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Identification of contractile $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ receptors in rat intrapulmonary artery using selective ligands

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Running title: P2Y receptor-mediated pulmonary artery contraction

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Abbreviations:
IPA, intrapulmonary arteries
ATP, adenosine 5'-triphosphate
ADP, adenosine 5'-diphosphate
UDP, uridine 5'-diphosphate
UDP-glucose, uridine 5'-diphosphate glucose
MRS2365, (N)-methanocarba-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-[(1*E*)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid
Suramin, 8,8'-[Carbonyl*bis*[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 μm) mounted on a wire myograph. Expression of P2Y receptor subtype expression was determined using RT-PCR with receptor-specific oligonucleotide primers. The selective $P2Y_1$ agonist MRS2365 induced small, concentration-dependent contractions that were inhibited by the $P2Y_1$ antagonist MRS2179. Contractions evoked by ATP were unaffected by MRS2179, but inhibited by about one third by the $P2Y_{12}$ antagonist AR-C69931MX. Combined blockade of P2X1 and $P2Y_{12}$ receptors virtually abolished the response to ATP. ADP also evoked contractions that were abolished by AR-C69931MX. The selective $P2Y_6$ receptor agonist, PSB 0474, evoked concentration-dependent contractions and was approximately 3-times more potent than UDP, but the $P2Y_{14}$ agonist, UDP-glucose, had no effect. Contractions evoked by UDP were inhibited by the $P2Y_6$ receptor antagonist, MRS2578, but not the $CysLT_1$ antagonist, MK571. Higher concentrations of MRS2578 inhibited contractions to KCl and so were not studied further. mRNA for $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ receptors was identified. Our working model is that $P2Y_{12}$ and P2X1 receptors are present in rat intrapulmonary arteries and together mediate ATP-induced vasoconstriction. Contractile $P2Y_6$, but not $P2Y_{14}$ or $CysLT_1$ 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 $P2Y_6$ 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 $P2Y_{12}$ receptors (Bodor et al., 2003), which were reported to mediate contraction of human isolated arteries (Wihlborg et al., 2004; Högberg et al., 2010).

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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)-methanocarba-2-MeSADP (MRS2365) is a highly selective and potent $P2Y_1$ agonist (Ravi et al., 2002; Chhatriwala et al., 2004), N6-methyl 2'-deoxyadenosine 3', 5'-bisphosphate (MRS2179), a potent, competitive $P2Y_1$ 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 $P2Y_{12}$ antagonist (Ingall et al., 1999). $P2Y_6$ 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 $P2Y_6$ over $P2Y_2$ receptors and is essentially inactive at the $P2Y_4$ and $P2Y_{14}$ receptors (El-Tayeb et al., 2006; Gao et al., 2010) and *N*,*N*"-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea (MRS2578), a potent and insurmountable $P2Y_6$ antagonist (Mamedova et al., 2004).

The aim of the present study was to use these subtype-selective ligands to determine the contributions of $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ receptors to the constriction of rat IPA elicited by ATP and UDP. In addition, the $P2Y_{14}$ 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 $P2Y_{14}$ 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 $CysLT_1$ receptor (Mellor et al., 2001, 2002). Thus the contributions of $P2Y_{14}$ and $CysLT_1$ receptors to the action of UDP were also investigated. Finally, this pharmacological approach was complemented by analysis of the expression of each P2Y subtype.

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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_2PO_4 0.5, NaH_2PO_4 0.5, $MgCl_2$ 1, glucose 11, $CaCl_2$ 1.8, titrated to pH 7.3 with NaOH and bubbled with "medical air" (21% O_2, 5% CO_2, 74% N_2). 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 37ºC. 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 $P2Y_1$ agonist MRS2365 and $P2Y_6$ agonist PSB 0474 were applied using the same protocol. To determine the effects of MRS2578, 3-[[[3-[(1*E*)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid (MK571) and 8,8'-[Carbonyl*bis*[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: $P2Y_1$ forward TCCTCTTCATTCCGATGTGCC, reverse TCTTCTTCTTGAGCCTGCCCA, 391 bp; $P2Y_6$

forward TGCTTGGGTGGTATGTGGAGT, reverse TGTTGTGTGAAGTAGAAGAGGATA, 498 bp; $P2Y_{12}$ forward GGCCTTCATGTTCCTGCTGTC, reverse GGGTGCTCTCCTTCACGTAGAAC, 404 bp. The mix was placed in a DNA Thermal Cycler (Perkin Elmer, UK) and the following protocol applied: 10 min at 95ºC followed by 35 cycles of 90 sec at 95ºC, 30 sec at 52ºC, 90 sec at 68ºC and a final extension step of 10 min at 68ºC. 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_2 salt), ADP (Na salt), UDP (Na_2 salt), UDP-glucose (Na_2 salt), PSB 0474 (Na_2 salt), acetylcholine chloride (Sigma, UK), MRS2179 (Na_4 salt), MRS2365 (Na_3 salt), 4,4',4",4"'-[Carbonylbis(imino-5,1,3-benzenetriyl-*bis*(carbonylimino))]*tetrakis*-1,3-benzenedisulfonic acid, (Na_8 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 -20ºC, 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^+ 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.

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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 $P2Y_1$ receptors are involved, their presence in rat IPA was studied initially using MRS2365, a highly potent and selective $P2Y_1$ 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 ± 13 mg (n=9), which was equivalent to 23.6 ± 4.4% of the response to 40 mM KCl. In contrast, at the same concentration, ATP evoked contractions that were equivalent to 50.3 ± 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 $P2Y_1$ receptors in rat IPA.

ATP-sensitive receptors - antagonist studies. The contribution of the $P2Y_1$ receptors to the ATP response was then investigated using MRS2179, a selective $P2Y_1$ 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 ± 3.2 % of control, n=6). In contrast, it reduced significantly the response to MRS2365 (100 μM) by 84.5 ± 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 ± 3.7 % of control, n=5) or UDP (300 μM) (99.3 ± 4.1 % of control, n=6).

$P2Y_{12}$ 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 $P2Y_{12}$ 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_{50} of 9.9 nM (95% confidence limits = 2.0-49.8 nM), Hill slope of 0.97 and maximum inhibition = 33.3 ± 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 ± 3.7 % of control, n=6) or UDP (300 μM) (95.7 ± 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 $P2Y_{12}$ receptors with the P2X1 antagonist, NF449 (30 μM) (Syed et al., 2010; Syed and Kennedy, 2011) and AR-C69931MX (1 μM) (Figure 2c,d).

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ATP is a weak antagonist at $P2Y_{12}$ receptors and would be expected to inhibit rather than stimulate $P2Y_{12}$ 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 $P2Y_{12}$ receptors. We hypothesised, therefore, that ATP was rapidly converted to ADP in rat IPA and that it was the diphosphate that acted at the $P2Y_{12}$ 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 $P2Y_{12}$ receptors appear to be functionally expressed in rat IPA and, together with P2X1 receptors, mediate the response to ATP.

UDP-sensitive receptors - agonist studies. Of the eight P2Y receptors, UDP is an agonist at the $P2Y_6$ and $P2Y_{14}$ 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 $P2Y_6$ 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_{50} values could not be determined. The concentration of agonists that induced 40% of the response to KCl (40 mM) (EC_{40K}) 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_{40K} 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 $P2Y_{14}$ receptors in rat IPA was tested by applying the selective $P2Y_{14}$ agonist, UDP-glucose. Maximal activation of the rat $P2Y_{14}$ 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 $P2Y_6$, but not $P2Y_{14}$ receptors in rat IPA.

UDP-sensitive receptors - antagonist studies. In the next series of experiments the effects of the $P2Y_6$ 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 ± 12.0 % of control,

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n=5), but higher concentrations depressed significantly the contractions to KCl (40 mM) (1 μM; 81.2 ± 5.9 % of control, n=6, $P<0.05$; 10 μM; 79.0 ± 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 $P2Y_6$ 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 μM) were 99.9 ± 1.0 % of those in its absence (n=8). Thus these data indicate that 1 and 10 μM 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 $P2Y_6$ receptors make an important contribution to UDP-evoked contraction of rat IPA.

To investigate a potential agonist action of UDP at the cysteinyl leukotriene $CysLT_1$ receptor, the effects of the $CysLT_1$ antagonist, MK571, were investigated. At 1 μM this drug is maximally effective at $CysLT_1$ 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 μM), KCl (40 mM) (Figure 4c) or ATP (300 μM) (102.8 ± 8.3 % of control, n=6). Thus the $CysLT_1$ 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 $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ 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 P2X1 and $P2Y_{12}$ receptors. $P2Y_1$ 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 $P2Y_6$ receptor as one of the sites through which UDP evokes vasoconstriction. In contrast, the data show that $P2Y_{14}$ receptors do not contribute to constriction and we found no role for $CysLT_1$ 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 $P2Y_{12}$ and P2X1 receptors. Low concentrations of the $P2Y_{12}$ antagonist, AR-C69931MX, depressed contractions evoked by ATP by about one third. The high potency of AR-C69931MX (IC_{50} = 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) $P2Y_{12}$ receptors. In addition, the ATP response was virtually abolished by combined blockade of P2X1 and $P2Y_{12}$ receptors, consistent with our earlier conclusion that more than half of the response to ATP is mediated by P2X1 receptors (Chootip et al., 2002, 2005). Since ATP is a weak antagonist at $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ 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 $P2Y_1$ and $P2Y_{12}$ receptors to induce aggregation (Robson et al., 2006).

The $P2Y_{12}$ 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. $P2Y_{12}$ 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 $P2Y_{12}$ receptors in vascular smooth muscle.

In this study the selective, potent $P2Y_1$ agonist, MRS2365, elicited concentration-dependent contractions of rat IPA that were inhibited by the $P2Y_1$ antagonist, MRS2179, indicating functional $P2Y_1$ receptor expression in rat IPA. The contractions were, however, small and micromolar concentrations of MRS2365 were required. MRS2365 is the most potent human $P2Y_1$ agonist currently available and active in the low nanomolar range at recombinant and native $P2Y_1$ receptors (Chhatriwala 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 $P2Y_1$ 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 $P2Y_1$ 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 $P2Y_6$ receptors. The selective $P2Y_6$ 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 $P2Y_6$ receptor antagonist, MRS2578, depressed the peak UDP response. MRS2578 is highly selective for the $P2Y_6$ receptor, with little or no effect at $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ 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_{50} (98 nM) obtained at the $hP2Y_6$ 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 $P2Y_6$ 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 $P2Y_6$ receptors. Thus care must be taken when using MRS2578 at these concentrations.

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Our previous studies showed that UDP acts not only at $P2Y_6$ 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 $P2Y_{14}$ receptor, as a high concentration of the endogenous $P2Y_{14}$ agonist, UDP-glucose (Carter et al., 2009), had no effect on IPA tone. The $CysLT_1$ receptor can also be ruled out since the potent, competitive $CysLT_1$ antagonist, MK571, did not antagonise UDP, at a concentration that abolished activation of the $CysLT_1$ 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 $P2Y_1$, $P2Y_6$, and $P2Y_{12}$ receptors, consistent with earlier reports of $P2Y_6$ 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-$P2Y_1$ 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 P2X1 receptors and indirectly at $P2Y_{12}$ receptors following breakdown to ADP, to evoke vasoconstriction of rat IPA. $P2Y_1$ receptors may also be functionally expressed, but at low levels and are not activated by ATP. In addition, UDP acts in part at $P2Y_6$, but not $P2Y_{14}$ or $CysLT_1$ receptors, to evoke vasoconstriction of rat IPA. The remaining site(s) of action of UDP remains to be determined.

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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.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Fumagalli M, Gachet C, Jacobson KA, and Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* **58:** 281-341.

Adnot S, Raffestin B, Eddahibi S, Braquet, P, and Chabrier PE (1991) Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. *J Clin Invest* **87:** 155-162.

Baek EB, Yoo HY, Park SJ, Kim HS, Kim SD, Earm YE, and Kim SJ (2008) Luminal ATP-induced contraction of rabbit pulmonary arteries and role of purinoceptors in the regulation of pulmonary arterial pressure. *Pflugers Arch* **457:** 281-291.

Barnes PJ and Liu SF (1995) Regulation of pulmonary vascular tone. *Pharmacol Rev* **47:** 87-131.

Bodor ET, Waldo GL, Hooks SB, Corbitt J, Boyer JL and Harden TK (2003) Purification and functional reconstitution of the human $P2Y_{12}$ receptor. *Mol Pharmacol* **64:** 210-216.

Boyer JL, Mohanram A, Camaioni E, Jacobson KA, and Harden TK (1998) Competitive and selective antagonism of $P2Y_1$ receptors by N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. *Br J Pharmacol* **124:** 1-3.

Burnstock G and Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* **16:** 433-440.

Burnstock G and Kennedy C (1986) A dual function for adenosine triphosphate in the regulation of vascular tone: excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ Res* **58:** 319-330.

Burnstock G and Kennedy C (2011) P2X receptors in health and disease, in: *Adv Pharmacol* (Jacobson KA, Linden J eds) **61:** 333-372, Academic Press, Burlington.

Camaioni E, Boyer JL, Mohanram A, Harden TK, and Jacobson KA (1998) Deoxyadenosine-bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors. *J Med Chem* **141:** 183-190.

Carter RL, Fricks IP, Barrett MO, Burianek LE, Zhou Y, Ko H, Das A, Jacobson KA, Lazarowski ER, and Harden TK (2009) Quantification of G_i-mediated inhibition of adenylyl cyclase activity reveals that UDP is a potent agonist of the human $P2Y_{14}$ receptor. *Mol Pharmacol* **76:** 1341-1348.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275:** 10767-10771.

Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA, and Harden TK (2004) Induction of novel agonist selectivity for the ADP-activated $P2Y_1$ receptor versus the ADP-activated $P2Y_{12}$ and $P2Y_{13}$ receptors by conformational constraint of an ADP analog. *J Pharmacol Exp Ther* **311:** 1038-1043.

Chootip K, Gurney AM, and Kennedy C (2005) Evidence for multiple P2Y receptors coupled to calcium-dependent, chloride channels in smooth muscle cells of the rat pulmonary artery. *Respir Res* **26:** 124.

Chootip K, Ness K, Wang J, Gurney AM, and Kennedy C (2002) Regional variation in P2 receptor expression in the rat pulmonary arterial circulation. *Br J Pharmacol* **137:** 637-646.

Cusack NJ and Hourani SMO (1996) Adenosine 5'-diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of stimulated adenylate cyclase are mediated by different receptors. *Br J Pharmacol* **76:** 221-227.

Dinh-Xuan AT, Higenbottam TW, Clelland CA, Pepke-Zaba J, Cremona G, Butt AY, Large SR, Wells FC, and Wallwork J (1991) Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N Eng J Med* **324:** 1539-1547.

El-Tayeb A, Qi A, and Müller CE (2006) Synthesis and structure-activity relationships of uracil nucleotide derivatives and analogues as agonists at human $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. *J Med Chem* **49:** 7076-7087.

Erlinge D and Burnstock G (2008) P2 receptors in cardiovascular regulation and disease. *Pur Sig* **4:** 1-20.

Evans RJ and Kennedy C (1994) Characterisation of P_2-purinoceptors in the smooth muscle of the rat tail artery; a comparison between contractile and electrophysiological responses. *Br J Pharmacol* **113:** 853-860.

Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S, Lane P,Bawagan H, Herrity N, Murphy K, Sarau HM, Ames RS, Wilson S, Livi GP, and Chambers JK (2001) Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. *Genomics* **78:** 124-128.

Gao ZG, Ding Y, and Jacobson KA (2010) UDP-glucose acting at $P2Y_{14}$ receptors is a mediator of mast cell degranulation. *Biochem Pharmacol* **79:** 873-879.

Govindan S, Taylor EJ, and Taylor CW (2010) Ca^{2+} signalling by P2Y receptors in cultured rat aortic smooth muscle cells. *Br J Pharmacol* **160:** 1953-1962.

Gui Y, Walsh MP, Jankowski V, Jankowski J, and Zheng XL (2008) Up_4A stimulates endothelium-independent contraction of isolated rat pulmonary artery. *Am J Physiol* **294:** L733-L738.

Hartley SA, Kato K, Salter KJ, and Kozlowski RZ (1998) Functional evidence for a novel suramin-insensitive pyrimidine receptor in rat small pulmonary arteries. *Circ Res* **83:** 940-946.

Hassėssian H and Burnstock G (1995) Interacting roles of nitric oxide and ATP in the pulmonary circulation of the rat. *Br J Pharmacol* **114:** 846-850.

Högberg C, Svensson H, Gustafsson R, Eyjolfsson A, and Erlinge D (2010) The reversible oral $P2Y_{12}$ antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature. *Int J Cardiol* **142:** 187-192.

Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, Humphries RG, Leff P, Clegg JA, Smith JA, and Tomlinson W (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem* **42:** 213-220.

Jernigan NL, Broughton BR, Walker BR, and Resta TC (2006) Impaired NO-dependent inhibition of store- and receptor-operated calcium entry in pulmonary vascular smooth muscle after chronic hypoxia. *Am J Physiol* **290:** 517-525.

Jones TR, Zamboni R, Belley M, Champion E, Charette L, Ford-Hutchinson AW, Frenette R, Gauthier J-Y, Leger S, Masson P, McFarlane CS, Piechuta H, Rokach J, Williams H, Young RN, DeHaven RN, and Pong SS (1989) Pharmacology of L-660,711 (MK-571): a novel potent and selective leukotriene D_4 receptor antagonist. *Can J Physiol Pharmacol* **67:** 17-28.

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela P, Voigt M, and Humphrey PPA (2001). International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev* **53:** 107-118.

Kennedy C and Leff P (1995) How should P_{2X}-purinoceptors be characterised pharmacologically? *Trends in Pharmacol Sci* **16:** 168-174.

Kubista H, Lechner SG, Wolf AM, and Boehm S (2003) Attenuation of the P2Y receptor-mediated control of neuronal Ca^{2+} channels in PC12 cells by antithrombotic drugs. *Br J Pharmacol* **138:** 343-350.

Lommatzsch M, Cicko S, Müller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Dürk T, Zissel G, Ferrari D, Di Virgilio F, Sorichter S, Lungarella G, Virchow JC, and Idzko M (2010) Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **181:** 928-934.

Liu SF, McCormack DG, Evans TW, and Barnes PJ (1989a) Characterization and distribution of P2-purinoceptor subtypes in rat pulmonary vessels. *J Pharmacol Exp Ther* **251:** 1204-1210.

Liu SF, McCormack DG, Evans TW, and Barnes PJ (1989b) Evidence for two P_2-purinoceptor subtypes in human small pulmonary arteries. *Br J Pharmacol* **98:** 1014-1020.

Lu W, Reigada D, Sévigny J, and Mitchell CH (2007) Stimulation of the $P2Y_1$ receptor up-regulates nucleoside-triphosphate diphosphohydrolase-1 in human retinal pigment epithelial cells. *J Pharmacol Exp Ther* **323:** 157-164.

Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams DL Jr, Ford-Hutchinson AW, Caskey CT, and Evans JF (1999) Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. *Nature* **399:** 789-793.

Macfarlane DE and Mills DCB (1975) The effects of ATP on platelets: evidence against the central role of released ADP in primary aggregation. *Blood* **46:** 309-319.

Mam V, Tanbe AF, Vitali SH, Arons E, Christou HA, and Khalil RA (2010) Impaired vasoconstriction and nitric oxide-mediated relaxation in pulmonary arteries of hypoxia- and monocrotaline-induced pulmonary hypertensive rats. *J Pharmacol Exp Ther* **332:** 455-462.

Mamedova LK, Joshi BV, Gao ZG, Von Kugelgen I, and Jacobson KA (2004) Diisothiocyanate derivatives as potent, insurmountable antagonists of $P2Y_6$ nucleotide receptors. *Biochem Pharmacol* **67:** 1763-1770.

McCormack DG, Barnes PJ, and Evans TW (1989) Purinoceptors in the pulmonary circulation of the rat and their role in hypoxic vasoconstriction. *Br J Pharmacol* **98:** 367-372.

Mellor EA, Austen KF, and Boyce JA (2002) Cysteinyl leukotrienes and uridine diphosphate induce cytokine generation by human mast cells through an interleukin 4-regulated pathway that is inhibited by leukotriene receptor antagonists. *J Exp Med* **195:** 583-592.

Mellor EA, Maekawa A, Austen KF, and Boyce JA (2001) Cysteinyl leukotriene receptor 1 is also a pyrimidinergic receptor and is expressed by human mast cells. *Proc Natl Acad Sci* **98:** 7964-7969.

Mitchell C, Syed NH, Gurney AM, and Kennedy C (2012) A Ca^{2+}-dependent chloride current and Ca^{2+} influx via $Ca_v1.2$ ion channels play major roles in P2Y receptor-mediated pulmonary vasoconstriction. *Br J Pharmacol* **166:** 1503-1512.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA, and Harden TK (1998) Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol* **54:** 1118-1123.

Ravi RG, Kim HS, Servos J, Zimmermann H, Lee K, Maddileti S, Boyer JT, Harden TK, and Jacobson KA (2002) Adenine nucleotide analogues locked in a northern methanocarba conformation: Enhanced stability and potency as $P2Y_1$ receptor agonists. *J Med Chem* **45:** 2090-2100.

Robson SC, Sévigny J, and Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Pur Sig* **2:** 409-430.

Rubino A and Burnstock G (1996) Evidence for a P2-purinoceptor mediating vasoconstriction by UTP, ATP and related nucleotides in the isolated pulmonary vascular bed of the rat. *Br J Pharmacol* **118:** 1415-1420.

Rubino A, Ziabary L, and Burnstock G (1999) Regulation of vascular tone by UTP and UDP in isolated intrapulmonary arteries. *European J Pharmacol* **370:** 139-143.

Sprague RS, Ellsworth ML, Stephenson AH, and Lonigro AJ (1996) ATP: the red blood cell link to NO and local control of the pulmonary circulation. *Am J Physiol* **271:** H2717-H2722.

Sprague RS, Olearczyk JJ, Spence DM, Stephenson AH, Sprung RW, and Lonigro AJ (2003) Extracellular ATP signaling in the rabbit lung: erythrocytes as determinants of vascular resistance. *Am J Physiol* **285:** H693-H700.

Syed NH and Kennedy C (2012) Pharmacology of P2X receptors. *WIREs Membr. Transp. Signal.*, **1:** 16–30. doi: 10.1002/wmts.1.

Syed NH, Tengah A, Paul A, and Kennedy C (2010) Characterisation of P2X receptors expressed in rat pulmonary arteries. *European J Pharmacol* **649:** 342-348.

Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, Soga T, Matsushime H, and Furuichi K (2001) Molecular cloning of the platelet P2TAC ADP receptor: pharmacological comparison with another ADP receptor, the $P2Y_1$ receptor. *Mol Pharmacol* **60:** 432–439.

Vial C and Evans RJ (2002) $P2X_1$ receptor-deficient mice establish the native P2X receptor and a $P2Y_6$-like receptor in arteries. *Mol Pharmacol* **62**: 1438-1445.

Vial C, Fung CY, Goodall AH, Mahaut-Smith MP, and Evans RJ (2006) Differential sensitivity of human platelet P2X1 and $P2Y_1$ receptors to disruption of lipid rafts. *Biochem Biophys Res Comm* **343:** 415-419.

Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T, and Erlinge D (2004) ADP receptor $P2Y_{12}$ is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol* **24:** 1810-1815.

Footnotes

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2) CM and NS contributed equally to the study.

Figure Legends

Figure 1. The effect of drugs active at $P2Y_1$ 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.

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Figure 5. P2Y receptor RT-PCR products. a) Agarose gel electrophoresis of RT-PCR products from rat IPA using specific oligonucleotide primers for rat $P2Y_1$, $P2Y_6$ and $P2Y_{12}$ 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

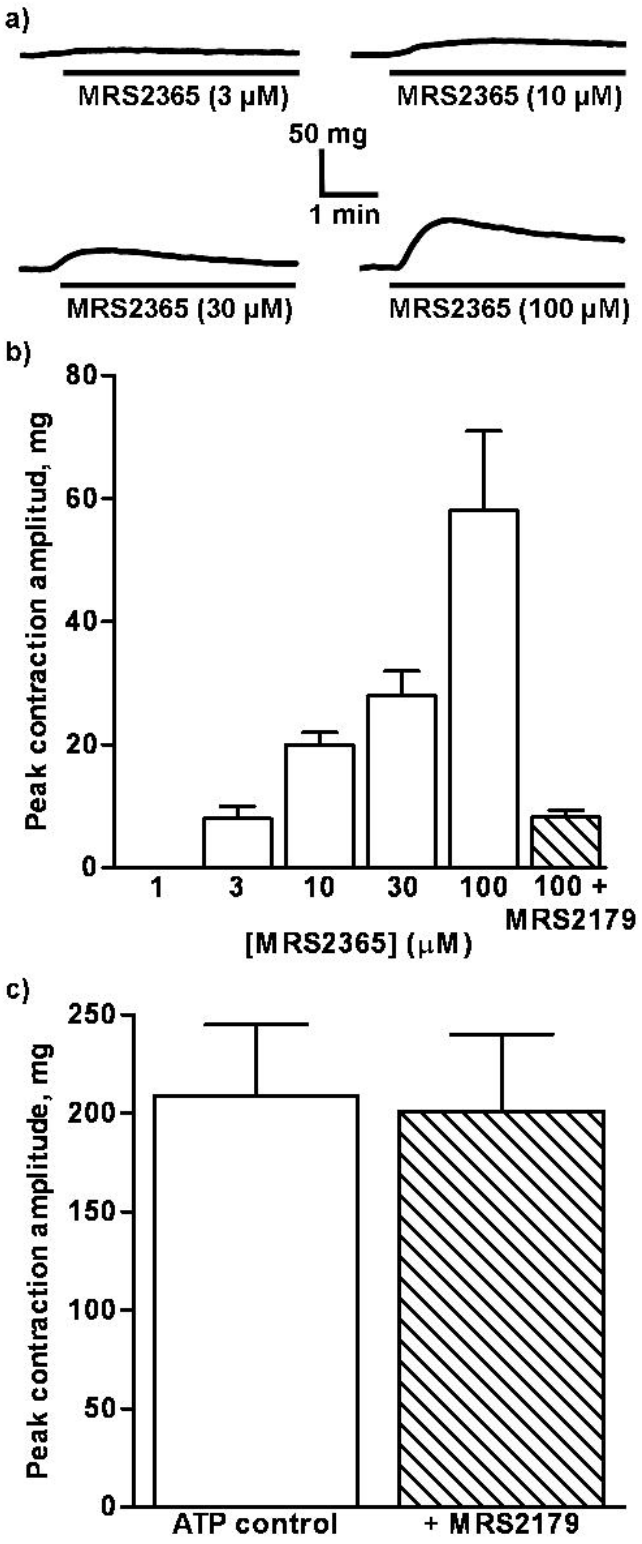
a)
MRS2365 (3 µM)
MRS2365 (10 µM)
50 mg
1 min
MRS2365 (30 µM)
MRS2365 (100 µM)
b)
Peak contraction amplitud, mg
80
60
40
20
0
1
3
10
30
100
100 + MRS2179
[MRS2365] (µM)
c)
Peak contraction amplitude, mg
250
200
150
100
50
0
ATP control
+ MRS2179

Figure 2

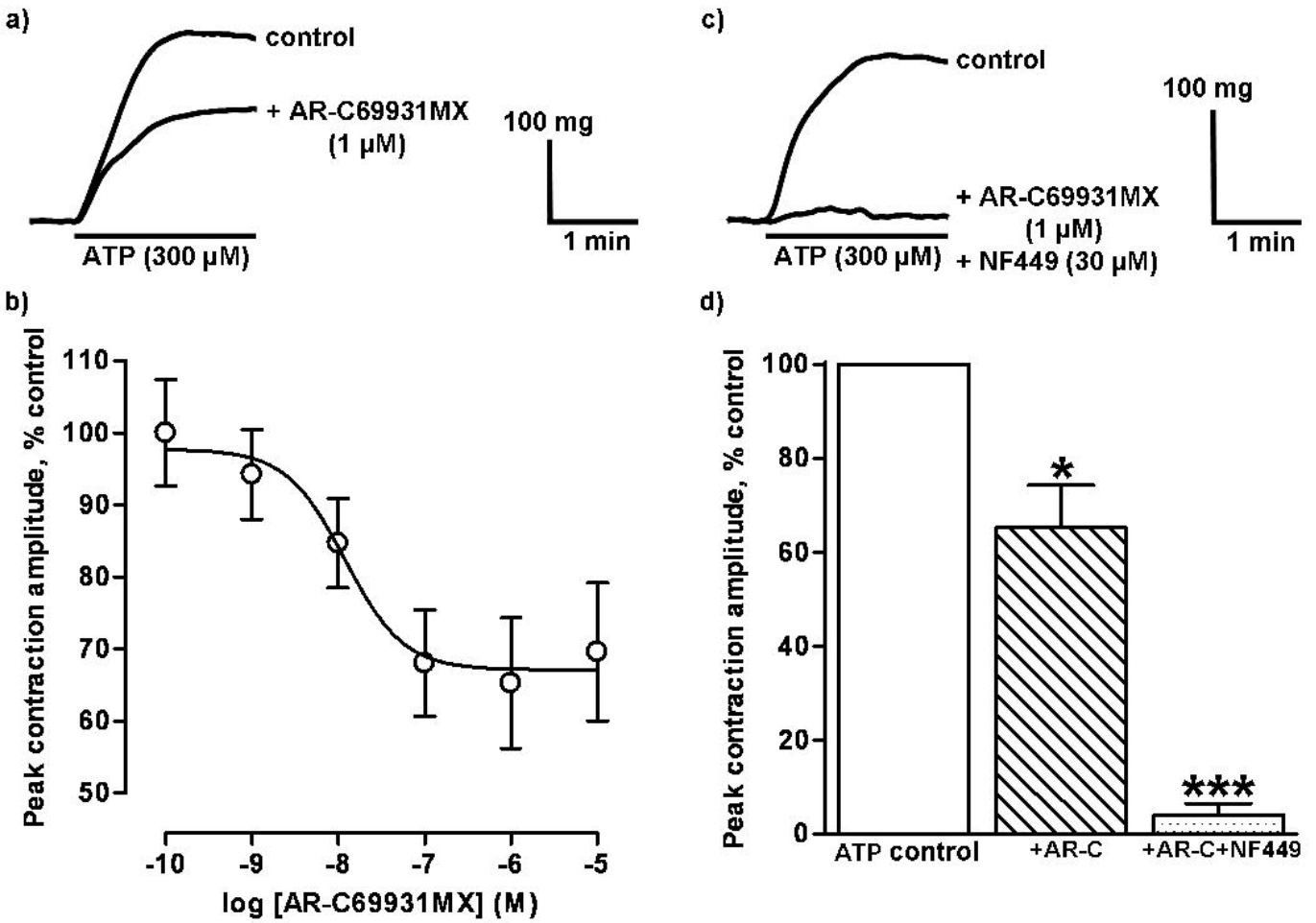
a)
control
+ AR-C69931MX
(1 μM)
100 mg
1 min
ATP (300 μM)
c)
control
100 mg
+ AR-C69931MX
(1 μM)
+ NF449 (30 μM)
1 min
ATP (300 μM)
b)
Peak contraction amplitude, % control
110
100
90
80
70
60
50
-10
-9
-8
-7
-6
-5
log [AR-C69931MX] (M)
d)
Peak contraction amplitude, % control
100
80
60
40
20
0
*
***
ATP control
+AR-C
+AR-C+NF449

Figure 3

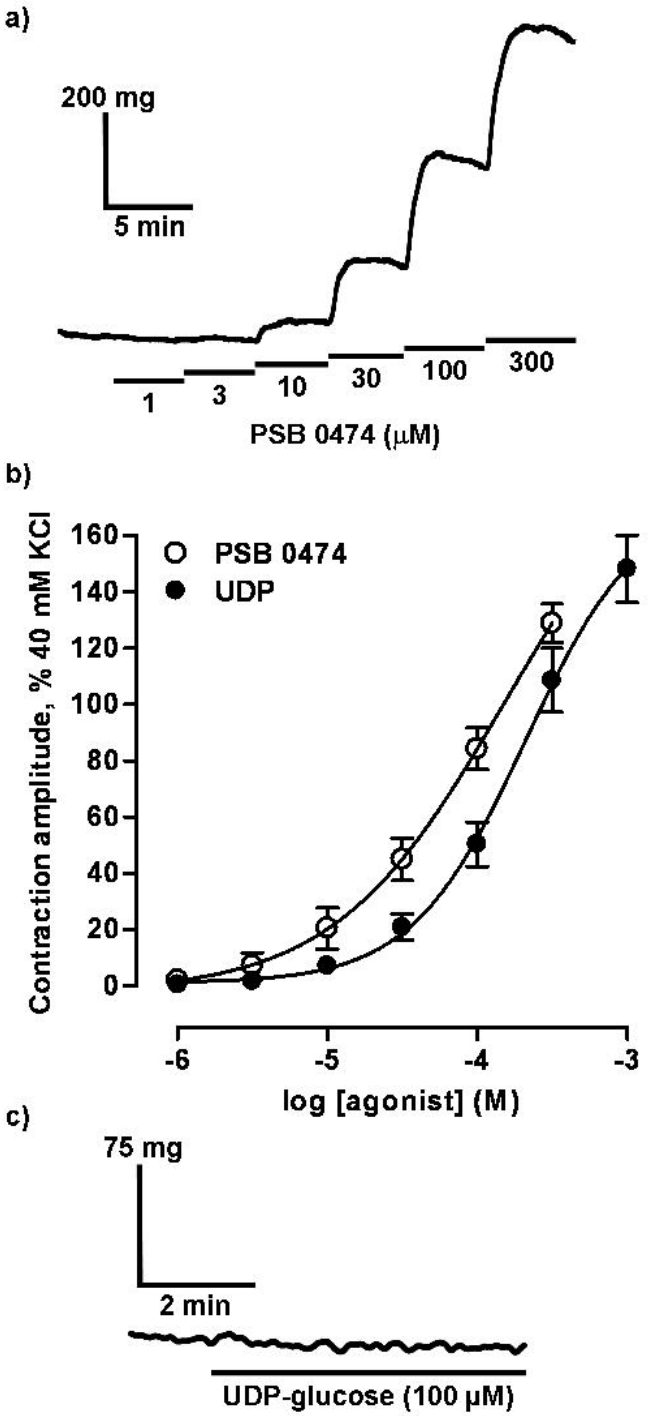
a)
200 mg
5 min
1
3
10
30
100
300
PSB 0474 (μM)
b)
Contraction amplitude, % 40 mM KCl
160
140
120
100
80
60
40
20
0
PSB 0474
UDP
-6
-5
-4
-3
log [agonist] (M)
c)
75 mg
2 min
UDP-glucose (100 μM)

Figure 4

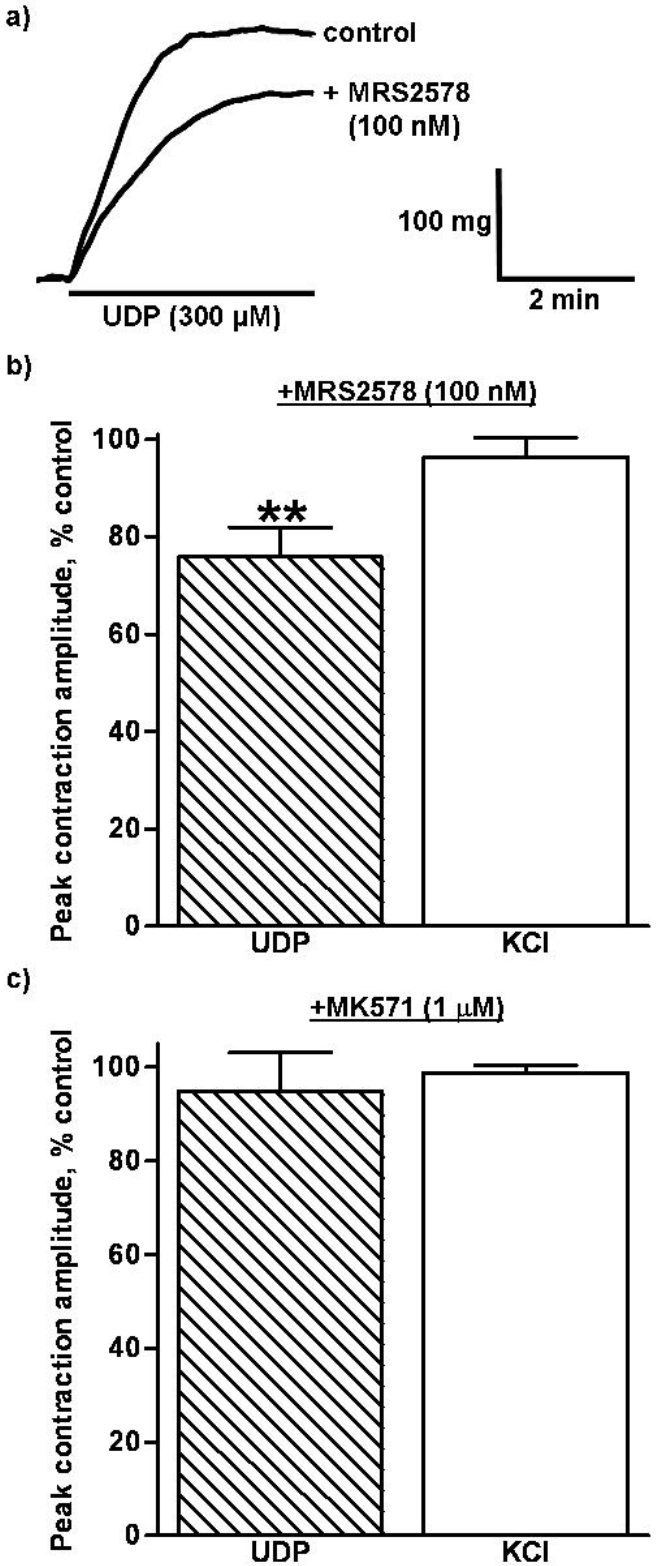
a)
control
+ MRS2578
(100 nM)
100 mg
2 min
UDP (300 µM)
b)
+MRS2578 (100 nM)
Peak contraction amplitude, % control
100
80
60
40
20
0
**
UDP
KCl
c)
+MK571 (1 µM)
Peak contraction amplitude, % control
100
80
60
40
20
0
UDP
KCl

Figure 5