Regional variation in P2 receptor expression in the rat pulmonary arterial circulation

[1,2]K. Chootip, [1,3]K.F. Ness, [1]Y. Wang, [1]A.M. Gurney & *,[1]C. Kennedy

[1]Department of Physiology and Pharmacology, University of Strathclyde, Strathclyde Institute for Biomedical Sciences, John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR

1   The P2 receptors that mediate contraction of the rat isolated small (SPA, 200–500 μm i.d.) and large (LPA, 1–1.5 mM i.d.) intrapulmonary arteries were characterized.

2   In endothelium-denuded vessels the contractile order of potency was α,β-methyleneATP (α,β-meATP) >> UDP = UTP = ATP = 2-methylthioATP > ADP in the SPA and α,β-meATP = UTP ≥ UDP > 2-methylthioATP, ATP >> ADP in the LPA. α,β-meATP, 2-methylthioATP and ATP had significantly greater effects in the SPA than the LPA ($P < 0.001$), but there was no difference in the potency of UTP or UDP between the vessels.

3   In the SPA, P2X$_1$ receptor desensitisation by α,β-meATP (100 μM) inhibited contractions to α,β-meATP (10 nM–300 μM), but not those to UTP or UDP (100 nM–300 μM). In the LPA, prolonged exposure to α,β-meATP (100 μM) did not desensitize P2X receptors.

4   Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin and reactive blue 2 (RB2) (30–300 μM) inhibited contractions evoked by α,β-meATP. UTP and UDP were potentiated by PPADS, unaffected by RB2 and inhibited, but not abolished by suramin. 1 and 3 mM suramin produced no further inhibition, indicating suramin-resistant components in the responses to UTP and UDP.

5   Thus, both P2X and P2Y receptors mediate contraction of rat large and small intrapulmonary arteries. P2Y agonist potency and sensitivity to antagonists were similar in small and large vessels, but P2X agonists were more potent in small arteries. This indicates differential expression of P2X, but not P2Y receptors along the pulmonary arterial tree.
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Introduction

P2X and P2Y receptors mediate the extracellular actions of nucleotides such as adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) and can play an important role in the control of vascular tone (Boarder & Hourani, 1998). P2X receptors are ligand-gated cation channels, seven mammalian subtypes (P2X$_{1-7}$) of which have been cloned (Khakh et al., 2001). The P2X$_1$ appears to be the predominant P2X subunit expressed in vascular smooth muscle, although mRNA and/or antibody staining for other P2X subunits has also been detected, in a vessel-specific manner (Collo et al., 1996; Vulchanova et al., 1996; Bo et al., 1998a, b; Nori et al., 1998; Phillips et al., 1998; Phillips & Hill, 1999; Hansen et al., 1999; Gitterman & Evans, 2000; Lewis et al., 2000). Smooth muscle P2X$_1$ receptors mediate a vasoconstriction that depends upon the influx of extracellular calcium ions through the P2X$_1$ receptor ion channel and via

L-type calcium channels opened by cell depolarization (Kennedy et al., 1997). P2Y receptors are heptahelical, G protein-coupled receptors and seven subtypes (P2X$_{1,2,4,6,11,12,13}$) have been cloned in mammals (Boarder & Webb, 2001; Communi et al., 2001). Of these, mRNA for the P2Y$_{1,2,4,6}$ subtypes has been detected in vascular smooth muscle (Erlinge et al., 1998; Harper et al., 1998; Hartley et al., 1998; Lewis et al., 2000). The most common responses to vascular P2Y receptor activation are contraction via smooth muscle receptors and vasodilation via endothelial receptors (Burnstock & Kennedy, 1986; McLaren et al., 1998a; Ralevic & Burnstock, 1998).

The pulmonary circulation is a low-pressure, low-resistance circuit that regulates blood flow to the gas exchanging surfaces in the lung. P2 receptor agonists are vasoactive in the pulmonary vascular bed of a variety of species, including humans (Barnes & Liu, 1995). In rat lung, P2 receptor agonists evoke vasoconstriction at resting tone and vasodilation if muscle tone is first raised (McCormack et al., 1989; Hasséssian & Burnstock, 1995; Rubino & Burnstock, 1996). Similarly, in large diameter branches of rat isolated intrapulmonary artery, P2 receptor agonists are contractile

*Author for correspondence; E-mail: c.kennedy@strath.ac.uk
Current addresses: [2]Department of Physiology, Faculty of Medicine, Naresuan University, Phitsanulok 65000, Thailand; [3]Centre for Cardiovascular Science, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD

at resting tone and relaxant at raised tone (Liu *et al.*, 1989; Rubino *et al.*, 1999). However, which P2 receptor subtypes mediate these effects is unclear. Furthermore, there are inconsistencies in the published data. For example, ATP was reported to be equipotent with UTP as a vasoconstrictor in the perfused rat lung (Rubino & Burnstock, 1996), but a weaker agonist in rat large intrapulmonary artery *in vitro* (Rubino *et al.*, 1999). Also, the P2 receptor antagonist suramin had no effect on the responses to UTP in the perfused lung and isolated large intrapulmonary artery, whereas it inhibited contractions evoked in isolated small intrapulmonary artery (Hartley *et al.*, 1998). These differences are likely to reflect the differential expression of multiple P2 receptor subtypes in smooth muscle and endothelial cells along the pulmonary arterial tree.

In order to understand fully the vasoactive effects of P2 receptors in the intact pulmonary vascular bed, it is clearly essential to know which P2 subtypes are expressed in different regions of the vascular tree and what type of response they mediate. Therefore, the aim of this study was to characterize and compare under identical conditions the properties of the P2 receptors present in isolated vessels from different regions of the rat pulmonary vascular tree. To do so, we isolated segments of small (SPA, 200 – 550 μm i.d.) and large (LPA, 1.0 – 1.5 mm i.d.) intrapulmonary arteries and determined the effects of the agonists ATP, UTP, uridine 5′-diphosphate (UDP), adenosine 5′-diphosphate (ADP), α,β-methyleneATP (α,β-meATP) and 2-methylthioATP (2-meSATP), which between them act at all known P2 receptor subtypes. Also, we determined the effects of the P2 receptor antagonists, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin and reactive blue 2 (RB2) against each agonist. A preliminary account of these results has been published (Chootip *et al.*, 2000).

Methods

Male Sprague-Dawley rats (150 – 250 g) were sacrificed by cervical dislocation and exsanguination. Following thoracotomy, the heart and lungs were removed *en bloc*, the lungs separated and intrapulmonary arteries of internal diameter 200 – 500 μm (SPA) and 1.0 – 1.5 mm (LPA) dissected out. The arteries were cleaned of connective tissue, cut into rings 5 mm long and mounted horizontally in 1 ml baths on a pair of intraluminal wires. Tissues were allowed to equilibrate under a resting tension of 0.5 g (SPA) and 1.0 g (LPA) for 60 min at 37°C in a salt solution composed of (mM): NaCl 122; KCl 5; N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulphonic acid] (HEPES) 10; KH$_2$PO$_4$ 0.5; NaH$_2$PO$_4$ 0.5; MgCl$_2$ 1; glucose 11; CaCl$_2$ 1.8, titrated to pH 7.3 with NaOH and bubbled with air (21% O$_2$, 5% CO$_2$, 74% N$_2$). Tension was recorded with Grass FT03 isometric force transducers connected to a MacLab/4e system, using Chart 3.3 software (AD Instruments).

After equilibration, the integrity of the smooth muscle was determined by applying 200 nM phenylephrine twice. The presence of a functional endothelium was assessed by determining the degree of relaxation to acetylcholine (10 μM) in vessels precontracted with phenylephrine. When appropriate the endothelium was removed by gently rubbing the vessel lumen with a stainless steel wire. Removal of the

endothelium was confirmed by loss of the relaxant response to acetylcholine.

Experimental protocols

Cumulative concentration – response curves to α,β-meATP, 2-meSATP, ATP, UTP, UDP and ADP (O'Connor *et al.*, 1990) and to uridine and adenosine (10 nM – 300 μM) were obtained in endothelium-intact and -denuded rings at resting tone. Contractions generally took 1 – 4 min to reach a plateau and were expressed as a percentage of the contraction induced by 40 mM K$^+$, which was applied by replacement of HEPES – buffer solution in the same preparation.

To induce desensitization of P2X receptors, 100 μM α,β-meATP was applied, evoking a large contraction. Once tension had returned to baseline, a further 100 μM α,β-meATP was added. α,β-meATP was then washed out, and when re-applied 2 min later, no contraction or contraction less than 10% of that to 40 mM K$^+$ was seen, confirming that the P2X receptors were desensitized. α,β-meATP was again washed out and 2 min later the test agonist administered. The control responses to a test agonist were obtained at the same time in separate pulmonary artery rings from the same animal, but without pre-exposure to α,β-meATP. The effects of P2 receptor antagonists were investigated by incubating tissues for 30 min with PPADS, suramin or RB2 (30 – 300 μM) and P2 receptor agonists were then applied. Control responses to the agonists were obtained in separate tissues from the same animal. In a series of experiments designed to determine the effects of high concentrations of suramin, three consecutive contractions were obtained to 300 μM UTP or UDP. Suramin (1 or 3 mM) was then applied to the tissue for 30 min and the agonist then reapplied.

Drugs and solutions

Phenylephrine hydrochloride, acetylcholine chloride, ATP (magnesium salt), ADP (potassium salt), UDP (sodium salt), UTP (sodium salt), adenosine hemisulfate and uridine (Sigma, U.K.), α,β-meATP (lithium salt), suramin hexasodium, 2-meSATP tetrasodium, PPADS tetrasodium (RBI, U.S.A.) and RB2, also known as Basilen Blue E-3G (Tocris, U.K.) were dissolved in distilled water as 10 or 100 mM stock solutions. 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

Where possible, log concentration – response plots were fitted to the data by logistic (Hill equation), non-linear regression analysis (Graphpad Prism, San Diego, U.S.A.). Except for α,β-meATP, the agonist concentration – response curves did not reach a maximum and so EC$_{50}$ values could not be calculated. Therefore, the potency of these agonists was compared at the level of contraction equivalent to 40% of the response to 40 mM K$^+$ (EC$_{40K}$). Values in the text and figures refer to mean $\pm$ s.e.mean or geometric mean with 95% confidence limits for EC$_{50}$ and EC$_{40K}$ values. Data were compared by paired or unpaired *t*-tests or one-way analysis of variance and Tukey's comparison as appropriate. Differences were considered significant when $P < 0.05$.

Results

Contractions to P2 receptor agonists

In endothelium-intact SPA at resting tone, all P2 agonists tested evoked concentration–dependent contractions, with α,β-meATP being the most potent ($EC_{50} = 0.93$ μM, 95% confidence limits $= 0.48 - 1.80$ μM). UTP, ATP and 2-meSATP were equipotent at the EC_{40K} level, but about 50 times less potent than α,β-meATP (Table 1). UDP was 227 times less potent than α,β-meATP, whilst ADP was even less potent, and at the highest concentration used (300 μM) evoked a contraction that was only $34 \pm 7\%$ ($n = 4$) of the response to 40 mM K^+. Removal of the endothelium had no significant effect on contractions evoked by any P2 receptor agonist except UDP, which was potentiated by about 5 fold ($P < 0.01$). Thus, in the absence of the endothelium the rank order of potency was α,β-meATP $>>$ UDP $=$ UTP $=$ ATP $=$ 2-meSATP $>$ ADP (Figure 1a, Table 1).

In the endothelium-intact LPA at resting tone, α,β-meATP, UTP and UDP evoked relatively small contractions and only those to UDP consistently reached the EC_{40K} level. 2-meSATP, ATP and ADP were essentially inactive (Table 1). Removal of the endothelium substantially and significantly ($P < 0.05$) increased the amplitude of contractions evoked by α,β-meATP (100–300 μM), UTP (300 μM) and UDP (300 μM) such that the EC_{40K} level was now reached by all three agonists (Figure 1b, Table 1). Contractions to 2-meSATP (100–300 μM), ATP (300 μM) (Figure 1b) and phenylephrine (200 nM, $n = 5$, not shown) were also significantly ($P < 0.05$) increased, but the nucleotides' concentration–response curves did not reach the EC_{40K} level. ADP remained inactive ($n = 4$). Thus, the rank order of agonist potency in the LPA in the absence of endothelium was α,β-meATP $=$ UTP $\geqslant$ UDP $>$ 2-meSATP, ATP $>>$ ADP.

In endothelium-denuded vessels α,β-meATP, 2-meSATP and ATP had significantly greater effects in the SPA than the LPA ($P < 0.001$), but there was no significant difference in the potency of UTP or UDP between the vessels (Table 1). Neither adenosine nor uridine (0.01–300 μM), breakdown products of ATP and UTP/UDP, evoked contractions in endothelium-intact or -denuded SPA and LPA ($n = 4$ each). These data indicate that both P2X and P2Y receptors are expressed in the smooth muscle of rat SPA and LPA and that the potency of P2X agonists is greater in the SPA.

Effects of prolonged administration of α,β-meATP

α,β-meATP induces desensitization of the P2X$_1$ receptor, the predominant P2X receptor subtype present in smooth muscle (see Kennedy, 2001). In the SPA, the contraction evoked by prolonged exposure to α,β-meATP (100 μM) decayed to basal tension over 10–15 min (Figure 2a). Further application of α,β-meATP (100 μM) had no effect. When α,β-meATP was washed out and reapplied 2 min later, there was still no response, indicating that it had desensitized the P2X
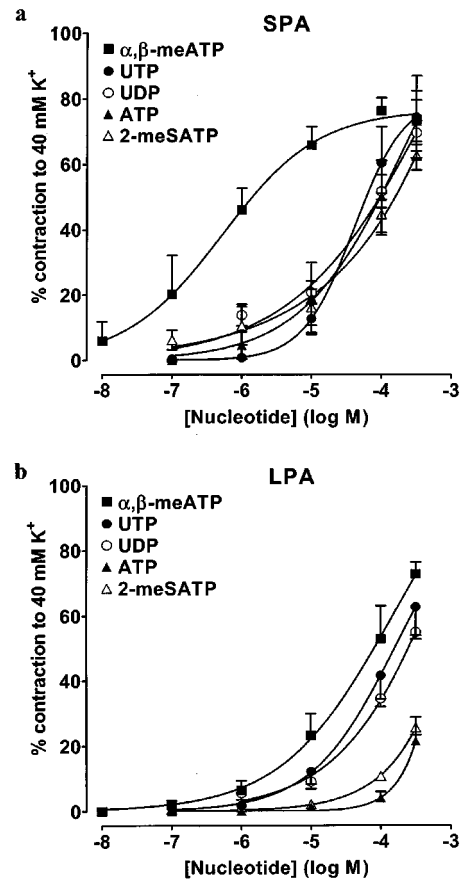

Figure 1 Contractions of rat isolated SPA and LPA to P2 receptor agonists. Cumulative concentration-response curves are shown for contractions to α,β-meATP (10 nM–300 μM), UTP (0.1–300 μM), UDP (0.1–300 μM), ATP (0.1–300 μM) and 2-meSATP (0.1–300 μM) at resting tone in endothelium-denuded rat (a) SPA and (b) LPA. Each point is the mean of 5–8 experiments. Vertical bars indicate s.e.mean and are smaller than the symbol in some cases.

Table 1 The potency of P2 receptor agonists in evoking contractions of SPA and LPA

| | SPA | | LPA | |
Agonist	+ endothelium	− endothelium	+ endothelium	− endothelium
α,β-meATP	0.9 (0.4–2.2) ($n=9$)***	0.7 (0.1–2.6) ($n=5$)**	>300 ($n=5$)	32 (8–132) ($n=5$)§
UTP	42 (30–57) ($n=7$)	47 (18–120) ($n=6$)	>300 ($n=6$)	73 (35–151) ($n=7$)
UDP	204 (150–275) ($n=6$)††	43 (19–98) ($n=6$)	167 (94–298) ($n=9$)	128 (92–178) ($n=9$)
ATP	47 (12–182) ($n=5$)	51 (9–297) ($n=6$)	no effect ($n=5$)	>300 ($n=7$)
2-meSATP	50 (12–200) ($n=5$)	63 (18–223) ($n=5$)	no effect ($n=5$)	>300 ($n=5$)

Values shown are EC_{40K} with 95% confidence limits (μM) for P2 agonist-induced contractions of rat SPA and LPA in the presence and absence of an intact endothelium. **$P < 0.001$, ***$P < 0.0001$ for EC_{40K} values of α,β-meATP *versus* UTP, UDP, ATP and 2-meSATP in SPA. ††$P < 0.01$ for UDP EC_{40K} values in endothelium-intact SPA versus endothelium-denuded SPA. §$P < 0.001$ for α,β-meATP EC_{40K} values in endothelium-denuded LPA versus endothelium-denuded SPA.
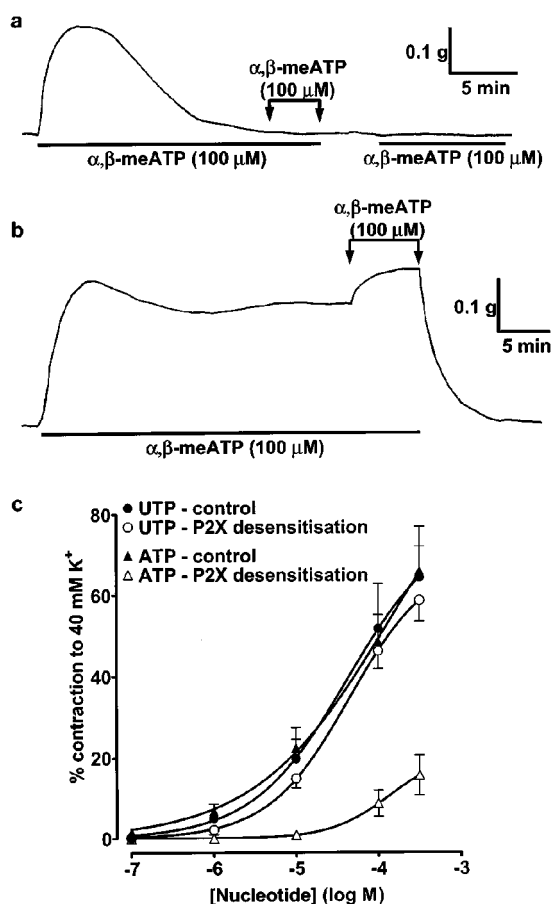
Figure 2 Effects of prolonged exposure to α,β-meATP in rat SPA and LPA. (a) SPA: α,β-meATP (100 μM), added as indicated by the horizontal bars, evoked a contraction that was not maintained. Once tension had returned to baseline, further addition of α,β-meATP (100 μM) had no effect. The drug was washed out and when α,β-meATP (100 μM) was added 2 min later no contraction was seen, confirming that the $P2X_1$ receptors had been desensitized. (b) LPA: α,β-meATP (100 μM) evoked a contraction that was sustained for at least 30 min. The drug was washed out and when α,β-meATP (100 μM) was re-added, a maintained contraction of similar amplitude was seen. (c) Cumulative concentration-response curves are shown for contractions to UTP and ATP (0.1–300 μM) at resting tone in SPA before (closed symbols) and after (open symbols) prolonged exposure to α,β-meATP. Each point is the mean of 5–6 experiments. Vertical bars indicate s.e.mean and are smaller than the symbol in some cases.

receptors in the SPA. Contractions to subsequent application of 10 nM – 10 μM α,β-meATP were abolished and those to 100–300 μM virtually abolished. Following the same protocol, responses to ATP (Figure 2c), 2-meSATP (0.1–300 μM, not shown), and ADP (1–300 μM, not shown) were inhibited in a similar manner. However, contractions evoked by UTP (Figure 2c) and UDP (1–300 μM, not shown) were unaffected by P2X receptor desensitization. This is consistent with the expression of both P2X and P2Y receptors in rat SPA.

In contrast to the SPA, α,β-meATP (100 μM) evoked a contraction in the LPA, which reached a peak within 2–3 min and was reasonably well maintained for at least 30 min (Figure 2b). Further application of α,β-meATP (100 μM) induced further contraction. After the agonist was washed out, re-application of α,β-meATP still induced a

sustained contraction. Similar responses were seen in endothelium-intact ($n=5$) and -denuded ($n=6$) LPA. Thus, in this tissue, α,β-meATP acts mainly at P2X receptors that do not desensitize and so the effects of P2X receptor desensitization on the responses to the other agonists was not studied.

Effects of P2 receptor antagonists in SPA

To further characterize the P2 receptors expressed in rat SPA and LPA, we next determined the ability of several P2 receptor antagonists to inhibit contractions evoked by α,β-meATP, which only acts at P2X receptors, and UTP and UDP, which only act at P2Y receptors. Thirty and 100 μM PPADS (Figure 3a), suramin (Figure 4a) and RB2 (Figure 5a) caused a progressive rightward shift of the concentration–response curve to α,β-meATP and significantly increased its EC_{40K} value (Table 2). The maximum response to α,β-meATP also appeared to decrease. Three hundred μM PPADS and suramin evoked a further rightward shift and depression of the α,β-meATP curve, such that the EC_{40K} level was not reached. Higher concentrations of RB2 were not used as these also depressed contractions evoked by phenylephrine (not shown). Thus, α,β-meATP evokes contraction of the SPA *via* a receptor that is sensitive to PPADS, suramin and RB2.

In contrast to α,β-meATP, the vasoconstrictor responses to UTP and UDP (Figure 3b,c) were potentiated by PPADS (30–300 μM) and their EC_{40K} values were significantly reduced (Table 2). Suramin (30–100 μM) caused a progressive rightward shift of their concentration–contraction curves (Figure 4b,c) and significantly increased their EC_{40K} values (Table 2), although increasing the concentration of suramin to 300 μM (Figure 4b,c) and to 1 or 3 mM produced no further inhibition ($n=5-7$, not shown). Finally, RB2 (30–100 μM) had no effect on contractions evoked by UTP and UDP (Figure 5b,c, Table 2). Thus, both UTP and UDP evoke contraction of the SPA *via* two receptors, one suramin-sensitive and the other suramin-insensitive, neither of which are antagonised by PPADS or RB2.

Effects of P2 receptor antagonists in LPA

The effects of PPADS, suramin and RB2 against each agonist in the LPA were qualitatively the same as those in the SPA, although there were some quantitative differences with α,β-meATP (Table 3). Thus, PPADS again caused a progressive rightward shift of the concentration–response curve to α,β-meATP, but produced a greater depression of the curve such that in the presence of 100 and 300 μM PPADS the EC_{40K} level was not reached. However, PPADS potentiated the contractions to UTP and UDP to a similar extent in the LPA as in the SPA. Suramin also caused a greater depression of the concentration–response curve to α,β-meATP in the LPA and 300 μM suramin abolished the contractions. As in the SPA, suramin (100 and 300 μM) inhibited contractions to UTP and UDP to a similar extent, and increasing the concentration of suramin to 1 and 3 mM produced no further inhibition. Finally, similar to PPADS and suramin, RB2 produced a greater depression of the concentration–response curve to α,β-meATP in the LPA
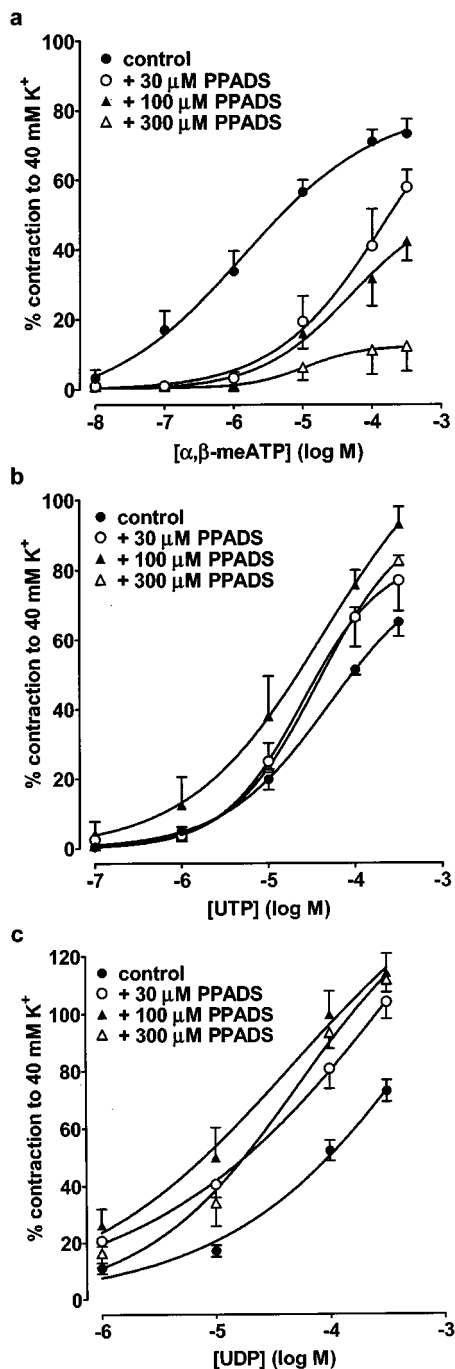
Figure 3 The effect of PPADS on contractions evoked by α,β-meATP, UTP and UDP in rat SPA. Cumulative concentration-response curves are shown for contractions of rat SPA evoked by (a) α,β-meATP (10 nM – 300 μM), (b) UTP (0.1 – 300 μM) and (c) UDP (1 – 300 μM) in the absence and presence of PPADS (30 – 300 μM). Each point is the mean of 4 – 6 experiments. Vertical bars indicate s.e.mean. The effects of α,β-meATP and UTP were studied in endothelium-intact tissues, whereas endothelium-denuded tissues were used when studying UDP.

Figure 4 The effect of suramin on contractions evoked by α,β-meATP, UTP and UDP in rat SPA. Cumulative concentration-response curves are shown for contractions of rat SPA evoked by (a) α,β-meATP (10 nM – 300 μM), (b) UTP (0.1 – 300 μM) and (c) UDP (1 – 300 μM) in the absence and presence of suramin (30 – 300 μM). Each point is the mean of 4 – 10 experiments. Vertical bars indicate s.e.mean. The effects of α,β-meATP and UTP were studied in endothelium-intact tissues, whereas endothelium-denuded tissues were used when studying UDP.

compared with the SPA. However, RB2 was ineffective against UTP and UDP.

Thus, α,β-meATP evokes contraction of the LPA *via* a receptor that is sensitive to PPADS, suramin and RB2, bu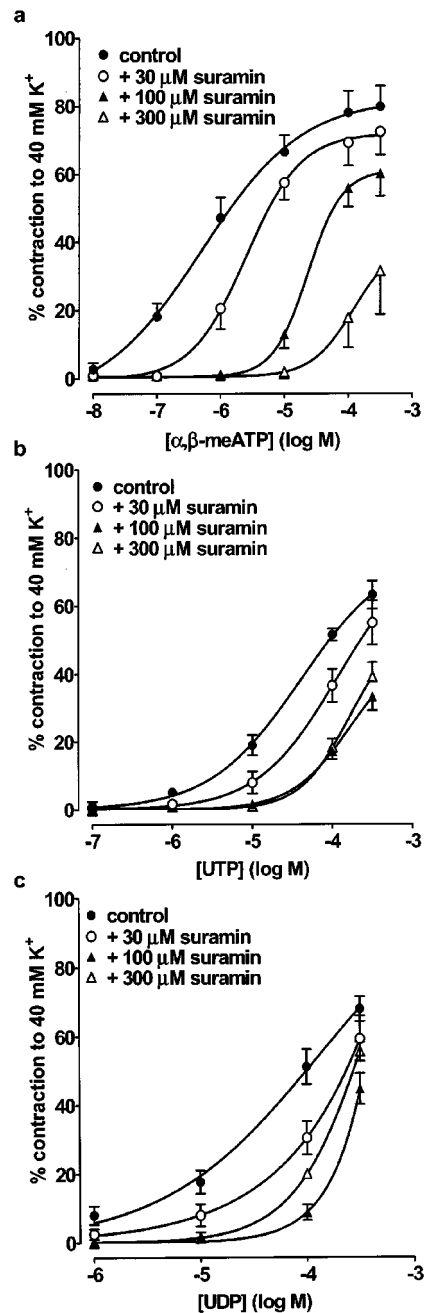t the antagonists depress the agonist concentration – response curve to a greater extent than in the SPA. Similar to the SPA, UTP and UDP evoke contraction of the LPA *via* two receptors, one suramin-sensitive and the other suramin-insensitive, neither of which is antagonized by PPADS or RB2.

Table 2 Effect of P2 receptor antagonists on responses to α,β-meATP, UTP and UDP in rat SPA

| Agonist | [Antagonist] (μM) | *Agonist EC$_{40K}$ (μM)* | | |
		+ PPADS	*+ Suramin*	*+ RB2*
α,β-meATP	0	1.5 (0.2 – 10.3)	0.7 (0.2 – 2.1)	0.7 (0.1 – 3.7)
	30	41.9 (7.1 – 244.3)*	3.3 (1.3 – 8.5)*	3.6 (0.6 – 24.6)*
	100	130.0 (20.6 – 822.2)**	45.5 (23.4 – 88.7)***	23.3 (5.3 – 102)*
	300	>300	>300	nd
UTP	0	39.8 (24.0 – 66.1)	45.7 (37.2 – 55.0)	66.1 (17.4 – 251.2)
	30	25.1 (15.8 – 38.9)	117.5 (60.3 – 223.9)††	64.6 (20.9 – 245.5)
	100	7.4 (1.3 – 42.7)††	>300	58.9 (24.5 – 141.3)
	300	15.9 (11.8 – 21.9)††	>300	nd
UDP	0	50.1 (33.1 – 74.1)	49.0 (31.6 – 75.9)	79.4 (56.8 – 109.6)
	30	9.5 (3.7 – 24.5)§	177.8 (69.2 – 457.1)§	97.9 (43.7 – 223.9)
	100	6.0 (1.0 – 33.9)§§	257.0 (183.0 – 354.8)§§	91.2 (46.8 – 177.8)
	300	13.0 (6.5 – 25.1)§§	186.2 (151.4 – 229.1)§	nd

Values shown are EC$_{40K}$ (μM) with 95% confidence limits; $n = 4 - 10$ in each case. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for α,β-meATP EC$_{40K}$ values, ††$P < 0.01$ for UTP EC$_{40K}$ values and §$P < 0.05$, §§$P < 0.01$ for UDP EC$_{40K}$ values, each in the presence of antagonists compared with control. nd = not determined. As the endothelium has no effect on the potency of α,β-meATP and UTP in SPA, endothelium-intact tissues were used with these agonists, whereas endothelium-denuded tissues were used when studying UDP.

Table 3 Effect of P2 receptor antagonists on responses to α,β-meATP, UTP and UDP in rat LPA

| Agonist | [Antagonist] (μM) | *Agonist EC$_{40K}$ (μM)* | | |
		+ PPADS	*+ Suramin*	*+ RB2*
α,β-meATP	0	28.2 (13.8 – 60.3)	19.1 (6.6 – 55.0)	27.5 (20.0 – 38.9)
	30	72.4 (32.4 – 166.0)*	27.5 (11.5 – 64.6)	>300
	100	>300	>300	>300
	300	>300	>300	nd
UTP	0	64.6 (27.5 – 147.9)	79.4 (33.9 – 190.5)	75.9 (42.7 – 131.8)
	30	32.4 (12.6 – 83.2)	67.6 (21.4 – 213.8)	125.9 (33.9 – 467.7)
	100	12.6 (2.8 – 57.5)†	>300	182.0 (87.1 – 380.2)
	300	10.5 (5.5 – 20.0)†	>300	nd
UDP	0	95.5 (53.1 – 173.8)	162.2 (182.0 – 75.9)	89.1 (40.8 – 194.5)
	30	64.3 (29.9 – 138.0)	182.0 (72.4 – 457.1)	161.4 (55.6 – 468.8)
	100	35.7 (4.1 – 312.6)§	>300	143.9 (39.5 – 522.4)
	300	19.2 (9.7 – 37.8)§	>300	nd

Values shown are EC$_{40K}$ (μM) with 95% confidence limits; $n = 4 - 11$ in each case. *$P < 0.05$ for α,β-meATP EC$_{40K}$ values, †$P < 0.05$ for UTP EC$_{40K}$ values and $P < 0.05$ for UDP EC$_{40K}$ values, each in the presence of antagonists compared with control. nd = not determined. All experiments were carried out in endothelium-denuded tissues.

Discussion

This study shows that purine and pyrimidine nucleotides evoke contraction of the rat SPA and LPA *via* multiple P2X and P2Y receptor subtypes. Contractions evoked by the P2X receptor agonist α,β-meATP were transient in the SPA, but tonic in the LPA. The P2Y receptor agonists UTP and UDP both appeared to act *via* two receptors in both vessels, although it is not clear if they were acting at the same receptors. Also, α,β-meATP was about 30 times more potent in the SPA than in the LPA, whereas UTP and UDP were equipotent in the SPA and LPA. Thus, the relative functional expression of P2X and P2Y receptors varies along the pulmonary arterial tree.

P2X receptors in rat pulmonary arteries

In this study, P2X receptor agonists appeared to act at the P2X$_1$ receptor to evoke vasoconstriction of the SPA. The rank order of potency was α,β-meATP > > 2-meSATP = ATP > ADP, and responses to each agonist were abolished or virtually abolished by prolonged exposure to α,β-meATP.

This is essentially the pharmacological profile of the P2X receptor in whole tissues, as originally described by Burnstock & Kennedy (1985). Also, PPADS, suramin and RB2 all caused substantial antagonism. Similar responses have been reported in the rat perfused pulmonary vascular bed (McCormack *et al.*, 1989; Hasséssian & Burnstock, 1995; Rubino & Burnstock, 1996).

Of the known recombinant P2X receptors, α,β-meATP is an agonist at the P2X$_1$ and P2X$_3$ subunits only, and PPADS and suramin are antagonists at both of these (Ralevic & Burnstock, 1998; Khakh *et al.*, 2001). However, the P2X$_3$ subunit has a very restricted distribution and is absent from smooth muscle cells (Collo *et al.*, 1996). In contrast, the P2X$_1$ subunit is expressed highly in arterial smooth muscle (Collo *et al.*, 1996; Vulchanova *et al.*, 1996; Bo *et al.*, 1998a, b; Nori *et al.*, 1998; Phillips *et al.*, 1998; Phillips & Hill, 1999; Hansen *et al.*, 1999; Gitterman & Evans, 2000; Lewis *et al.*, 2000). Thus, the P2X$_1$ receptor is most likely to mediate the vasoconstriction evoked by P2X receptor agonists in SPA.

In the LPA the same rank order of agonist potency was seen, and PPADS, suramin and RB2 all antagonized
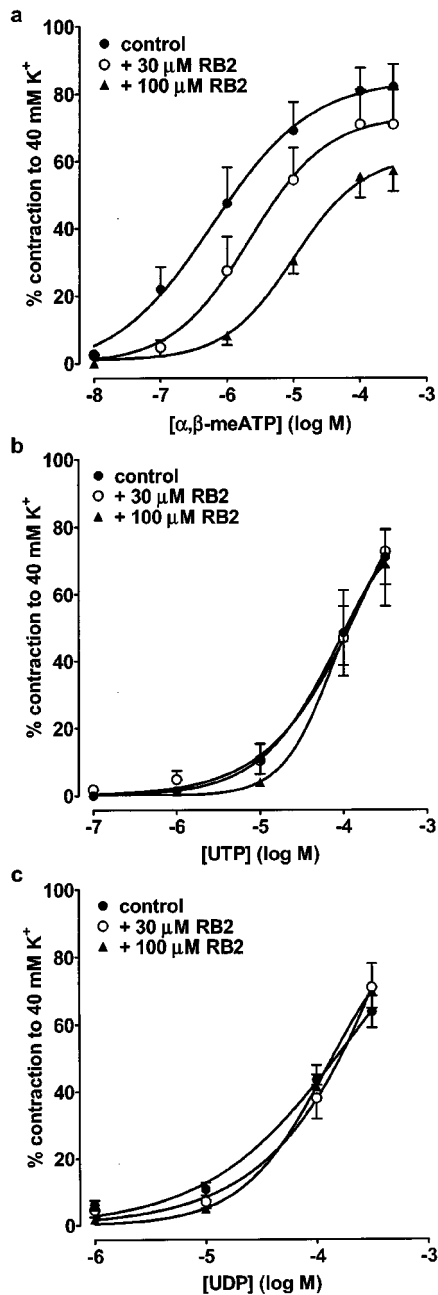
Figure 5 The effect of RB2 on contractions evoked by α,β-meATP, UTP and UDP in rat SPA. Cumulative concentration-response curves are shown for contractions of rat SPA evoked by (a) α,β-meATP (10 nM – 300 μM), (b) UTP (0.1 – 300 μM) and (c) UDP (1 – 300 μM) in the absence and presence of RB2 (30 – 100 μM). Each point is the mean of 4 – 6 experiments. Vertical bars indicate s.e.mean. The effects of α,β-meATP and UTP were studied in endothelium-intact tissues, whereas endothelium-denuded tissues were used when studying UDP.

contractions to α,β-meATP. However, the agonists were substantially less potent in the LPA than in the SPA. Furthermore, α,β-meATP induced a maintained contraction of the LPA. This was an unexpected finding, as in most arteries prolonged exposure to α,β-meATP induces P2X receptor desensitization (Kennedy *et al.*, 1997). Non-desensitizing responses to α,β-meATP have been reported in only a few cases; in the human placenta arterial bed

(Dobronyi *et al.*, 1997; Ralevic *et al.*, 1997) and rat renal vessels (Inscho *et al.*, 1998; Van Der Giet *et al.*, 1999).

Which P2X receptor subunit(s) mediates these maintained responses is unclear as α,β-meATP is not an agonist at the non-desensitizing recombinant P2X receptors (P2X$_{2,4,5,6,7}$) (Khakh *et al.*, 2001). It may be that the P2X$_1$ subunit is non-desensitizing in the LPA due to a post-translational modification or expression of a splice variant. That this could occur is implied by the finding that inhibiting phosphorylation of the P2X$_2$ subunit converts it from a rapidly- to a slowly-desensitizing form (Boué-Grabot *et al.*, 2000). Alternatively, the P2X$_1$ subunit may form a non-desensitizing heteropolymer with another subunit. A precedent for this mechanism is the report that when co-expressed, the P2X$_1$ and P2X$_5$ subunits form a channel that is activated by α,β-meATP in a slowly desensitizing manner and antagonized by PPADS and suramin (Torres *et al.*, 1998; Haines *et al.*, 1999). However, to date, the presence of only P2X$_1$, P2X$_2$, and P2X$_4$ subunits has been established in rat pulmonary artery smooth muscle (Nori *et al.*, 1998; Hansen *et al.*, 1999). The presence of P2X$_5$ receptors has not been investigated, although low levels of P2X$_5$ mRNA and protein have been reported in other arteries (Bo *et al.*, 1998b; Phillips *et al.*, 1998; Phillips & Hill, 1999; Gitterman & Evans, 2000; Lewis *et al.*, 2000). Finally, an alternative possibility is that α,β-meATP may act in the LPA at a P2X receptor subunit that has yet to be cloned.

P2Y receptors in rat pulmonary arteries

The current study showed that UTP and UDP both act *via* P2Y receptors, but not P2X receptors, to elicit contraction of rat SPA and LPA. We have reported previously that high concentrations of UTP activates P2X$_1$ receptors in rat tail artery (McLaren *et al.*, 1998a, b), but here the UTP- and UDP-evoked contractions of rat SPA were unaffected by P2X$_1$ receptor desensitization. Also, PPADS and RB2 inhibited responses to α,β-meATP, but not UTP or UDP in both SPA and LPA. This is consistent with previous reports of PPADS-, RB2- and α,β-meATP-resistant contractions to UTP and UDP in the rat perfused pulmonary bed (Rubino & Burnstock, 1996) and rat isolated pulmonary artery branches (Hartley *et al.*, 1998; Rubino *et al.*, 1999). Thus, UTP and UDP only act *via* P2Y receptors in rat SPA and LPA.

In this study UTP and UDP both acted *via* two P2Y receptors, although it is not clear if they acted at the same two P2Y receptors. In either case, suramin is an antagonist at one site, but not the other, and neither is antagonized by PPADS or RB2. Of the recombinant rat P2Y receptors UTP and UDP are agonists at the P2Y$_2$, P2Y$_4$ and P2Y$_6$ subtypes only (Boarder & Webb, 2001). UTP is a potent agonist and UDP is inactive at the P2Y$_2$ and P2Y$_4$ receptors, while UDP is a potent agonist at the P2Y$_6$ subtype. UTP has been reported to be an agonist at the P2Y$_6$ receptor, although it is substantially less potent than UDP (Nicholas *et al.*, 1996; Filippov *et al.*, 1999) and can also act indirectly at the P2Y$_6$ subtype after dephosphorylation to UDP (Nicholas *et al.*, 1996).

Our data are consistent with the P2Y$_2$ receptor as one of the sites of action of UTP, as it is suramin-sensitive and PPADS-insensitive (Charlton *et al.*, 1996a; Chen *et al.*, 1996a), as was seen here. The identity of the suramin-

resistant UTP receptor is less clear. Both the $P2Y_4$ (Charlton *et al.*, 1996b; Bogdanov *et al.*, 1998) and $P2Y_6$ receptors (Chang *et al.*, 1995; Robaye *et al.*, 1997) are largely insensitive to suramin and so are possible sites of action. On the other hand, PPADS and RB2 are reported to antagonize both of these (Chang *et al.*, 1995; Robaye *et al.*, 1997; Bogdanov *et al.*, 1998), but did not inhibit UTP-evoked contractions in rat SPA and LPA. Indeed, in our study PPADS potentiated the responses to UTP (and UDP). This was probably due to PPADS inhibiting their breakdown, as it is known to inhibit ecto-nucleotidases present in smooth muscle (Khakh *et al.*, 1995) and other tissues (Chen *et al.*, 1996b; Grobben *et al.*, 2000). Consistent with this idea, PPADS and the ecto-nucleotidase inhibitor ARL 67156 potentiated contractions to UTP in the rat isolated tail artery in a similar manner (McLaren *et al.*, 1998a).

A similar lack of inhibition of UTP by PPADS and suramin was seen in the rat perfused pulmonary bed (Rubino & Burnstock, 1996) and PPADS, suramin and RB2 were ineffective in rat isolated pulmonary artery branches (Rubino *et al.*, 1999). This is consistent with our conclusion that none of the cloned P2Y receptors appears to have the same antagonist sensitivity as the second site of action of UTP, which we report in rat SPA and LPA. Thus, UTP may act at a novel, uncloned receptor in the rat SPA and LPA. However, it should be noted that in most cases the interaction of putative antagonists with cloned P2Y receptors has not been extensively characterized and there are numerous contradictions in the published literature (see Suarez-Huerta *et al.*, 2001 for examples). Further studies are required and the $P2Y_4$ and $P2Y_6$ receptors are still potential candidates for the second site of action of UTP in rat SPA and LPA.

The present data indicate that the $P2Y_6$ receptor is probably the suramin-insensitive site of action of UDP in rat SPA and LPA, as UDP is an agonist at the cloned, suramin-insensitive $P2Y_6$ subtype (Border & Webb, 2001). Consistent with this, Hartley *et al.* (1998) identified mRNA for the $P2Y_6$ receptor in rat SPA. However, the same reservations regarding the lack of antagonism by PPADS and RB2 discussed above have to be noted. The identity of the suramin-sensitive site of action of UDP is less clear, since as noted above, of the recombinant P2Y receptors, UDP only acts at the $P2Y_6$ subtype. Thus, UDP may act at a novel,

uncloned receptor in the rat SPA and LPA. Alternatively, the suramin-insensitive effects of UDP may be mediated by the $P2Y_2$ receptor after extracellular conversion to UTP by ecto-nucleoside diphosphokinase (eNDPK) in vascular smooth muscle. At present, selective eNDPK inhibitors are not available and so this possibility cannot be tested. However, such a conversion of UDP to UTP has been proposed to explain, in part, apparent agonist actions of UDP at $P2Y_2$ receptors expressed in a cell line (Nicholas *et al.*, 1996).

Ideally, native receptors should be characterized on the basis of the actions of stable and selective agonists and antagonists, but at present such compounds are not available for $P2Y_{2,4,6}$ receptors. Nonetheless, these studies indicate that UTP most likely acts *via* suramin-sensitive $P2Y_2$ and suramin-insensitive $P2Y_4$ or $P2Y_6$ receptors to elicit contraction of rat SPA and LPA. Likewise, UDP probably acts *via* $P2Y_6$ receptors and $P2Y_2$ receptors. However, for both agonists an action at a novel, uncloned P2Y receptor cannot be ruled out.

In summary, the results of the present study indicate the presence of multiple subtypes of contractile P2X and P2Y receptors in rat SPA and LPA smooth muscle cells and suggests that the functional expression of these receptors varies along the pulmonary arterial tree. A similar conclusion was reached for the rat mesenteric arterial bed, although in that case the potency of both P2X and P2Y receptor agonists varied (Gitterman & Evans, 2000). The responses of the rat perfused lung to P2X receptor agonists, particularly the desensitization evoked by α,β-meATP (McCormack *et al.*, 1989; Rubino & Burnstock, 1996), more closely resembles those seen in the isolated SPA than in the LPA in the present study. This is consistent with small vessels exerting a greater effect than large vessels on pulmonary arterial pressure (Barnes & Liu, 1995). Thus, although studies on isolated arteries, particularly large arteries, have provided a substantial amount of information about receptor expression and mechanisms of action in general, care must be taken in extrapolating the data to the intact vascular bed.

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