

Identification of contractile P2Y<sub>1</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> receptors in rat intrapulmonary artery using  
selective ligands

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**Running title:** P2Y receptor-mediated pulmonary artery contraction

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**Number of text pages:** 21

**Number of tables:** 0

**Number of figures:** 5

Number of references: 58

Number of words in Abstract: 233

Number of words in Introduction: 749

Number of words in Discussion: 1,220

**Abbreviations:**

IPA, intrapulmonary arteries

ATP, adenosine 5'-triphosphate

ADP, adenosine 5'-diphosphate

UDP, uridine 5'-diphosphate

UDP-glucose, uridine 5'-diphosphate glucose

MRS2365, (N)-methanocarpa-2-MeSADP

MRS2179, N6-methyl 2'-deoxyadenosine 3', 5'-bisphosphate

AR-C69931MX, [N6-(2-methylthiomethyl)-2-(3,3,3-trifluoropropylthio)-, -dichloro-methylene ATP

PSB 0474, 3-(2-Oxo-2-phenylethyl)-UDP

MRS2578, N,N"-1,4-Butanediylbis[N'-(3-isothiocyanatophenyl)thiourea (MRS2578),

MK571, 3-[[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid

Suramin, 8,8'-[Carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid

**Section:** Cardiovascular

**Abstract:**

ATP and UDP constrict rat intrapulmonary arteries, but which receptors mediate these actions is unclear. Here we used selective agonists and antagonists, along with measurements of P2Y receptor expression, to characterise the receptor subtypes involved. Isometric tension was recorded from endothelium-denuded rat intrapulmonary artery rings (i.d. 200-500  $\mu\text{m}$ ) mounted on a wire myograph. Expression of P2Y receptor subtype expression was determined using RT-PCR with receptor-specific oligonucleotide primers. The selective P2Y<sub>1</sub> agonist MRS2365 induced small, concentration-dependent contractions that were inhibited by the P2Y<sub>1</sub> antagonist MRS2179. Contractions evoked by ATP were unaffected by MRS2179, but inhibited by about one third by the P2Y<sub>12</sub> antagonist AR-C69931MX. Combined blockade of P2X<sub>1</sub> and P2Y<sub>12</sub> receptors virtually abolished the response to ATP. ADP also evoked contractions that were abolished by AR-C69931MX. The selective P2Y<sub>6</sub> receptor agonist, PSB 0474, evoked concentration-dependent contractions and was approximately 3-times more potent than UDP, but the P2Y<sub>14</sub> agonist, UDP-glucose, had no effect. Contractions evoked by UDP were inhibited by the P2Y<sub>6</sub> receptor antagonist, MRS2578, but not the CysLT<sub>1</sub> antagonist, MK571. Higher concentrations of MRS2578 inhibited contractions to KCl and so were not studied further. mRNA for P2Y<sub>1</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> receptors was identified. Our working model is that P2Y<sub>12</sub> and P2X<sub>1</sub> receptors are present in rat intrapulmonary arteries and together mediate ATP-induced vasoconstriction. Contractile P2Y<sub>6</sub>, but not P2Y<sub>14</sub> or CysLT<sub>1</sub> receptors are also present and are a major site through which UDP evokes constriction.

## Introduction

The endogenous nucleotides adenosine 5'-triphosphate (ATP) and uridine 5'-diphosphate (UDP) act at P2X and P2Y receptors in the cardiovascular system to modulate arterial pressure (Burnstock and Kennedy, 1986, 2011; Erlinge and Burnstock, 2008). Both types of P2 receptor are expressed in human pulmonary arteries (Liu et al., 1989b) and there is growing evidence that they contribute to the regulation of pulmonary vascular tone *in vivo*. For example, they may be activated by ATP, released from red blood cells on their passage through the lungs (Sprague et al., 1996; 2003). In healthy pulmonary arteries the main effect of ATP is to promote vasodilation via endothelial P2Y receptors, which induce release of nitric oxide (Erlinge and Burnstock, 2008). This contributes to the maintenance of low pulmonary vascular resistance, essential for delivery of deoxygenated blood to the alveoli (Barnes and Liu, 1995).

Nucleotides also act at smooth muscle P2X and P2Y receptors to evoke pulmonary vasoconstriction (McCormack et al., 1989; Liu et al., 1989a,b; Hasséssian and Burnstock, 1995; Rubino and Burnstock, 1996; Hartley et al., 1998; Rubino et al., 1999; Chootip et al., 2002, 2005; Jernigan et al., 2006; Syed et al., 2010; Mitchell et al., 2012). These effects are likely to become more pronounced in conditions where endothelium-dependent relaxation is impaired, such as hypoxia- or monocrotaline-induced pulmonary hypertension (Adnot et al., 1991; Mam et al, 2010) and chronic obstructive pulmonary disease (Dinh-Xuan et al., 1991). This may be of particular concern in chronic obstructive pulmonary disease, where extracellular ATP levels in the lung are elevated (Lommatzsch et al., 2010). The smooth muscle receptors may also contribute to the acute pulmonary vasoconstriction evoked in hypoxic conditions, as the response in perfused rabbit lungs was inhibited by a P2 antagonist (Baek et al., 2008). Thus P2 receptors are clearly implicated in the control of pulmonary arterial tone under both physiological and a number of pathophysiological conditions.

Multiple P2 receptor subtypes mediate the actions of ATP and UDP (Burnstock and Kennedy, 1985; Khakh et al., 2001; Abbracchio et al., 2006). We reported that ATP constricts rat intrapulmonary arteries (IPA) via P2X1 receptors and an unidentified P2Y receptor, whilst UDP acts at two P2Y subtypes, one of which may be the P2Y<sub>6</sub> receptor (Chootip et al., 2002, 2005). These early studies were, however, limited by the very poor selectivity of the agonists and antagonists available. In addition, nucleotides are rapidly dephosphorylated by ectoenzymes in vascular smooth muscle (Evans and Kennedy, 1994; Kennedy and Leff, 1995; Robson et al., 2006), which decreases their apparent potency and can lead to the production of metabolites that are active at other receptor subtypes. For example, the initial metabolite produced from ATP is adenosine 5'-diphosphate (ADP), an agonist at P2Y<sub>12</sub> receptors (Bodor et al., 2003), which were reported to mediate contraction of human isolated arteries (Wihlborg et al., 2004; Högberg et al., 2010).

A much clearer identification of the individual P2Y receptor subtypes that mediate the actions of nucleotides is now possible due to the recent development of several compounds with P2Y receptor subtype-selectivity. For example, (N)-methanocarpa-2-MeSADP (MRS2365) is a highly selective and potent P2Y<sub>1</sub> agonist (Ravi et al., 2002; Chhatriwala et al., 2004), N6-methyl 2'-deoxyadenosine 3', 5'-bisphosphate (MRS2179), a potent, competitive P2Y<sub>1</sub> antagonist (Boyer et al., 1998; Camaioni et al., 1998) and [N6-(2-methylthiomethyl)-2-(3,3,3-trifluoropropylthio)-, -dichloro-methylene ATP (AR-C69931MX), a highly selective and potent P2Y<sub>12</sub> antagonist (Ingall et al., 1999). P2Y<sub>6</sub> receptors can now also be probed using 3-(2-Oxo-2-phenylethyl)-UDP (PSB 0474, also known as 3-phenacyl UDP), which displays >500-fold selectivity as an agonist at P2Y<sub>6</sub> over P2Y<sub>2</sub> receptors and is essentially inactive at the P2Y<sub>4</sub> and P2Y<sub>14</sub> receptors (El-Tayeb et al., 2006; Gao et al., 2010) and *N,N'*-1,4-Butanediylbis[*N'*-(3-isothiocyanatophenyl)thiourea (MRS2578), a potent and insurmountable P2Y<sub>6</sub> antagonist (Mamedova et al., 2004).

The aim of the present study was to use these subtype-selective ligands to determine the contributions of P2Y<sub>1</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> receptors to the constriction of rat IPA elicited by ATP and UDP. In addition, the P2Y<sub>14</sub> receptor was previously excluded due to reports that it was insensitive to UDP (Chambers et al., 2000; Freeman et al., 2001), but recent re-examination of its pharmacological properties revealed that UDP is actually a potent P2Y<sub>14</sub> agonist (Carter et al., 2009). A further issue is that UDP has also been proposed to be an agonist at the phylogenetically-related cysteinyl leukotriene CysLT<sub>1</sub> receptor (Mellor et al., 2001, 2002). Thus the contributions of P2Y<sub>14</sub> and CysLT<sub>1</sub> receptors to the action of UDP were also investigated. Finally, this pharmacological approach was complemented by analysis of the expression of each P2Y subtype.

## Materials and Methods

**Organ bath studies.** Male Sprague-Dawley rats (200-250 g) were killed by cervical dislocation and exsanguination. The procedures used were as humane as possible and comply with national guidelines for animal care. The heart and lungs were removed *en bloc* and placed in a solution composed of (mM); NaCl 122, KCl 5, N-[2-hydroxyethyl] piperazine-N'-(2-ethanesulfonic acid) (HEPES) 10, KH<sub>2</sub>PO<sub>4</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.5, MgCl<sub>2</sub> 1, glucose 11, CaCl<sub>2</sub> 1.8, titrated to pH 7.3 with NaOH and bubbled with "medical air" (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>). IPA of internal diameter 200-500 µm were dissected, cleaned of connective tissue and their endothelium removed gently by passing a needle and thread through the lumen. They were then cut into 5 mm rings, mounted horizontally on a pair of intraluminal wires in 1 ml organ baths and equilibrated under a resting tension of 0.5 g for 60 min at 37°C. Tension was recorded with Grass FT03 isometric force transducers, connected to a PowerLab/4e system, using Chart 4.2 software (AD Instruments, UK).

**Experimental protocols.** Drugs were directly added to the tissue bath and washed out by replacement with drug-free solution. Removal of endothelium was confirmed by loss of relaxation to acetylcholine (10 µM) when applied to vessels pre-contracted with ATP or UDP. Preliminary experiments showed that 6 repeated additions of ATP or UDP for 5 min at 30 min intervals elicited highly reproducible contractions. The P2Y<sub>1</sub> agonist MRS2365 and P2Y<sub>6</sub> agonist PSB 0474 were applied using the same protocol. To determine the effects of MRS2578, 3-[[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid (MK571) and 8,8'-[Carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid (suramin), control responses to an agonist were obtained. Arteries were then incubated with the antagonist for 20 min and the agonist re-administered. When studying the effects of AR-C69931MX, this protocol was followed three times, using three, progressively higher concentrations of AR-C69931MX.

**P2Y receptor mRNA expression.** Total RNA was prepared from endothelium-denuded IPA of 11 rats (wet tissue weight = 146.6 mg) using a Total RNeasy Midi kit (Qiagen, CA, USA), according to the manufacturer's protocol. The RNA concentration (42.5 µg/100 µl) was determined spectrophotometrically using a Genequant II RNA/DNA calculator (Pharmacia). cDNA was synthesised using 5 µg RNA and Superscript III reverse transcriptase (200 Units) (Invitrogen). The cDNA was added to a HotStarTaq DNA polymerase (Qiagen) PCR reaction mix containing 10 pmol/µl of appropriate forward (5'-3') and reverse (5'-3') primers (MWG-Biotech), each pair designed to selectively recognise a particular P2Y receptor, as follows: P2Y<sub>1</sub> forward TCCTCTTCATTCCGATGTGCC, reverse TCTTCTTCTTGAGCCTGCCA, 391 bp; P2Y<sub>6</sub>

forward TGCTTGGGTGGTATGTGGAGT, reverse TGTTGTGTGAAGTAGAAGAGGATA, 498 bp; P2Y<sub>12</sub> forward GGCCTTCATGTTCTGCTGTC, reverse GGGTGCTCTCCTTCACGTAGAAC, 404 bp. The mix was placed in a DNA Thermal Cycler (Perkin Elmer, UK) and the following protocol applied: 10 min at 95°C followed by 35 cycles of 90 sec at 95°C, 30 sec at 52°C, 90 sec at 68°C and a final extension step of 10 min at 68°C. PCR products were separated on a 1.5% w/v agarose gel and visualised by ethidium bromide staining. The bands were then purified and the sequence of each P2Y subtype confirmed using a BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Warrington, UK) and an Applied Biosystems 3100 Avant Genetic Analyser.

**Drugs and solutions.** ATP (Na<sub>2</sub> salt), ADP (Na salt), UDP (Na<sub>2</sub> salt), UDP-glucose (Na<sub>2</sub> salt), PSB 0474 (Na<sub>2</sub> salt), acetylcholine chloride (Sigma, UK), MRS2179 (Na<sub>4</sub> salt), MRS2365 (Na<sub>3</sub> salt), 4,4',4",4"-[Caronylbis(imino-5,1,3-benzenetriyl-*bis*(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid, (Na<sub>8</sub> salt) (NF449) (Tocris, UK), suramin hexasodium (RBI, USA), AR-C69931MX (The Medicines Company, USA) and MK571 (Na salt) (Biomol, UK) were dissolved in distilled water as 1, 10 or 100 mM stock solutions and diluted in HEPES-based buffer before applying to the tissues. MRS2578 (Tocris, UK) was dissolved in DMSO as a 10 mM stock solution, immediately frozen and stored at -20°C, as recommended by the supplier. On the day of use it was diluted in HEPES-based buffer before applying to the tissues. Isotonic 40 mM K<sup>+</sup> solution was prepared by replacing NaCl with an equimolar amount of KCl in HEPES-buffered solution to maintain osmolarity of the solution.

**Data Analysis.** Contractions are expressed as mg tension, percentage of the control response produced by a given agonist or percentage of the contraction evoked by KCl (40 mM), as appropriate. Data are shown as mean ± S.E.M. When appropriate, log concentration-response curves were fitted to the data by logistic (Hill equation), nonlinear regression analysis (Prism; GraphPad, San Diego, CA). Data were compared using Student's paired or unpaired t tests, as appropriate. Values of  $P < 0.05$  were considered to be statistically significant.

## Results

**ATP-sensitive receptors - agonist studies.** We showed previously that ATP evokes concentration-dependent contractions of rat IPA via P2X1 receptors and an, as yet, unidentified P2Y receptor (Chootip et al., 2002, 2005). To determine if P2Y<sub>1</sub> receptors are involved, their presence in rat IPA was studied initially using MRS2365, a highly potent and selective P2Y<sub>1</sub> agonist. MRS2365 (1 - 100 μM) induced small, concentration-dependent contractions of rat IPA that reached a peak within 1-2 min and then slowly declined (Figure 1a,b). At the highest concentration used (100 μM), the mean contraction amplitude was  $58 \pm 13$  mg (n=9), which was equivalent to  $23.6 \pm 4.4\%$  of the response to 40 mM KCl. In contrast, at the same concentration, ATP evoked contractions that were equivalent to  $50.3 \pm 11.0\%$  of the response to 40 mM KCl (Chootip et al., 2002). Due to the small quantity of MRS2365 available, a complete concentration-response curve could not be constructed, but it appears that MRS2365 is less efficacious than ATP at eliciting contractions of rat IPA and these data are consistent with the presence of contractile P2Y<sub>1</sub> receptors in rat IPA.

**ATP-sensitive receptors - antagonist studies.** The contribution of the P2Y<sub>1</sub> receptors to the ATP response was then investigated using MRS2179, a selective P2Y<sub>1</sub> antagonist. MRS2179 (10 μM) had no effect on basal tone and as shown in Figure 1c, no significant effect on contractions evoked by ATP (300 μM) ( $95.8 \pm 3.2\%$  of control, n=6). In contrast, it reduced significantly the response to MRS2365 (100 μM) by  $84.5 \pm 5.3\%$  ( $P < 0.01$ , n=4) (Figure 1b). At the same concentration, MRS2179 also had no significant effect on contractions elicited by KCl (40 mM) ( $97.0 \pm 3.7\%$  of control, n=5) or UDP (300 μM) ( $99.3 \pm 4.1\%$  of control, n=6).

P2Y<sub>12</sub> receptors were reported to contract human isolated arteries (Wihlborg et al., 2004; Högberg et al., 2010), therefore, the effects of AR-C69931MX, a potent and selective P2Y<sub>12</sub> antagonist, were determined. AR-C69931MX (0.1 nM - 10 μM) had no effect on rat IPA basal tone, but inhibited contractions produced by ATP (300 μM) in a concentration-dependent fashion, with an IC<sub>50</sub> of 9.9 nM (95% confidence limits = 2.0-49.8 nM), Hill slope of 0.97 and maximum inhibition =  $33.3 \pm 4.1\%$  (Figure 2a,b). In contrast, a maximally effective concentration of AR-C69931MX, (1 μM), had no significant effect on contractions elicited by KCl (40 mM) ( $94.5 \pm 3.7\%$  of control, n=6) or UDP (300 μM) ( $95.7 \pm 1.5\%$  of control, n=3). Our previous data indicated that P2X1 receptors mediate more than half of the response to ATP (Chootip et al., 2002, 2005) and consistent with this, the contractions elicited by ATP (300 μM) were virtually abolished by combined blockade of P2X1 and P2Y<sub>12</sub> receptors with the P2X1 antagonist, NF449 (30 μM) (Syed et al., 2010; Syed and Kennedy, 2011) and AR-C69931MX (1 μM) (Figure 2c,d).



ATP is a weak antagonist at P2Y<sub>12</sub> receptors and would be expected to inhibit rather than stimulate P2Y<sub>12</sub> receptors (Macfarlane and Mills, 1975; Cusack and Hourani, 1982; Bodor et al., 2003). It is well known, however, that ATP is rapidly dephosphorylated by ectoenzymes in vascular smooth muscle (Kennedy and Leff, 1995; Robson et al., 2006) and the initial metabolite produced is ADP, the natural ligand of P2Y<sub>12</sub> receptors. We hypothesised, therefore, that ATP was rapidly converted to ADP in rat IPA and that it was the diphosphate that acted at the P2Y<sub>12</sub> receptor to induce the contractile response. Consistent with this hypothesis, ADP (1 mM) evoked contractions of rat IPA and these were abolished by AR-C69931MX (1 μM) (n=4). Thus contractile P2Y<sub>12</sub> receptors appear to be functionally expressed in rat IPA and, together with P2X<sub>1</sub> receptors, mediate the response to ATP.

**UDP-sensitive receptors - agonist studies.** Of the eight P2Y receptors, UDP is an agonist at the P2Y<sub>6</sub> and P2Y<sub>14</sub> subtypes (Abbracchio et al., 2006; Carter et al., 2009), so initial studies examined the actions of agonists that are selective for these two P2Y subtypes. Cumulative addition of the selective P2Y<sub>6</sub> agonist, PSB 0474 (1 - 300 μM), evoked concentration-dependent contractions of rat IPA that reached a peak within a couple of minutes (Figure 3a,b). The concentration-response curve did not reach a maximum over the concentration range tested and so EC<sub>50</sub> values could not be determined. The concentration of agonists that induced 40% of the response to KCl (40 mM) (EC<sub>40K</sub>) was, therefore, calculated (Chootip et al., 2002) and found to be 27.3 μM (95% confidence limits = 6.8-47.7 μM, n=4). UDP (1 μM - 1 mM) also evoked concentration-dependent contractions of rat IPA, with an EC<sub>40K</sub> of 85.5 μM (95% confidence limits = 58.8-112.2 μM, n=11), (Figure 3b), which was significantly higher than the value for PSB 0474 ( $P < 0.05$ ).

The presence of contractile P2Y<sub>14</sub> receptors in rat IPA was tested by applying the selective P2Y<sub>14</sub> agonist, UDP-glucose. Maximal activation of the rat P2Y<sub>14</sub> receptor has been shown to occur at sub-micromolar concentrations of UDP-glucose (Chambers et al., 2000; Freeman et al., 2001), yet, as shown in Figure 3c, even at 100 μM UDP-glucose did not elicit vasoconstriction (n=4). Thus these data are consistent with the presence of contractile P2Y<sub>6</sub>, but not P2Y<sub>14</sub> receptors in rat IPA.

**UDP-sensitive receptors - antagonist studies.** In the next series of experiments the effects of the P2Y<sub>6</sub> receptor antagonist, MRS2578, were determined. MRS2578 (100 nM) had no effect on the basal tone of rat IPA, but reduced significantly the peak responses to UDP (300 μM) ( $P < 0.05$ ) by about 25% (Figure 4a,b). At the same concentration, MRS2578 had no significant effect on contractions evoked by KCl (40 mM) (Figure 4b) or ATP (300 μM) ( $95.4 \pm 12.0$  % of control,

n=5), but higher concentrations depressed significantly the contractions to KCl (40 mM) (1  $\mu$ M;  $81.2 \pm 5.9$  % of control, n=6,  $P < 0.05$ ; 10  $\mu$ M;  $79.0 \pm 4.2$  % of control, n=9,  $P < 0.01$ ).

The inhibitory effect of MRS2578 against KCl could, in theory, be because KCl induced the release of nucleotides, which then acted at the P2Y<sub>6</sub> receptor and so contributed to the KCl-evoked contraction. To address this possibility, the ability of the P2Y antagonist, suramin, to inhibit the response to KCl was determined at a concentration that substantially inhibited contractions of rat IPA evoked by ATP, UDP and UTP (Chootip et al., 2002, 2005). Contractions evoked by KCl (40 mM) in the presence of suramin (100  $\mu$ M) were  $99.9 \pm 1.0$  % of those in its absence (n=8). Thus these data indicate that 1 and 10  $\mu$ M MRS2578 act at an additional site to depress smooth muscle contractility, and so their effects against UDP were not studied. Nonetheless, it is clear that P2Y<sub>6</sub> receptors make an important contribution to UDP-evoked contraction of rat IPA.

To investigate a potential agonist action of UDP at the cysteinyl leukotriene CysLT<sub>1</sub> receptor, the effects of the CysLT<sub>1</sub> antagonist, MK571, were investigated. At 1  $\mu$ M this drug is maximally effective at CysLT<sub>1</sub> receptors (Jones et al., 1989; Lynch et al., 1999; Mellor et al., 2001), but had no significant effect on basal tone or the peak amplitude of contractions evoked by UDP (300  $\mu$ M), KCl (40 mM) (Figure 4c) or ATP (300  $\mu$ M) ( $102.8 \pm 8.3$  % of control, n=6). Thus the CysLT<sub>1</sub> receptor does not appear to be involved in the contractile actions of UDP.

**P2Y receptor expression.** In an attempt to correlate the pharmacological data with P2Y receptor expression, mRNA was extracted from rat IPA and subjected to RT-PCR using subtype-specific primers. Single PCR products of the predicted size for the P2Y<sub>1</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> receptors were amplified (Figure 5) and the identity of each amplicon confirmed by sequencing.

## Discussion

By using novel, selective pharmacological tools this study has shown that ATP evokes vasoconstriction of the rat IPA via P2X<sub>1</sub> and P2Y<sub>12</sub> receptors. P2Y<sub>1</sub> receptors also appear to be functionally expressed in the smooth muscle of this tissue, but at a low level and play no role in the response to ATP. In addition, we have clearly identified the P2Y<sub>6</sub> receptor as one of the sites through which UDP evokes vasoconstriction. In contrast, the data show that P2Y<sub>14</sub> receptors do not contribute to constriction and we found no role for CysLT<sub>1</sub> receptors in the actions of UDP. Thus we now have a much improved knowledge of the receptors through which the endogenous nucleotides ATP and UDP evoke vasoconstriction of the rat IPA.

**Sites of action of ATP.** These experiments indicate that ATP induces vasoconstriction of rat IPA by a combined action at P2Y<sub>12</sub> and P2X<sub>1</sub> receptors. Low concentrations of the P2Y<sub>12</sub> antagonist, AR-C69931MX, depressed contractions evoked by ATP by about one third. The high potency of AR-C69931MX (IC<sub>50</sub> = 9.9 nM), is consistent with its inhibitory effects at low nanomolar concentrations at native (Ingall et al., 1999; Kubista et al., 2003) and recombinant (Takasaki et al., 2001) P2Y<sub>12</sub> receptors. In addition, the ATP response was virtually abolished by combined blockade of P2X<sub>1</sub> and P2Y<sub>12</sub> receptors, consistent with our earlier conclusion that more than half of the response to ATP is mediated by P2X<sub>1</sub> receptors (Chootip et al., 2002, 2005). Since ATP is a weak antagonist at P2Y<sub>12</sub> receptors (Macfarlane and Mills, 1975; Cusack and Hourani, 1982; Bodor et al., 2003), it is very likely that ATP does not act directly at the P2Y<sub>12</sub> receptors, but instead is dependent upon breakdown to ADP by membrane-bound ectonucleotidases in the vascular smooth muscle (Kennedy and Leff, 1995; Robson et al., 2006). In support of this hypothesis, ADP, an agonist at P2Y<sub>12</sub> receptors (Takasaki et al., 2001; Bodor et al., 2003), contracted the rat IPA here in an AR-C69931MX-sensitive manner. This indirect effect of ATP, via ADP, is not unique and is also seen physiologically when ATP released from platelet dense granules is dephosphorylated by ectonucleotidases to produce ADP, which in turn acts at P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors to induce aggregation (Robson et al., 2006).

The P2Y<sub>12</sub> receptor has previously been reported to mediate contraction of human internal mammary and pericardial fat arteries and mouse aorta (Wihlborg et al., 2004; Högberg et al., 2010), but not mouse mesenteric artery (Vial and Evans, 2002). If it has widespread contractile effects in the vasculature then this could be of therapeutic importance. P2Y<sub>12</sub> antagonists, such as clopidogrel, are used clinically to inhibit platelet aggregation and if they were to also reduce vasospasm then this dual effect could be beneficial to patients suffering from vascular diseases, including pulmonary vascular disease. Further studies are needed to characterise fully the distribution of contractile P2Y<sub>12</sub> receptors in vascular smooth muscle.

In this study the selective, potent P2Y<sub>1</sub> agonist, MRS2365, elicited concentration-dependent contractions of rat IPA that were inhibited by the P2Y<sub>1</sub> antagonist, MRS2179, indicating functional P2Y<sub>1</sub> receptor expression in rat IPA. The contractions were, however, small and micromolar concentrations of MRS2365 were required. MRS2365 is the most potent human P2Y<sub>1</sub> agonist currently available and active in the low nanomolar range at recombinant and native P2Y<sub>1</sub> receptors (Chhatiwala et al., 2004; Lu et al., 2007; Govindan et al., 2010). The need for substantially higher concentrations of MRS2365 here to elicit small contractions suggests that whilst P2Y<sub>1</sub> receptors may be functionally expressed in rat IPA smooth muscle cells, it appears to be at a low level. Consistent with this, MRS2179 had no effect on ATP-induced contractions. ATP is a partial agonist at P2Y<sub>1</sub> receptors and so has little or no agonist action at low levels of receptor expression (Palmer et al., 1998).

**Sites of action of UDP.** The present study shows conclusively for the first time that UDP induces vasoconstriction of rat IPA via P2Y<sub>6</sub> receptors. The selective P2Y<sub>6</sub> agonist, PSB 0474 (El-Tayeb et al., 2006; Gao et al., 2010), evoked concentration-dependent contractions of rat IPA and was approximately three times more potent than UDP. Furthermore, a low concentration (100 nM) of the potent P2Y<sub>6</sub> receptor antagonist, MRS2578, depressed the peak UDP response. MRS2578 is highly selective for the P2Y<sub>6</sub> receptor, with little or no effect at P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>11</sub> receptors (Mamedova et al., 2004). This is consistent with, and extends our and others' previous pharmacological data obtained using the non-selective P2 receptor antagonists, suramin, PPADS and reactive blue 2 (Rubino and Burnstock, 1996; Hartley et al., 1998; Rubino et al., 1999; Chootip et al., 2002).

The maximal concentration of MRS2578 that could be used (100 nM) is very close to the IC<sub>50</sub> (98 nM) obtained at the hP2Y<sub>6</sub> receptor expressed in human 1321N1 astrocytoma cells (Mamedova et al., 2004), suggesting that higher concentrations of MRS2578 would depress the UDP-evoked contractions of rat IPA even further. The total contribution of P2Y<sub>6</sub> receptors to the UDP-evoked vasoconstriction could not be determined, however, because at higher concentrations (1 and 10 μM) the antagonist also inhibited contractions evoked by KCl, indicating a non-specific action of MRS2578 at other sites to depress smooth muscle contractility. This is, perhaps, not surprising as MRS2578 contains isothiocyanate groups, which, as potent electrophiles, can react chemically with nucleophilic groups on proteins (Mamedova et al., 2004). This is the first study that we are aware of, however, where appropriate control experiments have been reported, i.e. determination of the effect of MRS2578 against a contractile agent that acts independently of P2Y<sub>6</sub> receptors. Thus care must be taken when using MRS2578 at these concentrations.

Our previous studies showed that UDP acts not only at P2Y<sub>6</sub> receptors to constrict rat IPA, but also via another, as yet, unidentified P2Y subtype (Chootip et al., 2002, 2005). Here we show that the second site is not the P2Y<sub>14</sub> receptor, as a high concentration of the endogenous P2Y<sub>14</sub> agonist, UDP-glucose (Carter et al., 2009), had no effect on IPA tone. The CysLT<sub>1</sub> receptor can also be ruled out since the potent, competitive CysLT<sub>1</sub> antagonist, MK571, did not antagonise UDP, at a concentration that abolished activation of the CysLT<sub>1</sub> receptor (Jones et al., 1989; Lynch et al., 1999; Mellor et al., 2001). Thus the identity of the second site at which UDP acts to constrict rat IPA remains to be clarified and this will probably depend upon the development of selective antagonists at other P2Y receptor subtypes.

**P2Y receptor expression.** Rat IPA express mRNA for P2Y<sub>1</sub>, P2Y<sub>6</sub>, and P2Y<sub>12</sub> receptors, consistent with earlier reports of P2Y<sub>6</sub> mRNA expression (Hartley et al., 1998; Gui et al., 2008). Unfortunately it was not possible to correlate mRNA with protein expression, as in preliminary, control experiments, commercially-available antibodies showed staining in cells that do not express endogenous P2Y receptors. An anti-P2Y<sub>1</sub> antibody has also been reported to be non-specific (Vial et al., 2006).

**Summary.** In conclusion, our working model is that ATP acts directly at P2X<sub>1</sub> receptors and indirectly at P2Y<sub>12</sub> receptors following breakdown to ADP, to evoke vasoconstriction of rat IPA. P2Y<sub>1</sub> receptors may also be functionally expressed, but at low levels and are not activated by ATP. In addition, UDP acts in part at P2Y<sub>6</sub>, but not P2Y<sub>14</sub> or CysLT<sub>1</sub> receptors, to evoke vasoconstriction of rat IPA. The remaining site(s) of action of UDP remains to be determined.

**Acknowledgements.** We thank Dr. Rothwelle Tate for his invaluable guidance in the RT-PCR experiments.

#### **Authorship Contributions**

*Participated in research design:* Mitchell, Syed, Tengah, Gurney, Kennedy.

*Conducted experiments:* Mitchell, Syed, Tengah.

*Performed data analysis:* Mitchell, Syed, Tengah, Kennedy.

*Wrote or contributed to the writing of the manuscript:* Mitchell, Syed, Tengah, Gurney, Kennedy.

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## Footnotes

This study was supported by the British Heart Foundation (grant no. FS/04/070 to CK/AMG).

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2) CM and NS contributed equally to the study.

## Figure Legends

**Figure 1.** The effect of drugs active at P2Y<sub>1</sub> receptors. a) Traces show contractions evoked by MRS2365 (3 - 100 μM) in a rat isolated IPA. b) Open columns show the mean peak amplitude of contractions produced by MRS2365 (1 - 100 μM) (n=5-9). The cross-hatched column is the response to MRS2365 (100 μM) in the presence of MRS2179 (10 μM) (n=4). c) The mean peak amplitude of contractions evoked by ATP (300 μM) in the presence of MRS2179 (10 μM) for 20 min is shown. Vertical lines indicate S.E.M. (n=6).

**Figure 2.** Inhibition of contractions induced by ATP. a), c) The superimposed traces show typical contractions of rat isolated IPA evoked by ATP (300 μM) before (upper traces) and after (lower traces) incubation for 20 min with a) AR-C69931MX (1 μM) and c) AR-C69931MX (1 μM) plus NF449 (30 μM). ATP was applied as indicated by the solid bars. b) The mean peak amplitude of contractions evoked by ATP (300 μM) in the presence of AR-C69931MX (0.1 nM - 10 μM) (n=4-6). Vertical lines show S.E.M. The curve represents the fit of the Hill equation to the data. d) The mean peak amplitude of contractions evoked by ATP (300 μM) in the presence of AR-C69931MX (1 μM) (cross-hatched column) (n=4) and AR-C69931MX (1 μM) plus NF449 (30 μM) (speckled column) (n=6) are shown. \*  $P < 0.05$ , \*\*\* $P < 0.001$  for responses to ATP in the presence of antagonists compared to in their absence.

**Figure 3.** The actions of pyrimidine nucleotide agonists. a) The trace shows typical contractions of rat isolated IPA evoked by PSB 0474 (1 - 300 μM), applied cumulatively, as indicated by the solid bars. b) The mean peak amplitude of contractions evoked by PSB 0474 (1 - 300 μM) (n=4) and UDP (1 μM - 1 mM) (n=11), normalised as a percentage of the response to KCl (40 mM), is shown. Vertical lines indicate S.E.M. The curves represent the fit of the Hill equation to the data. c) The typical lack of effect of UDP-glucose (300 μM) on tone is shown.

**Figure 4.** The effects of putative antagonists. a) The superimposed traces show typical contractions of rat isolated IPA evoked by UDP (300 μM) before (upper trace) and after incubation with MRS2578 (100 nM) for 20 min (lower trace). UDP was applied as indicated by the solid bar. The mean peak amplitude of contractions evoked by UDP (300 μM) (cross-hatched columns) and KCl (40 mM) (open columns) in the presence of b) MRS2578 (100 nM) and c) MK571 (1 μM) expressed as a percentage of control, is shown. Vertical lines indicate S.E.M. n=6 for KCl in both; n= 4 and 6 for UDP in b) and c) respectively. \*\*  $P < 0.01$  for response to UDP in the presence of MRS2578 compared to in its absence.

**Figure 5.** P2Y receptor RT-PCR products. a) Agarose gel electrophoresis of RT-PCR products from rat IPA using specific oligonucleotide primers for rat P2Y<sub>1</sub>, P2Y<sub>6</sub> and P2Y<sub>12</sub> receptors are shown. Each pair of primers yielded bands in the presence of reverse transcriptase (lanes 1, 3, 5), but not in its absence (lanes 2, 4, 6). The markers on the left hand side show band size (base pairs).

Figure 1

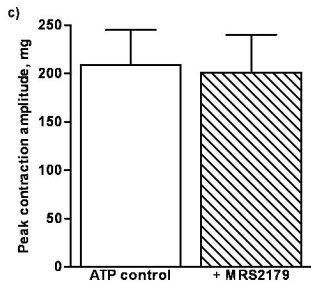
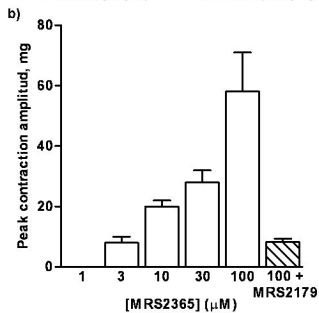
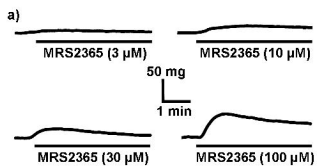


Figure 2

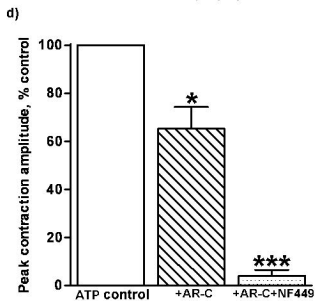
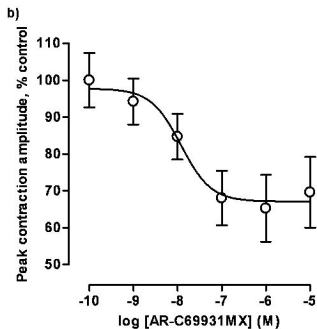
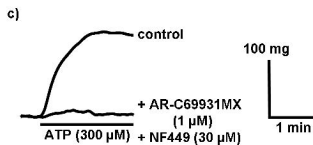
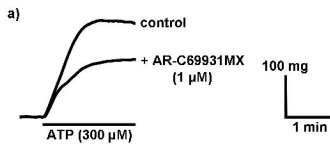


Figure 3

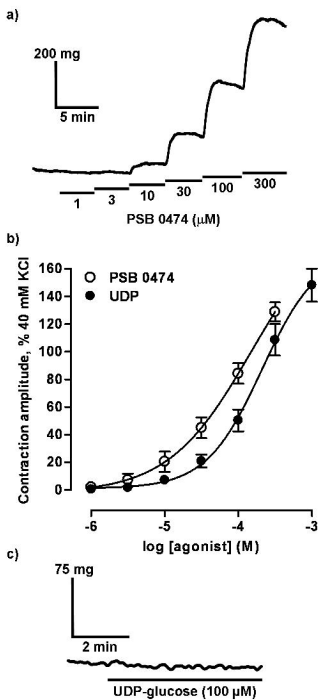




Figure 4

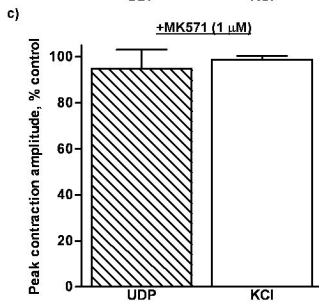
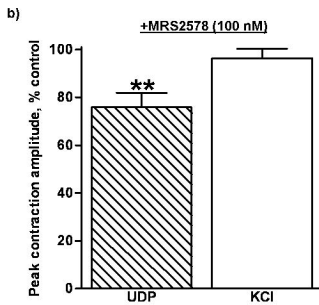
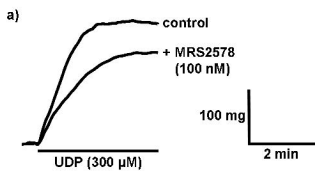


Figure 5

