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Co-existence of P_{2Y}- and PPADS-insensitive P_{2U}-purinoceptors in endothelial cells from adrenal medulla

[1]Jesús Mateo, Maria Teresa Miras-Portugal & Enrique Castro

Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, E-28040 Madrid, Spain

1 We have studied the effects of purinoceptor stimulation on Ca^{2+} signals in bovine adrenomedullary endothelial cells. $[Ca^{2+}]_i$ was determined with the fluorescent probe fura-2 both in population samples and in single, isolated, endothelial cells in primary culture and after subculturing.

2 In endothelial cells, maintained in culture for more than one passage, several purinoceptor agonists elicited clear $[Ca^{2+}]_i$ transient peaks that remained in the absence of extracellular Ca^{2+}. Adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) were equipotently active, with EC_{50} values of 8.5 ± 0.9 μM and 6.9 ± 1.5 μM, respectively, whereas 2-methylthioadenosine 5′-triphosphate (2MeSATP), adenosine 5′-(α,β-methylene)triphosphate (α,β-MeATP) and adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) were basically inactive. Adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) was a weak agonist. The apparent potency order was UTP = ATP > ADPβS > > 2MeSATP > α,β-MeATP.

3 Cross-desensitization experiments revealed that UTP or ATP, added sequentially at concentrations of maximal effect, could completely abolish the $[Ca^{2+}]_i$ response to the second agonist. ADPβS exerted only a partial desensitization of the response to maximal ATP, in accordance with its lower potency in raising $[Ca^{2+}]_i$.

4 The effect on $[Ca^{2+}]_i$ of 100 μM ATP in subcultured cells was reduced by only 25% with 100 μM suramin pretreatment and was negligibly affected by exposure to 10 μM pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS). The concentration-effect curve for ATP was not significantly affected by PPADS, but was displaced to the right by a factor of 6.5 by 100 μM suramin.

5 In primary cultures, clear $[Ca^{2+}]_i$ responses were elicited by 2MeSATP. Suramin totally and selectively blocked 2MeSATP responses, whereas UTP-evoked $[Ca^{2+}]_i$ transients were mainly unaffected by suramin or PPADS. Over 80% of cells tested showed responses to both 2MeSATP and UTP. The $[Ca^{2+}]_i$ response to UTP was not desensitized in the presence of 2MeSATP.

6 ATP and UTP stimulated the release of preloaded [^{3}H]-arachidonic acid ([^{3}H]-AA), both in the presence and in the absence of extracellular Ca^{2+}, by approximately 135% with respect to basal levels. Suramin and PPADS enhanced, rather than inhibited, the [^{3}H]-AA releasing effect of ATP by 2.5 times. Suramin also potentiated the effect of the calcium ionophore A23187.

7 These results indicate that endothelial cells from adrenomedullary capillaries co-express both P_{2Y}- and P_{2U}-purinoceptors. P_{2Y}-purinoceptors are lost in culture with the first passage of the cells. The P_{2U}-purinoceptor subtype present in these cells is insensitive to PPADS and thus similar to that found in aortic endothelial cells.

Keywords: Adrenomedullary endothelial cells; cytosolic calcium; Fura-2; purinoceptors; pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS); suramin; arachidonic acid release

Introduction

The adrenal medulla tissue is formed by a close packing of catecholamine-secreting chromaffin cells grouped around a dense network of capillary vessels (Coupland & Selby, 1976). This high vascularization provides a rapid output pathway into the main bloodstream for the hormones secreted by the gland. The endothelial cells that form the walls of adrenomedullary capillaries are thought to play an important role in the transport of secreted catecholamines into the blood (Banerjee *et al.*, 1985). Furthermore, endothelial cells participate in the regulation of local blood flow (Boeynaems & Pearson, 1990), which is known to increase in the adrenal gland upon stimulation of chromaffin cells and thus facilitates the exporting of secreted products (Jordan *et al*, 1989). In particular, it is now well established that, under stimulation with vasoactive compounds such as bradykinin, adenosine 5′-triphosphate (ATP) or histamine there is a cytosolic free Ca^{2+} increase in endothelial cells which results in the generation and release of various paracrine factors that relax as well as contract vascular smooth muscle cells (Newby & Henderson, 1990; Graier *et al.*, 1994). The relaxing factors include endothelium-derived relaxing factor (EDRF), which has been demonstrated to be nitric oxide (NO) or a nitric oxide-containing compound (Kelm *et al.*, 1988; Rosenblum, 1992) and prostacyclin (PGI_2) (Gosink & Forsberg, 1993). Endothelial cells can also release vasoconstrictor peptides, the endothelins, which increase smooth muscle Ca^{2+} concentration, resulting in a potent vasoconstriction (Yanagisawa *et al.*, 1988). In addition to these vasomotor actions, released factors can interact with chromaffin cells and modulate the secretion of catecholamines (Takeuchi *et al.*, 1992; Torres *et al.*, 1994).

In addition to catecholamine hormones, chromaffin cells store and secrete high amounts of ATP (Rojas *et al.*, 1985) and other purine-containing compounds (Rodriguez del Castillo *et al.*, 1988; Pintor *et al.*, 1991). Endothelial cell activity is potently enhanced by extracellular ATP (Hallam & Pearson, 1986; Pirotton *et al.*, 1987). Thus, the ATP secreted from chromaffin cells may be essential for efficient catecholamine transfer to the bloodstream. ATP exerts its extracellular actions through a variety of purinoceptors. These had been classified into P_{2X}/P_{2Y} subtypes based on differences in potency of the key agonists 2-methylthioadenosine 5′-triphosphate

[1]Author for correspondence at: Dept. Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Univ. Complutense de Madrid, E-28040 Madrid, Spain.

(2MeSATP) and adenosine 5'-(α,β-methylene)triphosphate (α,β-MeATP). P_{2X}-purinoceptors are defined by an agonist potency order of α,β-MeATP > ATP > 2MeSATP, while P_{2Y}-purinoceptors are characterized by an order 2MeSATP > ATP > α,β-MeATP (Burnstock & Kennedy, 1985). In addition to these classes, the P_{2U}-purinoceptor type was introduced to account for the ATP receptors also activated by uridine 5'-triphosphate (UTP) but not by 2MeSATP, with an apparent potency order UTP = ATP > > 2MeSATP > α,β-MeATP (O'Connor, 1992). In the light of molecular biology data, P_{2X}-purinoceptors are now indentified as ATP-gated channels (Suprenant *et al.*, 1995), and P_{2Y}-purinoceptors are members of the seven transmembrane segments, G-protein coupled, receptor family, together with P_{2U}-purinoceptors (Barnard *et al.*, 1994; Boarder *et al.*, 1995). This family has been functionally reclassified as P_{2Y1} to P_{2Y7} by Burnstock, with P_{2Y2} being the old P_{2U}, although the utility of this classification has been discussed (Abbracchio & Burnstock, 1994; Boarder *et al.*, 1994). In the absence of a better scheme, the several P_{2Y} family receptor clones are being numbered sequentially in chronological order from P_{2Y1} to P_{2Y6}. For the sake of clarity, in this paper we refer to nucleotide actions as P_{2Y}- or P_{2U}-mediated according to the effects of 2MeSATP and UTP respectively, while reserving P_{2Yn} nomenclature for the receptor molecules of known cloned sequences.

The identity of purinoceptor subtypes present in endothelial cells depends on the origin of the tissue. In endothelial cells from microvessels of rat brain (Frelin *et al.*, 1993) and rabbit myocardium (Mannix *et al.*, 1993) only P_{2U}-purinoceptors are present. On the other hand, the endothelial cell line AG4762 displays P_{2Y}-purinoceptors exclusively (Allsup & Boarder, 1990). Both purinoceptor subtypes have been shown to coexist in bovine aortic endothelial cells (Motte *et al.*, 1993; Wilkinson *et al.*, 1993). With respect to bovine adrenomedullary endothelial cells (BAMEC), ATP has been shown to stimulate the accumulation of inositol phosphates and the release of NO and prostacyclin (Forsberg *et al.*, 1987; Gosink & Forsberg, 1993). The ATP receptor present in these cells has been identified as an 'atypical' P_{2Y}-purinoceptor (Allsup & Boarder, 1990), or a P_{2U}-purinoceptor (Purkiss *et al.*, 1993). Our previous studies in these cells (Castro *et al.*, 1994) revealed responses to 2MeSATP that could be interpreted as mediated by a mixed P_{2Y}/P_{2U} population or, alternatively, indicating that 2MeSATP was a partial agonist on P_{2U} receptors. Thus, we have carried on with these studies to clarify the purinoceptors involved in the actions of ATP in endothelial cells from adrenal medulla. These results have been partially presented in abstract form (Castro *et al.*, 1995b).

Materials and methods

Isolation and culture of endothelial cells

Suspensions of bovine adrenomedullary cells were obtained by collagenase digestion of adrenal medulla tissue as described by Miras-Portugal *et al.* (1985). Endothelial cells were separated from chromaffin cells by differential plating as described by Banerjee *et al.* (1985) with minor modifications. Briefly, dissociated cells were seeded in 75-cm^2 culture flasks (Falcon) at a density of 30×10^6 cells per 30 ml of culture medium (Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 100 μg ml^{-1} kanamycin, and 2.5 μg ml^{-1} amphotericin), at 37°C in 5% CO_2 and 95% air. After a settling period of 3 h, the unattached cells and medium were removed. The remaining adherent cells were washed twice and maintained in culture with 15 ml of the same medium described above. When the cells reached confluence (approx. one week), they were subcultured by trypsinization with 0.05% trypsin and 0.002% EDTA in phosphate-buffered saline without added Ca^{2+} and Mg^{2+}. Washed cells were seeded into 75-cm^2 flasks ($1-1.5 \times 10^6$ cells per 15 ml of culture medium) or into 24-well Costar Petri dishes (approx. 5×10^4 cells per well) and incubated in 5% CO_2 and 95% air at 37°C. The cultures were used immediately after reaching confluence. Cultures between 2th–6th passages were usually employed. For microfluorometry experiments the cells were plated on round (15 mm diameter) glass coverslips placed in 35 mm Petri dishes.

Measurements of cytosolic [Ca^{2+}] in endothelial cell populations

Cytosolic Ca^{2+} concentration was determined with the fluorescent indicator fura-2 (fluo-3 for the experiments in which suramin was present). Endothelial cells were collected from confluent cultures in 75-cm^2 flasks by trypsinization as described above. Cells were washed and resuspended in Locke's solution (composition in mM: NaCl 140, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, D-glucose 5.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10.0, pH 7.4 with NaOH). Cells were loaded by incubation with 5 μM fura-2/AM for 45 min at 37°C in Locke's solution containing 1 mg ml^{-1} bovine serum albumin (BSA). After the loading period, cells were washed twice with fresh Locke's and resuspended at a density of 10^6 cells ml^{-1}. The recordings were made in 1.5-ml samples containing a cell suspension of approx. 10^6 cells ml^{-1} in thermostated and stirred cuvettes, in a Perkin-Elmer LS-50B fluorometer. Fluorescence intensity was determined with an excitation wavelength of 340 nm and an emission wavelength of 510 nm for fura-2 (490 nm and 525 nm respectively for fluo-3). Fluorescence traces were calibrated individually by releasing intracellular dye content with Triton X-100 (0.3%) and determining dye fluorescence in the presence of a mixture of EGTA/Tris ([Ca^{2+}] < 0.2 nM) and 2.5 mM Ca^{2+} to calculate F_{min} and F_{max}, respectively. [Ca^{2+}]$_i$ was derived from fluorescence traces following the equation of Grynkiewicz *et al.* (1985). The dissociation constant was assumed to be 224 nM for fura-2 and 400 nM for fluo-3 (Kao *et al.*, 1989). Additions to the cuvette were made with Hamilton syringes from at least 100 fold concentrated stock solutions to avoid large volume variations.

Microfluorometry

The [Ca^{2+}]$_I$ was recorded from single adrenal endothelial cells essentially as described previously by Castro *et al.* (1994) by use of a multiple excitation microfluorescence system (Cairn Research LTD, Kent, U.K.). Cells attached to coverslips were loaded by incubation with 5 μM fura-2/AM for 45 min at 37°C in Locke's solution containing 1 mg ml^{-1} BSA as in the previous case. Loading was stopped by removing the coverslip from the fura-2 solution and storing it in Locke's solution supplemented with 1 mg ml^{-1} of BSA until use. The coverslip was glued to a perspex piece, forming the bottom of a small (34 μl) perifusion chamber on the stage of a Nikon Diaphot microscope equipped with epifluorescence optics. The cells were illuminated alternately either at 340 and 380 nm and the emitted fluorescence was driven to the photomultiplier after passing through a 510 nm band-pass interference filter. The measuring field was routinely centered on the cell of interest by means of a rectangular diaphragm placed on the emission path blocking all incoming light except that from the selected cell. The ratio F_{340}/F_{380} trace is presented directly, reflecting changes in [Ca^{2+}]$_i$. Cells were perifused continuously with Locke's medium at ≈1–2 ml min^{-1}. Drugs were applied by rapidly changing the perifusion medium.

Measurements of [^{3}H]-arachidonic acid release

Cells grown in 24-well plates were labelled with [^{3}H]-arachidonic acid (0.5 μCi per well per 1 ml of medium) in DMEM to isotopic equilibrium (18–24 h) at 37°C. To eliminate unincorporated radioactivity, cells were washed twice for 30 min with 1 ml Locke's solution plus BSA (0.2%). [^{3}H]-arachidonic acid release was stimulated by incubation of the cells for 5 min with the tested agonist. The incubation medium (200 μl) was

collected and transferred to a scintillation vial and cells incubated for a further 25 min period in 300 μl of fresh medium (without agonist). This medium was collected and added to the scintillation sample. This procedure was followed to avoid interference by accumulation of hydrolysis products from nucleotide agonists.

Materials

Fura-2/AM and fluo-3/AM were from Molecular Probes (Eugene, OR, U.S.A.). UTP, adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), bradykinin and collagenase were from Boerhinger (Mannheim, Germany). 2MeSATP, adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and suramin were obtained from Research Biochemicals Inc. (Natick, Mass., U.S.A.). ATP, ADP, α,β-MeATP, adenosine 5′-tetraphosphate (Ap_4), trypsin, and calcium-ionophore A23187 were supplied by Sigma (St. Louis, MO, U.S.A.). [5,6,8,9,11,12,14,15-^{3}H(n)]-arachidonic acid ([^{3}H]-AA, 200 Ci mmol^{-1}) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum were from GIBCO BRL (U.K.). Antibiotics were supplied by Flow Laboratories Ltd. (Irvine, CA, U.S.A.). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was a gift from Charles H.V. Hoyle (University College London, London, U.K.). All other reagents were from Merck (Darmstadt, Germany).

Statistical analysis

Data are presented as mean ± s.e.mean of at least four determinations in different cell cultures. Significant differences were determined by Student's *t* test or one-way ANOVA, as required. When appropriate, single experiment traces are pre-

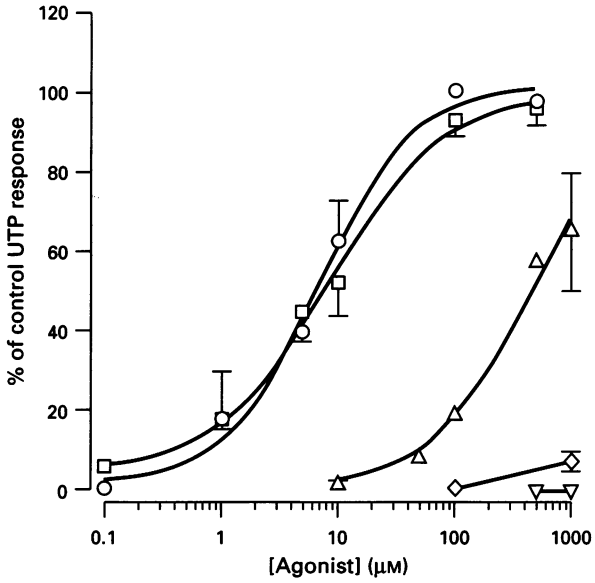

Figure 2 Concentration-effect curves for the increase of $[Ca^{2+}]_i$ elicited by purinoceptor agonists. Endothelial cells loaded with fura-2 were stimulated with different purinoceptor agonists: ATP (□), UTP (○), ADPβS (△), 2MeSATP (◇) and α,β-MeATP (▽). Data represent average maximal $[Ca^{2+}]_i$ changes (difference between peak and resting $[Ca^{2+}]_i$ levels) evoked by increasing concentrations of agonists, normalized within each experiment to the $[Ca^{2+}]_i$ response to 100 μM UTP in the same experimental batch. Vertical lines represent the s.e.mean of three to six experiments.

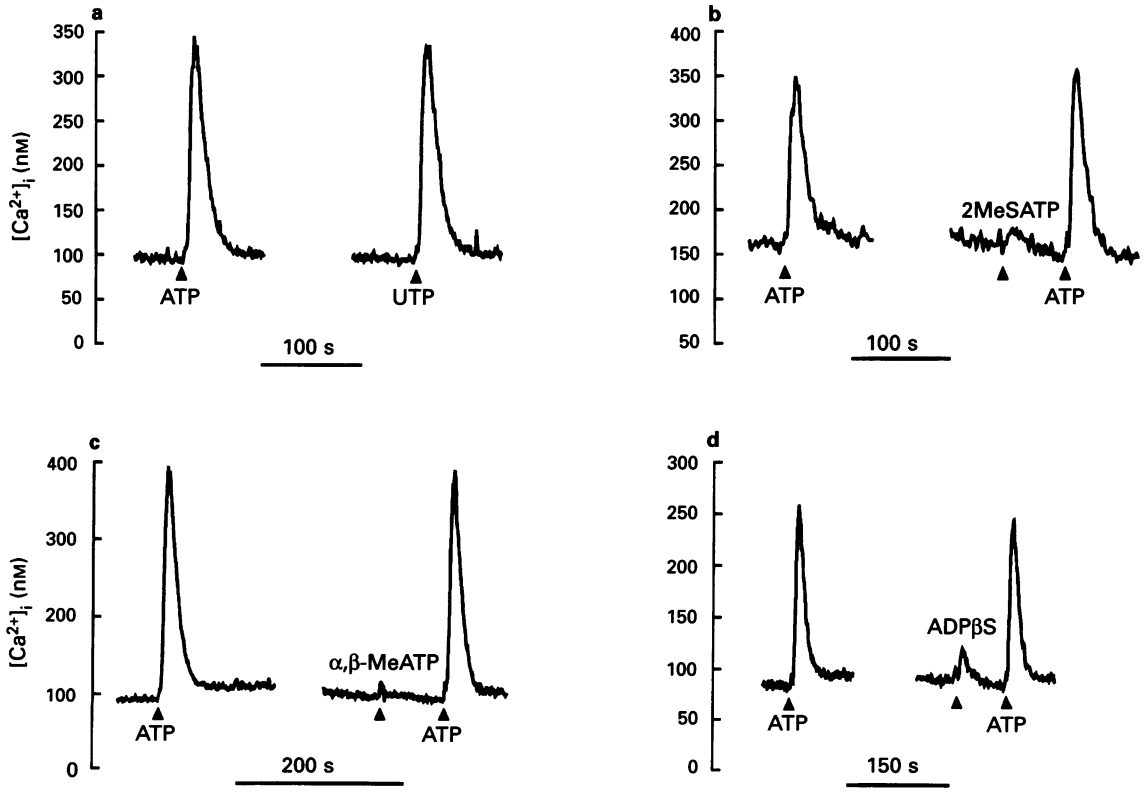

Figure 1 $[Ca^{2+}]_i$ responses after purinoceptor stimulation in endothelial cell populations. Each trace corresponds to a single sample of approximately 10^6 cells loaded with fura-2. Drugs were added at the point indicated by the arrowhead and remained in contact with the cells for the rest of the experiment. (a) ATP and UTP (both tested at 100 μM) were equipotent agonists. (b) 2MeSATP (1 mM) was almost totally ineffective. The presence of healthy ATP receptors was confirmed in the same sample. (c) Similar experiment performed with α,β-MeATP (1 mM). (d) ADPβS (100 μM) was a weak agonist. Each panel corresponds to different cultures to show the variability in the response to ATP.

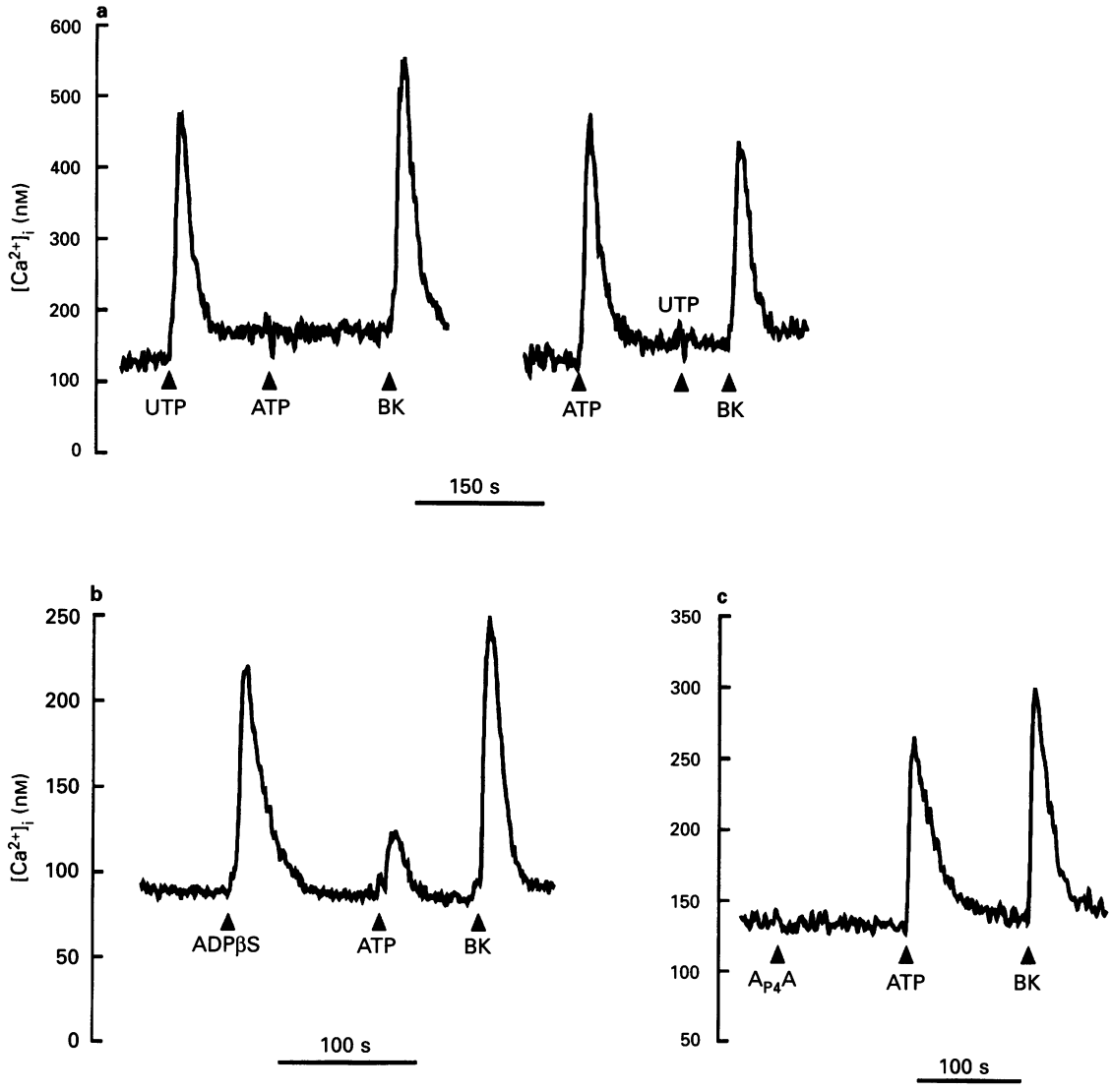

Figure 3 Cross-desensitization between purinoceptor agonists. Each trace represents a single experiment carried out in the same conditions as above. Drugs were added at the arrowheads and remained present for the rest of the experiment. (a) UTP (100 μM) completely blocked the response to a second challenge with ATP (100 μM), added after $[Ca^{2+}]_i$ had returned to near basal levels in the continuous presence of UTP and *vice versa*. (*b*) ADPβS (1 mM) did not abolish completely the response to ATP (100 μM). (c) Ap_4A (100 μM) was ineffective in eliciting a rise in $[Ca^{2+}]_i$ and did not affect the response to ATP (100 μM). In each case, the response to 1 μM bradykinin (BK) was preserved in spite of the presence of both purine compounds.

sented in the figures. They are representative of at least six other experiments with equivalent results. EC_{50} values were derived from nonlinear fittings to a logistic curve of the concentration-effect data.

Results

Effects of purinoceptor agonists on intracellular $[Ca^{2+}]$ in endothelial cell populations

To characterize the ATP receptor type present in these cells we measured $[Ca^{2+}]_i$ transients in response to ATP and various analogues. As can be seen in Figure 1, UTP evoked $[Ca^{2+}]_i$ responses of the same magnitude as ATP. In contrast, the potent P_{2Y} agonist 2MeSATP showed a very weak stimulant effect on $[Ca^{2+}]_i$, even at 1 mM, and α,β-MeATP, a P_{2X}-selective agent, was completely without effect on these cells. The ADP analogue ADPβS was able to elevate $[Ca^{2+}]_i$ but it was approximately 25 times less effective than ATP at the same concentration. The effect of purinoceptor agonists was dependent on the mobilization of intracellular stores of Ca^{2+} since clear $[Ca^{2+}]_i$ transients were recorded upon stimulation of cells with ATP in medium containing EGTA to buffer $[Ca^{2+}]_o$ at 100 nM (data not shown). The concentration-effect curves for these agonists are depicted in Figure 2. ATP and UTP were equipotent, with calculated EC_{50} values of 8.5 ± 0.9 μM and 6.9 ± 1.5 μM, respectively. ADPβS was considerably less potent, with an EC_{50} of 455 ± 45 μM. The Hill indexes close to 1 (0.87 ± 0.23, 1.08 ± 0.33 and 0.99 ± 0.1 for ATP, UTP and ADPβS, respectively) indicated that endothelial cells in these conditions expressed only one subtype of purinoceptor. To test if these agonists were acting at the same receptor, we carried out cross-desensitization experiments. A sample of cells was stimulated with a first agonist at a high concentration and after the $[Ca^{2+}]_i$ level returned to near basal levels, it was challenged again with a second agonist, in the continuous presence of the first (not washed out). ATP and UTP completely prevented the action of each other in a second challenge (Figure 3), but did not affect the $[Ca^{2+}]_i$ transients evoked by the non-related agent bradykinin. The weaker agonist ADPβS was also able to desensitize the response to a later stimulation with ATP, but again with less potency than produced by UTP. Neither α,β-MeATP nor 2MeSATP, tested

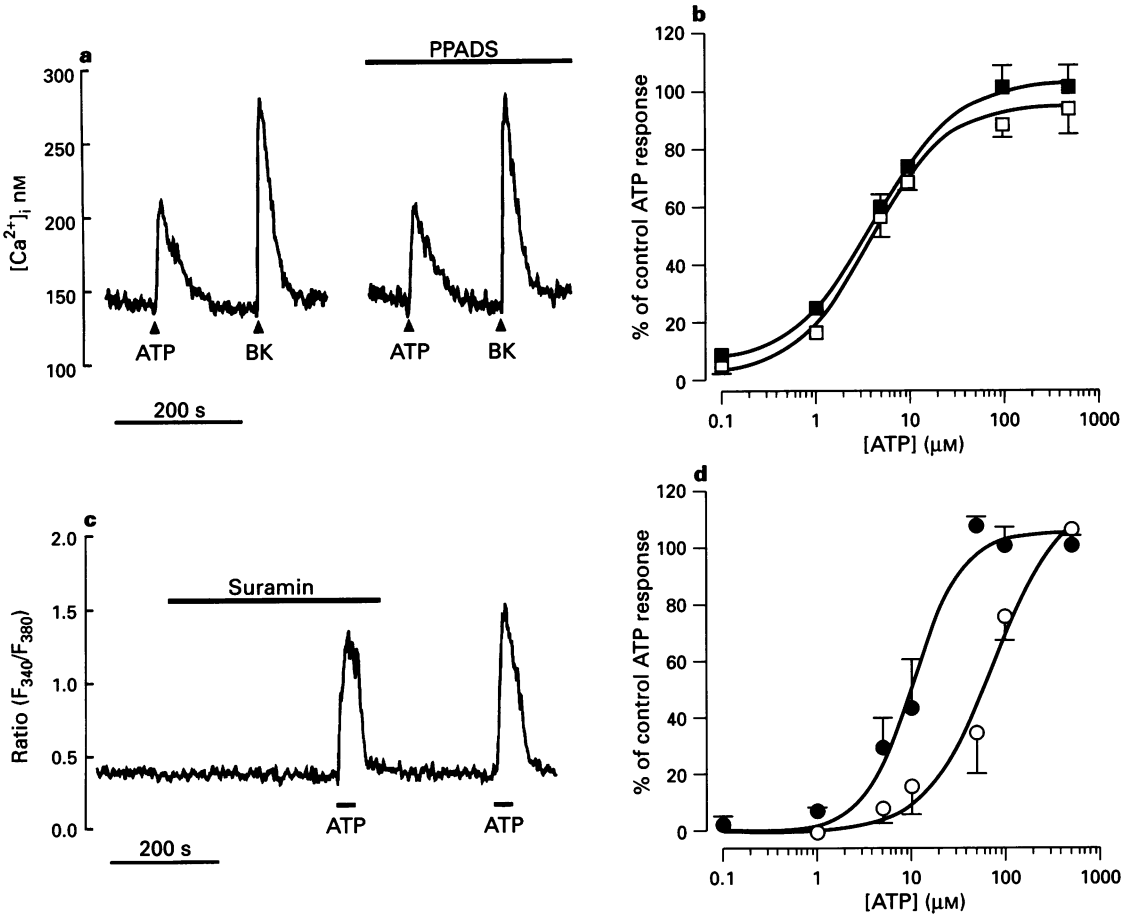

Figure 4 Effect of purinoceptor antagonists on $[Ca^{2+}]_i$ increases in endothelial cells. (a) Cell populations; the response to 100 μM ATP was unaffected by the presence of 10 μM pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), added to the cuvette 1 min before ATP challenge. BK, 1 μM bradykinin. (b) Concentration-effect curves for ATP in the absence (■) and in the presence (□) of 10 μM PPADS. Control response was arbitrarily chosen as the $[Ca^{2+}]_i$ increase elicited by 100 μM ATP. (c) Single cell microfluorometry: exposure to suramin (100 μM) for 5 min before challenging the cell with 100 μM ATP (in the continuous presence of suramin) slightly reduced the ATP-elicited ratio increase. (d) Concentration-effect curves for ATP in the absence (●) and in the presence (○) of 100 μM suramin. Individual curves were constructed in 4 different cells and responses normalized to the effect of 100 μM ATP before data pooling.

at 1 mM, reduced significantly the response to ATP (Figure 1).

We also tested the effect of other naturally occurring purine compounds that may act in endothelial cells. The diadenosine polyphosphate Ap_4A was completely ineffective both in elevating $[Ca^{2+}]_i$ and in preventing the response to a second challenge with ATP (Figure 3c). Adenosine, the last hydrolysis product of adenine nucleotides, and Ap_4, the accumulative product of Ap_5A hydrolysis by ectonucleotidases, were also assayed with negative results (data not shown).

Effects of P_2 antagonists on ATP-evoked $[Ca^{2+}]_i$ transients in endothelial cells

Two putative P_2 antagonists, suramin and PPADS, were tested to try to inhibit $[Ca^{2+}]_i$ transients evoked by ATP in endothelial cells. The $[Ca^{2+}]_i$ peak elicited by 100 μM ATP was not reduced by 10 μM PPADS (Figure 4a). Furthermore, Figure 4b shows that the addition of 10 μM PPADS did not significantly displace the concentration-effect curve for ATP in these cells, thus indicating that PPADS is not an antagonist of this action.

Suramin interfered with fluorescence collected from endothelial cells due to absorbed/quenched fura-2 fluorescence at short wavelengths. This phenomenon was probably due to suramin binding to proteins released from damaged cells that accumulate in the cuvettes (Middaugh *et al.*, 1992). Using the Ca^{2+} indicator fluo-3 (which is excited at longer wavelengths) to avoid this problem, suramin 100 μM reduced the response to 100 μM ATP by 24.5±4.7%. The interference by suramin was not observed in microfluorometry experiments, where cells were continuously washed. Figure 4c shows that by using fura-2 as indicator in this way, a similar inhibition could be achieved. Suramin 100 μM displaced the concentration-effect for ATP to the right by approximately 6.5 times indicating a moderate antagonistic effect (Figure 4d).

Effects of ATP and analogues on $[Ca^{2+}]_i$ in single endothelial cells

In previous studies (Castro *et al.*, 1994) we found that 2MeSATP was a potent partial agonist in subconfluent endothelial cells in primary culture, in open conflict with the results presented above. Thus, we tested the effect of purinoceptor agonists on subconfluent cells (days 1–3 of culture) from first passage and those following. 2MeSATP was completely ineffective in evoking $[Ca^{2+}]_i$ rises in these conditions, as was Ap_4A (Figure 5a). No cell was found to respond to 2MeSATP in those cultures in accordance with the lack of effect of this agonist in population experiments. In contrast, when primary cultures of endothelial cells were studied, a complex picture arose. Figure 5b shows typical responses from cells in those cultures. Some cells exhibited equipotent responses to ATP and 2MeSATP, while they were non-responsive to UTP (Figure 5b, cell 1). In other cells, ATP, UTP and 2MeSATP were all agonists elevating $[Ca^{2+}]_i$, although with different potencies (Figure 5b, cell 2). Figure 6 shows a typical cell responding to

ATP, 2MeSATP and UTP. The $[Ca^{2+}]_i$ response elicited by 2MeSATP was completely blocked in the presence of suramin but the response to UTP in the same cell was not significantly affected by PPADS and only slightly reduced by suramin. On the other hand, the $[Ca^{2+}]_i$ transient induced by UTP remained unchanged after a previous challenge with 2MeSATP.

Stimulation of [^{3}H]-arachidonic acid release by purinoceptor agonists

Some vasoactive compounds have been shown to activate a Ca^{2+}-sensitive phospholipase A_2 (PLA_2) and to stimulate the release of AA as a previous step to the production of prostacyclin by endothelial cells. Previous finding have shown ATP-stimulated formation of prostacyclin in endothelial cells from the adrenal medulla (Forsberg *et al.*, 1987; Gosink & Forsberg, 1993). Thus we tested if ATP elicited the generation and release of AA in these cells. Purinoceptor agonists that elevated $[Ca^{2+}]_i$ in subcultured endothelial cells, such as ATP and UTP, enhanced the production and release of [^{3}H]-AA to 135.5±6.5% ($n=6$) and 135.9±14.5 ($n=3$), respectively, with respect to basal production (Figure 7). The ionophore A23187, which also increased the $[Ca^{2+}]_i$ levels, was a potent stimulator of the release of arachidonate (156.6±13.9%, $n=4$). In contrast, the P_{2Y}-agonist 2MeSATP, which failed to elevate $[Ca^{2+}]_i$ in these cells, was not able to enhance the release of [^{3}H]-AA over basal levels (105.8±4.2%, $n=3$). The concentration-effect curve for the stimulation of [^{3}H]-AA release by ATP (Figure 7 inset) was similar to that of ATP in increasing $[Ca^{2+}]_i$. The calculated EC_{50} value for this process was 2.0 μM. When extracellular Ca^{2+} was buffered to 100 nM by addition of EGTA, there was a very clear (64%) reduction in the [^{3}H]-AA release response to A23187, while there was only a non-significant decrease in the response of ATP or UTP.

Neither suramin nor PPADS were able to act as purinoceptor antagonists on ATP-stimulated [^{3}H]-AA release. In contrast, as shown in Figure 8, the response of ATP was enhanced 2.5 times by these compounds. This action was not restricted to the nucleotide-stimulated response, since suramin, but not PPADS, produced *per se* a significant increase in the basal release of [^{3}H]-AA (to 119±5%) with respect to basal levels, and a doubling of the potentiation of [^{3}H]-AA release elicited by the ionophore A23187.

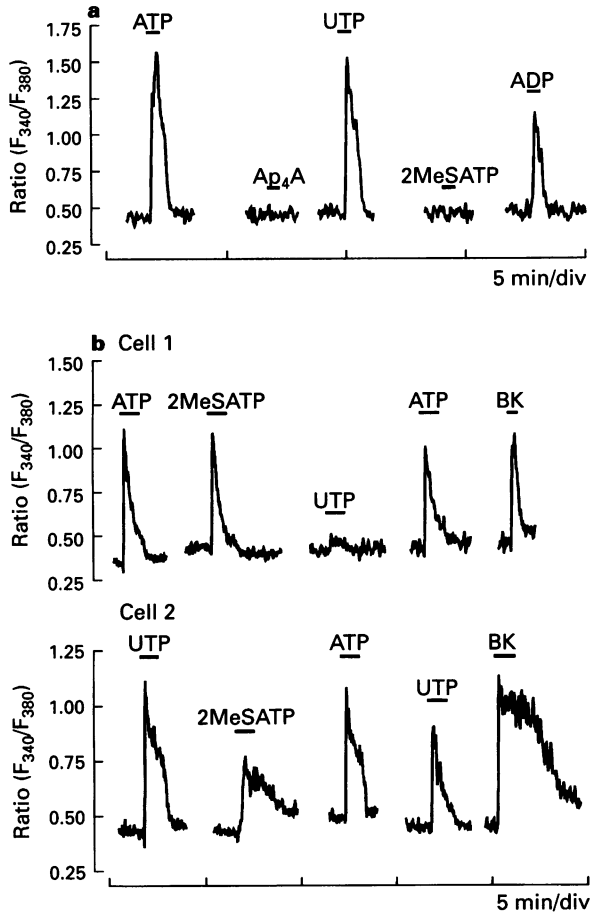

Figure 5 Responses to purinoceptor agonists in single endothelial cells. Fura-2 fluorescence ratio (F_{340}/F_{380}) traces are presented for representative cells. Fluorescence ratio increases with rising $[Ca^{2+}]_i$ (see Methods). The cells were continuously perfused with fresh medium, and drugs were applied only during the period indicated by the horizontal bars. The cell tested was allowed to rest for at least 5 min between successive challenges (trace breaks). (a) Subcultured endothelial cells. Ap_4A and 2MeSATP, both tested at 100 μM, were completely inactive, while ADP (1 mM) was weaker than ATP and UTP (100 μM). Equivalent results were obtained in 10 out of 10 cells tested. (b) Endothelial cells in primary culture. Cell 1: cell almost equally sensitive to ATP and 2MeSATP (100 μM) but very weakly responsive to UTP (100 μM). Cell 2: Cell displaying potent responses to ATP and UTP (100 μM) and lower, but clear, response to 2MeSATP (also 100 μM). BK, 1 μM bradykinin for comparison purposes. These two are representative of other 23 endothelial cells showing co-existing UTP and 2MeSATP responses, of a total number of 30 cells tested. The remaining 7 cells, displayed responses to UTP but not to 2MeSATP.

Discussion

Endothelial cells from different vessels seem quite heterogeneous in the receptor set they display in their membranes. In our case, cells obtained from the adrenal medulla and later subcultured show a single purinoceptor subtype that we have identified as P_{2U} based on its sensitivity to ATP and UTP as agonists and its lack of response to 2MeSATP. The experiments of cross-desensitization indicate that these compounds act at a common receptor site. Particularly, the fact that exposure to ATP can fully desensitize the response to UTP which means that all receptors activated by UTP are also sensitive to ATP. Thus, the responses to UTP are mediated by a purine receptor and not by a co-existing pyrimidine receptor, as in other tissues (Seifert & Schultz, 1989; Lazarowski & Harden, 1994). The apparent potency order for purinoceptor agonists at this site is UTP=ATP>ADPβS>>2MeSATP>α,β-MeATP, which fits the generic description of a P_{2U}-purinoceptor (O'Connor, 1992). The fact that subcultured cells display an homogeneous purinoceptor population allows us to consider unequivocally ADPβS as a P_{2U}-purinoceptor agonist, in addition to its activity at the P_{2Y}-purinoceptor.

An increasing number of metabotropic P_2-purinoceptors are being cloned (Boarder *et al.*, 1995; Chang *et al.*, 1995; Communi *et al.*, 1995a). Except for P_{2Y1} and P_{2Y5} receptors, where UTP is almost inactive, all other cloned purinoceptors are more or less sensitive to uridine nucleotides. However, the detailed pharmacological profile of the purinoceptor present in bovine adrenomedullary endothelial cells (BAMEC) seems different from several UTP-sensitive purinoceptors cloned so far: a P_{2Y3} receptor from chick brain favours ADP over ATP (Barnard *et al.*, 1994) and the P_{2Y4} (Communi *et al.*, 1995a) and P_{2Y6} (Chang *et al.*, 1995) receptors are more sensitive to uridine nucleotides compared to adenine, with ATP being a weak agonist (better considered as pyrimidinoceptors). P_{2Y2} receptors cloned from the mouse (Lustig *et al.*, 1993), man (Parr *et al.*, 1994) and rat (Rice *et al.*, 1995) are activated by ATP and UTP with roughly the same potency, and other agonists are less potent, as in endothelial cells from adrenal medulla and from most other tissues. It can be concluded that P_{2U} actions are most probably mediated by P_{2Y2}-type receptors. However, there are some discrepancies between the pharmacological profile of P_{2Y2}-purinoceptor clones identified so far and the P_{2U} effects in BAMEC. The P_{2Y2}-receptor cloned from human airway epithelium is very sensitive to

diadenosine polyphosphates such as Ap_4A (potency order UTP = ATP $\geqslant Ap_4A$ > ATPγS > > 2MeSATP > α,β-MeATP; Lazarowski *et al.*, 1995), while Ap_4A is completely inactive in increasing $[Ca^{2+}]_i$ in BAMEC. On the other hand, the responses to ATP and UTP in mouse neuroblastoma NG108-15 cells (from which P_{2Y2} was originally cloned) are inhibited by suramin and PPADS (Reiser, 1995), whereas the P_{2U}-mediated $[Ca^{2+}]_i$ peaks, elicited by purinoceptor stimulation in adrenal endothelial cells, were not antagonized by PPADS at concentrations similar to those used by Reiser (10 μM and 50 μM).

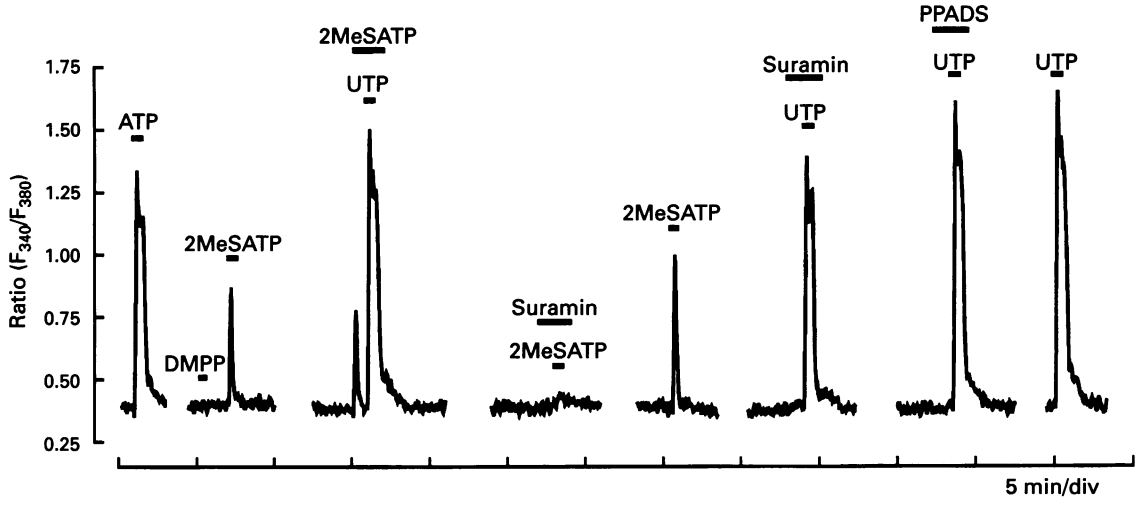

Figure 6 Pharmacological discrimination of P_{2U}- and P_{2Y}-purinoceptor-mediated actions in a single endothelial cell. Fura-2 ratio traces recorded successively from the same cell, representative of various experimental procedures replicated in several other cells (indicated by *n*). ATP and UTP used at 50 μM, 2MeSATP and suramin at 100 μM, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) at 50 μM and 1,1-Dimethyl-4-phenylpiperazinium (DMPP) at 10 μM. Lack of response to DMPP indicated that it was an endothelial, not chromaffin, cell. UTP elicited a potent $[Ca^{2+}]_i$ rise in the presence of 2MeSATP, applied 1 min before starting the UTP pulse (no cross-desensitization, $n=7$). The response to 2MeSATP was totally and reversibly blocked by suramin ($n=5$). The $[Ca^{2+}]_i$ peak induced by UTP was negligibly affected by suramin ($n=6$) or PPADS ($n=5$).

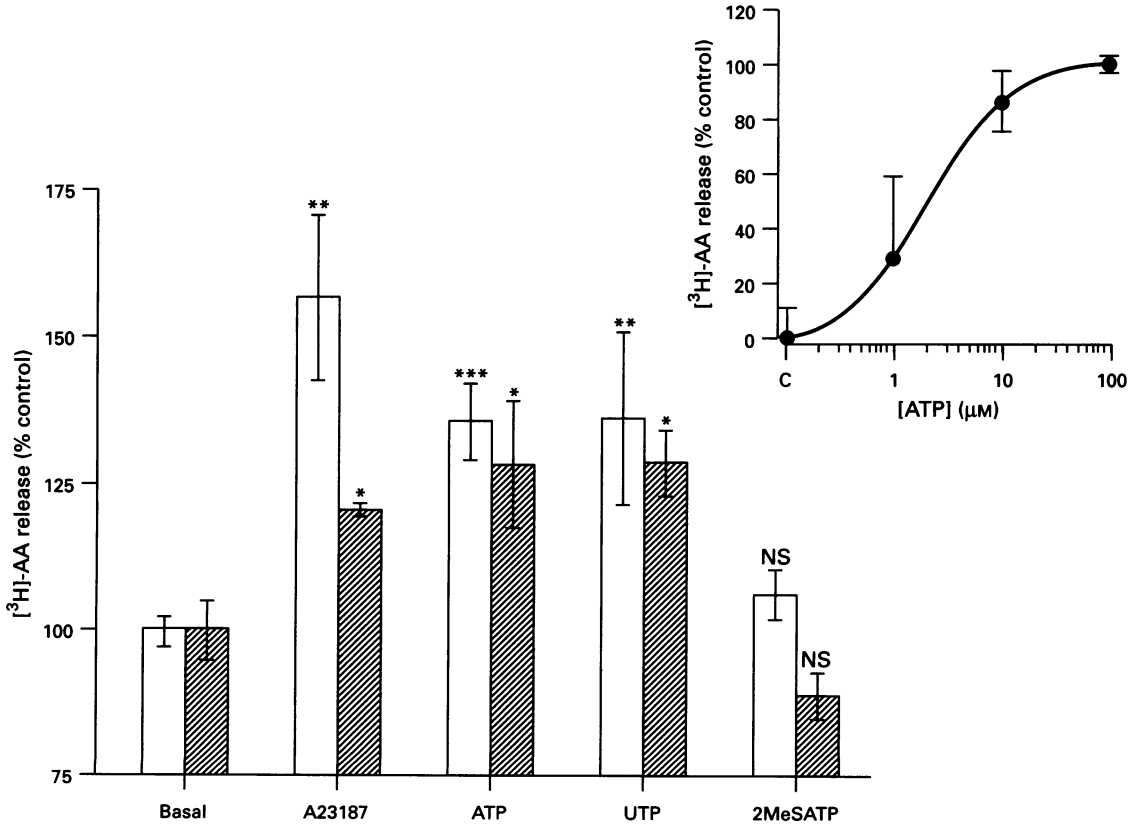

Figure 7 Ca^{2+}-dependent release of [^{3}H]-arachidonic acid ([^{3}H]-AA) from endothelial cells. The stimulation of [^{3}H]-AA release by each compound indicated in the horizontal axis was determined in medium containing 2.5 mM Ca^{2+} (open columns) or in medium buffered to 100 nM $[Ca^{2+}]_o$ with EGTA (hatched columns). Basal release levels amounted 430 $\pm$ 52 and 570 $\pm$ 55 c.p.m. respectively in the absence and in the presence of EGTA. ATP, UTP and 2MeSATP, 100 μM; A23187 10 μM. Each column represents the mean of 4 experiments performed in duplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ statistically significant differences with respect to corresponding control (with or without Ca^{2+}). Inset, concentration-effect curve for the stimulation of [^{3}H]-AA release by ATP normalized to the release obtained at 100 μM ATP.

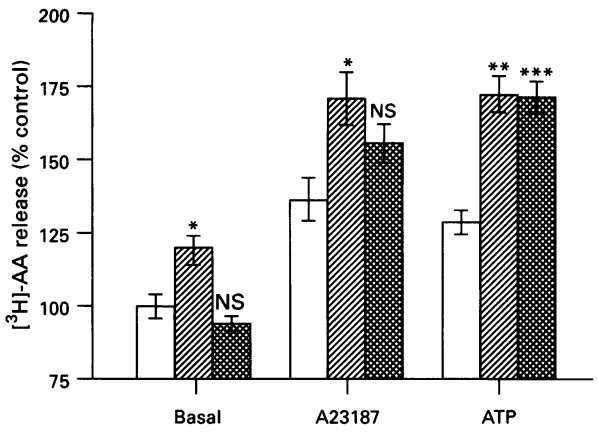

Figure 8 Effect of purinoceptor antagonists on [^{3}H]-arachidonic acid ([^{3}H]-AA) release. The release of [^{3}H]-AA elicited by 10 μM A23187 and 100 μM ATP was determined in the presence of 100 μM suramin (hatched columns) or 10 μM PPADS (cross-hatched columns). Each column represents the mean of 4 experiments performed in duplicate. $*P<0.05$, $**P<0.01$, $***P<0.001$ statistically significant differences with respect to corresponding control (in the absence of P_2-antagonist, open columns).

Are these differences due to particular properties of the bovine P_{2Y2}-receptor (a species difference) or do they represent a genuine receptor difference? P_{2U} effects resistant to antagonists suramin or PPADS are not restricted to the bovine adrenal medulla: they have been observed in bovine aortic endothelial cells (Brown *et al.*, 1995), bovine chromaffin cells (Castro *et al.*, 1995a), and more importantly, in guinea-pig myenteric glia (Kimball & Mulholland, 1996) and rat sympathetic ganglia (Connolly, 1995). At present, we do not know if this pharmacologically defined action represents a novel receptor molecule or simply a variation of the binding properties of the P_{2Y_2} receptor imposed by secondary factors. Our work points out the necessity to use antagonists, in addition to the more usual agonists, to characterize purinoceptors, specifically those newly cloned.

In addition to the P_{2U} purinoceptor already described, BAMEC in primary culture, but not after subsequent subculturing, also express a typical P_{2Y}-purinoceptor which is lost within the first cycle of division *in vitro*. This finding strongly suggests the co-existence of both receptors in the intact tissue, since the activation of a fast and transient expression of P_{2Y} receptors seems much less likely than their gradual decline after removal of cells from the highly specialized environment of the adrenal medulla. ATP responses in endothelial cells were considered to be mediated by P_{2Y} purinoceptors (Boeynaems & Pearson, 1990), but in recent years more importance has been given to P_{2U}-purinoceptors. More detailed studies have revealed the coexistence of P_{2Y}- and P_{2U}-purinoceptors in bovine aortic endothelial cells (Motte *et al.*, 1993; Brown *et al.*, 1995). The discrimination between P_{2Y}- and P_{2U}-mediated responses is helped by selective antagonists. Our results emphasize that suramin and PPADS can be used specifically to block P_{2Y}-receptors in the presence of P_{2U} receptors. P_{2U}- and P_{2Y}-receptors are not segregated into separated endothelial cell subpopulations. Experiments with single cells demonstrate that the majority of the cells ($\approx$80%) co-express the two receptor subtypes simultaneously, although the existence of small subpopulations of cells expressing only one purinoceptor subtype cannot be excluded. Identical results were obtained by Communi *et al.* (1995b) in aortic cells. Our finding that P_{2Y} receptors can be lost with time in culture may suggest an unifying hypothesis, general co-expression and selective loss of P_{2Y}- or P_{2U}-purinoceptors, to explain the known heterogeneity of P_2-purinoceptors expressed by endothelial cells from different tissues. In this way, the 'atypical' purinoceptor found by Allsup & Boarder (1990) should be regarded as an unresolved mix of P_{2Y}- and P_{2U}-receptors. On the other hand, their later results showing a homogeneous P_{2U} population in these cells (Purkiss *et al.*, 1993) probably reflects the use of older cultures.

Both receptors are coupled to the same signalling mechanism: cytosolic Ca^{2+} increases originated from IP_3-sensitive pools; so, they may be considered as redundant. Nevertheless, co-expression of P_{2Y}- and P_{2U}-receptors is not uncommon, having been observed in rat hepatocytes (Keppens, 1993), rat osteoblasts (Gallinaro *et al.*, 1995) and astroglia (Brunner & Murphy, 1993), in addition to aortic and adrenomedullary endothelial cells. So, the co-expression of P_{2U}- and P_{2Y}-receptors resembles more the rule than the exception. The biological significance of having two receptors mediating the same response is unclear. In aortic endothelial cells there are differences in the desensitization by PKC-activation and the G protein coupling of both receptors (Motte *et al.*, 1993). We have also observed that $[Ca^{2+}]_i$ peaks elicited by 2MeSATP tend to be more transient than those generated by UTP; but the functional extent of these differences for the physiology of the endothelium is unknown. There is also the possibility that these two receptors are differentially distributed to the basal versus luminal surfaces of the cell, although we have no evidence of such spatial heterogeneity.

Endothelial cells in general, and BAMEC in particular, have been shown to secrete NO and prostacyclin in response to purinoceptor stimulation (Newby & Henderson 1990; Gosink & Forsberg, 1993). The rate limiting step for the formation of eicosanoid compounds, such as prostacyclin, seems to be the formation and release of free AA (Samuelsson *et al.*, 1987). Here we have shown that purinoceptor stimulation effectively increases the mobilization of AA in BAMEC. Thus, $[Ca^{2+}]_i$ signals activated by purinoceptor stimulation are effectively coupled to endothelial cell function. In addition to the effects on vascular tone, NO and eicosanoids modulate the activity of neighbouring ATP-releasing chromaffin cells (Takeuchi *et al.*, 1992; Torres *et al.*, 1994; Rodriguez-Pascual *et al.*, 1996) closing a local feedback regulatory circuit between chromaffin and endothelial cells.

Suramin and PPADS do not antagonize the AA releasing effect of ATP, as would be expected from the pharmacology of the P_{2U}-receptor present in these cells, but paradoxically they enhance AA release induced by ATP and UTP. Suramin is a known inhibitor of ecto-ATPases that degrade ATP (Bailey & Hourani, 1994). So, in the presence of suramin the ATP lifetime in the extracellular medium is prolonged and, presumably, its actions are potentiated. Our results suggest that PPADS also has some ATPase inhibitory activity in endothelial cells, as has been demonstrated for ecto-ATPase from smooth muscle (Ziganshin *et al.*, 1995). However, suramin also potentiates the AA releasing effect of A23187, which indicates that there should be a direct effect on Ca^{2+}-dependent production of AA beyond any effect on nucleotide lifetimes. In fact, suramin has many other actions besides purinoceptor antagonism, which are claimed to be the basis of its antineoplasic effects (Stein, 1993). Suramin potently inhibits diacylglycerol (DAG) kinase, reducing DAG recycling (Kopp & Pfeiffer, 1990) which accumulates and can serve as substrate for AA generation by the DAG lipase (Whatley *et al.*, 1990). Furthermore, suramin also inhibits several protein tyrosine-phosphatases (Ghosh & Miller, 1993), increasing the steady level of protein tyrosine phosphorylation (Cardinali *et al.*, 1992). In this way, suramin can potentiate AA formation via phosphorylation-dependent phospholipase C (PLC)-gamma (Kim *et al.*, 1991). Any of these mechanisms may explain the enormous potentiating effect of suramin.

The results presented in this paper demonstrate the co-expression of P_{2Y}- and P_{2U}-purinoceptors in microvessel endothelial cells from adrenal medulla. Both suramin and PPADS are useful tools as they are more selective antagonists on P_{2Y}- than on P_{2U}-purinoceptors, allowing the discrimination of ATP-induced effects.

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