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Faculty of Medicine and Health Sciences  
School of Biomedical Sciences



Nucleotide regulation of vascular  
system

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Thesis submitted to the University of  
Nottingham

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*This thesis is  
dedicated  
To the souls of my  
grandmother  
Tamam and my  
grandfather Ali*

## **Abstract**

Previously, acyl derivatives of CoA were shown to antagonise human native and recombinant P2Y<sub>1</sub> purine receptors. The main aim of this thesis was to study the effect of these endogenous nucleotide derivatives at endogenous P2Y<sub>1</sub> receptors in blood vessels. Using isometric tension recordings, CoA, acetyl-CoA and palmitoyl-CoA (PaCoA) appeared to show selectivity for P2Y<sub>1</sub> receptors (over P2Y<sub>2</sub> and adenosine receptors) in the rat isolated thoracic aorta, with PaCoA being the most potent of the CoA derivatives used.

In porcine isolated mesenteric arteries (PMA) and porcine isolated coronary arteries (PCA), isometric tension recordings indicated that ADP mediated endothelium-dependent and endothelium-independent relaxations, respectively. Relaxations in PMA were blocked by the P2Y<sub>1</sub> receptor antagonist MRS2500 and PaCoA, whilst these were ineffective against ADP relaxations in the PCA.

A FlexStation was used to monitor calcium responses in native HEK293 cells expressing P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors using ADP and UTP, respectively. Responses to UTP were not significantly altered in the presence of PaCoA. In contrast, ADP-evoked

responses were significantly inhibited in the presence of either MRS2500 or PaCoA.

These data raise the possibility of an endogenous selective antagonism of P2Y<sub>1</sub> receptors via CoA compounds, irrespective of species or cellular environment.

Nicotinamide adenine dinucleotide (NAD) is an intracellular nucleotide which has been identified as an agonist at P2Y<sub>1</sub>, P2Y<sub>11</sub>, P2X and adenosine receptors. NAD evoked endothelium-independent concentration-dependent contractions of the pre-contracted PMA, which were unaltered in the presence of PaCoA. In contrast,  $\alpha\beta$ -methylene ATP (a desensitizing P2X receptor agonist) significantly reduced these responses suggesting the involvement of P2X<sub>1</sub>-like receptors.

In both RTA and PCA, NAD evoked endothelium-independent concentration-dependent relaxations of the pre-contracted vessels, which were attenuated by SCH58261, but not PaCoA, which suggests the involvement of smooth muscle A<sub>2A</sub> receptors. These results together emphasise the possibility of a tissue and receptor-specific role of NAD as an endogenous extracellular nucleotide in purinergic signalling.

## Publications

1. Alexander, SPH., eEfaishat, E. & Ralevic, V. (2008). Effect of Coenzyme A derivatives on P2Y<sub>1</sub> purine receptor-mediated vasorelaxations in the rat isolated thoracic aorta.  
<http://www.pa2online.org/abstract/abstract.jsp?abid=29156>
2. Alefishat, E., Ralevic, V. & Alexander, SPH. (2009). Effect of palmitoyl Co A on P2Y<sub>1</sub> receptor-mediated calcium responses in HEK cells.  
<http://www.pa2online.org/abstract/abstract.jsp?abid=29494>
3. Alefishat, E., Ralevic, V. & Alexander, SPH. (2009). Effects of coenzyme A derivatives on P2Y receptor-evoked calcium responses in HEK cells.  
<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837821/>
4. Alefishat, E., Alexander, SPH. & Ralevic, V. (2010). Palmitoyl CoA antagonism of ADP-induced relaxation of rat thoracic aorta.  
<http://onlinelibrary.wiley.com/doi/10.1111/j.1742-7843.2010.00600.x/pdf>
5. Alefishat, E., Alexander, SPH. & Ralevic, V. (2010). Effect of palmitoyl CoA on ADP-evoked vasorelaxations in porcine isolated coronary and mesenteric arteries.  
[http://www.fasebj.org/cgi/content/meeting\\_abstract/24/1\\_MeetingAbstracts/1b426](http://www.fasebj.org/cgi/content/meeting_abstract/24/1_MeetingAbstracts/1b426)
6. Alefishat, E., Alexander, SPH. & Ralevic, V. (2010). The effects of palmitoyl CoA on NAD-evoked vasorelaxations in the porcine isolated coronary artery.  
<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3083131/>
7. Alefishat, E., Alexander, SPH. & Ralevic, V. (2011). NAD-evoked responses in porcine isolated mesenteric arteries.  
[http://www.fasebj.org/cgi/content/meeting\\_abstract/25/1\\_MeetingAbstracts/616.28](http://www.fasebj.org/cgi/content/meeting_abstract/25/1_MeetingAbstracts/616.28)

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## Abbreviations

$\alpha,\beta$ -meATP	$\alpha,\beta$ - methyleneATP
ACBPs	Acyl-CoA binding proteins
AcCoA	Acetyl CoA
ANOVA	Analysis of variance
ATP	Adenosine 5-triphosphate
ADP	Adenosine 5-diphosphate
BSA	Bovine serum albumin
cADPR	Cyclic ADP Ribose
$[Ca^{2+}]_i$	Intracellular calcium
CoA	Coenzyme A
EDHF	Endothelium-derived hyperpolarizing factor
EMEM	Eagle's Minimum Essential Medium
FCS	Fetal calf serum
FABPs	Fatty acid binding proteins
GPCRs	G-protein-coupled receptors
IP <sub>3</sub>	Inositol triphosphate
NAD	Nicotinamide adenine dinucleotide
NAADP	Nicotinic acid adenine dinucleotide phosphate
NO	Nitric oxide
OlCoA	Oleoyl CoA
PaCoA	Palmitoyl CoA
PLC	Phospholipase C
PBS	Phosphate buffered saline
PCA	Porcine coronary artery
PMA	Porcine mesenteric artery
PPARs	Peroxisome proliferator-activated receptors
RTA	Rat thoracic aorta
UTP	Uridine-5-triphosphate

## 1.1 A Brief Historical Introduction

It was in 1929 when the extracellular signalling function of purine nucleotides and nucleosides was first recognized, with the report of Drury and Szent-Gyorgi, who showed that adenosine and adenosine 5'-monophosphate (AMP) can have potent physiological effects such as slowing the heart rate, dilatation of the coronary vessels and inhibition of intestinal movements (Drury and Szent-Gyorgyi, 1929). This aroused interest in this group of compounds and the work was extended by a study of the structure-activity relationships of adenine compounds by Gillespie. He showed amongst other things that the removal of phosphate from AMP does not lessen its activity so markedly as deamination and that the biological activity disappears from the compounds when the pentose is split from the purine base (Gillespie, 1934).

A number of other studies followed, showing diverse effects of purines on the cardiovascular system and were reviewed in 1950 (Green and Stoner, 1950). In 1959, Holton was the first to present a role for ATP as a transmitter in the nervous system by demonstrating its release during antidromic stimulation of sensory nerves supplying the rabbit ear artery (Holton, 1959)

The interest in this family of compounds expanded to include the pyrimidine nucleotides through investigation of the cardiovascular effects of uridine triphosphate (UTP), with these early studies showing that purines and pyrimidines have characteristic vascular activity (Hashimoto et al., 1964, Sakai et al., 1979).

Several purine dinucleotides such as  $\beta$ -nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) have also been shown to have potent actions on diverse tissues. NAD was recently shown to be released in a variety of smooth muscle tissues during stimulation of sympathetic nerves terminals in canine isolated mesenteric artery, blood vessels, urinary bladder and murine colonic muscles and was described as a neurotransmitter or neuromodulator (Smyth et al., 2004, Smyth et al., 2006, Breen et al., 2006, Mutafova-Yambolieva et al., 2007).

In 1957 Boettge published an extensive review describing the physiological and pharmacological significance of adenylyl compounds in humans (Boettge et al., 1957). This was followed by a hypothesis proposed in 1963 by Berne, which suggested that adenosine was the physiological mediator of the coronary vasodilatation associated with myocardial hypoxia (Berne, 1963). Burnstock presented an alternative hypothesis that ATP released from endothelial cells acts via endothelial P<sub>2</sub> receptors to cause a release of nitric oxide (NO) which results in

vasodilatation, and that adenosine is involved only in the longer-lasting component of reactive hyperaemia (Burnstock, 1993).

It was in the early 1960s that non-adrenergic, non-cholinergic (NANC) neurotransmission was first noticed and many studies were carried out in order to try to identify the nature of this response in different tissues such as guinea-pig taenia Coli (Burnstock et al., 1963, Burnstock et al., 1964, Burnstock et al., 1966, Martinson and Mauren, 1963). Evidence was presented that ATP was the molecule released from NANC nerves and responsible for NANC neurotransmission. This was followed later with the proposal of the term 'purinergic' stating that adenosine triphosphate or a related nucleotide is the transmitter involved in NANC and sympathetic neurotransmission in a wide variety of systems (Burnstock et al., 1970, Burnstock et al., 1972, Burnstock, 1972).

The purinergic hypothesis met with huge resistance from many scientists since ATP was considered unlikely to have this role as an extracellular signalling molecule. ATP was known as an intracellular molecule and source of energy involved in a variety of metabolic cycles (Burnstock, 2006). It was almost 25 years later that the purinergic hypothesis became widely accepted (Burnstock, 1997).

Purinergic receptors were first introduced by Burnstock in 1976 (Burnstock, 1976). In 1978, he set the basis for distinguishing two types of purinoceptors. He suggested that these receptors should be classified



into two different types; P1 purinoceptors, at which adenosine is the primary ligand, and P2 purinoceptors, at which ATP and ADP are the principal ligands (Burnstock, 1978). P2 receptors are now known to be activated also by UTP, UDP, UDP-glucose and other nucleotide sugars. Both P1 and P2 receptors may involved in physiological responses to ATP and ADP since adenosine can be derived from them in metabolic breakdown.

P1 and P2 receptors for purines and pyrimidines are now known to be expressed in a wide range of biological systems including the central and peripheral nervous systems, respiratory, gastrointestinal and cardiovascular systems and mediate a variety of diverse effects including regulation of contractility, neurotransmission, immune function and cell migration and proliferation.

## **1.2 Purine Receptors**

### **1.2.1 P1 Receptors**

Four subdivisions of P1 receptors have been identified; namely A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> (Table 1). All P1 receptors are G protein-coupled receptors with 7 transmembrane domains which are connected by extracellular and intracellular hydrophilic loops. The seven transmembrane domains are composed of hydrophobic amino acids, each having 21-28 amino acids combining together to form an  $\alpha$ -helix, with the N-terminal lying on the extracellular side and the C-terminal lying on the intracellular side of the membrane (Ralevic and Burnstock, 1998). Their signal transduction mechanism is through multiple intracellular effectors in response to nucleoside activation. The main distribution, agonists, antagonists and G-protein coupling for each subtype is shown in Table 1.

### **1.2.2 P2 Receptors**

P2 receptors are divided into two classes based on whether they are ligand-gated ion channels, named P2X receptors (ionotropic), or are coupled to G proteins, named P2Y receptors (metabotropic). This was

based on studies of the mechanisms of signal transduction and the cloning of receptors (Abbracchio and Burnstock, 1994, Fredholm et al., 1994) (Table 1). Unfortunately, pharmacological characterisation of endogenous P2 receptors is limited by overlap in their agonist selectivities, general lack of selective antagonists and metabolic interaction of nucleosides and nucleotides.

Studies of native P2 receptors had shown significant heterogeneity in the pharmacological responses indicating the presence of different subtypes of P2 receptor. To date, there are seven subtypes of the P2X (P2X<sub>1-7</sub>) family and eight subtypes of the P2Y (P2Y<sub>1,2,4,6,11,12,13,14</sub>) family; the missing numbers represent either receptors having partial sequence homology to P2Y receptors but having no functional evidence of activation by nucleotides, or they are non-mammalian orthologues. The cloning of the first two P2 receptors, P2Y<sub>1</sub> and P2Y<sub>2</sub> provided the basis for a revision in receptor nomenclature and a basis on which to identify native P2 receptor subtypes (Webb et al., 1993, Lustig et al., 1993). The P2Y<sub>12</sub> receptor found principally on platelets was not cloned until more recently (Hollopeter et al., 2001); it has only 19% homology with the other P2Y receptor subtypes hence it was proposed that it represented one of a subgroup of P2Y receptors, including P2Y<sub>13</sub> and P2Y<sub>14</sub>; for this subgroup signal transduction is entirely through adenylate cyclase (Abbracchio et al., 2003). Therefore it has been suggested that P2Y receptors can be subdivided into two subgroups; one that includes P2Y<sub>1,2,4,6,11</sub>, the other includes P2Y<sub>12,13,14</sub>;

this largely depends on the structural and phylogenetic criteria (Abbracchio et al., 2003).

### **1.2.2.1 P2X Receptors**

There are seven P2X receptor subtypes (P2X<sub>1-7</sub>) that have been cloned. P2X receptors are ATP-gated ion channels that mediate rapid and selective permeability to cations; Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> (North, 1996, Bean, 1992, Dubyak and el-Moatassim, 1993). This goes with their role as mediators of fast excitatory neurotransmission to ATP in the central and peripheral nervous systems. The receptor subunits are composed of two transmembrane domains, an extracellular loop and two intracellular domains. The extracellular loop is composed of 10 cysteine residues, 14 glycine residues and 2-6 N-linked glycosylation sites. The extracellular loop contains the ATP binding site (Jiang et al., 2000) and a site where a potential antagonist can bind (Garcia-Guzman et al., 1997). It has a hydrophobic H5 region that is located on the entrance to the pore. This area could have a role in the regulation and modulation of the channel through various ions such as Cu<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup>. Disulfide bridges may form the structural constraints needed to couple the ATP-binding site to the ion pore.

The subunits of the P2X receptors can be homomeric (composed of identical subunits) or heteromeric (composed of different subunits) and their properties vary with each individual subunit. For example it has been shown that P2X<sub>2</sub> and P2X<sub>3</sub> proteins can combine to form a functional P2X<sub>2/3</sub> heteromeric receptor (Lewis et al., 1995, Ralevic and Burnstock, 1998, Radford et al., 1997). Functional P2X receptors appear to be composed of three subunits (Stoop et al., 1999, Nicke et al., 1998, Jiang et al., 2003). The signal transduction mechanisms for the receptor seem to start after the activation of the P2X receptor where a rapid nonselective passage of cations (Na<sup>+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>) crosses the cell membrane which results in the generation of excitatory junction potentials and an increase in Ca<sup>2+</sup> levels inside the cell ending in membrane depolarisation (Bean, 1992). If this depolarisation reaches a threshold it will lead to the activation of L-type voltage gated Ca<sup>2+</sup> channels in addition to calcium-stimulated tyrosine kinases which activate MAP kinases that modulate transcriptional processing (Boarder and Webb, 2001a). P2X receptors are located in various tissues in the body; smooth muscle cells, nerves, endothelial cells in blood vessels, immune cells, glands and glial cells. Table 1.1 shows the main distribution, agonists, antagonists and signal transduction mechanism for each subtype of P1 and P2 receptor.

**Table 1.1** Characteristics of purine-regulated receptors.

Receptor	Main Distribution	Selective agonists	Selective antagonists	Principle transduction
P1 (adenosine)				
A <sub>1</sub>	Brain, spinal cord, testis, heart, autonomic nerve terminals	CPA, CCPA, S-ENBA,  GR79236	PSB36, DPCPX,  SLV320	G <sub>i/o</sub>
A <sub>2A</sub>	Brain, heart, lungs, spleen, blood vessels	CGS21680, HENECA, ATL-146e	SCH58261, ZM241385, KW 6002 SCH442416	G <sub>s</sub>
A <sub>2B</sub>	Large intestine, bladder, blood vessels	Bay60-6583	PSB603, MRS1754, MRS1706, PSB1115	G <sub>s</sub>
A <sub>3</sub>	Lung, liver, brain, testis, heart	2-CI-IB-MECA, IB-MECA	MRS1220, VUF5574, MRS1523, MRS1191	G <sub>i/o</sub>
P2X				
P2X <sub>1</sub>	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	L-βγ-meATP, αβ-meATP,  BzATP	TNP-ATP (non-selective), IP <sub>5</sub> I, NF023, NF449	Intrinsic cation channel (Ca <sup>2+</sup> and Na <sup>+</sup> )
P2X <sub>2</sub>	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	-	-	Intrinsic ion channel (particularly Ca <sup>2+</sup> )
P2X <sub>3</sub>	Sensory neurons, NTS, some sympathetic neurons	αβ-meATP, BzATP	TNP-ATP (non-selective),  A317491, RO3	Intrinsic cation channel
P2X <sub>4</sub>	CNS, testis, colon	-	-	Intrinsic ion channel (especially Ca <sup>2+</sup> )
P2X <sub>5</sub>	Proliferating cells in skin, gut, bladder, thymus, spinal cord	-	-	Intrinsic ion channel
P2X <sub>6</sub>	CNS, motor neurons in spinal cord	-	-	Intrinsic ion channel
P2X <sub>7</sub>	Apoptotic cells in, for example, immune cells, pancreas, skin	-	Brilliant Blue G, A804598, A839977, decavanadate, KN62, A740003, A438079	Intrinsic cation channel and a large pore with prolonged activation

P2Y				
P2Y <sub>1</sub>	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	2-MeSADP, ADP $\beta$ S, MRS2365	MRS2179, MRS2500, MRS2279, PIT	G <sub>q/11</sub>
P2Y <sub>2</sub>	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	2-thio-UTP, UTP $\gamma$ S, Ap4A	-	G <sub>q/11</sub>
P2Y <sub>4</sub>	Endothelial cells	UTP $\gamma$ S	ATP	G <sub>q/11</sub>
P2Y <sub>6</sub>	Some epithelial cells, placenta, T cells, thymus	3-Phenacyl-UDP, UDP	MRS2578 MRS2567	G <sub>q/11</sub>
P2Y <sub>11</sub>	Spleen, intestine, granulocytes	ARC67085, NAD, NAADP,	NF157	G <sub>s</sub> , G <sub>q/11</sub>
P2Y <sub>12</sub>	Platelets, glial cells	ADP, 2-MeSADP	ATP, ARL66096	G <sub>i/o</sub>
P2Y <sub>13</sub>	Spleen, brain, lymph nodes, bone marrow	-	MRS2211	G <sub>i/o</sub>
P2Y <sub>14</sub>	Placenta, adipose tissue, stomach, intestine, discrete brain regions	MRS2690	-	G <sub>i/o</sub>

Shown are receptor subtypes for purines and pyrimidines: distribution, agonists, antagonists, and transduction mechanisms. BBG, Brilliant blue green; BzATP 2'- & 3'-O-(4-benzoyl-benzoyl)-ATP; CCPA, chlorocyclopentyl adenosine; CPA, cyclopentyladenosine; CTP, cytosine triphosphate; IP<sub>3</sub>, inosine triphosphate; Ip5I, di-inosine pentaphosphate; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5'-Nethylcarboxamido adenosine; PLC, phospholipase C; RB2, Reactive blue 2. P2X receptor subtype agonist potencies are based on rat preparations, while P1 and P2Y receptor subtype agonist potencies are based on human preparations. Adapted from (Burnstock, 2007).

### **1.2.2.2 P2Y Receptors**

P2Y receptors are G protein-coupled receptors composed of 308-377 amino acids. They have seven transmembrane domains with hydrophilic loops connecting them on the extracellular and intracellular sides, they have an extracellular N terminus and intracellular C terminus which has binding sites for protein kinases. There are great levels of homology in the transmembrane-spanning region sequences but there is a great structural diversity in the intracellular loops and the C terminus between the subtypes which leads to a variation in the extent of G protein coupling. Stoichiometric investigation of this receptor have indicated that the P2Y<sub>1</sub> receptor is able to heterodimerise with the A<sub>1</sub> receptor (Yoshioka et al., 2002).

Primary sequencing of the P2Y<sub>1</sub> receptor and the use of the structural homologue rhodopsin has characterised positively charged amino acid residues in transmembrane regions 3, 6, and 7 that can be involved in ligand binding by electrostatic interactions with the phosphates of ATP (and presumably ADP) (Van Rhee et al., 1995, Jiang et al., 1997). Mutagenesis of the P2Y<sub>2</sub> receptor to convert positively charged amino acids in transmembrane regions 6 and 7 to neutral amino acids causes a 100- to 850-fold decrease in the potency of ATP and UTP,



which indicates a role for these amino acids in binding purines and pyrimidines (Erb et al., 1995).

P2Y receptors act via G protein coupling. The G proteins can be classified according to their sequence homology and function into 4 families:  $G_s$ ,  $G_{q/11}$ ,  $G_{i/o}$ ,  $G_{12/13}$ . Each family binds predominantly to different second messenger signalling pathways (Neves et al., 2002).

Although most P2Y receptors act via G protein coupling to activate phospholipase C (PLC) (Boyer and Harden, 1989), there are examples where the P2Y receptor is coupled to the inhibition of adenylyl cyclase (Boyer et al., 1996b, Boyer et al., 1993, Webb et al., 1996, Boyer et al., 1994, Boyer et al., 1995). The activation of PLC can eventually lead to the formation of inositol 1,4,5-trisphosphate ( $IP_3$ ) and mobilization of intracellular  $Ca^{2+}$  and the activation of other pathways such as protein kinase C (PKC), mitogen activated protein kinase pathways, phospholipase  $A_2$ , nitric oxide synthase and calcium dependent potassium channels. Since there are second messenger systems and/or ionic conductances mediated by G protein coupling, the response time of P2Y receptors is longer than that mediated by P2X receptors.

P2Y purine receptors have a wide distribution and mediate responses in various tissues. For example, P2Y receptors are found in different regions of kidney glomeruli, tubules and collecting ducts (Eltze and Ullrich, 1996, Dockrell et al., 2001, Cuffe et al., 2000, Bailey et al., 2000, van Der Weyden et al., 2000, Cha et al., 1998, Huber-Lang et al., 1997). P2Y receptors are present in the brain, on both presynaptic sites and on glial cells (Webb and Barnard, 1999, Zundorf et al., 2001, Moran-Jimenez and Matute, 2000, Moore et al., 2000, Schafer and Reiser, 1999, Neary et al., 1999). They are also present throughout the cardiovascular system. The main distribution of the different subtypes of P2Y receptor is shown in Table 1.1.

#### **1.2.2.2.1 P2Y<sub>1</sub> Receptors**

P2Y<sub>1</sub> receptors were first cloned from chick brain (Webb et al., 1993). Table 2 shows the cloned P2Y<sub>1</sub> receptors from different species. While the activation of P2Y<sub>1</sub> receptors is mostly associated with an increase in PLC activity (Boyer and Harden, 1989), there are examples where the P2Y<sub>1</sub> receptor is coupled to inhibition of adenylyl cyclase activity (Boyer et al., 1993, Boyer et al., 1994, Boyer et al., 1995, Boyer et al., 1996b). Some studies on glioma C6-2B and C6 cells have shown the presence of a P2Y<sub>1</sub>-like receptor that is coupled to the inhibition of adenylyl cyclase but is not effective in the activation of inositol phosphate accumulation (Schachter et al., 1996, Schachter et al., 1997a). This resulted in the proposal that two different types of P2Y<sub>1</sub>-like receptors exist; one coupled to PLC and another one coupled to adenylyl cyclase. Webb et al. (1996) concluded that a single P2Y<sub>1</sub>

receptor may be involved in different signalling pathways in different cell types, and that the two pathways do not coincide with each other (Webb et al., 1996).

P2Y<sub>1</sub> receptors respond to a variety of agonists: adenosine 5'-O-thiodiphosphate (ADPβS), 2-methylthio-ATP (2MeSATP), 2-methylthio-ADP (2MeSADP) and ADP all have greater potency than ATP. The P2Y<sub>1</sub> receptor is more sensitive to adenine nucleotide diphosphates than to triphosphates, while uridine di- and triphosphates are inactive (Leon et al., 1997, Boarder and Hourani, 1998). In contrast, the P2Y<sub>2</sub> receptor is activated by both ATP and UTP, while 2MeSATP and ADP have no effect. P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors show some selectivity for UTP and UDP, respectively, while the P2Y<sub>11</sub> receptor is selective for ATP but not UTP (Boarder and Hourani, 1998, Nicholas et al., 1996b, Webb et al., 1998). NAD also was shown to be an agonist at the P2Y<sub>1</sub> receptor (Mutafova-Yambolieva et al., 2007).

Heterogeneity in ligand binding at purine receptors includes both agonist and antagonists binding profiles. Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) acts as an antagonist at the P2Y<sub>1</sub> receptor. In contrast, the P2Y<sub>2</sub> receptor was shown to have species variation in sensitivity to this compound (Rayment et al., 2007a). Recombinant P2Y<sub>1</sub> receptors cloned from different species and tissues show different relative potencies to ATP and ADP as do their endogenous counterparts (Ralevic and Burnstock, 1998) (Table 1.2).

In blood vessels, P2Y<sub>1</sub> receptors are found mainly on the endothelium, where they cause vasorelaxation through Ca<sup>2+</sup>-dependent activation of endothelial nitric oxide synthase (NOS) and generation of endothelium-dependent hyperpolarizing factor (EDHF) (Ralevic and Burnstock, 1998) (Figure 1.1). In contrast, contractile P2Y<sub>1</sub> receptors are expressed in vascular smooth muscle cells (Pacaud et al., 1995, Erlinge et al., 1998), since P2Y<sub>1</sub> receptors act primarily through activation of G<sub>q/11</sub> G-proteins which will lead to the elevation of [Ca<sup>2+</sup>]<sub>i</sub> and lead to smooth muscle contraction, rather than relaxation.

Although ADP has been shown to produce a direct endothelium-independent vasodilatation in various tissues such as rabbit hepatic artery and human pulmonary artery, it is not clear whether this occurs through the P2Y<sub>1</sub> receptor (Kennedy and Burnstock, 1985, Brizzolara and Burnstock, 1991, Liu et al., 1989, Burnstock and Warland, 1987). A study investigated the mechanism of ADP-induced relaxation of porcine coronary artery and suggested that ADP mediates relaxation via a novel mechanism that involves adenine nucleotide evoked adenosine release and the subsequent activation of A<sub>2A</sub> receptors (Rayment et al., 2007b). NAD has also been reported to evoke the release of adenosine in rat vas deferens, guinea-pig taenia coli and guinea-pig bladder (Stone, 1981).

P2Y<sub>1</sub> receptors are colocalized in some cells with P2Y<sub>2</sub> receptors; while ATP is a common agonist, the receptors may be distinguished as ADP is selective for the P2Y<sub>1</sub> receptor, and UTP acts only at the P2Y<sub>2</sub> receptor. Endothelial cells express P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors and may express P2Y<sub>4</sub> receptors at which UTP is also an agonist (Burnstock, 2004). It was concluded that more than one subtype of P2Y receptor can regulate responses in an individual endothelial cell (Boarder and Hourani, 1998).

**Table 1.2** Cloned P2Y<sub>1</sub> receptors.

cDNA library source	Agonist activity	References
Human brain	2MeSATP > ATP >> UTP	Schachter et al., 1996
Human prostate and ovary	2MeSATP > ATP = ADP	Janssens et al., 1996
Human placenta	_____	Le'on et al., 1995, 1997
Human HEL cells	_____	Ayyanathan et al., 1996
Bovine endothelium	2MeSATP = ADP > ATP >> UTP	Henderson et al., 1995
Rat insulinoma cells	2MeSATP > 2Cl-ATP > ATP ( $\alpha\beta$ -meATP inactive)	Tokuyama et al., 1995
Rat ileal myocytes	2MeSATP = 2ClATP > ADP > ATP (UTP inactive)	Pacaud et al., 1996
Mouse insulinoma cells	_____	Tokuyama et al., 1995
Turkey brain	2MeSATP > ADP > ATP (UTP inactive)	Filtz et al., 1994
Chick brain	2MeSATP > ATP > ADP (UTP inactive)	Webb et al., 1993b

Adapted from Ralevic and Burnstock, 1998.

In pancreatic  $\beta$  cells, P2Y<sub>1</sub> receptors are involved in insulin secretion (Loubatieres-Mariani and Chapal, 1988). P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2X receptors have been identified in platelets (Kunapuli and Daniel, 1998). P2Y<sub>1</sub> receptors are present on non-myelinating Schwann cells while P2Y<sub>2</sub> receptors are found on myelinating Schwann cells (Mayer et al., 1998).

P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors have also been reported to be present on the membranes of rat liver isolated mitochondria and it was hypothesized that P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors played a role in internal mitochondrial signalling and may contribute to calcium homeostasis (Belous et al., 2004, Belous et al., 2006). ATP was shown to act intracellularly through the mitoK<sub>ATP</sub> channel, ATP-regulated Ca<sup>2+</sup> channels and ATP-dependent anion channels (Belous et al., 2004, Brookes et al., 2004). It has also been shown that P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>12</sub> receptors are present in mitochondria of rat astrocytes and glioma C6 cells (Krzeminski et al., 2007). Whether these intracellular P2Y receptors contribute to signalling by extracellular ATP has not yet been identified.

Desensitization of P2Y<sub>1</sub> receptors have been reported to involve phosphorylation by protein kinases and uncoupling from the associated G protein (Hardy et al., 2005, Rodriguez-Rodriguez et al., 2009). Studying P2Y<sub>1</sub> receptors in turkey erythrocyte membranes showed that desensitization with a  $t_{1/2}$  of 15 minutes occurred which

was heterologous and involved multiple mechanisms, but not PKC or intracellular  $\text{Ca}^{2+}$  (Galas and Harden, 1995).

### **1.3 Therapeutic significance of P2Y receptors**

It was suggested in several studies that modulators of members of the P2Y family of receptors can have therapeutic potential for the treatment of a variety of disorders such as cancer, cystic fibrosis, diabetes, and treatment of ischemia-reperfusion injury (Abbracchio and Burnstock, 1994, Burnstock and Williams, 2000).

For P2Y<sub>1</sub> receptors, their distribution and biological effects has revealed considerable information about their physiological significance. P2Y<sub>1</sub> receptors are widely spread among human organs (Janssens et al., 1996). They have been identified on microglia (Norenberg et al., 1994), and on astrocytes (Salter and Hicks, 1995). Extracellular ATP activates microglia and astrocytes through P2Y<sub>1</sub> receptors and leads directly to the release of inflammatory mediators . (Fischer and Krugel, 2007) and astrocytes are believed to play a role in the progression of Alzheimer's disease and other CNS inflammatory disorders like multiple sclerosis and stroke.



P2Y<sub>1</sub> receptors mediate many other effects including glycogenolysis in rat hepatocytes (Keppens and De Wulf, 1991), insulin secretion from pancreatic  $\beta$ -cells (Bertrand et al., 1987, Hillaire-Buys et al., 1991, Hillaire-Buys et al., 1993, Hillaire-Buys et al., 1994), gluconeogenesis in renal cortical tubules (Cha et al., 1995), and renin secretion in renal cortical slices (Churchill and Ellis, 1993a, Churchill and Ellis, 1993b, Jin et al., 1998)

Platelets are known to express P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2X<sub>1</sub> receptors (Hollopeter et al., 2001). P2Y<sub>1</sub> receptors mediate platelet shape change and aggregation (Daniel et al., 1998, Hechler et al., 1998). There are established therapeutic drugs targetting purinergic antithrombotic drugs. The purinergic antithrombotic drugs clopidogrel and ticlopidine reduce the risks of recurrent strokes and heart attacks, especially when combined with aspirin (Kunapuli et al., 2003, Kam and Nethery, 2003, Boeynaems et al., 2005). These drugs are antagonists at the P2Y<sub>12</sub> receptor that mediates platelet aggregation (Gachet, 2001, Matsagas et al., 2003). A highly potent and selective antagonist for the P2Y<sub>1</sub> receptor, MRS2500, has been shown to have antiaggregatory activity on human platelets (Cattaneo et al., 2004).

ATP, ADP, UTP, and adenosine are released from platelets and endothelial cells by a variety of mechanisms (Kunapuli, 1998, Lazarowski et al., 2003). ATP and ADP are released in the heart during ischemia from cardiac myocytes, endothelial cells, red blood cells, platelets and sympathetic nerves (Gordon, 1986, Burnstock, 1989, Ralevic and Burnstock, 1998, Burnstock, 1993). The distribution of

P2Y<sub>1</sub> receptors on the vascular endothelium indicates a role in the regulation of vascular tone, Figure 1. The administration of an intravenous injection of MRS2179 (a selective P2Y<sub>1</sub> antagonist) was reported to result in prolonged bleeding time and inhibition of rat platelet aggregation in response to ADP (Baurand et al., 2001). In addition, mice treated with MRS2179 and P2Y<sub>1</sub>-deficient mice both showed a significant decrease in arterial thrombosis (Lenain et al., 2003). Olivecrona et al. (2004) showed that *in vivo* administration of MRS2179 in pigs caused a selective inhibition of the P2Y<sub>1</sub> receptor and of the 2-MeSADP mediated coronary flow increase. They showed that MRS2179 significantly reduced the increase in coronary flow caused by 2-MeSADP or reactive hyperemia in coronary arteries with no significant differences in blood pressure (Olivecrona et al., 2004).

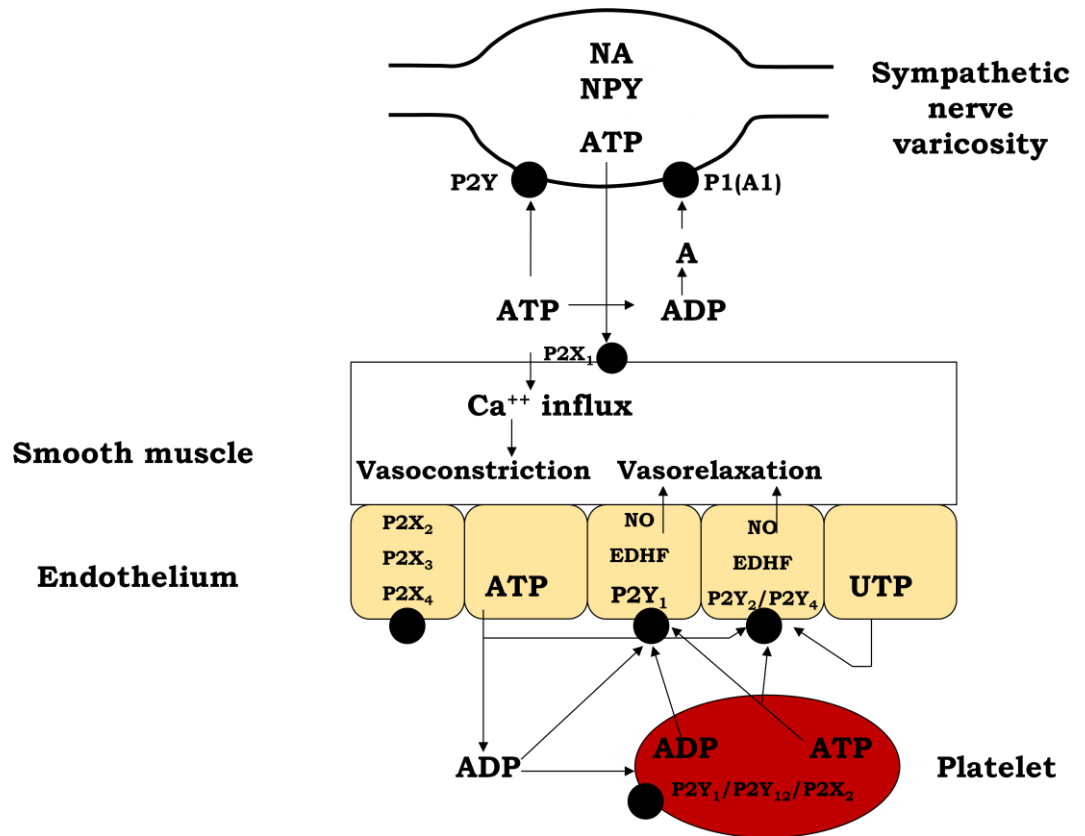
**Figure 1.1** Purinergic signalling controlling vascular tone.

Figure 1.1 Purinergic signalling controlling vascular tone. A diagram illustrating the main receptor subtypes for purine and pyrimidines in blood vessels. Perivascular nerves release ATP as a cotransmitter: ATP is released with NA and neuropeptide Y (NPY) from sympathetic nerves to act on smooth muscle P2X<sub>1</sub> receptors resulting in vasoconstriction. P1(A<sub>1</sub>)-purine receptors on nerve terminals of sympathetic nerves mediate adenosine (arising from enzymatic breakdown of ATP) modulation of neurotransmitter release. Endothelial cells release ATP and UTP during shear stress and hypoxia to act on P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> purine receptors, leading to the production of NO and EDHF and subsequent vasodilatation. ATP, following its release from aggregating platelets, also acts on these endothelial receptors. Platelets possess P2Y<sub>1</sub> and P2Y<sub>12</sub> ADP-selective purine receptors as well as P2X<sub>1</sub> receptors. For clarity, adenosine receptors on platelets and the smooth muscle have been omitted.

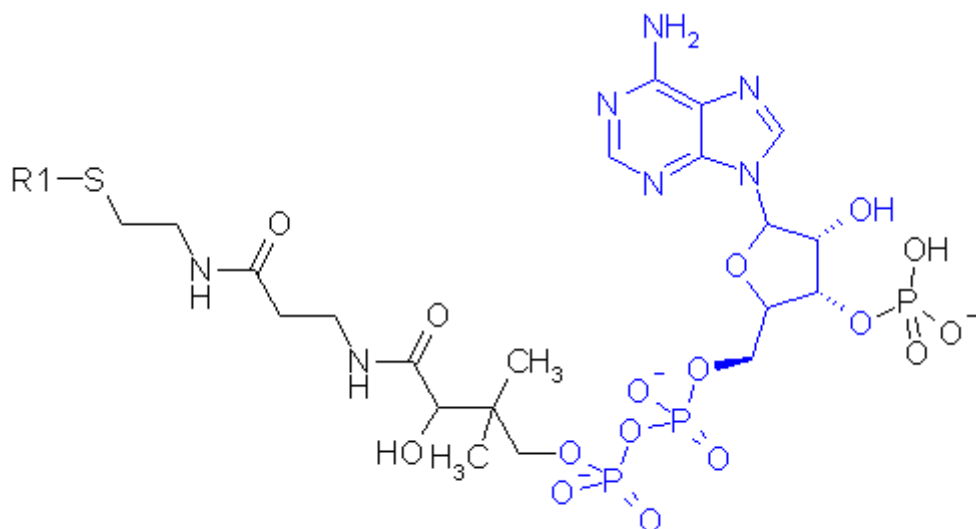
## 1.4 Acyl CoA derivatives as endogenous modulators

Fatty acids and related lipids are engaged in cellular metabolism as acyl-CoAs. CoA and its acyl derivatives are essential cofactors in all living organisms (Leonardi et al., 2005). Long-chain acyl-CoA esters can act as both substrates and intermediates in metabolism and as regulators of various intracellular functions. The structure of CoA, and its similarity to ADP, is shown in Figure 1.2. In the case of acetyl-CoA (AcCoA) (Figure 1.2b), palmitoyl-CoA (PaCoA) (Figure 1.2c) and oleoyl-CoA (OlCoA) (Figure 1.2d) cleavage of the thioester bond of the CoA group yields acetic acid (2:0) (number of carbons/number of double bonds), palmitic acid (16:0) and oleic acid (18:1), respectively. Palmitic acid is the starting substrate for the synthesis of longer chain fatty acids and unsaturated fatty acids into triglycerides, phospholipids and cholesterol esters.

Long chain acyl-CoAs are synthesized on the cytosolic face of many intracellular membranes resulting in a cytosolic pool where they are involved in various processes in the cell. Furthermore, fatty acid transport proteins (FATPs) have intrinsic acyl-CoA synthetase activity (Uchida et al., 1996, Steinberg et al., 1999a, Steinberg et al., 1999b, Coe et al., 1999). Acyl-CoA levels vary significantly in different metabolic condition such as diabetes, fasting, fat/glucose feeding and

digestion of hypolipidaemic drugs; it was suggested that 20-40% of the total fatty acyl pool is cytosolic (Oram et al., 1975). The total cellular concentration of long chain acyl CoA esters was shown to be in the range of 5-160  $\mu\text{mol/L}$ , and acyl CoA binding proteins (ACBPs) and fatty acid binding proteins (FABPs) keep the levels of free acyl CoA derivatives in a range of 0.1-200 nM intracellularly (Knudsen et al., 2000).

**Figure 1.2** Coenzyme A and its derivatives. In blue is the fraction of the Coenzyme A structure that is similar to adenosine diphosphate (ADP).



	a. Coenzyme A	b. Acetyl CoA	c. Palmitoyl CoA	d. Oleoyl CoA
R1=	H	Acetate	Palmitate	Oleate
Molecular Weight [g/mol]	767.53	832.56	1005.94	1031.98
Molecular Formula	C <sub>21</sub> H <sub>36</sub> N <sub>7</sub> O <sub>16</sub> P <sub>3</sub> S	C <sub>23</sub> H <sub>38</sub> N <sub>7</sub> NaO <sub>17</sub> P <sub>3</sub> S	C <sub>37</sub> H <sub>66</sub> N <sub>7</sub> O <sub>17</sub> P <sub>3</sub> S	C <sub>39</sub> H <sub>68</sub> N <sub>7</sub> O <sub>17</sub> P <sub>3</sub> S

Acyl-CoA is the ligand of acyl-CoA binding protein (ACBPs) that bind C14-C22 acyl-CoA esters with high affinity and specificity (Mogensen et al., 1987, Kragelund et al., 1999). ABPs were shown to mediate the intermembrane transport of acyl-CoAs (Rasmussen et al., 1994). ACBPs bind acyl-CoA with 3-4 times higher affinity than that of fatty acid binding proteins (FABPs) (Faergeman and Knudsen, 1997, Schroeder et al., 2008). Knockdown of ACBP in different cell lines caused disturbances in particular cellular functions such as growth arrest, impairment in adipocyte differentiation and lethality (Neess et al., 2011). In addition, overexpression of ACBP resulted in accumulation of triacylglycerol and some other lipid classes in the liver of transgenic mice (Neess et al., 2011). Furthermore, ACBPs were shown to affect the pool size of acyl-CoA (Schroeder et al., 2008). In malignant oesophageal tissue, ACBP genes were shown to be localised to endothelial cells in arteries within the connective tissue, while they were absent from these cells in the normal oesophageal submucosa (McCabe and Innis, 2005).

Palmitoyl CoA hydrolase is an enzyme which utilizes acyl-CoAs as substrates and may affect the intracellular concentration of these compounds. The enzyme catalyzes the hydrolysis of acyl-CoAs from 6-18 carbons. In the brain, long-chain acyl-CoA hydrolyzing activity of acylCoA hydrolase is much higher than that in any other organ in the body (Kurooka et al., 1972, Katoh et al., 1987, Kuramochi et al., 2002). The catalytic and molecular properties of the long-chain acyl-

CoA hydrolase present in rat and human brain was clarified (Broustas and Hajra, 1995, Broustas et al., 1996, Yamada et al., 1999b). In addition, the genomic organization of the human brain acyl-CoA hydrolase has also been described (Yamada et al., 1999a). It was found that the long-chain acyl-CoA hydrolase present in the brain is well-conserved in man and the rat (Yamada et al., 1999a). Although the potency of acyl-CoA hydrolases in the modulation of intracellular concentrations of acyl-CoAs may provide a regulatory mechanism for various cellular functions, the significance for any physiological function of brain acyl-CoA hydrolase is still unclear (Yamada, 2005).

Long chain acyl-CoA esters were reported to have high affinity for membrane phospholipid bilayers (Powell et al., 1985). Acyl-CoAs were shown to associate with cell membranes by insertion of the fatty acyl chain into the bilayer core (Boylan and Hamilton, 1992, Peitzsch and McLaughlin, 1993). In this regard, PaCoA may be classified as other membrane associating surfactants such as lysolipids (Requero et al., 1995b). The equilibrium partitioning of PaCoA into membranes is described by the partition constant  $K_p$  which is for PaCoA in the order of  $1 \times 10^5 \text{ M}^{-1}$  (Peitzsch and McLaughlin, 1993, Requero et al., 1995a). The membrane partitioning of acyl-CoAs has previously been studied and OIcCoA was shown to have no transbilayer movement in egg phosphatidylcholine vesicles (Boylan and Hamilton, 1992). Banhegyi et al. showed that PaCoA permeabilized rat liver microsomal vesicles (Banhegyi et al., 1996), however, other studies found no solubilization



or leakage of egg phosphatidylcholine vesicles by PaCoA (Requero et al., 1995b).

It was found that the oleoyl chain (18 carbons) effectively anchors the large CoA molecule to the phospholipid bilayer, while the octanoyl chain (8 carbon) results in only weak and transient binding of the CoA so it was suggested that the length of the acyl chain can control the affinity of the acyl-CoA compounds for membranes, and also their residence time in these membranes (Boylan and Hamilton, 1991).

There are no reports describing the presence of acyl-CoA extracellularly, although it has been shown that PaCoA synthetase is present on the external surface of rat isolated hepatocytes (de Groote et al., 1996). However, in certain pathophysiological conditions such as heart ischaemia, endurance training, diabetes and acute ischaemic stroke (van Breda et al., 1992, Pelters et al., 1999, Sambandam and Lopaschuk, 2003, Wunderlich et al., 2005), the cellular expression of FABPs is increased, leading to an overflow of these proteins into the blood such that the presence of these FABPs have been suggested to be markers of cellular damage. This raises the possibility that the FABP may also release the bound ligand into the serum. It was also reported that in certain pathophysiological states, high levels of acyl-CoA accumulate and can cause irreversible damage to cellular membranes such as in heart muscle after ischemia and in liver during starvation (Bortz and Lynen, 1963, Whitmer et al., 1978).

The significance of studies on the effect of acyl-CoAs *in vitro* and their relevance to the situation *in vivo* has been questioned in some reports, where it was postulated that the effects of these compounds on cellular functions are caused by their detergent-like properties, resulting in rather nonspecific effects, without acting as direct homeostatic modifiers of enzymes (Srere, 1965, Taketa and Pogell, 1966, Shafrir and Ruderman, 1974). However several other later reports have contradicted this and shown that acyl-CoA may play a regulatory role *in vivo* under certain circumstances (as shown below).

Fatty acyl-CoA derivatives have been reported to be potent modulators of a variety of cellular functions, activity of transporters, receptors and enzymes (Schmidt, 1989). Acyl-CoA compounds were shown to inhibit adenine nucleotide translocation through inhibiting the adenine nucleotide translocase activity which was reported in heart and liver (McLean et al., 1971, Pande and Blanchaer, 1971, Shug et al., 1971, Lerner et al., 1972, Harris et al., 1972, Woldegiorgis et al., 1982). This can result in lowering of the energy charge of the cell, which may affect both muscle contraction and electrical conduction in heart (Shug et al., 1975).

Furthermore acyl-CoAs modulate the activity of some other enzymes, receptors and transporters such as glucokinase (Tippett and Neet, 1982b, Tippett and Neet, 1982a), carnitine palmitoyltransferase I (Agius et al., 1987), glucose-6-phosphatase (Fulceri et al., 1993a) and the nuclear thyroid hormone receptor (Li et al., 1990). PaCoA inhibited the activity of long chain acylCoA synthetase in a reversible manner

(Pande, 1973). Both secreted and cellular proteins of the endoplasmic reticulum require acyl-CoA derivatives for their acylation (Olson et al., 1985, Simonis and Cullen, 1986). This reaction appears to have an important role in intracellular membrane trafficking (Pfanner et al., 1989). Acyl-CoA derivatives were also shown to bind to the orphan nuclear hormone receptors hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ) which may influence expression of HNF-4 $\alpha$ -controlled genes (Hertz et al., 1998).

Deeny et al. (1992) showed that acyl-CoA derivatives have a role also in the intracellular handling of Ca<sup>2+</sup> (Deeney et al., 1992). They showed that long-chain acyl-CoA derivatives have a role in the cytosolic balance of free Ca<sup>2+</sup> by the activation of ATP-dependent Ca<sup>2+</sup> accumulation (Deeney et al., 1992). It was also shown that acyl-CoA derivatives reduced the GTP-induced Ca<sup>2+</sup> release from liver microsomes (Comerford and Dawson, 1993). CoA and its fatty acyl derivatives were also shown to mobilize Ca<sup>2+</sup> and play a role in regulating Ca<sup>2+</sup> fluxes from the intracellular pool in liver (Fulceri et al., 1993b).

### **1.4.1 Acyl-CoA in pathophysiological states**

Normally fatty acids are mainly used in well-oxygenated hearts (Neely et al., 1972, Neely and Morgan, 1974); during ischemia, the myocardium is forced to use anaerobic oxidation to oxidize glucose and stores fatty acids as triglycerides (Scheuer and Brachfeld, 1966). This suggests that a decrease in fatty acid oxidation after myocardial ischemia would lead to an accumulation of long chain acyl-CoA esters in heart muscle. Schwartz et al. reported that the failure of myocardial cells to oxidise large amounts of fatty acids in ischaemic heart can be noticed by the rise in acyl carnitine and acyl-CoA concentration in the mitochondria, while in the cytoplasm, the increase in acyl-CoA concentration can be caused by free carnitine deficiency in ischaemic myocardium (Schwartz et al., 1973).

Furthermore, in pathological conditions such as type 2 diabetes and obesity, high circulating levels of free fatty acids lead to accumulation of acyl-CoAs in the cytosol (Golay et al., 1986, Reaven et al., 1988, Corkey et al., 2000), indicating that this class of compounds may contribute to the pathophysiology of obesity and susceptibility to type 2 diabetes (Riedel and Light, 2005). Chronic free fatty acids exposure results in increased levels of acyl-CoAs within  $\beta$ -cells, adding to the decreased insulin output through many proposed mechanisms (Corkey et al., 2000) including that acyl-CoAs increase  $K_{ATP}$  channel activity (Larsson et al., 1996, Branstrom et al., 1997, Riedel et al., 2003).

### **1.4.2 Effects of chain length and degree of saturation on acyl-CoA activity**

Branstorm et al. reported acyl-CoAs as potent activators of the  $K_{ATP}$  channel in human pancreatic  $\beta$ -cells; they also studied the effect of chain length of acyl-CoA and found that that only acyl-CoAs with 12 carbons or more were active and reported a maximal activation of  $K_{ATP}$  channel by PaCoA (C16:0) and noted that there was no further increase following addition of OlCoA (C18:1). They also found that long chain acyl-CoA stimulated  $K_{ATP}$  channel activity in human pancreatic  $\beta$ -cells in both the inside-out and whole cell preparations which led them to the conclusion that these compounds can have an important modulatory role of human  $\beta$ -cell electrical activity under both physiological and pathophysiological conditions (Branstrom et al., 2004).

Some other reports studied the effect of side chain length and the degree of saturation of acyl-CoAs and found that increased acyl-CoA side-chain length and saturation led to increased  $K_{ATP}$  channel activity. They concluded that it is possible that dietary fat composition may affect the severity of impaired glucose-stimulated insulin secretion via differential activation of  $\beta$ -cell  $K_{ATP}$  channels (Riedel and Light, 2005).

Back in 1976, acyl-CoA was reported to inhibit ADP-induced platelet aggregation (Lin et al., 1976). A number of CoA derivatives were investigated in this study; PaCoA 16:0 and other 14:0, 18:0, 18:1 and 18:2 derivatives; all these derivatives were more potent than CoA. OlCoA and other acylCoA derivatives were also reported to inhibit ADP and thrombin-induced platelets aggregation (Lascu et al., 1988). A more recent study has investigated in more details the effects of CoA derivatives of different chain length and saturation in platelets (Manolopoulos et al., 2008) and this is discussed in the next section on modulation of P2Y<sub>1</sub> purine receptors by acyl-CoA.

#### **1.4.3 Modulation of P2Y<sub>1</sub> purine receptors by acyl-CoA**

The structural similarity between the endogenous P2Y<sub>1</sub> receptor ligand (ADP) and CoA compounds (Figure 2) made it interesting to investigate the effect of these compounds on this receptor. Coddou et al., (2003) studied whether CoA and CoA derivatives, endogenously derived from fatty acids (CoA, AcCoA and PaCoA) or from metabolism of drugs (nafenopin-CoA (NafCoA), ciprofibroyl-CoA (CipCoA)), were antagonists at recombinant human P2Y receptors expressed in *Xenopus laevis* oocytes (Coddou et al., 2003). CoA was found to antagonize the ATP-gated currents evoked via the P2Y<sub>1</sub> receptor in a concentration-dependent manner. They also found that of all the CoA compounds investigated, NafCoA was the most potent antagonist. In their study, they suggested from the structures of the CoA derivatives a possible

interaction of the CoA moiety with the P2Y<sub>1</sub> purine binding site. In addition, they suggested an additional binding site; a hydrophobic pocket close to the ligand-binding domain which may involve hydrophobic amino acid residues to interact with the hydrophobic acyl-substituent. It was reported that the purine binding site for P2Y<sub>1</sub> receptors has several amino acids on the transmembrane domains 3, 6, 7 and charged amino acids that are part of the extracellular loops 2 and 3 (Jiang et al., 1997, Hoffmann et al., 1999). Furthermore Major et al. (1999) reported the possibility of a hydrophobic pocket close to the the P2Y<sub>1</sub> receptor ligand binding site; they found that increasing the size of substituents at the C2 or C8 adenine in ATP ligands enhanced the affinity of these ligands towards the P2Y<sub>1</sub> receptor (Major et al., 1999).

In the Coddou et al. (2003) study, they also concluded that both higher hydrophobicity and higher bulkiness (lesser flexibility) are important for higher antagonist potency. This explains why the addition of only two carbons to CoA, as in AcCoA, does not increase the potency much compared to CoA because of the lack of bulkiness. This importance of the side chain length and hydrophobicity on the potency of acyl-CoAs agrees with some other previous reports which looked at the effects of side chain length and the degree of saturation of acyl-CoAs and found that increased acyl-CoA side-chain length and saturation led to increased potency of acyl-CoA compounds at different functions, such as in the activation of K<sub>ATP</sub> channel (Riedel and Light, 2005).

Coddou et al. also tested the selectivity of acyl-CoA derivatives against P2X and P2Y<sub>2</sub> receptors and showed that none of the CoA derivatives interacted with these receptors. They also investigated the action of these compounds against 5-HT<sub>2</sub> receptors, since both the P2Y<sub>1</sub> and the 5-HT<sub>2</sub> receptors are coupled to G<sub>q/11</sub> and activate a Ca<sup>2+</sup> dependent chloride channel, and concluded that these compounds probably interact with the P2Y<sub>1</sub> protein rather than with the signaling pathway (Coddou et al., 2003). This study was done on human recombinant P2Y<sub>1</sub> receptors.

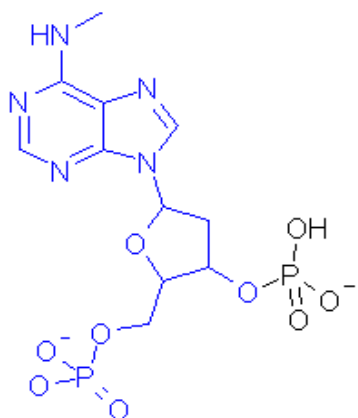
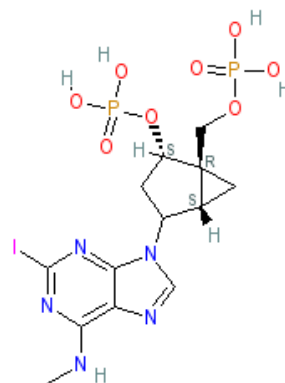
Other studies examined the effect of a number of CoA derivatives at endogenous P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors on platelets; they studied the effect of PaCoA (16:0) in depth and showed that PaCoA is an antagonist mainly at P2Y<sub>1</sub>, but also at P2Y<sub>12</sub> receptors (Manolopoulos et al., 2008). Furthermore, they studied the effect of chain length and saturation and found that compounds with saturated acyl groups containing 16-18 carbons were the most effective. It was concluded that acyl-CoA compounds may contribute as endogenous modulators of platelet function (Manolopoulos et al., 2008). The conclusion of Manolopoulos et al. about the effects of chain length on acyl-CoA activity also agrees with previous literature (Coddou et al., 2003, Riedel and Light, 2005).

The effects of acyl-CoA at endogenous P2Y receptors in blood vessels are unknown.



There are a number of selective antagonists described for the P2Y<sub>1</sub> receptor, such as MRS2179 (Figure 1.3a), which has an IC<sub>50</sub> of 0.3 μM at P2Y<sub>1</sub> receptors (Moro et al., 1998), MRS2279 with an IC<sub>50</sub> of 51.6 nM (Boyer et al., 2002) and MRS2500 (Figure 1.3b) with an IC<sub>50</sub> value of 0.95 nM (Hechler et al., 2006). Nevertheless, it would still be useful to identify new compounds which may possess a greater half life *in vivo* and have a greater selectivity and potency at the P2Y<sub>1</sub> receptor. It is interesting to note that, like CoA and its derivatives, the P2Y<sub>1</sub> receptor antagonists MRS2179, MRS2279 and MRS2500 have structural resemblance to the endogenous P2Y<sub>1</sub> receptor agonist ADP.

**Figure 1.3** Chemical structure of MRS2179 (a) and MRS2500 (b), potent and selective antagonists of the P2Y<sub>1</sub> receptor.

**a****b**

**Aims and Objectives**

The aim of present study was to investigate the effect of the endogenous “nucleotide”, PaCoA, on P2Y<sub>1</sub> purine receptor-mediated responses in blood vessels of different species, namely the rat thoracic aorta (RTA), porcine mesenteric artery (PMA), porcine coronary artery (PCA), and in human embryonic kidney (HEK) cells using ADP as agonist. The effect of CoA and acyl-CoA, AcCoA and OlCoA, were also tested. In addition, the present study aimed to test the selectivity of these compounds at these P2Y<sub>1</sub> receptors. Furthermore, it was aimed to investigate the effect of another endogenous nucleotide, NAD, on P1 and P2 receptors in RTA, PMA and PCA. Unlike ATP and ADP, the effect of NAD on purine receptors is not well explored.

## **Chapter Two**

### **Effects of acyl-CoA on P2Y receptor-mediated vasorelaxations in rat thoracic aorta**

## 2.1 Introduction

The effect of coenzyme A (CoA) and CoA derivatives were investigated and shown to be antagonists at recombinant human P2Y receptors expressed in *Xenopus laevis* oocytes (Coddou et al., 2003). CoA derivatives were found to antagonize the ATP-evoked currents mediated through the P2Y<sub>1</sub>, but not the P2Y<sub>2</sub>, receptor in a concentration-dependent manner (Coddou et al., 2003). Coddou et al. also found that of all the CoA compounds investigated (CoA, nafenopin-CoA, ciprofibril-CoA, acetyl CoA (AcCoA) and palmitoyl CoA (PaCoA)), nafenopin CoA was the most potent antagonist. This experiment was conducted using human recombinant P2Y<sub>1</sub> receptors. Another study examined the effect of acyl-CoA at endogenous P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors on human platelets and concluded that PaCoA acted mainly at P2Y<sub>1</sub>, but also evoked a partial antagonism at P2Y<sub>12</sub> receptors and that it may function as an endogenous modulator of platelet function (Manolopoulos et al., 2008). The effects of these compounds at endogenous P2Y receptors in blood vessels are unknown.

ADP induced relaxations are mediated by P2Y<sub>1</sub> receptors on the endothelium of the rat thoracic aorta (Bultmann et al., 1998). The present study investigated, using the rat thoracic aorta, whether the relaxation mediated by ADP through the P2Y<sub>1</sub> receptor in this tissue can be blocked with CoA and its derivatives, AcCoA and PaCoA.

Furthermore, it aimed to test the selectivity of these compounds for P2Y<sub>1</sub> receptors, given that it has previously been shown that P2Y<sub>1</sub>, P2Y<sub>2</sub> and A<sub>2</sub> receptors are expressed on the endothelium of the rat thoracic aorta (Rose-Meyer and Hope, 1990, Hansmann et al., 1997).

## **2.2 Materials and Methods**

### **2.2.1 Rat thoracic aorta preparation and isometric tension recording of ADP and UTP responses**

Male Wistar rats (200-250 g), obtained from Charles River (England, UK), were used in this study. After stunning, they were killed by cervical dislocation. The rat thoracic aorta (RTA) was removed and placed in oxygenated Krebs-Henseleit solution (composition mmol/l: NaCl 118.4, KCl 4.7, MgSO<sub>4</sub> 1.25, CaCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 24.9, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11.1) in order to rinse off any blood. RTA were then placed on a paraffin plate and covered with fresh Krebs-Henseleit solution and fine dissection was carried out to remove any excess connective tissue. Vessels were then cut into rings (3-4 mm in length). Sections from similar locations on each of the vessels used were taken each time for consistency.

The rings were mounted gently (to make sure not to damage the endothelium) between two stainless steel wire supports one of which was attached by a cotton thread to an isometric force transducer (Abingdon, Oxfordshire, UK) which measured the tension in the vessels through its connection to a Power Lab bridge amplifier (AD instrument, Abingdon, Oxfordshire, UK). The other wire was linked to a glass support.

Rings suspended on the wire supports were put in water-jacketed organ baths which were maintained at a temperature of 37 °C by a circulating water heater. Baths were previously filled with 15 ml oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs-Henseleit buffer solution. Mounted rings were initially tensioned to 1 g and allowed to equilibrate for 30 min after which a further 1 g of tension was added and the rings left to equilibrate for another 30 min.

After the equilibration period, KCl (60 mM) was added and the contraction noted once it reached a plateau (usually 15 min). The rings were then washed and left for 20 min to relax, after which, the same process was repeated. After contraction to the second exposure to KCl, the rings were washed and left for 1 h to relax. Only tissues which contracted to KCl to at least 0.4 g were used. The second contraction to KCl is the only one that was measured.

After the 1 h relaxation period and in order to investigate the relaxant properties of the agonists used, the rings were precontracted with methoxamine (0.3-2.0 μM) (Sigma Chemical Company) to a tension of 55-75 % of the KCl contraction. Stepwise cumulative addition of agonist (ADP, UTP) (Sigma Chemical Company) to the preparations was then carried out. Each concentration of agonist was left in contact with the tissue for 3-6 min to reach a point where no further



relaxation was seen. At this point, the next concentration of agonist was added.

For checking whether the responses were endothelium dependent or endothelium-independent, denudation was achieved by rubbing the lumen gently with forceps, and acetylcholine (100 nM) was used to assess success in removing the endothelium. Any vessel with a relaxation in response to acetylcholine of less than 10% of the methoxamine contraction was considered a denuded vessel.

Time control experiments were performed using the same protocol; after contracting vessels with methoxamine they were left for an hour to check for any loss of tone.

### **2.2.2 Effect of CoA and acyl-CoA on responses to ADP and UTP**

To investigate the possible antagonistic effects of PaCoA, AcCoA, CoA (Sigma Chemical Company) and MRS2179 (Tocris) on responses to ADP or UTP, these compounds were added after the 1 h relaxation period 10 min before methoxamine addition. Relaxation–response curves to ADP and UTP were then generated as described previously. Only a single antagonist was investigated in any one blood vessel ring.

In studying the reversibility of the PaCoA antagonism, rings were tensioned, equilibrated, KCl added and precontracted using the same protocol described above. Three rounds of addition of ADP (100  $\mu$ M) were performed. In the first round, in the absence of PaCoA, ADP (100  $\mu$ M) was added and the response was recorded, after which rings were washed out and left for 1hr. Then PaCoA (10  $\mu$ M) was added 10 min before recontracting with methoxamine, then ADP (100  $\mu$ M) was added to the organ bath and responses were recorded again. The rings were then washed again and left for 1 hr after which they were recontracted with methoxamine again and the response to the same concentration of ADP was recorded to check the reversibility of the PaCoA antagonism. Control experiments were performed using a similar protocol, but in the absence of PaCoA.

In separate experiments to check for any direct effect of PaCoA, 10  $\mu$ M was added after contracting the vessels with methoxamine and left in the organ bath for 1 hr. Readings were taken every 6 min to check for any change in tone.

### **2.2.3 Materials**

The supplier for all chemicals in this work was British Drug Houses (BDH, UK) unless otherwise stated. Drugs were dissolved in distilled water.

### **2.2.4 Statistical analysis**

The data were viewed and recorded using Chart for Windows v4.1. Results are expressed as mean  $\pm$  SEM. Two way ANOVA was used for statistical comparisons with Bonferroni post-hoc test. A P value  $< 0.05$  was taken as statistically significant.

## 2.3 Results

Addition of KCl (60 mM) produced a sustained contractile response of RTA. The mean tissue response to KCl addition was  $0.88 \pm 0.02$  g (n=39). Methoxamine caused a sustained contraction to 55-80% of the KCl response. The mean bath concentration of methoxamine required to produce this level of contraction was  $1.3 \pm 0.11$   $\mu$ M (n= 39).

### 2.3.1 Effect of ADP and UTP in rat thoracic aorta

ADP and UTP (0.1  $\mu$ M- 1mM) were added to the organ bath cumulatively. Both elicited concentration dependent relaxations in aortic rings precontracted with methoxamine (Figure 2.1).

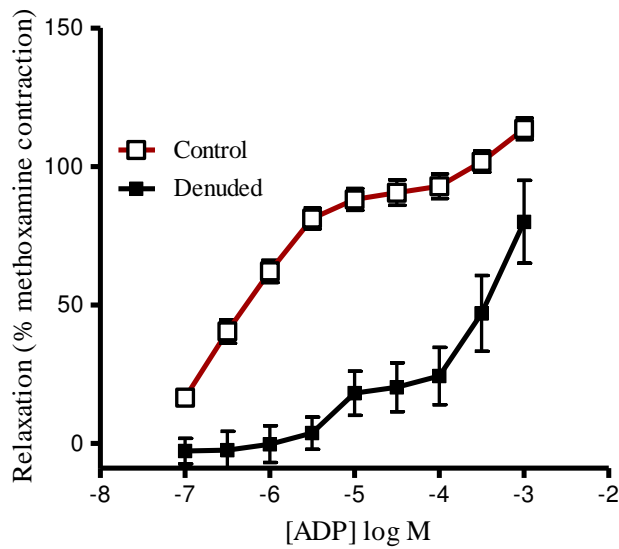
Figures 2.1a and 3 show the relaxation of rat aorta to ADP; trying to use 2 site curve fitting (Prism) to fit lines to the data was unsuccessful. One site curve fitting indicated a complete reversal of the methoxamine contraction by ADP with maximal relaxation observed typically at concentrations of 300  $\mu$ M or greater. The response to ADP did not reach a maximum, but almost fully reversed the methoxamine-induced precontraction so approximate values of  $R_{max}$  ( $103 \pm 4$ ),  $pEC_{50}$  ( $6.00 \pm 0.12$ ) and Hill slope ( $0.81 \pm 0.07$ ) (n=19) were calculated. Removing the endothelium caused a significant inhibition of the ADP-induced responses (Figure 2.1a).

In contrast, the response to UTP was biphasic with a peak relaxation at 3  $\mu$ M; concentrations greater than this caused a reduced relaxation of the aortic rings (Figure 2.1b). As noticed from the curve, the slope was very steep unlike the case with ADP. Non linear analysis for responses up to a concentration of 3  $\mu$ M of UTP resulted in  $R_{\max}$  values of  $94 \pm 5$ , a  $pEC_{50}$  value of  $6.64 \pm 0.04$  and Hill slope of  $2.02 \pm 0.20$  (n=8).

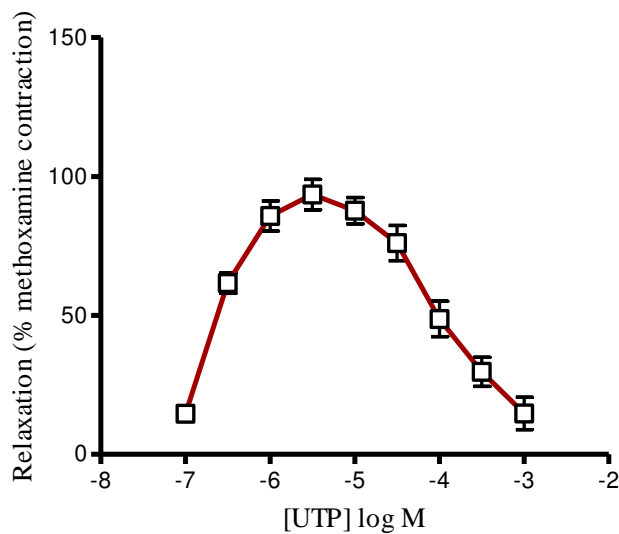
Acetylcholine (100 nM) was used to assess endothelium removal. Any relaxation in response to acetylcholine of less than 10% of the methoxamine contraction was considered a success. There was a very slight relaxation ( $2 \pm 0.4$  %, n = 6) in response to acetylcholine in denuded vessels compared to the profound relaxation evoked in controls ( $65 \pm 0.4$  %, n = 10).

**Figure 2.1.** Responses of the rat thoracic aorta evoked by a. adenosine diphosphate (ADP), in endothelium-intact and -denuded vessels, and b. uridine triphosphate (UTP) in endothelium-intact vessels. Vessels were precontracted with methoxamine. Data for ADP and UTP are mean  $\pm$  SEM (n=6-19, n= 8, for a and b, respectively).

a



b



### 2.3.2 Effect of CoA, acyl-CoA and MRS2179 on relaxations to ADP

In the presence of PaCoA (10  $\mu$ M), AcCoA (10  $\mu$ M) and CoA (10  $\mu$ M), the mean bath concentrations of methoxamine required to elicit 55-75% of the KCl contraction were unchanged when compared to the control ( $P > 0.05$ , one way ANOVA):  $1.42 \pm 0.18 \mu\text{M}$ ,  $n = 13$ ;  $1.56 \pm 0.16 \mu\text{M}$ ,  $n = 12$ ;  $1.39 \pm 0.17 \mu\text{M}$ ,  $n = 11$ , for PaCoA, AcCoA and CoA, respectively.

There was a trend for a rightward by CoA to the concentration:relaxation response curve to ADP (Figure 2.2a). 1  $\mu$ M, 3  $\mu$ M and 10  $\mu$ M CoA did not cause significant shift in the response to ADP (Table 2.1). Higher concentrations of CoA were not used due to cost considerations.

AcCoA also caused a rightward shift in the concentration:relaxation response curve to ADP (Figure 2.2b). Analysis showed that a concentration of 10  $\mu$ M AcCoA caused a 5-fold shift in the relaxations to ADP (Table 2.1). Concentrations of 1  $\mu$ M and 3  $\mu$ M CoA had no significant effect on the response to ADP.

For PaCoA, a concentration dependent rightward shift in the relaxation response curve to ADP was found (Figure 2.2c, 2.3). Schild analysis showed that concentrations of 1, 3, 10  $\mu$ M PaCoA caused

shifts of 18-, 55- and 331-fold of the response to ADP, respectively (Table 2.1).

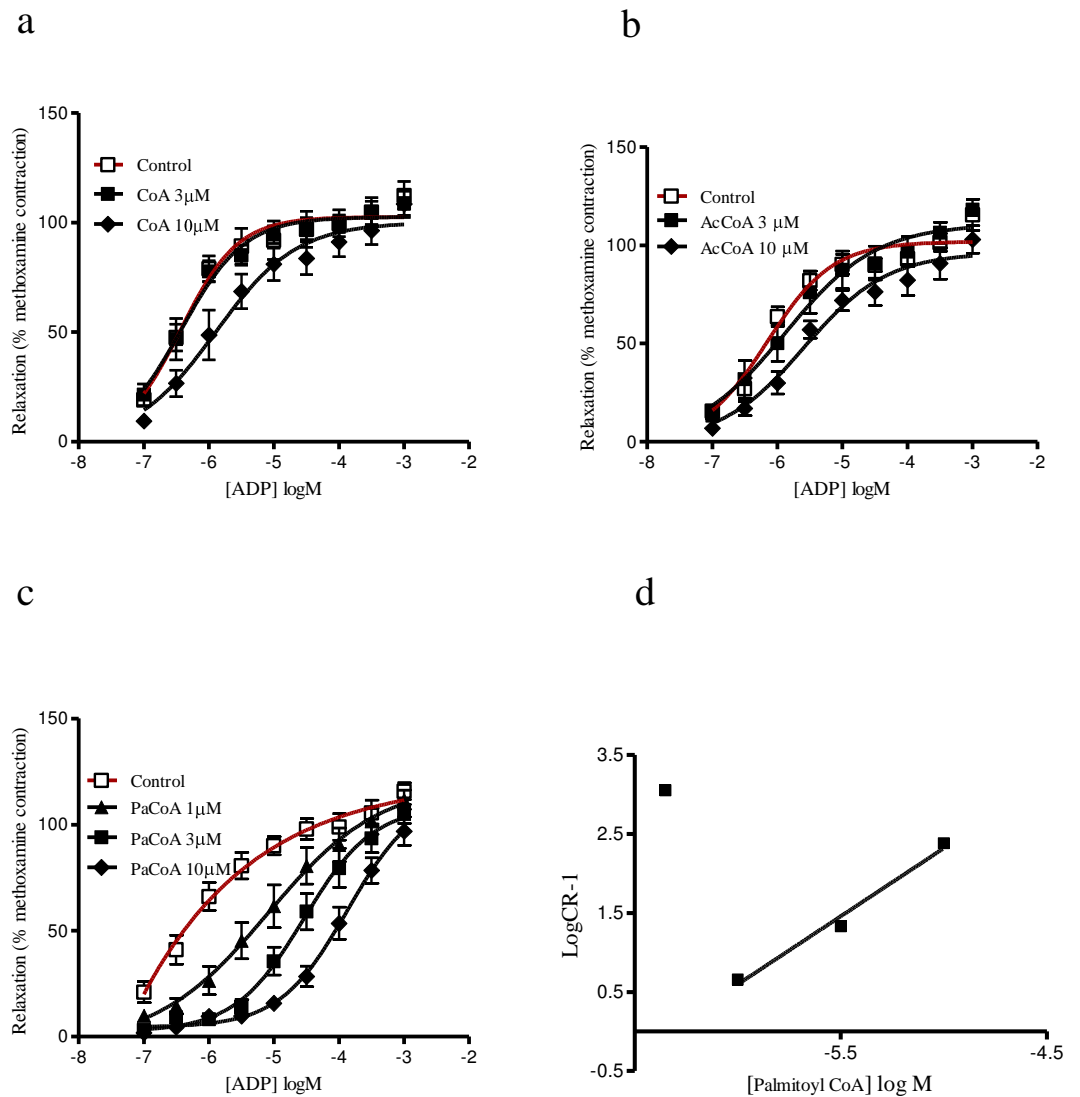
Figure 2.2d shows the Schild plot for PaCoA; the calculated slope was  $1.73 \pm 0.4$  with  $pA_2$  value of  $6.44 \pm 0.12$ . A  $\log pK_B$  value of  $5.32 \pm 0.25$  for AcCoA was calculated (n =5).

MRS2179 (1 $\mu$ M) caused a 29-fold rightward shift of the ADP response (Figure 2.4, Table 2.1), allowing calculation, using the Gaddum transformation, of an apparent  $pK_B$  value of  $7.32 \pm 0.17$  (n= 5).

We tested the reversibility of the PaCoA (10  $\mu$ M) inhibition of the response to ADP (100  $\mu$ M), which was shown to be reversible (Figure 2.5). The direct effect of PaCoA was assessed and it was shown that PaCoA had no direct effect on the RTA when compared to time controls (Figure 2.6).



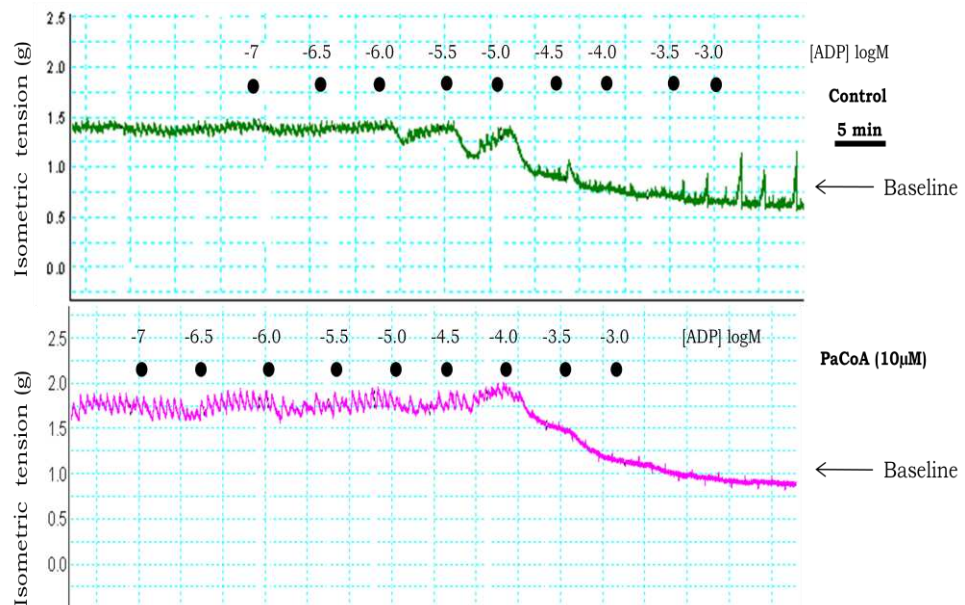
**Figure 2.2** Adenosine diphosphate (ADP) -evoked relaxation of the rat thoracic aorta in the presence of a. coenzyme A (CoA), b. acetyl CoA (AcCoA) and c. palmitoyl CoA (PaCoA). d. shows the Schild plot for these curves. Vessels were precontracted with methoxamine. Data are shown as mean  $\pm$  SEM (n= 5-6, n= 6-7, n= 7, respectively). In figure (a) and (b) the data for 1 $\mu$ M CoA and AcCoA were removed for clarity.



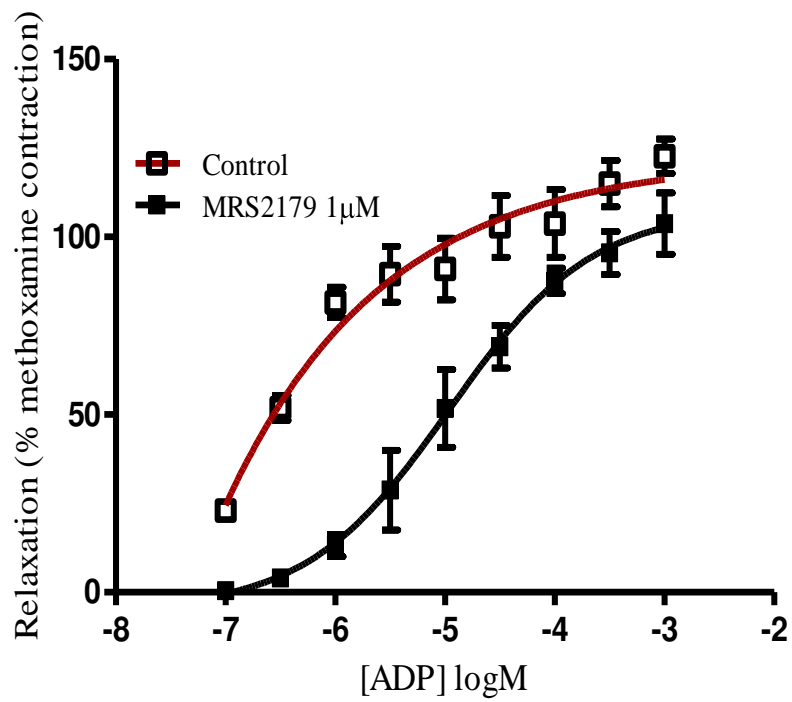
**Table 2.1.** Effect of coenzyme A (CoA), acetyl CoA (AcCoA), palmitoyl CoA (PaCoA) and MRS2179 on ADP evoked relaxations of the rat thoracic aorta. Results are mean  $\pm$  SEM.

Table 1	pEC <sub>50</sub>	Rmax	Hill slope
ADP (control) (n=5)	6.37 $\pm$ 0.06	104 $\pm$ 6	0.99 $\pm$ 0.15
ADP+ CoA (1 $\mu$ M) (n=6)	6.19 $\pm$ 0.10	111 $\pm$ 4	0.87 $\pm$ 0.19
ADP+ CoA (3 $\mu$ M) (n=6)	6.44 $\pm$ 0.11	102 $\pm$ 5	1.21 $\pm$ 0.20
ADP+ CoA (10 $\mu$ M) (n=6)	5.74 $\pm$ 0.29	106 $\pm$ 7	0.84 $\pm$ 0.16
ADP (control) (n=6)	6.19 $\pm$ 0.07	101 $\pm$ 4	1.0 $\pm$ 0.16
ADP+ AcCoA (1 $\mu$ M) (n=7)	6.00 $\pm$ 0.14	98 $\pm$ 6	1.06 $\pm$ 0.18
ADP+ AcCoA (3 $\mu$ M) (n=7)	5.54 $\pm$ 0.50	112 $\pm$ 6	1.07 $\pm$ 0.17
ADP+ AcCoA (10 $\mu$ M) (n=7)	5.51 $\pm$ 0.15*	102 $\pm$ 14	0.70 $\pm$ 0.11
ADP (control) (n=7)	6.09 $\pm$ 0.20	113 $\pm$ 7	0.97 $\pm$ 0.30
ADP+ PaCoA (1 $\mu$ M) (n=7)	4.84 $\pm$ 0.36*	128 $\pm$ 7	0.64 $\pm$ 0.08
ADP+ PaCoA (3 $\mu$ M) (n=7)	4.35 $\pm$ 0.26**	122 $\pm$ 7	0.81 $\pm$ 0.07
ADP+ PaCoA (10 $\mu$ M) (n=7)	3.57 $\pm$ 0.31***	127 $\pm$ 16	0.80 $\pm$ 0.12
ADP (control) (n=5)	6.37 $\pm$ 0.37	125.1 $\pm$ 10	0.71 $\pm$ 0.21
ADP+ MRS2179 (1 $\mu$ M) (n=5)	5.07 $\pm$ 0.26*	102.3 $\pm$ 9	1.07 $\pm$ 0.07

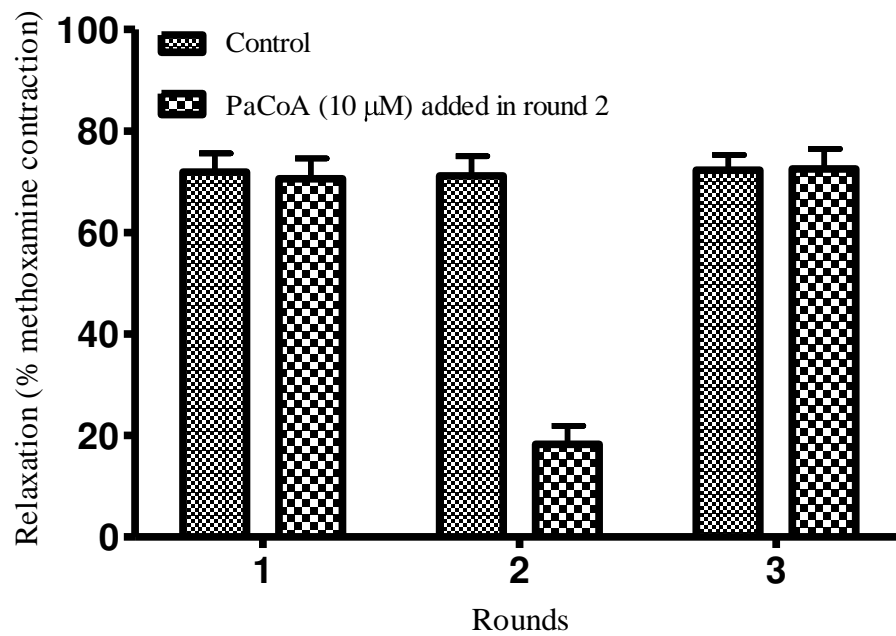
**Figure 2.3.** A representative trace for the response of the rat thoracic aorta to adenosine diphosphate (ADP) in the absence and presence of palmitoyl coenzyme A (PaCoA). Vessels were precontracted with methoxamine.



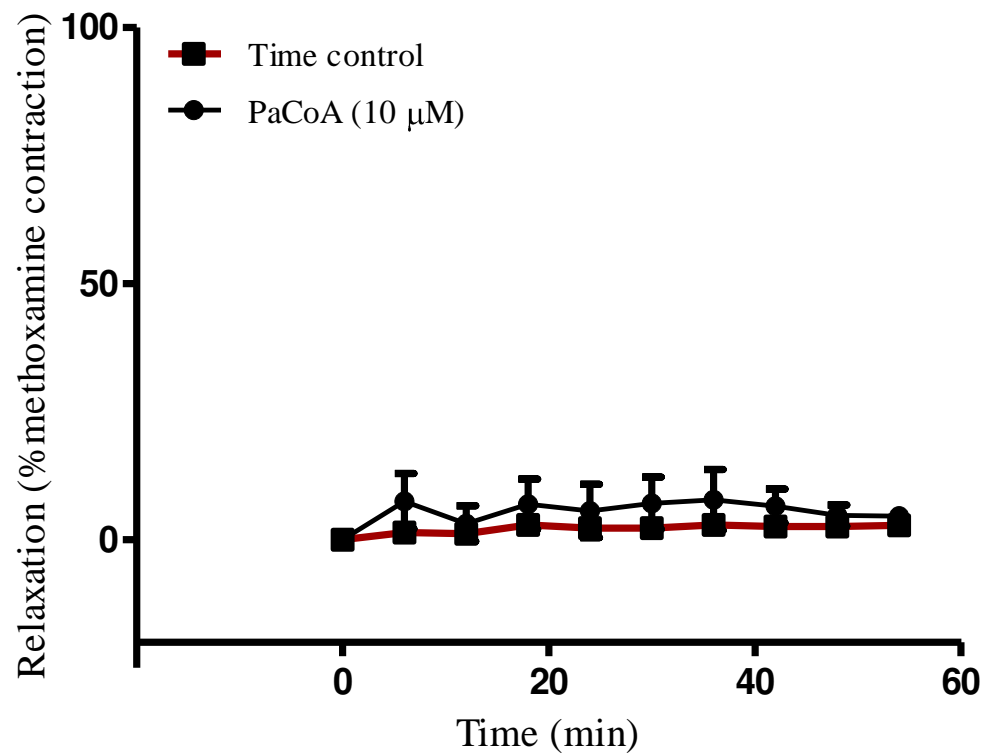
**Figure 2.4.** The effect of MRS2179 on the relaxation of the rat thoracic aorta by adenosine diphosphate (ADP). Vessels were precontracted with methoxamine. Data are mean  $\pm$  SEM, (n=5).



**Figure 2.5.** Reversibility of PaCoA-mediated inhibition of ADP-evoked relaxation of the rat thoracic aorta. After a control ADP relaxation and washout (round 1), ADP was added in the presence of palmitoyl CoA (PaCoA) (round 2) and after the washout of PaCoA (round 3). Control experiments were performed in the absence of PaCoA. Vessels were precontracted with methoxamine. Data are shown as mean  $\pm$  SEM (n= 8).



**Figure 2.6.** The effect of palmitoyl coenzyme A (PaCoA) in the rat thoracic aorta compared to time control. Readings were taken every 6 minutes. Vessels were precontracted with methoxamine. Results are shown as mean  $\pm$  SEM (n= 4-5).



### **2.3.3 Effect of CoA, acyl-CoA and MRS2179 on relaxations to UTP**

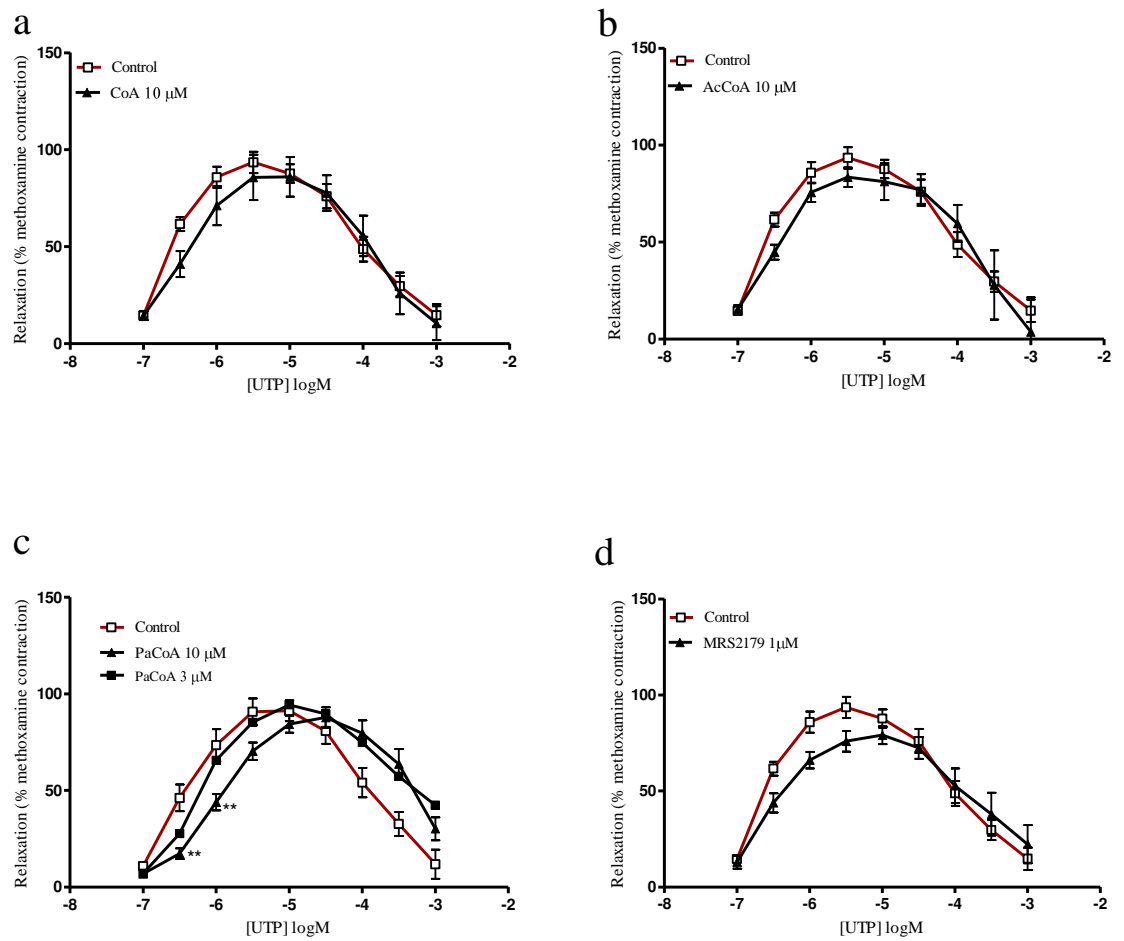
In order to examine the selectivity of CoA analogues, UTP was employed as a vasorelaxant. When analyzing the data, this was done up to the concentration of 3  $\mu\text{M}$  of UTP (at higher concentrations UTP started to elicit contraction).

Statistical analysis using two way ANOVA indicated that neither CoA (10  $\mu\text{M}$ ) nor AcCoA (10  $\mu\text{M}$ ) had a significant effect on the relaxation response to UTP (Figure 2.7a, b).

For PaCoA, a concentration-dependent rightward shift in the relaxation response curve to UTP was observed (Figure 2.7c). There was no significant effect of 3  $\mu\text{M}$  PaCoA, while 10  $\mu\text{M}$  PaCoA caused a 3-fold shift at concentrations of 0.3 and 1.0  $\mu\text{M}$  of UTP.

MRS2179 (1  $\mu\text{M}$ ) caused no significant shift in the UTP relaxation response (two way ANOVA, Figure 2.7d).

**Figure 2.7.** The response of the rat thoracic aorta to uridine triphosphate (UTP) in the presence of a. coenzyme A (CoA), b. acetylCoA (AcCoA), c. palmitoyl CoA (PaCoA) and MRS2179 (n= 6-8, n= 6-8, n=8-10, n=8-12, respectively). Vessels were precontracted with methoxamine. Results are shown as mean  $\pm$  SEM. (\*\*) indicate significant shift.





## **2.4 Discussion**

In the present study, agonists for P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors, ADP and UTP respectively, were used to examine effects of acyl-CoA derivatives on the relaxation of precontracted RTA. The results indicate that, of the three acyl CoA derivatives used in this study (CoA, AcCoA and PaCoA), the most potent CoA derivative is PaCoA acting as an antagonist with apparent selectivity for the P2Y<sub>1</sub> receptor in RTA.

### **2.4.1 Effect of ADP and UTP in rat thoracic aorta**

Concentration-dependent relaxation was produced in the presence of ADP or UTP. It was previously shown that UTP produced relaxation through P2Y<sub>2</sub> receptors on the endothelium of the rat thoracic aorta (Dol-Gleizes et al., 1999) and in the rat mesenteric arterial bed.(Buvinic et al., 2002) This contrasts with the conclusions of Guns et al. 2006 investigating mouse thoracic aorta, where they suggested that the P2Y<sub>2</sub> receptor had no major role in UTP-evoked relaxation and that the vasodilator effect of UTP was probably mediated mainly by a P2Y<sub>6</sub>-like receptor (Guns et al., 2006).

ADP was shown to induce relaxations mediated by P2Y<sub>1</sub> receptors on the endothelium of the rat thoracic aorta (Dol-Gleizes et al., 1999), rat mesenteric bed (Ralevic et al., 1995) and mouse aorta (Guns et al.,

2005). In the current study, we confirmed that the response to ADP was endothelium-dependent, since removing the endothelium evoked a significant inhibition of these relaxations.

The relaxations mediated by ADP, as shown in Figure 1a, were shallow and obviously different from relaxations mediated by UTP which were very steep (Figure 1b). This suggests that these two ligands mediate relaxations via different receptors, consistent with evidence that ADP acts as an agonist at P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors and that UTP is active at P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors (von Kugelgen, 2006).

In the present study, the pEC<sub>50</sub> value of ADP was found to be 6.00, similar to the pEC<sub>50</sub> value of 6.2 reported for the rat thoracic aorta previously (Dol-Gleizes et al., 1999). It is also similar to that found in mouse thoracic aorta (6.22) (Guns et al., 2005).

Responses evoked by UTP were biphasic, which may indicate that there are multiple elements for this response. The agonist potency (pEC<sub>50</sub>) of UTP in the current study was 6.64. A pEC<sub>50</sub> value of 5.83 was reported for UTP in the rat thoracic aorta (Dol-Gleizes et al., 1999); in the mouse thoracic aorta, a pEC<sub>50</sub> value of 6.46 was found (Guns et al., 2006). The authors suggested that responses were mediated through P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors, respectively. In the former study (Dol-Gleizes *et al.*, 1999), non-selective agonists and antagonists were used to define the nature of the receptor, while P2Y<sub>2</sub> receptor-

null mice allowed a more defensible identification in the latter investigation (Guns *et al.*, 2006). In the current study the pharmacology of the UTP responses was not analysed further. Whether UTP is acting through P2Y<sub>2</sub> or P2Y<sub>6</sub> receptors could be confirmed by the use of the P2Y<sub>6</sub> receptor-selective antagonists, MRS2578 or MRS2567 (Mamedova *et al.*, 2004).

At concentrations higher than 3  $\mu$ M, UTP-evoked relaxations were reversed; this may be due to an action on P2Y<sub>2</sub> receptors on the smooth muscle (Eltze and Ullrich, 1996). This response of UTP was not investigated in the current study and all the analysis of the effects of antagonists on the UTP response was performed up to the concentration of 3  $\mu$ M. This response for UTP can be tested by removing the endothelium to check the effect on these responses. Although AR-C118925XX is an example from the literature of a selective P2Y<sub>2</sub> receptor antagonist with submicromolar activity (Meghani, 2002), there are no commercially available P2Y<sub>2</sub> antagonists.

#### **2.4.2 Effect of acyl-CoA on concentration-dependent relaxation observed with ADP**

Antagonism evoked by PaCoA was found to be reversible, which agrees with what was reported in *Xenopus laevis* oocytes (Coddou *et al.*, 2003). PaCoA was found to have no direct effect on the RTA, suggesting there was no tonic activation of the target receptor or

activation by PaCoA of other targets in the absence of ADP. PaCoA was more potent than CoA and AcCoA as an antagonist at P2Y<sub>1</sub> receptors. This agrees with the findings in *Xenopus laevis* oocytes and human platelets (Coddou et al., 2003, Manolopoulos et al., 2008). 10 μM PaCoA caused an approximately 330-shift of ADP-evoked relaxations, while there was a 4- and 5-fold shift in the presence of 10 μM CoA and AcCoA, respectively. This increase in potency appears to be due to the increase in hydrophobicity from CoA, AcCoA to PaCoA (Coddou et al., 2003). The nucleotide moiety of the CoA compounds exhibits structural similarities with ADP, which suggests a ligand binding domain focussed on the purine binding site. In addition to this binding site, it may be that there is a second binding site, a hydrophobic pocket adjacent to the purine binding site to accommodate the lipophilic acyl-substituent; this pocket would help stabilize the interaction of the antagonist with the receptor (Coddou et al., 2003) (Chapter One). This could be tested using site-directed mutagenesis where alanine scanning could be performed to identify the site of action of PaCoA (as well as CoA and other CoA derivatives).

MRS2179 (1 μM) is a selective competitive antagonist at the P2Y<sub>1</sub> receptor (Boyer et al., 2002, Buvinic et al., 2002). In the presence of MRS2179, relaxations to ADP were significantly inhibited. The pK<sub>B</sub> value for MRS2179 obtained (7.32) is similar to the one reported in human recombinant P2Y<sub>1</sub> receptors expressed in *Xenopus laevis* oocytes where it was reported to be 7.7 (Coddou et al., 2003). The pK<sub>B</sub> value calculated was higher than the one reported for turkey

erythrocytes ( $pK_B = 6.46$  (Boyer et al., 1996a);  $6.99$  (Boyer et al., 1998). In the rat mesenteric arterial bed, a value of  $8.03$  for the  $pK_B$  was reported (Buvinic et al., 2002). The differences in affinity are presumably due to differences in the species or tissues used. Methodological differences may also be influential, since the study in turkey erythrocytes assessed adenine nucleotide-promoted inositol phospholipid hydrolysis response of turkey erythrocyte membranes; in the rat arterial mesenteric bed, changes in perfusion pressure were measured; while ATP activated currents were studied in *Xenopus laevis* oocytes.

#### **2.4.3 Effects of acyl-CoA on concentration-dependent relaxations to UTP**

CoA ( $10 \mu\text{M}$ ) and AcCoA ( $10 \mu\text{M}$ ) had no significant effect on the UTP relaxations, while PaCoA ( $10 \mu\text{M}$ ) caused a significant rightward shift of these relaxations. This shift was small (3-fold) when compared to the 330-fold shift PaCoA caused to the ADP-evoked relaxations via  $P2Y_1$  receptors. This indicates that these compounds are reasonably selective for  $P2Y_1$  versus  $P2Y_2$  receptors. This is consistent with the effect of these compounds at recombinant  $P2Y_1$  and  $P2Y_2$  receptors in *Xenopus* oocytes (Coddou et al., 2003).

MRS2179 had no significant effect on UTP-evoked relaxations, consistent with responses to UTP acting through a target other than the P2Y<sub>1</sub> receptor (Buvinic et al., 2002).

#### **2.4.4 Acyl-CoA as an endogenous modulators**

The relevance of *in vitro* studies of acyl-CoA to the situation *in vivo* has been questioned in some reports because of its detergent-like properties, which can result in rather nonspecific effects (Srere, 1965, Taketa and Pogell, 1966) (Chapter One). However, several other later reports have contradicted this and shown that acyl-CoA may play a regulatory role *in vivo* under certain circumstances since many enzymes are inhibited by acyl-CoA below the critical micelle concentration at which they have detergent-like properties (Harris et al., 1972, Lerner et al., 1972, Tippett and Neet, 1982b, Agius et al., 1987, Li et al., 1990, Hertz et al., 1998, Boylan and Hamilton, 1992) (Chapter One).

This maintenance of low concentrations of acyl-CoA is achieved via the fact that long chain acyl-CoA have high affinity for phospholipid bilayers; this helps to keep low levels of acyl-CoA (Powell et al., 1985) (Chapter One). In addition, acyl-CoA binding proteins (ACBPs) and fatty acid binding proteins (FABPs) keep the level of free acyl-CoA low (Chapter One). It was found that the length of the acyl chain can control the affinity of the acyl-CoA compounds for membranes, and

also their residence time in these membranes (Boylan and Hamilton, 1992) (Chapter One). It is possible that this can explain the higher potency of PaCoA as an antagonist at membrane P2Y<sub>1</sub> receptors compared to AcCoA and CoA.

There is no evidence about whether acyl-CoA is present extracellularly under normal physiological conditions although it has been shown that PaCoA synthetase is present on the external surface of rat isolated hepatocytes (de Groote et al., 1996). However in certain pathophysiological conditions such as heart ischaemia the cellular expression of FABPs is increased, leading to an overflow of these proteins into the blood and may also release the bound ligand into the serum (Chapter One). In addition, it was also reported that in certain pathophysiological states such as fasting, ischaemia and hypoxia, high levels of acyl-CoA accumulate and can cause irreversible damage to cellular membranes (Bortz and Lynen, 1963, Whitmer et al., 1978) (Chapter One). This indicates that this class of compounds may contribute to some pathophysiological conditions (Chapter One). The possibility that these compounds may be released during pathophysiological conditions was also reported (Glatz et al., 1993, Vork et al., 1993a, Vork et al., 1993b). Once released, it is possible that acyl-CoA derivatives will act as endogenous modulators on many levels such as platelet function and vascular regulation (through P2Y<sub>1</sub> receptors) which may implicate a key role of these compounds. The present study considered only the effect of CoA and its derivatives on P2Y<sub>1</sub>-mediated blood vessel relaxation, but the vascular P2Y<sub>1</sub> receptors

is also involved in regulation of endothelium proliferation and migration (Shen and DiCorleto, 2008).

CoA and its acyl CoA derivatives were shown to be antagonists at human recombinant P2Y<sub>1</sub> receptors expressed in *Xenopus laevis* oocytes. (Coddou et al., 2003). Also the effect of acyl derivatives of CoA compounds was examined at endogenous P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors on platelets leading to the conclusion that PaCoA is an antagonist at P2Y<sub>1</sub> and has a partial antagonism at P2Y<sub>12</sub> receptors and that it may function as an endogenous modulator of platelet function (Manolopoulos et al., 2008). The effect of chain length has been studied and it was found that increased acyl-CoA side-chain length increased the potency of these compounds as antagonists at P2Y<sub>1</sub> receptors (Coddou et al., 2003, Manolopoulos et al., 2008, Chapter One). The effect of the degree of saturation of acyl-CoAs chain was also studied and it was reported that the higher the degree of saturation the higher the potency will be for these compounds (Riedel and Light, 2005 Manolopoulos et al., 2008, Chapter One). In the present study, PaCoA was shown to have higher potency as an antagonist at P2Y<sub>1</sub> receptors compared to the shorter-chain acyl-CoAs used, AcCoA and CoA, in RTA. This agrees with the above reports about the effect of chain length of these compounds. The present study also supports suggestions that these compounds can act as endogenous modulators of cell-surface receptors.



Looking at previous findings of effects of acyl-CoA derivatives (Chapter One), and looking at our findings of the effect of PaCoA in blood vessels it is clear that these compounds modulate several cellular functions, receptors, enzymes and other cellular processes and should be considered for further investigations trying to determine their patho/physiological significance. In blood vessels it may be especially relevant to consider the role of these compounds in pathophysiological conditions such as hypertension and they may prove to be relevant in the design of novel compounds that degrade or bind and inactivate acyl-CoA compounds.

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## **Chapter Three**

### **Effect of palmitoyl CoA on ADP-evoked vasorelaxations in porcine isolated coronary and mesenteric arteries**

### 3.1 Introduction

In the rat thoracic aorta, using isometric tension recording, we examined the effects of CoA, AcCoA and PaCoA at vasorelaxant endothelial P2Y<sub>1</sub> receptors and showed that these compounds are antagonists at P2Y<sub>1</sub> receptors with PaCoA being the most potent (Chapter 2). It is known that species differences can affect both agonist and antagonist potencies. For example PPADS was shown to be inactive at smooth muscle P2Y<sub>1</sub>-like receptors in rabbit mesenteric artery (Ziganshin et al., 1994), but it blocked those in the rat mesenteric arterial bed (Ralevic and Burnstock, 1996). Also significant pharmacological differences concerning the antagonist potencies between the human and rat P2X<sub>4</sub> receptors were identified (Garcia-Guzman et al., 1996). Therefore, we wanted to investigate whether PaCoA would have a similar antagonistic effect in other different species and different vessels, so we moved on from rat to pig tissue and used two different vessels, specifically porcine mesenteric artery (PMA) and porcine coronary artery (PCA).

ADP mediates endothelium-dependent vasodilatation via activation of P2Y<sub>1</sub> receptors (Nicholas et al., 1996a, Guns et al., 2005); such as in rat and mouse mesenteric arteries (Buvinic et al., 2002, Harrington et al., 2007). Little is known about P2Y<sub>1</sub> in PMA. Endothelial P2Y<sub>1</sub> receptors have also been shown to mediate responses to ADP in PCA (Olivecrona et al., 2004). PCA are also reported to express A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptors (Merkel et al., 1992, Abebe et al., 1994, Monopoli et al., 1994, Balwierczak et al., 1991). In a variety of species, coronary

arteries express vasorelaxant P2Y receptors on the smooth muscle cells (Keef et al., 1992, Corr and Burnstock, 1994, Simonsen et al., 1997).

In both tissues (PMA and PCA), we started first by generally characterising P2Y<sub>1</sub> receptors using ADP as an agonist. Then a selective P2Y<sub>1</sub> antagonist (MRS2500) was used to confirm or exclude any involvement of P2Y<sub>1</sub> receptors (Kim et al., 2003). Also, by removing the endothelium in both PMA and PCA, it was hoped to identify whether the receptor involved was located on the endothelium or smooth muscle.

The main aim of the present study was to expand our findings in the rat thoracic aorta by showing that PaCoA acts as an antagonist of P2Y<sub>1</sub>-mediated, ADP-evoked relaxations in another species and in different vessels, specifically in PMA and PCA. Therefore we aimed to characterise the effect of ADP on PMA and PCA and to investigate whether PaCoA can block the responses mediated by ADP in these tissues.

## **3.2 Materials and Methods**

### **3.2.1 Porcine mesenteric artery and porcine coronary artery preparation and isometric tension recordings of ADP responses**

Porcine mesenteries and hearts, obtained from a local abattoir (Woods abattoir, Clipstone, Mansfield, Nottinghamshire), were placed in oxygenated Krebs-Henseleit solution at 4°C before being transported to the laboratory. First order porcine mesenteric arteries (PMA) or the interior descending branch of the coronary artery (PCA) were dissected out, placed in oxygenated Krebs-Henseleit solution in order to rinse off any blood, and kept overnight at 4°C. The next day, the vessels were placed on a paraffin plate and covered with fresh Krebs-Henseleit solution and fine dissection was carried out to remove any excess connective tissue. Vessels were then cut into rings (3-4 mm in length). Sections from similar locations on each of the vessels were taken each time for consistency.

PMA and PCA rings were mounted for isometric tension recording as described in Chapter Two. Mounted rings were tensioned to 10 g, left to relax for 1 h before KCl (60 mM) was added and the contraction recorded once it reached a plateau. The rings were then washed and left for 20 min to relax, after which, the same process was repeated. After contraction to the second exposure to KCl, the rings were washed and left for 1 h to relax. Only tissues which contracted to KCl

to at least 4.0 g on the second exposure were used. Only values for the second contraction to KCl are reported.

For precontraction, U46619 (11 $\alpha$ , 9 $\alpha$ -epoxymethano-PGH<sub>2</sub> (a thromboxane A<sub>2</sub> analogue) was used (30- 763 nM) to achieve a tension of 50-75% of the KCl-induced contraction. Stepwise cumulative addition of agonist (ADP, 0.1  $\mu$ M- 1 mM) (Sigma Chemical Company, UK) to the preparations was then carried out as described in Chapter Two.

Endothelium denudation was achieved by rubbing the lumen gently with forceps; any vessel with a relaxation in response to substance P (10 nM, Sigma Chemical Company) of less than 10% of the U46619-evoked contraction was considered a denuded vessel. In control vessels, substance P caused a rapid, transient relaxation that was absent in denuded vessels. Time control experiments were performed with the same protocol; after contracting with U46619, vessels were left for an hour to check for any loss of tension. Blood vessel tension was measured every 6 minutes.

### **3.2.2 Effect of PaCoA on responses to ADP**

To investigate the possible antagonistic effects of PaCoA (10  $\mu$ M, Sigma Chemical Company) and MRS2500 (1  $\mu$ M, a selective P2Y<sub>1</sub> receptor

antagonist, Tocris-Cookson, UK) on responses to ADP, these compounds were added 10 min before U46619 addition.

To check for any direct effect of PaCoA, 10  $\mu$ M was added after contracting the vessels with U46619 and left in the organ bath for an hour. Blood vessel tension was then measured every 6 minutes.

### **3.2.3 Immunohistochemical Staining**

Porcine mesenteric and coronary arteries were dissected and cleaned from any excess connective tissue and cut into 4-5 mm rings. Blood vessel rings were fixed in 4% paraformaldehyde and kept overnight at 4°C. The rings were then washed in phosphate-buffered saline (PBS) (Sigma Chemical Company) and frozen with a few drops of OCT mounting solution and 10  $\mu$ m thick slices were created using a microtome and transferred to slides and stored at -80°C.

Whole-mount segments of porcine coronary and mesenteric arteries were stained using the standard indirect immunofluorescence technique, where the slides were removed from -80°C freezer and allowed to equilibrate at room temperature for 30 min. Triton X (Sigma Chemical Company) was used as a permeabilizing agent in PBS + 0.1% bovine serum albumin (BSA) and left for 20 min. Rings were then washed out 3 times using PBS+ 0.1% BSA after which human serum (Sigma Chemical Company) was used to block any non-specific binding in the samples at room temperature for 30 min. After that

human serum was aspirated and slices were washed out 3 times, then 50 µl primary rabbit antibody anti-P2Y<sub>1</sub> was added at (1:50) dilution in human serum. Samples were incubated overnight at 4°C. Then they were washed with PBS+0.1 % BSA 3 times. The secondary antibody anti rabbit IgG (FITC) (1:50) (Sigma Chemical Company) was diluted in PBS+0.1% BSA and added to the tissue slices for P2Y<sub>1</sub> receptor detection. Then the slides were incubated at 37°C for 30 min, followed by washing with PBS+ 0.1% BSA 3 times. The slices were then covered with vector shield mounting solution and glass cover slips. Samples were visualized using fluorescence microscopy using an objective magnification of 40X. A parallel set of controls for each slide was performed, in the control slides there was no addition for the primary antibody.

### **3.2.4 Materials**

The supplier for all chemicals in this work was British Drug Houses (BDH, UK) unless otherwise stated. Antibodies were obtained from Calbiochem/Merck Biosciences (Nottingham, UK, anti-A2A Ab) and SIGMA (UK, anti-P2Y<sub>1</sub> Ab).

### **3.2.5 Statistical analysis**

Results are expressed as mean ± SEM. Two way ANOVA was used for statistical comparisons with Bonferroni post-hoc test. A P value < 0.05 was taken as statistically significant.



### **3.3 Results**

Addition of KCl (60 mM) produced a sustained contractile response of PMA and PCA segments; the mean tissue response to KCl addition was  $8.9 \pm 0.32$  g (n=25) and  $10.2 \pm 0.62$  g (n=30), respectively.

#### **3.3.1 Effect of ADP in porcine precontracted mesenteric (PMA) and coronary arteries (PCA)**

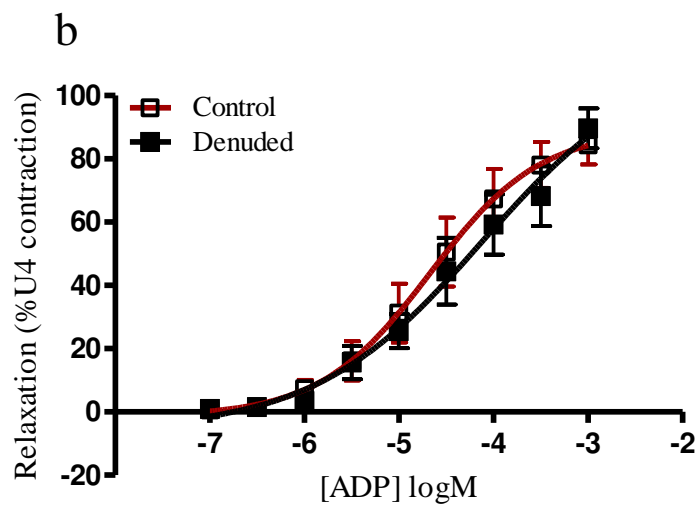
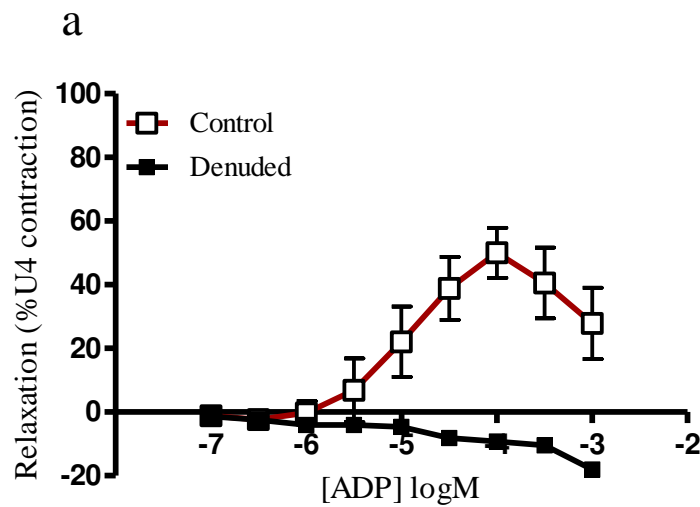
ADP (0.1  $\mu$ M-1 mM) elicited a concentration dependent relaxation of PMA (Figure 1a); maximal relaxation by ADP was observed at a concentration of 100  $\mu$ M. Concentrations of ADP greater than this caused a reduced relaxation of the PMA rings (Figures 3.1a, 3.2). Non linear analysis of these responses resulted in an estimates of  $R_{max}$  of  $54 \pm 8$  %, with a  $pEC_{50}$  value of  $6.91 \pm 0.19$  and Hill slope of  $2.0 \pm 0.21$  (n=8). Removing the endothelium abolished the ADP induced responses (Figure 3.1a). In the absence of endothelium a small contractile response was observed at the highest concentrations of ADP (Figures 3.1a, 3.2).

ADP (0.1  $\mu$ M-1 mM) elicited a concentration dependent relaxation of PCA (Figure 3.1b); maximal relaxation by ADP was observed at the highest concentration (1 mM). Non linear analysis for these responses resulted in estimates of  $R_{max}$  of  $94 \pm 7$ , with a  $pEC_{50}$  value of  $4.57 \pm 0.25$  and Hill slope of  $1.30 \pm 0.12$  (n=10). Removing the endothelium

had no significant effect on the ADP induced responses in this tissue (Figure 3.1b).

In both PMA and PCA, endothelium removal was assessed using substance P (10 nM). Any relaxation in response to substance P of less than 10% of the U46619 contraction was considered a success with regard to endothelium removal. In denuded PMA and PCA, substance P evoked relaxations of  $1.43 \pm 0.84$  % (n = 9) and  $3.05 \pm 1.26$  % (n = 7), respectively. While in endothelium intact PMA and PCA substance P elicited relaxations of  $54 \pm 3$  % (n = 8) and  $50 \pm 4$  % (n = 10), respectively.

**Figure 3.1.** Response evoked by adenosine diphosphate (ADP) in a. porcine mesenteric artery, b. Porcine coronary artery. Responses were evaluated in endothelium intact vessels (Control, n = 8-10) and in those in which the endothelium had been removed (Denuded, n = 7-9). Vessels were precontracted with U46619. Results are mean  $\pm$  SEM.



### **3.3.2 Effect of PaCoA on responses to ADP in porcine mesenteric arteries (PMA)**

PaCoA (10  $\mu$ M) abolished the relaxation response to ADP in mesenteric arteries (Figure 3.2, 3.3a). MRS2500 (10  $\mu$ M), a selective P2Y<sub>1</sub> antagonist, also abolished the ADP evoked relaxation (Figure 3.3b).

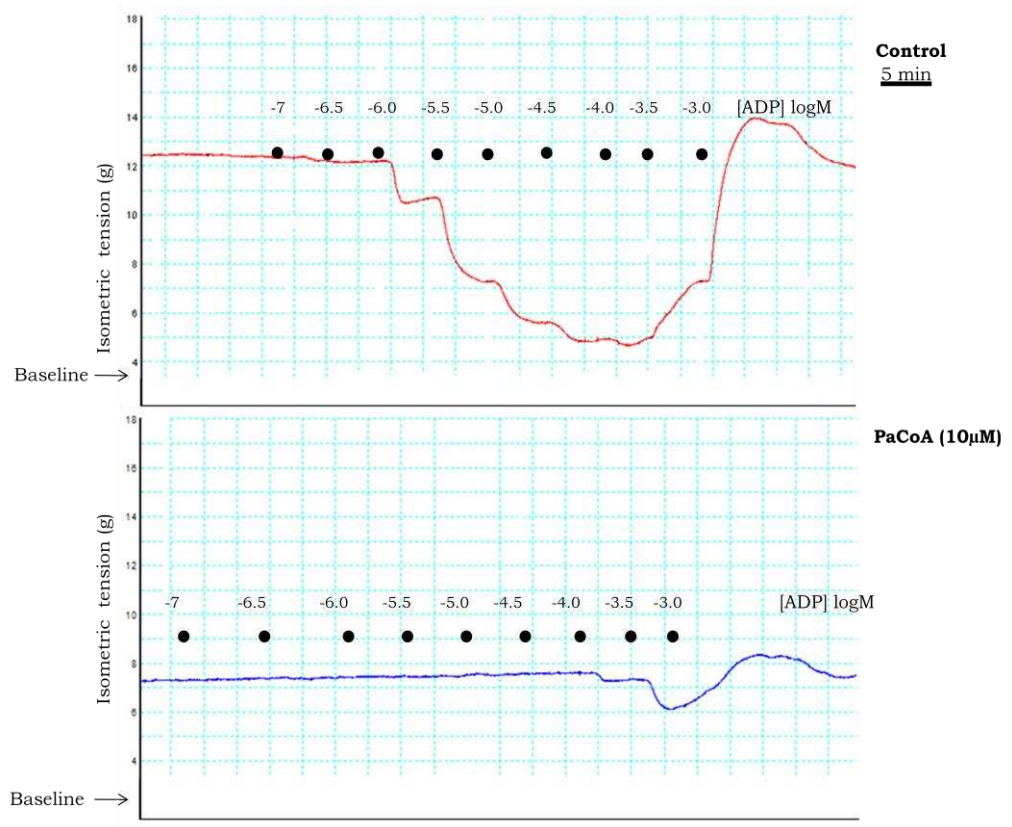
The possible direct effect of PaCoA (10  $\mu$ M) on vessel tone was investigated in the U46619-contracted PMA and was found to be without significant effect compared to the time control (Figure 3.5).

U46619 caused a sustained contraction to  $56 \pm 4$  % (n=8) of the KCl response. In the absence of antagonists, the concentration of U46619 required to produce this level of contraction was  $36 \pm 4$  nM (n= 8). In the presence of PaCoA (10  $\mu$ M) and MRS2500 (10  $\mu$ M) the U46619-induced contraction was  $61 \pm 2$  % (n=10) and  $65 \pm 9$  % (n=4), respectively, of the KCl-induced responses. The concentration of U46619 required to induce precontraction was unchanged ( $P > 0.05$ , one way ANOVA). For the direct effect and time control experiments, the U46619-induced contraction was  $51 \pm 9$  % (n= 4) and  $56 \pm 7$  % (n= 4), respectively, of the KCl-induced response. The concentration of U46619 required to induce precontraction was unchanged compared to the control in both time control and direct effect experiments ( $P > 0.05$ , one way ANOVA).

### **3.3.2.1 Immunohistochemical localization of P2Y<sub>1</sub> receptors in PMA**

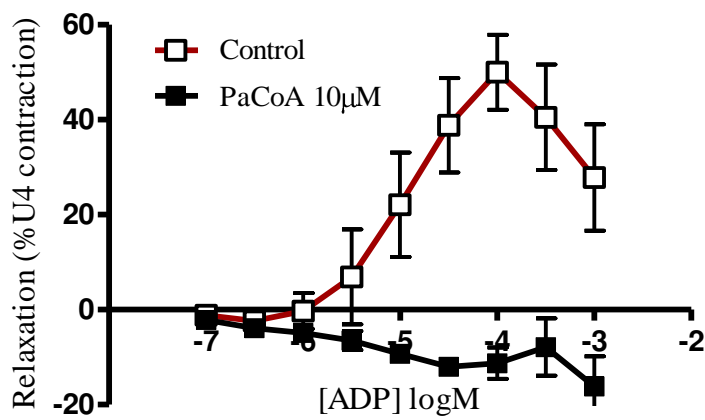
Since the ADP-mediated relaxations were blocked by P2Y<sub>1</sub> receptor antagonists in PMA, the expression of P2Y<sub>1</sub> receptors was investigated using immunohistochemistry. P2Y<sub>1</sub>-like immunoreactivity was observed in PMA on the endothelium and smooth muscle (Figure 3.4).

**Figure 3.2.** A representative trace for the response of the porcine mesenteric artery to adenosine diphosphate (ADP) in the absence and presence of palmitoyl coenzyme A (PaCoA). Vessels were precontracted with U46619.

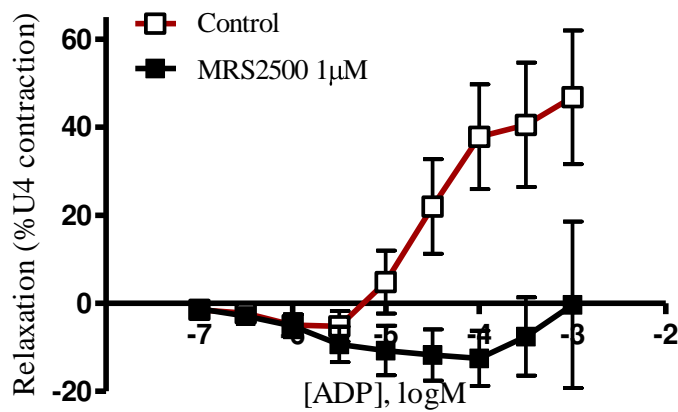


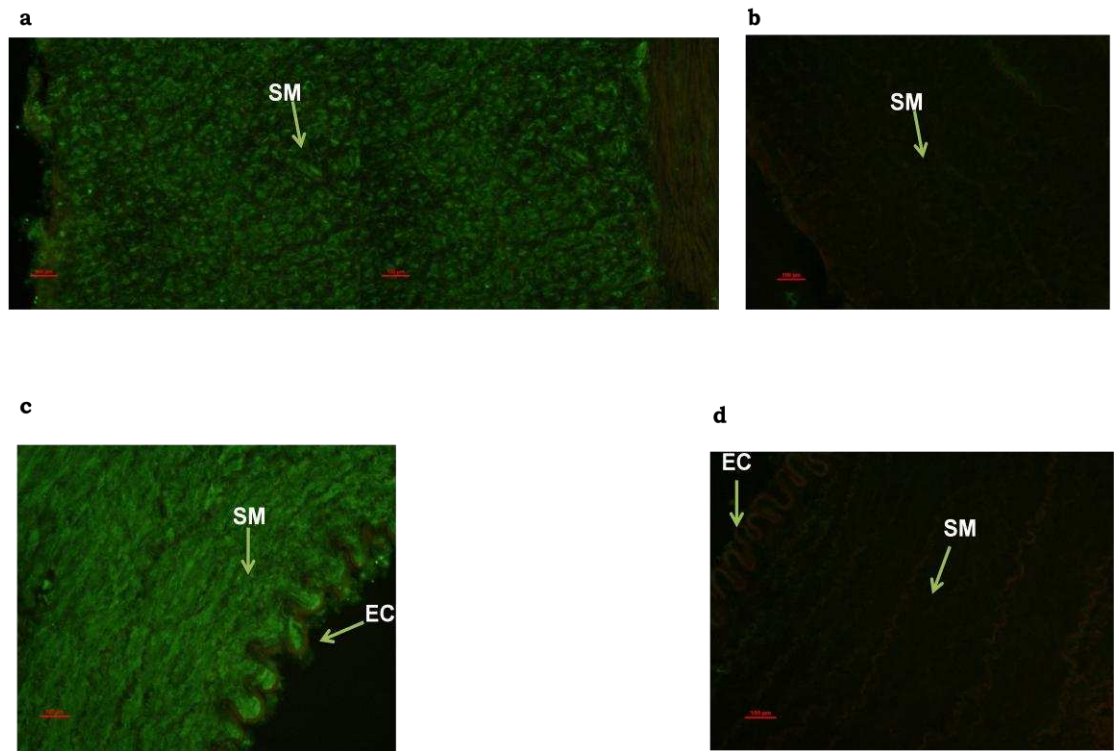
**Figure 3.3** The response of the porcine mesenteric artery to adenosine diphosphate (ADP) in the presence of a. palmitoyl coenzyme A (PaCoA), b. MRS2500. Vessels were precontracted with U46619. Results are shown as mean  $\pm$  SEM (n=8-10, n=5-4, respectively).

a



b

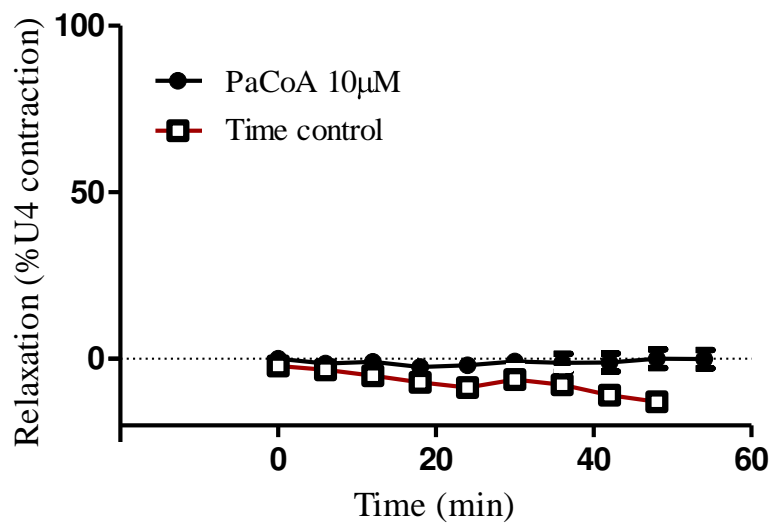




**Figure 3.4.** P2Y<sub>1</sub>- like immunostaining of the first order porcine mesenteric arteries on a. longitudinal sections and c. cross sections of the vessels accompanied by a control which had no primary antibody to P2Y<sub>1</sub> receptors in b. longitudinal section and d. cross section of the vessels. Smooth muscle (SM) and endothelial cells (EC) are indicated. Scale bar = 100  $\mu$ m.



**Figure 3.5.** The effect of palmitoyl coenzyme A (PaCoA) on contractile responses of the porcine mesenteric artery compared to a time control. Readings were taken every 6 minutes. Vessels were precontracted with U46619. Results are shown as mean  $\pm$  SEM (n= 4, n=4). In the time control group, error bars fall within the symbol.



### **3.3.3 Effect of PaCoA on relaxations to ADP in porcine coronary artery segments**

Although PaCoA (10  $\mu$ M) failed to evoke a significant effect on the response to ADP (Figure 3.6, 3.7a), there was a trend for a rightward shift in the ADP response curve in the presence of PaCoA. MRS2500 (10  $\mu$ M), a selective P2Y<sub>1</sub> antagonist, also had no significant effect on the ADP evoked responses (Figure 3.7b).

The direct effect of PaCoA (10  $\mu$ M) was investigated and it was found that PaCoA caused a significant vasorelaxation of the U46619-contracted PCA compared to the time control ( $P < 0.05$ , two way ANOVA) (Figure 3.9). The PaCoA direct effect was slow; it took around 20 min to evoke significant relaxations in response to PaCoA compared to the time control.

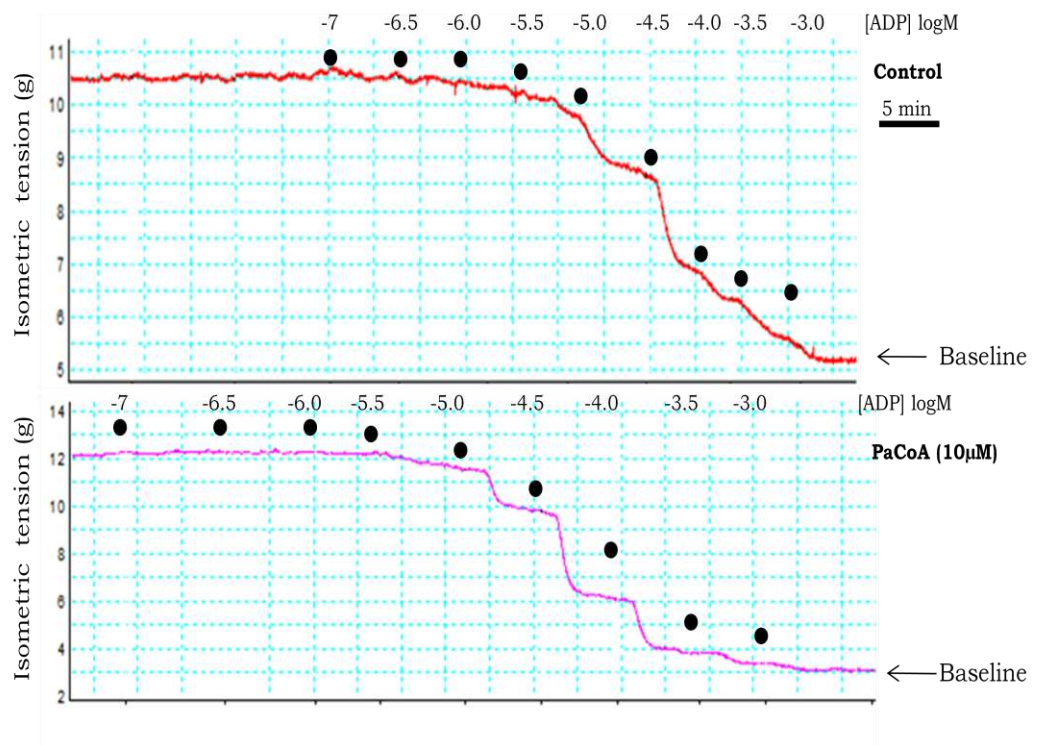
U46619 in the coronary artery caused a sustained contraction to  $59 \pm 4$  % (n=8) of the KCl response. The mean bath concentration of U46619 required to produce this level of contraction was  $13.0 \pm 2.5$  nM (n= 8). In the presence of PaCoA (10  $\mu$ M) and MRS2500 (10  $\mu$ M), the mean bath concentration of U46619 required to elicit  $53 \pm 4$  % (n=10) and  $56 \pm 7$  % (n=4), respectively, of the KCl contraction was unchanged ( $P > 0.05$ , one way ANOVA).

For the direct effect and time control experiments the U46619-induced contraction was  $60 \% \pm 4$  (n= 8) and  $50 \% \pm 8$  (n= 4), respectively, of the KCl-induced response. The concentration of U46619 required to induce precontraction was unchanged compared to the control in both time control and direct effect experiments ( $P > 0.05$ , one way ANOVA).

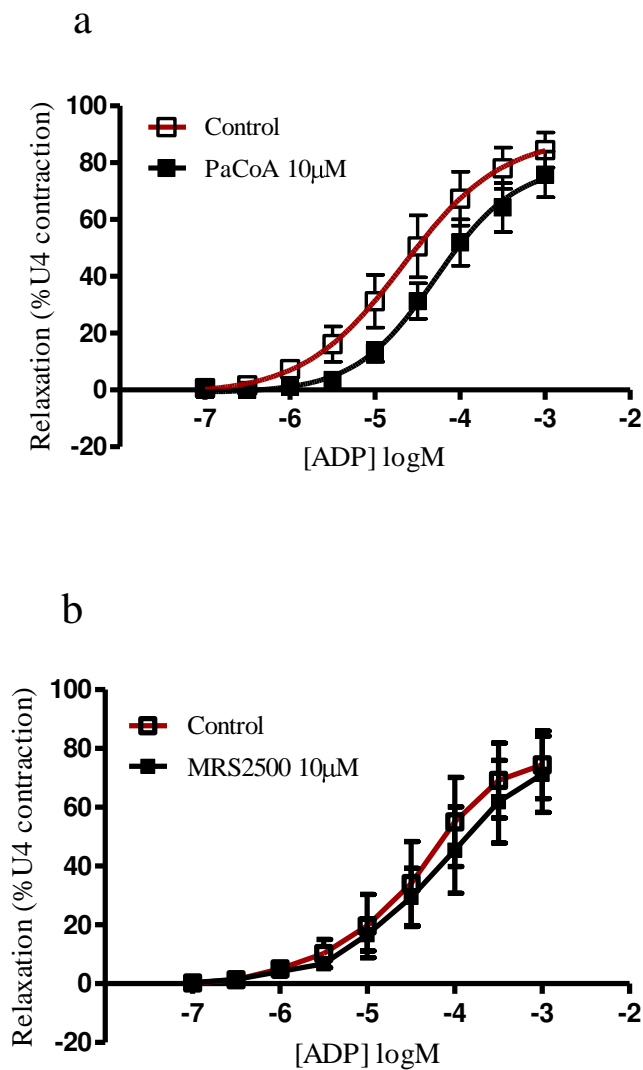
#### **3.3.3.1 Immunohistochemical localization of P2Y<sub>1</sub> receptors in PCA**

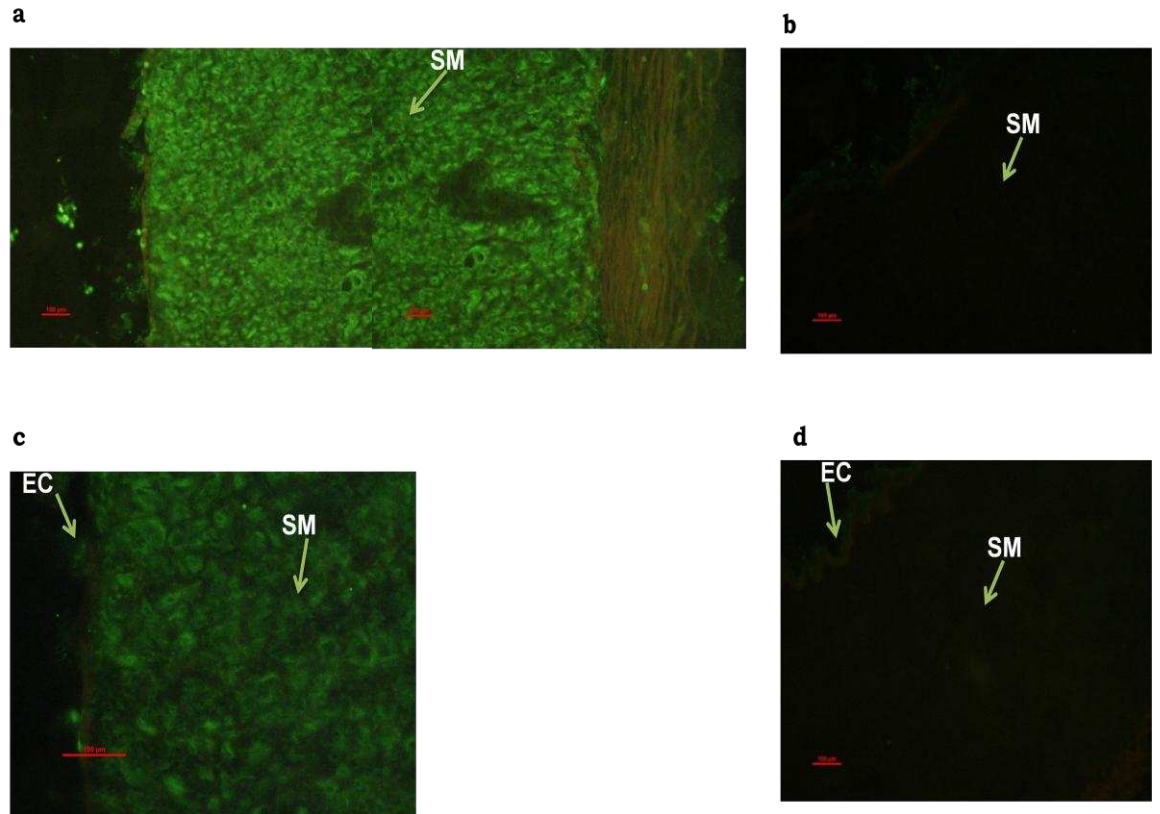
The expression of P2Y<sub>1</sub> receptors in PCA was investigated using immunohistochemistry, and immunoreactivity for P2Y<sub>1</sub> receptor was observed in PCA on the smooth muscle (Figure 3.8).

**Figure 3.6.** A representative trace for the response of the porcine coronary artery to adenosine diphosphate (ADP) in the absence and presence of palmitoyl coenzyme A (PaCoA). Vessels were precontracted with U46619.



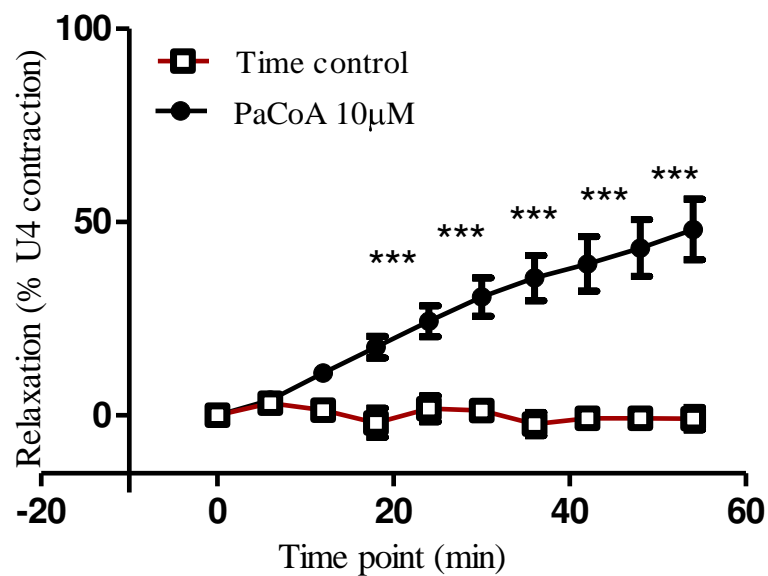
**Figure 3.7.** The response of the porcine coronary artery to adenosine diphosphate (ADP) in the presence of a. palmitoyl coenzyme A (PaCoA), b. MRS2500. Arteries were precontracted with U46619. Results are shown as mean  $\pm$  SEM (n=10, n=4, respectively).





**Figure 3.8.** P2Y<sub>1</sub>- like immunostaining of porcine coronary arteries a. longitudinal sections and c. cross sections of the vessels accompanied by a control which had no primary antibody to P2Y<sub>1</sub> receptors in b. longitudinal section and d. cross section of the vessels. Smooth muscle (SM) and endothelial cells (EC) are indicated. Scale bar = 100 μm.

**Figure 3.9.** The effect of palmitoyl coenzyme A (PaCoA) in the porcine coronary artery compared to time control, readings were taken every 6 minutes. Vessels were precontracted with U46619. Results are shown as mean  $\pm$  SEM (n=8, n=4, for PaCoA and time control data, respectively). \*\*\* P < 0.001.



### **3.4 Discussion**

PaCoA was used to characterise the effects of ADP on the PMA and PCA. We found that PaCoA and the selective P2Y<sub>1</sub> receptor antagonist MRS2500 abolished the ADP-evoked relaxations through the P2Y<sub>1</sub> receptor in PMA. In contrast, PaCoA and MRS2500 had no significant effect on ADP-evoked responses in PCA.

#### **3.4.1 Effect of PaCoA and MRS2500 on ADP evoked responses in porcine mesenteric arteries**

In the current study, ADP was observed to cause an endothelium-dependent vasorelaxation at concentrations up to 100 µM, after which there was a reduced vasorelaxation in PMA. A pEC<sub>50</sub> value of 6.9 was calculated for ADP in the PMA, which is similar to the value calculated for ADP activation of P2Y<sub>1</sub> receptors (6.8) in the rat mesenteric arterial bed (Buvinic et al., 2002). The involvement of P2Y<sub>1</sub> receptors was indicated since MRS2500 (1 µM) abolished the ADP evoked relaxations. To characterise further these ADP responses, PaCoA (10 µM) was used; it abolished the ADP-evoked relaxations in the PMA indicating that these relaxations were mediated through P2Y<sub>1</sub> receptors. There was no direct effect of PaCoA on these vessels.



This is consistent with our finding in the rat thoracic aorta where PaCoA acted as a potent P2Y<sub>1</sub> antagonist (Chapter 2).

Relaxations to ADP in the PMA were abolished by removal of the endothelium. ADP acts as an agonist at endothelial P2Y<sub>1</sub> receptors producing vasodilatation in several blood vessels (Ralevic and Burnstock, 1998), while ATP acts as a partial agonist at P2Y<sub>1</sub> receptors and as an agonist at P2Y<sub>2</sub>, P2Y<sub>6</sub> and P2X receptors (Ralevic and Burnstock, 1998). Buvinic et al. and Guns et al. showed that, in rat mesenteric arteries and mouse thoracic aortae, respectively, the vasodilator effects of ADP, but not ATP, were abolished by the P2Y<sub>1</sub> receptor antagonist MRS2179. On that basis, they suggested that ADP activates endothelial P2Y<sub>1</sub> receptors in these blood vessels, while ATP does not (Buvinic et al., 2002, Guns et al., 2006).

In our study, the ADP evoked vasorelaxation was endothelium dependent, consistent with the literature, showing that as in most vessels ADP relaxations were mediated through P2Y<sub>1</sub> receptors on the endothelium. Although not studied here, the mechanism of endothelium dependent vasorelaxation to ADP is likely to involve nitric oxide and EDHF, as shown for endothelial P2Y<sub>1</sub> receptors in rat mesenteric arteries (Malmsjo et al., 1999, Buvinic et al., 2002).

In endothelium-denuded vessels, there was a trend for a modest contraction in response to ADP. This may indicate contamination with ATP, possibly activating a receptor on the smooth muscle which may be P2X<sub>1</sub>, since it is unlikely for ADP to act directly at P2X<sub>1</sub> receptors (Mahaut-Smith et al., 2000, Vial et al., 2003) (Figure 3.1a). This likely accounts for the reduced vasorelaxation observed in endothelial-intact vessels at high concentrations of ADP. Contamination by ATP could be investigated by using an enzyme such as hexokinase which selectively hydrolyzes ATP (for example, (Harper et al., 1998)), checking the purity of the ADP (HPLC) or using a selective P2X<sub>1</sub> receptor antagonist such as NF449 (Braun et al., 2001).

Similar responses were noticed by Harrington et al. (2007) who showed that, in mouse mesenteric arteries, ADP caused a dilatation followed by contraction at concentrations 10  $\mu$ M and above; the dilator effects were endothelium dependent, indicating that they were P2Y receptor-mediated while the constrictor effects were endothelium independent and mediated by P2X<sub>1</sub> receptors. They concluded the latter response was caused by contamination with ATP (Harrington et al., 2007).

There was still a trend, although not significant, for a contraction of the PMA at concentrations of 300  $\mu$ M ADP or

above in the presence of PaCoA, similar to what was seen in the control ADP responses, which may also indicate that these contractile responses are mediated through a distinct target and may be caused by ATP contamination activating (most likely) P2X<sub>1</sub> receptors (as discussed above). The fact that PaCoA did not significantly affect these P2X-like contractile responses is also consistent with the fact that PaCoA did not act as an antagonist at recombinant P2X<sub>1</sub> receptors (Coddou et al., 2003).

In an effort to detect P2Y<sub>1</sub>-like expressed in PMA, using immunohistochemistry, P2Y<sub>1</sub>-like immunoreactivity was observed in PMA. However immunoreactivity was observed on both endothelium and smooth muscle. This may indicate a lack in functional effects of P2Y<sub>1</sub> receptors on smooth muscle as our results indicate that the ADP-mediated relaxations were endothelium dependent and were blocked by P2Y<sub>1</sub> antagonists.

In summary, our study in the PMA showed that ADP evokes endothelium-dependent vasorelaxation which was abolished by PaCoA and MRS2500 and is mediated through P2Y<sub>1</sub> receptor. PaCoA had no direct effect on PMA. At higher concentrations, ADP evoked vasoconstriction which may be mediated through contaminating ATP, although this needs to be confirmed using a selective P2X<sub>1</sub> receptor antagonist.

### **3.4.2 Effect of PaCoA and MRS2500 on ADP-evoked responses in porcine coronary arteries**

We have studied the effect of PaCoA on P2Y<sub>1</sub> receptor-mediated ADP evoked relaxations in rat thoracic aorta and porcine mesenteric artery, where PaCoA inhibited these responses in both tissues. In this Chapter, we also studied the effect of PaCoA on the ADP evoked responses in the PCA.

ADP elicited a concentration dependent endothelium independent relaxation of the PCA. ADP evokes relaxations through endothelial P2Y<sub>1</sub> receptors in a variety of species and blood vessels. In this study, the potency of ADP (pEC<sub>50</sub> 4.6) in PCA was much lower than that observed in the PMA (pEC<sub>50</sub> = 6.9) and to that in the rat thoracic aorta (pEC<sub>50</sub>= 6.0); in both the PMA and the RTA, the ADP response is clearly mediated via an endothelial P2Y<sub>1</sub> receptor. The ADP potency in PCA observed here is similar to previous findings from this laboratory, where ADP potency was found to be 5.3 in the PCA (Rayment et al., 2007b). The maximum response obtained here (94%) was also similar to the maximal response previously observed (89%) (Rayment et al., 2007b). The small difference in pEC<sub>50</sub> values could be due to differences in age or sex of the animals between the studies. It was reported that ADP mediates endothelium-independent relaxation via a novel mechanism involving release

of adenosine and activation of  $A_{2A}$  receptors independently of  $P2Y_1$  receptors in the PCA (Rayment et al., 2007b). This indicates that the PCA is different from other vessels in that ADP does not exert vasorelaxation through endothelial  $P2Y_1$  receptors as it does in other blood vessels, including the RTA and PMA studied here. This may explain the difference in the  $pEC_{50}$  values we found in PMA, RTA and PCA.

Another possibility, other than that ADP may be triggering the release of adenosine which then activates  $A_{2A}$  receptor (Rayment et al., 2007b), is that ADP may be broken down to adenosine which then mediates vasorelaxations through  $A_{2A}$  receptors known to be expressed on PCA (Merkel et al., 1992). This possibility was tested by Rayment et al. (2007b) in the PCA using a variety of enzyme inhibitors, after which they concluded that there was no role for extracellular hydrolysis of ADP in the generation of adenosine. In addition, Rayment et al showed that relaxations to the hydrolysis-resistant analogue of ADP,  $ADP\beta S$ , were insensitive to MRS2179 (a selective  $P2Y_1$  receptor antagonist) and were blocked by  $A_{2A}$  receptor antagonists, ZM241385 and SCH58261 (Rayment et al., 2007b).

As part of the characterization of these ADP-evoked relaxations, we investigated the effect of PaCoA, which failed to affect these responses, indicating that they are not mediated through  $P2Y_1$

receptors. To confirm this, MRS2500 was used, a better established P2Y<sub>1</sub> receptor antagonist, was used, which also had no significant effect on these relaxations.

In an attempt to detect P2Y<sub>1</sub> receptors expressed in PCA, using immunohistochemistry, P2Y<sub>1</sub>-like immunoreactivity was observed in PCA. Immunoreactivity was observed on the smooth muscle. Rayment et al. reported the expression of P2Y<sub>1</sub> receptors on the PCA smooth muscles (Rayment et al., 2007b). Since activation of smooth muscle P2Y<sub>1</sub> receptors is thought to lead to the activation of G<sub>q/11</sub> G-proteins which will elevate [Ca<sup>++</sup>]<sub>i</sub>, this is expected to lead to smooth muscle contraction, unlike relaxation seen when this receptor is activated on the endothelium (Rayment et al., 2007). Our findings in PCA agree with their findings that the ADP- mediated relaxations in PCA are not mediated via P2Y<sub>1</sub> receptors. This may indicate a lack of functional P2Y<sub>1</sub> receptors in PCA as our results indicate that the ADP-mediated endothelium independent relaxations were not affected with P2Y<sub>1</sub> antagonists. P2Y<sub>1</sub>-like receptors were also reported to be expressed in human coronary artery smooth muscle in cultured cells (Strobaek et al., 1996).

Interestingly, PaCoA significantly relaxed the U46619-contracted PCA in the absence of adenine nucleotides. The response was

slow (20 min), compared to the time needed for ADP to cause relaxations (3-5 min), perhaps suggesting a direct action on the smooth muscle. Although the mechanism by which PaCoA mediated these relaxations was not investigated, acyl CoA compounds have been reported to evoke responses in other tissues; for example, in the rat hippocampus, fatty acyl-CoAs enhance glutamate release (Zhang et al., 2000). Additionally, fibrates, which are metabolized either as acyl-CoA derivatives or as glucuronide conjugates, were reported to have an effect on coagulation by inhibiting hepatic fibrinogen synthesis; an effect thought to be mediated by PPAR $\alpha$  (peroxisomal proliferator-activated receptors) (Kockx et al., 1999). PPAR-evoked relaxations have been described in a number of smooth muscle preparations (see (O'Sullivan et al., 2009) for a review of endocannabinoids acting at PPARs). The mechanism of this direct, tissue-dependent relaxatory effect of PaCoA awaits further characterization. It should be noted that combining PaCoA and ADP in the PCA, did not result in augmentation of the relaxation to ADP (see Figure 3.4a); instead it was slightly reduced.

In summary, therefore, ADP evoked an endothelium independent relaxation of the PCA, which was not mediated through P2Y $_1$  receptors since neither PaCoA nor MRS2500 significantly affected these responses. PaCoA directly relaxed the

precontracted PCA, although the mechanism of this action is yet to be identified.

### **3.4.3 Responses in porcine mesenteric and coronary arteries**

The characteristic endothelium-dependent vasorelaxation to ADP observed in the PMA through P2Y<sub>1</sub> receptors is in contrast with the atypical response to ADP in the PCA which was endothelium-independent, independent of P2Y<sub>1</sub> receptors and likely to be through enhancing the release of adenosine which then acts through A<sub>2A</sub> adenosine receptors (Rayment et al., 2007b). Since two vessels from the same species (pigs) were being compared, then these differences cannot be due to species differences, but rather due to differences in the vessels themselves.

In the majority of literature ADP-evoked relaxations are mediated by P2Y<sub>1</sub> receptors located on the endothelium; activation of endothelial P2Y receptors was reported to lead to vasodilatation, while activation of P2Y receptors on the smooth muscle evokes vasoconstriction (Boarder and Hourani, 1998, Kunapuli and Daniel, 1998, Ralevic and Burnstock, 1998, von Kugelgen, 2006). In PCA there are also a number of reports of adenine nucleotide-evoked relaxation through smooth muscle P2Y receptors, especially in coronary arteries in different



species; Keef et al. reported P2Y mediated relaxation in guinea pig and rabbit coronary artery on the smooth muscle (Keef et al., 1992), Saetrum Opgaard and Edvinsson reported relaxatory responses in human coronary artery via P2Y receptors on smooth muscle (Saetrum Opgaard and Edvinsson, 1997). This kind of relaxatory response via smooth muscle P2Y receptors was also noticed in rabbit and lamb coronary artery (Corr and Burnstock, 1994, Simonsen et al., 1997). Rayment et al. (2007b) speculated in their report that the endothelium-independent action of ADP to evoke the release of adenosine may be a common property in the coronary arteries of these species.

The direct relaxatory effect of PaCoA observed in the PCA is also in contrast with its action in the PMA and RTA where it did not cause any significant response. Whether this response is mediated through PPARs is unclear and in need of further characterization.

In this Chapter, ADP responses in two different blood vessels, PMA and PCA, were assessed. PaCoA and MRS2500 abolished the ADP-evoked P2Y<sub>1</sub> receptor-mediated relaxations in PMA with no direct effect of PaCoA on these vessels. In the PCA, ADP-evoked relaxations were not mediated through P2Y<sub>1</sub> receptors and PaCoA had no significant effect on ADP-evoked responses, although it caused a significant relaxation of the precontracted

blood vessels. These findings highlight a significant difference between these two blood vessels and re-emphasize the special features of coronary arteries.

## **Chapter Four**

### **Effects of acyl-CoA on P2Y receptor-evoked calcium responses in HEK cells**

## 4.1 Introduction

Coddou et al. (2003) studied whether CoA and CoA derivatives were antagonists at human recombinant P2Y receptors. CoA, drug-derived CoA derivatives and CoA derivatives derived from endogenous fatty acids were found to antagonize ATP evoked responses at the P2Y<sub>1</sub> receptor, but not at the P2Y<sub>2</sub> receptor. Acyl derivatives of CoA were also found to act as antagonists at endogenous P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors on human platelets (Manolopoulos et al., 2008). Using isometric tension recording, we examined the relevance of these findings to the regulation of vascular contractility by studying the effects of CoA, AcCoA and PaCoA at vasorelaxant endothelial P2Y<sub>1</sub> receptors in the rat thoracic aorta, and showed that these compounds are antagonists at P2Y<sub>1</sub> receptors with PaCoA being the most potent (Chapter 2). In further isometric tension experiments, we tested the effect of PaCoA on the ADP evoked relaxation in porcine mesenteric artery where PaCoA abolished these relaxations (Chapter 3).

The effect of acyl derivatives of CoA at endogenous P2Y<sub>1</sub> receptors in human cell lines is unknown. The main aim of the present study was to investigate, using HEK cells, whether the increase in intracellular calcium mediated by ADP through the P2Y<sub>1</sub> receptor can be blocked with coenzyme A and its derivatives; AcCoA and PaCoA. Since the design of these experiment allowed us to use smaller amounts of the test compounds we examined the effect of another CoA derivative,

oleoyl CoA (OlCoA), to examine the effect of a longer chain acyl CoA as an antagonist at P2Y<sub>1</sub> receptors and compare the effect of the number of carbons in the acyl residue in AcCoA (acetic acid C2:0), PaCoA (palmitic acid C16:0) and OlCoA (oleic acid 18:1) on their antagonistic activity; the numbers in brackets indicate the number of carbons and double bonds, respectively. Since human embryonic kidney (HEK) cells endogenously express both P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors (Schachter et al., 1997b), we were able to test the selectivity of the observed effects of ADP at P2Y<sub>1</sub> versus effects mediated by UTP at the P2Y<sub>2</sub> receptor.

## **4.2 Methods**

### **4.2.1 Cell Culture**

HEK cells (passages: 27-35, 18-25) taken from the stocks of the School of Biomedical Sciences, University of Nottingham were grown in Eagle's Minimum Essential Medium (EMEM, Sigma Chemical Company) supplemented with 10% fetal calf serum (FCS, Sigma Chemical Company) and non-essential amino acids (Sigma Chemical Company).

Cells were grown at a temperature of 37°C in a humidified 95% air and 5% CO<sub>2</sub> atmosphere. All cell culture was carried out under sterile conditions in a class II laminar airflow cabinet.

### **4.2.2 Cell Passage**

Cells were maintained in 75 cm<sup>2</sup> polystyrene cell culture flasks. When confluent, cells were prewashed with 2 ml phosphate buffered saline (PBS, Sigma Chemical Company), the PBS was aspirated then 2 ml of 0.25% trypsin/EDTA (Sigma Chemical Company) was added to the cells and incubated for 5 min at 37°C. Eight ml fresh media were then added to resuspend the cells, which were transferred to sterile universal tubes and centrifuged at 1500 rpm for 5 min. The supernatant layer was discarded and the pellet was resuspended with 5 ml fresh media and the cells transferred to new flasks containing 20

ml fresh media at a split ratio of 1:5. Cells were cultured for 48 hours until they reached confluency.

#### **4.2.3 Preparation of 96 well cell plates**

Black-walled 96 well plates (Costar) were first treated with 100  $\mu$ l poly-L lysine (Sigma Chemical Company) for 20 min then washed once with PBS. Cells from one confluent flask were used to prepare the plate. First the flask was washed with 2 ml of PBS then 2 ml of trypsin/EDTA added and incubated for 5 min. Fresh media (8 ml) was added and the cell suspension was transferred to a tube and centrifuged at a speed of 1500 rpm for 5 min. The supernatant layer was discarded and the pellet was suspended in 5 ml fresh media; the cells were triturated with a pipette to ensure uniform distribution of cells. 1 ml of cell suspension was added to a sterile tube containing 24 ml of fresh media. The suspension was again triturated many times to ensure uniform distribution of cells, using a repeater pipette. 200  $\mu$ l of the cell suspension was transferred to each well. Cell plates were cultured for 48 hours to reach confluency.

#### **4.2.4 Measurement of changes in intracellular calcium, $[Ca^{2+}]_i$**

Changes in intracellular calcium,  $[Ca^{2+}]_i$ , were investigated using Fluo-4-AM (Invitrogen). The culture media was aspirated and replaced with

the Fluo-4-AM solution in the presence of EMEM media, 10% FCS and 2.5 mM probenecid (Sigma Chemical Company); 100  $\mu$ l was added to each well and the plates were incubated at 37°C with this solution for 45 min.

A loading buffer consisting of 250 mM probenecid in HEPES-buffered saline (HBSS) was used to wash out the excess dye after the 45 min incubation period; the wells were washed two times with this buffer each time using 100  $\mu$ l. After that 100  $\mu$ l of loading buffer solution or antagonists were added to control or antagonists wells respectively and incubated with the cells for 30 min after which intracellular calcium responses were recorded using a FlexStation II plate reader (Molecular Devices, USA).

All antagonist and agonist dilutions were made using the loading buffer solution. Fluorescence measurements were made at excitation wavelengths of 485 and 520 nm and an emission wavelength of 515 nm on the FlexStation II at 37°C. 20  $\mu$ l of agonists were added at 15 s. All added drugs remained in the well until the end of each experiment.

#### **4.2.5 Data analysis**

All data were first exported from FlexStation software to Excel, then analysed using both Prism version 5 and Excel. For analysis of levels



of  $[Ca^{2+}]_i$ , the mean of the initial fluorescence ratios was taken as a baseline (0-16 sec) and was subtracted from subsequent fluorescence ratios (16-40 sec). Data collected were from 6 or more different experiments and are presented as mean  $\pm$  SEM.

### 4.3 Results

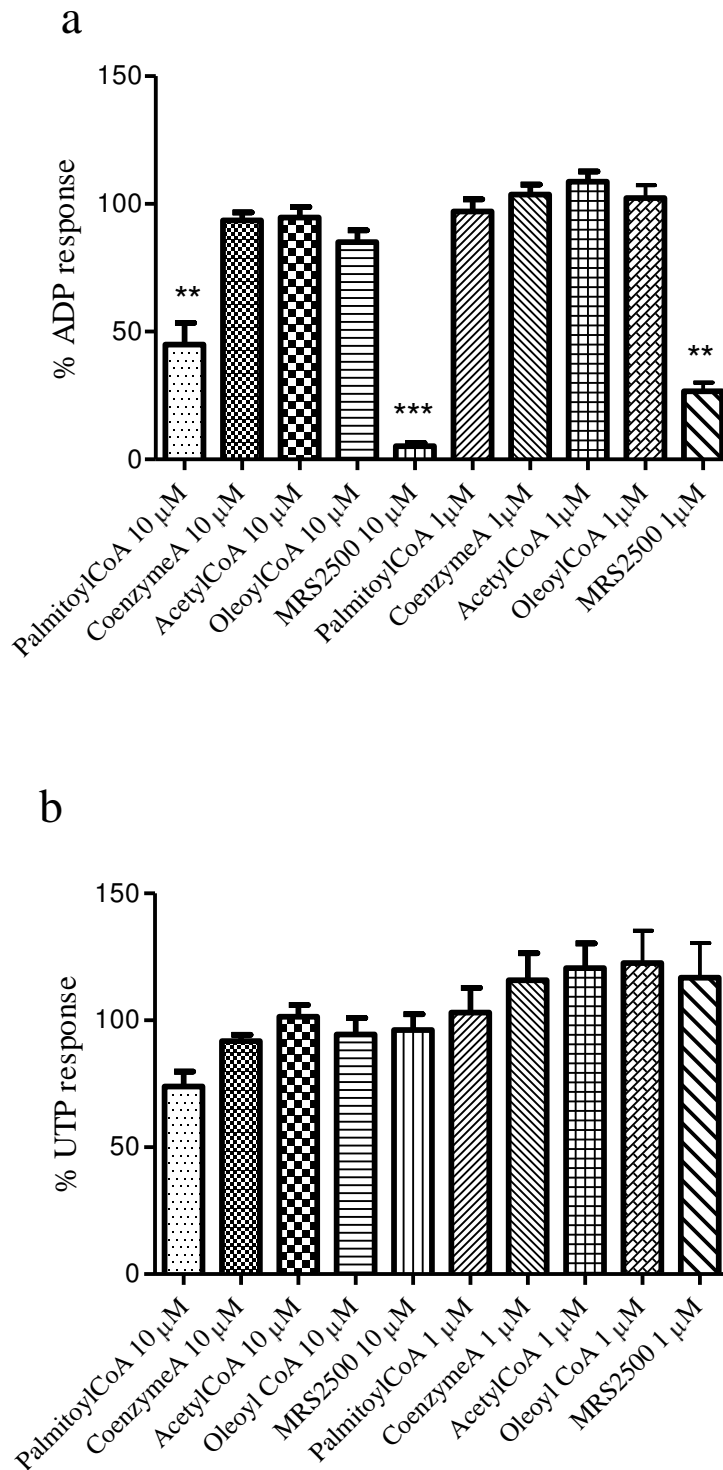
#### 4.3.1 Modulation of P2Y receptor-evoked elevations of $[Ca^{2+}]_i$ in HEK cells

ADP- and UTP-mediated elevations of  $[Ca^{2+}]_i$  were investigated in HEK cells using Fluo-4 AM. ADP, at 10  $\mu$ M, caused a transient response with a peak of  $32585 \pm 2364$  RFU (relative fluorescence units) over basal (n=11), which occurred within 25-30 seconds of drug administration. UTP, at 300  $\mu$ M, evoked a smaller elevation of  $[Ca^{2+}]_i$  with a mean of  $16228 \pm 2324$  RFU (n = 10), with a latency of 23-30 seconds. The response to UTP was  $49 \pm 4$  % of the response to ADP. These responses were used as controls, and effects of the other compounds were expressed as a percentage of the controls.

ADP-evoked responses were significantly inhibited in the presence of either 10  $\mu$ M PaCoA (residual response  $45 \pm 9$  % of control, n = 11) or 10  $\mu$ M MRS2500 (residual response  $5 \pm 1$  % of control, n = 7), but not in the presence of 1  $\mu$ M PaCoA (Figure 4.1a). Responses to ADP were not significantly altered in the presence of either 1 or 10  $\mu$ M CoA, AcCoA or oleoyl CoA.

Responses to UTP were not significantly altered in the presence of either 1 or 10  $\mu$ M PaCoA, CoA, AcCoA, OICoA or MRS2500 (Figure 4.1b).

**Figure 4.1.** The effect of CoA and its analogues and MRS2500 on  $[Ca^{2+}]_i$  responses to a. adenosine diphosphate (ADP), or b. uridine triphosphate (UTP) in HEK cells. Results are mean  $\pm$  SEM of the percentage of the response to 10  $\mu$ M ADP (n= 7-11) or 300  $\mu$ M UTP (n= 7-10), respectively.



### **4.3.2 Effect of palmitoyl CoA on adenine nucleotide-evoked $[Ca^{2+}]_i$ elevation in HEK cells**

ADP and ATP (0.1-100  $\mu$ M) evoked concentration-dependent calcium responses in HEK cells with  $pEC_{50}$  values of  $6.7 \pm 0.1$  ( $n = 3$ ) and  $5.6 \pm 0.1$  ( $n = 3$ ), respectively (Tables 4.1 and 4.2).

PaCoA (0.1-10  $\mu$ M) caused a concentration-dependent rightward shift in the calcium release evoked by ADP (Figure 4.2a).  $pEC_{50}$ ,  $R_{max}$  and Hill slope values for PaCoA are shown in Table 4.1. Schild analysis of the effects of PaCoA allowed calculation of an apparent  $pA_2$  value of  $7.2 \pm 0.2$  using ADP as the agonist, with a slope of  $1.4 \pm 0.2$  ( $n = 3$ , Figure 4.3a).

PaCoA (0.1-10  $\mu$ M) also produced a concentration-dependent rightward shift in the response curve to ATP (Figure 4.2b).  $pEC_{50}$ ,  $R_{max}$  and Hill slope values are reported in Table 4.2. Schild analysis gave a  $pA_2$  value of  $7.0 \pm 0.3$  and a slope of  $1.6 \pm 0.2$  ( $n = 3$ , Figure 4.3b). In order to calculate  $pA_2$  values, data for ADP and ATP were constrained, where the bottom was fixed at zero and the top was fixed to the maximum response evoked in the absence of antagonist for each individual experiment.

A further set of three experiments was carried out with an interval of a year after the previous experiments to increase the n numbers; in these PaCoA still significantly affected the calcium release evoked by ATP and ADP; the responses were abolished at a concentration of 1  $\mu$ M for both ADP and ATP in the presence of 10  $\mu$ M PaCoA, but PaCoA was less potent when compared to the first set of experiments.

**Table 4.1.** The effect of palmitoyl CoA on the calcium response evoked by ADP (0.1-100  $\mu$ M) in HEK cells

<b>Table</b>	pEC <sub>50</sub> value	R <sub>max</sub> (RFU)	Hill slope
ADP	6.74 $\pm$ 0.11	31062 $\pm$ 3706.95	1.17 $\pm$ 0.39
ADP + 0.1 $\mu$ M PaCoA	6.27 $\pm$ 0.11	31062	0.60 $\pm$ 0.15
ADP + 1 $\mu$ M PaCoA	5.28 $\pm$ 0.10	31062	0.94 $\pm$ 0.21
ADP + 10 $\mu$ M PaCoA	3.65 $\pm$ 0.22	31062	0.81 $\pm$ 0.12

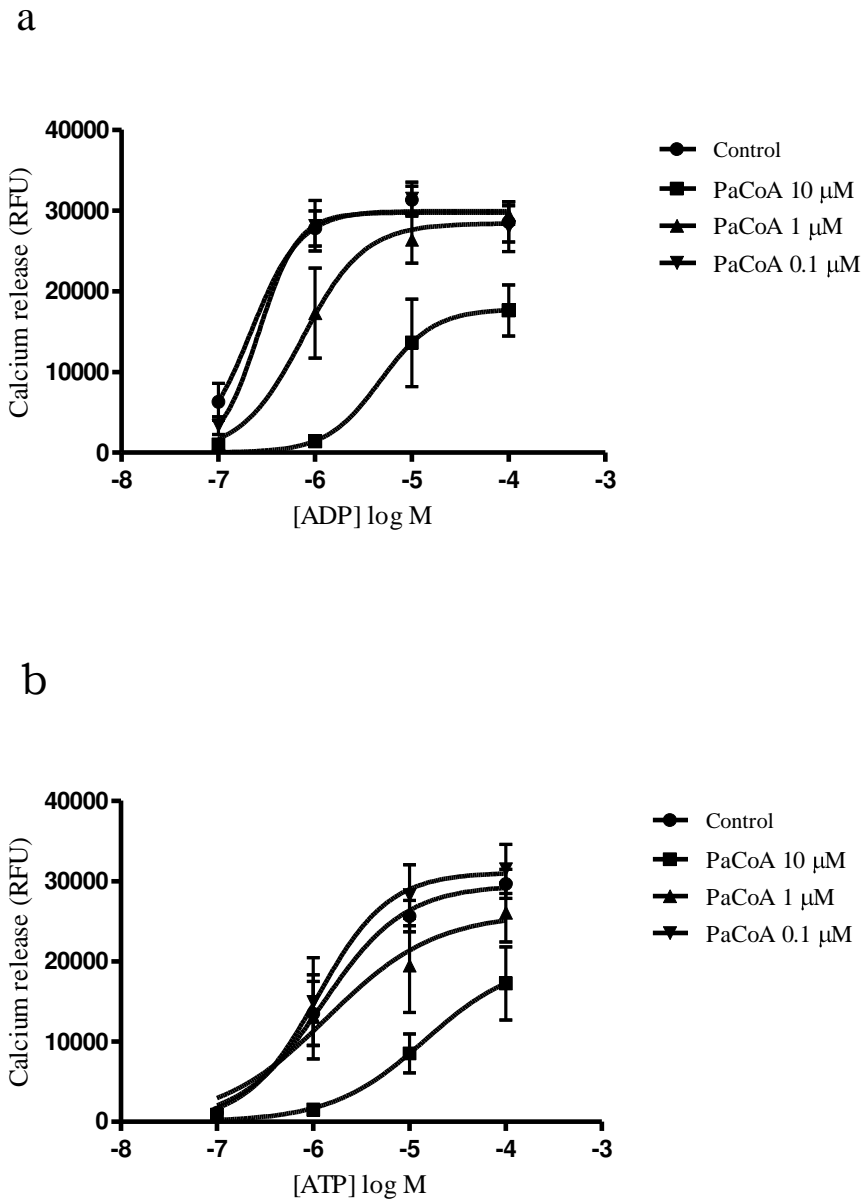
Data are mean  $\pm$  SEM, n=3.

**Table 4.2.** The effect of palmitoyl CoA on the calcium response evoked by ATP (0.1-100  $\mu$ M) in HEK cells

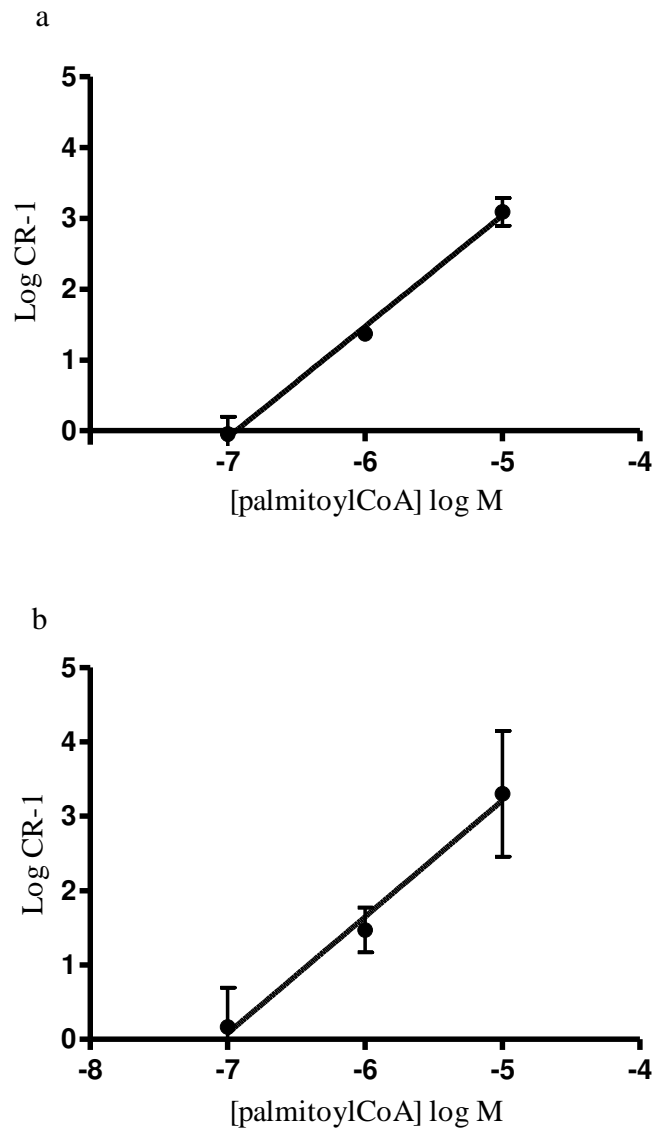
<b>Table</b>	pEC <sub>50</sub> value	Rmax (RFU)	Hill slope
ATP	5.56 $\pm$ 0.07	26520 $\pm$ 1999	1.55 $\pm$ 0.14
ATP + 0.1 $\mu$ M PaCoA	5.36 $\pm$ 0.02	26520	1.45 $\pm$ 0.25
ATP + 1 $\mu$ M PaCoA	4.40 $\pm$ 0.11	26520	0.82 $\pm$ 0.12
ATP + 10 $\mu$ M PaCoA	2.58 $\pm$ 0.55	26520	0.40 $\pm$ 0.13

Data are mean  $\pm$  SEM, n=3.

**Figure 4.2.** Effect of different concentrations of palmitoyl CoA on the calcium response of HEK cells to a. ADP, b. ATP. Results are mean  $\pm$  SEM, n= 6.



**Figure 4.3.** Schild plots for ADP in the presence of palmitoyl CoA (a) and ATP in the presence of palmitoyl CoA (b). Data are mean  $\pm$  SEM,  $n=3$ .





## **4.4 Discussion**

In this chapter, ADP and ATP were used to examine effects of CoA derivatives on the increase in intracellular calcium mediated by P2Y<sub>1</sub> receptors in HEK293 human embryonic kidney cells. To check the selectivity of these compounds, the effect of PaCoA on the UTP- (P2Y<sub>2</sub> receptor agonist) mediated increase in intracellular calcium was examined. The results indicate that, of the CoA analogues examined, PaCoA was the only CoA derivative that acts as an antagonist with apparent selectivity for the P2Y<sub>1</sub> receptor.

### **4.4.1 Effect of ADP, ATP and UTP on the elevation of [Ca<sup>2+</sup>]<sub>i</sub> in HEK cells**

Concentration-dependent elevation of [Ca<sup>2+</sup>]<sub>i</sub> was produced in the presence of ADP, ATP or UTP. It was previously shown that ADP, UTP and ATP induced an increase in the [Ca<sup>2+</sup>]<sub>i</sub> in HEK cells which express P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>4</sub> (Fischer et al., 2005, Bultmann et al., 1998).

At the P2Y<sub>1</sub> receptor, ADP is a more potent agonist than ATP, while ATP and UTP have equal potency as an agonist at P2Y<sub>2</sub> receptors and UTP is a more potent agonist at P2Y<sub>4</sub> than ATP (Ralevic and Burnstock, 1998).

In comparing responses to ADP, ATP and UTP, the potency of ADP was >10-fold more than that of ATP (pEC<sub>50</sub> of 6.7, 5.6, respectively). Maximal responses to UTP were 49% of those to ADP. These results are consistent with the fact that uridine nucleotides are mostly inactive at P2Y<sub>1</sub> and that P2Y<sub>1</sub> is more sensitive to adenine nucleotide diphosphates than to triphosphates (Alexander et al., 2006).

ADP was slightly more potent in HEK cells (pEC<sub>50</sub> of 6.7) compared to results obtained previously in the rat thoracic aorta (pEC<sub>50</sub> = 6.0, Chapter 2) and Dol-Gleizes et al. (1999) (pEC<sub>50</sub> of 6.2) (Dol-Gleizes et al., 1999). The difference in agonist potency might be caused by species differences. For example certain regions of the P2X<sub>7</sub> receptor, residues at positions 127 and 284, contribute to differences in agonist potency at rat, human and mouse P2X<sub>7</sub> receptors (Thompson et al., 2001, Young et al., 2007). It may also be due to the change from vascular tissue to cells or methodological differences, since the rat thoracic aorta was studied using isometric tension recording experiments while the HEK cell experiments involved measurement of changes in [Ca<sup>2+</sup>]<sub>i</sub> in monolayers using the FlexStation.

#### **4.4.2 Effect of CoA derivatives and MRS2500**

The present study found that PaCoA is a potent and selective antagonist of the native human P2Y<sub>1</sub> receptor in HEK cells,

while CoA and its derivatives AcCoA and OlCoA failed to have a significant antagonistic effect at these receptors. In the rat thoracic aorta, CoA or AcCoA caused a significant shift (4-fold, 5-fold, respectively) in the ADP-evoked relaxations through P2Y<sub>1</sub>, although the shift was relatively small (Chapter 2).

Coddou et al. (2003) suggested that the increase in potency of PaCoA, compared to CoA and AcCoA, may be due to possible interaction of the lipophilic acyl-substituent with a hydrophobic pocket close to the binding site. In addition they suggested that while hydrophobicity is important for the added antagonism, maybe bulkiness also plays a critical role. Despite having a double bond which reduces flexibility and should increase potency according to the Coddou et al paper, OlCoA does not follow this trend, as it had no significant effect on the ADP responses, although it has higher lipophilicity and bulkiness. Our findings agree with the conclusion of Manolopoulos et al. in platelets where they observed that CoA analogues containing saturated fatty acids provided greater inhibition than those with unsaturated fatty acids (Manolopoulos et al., 2008).

The selectivity of the CoA derivatives for the P2Y<sub>1</sub> receptors was confirmed, as responses to UTP were not significantly altered in the presence of PaCoA, CoA, AcCoA and OlCoA. This is consistent with the conclusion of Coddou et al (2003) at

recombinant P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors, and with the results in rat thoracic aorta (Chapter 2).

The results of Schild analysis for PaCoA showed apparent pA<sub>2</sub> values of 7.2 and 7.0, with slopes of 1.4 and 1.6 using ADP and ATP as agonists, respectively. In comparison with our previous investigations of the effects of PaCoA in the rat isolated aorta using ADP (pA<sub>2</sub> 6.44, slope 1.73), there is an increase in affinity, with a small reduction in slope, which may result from the change from a large tissue mass to a monolayer of cells. Species differences may also be responsible for such differences. For example several key residues have been identified to be responsible for the species differences in antagonist effects at the P2X<sub>7</sub> receptor between human and rat (Michel et al., 2008).

MRS2500 is the most potent and selective P2Y<sub>1</sub> receptor antagonist currently available (Kim et al., 2003). It displays 100-fold higher affinity compared with MRS2179 (Waldo et al., 2002, Kim et al., 2003) at human recombinant P2Y<sub>1</sub> receptors and inhibits platelet aggregation to ADP with an IC<sub>50</sub> of 0.95 nM (Cattaneo et al., 2004). In our study, although it was not possible to calculate a pK<sub>B</sub> value for MRS2500 as only a single ADP concentration was used, MRS2500 (10 μM) effectively blocked the ADP responses (residual response 5%). Responses to UTP were not significantly altered in the presence of MRS2500.

These results show that PaCoA is a reasonably potent antagonist at human native P2Y<sub>1</sub> receptors in HEK cells. This raises the possibility of an endogenous selective regulation of purinergic signalling involving inhibition of P2Y<sub>1</sub> receptors via CoA compounds.

## **Chapter Five**

**Effects of NAD on purine receptors-mediated responses in rat thoracic aorta and porcine isolated coronary and mesenteric arteries**

## 5.1 Introduction

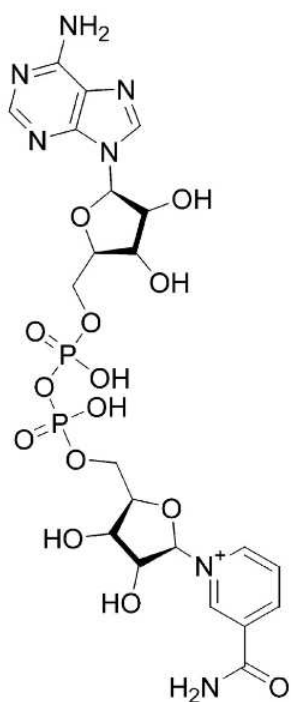
Nicotinamide adenine dinucleotide (NAD) (Figure 5.1) is released in a variety of smooth muscle tissues during stimulation of nerves including those in canine mesenteric artery and human urinary bladder (Smyth et al., 2004, Breen et al., 2006). Transporters for NAD have been identified that mediate both intercellular and intracellular transport of NAD through membranes.(De Flora et al., 2004). NAD has been identified as an agonist for P2Y<sub>1</sub> receptors in HEK cells (Mutafova-Yambolieva et al., 2007), P2Y<sub>11</sub> receptors in human granulocytes (Moreschi et al., 2006), P2X receptors in human monocytes (Grahner et al., 2009) and can also activate P1 adenosine receptors (Burnstock and Hoyle, 1985, Hoyle, 1990). In this chapter, responses to NAD in RTA, PMA and PCA were investigated using selective P1 and P2 receptor antagonists. In the previous chapters PaCoA was shown to act as an antagonist at P2Y<sub>1</sub> receptors in rat thoracic aorta (RTA) (Chapter Two) (Alexander et al., 2008), porcine mesenteric artery (PMA) (Chapter Three) (Alefisat et al., 2010) and in HEK cells (Chapter Four) (Alefisat et al., 2009). Hence, we also used PaCoA in this Chapter to characterise the responses to NAD in different blood vessels, specifically to investigate the possible involvement of P2Y<sub>1</sub> receptors.

It has previously been shown that vasorelaxant P2Y<sub>1</sub>, P2Y<sub>2</sub> and A<sub>2</sub> receptors are expressed on the endothelium of the RTA (Hansmann et al., 1997, Rose'Meyer and Hope, 1990). P2Y<sub>1</sub> receptors have also been

shown to evoke vasorelaxant responses in PCA (Olivecrona et al., 2004). PCA are also known to express  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors (Balwierczak et al., 1991, Merkel et al., 1992, Monopoli et al., 1994, Abebe et al., 1994). Little is known about purine receptor expression in the PMA. However, in Chapter Three, it was observed that ADP mediates responses through  $P2Y_1$  receptors in the PMA.

The main aim of the present study was to investigate the effects of NAD in isolated RTA, PMA and PCA and characterise the purine receptors involved in these responses. In addition, the effect of PaCoA on these NAD-mediated responses was studied.

**Figure 5.1** Chemical structure of nicotinamide adenine dinucleotide.





## 5.2 Materials and Methods

Porcine mesenteries and hearts, obtained from a local abattoir (Woods abattoir, Clipstone, Mansfield, Nottinghamshire) and male Wistar rats (200-250 g), obtained from Charles River (England, UK) were used in this study. PMA, PCA and RTA were dissected out, rings from PMA, PCA and RTA were mounted, tensioned, equilibrated, exposed to KCl and precontracted (using methoxamine for RTA and U46619 for PMA and PCA) for isometric tension recording as described in Chapters Two and Three. Stepwise cumulative addition of agonist (NAD, 0.1  $\mu$ M- 1 mM) (Sigma Chemical Company, UK) to the preparations was then carried out as described with ADP in Chapters Two and Three.

Endothelium denudation (by gentle rubbing) and time control experiments were performed using the same protocol described in Chapters Two and Three.

### 5.2.1 Effect of PaCoA on responses to NAD

To investigate the possible antagonistic effects of PaCoA (10  $\mu$ M, Sigma Chemical Company), CGS15943 (an adenosine receptor antagonist) (10  $\mu$ M) (SIGMA), SCH58261 (a selective  $A_{2A}$  receptor antagonist) (100 nM) (a gift from Schering Plough, Milan, Italy), suramin (a P2 receptor antagonist) (100 $\mu$ M) (Sigma Chemical Company, UK) and  $\alpha,\beta$ meATP (a P2X receptor agonist and desensitizing agent) (10  $\mu$ M) (SIGMA) on

responses to NAD, these compounds were added 10 min before U46619 or methoxamine addition.

### **5.2.2 Immunohistochemical Staining**

Porcine coronary arteries were stained using the standard indirect immunofluorescence technique as described in Chapter three, 50  $\mu$ l primary rabbit antibody A<sub>2A</sub> was added at (1:50) dilution in human serum was added to the slides. Samples were incubated overnight at 4°C. Then they were washed with PBS+0.1 % BSA 3 times. The secondary antibody anti mouse IgG (TRITC) (1:50) (Sigma Chemical Company) was diluted in PBS+ 0.1% BSA and added to the slides where we aimed for A<sub>2A</sub> receptor detection. Then the slides were incubated at 37°C for 30 min this was followed by washing with PBS+ 0.1% BSA 3 times. The slices were then covered with vector shield mounting solution and glass cover slips. Samples were visualized using fluorescence microscopy using an objective magnification of 40X. A parallel set of controls for each slide was performed, in the control slides there was no addition for primary antibody.

### **5.2.3 Materials**

The supplier for all chemicals in this work was British Drug Houses (BDH, UK) unless otherwise stated. All drugs were dissolved in water except for CGS15943 and SCH58261 were dissolved in DMSO.

Antibodies were obtained from Calbiochem/Merck Biosciences (Nottingham, UK, anti-A2A Ab).

#### **5.2.4 Statistical analysis**

Results are expressed as mean  $\pm$  SEM. Two way ANOVA was used for statistical comparisons with Bonferroni post-hoc test. A P value  $< 0.05$  was taken as statistically significant.

### 5.3 Results

Addition of KCl (60 mM) produced a sustained contractile response of segments of RTA, PMA and PCA. The mean tissue response to KCl addition in RTA, PMA and PCA was  $0.91 \pm 0.08$  g (n=35),  $9.21 \pm 0.43$  g (n=20), and  $9.96 \pm 0.20$  g (n=32), respectively.

#### 5.3.1 Effect of NAD in precontracted rat thoracic aorta, porcine mesenteric artery and porcine coronary arteries

In the RTA, NAD evoked concentration-dependent relaxations (Figure 5.1a, 5.3). The response to NAD did not reach a maximum response at concentrations up to 1 mM, but almost fully reversed the U46619-induced precontraction allowing the calculation of an approximate  $pEC_{50}$  value of  $4.24 \pm 0.19$  (n = 9). Endothelium removal resulted in no significant effect on the NAD induced relaxations (two way ANOVA,  $P > 0.05$ ) (Figure 5.1a).

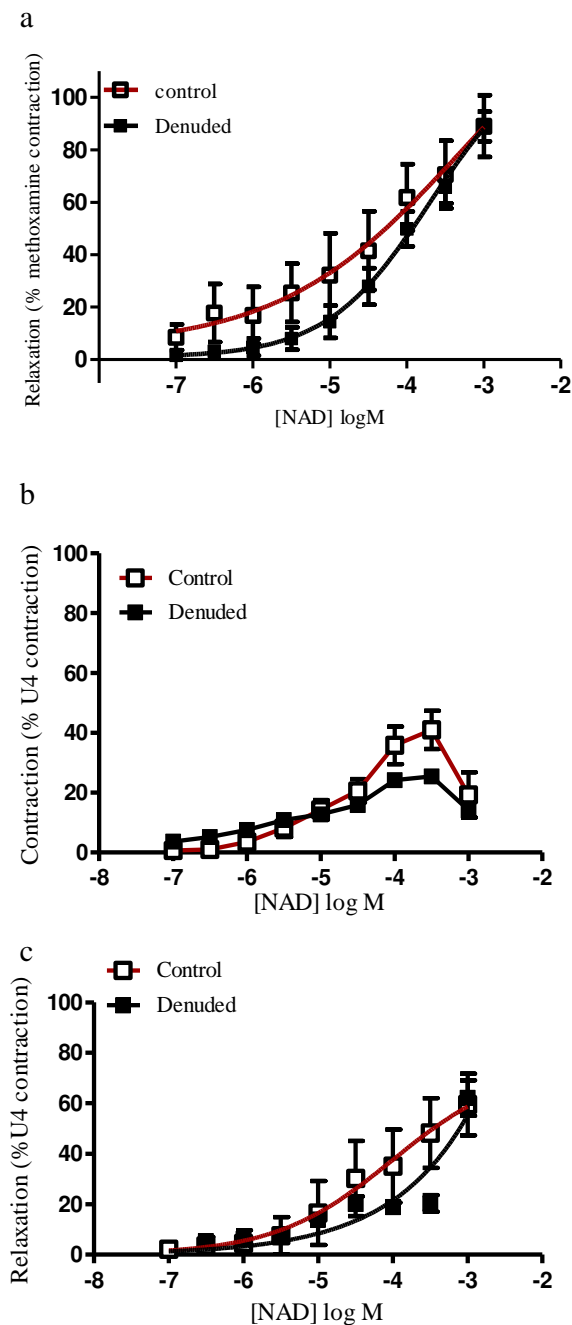
In PMA segments precontracted with U46619, NAD evoked concentration dependent contractions with a maximal contraction at 300  $\mu$ M (Figure 5.1b, 5.5). The highest concentration of NAD used (1 mM) caused a reduced contraction of the PMA. Non-linear analysis allowed computation of an  $R_{max}$  value of  $46 \pm 8$ ,  $pEC_{50}$  value of  $4.54 \pm$

0.22 and a Hill slope of  $1.05 \pm 0.12$  (n=12). Endothelium removal had no significant effect on the NAD-induced responses (Figure 5.1b).

In PCA segments precontracted with U46619, NAD evoked concentration dependent relaxations (Figure 5.1c). The response to NAD did not reach a maximum response so  $R_{max}$ ,  $pEC_{50}$  or Hillslope could not be calculated. Endothelium removal produced no significant effect on the NAD induced relaxations (Figure 5.1c).

In PMA and PCA segments, endothelium removal was assessed using substance P (10 nM), while acetylcholine (100 nM) was used to assess endothelium removal in the RTA. Any relaxation in response to substance P/acetylcholine of less than 10% of the U46619/methoxamine contraction was considered a success. In denuded PMA and PCA, there was an absence of the transient relaxation in response to substance P ( $6 \pm 1$  % (n = 6) and  $2 \pm 1$  % (n = 5), respectively) that was seen in control PMA and PCA segments ( $60 \pm 1$  % (n = 11) and  $70 \pm 1$  % (n = 6), respectively). In RTA there was a very slight relaxation ( $2 \pm 0.4$  %, n = 7) in response to acetylcholine in denuded vessels compared to the profound relaxation evoked in controls ( $65 \pm 0.4$  %, n = 10).

**Figure 5.1.** The effect of nicotinamide adenine dinucleotide (NAD) in segments from: a. rat thoracic aorta (RTA) b. porcine mesenteric artery (PMA), c. porcine coronary artery (PCA). Arteries were precontracted with U46619. Responses were evaluated in endothelium intact vessels (Control) and in those in which the endothelium had been removed (Denuded) in each of the RTA, PMA and PCA (n= 7-10, n= 6-11, n= 5-6, respectively). Results are mean  $\pm$  SEM.



### **5.3.2 Effect of P1 and P2 receptor antagonists on responses to NAD in the rat thoracic aorta**

Since NAD is able to act as an agonist at the P2Y<sub>1</sub> receptor, PaCoA was used to investigate whether NAD activates P2Y<sub>1</sub> receptors in PCA. PaCoA had no significant effect on the vasorelaxant response to NAD ( $P > 0.05$ , two way ANOVA) (Figure 2a).

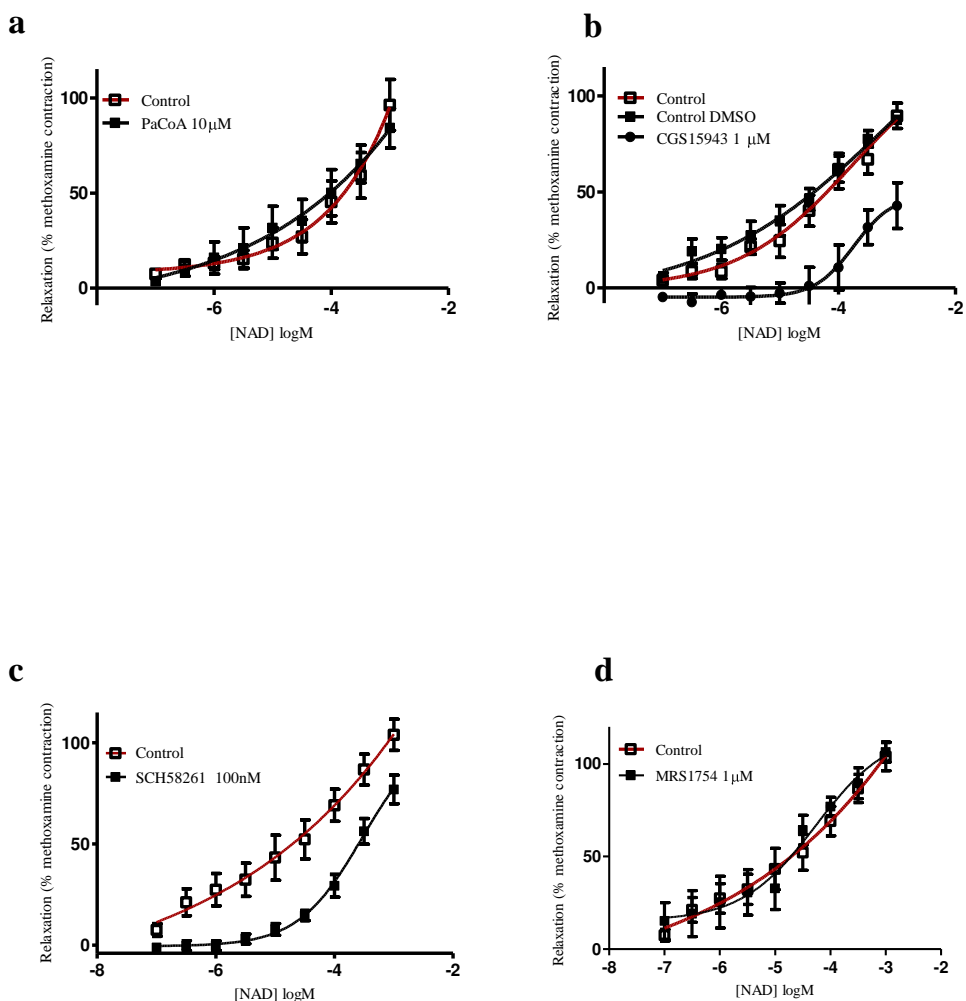
Since NAD is able to act as an adenosine receptor agonist, CGS15943 (a non-selective adenosine receptor antagonist, 1  $\mu$ M) was used, which significantly inhibited the NAD evoked relaxations (Figure 5.2b). SCH58261 (an A<sub>2A</sub> receptor-selective antagonist, 100 nM) was used, which also significantly decreased the NAD evoked relaxations with a calculated pKB value of  $7.25 \pm 0.24$  (Figure 5.2c, 5.3). The possible involvement of A<sub>2B</sub> receptors was also investigated using MRS1754 (a selective A<sub>2B</sub> receptor antagonist, 1  $\mu$ M), which had no significant effect on NAD-evoked relaxations (Figure 5.2d).

These experiments in rat thoracic aorta were carried out in methoxamine-precontracted tissues. In the absence of antagonists, methoxamine caused a sustained contraction to  $68 \pm 6\%$  (n=13) of the KCl response. The bath concentration of methoxamine required to produce this level of contraction was  $1.4 \pm 0.2 \mu$ M (n= 13). In the presence of PaCoA, SCH58261 and CGS15943, the level of tone was

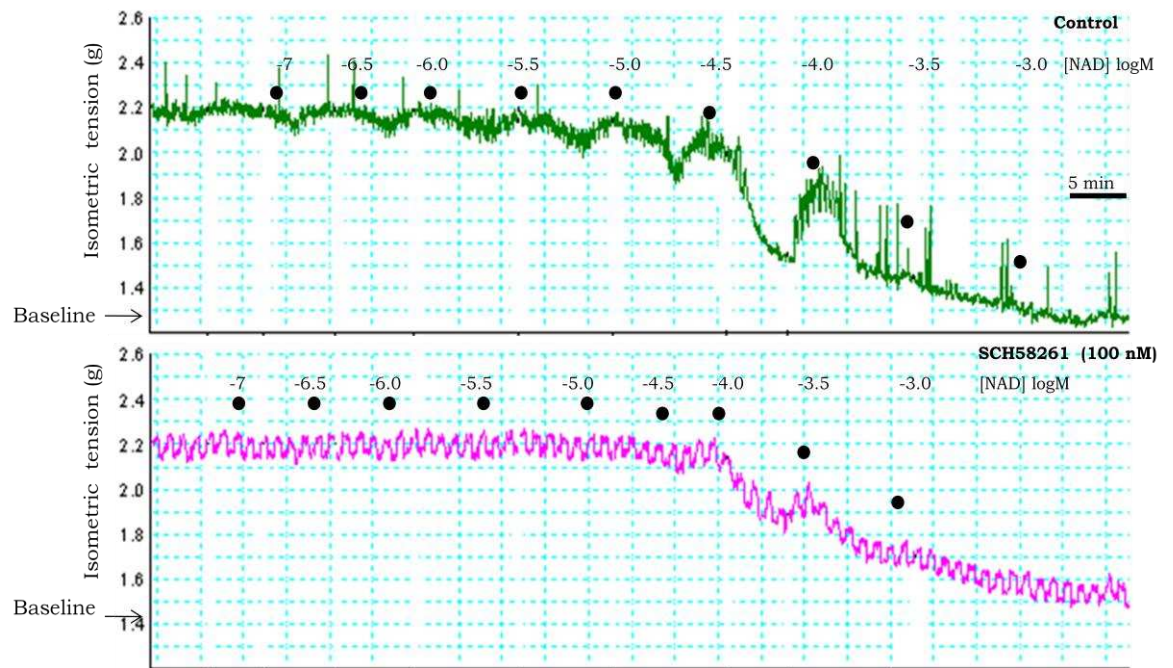
70 ± 6% (n= 6), 74 ± 5% (n = 15) and 59 ± 4% (n= 5) of the KCl contraction, respectively. There was no significant difference in the percentage of contraction to methoxamine in all used antagonists compared to the control ( $P > 0.05$  one way ANOVA). In the presence of PaCoA, SCH58261 and CGS15943, the mean bath concentration of methoxamine required was unchanged when compared to the control (one way ANOVA  $P > 0.05$ ).



**Figure 5.2.** Relaxatory responses in the rat thoracic aorta to nicotinamide adenine dinucleotide (NAD) in the presence of a. PaCoA, b. CGS15943, c. SCH58261 and d. MRS1754. Vessels were precontracted with methoxamine. Results are shown as mean  $\pm$  SEM (n=5-6, n=4-5, n=12-15 and n=4-8, respectively).



**Figure 5.3.** A representative trace for the relaxatory responses to cumulative addition of increasing concentrations of nicotinamide adenine dinucleotide (NAD) in the rat thoracic aorta in the absence and the presence of SCH58261. Vessels were precontracted with methoxamine.



### **5.3.3 Effect of P1 and P2 receptor antagonists on responses to NAD in porcine mesenteric artery**

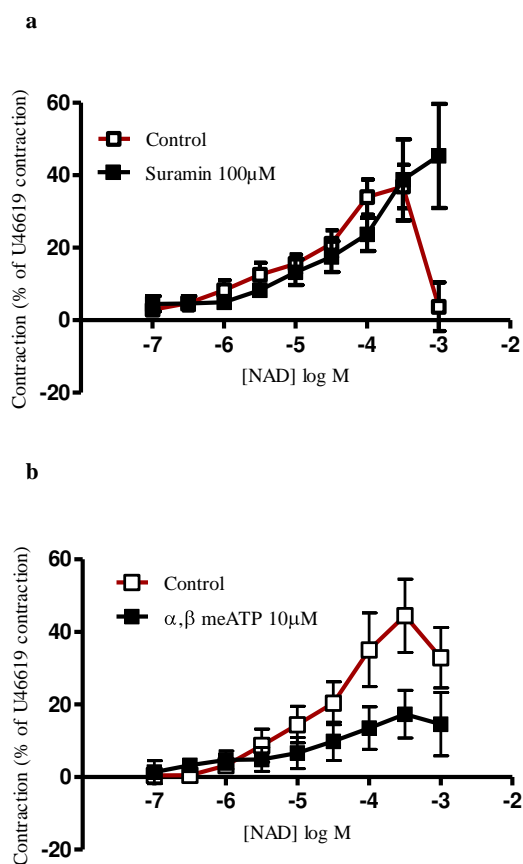
To test for any involvement of P2 receptors in the NAD-mediated contractile response, suramin (a P2 receptor antagonist, 100  $\mu\text{M}$ ) was used; it had no significant effect except at the highest NAD concentration where suramin prevented the reduction in NAD-evoked contraction (Figure 5.4a). In contrast,  $\alpha\beta$ -meATP (a P2X receptor desensitizing agonist, 10 $\mu\text{M}$ ) caused a significant inhibition of the NAD evoked responses (Figure 5.4b, 5.5). As  $\alpha\beta$ -meATP abolished the NAD response in some vessels an  $\text{EC}_{50}$  value could not be calculated. A mean contraction of  $45 \pm 10\%$  and  $17 \pm 7\%$  ( $n=7$ ,  $n=8$ , respectively) at 300  $\mu\text{M}$  NAD was found in the absence and presence of  $\alpha\beta$ -meATP, respectively.

Since NAD can also act as an agonist at the  $\text{P2Y}_1$  receptor, the effects of PaCoA was investigated. PaCoA at 10  $\mu\text{M}$  had no significant effect on the response to NAD ( $P > 0.05$ , two way ANOVA) (Figure 5.6a).

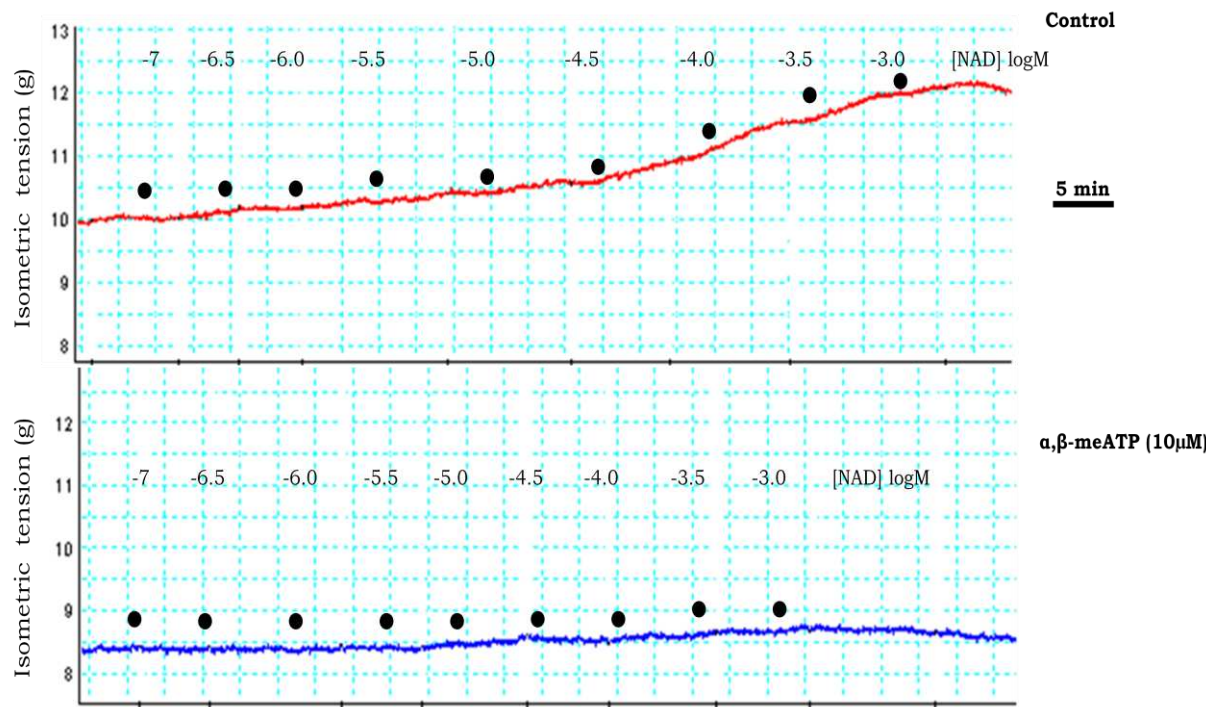
Since NAD can also act at P1 receptors, the effects of CGS15943 (a non-selective adenosine receptor antagonist, 10  $\mu\text{M}$ ) and SCH58261 (a selective  $\text{A}_{2\text{A}}$  receptor antagonist, 100 nM) were investigated; neither had any significant effect on the response to NAD (Figure 5.6b, c).

The above experiments in porcine mesenteric artery were carried out in vessels precontracted with U46619. In the absence of antagonists, U46619 caused a sustained contraction to  $75 \pm 4 \%$  ( $n= 7$ ) of the KCl response. The concentration of U46619 required to produce this level of contraction was  $131 \pm 18 \text{ nM}$  ( $n= 7$ ). In the presence of PaCoA, SCH58261, suramin and  $\alpha,\beta$ -meATP, U46619 elicited  $65 \pm 9 \%$  ( $n= 7$ ),  $63 \pm 5 \%$  ( $n= 4$ ),  $50 \pm 10\%$  ( $n= 4$ ) and  $58 \pm 3\%$  ( $n= 8$ ) of the KCl contraction, respectively, which was not significantly different from the control ( $P>0.05$ , one way ANOVA). The concentration of U46619 required to produce these contractions was unchanged ( $P> 0.05$ , one way ANOVA). In the presence of CGS15943, a higher concentration of U46619 ( $763 \pm 259 \text{ nM}$ ,  $n = 6$ ) was required to precontract the preparations compared to that used in the absence of CGS15943 ( $531 \pm 216 \text{ nM}$ ,  $n = 7$ ). There was no significant difference in the level of tone elicited in the absence and presence of GCS15943 at  $55 \pm 6\%$  ( $n = 7$ ) and  $49 \pm 10\%$  ( $n = 6$ ) of the KCl contraction, respectively.

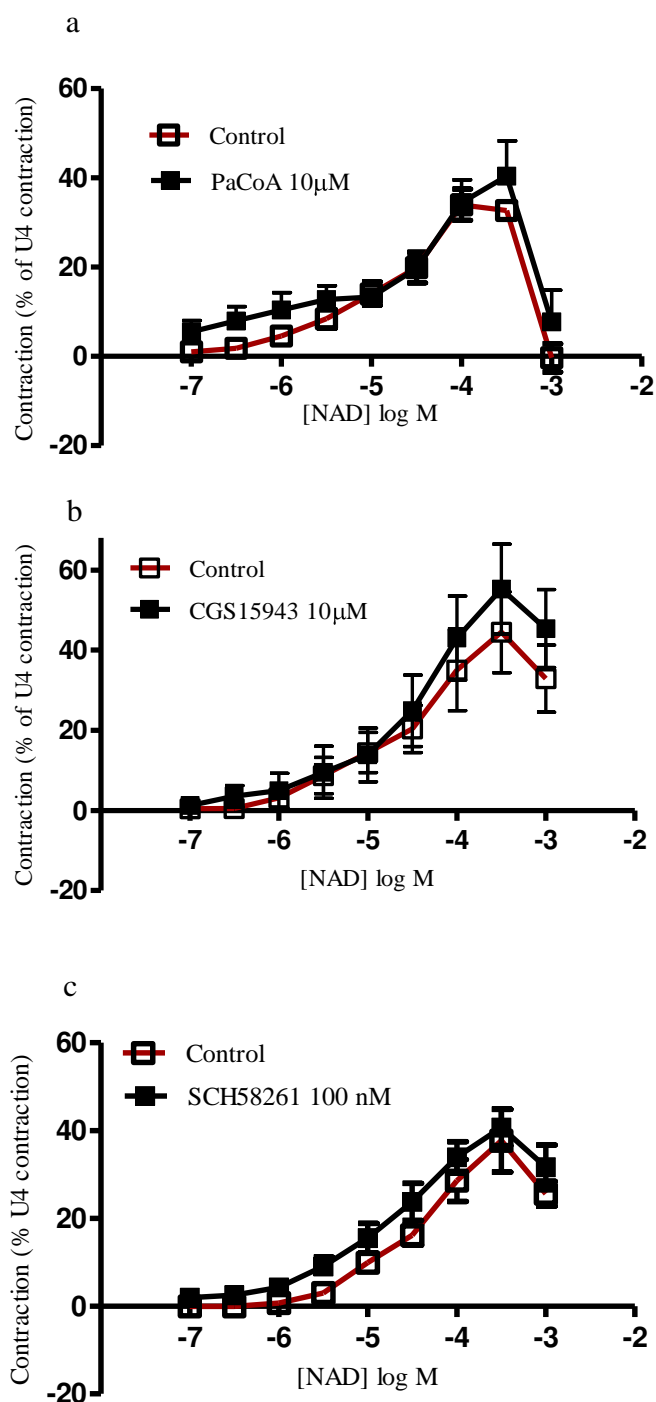
**Figure 5.4.** the contractile response of porcine mesenteric artery to nicotinamide adenine dinucleotide (NAD) in the presence of a. suramin, b.  $\alpha,\beta$ -meATP. The arteries had been precontracted with U46619. Results are shown as mean  $\pm$  SEM. (n=4, n= 7-8, respectively).



**Figure 5.5.** A representative trace for the contractile response to nicotinamide adenine dinucleotide (NAD) of porcine mesenteric artery in the absence and presence of  $\alpha,\beta$ -meATP. The arteries had been precontracted with U46619.



**Figure 5.6.** The porcine mesenteric artery contractile response to nicotinamide adenine dinucleotide (NAD) in the presence of a. PaCoA, b. CGS15943, c. SCH58261 (n=7, n=8, n=4, respectively). The arteries had been precontracted with U46619. Results are shown as mean  $\pm$  SEM.



#### **5.3.4 Effect of P1 and P2 receptor antagonists on responses to NAD in porcine coronary artery**

Since NAD can act as an agonist at the P2Y<sub>1</sub> receptor, PaCoA was used to investigate whether NAD activates P2Y<sub>1</sub> receptors in PCA. PaCoA had no significant effect on the vasorelaxant response to NAD ( $P > 0.05$ , two way ANOVA) (Figure 5.7a).

To characterise the NAD-evoked relaxations, we used CGS15943 (adenosine receptor antagonist) which, with the exception of the response to the highest concentration of NAD (1 mM), abolished the NAD evoked relaxations (Figure 5.7b). In the presence of CGS15943, contractions were observed at 10-100  $\mu$ M of NAD. SCH58261 (selective A<sub>2A</sub> receptor antagonist) (100 nM) abolished the NAD-evoked relaxations (Figure 5.7c, 5.8).

Since the NAD-mediated relaxations were blocked by A<sub>2A</sub> receptor antagonists, the expression of A<sub>2A</sub> receptors in PCA was investigated using immunohistochemistry; A<sub>2A</sub> receptor immunoreactivity was observed on the smooth muscle in PMA (Figure 5.9).

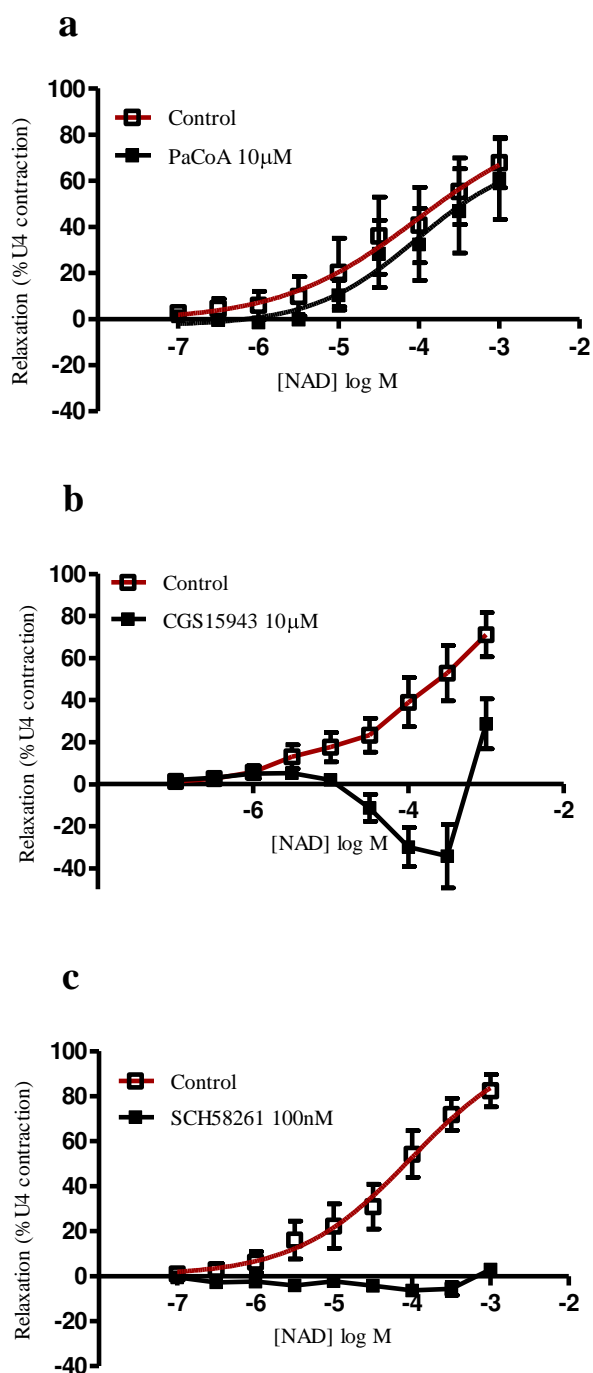
The above experiments in porcine coronary artery were carried out in U46619-precontracted tissues. In the absence of antagonists U46619 caused a sustained contraction to  $60 \pm 6\%$  (n=9) of the KCl response.



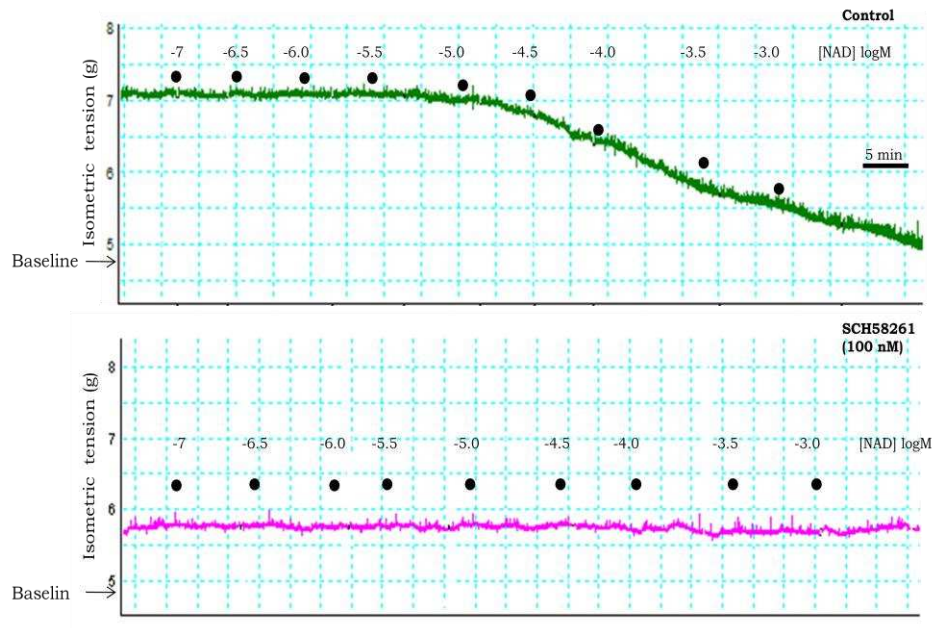
The bath concentration of U46619 required to produce this level of contraction was  $145 \pm 62$  nM (n= 9). In the presence of PaCoA and SCH58261 U46619 elicited  $65 \pm 5\%$  (n=5) and  $76 \pm 2\%$  (n=6) of the KCl contraction, respectively, which was not significantly different from the control (one way ANOVA  $P>0.05$ ). The mean bath concentration of U46619 required was unchanged (one way ANOVA  $P>0.05$ ).

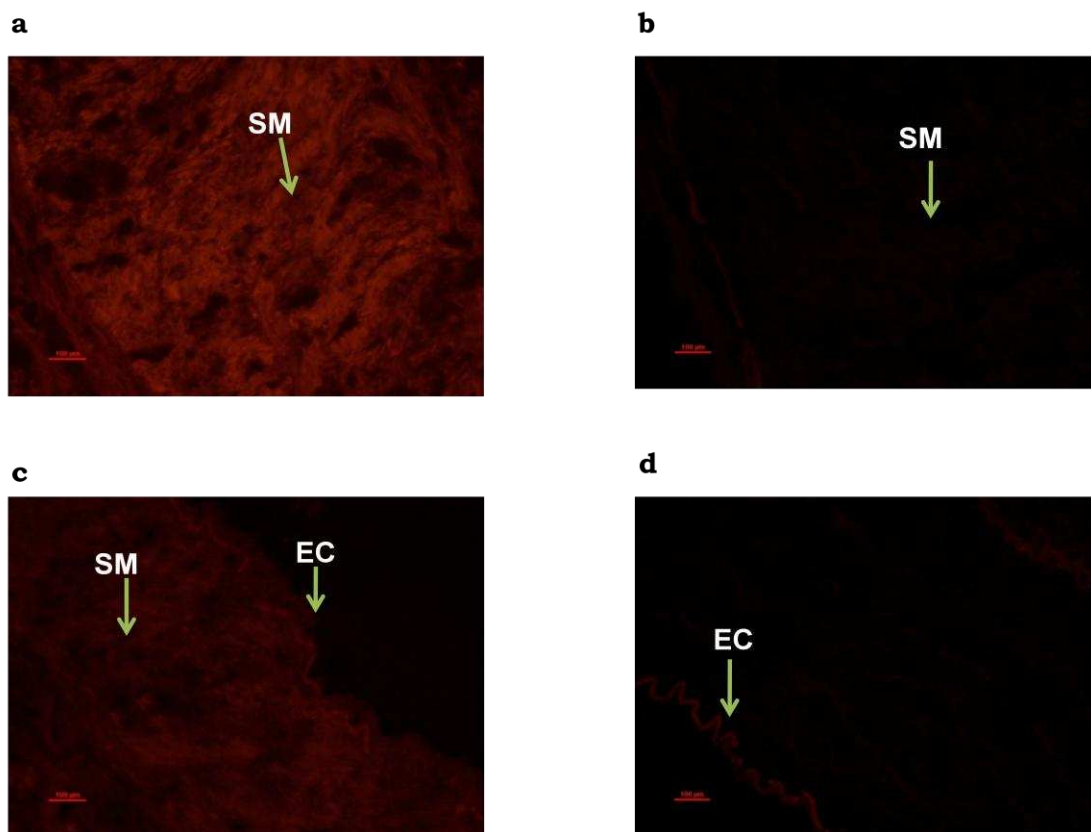
In the presence of CGS15943, a higher concentration of U46619 was required to precontract the PCA preparations; for these experiments  $175 \pm 79$  nM (n = 7) of U46619 was needed to achieve  $57 \pm 7\%$  (n = 7) of the KCl contraction in controls, while  $773 \pm 204$  nM (n = 6) of U46619 was needed to elicit  $24 \pm 4\%$  (n = 6) of the KCl contraction in the presence of CGS15943. Both the level of contraction to U46619 and the concentration of U46619 required to achieve that level were significantly different from the control ( $P < 0.05$ , one way ANOVA).

**Figure 5.7.** The porcine coronary artery relaxation to nicotinamide adenine dinucleotide (NAD) in the presence of a. PaCoA, b. CGS15943 c. SCH58261. Preparations were precontracted with U46619. Results are shown as mean  $\pm$  SEM (n=5, n=6, n=6 respectively).



**Figure 5.8.** A representative trace for the porcine coronary artery relaxation to nicotinamide adenine dinucleotide (NAD) in the absence and presence of SCH58261. Vessels were precontracted with U46619.





**Figure 5.9.** A representative  $A_{2A}$  receptor immunostaining of a porcine coronary artery in a. longitudinal sections and c. cross sections of the vessels accompanied by control which had no primary antibody to  $A_{2A}$  receptors in b. longitudinal section and d. cross section of the vessels. Smooth muscle (SM) and endothelial cells (EC) are indicated. Scale bar = 100  $\mu\text{m}$ .

## **5.4 Discussion**

The effects of NAD on the RTA, PMA and PCA were examined. NAD evoked vasorelaxations mediated through smooth muscle  $A_{2A}$  adenosine receptors in the RTA. In the PMA, NAD caused vasoconstrictions, which were mediated through P2X receptors. In the PCA, NAD evoked vasorelaxation through  $A_{2A}$  receptors on the smooth muscle. NAD, therefore evokes different effects (relaxation or contraction) in different blood vessels acting via different receptors.

### **5.4.1 Characterisation of NAD-evoked responses in rat thoracic aorta**

In the RTA, NAD caused endothelium independent vasorelaxations. PaCoA was used to see the effect it had on the NAD-evoked relaxations since NAD can act as an agonist at P2Y<sub>1</sub> receptors (Mutafova-Yambolieva et al., 2007). PaCoA had no significant effect on the NAD-evoked relaxations. The failure of PaCoA to inhibit the NAD-evoked relaxations in the RTA and the fact that these relaxations were endothelium independent indicates that P2Y<sub>1</sub> receptors do not mediate these vasorelaxations.

NAD was also shown to act as an agonist at P1 adenosine receptors (Hoyle, 1990, Burnstock and Hoyle, 1985), so P1 receptor antagonists were used to characterise the NAD-

mediated relaxations. We used CGS15943 (a non selective adenosine receptor antagonist) which significantly reduced the NAD evoked relaxations indicating the involvement of adenosine receptors. To determine which adenosine receptors mediate these responses, SCH58261 (a selective A<sub>2A</sub> receptor antagonist) was used. It significantly reduced the NAD-evoked relaxations in the RTA. This indicates that NAD-evoked relaxations in the RTA are mediated through A<sub>2A</sub> adenosine receptors.

The relaxations to NAD were not abolished in the presence of SCH58261 which may indicate the involvement of other adenosine receptors in addition to A<sub>2A</sub> adenosine receptors. NAD has previously been shown to act as an agonist at P1 receptors, without specification of which subtype was involved (Burnstock and Hoyle, 1985, Hoyle, 1990). Therefore, an A<sub>2B</sub> adenosine receptor antagonist, MRS1754, was used to check for any effect on the NAD-evoked relaxations. MRS1754 had no significant effect on the NAD-evoked relaxations which excludes the involvement of A<sub>2B</sub> adenosine receptors.

In the RTA, NAD had an approximate pEC<sub>50</sub> value of 4.24. This is similar to the pD<sub>2</sub> value reported by Burnstock and Hoyle (1985) for NAD-evoked relaxations in the guinea-pig taenia coli

at P1 receptors (4.18), but is somewhat different from the value identified by Mutafova-Yambolieva et al. (2007) in HEK cells at P2Y<sub>1</sub> receptors (6.1). NAD appears to act, therefore, at P1 receptors, specifically as an A<sub>2A</sub> receptor-selective agonist.

#### **5.4.2 Characterisation of NAD evoked responses in porcine mesenteric arteries**

In the current study in the PMA, NAD caused an endothelium-independent vasoconstriction up to a concentration of 300 µM above which it evoked a reduced contractile response. NAD has been identified as an agonist at P2Y<sub>1</sub> (Mutafova-Yambolieva et al., 2007), P2Y<sub>11</sub> (Moreschi et al., 2006), and various P2X receptors (P2X<sub>1</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub>) (Grahner et al., 2009) and can also activate P1 adenosine receptors (Hoyle, 1990, Burnstock and Hoyle, 1985).

There are no studies in the literature on the effect of NAD on the PMA. In the rat mesenteric arterial bed, Ralevic et al. showed that NAD, at concentrations above 50 nM, caused biphasic responses with a phase of constriction preceding the vasodilatation (Ralevic and Burnstock, 1998). It was suggested that it is unlikely that P1-purinoceptors mediate the vasodilator actions of NAD in rat mesenteric arterial bed; this was based on the conclusion of Blackburn et al. and Buss et al. that adenine

dinucleotides are largely resistant to degradation (Blackburn et al., 1987, Busse et al., 1988, Ralevic et al., 1995).

In this study, the P2 receptor antagonist suramin was used to characterise the responses to NAD. It had no significant effect except at the highest NAD concentration, where it prevented the reduced NAD-evoked vasoconstriction, implying a suramin-sensitive P2Y receptor-mediated relaxation solely at 1 mM NAD.  $\alpha\beta$ -meATP (a P2X receptor desensitizing agonist) caused a significant attenuation of the NAD-evoked contractions, suggesting activation of a suramin-insensitive P2X receptor. The responses to NAD were not completely abolished in the presence of the P2X desensitizing agent ( $\alpha,\beta$ -meATP at 10  $\mu$ M). It was reported previously that rat large mesenteric arteries have 25 - 100 fold lower sensitivity to  $\alpha\beta$ -meATP than smaller arteries (Gitterman and Evans, 2000). It was also reported that these rat large mesenteric arteries are insensitive to the P2 receptor antagonist suramin (Gitterman and Evans, 2000). It is likely, therefore, that, as relatively large blood vessels were used (first order PMA) in this study, that the NAD-evoked contractions were mediated through suramin-insensitive P2X receptors on the smooth muscle. Whether these responses are mediated through P2X<sub>1</sub> receptors could be confirmed using a selective antagonist such as NF449 (Rettinger et al., 2005). This agrees with the conclusion of Grahner et al. (2009) who showed that NAD acts



as an agonist at P2X receptors in human monocytes (Grahner et al., 2009).

Other potential reasons were investigated for the incomplete blockade evoked by  $\alpha,\beta$ -meATP of NAD-evoked contractile responses in the PMA. Since NAD can also act at P1 receptors, CGS15943 and SCH58261 were used. Neither had a significant effect on the response to NAD, confirming that these responses were not mediated through adenosine receptors.

The reduction in NAD evoked contraction which occurred at the highest concentration of NAD (1 mM) was blocked by suramin suggesting that it is mediated through P2 receptors. This needs further investigation as to whether a contaminant of the NAD, for example, ADP or ATP, may be responsible for this relaxation, or whether NAD itself activates suramin-sensitive P2Y receptors. Evidence from experiments conducted in the presence of PaCoA suggested that NAD fails to act through P2Y<sub>1</sub> receptors in this tissue.

There are no reported potency estimates for NAD acting through P2X receptors in the literature. In this study using PMA segments, the pEC<sub>50</sub> for NAD was 4.54 at P2X receptors. Burnstock and Hoyle (1985) reported a pD<sub>2</sub> value for P1-

mediated NAD evoked relaxations in the guinea-pig taenia coli of 4.18, while Mutafova-Yambolieva et al. (2007) found a pEC<sub>50</sub> value for NAD-evoked calcium responses through P2Y<sub>1</sub> receptors in HEK cells of 6.1. The potency differences presumably reflect simple differences in the receptors being activated.

#### **5.4.3 Characterisation of NAD-evoked responses in porcine coronary arteries**

In the current study, NAD caused an endothelium-independent vasorelaxation. Since NAD can act as an agonist at P2Y<sub>1</sub> receptors, the effects of PaCoA on the NAD evoked responses were tested. PaCoA had no significant effect on the NAD-evoked relaxations, indicating that P2Y<sub>1</sub> receptors do not mediate these vasorelaxations.

Since NAD can also act as an agonist at P1 adenosine receptors (Hoyle, 1990, Burnstock and Hoyle, 1985), adenosine receptor antagonists were employed to characterise the NAD-mediated responses in the PCA. CGS15943 inhibited these relaxations suggesting the involvement of adenosine receptors, without allowing identification of the particular subtype. The selective A<sub>2A</sub> receptor antagonist SCH58261 abolished NAD-evoked relaxations in the PCA, indicating that NAD evoked relaxations in PCA are mediated through A<sub>2A</sub> receptors.

#### **5.4.4 NAD responses in RTA, PMA and PCA**

Recently NAD has been increasingly reported as an extracellular signalling molecule, in addition to its intracellular role (Ziegler, 2000). The presence of a membrane-bound extracellular NADase implies that NAD may function as a physiological modulator of central neurotransmission (Khalmuradov et al., 1983, Snell et al., 1984). Degradation of NAD yields two second messengers that can induce increase of calcium from intracellular stores; cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (Bertheliet et al., 1998, Guse et al., 1997). NAD can be cleaved by ectoenzymes. Nucleotide phosphodiesterase/pyrophosphatase I (E-NPP, CD203 family) allows the production of AMP, which can be hydrolyzed to adenosine by ecto-5'-nucleotidase (CD73).

There are a number of shared properties between NAD and ADP. They both have significant roles in energy metabolism, they also act as substrates for extracellular enzymes which in turn generate a number of metabolites that may have signalling functions (Ziegler, 2005). For example, NAD serves as a substrate for mono-ADP-ribosyltransferases (ARTs) that catalyze the transfer of ADP-ribose from NAD to target proteins (Moss et al., 1990). NAD can also be used by CD38 and CD157; both have NAD-glycohydrolase and ADP-ribose cyclase activity,

resulting in the formation of ADP-ribose (ADPR) and cyclic ADP-ribose (cADPR) (Bertheliet al., 1998). Transmembrane transporters were identified for both NAD and cADPR (Bruzzone et al., 2001, Romanello et al., 2002, De Flora et al., 2004). These transporters mediate intracellular and intercellular trafficking of NAD and cADPR that enhances intracellular  $[Ca^{2+}]_i$  (De Flora et al., 2004).

Unlike ADP there are very few reports about intact NAD triggering intracellular events through surface receptors. NAD was reported to be released on nerve stimulation in blood vessels and urinary bladder in human, mouse and dog, and was demonstrated to have characteristics of a neurotransmitter (Breen et al., 2006, Mutafova-Yambolieva et al., 2007, Smyth et al., 2004). NAD was reported to act as an agonist at P2Y<sub>11</sub> receptor in granulocytes (Moreschi et al., 2006). Mutafova-Yambolieva et al. reported that NAD acts as an agonist at P2Y<sub>1</sub> receptors (Mutafova-Yambolieva et al., 2007). NAD was also reported to facilitate Ca<sup>2+</sup> influx, pore formation and cell death through acting as a substrate for mono-ADP-ribosyltransferase 2 (ART2), an enzyme that catalyzes ADP-ribosylation of P2X<sub>7</sub> receptors (Seman et al., 2003). An increase in  $[Ca^{2+}]_i$  in responses to extracellular NAD was reported in several cell types through the conversion of NAD into cADPR which in turns mobilises intracellular Ca<sup>2+</sup> (Sun et al., 1999, Verderio et al.,

2001, Esguerra and Miller, 2002, Romanello et al., 2002, De Flora et al., 2004). In human monocytes, exposure to NAD resulted in a rapid increase in  $[Ca^{2+}]_i$  caused by an influx of extracellular  $Ca^{2+}$  independent of intracellular  $Ca^{2+}$  (Gerth et al., 2004); in this tissue, the possibility of NAD acting through a degradation product was ruled out using selective inhibitors of CD38 and a stable NAD analogue. Interestingly, in this study the increase in  $[Ca^{2+}]_i$  was inhibited in the presence of ATP (Gerth et al., 2004). The possibility of NAD acting through an ATP receptor was investigated and led to the finding that NAD acts as an agonist at P2X receptors, an action inhibited in the presence of ATP which led to the speculation that since NAD and ATP have a similar mechanism to increase  $[Ca^{2+}]_i$  it is possible that ATP can control the fate of NAD by making it available to other pathways (Grahner et al., 2009).

In this study, NAD appeared to act through distinct receptors in different vascular beds. In the PMA, NAD appeared to act through P2X receptors, which agrees with the findings of Grahner et al. (2009) in human monocytes. Pfister et al. (2001) analyzed the NAD degradation products in human monocytes, and found that NAD was mainly degraded to ADP-ribose, nicotinamide, and minor amounts of AMP, ADP, and cADPR (Pfister et al., 2001). Adenosine was not detectable. If produced

sufficiently, all of these by-products have signalling functions mediated through different receptors. Based on this, Grahnert et al. (2009) concluded that NAD in human monocytes engages with P2X receptors as none of their by-products except ADP have been described to interact with P2X receptors. In the current study, it is not possible to confirm whether NAD acts directly at P2X receptors in the PMA or through its metabolites, mainly ADP. This can be confirmed using enzymes such as apyrase, a nucleoside diphosphatase that can catalyse the removal of the beta phosphate from ADP but fails to hydrolyze NAD (Babu et al., 2002).

In the RTA and PCA, it was observed that NAD evoked relaxations via  $A_{2A}$  receptors. This may be by acting directly at  $A_{2A}$  receptors, causing the release of adenosine or being broken down into adenosine. Blackburn et al. and Buss et al. suggested that adenine dinucleotides are largely resistant to degradation (Blackburn et al., 1987, Busse et al., 1988). This contrasts with the conclusion of Nikiforov et al. that  $NAD^+$  needs to be degraded outside the cell to serve as precursors of intracellular NAD (Nikiforov et al.). This also contrasts with the finding of Bruns (1980) who suggested that NAD needs to be broken down to act on adenosine receptors in human fibroblasts (Bruns, 1980).

These findings in the RTA and PCA agree with observations of Burnstock and Hoyle (1985) who reported that NAD acts in the guinea-pig taenia coli through P1 receptors. Using dipyridamole (a purine nucleoside uptake inhibitor) and 8-phenyltheophylline (a P1 receptor antagonist) they showed that the dominant mediator of NAD-induced relaxations is adenosine, and they reported two possibilities, which were that NAD either induces release of adenosine or is broken down to adenosine. They then concluded that NAD acts on P1 receptors probably indirectly, following its conversion to adenosine. NAD was reported to evoke the release of adenosine in rat vas deferens, guinea-pig taenia caecia and bladder (Stone, 1981).

NAD evoked endothelium independent vasorelaxation in the RTA and PCA through smooth muscle  $A_{2A}$  receptors. Whether these responses are caused by NAD activating  $A_{2A}$  receptors directly, by causing a release of adenosine or by being broken down into adenosine is in need of further characterisation. The role of adenosine could be confirmed by the use of the enzyme adenosine deaminase to degrade adenosine to inosine, a much less active metabolite. Furthermore, hydrolysis of NAD could be investigated using selective inhibitors of CD38 (an NAD-glycohydrolase), such as  $\beta$ -araF-NAD (Muller-Steffner et al., 1992). Radiolabelled NAD, such as carbonyl- $^{14}C$  NAD, could also be used to check the stability of this compound in contact with

vascular tissue. The use of HPLC with mass spectroscopy to determine extracellular levels of NAD and its metabolites could also be undertaken (Slominska et al., 2006).

The relaxations evoked by NAD were significantly reduced in the presence of SCH58261 in RTA, while SCH58261 abolished these NAD-evoked relaxations in PCA which may give an indication that in RTA the NAD evoked relaxations may involve other receptors that also mediate this relaxatory response.

In conclusion, there are clear differences in the actions of NAD in the PMA compared to the RTA and PCA; in both RTA and PCA, NAD acts through P1 receptors, while in PMA it appears to act through P2X receptors. The suggestion that NAD may be broken down to adenosine to act through P1 receptors may indicate higher levels of ectonucleotidases in RTA and PCA.



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## **Chapter Six**

### **General Discussion**

In this project, effects of acyl-CoA derivatives were examined on responses mediated by P2 and P1 receptors in RTA, PMA, PCA and HEK cells. Table 1 shows vasorelaxant purine receptors in the blood vessels used in the previous chapters, as characterised in our studies and as reported in literature. It can be seen that in the aorta and mesenteric arteries P2Y<sub>1</sub> receptors are expressed on the endothelium, but in coronary arteries these have only been reported on the endothelium in the pig (Olivecrona et al., 2004) but this is controversial (Rayment et al., 2007, Chapter Three, Five). P2Y<sub>2</sub> receptors also appear to be expressed on the endothelium of aorta and mesenteric arteries, but a recent study in mouse aorta raises the possibility that these may be P2Y<sub>6</sub> receptors (Guns et al., 2006). Nonetheless, the expression of at least two different vasorelaxant P2Y purine receptors in all three of the blood vessels allowed us to investigate the selectivity of CoA and its derivatives as possible P2Y<sub>1</sub> antagonists. In aorta, coronary and mesenteric arteries A<sub>2A</sub> and A<sub>2B</sub> receptors are expressed on both EC and SM (Table 6.1).

The present results indicate that, of all the acyl CoA derivatives used in this study (CoA, AcCoA, OlCoA and PaCoA), the most potent CoA derivative was PaCoA acting as an antagonist with

apparent selectivity for the P2Y<sub>1</sub> receptor in RTA, PMA and HEK cells.

Acyl-CoAs are essential intermediates in lipid biosynthesis and fatty acid metabolism. The possibility that acyl-CoAs play a physiologically significant role as endogenous modulators has received increasing attention (Chapters One and Two). Despite their non-specific effects which are evoked by their detergent properties, normally they are kept at low levels intracellularly by ACBPs and FABPs and these relatively low concentrations specifically and reversibly affect several transport and enzyme systems (Chapters One and Two).

Several studies have described different effects of acyl-CoA on a variety of cell functions and suggested that acyl-CoA can play a key regulatory role under certain circumstances (Chapter Two).

Table 6.1. Vasorelaxant purine receptors in aorta, coronary and mesenteric blood vessels in various species.

Vessels	Species	Receptors	location	Rferences
Aorta	guinea-pig	A <sub>2B</sub>	EC,SM	Hargreaves et al., 1991, Martin, 1992, Martin et al., 1993b, Gurden et al., 1993, Alexander et al., 1994
		P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>6</sub> , P2Y <sub>4</sub>	EC	Kaiser et al., 2002
	rat	A <sub>2A</sub> , A <sub>2B</sub>	EC, SM	conti et al 1993, Lewis et al., 1994, Monopoli et al., 1994, Prentice and Hourani, 1996, Chapter Five
		P2Y <sub>1</sub> , P2Y <sub>2</sub>	EC	Bultmann et al., 1998, Dol-Gleozes et al., 1999 Chapter Two
	mouse	A <sub>2A</sub> , A <sub>2B</sub>	EC,SM	Ponoth et al., 2009, Talukder et al., 2002, Ansari et al., 2006
		P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>6</sub>	EC	Beny et al., 2004, Guns et al., 2006
Coronary				
	pig	A <sub>2A</sub> , A <sub>2B</sub>	EC, SM	Balwierczak et al., 1991, Abeb et al., 1994, Monopoli et al., 1994, Rayment et al., 2007, Chapter Three, Chapter Five
		P2Y <sub>1</sub>	EC	Olivercrona et al., 2004
	lamb	P2Y	EC, SM	simonsen et al., 1997
	mouse	A <sub>2A</sub> , A <sub>2B</sub>	EC, SM	Feoktistov et al., 1997, Talukder et al., 2002
	bovine	A <sub>2A</sub>	---	Conti et al 1993
	guinea-pig isolated heart	A <sub>2A</sub>	---	Erga et al., 2000
mesenteric				
	pig	A <sub>2A</sub>	SM	Chapter Five
		P2Y <sub>1</sub>	EC	Chapter Three
	rat	A <sub>2A</sub>	EC, SM	Hiley et al., 1995
		A <sub>2B</sub>	SM	Rubino et al., 1995
		P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>4</sub>	EC	Malmsjo et al., 2000
		P2X	SM	Ralevic, 2002
	rabbit	A <sub>2A</sub>	EC	Balwierczak et al., 1991, de Brito et al., 2002
		P1, P2	SM	Mathieson and Burnstock, 1985
	mouse	P2X <sub>1</sub>	EC	Harrington et al., 2007
		P2Y <sub>6</sub>	EC	Koltsova et al., 2009

The extracellular existence of acyl-CoAs appears unlikely under normal physiological conditions. However, they may be released during pathophysiological conditions (Chapter Two). In this investigation, PaCoA was shown to act as a potent antagonist with apparent selectivity for P2Y<sub>1</sub> receptors (Chapters Two, Three, Four and Five). However, the mechanism by which PaCoA acts remains to be clarified. The simplest interpretation is that CoA compounds, because of their structural similarity with ADP, compete for the same binding site of the P2Y<sub>1</sub> receptor at the extracellular surface. The possibility of whether PaCoA can inhibit these receptors extracellularly and whether there is a possibility that transport proteins may move PaCoA to extracellular compartments under certain conditions is yet to be investigated. Another possibility is that acyl-CoA derivatives may act on the intracellular face of the plasma membrane to regulate P2Y<sub>1</sub> receptor function. In addition, acyl-CoAs were shown to associate with cell membranes by insertion of the fatty acyl chain into the bilayer (Powell et al., 1985). So whether the action of these compounds is facilitated by the fact that these compounds are actually integrated into the right place, in membranes, which make them available to interact with P2Y<sub>1</sub> receptors is an interesting possibility to investigate. Furthermore, it was suggested that in some cases ACBP donates

acyl-CoA directly to the site of action (Faergeman and Knudsen, 1997). However this ability to donate acyl-CoA does not always occur since AcCoA bound to ACBP was unable to inhibit AcCoA carboxylase (Rasmussen et al., 1994). So whether bound acyl-CoAs are still available for binding to some specific enzymes or even receptors is in need of further investigation.

The way by which these compounds act can be tested using isolated lipid rafts since P2Y<sub>1</sub> receptors have been shown to associate with lipid rafts (Volonte et al., 2007). By incubating these isolated lipid rafts with tritiated PaCoA and/or CoA, it should be possible to check for co-localization of P2Y<sub>1</sub> receptor with PaCoA which may help to explain the action of PaCoA as an antagonist at P2Y<sub>1</sub> receptors.

Another approach would be to make use of cells or cell fragments that natively express P2Y<sub>1</sub> receptors, such as platelets, and heterologously-expressed receptors in cells such as 1321N1 cells, an astrocytoma cell line that does not express endogenous purine receptors. Injecting these cells with a calcium-sensitive dye and PaCoA (or CoA, etc.) simultaneously and following any change in Ca<sup>2+</sup> signalling in response to extracellular ADP addition could be compared with the action of external PaCoA application on ADP addition. In whole cell patch clamp experiments Shumilina et al. have reported in Chinese hamster ovary cells and HEK cells the ability of endogenously

produced acyl-CoA to modulate the activity of  $K_{ATP}$  and Kir channels (Shumilina et al., 2006).

The use of mice in which the gene encoding for  $P2Y_1$  receptors is disrupted should allow confirmation of the target of ADP and PaCoA. In addition to the action of PaCoA on vascular cell-surface  $P2Y_1$  receptors, it will be useful to test the response of these compounds on mitochondrial  $P2Y_1$  receptors (Belous et al., 2004, Belous et al., 2006). Given intracellular nucleotide levels, it appears likely that these receptors are in a continuously activated state. Using isolated mitochondria, it should be possible to test the effects of the endogenous ligands, ADP and ATP, as well as PaCoA. It is conceivable that high intracellular levels of PaCoA allow the mitochondria to be protected from nucleotide-evoked desensitisation, which is only relieved when PaCoA is metabolised. The phenomenon observed of PaCoA-mediated inhibition of vascular, cell-surface  $P2Y_1$  receptors may just be a 'hangover' from the intracellular location and function of PaCoA.

*In vivo* administration of  $P2Y_1$  antagonists, MRS2179 and MRS2500, has been performed in pigs and mice, respectively, which gave further evidence for a role of the  $P2Y_1$  receptor in post ischemic hyperemia and thrombosis (Olivecrona et al.,

2004, Hechler et al., 2006). MRS2179 and MRS2500 significantly reduced the post-ischemic hyperemia and inhibited both systemic and localized arterial thrombosis, respectively; this supports the concept that targeting the P2Y<sub>1</sub> receptor can be a reasonable complement or alternative to current clinical management of reperfusion injury in the treatment of acute myocardial infarction and in antithrombotic therapy (Olivecrona et al., 2004, Hechler et al., 2006). PaCoA is an attractive lead for assessing these *in vivo* effects, since it was reported to have antiplatelet activity acting mainly at P2Y<sub>1</sub> receptors *ex vivo* and to be antagonist at recombinant P2Y<sub>1</sub> receptors (Coddou et al., 2003, Manolopoulos et al., 2008) and, in this study, to be an antagonist at endogenous vascular P2Y<sub>1</sub> receptors in RTA and PMA, as well as at native P2Y<sub>1</sub> receptors in HEK cells (Chapters Two, Three, Four and Five). If such actions are reproduced *in vivo*, PaCoA could be used as a lead compound for the development of even more selective and potent antagonists and possibly radioligands at the P2Y<sub>1</sub> receptor.

Although both the mechanism of action and the *in vivo* effects of acyl-CoA derivatives have yet to be clarified, it is an attractive field to explore and if these effects can be confirmed *in vivo*, there is a possibility that acyl-CoAs may add to the understanding and management of pathophysiological conditions such as hypertension, diabetes, thrombosis and



many more disorders. Interestingly, the action of PaCoA on blood vessels that was found in this study using RTA and PMA, where PaCoA was found to block the ADP-mediated relaxations at P2Y<sub>1</sub> receptors, may indicate a role in the severity of hypertension. If this can be demonstrated *in vivo*, it may suggest that CoA compounds could be used as leads in the design of molecules that may have the ability to prevent the interaction of endogenous PaCoA with P2Y<sub>1</sub>. On the other hand, the antiplatelet action of PaCoA at platelet P2Y<sub>1</sub> receptors, if found to be relevant *in vivo*, may suggest the use of PaCoA as a lead compound for the design of more potent compounds that can also bind and act as antagonists at P2Y<sub>1</sub> receptors in platelets and exert this antithrombotic activity observed with PaCoA.

In addition to the acyl-CoAs, another endogenous nucleotide derivative studied in this project was NAD. NAD is an essential coenzyme found in all cells. Intracellularly, NAD acts as an essential co-enzyme for the transfer of electrons in redox reactions and is also involved in other cellular processes such as acting as a substrate for several enzymes. Normally, the concentration of NAD depends on the balance between its release from cells and its enzymatic degradation. In human plasma, and in other extracellular fluids, the NAD concentration was reported to be in the range of 40 - 100 nM (Bruzzzone et al., 2001, De Flora et al., 2004). In certain pathophysiological states,

such as inflammation, levels of NAD were reported to be significantly higher in some tissues compared to those in plasma (Smyth et al., 2004, Krebs et al., 2005, Scheuplein et al., 2009). Extracellularly, NAD can elicit functional responses by binding to specific receptors, namely purinergic receptors and this may occur by direct actions of NAD or may involve metabolites generated by NAD hydrolysis, or through release of purines. NAD has been reported to have different signalling cascades and outcomes according to both cell type and environment; for example, in human granulocytes, NAD activates P2Y<sub>11</sub> receptor and results in cell activation (Moreschi et al., 2006). On the other hand, in gastrointestinal myocytes, NAD also activates P2Y<sub>11</sub> receptors and results in cell inhibition (Mutafova-Yambolieva et al., 2007). In the present study, NAD was found to act as an agonist at P2X receptors in PMA evoking vasoconstriction and an agonist at A<sub>2A</sub> receptors in RTA and PCA eliciting vasorelaxation (Chapter Five). It is tempting to speculate that the actions of NAD at A<sub>2A</sub> receptors are via adenosine generated from hydrolysis of NAD.

Enzymes such as CD38 can hydrolyse NAD and generate several metabolites such as ADPR and nicotinamide which may also be hydrolysed further into adenosine monophosphate and then converted to adenosine (Chapter Five). These metabolites and

metabolizing enzymes may also exert some potential extracellular and intracellular effects; for example adenosine can either bind to  $A_{2A}$  receptors or it can be used to reconstitute the intracellular nucleotide pool. So the hydrolysis of NAD can recycle these metabolites and also regulate signals mediated by purine receptors. The possibility that the extracellular pool of NAD and its metabolites may affect some intracellular functions has been explored in chronic lymphocytic leukemia cells. It was suggested that this extracellular network of NAD and its metabolites and the modulation of this network by hydrolyzing enzymes may contribute to modifying the local environment, making it favorable to the neoplastic cells, and that exploring this field may change therapeutic strategies for the management of lymphocytic leukemia (Vaisitti et al., 2011).

In this study, it was not determined whether the NAD responses in PMA, mediated through P2X receptors, and in PCA and RTA, through  $A_{2A}$  receptors, are a result of NAD acting directly at these receptors, through its metabolites or by evoking the release of nucleosides such as adenosine (Chapter Five). Although the reported plasma levels of NAD described above are lower than the range of concentrations we have used, levels of NAD resulting from cell disruption in some pathophysiological conditions may rise to relatively high levels. The action of NAD in PMA and PCA gives an indication of a probable application of

this compound; the relaxatory response in coronary arteries and the contraction in the mesenteric arteries may give a hint that these compounds may be used as a lead for compounds that may be used in the management of cardiovascular diseases such as angina and narrowing of coronary arteries.

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