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# Evidence that ATP acts at two sites to evoke contraction in the rat isolated tail artery

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**1.** The site(s) at which P2-receptor agonists act to evoke contractions of the rat isolated tail artery was studied by use of P2-receptor antagonists and the extracellular ATPase inhibitor 6-N,N-diethyl-D-$\beta,\gamma$-dibromomethyleneATP (ARL 67156).

**2** Suramin (1 $\mu$M – 1 mM) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (0.3 – 300 $\mu$M) inhibited contractions evoked by equi-effective concentrations of $\alpha,\beta$-methyleneATP ($\alpha,\beta$-meATP) (5 $\mu$M), 2-methylthioATP (2-meSATP) (100 $\mu$M) and adenosine 5′-triphosphate (ATP) (1 mM) in a concentration-dependent manner. Responses to $\alpha,\beta$-meATP and 2-meSATP were abolished, but approximately one third of the peak response to ATP was resistant to suramin and PPADS.

**3** Contractions evoked by uridine 5′-triphosphate (UTP) (1 mM) were slightly inhibited by suramin (100 and 300 $\mu$M) and potentiated by PPADS (300 $\mu$M).

**4** Desensitization of the $P2X_1$-receptor by $\alpha,\beta$-meATP abolished contractions evoked by 2-meSATP (100 $\mu$M) and reduced those to ATP (1 mM) and UTP (1 mM) to $15\pm3\%$ and $68\pm4\%$ of control.

**5** Responses to $\alpha,\beta$-meATP (5 $\mu$M) and 2-meSATP (100 $\mu$M) were abolished when tissues were bathed in nominally calcium-free solution, while the peak contractions to ATP (1 mM) and UTP (1 mM) were reduced to $24\pm6\%$ and $61\pm13\%$, respectively, of their control response.

**6** ARL 67156 (3 – 100 $\mu$M) potentiated contractions elicited by UTP (1 mM), but inhibited responses to $\alpha,\beta$-meATP (5 $\mu$M), 2-meSATP (100 $\mu$M) and ATP (1 mM) in a concentration-dependent manner.

**7** These results suggest that two populations of P2-receptors are present in the rat tail artery; ligand-gated $P2X_1$-receptors and G-protein-coupled P2Y-receptors.

**Keywords:** $P2X_1$-receptors; P2Y-receptors; ATP; UTP; PPADS; suramin; ARL 67156; rat tail artery

## Introduction

When adenosine 5′-triphosphate (ATP) is released as a cotransmitter with noradrenaline from perivascular sympathetic nerves, it acts at $P2X_1$-receptors, ligand-gated cation channels (see Buell *et al*., 1996; North, 1996), to evoke depolarization and contraction of vascular smooth muscle (see Burnstock, 1996; Sneddon *et al*, 1996). Vasoconstriction mediated via $P2X_1$-receptors is dependent upon influx of extracellular calcium ions through the $P2X_1$-receptor ion channel (Benham & Tsien, 1987; Kitajima *et al*., 1993; 1994; Pacaud *et al*., 1994; 1995; Loirand & Pacaud, 1995; Lagaud *et al*., 1996) and via L-type calcium channels opened by cell depolarization (Kitajima *et al*., 1993). Consequently, contractions mediated by $P2X_1$-receptors are abolished in the absence of extracellular calcium ions.

ATP can also act at other P2-receptors in arterial smooth muscle to raise cytoplasmic calcium levels independently of extracellular calcium ions. Instead, calcium ions are released from intracellular stores (see Abbracchio & Burnstock, 1994; Kennedy *et al*., 1997). However, in most cases it is not clear if this leads to vasoconstriction. Thus, the functions and properties of these receptors are poorly defined. These receptors are likely to be P2Y-receptors, linked by G-proteins to generation of inositol 1,4,5-trisphosphate ($IP_3$) and release of intracellular calcium ion stores (Harden *et al*., 1995). Consistent with this, uridine 5′-triphosphate (UTP), a potent agonist at several P2Y-receptor subtypes (Nicholas *et al*., 1996; Communi & Boeynaems, 1997), also causes generation of $IP_3$, release of internal calcium stores and vasoconstriction (Seifert & Schultz, 1989; Abbracchio & Burnstock, 1994; Harden *et al*., 1995).

The smooth muscle of the rat tail artery has a high density of $P2X_1$-receptors (Bo & Burnstock, 1993) and these mediate the neurogenic excitatory junction potentials (Sneddon & Burnstock, 1984; McLaren *et al*., 1995) and currents (Bao & Stjärne, 1993) which underlie the purinergic component of neurogenic contractions (Bao & Stjärne, 1993) in this tissue. The $P2X_1$-receptor agonists ATP, $\alpha,\beta$-methyleneATP ($\alpha,\beta$-meATP) and 2-methylthioATP (2-meSATP) activate fast inward currents via $P2X_1$-receptors in single rat tail artery smooth muscle cells and evoke contraction of the intact vessel (Evans & Kennedy, 1994).

Recently, we showed that UTP can also activate $P2X_1$-receptors in smooth muscle cells of the rat tail artery, but only at concentrations 100 times higher than those at which ATP is active (McLaren *et al*., 1997). However, UTP is equipotent with ATP at evoking contraction (Saïag *et al*., 1990; Evans & Kennedy, 1994). This suggests that UTP causes contraction of this artery via a site distinct from $P2X_1$-receptors. In some arteries UTP and ATP act via a common (non-$P2X_1$-) receptor (Eltze & Ullrich, 1996; Miyage *et al*., 1996), whereas in others, UTP acts at a receptor at which ATP is not an agonist (Von Kügelgen *et al*., 1987; Von Kügelgen & Starke, 1990; Ralevic & Burnstock, 1991; Windscheif *et al*., 1994; Rubino & Burnstock, 1996).

The aim of this study was to determine how many subtypes of P2-receptor mediate contraction of the rat isolated tail artery, using (i) the P2-receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), (ii) desensitization of the $P2X_1$-receptor and (iii) removal of extracellular calcium ions. Also, the influence of

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breakdown by ecto-ATPase on apparent agonist potency was studied with the ecto-ATPase inhibitor 6-N,N-diethyl-D-$\beta,\gamma$-dibromomethyleneATP (ARL 67156). A preliminary account of these results has been published (McLaren *et al*., 1996).

## Methods

Male Sprague Dawley rats (170–175 g) were killed by asphyxiation with $CO_2$ and subsequent cervical dislocation. The ventral tail artery was removed, cleaned of connective tissue and cut into rings 5–10 mm long (internal diameter $\approx$300 $\mu$m). These were mounted horizontally in 1 ml baths for tension recording by inserting two stainless steel wires into the lumen. The tissues were allowed to equilibrate under a resting tension of 0.5–0.75 g at 37°C for 1 h in a physiological salt solution of the following composition (mM): NaCl 140, KCl 5, $Na_2HPO_4$ 0.06, glucose 10, HEPES 10, $MgCl_2$ 1.2, $CaCl_2$ 2.5; titrated to pH 7.3 with NaOH (1 N) and bubbled continuously with 100% $O_2$. In one series of experiments the role of extracellular calcium ions in agonist-induced contractions was studied in a nominally calcium ion-free solution by omitting $CaCl_2$ from this solution. Tension was recorded with Grass FT03 transducers connected via a Quadbridge preamplifier to a MacLab/4e system using Chart 3.3 software (AD Instruments). In all preparations the endothelium was removed by gentle rubbing. Following precontraction with 1 $\mu$M noradrenaline, 10 $\mu$M acetylcholine did not produce vasodilatation, confirming that the endothelium had been removed.

Log concentration-response curves to P2-receptor agonists do not reach a maximum in this tissue (Evans & Kennedy, 1994; McLaren *et al*., 1996) and so $EC_{50}$ values cannot be calculated. Also, the low potency of ATP, 2-meSATP and UTP limit the extent to which their concentration-response curves can be shifted to the right in the presence of an antagonist. Therefore, the effects of suramin and PPADS were determined against single, equi-effective concentrations of $\alpha,\beta$-meATP (5 $\mu$M), 2-meSATP (100 $\mu$M), ATP (1 mM) and UTP (1 mM) (see Evans & Kennedy. 1994), so allowing the antagonism to be studied over as wide a concentration range as possible. Agonist response amplitude was consistent over the time-course of the experiments (data not shown).

The effects of PPADS and suramin on responses to exogenous agonists were examined in separate tissues. First, three reproducible, control responses to an agonist were obtained at 30 min intervals. The lowest concentration of PPADS or suramin used was then applied to the tissue for 30 min and the agonist readministered. This procedure was repeated until steady state inhibition was reached. Thereafter, progressively higher concentrations of antagonist were administered in the same manner until a clear maximum inhibition had been reached or until contractions were abolished.

To study the effect of desentitization of the $P2X_1$-receptor, control responses to ATP, 2-meSATP, UTP or noradrenaline were obtained. $\alpha,\beta$-meATP (30 $\mu$M) was then applied, evoking a large contraction. Once tension had returned to baseline a further 30 $\mu$M $\alpha,\beta$-meATP was added. This was repeated until $\alpha,\beta$-meATP did not evoke a response. The drug was washed out and when $\alpha,\beta$-meATP (30 $\mu$M) was reapplied 2 min later no contraction was seen, confirming that the $P2X_1$-receptors were desensitized. $\alpha,\beta$-meATP was again washed out and 2 min later the test agonist administered.

To study the role of extracellular calcium ions in agonist-induced contraction, control responses to the P2-receptor agonists or noradrenaline were obtained in the standard bathing solution. The tissues were then bathed in nominally calcium-free solution for 2 min before the test agonist was reapplied. Once the peak response was reached, the drugs were washed out and the tissues bathed in the calcium-containing solution.

Similarly, when the effects of ARL 67156 were studied, control agonist responses were obtained and increasing concentrations of ARL 67156 then applied to the tissue 10 min before subsequent reapplication of agonist (see Westfall *et al*., 1996).

### *Statistics*

Values in the text refer to mean$\pm$s.e.mean or geometric mean with 95% confidence limits for $IC_{50}$ values. Data were compared by Student's paired $t$ test or one way analysis of variance and Tukey's comparison as appropriate. Differences were considered significant when $P<0.05$. Concentration-inhibition response curves for the antagonists were fitted to the data by logistic (Hill equation), non-linear regression analysis (FigP, Biosoft, Cambridge, U.K.).

### *Drugs*

Acetylcholine chloride, ATP (disodium salt), $\alpha,\beta$-meATP (lithium salt), UTP (trisodium salt) (all Sigma, U.K.), 2-meSATP (Research Biochemicals Inc., U.S.A.), ARL 67156 (provided by Astra Charnwood) and suramin (Bayer, U.K.) were dissolved in distilled water and stored frozen as 100 mM stock solutions. PPADS (Tocris Cookson, U.K.) was dissolved in distilled water as a 10 mM stock solution and stored frozen in darkness. (−)-Noradrenaline bitartrate (Sigma, U.K.) was dissolved in acid saline (0.1% ascorbic acid, 0.9% NaCl) and frozen as a 100 mM solution.

## Results

### *Antagonism by suramin and PPADS*

$\alpha,\beta$-meATP (5 $\mu$M), 2-meSATP (100 $\mu$M) and ATP (1 mM) elicited contractions which peaked within 1 min and decayed in the continued presence of the agonist (Figures 1a,b, 2a,b, 3, 4 and 5). Contractions to UTP (1 mM) were often biphasic and always more maintained (Figures 1c, 2c, 3, 4 and 5).

Suramin (1 $\mu$M–1 mM, Figure 1) and PPADS (0.3–300 $\mu$M, Figure 2) inhibited the contractions to $\alpha,\beta$-meATP (5 $\mu$M), 2-meSATP (100 $\mu$M) and ATP (1 mM) in a concentration-dependent manner. PPADS was significantly more potent than suramin in each case ($P<0.05$, Table 1). The potency of PPADS against each agonist was not significantly different. However, suramin was 2–3 times more potent against $\alpha,\beta$-meATP and 2-meSATP than against ATP ($P<0.05$, Table 1). The slopes of the curves for both antagonists tended to be steep, with Hill slope values of around 2. However, the slope of the curve for suramin against ATP was notably steeper, with a Hill value of 3.9.

At the highest concentrations used, PPADS and suramin abolished contractions evoked by $\alpha,\beta$-meATP and 2-meSATP. By contrast, a component of the contraction elicited by ATP was clearly resistant to PPADS (30$\pm$5%) and suramin (36$\pm$6%). These values were not significantly different.

Responses to UTP (1 mM) were largely unaffected by the antagonists. Suramin (100 and 300 $\mu$M) caused a small, but significant inhibition of the contractions ($P<0.05$), but this was reversed at 1 mM suramin (Figure 1c,d). PPADS did not inhibit contractions to UTP at any concentration, but 300 $\mu$M PPADS produced significant potentiation ($P<0.05$, Figure 2c,d).

*Effects of α,β-meATP-induced desensitization*

Desensitization of the $P2X_1$-receptor by α,β-meATP abolished contractions evoked by 2-meSATP (100 μM) (control = 0.86 ± 0.36 g, $n = 6$, Figure 3a). In contrast, contractions evoked by ATP (1 mM) were greatly inhibited, but not abolished, with 15 ± 3% of the response remaining (control = 0.76 ± 0.29 g, test = 0.11 ± 0.04 g, $n = 5$, Figure 3b). This remaining response was not significantly different from the PPADS- and suramin-resistant responses to ATP.

Contractions in response to UTP (1 mM) were inhibited even less by desensitization of the $P2X_1$-receptor, with 68 ± 4% of the response remaining (control = 0.88 ± 0.28 g, test = 0.60 ± 0.20 g, $n = 6$, Figure 3c). Contractions to noradrenaline (3 μM) were unaffected by desensitization of the $P2X_1$-receptor (test = 97 ± 3% of control, $n = 6$, not shown).

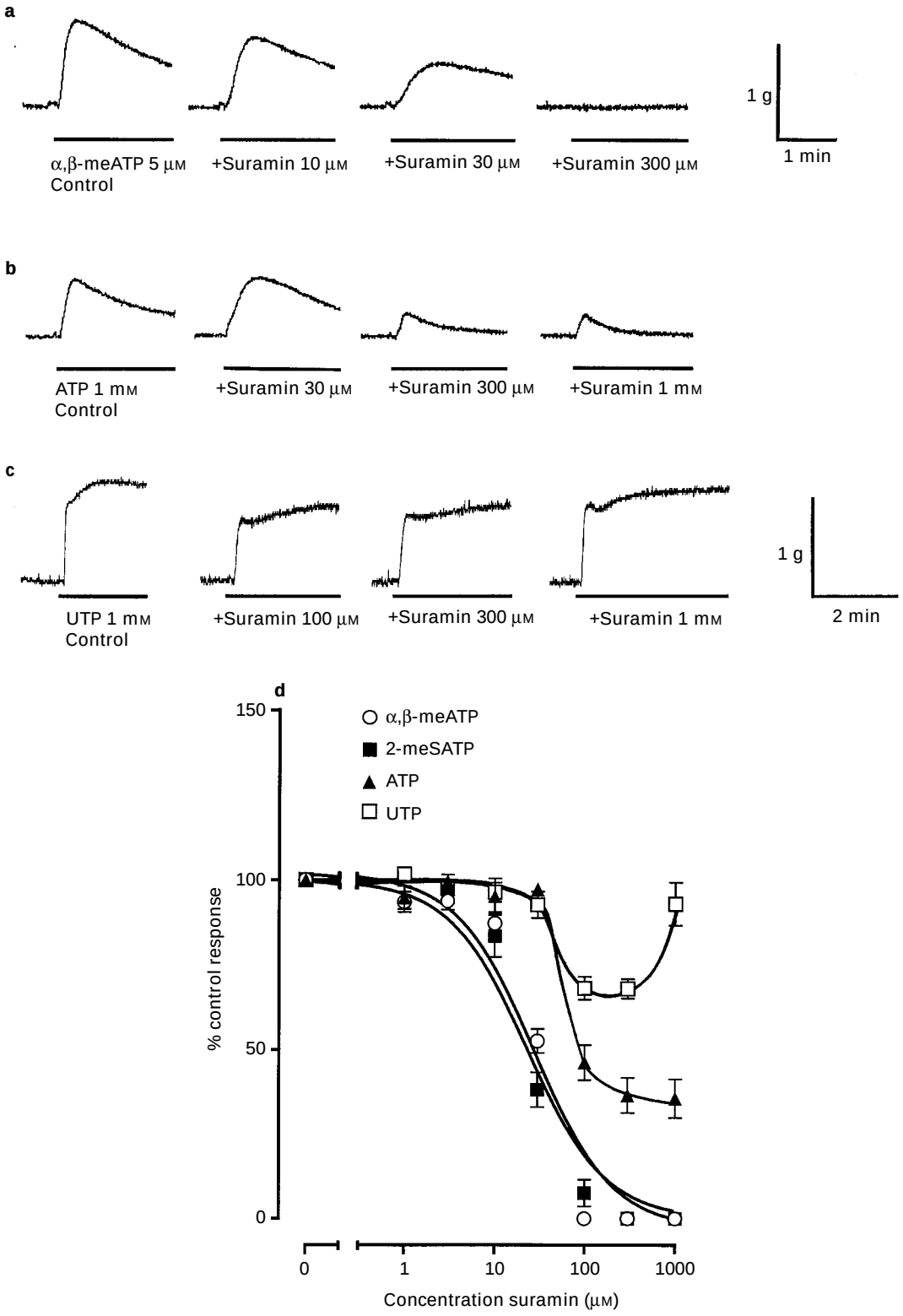


**Figure 1** The effect of suramin on contractions evoked by (a) α,β-meATP (5 μM), (b) ATP (1 mM) and (c) UTP (1 mM). Note the difference in time-scale for (c), compared with (a) and (b). (d) The graph shows mean data for the effect of suramin against these agonists and 2-meSATP (100 μM) ($n = 5–10$); vertical lines indicate s.e.mean.

*Effects of removal of external calcium ions*

Responses to α,β-meATP (5 μM) and 2-meSATP (100 μM) were abolished when tissues were bathed in nominally calcium-free solution ($n=4$, Figure 4a,b), while the peak contractions to ATP (1 mM) and UTP (1 mM) were reduced to $24\pm6\%$ ($P<0.01$, $n=4$, Figure 4c) and $61\pm13\%$ ($P<0.05$, $n=4$, Figure 4d), respectively, of their control response. For both agonists the remaining responses were not significantly different from those seen in the presence of PPADS or suramin, or following desensitization of the $P2X_1$-receptor. In the absence of external calcium ions contractions evoked by noradrenaline (3 and 30 μM) were also reduced to $37\pm4\%$ and $50\pm3\%$, respectively, of control values ($n=16$, not shown).

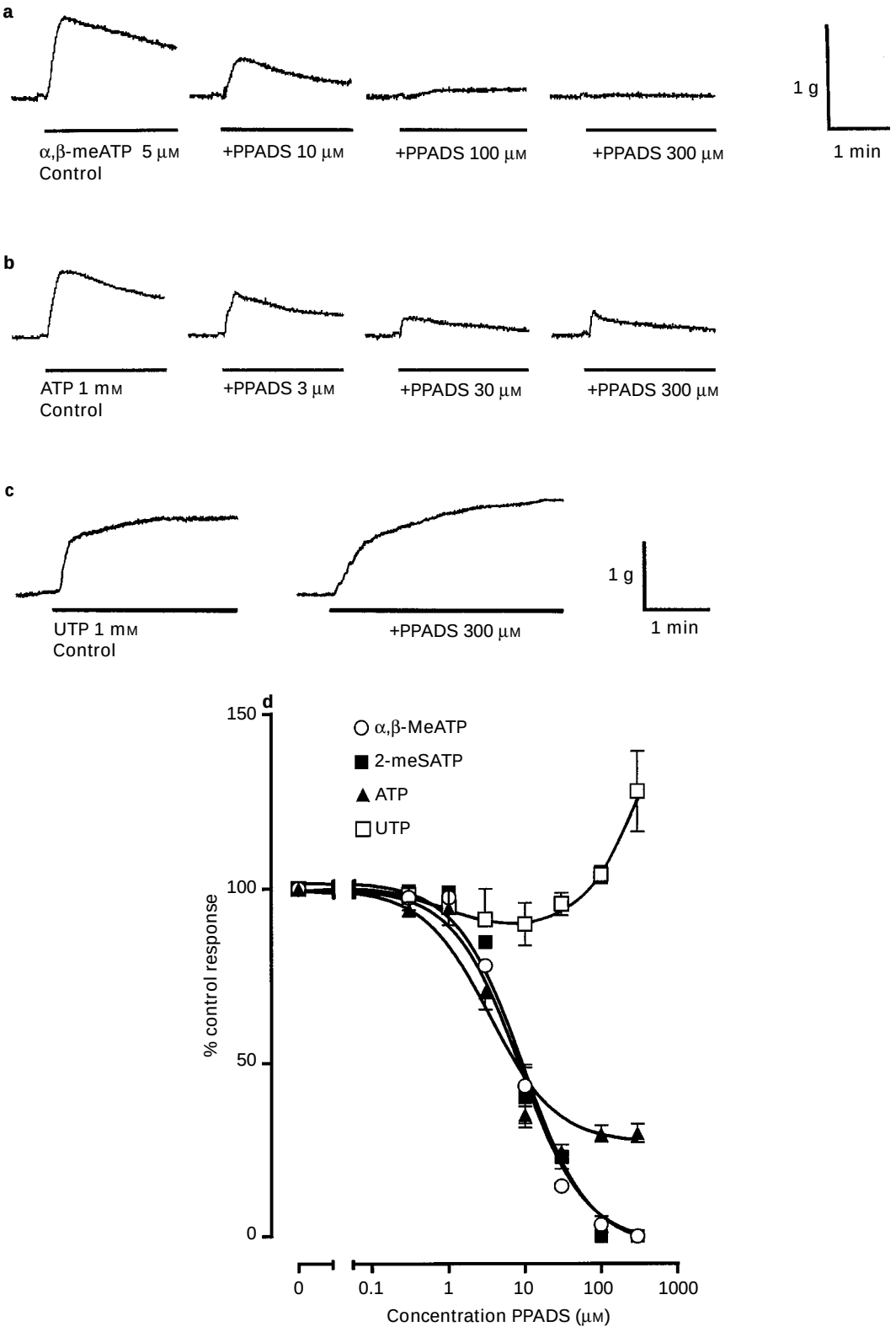


**Figure 2** The effect of PPADS on contractions evoked by (a) α,β-meATP (5 μM), (b) ATP (1 mM) and (c) UTP (1 mM). (d) The graph shows mean data for the effect of suramin against these agonists and 2-meSATP (100 μM) ($n=5-10$); vertical lines indicate s.e.mean.

*Effects of ARL 67156 on contractions*

ARL 67156 (3–100 $\mu$M) inhibited contractions evoked by $\alpha,\beta$-meATP (5 $\mu$M), 2-meSATP (100 $\mu$M) and ATP (1 mM) in a concentration-dependent manner (Figure 5). The inhibition was significant ($P<0.05$) with 30 and 100 $\mu$M ARL 67156 against $\alpha,\beta$-meATP and 2-meSATP and with 10–100 $\mu$M ARL 67156 against ATP. By contrast, responses to UTP (1 mM) were significantly potentiated by ARL 67156 (3–100 $\mu$M, Figure 5).

## Discussion

This study shows that at least two types of P2-receptor mediate vasoconstriction of the rat isolated tail artery. $P2X_1$-receptors, ligand-gated cation channels, appear to be the sole site of action of $\alpha,\beta$-meATP and 2-meSATP. They also mediate a substantial portion of the response to ATP, but only a small part of that to UTP. In contrast, one or more P2Y-receptors mediate the remainder of the contraction evoked by ATP and most of the response to UTP. Thus, the presence of multiple subtypes of P2-receptor must be taken into account when studying the actions of ATP and UTP.

In these experiments, contractions evoked by $\alpha,\beta$-meATP and 2-meSATP were abolished by desensitization of the $P2X_1$-receptor, suramin and PPADS. They were also dependent upon extracellular calcium ions. This is consistent with mediation by $P2X_1$-receptors. In previous studies in single smooth muscle cells from rat tail artery, $\alpha,\beta$-meATP and 2-meSATP activated suramin- and PPADS-sensitive, fast, transient, inward currents via $P2X_1$-receptors (Evans & Kennedy, 1994; McLaren *et al*., 1997). Also, the rise in intracellular calcium concentration seen in response to $P2X_1$-receptor activation is known to be dependent upon influx of extracellular calcium ions, (Kitajima *et al*., 1993; 1994; Pacaud *et al*., 1994; 1995; Loirand & Pacaud, 1995).

In the present study, ATP also acted via $P2X_1$-receptors to evoke vasoconstriction, as responses to ATP were greatly reduced by desensitization of the $P2X_1$-receptor, suramin and PPADS. ATP has previously been shown to activate $P2X_1$-receptors in dissociated smooth muscle cells of the rat tail artery (Evans & Kennedy, 1994; McLaren *et al*., 1997). However, ATP also activated a second receptor to evoke vasoconstriction, as about one third of the peak response

**Table 1** Potency of P2-receptor antagonists in the rat isolated tail artery

| | *α,β-meATP* (5 $\mu$M) | *2-meSATP* (100 $\mu$M) | *ATP* (1 mM) |
|---|---|---|---|
| PPADS | 8.1 (7.1–9.1) | 8.9 (5.8–12.0) | 3.5 (2.5–4.5) |
| Suramin | 29.9 (23.0–36.8) | 23.5 (21.1–25.9) | 64.1 (38.3–89.9) |

Values shown are $IC_{50}$ and 95% confidence limits ($\mu$M) for the antagonists; $n=5–10$ for each agonist.

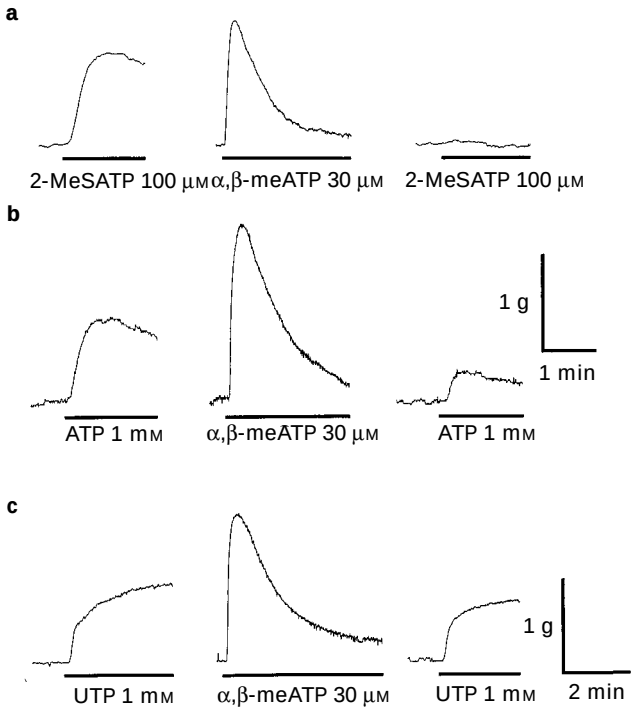


**Figure 3** The effect of desensitization of $P2X_1$-receptors by $\alpha,\beta$-meATP. Control responses were obtained to (a) 2-meSATP (100 $\mu$M), (b) ATP (1 mM) and (c) UTP (1 mM) (left-hand panel). $\alpha,\beta$-meATP (30 $\mu$M) was then applied (middle panel) and once tension had returned to baseline a further 30 $\mu$M $\alpha,\beta$-meATP was added (not shown). This was repeated until $\alpha,\beta$-meATP did not evoke a response; 2 min later the test agonist was readministered (right-hand panel). Note the difference in time-scale for UTP, compared with ATP and 2-meSATP.

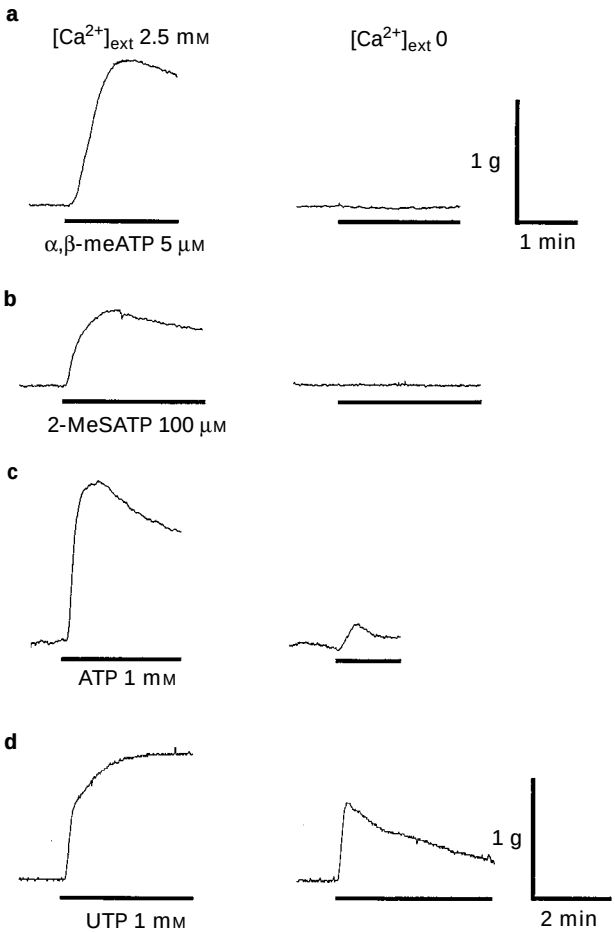


**Figure 4** The effect of removing extracellular $Ca^{2+}$ ions on contractions. Control responses were obtained to (a) $\alpha,\beta$-meATP (5 $\mu$M), (b) 2-meSATP (100 $\mu$M), (c) ATP (1 mM) and (d) UTP (1 mM) in normal extracellular bathing fluid (left-hand panel). Tissues were then bathed in nominally calcium-free solution for 2 min and then the test agonist was readministered (right-hand panel). Note the difference in time-scale for UTP.

was clearly resistant to the procedures which abolished responses to α,β-meATP and 2-meSATP acting via $P2X_1$-receptors. This response was seen in nominally calcium-free bathing solution, so it is likely that ATP acted at a P2Y-receptor to release calcium ions from intracellular stores. ATP has been shown to release calcium ions from intracellular stores in a number of arteris (see Abbracchio & Burnstock, 1994; Kennedy *et al*., 1997), but in most cases it was not clear if this then led to vasoconstriction.

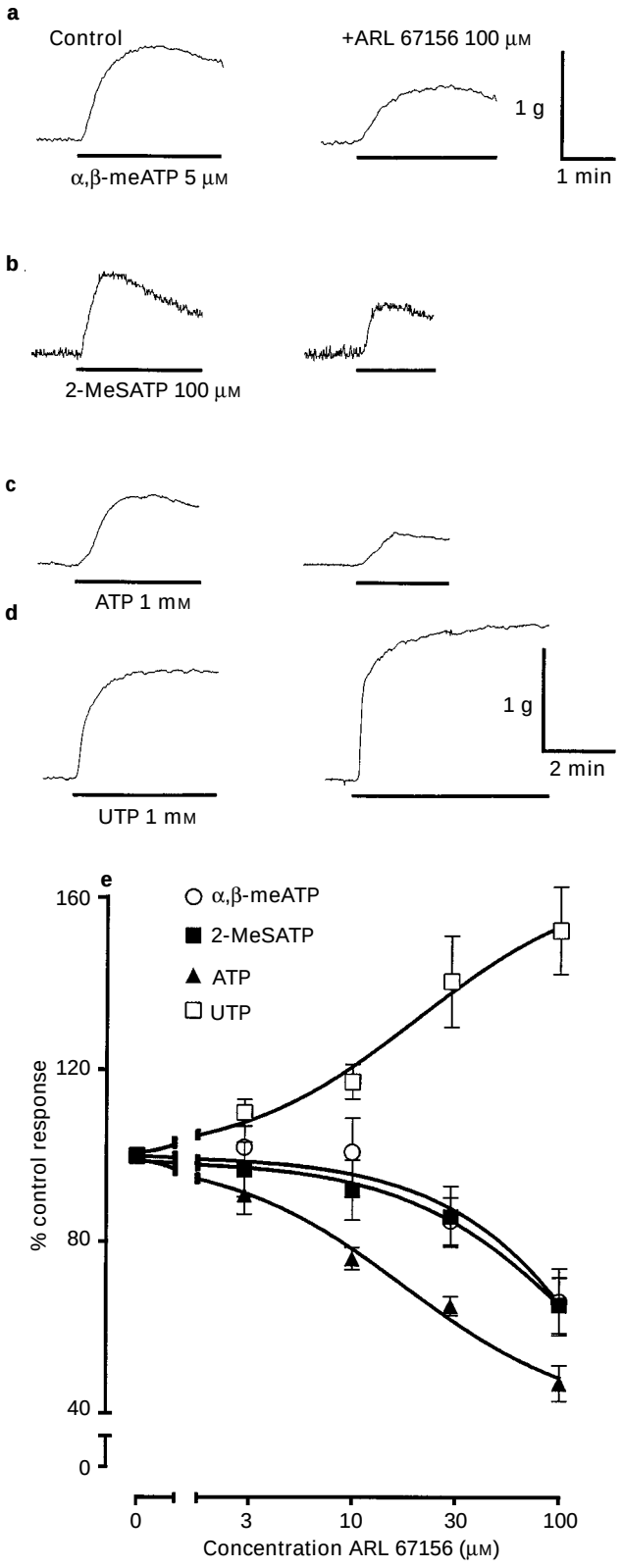


**Figure 5** The effect of ARL 67156 on contractions evoked by (a) α,β-meATP (5 μM), (b) 2-meSATP (100 μM), (c) ATP (1 mM) and (d) UTP (1 mM). Note the difference in time-scale for UTP. (e) The graph shows mean data ($n = 5-9$); vertical lines indicate s.e.mean.

Consistent with the presence of excitatory P2Y-receptors, contractions to UTP were only slightly reduced by $P2X_1$-receptor desensitization, suramin or removal of extracellular calcium ions and were not inhibited by PPADS. A similar profile has been seen in the rat mesenteric (Windscheif *et al*., 1994), pulmonary (Rubino & Burnstock, 1996) and renal (Eltze & Ullrich, 1996) vascular beds. Thus, $P2X_1$-receptors play only a small role in the response to UTP. We have previously shown that UTP has a low potency as an agonist at $P2X_1$-receptors in rat tail artery smooth muscle cells (McLaren *et al*., 1997). The present data indicate that G protein-coupled P2Y-receptors play a much greater role in the vasoconstriction evoked by UTP.

In the present study, responses to both ATP and UTP were still seen in the presence of high concentrations of suramin and PPADS, consistent with a common site of action. However, further studies are required to determine if this is so. A common excitatory receptor for UTP and ATP has been demonstrated in some (Eltze & Ullrich, 1996; Miyage *et al*., 1996), but not all (Von Kügelgen *et al*., 1987; Von Kügelgen & Starke, 1990; Ralevic & Burnstock, 1991; Windscheif *et al*., 1994; Rubino & Burnstock, 1996) arteries. UTP is an agonist at a number of the cloned P2Y-receptor subtypes (see Communi & Boeynaems, 1997 and references therein), of which the $P2Y_2$-receptor is the best characterized. ATP and UTP have a similar potency as agonists, and suramin and PPADS have weak or no antagonist action at this site (Charlton *et al*., 1996a,b; Nicholas *et al*., 1996). However, the other UTP-sensitive P2Y-receptor subtypes are not as well characterized and further studies are necessary, before their contribution to the responses to UTP and ATP can be determined.

These studies showed that the ecto-ATPase inhibitor ARL 67156 potentiated contractions evoked by UTP, consistent with breakdown of pyrimidine nucleotides by ecto-ATPase (see Plesner, 1995) and with inhibition of this breakdown by ARL 67156. The potentiating effect of PPADS on UTP may also be due to inhibition of ecto-ATPase (Khakh *et al*., 1995). In contrast, ARL 67156 inhibited contractions evoked by ATP, 2-meSATP and α,β-meATP. This was unexpected, as in other tissues ARL 67156, at the same concentrations used here, potentiates contractions to ATP and has no effect on those to the stable agonist α,β-meATP (Crack *et al*., 1995; Westfall *et al*., 1996; 1997). The simplest explanation is that in the rat tail artery ARL 67156 acts as an antagonist at $P2X_1$-receptors. ARL 67156 is also a $P2X_1$-antagonist in the rabbit ear artery, but at higher concentrations ($pA_2 = 3.3$). The reason for this difference is not known.

These experiments have shown that exogenous ATP acts at two types of P2-receptor to cause vasoconstriction of the rat isolated tail artery. However, it appears that only the $P2X_1$-receptor, a ligand-gated cation channel, is involved in the neurotransmitter actions of ATP in this tissue. The purinergic excitatory junction potentials and currents are abolished by desensitization of the $P2X_1$-receptor (Sneddon & Burnstock, 1984; Bao & Stjärne, 1993) and by suramin (Bao & Stjärne, 1993; McLaren *et al*., 1995), as is the purinergic component of neurogenic contractions (Bao & Stjårne, 1993). This implies that the G protein-coupled P2Y-receptor, which mediates the response to ATP, is not activated by neuronally-released

ATP. Instead, these receptors may mediate actions of ATP released from non-neuronal sources (see Burnstock & Kennedy, 1986).

In conclusion, ATP acts at two types of P2-receptor to cause vasoconstriction of the rat isolated tail artery. However, only the $P2X_1$-receptor is involved in the neurotransmitter actions of ATP. UTP also causes vasoconstriction at two P2-receptors, but mainly via a P2Y-receptor. This P2Y-receptor is likely to be a common site of action for ATP and UTP.

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