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Characterization of the P2 receptors on the human umbilical vein endothelial cell line ECV304

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1 To characterize the P2 receptors present on the human umbilical vein endothelial-derived cell line, ECV304, cytosolic Ca^{2+}, ($[Ca^{2+}]_c$), responses were recorded in single cells and in cell suspensions to a series of nucleotides and nucleotide agonists.

2 Concentration response curves were obtained in fura-2-loaded ECV304 cell suspensions, with EC_{50} values of 4.2 μM for ATP, 2.5 μM for UTP and 14 μM for adenosine-5′-*O*-(3-thio)triphosphate (ATPγS). EC_{50} values for 2-methylthioATP, ADP, adenosine-5′-*O*-(2-thio)diphosphate (ADPβS) and AMP were 0.5 μM, 3.5 μM, 15 μM and 4.7 μM respectively, but maximal $[Ca^{2+}]_c$ responses were less than those produced by a maximal addition of ATP/UTP. ECV304 cells were unresponsive to UDP and β,γ,methyleneATP.

3 Cross-desensitization studies on ECV304 cells suggested that ATP and UTP recognized the same receptor. However, ADP recognized a receptor distinct from the UTP-sensitive receptor and AMP recognized a third distinct receptor.

4 ECV304 $[Ca^{2+}]_c$ responses to 2-methylthioATP were inhibited in the presence of 30 μM pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), whereas $[Ca^{2+}]_c$ responses to UTP were unaffected by this treatment.

5 ECV304 cells responded to the diadenosine polyphosphate Ap_3A with rises in $[Ca^{2+}]_c$. Apparent responses to Ap_4A, Ap_5A and Ap_6A, were shown to be due to a minor nucleotide contaminant that could be removed by pre-treatment of the diadenosine samples with either alkaline phosphatase or apyrase.

6 ECV304 cells display a pharmacology consistent with the presence of at least two P2 receptors; a $P2Y_2$ receptor insensitive to the diadenosine polyphosphates and a $P2Y_1$ receptor sensitive to Ap_3A. In addition, ECV304 cells respond to AMP with increases in $[Ca^{2+}]_c$ *via* an as yet uncharacterized receptor.

Keywords: Adenine dinucleotides; diadenosine polyphosphate; ATP; UTP; Ca^{2+}; human; endothelial cells; P2 receptor

Introduction

Currently P2 receptors are subdivided into either ligand-gated cation channels (P2X receptors) or G-protein-coupled receptors (P2Y receptors) (Burnstock, 1996). In the vasculature, vasoconstriction is mediated *via* P2X and P2Y receptors present on smooth muscle and vasodilatation *via* P2Y receptors present on the endothelium (Boarder & Hourani, 1998). Subtypes of both P2Y and P2X receptors have been shown to be sensitive to the diadenosine polyphosphates as well as other nucleotides (Castro *et al*., 1994; Lazarowski *et al*., 1995; Pintor *et al*., 1996; Schachter *et al*., 1996).

The diadenosine polyphosphates are co-released with ATP and have been isolated from a variety of secretory vesicles, including the dense granules of platelets (Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983), chromaffin granules (Pintor *et al*., 1991) and brain synaptosomes (Pintor *et al*., 1992). The vasoactivity of the diadenosine polyphosphates has been documented in a variety of vascular systems inducing either vasoconstriction or vasodilatation depending on the specific vascular bed (Busse *et al*., 1988; Pohl *et al*., 1991; Ogilvie, 1992; Schlüter *et al*., 1994; Davies *et al*., 1995; Ralevic *et al*., 1995). Compared with the adenine mononucleotides with which they are co-released, diadenosine polyphosphates are longer lasting, potent vasoactive agents acting at P2 receptors (Busse *et al*., 1988; Lüthje & Ogilvie, 1988; Pohl *et al*., 1991; Ralevic *et al*., 1995).

Cultured bovine endothelial cells display sensitivity to the diadenosine polyphosphates Ap_3A and Ap_4A in some (Motte *et al*., 1996), although not in all cases studied (Chen *et al*., 1996; Mateo *et al*., 1996). As yet the actions of diadenosine polyphosphates on cultured human endothelial cells have not been studied.

We have characterized the nucleotide receptors present on the human umbilical vein endothelial-derived cell line ECV304 (Takahashi *et al*., 1990) with particular attention to their sensitivity to diadenosine polyphosphates. Since all of the cloned human P2Y receptors so far investigated induce Ca^{2+} release on agonist stimulation (Parr *et al*., 1994; Communi *et al*., 1995, 1996, 1997; Léon *et al*., 1997), cells were loaded with the Ca^{2+}-sensitive dye fura-2 and stimulated with the diadenosine polyphosphates as well as other nucleotides or nucleotide analogues to characterize the P2 receptors present.

The results indicate that ECV304 cells show $[Ca^{2+}]_c$ responses consistent with the presence of $P2Y_2$ - and $P2Y_1$ receptors. The diadenosine polyphosphate Ap_3A, elicited $[Ca^{2+}]_c$ responses *via* the putative $P2Y_1$ receptor, Ap_4A, Ap_5A and Ap_6A elicited no response at either the $P2Y_1$- or $P2Y_2$ receptor.

Methods

Materials

Experiments were performed at 37°C in HEPES-Buffered-Saline (HBS) composed of; NaCl, 145 mM; KCl, 5 mM;

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Na_2HPO_4, 1 mM; $MgSO_4$, 1 mM; HEPES, 10 mM; D-glucose, 10 mM; sulphinpyrazone, 200 μM; (to prevent dye leakage). Diadenosine polyphosphates and apyrase were purchased from Sigma Chemicals (Poole, Dorset, U.K.), alkaline phosphatase and hexokinase from Boehringer-Mannheim (Lewes, Sussex, U.K.), pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS) from Tocris-Cookson (Bristol, U.K.) and 2-methylthioATP from ICN Biomedicals (Thame, Oxon, U.K.). All other chemicals and reagents were purchased from Sigma Chemicals (Poole, Dorset, U.K.) or B.D.H. (Poole, Dorset, U.K.).

Cell culture

The human umbilical vein endothelial-derived cell line ECV304 (Takahashi *et al*., 1990), was maintained at 37°C in a 5% CO_2 humid incubator, in 80 cm^2 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B. All tissue culture reagents were purchased from Life Technologies Ltd., (Paisley, U.K.).

Measurement of cytosolic calcium ($[Ca^{2+}]_c$)

(i) Fluorescent imaging ECV304 cells were maintained on 22 mm glass coverslips at 37°C in a 5% CO_2 humid incubator, in 6 well plates containing culture medium. Cytosolic calcium was monitored by loading cells with 2 μM fura-2-AM (Molecular Probes, OR, U.S.A.) with 0.025% of the detergent Pluronic F-127 at 37°C for 45 min (in loading buffer consisting of HBS; 1 mM $CaCl_2$; 1% BSA). After loading, extracellular dye was removed by washing, and the cells then maintained in dye-free HBS. Coverslips were mounted in HBS on a thermostatted chamber (37°C) and viewed using a Nikon Diaphot inverted microscope. Cells were excited alternately at 340 and 380 nm using a PTI Deltascan Illuminator. Changes in fluorescence of individual cells were monitored at an emission of 510 nm using an intensified charge-coupled device (ICCD) camera (Photonic Science Ltd.). Data were viewed using a digital imaging system equipped with Image Master for Windows software (PTI). Photometric data were produced from images of individual cells. $[Ca^{2+}]_c$ was calculated from 340/380 nm ratio signals as described by Grynkiewicz *et al.* (1985). Reagents were added to the cells by continuous perfusion.

(ii) Cell suspensions Flasks of ECV304 cells were trypsinized by incubating cells at 37°C for 5 min with 1.2 ml Trypsin-EDTA Solution (1x) (Life Technologies Ltd., Paisley, U.K.). Cells were resuspended in 5 ml culture medium, then centrifuged for 3 min at 190 *g*. The pellet was resuspended in 5 ml loading media consisting of 2 μM fura 2-AM, 0.025% F127 in HBS; plus 1 mM $CaCl_2$; 1% BSA. The cells were loaded at 37°C for 50 min under constant agitation, centrifuged at 190 *g* for 3 min and then resuspended in 10 ml HBS.

For each experiment 1 ml of the cell suspension was sedimented for 15 s at low speed in a microcentrifuge, then resuspended in 1 ml of required buffer to remove extracellular dye. Cytosolic calcium was monitored by placing the cell suspension in a 37°C sample chamber. Fluorescence was measured using a PTI Deltascan dual excitation spectrofluorimeter, exciting alternately at 340 and 380 nm and collecting emission at 510 nm. Endothelial cell suspension $[Ca^{2+}]_c$ was calculated by calibrating the photometric 340/380 nm ratio trace as described for the monolayers.

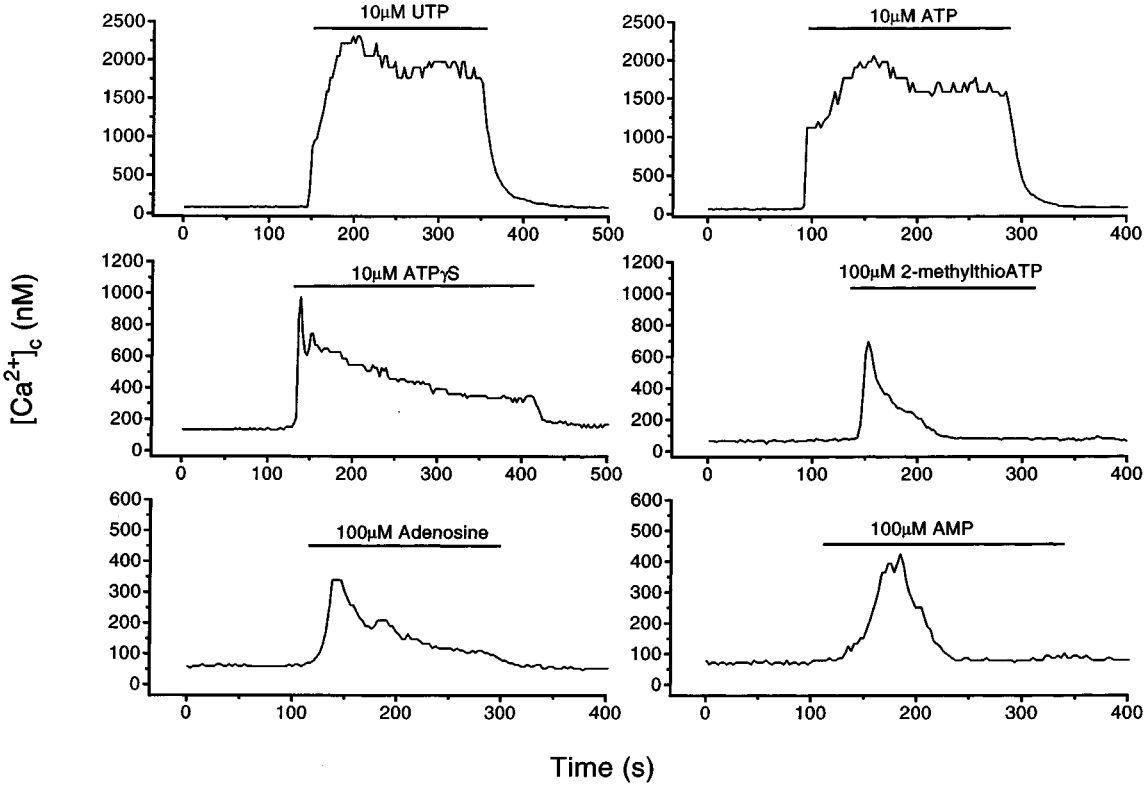

Figure 1 $[Ca^{2+}]_c$ responses in representative, single, fura-2-loaded ECV304 cells. Cells were perfused, in the presence of 1 mM Ca^{2+}, for 5 min with HBS and then with HBS plus agonist for the time indicated by the horizontal bar. The trace is representative of a minimum of three separate experiments, each with approximately 20 cells.

For experiments involving suramin, fluo 3 was used as suramin interferes with the fura 2 signal at 340 and 380 nm. Cells were treated in an identical manner to that used for fura 2 measurements, except that cell suspensions were incubated in the presence of 2 μM fluo 3-AM, 0.025% F127 in HBS; plus 1 mM $CaCl_2$; 1% BSA at 37°C for 20 min. The cells were then washed free of extracellular dye, as for fura 2-loaded cells and fluorescence was measured with excitation at 490 nm and emission collected at 526 nm.

Reagents were added to the cells by bolus addition to the sample chamber. Before any addition of agonist, cells were first mixed by pipette to measure any changes in $[Ca^{2+}]_c$. The resulting $[Ca^{2+}]_c$ responses were subtracted from the agonist induced $[Ca^{2+}]_c$ responses. EC_{50} values were calculated from 340/380 nm ratio values or 526 nm emission values using the computer program P-fit (Elsevier, Cambridge, U.K.). Data are in each case presented as mean ± s.e.mean.

Purification of diadenosine polyphosphates

The diadenosine polyphosphates and ATP were incubated in the presence of 20 units of alkaline phosphatase in a total volume of 100 μl dephosphorylation buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.5) for 30 min at 37°C. The samples were then made up to a total volume of 3 ml in HBS, plus 1 mM $CaCl_2$ and perfused over the cells. Samples treated with apyrase were incubated in the presence of 2 units apyrase in 100 μl HBS, 1 mM $CaCl_2$ in an identical manner to the alkaline phosphatase-treated samples. Samples of treated diadenosine polyphosphates were removed from the sample well and analysed by FPLC on a 1 ml Resource Q anion-exchange column using a linear elution gradient from 20 mM – 0.7 M NH_4HCO_3, pH 9.6.

Purification of UDP, ADP and ADPβS

ADP, ADPβS and UDP were incubated in the presence of 7 units of hexokinase in a total volume of 100 μl HBS for 60 min at 37°C. Samples of treated ADP, UDP and ADPβS were analysed by FPLC, in an identical manner to that used for diadenosine polyphosphates.

Results

ECV304 cells grown on glass coverslips responded to the nucleotides ATP, ATPγS, UTP, ADPβS, adenosine, AMP and 2-methylthioATP with a concentration dependent rise in $[Ca^{2+}]_c$. All cells tested were sensitive to ATP, ATPγS and UTP but only 78% of the cells tested responded to 2-methylthioATP, 47% to adenosine and 56% to AMP at maximal concentrations of agonist. ECV304 cells did not

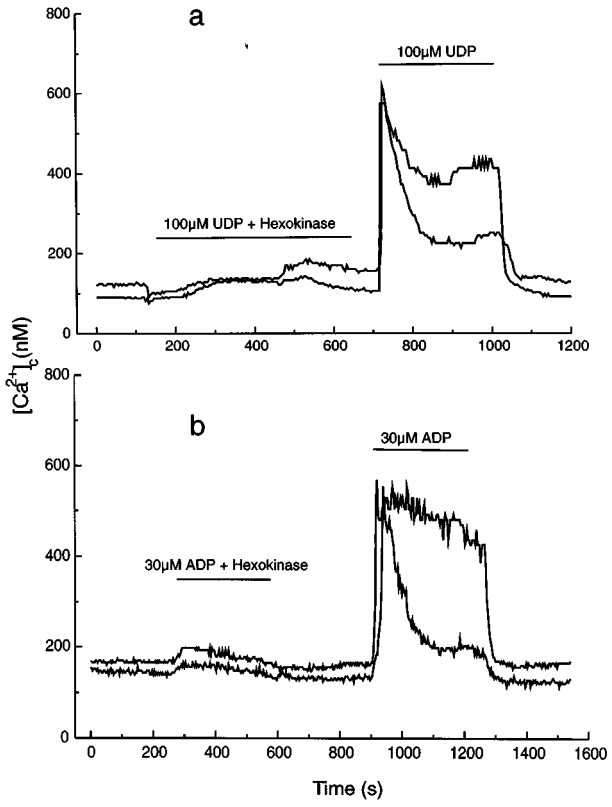

Figure 2 $[Ca^{2+}]_c$ responses in representative, single, fura-2-loaded ECV304 cells. Cells were perfused, in the presence of 1 mM Ca^{2+}, with HBS and then with HBS plus UDP or ADP for the time indicated by the horizontal bar. Samples of UDP and ADP were pretreated with hexokinase prior to perfusion as indicated. The trace is representative of three separate experiments, each with approximately 20 cells.

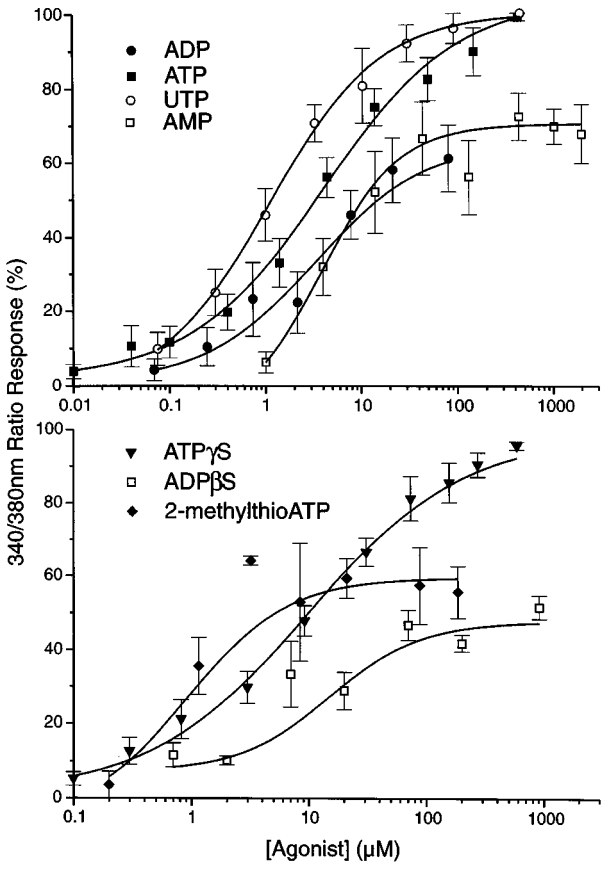

Figure 3 Agonist response curves for fura-2 loaded ECV304 cell suspensions. ECV304 cells were stimulated by the bolus addition of agonist to the sample cuvette and the maximum rise in the 340/380 nm ratio (Δ340/380 nm Ratio) plotted against the agonist concentration. Representative agonist response curves shown are expressed as a % of the maximal response for ATP/UTP for clarity. Measurements were always preceded by a sham addition to determine the extent of any apparent ratio responses caused by the mixing. Each data point represents the mean ± s.e.mean for a minimum of three separate experiments.

respond to 100 μM additions of β,γ-methyleneATP. Representative traces from individual responding cells are shown in Figure 1. Although cells appeared to respond to UDP, when cells were stimulated with samples of UDP treated with hexokinase to remove any contaminating UTP the responses were abolished, even though cells were still capable of responding to untreated samples, as shown in Figure 2a. Cells unable to respond to 2-methylthioATP were also found to be unable to respond to hexokinase-treated ADP, although these cells were still able to respond to untreated ADP, as shown in Figure 2b. When samples of hexokinase-treated ADP or UDP were analysed by FPLC, they ran as single peaks free of other contaminating nulceotides. The ADP and UDP peaks were at the same position in the elution profile as the major peak obtained by FPLC analysis of the untreated nucleotides, demonstrating that both ADP and UDP were unaffected by treatment with hexokinase.

Concentration response curves were obtained from fura-2 loaded cell suspensions by fluorescence photometry, giving EC_{50} values of 4.2 ± 0.8 μM for ATP, 2.5 ± 0.8 μM for UTP, 14 ± 4 μM for ATPγS. 2-methylthioATP, ADP, ADPβS and AMP gave EC_{50} values of 0.5 ± 1.0 μM, 3.5 ± 1.1 μM, 15 ± 1 μM and 4.7 ± 3.4 μM, respectively. When samples of ATPγS were treated with alkaline phosphatase in an identical manner to the diadenosine polyphosphates they gave identical responses to untreated samples, indicating that there was negligible contamination with other nucleotides. The concentration response curves for 2-methylthioATP, ADP, ADPβS and AMP revealed high-potency responses but with maximal 340/380 ratio responses lower than those obtained for ATP and UTP (Figure 3). These population responses gave maximal ratio responses $56\pm3\%$, $56\pm4\%$, $42\pm4\%$ and $67\pm5\%$ of the maximal ATP response, for 2-methylthioATP, ADP, ADPβS and AMP respectively (Figure 3). The presence of sulphinpyrazone in the incubation media did not influence the potency of UTP for the ECV304 cell receptor.

Cell suspensions were treated with supramaximal concentrations of nucleotides to test agonists for receptor cross-desensitization. Cells stimulated with 100 μM ATP were desensitized to a further addition of 100 μM ATP and also to an addition of 100 μM UTP (Figure 4a), suggesting that both ATP and UTP were recognized by the same receptor. However following a desensitizing addition of 100 μM UTP, a subsequent addition of ATP could still elicit a small response, (Figure 4b). Cells desensitized to UTP and ATP were still capable of responding to histamine, demonstrating that the ability to generate a $[Ca^{2+}]_c$ response was not impaired (Figure 4a and 4b). Cells desensitized to UTP were still able to respond to ADP (Figure 4c) and AMP (Figure 4d) indicating that both of these nucleotides recognize receptors distinct from the receptor sensitive to UTP.

ECV304 cell suspensions were treated with 100 μM suramin, just prior to the addition of agonist. Agonist response curves to UTP were indistinguishable from those obtained using untreated cells (Figure 5a). $[Ca^{2+}]_c$ responses in ECV304 cell suspensions, to UTP were also insensitive to 30 μM PPADS, a $P2X/P2Y_1$ receptor antagonist, (Figure 5b) (Lamprecht *et al*., 1992; Ziganshin *et al*., 1994; Brown *et al*., 1995).

Cell suspensions stimulated with either 2-methylthioATP (Figure 6a) or the diadenosine polyphosphate Ap_3A (Figure 6b) elicited a $[Ca^{2+}]_c$ response. The EC_{50} of Ap_3A-induced $[Ca^{2+}]_c$ responses in ECV304 cells was 3.4 ± 1.3 μM and maximal $[Ca^{2+}]_c$ responses were $47\pm4\%$ of those obtained with ATP/UTP. If the cells were first treated with 30 μM PPADS no $[Ca^{2+}]_c$ response was elicited either by an addition of 2-methylthioATP (Figure 6c) or Ap_3A (Figure 6d) although a subsequent UTP response was unaffected. Similar results were obtained with 100 μM suramin (data not shown). Ap_3A

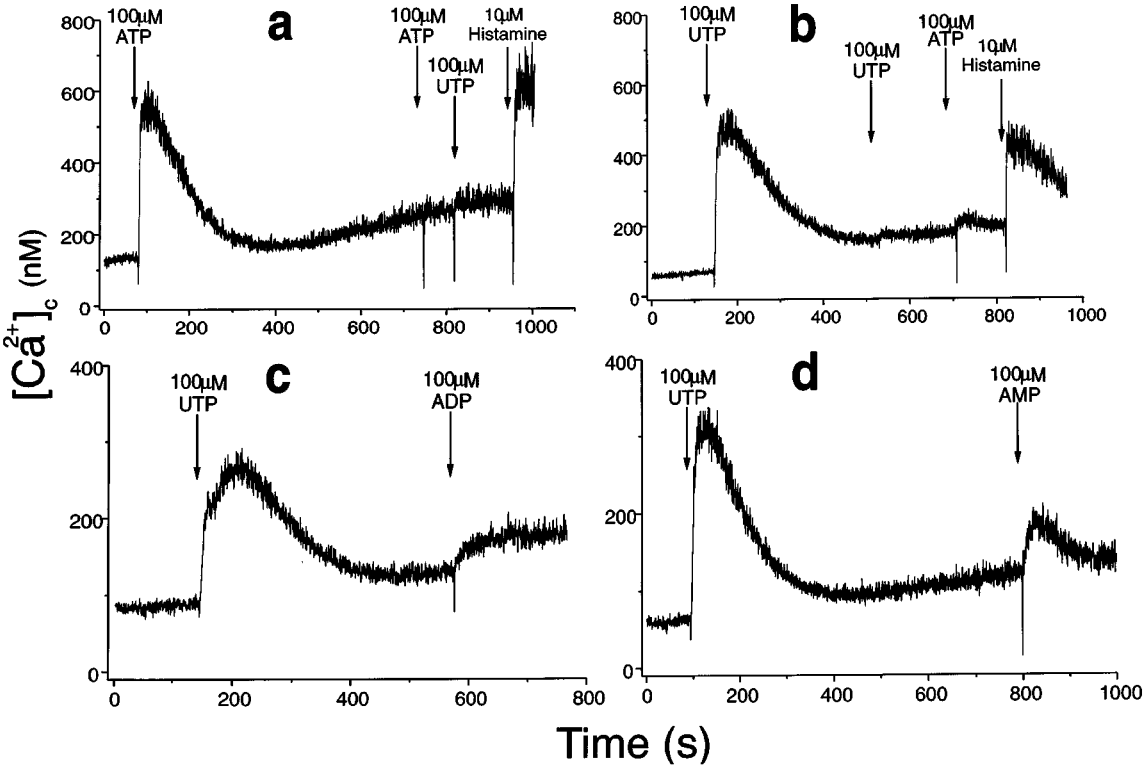

Figure 4 Cross-desensitization of $[Ca^{2+}]_c$ responses in fura-2 loaded, ECV304 cell suspensions. ECV304 cells suspended in HBS were stimulated by the bolus addition of agonist to the sample cuvette at the points indicated by the vertical arrows. Similar responses were obtained with single agonist additions.

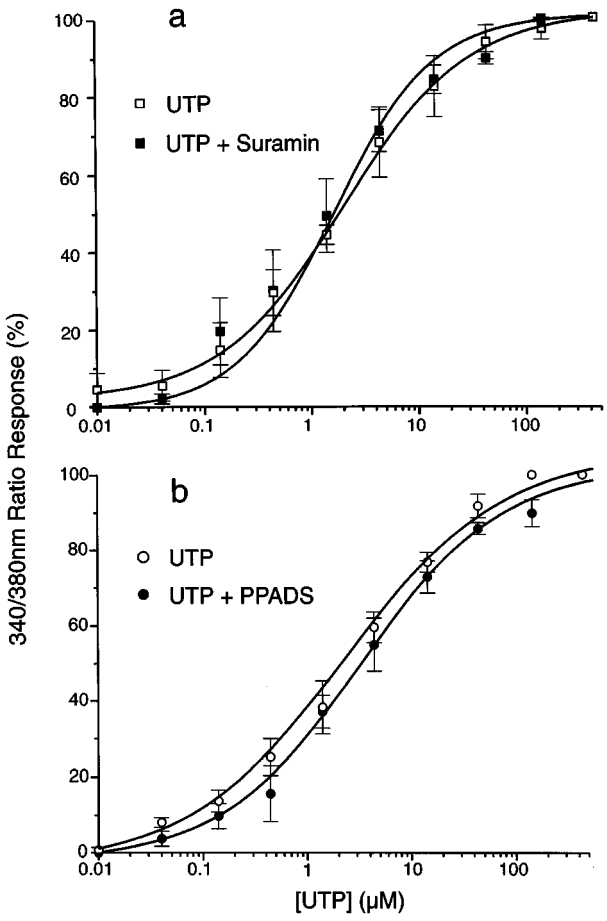

Figure 5 (a) Suramin inhibition of UTP $[Ca^{2+}]_c$ responses in fluo-3 loaded, ECV304 cell suspensions. Suramin (100 μM) was added to ECV304 cells prior to stimulation with agonist. (b) Inhibition of UTP $[Ca^{2+}]_c$ responses by PPADS in fura-2-loaded, ECV304 cell suspensions. PPADS (30 μM) was added to ECV304 cells prior to stimulation with agonist.

and 2-methylthioATP therefore recognize a suramin and PPADS-sensitive receptor. UTP recognizes a distinct receptor to that recognized by 2-methylthioATP and Ap_3A that is insensitive to both PPADS and suramin.

$[Ca^{2+}]_c$ responses to Ap_4A, Ap_5A and Ap_6A were only obtained at concentrations greater than 50 μM (results not shown). The low potency nature of the $[Ca^{2+}]_c$ responses to Ap_4A, Ap_5A and Ap_6A suggested that these responses could be due to a minor mononucleotide contaminant. When samples of Ap_4A, Ap_5A and Ap_6A were treated with alkaline phosphatase or apyrase prior to addition to the perfusion solution the $[Ca^{2+}]_c$ response was abolished. As a control, cells also failed to respond to 10 μM ATP treated with either apyrase or alkaline phosphatase but were still capable of responding to untreated samples of Ap_4A, Ap_5A or Ap_6A and ATP (for example Figure 7). $[Ca^{2+}]_c$ responses to Ap_3A were unaffected by alkaline phosphatase treatment. FPLC analysis demonstrated that any mononucleotide contamination was removed from stocks of Ap_4A, Ap_5A or Ap_6A by enzyme treatment leaving the diadenosines unaffected. When untreated samples of Ap_3A were analysed by FPLC no contamination with nucleotides was evident. It was concluded that the $[Ca^{2+}]_c$ responses seen in ECV304 cells to the diadenosine polyphosphates Ap_4A, Ap_5A or Ap_6A were solely due to a minor ATP contaminant but those to Ap_3A were mediated by a direct effect at a receptor.

Discussion

Our aim was to study the P2 receptors present on the human endothelial cell line, ECV304, with a particular emphasis on the effects of the diadenosine polyphosphates. In so doing we found that ECV304 cells express at least two P2 receptors.

The first, recognized both ADP and 2-methylthioATP with high affinity and was present in a proportion of the cell population. Cells unable to respond to 2-methylthioATP were also unable to respond to ADP, providing that the samples had been cleared of any nucleotide triphosphate contamination by hexokinase treatment. The response to ADP or 2-methylthioATP was unaffected by prestimulation with 100 μM UTP indicating that ADP and 2-methylthioATP are recognized by a receptor distinct to that which recognizes UTP. The response to 2-methylthioATP was inhibited by both PPADS and suramin, a property of the $P2Y_1$ receptor (Webb *et al.*, 1993; Brown *et al.*, 1995; Charlton *et al.*, 1996a). Likewise the $P2Y_1$ receptor recognizes both ADP and 2-methylthioATP with high affinity, but does not recognize UTP (Simon *et al.*, 1995; Léon *et al.*, 1997). The $P2Y_{11}$ receptor is also sensitive to 2-methylthioATP and ADP, but in contrast to the $P2Y_1$ receptor has only a low affinity for ADP (Communi *et al.*, 1997). This pharmacology therefore indicates that the $P2Y_1$ receptor rather than the $P2Y_{11}$ receptor is present in ECV304 cells. Consistent with the presence of an Ap_3A-sensitive $P2Y_1$ receptor on ECV304 cells, the cloned $P2Y_1$ receptor is also sensitive to Ap_3A (Pintor *et al.*, 1996; Schachter *et al.*, 1996).

All cells tested responded to ATP, ATPγS and UTP with a large rise in $[Ca^{2+}]_c$, sustained for the duration of stimulation with the agonist. A maximal concentration of ATP desensitized the cells to a further response to either ATP or UTP, demonstrating that both of these agonists were recognized by the same receptor. Agonist concentration response curves showed UTP and ATP to be approximately equipotent on ECV304 cells. When cells were stimulated with a maximal concentration of UTP, a further stimulation with UTP failed to generate a $[Ca^{2+}]_c$ response. Upon a subsequent addition of ATP, only a small rise in $[Ca^{2+}]_c$ was observed. This response could be attributed to either minor ADP contamination of the ATP stock or an ATP response acting on the putative $P2Y_1$ receptor. Since we found the UTP-sensitive receptor to have an equal affinity for both ATP and UTP, and to be insensitive to both ADP and 2-methylthioATP, the pharmacology is consistent with the presence of a $P2Y_2$ receptor (Lustig *et al.*, 1993; Parr *et al.*, 1994; Nicholas *et al.*, 1996). The additional presence of the $P2Y_4$ receptor cannot be discounted since the concentrations used to desensitize the cells to UTP and ATP are likely to desensitize $P2Y_4$ receptors in addition to $P2Y_2$ receptors (Communi *et al.*, 1995; Nicholas *et al.*, 1996). The lack of response to UDP would suggest that ECV304 cells do not express the $P2Y_6$ receptor (Communi *et al.*, 1996; Chang *et al.*, 1995).

We tested the sensitivity of the UTP response to the P2-antagonists, suramin and PPADS. $P2Y_2$ receptors are reported to be insensitive to PPADS and only partially sensitive to suramin (Wilkinson *et al.*, 1993; Chen *et al.*, 1996; Charlton *et al.*, 1996a), whereas the $P2Y_4$ receptor is insensitive to both of these compounds (Charlton *et al.*, 1996b). The $[Ca^{2+}]_c$ response to UTP in ECV304 cells was insensitive to inhibition by both PPADS and suramin. However we are cautious about using suramin as a diagnostic tool to distinguish between the $P2Y_2$- and $P2Y_4$ receptor-mediated responses in ECV304 cells, since the equal affinity shown by UTP and ATP indicates the presence of a $P2Y_2$ receptor. We suggest that the ECV304 cell

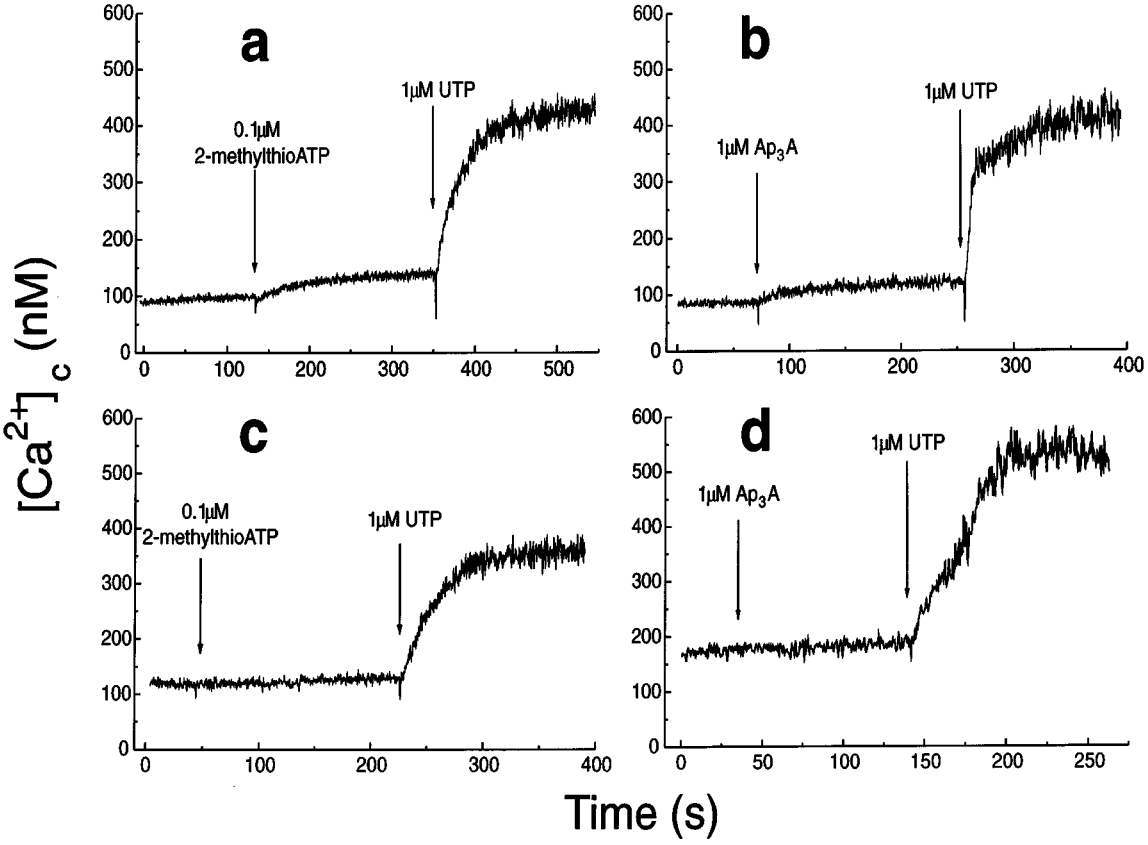

Figure 6 $[Ca^{2+}]_c$ responses in the presence or absence of PPADS. ECV304 cells were stimulated by the bolus addition of agonist to the sample cuvette at the points indicated by the arrows. Control responses to 2-methylthioATP and Ap_3A are shown in Figures 6a,b. PPADS (30 μM) was added to ECV304 cells in Figures 6c,d prior to stimulation with agonist. Similar responses were obtained with single agonist additions.

$P2Y_2$ receptor is insensitive to both PPADS and suramin under our experimental conditions.

When samples of diadenosine polyphosphates were treated with alkaline phosphatase, Ap_4A, Ap_5A and Ap_6A did not evoke a $[Ca^{2+}]_c$ response. Ap_5A and Ap_6A elicited no response at either the cloned $P2Y_1$ or $P2Y_2$ receptors (Lazarowski *et al.*, 1995; Pintor *et al.*, 1996; Schachter *et al.*, 1996). Ap_4A however, demonstrated a high potency at the cloned $P2Y_2$ receptor when expressed in human astrocytoma cells (Lazarowski *et al.*, 1995). The long time points used in that study mean that substantial amounts of monoadenine nucleotides could have accumulated from the hydrolysis of the diadenosines (Holler, 1992). However, this may not account entirely for the apparent high affinity of Ap_4A at this receptor, since Ap_5A and Ap_6A showed a very low affinity for the cloned $P2Y_2$ receptor. The cloned $P2Y_1$ receptor was also apparently sensitive to Ap_4A, although the responses were variable and possibly dependent on the expression system used (Pintor *et al.*, 1996; Schachter *et al.*, 1996).

Sensitivity to Ap_4A may be a consequence of the high expression levels of both the cloned $P2Y_1$ and $P2Y_2$ receptors. From our data it appears that when human endothelial cells express the endogenous $P2Y_1$ and $P2Y_2$ receptors these receptors are effectively insensitive to the diadenosine polyphosphates Ap_4A, Ap_5A and Ap_6A.

ECV304 cells also demonstrate a $[Ca^{2+}]_c$ response to AMP and adenosine. The receptor could be either the A_1, A_3 or A_{2B} adenosine receptor, since these P1 receptors are linked to an increase in $[Ca^{2+}]_c$ in addition to cAMP (Palmer & Stiles, 1995), however we have not attempted any further characterization.

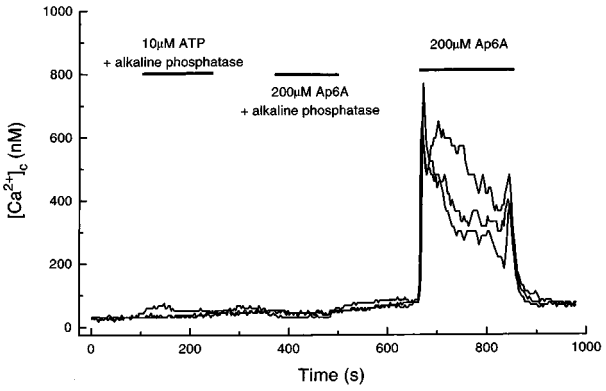

Figure 7 Ap_6A-evoked $[Ca^{2+}]_c$ responses in single, fura-2 loaded ECV304 cells. Cells were perfused in the presence of 1 mM Ca^{2+}, for 5 min with HBS and then with HBS plus agonist for the time indicated by the horizontal bar. Agonists treated with alkaline phosphatase were incubated at 37°C for 30 min prior to bolus addition to the sample well. The trace is representative of three separate experiments, each with approximately 20 cells. Similar results were obtained with Ap_4A and Ap_5A.

In conclusion, ECV304 cells exhibit a pharmacology consistent with the presence of P1 receptors and at least two P2Y receptor subtypes; a $P2Y_2$ receptor equally sensitive to

ATP and UTP and insensitive to the diadenosine polyphosphates and a $P2Y_1$ receptor sensitive to 2-methylthioATP, ADP and Ap_3A. During the preparation of this manuscript a report was published demonstrating the presence of mRNA transcripts for $P2Y_1$-, $P2Y_2$-, $P2Y_4$- and $P2Y_6$ receptors, in human umbilical vein endothelial cells (Jin *et al*., 1998). This confirms our conclusions about the presence of $P2Y_1$, $P2Y_2$ and possibly $P2Y_4$ receptors in ECV304 cells, however we found no pharmacological evidence for the presence of $P2Y_6$ receptors.

It should be noted that when working with diadenosine polyphosphates, care should be taken in confirming that a response to these compounds is not due to either a minor degradation product resulting from contamination of the original stock or degradation by extracellular enzymes. From these studies it seems likely that the diadenosine polyphosphates, with the exception of Ap_3A, do not elicit any vasodilatory effects directly at human endothelial $P2Y_2$- and $P2Y_1$ receptors but rather act *via* their degradation products.

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