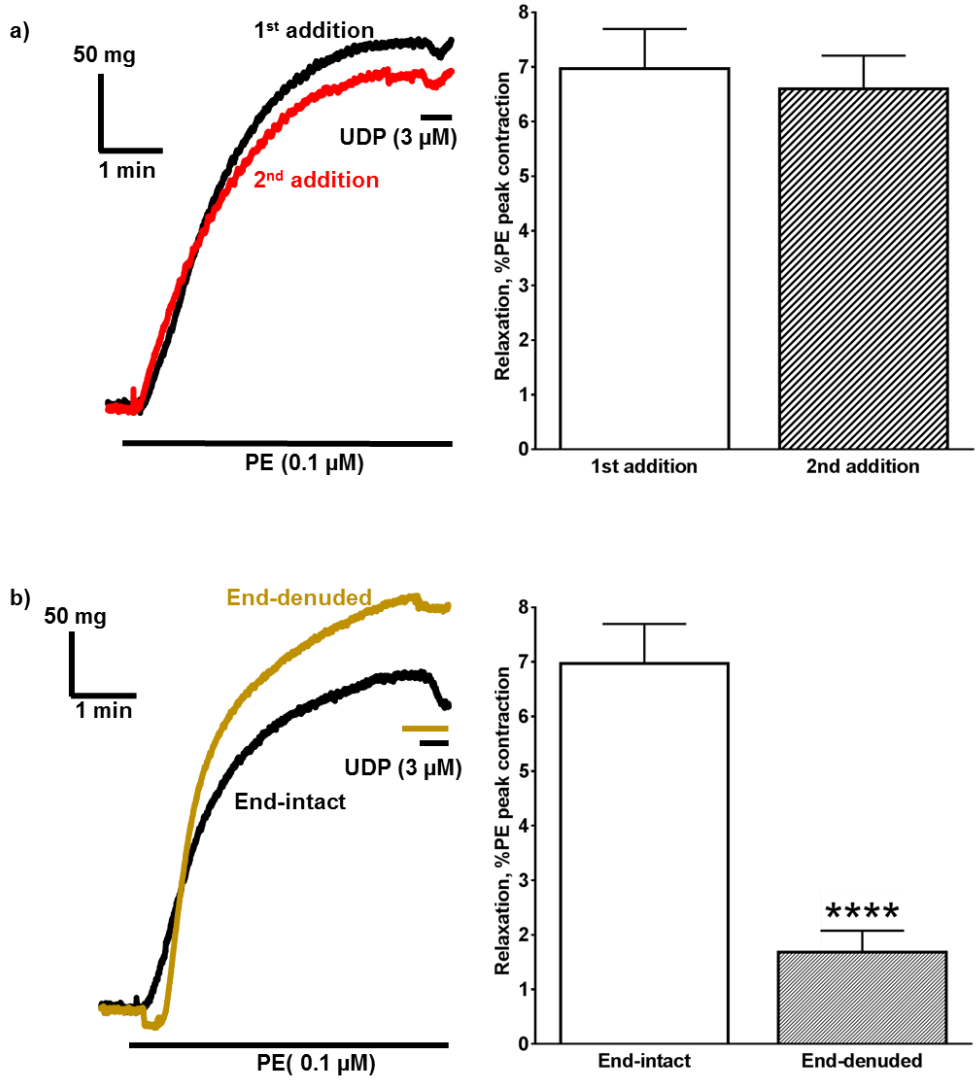


Figure 4.11: UDP-induced vasodilatation in endothelium-intact and -denuded rIPA. Left-hand panels: The superimposed traces show a) typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by two additions of UDP (3 µM), 30 min apart (1st addition - black, 2nd addition - red) and b) a comparison of relaxations seen in endothelium-intact (black) and -denuded (gold) rIPA. PE and UDP were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by UDP (3 µM) in a) endothelium-intact rIPA, and b) endothelium-intact and -denuded rIPA, expressed as a percentage of the contraction elicited by PE is shown. Vertical lines indicate SEM. n=6 each. **** $P < 0.0001$ for responses produced by UDP in endothelium-denuded rIPA compared to responses in endothelium-intact rIPA.

Finally, ADP (10 μM) also induced rapid, transient relaxation of the pre-contracted endothelium-intact rIPA (figure 4.12a), with a mean amplitude of 43 ± 2 mg (range = 14-70 mg, n=42), which was equivalent to 19.8 ± 1.2% of the PE-induced contraction (range = 7.9-36.0%). When ADP (10 μM) was added twice, 30 min apart, there was no significant difference in the mean amplitude of the responses (figure 4.12a), with the second being 103.8 ± 3.6% of that of the first (n=6). In endothelium-denuded tissues the relaxations were substantially and significantly smaller ($P < 0.0001$), with a mean amplitude of 8 ± 1 mg (n=6), equivalent to 2.2 ± 0.4% of the PE-induced contraction (figure 4.12).

Together, these data show that all four nucleotides evoke reproducible, relaxations of rIPA that are largely dependent upon an intact endothelial layer. Relaxations produced by ADP were significantly larger than those seen in response to ATP, UTP and UDP ($P < 0.0001$) (figure 4.13), whilst ATP had a greater effect than UDP, ($P < 0.0001$), but not UTP. There was no difference between the responses to UTP and UDP.

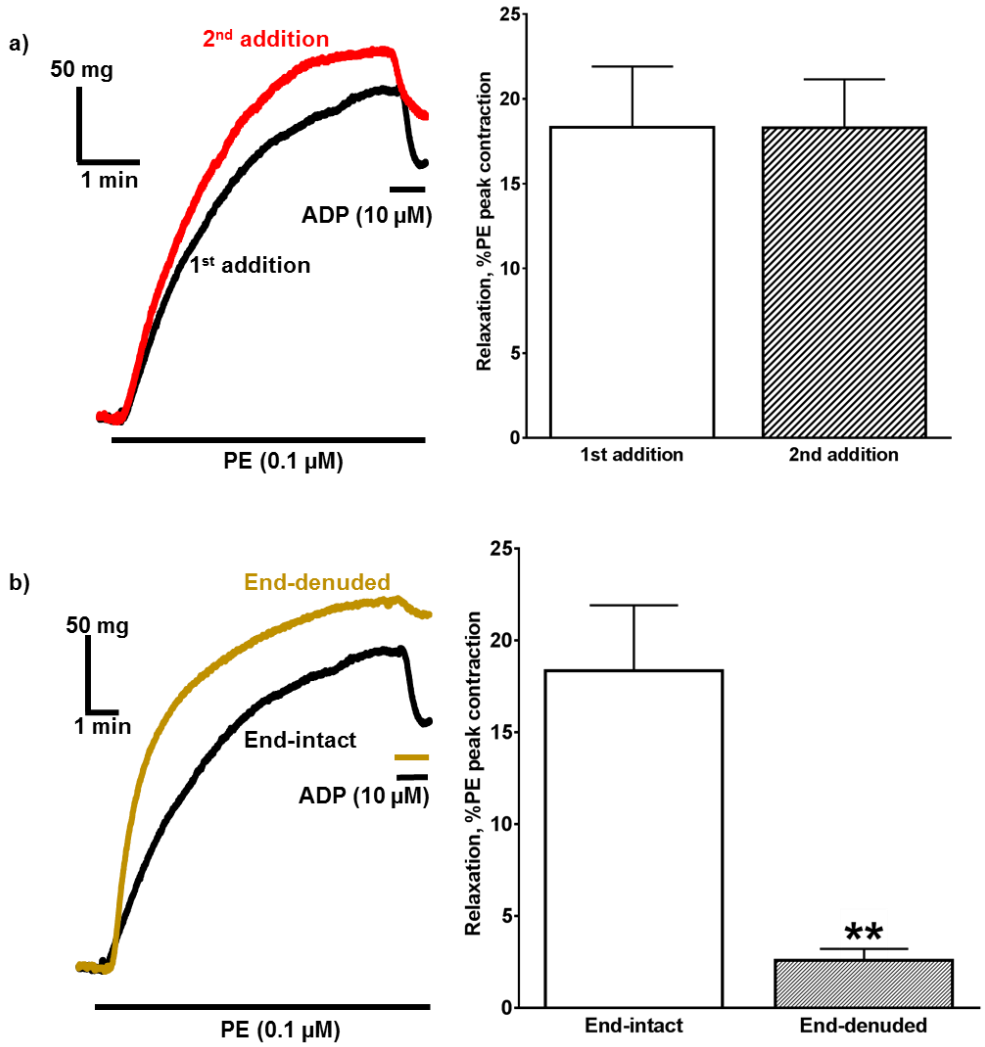


Figure 4.12: ADP-induced vasodilatation in endothelium-intact and -denuded rIPA Left-hand panels: The superimposed traces show a) typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by two additions of ADP (10 µM), 30 min apart (1st addition - black, 2nd addition - red) and b) a comparison of relaxations seen in endothelium-intact (black) and -denuded (gold) rIPA. PE and ADP were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by ADP (10 µM) in a) endothelium-intact rIPA, and b) endothelium-intact and -denuded rIPA, expressed as a percentage of the contraction elicited by PE is shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by ADP in endothelium-denuded rIPA compared to responses in endothelium-intact rIPA.

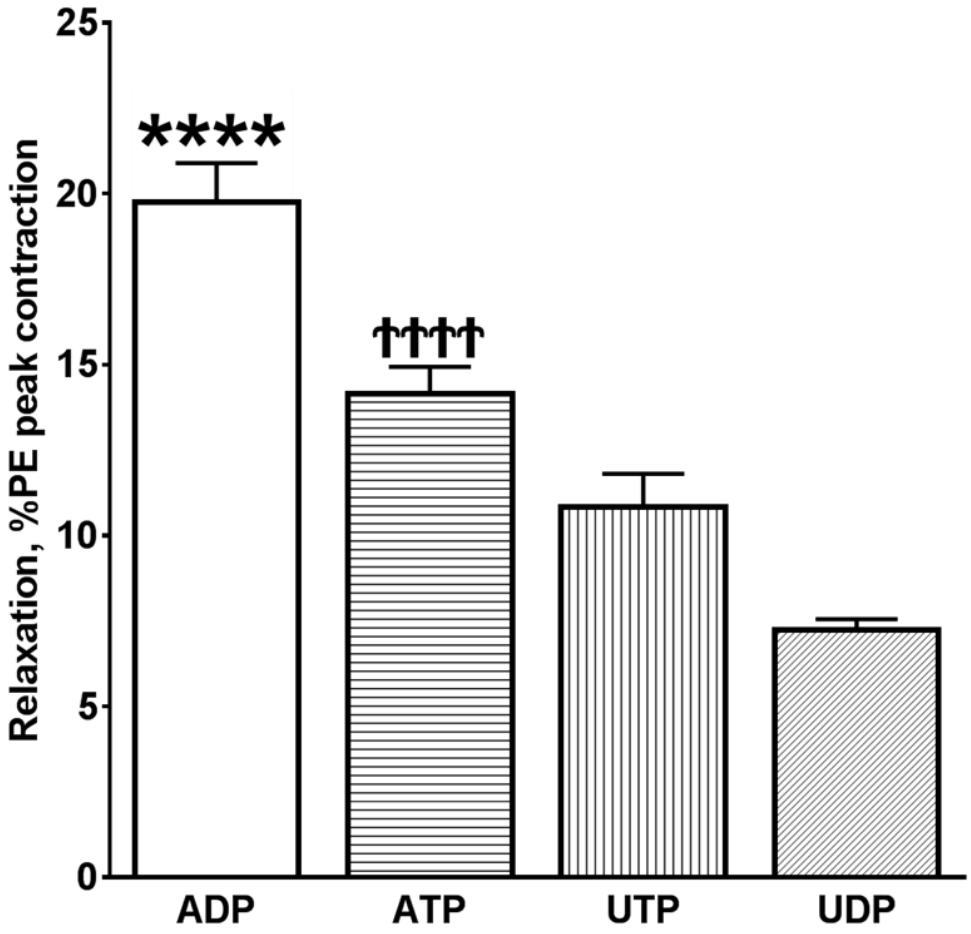


Figure 4.13: **Comparison between nucleotide-induced vasodilatation in endothelium-intact rIPA.** The mean peak amplitude of relaxations evoked by ADP (10 µM), ATP (10 µM) UTP (3 µM), UDP (3 µM) in endothelium-intact rIPA, expressed as a percentage of the contraction elicited by preadministration of PE (0.1 µM) are shown. Vertical lines indicate SEM. n=42 (ADP), n=37 (ATP), n=24 (UTP, UDP). **** $P < 0.0001$ for responses produced by ADP compared to those to ATP, UTP and UDP. †††† $P < 0.0001$ for responses produced by ATP compared to those to UDP.

4.2.3.2 Effects of AR-C118925XX on nucleotide-induced vasodilation

Next, the effect of AR-C118925XX (1 µM) on these nucleotide-evoked vasodilatations in the presence of an intact endothelium was investigated. When preincubated with the tissue for 20 min, AR-C118925XX had no effect on basal tone of rIPA or on contractions evoked by PE (0.1 µM) (time-matched control = 101.2 ± 2.2% of control, n=24; +ARC118925XX = 101.3 ± 1.6% of control, n=24). However, AR-C118925XX significantly reduced the vasodilation induced by UTP (3 µM) by 59.2 ± 3.4% (n=6; figure 4.14a) and that by UDP (3 µM) by 33.0 ± 6.1% (n=6; figure 4.14b). In contrast, at the same concentration, AR-C118925XX had no effect on the dilatation evoked by 10 µM ATP (102.9 ± 4.3% of control, n=6; figure 4.15a), or ADP (100.8 ± 4.4% of control, n=6; figure 4.15b). Thus $P2Y_2$ receptors appear to contribute substantially to the vasodilatation of rIPA evoked by UTP, less so to the UDP response and not at all to ATP and ADP relaxations.

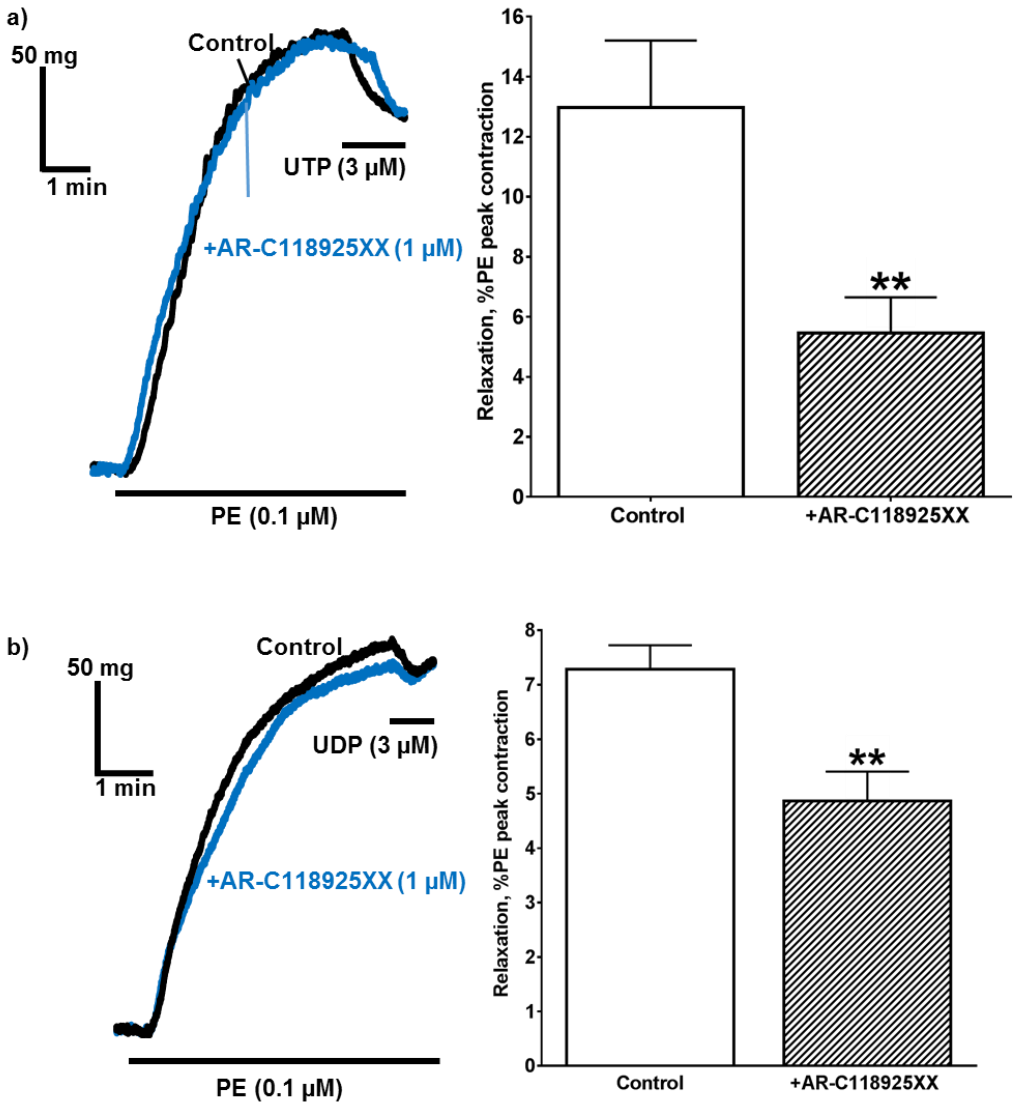


Figure 4.14: Effects of AR-C118925XX on relaxations evoked by UTP and UDP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) UTP (3 µM) and b) UDP (3 µM) in the absence (black) and presence (blue) of AR-C118925XX (1 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of the relaxations evoked by a) UTP (3 µM) and b) UDP (3 µM) alone (Control) and in the presence of AR-C118925XX (1 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by UTP and UDP in the presence of AR-C118925XX compared to in its absence.

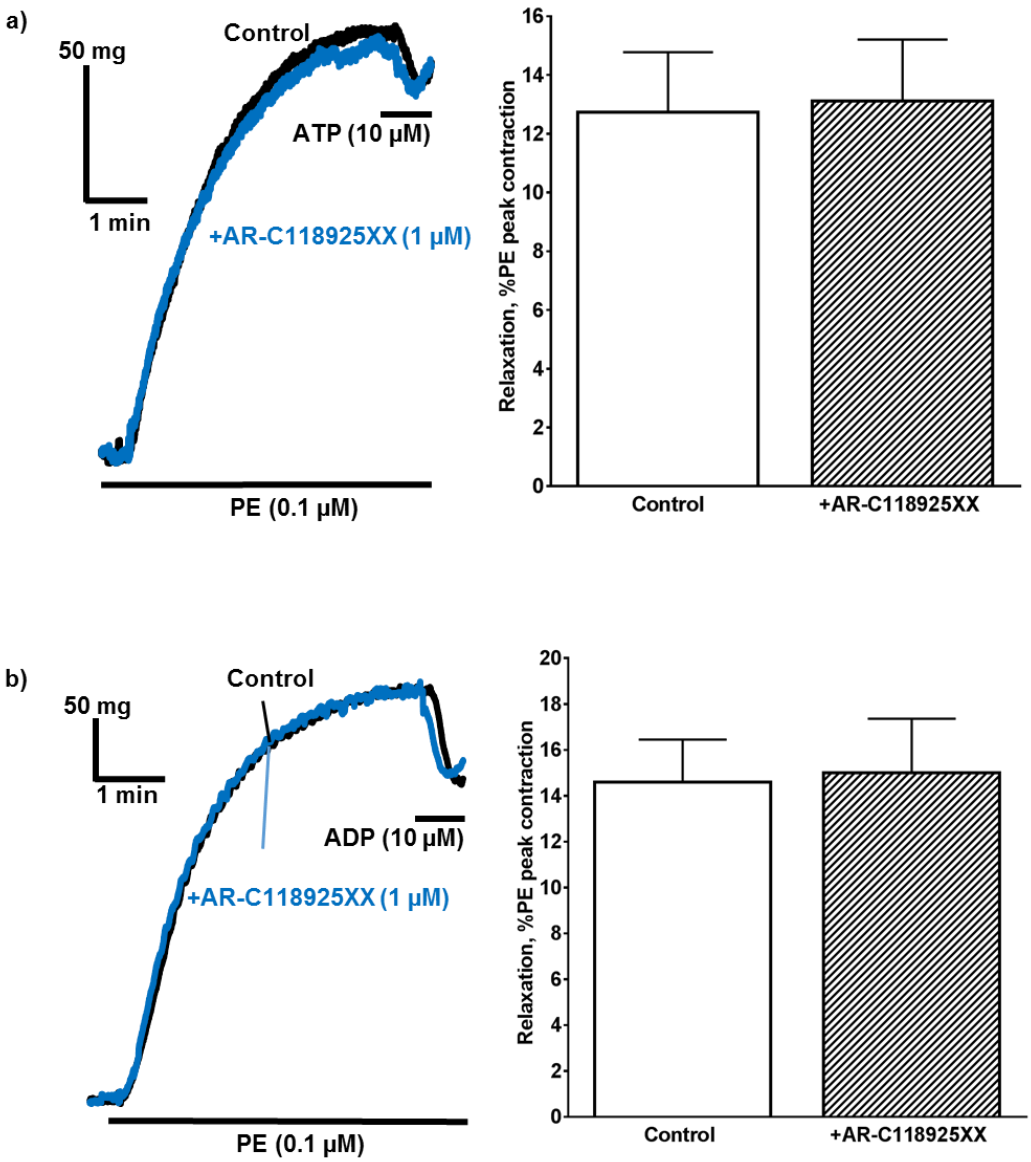


Figure 4.15: Effects of AR-C118925XX on relaxations evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of AR-C118925XX (1 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of the relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of AR-C118925XX (1 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each.

4.2.3.3 Which receptors mediate uridine nucleotide-induced vasodilatation?

The inability of a high concentration of AR-C118925XX to abolish the vasodilation induced by UTP indicates that approximately 40% of its effect may be mediated by $P2Y_4$ receptors, the other P2Y receptor subtype at which it is an agonist, and/or $P2Y_6$ and $P2Y_{14}$ receptors, where it can act after dephosphorylation to UDP by ecto-nucleotidases. Similarly, AR-C118925XX inhibited the response to UDP by only one third, and $P2Y_6$ and/or $P2Y_{14}$ receptors are the other potential sites of action. Therefore, in the next set of experiments, the role of $P2Y_6$ receptors in the UTP- and UDP-evoked relaxation of rIPA was investigated by determining the effects of the non-competitive $P2Y_6$ receptor antagonist, MRS2578 (1 μM). This concentration is ten times higher than the IC_{50} (98 nM) obtained at the $hP2Y_6$ receptor expressed in human 1321N1 astrocytoma cells (Mamedova *et al.,* 2004).

When preincubated with the tissue for 20 min, MRS2578 (1 μM) had no effect on basal tone of rIPA, though it slightly, but significantly inhibited the contractions evoked by PE (0.1 μM) (92.8 ± 1.9% of control, n=12, $P < 0.05$). MRS2578 had no effect on the relaxation evoked by UTP (3 μM) (102.5 ± 5.0% of control, n=6; figure 4.16a), but it significantly reduced that induced by UDP (3 μM) by almost half (55.2 ± 12.1% of control, n=6, $P < 0.01$; figure 4.16b). Higher concentrations of MRS2578 were not used because they have been reported to have actions at sites other than $P2Y_6$ receptors (Mitchell *et al*., 2012). Thus these data indicate that UDP-evoked relaxation appears to be mainly through $P2Y_6$ receptors and that the remaining site at which UTP elicits vasodilatation is not the $P2Y_6$ receptor.

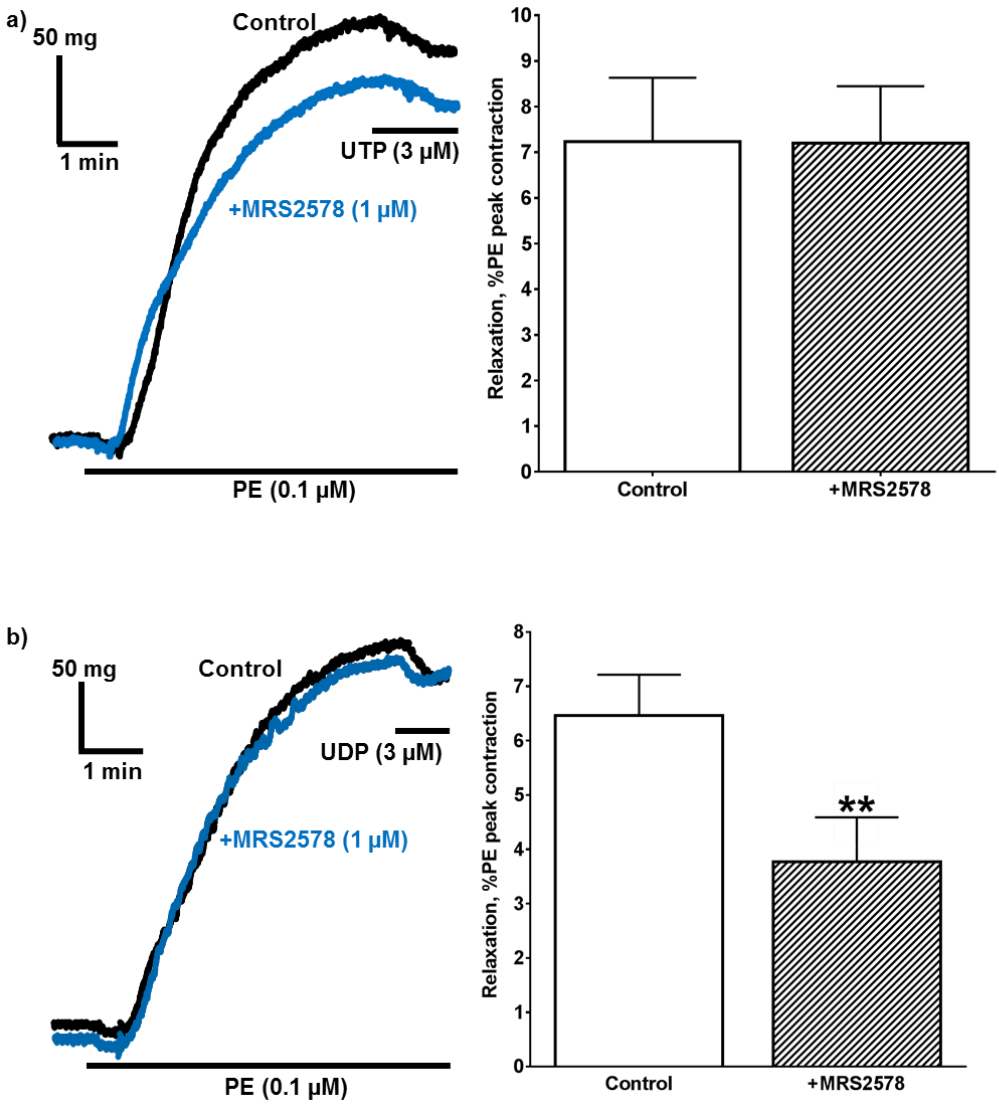


Figure 4.16: Effects of MRS2578 on relaxations evoked by UTP and UDP. Left-hand panels: The superimposed traces show typical relaxation of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) UTP (3 µM) and b) UDP (3 µM) in the absence (black) and presence (blue) of MRS2578 (1 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) UTP (3 µM) and b) UDP (3 µM) alone (Control) and in the presence of MRS2578 (1 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by UDP produced in the presence of MRS2578 compared to in its absence.

The presence of $P2Y_6$ receptors in rIPA were studied further using the selective $P2Y_6$ receptor agonists, INS48823 and P-UDP. INS4882 is a potent $hP2Y_6$ agonist, but has no appreciable activity at $hP2Y_1$, $hP2Y_2$ or $hP2Y_4$ receptors (Korcok *et al.,* 2005), while P-UDP has 500-fold selectivity at $hP2Y_6$ over $hP2Y_2$ receptors and is virtually inactive at the $hP2Y_4$ and $hP2Y_{14}$ receptors (El-Tayeb *et al.,* 2006; Gao *et al.,* 2010b). In the two tissues tested, INS48823 (100 nM - 3 µM) elicited small, but concentration-dependent relaxations and at 10 µM, vasoconstriction (figure 4.17a). Further experiments could not be carried out due to lack of time. Surprisingly, P-UDP (100 nM - 1 µM) evoked, at most, very small relaxations in precontracted rIPA and at 3 µM it tended to cause further vasoconstriction (figure 4.17b).

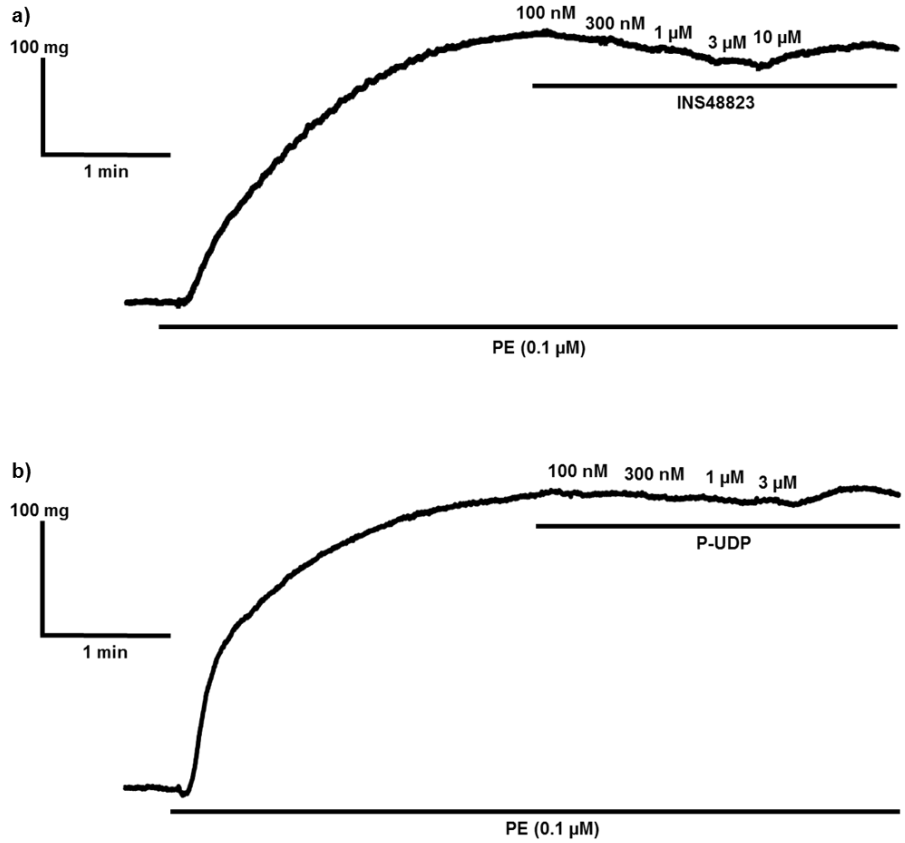


Figure 4.17: Effects of selective $P2Y_6$ agonists on rIPA at raised tone. The traces show the effects of a) INS48823 (100 nM - 10 µM and b) P-UDP (100 nM - 3 µM) on endothelium-intact rIPA that had been precontracted by PE (0.1 µM). INS48823 and P-UDP were added cumulatively, as indicated by the horizontal bars. The responses are typical of those seen in 2 (INS48823) and 8 (P-UDP) tissues.

Finally, the non-selective, non-competitive, allosteric P2Y antagonist, suramin, was used to investigate which other P2Y receptor subtypes UTP and UDP may act at to elicit vasodilation of rIPA. 300 µM was used as this concentration of suramin was maximally effective at inhibiting UTP- and UDP-evoked contractions of rIPA (Chootip *et al.,* 2002). When preincubated with the tissue for 20 min, suramin (300 µM) had no effect on basal tone of rIPA, but it significantly reduced contractions evoked by PE (0.1 µM) to 88.1 ± 2.0% of control, n=24, $P < 0.0001$. Surprisingly, however, suramin had no effect on the vasodilation evoked by UTP (3 µM) (99.5 ± 8.2% of control, n=6; figure 4.18a) and UDP (3 µM) (105.3 ± 5.7% of control, n=6; figure 4.18b).

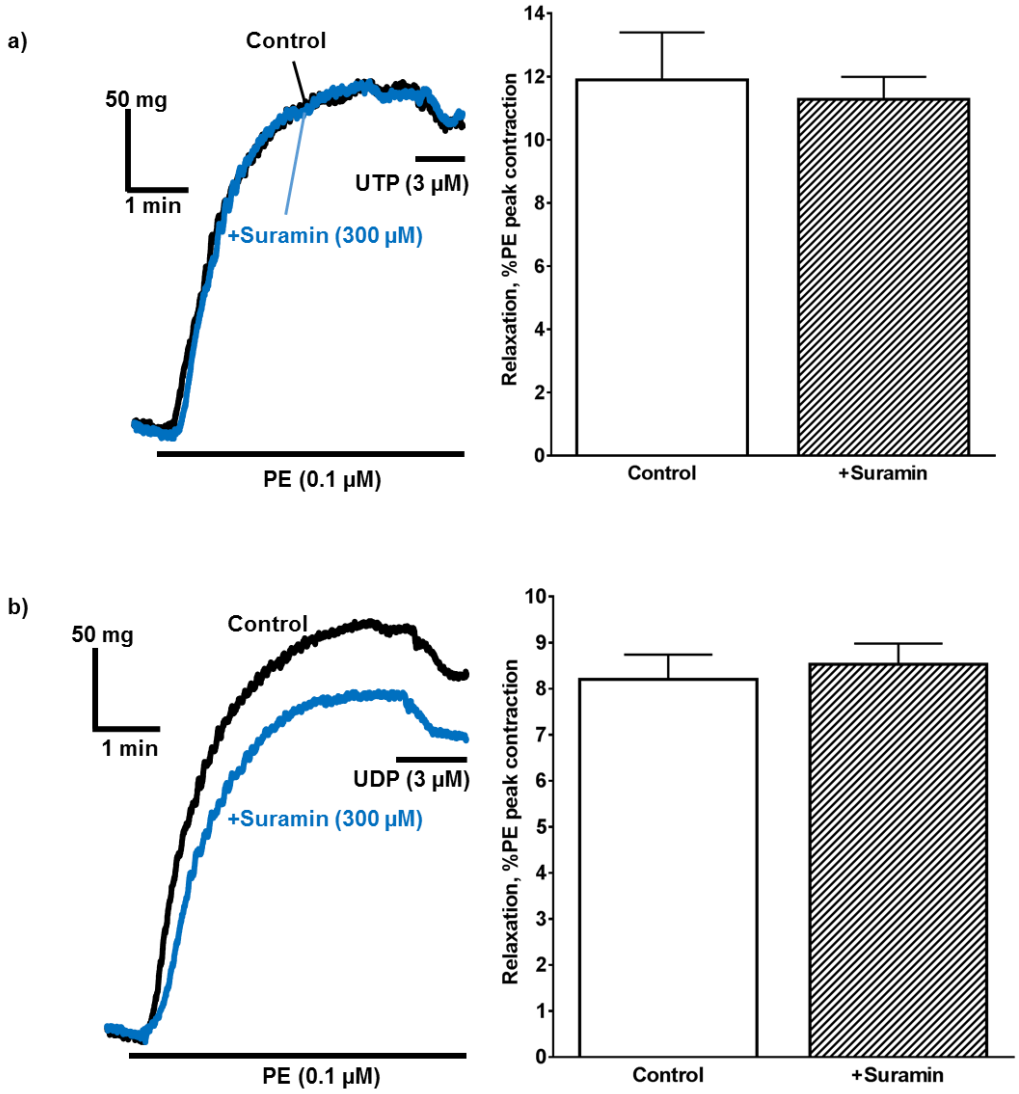


Figure 4.18: Effects of suramin on relaxations evoked by UTP and UDP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) UTP (3 µM) and b) UDP (3 µM) in the absence (black) and presence (blue) of suramin (300 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) UTP (3 µM) and b) UDP (3 µM) alone (Control) and in the presence of suramin (300 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each.

4.2.3.4 Which receptors mediate adenine nucleotide-evoked vasodilatation?

As reported in Figure 4.15b, AR-C118925XX had no effect on ADP-induced vasodilation, which is consistent with the inability of ADP to stimulate $P2Y_2$ receptors (Abbracchio *et al*., 2006; Jacobson *et al*., 2020). In contrast, ATP is a potent $P2Y_2$ receptor agonist, so the lack of effect of AR-C118925XX on ATP-induced vasodilation (figure 4.15a) was a surprise. Therefore, in the next series of experiments, the roles of other adenine-nucleotide-sensitive P2Y receptor subtypes were investigated. First, the effects of the selective $P2Y_1$ receptor antagonist, MRS2179 (10 μM), a concentration that is 100 times higher than its K_B at $hP2Y_1$ receptors (100 nM; Boyer *et al.*, 1998), were determined.

When preincubated with the tissue for 20 min, MRS2179 (10 μM) had no effect on basal tone of rIPA, though it slightly, but significantly inhibited the contractions evoked by PE (0.1 μM) (92.0 ± 0.9% of control, n=12, $P < 0.01$). MRS2179 also had no effect on the relaxations produced by ATP (10 μM) (104.5 ± 6.5% of control n=6; figure 4.19a), but significantly reduced those evoked by ADP (10 μM) by more than half (46.2 ± 10.0% of control, n=6, $P < 0.01$; figure 4.19b). Thus these data show that ADP, but not ATP acts through $P2Y_1$ receptors to induce vasodilation of rIPA.

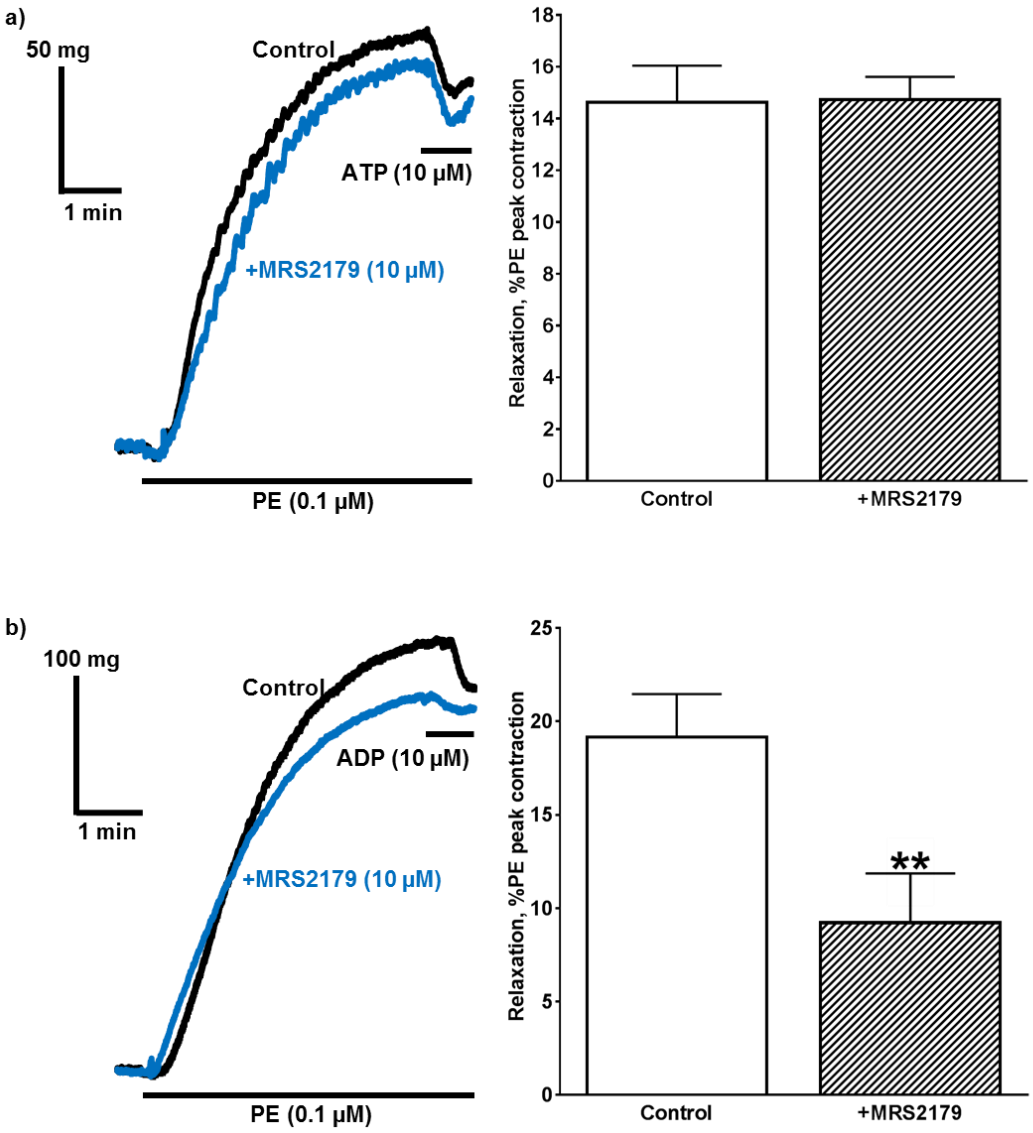


Figure 4.19: Effects of MRS2179 on relaxations evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of MRS2179 (10 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of MRS2179 (10 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by ADP in the presence of MRS2179 (10 µM) compared to in its absence.

The presence of $P2Y_1$ receptors in rIPA was studied further using MRS2365, a selective and potent $P2Y_1$ receptor agonist (Ravi *et al.,* 2002; Chhatriwala *et al.,* 2004). In two tissues MRS2365 (100 nM - 100 µM) produced large, transient, concentration-dependent relaxations of rIPA with EC_{50} values of 1.7 and 4.2 µM (figure 4.20). Further experiments could not be carried out due to lack of time. Nonetheless, these data are consistent with endothelial $P2Y_1$ receptors mediating substantial vasodilation.

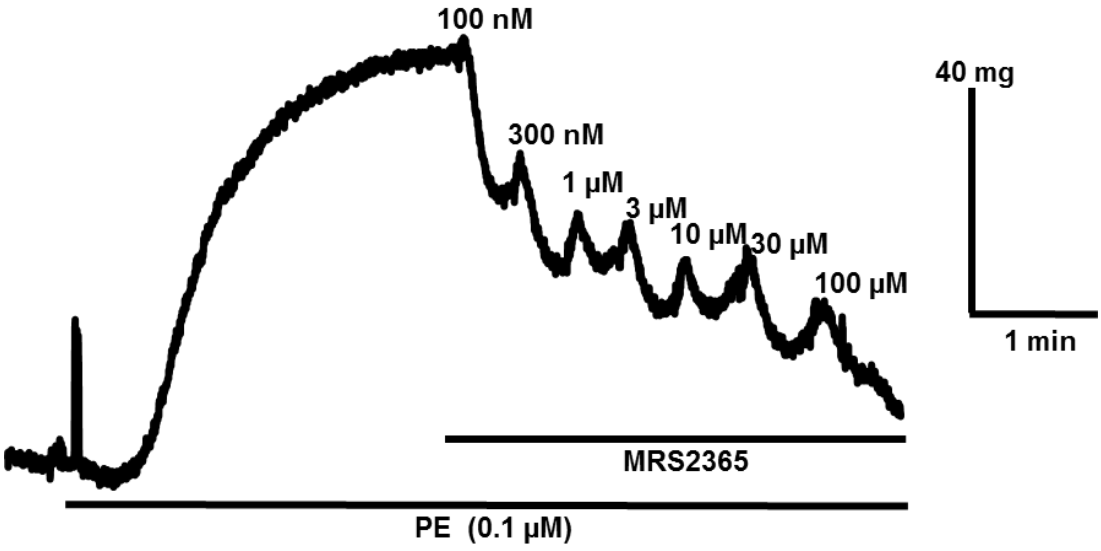


Figure 4.20: A selective $P2Y_1$ agonist elicits vasodilation of rIPA at raised tone. The traces show relaxation of PE (0.1 µM) precontracted, endothelium-intact rIPA induced by cumulative additions of MRS2365 (100 nM - 100 µM), as indicated by the horizontal bars. The responses are typical of those seen in 2 tissues.

Next, the effects of AR-C69913MX, an antagonist with low or sub-nM potency at $P2Y_{12}$ (Ingall *et al.,* 1999; Takasaki *et al.,* 2001; Hoffmann *et al.,* 2008) and $P2Y_{13}$ (Marteau *et al.,* 2003; Fumagalli *et al.,* 2004) receptors, on the ATP- and ADP-evoked relaxations of rIPA were investigated. When preincubated with the tissue for 20 min, AR-C69913MX (1 µM) had no effect on basal tone of rIPA, contractions evoked by PE (0.1 µM) (103.3 ± 4.1% of control, n=12) or on the relaxations evoked by ATP (10 µM) (104 ± 8.3% of control, n=6; figure 4.21a). However, AR-C69913MX (1 µM) significantly increased the amplitude of the relaxations evoked by ADP (10 µM) by 37.0 ± 6.6%, n=6; $P < 0.01$; figure 4.21b).

These data indicate that $P2Y_{12}$ and/or $P2Y_{13}$ receptors mediate vasoconstriction of rIPA, which partially counteracts the vasodilation induced by ADP. The inhibitory effect of MRS2179 on ADP-induced relaxations was, therefore, re-examined under conditions in which the counteractive contractions were blocked. Combined application of MRS2179 (10 µM) and AR-C69913MX (1 µM), significantly reduced the ADP-evoked relaxation to 50.9 ± 4.9% of control (n=6, $P < 0.001$; figure 4.22a, b), which was not significantly different from the effect produced by MRS2179 alone (46.2 ± 10.0% of control) as reported above in figure 4.19b. The results show that approximately 50% of the ADP-evoked vasodilatation is mediated through $P2Y_1$ receptors, and the remainder of the relaxation, and all of ATP's action, do not involve $P2Y_{12}$ or $P2Y_{13}$ receptors.

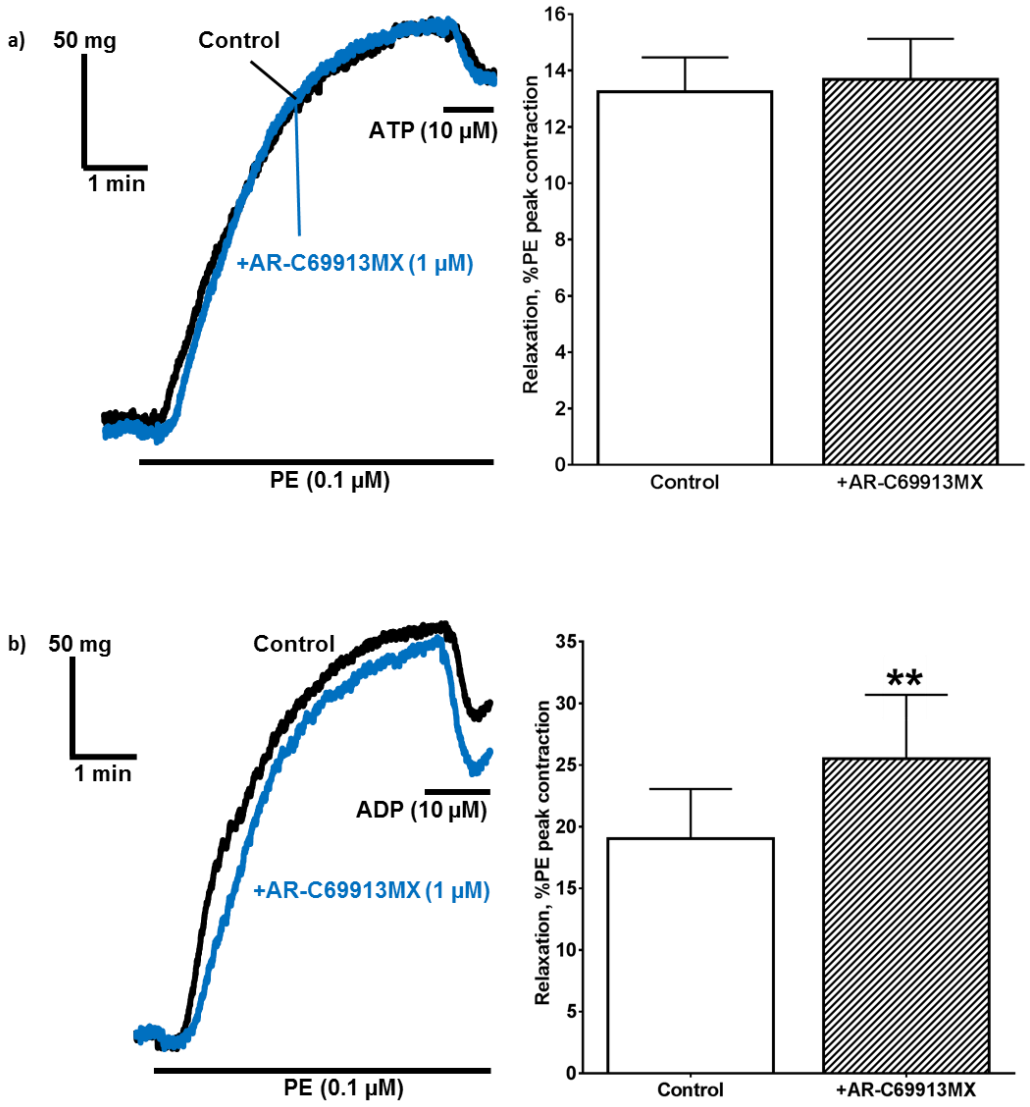


Figure 4.21: Effects of AR-C69913MX on relaxations evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of AR-C69913MX (1 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of AR-C69913MX (1 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each. ** $P < 0.01$ for responses produced by ADP in the presence of AR-C69913MX (1 µM) compared to in its absence.

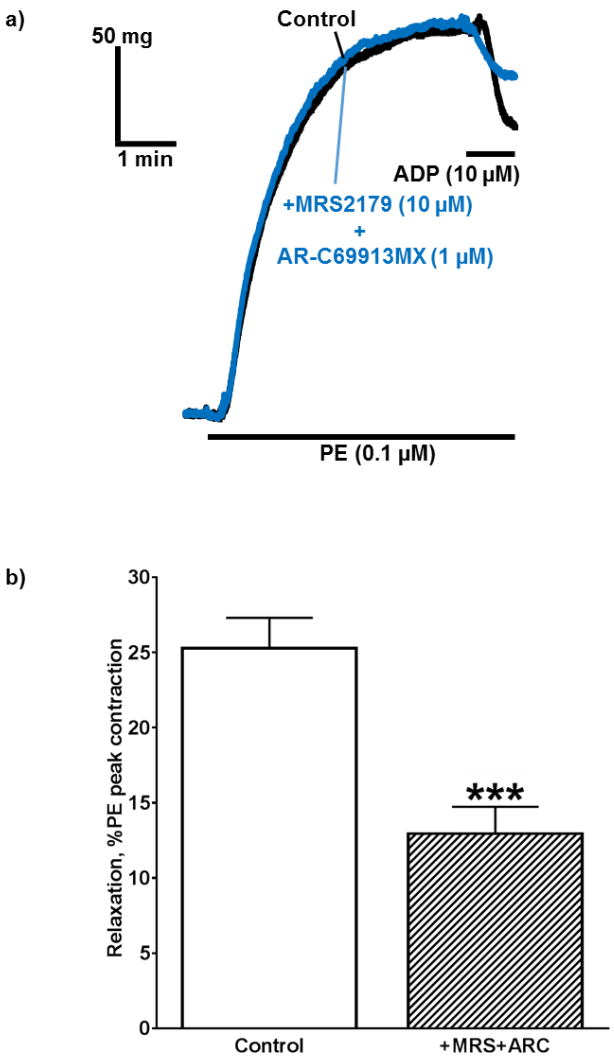


Figure 4.22: Inhibition of relaxations evoked by ADP. a) The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA by ADP (10 µM) in the absence (black) and (presence (blue) of MRS2179 (10 µM) plus AR-C69913MX (1 µM). Drugs were added as indicated by the horizontal bars. b) The mean peak amplitude of relaxations evoked by ADP (10 µM) alone (Control) and in the presence of MRS2179 (10 µM) plus AR-C69913MX (1 µM) (+MRS+ARC), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6. *** $P < 0.001$ for responses produced by ADP (10 µM) in the presence of MRS2179 (10 µM) plus AR-C69913MX (1 µM) compared to in their absence.

Next, the effects of the non-selective P2Y antagonists, suramin and PPADS were determined. Suramin (300 µM) significantly reduced the relaxations evoked by ATP (10 µM) to 66.9 ± 8% of control (n=6; figure 4.23a) and by ADP (10 µM) to 64 ± 7.8% of control (n=6; figure 4.23b). PPADS was used at a concentration of 100 µM, as this was maximally effective at inhibiting ATP-evoked contractions of rIPA (Chootip *et al.*, 2005). When preincubated with the tissue for 20 min, PPADS (100 µM) had no effect on basal tone of rIPA, but significantly increased the peak amplitude of contractions evoked by PE (0.1 µM) to 134.5 ± 6.0% of control (n=6, $P < 0.0001$). PPADS also reduced the relaxations evoked by ATP (10 µM) to 59.1 ± 10.7% of control (n=3; figure 4.24a) and by ADP (10 µM) to 23.3 ± 2.2% of control (n=3; figure 4.24b) and, but only the latter effect was significant ($P < 0.001$). Further experiments could not be carried out due to lack of time.

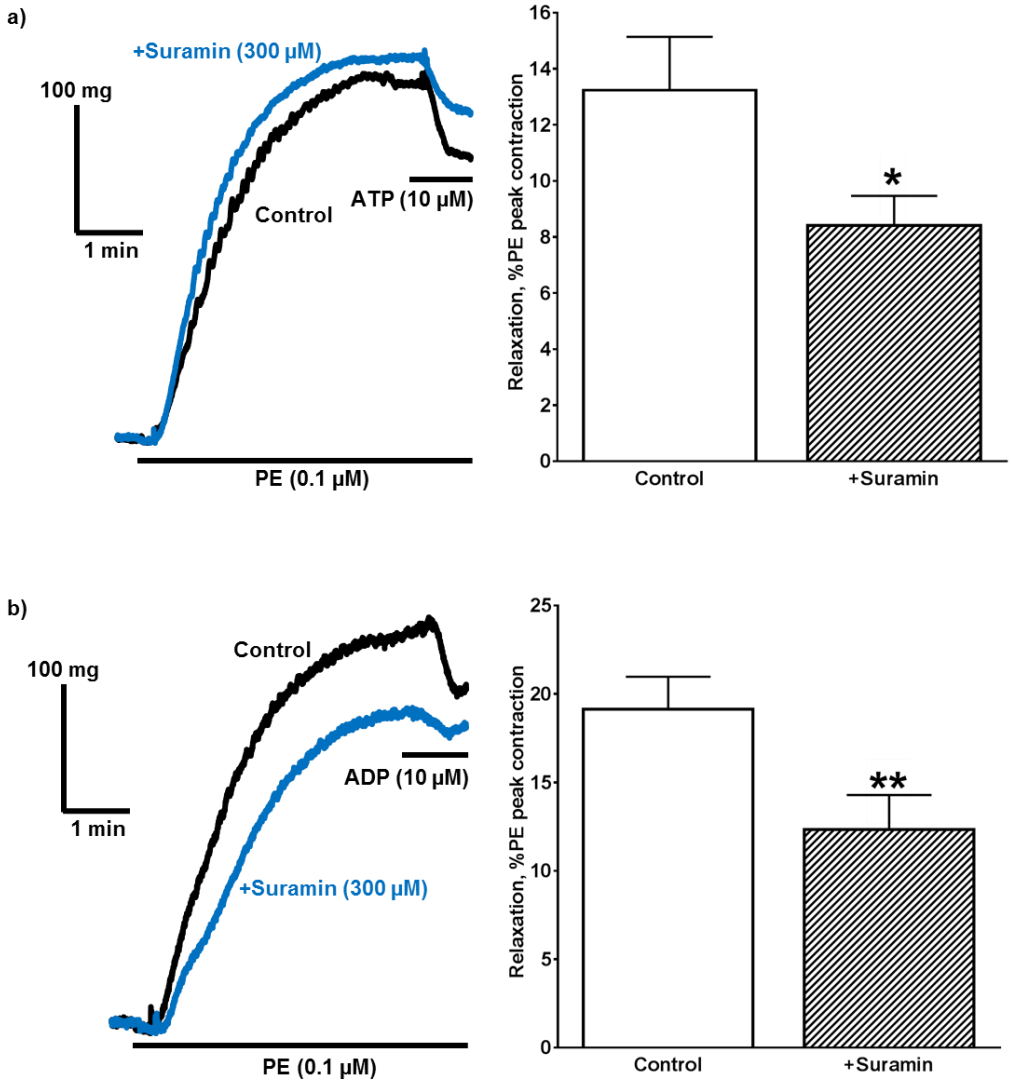


Figure 4.23: Effects of suramin on relaxations evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of suramin (300 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of suramin (300 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=6 each. * $P < 0.05$ and ** $P < 0.01$ for responses produced by ATP and ADP respectively, in the presence of suramin (300 µM) compared to in its absence.

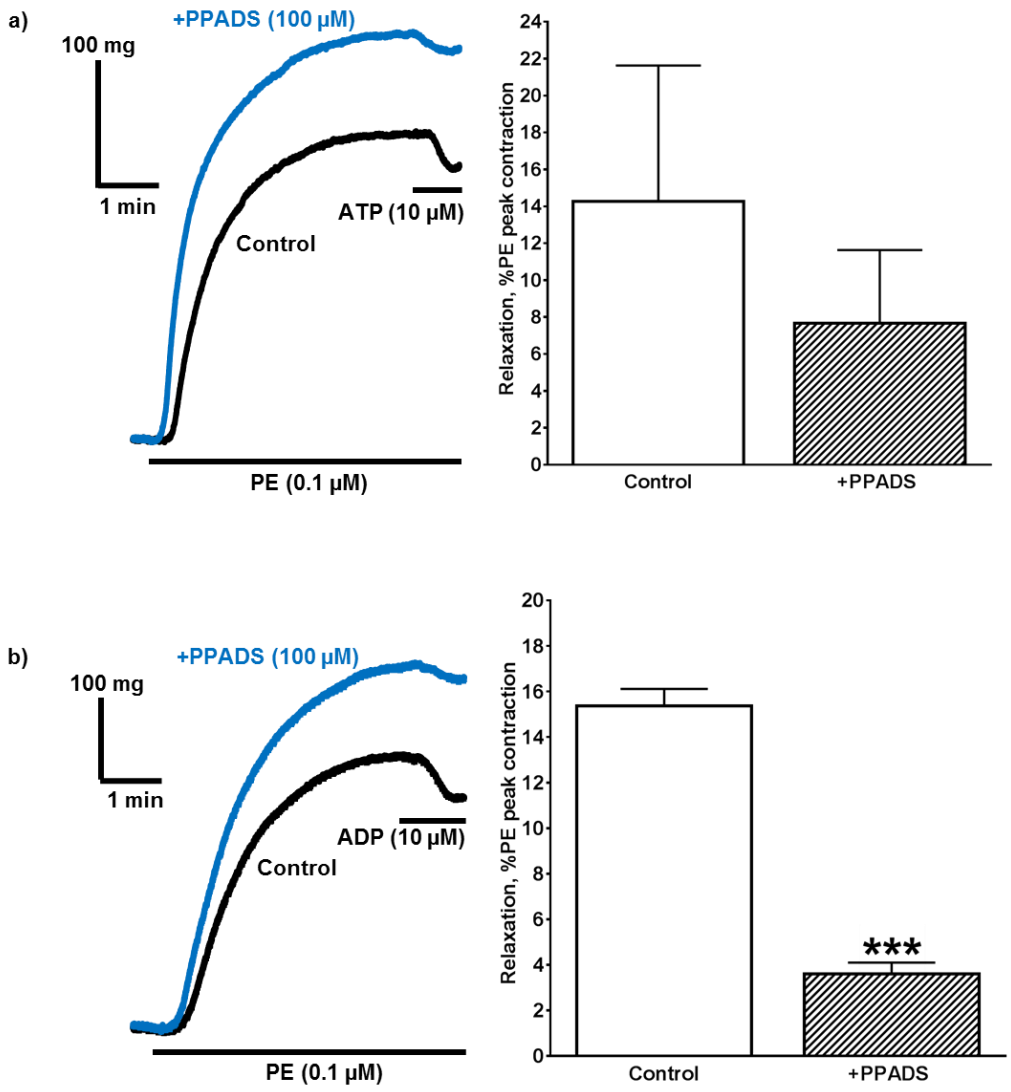


Figure 4.24: Effects of PPADS on relaxations evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of PPADS (100 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of PPADS (100 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=3 each. *** $P < 0.001$ for responses produced by ADP in the presence of PPADS (100 µM) compared to in its absence.

Finally, extracellular ATP and ADP can both be progressively dephosphorylated by a family of ecto-nucleotidases, leading to the production of adenosine, which can act at P1 receptors to elicit vasodilation (Burnstock and Kennedy, 1986; Rayment *et al.,* 2007; Alsaqati *et al.,* 2014). To address the possibility that ATP and ADP may indirectly induce vasodilatation of rIPA via P1 receptors, the effects of CGS15943 (1 µM), a potent P1 receptor antagonist, were determined. At this concentration CGS15943 has been shown to substantially block P1 receptors (Ongini *et al.*, 1999; Ilie *et al*., 2012; Sivaramakrishnan *et al.*, 2012).

When preincubated with the tissue for 20 min, CGS15943 (1 µM) had no effect on basal tone of rIPA, but significantly reduced the contractions evoked by PE (0.1 µM) to 49.2 ± 4.4% of control (n=7, $P < 0.0001$). CGS15943 (1 µM) had no effect on the relaxation evoked by ATP (10 µM; 105.5 ± 8.8% of control, n=4; figure 4.25a) and ADP (10 µM; 98.4 ± 13.5% of control, n=3; figure 4.25b). Thus the relaxations evoked by ATP and ATP do not involve breakdown to adenosine and stimulation of P1 receptors.

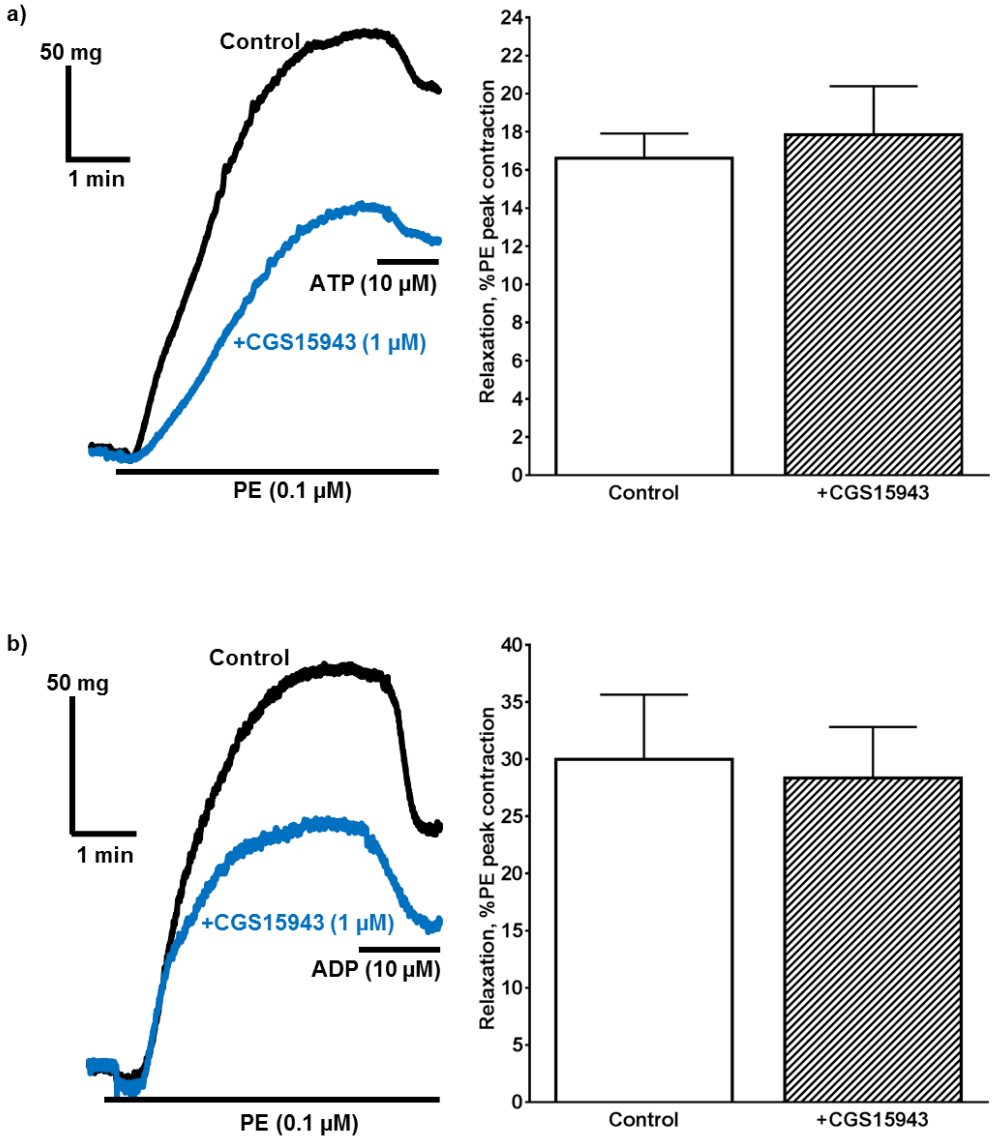


Figure 4.25: Effects of CGS15943 on relaxation evoked by ATP and ADP. Left-hand panels: The superimposed traces show typical relaxations of PE (0.1 µM) pre-contracted, endothelium-intact rIPA induced by a) ATP (10 µM) and b) ADP (10 µM) in the absence (black) and presence (blue) of CGS15943 (1 µM). Drugs were added as indicated by the horizontal bars. Right-hand panels: The mean peak amplitude of relaxations evoked by a) ATP (10 µM) and b) ADP (10 µM) alone (Control) and in the presence of CGS15943 (1 µM), expressed as a percentage of the contraction elicited by PE, are shown. Vertical lines indicate SEM. n=4 (ATP) and n=3 (ADP).

4.3 DISCUSSION

The data reported in this chapter demonstrated that UTP, ATP and UDP evoked reproducible contractions of rIPA at resting tone when applied repeatedly. These three nucleotides and ADP also elicited reproducible, endothelium-dependent vasodilation when the tone was raised by PE. This reproducibility enabled me to study which P2Y receptors underlie the actions of the nucleotides in the pulmonary vasculature. The selective $P2Y_2$ receptor antagonist, AR-C118925XX, inhibited relaxations induced by UTP and UDP, but not ATP or ADP and had no effect on any of the contractions. These results, together with the effects of several other selective and non-selective P2Y receptor agonists and antagonists, revealed that the profile of the individual P2Y receptor subtypes that mediate pulmonary vasoconstriction and vasodilation is complex.

4.3.1 Vasoconstriction of rIPA

Here, repeated addition of UTP, ATP, and ADP evoked vasoconstriction in endothelium-intact rIPA that were reproducible. However in endothelium-denuded IPA, a decrease in contraction over time was observed. It is unclear why this was so, but it may be an intact endothelium helps maintain artery integrity under *in vitro* conditions and the act of physically removing the endothelium reduced artery viability over time. O_2 levels can also affect tissue responses. In normoxic conditions (10-21% O_2) *),* K^+ channels play a key role in maintaining membrane hyperpolarisation, which limits voltage gated Ca^{2+} entry and further contraction. In hypoxic conditions (1-5% O_2), PASMCs depolarise due to dysfunction of voltage-gated K^+ channels, leading to an increase in intracellular Ca^{2+} thereby affecting the contractility of the PASMCs (Humbert *et al.,* 2004; Moudgil *et al.,* 2006). The present experiments, however, were carried out in normoxic conditions (constant bubbling of bathing solution with medical air (21% O_2, 5% CO_2 and 74% N_2), so hypoxia is unlikely to be a factor in the decrease in contraction amplitude.

AR-C118925XX did not affect basal tone of rIPA when added alone and also had no effect on contractions evoked by UTP, ATP, and UDP. The concentration of AR-C118925XX used, 1 µM, is 270 times greater than its K_B at $P2Y_2$ receptors (3.7 nM)

determined in the previous chapter and caused a substantial rightwards shift in the CRC produced by UTP and ATP in the Ca^{2+} bioassay, so would be expected to inhibit any component of the nucleotide-evoked contractions that was mediated by $P2Y_2$ receptors. Thus these experiments show that $P2Y_2$ receptors do not appear to be involved in contractions of rIPA evoked by UTP, ATP or UDP. This was a surprise, as a previous study in the Kennedy lab, Chootip *et al*., (2002), found that suramin, which is an antagonist at $P2Y_2$, but largely ineffective at $P2Y_4$ and $P2Y_6$ receptors, caused a progressive rightwards shift in the UTP and UDP CRC at 30 and 100 µM, but 300 µM suramin did not produce any further inhibition. In contrast, the contractions were unaffected by PPADS and RB2, which inhibit $P2Y_4$ and $P2Y_6$ receptors, but not $P2Y_2$ receptors. Thus, they suggested that UTP and UDP may act via two P2Y receptors in rIPA, the $P2Y_2$ receptors and either $P2Y_4$ or $P2Y_6$ receptors. In a subsequent study, the UDP-evoked contractions were partially inhibited by the low potency $P2Y_6$ antagonist, MRS2578, and the selective $P2Y_6$ agonist, PUDP, was more potent than UDP, consistent with this suggestion (Mitchell *et al*., 2012). In addition, MRS2578 inhibited UTP-evoked contractions to a similar extent (Mitchell and Kennedy, unpublished observations). Note that knockout of $P2Y_6$ receptors abolished UTP- and UDP-evoked contractions of mouse aorta (Kauffenstein *et al*., 2010), confirming that UTP can stimulate this subtype. Thus $P2Y_6$ receptors underlie at least a part of the contractile activity of UDP and UTP in rIPA, but the development of selective and potent $P2Y_4$ and $P2Y_6$ receptor antagonists is needed for the roles of these P2Y subtypes in the contractions of rIPA elicited by UTP and UDP to become clearer. $P2Y_{14}$ receptors are unlikely to contribute, as a high concentration of the $P2Y_{14}$ agonist, UDP-glucose, did not cause contraction of rIPA (Mitchell *et al*., 2012).

Interestingly, a 10-fold higher concentration of AR-C118925XX, 10 µM, virtually abolished contractions of rat small pulmonary veins induced by ATP (Henriquez *et al*., 2018). The lack of effect of the antagonist on contractions in my studies is unlikely to be because the concentration used, 1 µM, was too low, since this concentration did inhibit UTP-evoked vasodilation. UTP also causes vasoconstriction of rabbit (Baek *et al*. 2008) and pig (McMillan *et al*., 1999) pulmonary arteries, but the receptors mediating these actions have not been characterised pharmacologically.

The lack of effect of AR-C118925XX on contractions of rIPA evoked by ATP seen in the present study, is consistent with the reports by Chootip *et al*., (2005), in which the

responses were abolished by suramin and partially inhibited by PPADS, and by Mitchell *et al*., (2012), where they were unaffected by MRS2179, but inhibited by nearly 40% by AR-C69931MX, a $P2Y_{12/13}$ receptor antagonist, and virtually abolished by combined blockade of P2X1 and $P2Y_{12/13}$ receptors. AR-C69931MX was previously shown to abolish contractions of rIPA evoked by ADP, so ATP's action at $P2Y_{12/13}$ receptors may require that it be dephosphorylated to ADP (Mitchell *et al*., 2012). Thus my data confirm that $P2Y_2$ receptors play no role in ATP-induced contraction of rIPA. To the best of my knowledge, receptor subtype-selective antagonists have not been used to characterise how ATP causes pulmonary artery vasoconstriction in any other species.

4.3.2 Vasodilation of rIPA

In this study, ATP, ADP, UTP and UDP all evoked concentration-dependent relaxation of preconstricted rIPA, though the CRC were shallow and did not reach a clear maximum. This can arise if an agonist acts at more than one receptor and with different potencies to produce its effect and/or if agonist steady-state concentrations at the biophase next to the plasma membrane are not maintained due to agonist breakdown by ecto-enzymes or cellular uptake by transporters. Consistent with a role for agonist breakdown in the shallow CRC, knockout of ecto-nucleoside triphosphate diphosphohydrolase (CD39), an ecto-nucleotidase that dephosphorylates tri- and diphosphate nucleotidases (Robson *et al*., 2006), greatly potentiated contractions of mouse aorta evoked by exogenous UDP and UTP and greatly increased the slope of their CRC (Kauffenstein *et al*., 2010). Consequently, single concentrations of nucleotides that were in the EC_{50} -EC_{70} range were used from then on. Under these conditions, ATP and ADP produced relaxations that were larger than those produced by UTP and UDP. Furthermore, the responses to each were substantially reduced on removal of the endothelium. Rubino *et al*., (1999) also found that physical removal of the endothelium abolished vasodilation induced by ATP in rat isolated pulmonary arteries and Hasséssian and Burnstock, (1995) reported that inhibiting NO production with L-NAME converted ATP-induced vasodilation in the rat isolated, perfused pulmonary vascular bed to vasoconstriction. Thus all four nucleotides induce endothelium-dependent vasodilation of rIPA. Endothelium-dependent vasodilation to

ATP and ADP has also been reported in pulmonary arteries isolated from humans (Greenberg *et al*., 1987; Liu *et al.*, 1989; Dinh-Xuan *et al.*, 1991), to ATP in dogs (De Mey and Vanhoutte, 1982) and to ATP and UTP in rabbits (Qasabian *et al.,* 1997; Konduri *et al.,* 2004).

4.3.2.1 Receptors mediating the effects of UTP and UDP

In the previous chapter I reported that AR-C118925XX inhibited Ca^{2+} mobilisation in human EAhy926 endothelial cells, so my hypothesis here was that the vasodilation of rIPA elicited by UTP would also be inhibited by AR-C118925XX. This proved to be the case, as the UTP-evoked relaxations were reduced by ~60%. The UDP-evoked responses were also inhibited, though to a lesser extent (~33%). UDP is not an agonist at $P2Y_2$ receptors, so this was a surprise. The product data sheet provided by Sigma-Aldrich indicates that the UDP stock is "≥ 96.0%" pure". No further details are provided, but if the remaining 4% was entirely UTP then, based on the relaxant CRC generated in the initial experiments, the concentration of UTP that the tissue would be exposed to when UDP solution was added would be sufficient to produce relaxation. Alternatively, the AR-C118925XX-sensitive relaxation could reflect conversion of some of the added UDP to UTP by ecto-nucleoside diphosphokinase (NDPK) activity (see Kennedy *et al.*, 2000).

The AR-C118925XX-resistant component of the UTP-evoked vasodilation was not mediated via $P2Y_6$ receptors because the $P2Y_6$ antagonist, MRS2578, failed to inhibit it. In contrast, MRS2578 reduced the action of UDP by ~45%. It is likely that $P2Y_6$ receptors play an even greater role in the UDP-induced vasodilation, as the concentration of MRS2578 used, 1 µM, is submaximal (Mamedova *et al.,* 2004). Higher concentrations were not applied because they have been reported to have actions at sites other than $P2Y_6$ receptors (Mitchell *et al*., 2012). Consistent with the presence of endothelial $P2Y_6$ receptors, INS4882, a potent $hP2Y_6$ agonist that has no appreciable activity at $hP2Y_1$, $hP2Y_2$ or $hP2Y_4$ receptors (Korcok *et al*., 2005), also induced concentration-dependent relaxations. Surprisingly, another $P2Y_6$-selective agonist, P-UDP evoked only slight vasodilation. The reason for this discrepancy is unclear.

Unexpectedly, suramin was ineffective against both UTP and UDP, even though it is a $P2Y_2$ receptor antagonist. It is unclear why it did not mimic the effects of AR-C118925XX. $P2Y_4$ receptors are suramin-insensitive (Charlton *et al.,* 1996) and so might play a role in the antagonist-resistant components of vasodilation. However, as noted above, no selective $P2Y_4$ receptor antagonists are currently available. Regardless, the data reported in this chapter demonstrate that UTP and UDP both elicit endothelium-dependent vasodilation of rIPA, with UTP acting in part via $P2Y_2$ receptors and UDP in part via $P2Y_2$ and $P2Y_6$ receptors. Complete characterisation of the receptors involved is, however, limited by the off-site actions of higher concentrations of MRS2578 and the lack of selective $P2Y_4$ receptor antagonists. As noted above, UTP also induces endothelium-dependent vasodilation of rabbit isolated pulmonary artery (Qasabian *et al*., 1997; Konduri *et al*., 2004), but the receptors mediating this effect have not been studied. Thus at present, the data reported in this chapter are the most detailed pharmacological characterisation of UTP-sensitive, pulmonary endothelial P2Y receptors.

4.3.2.2 Receptors mediating the effects of ATP and ADP

In contrast to its inhibitory actions against UTP and UDP, AR-C118925XX had no effect on ADP-evoked relaxation of rIPA. This is consistent with the inability of ADP to stimulate $P2Y_2$ receptors (Abbracchio *et al*., 2006; Jacobson *et al*., 2020). Responses to ADP were, however, substantially inhibited by the selective $P2Y_1$ receptor antagonist, MRS2179, and the nonselective antagonists, suramin and PPADS, both of which are active at $P2Y_1$ receptors (Abbracchio *et al*., 2006; Jacobson *et al*., 2020). In addition, the selective $P2Y_1$ receptor agonist, MRS2365 (Ravi *et al*., 2002; Chhatriwala *et al*., 2004), induced similar large, concentration-dependent relaxations. ADP also stimulates $P2Y_{12}$ and $P2Y_{13}$ receptors, but the $P2Y_{12/13}$ receptor antagonist, AR-C69931MX, potentiated rather than inhibited the ADP-induced vasodilation. This is likely due to it preventing the counteractive vasoconstriction induced by ADP via smooth muscle $P2Y_{12/13}$ receptors (Mitchell *et al*., 2012). ADP can be broken down to adenosine by ecto-nucleotidases, but adenosine receptors, which are expressed in endothelial cells (Feoktistov *et al.,* 2002), do not contribute to ADP's actions, as they were unaffected by the potent and non-selective adenosine receptor

antagonist, CGS1593 (Ongini *et al*., 1999: Ilie *et al*., 2012; Sivaramakrishnan *et al.*, 2012). Together, these data indicate that $P2Y_1$ receptors are the major site of relaxation of rIPA by ADP and that $P2Y_{12/13}$ and adenosine receptors are not involved.

ATP is a potent $P2Y_2$ receptor agonist (Abbracchio *et al*., 2006; Jacobson *et al*., 2020), so the lack of effect of AR-C118925XX on ATP-induced vasodilation of rIPA was a surprise. ATP is a partial agonist at $P2Y_1$ receptors (Palmer *et al*., 1998), but unlike ADP, does not appear to act at them here, as MRS2179 also had no effect on ATP's actions. Likewise, the lack of inhibition by CGS1593 shows that adenosine receptors are not involved. ATP-induced relaxations were inhibited, however, by suramin and PPADS, so $P2Y_4$ and/or P2X4 receptors might play a role. Most P2X receptor subtypes are not expressed in endothelial cells, but there is strong evidence that endothelial P2X4 receptors contribute to flow-dependent control of vascular tone. Endothelial cells release ATP in response to the shear stress at their surface generated by blood flow (Bodin *et al.,* 1991) and ATP acts at P2X4 receptors to induce vasodilation (Yamamoto *et al.,* 2000). This response is greatly decreased by knockout of the P2X4 receptor and the blood pressure of these mice is higher than in wild-type animals (Yamamoto *et al.,* 2006). Reasonably selective P2X4 antagonists have become available in recent years (Illes *et al.,* 2021) and so could be used to determine the contribution of P2X4 receptors to the vasodilation of rIPA induced by ATP. At present, however, the receptor(s) through which ATP acts to cause vasodilation of rIPA remain uncertain.

4.3.3 Modulation of PE-induced vasoconstriction by purinergic antagonists

Several of the antagonists used here inhibited the vasoconstriction induced by PE, indicating that they may have inhibitory effects at sites other than purinergic receptors. This is true for MRS2578, which was previously shown to suppress KCl-evoked contractions of rIPA (Mitchell *et al*., 2012). It may not be the case, however, for suramin, as endogenous ATP has been shown to contribute to α_1-receptor-mediated vasoconstriction, following its release from vascular smooth muscle cells via pannexin-1 channels (Billaud *et al.,* 2011, 2015; Begandt *et al.,* 2017). Thus vasoconstriction of mouse thoracodorsal resistance arteries induced by PE was reduced by suramin and by pannexin-1 inhibitors, siRNA and knockdown. It was

associated with ATP release from smooth muscle cells, which was also suppressed by inhibition of pannexin-1. In contrast, 5-HT and endothelin-1 did not induce ATP release and the contractions they produced were unaffected by pannexin-1 inhibition. Pannexin-1 knockout also reduced PE-evoked contractions of mouse mesenteric artery (Kauffenstein *et al*., 2016). The inhibition of PE-evoked vasoconstriction of rIPA by suramin may, therefore, be due to it inhibiting the contractile action of released ATP, most likely at P2X1 receptors. The rIPA also expresses a low level of contractile $P2Y_1$ receptors (Mitchell *et al*., 2012), but they are unlikely to be involved, as MRS2179 had no effect on PE-evoked contractions. It is unclear why CGS1593 caused a large decrease or why PPADS potentiated the contractions.

In conclusion, UTP, UDP, ATP produced reproducible vasoconstriction on the rIPA at resting tone that were unaffected by AR-C118925XX, which indicates that contractile $P2Y_2$ receptors are not functionally expressed in rIPA VSMC. At raised tone, these agonists produced endothelium-dependent vasodilation that was reproducible and consistent with previous studies. UTP-evoked vasodilatation is mainly through $P2Y_2$ receptors and $P2Y_6$ receptors are not involved. UDP-evoked relaxation is mainly via $P2Y_6$ receptors and $P2Y_2$ receptors also appear to contribute to its action. ATP-induced endothelium-dependent relaxation is not via $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$ or adenosine receptors, but may be through $P2Y_4$ and/or P2X4 receptors. ADP elicited vasodilation mainly via $P2Y_1$ receptors and the remaining response is not mediated by $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$ or adenosine receptors. Together, these data extend our understanding of contractile purinergic receptors in rIPA and represent the most detailed pharmacological characterisation of the receptors that mediate endothelium-dependent vasodilation, but further studies with selective antagonists are needed to obtain a full understanding.

5 Characterisation of P2Y Receptors in Rat Tail Artery

5.1 INTRODUCTION

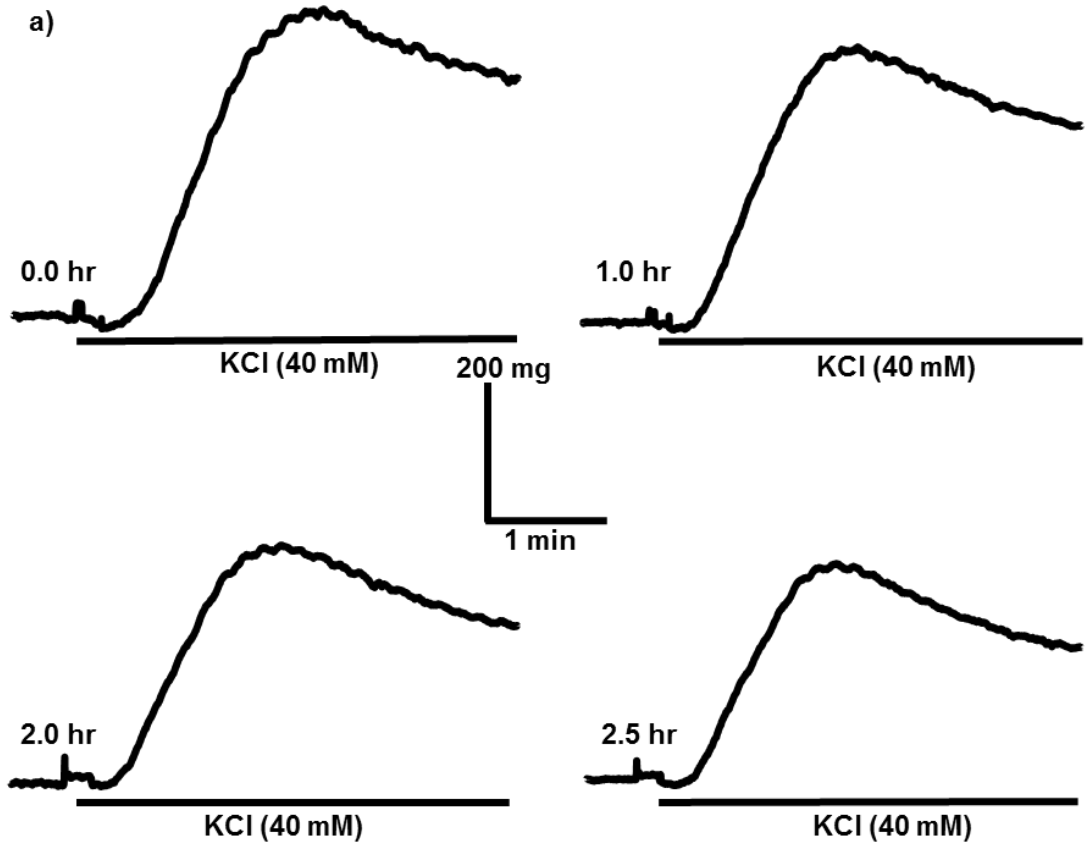
In the previous chapter the functional expression of P2Y receptors in pulmonary arteries was characterised. The pulmonary vascular bed is, however, a specialised low-resistance, low-pressure circuit that is designed to maximize the exchange of CO_2 and O_2 in the alveoli and which carries deoxygenated blood away from the heart. Thus conclusions reached based on data obtained in rIPA may not be applicable to systemic arteries and vascular beds, which have higher resistance and pressure and which carry oxygenated blood away from the heart. The rTA is a systemic artery that has been frequently used to study P2 receptor expression and function. For example, ATP has been shown to be an excitatory co-transmitter with NA from sympathetic nerves (Sneddon and Burnstock, 1985; McLaren *et al.,* 1995b), consistent with a high density of P2X1 receptors in the smooth muscle cells (Bo and Burnstock 1993) and the ability of ATP to elicit fast inward currents and vasoconstriction (Evans and Kennedy, 1994). ATP also induced vasoconstriction via Ca^{2+} release from the sarcoplasmic reticulum, indicating activation of P2Y receptors (McLaren *et al.,* 1995a). UTP-evoked vasoconstriction of rTA also requires Ca^{2+} release from the sarcoplasmic reticulum (McLaren *et al*., 1995a), as well as Ca^{2+} influx through $Ca_v1.2$ channels (Tengah *et al*., 2018). There are, however, no published reports on nucleotide-evoked vasodilation of rTA.

At present, the characterisation of the receptors through which these nucleotides (especially UTP) produce their effects in rTA has been difficult to determine due to the unavailability of selective P2 receptor subtype antagonists. The introduction of AR-C118925XX has now made it possible to determine the involvement of $P2Y_2$ receptors. Thus the primary aim of this set of experiments was, therefore, to determine if AR-C118925XX inhibited nucleotide-evoked vasoconstriction of rTA. The ability of nucleotides to elicit vasodilation was also studied.

5.2 RESULTS

5.2.1 Reproducibility of Nucleotide-Induced Contractions

The aim of the initial set of experiments was to determine if nucleotide-evoked contractions of rTA are reproducible. Previous studies in the Kennedy lab used only endothelium-denuded tissues, which produced large, reliable responses. Therefore, this approach was continued here. As in the previous chapter, KCl, which acts independently of receptors, was used as a control. When added to rTA for 5 min, six times at 30 min intervals, KCl (40 mM) evoked contractions that were of similar amplitude (figure 5.1). There was no significant difference between the amplitudes of the first contraction (503 ± 66 mg, n=7) and the subsequent responses.



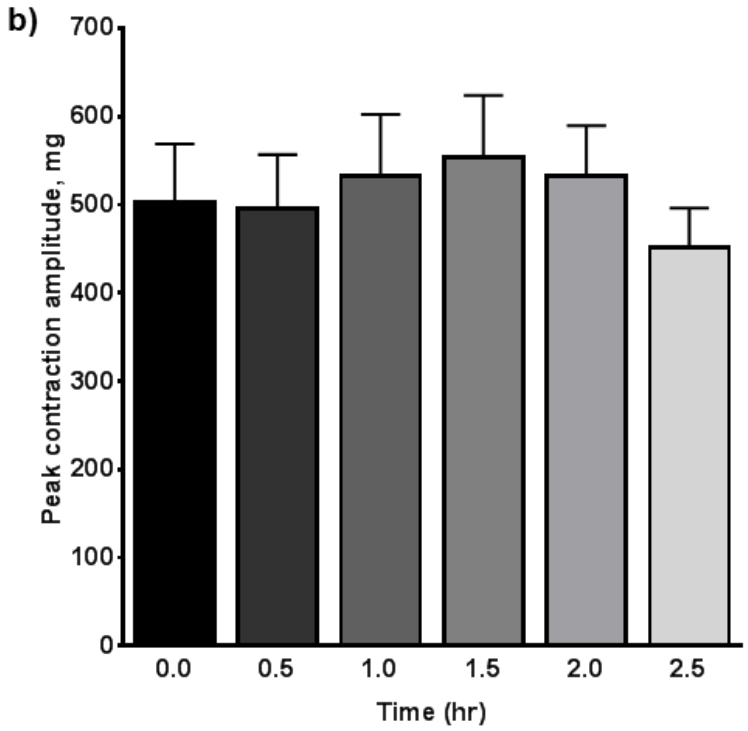


Figure 5.1: KCl-induced vasoconstriction of rTA. a) The traces show typical contractions of endothelium-denuded rTA evoked by addition of KCl (40 mM), as indicated by the horizontal bars, at 0.0, 1.0, 2.0 and 2.5 hr. b) The mean peak amplitude of contractions induced by KCl (40 mM) are shown. Vertical lines indicate SEM. n = 7.

When UTP (1 mM) was added to the rTA for 5 min, six times at 30 min intervals, it produced vasoconstrictions that were of similar amplitude throughout (figure 5.2) and there was no significant difference between the amplitudes of the first contraction (289 ± 24 mg, n=7) and the subsequent responses.

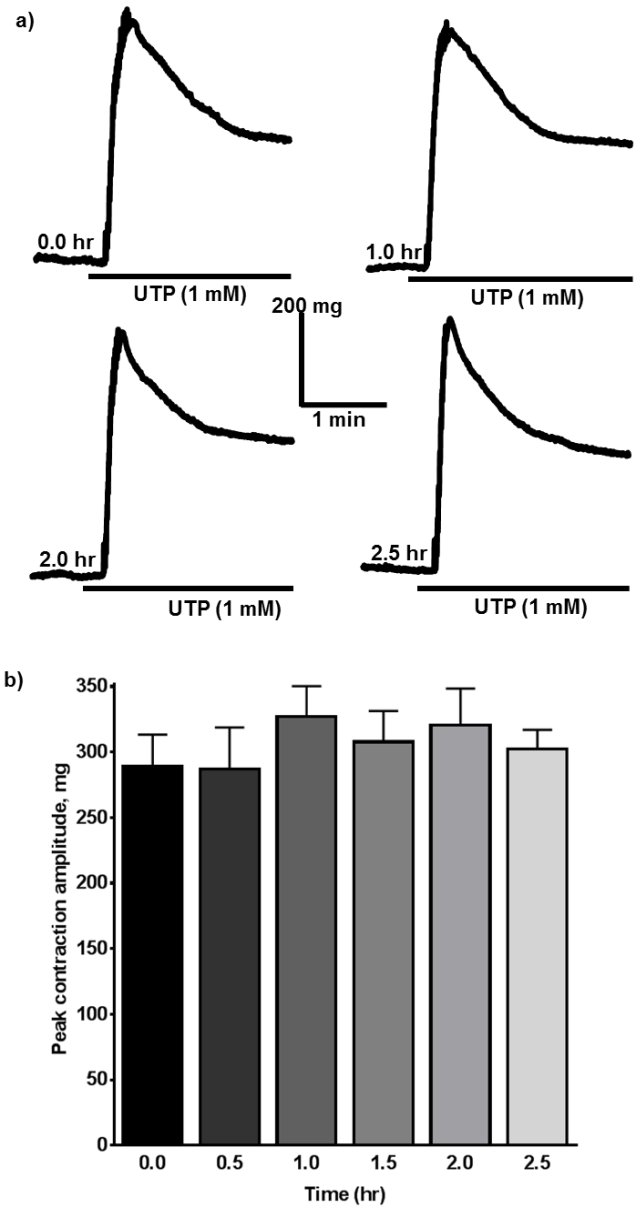


Figure 5.2: UTP-induced vasoconstriction of rTA. a) The traces show typical contractions of endothelium-denuded rTA evoked by addition of UTP (1 mM), as indicated by the horizontal bars, at 0, 1.0, 2.0 and 2.5 hr. b) The mean peak amplitude of contractions induced by UTP (1 mM) are shown. Vertical lines indicate SEM. n = 7.

ATP (1 mM) also produced contractions that were reproducible in the endothelium-denuded rTA when added for 5 min, six times at 30 min intervals (figure 5.3). There was no significant difference in the mean amplitudes of the response to the first addition (286 ± 33 mg, n=6) and the subsequent responses.

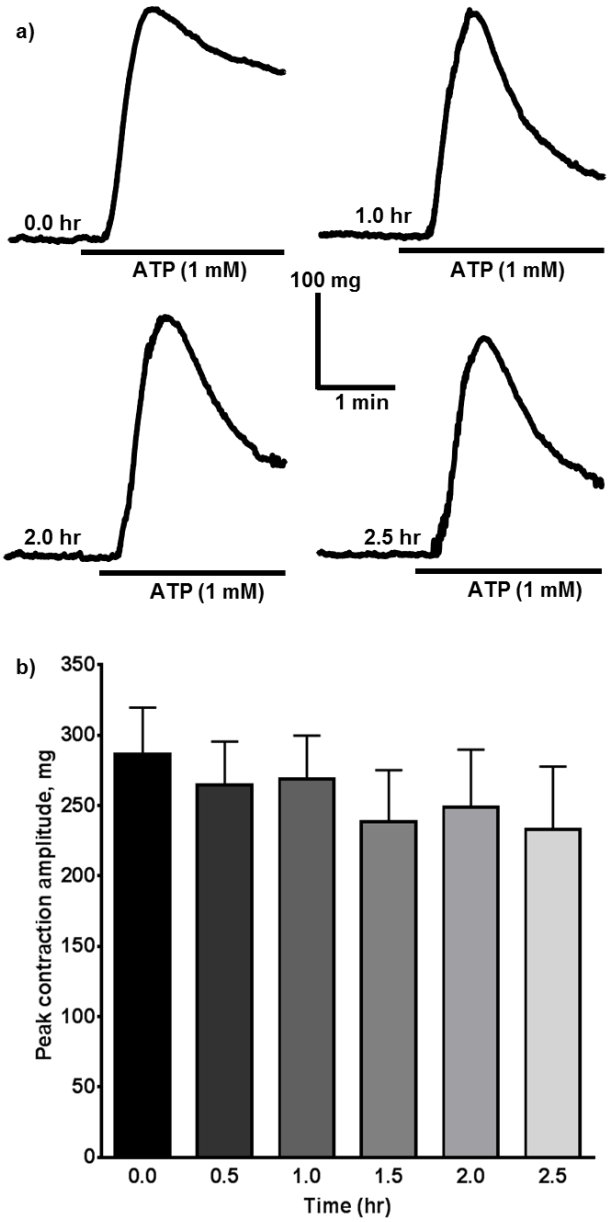


Figure 5.3: ATP-induced vasoconstriction of rTA. a) The traces show typical contractions of endothelium-denuded rTA evoked by addition of ATP (1 mM), as indicated by the horizontal bars, at 0, 1.0, 2.0 and 2.5 hr. b) The mean peak amplitude of contractions induced by ATP (1 mM) are shown. Vertical lines indicate SEM. n = 6.

Finally, when UDP (1 mM) was added to the endothelium-denuded rTA for 5 min, six times at 30 min intervals, it too produced contractions that were of similar amplitudes throughout (figure 5.4) and there was no significant difference in the amplitudes of the first contraction (249 ± 44 mg, n=6) compared to subsequent responses.

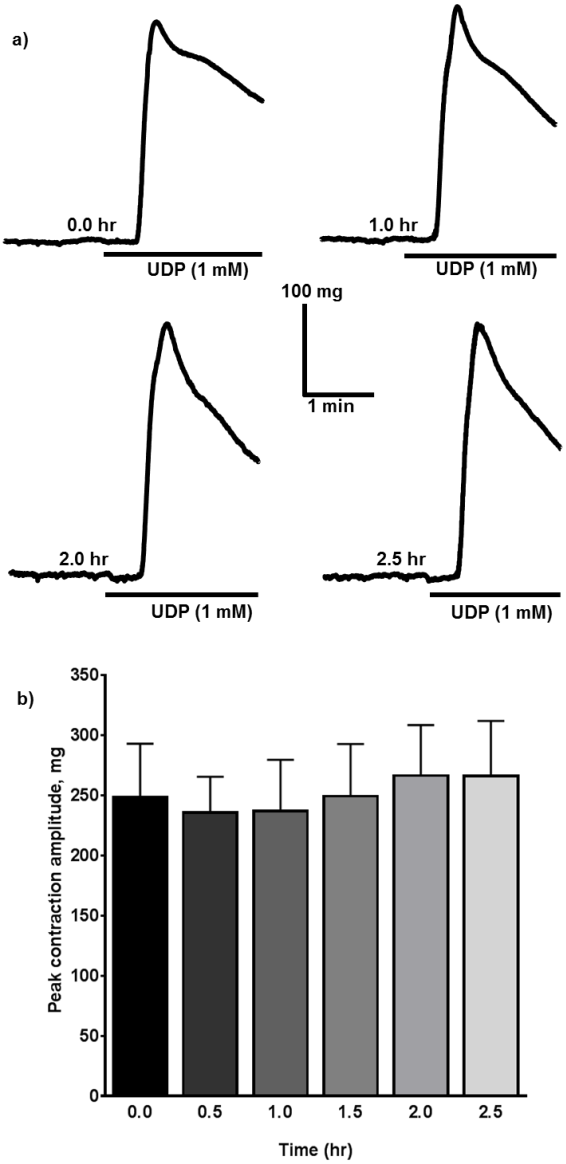


Figure 5.4: UDP-induced vasoconstriction of rTA. a) The traces show typical contractions of endothelium-denuded rTA evoked by addition of UDP (1 mM), as indicated by the horizontal bars, at 0.0, 1.0, 2.0 and 2.5 hr. b) The mean peak amplitude of contractions induced by UDP (1 mM) are shown. Vertical lines indicate SEM. n = 6.

5.2.2 Effects of AR-C118925XX on KCl- and Nucleotide-Induced Contractions

The data reported above show that UTP, ATP and UDP all evoke reproducible contractions of endothelium-denuded rTA. This enabled me to next proceed to characterise the effects of AR-C118925XX on the nucleotide-evoked contractions. It is notable in the published literature that 10 μM is the most commonly used concentration of AR-C118925XX (discussed in more detail in the General Discussion). In light of this and the lack of effect of 1 μM AR-C118925XX against nucleotide-evoked contractions of rIPA reported in chapter 4, the higher concentration was used in the following experiments in rTA.

When preincubated for 20 min, AR-C118925XX (10 μM) had no effect on basal tone or on contractions evoked by KCl (40 mM; figure 5.5), UTP (1 mM) (figure 5.6a), ATP (1 mM) (figure 5.6b) or UDP (1 mM) (figure 5.6c). Thus $P2Y_2$ receptors do not appear to contribute to contractions of rTA evoked by UTP, ATP and UDP.

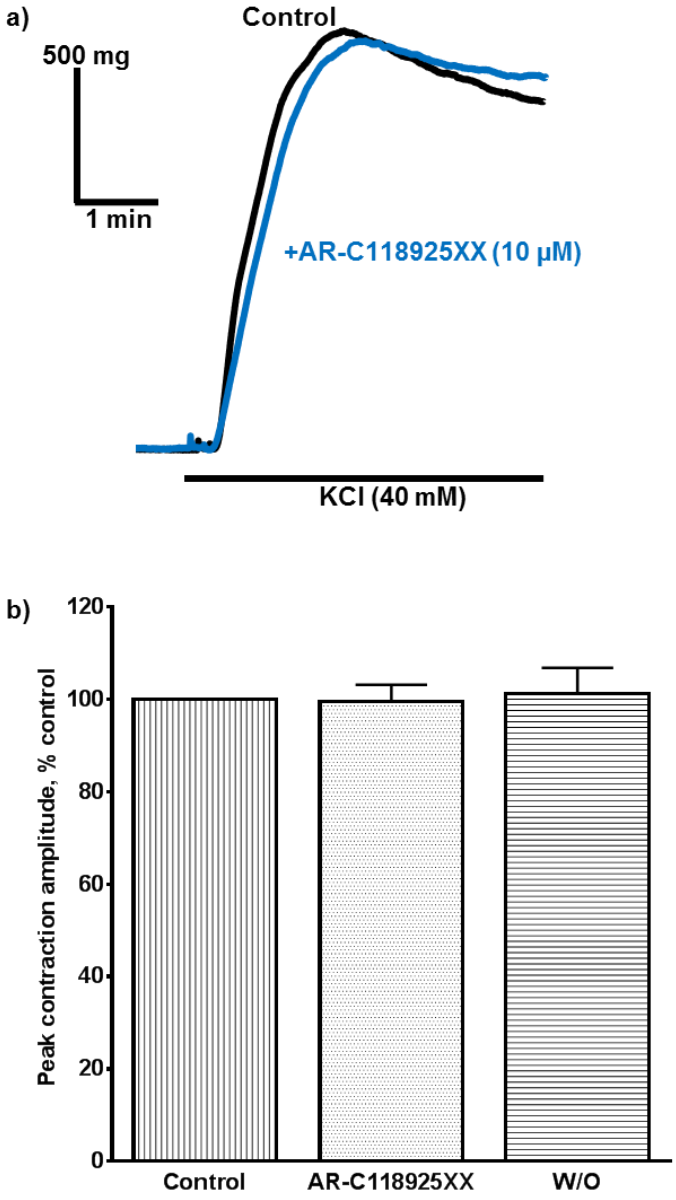
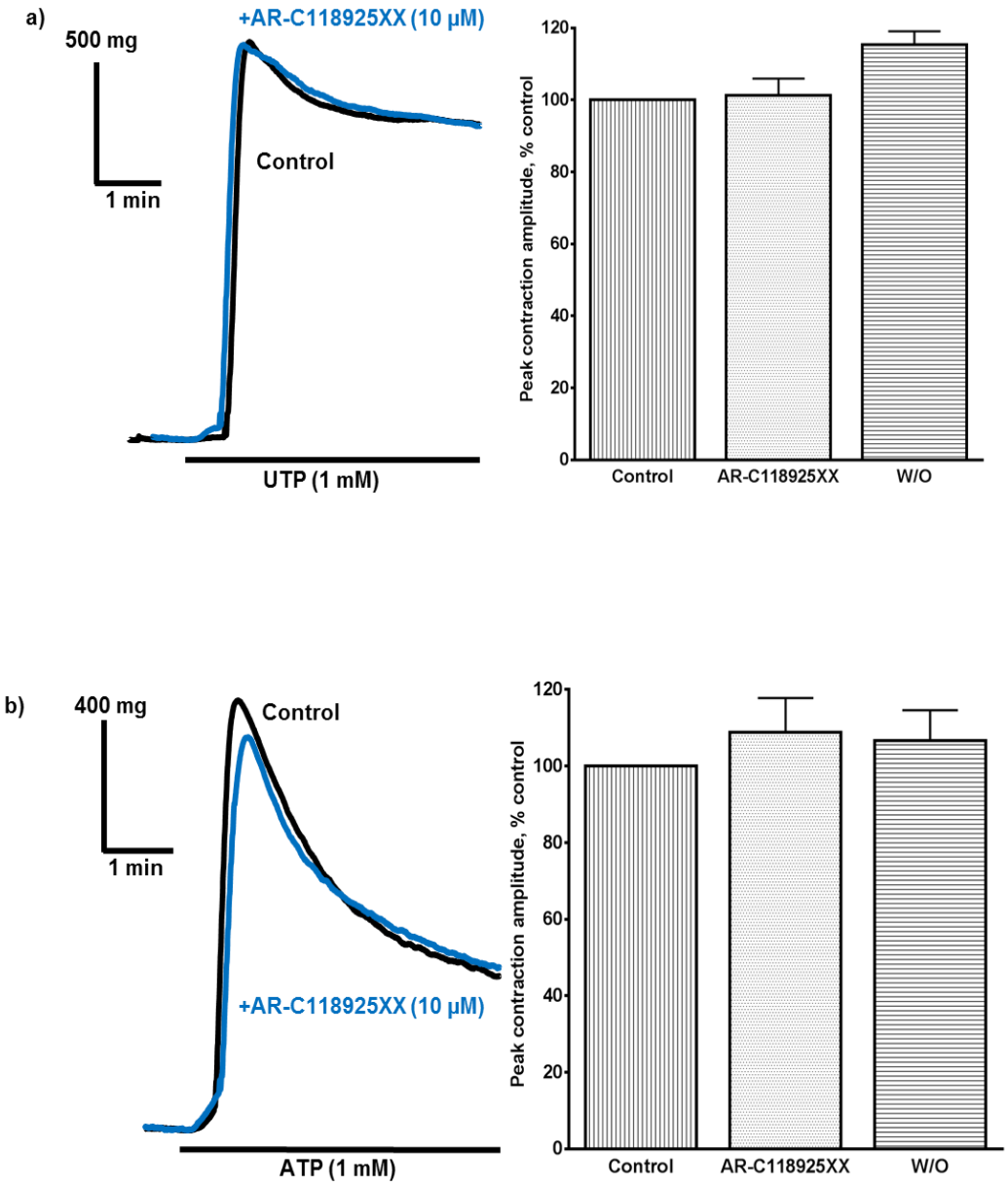


Figure 5.5: Effect of AR-C118925XX on contractions of rTA evoked by KCl. a) The superimposed traces show typical contractions of endothelium-denuded rTA evoked by addition of KCl (40 mM), as indicated by the horizontal bar, in the absence (black) and presence (blue) of AR-C118925XX (10 µM). b) The mean peak amplitude of contractions evoked by KCl (40 mM) alone (Control), in the presence (AR-C118925XX) and after washout of AR-C118925XX (10 µM) (W/O) are shown. Vertical lines indicate SEM. n=6.


a)
500 mg
1 min
+AR-C118925XX (10 µM)
Control
UTP (1 mM)
Peak contraction amplitude, % control
120
100
80
60
40
20
0
Control
AR-C118925XX
W/O
b)
400 mg
1 min
Control
+AR-C118925XX (10 µM)
ATP (1 mM)
Peak contraction amplitude, % control
120
100
80
60
40
20
0
Control
AR-C118925XX
W/O

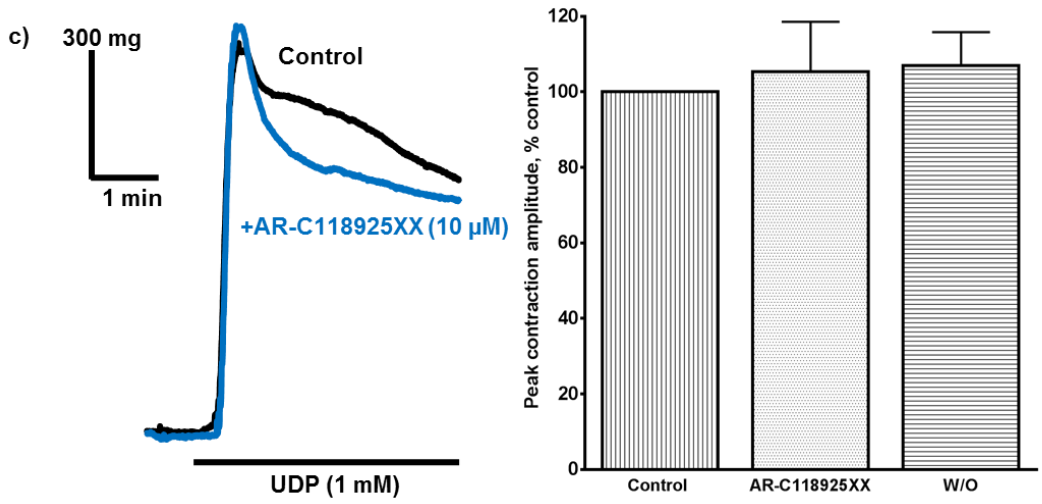


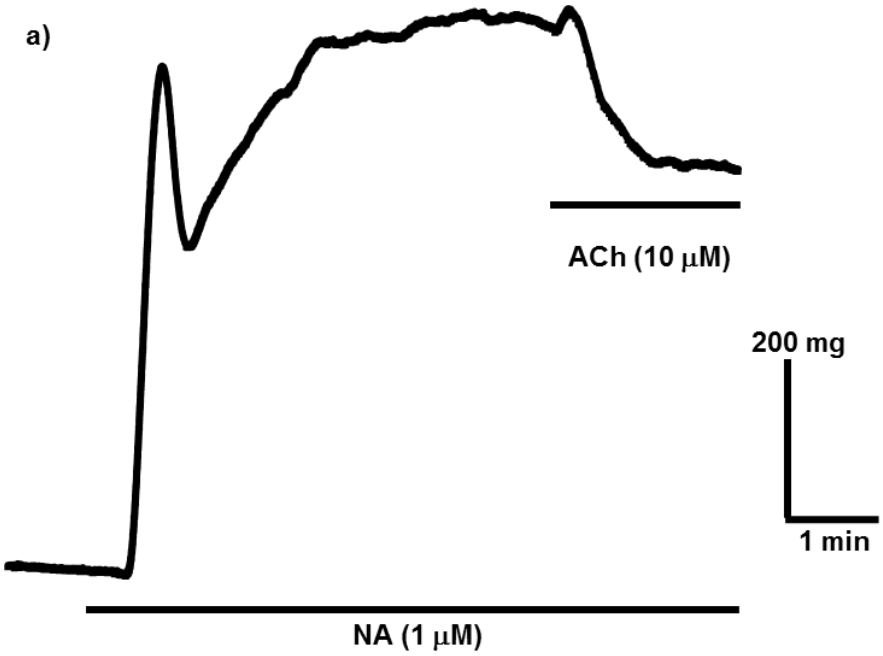
Figure 5.6: Effects of AR-C118925XX on contractions of rTA evoked by UTP, ATP and UDP. Left hand panels: The superimposed traces shows typical contractions of endothelium-denuded rTA evoked by addition of a) UTP (1 mM), b) ATP (1 mM) and c) UDP (1 mM), as indicated by the horizontal bars, in the absence (black) and presence (blue) of AR-C118925XX (10 µM). Right hand panels: The mean peak amplitude of the contractions evoked by a) UTP (1 mM), b) ATP (1 mM) and c) UDP (1 mM) alone, (Control), in the presence (AR-C118925XX) and on washout (W/O) of AR-C118925XX (10 µM) are shown. Vertical lines indicate SEM. n=4 (UTP), n=6 (ATP, UDP).

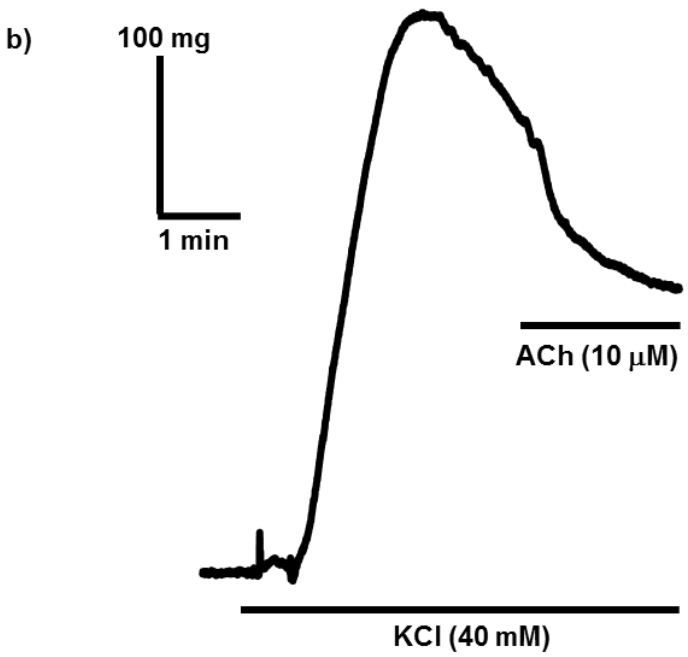
5.2.3 Effects of UTP, UDP and ATP at Raised Tone

In the final set of experiments the ability of nucleotides to elicit endothelium-dependent vasodilation of the rTA was investigated. First, tissues were precontracted with NA (1 µM) and ACh was then applied. NA-induced contractions reached peak amplitude (957 ± 113 mg, n=23) within 1-5 min. ACh then evoked biphasic responses, with small, transient contractions (36 ± 5 mg, n=23) being followed by small relaxations of variable amplitude (6.2 ± 1.5%, n=23; figure 5.7a). None of the nucleotides produced a clear relaxation in these tissues. Instead, UTP and ATP and UDP (10 µM - 1 mM) elicited concentration-dependent contractions (figures 5.8, 5.9). The responses were small and transient at the lower concentrations, whilst those to the highest concentrations of UTP and ATP were more maintained. At each concentration there was no significant difference between the amplitude of the responses evoked by the three nucleotides.

The inability of ACh to evoke a large relaxation of NA-precontracted tissues was a surprise, so its action on KCl-precontracted tissues was determined. Under these conditions ACh elicited a much larger vasodilation (37.1 ± 2.3%, n=23; figure 5.7b). A similar response was seen, however, in tissues in which the endothelium had been physically removed (32.2 ± 3.7%, n=13; figure 5.7c). When added to precontracted tissues from which the endothelium had not been removed, UTP, ATP and UDP (10 µM - 1 mM) did not elicit vasodilation, but again produced maintained, concentration-dependent contractions (figures 5.10, 5.11). At 1 mM, the contractions evoked by UDP were significantly smaller than those evoked by ATP ($P<0.01$).

These data indicated that P2Y receptors do not mediate endothelium-dependent vasodilation of the rTA.


a)
ACh (10 μM)
200 mg
1 min
NA (1 μM)


b)
100 mg
1 min
ACh (10 μM)
KCl (40 mM)

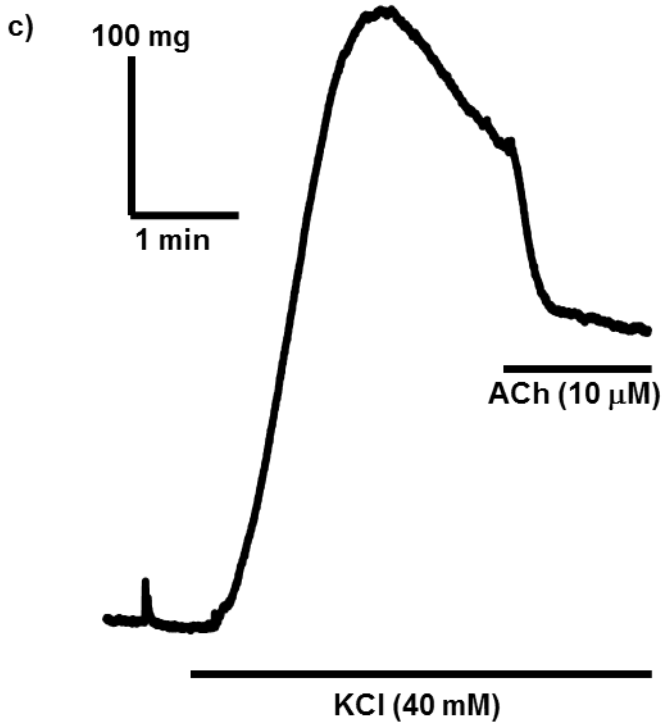


Figure 5.7: The effects of ACh on precontracted rTA. The traces show typical effects of ACh (10 μM) on endothelium-intact rTA precontracted by a) NA (1 μM and b) KCl (40 mM) and c) on endothelium-denuded rTA precontracted by KCl (40 mM), added as indicated by the horizontal bars.

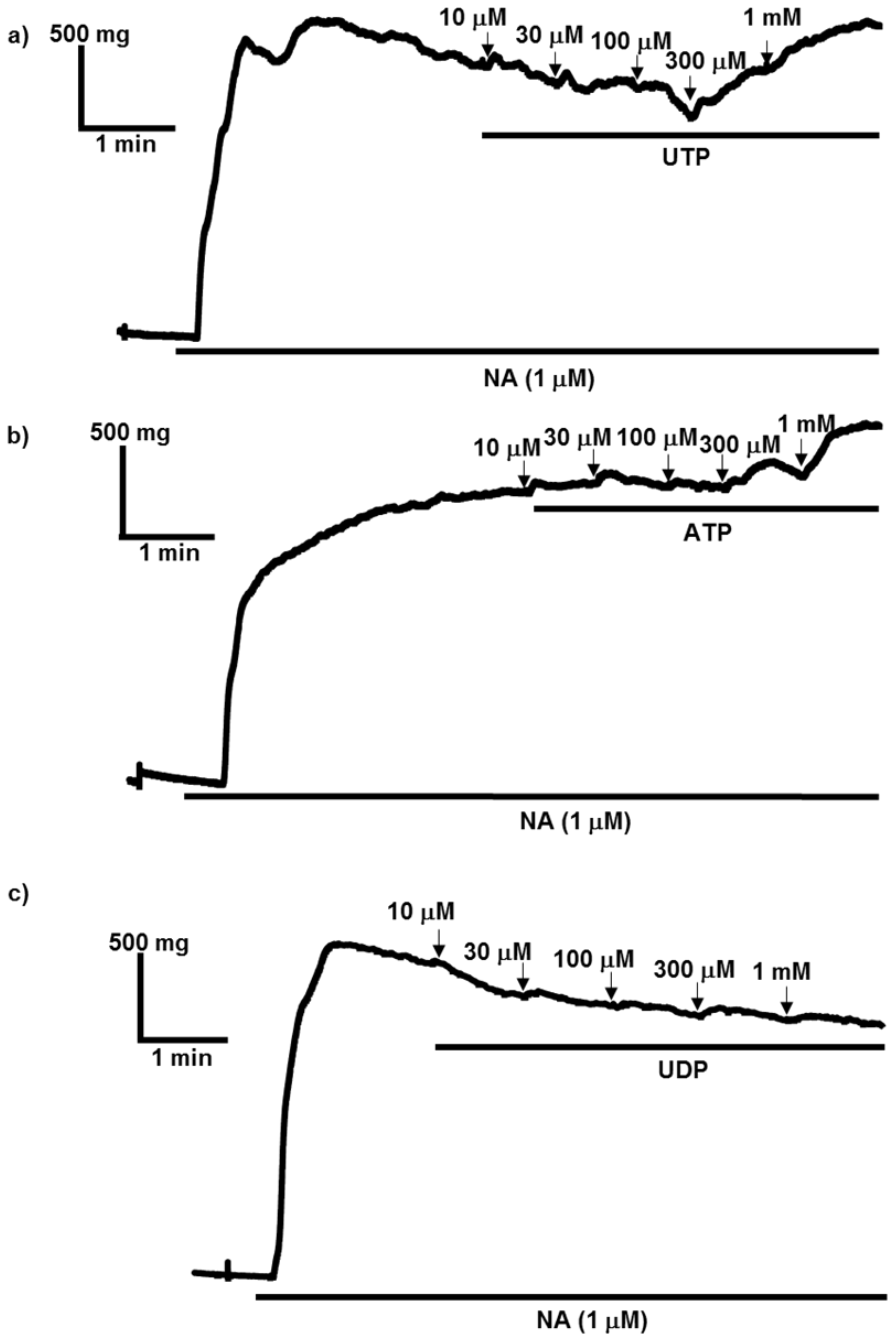


Figure 5.8: Nucleotides elicit vasoconstriction of rTA at raised tone. The traces show typical responses of NA (1 μM) pre-contracted, endothelium-intact rTA induced by cumulative addition of a) UTP, b) ATP and c) UDP (10 μM - 1 mM), as indicated by the horizontal bars and arrows.

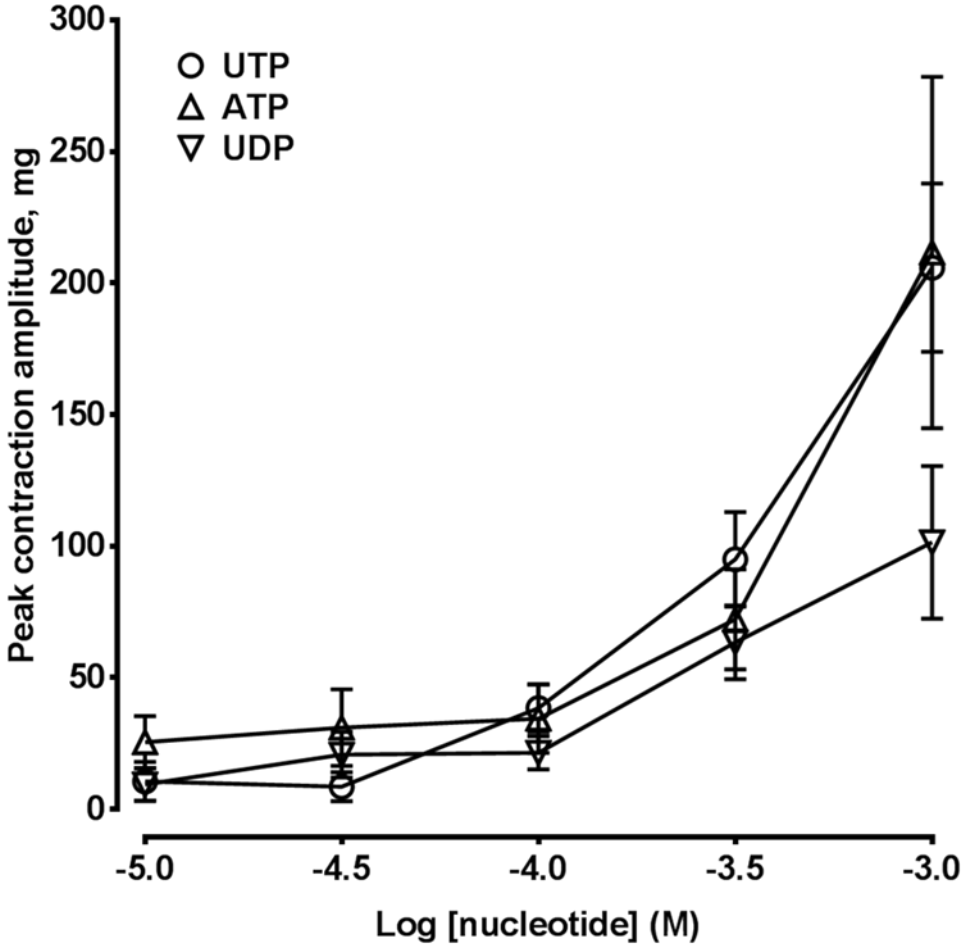


Figure 5.9: Nucleotides elicit concentration-dependent contraction of rTA at raised tone. The mean peak amplitude of contractions evoked by cumulative additions of UTP, ATP and UDP on NA (1 µM) pre-contracted endothelium-intact rTA is shown. Vertical lines indicate SEM, n=7 (UTP, UDP), n=6 (ATP).

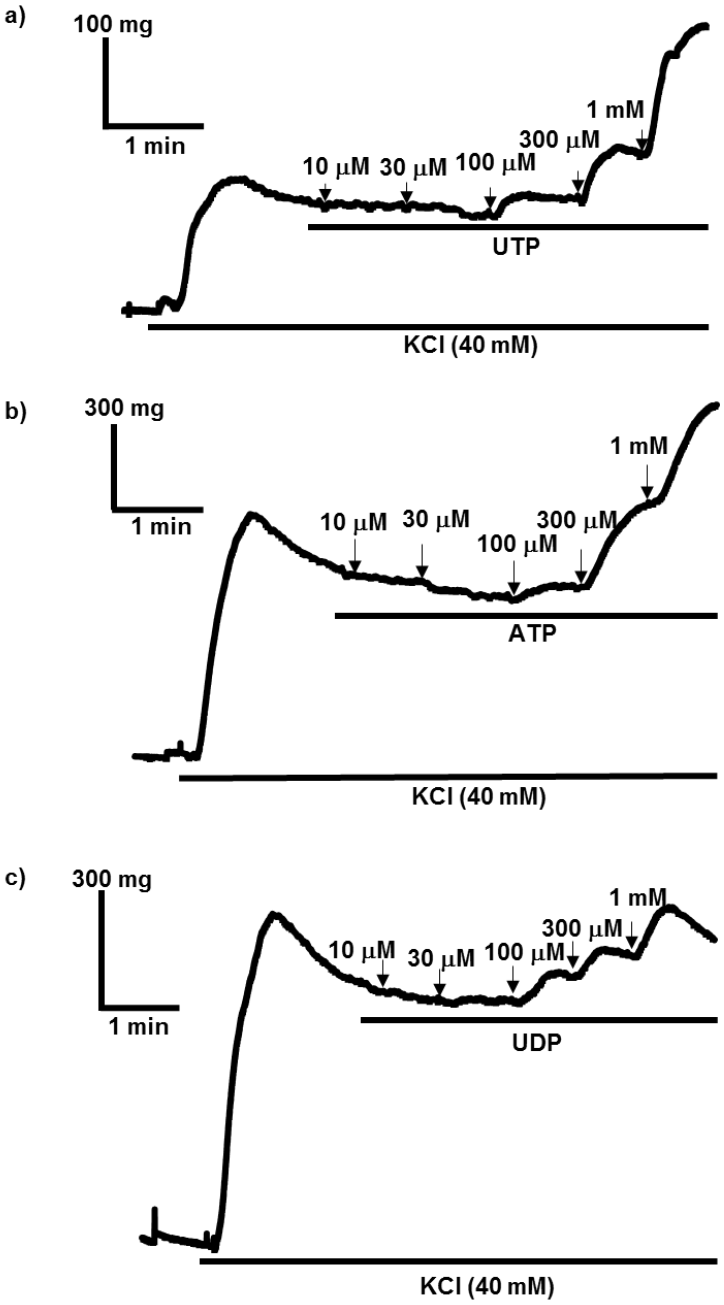


Figure 5.10: Nucleotides elicit vasoconstriction of rTA at raised tone. The traces show typical contractions of KCl (40 mM) pre-contracted, endothelium-intact rTA induced by cumulative addition of a) UTP, b) ATP and c) UDP (10 µM - 1 mM), as indicated by the horizontal bars and arrows.

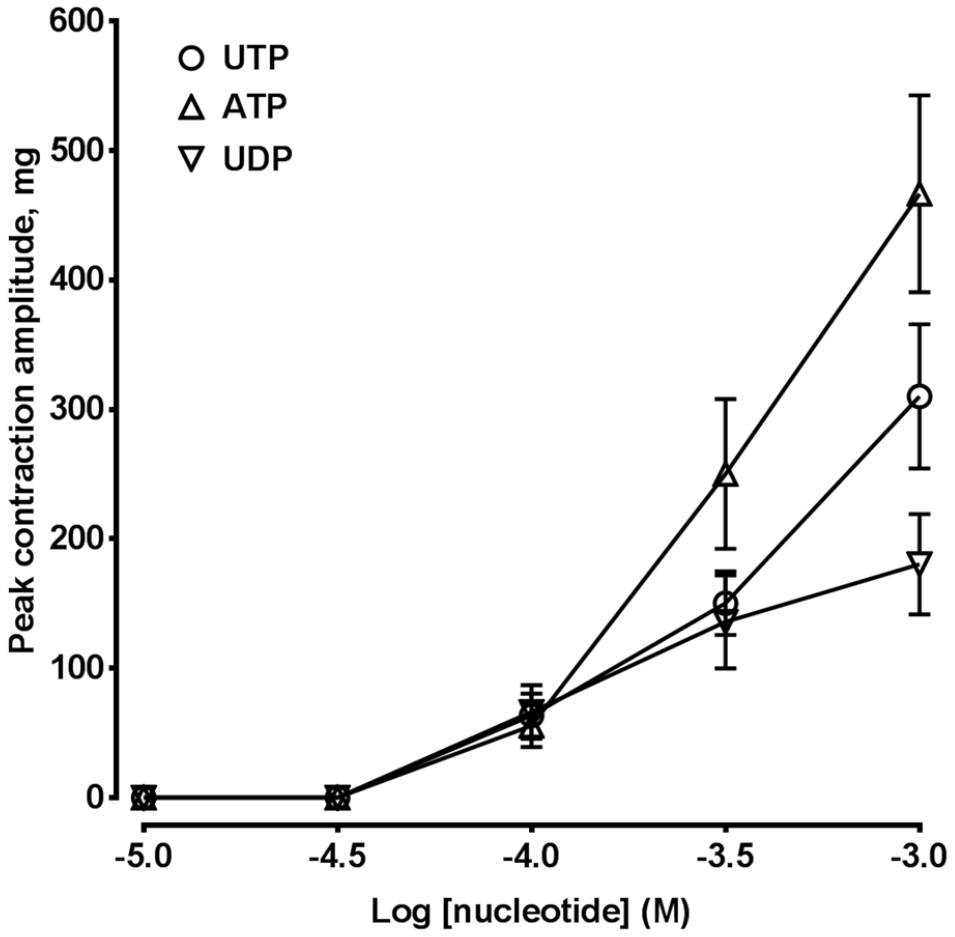


Figure 5.11: Nucleotides elicit concentration-dependent vasoconstriction of rTA at raised tone. The mean peak amplitude of contractions evoked by cumulative addition of UTP, ATP and UDP on KCl (40 mM) pre-contracted endothelium-intact rTA is shown. Vertical lines indicate SEM, n=8 (UTP, UDP), n=7 (ATP).

5.3 DISCUSSION

The results reported in this chapter show that UTP, UDP and ATP evoked contractions of endothelium-denuded rTA at resting tone, which were reproducible when the agonists were added repeatedly at 30 min intervals. This, therefore, was a suitable protocol for investigating the effects of AR-C118925XX on and the role $P2Y_2$ receptors in nucleotide-evoked vasoconstriction. However, the antagonist had no effect on the contractions. In addition, the nucleotides did not cause precontracted tissues to relax. Thus $P2Y_2$ receptors do not mediate vasoconstriction of the rTA and endothelial P2Y receptors do not mediate vasodilation of this tissue.

5.3.1 Responses at resting tone

AR-C118925XX did not alter the basal tone of rTA when added alone, mirroring the lack of effect in rIPA, 1321N1 and EAhy926 cells reported in chapters 3 and 4, and consistent with a high degree of specificity of AR-C118925XX for $P2Y_2$ receptors. The antagonist also had no effect on contractions evoked by UTP, ATP and UDP, which indicates that $P2Y_2$ receptors do not mediate vasoconstriction of rTA, even though moderate levels of $P2Y_2$ receptor-like immunoreactivity have been reported in rTA smooth muscle (Wallace *et al.,* 2006). The absence of inhibition by AR-C118925XX again mirrors what was seen in rIPA in chapter 4. It is also consistent with an earlier study in rTA by McLaren *et al*., (1995a) in which UTP-evoked contractions were only slightly inhibited by concentrations of suramin that block agonist action at $P2Y_2$ receptors. In addition, they found that the contractions were not inhibited by PPADS. $P2Y_4$ receptors are suramin- and PPADS-insensitive (Charlton *et al*., 1996) and so may be the site of action of UTP in rTA, though only weak $P2Y_4$ receptor-like immunoreactivity was observed in rTA smooth muscle (Wallace *et al.*, 2005). As noted a number of times in this thesis, the development of selective and competitive antagonists at other P2Y receptor subtypes is required in order to determine the site of action of UTP in rTA.

The ATP-induced contractions of rTA were also unaffected by AR-C118925XX in this study. McLaren *et al*., (1995a) found that a small component (15%) of ATP's action required Ca^{2+} release from the sarcoplasmic reticulum, indicating activation of P2Y

receptors, but the contractions were mediated predominantly by P2X1 receptors. This is consistent with a high density of P2X1 receptor expression in rTA smooth muscle (Bo and Burnstock, 1993) and the activation of fast inward currents by ATP in these cells (Evans and Kennedy, 1994). P2X1 receptors mediate the excitatory co-transmitter action of ATP from sympathetic nerves (Sneddon and Burnstock, 1985).

5.3.2 Responses at raised tone

In contrast to the vasodilation seen in rIPA, the present study found that UTP, ATP and UDP caused further contraction of the precontracted rTA, which was seen whether the tone was raised by NA or KCl. This was a surprise as endothelial P2Y receptor mRNA and protein expression and nucleotide-evoked, endothelium-dependent vasodilation have been reported in numerous blood vessels (Burnstock and Knight, 2004; Erlinge and Burnstock, 2008). NA also produced biphasic contraction of the rTA, this response is typical to NA where studies show biphasic response occurs as a result of activating the α_{-1A} and $_{-1B}$ adrenoceptors. ATP has also been proposed to be a co-transmitter of these responses (Burnstock and Sneddon, 1985; Jähnichen *et al.,* 2004).

Surprisingly, ACh had only a very small effect in tissues precontracted by NA. We cannot exclude that the endothelium was accidentally removed during the preparation of the tissue, but this is unlikely as the rTA is a thicker and more robust tissue than rIPA and ACh elicited large endothelium-dependent relaxations of rIPA in my hands (chapter 4). Interestingly, ACh did relax the rTA when it was precontracted by KCl, although a similar response was seen whether or not the endothelium had deliberately been physically removed. The most likely explanation is that muscarinic receptors in rTA smooth muscle cells are negatively coupled to the L-type VGCC that are activated by KCl-induced depolarisation and which mediate the Ca^{2+} influx that underlies vasoconstriction. The resultant decrease in Ca^{2+} influx would reverse the KCl-induced contraction. NA, acting at α_1-adrenoceptors, can activate L-type VGCC in some tissues, but it predominantly acts by inducing Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} sensitisation (Kennedy, 2015).

In conclusion, UTP, UDP, ATP produced reproducible vasoconstriction on the rTA at resting tone. At raised tone, these agonists also caused vasoconstriction.

Furthermore, the contractile responses evoked at resting tone were unaffected by AR-C118925XX, which indicates that contractile $P2Y_2$ receptors are not functionally expressed in rTA smooth muscle cells.

6 General Discussion

6.1 INTRODUCTION

In order to understand the roles of endogenous substances in influencing or controlling the activity of cells or tissues in health and disease, it is first necessary to identify the receptors through which they act. The roles of individual P2Y receptor subtypes in many of the actions of nucleotides, such as ATP and UTP, are, however, unclear due to the poor selectivity of most agonists and antagonists available. The data presented in this thesis demonstrated first that AR-C118925XX is a potent, selective and reversible $P2Y_2$ receptor antagonist. This enabled the functional expression of $P2Y_2$ receptors in vascular cells and tissues to then be studied and it was found that they mediate Ca^{2+} mobilisation in a human endothelial cell line and endothelium-dependent vasodilation of rat pulmonary arteries, but not a systemic artery, the rTA. In addition, $P2Y_2$ receptors did not contribute to nucleotide-evoked vasoconstriction of either type of artery. This shows that nucleotides modulate pulmonary arterial tone via $P2Y_2$ receptors expressed in endothelial, but not smooth muscle cells. Thus AR-C118925XX is a powerful new tool for determining the functions of $P2Y_2$ receptors in health and disease and identifying new therapeutic targets.

6.1.1 AR-C118925XX as a tool to identify functional expression of $P2Y_2$ receptors and potential physiological and pathophysiological roles

At the time my studies began AR-C118925XX had only just become commercially available and details of its potency had not been published. Nonetheless, a number of groups had already obtained the antagonist, mostly from Professor Christa Müller, a synthetic chemist at the University of Bonn, who had developed an improved method for its synthesis (Rafehi *et al.*, 2017a). Tocris Bioscience then started selling AR-C118925XX and unsurprisingly it has since been widely used and has helped identify the effects mediated by $P2Y_2$ receptors in a variety of cell types, tissues and disease models.

The cardiovascular system has been a major focus of research using AR-C118925XX. Consistent with the conclusion reached in this thesis that $P2Y_2$ receptors are functionally expressed in human umbilical vein endothelial cells, AR-C118925XX

(30 μM) substantially inhibited eNOS and Akt phosphorylation induced by endogenous ATP, released by fluid shear stress in bovine aortic endothelial cells (Wang *et al.,* 2015). A subsequent study in human umbilical artery endothelial cells found that Ca^{2+} transients and eNOS and Akt phosphorylation induced by Yoda-1, an agonist at the endothelial mechanosensitive PIEZO1 cation channel that is activated upstream of the flow-induced ATP release, were inhibited as well (Wang *et al.,* 2016). Thus $P2Y_2$ receptors appear to play an important role in endothelial mechanotransduction. AR-C118925XX also virtually abolished ATP-triggered Ca^{2+} transients in rat carotid artery endothelial cells at 1 μM (Lee *et al.,* 2018) and mouse cortical bEND.3 endothelial cells at 3 μM (Leong *et al.,* 2018). Interestingly, in a subsequent study the same group reported an effect of AR-C118925XX in bEND.3 endothelial cells that was independent of $P2Y_2$ receptors, as at 3 μM it induced Ca^{2+} influx downstream of store emptying, via an as yet unidentified novel class of Ca^{2+} transporter (Wu *et al.,* 2019). In addition, AR-C118925XX (10 μM) also partially reduced UTP-induced, endothelium-dependent relaxation of carotid arteries from spontaneous hypertensive and control Wistar-Kyoto rats (Matsumoto *et al.,* 2020). In contrast to the data in this thesis, Henriquez *et al*., (2018) found that AR-C118925XX was effective against vasoconstriction, as at 10 μM it abolished ATP-induced contractions of rat small pulmonary veins, so expression of contractile $P2Y_2$ receptors appears to be tissue specific. Finally, AR-C118925XX (10 μM) inhibited hypoxia-induced apoptosis of mouse cardiomyocytes by 51% (Cosentino *et al.,* 2012) and reduced by around 50% the cardioprotective effect of the $P2Y_2$ agonist, MRS2768, against hypoxia in rats (Hochhauser *et al.,* 2013), but at 1 μM had no effect against the positive inotropic action of UTP in mouse atria (Gergs *et al.,* 2018). Thus $P2Y_2$ receptors are a potential target for reducing cardiomyocyte damage during ischaemic/hypoxic stress.

AR-C118925XX has also been to investigate the role of $P2Y_2$ receptors in cancer. For example, $P2Y_2$ receptors were upregulated in pancreatic ductal adenocarcinoma samples obtained from human patients, which was associated with a poor prognosis (Hu *et al.,* 2019). Extracellular ATP levels were found to be much higher in the tumour microenvironment compared to paired adjacent non-tumour tissue, indicating a potential role for ATP. To test this hypothesis *in vivo*, the authors implanted pancreatic ductal adenocarcinoma cell lines subcutaneously in mice and found that AR-

C118925XX (10 mg/kg i.p.) slowed the rate of tumour progression and prolonged animal survival. This dose of AR-C118925XX also reduced tumour volume in mice implanted with an oral squamous cell cancer line (Woods *et al.,* 2020). In addition, AR-C118925XX (10 μM) reduced ATP activation of the inflammasome in the radiotherapy-resistant human breast cancer RT-R-MDA-MB-231 cell line, implicating $P2Y_2$ receptors in tumour progression (Jin *et al.,* 2018). At the same concentration AR-C118925XX also prevented communication between human breast cancer MCF-7 and MDA-MB-231 cells in response to mechanical wounding (Pratt *et al.,* 2018) and abolished UTP- and ATP-induced Ca^{2+} mobilisation and ERK1/2 and EGFR phosphorylation in oral and pharyngeal squamous cell carcinoma cell lines (Woods *et al*., 2020). Thus $P2Y_2$ receptors are a potential therapeutic target for several types of cancer.

$P2Y_2$ receptors might also be targeted for treatment of skin disorders. In dermal fibroblasts obtained from patients with cutaneous-type systemic sclerosis, AR-C118925XX (10 μM) abolished ATP-induced IL-6 release and p38 phosphorylation, whilst at 7 mg/kg i.p. it substantially reduced bleomycin-induced dermal fibrosis in mice *in vivo* (Perera *et al.,* 2019). A role in atopic dermatitis has been suggested, as AR-C118925XX (10 μM) inhibited IL-33 synthesis and release from human keratinocytes induced by house dust mite allergens (Dai *et al.,* 2020). This concentration also nearly abolished ATP- and UTP-elicited Ca^{2+} mobilisation in HaCaT human keratinocyte cells and AR-C118925XX slowed scratch wound closure in a cell monolayer, with an IC_{50} of 288 nM (McEwan *et al.,* 2021).

In human neutrophils AR-C118925XX (0.5 μM) almost abolished UTP- and ATPγS-induced superoxide production (Önnheim *et al.,* 2014) and at 1 μM abolished intracellular Ca^{2+} release evoked by ATPγS (Gabl *et al.,* 2016) and ATP-induced Ca^{2+} mobilisation and superoxide production (Lind *et al.,* 2019). Similarly, AR-C118925XX inhibited Ca^{2+} mobilisation evoked by UTP in DH82 canine macrophages in a concentration-dependent manner, with an IC_{50} of 245 nM and virtually abolished the response at 1 μM (Sophocleous *et al.,* 2020). AR-C118925XX (10 and 100 μM), however, had no effect on IL-6 release from peritoneal macrophages in response to LPS (1 mg/ml) in a mouse model of peritonitis and sepsis (caecal ligation and puncture), but the release was abolished by the selective $P2Y_1$ antagonist, MRS2279

(Dosch *et al.,* 2019). This is a good example of how selective antagonists can help differentiate between multiple sites of action of an agonist.

$P2Y_2$ receptors may also play a role in orofacial pain, as AR-C118925XX (10 μM) supressed UTP-induced Ca^{2+} mobilisation in mouse trigeminal ganglion satellite cells by 90% and reduced facial allodynia in a mouse model of sub-chronic trigeminal sensitisation in a dose-dependent manner, with full reversal at 7 mg/kg (Magni *et al.,* 2015). ARC118925XX (0.2 and 1 mg) also inhibited masseter muscle inflammatory hypersensitivity (Knezevic *et al.,* 2020). $P2Y_2$ receptors in salivary glands have been identified as a target for treating Sjörgren's syndrome, a chronic autoimmune disorder of the salivary and lacrimal glands characterised by lymphocyte infiltration and decreased saliva and tear production (Jasmer *et al.,* 2021). In B lymphocytes isolated from a mouse model of salivary gland inflammation, AR-C118925XX (10 μM) abolished UTP-evoked Ca^{2+} mobilisation, ERK phosphorylation and B cell migration. Systemic administration of AR-C118925XX (1 mg/kg i.p.) *in vivo* diminished the inflammation by ~50% and increased carbachol-evoked saliva production by 70-80%. The $P2Y_2$ receptor agonist, Diquafosol, is licenced in Japan, South Korea and China for treatment of dry-eye syndrome and acts by stimulating secretion of water, mucins and lipids as tears. The meibomian glands, which are present in the upper and lower eyelids were identified as a source of the lipids, as AR-C118925XX (3 μM) reduced Diquafosol-evoked Ca^{2+} mobilisation and cholesterol secretion from rabbit meibomian gland cells *in vitro* (Endo *et al.,* 2021).

$P2Y_2$ receptors have been proposed to play a role in diet-induced obesity, as in human adipose-derived mesenchymal cells, which undergo adipogenesis to expand adipose tissue, AR-C118925XX inhibited ATP- and UTP-evoked Ca^{2+} mobilisation with an IC_{50} ~1 μM (Ali *et al.,* 2018) and at 10 μM prevented differentiation and maturation of mouse preadipocytes and 3T3-L1 cells, a mouse preadipocyte cell line, into adipocytes (Zhang *et al.,* 2020). Finally, AR-C118925XX inhibited ATPγS-induced mucin section from human bronchial epithelial cells, with an IC_{50} of ~1 μM (Kemp *et al.*, 2004) and at 5 μM reduced by ~60% the Cl^- secretion from mouse colonic epithelial cells evoked by endogenously-released ATP (Lu *et al.,* 2019), but had no effect at 10 μM on NaCl-induced expression of the c-Fos gene in human retinal pigment epithelial cells (Kleiner *et al.*, 2018).

Although a range of concentrations of AR-C118925XX were used in the studies discussed above, the most common was 10 μM, which is ~2,700 times higher than the K_B calculated in our study and 270, 770 and 630 times than the K_B values reported in Rafehi *et al*., (2017 a,b) and Kindon *et al.,* (2017) respectively. The use of a high concentration increases the possibility of an action of AR-C118925XX at sites other than $P2Y_2$ receptors, but this is unlikely to be the case in the studies above, since at 10 μM, AR-C118925XX had no effect at 37 other receptors (Kemp *et al.,* 2004). Indeed, the only indications to date of an action at a site other than $P2Y_2$ receptors are blockade of recombinant human P2X1 (IC_{50} = 2.63 μM) and P2X3 receptors (IC_{50} = 0.819 μM; Rafehi *et al*., 2017a) and the activation of an unidentified novel class of Ca^{2+} transporter in mouse cortical bEND.3 endothelial cells by 3 μM AR-C118925XX (Wu *et al.,* 2019). Nevertheless, it is preferable, where possible, to avoid using higher concentrations of an antagonist than necessary.

6.1.2 Vascular P2Y receptors; functional expression and physiological/pathophysiological roles

A major aim of this thesis was to use AR-C118925XX and other antagonists to identify the P2Y receptors that mediate nucleotide-evoked vasoconstriction and vasodilation in rIPA and the findings and the conclusions reached are discussed in chapter 4. P2Y receptor mRNA and protein are, however, expressed in vascular smooth muscle and endothelial cells throughout the vascular system, with the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ subtypes being most commonly identified (Burnstock and Knight, 2004; Erlinge and Burnstock, 2008). Nucleotides have widespread actions on these cells and selective antagonists and receptor knockout or knockdown have helped determine the receptor subtypes that mediate some of these actions and identify potential novel therapeutic targets. Receptor knockout and knockdown are powerful techniques, but they have limitations, including cost, knockout being largely restricted to mice and knockdown often being incomplete. Potent, selective, competitive antagonists, like AR-C118925XX, have the advantages of ease of use and applicability in humans and are a powerful, complimentary tool for studying receptor function.

$P2Y_1$ receptors: Although the effect of $P2Y_1$ receptor knockout on the vascular actions of ADP and ATP has not, to the best of my knowledge, been reported, the selective antagonist, MRS2179, has been widely used. Similar to its effect on rIPA (chapter 4), MRS2179 inhibited the endothelium-dependent vasodilation induced by ADP in the coronary artery or coronary vascular bed of rats (Van der Giet *et al.,* 2002; Kaneshiro *et al.,* 2010), guinea-pigs (Gorman *et al.,* 2003), dogs (Bender *et al.,* 2011) and pigs (Zhou *et al.,* 2013), guinea-pig (Kaiser and Buxton, 2002) and mouse (Guns *et al.,* 2005) aorta, rat mesenteric bed (Buvinic *et al.,* 2002), rat pial arterioles (Xu *et al.,* 2005) and dog and monkey cerebral arteries (Geddawy *et al.,* 2010). Endothelium-dependent vasodilation of human left internal mammary arteries via $P2Y_1$ receptors was also identified using another $P2Y_1$ antagonist, MRS2216 (Wihlborg *et al.,* 2003). In contrast, MRS2179 had no effect on the endothelium-dependent vasodilation of rat mesenteric bed (Buvinic *et al*., 2002) or mouse aorta (Guns *et al*., 2005) induced by ATP, again similar to rIPA, but it shifted the ADP CRC rightwards in the aorta of $P2Y_2$ knockout mice (Guns *et al.,* 2006) and inhibited by ~50% the increase in coronary arterial blood flow elicited by ATP infusion in anaesthetised pigs (Olivecrona *et al.,* 2004). Thus, while ADP elicits endothelium-dependent vasodilation through $P2Y_1$ receptors, ATP appears to act via $P2Y_2$, and in some cases, $P2Y_1$ receptors.

Interestingly, Olivecrona *et al*., (2004) reported that MRS2179 also reduced the post-ischaemic increase in coronary arterial blood flow by ~50%, so $P2Y_1$ receptors appear to contribute to post-ischemic, coronary reactive hyperemia and could potentially be targetted to reduce reperfusion injury that occurs during angioplasty after acute myocardial infarction. Finally, MRS2179 has also been used to identify contractile $P2Y_1$ receptors located in vascular smooth muscle cells of human umbilical and chorionic vessels, which appeared to act via release of thromboxane A_2 (Buvinic *et al.,* 2006). These contractions were larger than the MRS2179-sensitive responses seen in rIPA (Mitchell *et al*., 2012).

$P2Y_{12/13}$ receptors: ADP is also agonist at $P2Y_{12}$ and $P2Y_{13}$ receptors and the $P2Y_{12}$/$P2Y_{13}$ receptor antagonists, ticagrelor and AR-C67085, inhibited contractions of endothelium-denuded human internal mammary and pericardial fat arteries and mouse aorta evoked by 2-meSADP (Wihlborg *et al*., 2004; Högberg *et al*., 2010).

Contractile $P2Y_{12}/P2Y_{13}$ receptors have also been identified in rIPA, where the $P2Y_{12}/P2Y_{13}$ receptor antagonist, AR-C69931MX, abolished contractions evoked by ADP (Mitchell *et al*., 2012). These data have yet to be backed up by receptor knockout experiments, but smooth muscle $P2Y_{12}$ and/or $P2Y_{13}$ receptors appear to mediate vasoconstriction.

$P2Y_2$ receptors: As discussed above, AR-C118925XX has been very useful in characterising actions mediated by vascular $P2Y_2$ receptors, as it reduced UTP-induced, endothelium-dependent relaxation of carotid arteries from spontaneous hypertensive and control Wistar-Kyoto rats (Matsumoto *et al.,* 2020) and associated endothelial signalling events, such as Ca^{2+} mobilisation (Lee *et al.,* 2018; Leong *et al.,* 2018) and eNOS and Akt phosphorylation (Wang *et al.,* 2015). Consistent with these data, knockdown of $P2Y_2$ receptors suppressed Ca^{2+} mobilisation evoked by ATP and UTP in human EAhy926 (Raqeeb *et al.,* 2011) and bovine aortic (Wang *et al.*, 2015) endothelial cells. The latter study also saw a large decrease in the Ca^{2+} mobilisation, phosphorylation of eNOS and Akt and tyrosine phosphorylation of SRC kinase, PECAM-1, and VEGFR-2 induced by fluid shear stress in bovine aortic endothelial cells. In addition, mice in which endothelial $P2Y_2$ receptors were selectively knocked out had raised mean arterial blood pressure (Wang *et al.*, 2015). Mesenteric arteries from these mice did not vasodilate in response to an increase in flow, unlike wild-type tissue, and had less phosphorylated eNOS. The same group then reported that both AR-C118925XX and $P2Y_2$ receptor knockdown substantially reduced signalling events evoked by the PIEZO-1 mechanosensitive channel agonist, Yoda-1, in human umbilical artery endothelial cells (Wang *et al.,* 2016). This, together with the demonstration of ATP release by Yoda-1 indicates that $P2Y_2$ receptors, activated by endogenously released ATP, mediate the vasodilation evoked by fluid sheer stress and that this lowers mean arterial blood pressure.

It is notable that $P2Y_2$ receptor knockout had no effect on endothelium-dependent vasodilation evoked by UTP in mouse aorta (Guns *et al.,* 2006) and coronary artery (Haanes *et al.,* 2016), but reduced responses to ATP and ATPγS in the aorta and abolished relaxations evoked by the selective $P2Y_2$ agonist, UTPγS in the coronary artery. Selective deletion of endothelial $P2Y_2$ receptors also produced a moderate rightwards shift of the ATPγS and UTPγS CRC in the aorta (Chen *et al.,* 2017). These data suggest that in in the absence of $P2Y_2$ receptors, UTP can act at other P2Y

subtypes to elicit vasodilation. Consistent with this possibility, the contribution of $P2Y_1$ receptors to the action of ATP was increased in $P2Y_2$ knockout mice (Guns *et al.*, 2006). The $P2Y_4$ receptor does not appear to be involved in the UTP response, however, as deletion had no effect (Guns *et al.,* 2005). In contrast, $P2Y_6$ receptors may play a role, since knockout produced a small rightwards shift in the UTP CRC in the aorta (Bar *et al.,* 2008) and a much larger shift in the coronary artery (Haanes *et al.*, 2016). It is also possible that deletion of one P2Y subtype *in vivo* leads to upregulation of another to compensate for the loss, but unfortunately, receptor expression levels were not measured in most of these studies, although Bar *et al.*, (2008) found that the amount of $P2Y_1$ receptor mRNA in mouse aorta was doubled by $P2Y_6$ receptor deletion. These knockout studies did not provide as clear a demonstration as would be liked of how UTP causes vasodilation, particularly in the mouse aorta. This situation is not unique, as knocking out each of the three subtypes individually had no effect against the positive inotropic action of UTP in mouse atria (Gergs *et al.,* 2018). Full characterisation of how UTP acts may require knocking out multiple P2Y subtypes at the same time and/or using AR-C118925XX and MRS2578 to characterise knockout-resistant responses pharmacologically.

Further insight into the signalling mechanisms underlying $P2Y_2$ receptor-mediated vasodilation was obtained in anaesthetised mice (Dominguez Rieg *et al.,* 2015). Bolus i.v. administration of the $P2Y_{2/4}$ agonist, INS45973, caused a fall in mean arterial blood pressure within seconds that was converted to an increase after $P2Y_2$ knockout. Heart rate was unchanged in both cases, suggesting that INS45973 acted directly on the peripheral resistance vessels. Deletion of $K_{Ca}3.1$, an intermediate conductance Ca^{2+}-activated K^+ channel, or connexin 37, which forms myoendothelial gap junctions between endothelial and smooth muscle cells, both reduced the fall in blood pressure induced by INS45973.

$P2Y_2$ receptors can also mediate vasoconstriction in vessels at resting tone, as AR-C118925XX abolished ATP-induced contractions of rat small pulmonary veins (Henriquez *et al*., 2018). Knockout of this subtype, however, had no effect on UTP-evoked contractions of mouse aorta (Kauffenstein *et al.,* 2010) or coronary artery (Haanes *et al.*, 2016), but aortic responses to ATPγS were substantially inhibited and coronary contractions to UTPγS were abolished. Similar to the vasodilation data discussed above, this suggests that UTP can act at other P2Y subtypes to elicit

vasoconstriction and this was confirmed by the virtual abolition of contractions of the aorta of $P2Y_6$ receptor knockout mice (Kauffenstein *et al.*, 2010). Note that responses to ATPγS were unaffected by deletion of the $P2Y_6$ receptor, indicating the presence of functional $P2Y_2$ receptors, so it is not clear why UTP did not act at them to evoke contraction.

Knockout of $P2Y_2$ receptors has revealed roles in cardiovascular disorders that are not directly related to their vasodilatory effect. Selectively deleting endothelial $P2Y_2$ receptors in $ApoE^{-/-}$ mice, a model of atherosclerosis, greatly reduced the number of atherosclerotic, fatty streak lesions in the aorta (Qian *et al.,* 2016; Chen *et al.,* 2017). This was associated with reduced endothelial expression of vascular cell adhesion molecule-1, which plays an important role in adhesion of leukocytes to endothelial cells and subsequent transendothelial migration, decreased transendothelial migration of monocytes and lower levels of the inflammatory cytokine, lymphotoxin α. Based on these and other data the authors hypothesised that endothelial injury in the early stages of atherosclerosis causes local release of nucleotides, which act at $P2Y_2$ receptors to produce lymphotoxin α, which in turn upregulates vascular cell adhesion molecule-1 expression, so promoting inflammation. Deleting the $P2Y_2$ receptor shifted the atherosclerotic plaque from an inflammatory phenotype to a more stable form. Consistent with this hypothesis, the plasma levels of ATP and ADP were higher in atherosclerotic patients than in control subjects in whom there was no clinical evidence of peripheral artery disease (Jalkanen *et al.,* 2015). Low expression of ecto-nucleoside triphosphate diphosphohydrolase (CD39), which dephosphorylates tri- and diphosphate nucleotides (Robson *et al.,* 2006), were associated with disease progression. Thus pharmacological blockade of $P2Y_2$ receptors is a potential novel therapy for inhibiting the development of atherosclerosis.

Global knockout of $P2Y_2$ receptors also produced mice with raised mean arterial blood pressure, which was associated with impaired excretion of Na^+ and water by the kidney, leading to increased blood volume (Rieg *et al.,* 2007, 2011; Pochynyuk *et al.,* 2010). So in addition to endothelial $P2Y_2$ receptors inducing vasodilation in response to fluid sheer stress, apical $P2Y_2$ receptors in the distal nephron also lower blood pressure by increasing Na^+ and water excretion.

$P2Y_4$ receptors: The lack of a selective antagonist means that there are no pharmacological data on the functions of vascular $P2Y_4$ receptors. Knockout of $P2Y_4$ receptors had no effect on the endothelium-dependent relaxation (Guns *et al.,* 2005) or on vasoconstriction (Kauffenstein *et al.*, 2010) of mouse aorta evoked by UTP or UDP.

$P2Y_6$ receptors: Although the $P2Y_6$ receptor antagonist, MRS2578, has been available since 2004 and has been used extensively, it has not been employed to successfully identify relaxant endothelial $P2Y_6$ receptors other than in rIPA, as reported in chapter 4. Conflicting data have been reported for the effects of $P2Y_6$ receptor knockout on vasodilation of the mouse aorta. Bar *et al.*, (2008) found that it caused a large rightwards shift in the UDP CRC and a small shift in the UTP CRC, whereas Nishimura *et al.*, (2016) saw no change in UDP-evoked relaxations. The reason for this difference is not known. Two reports agree, however, that $P2Y_6$ knockout had no effect on diastolic, systolic and mean arterial blood pressure (Kauffenstein *et al.,* 2016; Nishimura *et al.,* 2016), consistent with a lack of effect of MRS2578 (Nishimura *et al.*, 2016).

$P2Y_6$ receptors can also mediate vasoconstriction in some vessels at resting tone, as MRS2578 inhibited UDP-evoked contractions of rIPA at resting tone (Mitchell *et al.*, 2012), though UTP-evoked contractions of pig pancreatic (Alsaqati *et al.,* 2014b) and coronary (Abbas *et al.,* 2018) arteries were unaffected by MRS2578. In addition, knockout of the $P2Y_6$ receptor abolished contractions of mouse aorta evoked by UDP and UTP (Kauffenstein *et al.*, 2010), of coronary artery evoked by UDP (Haanes *et al.,* 2016) and of mesenteric artery elicited by UDP, UTP and UDPβS (Kauffenstein *et al.*, 2016). In cultured mesenteric artery smooth muscle cells, activation of the small G protein, RhoA, by UDP, UTP and UDPβS and UDP-induced Ca^{2+} mobilisation and phosphorylation of p38, ERK, JNK, myosin light chain and myosin light chain phosphatase at ser-696 and ser-853, were all abolished or greatly decreased by $P2Y_6$ receptor knockout. In contrast, Ca^{2+} mobilisation evoked by UTP was barely affected and that to ATP was unaffected. Ca^{2+} mobilisation induced by the $P2Y_6$ agonist, PUDP, in aortic smooth muscle cells was also abolished by knockout of the $P2Y_6$ receptor (Nishimura *et al.*, 2016).

Smooth muscle $P2Y_6$ receptors contribute to the development of myogenic tone that is evoked by a stepped increase in vascular perfusion pressure, as knockout reduced this response by about half (Kauffenstein *et al*., 2016). MRS2578 produced a similar decrease. In contrast, deletion of CD39 potentiated the myogenic tone and contractions evoked by exogenous UDP and UTP (Kauffenstein *et al*., 2010). The myogenic tone that develops during chronic heart failure induced by coronary artery ligation was also substantially inhibited by deletion of the $P2Y_6$ receptor, as was angiotensin II-induced hypertension (Kauffenstein *et al*., 2016). Nishimura *et al*., (2016) also found that $P2Y_6$ receptor knockout and MRS2578 inhibited angiotensin II-induced hypertension and showed that this is because $P2Y_6$ receptors form stable heterodimers with AT1 angiotensin II receptors, which mediate a rise in blood pressure, vascular remodeling, oxidative stress, and endothelial dysfunction. Induction of hypertension by angiotensin II also caused a decrease in the vascular expression and activity of CD39, which would reduce the breakdown of endogenous UDP and so potentiate its actions (Roy *et al.,* 2018). Thus the AT1/$P2Y_6$ dimer is a novel potential target for treating angiotensin II-related hypertension.

$P2Y_6$ receptors have also been proposed to play a role in the development of atherosclerosis (Stachon *et al.,* 2014). The receptor was upregulated in the aorta of low-density lipoprotein receptor-deficient mice that had been fed a high-cholesterol diet to induce atherosclerosis and knocking it out greatly reduced aortic atherosclerotic lesions. Deleting the $P2Y_6$ receptor also reduced the amount of lipid and number of macrophages present in plaques and increased the number of smooth muscle cells and the collagen content. Aortic expression of vascular cell adhesion molecule-1 expression and IL-6 were also reduced. So, like $P2Y_2$ receptors, $P2Y_6$ receptors appear to contribute to atherosclerosis by promoting inflammation and the development of aortic plaques and their pharmacological blockade is another potential novel therapy for treating this disorder.

$P2Y_{14}$ receptors: UDP and UDP-glucose are both agonists at the $P2Y_{14}$ receptors and the selective $P2Y_{14}$ receptor antagonist, PPTN, inhibited contractions of pig pancreatic (Alsaqati *et al.,* 2014a) and coronary (Abbas *et al*., 2018) arteries induced by UDP-glucose and the selective $P2Y_{14}$ receptor agonist, MRS2690. Interestingly, the responses in the former, but not the latter tissue were reduced by physical removal

of the endothelium and the contractions were dependent, at least in part, on endothelial production of thromboxane A_2, prostaglandin $F_{2\alpha}$ and endothelin-1.

6.1.3 Purinergic signalling in pulmonary arteries and disorders

The data reported in this thesis extend our understanding of the functional expression of purinergic receptors in the pulmonary artery, particularly $P2Y_2$ receptors. UTP, UDP, ATP produced reproducible contractions of the rIPA at resting tone that were unaffected by AR-C118925XX, which indicates that contractile $P2Y_2$ receptors are not functionally expressed in rIPA VSMC. At raised tone, these agonists and ADP produced endothelium-dependent vasodilation that was reproducible. UTP-evoked vasodilatation was mainly mediated through $P2Y_2$ receptors, and $P2Y_6$ receptors were not involved. UDP-evoked relaxation was mainly via $P2Y_6$ receptors and $P2Y_2$ receptors also appeared to contribute to its action. ATP-induced endothelium-dependent relaxation did not involve $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$ or adenosine receptors, but may be through $P2Y_4$ and/or P2X4 receptors. ADP elicited vasodilation mainly via $P2Y_1$ receptors and the remaining response is not mediated by $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$ or adenosine receptors.

At present, the contribution of these receptors to physiological signalling and pathophysiological conditions in the lungs is not well characterised. The rise in pulmonary blood pressure induced by acute hypoxia was inhibited by suramin, a broad spectrum P2X/P2Y receptor antagonist, in perfused rabbit lungs (Baek *et al.,* 2008) and by blocking $P2Y_1$ and $P2Y_{12}$ receptors with MRS2500 and cangrelor respectively, in pigs *in vivo* (Kylhammar *et al*., 2014). Beyond this, however, neither subtype-selective P2Y receptor antagonists nor knockout of individual subtypes have been used to investigate potential roles for nucleotide-mediated purinergic signalling in the development and progression of PAH and other pulmonary disorders, but several reports are consistent with a possible role (see Cai *et al*., 2020; Strassheim *et al*., 2020 for recent reviews). For example, CD39 is downregulated in pulmonary arterial endothelial cells of PAH (Helenius *et al.,* 2015; Visovatti *et al.,* 2016) and COPD (Aliagas *et al.,* 2018) patients. COPD is also associated with increased extracellular ATP levels (Lommatzsch *et al.*, 2010) and a reduction in ADP-induced pulmonary vasodilation (Dinh-Xuan *et al.,* 1991). Inducing chronic hypoxia in CD39

knockout mice had a greater effect than in wild-type animals, producing more extracellular ATP, a greater rise in PAP and greater right ventricular and arterial medial hypertrophy (Visovatti *et al.*, 2016). Intravenous infusion of the soluble ATPase/ADPase, apyrase, reversed the increase in PAP in CD39 knockout mice to the same level as seen in hypoxic wild-type control animals. Thus these results suggest that targeting purinergic signalling could provide a novel therapeutic approach for treating PAH and other pulmonary disorders.

6.2 Limitations to the study

During these studies several factors limited my ability to characterise which P2Y receptors modulate vascular tone in pulmonary arteries;

- There is still a lack of potent, selective, antagonists for most P2Y receptor subtypes. These compounds are needed to classify which P2Y subtypes are involved in the pathogenesis of PAH.
- Due to the pandemic, the delivery of some reagents was greatly delayed and because of this some experiments were put on hold.
- Time constraints prevented me from investigating various aspects, such as
    - Immunological staining of pulmonary artery to confirm endothelium-intact and –denuded tissues.
    - Determining if the experiments were under normoxic conditions at all times by measuring the O_2 levels.
    - Testing PPTN, a $P2Y_{14}$ antagonist, against UTP and UDP vasodilation and vasoconstriction, and increasing the concentration of AR-C118925XX to 10 µM, as other recent studies have used.
    - Determining if contractions of rTA evoked by repeated addition of NA were reproducible.
- Using the same tension while setting up rIPAs and rTAs. This may have played a role in responses.
- Excluding hypoxic conditions while studying the responses in pulmonary artery.
- The experiments were limited to animal tissues from normotensive animals. Experiments using tissues obtained from animal models of pulmonary hypertension and human tissues would greatly increase the scope of these studies.

6.3 Future experiments

The present study has provided strong evidence for the existence of endothelial P2Y receptors in the rIPA, together with a greater understanding of which receptor subtypes are involved in the relaxation of pulmonary arteries. However, a number of issues are yet to be resolved and the following experiments could be performed to gain more insight into P2Y receptor-mediated vasoconstriction and vasodilatation.

I. Pharmacological characterisation of the P2Y receptor subtypes could be developed, by;

a) Adding MRS2578 plus AR-C118925XX against UTP and UDP vasodilation to determine if their effects are additive.

b) Increasing the concentration of AR-C118925XX to 10 μM, as other studies have used, to see if this produces greater inhibition.

c) Investigating if AR-C118925XX inhibits responses evoked by P2Y receptor subtype-selective agonists.

d) Determining if 5-BDBD and BX430, P2X4 receptor antagonists, inhibit ATP-induced vasodilation.

II. Receptor function can also be studied using knockout animals and it would be interesting to determine the effects of knocking out multiple P2Y subtypes at the same time and using AR-C118925XX and MRS2578 to characterise knockout-resistant responses pharmacologically.

III. Human pulmonary arteries can be obtained from a local hospital, so it would be of great interest to carry out a similar pharmacological characterisation of the P2Y receptor subtypes functionally expressed in human endothelial and smooth muscle cells.

IV. The ultimate aim is to determine if altered purinergic signaling contributes to the development and progression of PAH and other pulmonary disorders and the role of $P2Y_2$ receptors could be investigated by:

a) Studying the effect of $P2Y_2$ receptors on pulmonary vascular cell proliferation.

b) Studying the effects of AR-C118925XX on the changes in blood pressure and tissue structure seen in animal models of PAH, including chronic hypoxia, in which rats are maintained under hypoxic conditions (10% O_2) for 2-3 weeks and hypertension induced by the alkaloid monocrotaline (Adnot *et al.,* 1991; Stenmark *et al.,* 2009). Both models show physical changes in the pulmonary vasculature similar to those seen in PAH, display sustained pulmonary vasoconstriction and a decreased sensitivity to vasodilators.
c) For the purpose of treatment, to investigate if AR-C118925XX can be administered chronically *in vivo*.

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