Br. J. Pharmacol. (1988), 95, 1181–1190

Regulation of P_{2y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration

Thomas D. Carter, *Trevor J. Hallam, †[1]Noel J. Cusack & [2]Jeremy D. Pearson

Section of Vascular Biology, MRC Clinical Research Centre, Harrow, Middx. HA1 3UJ; *Department of Cellular Pharmacology, Smith, Kline & French Research Ltd., The Frythe, Welwyn, Herts, AL6 9AR and †Department of Pharmacology, King's College, Strand, London WC2R 2LS

1 ATP and ATP analogues induced prostacyclin (PGI_2) secretion from human cultured umbilical vein endothelial cells.

2 The threshold active concentration for ATP was $\leqslant 1\ \mu M$. The rank order of potency of analogues was 2-chloroadenosine 5′-triphosphate (2-ClATP) > 2-methylthioadenosine 5′-triphospate (2-MeSATP) > ATP > ADP, while adenosine 5′-(α,β-methylene)triphosphonate, AMP and adenosine were inactive, indicating the presence of P_{2y}-purinoceptors.

3 In contrast to their actions on P_{2y}-receptors in guinea-pig taenia coli, isopolar analogues of 2-methylthioadenosine 5′-(β,γ-methylene)triphosphonate were less effective than ATP.

4 ATP and ATP analogues increased intracellular free calcium ions, $[Ca^{2+}]_i$, giving a rapid transient peak due predominantly to release from intracellular stores, followed by a maintained steady-state elevated level due to influx.

5 The dose-response curves for peak $[Ca^{2+}]_i$ induced by ATP, 2-ClATP and 2-MeSATP were very similar to those for PGI_2 production.

6 Elevations of $[Ca^{2+}]_i$, above a threshold value of 0.8–1 μM, were necessary for PGI_2 production in response to P_{2y}-receptor activation.

7 The dose relationships between PGI_2 release and peak $[Ca^{2+}]_i$ were equivalent whether $[Ca^{2+}]_i$ was raised by ionomycin or via P_{2y}-receptor activation by ATP or 2-ClATP, indicating that elevations of $[Ca^{2+}]_i$ provide the major, if not the exclusive intracellular pathway for P_{2y}-purinoceptor-mediated PGI_2 synthesis.

Introduction

Vascular endothelial cells modulate the activity of adjacent smooth muscle cells and platelets in response to a variety of stimuli, through the release of the vasoactive mediators endothelium-derived relaxant factor (EDRF; Furchgott & Zawadski, 1980) and prostacyclin (PGI_2; Moncada *et al.*, 1977). Extracellular adenosine 5′-triphosphate (ATP) has actions via P_2-purinoceptors on a variety of tissues (Burnstock & Kennedy, 1985; Gordon, 1986), but in particular it is a potent inducer of EDRF release in vascular tissue (De Mey & Vanhoutte, 1981; Gordon & Martin, 1983) and of PGI_2 release from perfused vascular beds and cultured endothelial cells (Schwartzman & Raz, 1982; Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1983; McIntyre *et al.*, 1985). However, the intracellular mechanisms that couple activation of P_2-purinoceptors to EDRF or PGI_2 synthesis are not known.

Several observations suggest that elevated levels of intracellular free calcium ions, $[Ca^{2+}]_i$, play an important role in PGI_2 production, the most compelling of which come from studies where $[Ca^{2+}]_i$ have been measured directly in endothelial cells loaded with the calcium-sensitive fluorescent dyes quin-2 or fura-2. Thus ATP, adenosine 5′-diphosphate (ADP), histamine, bradykinin and thrombin, which each induce endothelial PGI_2 release, also elevate $[Ca^{2+}]_i$ (Luckoff & Busse, 1986; Hallam & Pearson, 1986; Hamilton & Sims, 1987; Rotrosen & Gallin, 1987; Hallam *et al.*, 1988). By

[1] Present address: Nelson Research Centre, 1001 Health Sciences Road West, Irvine, CA 92715, U.S.A.
[2] Author for correspondence.

© The Macmillan Press Ltd 1988

concomitant measurement of $[Ca^{2+}]_i$ and PGI_2 release in adherent monolayers of fura-2 loaded human endothelial cells we found that elevation of $[Ca^{2+}]_i$ above a threshold value of 0.8–1.0 μM is necessary for thrombin-stimulated PGI_2 release (Hallam *et al.*, 1988).

In this study we have used a series of ATP analogues to characterize the P_2-purinoceptor on human umbilical vein endothelial cells that mediates PGI_2 production. We then determined that PGI_2 synthesis in response to activation of this receptor requires elevated $[Ca^{2+}]_i$ and, in addition, that this rise in $[Ca^{2+}]_i$ provides the major, if not the exclusive, transduction signal for prostanoid synthesis.

Methods

Cell culture

Endothelial cells were isolated and cultured from segments of human umbilical vein as previously described (Jaffe *et al.*, 1973; Hallam *et al.*, 1988). For experiments, cells from confluent primary cultures were detached by brief trypsinization, resuspended in growth medium and seeded at near confluent density in 16 mm diameter plastic wells of 24-well trays (Nunc) or on glass coverslips (22 mm × 11 mm). One to two days later, when confluent, cells were used for determination of PGI_2 release (on coverslips or in wells) or for measurement of $[Ca^{2+}]_i$ (on coverslips).

Determination of PGI_2 release

Confluent monolayers of cells in 16 mm diameter wells ($\simeq 10^5$ cells per well) were used for the replicate determination of PGI_2 release. The complete growth medium in each well was replaced with serum-free 20 mM HEPES-buffered Dulbecco's medium. After 30 min it was carefully removed and replaced with 1.2 ml of the same medium. Five minutes later 0.2 ml was removed and stored at 4°C. A further 0.2 ml aliquot was removed 25 min later followed immediately by the addition of 0.2 ml of medium containing a stimulatory agonist. A final sample was taken 5 min later. The stable hydrolysis product of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), was determined in each aliquot by specific radioimmunoassay (Ager *et al.*, 1982).

Basal PGI_2 release rates were measured over the period 5–30 min, thus eliminating the transient release caused simply by removing and replacing the medium; and stimulated release rates were measured over the 30–35 min period.

In some experiments PGI_2 release was determined concomitantly with $[Ca^{2+}]_i$ by subsampling the medium above coverslips of fura-2 loaded cells (see below).

Determination of $[Ca^{2+}]_i$

Confluent monolayers of cells on glass coverslips were loaded with fura-2 by incubating the cells with 1 μM fura-2 acetoxymethyl ester in Dulbecco's medium containing 20% (v/v) foetal calf serum for 45 min at 37°C (Hallam & Pearson, 1986). The coverslips were then transferred to a HEPES-buffered physiological saline of the following composition (in mM): NaCl 145, KCl 5, $MgSO_4$ 1, HEPES, 10, $CaCl_2$ 1, glucose 10; pH 7.4 at 37°C. Cells on coverslips were then allowed to cool to room temperature to minimize the loss of active dye through leakage from the cells, and used within 60 min. For each experiment a coverslip was placed diagonally in a quartz cuvette containing buffer. Fluorescence was recorded continuously (excitation wavelength 340 nm; emission 500 nm) in an Aminco-Bowman fluorescence spectrometer with a thermostatted cuvette holder at 37°C. Calibration of the fluorescent signal was as described by Hallam & Pearson (1986).

Assessment of enhanced permeability in response to ATP

Confluent monolayers of cells in 16 mm diameter wells were treated with various doses of ATP for 5 min using exactly the same experimental protocol as that for determination of stimulated PGI_2 release. Twenty five μl of 1 mM ethidium bromide were then added to each well for 5 min, after which the monolayers were rinsed in phosphate-buffered saline and examined by fluorescence microscopy using a Leitz Dialux microscope with an IT-2-auramine filter. Cells that are impermeable to the dye do not fluoresce, those rendered permeable have fluorescent nuclei (due to dye intercalated with DNA) and can be counted (Gomperts, 1983). For positive controls, cells were electropermeabilised (4 exposures of 2.0 KV cm^{-1}, $\tau \simeq 5\,\mu s$: Knight & Baker, 1982) before addition of ethidium bromide: this treatment rendered >95% of cells permeable.

Drugs

ATP, ADP, adenosine 5′-monophosphate (AMP), adenosine 5′-(α,β-methylene)-triphosphonate (APCPP), adenosine and histamine diphosphate were from Sigma Chemical Co.; fura-2 and ionomycin from Calbiochem; and mepyramine maleate from May and Baker. Adenosine 5′-O-(3-thiotriphosphate) (ATPγS) was from Boehringer Mannheim. 2-Chloroadenosine 5′-triphosphate (2-ClATP) and 2-methylthioadenosine 5′-triphosphate (2-MeSATP) were synthesized as described by Gough *et al.* (1973). The isopolar analogues 2-methylthioadenosine 5′-(β,

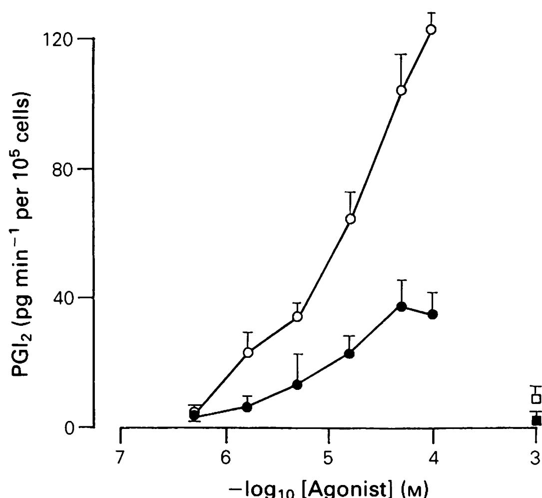

Figure 1 Dose-related prostacyclin (PGI_2) release from human umbilical vein endothelial cells in response to (○) ATP, (●) ADP, (□) AMP or (■) adenosine. Points show means of 4–10 observations, except 50 μM ATP (37 observations), and vertical lines indicate s.e.

γ-methylene) triphosphonate (2-MeSAPPCP), 2-methylthioadenosine 5'-(β,γ-dichloromethylene)triphosphonate (2-MeSAPPCCl_2P) and 2-methylthioadenosine 5'-(β,γ-difluoromethylene) triphosphonate (2-MeSAPPCF_2P) were synthesized as previously described by Cusack *et al.* (1987).

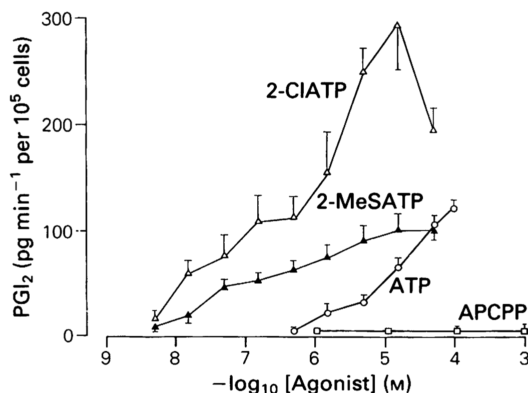

Figure 2 Dose-related prostacyclin (PGI_2) release from human umbilical vein endothelial cells in response to 2-chloroadenosine 5'-triphosphate (2-ClATP), 2-methylthioadenosine 5'-triphosphate (2-MeSATP), ATP or adenosine 5'-(α,β-methylene)-triphosphonate (APCPP). Points show means of 5–11 observations and vertical lines indicate s.e.

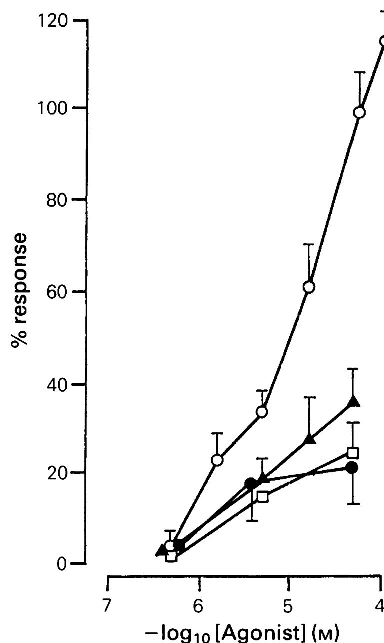

Figure 3 Dose-related prostacyclin (PGI_2) release from human umbilical vein endothelial cells in response to ATP (○), 2-methylthioadenosine 5'-(β,γ-difluoromethylene) triphosphonate (2-MeSAPPCF_2P; ▲), 2-methylthioadenosine 5'-(β,γ-dichloromethylene) triphosphonate (2-MeSAPPCCl_2P; ●) or 2-methylthioadenosine 5'-(β,γ-methylene) triphosphonate (2-MeSAPPCP; □). Points show means and vertical lines s.e. of 4–10 observations (ATP) or 5–9 observations (analogues), apart from single estimates for the 2 lower doses of 2-MeSAPPCP. The release is expressed as a % of that in response to 50 μM ATP.

Results

PGI_2 release

In initial experiments we determined that addition of ATP, ADP or ATPγs each caused a transient (2–3 min) burst of PGI_2 release from human umbilical vein endothelial cells. Thereafter the standardised protocol described in Methods was adopted, with stimulated PGI_2 release being estimated after 5 min exposure to agonist.

Figure 1 shows that ATP induced PGI_2 release in a dose-dependent manner, with a threshold active dose of < 1 μM. ADP caused dose-dependent release of PGI_2 with a similar minimally active dose, but responses were consistently $\simeq$30% of those to ATP. AMP and adenosine (up to 1 mM) were inactive.

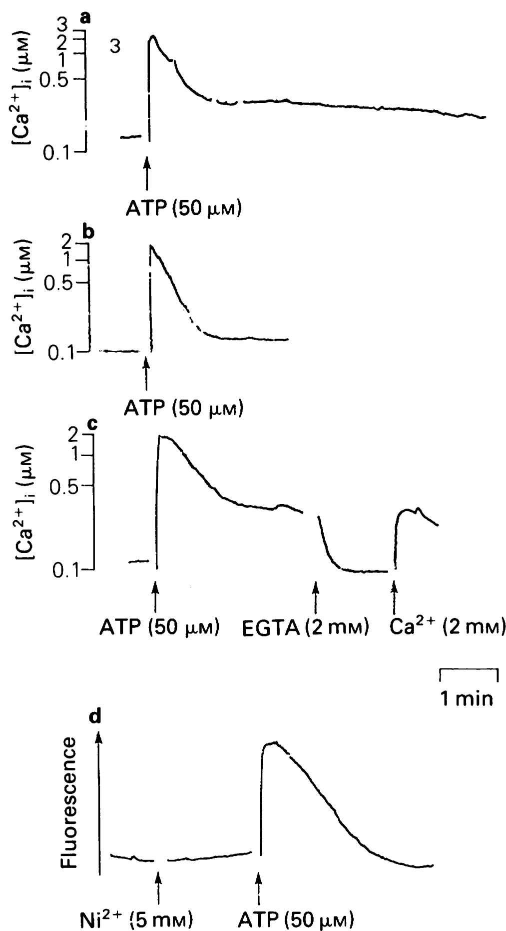

Figure 4 $[Ca^{2+}]_i$ responses to ATP in human umbilical vein endothelial cells. (a), (c) and (d) show examples of responses in the presence of extracellular Ca^{2+} (1 mM); (b) shows a response in the absence of extracellular Ca^{2+} (1 mM EGTA).

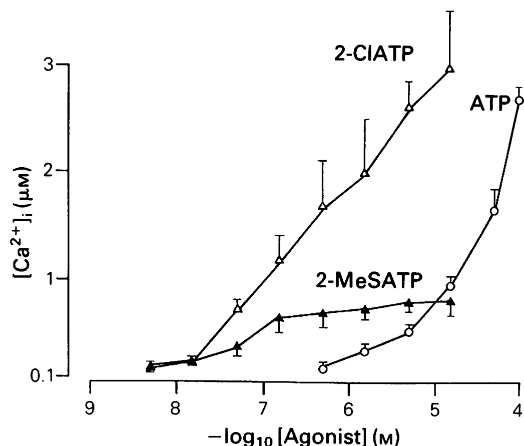

Figure 5 Dose-related peak elevations of $[Ca^{2+}]_i$ in human umbilical vein endothelial cells in response to 2-chloroadenosine 5'-triphosphate (2-ClATP), 2-methylthioadenosine 5'-triphosphate (2-MeSATP) or ATP. Points show means and vertical lines s.e. of 6–12 observations (ATP), or 4–7 observations (analogues).

2-C1ATP was the most potent analogue tested, with a threshold dose of 10 nM. Unlike the other analogues it apparently produced a higher maximal response than ATP (Figure 2), though it should be noted that at the highest tested dose (100 μM) the response to ATP was sub-maximal. 2-MeSATP was also considerably more potent than ATP at low doses, but the two were approximately equiactive at a concentration of 50 μM, because the dose-response curve was not parallel to that of ATP. APCPP (< 1 mM) was inactive (Figure 2). The isopolar analogue series 2-MeSAPPCP, 2-MeSAPPCl$_2$P and 2-MeSAPPCF$_2$P were all approximately equiactive, but substantially less effective than ATP (Figure 3).

P_2-purinoceptor-mediated elevation of $[Ca^{2+}]_i$

Resting levels of $[Ca^{2+}]_i$ in non-stimulated cells in 1 mM $[Ca^{2+}]$-containing buffer were 100 ± 4 nM (mean ± s.e., $n = 81$). Addition of ATP stimulated rapid dose-dependent increases in $[Ca^{2+}]_i$ that peaked within 10–15 s and then declined over 2–3 min to a steady-state elevated level that was evident for > 5 min thereafter (Figure 4a). When experiments were carried out in calcium-depleted medium, using buffer in which 1 mM EGTA replaced 1 mM $CaCl_2$, resting levels of $[Ca^{2+}]_i$ were not significantly different (113 ± 19 nM, $n = 12$), and stimulation by ATP resulted in an initial elevation of $[Ca^{2+}]_i$ similar to that found in the presence of external Ca^{2+}. However, over the next 1–2 min $[Ca^{2+}]_i$ declined to resting levels with no steady-state elevation (Figure 4b). In the continued presence of the agonist, the sustained elevated levels of $[Ca^{2+}]_i$ were regulated by extracellular $[Ca^{2+}]$: Figure 4c shows that removal or addition of extracellular Ca^{2+} caused rapid changes of $[Ca^{2+}]_i$ between elevated and resting levels. Addition of 5 mM $[Ni^{2+}]$ before stimulation with ATP in the presence of Ca^{2+} caused no detectable change in resting $[Ca^{2+}]_i$, but the subsequent response was analogous to that when EGTA was present, i.e. no sustained elevation of $[Ca^{2+}]_i$ occurred (Figure 4d). ATP-induced changes in $[Ca^{2+}]_i$ were not altered by pre-incubation for 5 min with 10 μM nifedipine (data not shown).

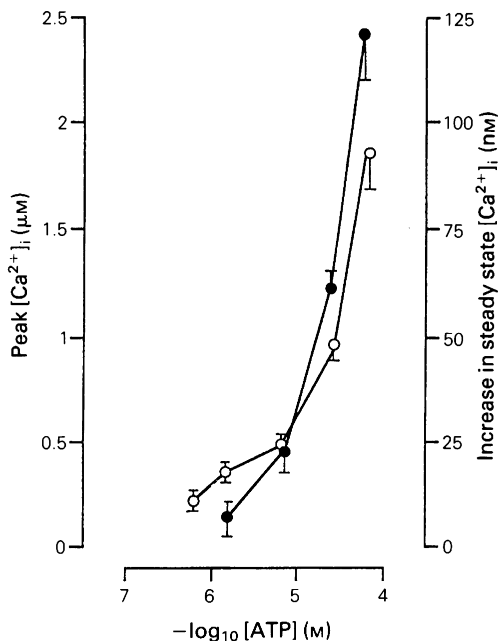

Figure 6 Comparison of dose-response relationships for peak elevations of $[Ca^{2+}]_i$ (○) and steady-state elevations of $[Ca^{2+}]_i$ (●) in human umbilical vein endothelial cells in response to ATP. Points show means and vertical lines s.e. of 6–12 observations (peak Ca values) and 3–10 observations (steady state). The steady state values are expressed as the increase above pre-stimulated levels and were measured 2 min after addition of ATP.

2-ClATP, 2-MeSATP and 2-MeSAPPCF$_2$P each stimulated transient changes in $[Ca^{2+}]_i$ with the same characteristics as those induced by ATP. The dose-response relationships between peak $[Ca^{2+}]_i$ and added ATP, 2-ClATP or 2-MeSATP are shown in Figure 5: 2-ClATP was about 100 × more potent than ATP. 2-MeSATP was intermediate in potency between 2-ClATP and ATP, whereas 2-MeSAPPCF$_2$P (not shown) was less potent than ATP: 15 μM 2-MeSAPPCF$_2$P raised $[Ca^{2+}]_i$ to 230 ± 25 nM ($n = 3$). Figure 6 demonstrates that the steady-state elevation of $[Ca^{2+}]_i$ was also related to the dose of agonist used.

Relationship beween PGI$_2$ release and elevated $[Ca^{2+}]_i$

The strong similarities between the potency and effectiveness of 2-ClATP, 2-MeSATP and ATP as stimuli for PGI$_2$ release and the elevation of $[Ca^{2+}]_i$ (cf. Figures 2 and 5) suggested that the two processes were causally linked. To determine whether elevated $[Ca^{2+}]_i$ was necessary for PGI$_2$ production in response to ATP and its analogues, endothelial cells were depleted of agonist-sensitive intracellular calcium pools, by pretreatment with histamine in the absence of extracellular Ca^{2+}, before testing responses to ATP.

Figure 7a demonstrates that in the presence of extracellular Ca^{2+} histamine (100 μM) produced a maximally detectable rise in $[Ca^{2+}]_i$, with a similar time-course to that induced by ATP, and that the sustained elevated level of $[Ca^{2+}]_i$ was rapidly abolished by the H$_1$-antagonist mepyramine. The data shown in Figure 7b confirm that if the antagonist was added first, histamine was completely inactive. Figure 7c illustrates the $[Ca^{2+}]_i$ response to 50 μM ATP in the absence of extracellular Ca^{2+}, and Figure 7d shows that the response was unchanged by pretreatment with mepyramine followed by histamine. In contrast (Figure 7e), in the absence of extracellular Ca^{2+}, ATP was unable to induce any rise in $[Ca^{2+}]_i$ when the cells were first exposed to histamine followed by mepyramine. When extracellular Ca^{2+} was introduced 3 min later, $[Ca^{2+}]_i$ levels rose over approximately 60 s to reach a steady-state elevated level of 290 ± 30 nM ($n = 6$).

The results obtained when concomitant measurements of $[Ca^{2+}]_i$ and PGI$_2$ release were made throughout this procedure are shown in Figure 8. Histamine caused transient elevation of $[Ca^{2+}]_i$ and PGI$_2$ synthesis over 2 min. Subsequent addition of mepyramine had no effect, and when ATP was then added no PGI$_2$ release or $[Ca^{2+}]_i$ elevation occurred. To ensure that pretreatment with histamine had not depleted arachidonate-rich phospholipids, leading to a general inability to synthesize PGI$_2$, extracellular Ca^{2+} and ionphore were added, which caused rapid elevation of $[Ca^{2+}]_i$ and further PGI$_2$ release.

Finally we examined the dose-response relationship between $[Ca^{2+}]_i$ and PGI$_2$ synthesis. Cells were exposed to graded doses (1 nM–3 μM) of ionomycin in the absence of extracellular Ca^{2+}. The resultant levels of 6-keto-PGF$_{1\alpha}$ accumulated after 5 min were then plotted against the corresponding peak $[Ca^{2+}]_i$ (Figure 9). No PGI$_2$ production was evident until $[Ca^{2+}]_i$ rose to 0.8–1.0 μM, whilst above this threshold PGI$_2$ release increased with increasing $[Ca^{2+}]_i$. Similar experiments were then performed with ATP (0.15–50 μM) and 2-ClATP (0.005–1.5 μM), yielding points that fell on the same Ca^{2+}-activation curve (Figure 9).

Assessment of enhanced permeability in response to ATP

Under the conditions outlined in the Methods section, exposure of cells to 50–500 μM ATP for

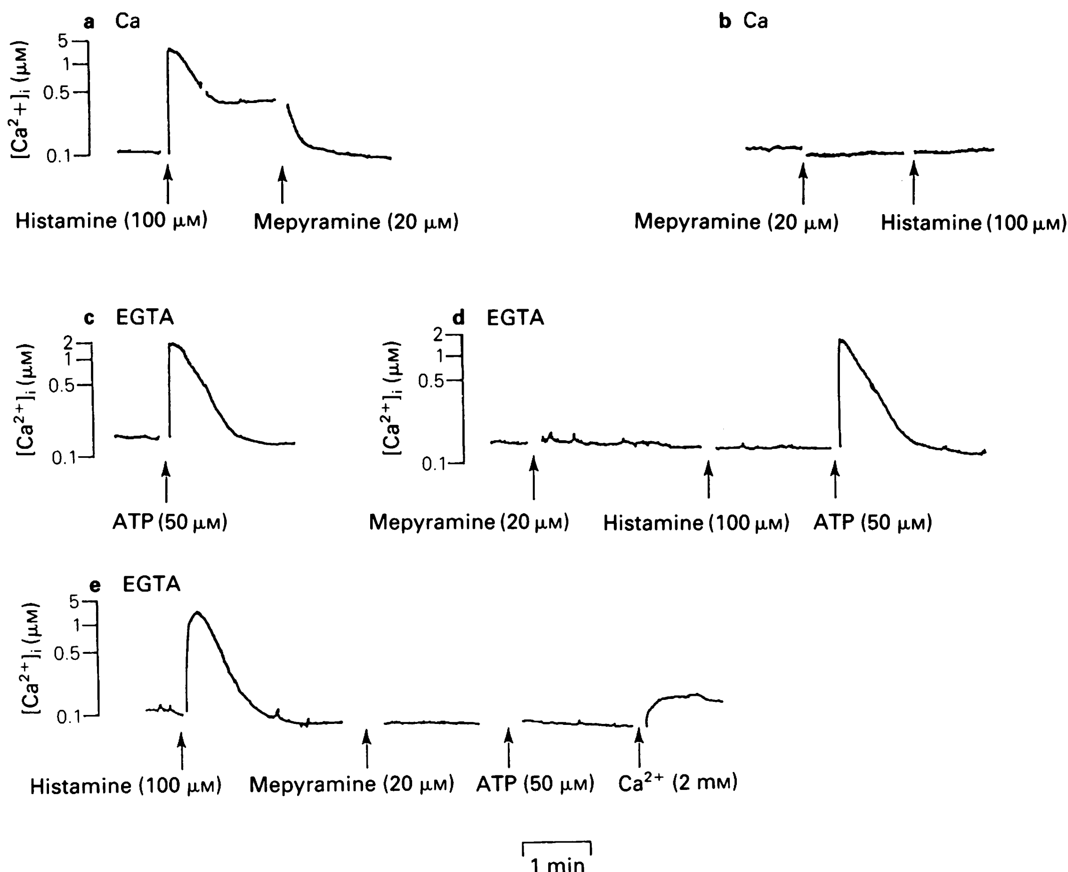

Figure 7 Depletion of internal Ca^{2+} stores in human umbilical vein endothelial cells by treatment with a maximally effective dose of histamine in the absence of external Ca^{2+}. Traces (a) and (b) demonstrate the ability of mepyramine to reverse or block the action of histamine in the presence of external Ca^{2+} (1 mM). Traces (c) and (d) demonstrate that histamine treatment after mepyramine in the absence of external Ca^{2+} (1 mM EGTA) does not alter responses to ATP. Trace (e) demonstrates that, after depletion of internal Ca^{2+} with histamine, ATP cannot increase $[Ca^{2+}]_i$ in the absence of external Ca^{2+} (1 mM EGTA).

5 min did not induce any permeability change detectable by ethidium bromide uptake.

Discussion

Characterization of the purinoceptor

Previous studies have shown that porcine aortic cells possess P_2-purinoceptors, apparently of the P_{2y}-subtype as defined by Burnstock & Kennedy (1985), which are linked to PGI_2 synthesis and EDRF production (Pearson *et al.*, 1983; Martin *et al.*, 1985; Needham *et al.*, 1987). In addition, in porcine and bovine endothelial cells P_2-receptor occupation is accompanied by transient elevations in $[Ca^{2+}]_i$ (Hallam & Pearson, 1986; Luckhoff & Busse, 1986). Characterization of P_2-purinoceptors on human endothelial cells has not been obtained previously. Furthermore, no study of the kinetics of the $[Ca^{2+}]_i$ changes following endothelial P_2-receptor occupation, their dependence on extracellular Ca^{2+}, and their relationship to PGI_2 or EDRF release has been attempted before.

Our results demonstrate that human umbilical vein cells synthesize PGI_2 in response to stimulation of P_2-purinoceptors, i.e. when exposed to ATP or ADP but not to AMP or adenosine (Burnstock, 1978). The response was transient regardless of whether the ATP analogues tested were susceptible or resistant (ATPγS; Cusack *et al.*, 1983) to hydro-

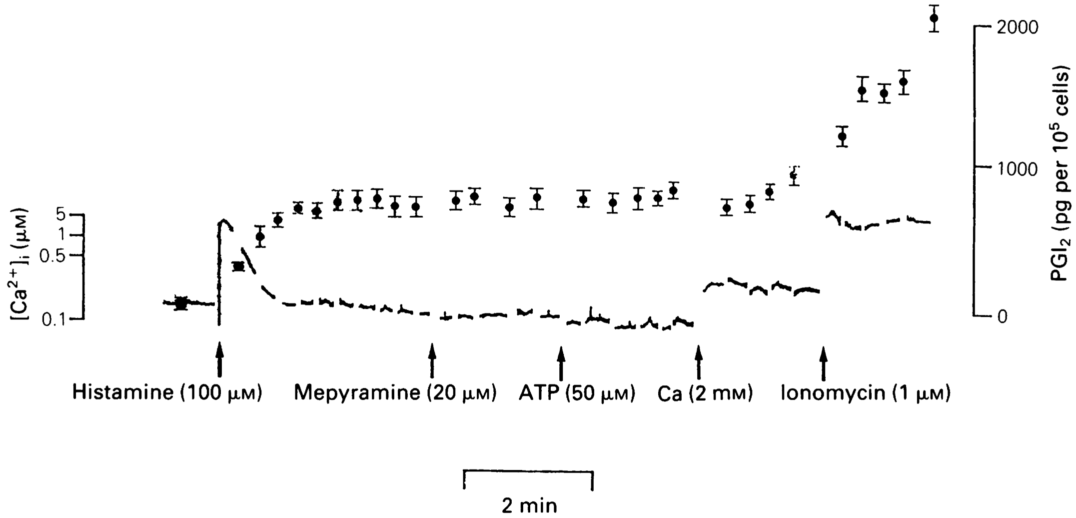

Figure 8 Correlation between $[Ca^{2+}]_i$ changes and prostacyclin (PGI_2) release. Cells were treated according to the protocol in Figure 7e, in the absence of external Ca^{2+} (1 mM EGTA). The $[Ca^{2+}]_i$ trace is a single example. PGI_2 release measurements show means and vertical lines s.e. from 8 replicate experiments.

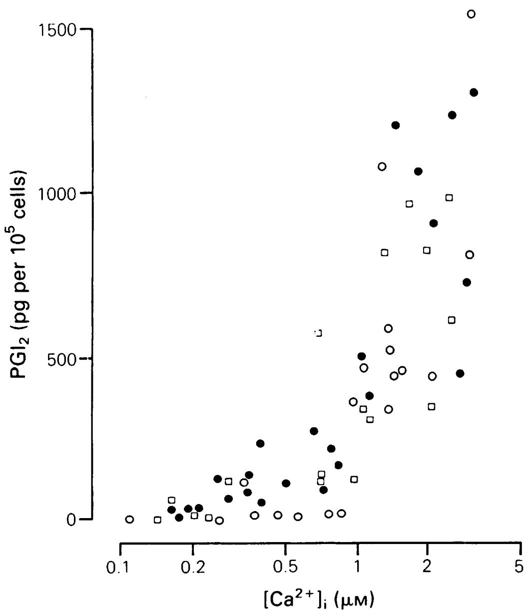

Figure 9 Dose-response relationship between peak $[Ca^{2+}]_i$ and prostacyclin (PGI_2) release in human umbilical vein endothelial cells in response to ionomycin (●), ATP (□) or 2-chloroadenosine 5′-triphosphate (○). 6-Keto-prostaglandin $F_{1\alpha}$ was measured 5 min after addition of the agonist.

lysis by ectonucleotidases, indicating that tachyphylaxis occurred. Other data from our laboratory demonstrate that this is due to events at or near the receptor (Toothill *et al.*, 1988). ATP was a less effective agonist in human than in porcine endothelial cells, giving release rates of 50 × basal in response to 50 μM ATP whereas porcine cells yielded up to 1000 × basal rate (Needham *et al.*, 1987).

The increased potency of 2-ClATP and 2-MeSATP, together with the lack of activity of APCPP, indicate that the purinoceptor is of the P_{2y}-subtype as classified by Burnstock & Kennedy (1985) and Gordon (1986), like the purinoceptor we previously described on porcine endothelium (Martin *et al.*, 1985; Needham *et al.*, 1987). It has recently been demonstrated that the rank order of potency of the isopolar analogues 2-MeSAPPCP, 2-MeSAPPCCl$_2$P, 2-MeSAPPCF$_2$P was correlated with their electronegativity when relaxing guinea-pig taenia coli, while their ability to constrict guinea-pig urinary bladder was not related to electronegativity (Cusack *et al.*, 1987). Thus 2-MeSAPPCF$_2$P was slightly more potent than ATP itself and 70 × more potent than 2-MeSAPPCP on taenia coli, and it was suggested that this feature might be a characteristic of P_{2y}-purinoceptors (Cusack *et al.*, 1988). In our experiments, however, this series of isopolar analogues was approximately equipotent and all were

less effective than ATP, indicating that the endothelial P_{2y}-purinoceptor does not have the same characteristics as that on taenia coli with respect to its response to these analogues.

In several cell types exogenous ATP non-selectively increases membrane permeability so that low M_R components (including ATP) can be released (Gordon, 1986). In the mast cell the pharmacological characterization of a P_2-receptor, which recognises ATP^{4-} and is linked to enhanced membrane permeability and histamine secretion (Cockcroft & Gomperts, 1980), has recently been described (Tatham et al., 1988). This receptor has some features that are reminiscent of the P_{2y}-purinoceptor; e.g. it is recognised by 2-substituted analogues and not, except at very high doses, by APCPP, though it is clearly a distinct P_2-purinoceptor type. Since we have previously noted purine release from perfused columns of porcine aortic endothelial cells cultured on microcarrier beads in response to 100 μM ATP (Needham et al., 1987), we were concerned to determine whether any enhanced permeability was detectable in our current experiments. The lack of any effect of ATP at doses of up to 500 μM demonstrates, first, that the dose-response curves for PGI_2 release obtained were not complicated by release of endogenous ATP. Second, it shows that endothelial P_{2y}-receptor occupation does not lead to enhanced membrane permeability of the type found in the mast cell, which has not been demonstrated previously.

P_{2y}-purinoceptor-mediated elevation of $[Ca^{2+}]_i$

ATP and its active analogues induced dose-dependent elevations of $[Ca^{2+}]_i$. In the presence of extracellular Ca^{2+}, the response consisted of two phases; an initial rapid and transient peak followed by a long-lasting plateau phase where the concentration achieved was also dose-related. We conclude that the first phase consists predominantly of discharge of Ca^{2+} from bound intracellular stores, because it was maintained in the absence of extracellular Ca^{2+} or when Ni^{2+} was added extracellularly in a concentration sufficient to block Ca^{2+} uptake (Hallam & Rink, 1985). We conclude that the second phase is due to influx of Ca^{2+} across the plasma membrane, because it was not found in the absence of extracellular Ca^{2+} or when Ca^{2+} influx was blocked by Ni^{2+}, and it could be reversibly inhibited by chelation of extracellular Ca^{2+} with EGTA.

Ca^{2+} influx was insensitive to pretreatment with a voltage-operated Ca^{2+} channel blocker (10 μM nifedipine). The influx of Ca^{2+} in response to histamine was rapidly terminated by the addition of an H_1-antagonist. We therefore presume that this Ca^{2+} influx in response either to histamine, ATP or thrombin (Hallam et al., 1988) is due to activation of a Ca^{2+} entry mechanism following agonist addition.

Relationship between PGI_2 release and elevated $[Ca^{2+}]_i$

ATP-induced PGI_2 release was transient and its extent was not markedly different in the presence or absence of extracellular $[Ca^{2+}]_i$, indicating that if raised $[Ca^{2+}]_i$ is involved in PGI_2 synthesis it is only the initial transient peak that is important. The similar dose-response curves for stimulation of $[Ca^{2+}]_i$ and of PGI_2 release by ATP or its 2-substituted analogues demonstrate that the two processes are closely related but do not indicate whether elevated $[Ca^{2+}]_i$ is necessary for PGI_2 synthesis. Cells were therefore depleted of intracellular Ca^{2+}, by challenge with a maximally effective dose of histamine in the absence of extracellular Ca^{2+}, and the histamine was then antagonized with mepyramine. Under these conditions ATP neither induced any change in $[Ca^{2+}]_i$ nor stimulated PGI_2 synthesis. The cells were still capable of PGI_2 synthesis when Ca^{2+} and ionomycin were added. Thus elevated $[Ca^{2+}]_i$ is required for ATP-induced PGI_2 synthesis.

$[Ca^{2+}]_i$ rose to a steady-state elevated level when external Ca^{2+} was replaced a few minutes after ATP in these experiments. The simplest interpretation (although others cannot be excluded) for this rise is that Ca^{2+} entry was stimulated and maintained in the presence of ATP. The lack of a full $[Ca^{2+}]_i$ transient response (with an initial peak of PGI_2 production) under these conditions implies that the internal store of Ca^{2+} cannot refill sufficiently quickly and/or, more plausibly, that the transduction mechanisms leading to its release rapidly become refractory to ATP. This is consistent with the rapid induction of homologous desensitization of agonist-mediated PGI_2 release in these cells (Toothill et al., 1988).

Induction of PGI_2 synthesis by ionomycin, i.e. by a mechanism solely dependent on elevation of $[Ca^{2+}]_i$, required a threshold $[Ca^{2+}]_i$ of 0.8–1 μM. The $[Ca^{2+}]_i/PGI_2$ dose-response relationships in response to either ATP or 2-ClATP were indistinguishable from that generated by ionomycin, demonstrating that elevation of $[Ca^{2+}]_i$ is the major, if not the only, stimulus for PGI_2 synthesis when endothelial P_{2y}-receptors are activated. Ionophores do not initiate endothelial inositol phospholipid turnover, whereas ATP does (Lambert et al., 1987; Pirotton et al., 1987). Thus while diacylglycerol formation, and hence protein kinase C modulated transduction pathways, may contribute to other P_{2y}-mediated

endothelial responses, they seem to be unimportant in initiating PGI_2 synthesis.

Conclusions

Human umbilical vein endothelial cells possess P_{2y}-purinoceptors linked to PGI_2 synthesis. P_{2y}-mediated PGI_2 release is due to transient elevation of $[Ca^{2+}]_i$ derived predominantly from intracellular bound stores, as we have previously found for thrombin-induced PGI_2 synthesis (Hallam *et al.*, 1988). P_{2y} agonists also stimulate Ca^{2+} influx. Whilst this influx contributes little to PGI_2 synthesis, it may well be relevant to the production of EDRF, which is longer-lasting and requires extracellular Ca^{2+} (Singer & Peach, 1984), and further experiments are needed to test this hypothesis.

We thank the Delivery Suite, Northwick Park Hospital, for their expert help in the collection of umbilical cords, and Professor B. Peskar (Ruhr University; Bochum) for his generous gift of 6-keto-prostaglandin $F_{1\alpha}$ antiserum. T.D.C. is the recipient of an M.R.C./Smith, Kline & French partnership research student award.

References

AGER, A., GORDON, J.L., MONCADA, S., PEARSON, J.D., SALMON, J.S. & TREVETHICK, M. (1982). Effects of isolation and culture on prostaglandin synthesis by porcine aortic endothelial and smooth muscle cells. *J. Cell. Physiol.*, **110**, 9–16.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: a multidisciplinary Approach*, ed. Staub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2 purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

COCKROFT, S. & GOMPERTS, B.A. (1980). The ATP^{4-} receptor of rat mast cells. *Biochem. J.*, **188**, 789–798.

CUSACK, N.J., PEARSON, J.D. & GORDON, J.L. (1983). Stereoselectivity of ectonucleotidases on vascular endothelial cells. *Biochem. J.*, **214**, 975–987.

CUSACK, N.J., HOURANI, S.M.O., LOIZOU, J.D. & WELFORD, L.A. (1987). Pharmacological effects of isopolar phosphate analogues of ATP on P_2 purinoceptors on guinea-pig taenia coli and urinary bladder. *Br. J. Pharmacol.*, **90**, 791–795.

CUSACK, N.J., WELFORD, L.A. & HOURANI, S.M.O. (1988). Studies of the P_2 purinoceptor using adenine nucleotide analogues. In *Adenosine and Adenine nucleotides: Physiology and Pharmacology*, ed. Paton, D.M. pp. 73–84, London: Taylor & Francis.

DE MEY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol.*, **316**, 347–355.

FURCHGOTT, R.F. & ZAWADSKI, J.R. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GOMPERTS, B.D. (1983). Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. *Nature*, **306**, 64–66.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GORDON, J.L. & MARTIN, W. (1983). Endothelium-dependent relaxation of the pig aorta: relationship to stimulated ^{86}Rb efflux from isolated endothelial cells. *Br. J. Pharmacol.*, **79**, 531–541.

GOUGH, G.R., MAGUIRE, M.H. & SATCHELL, D.G. (1973). Three new adenosine triphosphate analogs. Synthesis and effects on isolated gut. *J. Med. Chem.*, **16**, 1188–1190.

HALLAM, T.J. & PEARSON, J.D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS Letts.*, **207**, 95–99.

HALLAM, T.J. & RINK, T.J. (1985). Agonists stimulate divalent cation channels in the plasma membrane of human platelets. *FEBS Letts.*, **186**, 175–179.

HALLAM, T.J., PEARSON, J.D. & NEEDHAM, L. (1988). Thrombin-stimulated elevation of human endothelial cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochem. J.*, **251**, 243–249.

HAMILTON, K.K. & SIMMS, P.T. (1987). Changes in cytosolic Ca^{2+} associated with von Willebrand factor release in human endothelial cells exposed to histamine. *J. Clin. Invest.*, **79**, 600–608.

JAFFE, E.A., NACHMAN, R.L., BECKER, C.G. & MINICK, G.R. (1973). Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.*, **52**, 2745–2756.

KNIGHT, D.E. & BAKER, P.F. (1982). Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membrane Biol.*, **68**, 107–140.

LAMBERT, T.L., RICHARD, S.K. & WHARTON, R.A. (1986). Bradykinin stimulation of inositol polyphosphate production in porcine aortic endothelial cells. *J. Biol. Chem.*, **26**, 15288–15293.

LUCKHOFF, A. & BUSSE, R. (1986). Increased free calcium in endothelial cells under stimulation with adenosine nucleotides. *J. Cell. Physiol.*, **126**, 414–420.

MARTIN, W., CUSACK, N.J., CARLETON, J.S. & GORDON, J.L. (1985). Specificity of P_2-purinoceptor that mediates endothelium-dependent relaxation of the pig aorta. *Eur. J. Pharmacol.*, **108**, 295–299.

McINTYRE, T.M., ZIMMERMAN, G.A., SATOH, K. & PRESCOTT, S.M. (1985). Cultured endothelial cells synthesise both platelet activating factor and prostacyclin in response to histamine, bradykinin and adenosine triphosphate. *J. Clin. Invest.*, **76**, 271–280.

MONCADA, S., HERMAN, A.G., HIGGS, E.A. & VANE, J.R. (1977). Differential formation of prostacyclin by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium. *Thromb. Res.*, **11**, 323.

NEEDHAM, L., CUSACK, N.J., PEARSON, J.D. & GORDON, J.L. (1987). Characteristics of the P_2 purinoceptor that

mediates prostacyclin production by pig aortic endothelial cells. *Eur. J. Pharmacol.*, **134,** 199–209.

PEARSON, J.D., SLAKEY, L.L. & GORDON, J.L. (1983). Stimulation of prostacyclin production through purinoceptors on cultured porcine endothelial cells. *Biochem. J.*, **214,** 273–276.

PIROTTON, S., ERNEUX, C. & BOEYNAEMS, J.M. (1987). Dual role of GTP binding proteins in the control of endothelial prostacyclin. *Biochem. Biophys. Res. Commun.*, **147,** 1113–1120.

ROTROSEN, G. & GALLIN, J.I. (1987). Histamine Type I receptor occupancy increases endothelial cytoplasmic calcium, reduces F-actin and promotes albumin diffusion across cultured endothelial monolayers. *J. Cell Biol.*, **103,** 2379–2387.

SCHWARTZMAN, M. & RAZ, A. (1982). Purinergic vs. peptidergic stimulation of lipolysis and prostaglandin generation in the perfused rabbit kidney. *Biochem. Pharmacol.*, **31,** 2453–2458.

SINGER, H.A. & PEACH, M.J. (1984). Calcium and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4,** (Suppl. II), 19–25.

TATHAM, P.E.R., CUSACK, N.J. & GOMPERTS, B.D. (1988). Characterisation of the ATP^{4-} receptor that mediates permeabilisation of rat mast cells. *Eur. J. Pharmacol.*, **147,** 13–21.

TOOTHILL, V.J., NEEDHAM, L., GORDON, J.L. & PEARSON, J.D. (1988). Desensitization of agonist-stimulated prostaglandin release in human umbilical vein endothelial cells. *Eur. J. Pharmacol.*, (in press).

VAN COEVORDEN, A. & BOEYNAEMS, J.M. (1984). Physiological concentrations of ADP stimulate the release of prostacyclin from bovine aortic endothelial cells. *Prostaglandins*, **27,** 615–626.

(Received June 15, 1988
Accepted July 26, 1988)