Identification of Contractile P2Y₁, P2Y₆, and P2Y₁₂ Receptors in Rat Intrapulmonary Artery Using Selective Ligands

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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 characterize 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 by using reverse transcription- polymerase chain reaction with receptor-specific oligonucleotide primers. The selective P2Y₁ agonist (N)-methylcarba-2- methylthioadenosine-5'-O-diphosphate (MRS2365) induced small, concentration-dependent contractions that were inhibited by AR-C69931MX. The selective P2Y₁ agonist 3-(2-oxo-2-phenylethyl)-UDP (PSB 0474) evoked concentration-dependent contractions and was approximately three times more potent than UDP, but the P2Y₁₂ agonist UDP-glucose had no effect. Contractions evoked by UDP were inhibited by the P2Y₁₂ receptor antagonist N,N'-1,4-butanediylbis-N'-(3-isothiocyanato- phenyl)thiourea (MRS2578), but not the cysteinyl leukotriene 1 (CysLT₁) antagonist 3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)(3-dimethylamino-3-oxopropyl)thio)methylthiopropanoic acid (MK571). Higher concentrations of MRS2578 inhibited contractions to KCl, so they were not studied further. mRNA for P2Y₁, P2Y₆, and P2Y₁₂ receptors was identified. Our working model is that P2Y₁₂ and P2Y₁ receptors are present in rat intrapulmonary arteries and together mediate ATP-induced vasoconstriction. Contractile P2Y₆ receptors, but not P2Y₁₂ or CysLT₁ receptors are also present and are a major site through which UDP evokes constriction.

Introduction

The endogenous nucleotides ATP and 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 receptors 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 and 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, which is essential for the 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 (Liu et al., 1989a,b; McCormack et al., 1989; Hassésson and Burnstock, 1995; and others). This study was supported by the British Heart Foundation [Grant FS/04/070] (to A.M.G. and C.K.). C.M. and N.S. contributed equally to this study.

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ABBREVIATIONS: IPA, intrapulmonary arteries; MRS2365, (N)-methylcarba-2-methylthioadenosine-5'-O-diphosphate; MRS2179, N⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate; AR-C69931MX, N⁶-(2-methylthiomethyl)-2-(3,3,3-trifluoropropyl)thio)dichloro-methylene ATP; PSB 0474, 3-(2-oxo-2-phenylethyl)-UDP; MRS2578, N,N'-1,4-butanediylbis-N'-(3-isothiocyanato-phenyl)thiourea; MK571, 3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)(3-dimethylamino-3-oxopropyl)thio)methylthiopropanoic acid; NF449, 4,4',4''-[carbonyl]bis(mino-5,1,3-benzenetriyl-bis(carbonylmino))tetraakis-1,3-benzenedisulfonic acid; RT, reverse transcription; PCR, polymerase chain reaction; CysLT₁, cysteinyl leukotrienes; bp, base pairs.
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 endothelial-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, because the response inperfused 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 pathological 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 P2 receptor, whereas UDP acts at two P2Y subtypes, one of which may be the P2Y6 receptor (Chootip et al., 2002, 2005). Those 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 ADP, an agonist at P2Y12 receptors (Bodor et al., 2003), which were reported to mediate the contraction of human isolated arteries (Wihlborg et al., 2004; Høgberg et al., 2010).

A much clearer identification of the individual P2Y receptor subtypes that mediate the actions of nucleotides is now possible because of the development of several compounds with P2Y receptor subtype selectivity. For example, (N)-methanocarba-2-methylthioadenosine-5′-O-diphosphate (MR2365) is a highly selective and potent P2Y1 agonist (Ravi et al., 2002; Chhatriwala et al., 2004), N6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MR2179) is a potent, competitive P2Y1 antagonist (Boyer et al., 1998; Camaioni et al., 1998), and N6-(2-methylthiomethyl)-2-(3,3,3-trifluoropropanylthio) dichloro-methylene ATP (AR-C69931MX) is a highly selective and potent P2Y12 antagonist (Ingall et al., 1999). P2Y6 receptors can now also be probed by using 3-(2-oxo-2-pyridylthio)dichloro-methylene ATP (AR-C69931MX) is a highly selective and potent P2Y12 antagonist (Ingall et al., 1999).

The aim of the present study was to use these subtype-selective ligands to determine the contributions of P2Y1, P2Y6, and P2Y12 receptors to the constriction of rat IPA elicited by ATP and UDP. In addition, the P2Y14 receptor was previously excluded because of reports that it was insensitive to UDP (Chambers et al., 2000; Freeman et al., 2001), but re-examination of its pharmacological properties revealed that UDP is actually a potent P2Y14 agonist (Carter et al., 2009). A further issue is that UDP has also been proposed to be an agonist at the phylogenetically related cysteiny1 leukotrienes 1 (CysLT1) receptor (Mellor et al., 2001, 2002). Thus the contributions of P2Y14 and CysLT1 receptors to the action of UDP were also investigated. Finally, this pharmacological approach was complemented by analysis 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 122 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM KH2PO4, 0.5 mM NaH2PO4, 1 mM MgCl2, 11 mM glucose, and 1.8 mM CaCl2 titrated to pH 7.3 with NaOH and bubbled with “medical air” (21% O2, 5% CO2, and 74% N2). IPA of i.d. 200 to 500 μm were dissected and cleaned of connective tissue, and their endothelium was 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 (Grass Instruments, Quincy, MA), connected to a PowerLab/4e system using Chart 4.2 software (ADInstruments, Ltd., Chalgrove, Oxfordshire, 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 precontracted with ATP or UDP. Preliminary experiments showed that six repeated additions of ATP or UDP for 5 min at 30-min intervals elicited highly reproducible contractions. The P2Y1 agonist MR3265 and P2Y6 agonist PSB 0474 were applied by using the same protocol. To determine the effects of MR32578, 3-(3-(2-(7-chloro-2-quinolinyl)-ethenyl)phenyl)3-(3-dimethylamino-3-oxopropyl)thio)methyl)thio anxious acid (MK571), and 8,8′-[carboxylbis[(3-dimethylamino-3-oxopropyl)thio]methyl]thio anxious acid (MRS2578), a potent and insurmountable P2Y6 antagonist, responses to an agonist were obtained. Arteries were then incubated with the antagonist for 20 min, and the agonist was readministered. 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 of 146.6 mg) by using a Total RNeasy Midi kit (QIAGEN, Valencia, CA), according to the manufacturer’s protocol. The RNA concentration (42.5 μg/100 μl) was determined spectrophotometrically using a Genequant II RNA/DNA calculator (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). cDNA was synthesized by using 5 μg of RNA and Superscript III reverse transcriptase (200 U) (Invitrogen, Carlsbad, CA). The cDNA was added to a HotStarTag DNA polymerase (QiAGEN) PCR mix containing 10 pmol/μl of appropriate forward (5′-3′) and reverse (5′-3′) primers (Eurofins MWG Operon, Huntsville, AL) with each pair designed to selectively recognize a particular P2Y receptor, as follows: P2Y1, forward TCCTTTCCATCTCGAGTGCC, reverse TCCTCTTCTTGGACCCTGCCCA, 391 bp; P2Y6, forward TGTCCTGGTATGTTGAGT, reverse TGGTGGTGAGTAGAAGAGGATA, 498 bp; and P2Y12, forward GGCTTTGCTGTTGCTGTC, reverse GGGTGCTCTTTACGATGAC, 404 bp. The mix was placed in a DNA Thermal Cycler (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the following protocol was applied: 10 min at 95°C followed by 35 cycles of 90 s at 95°C, 90 s at 52°C, 90 s 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 visualized by ethidium bromide staining. The bands were then purified, and the sequence of each P2Y subtype was confirmed by using a BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Warrington, UK) and an Applied Biosystems 3100 Avant Genetic Analyzer.

**Drugs and Solutions.** ATP (Na₂ salt), ADP (Na salt), UDP (Na₂ salt), UDP-glucose (Na₂ salt), PSB 0474 (Na₂ salt), acetylcholine chloride (Sigma Chemical, Poole, Dorset, UK), MRS2179 (Na₄ salt), MRS2365 (Na₃ salt), 4,4'/H₁₁₀₃₂₄₄',4'/H₁₁₀₃₂₄₄'/H₁₁₆₃₀-[carbonylbis(imino-5,1,3-benzetriyl-bis(carbonylimino))tetrazis-1,3-benzenedisulfonic acid, (Na₈ salt) (NF449) (Tocris Bioscience, Bristol, UK), suramin hexasodium (Sigma/RBI, Natick, MA), AR-C69931MX (The Medicines Company, Parsippany, NJ), and MK571 (Na salt) (Enzo Life Sciences, Inc., Farmingdale, NY) were dissolved in distilled water as 1, 10, or 100 mM stock solutions and diluted in HEPES-based buffer before application to the tissues. MRS2578 (Tocris Bioscience) was dissolved in dimethyl sulfoxide 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 application 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 milligrams of 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 Software, Inc., San Diego, CA). Data were compared by 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 whether P2Y₁ receptors are involved, their presence in rat IPA was studied initially by using MRS2365, a highly potent and selective P2Y₁ agonist. MRS2365 (1–100 μM) induced small concentration-dependent contractions of rat IPA that reached a peak within 1 to 2 min and then slowly declined (Fig. 1a and 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. Because of the small quantity of MRS2365 available, a complete concentration-response curve could not be constructed, but it seems that MRS2365 is less efficacious than ATP at eliciting contractions of rat IPA, and these data are consistent with the presence of contractile P2Y₁ receptors in rat IPA.

**ATP-Sensitive Receptors: Antagonist Studies.** The contribution of the P2Y₁ receptors to the ATP response was then investigated by using MRS2179, a selective P2Y₁ antagonist. MRS2179 (10 μM) had no effect on basal tone and, as shown in Fig. 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) (Fig. 1b). At the same concentration, MRS2179 also had no significant effect

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**Fig. 1.** The effect of drugs active at P2Y₁ 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).
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). P2Y₁₂ receptors have been reported to contract human isolated arteries (Wihlborg et al., 2004; Högberg et al., 2010); therefore, the effects of AR-C69931MX, a potent and selective P2Y₁₂ 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 IC₅₀ of 9.9 nM (95% confidence limits of 2.0–49.8 nM), Hill slope of 0.97, and maximum inhibition of 33.3 ± 4.1% (Fig. 2, a and 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 the combined blockade of P2X₁ and P2Y₁₂ receptors with the P2X₁ antagonist NF449 (30 μM) (Syed et al., 2010; Syed and Kennedy, 2012) and AR-C69931MX (1 μM) (Fig. 2, c and d).

ATP is a weak antagonist at P2Y₁₂ receptors and would be expected to inhibit rather than stimulate P2Y₁₂ 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 P2Y₁₂ receptors. We hypothesized, therefore, that ATP was rapidly converted to ADP in rat IPA and that it was the diphosphate that acted at the P2Y₁₂ 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 P2Y₁₂ receptors seem 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 P2Y₆ and P2Y₁₄ 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 P2Y₆ agonist PSB 0474 (1–300 μM) evoked

![Fig. 2. Inhibition of contractions induced by ATP.](https://image-source.com)
concentration-dependent contractions of rat IPA that reached a peak within a couple of minutes (Fig. 3, a and b). The concentration-response curve did not reach a maximum over the concentration range tested, so EC\text{50} values could not be determined. The concentration of agonists that induced 40% of the response to KCl (40 mM) (EC\text{40K}) was therefore calculated (Chootip et al., 2002) and found to be 27.3 \mu M (95% confidence limits of 6.8–47.7 \mu M; \text{n} = 4). UDP (1 \mu M-1 mM) also evoked concentration-dependent contractions of rat IPA, with an EC\text{40K} of 85.5 \mu M (95% confidence limits of 58.8–112.2 \mu M; \text{n} = 11), which was significantly higher than the value for PSB 0474 (P < 0.05).

The presence of contractile P2Y14 receptors in rat IPA was tested by applying the selective P2Y14 agonist UDP-glucose. Maximal activation of the rat P2Y14 receptor has been shown to occur at submicromolar concentrations of UDP-glucose (Chambers et al., 2000; Freeman et al., 2001), yet, as shown in Fig. 3c, even at 100 \mu M UDP-glucose did not elicit vasoconstriction (\text{n} = 4). Thus, these data are consistent with the presence of contractile P2Y6, but not P2Y14 receptors in rat IPA.

UDP-Sensitive Receptors: Antagonist Studies. In the next series of experiments the effects of the P2Y6 receptor antagonist MRS2578 were determined. MRS2578 (100 nM) had no effect on the basal tone of rat IPA, but significantly reduced the peak responses to UDP (300 \mu M) (P < 0.05) by approximately 25% (Fig. 4, a and b). At the same concentration, MRS2578 had no significant effect on contractions evoked by KCl (40 mM) (Fig. 4b) or ATP (300 \mu M) (95.4 ± 12.0% of control; \text{n} = 5), but higher concentrations depressed significantly the contractions to KCl (40 mM) (1 \mu M, 81.2 ± 5.9% of control, \text{n} = 6, P < 0.05; 10 \mu M, 79.0 ± 4.2% of control, \text{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 P2Y6 receptor and 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 ± 1.0% of those in its absence (\text{n} = 8). Thus, these data indicate that at 1 and 10 \mu M MRS2578 acts at an additional site to depress smooth muscle contractility, so its effects against UDP were not studied. Nonetheless, it is clear that P2Y6 receptors make an important contribution to UDP-evoked contraction of rat IPA.

To investigate a potential agonist action of UDP at the CysLT1 receptor, the effects of the CysLT1 antagonist MK571 were investigated. At 1 \mu M this drug is maximally effective at CysLT1 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) (Fig. 4c), or ATP (300 \mu M) (102.8 ± 8.3% of control; \text{n} = 6). Thus the CysLT1 receptor does not seem 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 P2Y6, P2Y10, and P2Y12 receptors were amplified (Fig. 5), and the identity of each amplicon was confirmed by sequencing.

Fig. 3. The actions of pyrimidine nucleotide agonists. a, the trace shows typical contractions of rat isolated IPA evoked by PSB 0474 (1–300 \mu M), applied cumulatively, as indicated by the solid bars, b, the mean peak amplitudes of contractions evoked by PSB 0474 (1–300 \mu M) (\text{n} = 4) and UDP (1 \mu M-1 mM) (\text{n} = 11), normalized as a percentage of the response to KCl (40 mM), are 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 \mu M) on tone is shown.
By using novel selective pharmacological tools we have shown that ATP evokes vasoconstriction of the rat IPA via P2X1 and P2Y12 receptors. P2Y1 receptors also seem to be functionally expressed in the smooth muscle of this tissue, but at a low level, and they play no role in the response to ATP. In addition, we have clearly identified the P2Y6 receptor as one of the sites through which UDP evokes vasoconstriction. In contrast, the data show that P2Y14 receptors do not contribute to constriction, and we found no role for CysLT1 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 P2Y12 and P2X1 receptors. Low concentrations of the P2Y12 antagonist AR-C69931MX depressed contractions evoked by ATP by approximately one-third. The high potency of AR-C69931MX (IC50 = 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) P2Y12 receptors. In addition, the ATP response was virtually abolished by the combined blockade of P2X1 and P2Y12 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). Because ATP is a weak antagonist at P2Y12 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 P2Y12 receptors, but instead depends on 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 P2Y12 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

Fig. 4. The effects of putative antagonists. a, the superimposed traces show typical contractions of rat isolated IPA evoked by UDP (300 μM) before (top) and after incubation with MRS2578 (100 nM) for 20 min (bottom). UDP was applied as indicated by the solid bar. b and c, the mean peak amplitude of contractions evoked by UDP (300 μM) (cross-hatched columns) and KCl (40 mM) (open columns) in the presence of MRS2578 (100 nM) (b) and MK571 (1 μM) (c) 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 with response in its absence.

Fig. 5. Agarose gel electrophoresis of RT-PCR products from rat IPA using specific oligonucleotide primers for rat P2Y1, P2Y6, and P2Y12 receptors is shown. Each pair of primers yielded bands in the presence of reverse transcriptase (lanes 1, 3, and 5), but not in its absence (lanes 2, 4, and 6). The markers on the left show band size (base pairs).
dense granules is dephosphorylated by ectypeoluidases 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; Högborg 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 fully characterize 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, however, were small, and micromolar concentrations of MRS2365 were required. MRS2365 is the most potent human P2Y<sub>1</sub> agonist currently available and is active in the low nanomolar range at recombinant and native P2Y<sub>1</sub> receptors (Chhatriwala et al., 2004; Lu et al., 2007; Govindan et al., 2010). The need for substantially higher concentrations of MRS2365 to elicit small contractions suggests that although P2Y<sub>1</sub> receptors may be functionally expressed in rat IPA smooth muscle cells, it seems 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, so it 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 nonselective P2 receptor antagonists, suramin, pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid, and reactive blue 2 (Rubino and Burnstock, 1996; Hartley et al., 1998; Rubino et al., 1999; Chhootip et al., 2002).

The maximal concentration of MRS2578 that could be used (100 nM) is very close to the I<sub>C50</sub> (98 nM) obtained at the human P2Y<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 nonspecific action of MRS2578 at other sites to depress smooth muscle contractility. This is, perhaps, not surprising because 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, because 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, because the potent, competitive CysLT<sub>1</sub> antagonist MK571 did not antagonize 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. This probably will depend on the development of selective antagonists at other P2Y receptor subtypes.

**P2Y Receptor Expression.** Rat IPA express mRNA for P2Y<sub>1</sub>, P2Y<sub>4</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, because 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 nonspecific (Vial et al., 2006).

**Summary.** In conclusion, our working model is that ATP acts directly at P2X1 receptors and indirectly at P2Y<sub>12</sub> receptors after breakdown to ADP to evoke vasoconstriction of IPA. P2Y<sub>12</sub> receptors may also be functionally expressed, but at low levels, and they are not activated by ATP. In addition, UDP acts in part at P2Y<sub>4</sub>, but not P2Y<sub>14</sub> or CysLT<sub>1</sub> receptors, to evoke vasoconstriction of rat IPA. The remaining sites of action of UDP remain to be determined.

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**Authorship Contributions**

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

**Conducted experiments:** Mitchell, Syed, and Tengah.

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

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

**References**


itive and selective antagonism of P2Y$_2$ receptors by ADP analog. 


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