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Characterization of the P_2 receptors in rabbit pulmonary artery

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1 We have identified the P_2 receptors mediating vasomotor responses in the rabbit pulmonary artery.

2 Neither ATP nor UTP contracted intact or endothelium-denuded rings. However, both relaxed intact rings of rabbit pulmonary artery that had been preconstricted with phenylephrine (pD_2 5.2 and 5.6, respectively).

3 The vasodilator effect of UTP was endothelium-dependent and abolished by the nitric oxide synthase inhibitor N^G-nitro-L-arginine (L-NOARG).

4 The vasodilator effect of ATP was only partially inhibited by removal of endothelium or addition of L-NOARG, suggesting an additional direct effect on vascular smooth muscle.

5 The endothelium-dependent vasodilator responses to UTP and ATP were competitively antagonized by suramin.

6 Preconstricted, endothelium-denuded rings were also relaxed by 2-methylthio ATP (pD_2 6.6), a P_{2Y} receptor agonist.

7 Ca^{2+}-mobilizing P_{2U} receptors were identified on smooth muscle cells on the basis of single cell responses to ATP (pD_2 7.8) and UTP (pD_2 7.9; 6.7 in the presence of 100 μM suramin).

8 There was no evidence of a Ca^{2+}-mobilizing P_{2Y} receptor in these cultured cells.

9 The data suggest the presence of (i) a suramin-sensitive P_{2U} receptor on endothelial cells that induces vasorelaxation through NO release, (ii) a suramin-sensitive P_{2U} receptor on cultured smooth muscle cells that mobilizes Ca^{2+} but is not coupled to vasomotor responses and (iii) a putative P_{2Y} receptor on vascular smooth muscle cells that induces relaxation via a Ca^{2+}-independent signal transduction pathway.

Keywords: P_{2U} receptor; P_{2Y} receptor; pulmonary artery; nitric oxide; intracellular calcium

Introduction

Among the known regulators of vascular smooth muscle tone, extracellular adenosine 5′-triphosphate (ATP) and other nucleotides have been shown to mediate receptor-dependent relaxation via receptors on endothelial cells as well as receptor-dependent constriction via receptors on vascular smooth muscle cells. Various receptors for extracellular nucleotides have been described (Dalziel & Westfall, 1994; Fredholm *et al*., 1994; Harden *et al*., 1995). Receptors for extracellular ATP have been classified as P_2 receptors to distinguish them from adenosine-selective P_1 receptors. P_2 receptor subtypes can be distinguished on the basis of their signalling mechanism (Abbracchio & Burnstock, 1994). Members of the P_{2X} receptor family are ligand-gated, Ca^{2+}-permeable ion channels (Chen *et al*., 1995). They may be distinguished from G protein-coupled receptors, including the P_{2Y} and P_{2U} receptors, which in some cases activate phosphoinositide-specific phospholipase C (Barnard *et al*., 1994). Distinctions between different receptors have also been made on the basis of agonist selectivity, although this is proving less useful as the number of cloned P_2 receptors increases. In general, P_{2X} receptors respond selectively to α,β-methylene ATP (AMP-CPP), or in some cases 2-methylthio ATP (2-MeSATP) (Iles & Norenberg, 1993). However, recently, novel P_{2X} receptors have been described for which neither AMP-CPP nor 2-MeSATP are selective agonists (Bo *et al*., 1995). Furthermore, the P_{2Z} cytolytic receptor which responds selectively to 3′-O-(4-benzoyl)benzoylATP but not AMP-CPP or 2-MeSATP has been shown recently to belong to the P_{2X} receptor subgroup (Suprenant *et al*., 1996). P_{2Y} receptors respond selectively to 2-MeSATP or adenosine 5′-O-2-thiophosphate (ADPβS) whereas P_{2U} receptors respond to the pyrimidine nucleotide uridine 5′-triphosphate (UTP) as well as ATP (O'Connor *et al*., 1991; Erb *et al*., 1993). In addition to the P_2 receptors, members of a separate family of pyrimidine nucleotide receptors that are activated by UTP but not by purine nucleotides have been described (Seifert & Schultz, 1989) and cloned (Chang *et al*., 1995; Communi *et al*., 1995; Nguyen *et al*., 1995).

Although varying between species and vascular bed, several of these nucleotide receptor subtypes have been implicated in the control of vascular smooth muscle tone (Boeynaems & Pearson, 1990; O'Connor *et al*., 1991). On smooth muscle cells these include P_{2X} receptors (e.g. in rabbit ear artery, Benham & Tsien, 1987) and receptors selective for UTP (e.g. in rabbit basilar artery, von Kügelgen & Starke, 1990). On endothelial cells these include P_{2Y} receptors (e.g. in porcine aorta, Martin *et al*., 1985) and P_{2U} receptors. P_{2U} receptors co-exist with P_{2Y} receptors on bovine (O'Connor *et al*., 1991; Wilkinson *et al*., 1993; 1994) and rat (O'Connor *et al*., 1991) aortic endothelial cells. Activation of both P_{2Y} (Allsup & Boarder, 1990) and P_{2U} (Communi *et al*., 1995) receptors on bovine aortic endothelial cells cause prostacyclin release. Although P_{2Y} receptors have been implicated in NO release (O'Connor *et al*., 1991), there is some doubt as to whether P_{2U} receptors also mediate NO release.

P_2 receptors may play a significant role in the regulation of pulmonary vascular tone under conditions of high flow through release of NO (Hassessian *et al*., 1993; Hassessian & Burnstock, 1995). As part of our studies on pulmonary hypertension, we have now examined the role of P_2 receptors in the regulation of vascular smooth muscle tone in the rabbit pulmonary artery (RPA).

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Methods

Animals

Main pulmonary arteries were obtained from heparinized (500 u, i.v.) male and female NZW rabbits (<1.5 kg) after Nembutal anaesthesia (120 mg pentobarbitone Na, Boehringer Ingelheim, Sydney) according to a protocol approved by the Garvan/St Vincent's Hospital Animal Experimentation and Ethics Committee.

Organ bath experiments

Endothelium was removed from alternate rings (diameter 3-4 mm) by gentle rubbing of the luminal surface with a wooden swab stick. Adherent tissue was cleared and 2 mm wide rings were suspended at 37°C in 10 ml jacketed organ baths containing freshly prepared Krebs-bicarbonate solution (composition in mM NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.18, glucose 5, $NaHCO_3$ 25 and $CaCl_2.2H_2O$ 2.54) continuously gassed with 5% CO_2 and 95% O_2. A basal tension of 2.00 ± 0.05 g was chosen after preliminary studies of the contractile response to KCl. Isometric tension was measured by Grass FT03 force transducers (Quincy, MA U.S.A.) coupled to a MacLab data acquisition system (Analog Digital Instruments, Sydney, Australia). In cumulative concentration-response experiments, drugs were administered sequentially after the tension had stabilized ($\leqslant$2 min), whereas in bolus experiments a 30 min washout and re-equilibration period was used.

Tissue culture

Rabbit pulmonary artery smooth muscle cells (RPASMC) were obtained from the cleared vessels which were washed several times in Hanks Balanced Salt Solution (GIBCO, Life Technologies, Melbourne) and incubated in 1000 u ml^{-1} collagenase type II (Sigma Chemical Co, Sydney) for 30 min at 37°C to remove endothelium. Strips of muscle media were carefully peeled off with watchmakers forceps, diced and incubated in a solution containing 1000 u ml^{-1} collagenase type II and 60 u ml^{-1} porcine pancreatic elastase (Calbiochem-Novabiochem, Sydney) for 2–3 h at 37°C with periodic pipetting. Either the tissue explants or dispersed cells were seeded in Dulbecco's Modified Eagles Medium (DMEM) supplemented with penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}), fresh L-glutamine (4 mM, all from GIBCO) and 10% foetal bovine serum (PA Biologicals, Sydney). Cultured cells expressed smooth muscle actin and were negative for Factor VIII: RAg, an endothelial cell marker. The cells (2×10^5/well) at passage 2–3 were plated onto glass coverslips at least 3 days before an experiment and serum-deprived for 24 h in 4% Monomed (Commonwealth Serum Laboratories, Melbourne) in DMEM before the intracellular calcium assays.

Intracellular calcium assays

RPASMC on glass coverslips were incubated for 30 min with 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM; stock solution 1 mM in dimethylsulphoxide; Molecular Probes) in 4% Monomed and washed twice in perfusion solution (composition in mM: NaCl 145, KCl 5, HEPES 10, $MgCl_2$ 1.2, $CaCl_2$ 1 and 0.1% bovine serum albumin; pH 7.45). The loaded cells were mounted on the stage of a Nikon Diaphot microscope, modified for microfluorescence studies as previously described (Gibb *et al*., 1994), and superfused with perfusion solution at 37°C by a peristaltic pump (Ismatech, Extech Equipment, Melbourne). The ratio of fluorescence emission at 510 nm by Fura-2 at excitation wavelengths of 340 nm and 380 nm was used to measure $[Ca^{2+}]_i$ as previously described (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K\frac{(R - R_{min})}{(R_{max} - R)} \cdot \frac{(S_{f2})}{(S_{b2})}$$

Where: $K = 224$ nM (Fura-2 at 37°C); R_{min} = ratio value in Ca^{2+} free conditions; R_{max} = ratio value at a maximal Ca^{2+} concentration; S_{f2} = 380 nm reading in low Ca^{2+} conditions (corrected for background); S_{b2} = 380 nm reading in high Ca^{2+} conditions (corrected for background). R_{min} and S_{f2} were determined at the end of an experiment by perfusing the cells with Ca^{2+}-free perfusion solution containing 5 mM EGTA with ionomycin (2 μM, Carbiochem, Sydney). R_{max} and S_{b2} were then determined by the addition of 5 mM $CaCl_2$ in ionophore-containing perfusion solution.

Drugs

Stock solutions of ATP (disodium salt), UTP (monosodium salt), AMP-CPP (lithium salt), 2-MeSATP (tetrasodium salt, RBI, Natick, MA, U.S.A.) and suramin (also from RBI) were made up in Milli-Q water and stored at −20°C. Stock solutions of (−)-phenylephrine HCl, acetylcholine percholate and N^G-nitro-L-arginine (L-NOARG) were freshly prepared with Milli-Q water. For organ bath experiments they were diluted in Krebs-bicarbonate solution and for intracellular calcium experiments, in perfusion solution. All drugs were kept on ice and, unless otherwise stated, were purchased from Sigma.

Data analysis

Organ bath results are presented as means ± s.e. mean. Comparisons between concentration-response curves were performed by 2-way analysis of variance (ANOVA) and where differences were detected, unpaired *t* tests were used to compare individual data points. Graphs were obtained by fitting the results to a 4 parameter logistic function (SigmaPlot, Jandel Scientific, U.S.A.). Concentration-response data from the intracellular calcium experiments were plotted as the mean ± s.e. mean and fitted to the Hill equation to obtain the EC_{50} and Hill coefficient (n_H).

$$E = \frac{E_{max} \cdot [A]^{n_H}}{K + [A]^{n_H}}$$

Where: [A] = free agonist concentration; E = effect; E_{max} = maximum effect; K = equilibrium dissociation constant and n_H = Hill coefficient.

Note that: $$EC_{50} = (K)\frac{1}{n_H}$$

Results

Phenylephrine was used to preconstrict the RPA rings after initial experiments had confirmed comparable, sustained contraction in the presence or absence of endothelium and identified a concentration (1 μM) that produced approximately 80% maximal contraction. Before the addition of the selected P_2 receptor agonists, the presence of endothelium was tested functionally by use of the endothelium-dependent vasodilator, acetylcholine (10^{-9}–10^{-4} M). At low concentrations (10^{-9} – $10^{-6.5}$ M) this produced concentration-dependent relaxation of the RPA rings with endothelium but contraction at higher concentrations (10^{-6}–10^{-4} M), as seen in endothelium-denuded rings and previously described (data not shown) (Altiere *et al*., 1994; Corr & Burnstock, 1994).

The effect of P_2 agonists on rabbit isolated pulmonary artery with and without endothelium

None of the agonists tested (ATP, AMP-CPP, 2-MeSATP, UTP) induced contraction of the RPA rings over a wide range

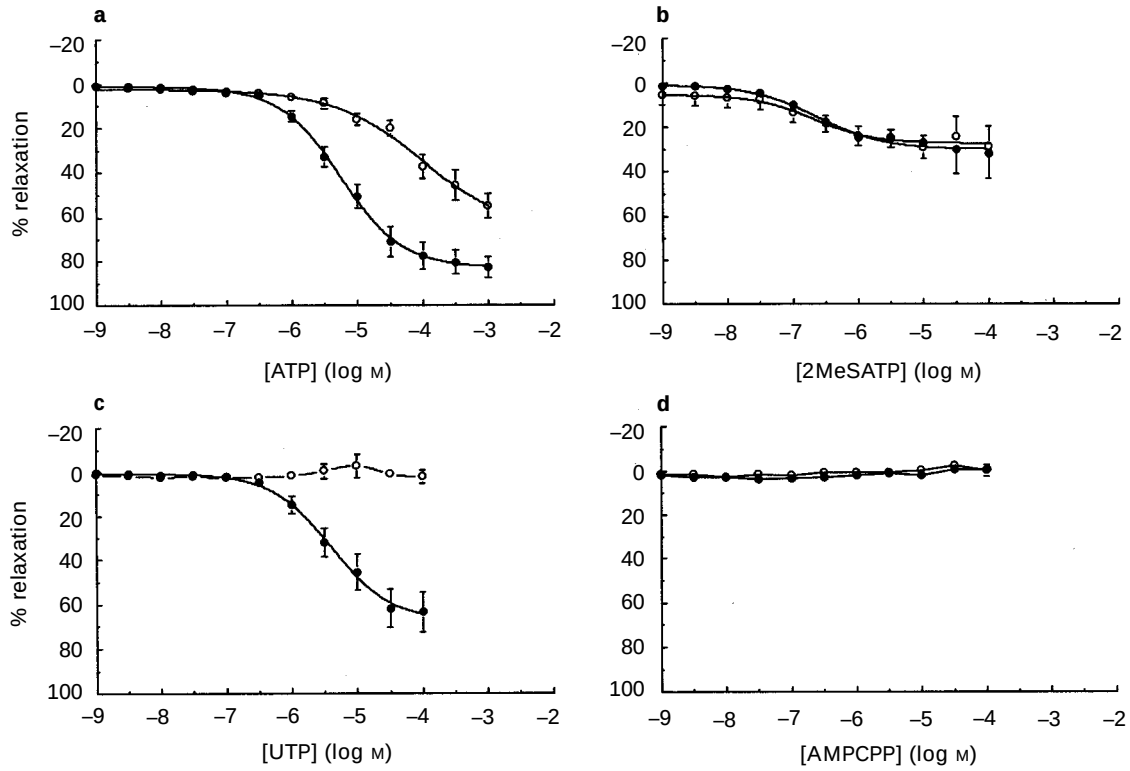

Figure 1 Responses of preconstricted rings from rabbit pulmonary artery to ATP (a), 2-methylthio ATP (2-MeSATP) (b), UTP (c) and α,β-methylene ATP (AMP-CPP) (d) in the presence (●) and absence (○) of endothelium ($n=5$). Vertical lines show s.e.mean.

of concentrations ($10^{-9}-10^{-5}$ M) (data not shown). There was no evidence of desensitization, as no significant contractile response was observed when 10^{-5} M AMP-CPP, 2-MeSATP, and UTP, or 10^{-3} M ATP were added as bolus doses.

The effect of P_2 agonists on preconstricted rabbit pulmonary artery with and without endothelium

ATP induced concentration-dependent relaxation of endothelium-containing and endothelium-denuded RPA rings preconstricted with phenylephrine (pD_2 5.2 and ~4.0, respectively, assuming a similar maximum response; Figure 1a). The response was significantly greater in the presence of endothelium. 2-MeSATP induced a small concentration-dependent relaxation which did not differ between rings with and without endothelium (pD_2 6.7 and 6.6, respectively) (Figure 1b). UTP induced endothelium-dependent relaxation (pD_2 5.6) (Figure 1c) which was comparable to ATP, but did not induce endothelium-independent relaxation. AMP-CPP had no effect, either in the presence or absence of endothelium (Figure 1d). These results suggest that the endothelium-dependent dilator response was mediated through a P_{2U} receptor. In addition, there was evidence for an endothelium-independent dilator response apparently mediated through a P_{2Y} receptor on smooth muscle cells.

The effect of L-NOARG on P_2-mediated vasodilatation in the rabbit pulmonary artery

The mechanism of the putative P_{2U} dilator response was investigated by use of a competitive antagonist of nitric oxide synthase, L-NOARG. Addition of L-NOARG (30 μM) to the preconstricted rings had no effect on tension alone. Before the addition of L-NOARG to the treated group of rings, there was no significant difference in the response to acetylcholine and UTP compared to the group left untreated. The endothelium-dependent dilator response to UTP (pD_2 5.6) was almost abolished by L-NOARG (Figure 2a) whereas L-NOARG shifted the ATP concentration-response curve (Figure 2b) to the right (pD_2 4.9). Nevertheless, there was still a significant dilator response to high concentrations of ATP, even in the presence of 30 μM L-NOARG.

The effect of suramin on P_2-mediated vasodilatation in the rabbit pulmonary artery

The receptor mediating the vasodilator responses to ATP and UTP in the presence of endothelium was characterized with suramin. Suramin (100 μM) induced a parallel shift to the right in the concentration-response curve for both nucleotides (Figure 2c and 2d). pD_2 values for UTP in the absence and presence of suramin were 6.1 and 4.6, respectively ($P<0.0001$); the corresponding pD_2 values for ATP were 5.6 and 4.3 ($P<0.0001$). Suramin (100 μM) had no effect on ATP-induced vasodilatation in endothelium-denuded RPA rings (data not shown). Its effect on 2-MeSATP-induced vasodilatation, either with or without endothelium, was equivocal due to the relatively small response to this agonist (data not shown).

The effect of P_2 agonists on Ca^{2+} responses in rabbit cultured pulmonary artery smooth muscle cells

The intracellular Ca^{2+} response of cultured RPASMC to P_2 agonists was investigated in dispersed cells, given the difficulties in interpreting relative potencies in tissue preparations with significant ectonucleotidase activity. There was no significant difference between the responses of explanted and dispersed cells under the conditions used (data not shown). Whereas AMP-CPP and 2-MeSATP consistently failed to induce a rise in $[Ca^{2+}]_i$ (Figure 3a), ATP and UTP produced concentration-dependent increases in $[Ca_{2+}]_i$ (Figure 4). Their pD_2 values were comparable (7.8 and 7.9, respectively) and in each case the Hill coefficients were close to unity (1.13 and 0.95, respectively). Again suramin (100 μM) induced a significant parallel shift to the right of the concentration-response curve for each agonist (to pD_2 6.7 in the case of UTP, data not shown).

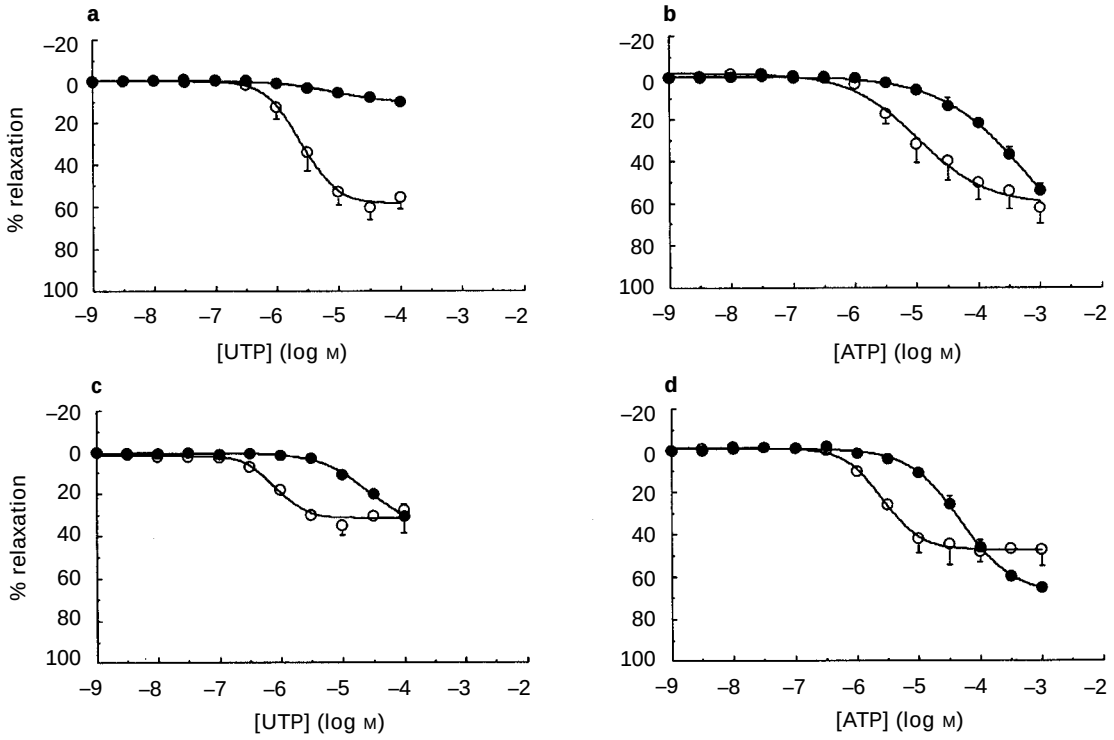

Figure 2 Relaxation of preconstricted rings from rabbit pulmomary artery by UTP and ATP in the presence (●) and absence (○)

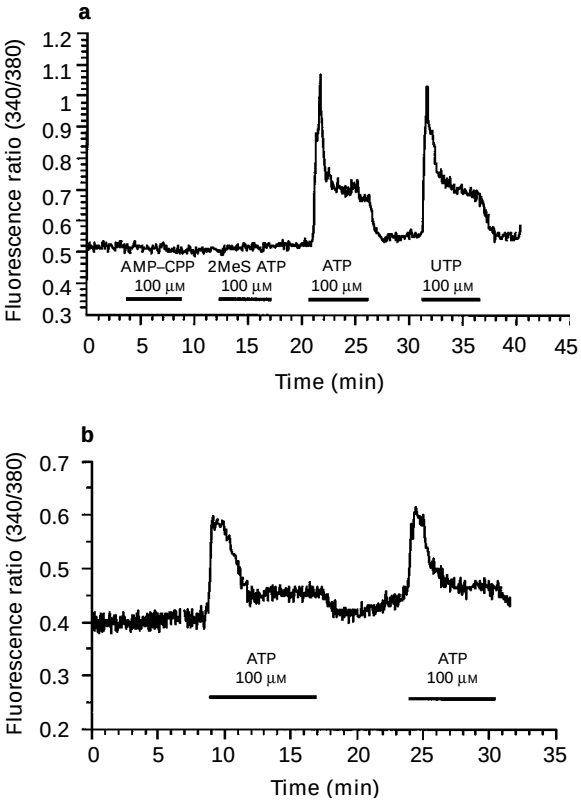

Figure 3 (a) Changes in $[Ca^{2+}]_i$ in cultured smooth muscle cells from rabbit pulmonary artery in response to 100 μM AMP-CPP, 2-MeSATP, ATP and UTP (representative of 4 experiments). (b) Failure of ATP to desensitize the Ca^{2+}-mobilizing response to ATP in smooth muscle cells (representative of 3 experiments).

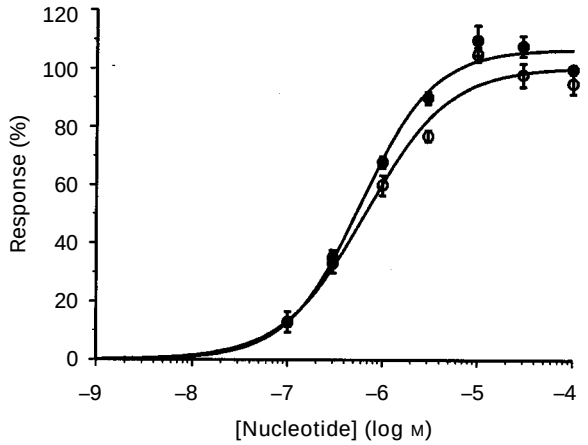

Figure 4 Concentration-response curves of $[Ca^{2+}]_i$ transients in cultured smooth muscle cells from rabbit pulmonary artery induced by ATP (○) and UTP (●) ($n=6$ experiments, 4 rabbits). Results are expressed as a percentage of the response to the maximum concentration of each nucleotide. Vertical lines show s.e.mean.

The responses demonstrated a typical 'peak and plateau' pattern (Figure 3). The plateau phase was abolished in Ca^{2+}-free perfusion solution, suggesting that it was generated by the influx of extracellular Ca^{2+} (data not shown). Since the peak response was minimally affected it was probably generated from intracellular stores. There was no evidence of homologous desensitization (Figure 3b), whereas concomitant experiments with endothelin-1 did demonstrate homologous desensitization of its receptor (data not shown).

Discussion

There is no evidence from these experiments that P_2 receptors mediate vasoconstriction in the RPA. In addition to cumulative concentration-response experiments in the presence or absence of endothelium, we used single boluses of relatively high agonist concentrations to abrogate the possible effect of receptor desensitization. In particular, concentrations of AMP-CPP previously shown to induce significant contraction in the rabbit coronary artery (10 μM) (Corr & Burnstock, 1994) failed to elicit a response attributable to a P_{2X} receptor. This contrasts with other well-characterized vascular beds in the rabbit where P_{2X}-mediated vasoconstriction has been described, including the isolated coronary (Corr & Burnstock, 1994), mesenteric (Mathieson & Burnstock, 1985) and ear arteries (Kennedy & Burnstock, 1985a) as well as the isolated, perfused hepatic arterial circulation (Ralevic *et al*., 1991). However, studies on the rabbit basilar artery also failed to provide evidence of P_{2X}-mediated vasoconstriction (von Kügelgen & Starke, 1990). Instead vasoconstriction in that vessel appeared to be mediated by at least two receptors for UTP, of which one was probably a P_{2U} receptor.

In the RPA both ATP and UTP induced vasodilatation through an endothelial P_{2U} receptor coupled to nitric oxide synthase. Inhibition of NO production has been shown previously to attenuate the vasodilator effects of ATP and UTP in the rat mesenteric arterial bed, although it was possible that UTP acted through additional vasodilator mechanisms (Ralevic & Burnstock, 1991). Again, in the rat pulmonary circulation, NO release by P_2 receptor agonists attenuates increases in perfusion pressure induced by high flow (Hassessian *et al*., 1993; Hassessian & Burnstock, 1995). However, this effect did not appear to be mediated by a P_{2U} receptor. Direct comparison of our results with these studies on resistance vessels in the rat is difficult.

The RPA P_{2U} receptor is probably linked to activation of nitric oxide synthase through increases in inositol trisphosphate (Pfeilschifter, 1990) and intracellular Ca^{2+} (Conigrave & Jiang, 1995). Rises in $[Ca^{2+}]_i$ have been demonstrated in bovine pulmonary artery endothelial cells (Lustig *et al*., 1992). It is evident that additional mechanisms are involved in the vasodilator response to ATP, for its effect was not completely abrogated by L-NOARG. These may include prostacyclin (PGI_2) production, as demonstrated in several systems (Needham *et al*., 1987; Demolle *et al*., 1988; Allsup & Boarder, 1990), or a direct action on SMC. However, in the rabbit perfused lung PGI_2 has been shown to increase pulmonary vascular resistance through stimulation of thromboxane A_2 synthesis (Kaapa *et al*., 1991).

ATP also induces vasodilatation directly through an action on SMC which is evident when the endothelium is removed. This response is comparable to that seen when endothelial nitric oxide synthase is inhibited. It is not mediated by a P_{2U} receptor, but may be mediated by a P_{2Y} receptor. However, the effect of ATP on endothelium-independent relaxation was not clearly antagonized by suramin and its maximal effect was greater than 2-MeSATP. The right shift of the ATP concentration-response curve relative to 2-MeSATP in the absence of endothelium may be accounted for by the higher affinity of 2-MeSATP for the P_{2Y} receptor. SMC P_{2Y} receptors have been demonstrated in the rabbit isolated portal vein (Kennedy & Burnstock, 1985b) as well as the coronary (Corr & Burnstock, 1994), mesenteric (Mathieson & Burnstock, 1985) and hepatic arteries (Brizzolara & Burnstock, 1991). In addition, a vasodilator contribution from a P_{2Y} receptor on SMC could not be excluded in the isolated perfused hepatic artery where there are also P_{2Y} receptors on the endothelium coupled to nitric oxide production (Ralevic *et al*., 1991).

The P_2 receptors were reclassified in the light of evidence that the rank orders of agonist potency, according to which they were previously characterized, have been biased by the differential sensitivity of the ligands to ectonucleotidases (Suprenant *et al*., 1996). The susceptibility of ATP and 2-MeSATP to ectonucleotidases resulted in an underestimation of their potency on the P_{2X} receptor relative to AMP-CPP which is relatively resistant. Examples of each of the main P_2 receptor types have been cloned, sequenced and expressed. Further reclassification is inevitable as subtype-specific antagonists are developed. In the meantime we have attempted to characterize the receptor mediating endothelium-dependent vasodilatation by use of suramin, a non-selective P_2 receptor antagonist which has been shown in different systems to inhibit P_{2X}, P_{2Y} (Dalziel & Westfall, 1994) and P_{2U} (Erb *et al*., 1993; Chang *et al*., 1995; Communi *et al*., 1995; Nguyen *et al*., 1995) responses. In our experiments on the RPA it appears to act as a P_{2U} antagonist.

We have investigated responses in cultured cells where biophase ectonucleotidases are much less likely to influence responses. Due to the difficulty in obtaining endothelial cell preparations of sufficient purity from RPA, these experiments have been restricted to SMC. P_{2X} and P_{2Y} receptor agonists failed to induce a rise in $[Ca^{2+}]_i$ in cultured RPASMC, but ATP and UTP were equipotent, presumably acting via a P_{2U} receptor which again was suramin-sensitive. Although contractile responses have been attributed to P_{2U} receptors previously, we did not detect such a response in our organ bath experiments. Nor was there evidence of endothelium-independent dilatation mediated through the P_{2U} receptor. It is, therefore, likely that the RPASMC P_{2U} receptor either becomes expressed during culture or is inherently uncoupled from vasomotor responses.

Phenotypic modulation *in vivo* and *in vitro* has been well described in rabbit aortic SMC (Campbell *et al*., 1988). The change from a 'contractile' to a 'synthetic' phenotype is accompanied not only by changes in the contractile and metabolic 'machinery' but also by changes in receptor expression. For instance, in rat aortic smooth muscle cells the endothelin B receptor is up-regulated at the expense of the A receptor (Eguchi *et al*., 1994) and P_{2X} responses are lost while P_{2Y} responses are acquired (Pacaud *et al*., 1995). It is possible that expression of the P_{2U} receptor may also be altered during phenotypic modulation *in vitro*, a process thought to parallel pathophysiological processes observed in systemic and pulmonary arteries *in vivo*. Progress in delineating the mechanisms will depend on further molecular characterization of the rabbit P_2 receptor subtypes.

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