Constitutive Release of Purines in Control of Vascular Contractility

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Abstract

Most large and medium sized arteries are surrounded by perivascular adipose tissue (PVAT). PVAT was once considered a passive structural component of the vascular wall, not an active regulator of vascular homeostasis. However, PVAT releases several active mediators that have a paracrine effect on vascular tone. Adipocytes express P2X and P2Y receptors, whose activation influences a number of adipocyte functions including the release of adipokines. Adipocytes can also release nucleotides to control adipocyte activity. It is unknown whether this occurs in PVAT and alters the tone of the adjacent vasculature. The constitutive release of purines in blood vessels is relatively unknown. However, it is important to understand vascular control mechanisms to offer new therapeutic approaches for cardiovascular diseases such as hypertension. The main purpose of this study was to determine if nucleotides (ATP/UTP) are released constitutively from PVAT to regulate the vascular tone of porcine mesenteric artery. Further investigation was performed to examine the possibility that P2X, P2Y, and adenosine receptors may contribute to the vasorelaxant effect induced by AR-C118925XX (a $P2Y_2$ receptor antagonist). Additionally, I investigated whether the nucleotides ATP and UTP activate P2Y receptors to release adiponectin and leptin from 3T3-L1 adipocytes. I also examined whether the anti-contractile effect of PVAT in response to ATP and UTP in porcine splenic arteries may be associated with adiponectin release.

Isometric tension recordings were performed using mesenteric arteries obtained from large white hybrid pigs with and without PVAT. A direct vasorelaxant effect of suramin (P2 receptor antagonist), AR-C118925XX and MSG228 ($P2Y_2$ receptor antagonists) was found on U46619-pre-constricted vascular tone of porcine mesenteric arteries. Apyrase (metabolises nucleotides) abolished the vasorelaxant response of pre-

contracted mesenteric arteries to AR-C118925XX. Additionally, apyrase caused a dual effect of contraction and relaxation in pre-contracted mesenteric arteries. ARL67156 (ectonucleotidases inhibitor) directly caused an initial small contraction in U46619- pre-constricted mesenteric artery tone. Further experiments demonstrated that the connexin/pannexin channel inhibitors carbenoxolone and probenecid also caused a relaxation of U46619-pre-constricted vascular tone in porcine mesenteric arteries.

In this study, evidence ruled out the involvement of PVAT, endothelium and P2X and adenosine receptors in nucleotides release and responses to P2 receptor antagonists, suggesting that nucleotide release and actions involves the vascular smooth muscle. Relaxation of AR-C118925XX was also observed in further vascular beds and species in this study, specifically porcine coronary and splenic arteries, as well as rat mesenteric arteries.

Extracellular ATP was measured by luminescence directly from the Krebs solution in porcine mesenteric arteries. It appears that isolated mesenteric arteries are capable of releasing ATP and the presence of PVAT in this experiment increased the amount of ATP released from isolated mesenteric arteries. However, there were no differences in the effects of P2 receptor antagonists in the presence or absence of PVAT on mesenteric artery tone.

The present study also identified the role of extracellular nucleotides ATP and UTP in adiponectin release from 3T3-L1 adipocytes via activation of $P2Y_2$ receptors. Adiponectin release was not regulated by constitutive activation of $P2Y_2$ receptors. In isolated arteries, the presence of PVAT in splenic, but not mesenteric, arteries decreased the level of contraction induced by ATP and UTP. The anti-contractile effects of PVAT on porcine splenic arteries in response to ATP and UTP might be mediated by adiponectin and other relaxant factors.

Together, these results demonstrated that endogenous ATP/UTP is released constitutively from vascular smooth muscle via connexins and pannexins to act on vasocontractile $P2Y_2$ receptors.

Publications

1- Alqarni A, Maneesai P, Dunn WR, Ralevic V. (2019).
Direct vasorelaxant effect of the selective $P2Y_2$ receptor antagonist AR-C118925XX in mesenteric arteries: constitutive release of purines in control of vascular contractility? Pharmacology 2019. Edinburgh International Conference Centre, Edinburgh, UK.

2- Alqarni A, Maneesai P, Dunn WR, Ralevic V. (2019).
Direct vasorelaxant effect of the selective $P2Y_2$ receptor antagonist AR-C118925XX in mesenteric arteries: constitutive release of purines in control of vascular contractility? UK Purine Club 10th Anniversary Symposium 2019, Sheffield, UK.

3- Alqarni A, Dunn WR, Ralevic V. (2021).
Role of UTP-sensitive P2Y receptors, P2X receptors and the endothelium in the vasorelaxant effect induced by AR-C118925XX. Pharmacology 2021, Online, Worldwide.

4- Alqarni A, Dunn WR, Stocks MJ, Ralevic V. (2021).
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Functional role of adenosine via K_{ATP} channels in cerebral capillary endothelial cells and pericytes. Purinergic Signalling, Journal club.

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Roles of purinergic receptors in adiponectin and leptin release in adipocytes. Pharmacology 2022. ACC Convention Centre, Liverpool, UK.

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Abbreviations

AC	Adenylyl cyclase
ADRF	Adipose-derived relaxing factor
BAT	Brown adipose tissues
BK_{Ca}	Calcium-activated potassium channels
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
DG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EDHF	Endothelium derived hyperpolarizing factor
EDRF	Endothelium derived relaxing factors
eNOS	Endothelial NO synthase
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase family
GPCRs	G-protein-coupled receptors
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
IP_3	Inositol 1,4,5-trisphosphate
MAPKs	Mitogen-activated protein kinases
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NO	Nitric oxide
NTPDase	Ectonucleoside triphosphate diphosphohydrolase
PGI_2	Prostacyclin
PKC	Protein kinase C
PLC	Phospholipase C
PVAT	Perivascular adipose tissue
PVCF	PVAT-derived contractor factor

PVRF	Perivascular-derived relaxing factors
sGC	Soluble guanylate cyclase
TNFα	Tumor necrosis factor alpha
TXA_2	Thromboxane
UCP	Uncoupling protein
VGCC	voltage-gated Ca^{2+} channels
VSMCs	Vascular smooth muscle cells
WAT	White adipose tissue

Chapter 1

General introduction

1.1 Blood vessel structure

The vascular system consists of a series of vessels that play a vital role in distributing the blood flow throughout the circulatory system. The blood vessels are divided into arteries, veins and capillaries. Arteries supply the organs with blood and carry oxygenated blood from the heart while veins carry deoxygenated blood back to the heart (Pugsley & Tabrizchi, 2000). Capillaries connect the smallest branches of vessels, arterioles and venules, and are responsible for exchanging oxygen, carbon dioxide, metabolites, and nutrients with tissues (Mazurek et al., 2017). The blood vessel wall in general consists of three main layers as follows: tunica intima, tunica media and tunica adventitia (Figure 1.1). The tunica intima or tunica interna, is an inner layer of endothelial cells, which lines the entire vasculature. A number of physiological and pathological functions are performed by the endothelium including modulation of vascular tone and blood flow, effects on cellular proliferation, immunity and inflammatory response regulation and blood coagulation. A number of substances and biologically active factors such as nitric oxide (NO), prostacyclin (PGI_2), thromboxane (TXA_2) and endothelium derived hyperpolarizing factor (s) (EDHF) are released by endothelium which modulate vasomotor function and affect homeostasis (Kang, 2014; Klabunde, 2011; Sandoo et al., 2010). NO for example is a key vasodilator released through the actions of locally released and circulating factors acting on endothelial receptors or in response to shear stress. NO also inhibits platelet adhesion and aggregation as well as suppressing abnormal vascular smooth muscle cell migration (Oparil et al., 2003).

The tunica media is the middle layer which is composed of smooth muscle cells and elastic connective tissue. In the tunica media of vessels, large or small, smooth muscle cells are the most common type of cells. The vascular smooth muscle cells (VSMCs) are primarily responsible for regulating blood vessel tone through vasoconstriction and vasodilation. During vasoconstriction, smooth muscle in tunica media contracts, causing the

lumen to narrow and upstream arterial pressure to increase. Vasodilation increases blood flow as smooth muscle relaxes, thus widening the lumen and decreasing systemic blood pressure. A balance between vasodilator and vasoconstrictor responses regulates vascular tone. The sympathetic nervous system innervates smooth muscle via post-ganglionic noradrenergic neurons (Brozovich et al., 2016; Goodwill et al., 2017). The outer layer (tunica adventitia or tunica externa) contains collagen, fibroblasts, lymphatics and perivascular nerves and provides strength and flexibility to the arterial wall. The capillaries are composed of a single layer of endothelial cells and a basement membrane without smooth muscle. Tunica media and tunica adventitia are thicker in arteries than veins (Klabunde, 2011; Regan et al., 2015).

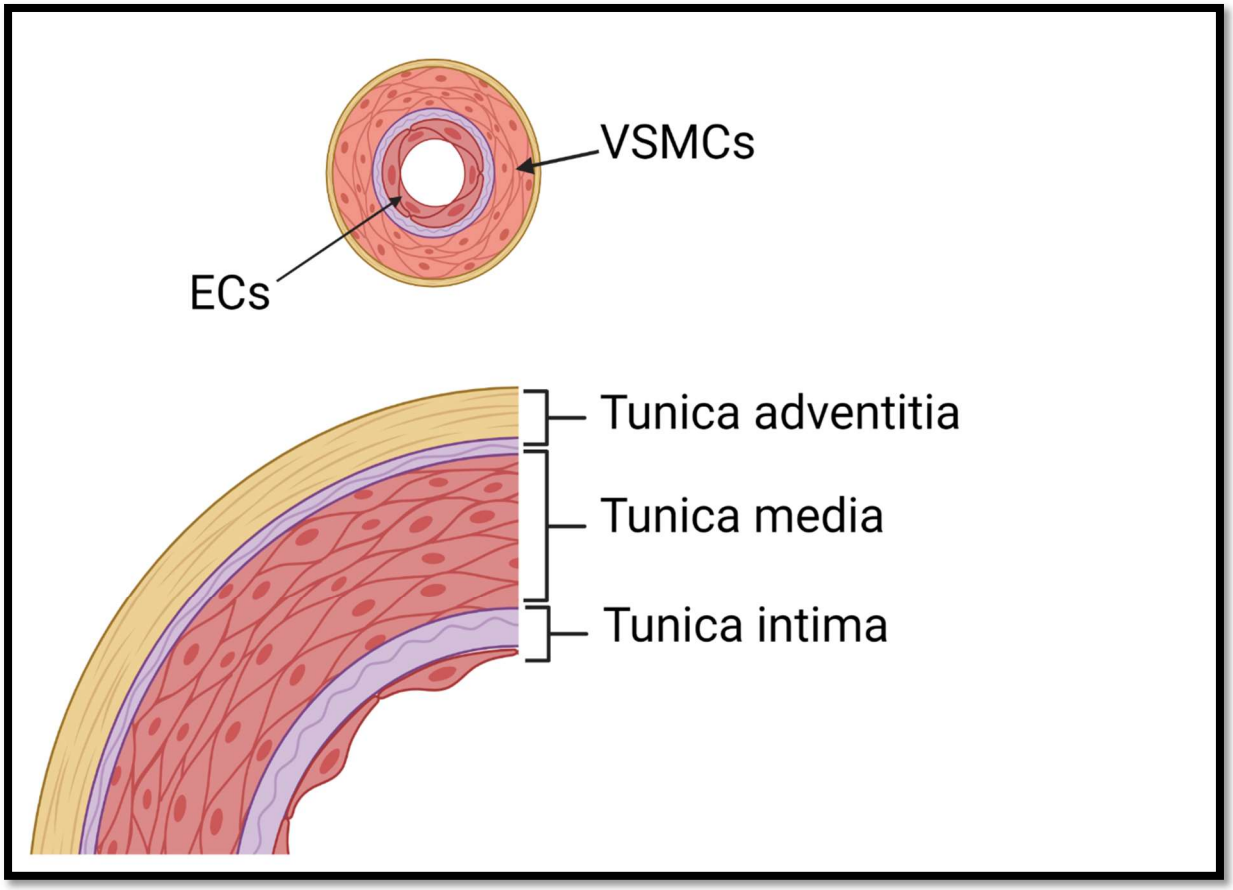


Figure 1.1: A diagram showing the three different layers of an artery wall, tunica intima, tunica media, and tunica adventitia. An arterial wall cross section showing the main vascular cells, VSMCs and ECs.

1.1.1 Regulation of vascular tone

Blood flow through the body is driven by arterial blood pressure, which is determined by total peripheral resistance and cardiac output. The length and the diameter of blood vessels control peripheral resistance. In addition to providing an important role for regional circulation of blood flow and tone, small resistance arteries (lumen diameter 50-400 μm) regulate blood pressure by controlling vascular resistance. The cardiac output which is the amount of blood pumped by the heart each minute, is determined by the heart rate and stroke volume. The control of vascular tone and vessel diameter are a very complex process controlled by neurogenic and endocrine factors (e.g. noradrenaline, angiotensin II, vasopressin), as well as local factors (e.g. endothelin, NO, acetylcholine and ATP) (Christensen & Mulvany, 2001; Gordan et al., 2015; Regan et al., 2015). VSMCs control blood pressure and blood distribution by dynamically contracting and relaxing. An increase in intracellular Ca^{2+} concentration triggers VSMC contraction. It is possible to increase intracellular free Ca^{2+} concentrations either by releasing Ca^{2+} from intracellular stores in the sarcoplasmic reticulum and/or by allowing Ca^{2+} to enter the extracellular space through plasma membrane Ca^{2+} channels, particularly via L-type voltage-gated calcium channels. Calmodulin binds Ca^{2+} in the cytosol forming the Ca^{2+}–calmodulin complex and activating myosin light chain (MLC) kinase (Figure 1.2). Consequently, MLC is phosphorylated and a cross-bridge is formed with actin, which leads to smooth muscle contraction (Gao et al., 2013; Zhao et al., 2015). In addition to increasing extracellular Ca^{2+} influx, in response to some agonists such as hormones or neurotransmitters through GPCRs, release of intracellular Ca^{2+} can be stimulated through IP_3 receptors by activating phospholipase C, which in turn forms inositol 1,4,5-trisphosphate (IP_3), which triggers Ca^{2+} release from the intracellular Ca^{2+} stores in the sarcoplasmic reticulum (Kuo & Ehrlich, 2015; Webb, 2003). Store-operated calcium channels (SOCs) are also important calcium signalling pathways. SOCs are mainly activated when intracellular calcium

stores from sarcoplasmic reticulum are depleted (Parekh & Putney Jr, 2005). In addition, Ca^{2+}-independent pathways are involved in contraction of VSMCs through Rho protein kinase pathways, a family of small GPTases mainly located at the plasma membrane of VSMCs. Rho kinase activation causes the inhibition of MLC phosphatase. This promotes a higher degree of myosin light chain phosphorylation, which stimulates smooth muscle contractions (Fukata et al., 2001).

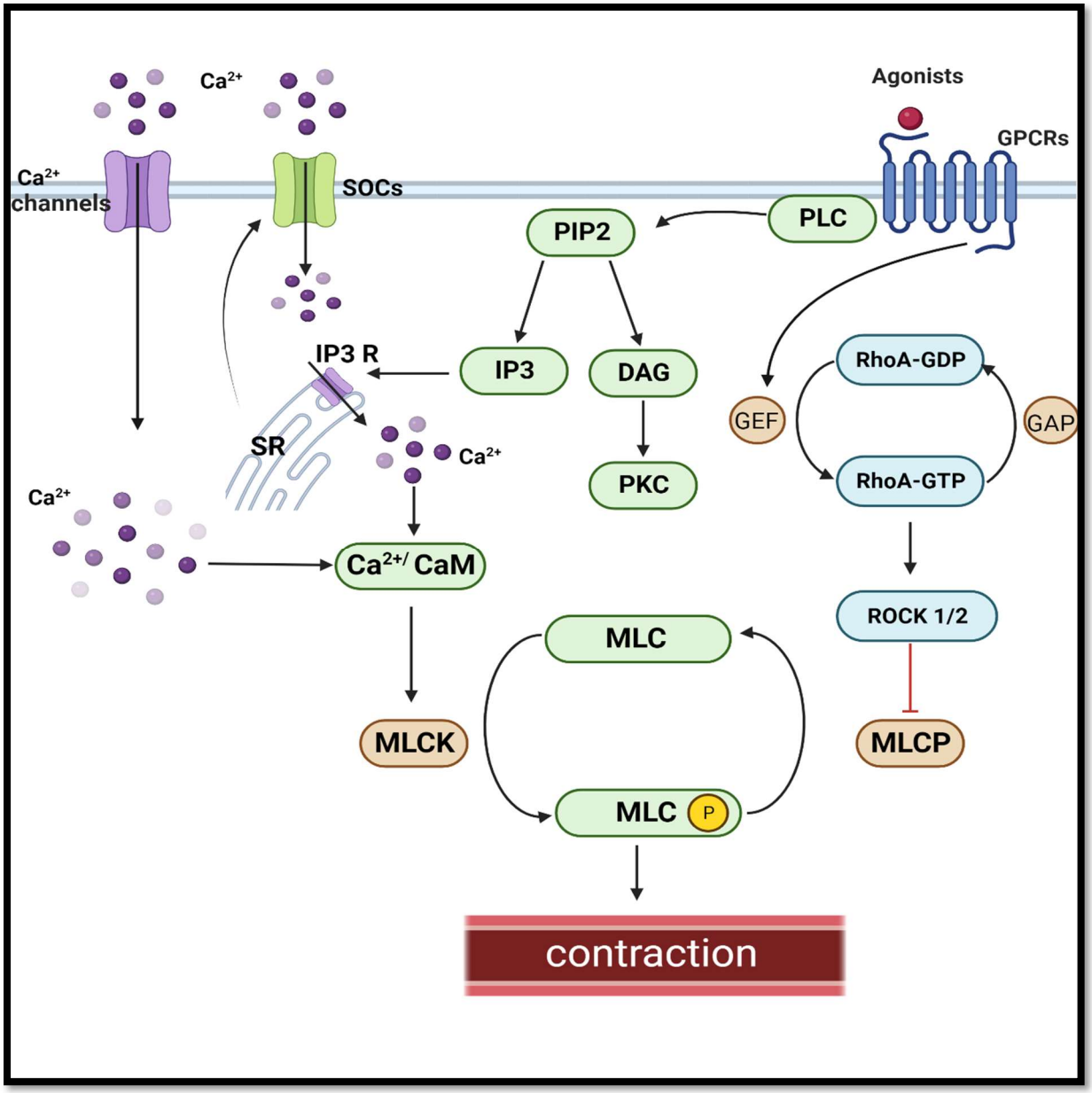


Figure 1.2: Vascular smooth muscle contraction mechanism. Ca^{2+} enters the extracellular space through plasma membrane Ca^{2+} channels or by

coupling through a GPCRs, the cell increases phospholipase C (PLC) activity following this binding. Diacylglycerol (DG) and inositol 1, 4, 5-trisphosphate (IP_3) are potent second messengers produced by the membrane lipid phosphatidylinositol 4, 5-bisphosphate by PLC. The DG, together with Ca^{2+}, activates protein kinase C (PKC), which phosphorylates specific target proteins. PKC promotes contractions in smooth muscles. An increase in intracellular Ca^{2+} levels is further induced by IP_3 by releasing Ca^{2+} from the sarcoplasmic reticulum. Myosin light chain kinase (MLCK) is activated when calcium binds to calmodulin. A contraction is induced when myosin light chain (MLC) is phosphorylated by (MLCK). In addition, in response Ca^{2+} reduction in sarcoplasmic reticulum*, SOCs are activated. The second mechanism involves activating a guanine nucleotide exchange factor (GEF) that converts Rho-GDP into Rho-GTP via the GPCR. MLCP is inhibited and contractions are induced by ROCK/2 kinases activated by Rho.*

Relaxation of smooth muscles is caused either by the removal of contractile stimuli, or by substances that directly inhibit contractility. Endogenous and exogenous substances can bind to GPCRs on the endothelium, stimulating EDHF and producing NO, PGI_2, and other diffusible mediators such as hydrogen peroxide (H_2O_2). With regard to EDHF myoendothelial gap junction (MEGJ), electrically couple between VSMCs and endothelial cells, such that the hyperpolarizing current can reach adjacent VSMCs, ultimately causing hyperpolarization and relaxation. Increasing intracellular Ca^{2+} concentration in the endothelium contributes to activation of these vasodilator pathways. For example, by increasing Ca^{2+} concentration, calcium–calmodulin complexes will be formed, which activate the calmodulin-binding domain of the endothelial NO synthase (eNOS), resulting in catalysing the breakdown of L-arginine into NO (Quillon et al., 2015). Endothelial cells release NO which targets smooth muscle cells and relaxes them by activating a soluble guanylate cyclase (sGC) and elevating intracellular cyclic guanosine monophosphate (cGMP) levels, resulting in the activation of cGMP-dependent protein kinases (PKG). The cGMP/PKG

signalling works by dilating blood vessels through a number of pathways including activation of calcium-activated potassium channels (BK_{Ca}) resulting in the loss of intracellular potassium and hyperpolarization of the plasma membrane and decreased calcium influx through voltage-gated calcium channels. The cGMP/PKG also controls intracellular calcium levels through regulation of sarcoplasmic reticulum calcium release by phosphorylation of IP_3 receptors (Francis et al., 2010; Lincoln et al., 2001). In addition, a major role is played by cyclooxygenase (COX) in vascular endothelium in the production of PGI_2. Adenylyl cyclase (AC) is stimulated by PGI_2 by binding to the prostacyclin receptor (IP) on the VSMC membrane, which leading to conversion of ATP to cyclic adenosine monophosphate (cAMP), activating PKA and causing vasodilation (Barac & Panza, 2009) (Figure 1.3).

KCl is commonly used as a depolarizing agent and as a standard tool-set used in cardiovascular isometric tension experiments. KCl depolarizes muscle cells by increasing extracellular potassium concentrations. As a result of this depolarization, muscle contractions are triggered and calcium influx is increased through VGCC (Ratz et al., 2005).

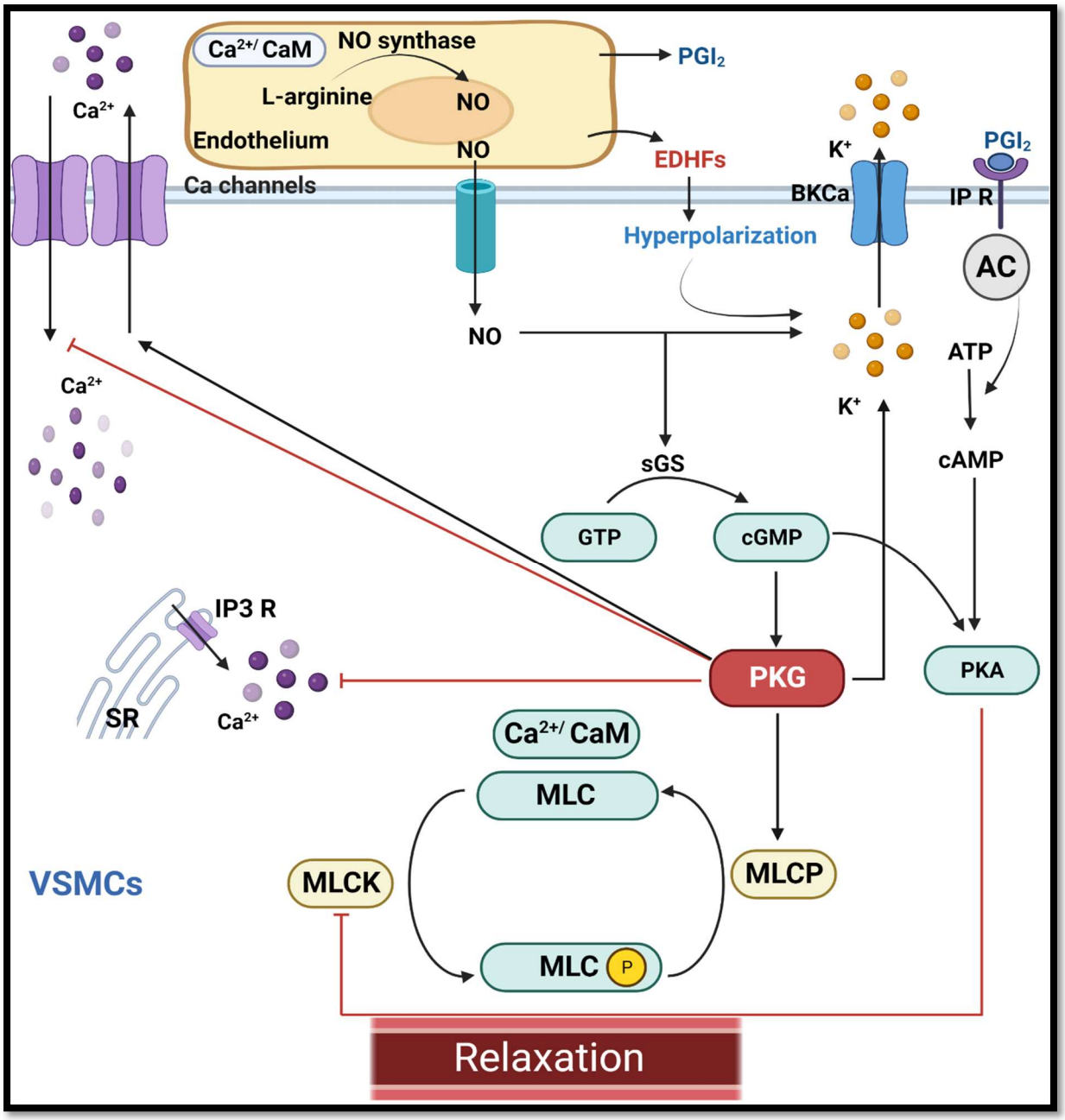


Figure 1.3: Vascular smooth muscle relaxation mechanism. A response to external neuronal, humoral or mechanical stimulation calcium-calmodulin complexes will be formed as Ca^{2+} concentration increases, activating the calmodulin-binding domain of endothelial NO synthase (eNOS) to catalyze the breakdown of L-arginine into NO. As NO diffuses to nearby smooth muscle cells (VSMC), it activates soluble guanylate cyclase (sGC), increasing intracellular cGMP production. The cGMP-dependent protein kinase (PKG), which regulates many target proteins, is activated by cGMP. Relaxation requires a decrease in intracellular Ca^{2+} concentration, a

decrease in intracellular K^+ levels and an increase in MLC phosphatase (MLCP) activity. Endothelial cells produce PGI_2. The prostacyclin receptor (IP) is the main target of PGI_2. Adenylyl cyclase (AC) and cAMP are increased by PGI_2/IP coupling to G_s. In turn, activation of cAMP can trigger the activation of PKA and the inhibition of MLCK, leading to relaxation.

1.2 Purinergic receptors

Nucleotide and nucleoside receptors are widely expressed in various organs. Purinergic signalling plays an important role in controlling many biological functions through activation of extracellular purine and pyrimidine receptors, which are expressed throughout the body system including cardiovascular, central and peripheral nervous, respiratory and gastrointestinal systems. Many types of cells within the vascular system express purinergic receptors, including endothelial cells, smooth muscle cells, platelets and immune cells. A variety of vascular functions are regulated by purinergic receptors, such as vascular permeability, vascular cell remodelling, proliferation and migration, platelet activation, endothelium function and contractility. Several vascular diseases including atherosclerosis, hypertension and thrombosis are thought to be caused by the dysfunction of these receptors (Burnstock & Ralevic, 2014; Ralevic & Dunn, 2015). Currently, fifteen different purinergic receptors for adenine and uridine nucleotides and nucleotide sugars have been identified in mammals. The original nomenclature of the purinergic receptors was introduced in 1978 by Burnstock. This classification divided receptors into P1 for adenosine and P2 for ATP and ADP. Adenosine P1 receptors are divided into subtypes referred to as A_1, A_{2A}, A_{2B} and A_3 which all belong to the G protein-coupled receptor (GPCR) superfamily. P2 receptors were then classified into two types P2X and P2Y receptors. P2X receptors are ligand-gated ion channel receptors and comprise seven receptor subtypes P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7 which are activated in response to extracellular ATP. P2Y receptors are typical GPCR and include eight mammalian subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and

$P2Y_{14}$. P2Y receptors are activated by a variety of extracellular nucleotides including ATP, ADP, UTP, UDP and UDP-glucose (Table 1.1) (Fredholm et al., 2021; Illes et al., 2021; Jacobson et al., 2020). Purinergic receptors have been identified in various porcine tissues, including A_1, A_2, P2X1 and $P2Y_2$ (Alefishat et al., 2015; Shatarat et al., 2014; Sun et al., 2019). The present thesis focusses on the role of purines in regulating vascular contractility.

Table 1.1: Adenosine, purine and pyrimidine nucleotides acting at purinergic receptors.

Agonist	Receptor	Reference
Adenosine	A_1, A_{2A}, A_{2B}, A_3	(Fredholm et al., 2021; Illes et al., 2021; Jacobson et al., 2020)
ATP	P2X1-P2X7,$P2Y_1$ $P2Y_2$, $P2Y_{11}$	
ADP	$P2Y_1$, $P2Y_{12}$, $P2Y_{13}$	
UTP	$P2Y_2$, $P2Y_4$, $P2Y_6$	
UDP	$P2Y_6$	
UDP-glucose	$P2Y_{14}$	

1.2.1 Adenosine Receptors

Adenosine is the main agonist at P1 receptors; therefore, the term adenosine receptor is used to indicate these receptors. Adenosine is an endogenous nucleoside produced mainly from the metabolism of ATP. There are four different adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). These receptors are widely distributed throughout the body. Adenosine signalling through these receptors is mainly mediated through adenylyl cyclase activation or inhibition. A_1 and A_3 receptors are coupled to the Gi/o protein and α-subunits which inhibit intracellular cyclic adenosine monophosphate

(cAMP) production and results in reduction of PKA activity. A_{2A} and A_{2B} receptors interact with Gs/olf proteins, which stimulate intracellular cAMP production and results in stimulation of PKA. Furthermore, adenosine receptors can stimulate further pathways involving phospholipase C (PLC), mitogen-activated protein kinases (MAPKs) and Ca^{2+}. Adenosine receptors are involved in regulation of cardiac contractility, heart rate and vascular tone (Fredholm et al., 2001; Jacobson & Gao, 2006).

Adenosine receptors have been reported to cause vasoconstriction or vasodilation in different blood vessels and species (Ralevic, 2009). The adenosine vasorelaxant effects are mediated mainly by A_{2A} and A_{2B} receptors which are distributed in both the smooth muscle and endothelium (Headrick et al., 2013; Kemp & Cocks, 1999; Ralevic & Burnstock, 1998). It has been reported that endothelium plays a crucial role in adenosine-induced relaxation in a number of blood vessels such as the aorta (Arsyad & Dobson, 2016; Lewis et al., 1994). There is evidence that adenosine-induced vasodilation is associated with the activation of endothelial NO, prostanoids and hyperpolarizing factors (Mustafa et al., 2009; Ray & Marshall, 2006). In porcine vessels, it has been shown that adenosine generates concentration-dependent relaxation in mesenteric and coronary arteries and retinal arterioles (Alefishat et al., 2015; Riis-Vestergaard et al., 2014; Sun et al., 2019). It has been shown that adenosine stimulates K_{ATP} channels and induces hyperpolarization in mesenteric arteries smooth muscle and cerebral capillary endothelium by activating AC-cAMP-PKA pathways (Kleppisch & Nelson, 1995; Sancho et al., 2022). In addition to vosorelaxation, vasoconstriction mediated by adenosine receptors has been reported in several vascular beds. For example, contraction responses have been shown to be mediated by an A_1 receptors in mice aorta and afferent arterioles (Hansen et al., 2003; Kunduri et al., 2013).

1.2.2 P2X Receptors

It is well established that P2X receptors are widely expressed throughout arteries and veins and are involved in modulation of vascular tone. P2X

receptors are cell surface ligand-gated ion channels activated in response to extracellular ATP. Seven subunits of the P2X receptors have been cloned and recognised (P2X1-7) with 35–54% similarity in sequence. All P2X subunits have a common topology with two hydrophobic transmembrane domains with a large extracellular ATP-binding site loop and intracellular cytoplasmic amino and carboxyl termini containing binding sites of protein kinases. It is possible for P2X receptor subunits to form functional trimeric homomers (containing the same subunits) for example, P2X1 and P2X7 or heteromers (containing different subunits) such as P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6 and P2X4/7 and the properties are different for each (Browne, 2012; Coddou et al., 2011; Lewis & Evans, 2001; North, 2002). In response to ATP binding to the extracellular loop of P2X receptors, there is a conformational change in the transmembrane channel promoting efflux of K^+ and influx of Na^+ and Ca^{2+} through voltage-gated Na^+ and Ca^{2+} channels respectively, leading to membrane depolarization and vasoconstriction. Sympathetic nerves are an important source of ATP acting at vascular smooth muscle P2X receptors but other sources include contracting smooth muscle cells and damaged cells (Figure 1.4) (Li et al., 2010; Rummery et al., 2007). P2X1 and P2X3 receptors undergo rapid desensitization followed by long recovery periods until they can be activated again. P2X1 and P2X3 desensitization takes place within milliseconds, whereas P2X2, P2X4, P2X5 and P2X7 desensitize more slowly, taking 100 to 1000 times longer (Rettinger & Schmalzing, 2003).

There are two predominant types of P2X receptors expressed in smooth muscle cells in the vasculature: P2X1 and P2X4. P2X7 receptors are also present. In smooth muscle cells of the vascular system, P2X2, P2X3, P2X5, and P2X6 are either not expressed or rarely expressed (Harhun et al., 2015; Lewis & Evans, 2001). The contractile response which is mediated by P2X receptors activation has been shown in various blood vessels such as rat isolated pulmonary artery and vein (Liu et al., 1989), renal (Guan et al., 2007) and rat mesenteric vascular bed (Gitterman & Evans, 2000). This

vasoconstriction induced by ATP seems to be mediated mainly by P2X1 receptors as shown in mesenteric arteries of P2X1 receptors-deficient mice (Vial & Evans, 2002). In addition, immunohistochemical studies and real-time PCR and western blot assays have shown that P2X1 receptors are highly expressed in vascular smooth muscle (Lewis & Evans, 2000; Wang et al., 2002). Moreover, rat immunohistochemical studies have shown a higher degree of expression of P2X1 receptors in medium and small arteries than in large arteries (Lewis & Evans, 2001). Porcine mesenteric arteries are known to express contractile P2X1-like receptors sensitive to the metabolically stable analogue α,β-meATP (Shatarat et al., 2014). Furthermore, in porcine pancreatic arteries, there is evidence that P2X1 receptors are responsible for the contraction induced by ATP and α,β-meATP. P2X4 receptors have been shown to be coexpressed with P2X1 in vascular smooth muscle cells of rat cerebral arteries (Harhun et al., 2015) and human omental arteries (Nichols et al., 2014). Moreover, pyrimidine nucleotides have been shown to induce contraction through activation of P2X receptors. For instance UTP can induce artery contraction via actions at vascular smooth muscle P2X1 receptors (Froldi et al., 1997; McLaren, Sneddon, et al., 1998). However, this effect might be due to contamination of the UTP stock.

The functional roles of the vascular smooth muscle cells P2X4 receptors remain unclear. There is some evidence that $P2X_4$ receptors are expressed in endothelial cells in addition to the vasorelaxant P2Y receptors. P2X4 has a high level of expression in human umbilical vein endothelial cells and contributes to vasodilation (Wang et al., 2002). ATP release, in response to stimulation by fluid shear stress mediated Ca^{2+} influx and resulted in increased intracellular Ca^{2+} concentrations and endothelium-derived relaxing factors (EDRF) production such as NO and PGI_2 release (Yamamoto et al., 2000). Apyrase, which hydrolyses ATP and ADP, caused a reduction in shear stress-induced Ca^{2+} levels in endothelium of human pulmonary arteries which led the authors to conclude that P2X4 receptors are

stimulated by ATP released in response to stimulation by shear stress in endothelial cells (Yamamoto & Ando, 2004). In addition, a study in P2X4-knockout mice has shown that Ca^{2+} influx and NO production were diminished and an increase in blood pressure was evident, suggesting that P2X4 receptors in the endothelium are essential for controlling vascular function and blood pressure (Yamamoto et al., 2006).

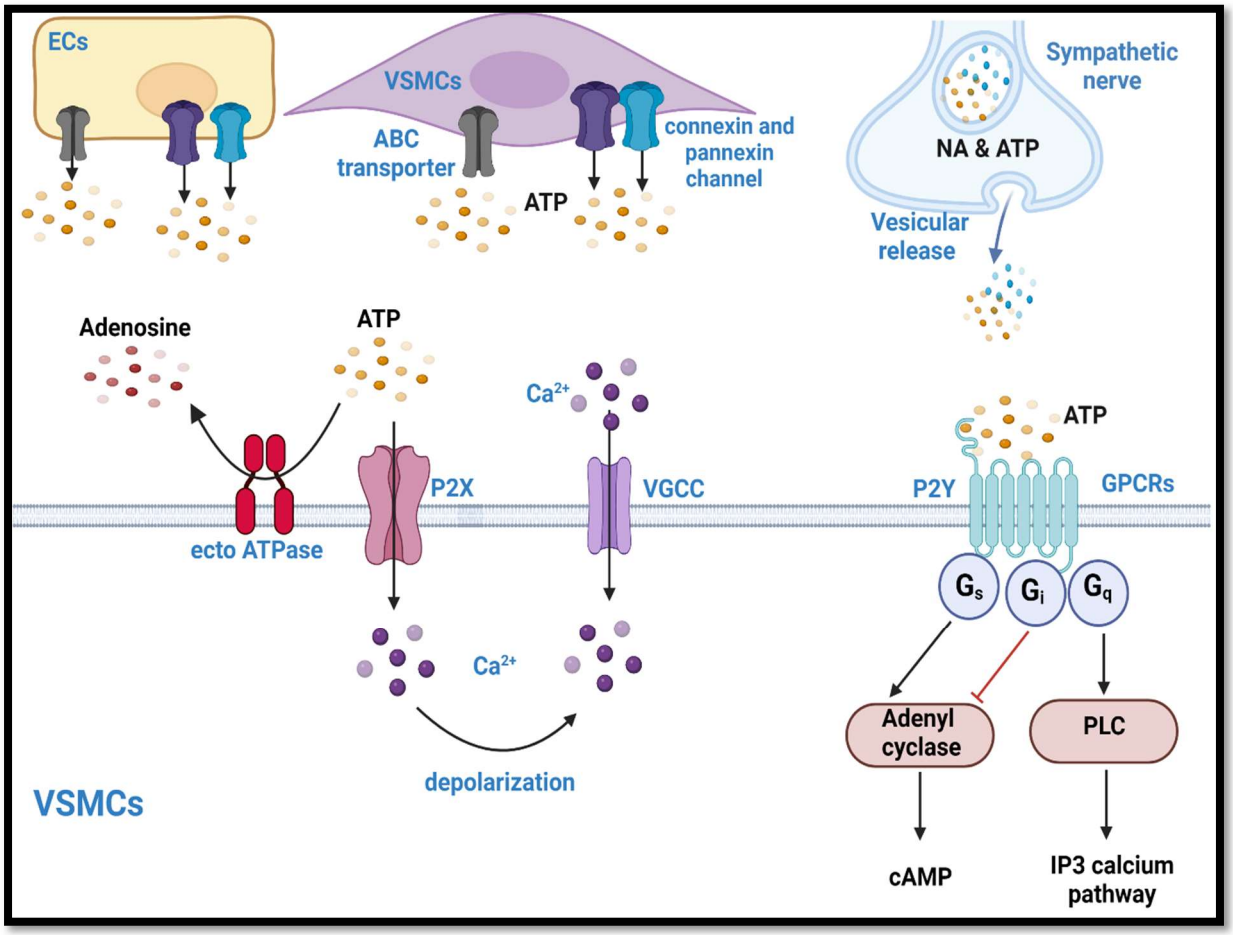


Figure 1.4: An illustration showing ATP release from sympathetic nerve, VSMCs or ECs via a number of mechanisms including exocytosis, connexin and pannexin channels or ABC transporters. Cell surface ectonucleotidases hydrolyses ATP. Signalling pathways of GPCR P2Y receptors in response to extracellular nucleotides via PLC and adenyl cyclase to increase IP_3 and cAMP production. Binding of ATP to ligand-gated ion channel P2X receptors promoting influx Ca^{2+} through voltage-gated Ca^{2+} channels (VGCC) leading to membrane depolarization and vasoconstriction.

1.2.3 P2Y Receptors

P2Y receptors are GPCRs which are extracellular receptors for purine and pyrimidine nucleotides. P2Y receptors can be activated by a variety of extracellular nucleotides including ATP, ADP, UTP, UDP and UDP-glucose. A total of eight mammalian P2Y receptor subunits have been found ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) (Jacobson et al., 2020). P2Y receptors are a family of purinergic GPCRs which consist of seven hydrophobic transmembrane domains connected by an extracellular amino terminus and an intracellular carboxyl-terminus. The extracellular loops are involved in nucleotide ligand binding while the intracellular loops are involved in signalling transmission, including the G protein activation (Jacobson et al., 2012; von Kuegelgen & Hoffmann, 2016). The P2Y receptors are further classified according to their endogenous agonists into adenine nucleotide-preferring receptors which are mostly responsive to ATP and ADP (human and rodent $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$), uracil nucleotide-preferring receptors (human $P2Y_4$ and $P2Y_6$) and mixed selectivity receptors (human and rodent $P2Y_2$ and rodent $P2Y_4$) (Ralevic & Burnstock, 1998; von Kügelgen, 2006). Additionally, it is also possible to subdivide P2Y receptors into two groups based on the specific type of G protein coupling. The first subtype includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors which are coupled through $G_{q/11}$ protein via phospholipase C (PLC) pathway. This results in increased synthesis of inositol triphosphate (IP_3) and causes release of Ca^{2+} from the endoplasmic reticulum leading to increased intracellular Ca^{2+} level and activation of protein kinase C (PKC). The second subtype includes $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors which are coupled to G_i protein and that inhibit the activity of adenylyl cyclase, thereby reducing intracellular cyclic adenosine monophosphate (cAMP). The $P2Y_{11}$ can also couple to G_s protein and activate adenylyl cyclase and increase the intracellular cAMP (Abbracchio et al., 2006; von Kügelgen, 2006).

In both vascular smooth muscle and endothelium, P2Y receptors are expressed and play important roles in regulation of blood vessels tone (Wang et al., 2002). The principal functional P2Y receptors found in endothelial cells of blood vessels are $P2Y_1$ receptors (activated by ATP and ADP), $P2Y_2$ (equally activated by UTP and ATP) and $P2Y_6$ (activated by UDP) (Ralevic, 2009; Ray et al., 2002). $P2Y_1$ and $P2Y_2$ receptors have been shown to be expressed in various human and rodent endothelial vascular beds including human umbilical vein (Wang et al., 2002), human internal mammary artery, radial artery, saphenous vein (Ray et al., 2002) and rat mesenteric artery (Buvinic et al., 2002; Mistry et al., 2003). Endothelial P2Y receptors mediate vascular relaxation in response to extracellular nucleotides by release of prostaglandins and endothelium-derived hyperpolarizing factor (EDHF) (Mistry et al., 2003; Wihlborg et al., 2003). Moreover, activation of $P2Y_2$ receptors by ATP and UTP in human umbilical vein endothelial cells (HUVECs) resulted in Ca^{2+} mobilization and mediating membrane hyperpolarization and NO production (Raqeeb et al., 2011). In the rat mesenteric artery, binding of 2-MeSATP and UTP to $P2Y_1$ and $P2Y_2$ receptors mediated vasodilatation through increase in NO release and accumulation of cyclic guanosine monophosphate (cGMP) (Buvinic et al., 2002). In human and bovine endothelial cells, $P2Y_2$ receptors through $G_{q/11}$ have been shown to mediate fluid shear stress acting on the endothelium to induce the intracellular Ca^{2+} concentration and activation of the endothelial NO synthase (eNOS). $P2Y_2$ $G_{q/11}$ deficient mice have shown a lack of flow-induced vasodilation associated with hypertension and reduced eNOS formation (Wang et al., 2015).

There are many studies which have demonstrated the existence of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in vascular smooth muscle cells. Generally, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in vascular smooth muscles have been shown to mediate contraction and contribute in the regulation of vascular tone. This vasoconstriction has been shown in a range of vascular beds in different species including human coronary arteries mainly by activation of

$P2Y_2$ and $P2Y_4$ receptors by the stable pyrimidine agonists ATPγS and UTPγS (Malmsjö, Hou, et al., 2000). Moreover, it has been reported that ATP and UTP induce contraction with similar potencies in rat small pulmonary veins. The contractile response in this study to extracellular ATP is mediated by smooth muscle $P2Y_2$ receptors, and it was found that suramin and AR-C118925 inhibited contraction induced by ATP (Henriquez et al., 2018). According to a study, UTP and MRS2768, a selective $P2Y_2$ agonist, induce vasoconstriction of porcine pancreatic arteries via $P2Y_2$ receptors (Alsaqati et al., 2014). Moreover, it has been reported that $P2Y_2$ receptor in porcine coronary artery smooth muscle is the mediator of the vasoconstrictor property of UTP (Rayment et al., 2007). In addition, in rat mesenteric artery, the role of contractile $P2Y_2$ and $P2Y_4$ receptors was confirmed and ATPγS and UTPγS were shown to be more potent than endogenous ATP and UTP due to ectonucleotidase activity (Malmsjö, Adner, et al., 2000).

$P2Y_6$ receptors are expressed in vascular tissues, and are activated by UDP and UTP. There is a greater affinity for UDP than UTP for the $P2Y_6$ receptor, and the nucleotides of adenine are mostly inactive (von Kügelgen, 2019). $P2Y_6$ receptors activated by the selective agonist UDPβS, have been reported to mediate contraction in human cerebral arteries and rat mesenteric artery (Malmsjö, Adner, et al., 2000; Malmsjö et al., 2003). In addition, it has been reported that myogenic tone is maintained through activation of $P2Y_6$ receptors in response to endogenous pyrimidine nucleotides release in mesenteric resistance arteries, and via a direct mechanical activation of $P2Y_4$ and $P2Y_6$ receptors without endogenous nucleotides release in cerebral arterioles (Brayden et al., 2013; Kauffenstein et al., 2016). $P2Y_{12}$ receptors in vascular smooth muscle have also been shown to mediate contraction after stimulation with ADP in human coronary and rat intrapulmonary arteries (Mitchell et al., 2012; Wihlborg et al., 2004). Additionally, $P2Y_{14}$ receptors activated by UDP-glucose have been reported to induce contraction in mouse and porcine coronary and cerebral arteries and in porcine pancreatic arteries through

cAMP-dependent mechanisms (Abbas et al., 2018; Alsaqati et al., 2014; Haanes & Edvinsson, 2014). The pyrimidine nucleotides mediate contraction via different signal transduction pathways including coupling to $G_{\alpha q/11}$, IP_3 to induce Ca^{2+} release from endoplasmic reticulum, promoting Ca^{2+} influx through voltage-gated Ca^{2+} channels and Rho kinase and protein kinase C pathway (Sugihara et al., 2011; Tengah et al., 2018).

A recognised limitation in the study of P2 purine receptors has been the complex pharmacological profiles of endogenous nucleotides which can stimulate more than one P2 receptor subtypes. Furthermore, there is a lack of specific antagonists at some P2 receptor subtypes. The broad-spectrum P2 receptor antagonist suramin is known to inhibit P2X and P2Y receptors. Generally, suramin has been non-competitive with low potency for inhibiting the P2 receptor subtypes (Ralevic & Burnstock, 1998). Suramin has been used clinically to treat African sleeping sickness and as an antiparasitic drug. Although suramin targets a wide range of biological processes and signalling pathways, its potential applications are numerous and diverse (Wiedemar et al., 2020). Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is another non-selective P2 receptor antagonist that affects most P2X and P2Y receptor subtypes. PPADS has been widely used to study the physiological and pathological roles of these receptors. Its potency and selectivity can vary depending on the receptor subtype and experimental conditions (Ralevic & Burnstock, 1998). Aside from the broad spectrum P2 receptor antagonists, there are relatively potent and selective antagonists for some P2X and P2Y receptor subtypes (Table 1.2). A selective antagonist for $P2Y_2$ receptors, AR-C118925XX, was developed with submicromolar potency at recombinant human $P2Y_2$ receptors in CHO cells (IC_{50} 0.0721 ± 0.0124 μM vs 500 nM UTP, and 0.0574 ± 0.0196 μM vs 500 nM ATP) (Rafehi et al., 2017) and micromolar potency at inhibiting both mucin secretion from differentiated human bronchial epithelial cells (IC_{50} ~1 μM vs 100 μM ATPγS) (Kemp et al., 2004) and calcium responses in primary human in vitro-differentiated adipocytes

(IC_{50} 0.683±116 μM vs ATP) (Ali et al., 2018). AR-C118925XX was inactive at all other P2Y receptors at 10 μM (Kemp et al., 2004; Rafehi et al., 2017). In human vascular endothelial cells, AR-C118925XX (10 nM to 1 μM) did not affect intracellular Ca^{2+} directly, but shifted the UTP concentration-response curve rightward without changing maximum response (Muoboghare et al., 2019). However, it also antagonised recombinant human P2X1 (IC_{50} 2.63 ± 0.49 μM) and P2X3 receptors (IC_{50} 0.819 ± 0.102 μM), thus making it about 50-fold and 14-fold selective for the $P2Y_2$ vs the P2X1 and P2X3 receptors respectively (Rafehi et al., 2017). AR-C118925XX was shown to be inactive at 35 other (non-purinergic) receptors at 10 μM (Kemp et al., 2004). The commercial availability of AR-C118925XX has opened up opportunities to increase our understanding of the physiological roles of $P2Y_2$ receptors. MSG228 (compound 17) is a compound developed based on AR-C118925 and shown to be selective and high-affinity for human $P2Y_2$ receptor in calcium mobilization (PKd = 6.51) (Conroy et al., 2018).

Table 1.2: Selective antagonists for P2X and P2Y receptors.

Receptor	Selective Antagonist	Reference
P2X1	NF449 NF023 NF279 PSB-2001 PSB-2014	(Burnstock, 2007; El - Ajouz et al., 2012; Illes et al., 2021; Tian et al., 2020)
P2X2	NF770	(Wolf et al., 2011)
P2X3	A-317491 Gefapixant (AF-219)	(Illes et al., 2021)
$P2Y_1$	MRS 2179 MRS 2279 MRS 2500	(Kim et al., 2003; von Kügelgen & Hoffmann, 2016)

$P2Y_2$	AR-C118925XX	(Kemp et al., 2004)
$P2Y_6$	MRS 2578	(Mamedova et al., 2004)
$P2Y_{12}$	Clopidogrel Ticagrelor AR-C69931	(Huang et al., 2000; Savi et al., 2001; Springthorpe et al., 2007)
$P2Y_{14}$	PPTN	(Barrett et al., 2013)

1.2.4 Nucleotides release

Almost all tissues and cells show regulated release of ATP, which is accompanied by the release of UTP in some cases (Lazarowski, 2012). It is well established that ATP is co-released with noradrenaline from the perivascular sympathetic nerve terminals and functions as a neurotransmitter and to evoke excitatory junction potentials and vasoconstriction (Burnstock, 2009). ATP may also be released from other cell types including sensory nerves, erythrocytes, leucocytes, and platelets, and during inflammation and injury (Gorini et al., 2013). ATP and UTP are released from endothelial cells in response to hypoxia and shear stress (Erlinge & Burnstock, 2008). However, there is limited knowledge about the presence of UTP and other pyrimidines in synaptic vesicles. UTP and UDP have shown to be stored in granules of chromaffin cells and platelets and are present at about 10% of the ATP concentration (Anderson & Parkinson, 1997). In most cell types the intracellular concentration of ATP ranges from 1 to 10 mM whereas, in normal conditions, ATP at the extracellular is kept at the subnanomolar range (Gorini et al., 2013). Release of UTP was measured in medium bathing resting cultures of a selection of cell lines including platelets and leukocytes, airway epithelial cells and rat astrocytes. The extracellular UTP concentrations were found in the range from 1 to 10 nM, around one-third of the ATP concentration

(Lazarowski & Harden, 1999). It has been reported that both osteoclasts and osteoblasts constitutively release ATP (Brandao-Burch et al., 2012).

Regarding constitutive nucleotide release and activation of vascular P2 receptors, in vivo studies have shown that ATP is tonically released from glial cells in rat retinal arterioles and acts on P2X1 receptors on vascular smooth muscle cells to cause constriction; in this study removal of endogenous nucleotides with apyrase dilated the blood vessels and ectonucleotidase inhibition (to prevent nucleotidase metabolism) caused constriction; furthermore suramin and PPADS (P2 receptor antagonists) and NF023 (P2X1 receptor antagonist) dilated the arteries, consistent with a tonic release of ATP causing contraction (Kur & Newman, 2014). There is also evidence that pyrimidines are released in response to changes in arterial pressure to cause contraction (the myogenic response) in mouse isolated small mesenteric arteries; here $P2Y_6$ receptor deletion ($P2Y_6$-/- mice) and antagonism (MRS2578) blocked the myogenic contractile response whereas ectonucleotidase inhibition potentiated it, consistent with nucleotide auto/paracrine activation of vascular $P2Y_6$ receptors (Kauffenstein et al., 2016). It has also been shown that myogenic tone of cerebral arterioles is mediated via direct mechanical activation of $P2Y_4$ and $P2Y_6$ receptors rather than the release of endogenous nucleotides (Brayden et al., 2013). It has been found that nucleotides can be released constitutively from adipocytes themselves to regulate adipocyte function, primarily via activation of adipocyte $P2Y_2$ receptors (Ali et al., 2018; Rossato et al., 2022).

A number of mechanisms have been proposed to control ATP and other nucleotides release including connexin hemichannels and pannexin channels. Connexin and pannexin channels play significant roles in regulation of purinergic transmission throughout the body (Lohman et al., 2012). Connexins are intercellular gap junction channels that allow diffusion of ions and small molecules including ATP between cells in vertebrates. Connexins come together in groups of six and form pores

named connexons or hemichannels (Sáez & Leybaert, 2014). A connexon hemichannel in the plasma membrane of one cell docks with a connexon hemichannel in the plasma membrane of adjacent cells. Docking of two connexon hemichannels creates intercellular gap junction channels that directly connects the cytoplasm of the two cells (Sáez et al., 2003). However, the connexin may remain undocked at the plasma membrane and mediate communication between the intercellular and the extracellular compartments of an individual cell (Harris, 2007). To date, about 20 connexin genes have been recognised in mice and 21 connexin isoforms have been identified in humans (Söhl & Willecke, 2004). All connexin subunits have a common structure with four transmembrane domains with two extracellular loops, an intracellular loop and intracellular cytoplasmic amino and carboxyl termini (Thévenin et al., 2013). Connexin proteins are generally named in terms of their specific molecular weights in kDa for example Cx37 is the connexin protein of around 37 kDa (Sohl & Willecke, 2003). Connexin hemichannels in the plasma membrane are considered to be closed under normal physiological conditions but they can be activated in response to several stimuli including decreases in extracellular Ca^{2+} concentration, membrane depolarization and mechanical stimulation (Patel et al., 2014; Sáez & Leybaert, 2014).

In addition to connexin, another protein family named pannexin was identified. Pannexins were initially proposed to form gap junctions between adjacent cells due to the structural similarity to connexins. However, pannexins only function as channels on the plasma membrane of cells and permit the passage of ions and molecules (less than 1 kDa) between the cytoplasm and the extracellular compartment (Sosinsky et al., 2011). Pannexins share no sequence homology to the connexins but they have a similar topology. In general, pannexins comprise four transmembrane domains, two extracellular loops and one intracellular loop with amino and carboxyl termini (Penuela et al., 2013). Three pannexin proteins have been cloned in rodent and human genomes, Panx1, Panx2 and Panx3. Panx1 and

Panx3 isoforms seem to assemble into hexamers, whereas Panx2 could form heptamers or octamers (Panchin, 2005). It has been shown that unlike connexin hemichannels, pannexin channels open under normal physiological condition. Pannexin channels can be activated in response to different stimuli including membrane depolarization, hypoxia, shear stress, and ATP binding to P2X and P2Y receptors (Bruzzone et al., 2003; Locovei et al., 2006; Pelegrin & Surprenant, 2006).

In the vascular smooth muscle and endothelial cells, four connexin isoforms have been found: Cx37, Cx40, Cx43, and Cx45 (Haefliger et al., 2004; Hakim et al., 2008). In addition, it has been reported that endothelial cells express Cx32 (Okamoto et al., 2009). Several studies have investigated ATP release via these connexin hemichannels on VSMCs and ECs. It has been reported that mechanical stimulation induced ATP release via connexin hemichannels in corneal endothelial cells. In this study, ATP release promoted calcium wave propagation. Also, ATP release was inhibited by connexin hemichannels blockers flufenamic acid and peptide Gap26 (Gomes et al., 2005). Another study has reported that Cx43 hemichannels mediated ATP release from human microvascular endothelial cells. This study revealed that release of ATP from these cells was significantly reduced by hypoxia (Faigle et al., 2008). Another study provided experimental evidence that connexin hemichannels control vascular tone in rat mesenteric arteries. In this study, blocking peptide of Cx43 hemichannels resulted in reduction of norepinephrine-induced contraction and calcium oscillations. Also, ATP release from smooth muscle cells was inhibited by blockage of Cx43 hemichannels (Bol et al., 2017).

Pannexin channels are also expressed in vascular cells. A number of studies have examined the involvement of pannexin channels in the release of ATP in different cells. It has been shown that ATP can be released via Panx1 channels upon sympathetic stimulation (phenylephrine) in mouse thoracodorsal resistance arteries and cultured human coronary arterial VSMCs. ATP then activates purinergic receptors on neighbouring cells and

increases vascular tone. In this study, pannexin channels blockers mefloquine, probenecid, and 10Panx1 were highly effective in decreasing the contractile response induced by phenylephrine (Billaud et al., 2011). It has also been reported that ATP can be released through Panx1 channels in response to thrombin in human umbilical vein endothelial cells. In this study, carbenoxolone, connexin hemichannels and Panx1 channel inhibitor, significantly inhibited thrombin-mediated ATP release (Gödecke et al., 2012).

1.2.5 Metabolism of nucleotides

Extracellular nucleotides including ATP, ADP, UTP and UDP have important roles in regulation of blood vessels functions by activation of P2X and P2Y receptors. These nucleotides are inactivated by enzymes called ectonucleotidases. These are cell surface metabolizing enzymes that rapidly hydrolyse extracellular nucleotides to respective nucleosides and free phosphate. Ectonucleoside triphosphate diphosphohydrolase (NTPDase) family hydrolyses tri and diphosphate nucleotides including ATP and ADP to produce AMP. A member of the ectonucleotide pyrophosphatase/phosphodiesterase family (ENPP) can hydrolyse ATP directly into AMP. Cell surfaces contain ectonucleotidases such as NTPDase1, NTPDase2, NTPDase3 and NTPDase8, as well as ENPP1 and ENPP3. A number of NTPDase enzymes are found intracellularly including NTPDase4, NTPDase5, NTPDase6, and NTPDase7 (Robson, Sévigny, et al., 2006). Ecto-5'-nucleotidase (CD73) is the main enzyme responsible for the conversion of AMP to adenosine (Figure 1.5). NTPDase can also hydrolyse other nucleotide including UTP to UDP to UMP to uridine (Baqi, 2015; Yegutkin, 2008; Zimmermann, 2000). The expression and activity of ectonucleotidases has been identified in blood vessels (Yegutkin, 2008). NTPDase1/CD39 protein is expressed in the vascular endothelium, platelets and leukocytes (Robson, Sevigny, et al., 2006; Zimmermann, 2000). It has been reported that shear stress imposed on the vascular endothelium induces release of ATP accompanied by an extracellular rise in the activity

of enzymes that hydrolysis both ATP (ATPases) and AMP (5'-nucleotidases) (Yegutkin et al., 2000). ARL 67156 is a competitive antagonist of NTPDase, and it was developed to inhibit NTPDase, which catalyses the hydrolysis of extracellular ATP to ADP and ADP to AMP. The potency of ARL 67156 against NTPDase1 and NTPDase3 and ENPP1 is moderate, but its inhibition of NTPDase2 and NTPDase8, ENPP3 and ecto-5′-nucleotidase is less (Levesque et al., 2007). It has been reported that ARL 67156 increased ATP responses and inhibited the ecto-ATPase activity in the rabbit ear artery (Crack et al., 1995) and guinea-pig vas deferens (Westfall et al., 1996). In addition, ARL 67156 has been shown to potentiate contractions elicited by UTP in the rat isolated tail artery (McLaren, Burke, et al., 1998).

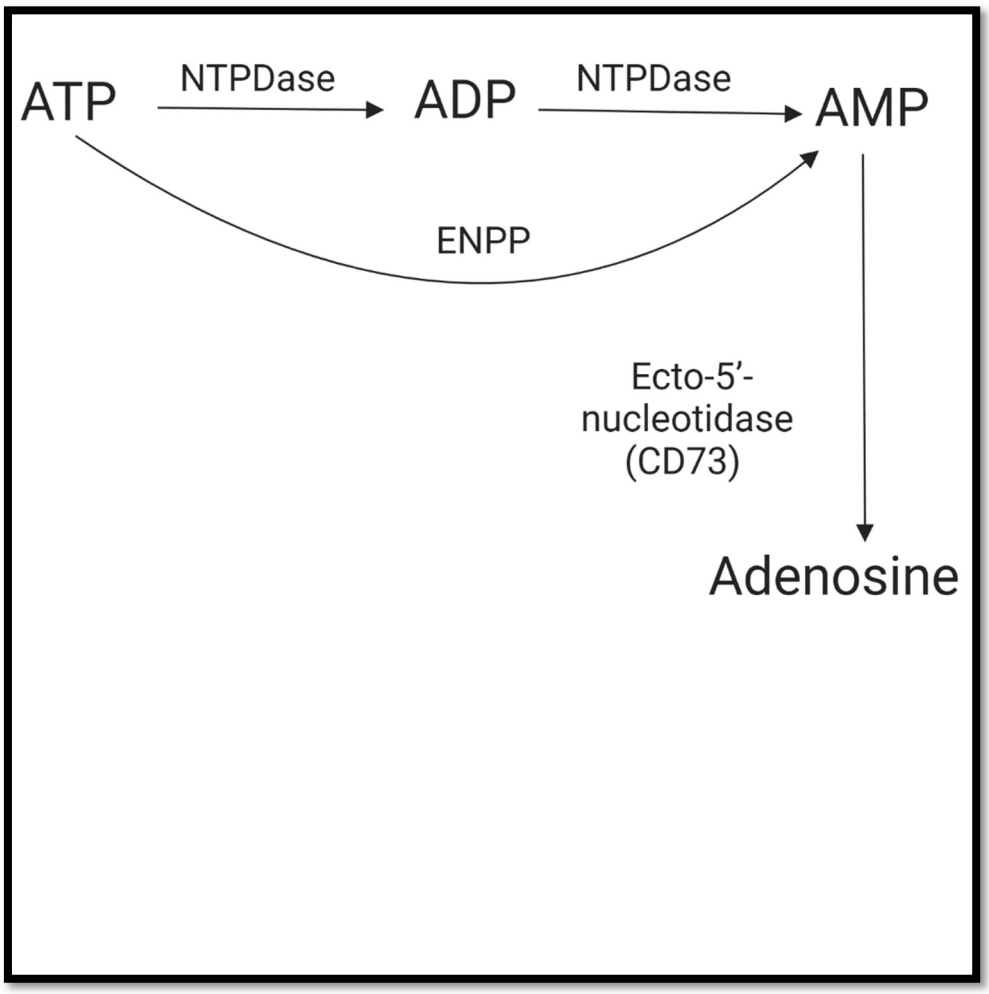


Figure 1.5: The adenine nucleotide-hydrolysing pathway by ectonucleotidases. ATP can be hydrolysed directly to AMP by ectonucleotide

pyrophosphatase/phosphodiesterase (ENPP). ATP can also be hydrolysed by ectonucleoside triphosphate diphosphohydrolase (NTPDase) to ADP. ADP is hydrolysed by NTPDase to produce AMP. AMP is converted to adenosine by ecto-5'-nucleotidase (CD73).

1.3 Adipose tissues

Adipose tissue is the main endocrine organ that regulates energy equilibrium and lipid storage. Adipose tissue produces adipokines, which are bioactive factors that regulate several metabolic functions and communicate with other organs. Adipose tissue dysfunction is an important factor in obesity and its related diseases such as insulin resistance, cardiovascular disease and diabetes. Adipocytes, pre-adipocytes, macrophages, T cells, fibroblasts, nerves and endothelial cells are among the cell types in the adipocyte tissue (Rosen & MacDougald, 2006; Victorio et al., 2016). There are two main types of adipose tissues in mammalian species, identified based on colour i.e. white adipose tissue (WAT) and brown adipose tissues (BAT). These two types of adipose tissue have different colours, structures, metabolic roles, biological characteristics and gene expression forms (Cedikova et al., 2016; Park et al., 2014). A normal adult's body weight is approximately 20% WAT, which works mainly as an energy storage for triglycerides (Gil-Ortega et al., 2015). When the body requires energy during exercise or fasting and there is not enough stored carbohydrate, triglycerides are hydrolysed into glycerol and free fatty acids and released into the blood in a process called lipolysis. WAT can also be involved in endocrine processes, through the release of adipokines such as leptin, adiponectin, resistin and TNF-α (Gil-Ortega, Somoza, Huang, Gollasch, & Fernandez-Alfonso, 2015; Medina-Gomez, 2012). WAT is typically characterised by a yellow spherical morphology with a single unilocular large lipid droplet, enclosed by a thin layer of cytoplasm, with few mitochondria and small smooth endoplasmic reticulum. White adipose tissue can be classified into visceral or intra-abdominal white adipose tissue (vWAT), and subcutaneous white adipose tissue (sWAT). Among the fatty

deposits in the visceral are those in the retroperitoneum, the omentals, the mesenteric, the epicardial, and the gonadal area (Cedikova et al., 2016; Medina-Gomez, 2012).

Brown adipose tissue (BAT) is considered as the essential source of non-shivering thermogenesis to regulate body temperature by enhancing lipid metabolism. The thermogenic mechanism in BAT occurs due to the biochemical property of the mitochondria transport protein named uncoupling protein (UCP). UCP catalyses proton conductance and uncouples the respiratory chain from ATP synthesis through raising the permeability of the inner membrane of mitochondria, consequently, allowing the energy dissipation as heat. The thermogenic process is controlled by the sympathetic nervous system via noradrenaline acting on β3-adrenoceptors (Cannon & Nedergaard, 2004; Giralt & Villarroya, 2013; Klaus, 1997). BAT is normally smaller in shape than WAT and characterised by an oval nucleus located centrally surrounded by a large cytoplasm with multilocular fat droplets, and high mitochondrial abundance (Stephens et al., 2011). In rodents and large mammals, BAT is mainly spread around the interscapular region (upper back), axillary and perirenal fat. In general, BAT displays more vascularization and innervation than WAT. BAT and WAT receive sympathetic nervous innervation (Park et al., 2014; Szasz et al., 2013). A third type of adipocyte has been recognised, called brite (brown in white) or beige adipocytes. The brite/beige adipocytes are brown adipocytes appearing within WAT and have mixed characteristics of both white and brown adipocytes. In the basal state, adipocytes of brite/beige adipocyte show a resemblance to those of WAT including a single large unilocular shape and the lack of UCP1 expression, while in response to stimulation, brite/beige adipocyte behaves more like BAT with multilocular fat droplets and UCP1 expression in a process known as browning. WAT can be transformed to brite/beige adipocytes by specific stimuli, such as cold exposure, catecholamines, β-adrenergic receptor agonists, fibroblast growth factor-21(FGF21), thiazolidinedione, peroxisome proliferator-

activated receptor gamma (PPAR-γ) agonist and polypeptide hormone irisin (Cedikova et al., 2016; Park et al., 2014; Rosenwald et al., 2013).

1.3.1 Perivascular adipose tissue (PVAT)

Perivascular adipose tissue (PVAT) is a local adipose tissue surrounding most blood vessels, excluding capillaries and pulmonary and cerebral blood vessels. PVAT has a different phenotype from other adipose tissues, and it is broadly distributed over large arteries, veins and small vessels and skeletal muscle microvessels (Gil-Ortega et al., 2015). In addition, PVAT has been found within organs such as the kidney in the renal sinus around the renal vasculature (Restini et al., 2018). PVAT is highly likely to be the tunica adiposa, a fourth layer lining the vessel wall. PVAT lies directly on the external side of the adventitial layer without any anatomical barriers or elastic lamina between them which facilitates transient actions of bioactive cytokines that are released by PVAT into the adjacent blood vessel wall (Rajsheker et al., 2010; Szasz & Webb, 2012). These soluble factors can also travel between PVAT and blood vessel wall layers by direct diffusion or by the vasa vasorum (Gil-Ortega et al., 2015).

Adipocytes are the most abundant cells in PVAT. Based on the adipose tissue categories, PVAT can be white, brown adipose tissues or mixed PVAT. For example human and rodent mesenteric PVAT is characterised by unilocular WAT, whereas abdominal aortic PVAT is considered as a mixed PVAT which mostly resembles the classic BAT with multilocular fat droplets (Ramirez et al., 2017; Szasz et al., 2013). Thoracic aortic PVAT is a classic BAT, while PVAT in human coronary artery is categorized as WAT (Brown et al., 2014; Xia & Li, 2017). Based on its location and the type of vessel, PVAT exhibits morphological, anatomical, and developmental differences. Besides adipocytes, PVAT is comprised of further cells called the stromal vascular fraction (SVF), containing lymphocytes, macrophages, fibroblasts, mesenchymal stem cells and vasa vasorum endothelial cells. The SVF cells play an important role in regulating the secretion of adipokines and other bioactive materials from PVAT (Mariman & Wang, 2010; Szasz et al., 2013).

Until recently, PVAT was considered only as a connective tissue that provides vascular structural support and mechanical protection of the adjacent vascular beds, in addition to energy storage (WAT) and non-shivering thermogenic (BAT) functions (Szasz et al., 2013). PVAT has routinely been removed for isolated blood vessel functional studies. However, Soltis and Cassis (1991) reported the first study which showed that PVAT decreased the contractile response to adrenaline in the isolated rat thoracic aorta. They stated that this anti-contractile effect was caused by the uptake of adrenaline by the surrounding adipose tissue (Soltis & Cassis, 1991). More recent studies have shown that PVAT reduces the vasocontractile effect to further agonists, including phenylephrine, serotonin, angiotensin II and endothelin-1, due to releasing a transferable adipose-derived relaxing factor (ADRF) (Lohn et al., 2002). Since then, a number of studies have sought to examine the vascular role of PVAT, and have confirmed that PVAT is a metabolically active organ which participates in vascular tone regulation and homeostasis by producing a wide range of bioactive molecules (Britton & Fox, 2011; Brown et al., 2014). The main substances released from PVAT are adipokines including (adiponectin, leptin, resistin, adrenomedullin and visfatin) and cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-1, interleukin-6, interleukin-8, monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1)). In addition to adipocytokines, PVAT releases reactive oxygen species (ROS) such as H_2S, NO, superoxide, and H_2O_2. Furthermore, components of the renin-angiotensin system (RAS) including angiotensinogen, angiotensin I & II, angiotensin (1-7), ACE1 and ACE2 are released by PVAT (Szasz et al., 2013; Szasz & Webb, 2012). These mediators work as autocrine factors to regulate PVAT metabolic process and perform paracrine/endocrine effects on blood vessels through controlling cellular process to alter vascular function, peripheral resistance and vascular tone (Cheng et al., 2018; Gollasch, 2012).

A number of these mediators can induce vasodilation (anti-contractile effects) and are known as adipocyte-derived relaxing factors (ADRF) or perivascular-derived relaxing factors (PVRF) (Figure 1.6) (Ramirez et al., 2017; Xia & Li, 2017). PVRF include leptin, adiponectin, interleukin 6, interleukin-1, TNF-alpha, angiotensin 1-7 and vascular endothelial growth factor (VEGF) (Fernández-Alfonso et al., 2011; Gollasch, 2012). The release of PVRF causes vascular tone reduction by different processes including endothelium dependent mechanisms (NO release), and activation of K^+ channels in vascular smooth muscle (Gao et al., 2007). Another mechanism for the relaxant effects caused by PVRF in rat aorta is related to ATP-dependent K^+ (K_{ATP}) channel and tyrosine kinase in a mechanism not dependent on endothelial NO synthesis (Lohn et al., 2002). A study in human internal thoracic arteries has shown that PVRF induces relaxation through activation of calcium-dependent potassium channels (Kca) (Gao et al., 2005). Further findings proposed that PVAT regulates arterial tone through stimulation of voltage-dependent, delayed-rectifier K^+ (Kv) channels which trigger hyperpolarization of the vascular smooth cell membrane (Verlohren et al., 2004). In addition, other evidence has suggested that the activation of myocyte large-conductance Ca^{2+} activated K^+ (BK_{Ca}) channels through PVAT-derived adiponectin has a role in reducing of vascular tone (Lynch et al., 2013). PVRF has been reported to act through endothelium independent and endothelium-dependent relaxation mechanisms on rat thoracic aorta via H_2O_2 and NO release and subsequent activation of soluble guanylyl cyclase (sGC) and K_{Ca} channels (Gao et al., 2007).

PVAT has also been reported to release contractile factors referred to as adipocyte-derived contractor factors (ADCF) or PVAT-derived contractor factor (PVCF) (Ramirez et al., 2017). In 2006 Gao et al. reported that PVAT enhanced the arterial contraction of rat superior mesenteric artery to perivascular nerve stimulation. This response involved the release of superoxide after activation of tyrosine kinase and the MAPK/ERK pathway

(Gao et al., 2006). An additional study in the rat mesenteric artery has demonstrated a role for angiotensin II released by PVAT in the potentiation of EFS-induced contraction (Lu et al., 2010). Another study has reported that PVAT inhibits coronary endothelial NO production by a PKC-β-dependent mechanism, which induces phosphorylation of endothelial NO synthase and Thr495 residue (Payne et al., 2009).

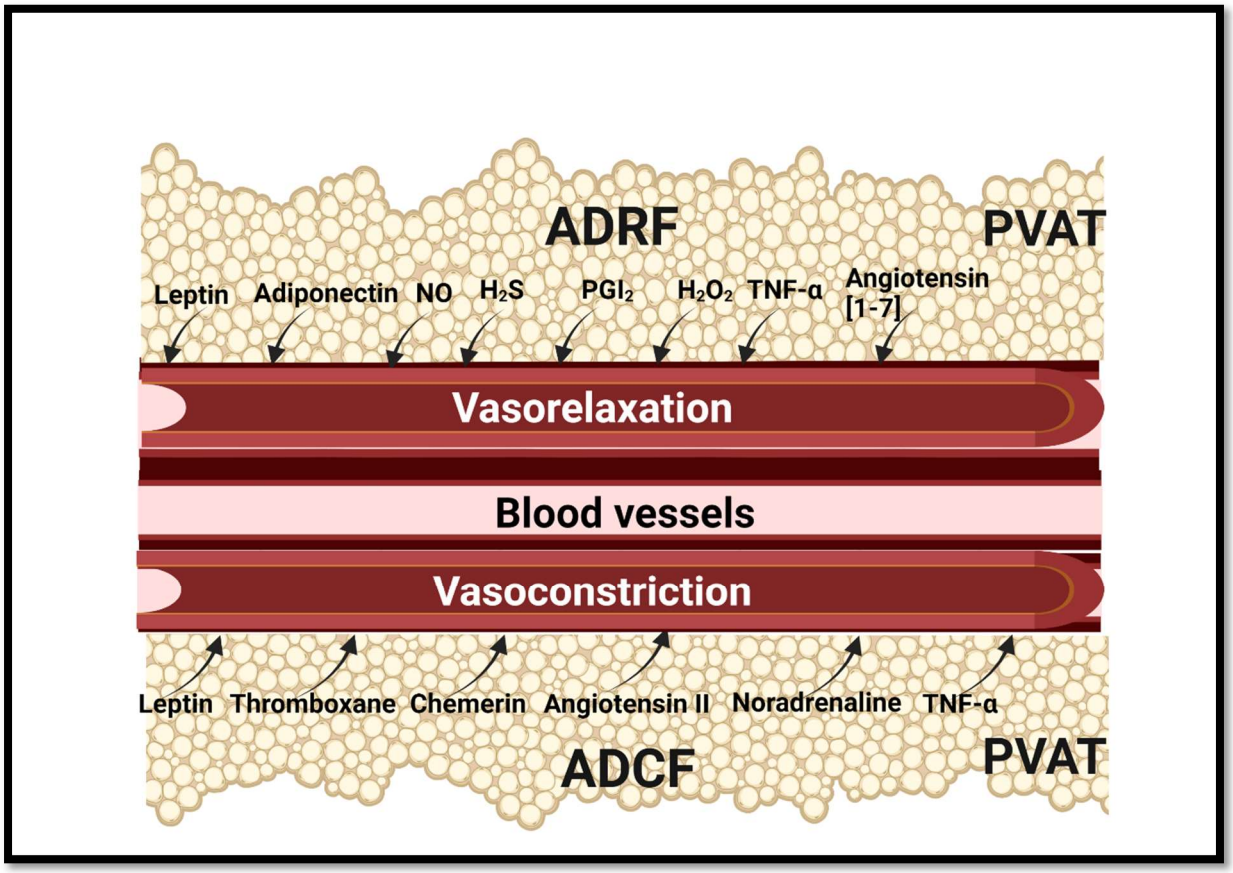


Figure 1.6: The effects of adipocyte derived relaxing factor (ADRF) and adipocyte derived contractor factor (ADCF) released from PVAT in the contractility of adjacent blood vessels. A number of ADRF such as leptin, adiponectin, nitric oxide (NO), prostacyclin (PGI_2), angiotensin (1-7), TNF-α, H_2O_2 and H_2S induce vasorelaxation. In addition, ADCF such as leptin, chemerin, thromboxane, angiotensin II and noradrenaline cause vasoconstriction.

The sympathetic nervous system (SNS) innervates both WAT and BAT. It has been reported that five adrenergic receptor subtypes (α1, α2, β1, β2

and β3) are expressed in adipocytes and are involved in the adrenergic regulation of white and brown adipose tissue function (Lafontan & Berlan, 1993). Noradrenaline released from the postganglionic nerve endings of the SNS modulates lipolysis of WAT, and stimulates UCP-1 synthesis to regulate non-shivering thermogenesis in BAT through β3-adrenoceptors (Brito et al., 2008). It has been shown that β1, β2, and β3 adrenoceptors in WAT stimulate lipolysis whereas α2 receptors inhibit lipolysis (Lafontan & Berlan, 1993). PVAT, in human saphenous vein receives sympathetic innervation as shown using immunostaining (Dashwood & Loesch, 2011). Activation of electrical field stimulation (EFS) of sympathetic nerves in PVAT activates β3 adrenoceptors causing the release of the anti-contractile factor adiponectin from PVAT (Saxton et al., 2018). Sensory nerves also are expressed in both WAT and BAT (Bartness et al., 2010). In addition, it has been reported recently that sensory nerves are present in the PVAT surrounding rat mesenteric arteries. The PVAT is essential for sensory neurogenic vasodilation involving calcitonin gene-related peptide (CGRP) release and crosstalk with leptin release (Abu Bakar et al., 2017).

1.3.2 Adipokines

It is believed that adipocytes secrete a wide range of adipokines that regulate adipocyte function in an autocrine/paracrine manner and affect whole body homeostasis among them are energy balance, insulin sensitivity, inflammation and immunity (Deng & Scherer, 2010).

1.3.2.1 Leptin

The first adipokine identified was leptin, one of the most important hormones released by adipose tissue. Leptin, 167 amino acid peptide hormone with a molecular weight of 16 kD and a product of the Ob gene, plays a critical role in energy homeostasis and appetite control. Apart from regulating energy expenditure, leptin is also responsible for regulating insulin sensitivity, glucose uptake, immune function and bone metabolism (Münzberg & Morrison, 2015; Zhang et al., 1994). The effects of leptin are

mediated by binding to leptin receptors (ObRs) expressed mainly in hypothalamus. In addition, there are leptin receptors throughout the body including adipose tissue, liver and pancreatic cells indicating that leptin has endocrine, paracrine and autocrine functions. There are six isoforms of ObRs (a, b, c, d, e and f) based on their intracellular domain lengths (Gorska et al., 2010). A significant correlation exists between serum leptin levels and adipose tissue mass. The amount of circulating leptin decreases during fasting or energy restriction, but increases after feeding. It is well known that obesity is associated with hyperleptinemia, and most obese people are intolerant to or resistant to leptin. Many factors regulate leptin expression and secretion including inflammatory cytokines, glucocorticoids, insulin and norepinephrine release (Dardeno et al., 2010; Münzberg & Morrison, 2015; Obradovic et al., 2021).

It has been shown that leptin and its receptors are expressed in the vasculature in both endothelial and vascular smooth muscle (Schroeter et al., 2007). In terms of its effects on the vasculature, leptin has been reported to have several conflicting effects. Cell proliferation and growth of vascular smooth muscle have been observed to be both stimulated and inhibited by leptin (Bohlen et al., 2007; Huang et al., 2010). It has been shown that leptin contributes to the development of atherosclerosis, vascular resistance and vascular permeability (Bodary et al., 2005; Cao et al., 2001). In addition, endothelin-1 secretion, sympathetic nerves and renin-angiotensin systems functions can be regulated by leptin, the net effect of which is to cause vasoconstriction and consequently increasing blood pressure (Dubey & Hesong, 2006). However other evidence suggests that leptin induces vasorelaxation both through independent and direct mechanisms (Benkhoff et al., 2012; Mohammed et al., 2007; Momin et al., 2006). A study conducted in vivo in rats showed that leptin intravenous infusion leads to lower arterial pressure by increasing levels of NO (Frühbeck, 1999). Moreover, leptin-induced vasodilation in isolated rat aorta is mediated by NO release via phosphorylation of endothelial NO

synthase (eNOS) at Ser1177 by activating Akt (Sahin et al., 2009; Vecchione et al., 2002). In human saphenous veins and internal mammary arteries, leptin is thought to cause vasodilation without affecting endothelium or NO (Momin et al., 2006). In addition, leptin-induced vasodilation in human coronary arteries may not be mediated by NO as well (Matsuda et al., 2003).

1.3.2.2 Adiponectin

Adiponectin was recognized for the first time in 1995 (Scherer et al., 1995). The peptide adiponectin which is a 244 amino acid protein with a molecular weight of 30 kD, is the most abundant peptide secreted by adipocytes. There are three receptors for adiponectin known as AdipoR1, AdipoR2 and T-cadherin. Adiponectin is an insulin-sensitizing hormone, stimulating glucose and fatty acid metabolism and reducing liver glucose production. Besides its essential role in metabolic regulation, adiponectin also plays a significant role in proliferation, inflammation and oxidative stress (Achari & Jain, 2017; Polito et al., 2020). Adiponectin levels decrease with obesity and are positively correlated with insulin sensitivity (Rasmussen et al., 2006). In the vasculature, adiponectin has direct vasodilatory effects, inhibits VSMC proliferation and mediates PVAT anti-contractility (Fésüs et al., 2007; Wang et al., 2005). The first demonstration of adiponectin's vasodilator properties was in rat and mouse aortic and mesenteric arteries, in which adiponectin inhibited serotonin-induced contractions. The relaxation response was endothelium-independent and a voltage-gated potassium (Kv) channel in the VSMC appears to be responsible for this effect (Fésüs et al., 2007). Adiponectin is also known to directly activate large conductance calcium activated potassium (BK) channel in rat and mouse mesenteric arteries (Lynch et al., 2013; Weston et al., 2013). The presence of AdipoR1 and AdipoR2 receptors was associated with increased NO production caused by adiponectin in aortic endothelial cells (Tan et al., 2004). It has been shown that in healthy PVAT around human gluteal small arteries, adiponectin promotes vasodilation by increasing the bioavailability

of NO. After incubation with adiponectin blocking peptide, healthy PVAT from the vessels lost its anti-contractile effect. As compared to healthy vessels, obese arteries exhibited no anti-contractile effect. Additionally in this study, PVAT was found to have an anti-contractile effect in isolated mesenteric arteries of healthy rats, but this effect was abolished by adiponectin blocking peptides. The exogenous addition of adiponectin to the pre-contracted isolated vessels induced vasodilatation (Greenstein et al., 2009). It has been reported that AMPK has a critical role in maintaining the anti-contractile effects of mice thoracic aortic PVAT. Adiponectin's generation and vascular effects are also affected by a lack of AMPK isoforms in the vascular wall (Almabrouk et al., 2017).

1.3.3 Purinergic receptors in adipose tissue

As mentioned earlier, PVAT includes both white and brown adipose tissue, which are mainly responsible for energy storage and non-shivering thermogenesis respectively. It has also been reported that PVAT has a local effect on vascular smooth muscle cell contractility via the release of a number of bioactive substances and adipokines. In adipose tissue, all four subtypes of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) are expressed. Signalling of adenosine in adipocytes via its receptors has been shown to enhance insulin-stimulated glucose uptake, lipogenesis, and suppress lipolysis (Gnad et al., 2014; Johansson et al., 2008; Tozzi & Novak, 2017). It has been reported that A_1 receptors activation increases leptin levels in the blood in vivo studies and leptin secretion from isolated adipose tissue, indicating that A_1 receptors stimulate leptin secretion directly from fat cells (Rice et al., 2000). Adenosine is produced after the breakdown of ATP in rat isolated white adipocytes and has been shown to activate the A_1 receptor via the PLC-PKC pathway and promote insulin-induced leptin release (Cheng et al., 2000). The A_{2A} receptor mediated lipolysis and thermogenesis induced by adenosine in brown adipocytes. In addition, in mice, A_{2A} receptor antagonists reduce obesity induced by excessive fat intake. A_{2A} receptor agonists improve glucose homeostasis and adipose

tissue inflammation in obese mice, suggesting potential therapeutic applications (DeOliveira et al., 2017; Gnad et al., 2014).

Adipose tissue also expresses a number of P2 receptors including (P2X1–7, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$). There is evidence that P2X and P2Y receptors play a role in regulating adipogenesis, glucose transport, inflammation, as well as adipokines secretion in adipose tissues (Bulloch & Daly, 2014; Tozzi & Novak, 2017). In white adipocytes from rats, both ATP and UTP have been shown to increase the intracellular Ca^{2+} concentration by activation of $P2Y_2$ and $P2Y_{11}$ receptors via the cAMP-PKA signalling pathway. Moreover, in human adipocytes, ATP, ATPγS, UTP and α,β-meATP increased Ca^{2+} suggesting P2X and P2Y have a functional role (Rossato et al., 2022). Another study has shown that $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors are the only purinergic receptors that are functionally involved in intracellular Ca^{2+} responses induced by ATP, ADP and UTP in human adipocytes. This study also noted that selective antagonism of $P2Y_2$ receptors by AR-C118925XX or their knockdown caused a significant decrease in intracellular calcium concentration and increase in cellular cAMP, which led the authors to conclude that autocrine activation of $P2Y_2$ receptors is essential in regulation of basal lipolysis (Ali et al., 2018). There is evidence that $P2Y_2$ receptors are critical for regulating adipokine production and the promotion of adipogenesis and inflammation. In this study, a high-fat diet led to an increase in insulin, leptin and adiponectin levels in serum samples collected from wild type mice, whereas these increases were not detected in mice with $P2Y_2$ knock-outs (Zhang et al., 2020). $P2Y_{11}$ receptor activation has been shown to inhibit insulin-induced leptin production and stimulation of lipolysis (Lee, Jun, et al., 2005). $P2Y_1$ receptors have been reported to be expressed in rat white adipose tissue to contribute to leptin secretion after stimulation by ADP. The level of leptin was lower in mice deficient $P2Y_1$ receptor and when $P2Y_1$ receptor is inhibited by MRS2500 under insulin-stimulated and basal conditions (Laplante et al., 2010). It has been shown in cardiac adipocytes that adiponectin secretion was inhibited by UTP or

MRS4062, and $P2Y_4$ receptor knockout mice showed higher levels of adiponectin secretion during hypoxia (Lemaire et al., 2017).

P2X7 receptors mediate adipogenesis and lipid metabolism in age and sex-dependent ways (Beaucage et al., 2014). It has been reported that the P2X7 receptors play a role in inflammatory activation in adipose tissue. Activation of P2X7 receptors which are functionally expressed in human adipocytes modulates the release of inflammatory cytokines including TNFα, IL-6 and PAI-1 in human visceral and subcutaneous adipose tissue (Madec et al., 2011). It has been shown in PVAT surrounding a small mesenteric artery that P2X7 receptor and inflammasome components are expressed at increased levels in smokers, enhancing inflammatory cytokines production including IL-1b and IL-18 (Rossi et al., 2014). In brown adipose tissues, ATP and UTP have also been reported to increase intracellular Ca^{2+} concentration (Lee, Vielhauer, et al., 2005). UCP-1 expression and browning were enhanced by $ATP\gamma S$ in both wild type and mice lacking β-adrenergic receptors during low adaptive thermogenesis (Razzoli et al., 2016).

Nucleotides can be released from the adipocytes themselves to regulate adipocyte function, primarily via activation of adipocyte $P2Y_2$ receptors (Ali et al., 2018; Rossato et al., 2022). In addition, adipocytes are not the only possible source of endogenous purines acting at adipocyte P2 receptors. Sympathetic nerves, via noradrenaline acting at β-adrenoceptors, have long been recognised to regulate adipose tissue function e.g. mobilizing fatty acids and adipokines production and secretion from WAT (Ahmadian et al., 2010; Rayner, 2001) and in non-shivering thermogenesis of BAT (Hondares et al., 2011). However, despite ATP being an established cotransmitter with noradrenaline, it is not known whether ATP and (UTP) released from sympathetic nerves innervating PVAT has a role in regulation of adipocyte functions through activation of adipocyte P2 receptors including $P2Y_2$.

1.4 Aims

There is relatively little knowledge about the constitutive release of purines in blood vessels, although this is important for basic understanding of vascular control mechanisms, but also the pathophysiology of cardiovascular diseases such as hypertension for which it may offer new therapeutic approaches. PVAT surrounds most of the large and medium arteries. Prior to its recognition as an active regulator of vascular homeostasis, PVAT was considered a passive structural component of the vascular wall. There is no doubt that PVAT releases many active mediators that have a paracrine effect on vascular tone (Brown et al., 2014). In adipocytes, P2Y and P2X receptors are expressed, whose activation influences lipolysis and regulates the release of adipokines (Tozzi & Novak, 2017). The role of these receptors expressed in PVAT in the regulation of adjacent vasculature is less understood. In addition, it is possible for adipocytes themselves to release nucleotides to regulate adipocyte activity (Ali et al., 2018). Whether this occurs in PVAT and alters tone of the adjacent vasculature is unknown. Therefore, the aim of this study was to investigate if nucleotides (ATP/UTP) are released constitutively from PVAT to regulate the vascular tone of mesenteric arteries. In addition, the current study had the following specific aims:

- To investigate whether P2 receptor antagonists have direct effects on vascular tone as an indication of constitutive release of endogenous nucleotides.
- To investigate whether the relaxation responses induced by P2 receptor antagonists in the mesenteric artery are due to nucleotides released via connexin and pannexin channels. Also, to study the role of the vascular endothelium as a possible source of the nucleotides.
- The possible involvement of other P2X, P2Y and adenosine receptors in the vasorelaxant effect induced by AR-C118925XX ($P2Y_2$ receptor antagonist) was also investigated.

- To study whether the nucleotides ATP and UTP act on P2Y receptors on adipocytes to release adiponectin and leptin. The findings led me to investigate if adiponectin released from PVAT contributes to the anti-contractile action of PVAT.

Chapter 2

Materials and Methods

2.1 Porcine tissue preparation

Mesenteries, hearts and spleens were obtained from large white hybrid pigs of both sexes from a local abattoir in Nottingham. The pigs were around 22 weeks old and weighed around 70 kg. The mesentery was isolated directly from the intestines and placed in a container of an ice-cold Krebs-Henseleit solution (NaCl 118, KCl 4.8, MgSO4 1.1, NaHCO3 25, KH2PO4 1.2, D-glucose 12 and CaCl2 1.25 mM) and was aerated with 95% O2 and 5% CO2. Then, the superior mesenteric artery and the first order branch arteries were dissected from the surrounding fat. They were then placed in a universal tube containing a pre-gassed Krebs-Henseleit solution and stored overnight at 4°C. In the coronary and splenic artery experiments, the anterior proximal artery and a sample of the dorsal branch, respectively, were dissected and stored in a Krebs-Henseleit solution at 4°C overnight in the refrigerator.

On the second day, a fine dissection was conducted to isolate the first order branch artery (large mesenteric artery) and prepare the artery rings. All surrounding fat was removed from the PVAT-free mesenteric artery segments (Figure 2.1). The same procedure was conducted to clear the porcine coronary and the splenic arteries of any surrounding connective tissue and fat. Arteries with and without PVAT were cut into rings around 5 mm in length. In experiments investigating the effect of PVAT, around 350 mg of surrounding fat remained attached to the 5 mm artery segments (Figure 2.2). In the endothelium-denuded mesenteric arteries, the endothelium was carefully removed with forceps by rubbing it against the internal surface. At the end of the experiments, the successful removal of endothelium was confirmed by the absence of a relaxation response to bradykinin in all U46619-pre-constricted endothelium denuded mesenteric artery segments.

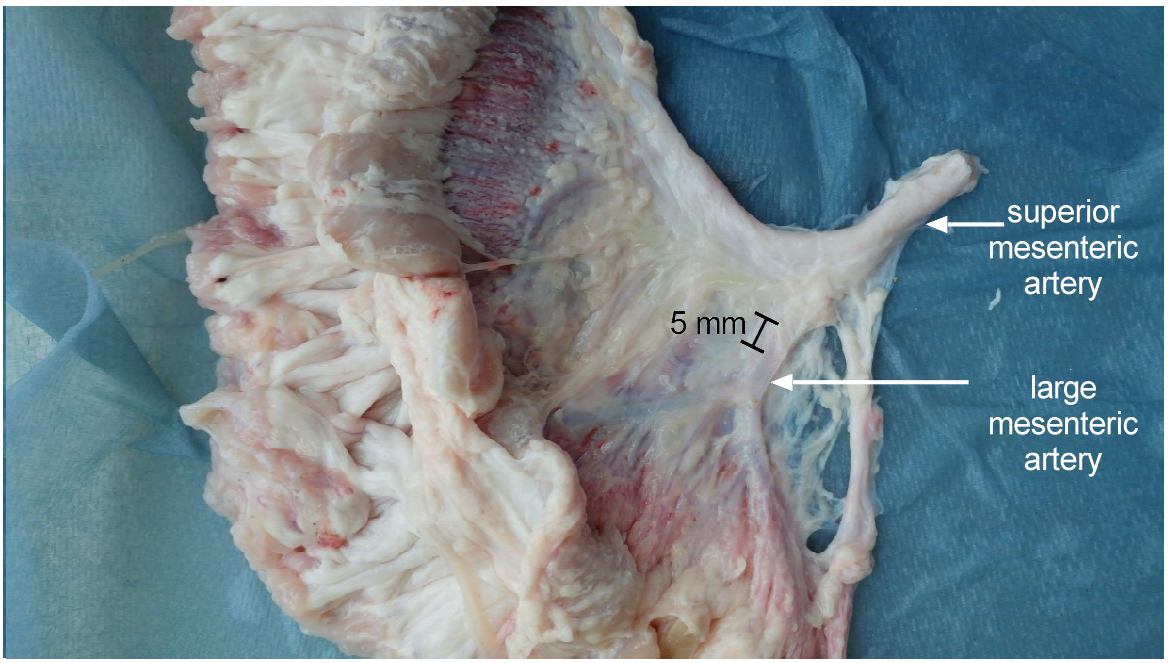


Figure 2.1: The porcine-isolated mesenteric arterial bed shows the superior mesenteric artery and the large mesenteric artery. A fine dissection was conducted to isolate the first order branch artery (large mesenteric arteries, which were used in this study) and prepare the artery rings.

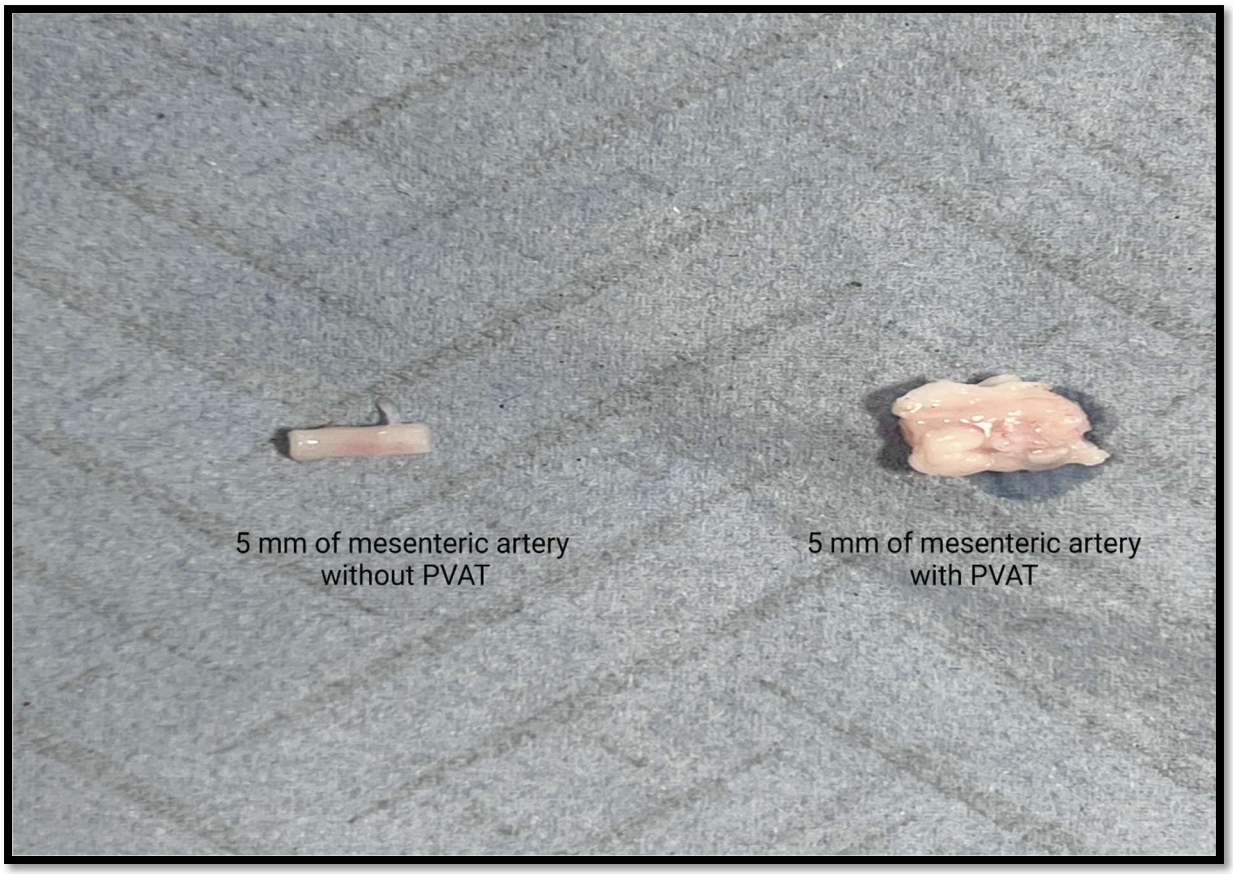


Figure 2.2: Rings of arteries with and without PVAT were cut at a length of around 5 mm, and around 350 mg of surrounding fat remained attached to the 5 mm artery segments.

2.2 Isometric tension recording

The tissues were then placed in 10 ml jacketed organ baths containing an oxygenated (95% O2 and 5% CO2) Krebs-Henseleit solution, which was maintained at 37°C using a water heater circulator (Techne Circulator C-85A). Then, the arterial rings were mounted onto a glass rod containing a stainless steel hook, with another hook attached to the tissue, and were then connected to an isometric force transducer (Figure 2.3). A Quad Bridge amplifier unit was used to allow the PowerLab (AD Instruments) to connect to the transducers. Tension was recorded by using LabChart data analysis software (AD Instruments version 7) on a Windows XP PC. The transducers were calibrated by using a 20 g weight, and then an initial tension of 10 g was applied to the artery segments. After applying the initial tension to all

segments, they were allowed to equilibrate for about 50 minutes and then relax to a final resting tension of between 1-2 g. When a stable baseline was obtained, the tissues viability and activity were tested with two responses to 60 mM KCl. Once the tone of the tissues had returned to the baseline and had stabilised, the thromboxane A2 (TP) receptor agonist U46619 (10 nM to 90 nM) was added to the artery segments to elicit a contraction of about 40-60% of the second KCl response.

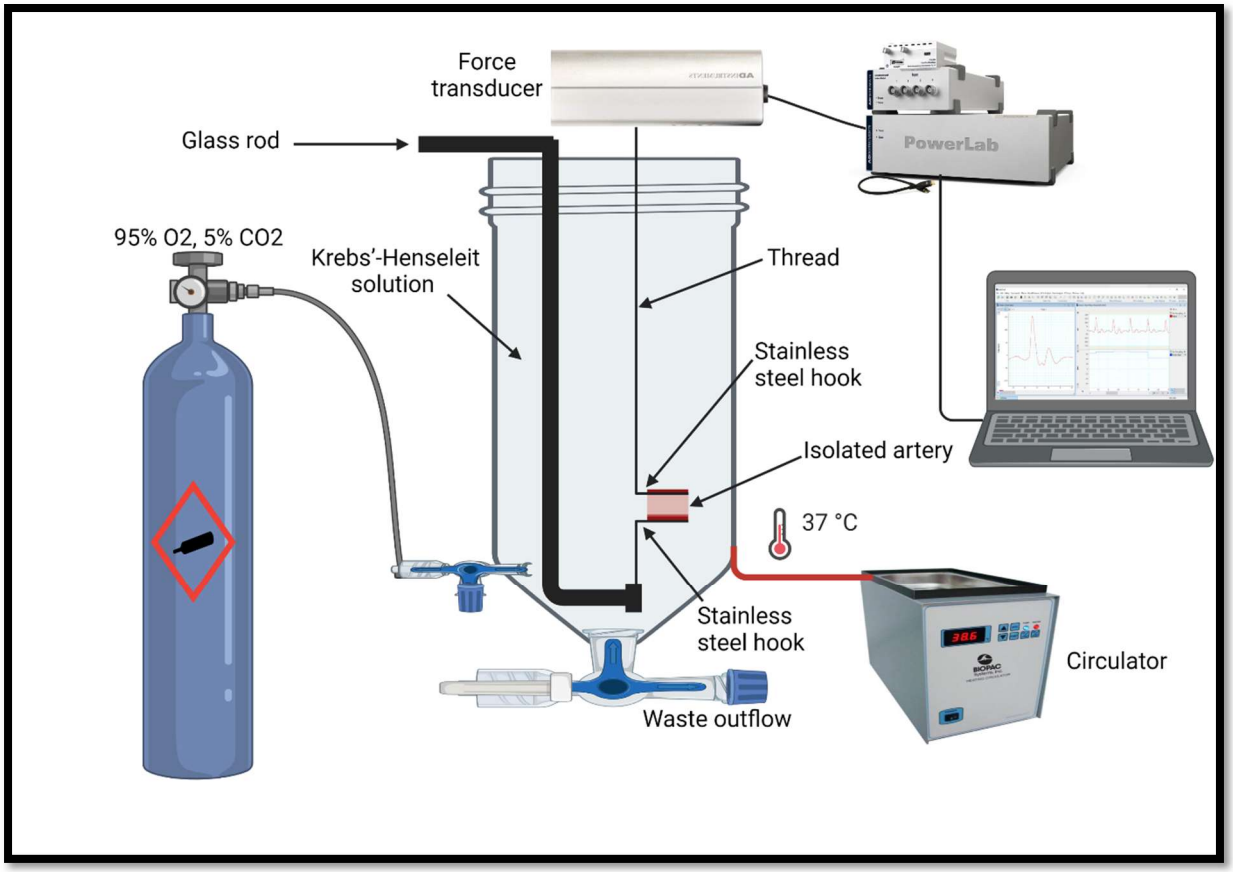


Figure 2.3: An isolated tissue set up showing a 10 ml Krebs-Henseleit solution maintained at 37°C in oxygenated (95% O2 and 5% CO2) organ baths. A stainless steel hook was attached to a glass rod with arterial rings. Another hook was attached to the tissue and was connected by a thread to an isometric force transducer. The PowerLab was connected to the transducers using a Quad Bridge amplifier unit. The tension was recorded using LabChart data analysis software.

2.3 Experimental protocols

2.3.1 Effect of purinergic receptor antagonists on U46619-induced contractions of porcine arteries

To determine the effect of P2 receptor antagonists, the P2-purinoceptor antagonists suramin (100 μM), AR-C118925XX (1 μM and 10 μM), MSG228 (1 μM and 10 μM), MRS2578 (10 μM) and NF449 (30 μM) were added directly to the stable tone of the U46619-pre-constricted mesenteric artery segments and incubated for 60 minutes. Theophylline (10 μM) was also incubated to study the role of adenosine receptors. Some mesenteric arteries were incubated with P2 receptor antagonists in the presence of the attached PVAT. In some experiments, U46619-pre-constricted coronary and splenic arteries were incubated for 60 minutes with AR-C118925XX (10 μM).

A different set of experiments were done to study the functional role of the endothelium in response to AR-C118925XX. AR-C118925XX (10 μM) was added to endothelium denuded vessel segments of the mesenteric arteries and incubated for 60 minutes.

In a different set of experiments, porcine-isolated mesenteric arteries were pre-contracted using KCl (20 to 30 mM), phenylephrine (0.5 to 1 μM) or endothelin (10 to 20 nM) to about 40-60% of the second KCl response. Phenylephrine and endothelin produced an unstable increase in tone of the mesenteric arteries. However, a stable contractile tone in the mesenteric arteries was achieved with KCl (20 to 30 mM). AR-C118925XX (10 μM) was incubated for 60 minutes in the KCl-pre-constricted mesenteric arteries.

Dimethyl sulfoxide (DMSO) (0.1% v/v) or distilled water (10 μl) was added to the adjacent artery segment from the same animal as a control.

2.3.2 Effect of connexin and pannexin channel blockers on U46619-induced contractions of porcine mesenteric artery

The connexin and pannexin channel blockers carbenoxolone and probenecid (100 µM) were added directly to the pre-constricted tone of mesenteric artery segments and were incubated for 60 minutes. Dimethyl sulfoxide (DMSO) (0.1% v/v) or distilled water (10 µl) was added to an adjacent mesenteric artery segment from the same animal as a control. In some experiments, AR-C118925XX (10 µM) was added to U46619-pre-constricted porcine mesenteric arteries together with either carbenoxolone (100 µM) or probenecid (100 µM).

To investigate whether carbenoxolone and probenecid had a damaging effect on porcine mesenteric arteries smooth muscle and endothelium, KCl (60 mM) and bradykinin (10 µM) were added at the end of the experiments.

2.3.3 Effect of AR-C118925XX on vascular tone in porcine isolated mesenteric arteries desensitised with UTP and α,β-meATP

P2Y receptors were desensitised by preincubation with UTP (300 µM). UTP was added several times (3 to 4 times), with an interval of 20 to 30 minutes between each addition. UTP (300 µM) was added to the U46619-pre-constricted porcine mesenteric arteries, without washout between additions. Once stable tone was achieved, the $P2Y_2$ receptor antagonist AR-C118925XX (10 µM) was incubated. A different set of experiments was conducted to desensitise the P2X1 receptors using α,β-meATP, which was added (10 µM) at basal tone. The tone of mesenteric arteries was then raised by U46619. After a stable pre-contractile tone had been achieved with U46619, AR-C118925XX (10 µM) was added for 60 minutes.

2.3.4 Effect of exogenous ATP and UTP on vascular tone in porcine-isolated mesenteric arteries

AR-C118925XX (10 µM) was incubated for 20 minutes before the cumulative addition of ATP and UTP (10 µM-1 mM) to study the effect of

exogenous nucleotides in U46619-precontracted porcine mesenteric arteries with and without PVAT.

The effect of the different concentrations of the exogenous ATP and UTP on the U46619-pre-constricted vascular tone in porcine mesenteric arteries was studied. A combination of ATP and UTP (100 μM, 300 μM and 1 mM), and single additions of ATP (100 μM, 300 μM and 1 mM) and UTP (100 μM, 300 μM and 1 mM) were studied.

A different set of experiments was conducted to study the effect of PVAT on the contractile response of exogenous nucleotides. Cumulative concentrations of ATP and UTP (10 μM-1 mM) were added to the U46619-pre-constricted mesenteric and splenic arteries.

2.3.5 Effect of ecto-ATPase and ecto-ATPase inhibitor on vascular tone in porcine isolated mesenteric arteries

Apyrase (5 and 10 units/ml) was incubated with both PVAT and PVAT-free U46619-pre-constricted mesenteric arteries. The incubation time was extended by 120 minutes because apyrase 10 units/ml produced dual effects on the pre-contractile tone. In another set of experiments, apyrase (10 units/ml) was also added at the basal tone before pre-contraction with U46619 in PVAT and PVAT-free mesenteric arteries. After a stable pre-contractile tone had been achieved with U46619, AR-C118925XX (10 μM) was added for 60 minutes.

ARL67156 (100 μM), which is an ecto-ATPase inhibitor, was added for 60 minutes to both U46619-pre-constricted PVAT and PVAT-free mesenteric arteries.

2.3.6 Contraction response of ATP and UTP in porcine splenic arteries with and without PVAT

Cumulative concentrations of ATP and UTP (10 μM-1 mM) were added to the U46619-pre-constricted splenic arteries to study the effect of PVAT on the contractile response of exogenous nucleotides.

To study the effect of adiponectin on the vascular tone of splenic arteries, AdipoRon (1 to 100 μM), an adiponectin receptor agonist, was added cumulatively to U46619-pre-constricted vascular tone of splenic arteries with PVAT. Additionally, anti-adiponectin antibody (ABIN3208272, Antibodies-online) was incubated with splenic artery with PVAT and its effect on the PVAT-induced depression of responses to ATP and UTP was studied.

2.3.7 Determination of ATP concentration in porcine isolated mesenteric arteries

To determine the amount of ATP released into a Krebs-Henseleit solution by PVAT and PVAT-free mesenteric arteries, luminescence method was used. Concentrations of ATP in the Krebs–Henseleit solution were measured using the Luminescent ATP Detection Assay Kit (ab113849, Abcam, Cambridge, UK). Luminescence was measured using an ATP assay in accordance with the manufacturer's instructions (Luminescent ATP Detection Assay Kit (Ab113849), 2022). ATP assays are based on the production of light caused by ATP reacting with firefly luciferase and luciferin. In the cell, the emission of light is proportional to the concentration of ATP as follows:

ATP + D-Luciferin + O2 Oxyluciferin + AMP + PPi + CO2 + Light

Segments of PVAT and PVAT-free mesenteric arteries were prepared, and the average weight of PVAT was about 350 mg remain attached to 5 mm mesenteric artery segments. Segments of isolated-mesenteric arteries were incubated in a 1 ml tube containing an oxygenated (95% O2 and 5% CO2) Krebs-Henseleit solution, which was maintained at 37°C for 20 minutes. ATP release was measured in isolated-mesenteric arteries with and without PVAT and in the presence and absence of apyrase (10 units/ml) and U46619 (50 nM). Samples were collected, and 50 μl volume of each sample and ATP working standards were transferred to opaque 96-well plates. 50 μl of detergent and substrates were added to the samples and

standards. Luminescence was measured by a luminescence microplate reader. An ATP standard curve was used to calculate the ATP concentration in each sample. Based on the standard curve, the range was 0.001 µM-10 µM (Figure 2.4). Results were expressed as nanomolar (nM) of the ATP released.

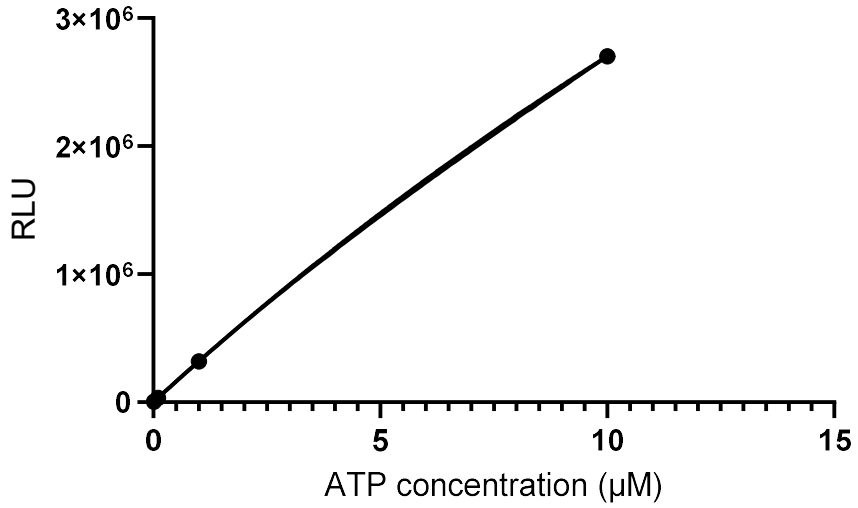


Figure 2.4: An example of ATP standard curve of Luminescent ATP Detection Assay Kit (Ab113849) used in this experiment. The range was 0.001 µM-10 µM.

2.4 Cell culture

3T3-L1 mouse preadipocytes (ATCC-CL-173) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). 3T3-L1 cells (passage 9 to 10) were cultured in basal medium 1 (BM1). This contained Dulbecco's Modified Eagle Medium (DMEM) (Sigma D6546), with 10% new-born calf serum (NCS) (Sigma N4637), 100 units/mL of penicillin, 100 mg/mL of streptomycin (P/S) (Sigma PO781) and 2 mM of L-glutamine (Sigma G7513) in a humidified incubator with 5% CO2 at 37°C, as recommended by the supplier (Table 2.1). In a T75 cm2 flask, 3T3-L1 cells were grown to 70% to 80% confluence. Afterwards, the cells were washed with phosphate-buffered saline (PBS) before being trypsinised with 1% trypsin and subcultured at a 1:5 ratio.

In a 6-well cell culture plate and at a density of 3 x 10^4 3T3-L1, adipocytes were seeded. An automated cell counter, BIO-RAD TC20TM, with 50% Trypan Blue, was used to count the cells. 3T3-L1 adipocytes were treated with prodifferentiator agents to transform from their fibroblastic phenotype to an adipocyte phenotype (Zebisch et al., 2012). 3T3-L1 adipocytes were grown to confluence, and the differentiation was stimulated by differentiation medium 1 (DM1). DM1 contains DMEM, 10% fetal bovine serum (FBS) (Sigma F9665), P/S, L-glutamine, 1 µg/ml of insulin (Sigma 15523), 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX) (Sigma 17018), 0.25 of µM dexamethasone (Sigma D1756) and 2 µM of rosiglitazone (Sigma R2408). 3T3-L1 adipocytes were incubated with DM1 for 48 hours. After 48 hours, the media was replaced with differentiation medium 2 (DM2), which contains DMEM, 10% FBS, P/S, L-glutamine and 1 µg/ml of insulin. 3T3-L1 adipocytes were incubated with DM2 for 48 hours. This was followed by changing medium to basal medium 2 (BM2), which contains DMEM, 10% FBS, P/S and L-glutamine. The cells were maintained by changing the medium every 24 and 48 hours for about 7 days. A small number of intracellular lipid droplets appeared around day 7 and increased in size over the next few days, and at 13-14 days post-induction of differentiation, almost all the cells were filled with lipid droplets of various sizes (Figure 2.5).

Table 2.1: 3T3-L1 adipocyte growing and differentiation medium components.

Type of medium	Components	Days	Durations
Basal medium 1 (BM1)	DMEM NCS P/S	Day 0-3	48-72 hours

Differentiation medium 1 (DM1)	DMEM FBS P/S IBMX Dexamethasone Rosiglitazone Insulin	Day 3-5	48 hours
Differentiation medium 2 (DM2)	DMEM FBS P/S Insulin	Day 5-7	48 hours
Basal medium 2 (BM2)	DMEM FBS P/S	Day 7-14	7 days
Basal medium 3 (BM3)	DMEM P/S	Day 15	24 hours

2.4.1 Oil red O staining of 3T3-L1 adipocytes

Differentiated and undifferentiated 3T3-L1 cells were stained with oil red O to confirm the presence of lipid droplets. The culture medium was removed from the 6-well plate, and PBS was used to wash the 3T3-L1 fibroblasts and adipocytes. A fixative solution of 4% (v/v) paraformaldehyde was then applied to the cells for 20 minutes at room temperature. An oil red O solution of 0.5% isopropanol (Sigma, O1391) was used. To prepare a fresh working solution, a stock solution was diluted with distilled water (3:2), and the solution was filtered through a 0.2 µm filter. At room temperature, an oil red O working solution was applied to the cells for 1 hour. After washing

3 times with PBS, the cells were incubated for 2 minutes with 60% isopropanol. In the final step, the 3T3-L1 cells were washed with PBS, and the stained lipid droplets were visualised under a light microscope.

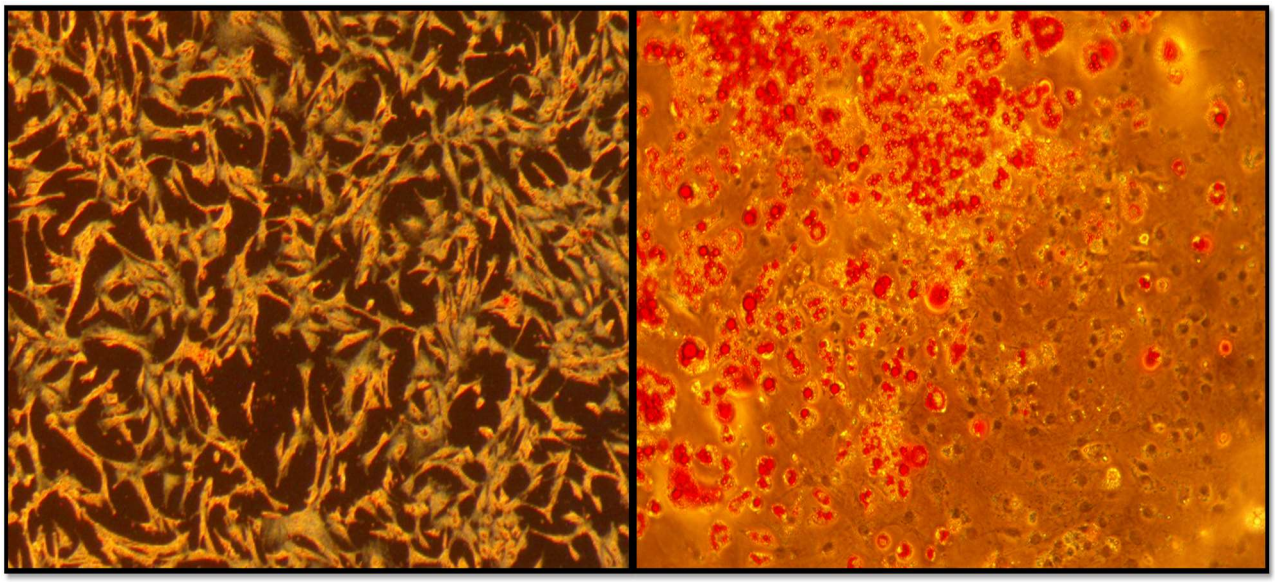


Figure 2.5: (Left) 3T3-L1 fibroblast before differentiation; (right) 14 days post-induction of differentiation, stained with oil red O to identify lipid droplets.

2.4.2 Treatment of 3T3-L1 adipocytes

The 3T3-L1 adipocytes were cultured in 6-well plates until day 15 of differentiation at 37°C in 5% CO2. Following differentiation for 15 days, the medium was changed to basal medium 3 (BM3), which contains DMEM and P/S without FBS, and experiments were conducted. To allow adipokines to accumulate, BM3 was changed to minimal amounts (1ml). As serum-free was used to incubate 3T3-L1 adipocytes with various drugs, it was assumed that the presence of serum could lead to conflicting results because it could contain adipokines, which can interfere with ELISA kits. The following reagents were added directly to the BM3: ATP (300 µM), UTP (300 µM), ADP (300 µM), UDP (300 µM), apyrase (10 units/ml), suramin (100 µM), AR-C118925 (10 µM), MSG228 (10 µM), NF449 (30 µM), MRS2578 (10 µM), insulin (170 nM) and DMSO (0.1% v/v). The cells were then incubated for

24 hours. In addition, the effect of AR-C118925XX and MSG228 (10 μM) on ATP- and UTP-induced adiponectin release was studied. The treated medium was collected and stored at -80°C for ELISA testing.

2.4.3 Measurement of adiponectin and leptin concentrations

In accordance with the instructions provided by the manufacturer, both adiponectin and leptin concentrations were measured using ELISA kits. A mouse adiponectin ELISA Kit (ab108785, Abcam, Cambridge, UK) was used to measure adiponectin levels in the media. Based on the standard curve, the range was 0.391 ng/ml-25 ng/ml, and the sensitivity was 0.62 ng/ml. Results were expressed as nanograms (ng) of adiponectin released per ml of media solution. Media leptin levels were also measured using a mouse leptin ELISA Kit (ab100718, Abcam, Cambridge, UK). The standard curve showed that the range was 4.1 pg/ml-1000 pg/ml, and sensitivity was 4 pg/ml. Results were expressed as picograms (pg) of leptin released per ml of media solution.

2.5 Chemicals

Table 2.2: Concentrations and solvents of drugs and chemicals used in this study.

Drug	Stock concentration	Final concentration	Solvent
KCl	3 M	60 mM	H_2o
U46619	10 μM	10 nM to 90 nM	methyl acetate
ATP	1 M	10 μM to 1 mM	H_2o
UTP	1 M	10 μM to 1 mM	H_2o
ADP	100 mM	300 μM	H_2o
UDP	100 mM	300 μM	H_2o

Suramin	100 mM	300 µM	H_2o
AR-C118925XX	10 mM	1 and 10 µM	DMSO
MSG228	10 mM	1 and 10 µM	DMSO
NF449	10 mM	30 µM	H_2o
α,β-meATP	10 mM	10 µM	H_2o
MRS2578	10 mM	10 µM	DMSO
Theophylline	10 mM	10 µM	H_2o
Apyrase	100 units/ml	5 and 10 units/ml	H_2o
ARL67156	100 mM	100 µM	H_2o
Carbenoxolone	100 mM	100 µM	H_2o
Probenecid	100 mM	100 µM	DMSO
Bradykinin	100 mM	10 µM	H_2o
AdipoRon	1 mM	1 to 100 µM	DMSO
Insulin	1 mg/ml	170 nM and 1 ug/ml	-----
3-isobutyl-1methylxanthine (IBMX)	0.5 mM	50 mM	DMSO
Dexamethasone	25 µM	0.25 µM	DMSO
Rosiglitazone	200 µM	2 µM	DMSO

All the drugs were obtained from Sigma-Aldrich UK, except for U46619, AR-C118925XX, α,β-meATP, NF449, bradykinin, MRS2578 and ARL67156, which were obtained from Tocris Bioscience UK. MSG228 was provided by Michael Stocks, School of Pharmacy, University of Nottingham.

2.6 Statistical analysis

For the organ bath pharmacology results, the data were expressed as a percentage of relaxation of the U46619-induced tone. Contractile responses were expressed as percentages of 60 mM KCl-induced contractions. All results were expressed as means ± SEM; a minimum of six independent experiments, with n representing the number of animals used. For statistical analysis, a two-way analysis of variance (ANOVA) with multiple comparisons Sidak's post hoc test was used. $P<0.05$ was considered statistically significant. Also, comparing two groups was done using the Student's t-test.

For the cell culture results, these data were expressed as (ng) and (pg) of adiponectin and leptin released per ml of media solution, respectively. All results were expressed as means ± SEM of five independent experiments, where n is the number of different passages through which 3T3-L1 fibroblasts were differentiated into adipocytes. For statistical analysis, a one-way analysis of variance (ANOVA) with multiple comparisons Tukey's test was used. $P<0.05$ was considered statistically significant. Calculations, statistical analysis and data plotting were performed with Microsoft Excel and GraphPad Prism version 9.

Chapter 3

The possible release of nucleotides from PVAT in regulating vascular tone in porcine mesenteric arteries

3.1 Introduction

It is well established that PVAT has an important role in vascular homeostasis. PVAT has a local effect on vascular smooth muscle cell contractility via the release of a number of bioactive substances and adipokines (Brown et al., 2014; Chang et al., 2020). It is generally accepted that PVAT is anti-contractile, with most studies showing that when it is removed, vasoconstriction to stimuli is increased in the PVAT-free blood vessels; however, PVAT can also induce vasocontractile effects (see Chapter 1). Knowledge about mediators released from PVAT in the regulation of blood vessel contractility is important however, the mechanisms by which these mediators exert their effects remain unclear.

P2Y and P2X receptors are expressed in the vasculature in both endothelial and vascular smooth muscle cells and participate in vascular tone regulation. A number of P2 receptors are also expressed in adipose tissues and have a variety of roles including lipolysis, lipogenesis and adipokine release, which is mediated through the binding of nucleotides to these receptors (Tozzi & Novak, 2017). Constitutive ATP release from the adipocytes themselves and subsequent activation of $P2Y_2$ receptors has been shown to regulate basal lipolysis in human adipocytes (Ali et al., 2018). It has been reported recently that basal ATP release from adipocytes mediated inflammatory cytokine secretion via activation of adipocyte P2 receptors (Rossato et al., 2022). Whether this occurs in PVAT and alters tone of the adjacent vasculature is unknown.

Adipocytes are not the only possible source of endogenous nucleotides acting at adipocyte P2 receptors. Sympathetic nerves, via noradrenaline acting at β-adrenoceptors, have long been recognised to regulate adipose tissue function e.g. mobilizing fatty acids and adipokines production and secretion from WAT (Ahmadian et al., 2010; Rayner, 2001) and in non-shivering thermogenesis of BAT (Hondares et al., 2011). ATP may also be released from other cell types including sensory nerves, erythrocytes, leucocytes, and platelets, and during inflammation and injury (Gorini et al.,

2013). However, despite ATP being an established cotransmitter with noradrenaline, it is not known whether ATP and UTP released from sympathetic nerves innervating PVAT has a role in regulation of adipocyte functions through activation of adipocyte P2 receptors including $P2Y_2$.

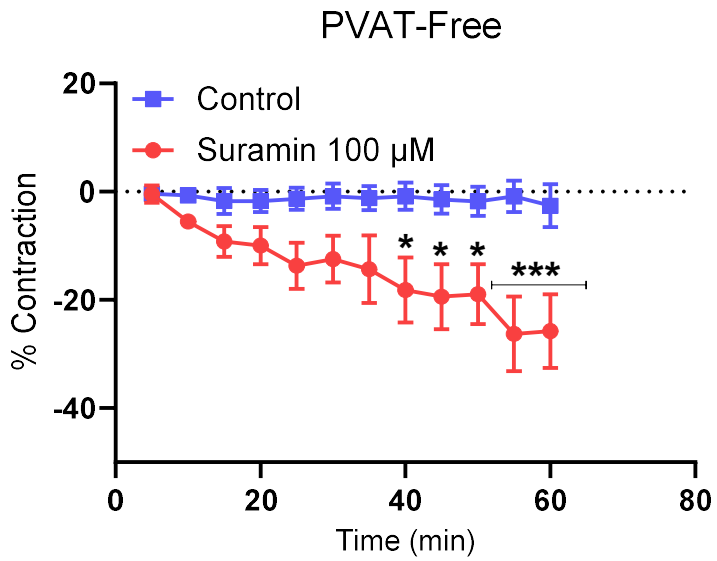
mRNA for multiple P2X and P2Y receptor subtypes has been shown in human visceral and subcutaneous adipose tissue (Rossato et al., 2022). The present study was especially interested in the $P2Y_2$ receptor because of evidence that it has a functional role in cultured adipocytes in regulating basal lipolysis (Ali et al., 2018). ATP and UTP bind equally to $P2Y_2$ receptors which are distributed in the vascular smooth muscle and endothelium (Burnstock & Ralevic, 2014; Kennedy et al., 2013). Both ATP and UTP can induce vasoconstriction and/or vasodilation depending on the blood vessel and experimental conditions (see Chapter 1). Recognised limitations in the study of P2 receptors are that nucleotides can activate more than one P2 receptor subtype. In addition, these nucleotides are rapidly hydrolysed to their respective nucleotides/nucleosides by ectonucleotidases. Furthermore, there is a lack of selective P2 receptor antagonists for a number of receptors. In this study, two selective $P2Y_2$ receptor antagonists, AR-C118925XX and MSG228, were used in addition to the broad-spectrum P2 receptor antagonist suramin.

Therefore, the aim of this chapter was to examine if nucleotides (ATP/UTP) are released constitutively from PVAT to regulate the vascular tone of mesenteric arteries.

3.2 RESULTS

3.2.1 Effect of suramin on vascular tone in porcine isolated mesenteric arteries

To investigate the possibility that there is a constitutive release of the nucleotides ATP/UTP involved in regulation of the tone of isolated mesenteric artery segments, the non-selective P2 receptor antagonist suramin (100 μM) was incubated for 60 min, after pre-contraction with U46619, in both PVAT and PVAT-free porcine mesenteric arteries. Suramin (100 μM) caused significant relaxation of U46619-pre-constricted vascular tone in porcine mesenteric arteries with and without PVAT. The relaxation response at 60 min to suramin in PVAT-free mesenteric arteries was 25.7 ± 6.8% (n = 7) which was significantly different to relaxation due to its solvent DMSO, at 2.6 ± 3.9% (n=7). Suramin also caused a relaxation response in mesenteric arteries with PVAT of 21.10 ± 3.9% (n = 8) at 60 min, significantly different to the relaxation induced by its solvent DMSO, which was 7.2 ± 3.2% (n = 8) (Figure 3.1).



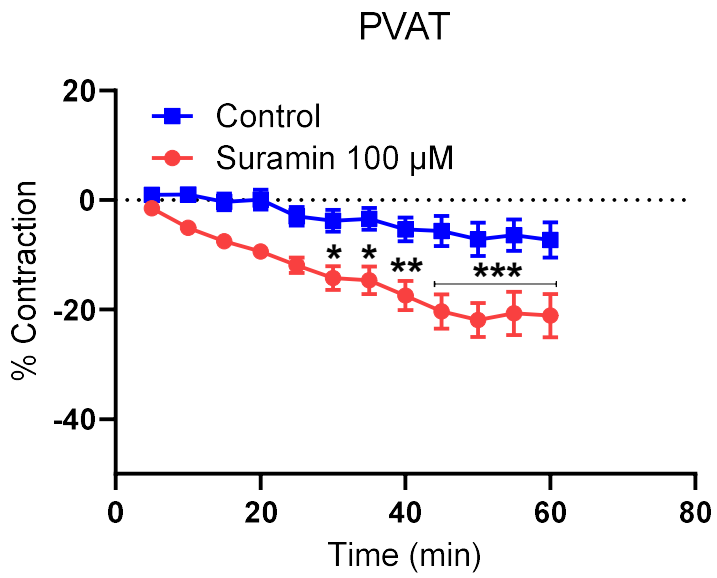


*Figure 3.1: The effects of incubating suramin (100 µM) for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Distilled water (20 µl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 7-8). Compared to the controls * P<0.05, ** P< 0.01 and *** P <0.001, 2-way ANOVA followed by Sidak's post hoc test.*

3.2.2 Effect of AR-C118925XX on vascular tone in porcine isolated mesenteric arteries

To investigate the possibility that nucleotides ATP and UTP are released constitutively from PVAT, porcine isolated mesenteric artery segments, with and without PVAT, were incubated for 60 min with the $P2Y_2$ receptor antagonist AR-C118925XX (1 µM and 10 µM) after pre-constriction with U46619. AR-C118925XX at 1 µM did not alter the vascular tone in porcine mesenteric arteries with and without PVAT and there was no significant difference in tone of the arteries in the presence of AR-C118925XX 1 µM and the control DMSO (0.1% v/v) (n=6) (Figure 3.2). AR-C118925XX at 10 µM caused a significant relaxation of U46619-pre-constricted vascular tone in both PVAT and PVAT-free porcine mesenteric arteries (Figures 3.3). The relaxation responses at 60 min to AR-C118925XX 10 µM in PVAT and PVAT-free porcine mesenteric arteries were 25.8 ± 7.1% (n = 9) and 25.0 ±

7.8% (n = 8), respectively. The time course and relaxation response to AR-C118925XX (10 μM) was very similar to that produced by suramin.

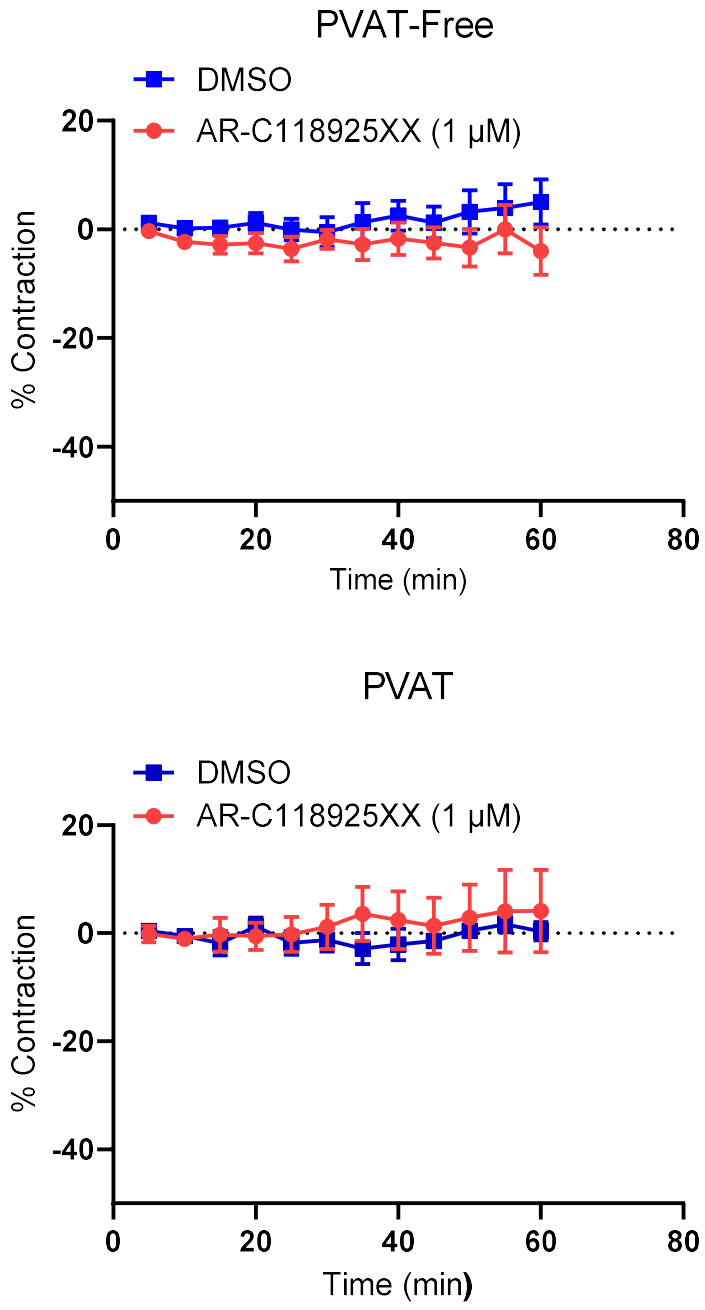
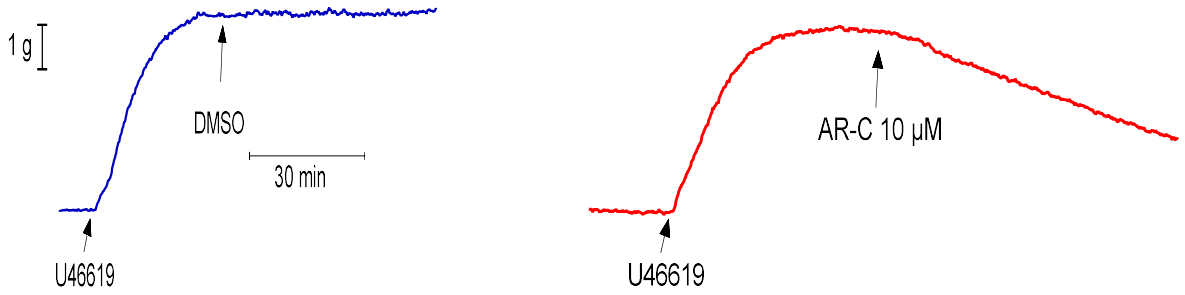
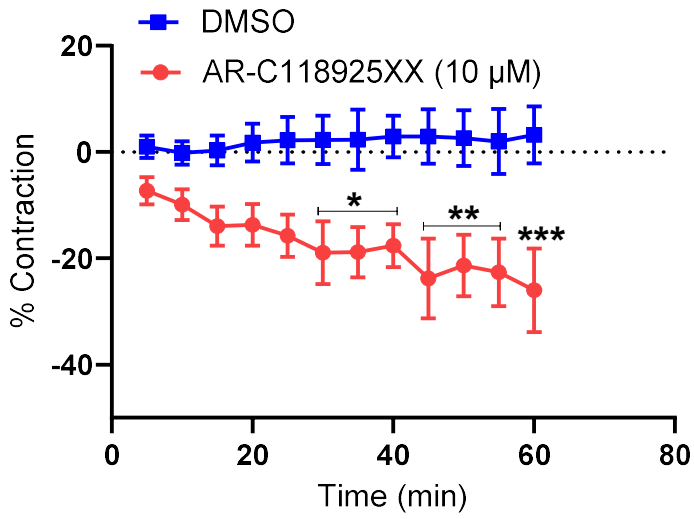


Figure 3.2: The effects of incubating AR-C118925XX (1 μM) for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

(A)



(B) PVAT-Free



(C) PVAT

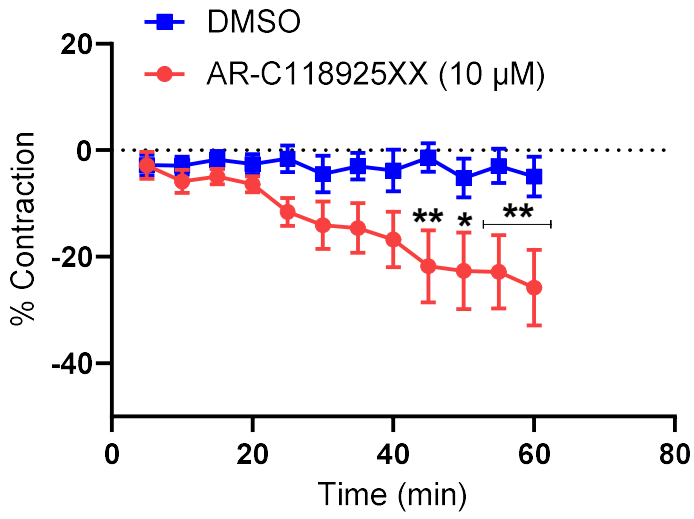


*Figure 3.3: (A) Representative traces showing the effects of incubating AR-C118925XX (10 μM) and DMSO for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries without PVAT. The effects of incubating AR-C118925XX (10 μM) for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT (B) and (C). DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8-9). Compared to the controls * P<0.05, ** P< 0.01 and *** P <0.001, 2-way ANOVA followed by Sidak's post hoc test.*

3.2.3 Effects of the selective $P2Y_2$ receptor antagonist MSG228 on vascular tone in porcine isolated mesenteric arteries

To further investigate if there is a constitutive release of nucleotides involved in regulation of the tone of isolated mesenteric artery, another selective antagonist of $P2Y_2$ receptor MSG228 (1 and 10 μM) was incubated for 60 min. There was no significant difference in the vascular tone in porcine mesenteric arteries in the presence of MSG228 1 μM and the control DMSO (0.1% v/v) (Figure 3.4, A). In porcine mesenteric arteries without PVAT pre-contracted with U46619, 10 μM MSG228 elicited relaxation of 31.2 ± 6.5% at 60 min (n=8) which was significantly different to relaxation due to its solvent DMSO (0.1% v/v), at 14.4 ± 2.7% (n=8) (Figure 3.4, B).

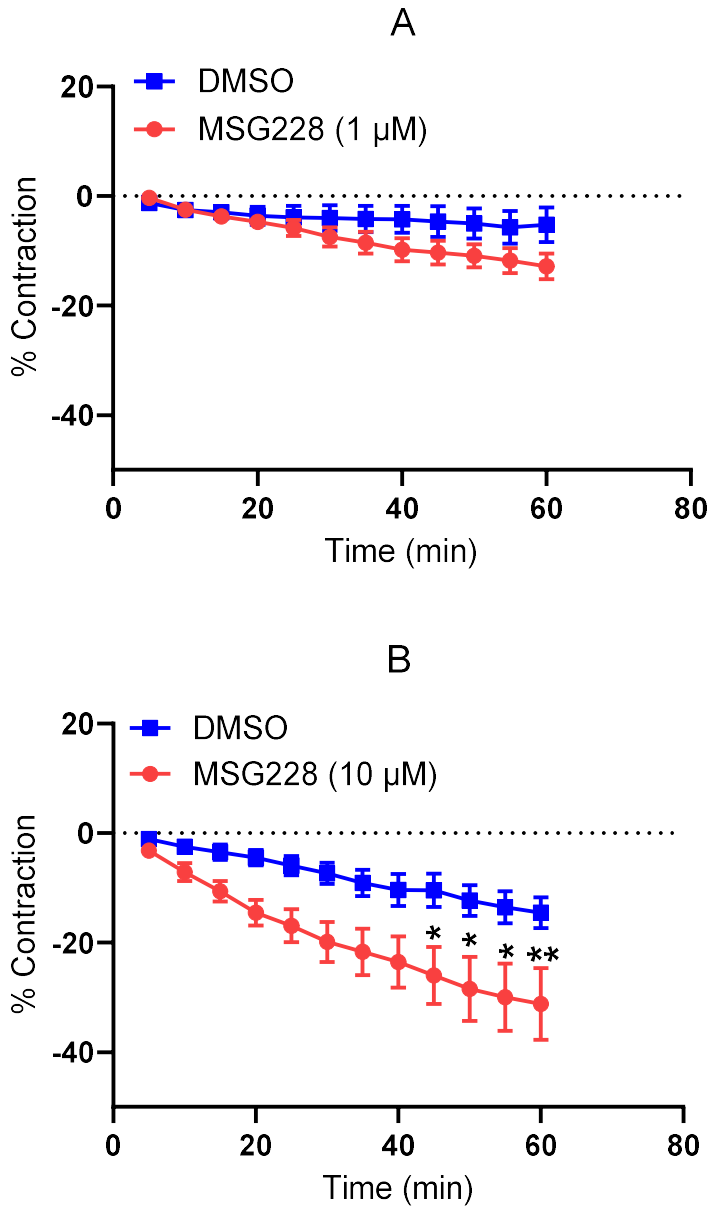


*Figure 3.4: The effects of incubating MSG228 (1 μM) (A) and (10 μM) (B) for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries without PVAT. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8 and 10). Compared to the controls * P<0.05 and ** P< 0.01, 2-way ANOVA followed by Sidak's post hoc test.*

3.2.4 Effect of exogenous ATP and UTP on vascular tone in porcine isolated mesenteric arteries

Cumulative concentration–response curves of ATP and UTP were used to study the effect of exogenous nucleotides in porcine mesenteric arteries. Cumulative concentrations of ATP and UTP (10 µM to 1 mM) were added to U46619-pre-constricted mesenteric arteries after 20 minutes of incubation with AR-C118925XX (10 µM) or DMSO (0.1% v/v) in both PVAT and PVAT-free segments. Both ATP and UTP induced concentration-dependent contraction in both PVAT and PVAT-free porcine mesenteric arteries, but AR-C118925XX (10 µM) did not significantly affect this response (Figure 3.5, 3.6, 3.7 and 3.8). In addition, single concentrations of ATP (300 µM) and UTP (300 µM) induced contraction in the presence and absence of AR-C118925XX (10 µM) (Figure 3.9).

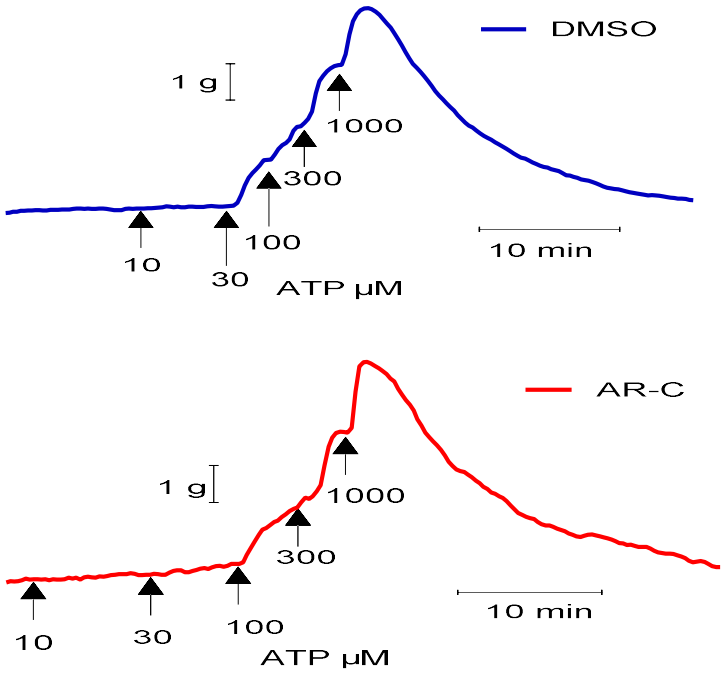


Figure 3.5: An example of representative traces showed cumulative concentration-response curves of ATP (10 µM to 1 mM) in the presence of AR-C118925XX (10 µM) or DMSO (0.1% v/v) in U46619-pre-constricted porcine isolated mesenteric arteries without PVAT.

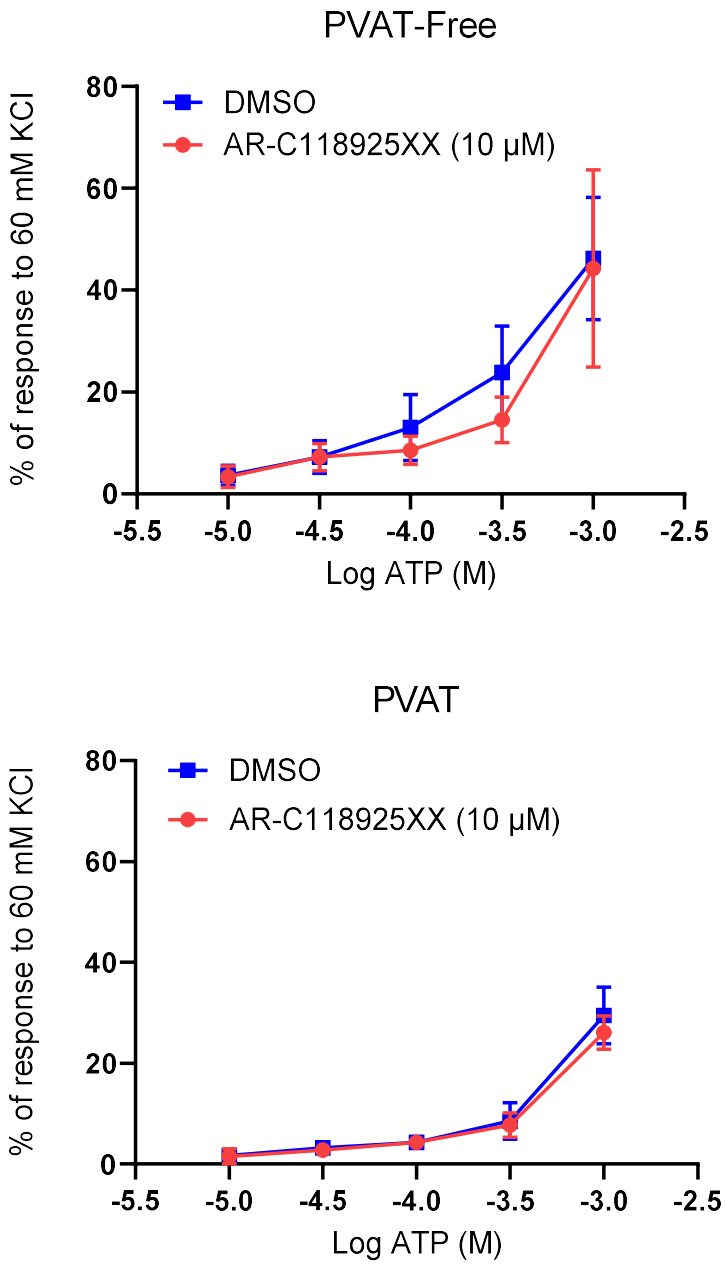


Figure 3.6: Cumulative concentration-response curves of ATP (10 µM to 1 mM) in the presence of AR-C118925XX (10 µM) or DMSO (0.1% v/v) in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

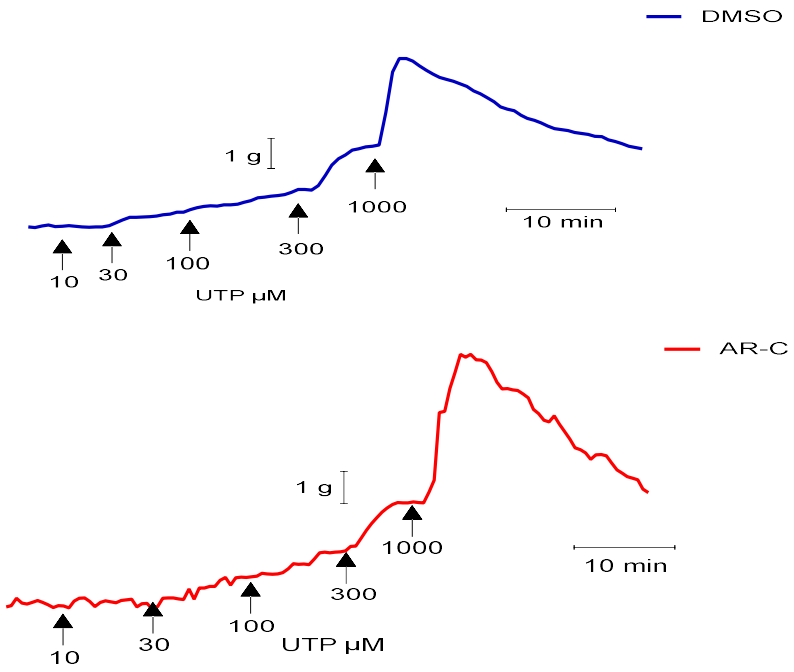
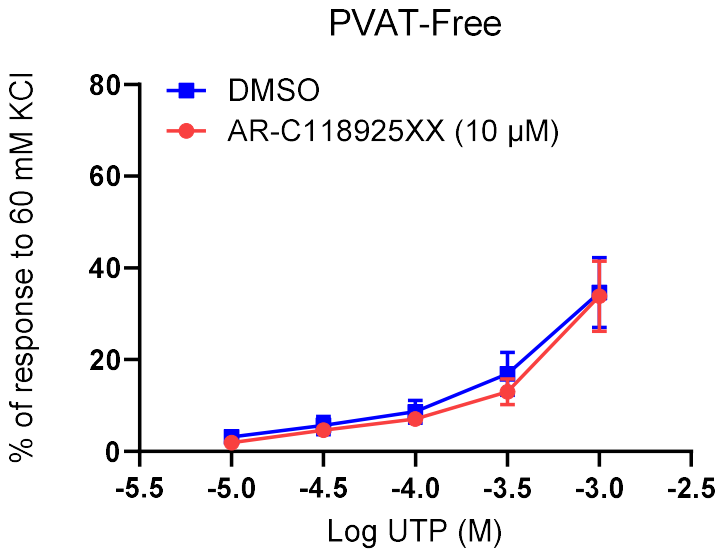


Figure 3.7: An example of representative traces showed cumulative concentration-response curves of UTP (10 µM to 1 mM) in the presence of AR-C118925XX (10 µM) or DMSO (0.1% v/v) in U46619-pre-constricted porcine isolated mesenteric arteries without PVAT.



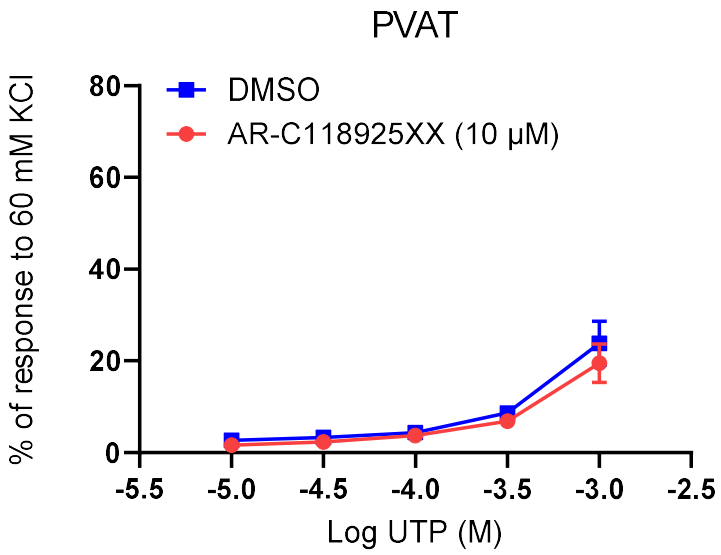
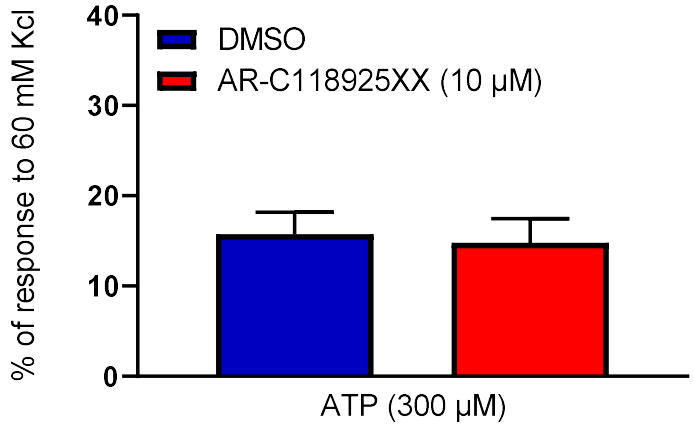


Figure 3.8: Cumulative concentration-response curves of UTP (10 µM to 1 mM) in the presence of AR-C118925XX (10 µM) or DMSO (0.1% v/v) in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).



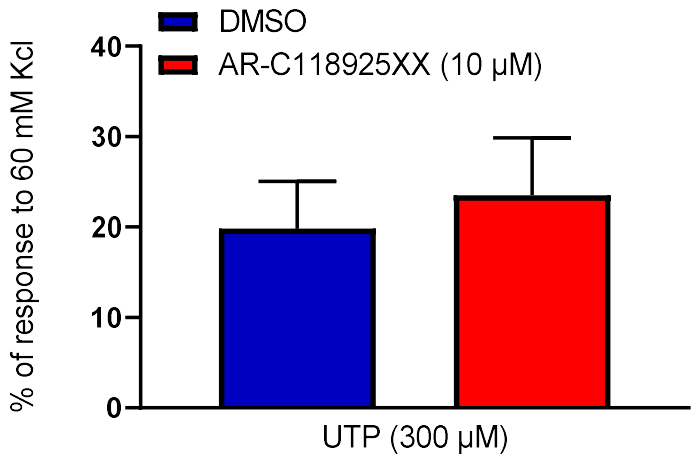


Figure 3.9: The effects of single concentrations of ATP and UTP (300 µM) in the presence of AR-C118925XX (10 µM) or DMSO (0.1% v/v) in U46619-pre-constricted porcine isolated mesenteric arteries without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

Upon reaching the maximal contractile response induced by ATP and UTP, this response was followed by relaxation of vascular tone of mesenteric arteries and a return to baseline. The time (t50) at which the peak response was reversed by 50% was measured in control conditions and in the presence of AR-C118925XX (10 µM). AR-C118925XX altered the time of recovery after the contractile response induced by ATP and UTP (300 µM), and caused a more rapid relaxation of the vascular tone of mesenteric artery. In the presence of AR-C118925XX (10 µM), t50 for ATP was 4.18 ± 0.39 min (n=7) which was significantly different to t50 for ATP in the control conditions, at 6.26 ± 0.79 min (n=7) (Figure 3.10). AR-C118925XX (10 µM) also significantly decreased t50 for UTP which was 9.62 ± 0.53 min (n=6) compared to t50 for UTP in the control conditions, at 13.25 ± 1.14 min (n=6) (Figure 3.11).

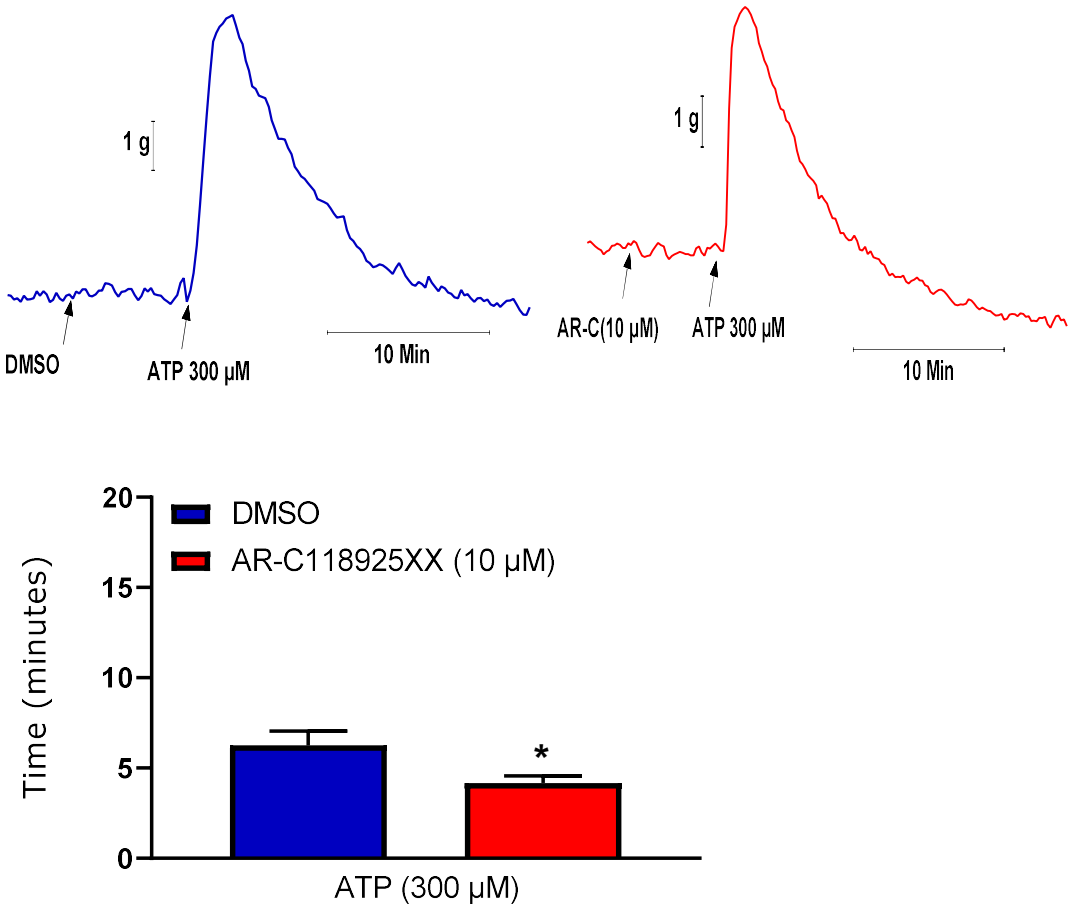


*Figure 3.10: Time t50 (minutes) at which 50% of the peak relaxation response was achieved after the maximum contractile response induced by ATP (300 µM) with AR-C118925XX (10 µM) or DMSO (0.1% v/v). Data are expressed as (minutes) ± SEM (n = 7). Compared to the controls * P<0.05, using two-tailed, unpaired Student's t-test*

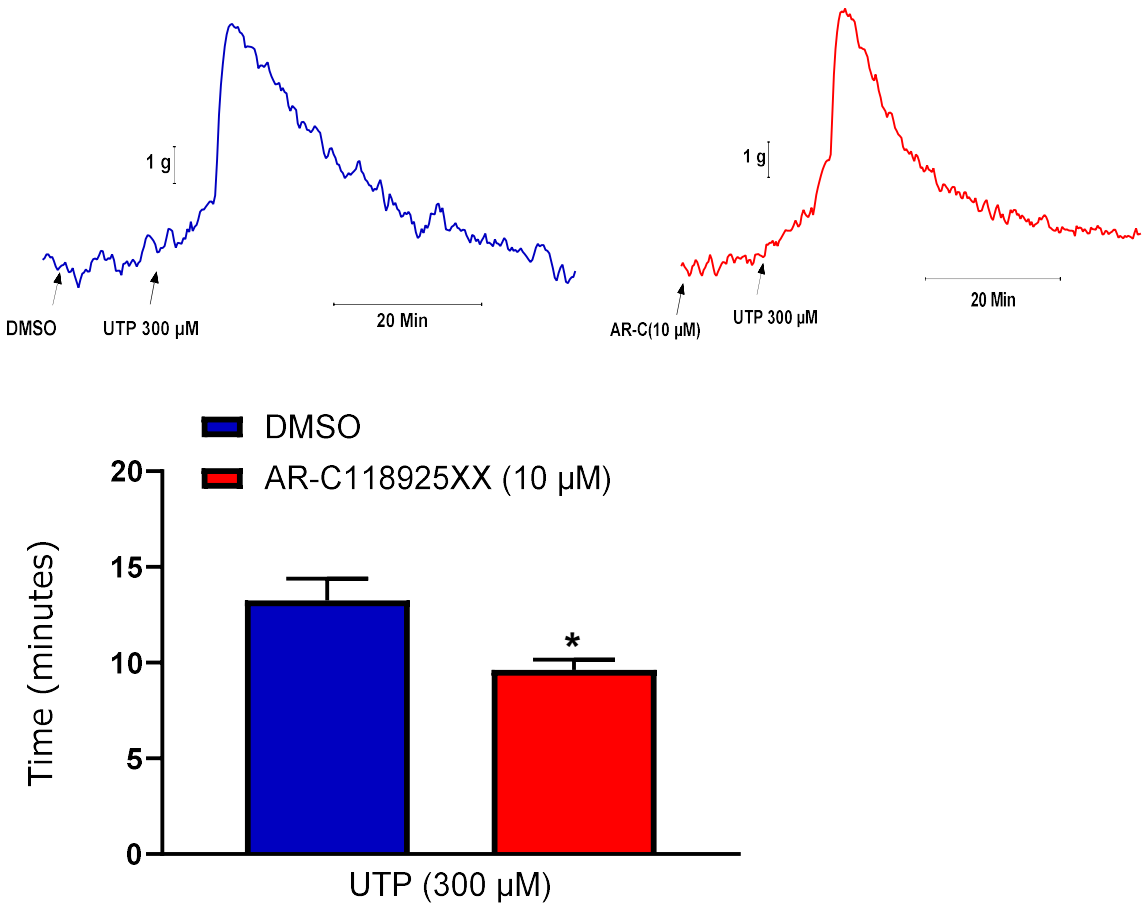


*Figure 3.11: Time t50 (minutes) at which 50% of the peak relaxation response was achieved after the maximum contractile response induced by UTP (300 µM) with AR-C118925XX (10 µM) or DMSO (0.1% v/v). Data are expressed as (minutes) ± SEM (n = 6). Compared to the controls * P<0.05, using two-tailed, unpaired Student's t-test.*

3.2.5 Effect of combination of ATP and UTP in porcine isolated mesenteric arteries

The effect of different concentrations of exogenous nucleotides ATP and UTP on the U46619-pre-constricted vascular tone in porcine mesenteric arteries was studied. A combination of ATP and UTP, and ATP and UTP alone caused contraction of mesenteric arteries. There was no significant difference in the contraction responses induced by a combination of ATP and UTP, and ATP and UTP alone (Figure 3.12).

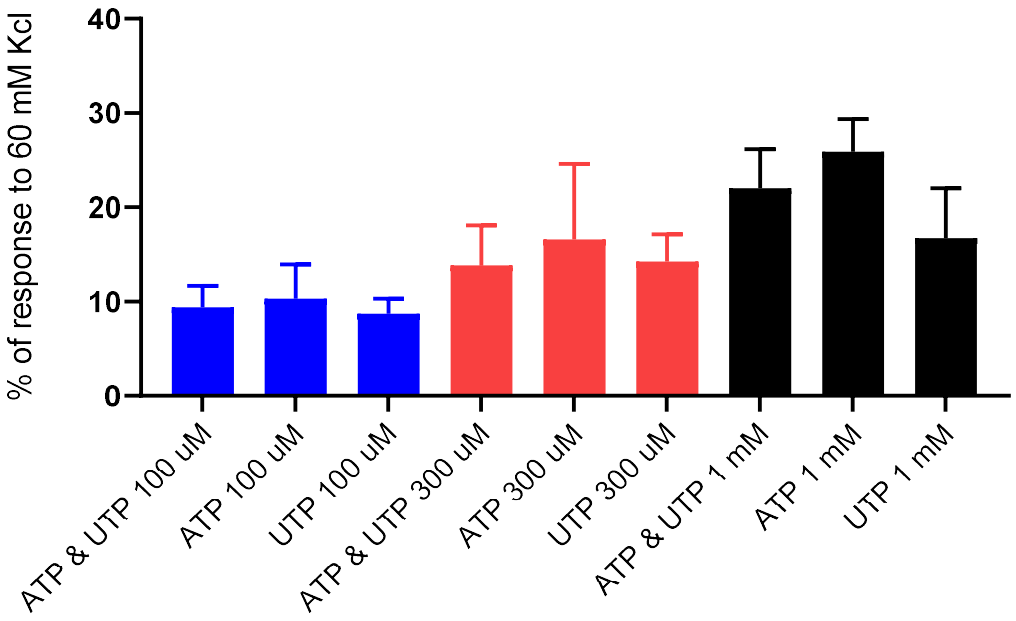


Figure 3.12: Contractile responses induced by different concentrations of a combination of ATP and UTP and ATP and UTP alone in U46619-induced tone of mesenteric arteries. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

3.2.6 Effect of ecto-ATPase, apyrase, and ecto-ATPase inhibitor ARL67156, on vascular tone in porcine isolated mesenteric arteries

To further investigate the possible ongoing release of nucleotides, the effects of apyrase (metabolises nucleotides) on tone of the pre-contracted arteries was studied. Apyrase (5 and 10 units/ml) was incubated with both PVAT and PVAT-free mesenteric arteries. Apyrase (5 units/ml) had no direct effect on U46619-pre-constricted vascular tone in both PVAT and PVAT-free porcine mesenteric arteries (Figure 3.13). Addition of apyrase (10 units/ml) to U46619-pre-constricted vascular tone of mesenteric arteries caused complex alterations in the tone. There was a relaxation during the first 5 minutes of incubation, followed by contraction for about one hour. The maximum contraction induced by apyrase (10 units/ml) in PVAT-free mesenteric arteries was 36.33 ± 3.9% (n = 6), and 29.6 ± 4.9% (n = 6) in PVAT mesenteric arteries (Figure 3.14). Then, apyrase evoked relaxation of U46619-pre-constricted vascular tone in some PVAT and PVAT-free

arteries (Figure 3.14) although this was not significantly different to the control when measured at 120 min. The relaxation response induced by apyrase (10 units/ml) in PVAT-free mesenteric arteries was 9.9 ± 17.4% (n = 6) compared to 4.0 ± 0.48% (n = 6) in the control condition. In PVAT mesenteric arteries, the relaxation response was 8.11 + 11.6% (n=6) compared to 2.1 + 1.8% (n= 6) in the control condition.

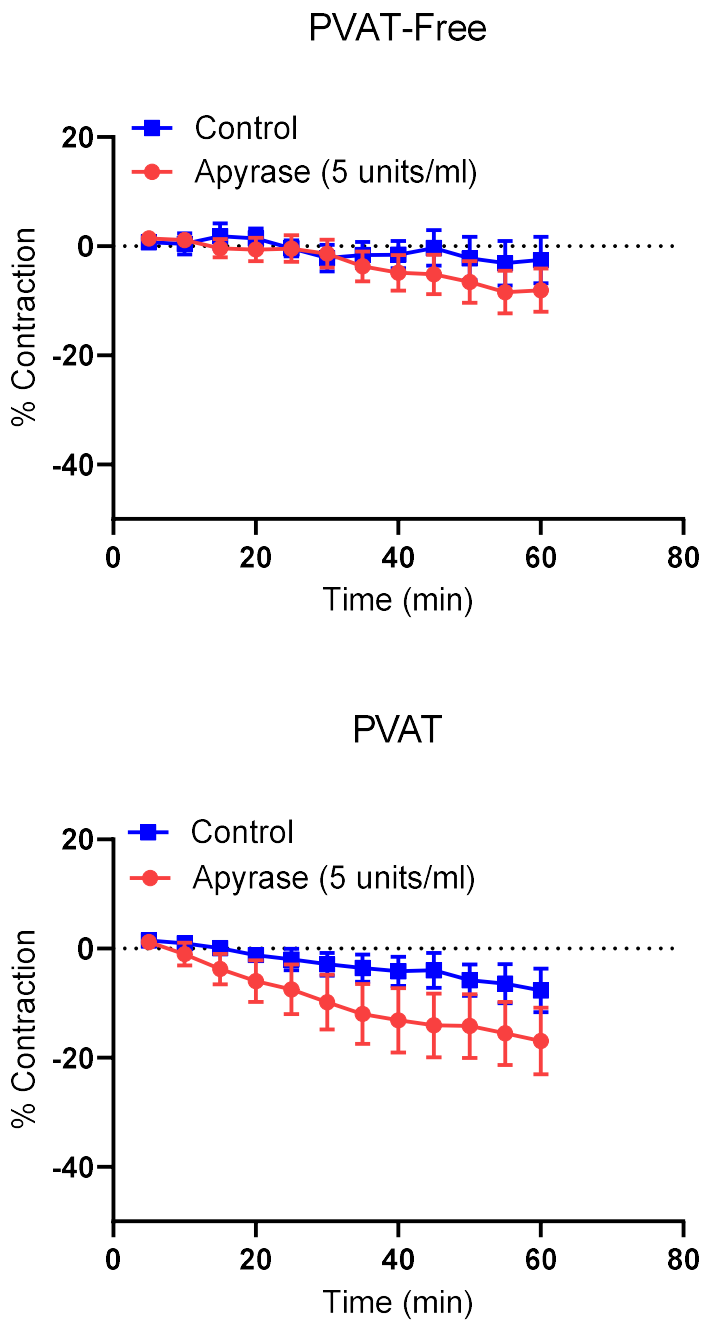
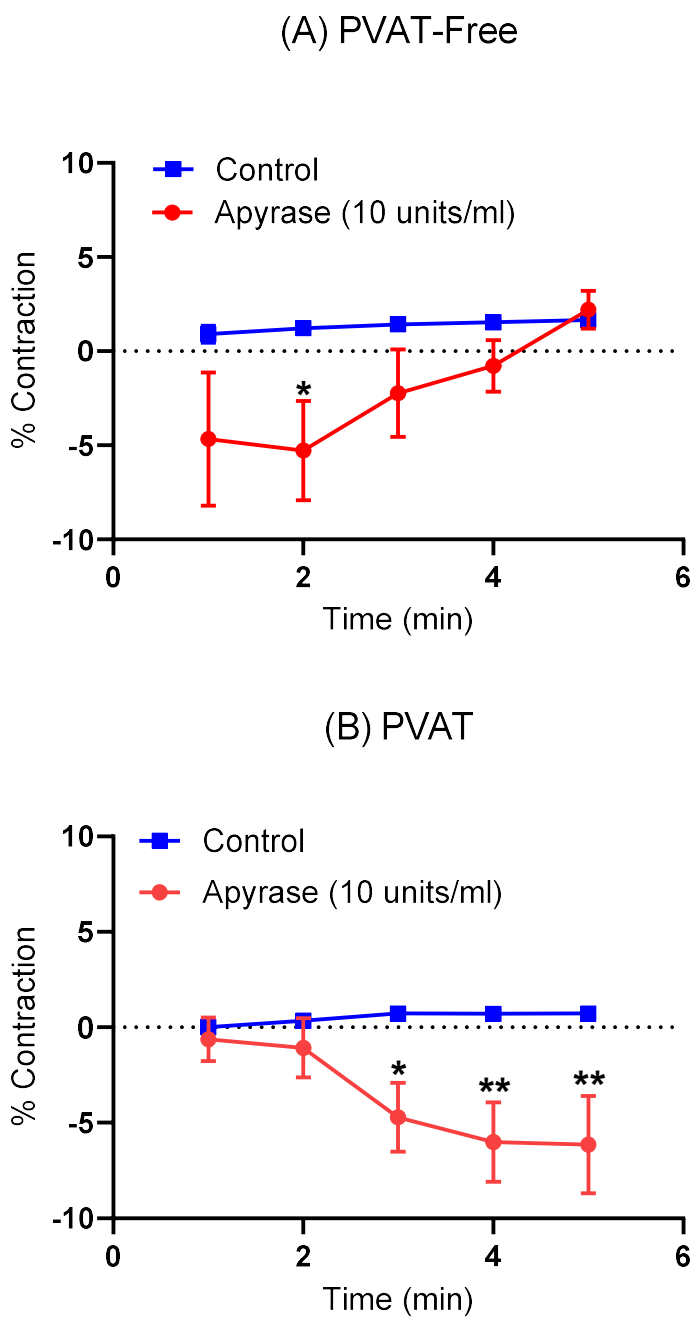
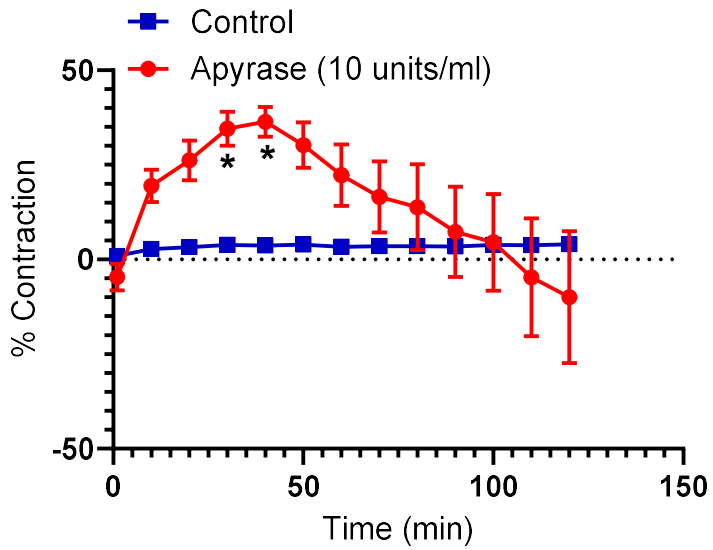


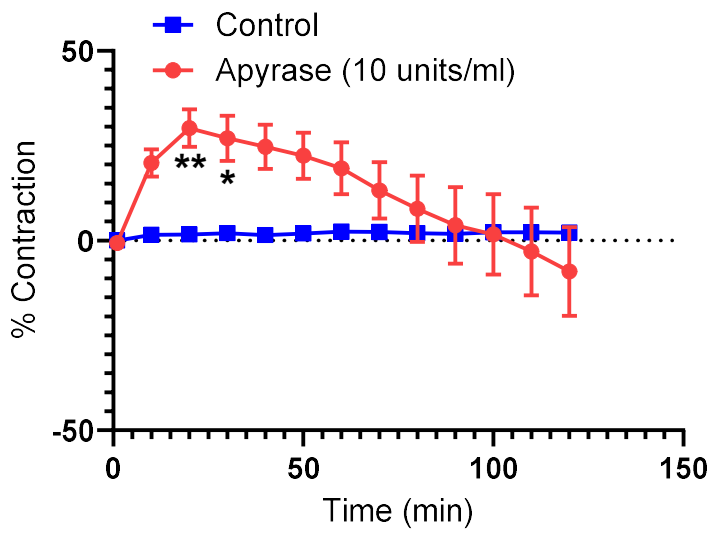
Figure 3.13: The effects of incubating exogenous ectonucleotidases apyrase (5 units/ml) for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Distilled water (10 µl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8).



(C) PVAT-Free



(D) PVAT



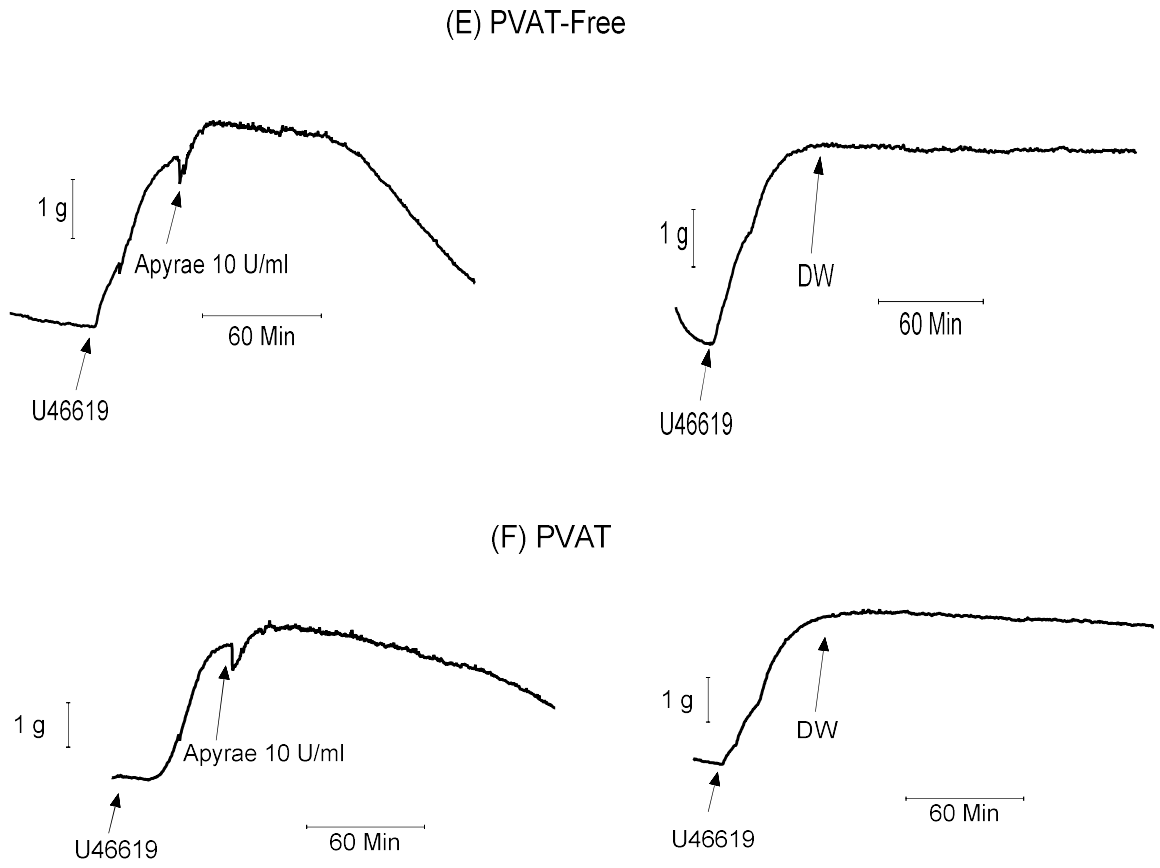


Figure 3.14: (A), (B) The effects of exogenous ectonucleotidases apyrase (10 units/ml) for 5 minutes, (C) and (D) for 2 hours, in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. (E), (F) Representative traces showing the response of U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT to apyrase (10 units/ml). Distilled water (10 µl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8).

Apyrase (10 units/ml) was also added at basal tone before pre-contraction with U46619 in PVAT and PVAT-free mesenteric arteries to investigate whether metabolism of endogenous nucleotides would affect the vasomotor response to AR-C118925XX. After a stable pre-contractile tone had been achieved with U46619, AR-C118925XX (10 µM) was added for 60 minutes. In this experiment, AR-C118925XX had no significant effect on the tone of U46619-pre-constricted porcine mesenteric arteries (n=8) (Figure 3.15).

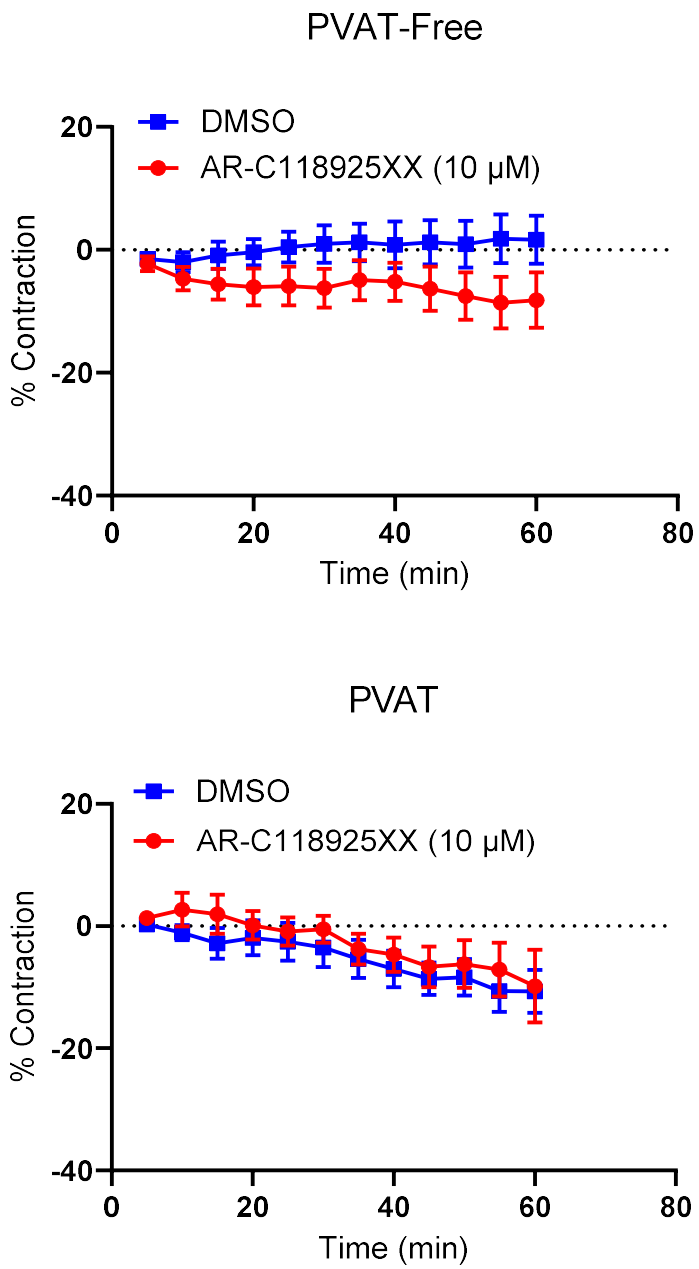
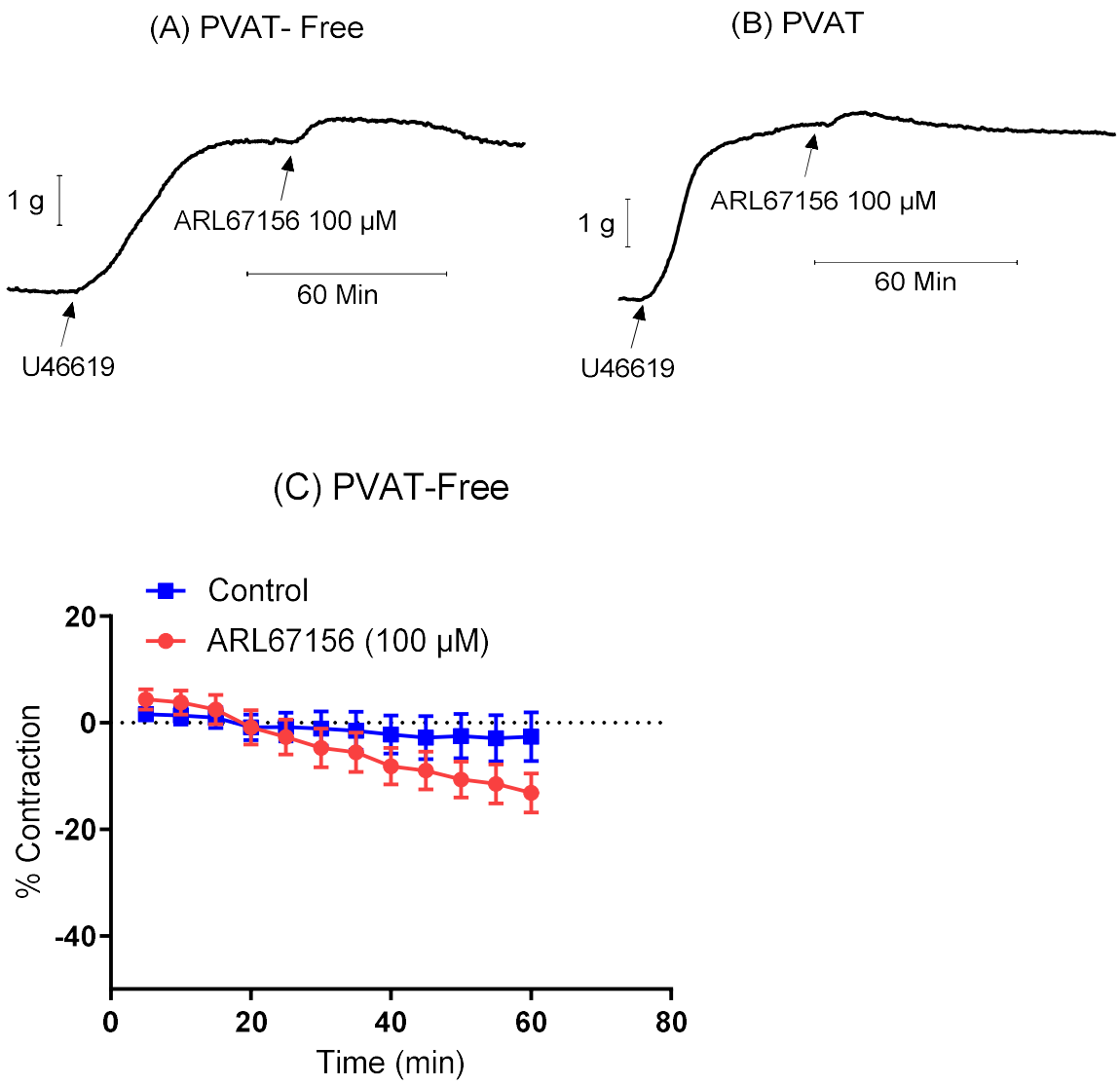


Figure 3.15: Effect of apyrase (10 units/ml) on AR-C118925XX (10 µM) responses in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. AR-C118925XX (10 µM) was incubated for 60 minutes and DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6-8).

ARL67156 (100 μM) which is an ecto-ATPase inhibitor, was added for 60 minutes to both PVAT and PVAT-free mesenteric arteries. ARL67156 at the first 10 to 20 minutes of incubation caused a small contraction of U46619-pre-constricted vascular tone in both PVAT and PVAT-free porcine mesenteric arteries. The maximum contraction induced by ARL67156 (100 μM) in PVAT-free mesenteric arteries was 4.4 ± 1.9% (n = 8), and 6.5 ± 2.7% (n = 6) in PVAT mesenteric arteries (Figure 3.16). Although this was modest and so not statistically significantly different to the control.



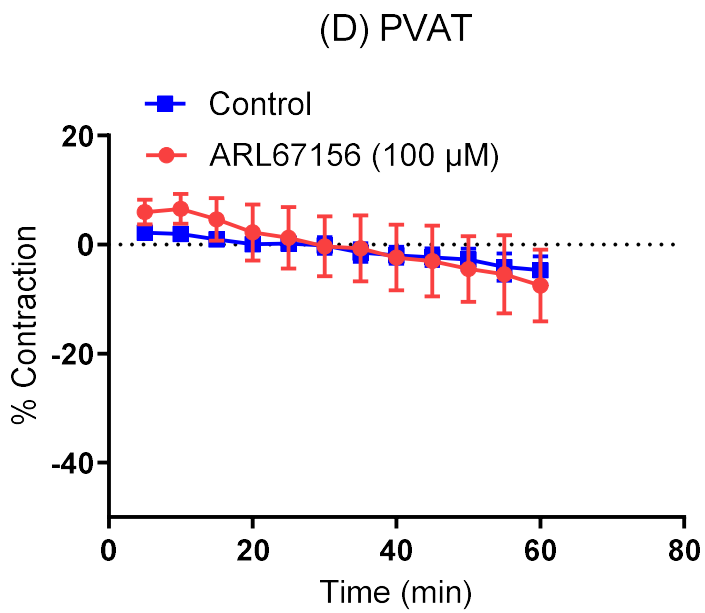


Figure 3.16: (A) and (B) Representative traces showing the response of mesenteric arteries with and without PVAT to ARL67156 (100 µM). (C) and (D) The effects of incubating ARL67156 (100 µM), ecto-ATPase inhibitor, for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Distilled water (10 µl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6-8).

3.3 Discussion

The aim of this thesis was to investigate the possibility that nucleotides ATP/UTP are released constitutively from PVAT to regulate vascular tone of mesenteric arteries. Thus, in this chapter, the effects of suramin (P2 receptor antagonist), AR-C118925XX and MSG228 ($P2Y_2$ receptor antagonists) on the U46619-pre-constricted vascular tone of mesenteric arteries in the presence and absence of PVAT were investigated. Next, the effects of exogenous ATP and UTP were examined. Lastly, the effects of ecto-ATPase, apyrase, and the ecto-ATPase inhibitor ARL67156, on vascular tone in PVAT and PVAT-free porcine mesenteric arteries were studied. It was found that AR-C118925XX and MSG228 at 10 μM and suramin at 100 μM caused relaxation of U46619-pre-constricted vascular tone in both PVAT and PVAT-free mesenteric arteries. In addition, exogenous ATP and UTP induced concentration-dependent contraction in PVAT and PVAT-free mesenteric arteries. AR-C118925XX did not significantly affect the maximum responses to ATP and UTP but it reduced the time to recovery from the response. Apyrase and ARL67156 also altered U46619-pre-constricted mesenteric artery tone. The presence of PVAT did not influence mesenteric artery contractility. Responses to P2 receptor antagonists, ATP and UTP, and apyrase were similar in the presence and absence of PVAT.

Suramin, is a broad-spectrum P2 receptor antagonist which has been reported to antagonize various recombinant and endogenous P2X and P2Y receptors and this inhibition was mostly non-competitive with low potency (Ralevic & Burnstock, 1998). AR-C118925XX is a selective $P2Y_2$ receptor antagonist which is inactive at all other P2Y receptors at a concentration of 10 μM and approximately 50-fold selective for the $P2Y_2$ over the P2X1 receptor (Rafehi et al., 2017). MSG228 is another selective $P2Y_2$ receptor antagonist. MSG228 is one of the compounds that was developed based on AR-C118925 and shown to be selective and have high-affinity for $hP2Y_2$ receptors (PKd = 6.50) in calcium mobilization activities (Conroy et al.,

2018). In this study, suramin induced a relaxation of U46619-pre-constricted vascular tone in both PVAT and PVAT-free arteries. AR-C118925XX and MSG228 at a concentration of 10 μM also induced a relaxation of U46619-pre-constricted vascular tone in both PVAT and PVAT-free porcine mesenteric arteries with a similar time course and amplitude as suramin (~25% relaxation at 60 min), suggesting that the observed effects are not due to non-specific actions of the antagonists. It is not completely clear why these antagonists have a slow relaxation response, but it could be related to the distribution of P2 receptors and the permeability of these antagonists at different layers of the vascular wall.

ATP release from adipocytes has been shown to be through pannexin-1 channels; the ATP then acts in an auto/paracrine role to promote different functions in the adipocytes including modulation of insulin-stimulated glucose uptake and lipolysis (Adamson et al., 2015; Tozzi et al., 2020). ATP release from adipocytes can be regulated by adrenergic agonists, glucose and insulin (Tozzi et al., 2020). It has been reported also that ATP is constitutively released from adipocytes and subsequent activation of $P2Y_2$ receptors has been shown to regulate basal lipolysis in human adipocytes (Ali et al., 2018). Furthermore, basal ATP release has been shown recently to regulate inflammatory cytokine secretion (IL-6) through P2 receptor activation in human adipocytes (Rossato et al., 2022). The expression of functional P2X and P2Y receptors in adipose tissues has been identified, showing that extracellular nucleotides modulate the functions and activities of these tissues (Tozzi & Novak, 2017). PVAT also releases factors including adipokines, which are crucial in regulating vascular tone. PVAT has been reported to induce an anti-contractile effect in a number of vascular beds through ADRF (see Chapter 1). However, contractile responses in the presence of PVAT via ADCF have also been shown (Gao et al., 2006; Ramirez et al., 2017; Xia & Li, 2017). However, the effects observed in this chapter show that the source of nucleotides was not the mesenteric artery

PVAT, and the most likely source of nucleotides was smooth muscle or endothelium (investigated further in Chapter 4).

ATP is released from sympathetic nerves, vascular smooth muscle cells and endothelial cells and maintains the vascular tone of adjacent vasculature through activation of P2X and P2Y receptors (Burnstock, 1990; Erlinge & Burnstock, 2008). ATP may also be released from other cell types relevant to the regulation of vascular tone including sensory nerves, erythrocytes, leucocytes, and platelets, and during inflammation and injury (Gorini et al., 2013). Tonic release of ATP and its actions at P2X1 receptors have been shown to be involved in control of vascular tone in the in vivo rat retina (Kur & Newman, 2014). Furthermore, it has been shown that stretch of vascular cells caused by intraluminal pressure stimulates the release of endogenous pyrimidine nucleotides that promote the contraction via $P2Y_6$ receptor activation and maintains myogenic tone of mesenteric arteries (Kauffenstein et al., 2016). Constitutively release of ATP has been also reported in other cell types including osteoclasts and osteoblasts, olfactory epithelium and monocytes (Brandao-Burch et al., 2012; Hayoz et al., 2012; Sivaramakrishnan et al., 2012).

If nucleotide release is involved in the vasorelaxant effects of suramin, AR-C118925 and MSG228, then ATP and UTP would be expected to be vasoconstrictors of porcine mesenteric arteries. Both ATP and UTP have dual effects, vasoconstriction and/or vasodilation depending on the species, blood vessel and experimental conditions (Burnstock, 2008). In the present study, in U46619-pre-constricted porcine mesenteric arteries, both exogenous ATP and UTP caused vasoconstriction only. A combination of ATP and UTP also evoked vasoconstriction. This is consistent with the possibility that endogenous ATP/UTP is released constitutively to act at contractile P2 receptors. The equal potency of ATP and UTP indicates a possible involvement of $P2Y_2$ receptors (Jacobson et al., 2020). The failure of AR-C118925 to block ATP and UTP concentration-dependent contractions was, therefore, surprising. AR-C118925 did, however, decrease the time to

recovery after the peak of the response to single concentrations of ATP/UTP indicating an involvement of $P2Y_2$ receptors. This reduction in the duration of the ATP/UTP contractile response in the presence of AR-C118925 could be due to either inhibition of binding of the exogenous nucleotides to $P2Y_2$ receptors, or functional antagonism caused by vasorelaxation due to AR-C118925 (due to antagonism of endogenous nucleotide $P2Y_2$ mediated vasocontraction).

Regarding the AR-C118925-resistant component of exogenous ATP/UTP-mediated contraction, $P2Y_2$ receptors are just one of several contractile P2 receptors expressed in blood vessels. Porcine mesenteric arteries express rapidly desensitizing contractile receptors sensitive to a,b-meATP, likely P2X1 (see Chapter 4). Contractile P2X receptors activated by ATP (predominantly P2X1) are expressed in most blood vessels including porcine and rat mesenteric arteries (Gitterman & Evans, 2000; Liu et al., 1989; Shatarat et al., 2014). UTP can also cause artery contraction via actions at vascular smooth muscle P2X (P2X1) receptors (Froldi et al., 1997; McLaren, Sneddon, et al., 1998). Regarding the UTP contractions, UTP acts mainly on $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Burnstock, 2007). It has been reported that $P2Y_6$ receptor in mouse aorta is the mediator of vasoconstriction to UTP and UDP (Kauffenstein et al., 2010). Therefore, it is possible that $P2Y_4$, $P2Y_6$ and/or P2X receptors, as well as $P2Y_2$ receptors, are involved in the contractile responses to exogenous UTP/ATP in the porcine mesenteric artery.

There is an apparent disconnect between AR-C118925-mediated inhibition of endogenous nucleotide contraction (leading to vasorelaxation) and its failure to block most of the exogenous nucleotide (ATP/UTP) response. The reason is not entirely clear but it may involve the relative concentrations of endogenous vs exogenous nucleotides, diffusion (or limited diffusion) across the many cell layers of the media, and region specific expression of P2 receptors throughout the blood vessel wall. Specifically, AR-C118925 likely diffuses though the many smooth muscle cell layers of the media to

antagonise endogenously activated $P2Y_2$ receptors, expressed throughout the media (Haanes et al., 2016; Wallace et al., 2006; Wang et al., 2003), leading to its slow relaxation response (Figure 3.3). In contrast, the rapidly achieved high concentrations of exogenously applied ATP/UTP may act at AR-C118925-insensitive P2X receptors located at the adventitial surface to evoke rapid contraction (Figures 3.8, 3.9) before being quickly metabolised by ectonucleotidases. In this respect immunohistochemical labelling has shown clustering of P2X1 receptors at the adventitial surface of the media (in association with nerve varicosities) (Hansen et al., 1999). However others have reported a more even distribution of P2X1 and P2X4 receptor immunoreactivity throughout the blood vessel wall; P2X7 receptor immunoreactivity was associated with the outer adventitial layer (P2X2, P2X3 and P2X6 receptor immunoreactivity was not detected) (Lewis & Evans, 2001). Heteromeric receptor formation may also contribute to the AR-C118925-insensitive exogenous nucleotide responses.

If there is an ongoing release of endogenous nucleotides which contributes to contractile tone of porcine mesenteric arteries, it would be expected that apyrase (metabolises nucleotides) would have an effect on tone of the pre-contracted arteries. The addition of apyrase (10 units/ml) in this study caused a dual effect of contraction and relaxation in pre-contracted mesenteric arteries. I also observed that the vasorelaxant response of the pre-contracted mesenteric arteries to AR-C118925XX was abolished in the presence of apyrase (10 units/ml), likely due to the absence of endogenous ATP or UTP. Therefore, hydrolysis of endogenous nucleotides by apyrase produced the respective nucleotide forms (ADP or UDP) which could bind to P2X or P2Y receptors expressed in smooth muscles or endothelium to account for its vasomotor effects. The porcine mesenteric artery is known to express vasorelaxant $P2Y_1$ receptors sensitive to ADP (Alefishat et al., 2013). Apyrase did not cause a similar effect on tone of the pre-contracted arteries as the P2 receptor antagonists (relaxation) because apyrase induces endogenous nucleotides metabolism to different nucleotide

subtypes. Apyrase (1 and 10 units/ml) has been observed to reduce phenylephrine-induced constriction in mouse pressurised thoracodorsal resistance arteries (Billaud et al., 2011). In addition, apyrase in rat retina reduced endogenous ATP levels and dilated retinal arterioles by approximately 40% (Kur & Newman, 2014). It has been reported that apyrase supressed shear stress-induced Ca^{2+} influx in endothelial cells of human pulmonary artery (Yamamoto et al., 2003).

ATP and UTP are readily hydrolysed and the possible involvement of ectonucleotidase inhibition was also considered regarding relaxations to AR-C118925XX and suramin. At 10 μM AR-C118925XX acts as an ectonucleotidases inhibitor (Rafehi et al., 2017). In addition, it has been reported that suramin inhibits ecto-ATPase (Chen et al., 1996; Crack et al., 1994). Thus, both antagonists could cause vasorelaxation by enhancing possible vasorelaxant effects of endogenous ATP/UTP at P2 receptors in porcine mesenteric arteries. However, the ecto-ATPase inhibitor ARL67156 had no significant effect on artery tone suggesting that an involvement of ectonucleotidases is not a major mechanism in the relaxation response to the two antagonists. Interestingly, ARL67156 caused an initial small contraction of the mesenteric artery, which is consistent with the possibility that there is an endogenous release of vasocontractile ATP or UTP, the levels and therefore actions of which are increased by ARL67156. Nucleotide metabolism is regulated at the surface of VSMCs by ectonucleotidases, which contributes to the local regulation of vascular tone. It has been reported that a lack of NTPDase1 increased vessel contractility in the thoracic aortas. Contractions induced by exogenous ATP, UTP, ADP and UDP were potentiated in the preparation of NTPDase1 knockout mice. The ARL67156 also potentiated UDP-induced contractions in mice with NTPDase1 (Kauffenstein et al., 2010). An increase in myogenic tone of mouse mesenteric arteries is associated with reduced extracellular nucleotide hydrolysis caused by ARL67156 (Kauffenstein et al., 2016).

In conclusion, these data report a direct vasorelaxant effect of P2 receptor antagonists on vascular tone of porcine pre-contracted mesenteric arteries. In addition, these data indicate that hydrolysis of constitutively released nucleotides with apyrase induced relaxation and contraction of pre-constricted mesenteric arteries. The evidence, thus far, suggests that this does not involve PVAT. It points to an involvement of endogenously released ATP or UTP, acting via vasocontractile P2X or P2Y receptors, which are blocked by the antagonists to cause vasorelaxation.

Chapter 4

Investigating the role of the endothelium and connexin and pannexin channels in the vasorelaxant effect induced by AR-C118925XX, and the selective involvement of $P2Y_2$ receptors

4.1 Introduction

The endothelium is a significant source of ATP released under basal conditions and in response to hypoxia, fluid shear stress and ischemia, and maintains vascular tone through activation of vasodilator endothelial P2 receptors (To et al., 2015; Yamamoto et al., 2006). A number of mechanisms have been proposed for ATP and other nucleotides release including connexin hemichannels and pannexin channels (Lohman et al., 2012). It has been reported that mechanical stimulation induced ATP release via connexin hemichannels in corneal endothelial cells. In this study, ATP release promoted calcium wave propagation, and ATP release was inhibited by the connexin hemichannel blockers flufenamic acid and peptide Gap26 (Gomes et al., 2005). Cx43 hemichannels-mediated ATP release from human microvascular endothelial cells was significantly reduced by hypoxia (Faigle et al., 2008). Another study provided evidence that connexin hemichannels control vascular tone in rat mesenteric arteries. In this study, use of a Cx43 hemichannel blocking peptide resulted in reduction of norepinephrine-induced contraction and calcium oscillations. Also, ATP release from smooth muscle cells was inhibited by blockage of Cx43 hemichannels (Bol et al., 2017).

In addition to connexin hemichannels, it has been shown that ATP can be released via Panx1 channels upon sympathetic stimulation (phenylephrine) in mouse thoracodorsal resistance arteries and cultured human coronary arterial VSMCs. ATP then activates purinergic receptors on neighbouring cells and causes contraction. In this study, the pannexin channel blockers mefloquine, probenecid, and 10Panx1 were highly effective in decreasing the contractile response induced by phenylephrine (Billaud et al., 2011). It has also been reported that ATP can be released through Panx1 channels in response to thrombin in human umbilical vein endothelial cells. In this study, carbenoxolone, a connexin hemichannel and Panx1 channel inhibitor, significantly inhibited thrombin-mediated ATP release (Gödecke et al., 2012). If there is an ongoing release of endogenous nucleotides

which contributes to contractile tone of mesenteric arteries, it would be expected that connexin and pannexin channel blockers would have a similar effect on tone of the pre-contracted arteries to the P2 receptor antagonists as reported in Chapter 3, i.e. that they would also cause a relaxation.

In Chapter 3, I found that both suramin (100 μM), a non-selective P2 receptor antagonist, and AR-C118925XX and MSG228 (10 μM), selective $P2Y_2$ receptor antagonists, caused a significant relaxation of U46619-pre-constricted vascular tone in porcine mesenteric arteries with and without PVAT. In addition, data in Chapter 3 showed that there was no significant difference between the responses of PVAT and PVAT-free mesenteric arteries to P2 receptor antagonists, exogenous nucleotides, apyrase and ARL67156. Therefore, the present chapter has focused on PVAT-free mesenteric arteries only.

The principle aim of this chapter was to examine whether the relaxation responses induced by P2 receptor antagonists in the mesenteric artery are due to nucleotides released via connexin and pannexin channels. Also, to study the role of endothelium as a possible source of the nucleotides. AR-C118925XX, at 10 μM, is selective for $P2Y_2$ receptors with no action at other P2Y receptors but it has been shown to antagonise human recombinant P2X1 and P2X3 receptors at this concentration (Rafehi et al., 2017). Therefore, the possible involvement of other P2X, P2Y and adenosine receptors in the vasorelaxant effect induced by AR-C118925XX was also investigated.

4.2 RESULTS

4.2.1 Effect of AR-C118925XX on vascular tone in endothelium denuded porcine isolated mesenteric arteries

In order determine the functional role of the endothelium on the response to the selective $P2Y_2$ receptor antagonist, AR-C118925XX (10 μM), was added to endothelium denuded vessel segments. AR-C118925XX (10 μM) caused a relaxation of U46619-pre-constricted vascular tone in endothelium denuded porcine mesenteric arteries. The relaxation response in endothelium denuded arteries at 60 minutes was 18.43± 3.5% (n = 6) which was significantly different to relaxation due to its solvent DMSO (0.1% v/v), at 6.25± 2.9% (n=6) (Figure 4.1). In this experiment, the vasorelaxation response to AR-C118925XX was very similar in the absence or presence of the endothelium (n = 6) (Figure 4.2). Therefore, the response to AR-C118925XX, and possible endogenous nucleotides release, is largely endothelium-independent.

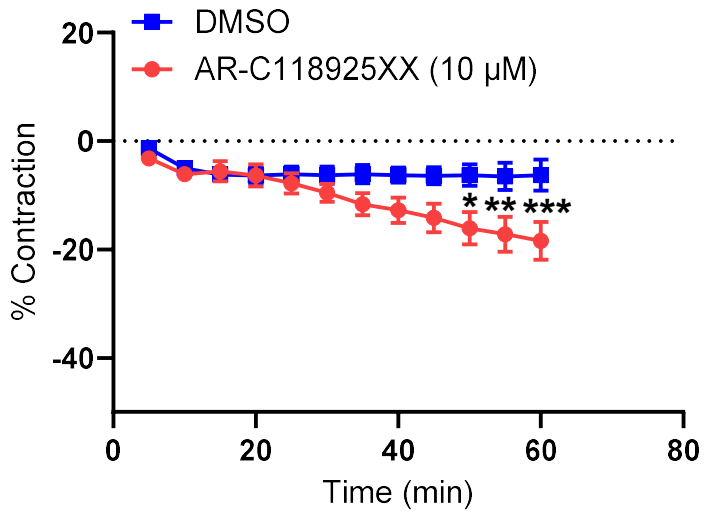


Figure 4.1: The effects of incubating AR-C118925XX (10 μM), for 60 minutes in U46619-pre-constricted endothelium denuded porcine isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean

*± SEM (n = 6). Compared to the controls * P<0.05, ** P< 0.01 and *** P <0.001, 2-way ANOVA followed by Sidak's post hoc test.*

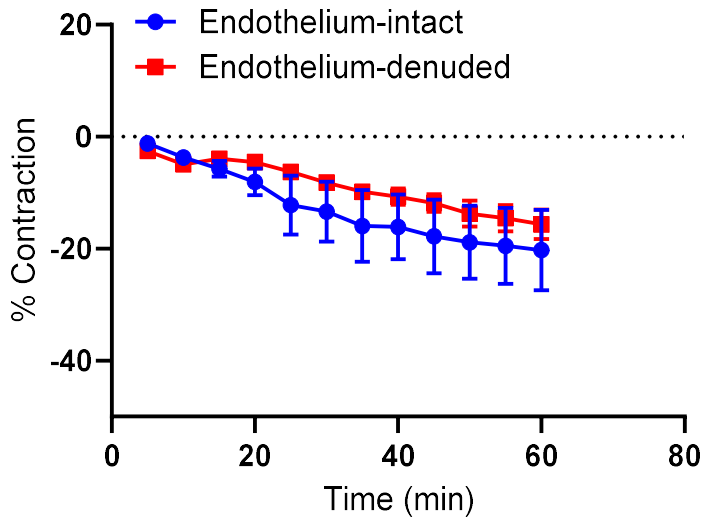


Figure 4.2: Effect of removal of the endothelium on AR-C118925XX (10 µM) responses. AR-C118925XX (10 µM) was incubated for 60 minutes in U46619-pre-constricted endothelium intact and denuded porcine isolated mesenteric arteries. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

4.2.2 Effect of AR-C118925XX on vascular tone of isolated mesenteric arteries pre-contracted with KCl, phenylephrine and endothelin

It is unclear whether the relaxant response to AR-C118925XX and possible nucleotide release is associated specifically with the U46619-induced contraction. In order to study this, porcine isolated mesenteric arteries were pre-contracted using KCl (20 to 30 mM), phenylephrine (0.5 to 1 µM) and endothelin (10 to 20 nM) to about 40-60% of the second KCl response. Phenylephrine and endothelin produced an unstable increase in tone of the mesenteric arteries and so could not be studied further. However, a stable contractile tone in mesenteric arteries was achieved with KCl (20 to 30 mM). AR-C118925XX (10 µM) was incubated for 60 minutes in KCl-pre-constricted mesenteric arteries only. AR-C118925XX (10 µM) elicited relaxation of 10.48 ± 5.2% at 60 min (n=6) which was significantly

different to relaxation due to its solvent DMSO (0.1% v/v), at 1.9 ± 3.2% (n=6) (Figure 4.3).

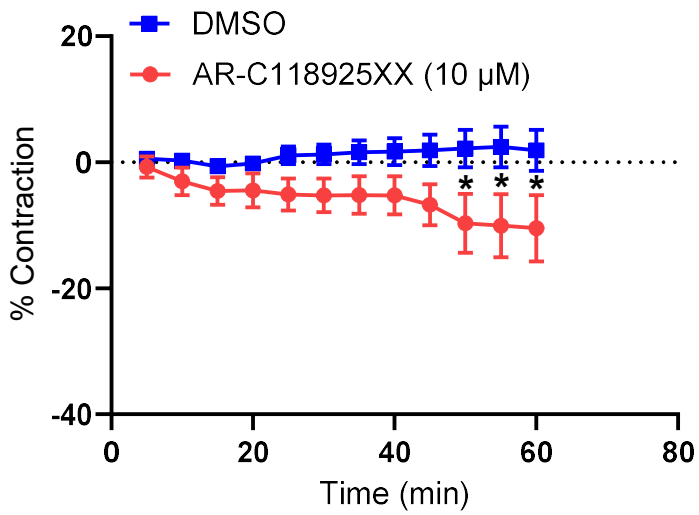


*Figure 4.3: The effects of incubating AR-C118925XX (10 µM), for 60 minutes in KCl-pre-constricted porcine isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6). Compared to the controls * P<0.05, 2-way ANOVA followed by Sidak's post hoc test.*

The effect of AR-C118925XX on the basal tone of mesenteric arteries was also studied. AR-C118925XX (10 µM) elicited relaxation on basal tone of mesenteric arteries. The relaxation response 30 minutes after the incubation of AR-C118925XX was 2.81 ± 0.3% (n=6) which was significantly different to relaxation due to its solvent DMSO, at 0.28 ± 0.8% (n=6) (Figure 4.4).

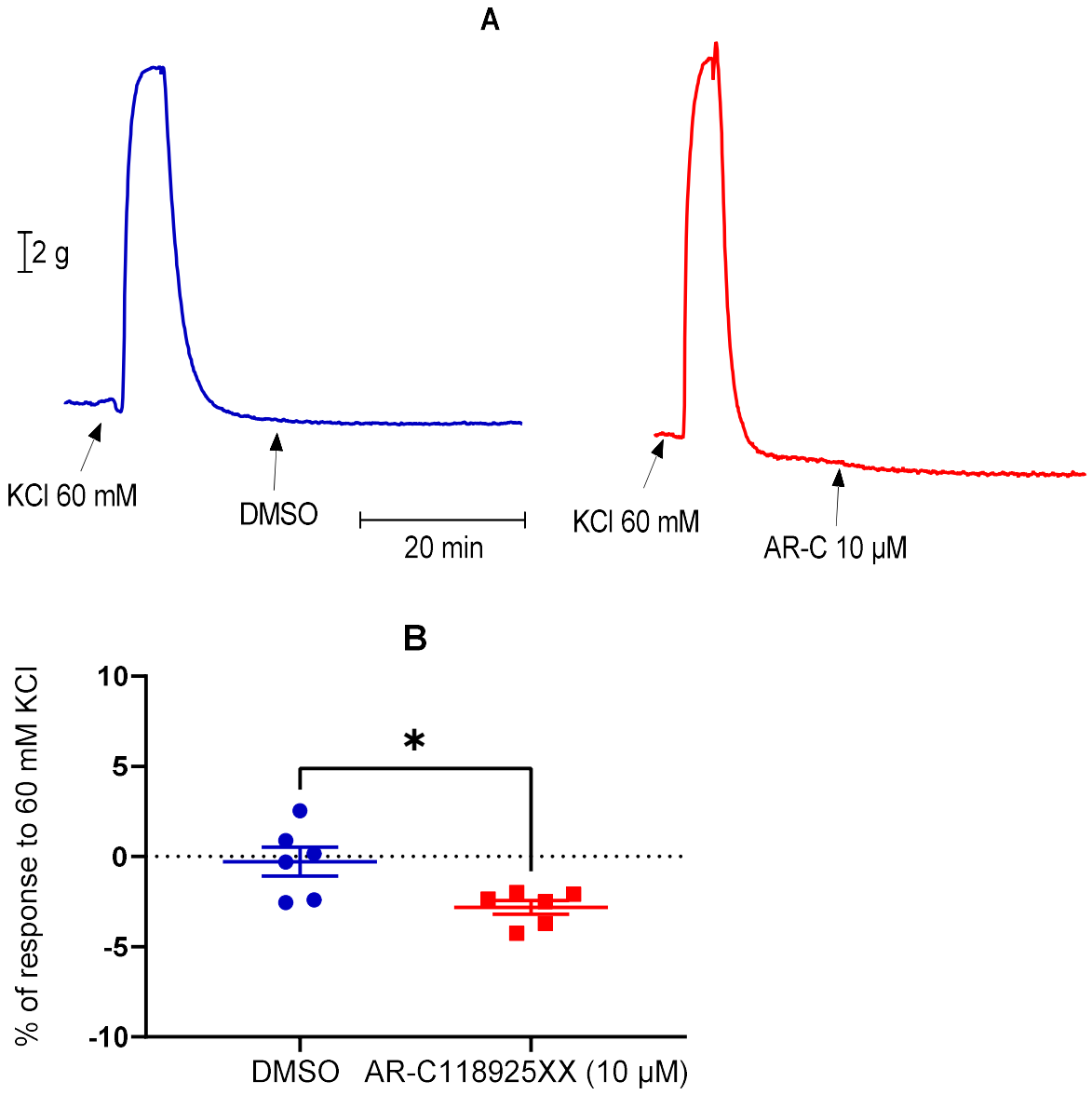


Figure 4.4: (A) Representative traces showing the effects of AR-C118925XX (10 µM) and DMSO on the basal tone of porcine mesenteric arteries. (B) The effects of incubating AR-C118925XX (10 µM) on the basal tone of porcine isolated mesenteric arteries at 30 minutes. DMSO (0.1% v/v) was added as a control. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6). Compared to the control * P<0.05*, Student's 2-tailed unpaired t-test.*

4.2.3 Effect of AR-C118925XX on vascular tone in porcine isolated mesenteric arteries desensitised with UTP

To find out whether AR-C118925XX acts at P2Y receptors to produce the vasorelaxation response, UTP (300 μM) was added to U46619-pre-constricted porcine mesenteric arteries to desensitise P2Y receptors (as described in Chapter 2). In this study, the first addition of UTP (300 μM) induced contraction 17.4 ± 3.1% (n = 6). The subsequent additions of UTP elicited smaller contractions 6.1 ± 1.18% (n=6) and 2.9 ± 0.64% (n=6) due to UTP induced a desensitisation of P2Y receptors in porcine mesenteric arteries (Figure 4.5). AR-C118925XX 10 μM had no significant effect on stable tone of U46619-pre-constricted porcine mesenteric arteries which were desensitised by UTP (n=6) (Figure 4.6).

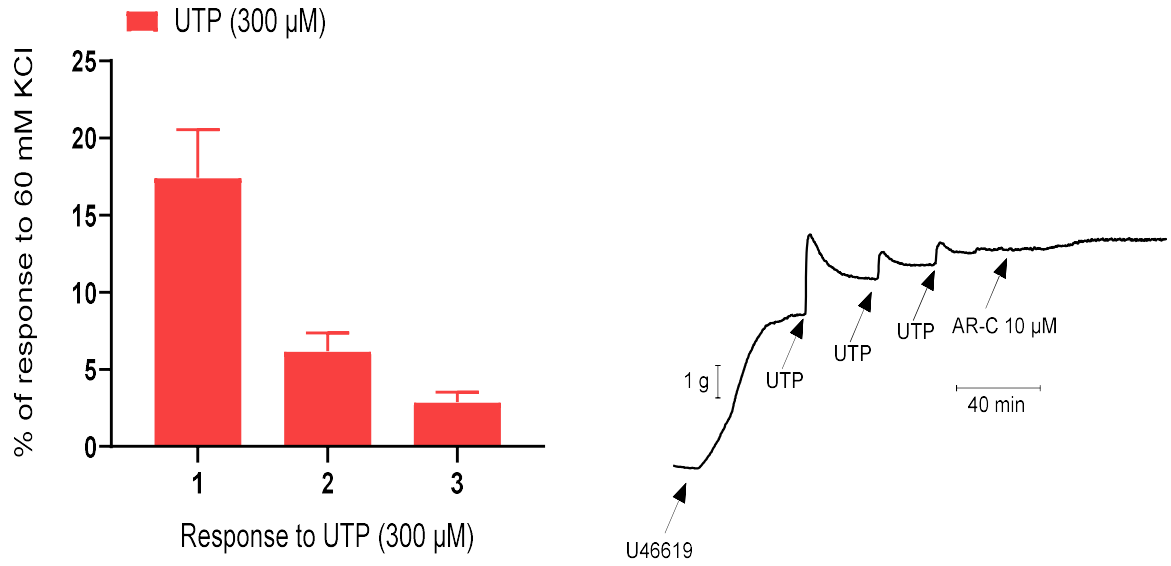


Figure 4.5: Contractile responses to multiple addition of UTP (300 μM), and a representative trace showing the effect of P2Y receptors desensitised by UTP on AR-C118925XX (10 μM) responses. UTP (300 μM) induced a desensitisation in U46619-pre-constricted porcine isolated mesenteric arteries. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6), were 1= 1st response to UTP, 2= 2nd response to UTP and 3= 3rd response to UTP (300 μM).

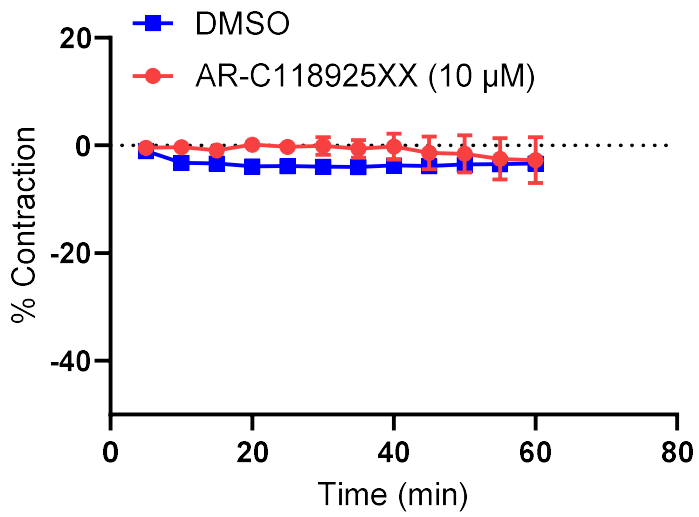


Figure 4.6: Effect of P2Y receptors desensitised by UTP on AR-C118925XX (10 µM) responses in U46619-pre-constricted porcine isolated mesenteric arteries. AR-C118925XX (10 µM) was incubated for 60 minutes and DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

4.2.4 Effect of MRS2578, a $P2Y_6$ receptor antagonist, on vascular tone in porcine mesenteric arteries

$P2Y_6$ receptors are expressed in VSMCs, and are activated by UDP and UTP (von Kügelgen, 2019). If UTP is released constitutively from vascular cells, it could activate $P2Y_6$ receptors. In order to study the possible involvement of $P2Y_6$ receptors in the vasorelaxant effect induced by P2 receptor antagonists, the selective $P2Y_6$ antagonist MRS2578 (10 µM) was incubated with U46619-pre-constricted mesenteric arteries. MRS2578 (10 µM) had no significant effect on U46619-pre-constricted porcine mesenteric arteries (n=6) (Figure 4.7).

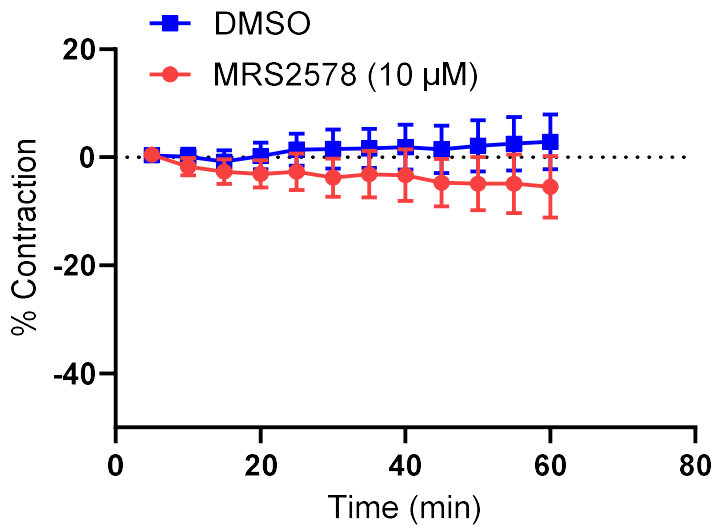


Figure 4.7: The effects of incubating MRS2578 (10 µM), for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

4.2.5 Involvement of P2X receptors in the vasorelaxant effect induced by AR-C118925XX in porcine isolated mesenteric arteries

In order to investigate the possible involvement of P2X receptors in the vasorelaxant effect induced by P2 receptor antagonists, α,β-meATP (10 µM), which is hydrolysis resistant (Martínez-Cutillas et al., 2014), was added at basal tone before pre-contraction with U46619 to desensitise P2X1 receptors. The contraction induced by α,β-meATP (10 µM) was 53.44 ± 4.7% (n=6). Once stable tone of U46619-pre-constricted was achieved, AR-C118925XX (10 µM) was added. Desensitizing P2X1 receptors using α, β-meATP (10 µM) had no effect on the relaxation response induced by AR-C118925XX (10 µM) 23 ± 3.1% (n=6) (Figure 4.8). NF449, which is a selective P2X1 receptor antagonist, was also incubated with U46619-pre-constricted mesenteric arteries to further study the possible involvement of P2X1 receptors in the vasorelaxant effect induced by AR-C118925XX. NF449 (30 µM) had no significant effect on U46619-pre-constricted vascular tone in porcine mesenteric arteries (n=8) (Figure 4.9).

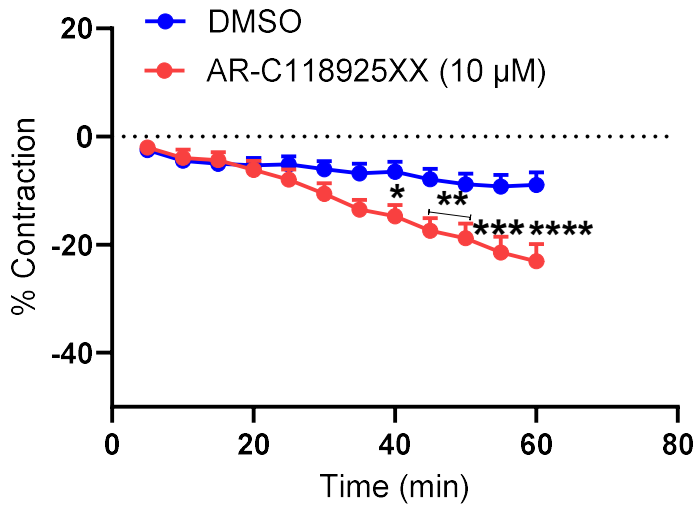


*Figure 4.8: Effect of P2X receptors desensitised by α,β-meATP on AR-C118925XX (10 µM) responses in U46619-pre-constricted porcine isolated mesenteric arteries. AR-C118925XX (10 µM) was incubated for 60 minutes and DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6). Compared to the controls * P<0.05, ** P< 0.01, *** P <0.001 and **** P<0.0001, 2-way ANOVA followed by Sidak's post hoc test.*

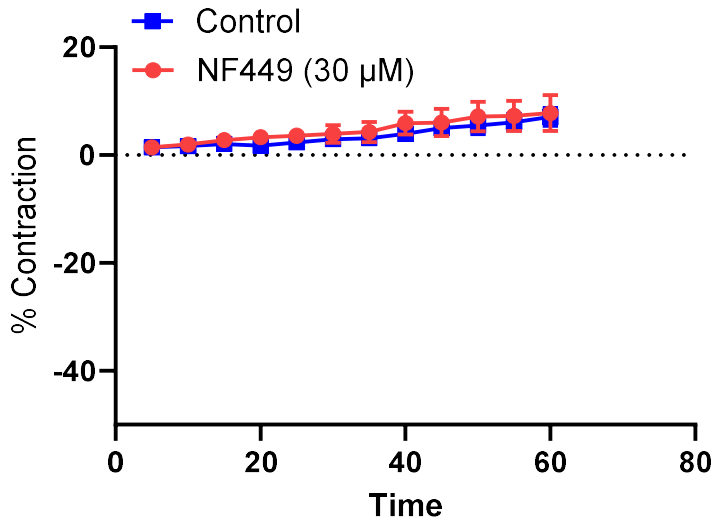


Figure 4.9: The effects of incubating NF449 (30 µM), for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries. DW (30 µl)

was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8).

4.2.6 Involvement of adenosine receptors in the vasorelaxant effect induced by AR-C118925XX in porcine isolated mesenteric arteries

To find out whether the vasorelaxant response induced by AR-C118925XX involves adenosine acting through adenosine receptors, a non-selective adenosine receptor antagonist, theophylline, was used. Theophylline (10 μM) had no significant effect on U46619-pre-constricted vascular tone in porcine mesenteric arteries (n=6) (Figure 4.10).

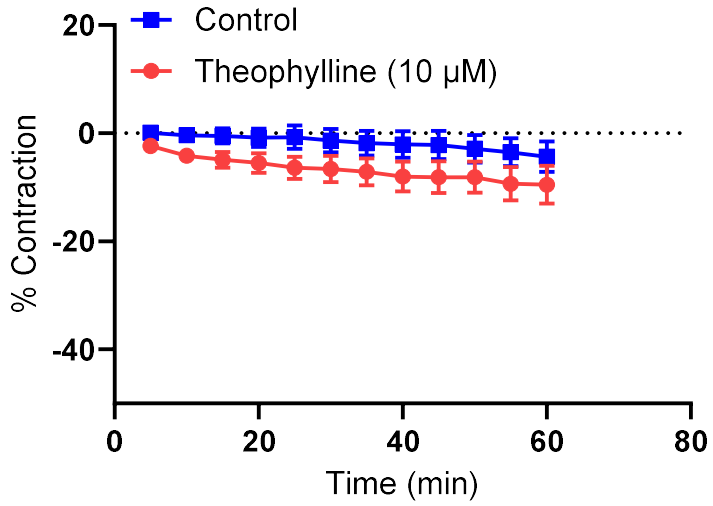


Figure 4.10: The effects of incubating theophylline (10 μM), for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries. DW (10 μl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

4.2.7 Effects of connexin and pannexin channel blockers on vascular tone in porcine isolated mesenteric arteries

In order to further examine the hypothesis that nucleotides are released constitutively, porcine isolated mesenteric artery segments were incubated for 60 min with the connexin and pannexin channels blockers carbenoxolone (100 μM) and probenecid (100 μM) after pre-contraction

with U46619. Carbenoxolone (100 µM) and probenecid (100 µM) caused a significant relaxation of U46619-pre-constricted vascular tone in endothelium intact and denuded porcine mesenteric arteries. The relaxation responses at 60 min to the combination of carbenoxolone (100 µM) and probenecid (100 µM) was 67.3 ± 3.3% (n = 8) (Figure 4.11). The relaxation response at 60 min to carbenoxolone (100 µM) alone in endothelium intact porcine mesenteric arteries was 32 ± 5.3% (n= 7), and 33.8 ± 3.5% (n= 6) in endothelium denuded porcine mesenteric arteries (Figure 4.12). In addition, probenecid (100 µM) induced a relaxation of U46619-pre-constricted vascular tone in both endothelium intact and denuded porcine mesenteric arteries 25.2 ± 4.8% (n= 6) and 23.3 4.8% (n= 6) respectively (Figure 4.13). As a result, there was no difference in the response to carbenoxolone or probenecid between porcine mesenteric arteries with an intact endothelium and those with a denuded endothelium.

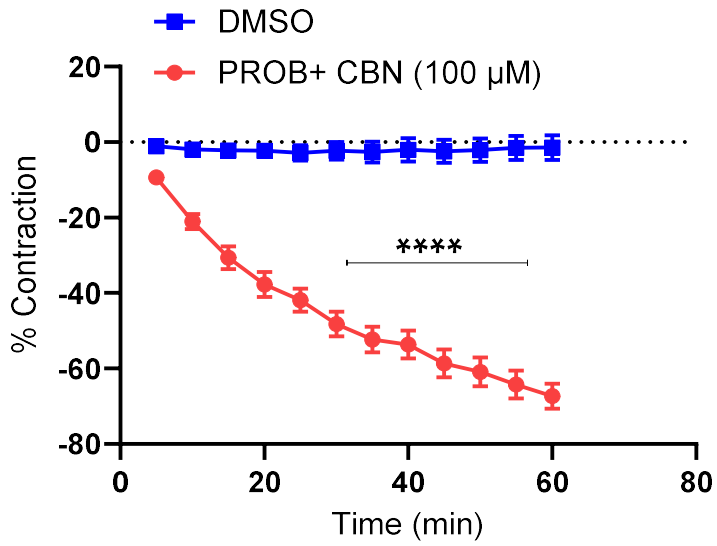


*Figure 4.11: The effects of incubating a combination of carbenoxolone (100 µM) and probenecid (100 µM), for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 8). Compared to the controls **** = P<0.0001, 2-way ANOVA followed by Sidak's post hoc test.*

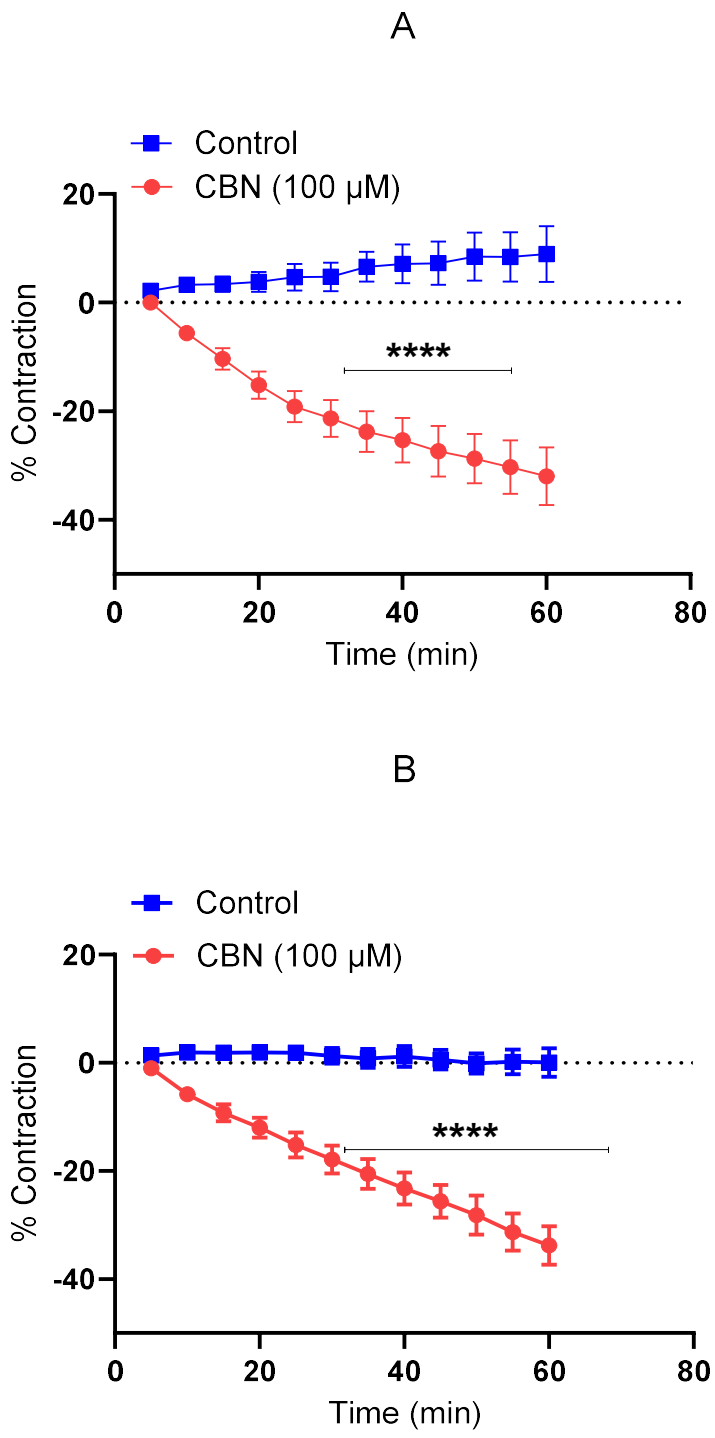


*Figure 4.12: The effects of incubating carbenoxolone (100 µM), for 60 minutes in U46619-pre-constricted endothelium intact (A) and denuded (B) porcine isolated mesenteric arteries. DW (10 µl) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 7 and 6). Compared to the controls **** = P<0.0001, 2-way ANOVA followed by Sidak's post hoc test.*

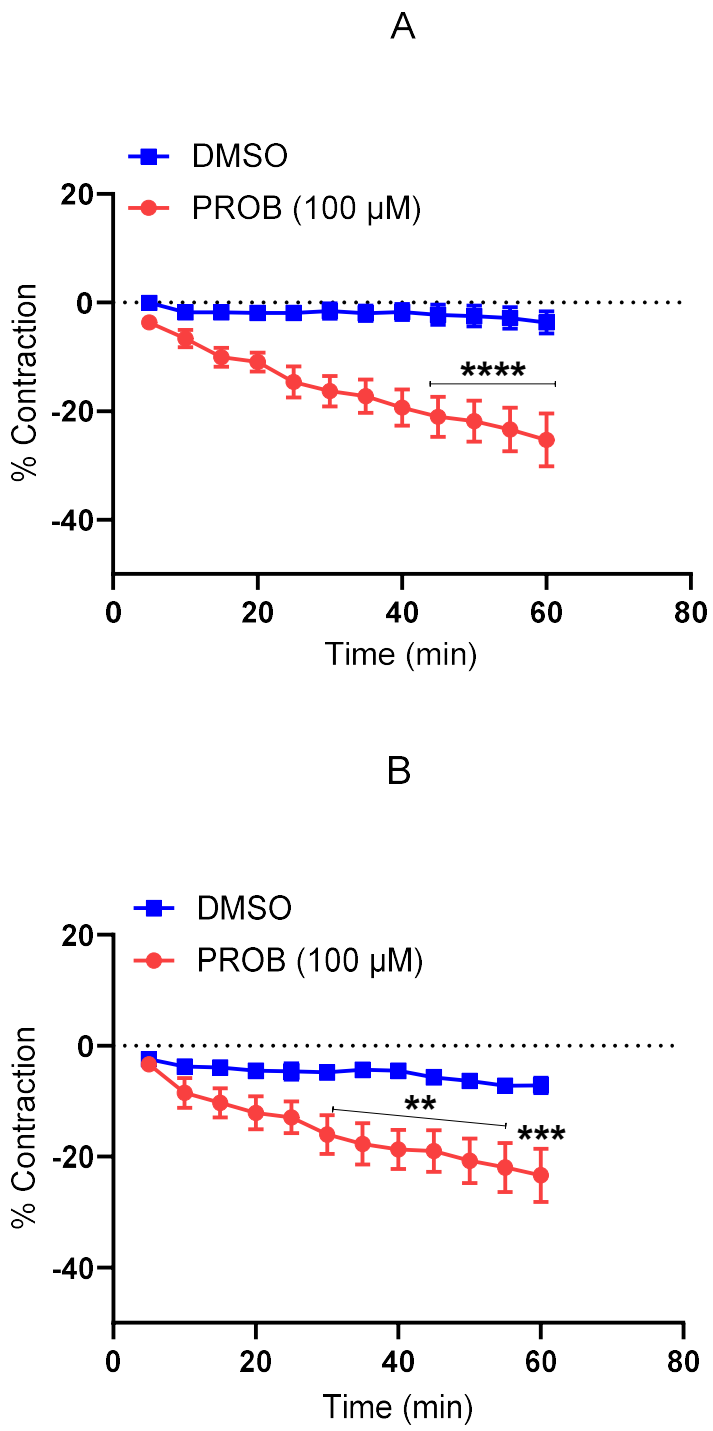
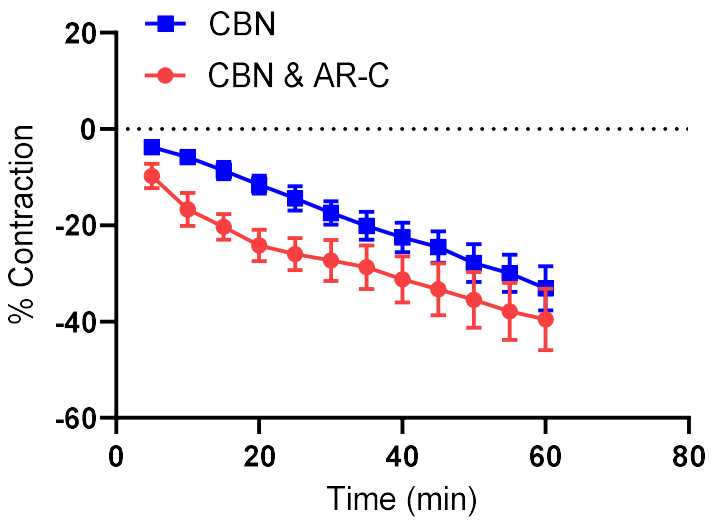


*Figure 4.13: The effects of incubating probenecid (100 µM), for 60 minutes in U46619-pre-constricted endothelium intact (A) and denuded (B) porcine isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6). Compared to the controls ** P< 0.01, *** P <0.001 and **** P<0.0001, 2-way ANOVA followed by Sidak's post hoc test.*

In order to further investigate the hypothesis that nucleotides are released via connexin and pannexin channels, acting via vasocontractile P2Y receptors to maintain the vascular tone of mesenteric arteries, AR-C118925XX (10 μM) was added to U46619-pre-constricted porcine mesenteric arteries together with either carbenoxolone (100 μM) or probenecid (100 μM). The addition of carbenoxolone and probenecid together with AR-C118925XX caused no further effect compared to carbenoxolone and probenecid alone. Carbenoxolone (100 μM) with AR-C118925XX (10 μM) elicited relaxation of 39.5 ± 6.4% (n=8) which was not significantly different to relaxation by carbenoxolone (100 μM) alone at 33.1 ± 4.6% (n=8). Probenecid (100 μM) with AR-C118925XX (10 μM) also caused relaxation of U46619-pre-constricted vascular tone of porcine mesenteric arteries 46.5 ± 5.3% (n= 8) which was not significantly different to relaxation by probenecid (100 μM) alone at 41.6 ± 7.6% (n=8) (Figure 4.14).

A



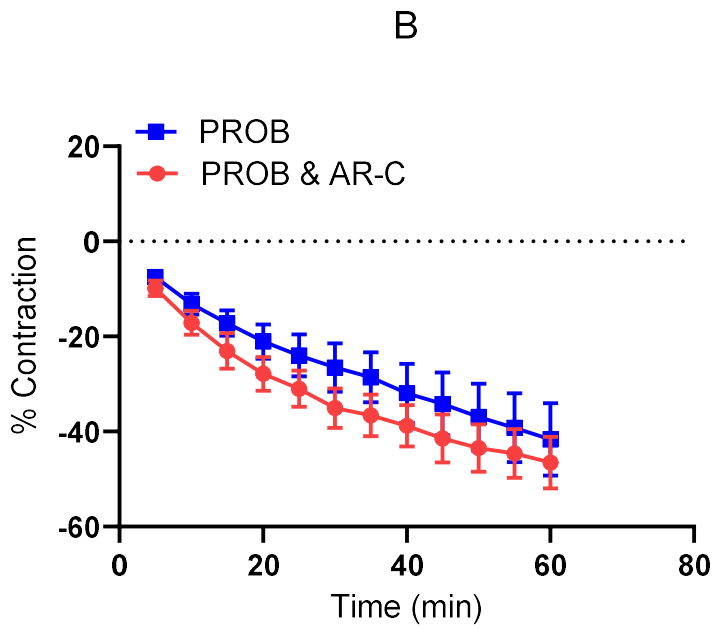


Figure 4.14: The effects of incubating AR-C118925XX (10 μM), for 60 minutes in U46619-pre-constricted porcine isolated mesenteric arteries together with carbenoxolone (100 μM) (A) and probenecid (100 μM) (B). The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6). P>0.10, 2-way ANOVA followed by Sidak's post hoc test.

In prolonged exposures, carbenoxolone and probenecid might have non-specific effects on blood vessels (Rubinstein et al., 2014; Ullian et al., 1996). In order to investigate that carbenoxolone and probenecid do not produce a damaging effect on porcine mesenteric artery smooth muscle and endothelium, KCl (60 mM) and bradykinin (10 μM) were added at the end of the experiments. Carbenoxolone and probenecid did not impair tissue contractile or relaxant function. KCl (60 mM) at the end of the experiments induced similar contraction to the second KCl response (n=6) (Figure 4.15). In addition, relaxation responses to bradykinin (10 μM) were not affected by incubation of porcine mesenteric arteries with carbenoxolone and probenecid (n=6) (Figure 4.16).

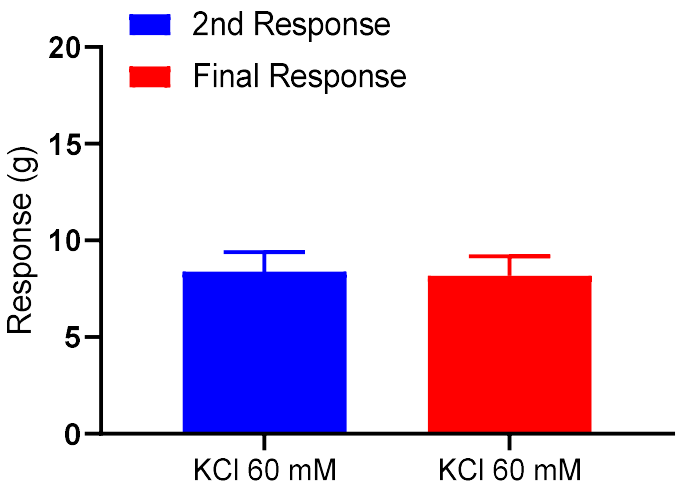


Figure 4.15: Contractile responses induced by KCl (60 mM) at basal tone. 2nd response is the maximal contraction induced by KCl before incubation with carbenoxolone and probenecid. Final response is the maximal contraction induced by Kcl after incubation with carbenoxolone and probenecid at the end of the experiments. Data are expressed as gram (g) tension and are mean ± SEM (n = 6).

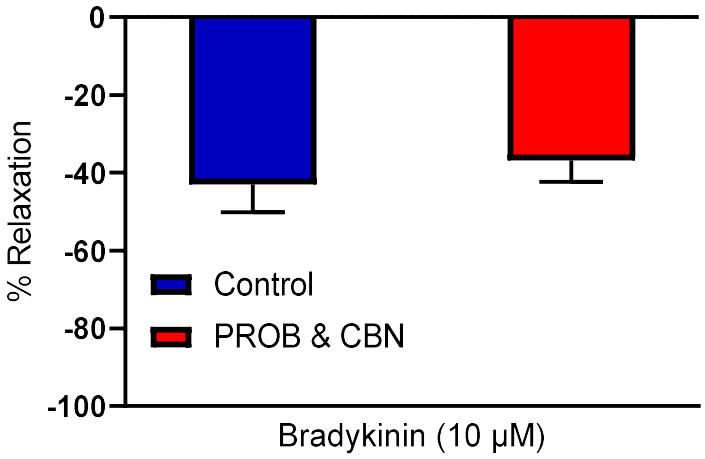


Figure 4.16: Relaxation responses to bradykinin (10 µM) in U46619-pre-constricted porcine isolated mesenteric arteries after incubation with carbenoxolone (100 µM) and probenecid (100 µM). DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

4.2.8 ATP concentration in porcine isolated mesenteric arteries

To further study the possibility that nucleotides are released tonically from mesenteric arteries, ATP levels were measured using luminescence directly from the Krebs'-Henseleit solution. In this study, ATP release was measured in isolated mesenteric arteries with and without PVAT and in the presence and absence of apyrase (10 units/ml) and U46619 (50 nM). The presence of PVAT in this experiment increased the amount of ATP released from isolated porcine mesenteric arteries (Figure 4.17). The basal ATP concentration in mesenteric arteries with PVAT was 36.77 ± 5.61 nM (n = 5) compared to 9.29 ± 1.22 nM (n = 5) in mesenteric arteries without PVAT. Stimulation of mesenteric arteries with U46619 (50 nM) had no significant effect on the concentration of ATP released compared to the basal release. Furthermore, mesenteric arteries with and without PVAT incubated with apyrase (10 units/ml) released less ATP compared to basal conditions, but this was not statistically significant (Figure 4.18).

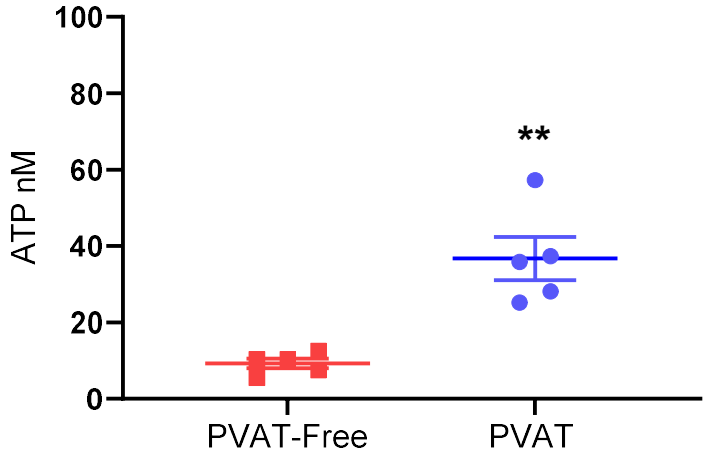


*Figure 4.17: Concentrations of ATP released from isolated mesenteric arteries with and without PVAT at the basal conditions. Data are expressed as concentrations of ATP (nM) ± SEM (n = 5). Compared to the PVAT-Free ** P< 0.01, Student's 2-tailed unpaired t-test.*

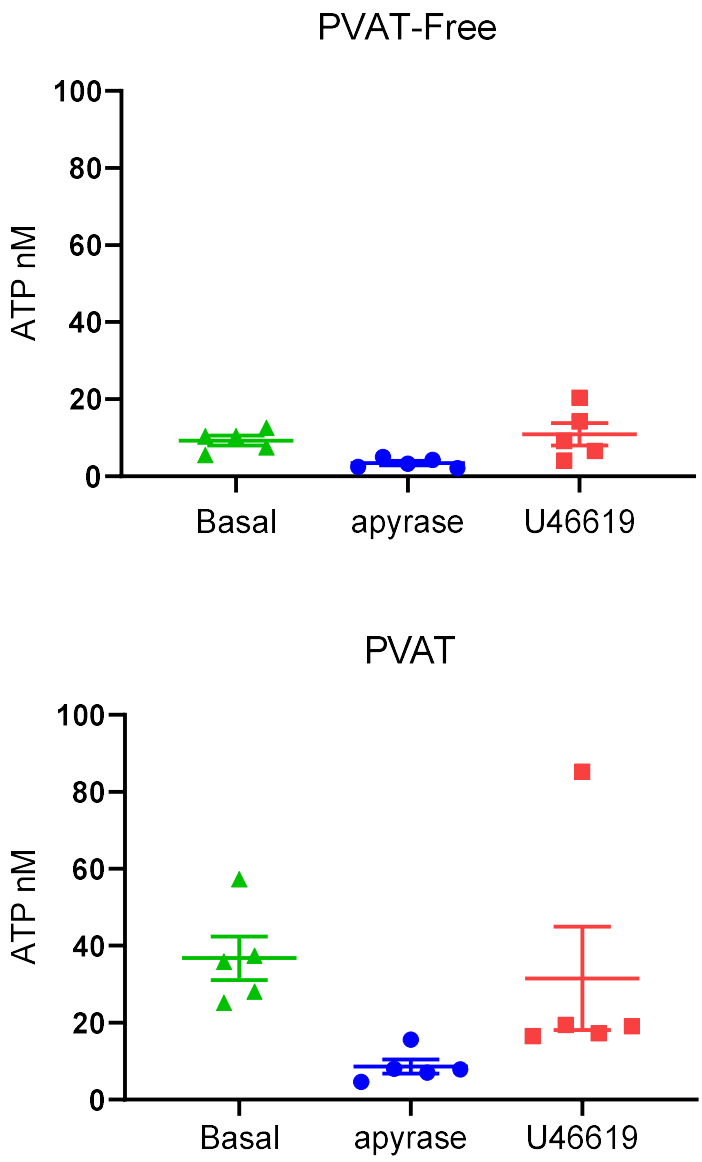


Figure 4.18: Concentrations of ATP released from isolated mesenteric arteries with and without PVAT. ATP levels were determined in the presence of apyrase (10 units/ml), U46619 (50 nM) and the basal release. Data are expressed as concentrations of ATP (nM) ± SEM (n = 5). Student's 2-tailed unpaired t-test.

4.2.9 Effect of AR-C118925XX on vascular tone in porcine isolated coronary and splenic arteries, and in human and rat mesenteric arteries

In order to determine how widespread the vascular effect of AR-C118925XX is in different porcine vascular beds, the effect of AR-C118925XX was studied in porcine coronary and splenic arteries. U46619-pre-constricted coronary and splenic arteries were incubated with AR-C118925XX (10 μM) for 60 minutes. AR-C118925XX (10 μM) induced a significant relaxation of U46619-pre-constricted vascular tone in porcine coronary and splenic arteries 25.8 ± 10.7% (n=6) and 25.25 ± 5.9% (n=4) respectively (Figure 4.19). In order to investigate its relevance across species human and rat mesenteric arteries were used. In human mesenteric arteries, University of Nottingham Human Tissue Research Licence (no 12265), vasopressin alone or a combination of U46619 and endothelin were used as vasoconstrictors (n=5). However, unstable vascular tone of the human mesenteric arteries was observed and all vasoconstrictors caused rhythmic contraction (Appendix A). My colleague Putcharawipa Maneesai performed and provided data on the rat mesenteric arteries in this study. In rat mesenteric arteries without PVAT, which were pre-contracted with U46619, 10 μM AR-C118925XX elicited relaxation of 70.79 ± 13.44% (n=4) which was significantly different to relaxation by its solvent, DMSO, at 16.92 ± 9.55% (n=4). 1μM AR-C118925XX also elicited relaxation of 15.03 ± 9.67% (n=3) in rat mesenteric arteries but it was not significantly different to relaxation by DMSO (Figure 4.20).

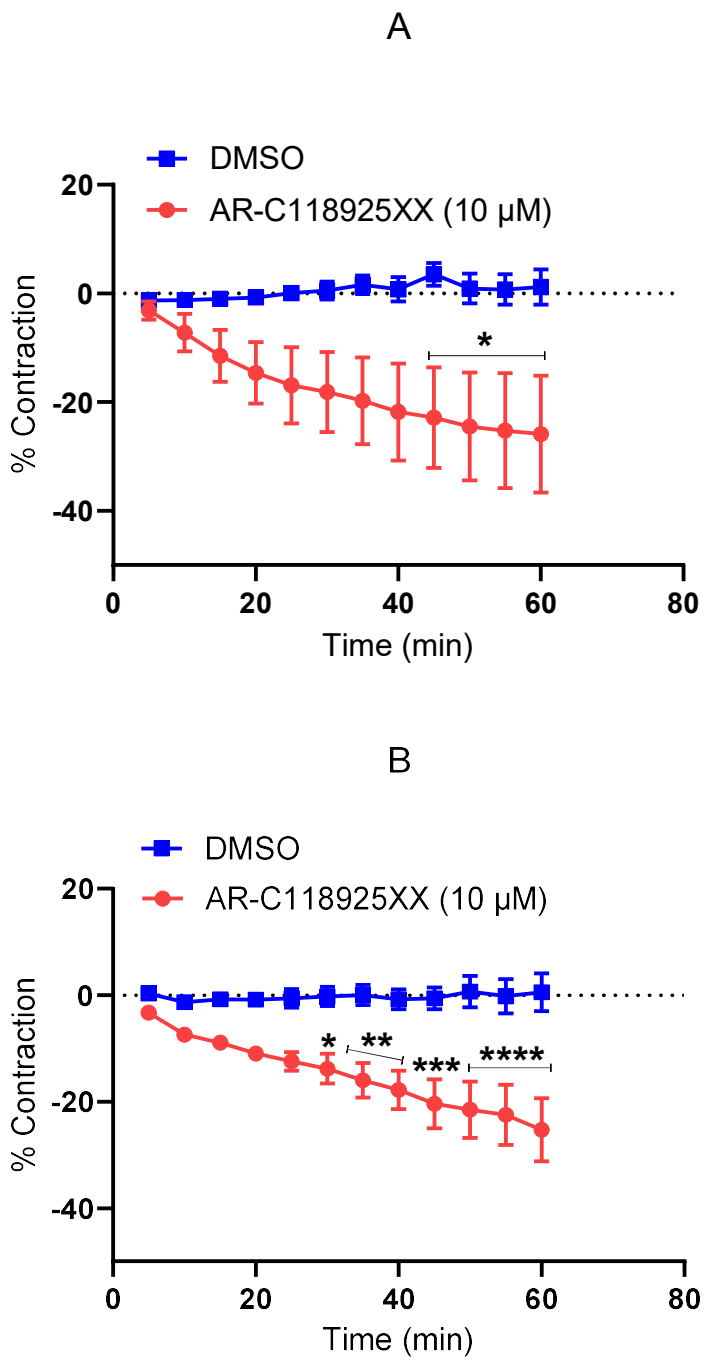


*Figure 4.19: The effects of incubating AR-C118925XX (10 µM), for 60 minutes in U46619-pre-constricted porcine isolated coronary (A) and splenic (B) arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6 & 4). Compared to the controls * = P<0.05, *** = P <0.001 and **** = P<0.0001, 2-way ANOVA followed by Sidak's post hoc test.*

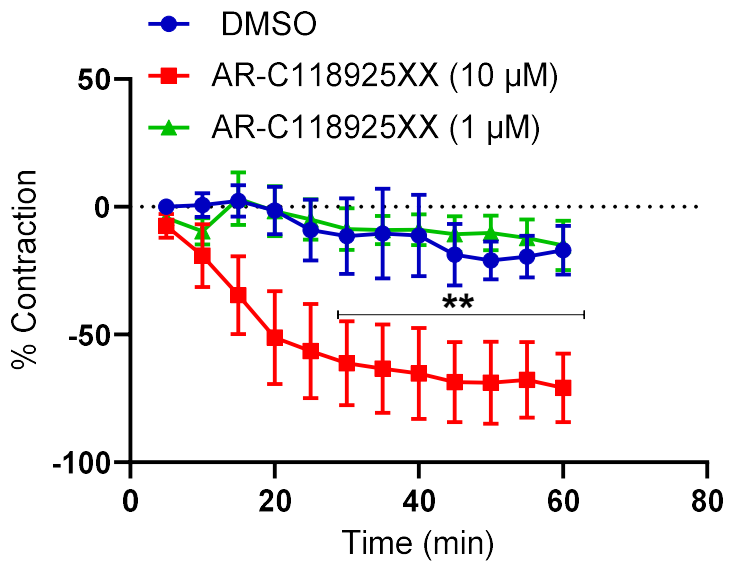


*Figure 4.20: The effects of incubating AR-C118925XX (1 and 10 µM), for 60 minutes in U46619-pre-constricted rat isolated mesenteric arteries. DMSO (0.1% v/v) was added as a control. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 4). Compared to the controls ** P< 0.01, 2-way ANOVA followed by Dunnett's multiple comparisons test.*

4.3 Discussion

The general aim of this study was to investigate the possibility that nucleotides are released constitutively from VSMCs and/or endothelium to regulate the vascular tone of mesenteric arteries. This study also investigated whether the relaxation responses induced by P2 receptor antagonists in the vascular tone of mesenteric artery are due to nucleotides release via connexin and pannexin channels. In this study the possible involvement of UTP-sensitive P2Y receptors, P2X and adenosine receptors in the vasorelaxant effect induced by AR-C118925XX was studied.

The main finding from this chapter is that pharmacological inhibition of $P2Y_2$ receptors by AR-C118925XX elicited relaxation in endothelium denuded mesenteric arteries, and the relaxation response to AR-C118925XX was not affected by endothelium removal. The possible release of endogenous nucleotides and the response to P2 receptor antagonists are therefore endothelium-independent, and the source of nucleotides is the vascular smooth muscle cells. Furthermore, the involvement of P2X1 receptors in the present study was ruled out by the lack of effect of α,β-meATP on responses to AR-C118925XX and the lack of a direct effect of the P2X1 receptor antagonist NF449. The absence of the relaxation response to AR-C118925XX after $P2Y_2$/$P2Y_4$ receptors desensitization by UTP is consistent with a specific effect on $P2Y_2$ receptors. I also observed that pharmacological inhibition of connexin and pannexin channels induced relaxation of U46619-pre-constricted vascular tone of mesenteric arteries.

A healthy endothelium provides vasodilator tone and inhibits platelet aggregation through the release of NO, PGI_2, and endothelium-derived hyperpolarizing factors (Mazurek et al., 2017). Endothelial cells are also a major source of nucleotides released during basal and stimulated conditions (Erlinge & Burnstock, 2008). It has been reported that ATP is constitutively released from human endothelial cells and activates P2 receptors in an autocrine/paracrine manner (Schwiebert et al., 2002). An increase in ATP concentration in endothelium has been shown in response to various stimuli

including hypoxia, ischaemia and shear stress (Bodin & Burnstock, 1995; Bodin & Burnstock, 2001). The current study examined the possible functional role of endothelium in the endogenous release of nucleotides and in the responses to the selective $P2Y_2$ receptor antagonist AR-C118925XX. However, the relaxation response to AR-C118925XX was not affected by endothelium removal. Thus, the possible endogenous nucleotides release and the response to P2 receptor antagonists might be endothelium-independent, and the most likely source of nucleotides is the vascular smooth muscle cells. Previous studies have shown that ATP can be released from vascular smooth muscle cells (Sedaa et al., 1990). In vascular smooth muscle cells, it has been reported that Panx1 channels release purines in response to phenylephrine stimulation, which control vascular tone via P2 receptors (Billaud et al., 2011).

Vascular smooth muscle cells are known to express a number of P2X and P2Y receptors. Generally, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in vascular smooth muscles have been shown to mediate contraction. UTP via $P2Y_2$ receptors mediates vasoconstriction in a range of vascular beds in different species including human coronary and cerebral arteries, porcine coronary artery and rat mesenteric artery (Malmsjö, Hou, et al., 2000; Malmsjö et al., 2003; Rayment et al., 2007). In addition to $P2Y_2$ receptors, UTP is a ligand for $P2Y_4$ receptors (Erb & Weisman, 2012). However, currently, there are no selective antagonists for $P2Y_4$ receptors. In this study, both ATP and UTP elicited concentration dependent vasoconstrictor responses in the porcine mesenteric arteries. To further support the possibility that nucleotides are constitutively released from smooth muscle cells and bind to the contractile P2Y receptors, UTP was applied three times to induce desensitization of $P2Y_2$/$P2Y_4$ receptors before adding AR-C118925XX. The absence of the relaxation response to AR-C118925XX after $P2Y_2$/$P2Y_4$ receptors desensitization by UTP in this experiment is consistent with specific effects on the $P2Y_2$ receptors.

Desensitization is a reduction in receptor ability to be stimulated over time which develops with high activation by agonist. Desensitization is a common feature of purinergic receptors which varies in speed of onset and recovery (resensitization) within the subtypes of these receptors (Gainetdinov et al., 2004; Giniatullin & Nistri, 2013). Desensitization of P2Y receptors, which are GPCRs, can be generally initiated by phosphorylation on serine and threonine residues in the intracellular domain and C terminus of the receptor. There are two known types of receptor desensitization, heterologous and homologous desensitization. In heterologous desensitization, receptor phosphorylation is induced by protein kinase PKA and PKC. For homologous desensitization, G protein-coupled receptor kinases (GRKs) involves in the specific phosphorylation of agonist-activated receptors which followed by the binding of β-arrestins. Binding of β-arrestins producing uncoupling from their G-proteins and receptor internalization (Giniatullin & Nistri, 2013; Nishimura et al., 2017). P2Y receptors have shown a different degree of interaction with β-arrestins translocation pattern upon stimulation by nucleotides. Stimulation of $P2Y_2$ receptors by UTP causes a strong interaction with both β-arrestins1 and β-arrestins2 while ATP only induces a strong interaction with β-arrestins 1 (Hoffmann et al., 2008). ATP and UTP cross-desensitization were used to investigate the mechanism of UTP-mediated vascular tone regulation and P2 characterization in bovine cerebral arteries (Miyagi et al., 1996). In addition, it has been reported that in human umbilical vein and bovine retina endothelial cells, the $P2Y_2$ receptor desensitizes with prior exposure to the extracellular UTP. Desensitization of $P2Y_2$ is manifested by a decrease in peak calcium responses when cells were previously activated by the UTP (Sanabria et al., 2008).

$P2Y_6$ receptors are expressed in vascular tissues, and are activated by UDP and UTP. There is a greater affinity for UDP than UTP for the $P2Y_6$ receptor, and the nucleotides of adenine are mostly inactive (von Kügelgen, 2019). It has been reported that UTP and UDP show different contractile and

relaxant responses in proximal and distal mouse coronary arteries through binding to $P2Y_2$ and $P2Y_6$ receptors, and that UTP can induce relaxation downstream after its hydrolysis to UDP, without affecting the contractile pyrimidine receptors (Haanes et al., 2016). Moreover, $P2Y_6$ receptor-knockout mice showed an absence of endothelium-dependent relaxation responses induced by UDP in thoracic aorta (Bar et al., 2008). $P2Y_6$ receptor in human cerebral arteries has been shown to mediate contraction, and it may possibly be a potential therapeutic target for cerebral vasospasm (Malmsjö et al., 2003). In addition, it has been reported that myogenic tone is maintained through activation of $P2Y_6$ receptors in response to endogenous pyrimidine nucleotides release in mesenteric resistance arteries, and via a direct mechanical activation of $P2Y_4$ and $P2Y_6$ receptors without endogenous nucleotides release in cerebral arterioles (Brayden et al., 2013; Kauffenstein et al., 2016). The myogenic tone in mesenteric arteries and human subcutaneous arteries was sensitive to inhibition of $P2Y_6$ receptor by the selective antagonist MRS2578 (Kauffenstein et al., 2016). The possible contribution of $P2Y_6$ receptors in this study was studied. However, the role of $P2Y_6$ receptors and UDP was ruled out due to the lack of effect in vascular tone of mesenteric arteries to MRS2578.

Most vascular smooth muscle cells express the P2X1 receptor subtype which has been identified to induce vasoconstriction in a number of vascular preparations (see Chapter 1). Porcine mesenteric arteries are known to express contractile P2X1-like receptors sensitive to the metabolically stable analogue α,β-methylene ATP (Shatarat et al., 2014). Although AR-C118925XX is selective for $P2Y_2$ receptors, its IC_{50} for P2X1 receptors is 2.63 μM (Rafehi et al., 2017), which means that when used at 10 μM as in the present study it would be expected to block P2X1 receptors (as well as $P2Y_2$ receptors). Thus, endogenous ATP may be constitutively released to activate P2X1 receptors to contribute to contractile tone, and when blocked by AR-C118925XX this leads to relaxation. Tonic release of ATP and its actions at P2X1 receptors have been shown to be involved in control of

vascular tone in the in vivo rat retina (Kur & Newman, 2014). In addition, there is some evidence that UTP can cause contraction via action at vascular smooth muscle P2X1 receptors (Froldi et al., 1997; McLaren, Sneddon, et al., 1998). P2X1 and P2X3 receptors can be desensitized by α,β-MeATP in hundreds of milliseconds (Rettinger & Schmalzing, 2003). In this study, response to AR-C118925XX was not affected by desensitization of P2X1 receptor by α,β-MeATP. However, it was difficult to prove that P2X1 receptors are desensitized with a single addition of α,β-MeATP. Pharmacological blockade of P2X1 receptors by NF449 did not alter the vascular tone of mesenteric arteries. This finding is consistent with a specific effect of the $P2Y_2$ receptor antagonists. It could have been useful to directly test AR-C118925XX and NF449 against contraction to a,b-MeATP which would strengthen the findings.

Extracellular ATP level is regulated by membrane ectonucleotidases which induce ATP hydrolysis to ADP, AMP and adenosine. As a result of ectoATPase actions, adenosine is produced and activates adenosine receptors, which are highly expressed in vascular cells and known to maintain vascular tone. Adenosine has a vasorelaxant effect mediated mostly by A_{2A} and A_{2B} receptors expressed in both smooth muscle and endothelium (Reiss et al., 2019; Zhang et al., 2021). The purpose of this experiment was to determine whether ATP released from VSMCs is hydrolysed, and whether adenosine is responsible for relaxing the mesenteric arteries. Theophylline, a non-selective antagonist of adenosine receptors, did not significantly alter vascular tone in the mesenteric arteries. The relaxation response is therefore induced by P2 receptor antagonists, but not by adenosine.

It is unclear in this study whether the nucleotides release is associated specifically with inhibition of U46619-induced contraction. AR-C118925XX also caused a relaxation response at basal tone, but the relaxation was small, due to the lack of tone in these preparations. However, another pre-contractile agent KCl was used in this study, and AR-C118925XX still

induced vascular relaxation of mesenteric arteries. This demonstrated that nucleotides are released during basal and contractile tone evoked by U46619 (and other vasoconstrictors) and suggests that these nucleotides provide a supporting role in vasocontractile function of porcine mesenteric arteries. In this study, AR-C118925XX also induced vascular relaxation in porcine coronary and splenic arteries. Additionally, AR-C118925XX induced vascular relaxation in rat mesenteric arteries. This suggests that constitutive release of nucleotides within the vasculature may be widespread occurring in multiple species and vascular beds. Investigation into the nucleotides release and their role in control of vascular tone via P2X and P2Y receptors may introduce potential therapeutic targets for the treatment of vascular disorders including hypertension.

Connexin and pannexin channels are expressed in vascular cells and involved in ATP release (Lohman et al., 2012). If there is an ongoing release of endogenous ATP which contributes to contractile tone of porcine mesenteric arteries, it would be expected carbenoxolone and probenecid, connexin and pannexin channel blockers, would have a similar effect on tone of the pre-contracted arteries to the $P2Y_2$ receptor antagonists i.e. that they would also cause a relaxation. This study confirmed that carbenoxolone and probenecid induced relaxation of U46619-pre-constricted vascular tone of mesenteric arteries. The result obtained using connexin and pannexin channel antagonists in the current study may suggest that nucleotides are released from vascular smooth muscle of mesenteric arteries via these channels since the responses were similar in the presence and absence of endothelium. Pharmacological blockage of connexin and pannexin channels and subsequent inhibition of ATP release has been reported in several vascular beds, e.g. carbenoxolone inhibited thrombin-mediated ATP release in human umbilical vein endothelial cells (Gödecke et al., 2012). In addition, ATP release from smooth muscle cells and vasoconstriction in rat mesenteric arteries were inhibited by blockage of Cx43 hemichannels (Bol et al., 2017). Activation of Panx1 in vascular

smooth muscle is thought to be able to regulate both sympathetic nerve constriction and blood pressure. The pannexin channels blockers mefloquine, probenecid, and 10Panx1 were highly effective in decreasing the contractile response induced by phenylephrine; mice with over-expression of Panx1 released more ATP following phenylephrine stimulation, and ATP hydrolysis by apyrase reduced EFS contractions (Billaud et al., 2011; Dunaway et al., 2022). Endothelial Panx1 has been reported to increase ATP release in mouse small pulmonary arteries acting via a $P2Y_2$ receptor-PKC signalling pathway activated transient receptor potential (TRPV4) channels. Consequently, the pulmonary arteries are dilated and the pulmonary arterial pressure is lowered (Daneva et al., 2021). Thus, in this study, the results obtained using connexin and pannexin channel antagonists support the hypothesis of nucleotides are released from mesenteric arteries and that this involves flux through pannexin/connexin channels. However, some connexin and pannexin channel blockers have shown effects on other targets such as Ca^{2+} and K^+ channels, gap-junction and P2X7 receptors (Willebrords et al., 2017).

To further support the hypothesis of ATP release, extracellular ATP was measured by luminescence in porcine mesenteric arteries under basal conditions. The presence of PVAT in this experiment increased the amount of ATP released. Additionally, stimulation of mesenteric arteries with U46619 had no effect on ATP release compared to basal release. This indicates that the release and vascular effect of ATP in the current study were not due to U46619 stimulation. In addition, apyrase caused a reduction in the amount of ATP released compared to basal conditions. Taken together, these data suggest that isolated mesenteric arteries are capable of releasing ATP to act in smooth muscle $P2Y_2$ receptors to modulate the vascular tone. Despite the finding in this study that preparations with PVAT release more ATP, the effects of P2 receptor antagonists in the raised tone of mesenteric arteries were similar in the presence and absence of PVAT. In addition, in this experiment, the ATP

concentrations were measured in mesenteric vessels in a tube without tensions. It is possible that this explains the low levels of ATP measured in the vessels. A higher amount of ATP may be released by tissues under tension. Extracellular ATP release at resting conditions has been observed in blood vessels including human umbilical vein endothelial cells (Gödecke et al., 2012; To et al., 2015). A majority of studies report very low (nanomolar range) basal extracellular ATP concentrations. The Background levels of ATP concentration and its gradients are high due to ATP concentration ranges in extracellular in nM vs mM in intracellular compartments (Burnstock, 2007; Yegutkin, 2014). However, the accumulation of spontaneous extracellular ATP was observed in some studies only when endogenous ecto-ATPase activity was inhibited (Daneva et al., 2021; Okada et al., 2006).

In conclusion, AR-C118925XX induced relaxation in endothelium-denuded mesenteric arteries by inhibiting $P2Y_2$ receptors. Consequently, the release of endogenous nucleotides and the response to P2 receptor antagonists are independent of the endothelium, and smooth muscle cells are the source of nucleotides. A specific effect of $P2Y_2$ receptor antagonists is consistent with the absence of the relaxation response to AR-C118925XX following UTP desensitization of $P2Y_2$/ $P2Y_4$ receptors. Based on the evidence, there appears to be a possible involvement of endogenously released ATP/UTP via connexins and pannexins acting via vasocontractile P2Y receptors.

Chapter 5

Investigating the region-specific anti-contractile effect of PVAT in porcine arteries, and the role of ATP and UTP in adipokines release from 3T3-L1 adipocytes

5.1 Introduction

Most of the peripheral vasculature is surrounded by PVAT which is mainly composed of white and brown adipocytes. Additionally, PVAT contains a stromal vascular fraction, containing lymphocytes, macrophages, fibroblasts, mesenchymal stem cells and vasa vasorum endothelial cells (Szasz & Webb, 2012). There is substantial evidence that PVAT releases adipokines and bioactive substances that affect the contractility of vascular smooth muscle cells. These mediators can induce vasodilation (anti-contractile effects) and include leptin, adiponectin, interleukin 6, interleukin-1, TNF-alpha, angiotensin 1-7 and vascular endothelial growth factor (VEGF) (Hillock-Watling & Gotlieb, 2022; Qi et al., 2018). In addition, it has been shown that adipokines influence a number of processes including energy, appetite, lipid and glucose metabolism, insulin and inflammation (Pereira & Alvarez-Leite, 2014). One of the well-characterized adipokines is leptin. Aside from inhibiting food intake and stimulating energy expenditure, leptin also induces both endothelium-dependent and endothelium-independent vasorelaxation (Nakagawa et al., 2002). Adipocytes also release adiponectin which exerts its effect through binding to its receptors AdipoR1 and AdipoR2. In addition to regulating glucose levels, lipid metabolism, and insulin sensitivity, adiponectin induces vasodilation in a number of vascular beds including rat mesenteric arteries and porcine coronary arteries (Greenstein et al., 2009; Omae et al., 2013).

It is well known that purinergic receptors are important for regulating the functions of adipocytes. In white and brown adipocytes a variety of P2X and P2Y receptor subtypes have been identified (Bulloch & Daly, 2014). P2X and P2Y receptors in adipose tissues have been reported to contribute in regulation of adipogenesis, glucose transport, inflammatory responses and leptin and adiponectin production (Bulloch & Daly, 2014; Tozzi & Novak, 2017). In white adipocytes from rats, both ATP and UTP have been shown to increase the intracellular Ca^{2+}concentration by activation of $P2Y_2$ and $P2Y_{11}$ receptors via the cAMP-PKA signalling pathway. $P2Y_{11}$ receptor

activation has been shown to inhibit insulin-induced leptin production and stimulation of lipolysis (Lee et al., 2005). In addition to $P2Y_2$ and $P2Y_6$ receptors, $P2Y_1$ receptors have been reported to be expressed in rat white adipose tissue to contribute to leptin secretion after stimulation by ADP (Laplante et al., 2010). It has been reported that the $P2X_7$ receptors play a role in inflammatory activation in adipose tissue. Activation (by ATP) of $P2X_7$ receptors expressed in human adipocytes modulates the release of inflammatory cytokines including TNFα, IL-6 and PAI-1 in human visceral (VAT) and subcutaneous adipose tissue (SAT) (Madec et al., 2011). The results of a study in human adipocytes indicate that $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors are the only purinergic receptors that are functionally involved in intracellular Ca^{2+} responses induced by ATP, ADP and UTP. This study also noted that selective antagonism of $P2Y_2$ receptors by AR-C118925XX or their knockdown caused a significant decrease in intracellular calcium concentration and increase in cellular cAMP, which led the authors to conclude that autocrine activation of $P2Y_2$ receptors is essential in regulation of basal lipolysis (Ali et al., 2018). Furthermore, in brown adipose tissue, ATP and UTP have also been reported to increase intracellular Ca^{2+} concentration (Lee et al., 2005).

In terms of understanding how purine nucleotides modulate PVAT functions, as well as how PVAT regulates the adjacent vasculature, there is still much to be understood. The aim of this chapter was to study whether the nucleotides ATP and UTP act on P2Y receptors on PVAT to release adiponectin and leptin. The outcome guided me to investigate if adiponectin released from PVAT contributes to the anti-contractile action of PVAT – for this I used porcine splenic arteries, because the preliminary studies in Chapter 3, which were confirmed in this chapter, showed no anti-contractile effect of PVAT on porcine mesenteric arteries.

5.2 RESULTS

5.2.1 Effect of PVAT in the contractile response of mesenteric arteries

To determine the effect of PVAT in the contractile response of mesenteric arteries, the vessels were contracted with U46619 in cumulative concentrations (1 nM to 300 nM). The response of mesenteric arteries to U46619 was not affected by the presence of PVAT. Response at maximum concentration of U46619 in the PVAT-free mesenteric arteries was 86.35 ± 13.8% (n= 6) compared to 99.87 ± 11.4% (n=6) in the mesenteric arteries with PVAT (Figure 5.1).

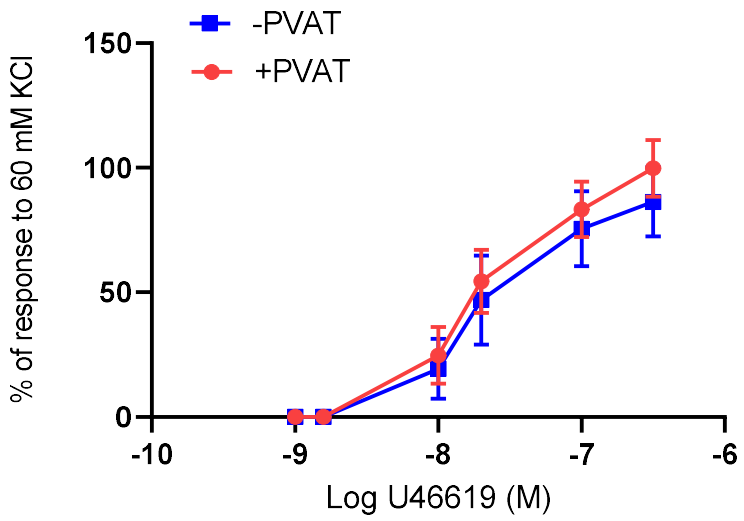


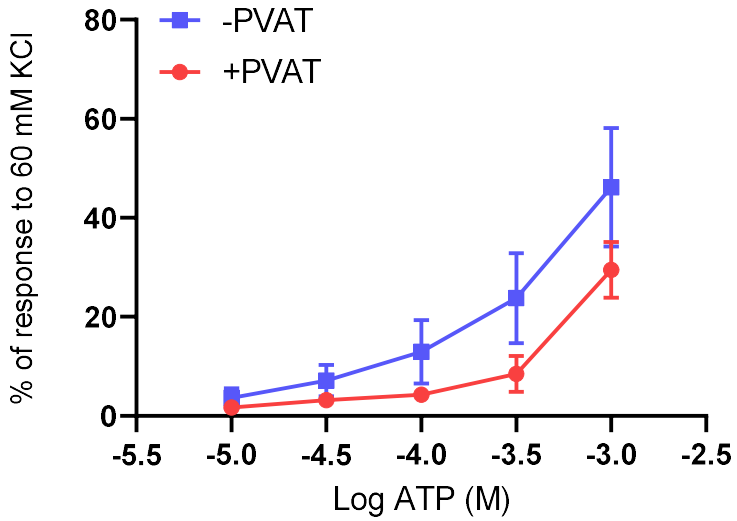
Figure 5.1: Cumulative concentration-response curves of U46619 (1 nM to 300 nM) of porcine isolated mesenteric arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

5.2.2 Contractile responses of ATP and UTP in mesenteric arteries with and without PVAT

To study the effect of ATP and UTP in mesenteric arteries with and without PVAT, the vessels were contracted with ATP and UTP in cumulative concentrations (10 µM to 1 mM). Both ATP and UTP produced concentration-dependent contractions. There were no significant

differences (P>0.10, 2-way ANOVA) between the responses to ATP and UTP in the mesenteric arteries with PVAT compared to the PVAT-free mesenteric arteries. Response at maximum concentration of ATP in the PVAT-free mesenteric arteries was 46.19 ± 11.9% (n= 6) compared to 29.4.87 ± 5.6% (n=6) in the mesenteric arteries with PVAT (Figure 5.2, A). Response at maximum concentration of UTP in the PVAT-free mesenteric arteries was 34.63 ± 7.5% (n= 6) compared to 23.83 ± 4.7% (n=6) in the mesenteric arteries with PVAT (Figure 5.2, B).

A



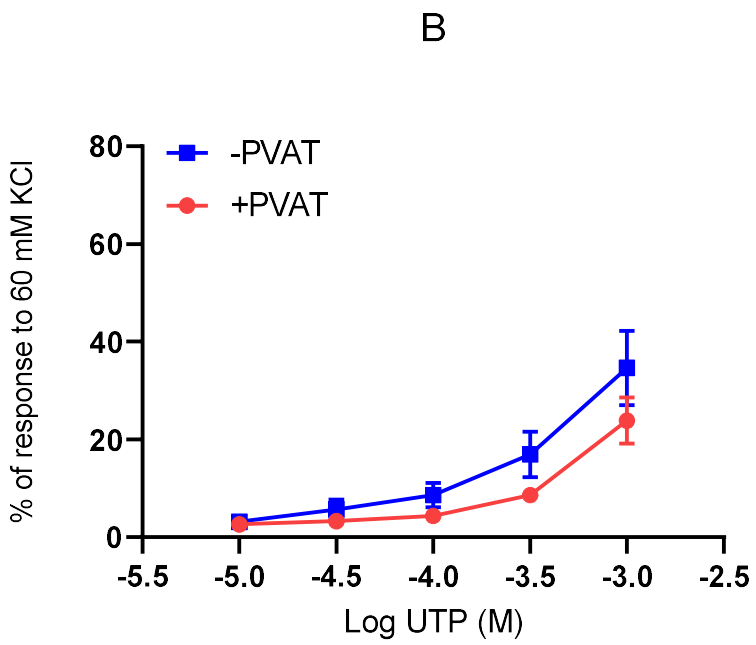


Figure 5.2: Cumulative concentration-response curves of ATP (A) and UTP (B) (10 μM to 1 mM) in U46619-pre-constricted porcine isolated mesenteric arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

5.2.3 Effect of PVAT in the contractile response of splenic arteries

Due to the fact that the vascular properties of PVAT differ according to anatomical location, the effect of PVAT in the contractile response of splenic arteries was studied. The vessels of splenic arteries with and without PVAT were contracted with U46619 in cumulative concentrations (1 nM to 300 nM). The response of splenic arteries to U46619 in the presence of PVAT was significantly diminished compared to vessels without PVAT. The response at the maximum concentration of U46619 in the PVAT-free splenic was 112.7 ± 13% (n= 7) compared to 68.02 ± 10% (n=7) in the splenic arteries with PVAT (Figure 5.3).

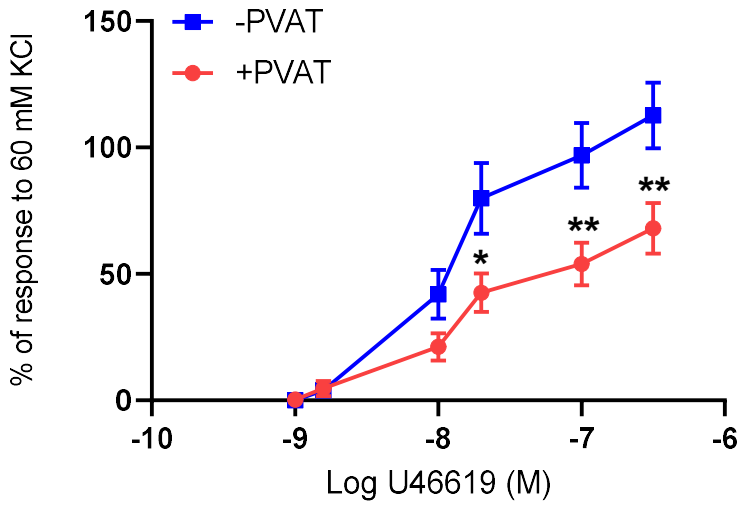


*Figure 5.3: Cumulative concentration-response curves of U46619 (1 nM to 300 nM) of porcine isolated splenic arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 7). Compared to the vessels with PVAT * P<0.05 and ** P< 0.01, 2-way ANOVA followed by Sidak's post hoc test.*

5.2.4 Contractile responses of ATP and UTP in splenic arteries with and without PVAT

To study the effect of ATP and UTP in splenic arteries with and without PVAT, the vessels were contracted with ATP and UTP in cumulative concentrations (10 µM to 1 mM). In the presence of PVAT, the contractile responses to ATP and UTP were significantly diminished. The response at the maximum concentration of ATP in the absence of PVAT was 29.75 ± 7.2% (n= 6) compared to 14.50 ± 2.8% (n=6) in the presence of PVAT (Figure 5.4, A). The response at the maximum concentration of UTP in the absence of PVAT was 38.14 ± 2.7% (n= 6) compared to 28.53 ± 2.7% (n=6) in the presence of PVAT (Figure 5.4, B). The effect of PVAT on the contractile response of splenic arteries induced by the stable analogue of ATP α,β-methylene ATP was also studied. In the absence of PVAT, the maximum contraction induced by α,β-me ATP 10 µM was 141.4 ± 10.8% (n= 7) which was significantly reduced in the vessels with PVAT, at 95.13 ± 3.08% (n=7) (Figure 5.5).

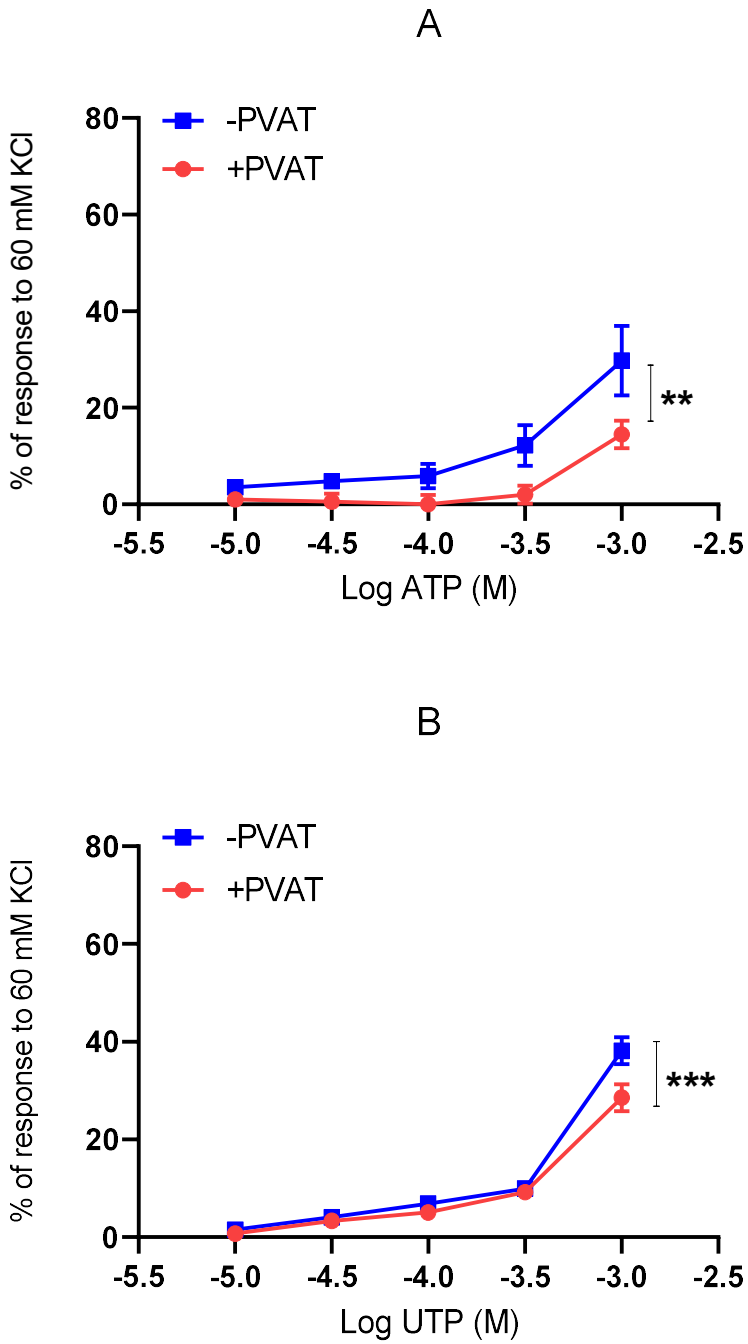


*Figure 5.4: Cumulative concentration-response curves of ATP (A) and UTP (B) (10 μM to 1 mM) in U46619-pre-constricted porcine isolated splenic arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6). Compared to the vessels with PVAT ** P< 0.01 and *** = P <0.001, 2-way ANOVA followed by Sidak's post hoc test.*

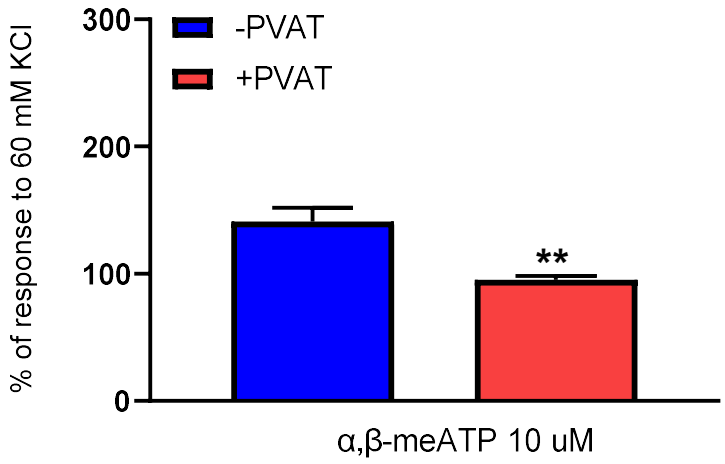


*Figure 5.5: The contractile response of α,β-methylene ATP 10 μM in porcine splenic arteries with and without PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 7). Compared to the vessels with PVAT ** P< 0.01, Student's 2-tailed unpaired t-test.*

5.2.5 Effects of nucleotides on the release of leptin from cultured 3T3-L1 adipocytes

Since PVAT reduced contraction induced by ATP and UTP, the possibility that these nucleotides stimulate the release of vasorelaxant molecules from PVAT was examined. It has been shown that leptin is one of the relaxant factors released from PVAT (Gálvez-Prieto et al., 2012). The levels of leptin released from cultured 3T3-L1 adipocytes into the media was studied using ELISA in the presence of exogenous ATP (300 μM), UTP (300 μM), ADP (300 μM), UDP (300 μM) and apyrase (10 units/ml). However, there was no significant difference in leptin release in 3T3-L1 adipocytes in the presence of ATP, UTP, ADP, UDP and apyrase compared to the control (n=5) (Figure 5.6).

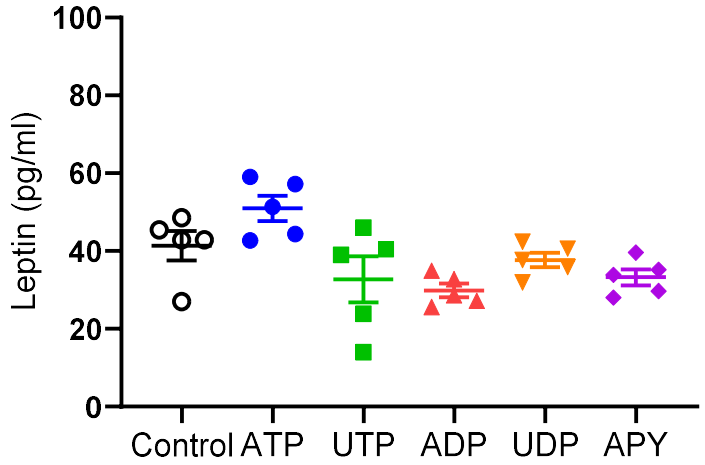


Figure 5.6: The levels of leptin released from cultured 3T3-L1 adipocytes into the media after 24 h of incubation in the presence of ATP (300 μM), UTP (300 μM), ADP (300 μM), UDP (300 μM), apyrase (APY, 10 units/ml) and the vehicle control. Data are expressed as pg of leptin released per ml of media and are means ± SEM (n =5).

5.2.6 Effects of P2 receptor antagonists on the release of leptin from cultured 3T3-L1 adipocytes

To investigate the roles of P2 receptors in the release of leptin from cultured 3T3-L1 adipocytes, cells were treated with suramin (100 μM), AR-C118925 (10 μM), MSG228 (10 μM), NF449 (30 μM), MRS2578 (10 μM) and insulin (170 nM). Suramin decreased leptin release 16.26 ± 3.0 pg/ml compared to 37.63 ± 0.9 pg/ml in the control (n=5). However, the other P2 receptor antagonists did not significantly affect the release of leptin (n=5). Insulin increased leptin release, and leptin concentration in the media was 60.44 ± 8.2 pg/ml compared to 37.63 ± 0.9 pg/ml in the control (n=5) (Figure 5.7).

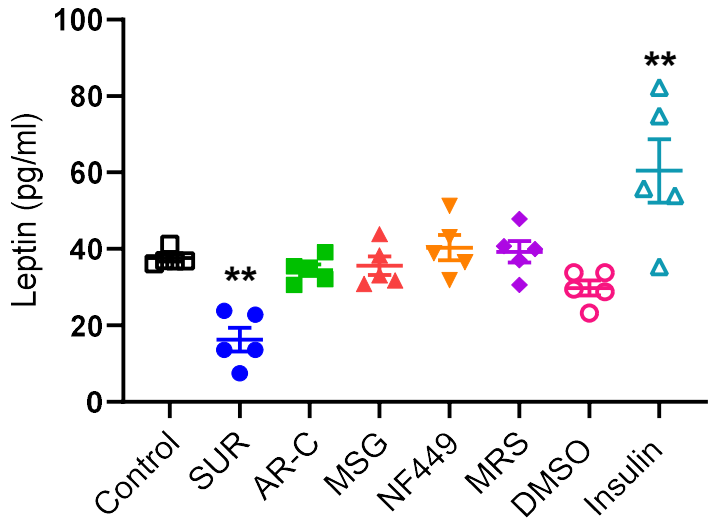


*Figure 5.7: The levels of leptin released from cultured 3T3-L1 adipocytes into the media after 24 h of incubation in the presence of suramin (100 μM), AR-C118925 (10 μM), MSG228 (10 μM), NF449 (30 μM), MRS2578 (10 μM), DMSO, insulin (170 nM) and the vehicle control. Data are expressed as pg of leptin released per ml of media and are means ± SEM (n =5). Compared to the control ** P< 0.01, one-way ANOVA followed by Tukey's multiple comparisons test.*

5.2.7 Effects of nucleotides on the release of adiponectin from cultured 3T3-L1 adipocytes

In order to determine whether nucleotides affect the release of adiponectin, the levels of adiponectin released from cultured 3T3-L1 adipocytes into the media was studied using ELISA in the presence of exogenous ATP (300 μM), UTP (300 μM), ADP (300 μM), UDP (300 μM) and apyrase (10 units/ml). ATP, UTP and UDP increased adiponectin release in adipocytes. Adiponectin concentrations in the media were 37.56 ± 0.71, 38.98 ± 1.5 and 35.5 ± 4.1 ng/ml in the presence of ATP, UTP and UDP respectively, compared to 24.98 ± 0.7 ng/ml in the control (n=5). There was no effect of apyrase on the secretion of adiponectin (n=5) (Figure 5.8).

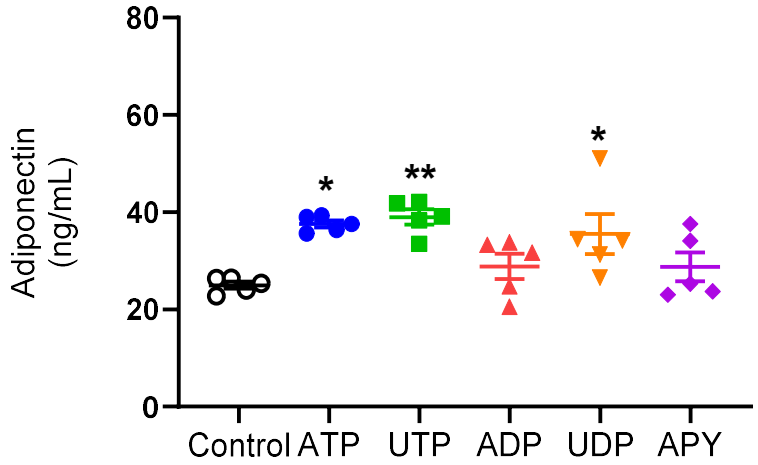


*Figure 5.8: The levels of adiponectin released from cultured 3T3-L1 adipocytes into the media after 24 h of incubation in the presence of ATP (300 μM), UTP (300 μM), ADP (300 μM), UDP (300 μM), apyrase (APY, 10 units/ml) and the vehicle control. Data are expressed as ng of adiponectin released per ml of media and are means ± SEM (n =5). Compared to the control * P<0.05 and ** P< 0.01, one-way ANOVA followed by Tukey's multiple comparisons test.*

5.2.8 Effects of P2 receptor antagonists on the release of adiponectin from cultured 3T3-L1 adipocytes

Since ATP, UTP and UDP induced the release of adiponectin from cultured 3T3-L1 adipocytes, the role of P2 receptors in the release of adiponectin was studied. 3T3-L1 adipocytes were treated with suramin (100 μM), AR-C118925 (10 μM), MSG228 (10 μM), NF449 (30 μM), MRS2578 (10 μM) and insulin (170 nM). Suramin (100 μM) increased adiponectin release 40.91 ± 2.2 ng/ml compared to 24.98 ± 0.7 ng/ml (n=5). Treatment of cells with AR-C118925XX, MSG228, MRS2578, NF449 and insulin did not significantly affect the release of adiponectin (n=5) (Figure 5.9).

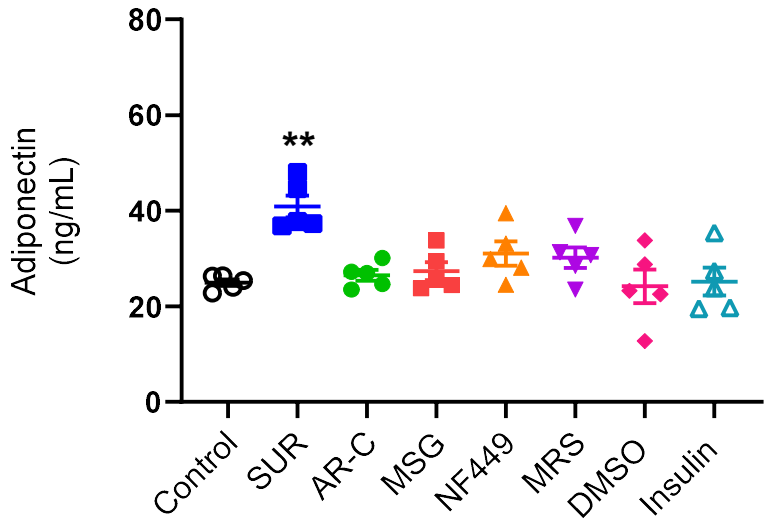


*Figure 5.9: The levels of adiponectin released from cultured 3T3-L1 adipocytes into the media after 24 h of incubation in the presence of suramin (100 μM), AR-C118925 (10 μM), MSG228 (10 μM), NF449 (30 μM), MRS2578 (10 μM), DMSO, insulin (170 nM) and the vehicle control. Data are expressed as ng of adiponectin released per ml of media and are means ± SEM (n =5). Compared to the control ** P< 0.01, one-way ANOVA followed by Tukey's multiple comparisons test.*

5.2.9 Effect of the selective $P2Y_2$ receptor antagonists AR-C118925XX and MSG228 on ATP and UTP-induced adiponectin release

In this result, the $P2Y_2$ receptor antagonists AR-C118925 and MSG228 did not affect the basal release of adiponectin (section 5.2.8). As ATP and UTP stimulated adiponectin release from cultured 3T3-L1 adipocytes, the effect of the selective $P2Y_2$ receptor antagonists AR-C118925XX and MSG228 (10 μM) on ATP and UTP-induced adiponectin release was studied. Both AR-C118925XX and MSG228 significantly inhibited ATP-induced adiponectin release from 3T3-L1 adipocytes. The adiponectin concentration in the media was found to be 24.75 ± 3.05 ng/ml (n=5) and 20.98 ± 1.7 ng/ml (n=5) with co-incubation of ATP and AR-C118925XX, and ATP and MSG228, respectively, compared to 37.42 ± 2.6 ng/ml (n=5) when ATP was incubated alone (Figure 5.10, A). Furthermore, UTP-induced adiponectin

release from 3T3-L1 adipocytes was significantly inhibited by incubation AR-C118925XX and MSG228. The adiponectin concentration in the media was found to be 17.26 ± 3.4 ng/ml (n=5) and 16.24 ± 1.9 ng/ml (n=5) when cells were treated with combination of UTP and AR-C118925XX and UTP and MSG228 respectively, compared to 40.95 ± 3.2 ng/ml (n=5) when cells were treated by UTP alone (Figure 5.10, B).

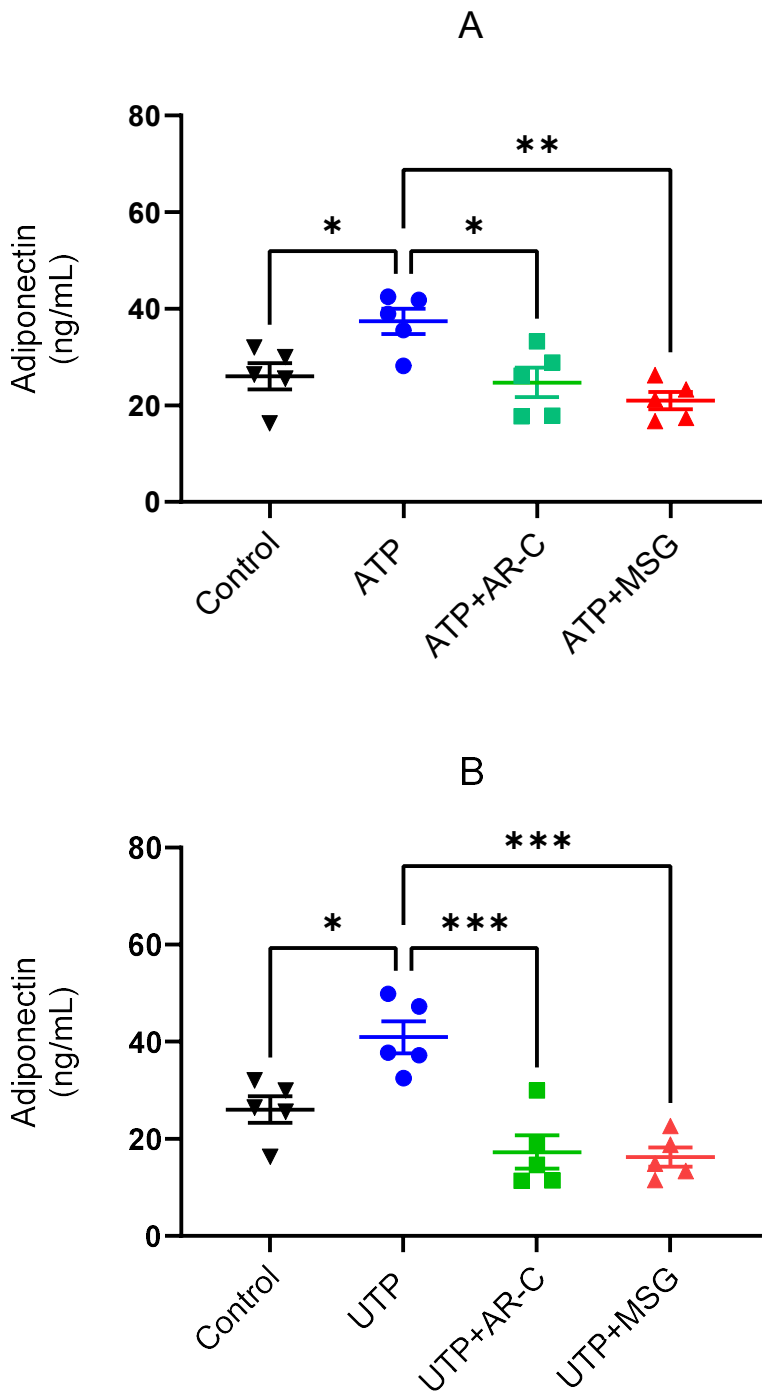
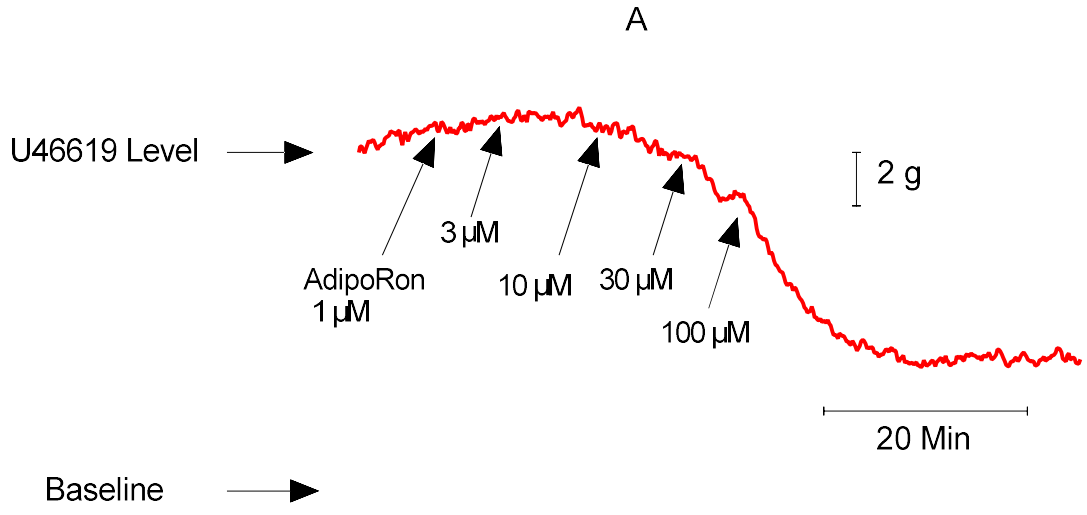


*Figure 5.10: Effect of AR-C118925XX and MSG228 on ATP and UTP-induced adiponectin release from cultured 3T3-L1 adipocytes after 24 h of incubation. (A) ATP (300 µM) was incubated alone or in a combination with AR-C118925XX (10 µM) and MSG228 (10 µM). (B) UTP (300 µM) was incubated alone or in a combination with AR-C118925XX (10 µM) and MSG228. Data are expressed as ng of adiponectin released per ml of media and are means ± SEM (n =5). Compared to the ATP or UTP alone * P<0.05, ** P< 0.01 and *** P <0.001, one-way ANOVA followed by Tukey's multiple comparisons test.*

5.2.10 Effect of AdipoRon on vascular tone in porcine isolated splenic arteries with PVAT

The results using cultured 3T3-L1 adipocytes showed that ATP and UTP induced adiponectin release via activation of $P2Y_2$ receptors. I hypothesised that adiponectin released from PVAT might produce vascular relaxation in splenic arteries to account for the anti-contractile effect of PVAT. Therefore, the effect of AdipoRon, an adiponectin receptor agonist, on vascular tone of splenic arteries with PVAT was determined. AdipoRon (1 to 100 µM) caused a relaxation of U46619-pre-constricted vascular tone in porcine splenic arteries. The relaxation response at maximum concentration of AdipoRon was found to be 48 ± 8.7% (n = 6) (Figure 5.11).



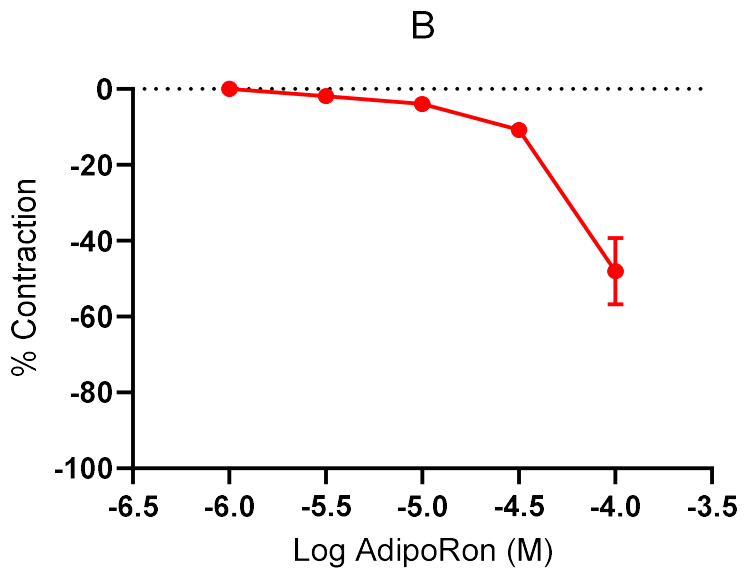


Figure 5.11: (A) Representative trace showing the effects of AdipoRon. (B) Effect of AdipoRon (1 to 100 μM) on U46619-pre-constricted vascular tone of porcine splenic arteries with PVAT. The data are expressed as a percentage of the U46619-induced tone and are mean ± SEM (n = 6).

5.2.11 Effect of anti-Adiponectin antibody on contractile responses induced by ATP and UTP in splenic arteries with PVAT

In splenic arteries, the contractile responses to ATP and UTP were significantly diminished in the presence of PVAT. This effect might be due to ATP and UTP acting on $P2Y_2$ receptors on PVAT to release adiponectin, which causes vasodilatation. In order to further examine this hypothesis, anti-adiponectin antibody was incubated with splenic artery with PVAT and its effect on the PVAT-induced depression of responses to ATP and UTP was studied. In this study, pre-incubation of anti-adiponectin antibody had no significant effect on the contractile response to ATP and UTP in splenic arteries (Figure 5.12).

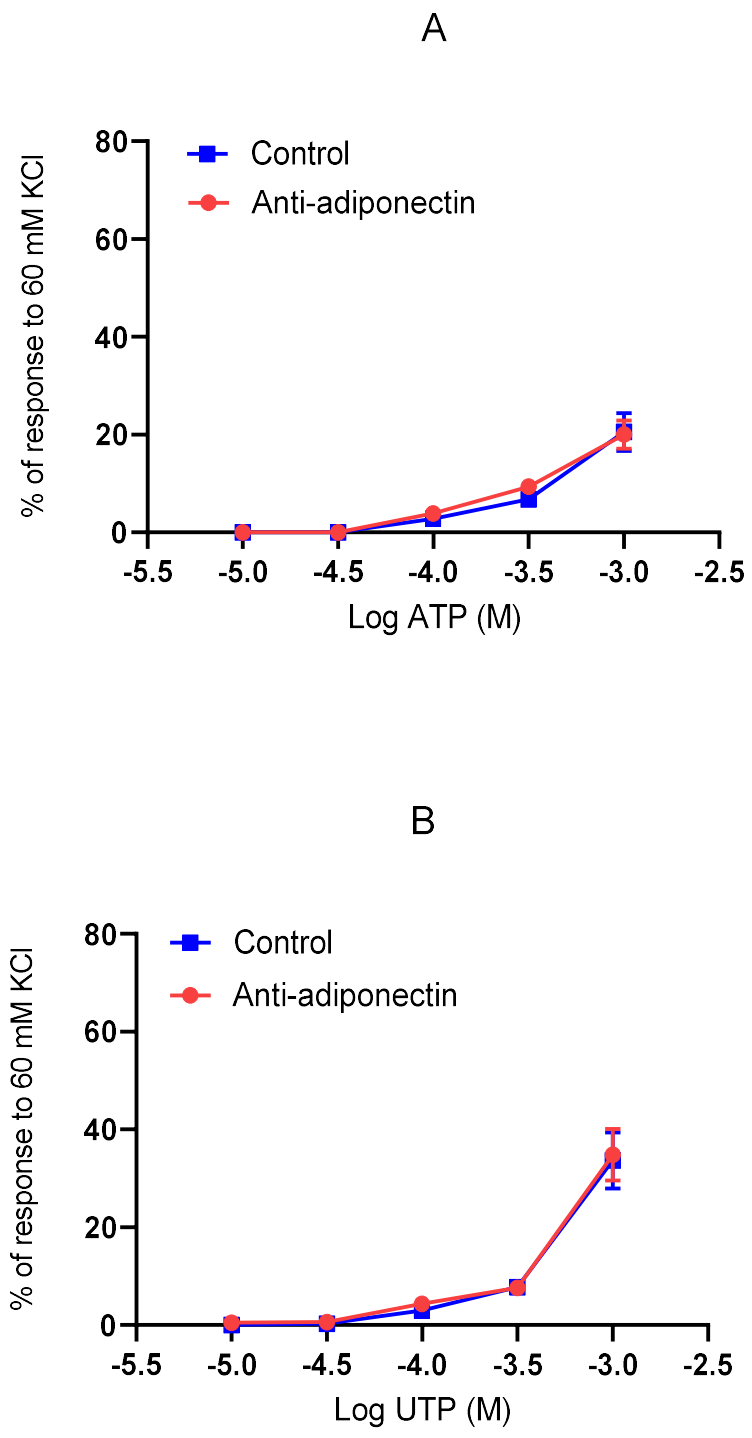


Figure 5.12: Effect of anti-adiponectin antibody on PVAT-induced relaxation in response to ATP (A) and UTP (B) (10 μM to 1 mM) in U46619-pre-constricted porcine isolated splenic arteries with PVAT. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 6).

5.3 Discussion

The key aim of this chapter was to study whether ATP and UTP stimulate adiponectin and leptin release from adipocytes by acting on P2Y receptors. This study also examined whether PVAT-induced relaxation of splenic arteries results from adiponectin release caused by ATP and UTP. The main finding from this chapter was that the presence of PVAT in splenic, but not mesenteric, arteries decreased the level of contraction induced by ATP, UTP and U46619. In addition, ATP and UTP increased adiponectin release in 3T3-L1 adipocytes through activation of $P2Y_2$ receptors.

PVAT was first studied by Soltis and Cassis who showed that PVAT had an anti-contractile effect to norepinephrine in isolated rat aorta (Soltis & Cassis, 1991). A transferable adipose-derived relaxing factor released by PVAT reduces the vasocontractile effects of agonists, including phenylephrine, serotonin, angiotensin II and endothelin-1 (Lohn et al., 2002). PVAT can release adipokines, NO and H_2S which influence vascular tone. In order to understand the mechanism of anti-contractile effects caused by perivascular-derived relaxing factors (PVRF), a variety of studies have been conducted. These studies have shown that the mechanisms for the relaxant effects caused by PVRF include endothelium-dependent mechanisms involving K^+ channel activation in vascular smooth muscle. The vasorelaxation effect could also occur by activating large-conductance Ca^{2+} activated K^+ (BK_{Ca}) channels in myocytes, as well as by activating soluble guanylyl cyclase (sGC) and K_{Ca} channels (Gao et al., 2005; Gao et al., 2007; Lohn et al., 2002). PVAT has been reported to cause vasocontraction in addition to anti-contractile effects by releasing PVAT-derived contractor factors including reactive oxygen species and angiotensin II (Ramirez et al., 2017). It has been reported that PVAT enhanced the arterial contraction of rat superior mesenteric artery to perivascular nerve stimulation which may involve angiotensin II (Gao et al., 2006; Lu et al., 2010).

In the current study, PVAT had no effect on the contractility of mesenteric arteries, and contractions to U46619, ATP and UTP were similar in PVAT-intact and denuded vessels. In contrast, in splenic arteries, there were anti-contractile effects in PVAT-intact vessels, and the contractile responses of splenic arteries to U46619, ATP, UTP and α,β-methylene ATP in PVAT-intact vessels were significantly diminished compared to vessels without PVAT. The current finding has clearly shown that the vascular properties of PVAT differ according to anatomical location and species. It is well-known that PVAT exhibits regional phenotypic and functional differences depending on the type of vascular bed or location within a vascular bed (Gil-Ortega, Somoza, Huang, Gollasch, & Fernández-Alfonso, 2015). In the thoracic aorta, PVAT primarily consists of brown adipose tissue, while in the abdominal aorta and mesenteric artery it consists mostly of white adipose tissue (Barp et al., 2021). It has been reported that there are different anti-contractile properties and NO production in rat abdominal and thoracic aortic PVAT. In the abdominal aorta, PVAT did not exhibit the same anti-contractile effects as phenylephrine did in the thoracic aorta. In addition, the abdominal PVAT expressed a lower level of endothelial NO synthase (eNOS) than the thoracic PVAT (Victorio et al., 2016). The contractile response to U46619 in mesenteric arteries of rats with intact PVAT has been reported to be diminished (Zaborska et al., 2016). This was performed in rats' mesentery, while this work was conducted in pigs' mesenteric vessels. This may explain why the results are contrasting. PVAT's anti-contractile property on the same vascular bed may be affected by some factors including gender and age. It has been reported that the anti-contractile property of PVAT was lost in male porcine coronary arteries, however, in female porcine coronary arteries PVAT inhibits contraction (Ahmad et al., 2017). In addition, it has been shown that age-related changes in NO bioavailability reduce PVAT's anti-contractile effects to U46619 and phenylephrine in rat mesenteric arteries (Lewis et al., 2015).

Purinergic receptors are well-known to regulate adipocyte functions. P2X and P2Y receptors in adipose tissues have been reported to contribute in regulation of adipogenesis, glucose transport, inflammatory responses and adipokine production (Bulloch & Daly, 2014; Tozzi & Novak, 2017). In white adipocytes from rats, both ATP and UTP have been shown to increase the intracellular Ca^{2+}concentration by activation of $P2Y_2$ and $P2Y_{11}$ receptors via cAMP-PKA signalling pathway. $P2Y_{11}$ receptor activation has been shown to inhibit insulin-induced leptin production and stimulation of lipolysis (Lee, Jun, et al., 2005). In another study in rat adipocytes ATP did not induce lipolysis and glucose transport but stimulated lipogenesis (Schödel et al., 2004). It has been reported that targeting $P2Y_1$ receptors through inhibiting or deleting $P2Y_1$ receptors results in a decrease in leptin production in mouse adipose tissues (Laplante et al., 2010). It has been reported that noradrenaline induced adiponectin exocytosis by triggering cAMP. In this study, ATP also stimulated adiponectin secretion via elevation of Ca^{2+} and activation of $P2Y_2$ receptors G_{q11}/PLC pathway (Musovic et al., 2021). In addition, adiponectin expression and secretion were inhibited by stimulation of $P2Y_4$ receptors by UTP or MRS4062 in cardiac adipocytes, and an increase in adiponectin secretion is observed in $P2Y_4$ knockout mice (Lemaire et al., 2017).

In the current study, extracellular nucleotides ATP, UTP and UDP induced adiponectin release from 3T3-L1 adipocytes. Treatment of cells with different P2 receptors antagonists had no effect in the basal release of adiponectin. It was not observed that adding apyrase to 3T3-L1 adipocytes lowered the level of adiponectin, indicating that the P2 receptors are not constitutively activated in regulating adiponectin release. Inhibition of $P2Y_2$ receptors by AR-C118925XX and MSG228 inhibited ATP and UTP-induced adiponectin release from 3T3-L1 adipocytes. Therefore, it was found that exogenous nucleotides, ATP and UTP activated the $P2Y_2$ receptor on adipocytes causing adiponectin to be released. A previous study in human adipocytes reported that the rate of basal lipolysis and intracellular cAMP

levels were increased by blocking $P2Y_2$ receptors with AR-C118925XX. Furthermore, as a result of $P2Y_2$ receptor antagonism, there was an acute increase in basal lipolysis that changed the pattern of adipokines release including an increase in adiponectin release (Ali et al., 2018). Another study has confirmed a significant role of $P2Y_2$ receptors in increasing resistance to obesity induced by high-fat diets in mice. It was observed that the levels of serum adiponectin in WT mice fed high-fat diets were significantly higher, whereas the levels of adiponectin in $P2Y_2$ receptor KO mice fed high-fat diets were not significantly raised. Pharmacological blockade of $P2Y_2$ receptors by AR-C118925XX and $P2Y_2$ KO mice in this study established a role of $P2Y_2$ receptors in regulating the production of adipokines and promoting adipogenesis and inflammation (Zhang et al., 2020).

The results from cultured 3T3-L1 adipocytes showed that ATP and UTP induced adiponectin release. The fact that adiponectin induces vasodilation led me to hypothesize that adiponectin released from PVAT in response to nucleotides could induce vascular relaxation in splenic arteries as a mechanism involved in the anti-contractile effect of PVAT. The activation of the receptor for adiponectin by AdipoRon promoted a relaxation of U46619-pre-constricted vascular tone in porcine splenic arteries. Due to the lack of selective antagonists of adiponectin receptors, an anti-adiponectin antibody was used. However, anti-adiponectin antibody had no significant effect on the contractile response to ATP and UTP in PVAT-intact splenic arteries; an increase in ATP and UTP contractions would have been expected if the antibody had blocked the actions of anti-contractile PVAT-derived adiponectin. The first demonstration of adiponectin's vasodilator properties was in rat and mouse aortic and mesenteric arteries, in which adiponectin inhibited serotonin-induced contractions. The relaxation response was endothelium-independent and a voltage-gated potassium (Kv) channel in the VSMC appears to be responsible for this effect (Fésüs et al., 2007). Adiponectin is also known to directly activate large conductance calcium activated potassium (BK) channel in rat and mouse mesenteric arteries

(Lynch et al., 2013; Weston et al., 2013). It has been shown that vessels with PVAT showed an anti-contractile effect in isolated mesenteric arteries of healthy rats, which was abolished after exposure to adiponectin blocking peptides. Pre-contracted vessels were rapidly dilated after exogenous adiponectin was added to the organ bath solution. Additionally, this study found that healthy PVAT around human small gluteal arteries had an anti-contractile effect accompanied by an increase in NO bioavailability, which was lost after treatment with an adiponectin blocking peptide. While, PVAT of arteries from obese humans lacked the anti-contractile properties of healthy vessels. The findings indicate that healthy PVAT produces anti-contractile effects through adiponectin release, which involves endothelial NO production (Greenstein et al., 2009). Thus, PVAT-induced relaxation of splenic arteries in response to nucleotides may be mediated by adiponectin and other relaxant factors. Clearly, much still remains to be understood about the level of adiponectin release and adiponectin receptor expression in splenic PVAT.

There is also evidence that leptin induces vasorelaxation in both an endothelium dependent and endothelium independent manner (Benkhoff et al., 2012; Momin et al., 2006). An in vivo study in rats showed that leptin intravenous infusion reduces arterial pressure by increasing NO levels (Frühbeck, 1999). In addition, vasodilation caused by leptin in isolated rat aorta is mediated by NO release via phosphorylation of endothelial NO synthase (eNOS) at Ser1177 by activating Akt (Sahin et al., 2009; Vecchione et al., 2002). It is believed that leptin causes vasodilation in human saphenous vein and internal mammary artery in a mechanism-independent of endothelium and NO (Momin et al., 2006). In addition, leptin-induced vasodilation in human coronary arteries may not be mediated by NO as well (Matsuda et al., 2003).

However, ATP, UTP, ADP and UDP in the current study had no significant effect on the levels of leptin. Also, Leptin release was not affected by different P2 receptor antagonists. It has been shown in rat white adipocytes

that ATP (300 μM) decreased insulin-induced leptin release at different incubation times (12, 24 and 48 hours) (Lee et al., 2005). In the current study, insulin increased the release of leptin, and this is consistent with the fact that there is a strong link between insulin and leptin secretion. For example, it has been reported in 3T3-L1 adipocytes that leptin secretion and mRNA levels are increased by insulin through cAMP stimulation (Tsubai et al., 2016). Suramin was the only P2 receptor antagonist that altered the basal release of leptin and adiponectin. Suramin affects leptin and adiponectin in opposite directions. This could be due to a different effect of suramin than on P2 receptors. Suramin is a non-specific P2 receptor antagonist, however, suramin is known to target a number of membrane channels and signalling pathways including Na, K-ATPase, PKC and protein tyrosine phosphatases (Wiedemar et al., 2020). Therefore, there I found no evidence for constitutive activation of P2 receptors in regulation of adiponectin and leptin release.

In conclusion, PVAT can release a variety of adipokines and mediators, with the ability to induce both vascular contractile and vasorelaxant effects. As only two adipokines were examined in the current study, more studies are needed to examine nucleotides' effects on other PVAT-derived relaxant or contractile factors such as NO, PGI_2, thromboxane and angiotensin II. In this chapter, the data showed that extracellular nucleotides ATP and UTP induce adiponectin release from 3T3-L1 adipocytes through activating $P2Y_2$ receptors. There was no evidence for constitutive activation of P2 receptors in regulation of adiponectin and leptin release. PVAT-induced anti-contractile actions in splenic arteries in response to nucleotides might be mediated by adiponectin and other relaxant factors.

Chapter 6

General Discussion

6.1 Overview of the main findings

As part of this thesis, nucleotides ATP and UTP were investigated to determine whether they are released constitutively by PVAT and whether this can affect the vascular tone of mesenteric arteries. This study found the following; the selective $P2Y_2$ receptor antagonists, AR-C118925XX and MSG228 at 10 μM and the non-selective P2 receptor antagonist suramin at 100 μM were effective in inducing relaxation of U46619-pre-constricted vascular tone in mesenteric arteries. Further experiments demonstrated that the vasorelaxant response of pre-contracted mesenteric arteries to AR-C118925XX was abolished in the presence of apyrase (which metabolises nucleotides). Additionally, apyrase and ARL67156 (ectonucleotidases inhibitor) directly altered U46619- pre-constricted mesenteric artery tone. Moreover, carbenoxolone and probenecid (connexin/pannexin channel inhibitors) also caused a significant relaxation of U46619-pre-constricted vascular tone in porcine mesenteric arteries. In this study, evidence ruled out the involvement of PVAT, endothelium and P2X and adenosine receptors in nucleotides release and responses to P2 receptor antagonists, suggesting that nucleotide release and actions involves the vascular smooth muscle. Relaxation of AR-C118925XX was also observed in other vascular beds and species, including porcine coronary and splenic arteries, as well as rat mesenteric arteries. Extracellular ATP was measured by luminescence directly from the Krebs solution in porcine mesenteric arteries. It appears that isolated mesenteric arteries are capable of releasing ATP and the presence of PVAT in this experiment increased the amount of ATP released from isolated mesenteric arteries. However, there were no differences in the effects of P2 receptor antagonists in the presence or absence of PVAT on mesenteric artery tone.

The present study also identified a role of extracellular nucleotides ATP and UTP in adiponectin release from 3T3-L1 adipocytes via activation of $P2Y_2$ receptors. Adiponectin release was not regulated by constitutive activation of $P2Y_2$ receptors. The anti-contractile effects of PVAT on porcine splenic

arteries in response to ATP and UTP might be mediated by adiponectin and other relaxant factors.

6.2 The selective involvement of $P2Y_2$ receptors and nucleotides release

I recorded the functional responses of isolated tissues using an isometric tension recording system (organ bath). This technique is useful in cardiovascular pharmacology and physiology experiments for a number of reasons. Among these are the ability to measure isometric vascular contraction or relaxation caused by increasing antagonist concentrations, and the ability to modulate agonist potency and efficacy in isolated blood vessel rings (Jespersen et al., 2015). This technique also allowed me to study the effects of PVAT on the contractility of blood vessel segments. Porcine vessels were used as a model in the current study for several reasons, including the fact that porcine vessels share many anatomical and physiological features with human vessels. There are many similarities between porcine and human vessels in terms of their size, structure and function, making them a suitable model for studying human cardiovascular function. Additionally, porcine vessels are readily available and easy to obtain, and because they are relatively large, it is easier to measure and manipulate physiological and pharmacological parameters (Suzuki et al., 2010; Tsang et al., 2016).

The diverse pharmacological profiles of endogenous nucleotides that stimulate more than one P2 receptor subtype have been recognised as a limitation in the study of P2 purine receptors. In addition, there is a possibility of multiple contractile and relaxant receptors present in a particular vascular bed depending on where they are expressed endothelium vs. smooth muscle and PVAT. There is an equal affinity between ATP and UTP for $P2Y_2$ receptors, but the two bind to more than one subtype of P2 receptor (Jacobson et al., 2020). Studies have shown that both nucleotides can have dual effects on vascular tone, contraction and relaxation depending on vascular type and experimental conditions (Burnstock, 2008). Generally, vasorelaxation occurs when nucleotides bind

to the P2 receptor subtype expressed in the endothelium, whereas vasoconstriction occurs when nucleotides bind to the P2 receptor subtype expressed in vascular smooth muscle (Erlinge & Burnstock, 2008). According to the current study in chapter 3 and 5, ATP and UTP induce only contractions in porcine mesenteric and splenic arteries. The contraction effect induced by these nucleotides in this study is consistent with the possibility that endogenous tonic release of ATP and UTP maintains the tone of the mesenteric arteries by binding to contractile $P2Y_2$ receptors in the vascular smooth muscle. The effects of ATP and UTP alone or in combination were studied using various exogenous concentrations. The addition of ATP or UTP alone or in combination produced contraction effects. However, presumably due to the rapid hydrolysis of these nucleotides, the exogenous concentrations (mM and μM) used were higher than the endogenous extracellular range (nM).

Several P2 receptor subtypes lack selective antagonists, making it difficult to determine their functions. The selective targeting of $P2Y_2$ receptors by AR-C118925XX has been demonstrated in several studies (Muoboghare et al., 2019; Rafehi et al., 2017). AR-C118925XX greatly enhances our ability to understand how $P2Y_2$ receptors contribute to maintaining vascular tone. In the current study, AR-C118925XX caused relaxation effects in a number of vascular beds, which may suggest $P2Y_2$ receptor activation after constitutive release of nucleotides. While, the maximum response to exogenous ATP and UTP was not significantly affected by AR-C118925XX, the time to recover from the response was significantly reduced. Reasons for this are unclear but could involve the relative concentrations of endogenous vs exogenous nucleotides, diffusion (or limited diffusion) across the many cell layers of the media, and region specific expression of P2 receptors throughout the blood vessel wall. It has been shown that AR-C118925XX had no effect on the contractions evoked by UTP and ATP in rat isolated intrapulmonary and tail arteries. In addition, ATP-induced vasodilation was not affected by AR-C118925XX, but UTP-induced

vasodilation was significantly reduced by AR-C118925XX in intrapulmonary arteries of rats (Dales et al., 2022). ATP-induced small pulmonary vein contraction was inhibited by AR-C118925XX in another study (Henriquez et al., 2018). Therefore, AR-C118925XX appears to act differently depending on the experimental conditions and the expression profile of $P2Y_2$ receptors in different vascular beds and species. It is possible that the distribution of $P2Y_2$ receptors and the permeability of AR-C118925XX in the different layers of the vascular wall may explain the slow relaxation response of AR-C118925XX in this study since $P2Y_2$ receptors might not be blocked immediately by AR-C118925XX. The lack of effect of AR-C118925XX on the maximum contractions induced by ATP and UTP may be explained by this if I consider that high concentrations of ATP and UTP would be rapidly achieved by their exogenous application, followed by their rapid metabolism, and so the exogenous nucleotides could mainly be acting on non-$P2Y_2$ receptors. The results indicated that the mesenteric arteries did respond rapidly to the contractile agents ATP and UTP and the response was relatively short-lived (Figures 3.8, 3.9). P2X1 receptor clustering at the adventitial surface has been described (Hansen et al., 1999) which might contribute to the lack of effect of AR-C118925XX on responses to the exogenous nucleotides. It would be interesting to investigate whether there is a region specific expression of P2 receptors, especially $P2Y_2$ and P2X1, throughout the mesenteric artery wall.

The selective $P2Y_2$ receptor antagonist AR-C118925XX was developed with submicromolar potency at recombinant human $P2Y_2$ receptors. A 10 μM concentration of AR-C118925XX showed inactivity at 35 other non-purinergic receptors (Kemp et al., 2004; Rafehi et al., 2017). It was necessary to use a high concentration of AR-C118925XX (10 μM) to block $P2Y_2$ receptors at a more intense level since there was no effect of 1 μM on the vascular tone of mesenteric arteries in this study. In a wide range of experiments, a concentration of 10 μM or above of AR-C118925XX has been

used as an antagonist of $P2Y_2$ receptors (Ali et al., 2018; Filiberto et al., 2022; Genovese et al., 2023; Róg et al., 2019).

This study did not examine $P2Y_2$ receptor expression in porcine mesenteric arteries. However, there is a well-known expression profile for $P2Y_2$ receptors in porcine coronary and rat mesenteric arteries (Buvinic et al., 2002; Ishida et al., 2011; Rayment et al., 2007). This study demonstrated that AR-C118925XX induced relaxation effects on the pre-contracted tone of porcine mesenteric and coronary arteries as well as rat mesenteric arteries. It was further demonstrated that $P2Y_2$ receptors play an important role in this study when MSG228 ($P2Y_2$ receptor antagonist) had similar effects to AR-C118925XX. Further experiments performed using UTP to desensitize $P2Y_2$/$P2Y_4$ receptors, I observed no relaxation response to AR-C118925XX, supporting the hypothesis that this antagonist specifically affected $P2Y_2$ receptors. There are currently no commercially available antagonists of $P2Y_4$ receptor, however, the $P2Y_4$ receptor is insensitive to suramin which discriminates between $P2Y_2$ and $P2Y_4$ receptor subtypes (Brunschweiger & Muller, 2006). In this study, suramin induced a similar relaxation effect to AR-C118925XX in porcine mesenteric arteries. The selective $P2Y_6$ receptor antagonist MRS2578 had no significant effect in the current study. AR-C118925XX responses also was not affected by desensitizing P2X1 receptors with α,β-MeATP. NF449 did not alter the vascular tone of mesenteric arteries when it blocked P2X1 receptors. A contractile P2X1 receptor has been found to exist on porcine mesenteric arteries that is sensitive to α,β-meATP (Shatarat et al., 2014).

Apyrase catalyses the sequential hydrolysis of nucleotides (Riewe et al., 2008). As long as ATP/UTP is being released, apyrase will affect the tone of pre-contracted arteries, in a similar manner to $P2Y_2$ receptor antagonists. The addition of apyrase to pre-contracted mesenteric arteries caused both contractions and relaxations. Also, AR-C118925XX had no vasorelaxant effect on pre-contracted mesenteric arteries treated with apyrase. In addition, there was a small contraction of the mesenteric artery in response

to ARL67156, suggesting that vasocontractile ATP or UTP was released endogenously. It has been reported that there was a significant increase in contractile responses to exogenous UTP in porcine coronary and ear arteries in response to ARL67156 (Rayment et al., 2007). It has been shown in in vivo that tonic level of constriction is provided by ATP in retinal arterioles. Apyrase lowered endogenous ATP levels, causing retinal arterioles to dilate, while an ATPase inhibitor increased them, causing vessels to constrict, which is similar to the current finding (Kur & Newman, 2014).

Further investigation as to the mechanism by which ATP and UTP are released tonically from mesenteric arteries in order to maintain vascular tone was conducted through inhibition of connexin and pannexin channels. According to my findings, pannexin and connexin channel blockers had similar effects on the contractile tone of pre-contracted arteries as $P2Y_2$ receptor antagonists, suggesting that there is a continuous release of endogenous ATP/UTP, through pannexin and or connexon channels which contributes to the contractile tone of porcine mesenteric arteries. Pharmacological blockage of connexin and pannexin channels and subsequent inhibition of ATP release were observed in various vascular beds (Bol et al., 2017; Gödecke et al., 2012). According to a recent study, ATP released from the endothelium via Panx1 activates smooth muscle $P2Y_2$ receptors in mouse aorta. In turn, this led to the release of pro-inflammatory cytokines, an increase in intracellular Ca^{2+} and the regulation of vascular remodelling (Filiberto et al., 2022).

ATP levels were measured under basal conditions in this study, suggesting that isolated mesenteric arteries can release ATP to activate smooth muscle $P2Y_2$ receptors, modulating vascular tone. Although, it is technically challenging to measure extracellular levels of UTP in vascular smooth muscle, the concentrations of UTP have been quantified using a reaction based on UDP-glucose pyrophosphorylase and HPLC coupled to radioenzymatic assays. Under resting conditions, UTP was detected in low nanomolar concentrations in most extracellular mediums. After mechanical

stimulation, however, UTP levels were elevated (Lazarowski & Harden, 1999). In both resting and mechanically stimulated conditions, an extracellular UTP/ATP ratio of 1:3 was observed (Lazarowski et al., 2003). Both ATP and UTP may have a common source and mechanism of release (Chapter 1). Thus, there is consistency in the results obtained from the inhibition of ATP and UTP binding to $P2Y_2$ receptors (AR-C118925XX, MSG228), the induction or inhibition of ATP and UTP metabolism (apyrase, ARL67156), and the blockage of the release of these nucleotides from connexin and pannexin channels (probencid, carbenoxelone). It seems unlikely that the observed effects are due to non-specific antagonist actions. Taken together, the previous observations indicate that endogenous ATP/UTP is released from vascular smooth muscle via connexins and pannexins acting on vasocontractile $P2Y_2$ receptors.

6.3 The interactions between nucleotides with PVAT-derived mediators

Historically, PVAT was seen simply as a structural protective layer that does not have any physiological function for the vessel walls it surrounds. Over the past few decades, many studies have been designed to investigate this relationship between PVAT and the vessel wall. These studies have demonstrated its importance, particularly in regulating vascular tone and revealing significant insights into vascular disease pathogenesis. PVAT plays an active role in vascular homeostasis and dysfunctions associated with obesity, hypertension, inflammation and coronary artery disease (Brown et al., 2014; Li et al., 2021). PVAT regulates vascular function by releasing adipokines, cytokines, reactive oxygen species, NO, and H_2S via endocrine and paracrine mechanisms (Xia & Li, 2017). In the present study, PVAT was investigated for its effect on blood vessel contractility and its role in nucleotides release. Chapter 3 provided evidence that porcine mesenteric PVAT has no effect on the contractility of mesenteric arteries. Therefore, PVAT did not represent the major source of nucleotides in the current study. This is because there were no differences in the responses to P2 receptor antagonists, ATP and UTP or apyrase in the presence or absence of PVAT.

Chapter 5 results showed that 3T3-L1 adipocytes were induced to release adiponectin by exogenous ATP and UTP. The inhibition of $P2Y_2$ receptors by AR-C118925XX and MSG228 inhibited ATP and UTP-induced adiponectin release from 3T3-L1 adipocytes. Addition of apyrase to 3T3-L1 adipocytes did not lower the level of adiponectin, indicating that the P2 receptors are not constitutively activated in regulating adiponectin release. Similarly serum adiponectin levels were significantly higher in WT mice fed high-fat diets, whereas $P2Y_2$ receptor knockout mice fed either high-fat or normal diets showed no significant increase in adiponectin levels (Zhang et al., 2020). It was found also that AR-C118925XX antagonized $P2Y_2$ receptors in human adipocytes and increased adiponectin release (Ali et al., 2018). The adipokines concentrations in these experiments were measured after 24 hours. It is unknown whether nucleotides will stay stable for 24 hours. It is possible that these nucleotides would have been metabolised. However, ATP and UTP-induced adiponectin release from 3T3-L1 adipocytes was inhibited by AR-C118925XX and MSG228, indicating that exogenous adipokines are stable. Future experiments could involve measuring adiponectin levels at different time points.

It was found in chapter 5 that PVAT-intact vessels showed anti-contractile effects and that contractile responses of splenic PVAT-intact arteries to U46619, ATP, UTP, and α,β-MeATP were significantly diminished compared to vessel without PVAT. PVAT, however, did not show any anti-contractile effects in the mesenteric arteries. In light of the conclusion from chapter 5 that ATP and UTP activate $P2Y_2$ receptors to release adiponectin from 3T3-L1 adipocytes, I investigated whether PVAT anti-contractile activity involves adiponectin released by PVAT in porcine splenic arteries. In addition, adiponectin is one of the most abundant adipokines produced by PVAT with anti-contractile and anti-inflammatory effects on the vascular wall (Yanai & Yoshida, 2019). AdipoRon activated the adiponectin receptor in porcine splenic arteries, resulting in a relaxation of U46619-pre-constricted vascular tone. Anti-adiponectin antibodies were used since there was no

selective antagonist for adiponectin receptors. Anti-adiponectin antibodies did not significantly affect ATP or UTP contractile responses in PVAT-intact splenic arteries; if the antibody had blocked the actions of anti-contractile PVAT-derived adiponectin, ATP and UTP contractions would have increased. The current study assessed the effect of nucleotides on the release of two adipokines only. There might be a role for other relaxant factors as well as adiponectin in PVAT-induced anti-contractile actions in splenic arteries in response to nucleotides. PVAT anti-contractile effects are mediated, however, by a number of factors including NO, PGI_2, angiotensin 1-7, H_2O_2 and H_2S (Brown et al., 2014). It has been shown that nucleotides and P2 receptors are important in controlling adipokines release from adipocytes. As an example, ATP stimulates adiponectin release by elevating Ca^{2+} and increasing $P2Y_2$ receptors via the G_{q11}/PLC pathway in 3T3-L1 adipocytes and inguinal white adipose tissue (Musovic et al., 2021). In order to regulate the tone of blood vessels, it is necessary to understand how nucleotides maintain PVAT release of relaxant and contractile factors.

6.4 Future studies

This thesis provides a detailed understanding of how nucleotides ATP and UTP are released constitutively and regulate vascular tone via $P2Y_2$ receptors. The present study provides clear evidence that targeting $P2Y_2$ receptors directly induce a vasorelaxation across a range of vascular beds and species including porcine mesenteric and coronary arteries as well as rat mesenteric arteries. In addition, the current study indicated that ATP and UTP activated $P2Y_2$ receptors in 3T3-L1 adipocytes to release adiponectin. A subsequent study showed in the splenic arteries that PVAT had an anti-contractile effect in response to ATP and UTP, and adiponectin receptor activation resulted in relaxation. There is, however, much to explore in the future based on the research presented here. The possibility that ATP and UTP may trigger the release of relaxant adipokines and mediators from PVAT through the activation of $P2Y_2$ receptors is one of the important area of interest. It would be interesting to conduct a more

comprehensive study using antagonists for $P2Y_2$ receptors and measure the concentrations of adipokines known to induce vasorelaxation. It will then be possible to explore the signalling pathways involved in this process. A further area of interest is the regulation of vascular tone by $P2Y_2$ receptors. In order to understand how AR-C118925 inhibited endogenous nucleotide contractions while not inhibiting most exogenous ATP and UTP responses, immunofluorescence could be used to study region-specific expression of P2 receptors throughout the blood vessel wall. Additionally, it is possible to carry out genetic knockouts of $P2Y_2$ receptors in vessels. The results of this study will confirm evidence for the basal release of ATP and UTP and the binding of these nucleotides to $P2Y_2$ receptors. Other P2 receptors, such as P2X1, may have functional roles that can be further explored. In addition, the direct vasorelaxation induced by AR-C118925XX and MSG228 suggests that $P2Y_2$ receptor antagonists may have therapeutic potential in the treatment of hypertension. Further research should be conducted to investigate whether $P2Y_2$ receptor antagonists have an effect on blood pressure in animal models of hypertension.

Purinergic receptors are not commonly studied in pigs, and there is a variety of species-specific molecular and cellular differences between pigs and rodents. Cloning the P2X and P2Y receptor genes from porcine vascular cells and understanding species cross-reactivity will be useful. It is possible to clone P2 receptors from porcine vascular tissues using recombinant DNA technology and HEK293 cells.

In this thesis, the proposed findings were derived from in vitro studies which could provide valuable information concerning the potential benefits of $P2Y_2$ receptor-mediated signalling in blood vessels to regulate vascular tone and blood pressure. Therefore, the physiological and clinical relevance of the findings requires further investigation. These findings provide meaningful data to justify follow-up studies in vivo to evaluate the role of nucleotides and P2 receptors and the mechanisms underlying their effects, which could lead to a novel hypertension and obesity therapeutic target.

6.5 Conclusion

In conclusion, the present study demonstrated that endogenous ATP and UTP are constitutively released via connexin and pannexin channels act via vasocontractile $P2Y_2$ receptors. These receptors are blocked by P2 receptors antagonists to cause vasorelaxation. Results from Chapter 3 showed a direct vasorelaxant effect of suramin, AR-C118925XX and MSG228 on the vascular tone of porcine pre-contracted mesenteric arteries. In addition, these data indicated that hydrolysis of endogenous nucleotides with apyrase induced relaxation and contraction of pre-constricted mesenteric arteries, while ectonucleotidase inhibition caused contraction (Figure 6.1). Exogenous addition of ATP and UTP induced contractions in pre-contracted mesenteric arteries. There was no significant effect of AR-C118925XX on the maximum response to ATP and UTP, but the time to recover from the response was significantly reduced.

Moreover, I demonstrated in chapter 4 that smooth muscle cells are the source of nucleotides and are independent of the endothelium in releasing endogenous nucleotides and responding to P2 receptor antagonists. After UTP desensitization of $P2Y_2$/$P2Y_4$ receptors, AR-C118925XX did not induce a relaxation response, consistent with a specific effect of $P2Y_2$ receptor antagonists. AR-C118925XX also caused relaxation in well-known $P2Y_2$ expression vascular beds, such as porcine coronary arteries and rat mesenteric arteries, which similarly induce relaxation. In addition, pannexin and connexin channel blockers had similar effects on the vascular tone of pre-contracted arteries as the $P2Y_2$ receptor antagonists, suggesting that porcine mesenteric arteries constantly release endogenous ATP and UTP. Also, the evidence from this chapter eliminated the possibility that P2X, $P2Y_6$ and adenosine receptors were involved in AR-C118925XX-induced relaxation.

As shown in chapter 5, ATP and UTP induce adiponectin release from 3T3-L1 adipocytes by activating $P2Y_2$ receptors. Adiponectin and leptin release was not regulated by the constitutive activation of P2 receptors. The anti-

contractile actions of PVAT in splenic arteries in response to nucleotides might be mediated by adiponectin and other relaxant factors.

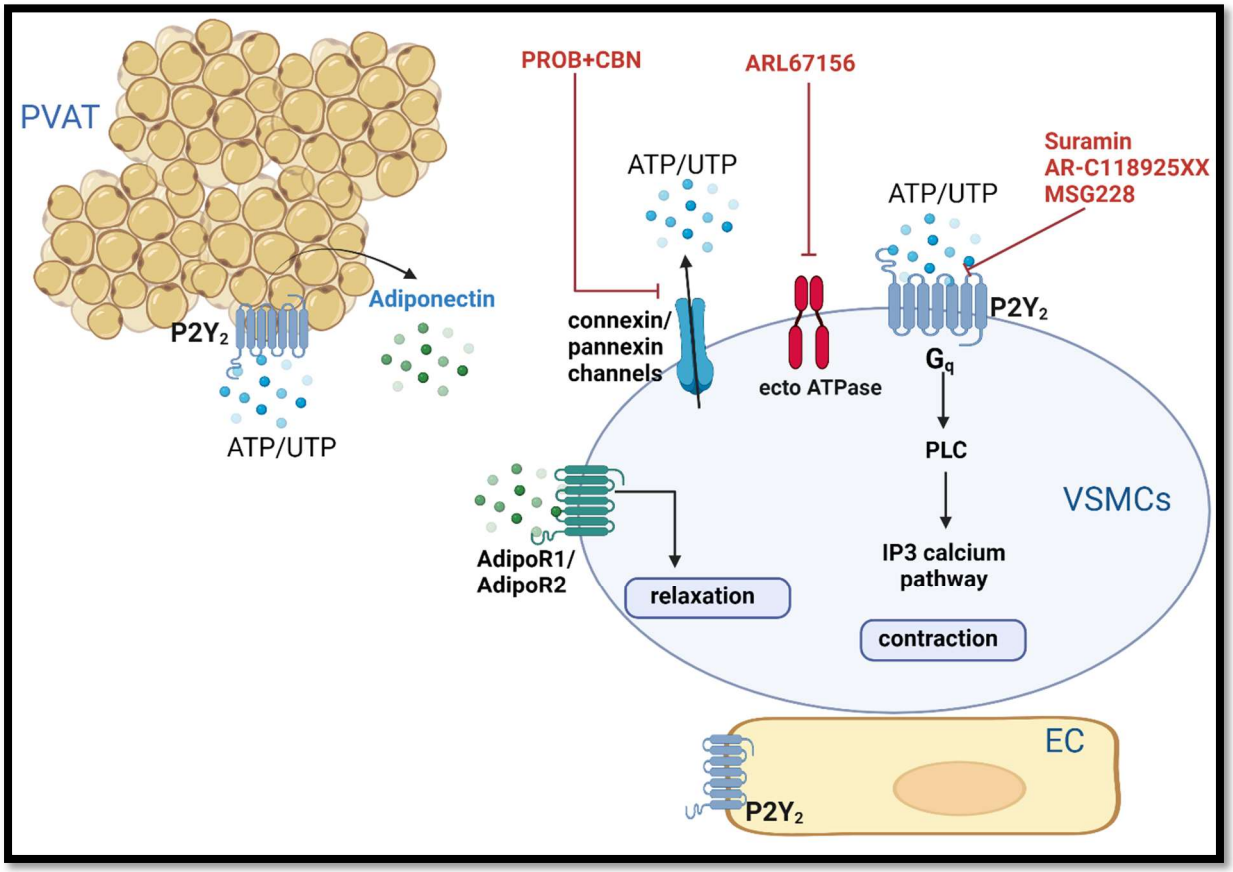


Figure 6.1: Hypothesized schematic diagram of ATP and UTP basal release from VSMCs and their subsequent binding to $P2Y_2$ receptors. In VSMCs, ATP and UTP are released through connexin and pannexin channels and bind to vasocontractile $P2Y_2$ receptors. Pharmacological antagonists of $P2Y_2$ receptors by suramin, AR-C118925XX and MSG228 induce vasorelaxation. Inhibition of connexin and pannexin channels by carbenoxolone and probenecid affects the extracellular level of ATP and UTP which also induces vascular relaxation. By targeting ectoATPase on the cell surface of VSMCs, ATP and UTP are also increased extracellularly. There was an endothelium-independent response to $P2Y_2$ receptor antagonists and the release of nucleotides. Both ATP and UTP activate $P2Y_2$ receptors in adipocytes to release adiponectin. It was shown that PVAT inhibited contractile activity in the splenic arteries in response to both ATP and UTP. I hypothesized that

binding of ATP and UTP to $P2Y_2$ receptors in PVAT induces adiponectin release which in turn binds to adiponectin receptors (AdipoR1 and AdipoR2) and induces vasorelaxation.

Appendices

A. Responses to KCl and other vasoconstrictors in human mesenteric arteries

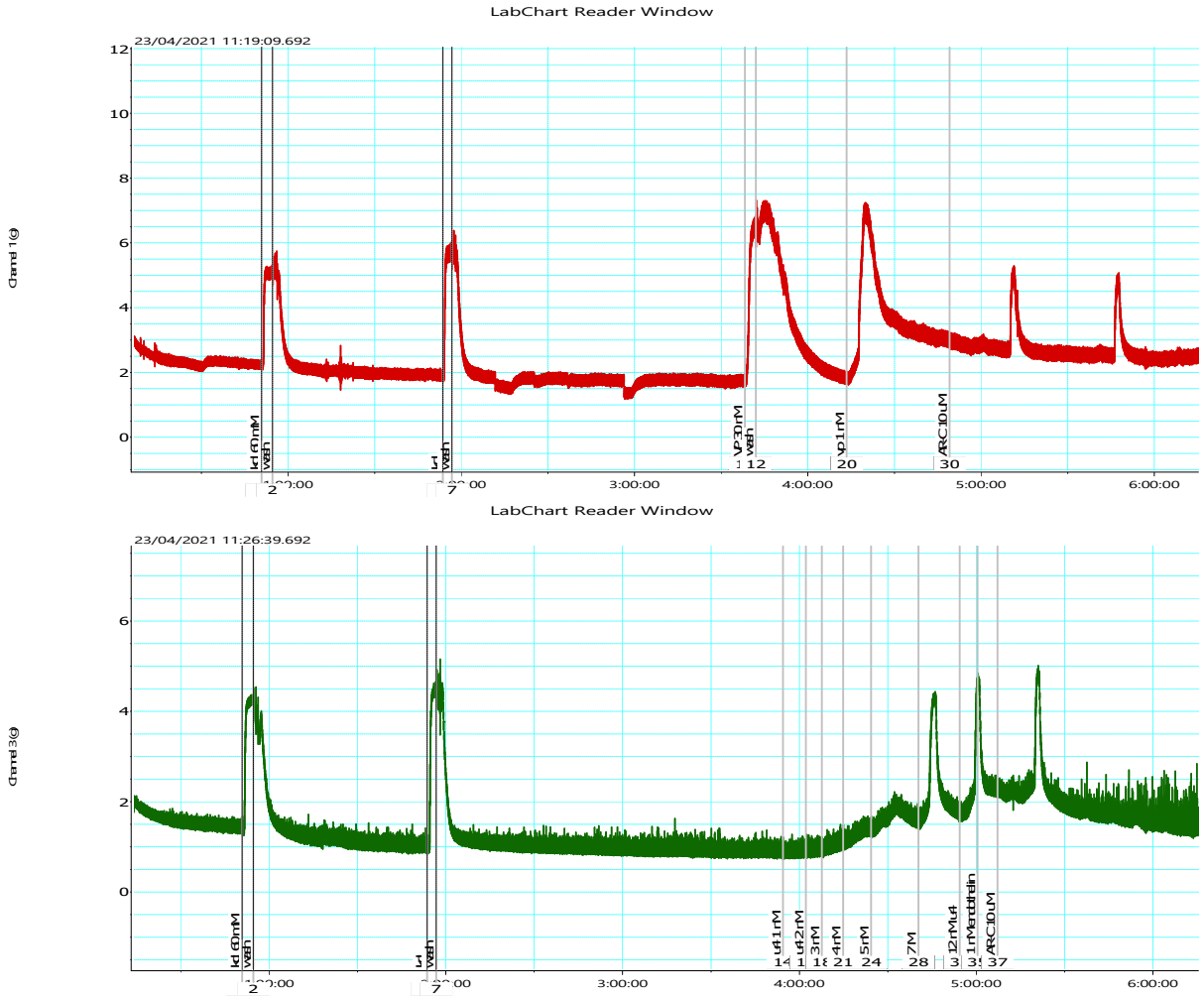


Figure 1: Representative traces showing responses to KCl and other vasoconstrictors in the human mesenteric arteries. Vasopressin alone or a combination of U46619 and endothelin were used as vasoconstrictors. Unstable vascular tone of the human mesenteric arteries was observed and all vasoconstrictors caused rhythmic contraction.

B. Effect of KCl and U46619 on vascular tone in porcine isolated mesenteric arteries

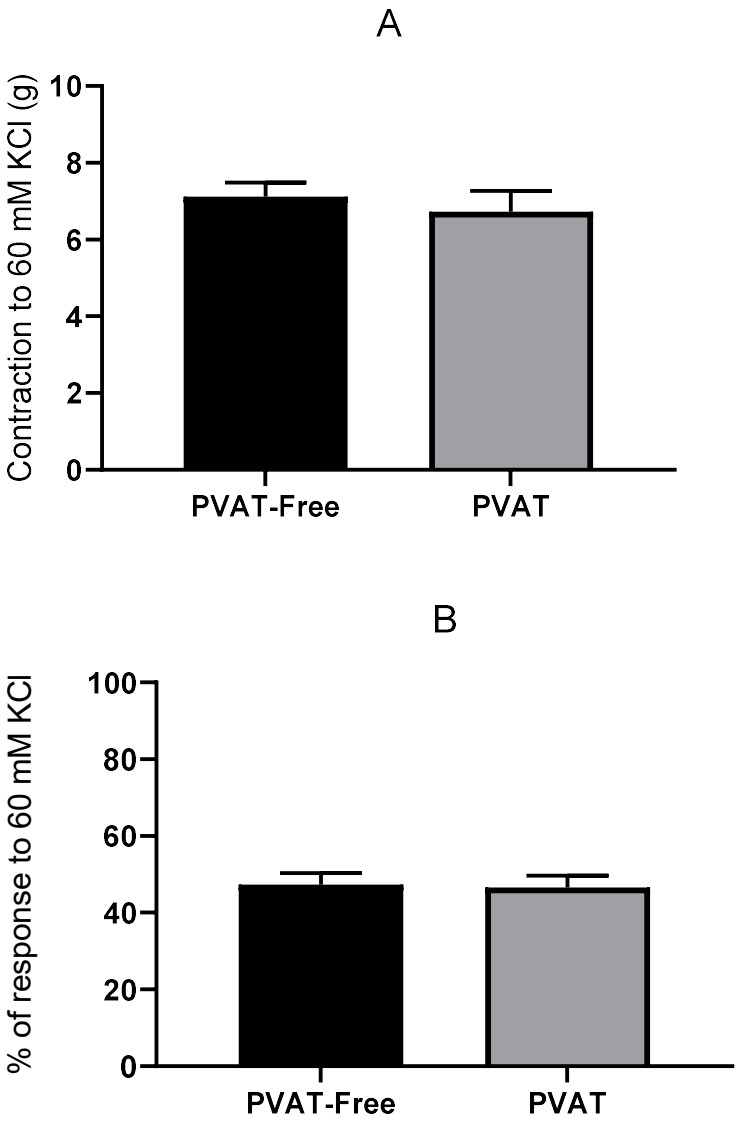


Figure 2: (A) Contractile responses induced by 60 mM KCl in PVAT and PVAT-free porcine mesenteric arteries. Data are expressed as gram and are mean ± SEM (n = 13). (B) Contractile responses induced by U46619 (20 nM to 60 nM) to a level of 40-60% of the second KCl response in PVAT and PVAT-free porcine mesenteric arteries. Data are expressed as % of response to 60 mM KCl and are mean ± SEM (n = 10).

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