British Journal of Pharmacology (1996) 117, 1341-1347

Stimulation by the nucleotides, ATP and UTP of mitogen-activated protein kinase in EAhy 926 endothelial cells

[1]A. Graham, A. McLees, C. Kennedy, *G.W. Gould & R. Plevin

Department of Physiology & Pharmacology, University of Strathclyde, Royal College, 204 George St, Glasgow G1 1XW and *Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, Davidson Building, University of Glasgow, University Avenue, Glasgow, G12 8QQ

1 We have investigated the characteristics of activation of the 42kDa isoform of mitogen-activated protein (MAP) kinase in response to various nucleotides in the endothelial cell line EAhy 926.

2 Adenosine 5'-triphosphate (ATP) in the concentration range 0.1-100 μM stimulated the rapid and transient tyrosine phosphorylation and activation of the 42 kDa isoform of MAP kinase in EAhy 926 endothelial cells which peaked at 2 min and returned to basal values by 60 min. ATP also stimulated a similar response in primary cultured bovine aortic endothelial cells.

3 Uridine 5' triphosphate (UTP) also stimulated the 42 kDa isoform of MAP kinase with similar potency to ATP (EC_{50} values 5.1 ± 0.2 μM for UTP; 2.9 ± 0.8 μM for ATP), whilst the selective P_{2Y}-purinoceptor agonist, 2-methylthioATP (2-meSATP) was without effect up to concentrations of 100 μM. In bovine aortic endothelial cells however, UTP and 2-meSATP both stimulated MAP kinase.

4 Pretreatment of cells for 24 h with 12-O tetradecanoyl phorbol 13-acetate resulted in the loss of the α and ε isoforms of protein kinase C (PKC) and virtual abolition of nucleotide-stimulated MAP kinase activity (>90% inhibition).

5 Preincubation for 30 min with the PKC inhibitor, Ro-31 8220 (10 μM) reduced MAP-kinase activation at 2 min but potentiated the response at 60 min.

6 Removal of extracellular calcium in the presence of EGTA reduced the MAP kinase activation in response to UTP by approximately 30-50%.

7 Pretreatment with pertussis toxin (18 h, 50 ng ml^{-1}) did not significantly affect the UTP-mediated activation of pp42 MAP kinase.

8 These results show that in the EAhy 926 endothelial cell line, nucleotides stimulate activation of MAP kinase in a protein kinase C-dependent manner through interaction with a P_{2U}-purinoceptor.

Keywords: ATP; UTP; MAP kinase; protein kinase C; EAhy 926 endothelial cells

Introduction

The adenine nucleotide, adenosine 5' triphosphate (ATP), has many actions in the cardiovascular system which are mediated through interaction of ATP with specific purinoceptors. Receptors for ATP have been classified into P_{2X}, P_{2Y}, P_{2U}, P_{2Z} and P_{2T} on the basis of agonist potency orders (Burnstock & Kennedy, 1985; Gordon, 1986; O'Connor *et al.*, 1991). In endothelial cells, ATP acts on an extracellular purinoceptor resulting in generation of second messengers such as inositol 1,4,5 trisphosphate ($InsP_3$), diacylglycerol (DAG) and increased intracellular Ca^{2+} levels (Boeynaems & Pearson, 1990). Cellular responses to ATP have largely been attributed to activation of P_{2Y}-purinoceptors (Kennedy, 1990) and many of these have been well characterized, for example the release of prostacyclin (Pearson *et al.*, 1983; Needham *et al.*, 1987) and nitric oxide (Kelm *et al.*, 1988) leading to smooth muscle relaxation (Martin *et al.*, 1985). However, recent reports have shown that another receptor, sensitive to both ATP and the pyrimidine nucleotide uridine 5' triphosphate (UTP), is present on endothelial cell types (O'Connor *et al.*, 1991; Motte *et al.*, 1993; Purkiss *et al.*, 1993) and this has been termed the P_{2U}-purinoceptor. Increases in $InsP_3$ levels have also been observed in response to UTP (Purkiss *et al.*, 1993). However, other intracellular signalling events in response to ATP and UTP within endothelial cells have not been characterized.

In addition to the events described above, within endothelial cells, stimulation with nucleotides leads to intracellular protein phosphorylation (Demolle *et al.*, 1988), alkalinization (Kitazono *et al.*, 1988) and expression of c-*fos* and c-*myc* (Boutherin-Falson *et al.*, 1990), events associated with the stimulation of DNA synthesis. ATP has been shown to increase thymidine incorporation in endothelial cells, an indicator of cell growth (Van Daele *et al.*, 1992), but the mechanism by which DNA synthesis is increased has not been elucidated. Tyrosine phosphorylation of cytosolic proteins has been shown to be important in relaying extracellular messages to the nucleus, thus promoting cell division (for review see Malarkey *et al.*, 1995). Within smooth muscle and fibroblast cell lines, tyrosine phosphorylation of the mitogen-activated protein (MAP) kinase family in response to growth factors and G-protein linked agonists is thought to be important in cellular growth and proliferation (Anderson *et al.*, 1990; Thomas, 1992; Davis, 1993). MAP kinase activation has been demonstrated in response to ATP in astrocytes (Neary *et al.*, 1994) and in response to ATP and UTP in renal mesangial cells (Huwiler & Pfeilschifter, 1994); however, the role of MAP kinase in endothelial cells has not yet been elucidated.

This study shows that in the EAhy 926 endothelial cell line, the nucleotides ATP and UTP activate MAP kinase and that this activation is regulated by protein kinase C (PKC), utilizes calcium-dependent and -independent mechanisms and does not involve activation of a G_i protein. A preliminary account of some of these results has been presented to the British Pharmacological Society (Graham *et al.*, 1995).

[1] Author for correspondence.

Methods

Cell culture

The EAhy 926 cell line derived from human endothelial cells (Edgell *et al.*, 1983) was maintained in Dulbecco's modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS), 27 mg ml^{-1} glutamine and penicillin/streptomycin (250 i.u. ml^{-1} and 250 mg ml^{-1}) and hypoxanthine, aminopterin, thymidine (HAT) supplement at 37°C in a humidified atmosphere of air/CO_2 (19:1). Bovine aortic endothelial cells were cultured in a similar manner to that described by Motte *et al.* (1993). Briefly, endothelial cells were detached from bovine aortae by collagenase digestion (0.2%, 20 min at 37°C) and cells resuspended in DMEM containing 20% FCS, 27 mg ml^{-1} glutamine and penicillin/streptomycin (250 i.u. ml^{-1} and 250 mg ml^{-1}) and fungizone. Cells were grown under the same conditions as the EAhy 926 cells and used between passages 3–8. All experiments were performed on cells grown to confluency on 6 cm^2 plates or 80 cm^2 flasks and made quiescent by serum deprivation for 48 h.

Cell stimulation

Cells were incubated in Hanks buffered saline containing 10 mM glucose and 1% (w/v) of bovine serum albumin (BSA) for 30 min then stimulated with agonist or vehicle for the appropriate times. The reaction was terminated by rapid aspiration followed by washing in ice cold 20 mM HEPES buffer pH 7.4 containing (mM): NaCl 150, NaF 50, $Na_4P_2O_7$ 10, EDTA 4, EGTA 2 and Na_3VO_4 2. Following the final aspiration, 0.5 ml of sodium dodecyl sulphate (SDS) sample buffer (63 mM Tris HCl, pH 6.8, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% glycerol, 2% SDS and 0.007% bromophenol blue), at 70°C was added and the sample passed repeatedly through a 21G needle. The samples were boiled for 5 min and then stored at −80°C until analysis.

Immunoblotting

Aliquots of each sample (30–50 μg of protein) were run on 10% polyacrylamide gel electrophoresis (SDS–PAGE) and then transblotted onto nitrocellulose. The nitrocellulose membranes were blocked for non-specific binding for 3 h in 50 mM Tris buffer pH 7.4, containing 150 mM NaCl, 0.2% (v/v) Tween (NaTT) and 3% (w/v) BSA, then incubated in NaTT containing 0.2% BSA and 200 ng ml^{-1} of rabbit polyclonal IgG anti-phosphotyrosine antibody overnight. The blots were rinsed in NaTT buffer, washed for 90 min (×6 changes of NaTT) and then incubated for 60 min in buffer containing 0.2% BSA and a donkey anti-rabbit IgG antibody conjugated to horse radish peroxidase (HRP) (1:10 000 dilution). The blots were then washed for 120 min (×8 changes of NaTT) and developed using the ECL detection system (Amersham). The blotting procedure was assessed for specificity for phosphotyrosine, appropriate antibody dilution and linearity of antibody binding with increasing protein concentrations (Saville *et al.*, 1994). For analysis of the activation of MAP kinase, a mouse monoclonal anti-MAP kinase antibody (MK12) and a donkey anti-mouse IgG antibody conjugated to HRP were employed. PKC α, β, δ, and ε were identified with specific polyclonal antibodies and detected by ECL after incubation with horseradish peroxidase (HRP)-conjugated donkey anti rabbit IgG antibody at a 1:10 000 dilution. Incubations were carried out as described above.

In vitro *MAP kinase assay*

Individual 80 cm^2 flasks of cells were made quiescent by serum deprivation for 48 h and stimulated with agonist for the appropriate times. The reaction was terminated by addition of 5 ml ice cold 150 mM NaCl. Flasks were washed with a further 5 ml NaCl and cells centrifuged at 1000 *g* for 5 min. The pellet was transferred to Eppendorf tubes and dispersed into 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM phenyl methyl sulphonyl fluoride, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin by repeated passage through a 26G needle. Samples were then centrifuged for 15 min at 25 000*g* and the supernatants assayed for MAP kinase activity with the epidermal growth factor (EGF) receptor peptide used as substrate (BIOTRAK MAP kinase assay kit, Amersham). All results are expressed as mean ± s.e.mean of 3 experiments.

Materials

All materials were of the highest commercial grades available. BIOTRAK MAP kinase assay kits, second antibodies, enhanced chemiluminescent (ECL) detection reagents and γ-[^{32}P]-ATP (3000 Ci $mmol^{-1}$) were purchased from Amersham International (Bucks, U.K.). Ro-31 8220 was a kind gift from Dr. G. Lawton (Roche, Welwyn Garden City, U.K.). All anti-phosphotyrosine, MAP kinase and PKC antibodies were from Affiniti Research Products Ltd. (Exeter, Devon, U.K.). All other chemicals were purchased from Sigma.

Data analysis

All blots were quantified using scanning densitometry. Concentration-response curve analysis was by an iterative curve fitting procedure (De Lean *et al.*, 1980).

Results

MAP kinase activation in response to ATP and UTP

In EAhy 926 cells, ATP (0.1–100 μM) stimulated the tyrosine phosphorylation of the 42kDa isoform of MAP kinase in a time- and concentration-dependent manner. Activation was transient, reaching a peak at 2 min, before declining towards basal values by 60 min (Figure 1a). The time course of tyrosine phosphorylation was closely matched by the shift in mobility of MAP kinase when the phosphotyrosine blots were reprobed with a specific MAP kinase antibody (Figure 1b). Stimulation of MAP kinase activity was further confirmed by assessing the *in vitro* phosphorylation of a specific MAP kinase substrate, the EGF receptor peptide. Addition of ATP resulted in a 3–4 fold increase in MAP kinase activity by this method (Table 1).

UTP also stimulated the tyrosine phosphorylation and activation of MAP kinase with similar kinetics to those observed for ATP (results not shown). The efficacy and potency of UTP and ATP were similar, maximum stimulation occurring at 30 μM for both ATP and UTP as shown in the concentration curves of Figure 2 and by *in vitro* kinase activity in Table 1. EC_{50} values of 2.9 ± 0.2 μM and 5.1 ± 0.8 μM ($n=3$) were obtained for ATP and UTP respectively. In contrast, Table 1 shows that the selective P_{2Y}-purinoceptor agonist 2-methylthioATP (2-meSATP) failed to stimulate MAP kinase activation significantly, up to a concentration of 100 μM (results not shown). In bovine cultured aortic endothelial cells, MAP kinase activation was observed in response to ATP and UTP within a similar concentration range and with similar kinetics (Figure 3a for UTP, results for ATP not shown). In addition, MAP kinase activation was also observed after stimulation of the cells with the selective P_{2Y}-purinoceptor agonist 2-meSATP (Figure 3b).

Desensitization of the MAP kinase response to UTP

The transient nature of the MAP kinase activation in response to UTP was further investigated by addition of agonist at much later time points, when the MAP kinase activation had returned to basal levels. Further stimulation with UTP (100 μM) was carried out, 2 h after the initial signal had returned to basal levels, and resulted in no reactivation of MAP

kinase when the agonist had not been washed off as shown in Table 2. In contrast, virtually full activation of MAP kinase in response to UTP was seen when the agonist was removed after 60 min. A similar phenomenon was observed when MAP kinase activation was assessed by immunoblotting (results not shown). Cross desensitization between ATP and UTP was also observed when cells were pretreated for 60 min with UTP (Figure 4). No desensitization of responses to lysophosphatidic acid (10 μM for 5 min) (LPA), which also acts through a G-protein linked receptor, was observed following UTP pretreatment (in pmol Pi min^{-1} mg^{-1} protein control = 3.88 ± 0.34, control + UTP pre = 5.05 ± 0.38, LPA = 10.69 ± 1.84, LPA + UTP pre = 10.72 ± 0.57). Activation of MAP kinase by both ATP and UTP was apparent to a similar magnitude to that seen in control cells when the cells were pretreated with 2-meSATP (Figure 4).

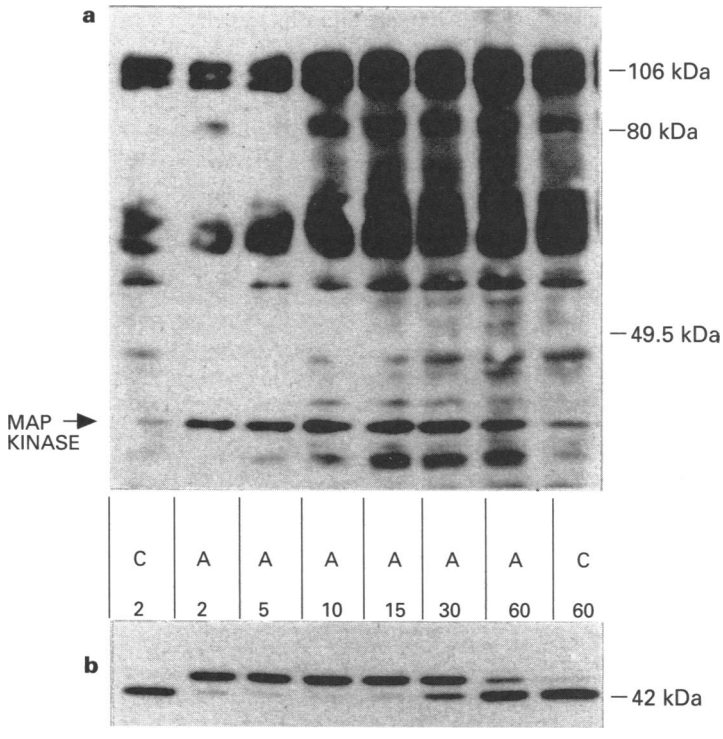

Figure 1 Time course of ATP-stimulated tyrosine phosphorylation of pp42 MAP kinase. EAhy 926 cells were incubated with ATP (100 μM) for 2–60 min and then assayed for phosphotyrosine content (a) as described in the methods section and the blots reprobed with a specific MAP kinase antibody to assess MAP kinase activation (b). The numbers represent stimulation periods in min. C represents control unstimulated cells and A represents ATP-stimulated cells. Molecular weight markers are shown in kDa. Each blot is representative of at least three others.

Table 1 *In vitro* MAP kinase activity in response to ATP, UTP and 2-meSATP in EAhy 926 endothelial cells

Sample	*MAP kinase activity* (pmol Pi min^{-1} mg^{-1} protein)
Control	2.53 ± 0.29
ATP	10.67 ± 2.03
UTP	10.92 ± 3.18
2meSATP	2.88 ± 0.52

Cells were incubated with 30 μM of UTP, ATP or 2meSATP for 2 min and cell extracts assayed for *in vitro* MAP kinase activity as outlined in the Methods section. Each value represents the mean ± s.e. mean of three experiments performed in duplicate.

Role of protein kinase C in the MAP kinase response to ATP and UTP

Pretreatment of the EAhy 926 cells for 24 h with the phorbol ester, 12-O tetradecanoyl 13-phorbol acetate (TPA, 100 nM),

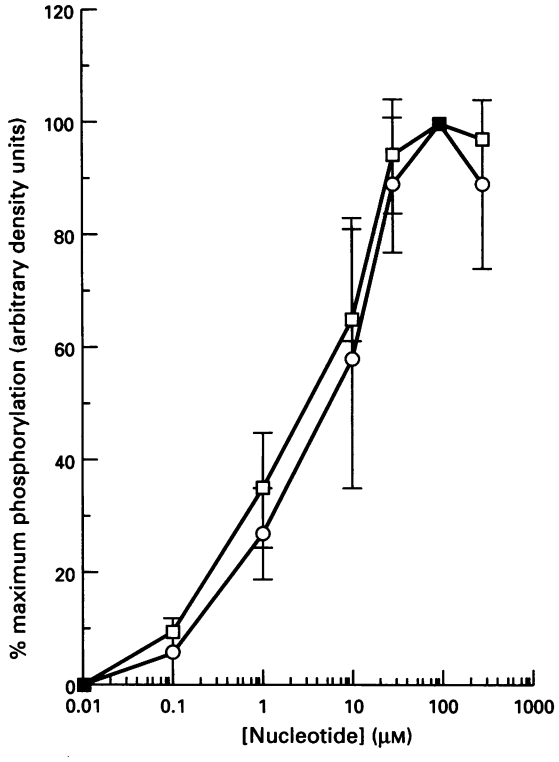

Figure 2 Concentration-response curves for nucleotide-induced activation of MAP kinase: Cells were incubated with increasing concentrations of UTP (○) or ATP (□) and MAP kinase activation assessed by Western blotting using a specific MAP kinase antibody as outlined in the Methods section. Results are expressed as mean ± s.e.mean where $n = 3$.

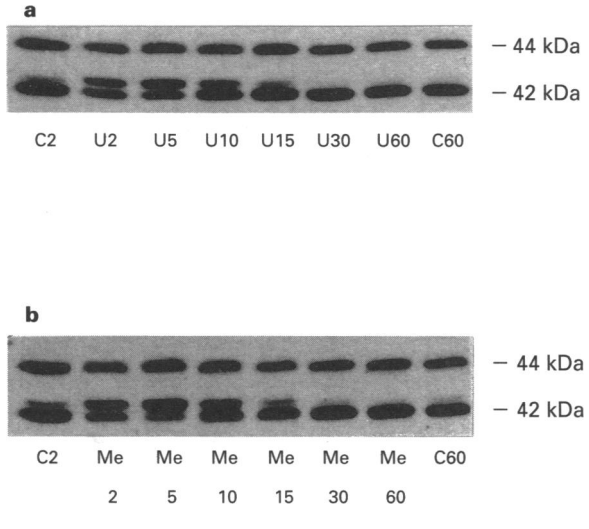

Figure 3 Time course of UTP- and 2-meSATP-stimulated activation of pp42 and pp44 MAP kinase in bovine aortic endothelial cells: primary endothelial cells (passages 3–8) were grown to confluency, made quiescent by serum deprivation for 48 h and incubated with UTP (10 μM) in (a) or 2-meSATP (Me, 30 μM) in (b) for 2–60 min. MAP kinase activation was assessed using specific MAP kinase antibodies. Numbers represent times in min. C = control. The molecular weights of the two isoforms are shown in kDa.

caused downregulation of PKCα and ε (Figure 5a and b respectively), and resulted in over 90% reduction in the activation of MAP kinase in response to maximal concentrations of both UTP, as shown in Figure 6, and ATP (results not shown) as assessed by Western blotting. Levels of PKC β and PKC δ were unaffected (Figure 5c and d). Preincubation for 24 h with the inactive phorbol ester, isophorbol 4α,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one, had no effect on expression of the PKC isoforms as shown in Figure 5. We also investigated the effects of inhibition of PKC using the selective PKC inhibitor Ro-31 8220 (Davis *et al.*, 1989). Preincubation for 30 min with Ro-31 8220 (10 μM) resulted in approximately 90% reduction in the MAP kinase signal at early time points. At 60 min, when the signal had returned to basal in the absence of Ro-31 8220, up to 40% of maximal MAP kinase activation was still evident in the presence of the PKC inhibitor (Figure 6); however, this may be due to activation of MAP kinase induced by Ro-31 8220.

Table 2 Desensitization of MAP kinase activation in response to UTP

Sample	*MAP kinase activity* (Fold stimulation)
Control	1
UTP 2 min	2.98 ± 0.51
UTP 60 min	1.24 ± 0.09
Pre no wash vehicle	0.82 ± 0.05
Pre no wash UTP	1.32 ± 0.36
Pre wash vehicle	0.78 ± 0.13
Pre wash UTP	2.72 ± 0.66

EAhy 926 endothelial cells were stimulated with UTP for 60 min (Pre) and then rechallenged with vehicle or UTP after a 2 h period. At the 60 min time point in some experiments agonist was washed off (wash), and this was compared to the effect after agonist was left in contact with the cells for the entire incubation period (no wash). Rechallenge with UTP was for 5 min. MAP kinase activity was measured as described in the Methods section. Experiments were carried out in duplicate and results are mean (± s.e.mean) where $n = 3$.

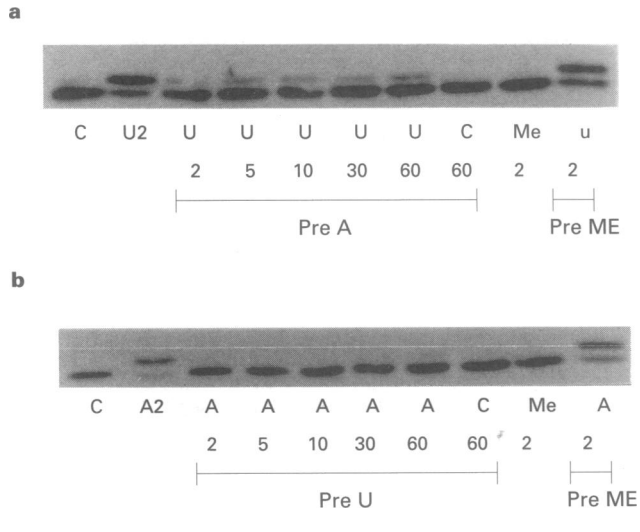

Figure 4 Cross desensitization of the MAP kinase response to ATP and UTP: (a) shows UTP-stimulated MAP kinase (U) and a time course for UTP following 60 min pretreatment with ATP (pre A). The response to UTP following 2-meSATP treatment (60 min) is also shown; (b) shows ATP-stimulated MAP kinase (A) and a time course for ATP following 60 min pretreatment with UTP (pre U). The response to ATP following 2-meSATP treatment (60 min) is also shown.

Effect of calcium on stimulation of MAP kinase by UTP

Removal of extracellular calcium reduced the activation of MAP kinase in response to maximal concentrations of UTP by approximately 30% at early times and this inhibition of activation increased to greater than 50% at 15 min as shown in Figure 7a. Raising intracellular calcium levels using the calcium ionophore A23187 caused no significant activation of MAP kinase over a 30 min period, and this was unaffected after pretreatment of the cells for 24 h with TPA (Figure 7b) to downregulate PKCα and ε.

Effect of preincubation with pertussis toxin on UTP-stimulated activation of MAP kinase

Previous work has shown that accumulation of inositol phosphates in response to ATP and UTP is inhibited when G_i is ADP-ribosylated with pertussis toxin (Boeynaems & Pearson, 1990). We therefore were interested in whether the stimulation of MAP kinase observed in EAhy 926 cells, occurs through a G_i dependent mechanism. Our results showed that pertussis toxin pretreatment (18 h, 50 ng ml^{-1}) had no effect on the activation of MAP kinase in response to UTP in EAhy 926 endothelial cells (Figure 8). Similar results were obtained with ATP (results not shown). Within this cell line it has previously been shown that responses to the G-protein linked receptor agonist, LPA are pertussis toxin-sensitive (McLees *et al.*, 1995) indicating that the pathway is intact in EAhy 926 cells and that pertussis toxin is functional within this cell type.

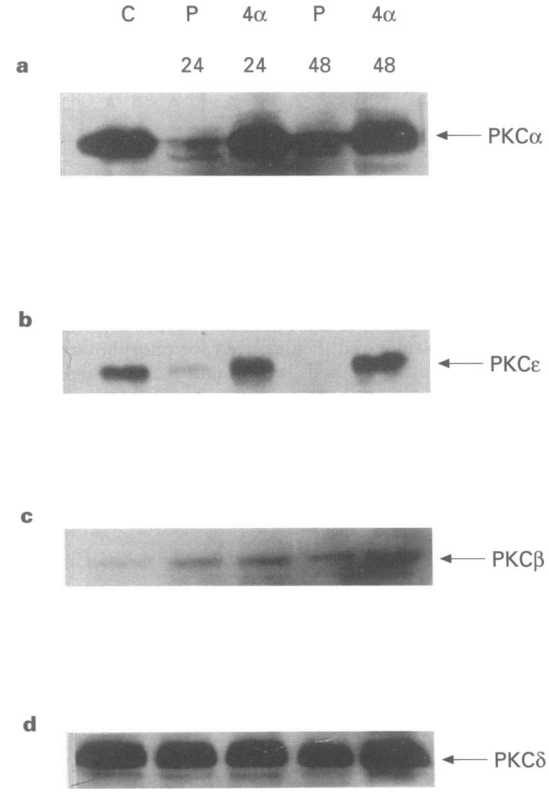

Figure 5 Down regulation of PKC using phorbol ester: (a) shows PKCα and (b) shows PKCε content after Western blotting with specific PKC antibodies in control cells (C) and after 24 h pretreatment with TPA (P24) or 48 h pretreatment with TPA (P48). Panels (c) and (d) show PKCδ and PKCβ content respectively after TPA down regulation. 4α represents pretreatment with the inactive phorbol ester. The arrowhead denotes the PKC isoform.

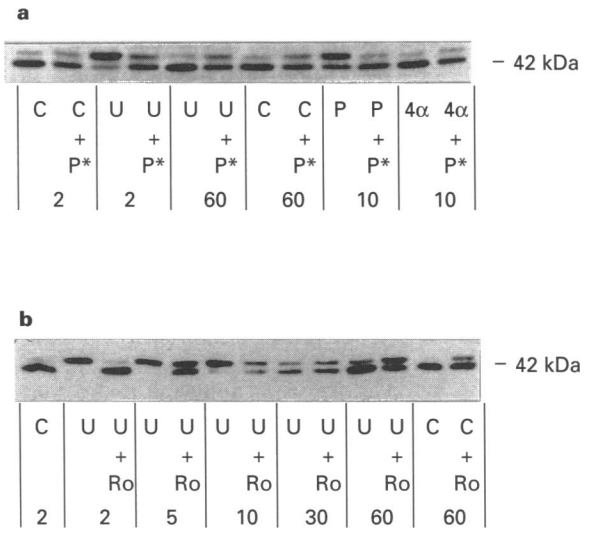

Figure 6 Effect of PKC down regulation and PKC inhibition upon UTP-stimulated activation of MAP kinase: (a) shows a MAP kinase shift blot of a 60 min time course to UTP (100 μM) in control cells (U) or in cells pretreated with 100 nM TPA for 24 h (U+P*). P represents cells stimulated for 10 min with TPA. (b) Shows a MAP kinase shift blot of a 60 min time course to UTP (U) in cells pretreated with 10 μM Ro-31 8220 (Ro). C represents unstimulated cells. The numbers represent times in min. Molecular weight markers are shown in kDa. Each blot is representative of at least three others.

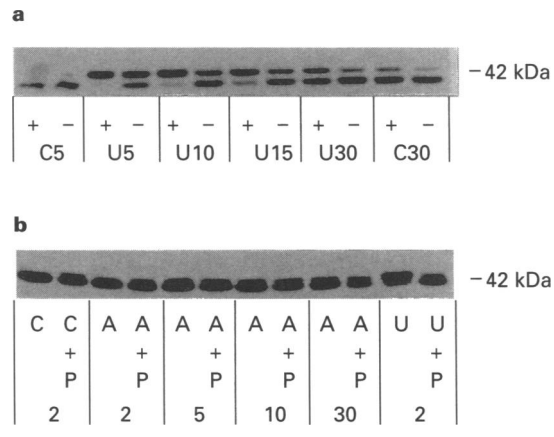

Figure 7 Effect of A23187 on MAP kinase activation and of extracellular Ca^{2+} removal on UTP-stimulated activation of pp42 MAP kinase in EAhy 926 cells. In (a) cells were preincubated in Hanks buffered saline containing glucose 10 mM (HBG) and 1.2 mM Ca^{2+} (+) or in the absence of extracellular Ca^{2+} and in the additional presence of 0.5 mM EGTA (–) for 30 min and then incubated with 100 μM UTP (U) for the times indicated. C represents unstimulated cells. (b) Shows the effects of TPA down regulation (P) on cells stimulated over 30 min with the calcium ionophore A23187 (10 μM) (A). Cell extracts were assayed for MAP kinase content as outlined in the Methods section. Each blot is representative of at least three others.

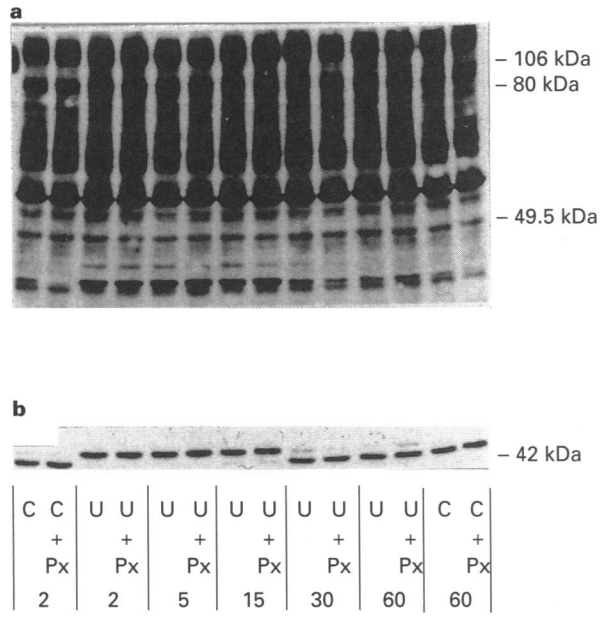

Figure 8 Effect of pertussis toxin pretreatment on UTP-stimulated tyrosine phosphorylation of MAP kinase: (a) Shows a phosphotyrosine blot of UTP-induced stimulation (100 μM) in control EAhy 926 cells (U) and in cells pretreated for 18 h with 50 ng ml^{-1} pertussis toxin (+Px). (b) Shows a reprobe of the same blot using a specific MAP kinase antibody. C represents unstimulated cells. The numbers represent time in min. Molecular weight markers are shown in kDa. Each blot is representative of at least three others.

Discussion

The results obtained in this study show firstly that MAP kinase activation occurs in a time- and concentration-dependent manner in the human endothelial cell line EAhy 926 in response to the nucleotide ATP. Within this cell type, activation is transient with maximal tyrosine phosphorylation occurring within 2 min and the signal declining towards basal levels by 60 min. We also found that UTP stimulated MAP kinase over a similar concentration-range. There is already evidence that the nucleotides ATP and UTP cause stimulation of MAP kinase activity in renal mesangial cells (Huwiler & Pfeilschifter, 1994) and in astrocytes (Neary *et al.*, 1994) but this is the first demonstration of MAP kinase stimulation in response to nucleotides in endothelial cells.

Various authors have described the presence of two types of receptor on endothelial cells, the P_{2Y}-purinoceptor, which responds to ATP and 2-meSATP, and a nucleotide receptor known as the P_{2U}-purinoceptor which responds to UTP and ATP but is insensitive to 2-meSATP (O'Connor *et al.*, 1991; Mannix *et al.*, 1993). The relative potencies of ATP and UTP, and the fact that the specific P_{2Y}-purinoceptor agonist, 2-meSATP, did not elicit a response in the EAhy 926 endothelial cells used in this study suggests the exclusive involvement of the P_{2U}-purinoceptor subtype in the activation of MAP kinase in this cell type. It is possible that this cell line may only express the nucleotide receptor (P_{2U}) as the bovine aortic endothelial cell line AG4762 is known to express only the P_{2Y}-purinoceptor (Allsup & Boarder, 1990), but this remains to be determined. However, the fact that 2-meSATP can activate MAP kinase in the primary bovine aortic endothelial cells supports this hypothesis. In addition, in preliminary experiments we found that ATP failed to reactivate MAP kinase following an initial 60 min stimulation with UTP and similarly, UTP failed to reactivate MAP kinase after preincubation with ATP, suggesting homologous desensitization of a single receptor subtype on this cell type. In support of this, cross desensitization of the effects of ATP and UTP on intracellular increases in calcium in endothelial cell have been documented (Mannix *et al.*, 1993; Briner & Kern, 1994).

In contrast to the signalling pathways which lead to MAP kinase activation, the phospholipase C and phospholipase D linked pathways are well described (Purkiss *et al.*, 1993; Pfeilschifter & Merriweather, 1993). PLC activation leads to generation of $InsP_3$, resulting in an increased level of intracellular Ca^{2+} and generation of DAG, the physiological activator of PKC. Within EAhy 926 cells the PKC activating

phorbol ester, TPA, produced activation of MAP kinase, an effect which could be completely abolished after down regulation of the α and ε isoforms of PKC. Under these conditions over 90% of the MAP kinase signal in response to UTP was abolished, implying that either PKCα or PKCε, or both are important in MAP kinase activation. In our experiments we also observed inhibition of the MAP kinase signal after preincubation with the PKC inhibitor, Ro-31 8220, and this evidence also supports a role for PKC in generation of the UTP-mediated MAP kinase signal. The mechanism of activation of MAP kinase in likely to involve the activation of raf-1 since previously it has been shown that PKCα can activate raf-1 *in vitro* (Kolch *et al.*, 1993). It has recently been reported that MAP kinase activation occurs on raising intracellular calcium in human umbilical vein endothelial cells (Fleming *et al.*, 1995); however, in EAhy 926 cells, we found no significant stimulation of MAP kinase in response to the calcium ionophore A23187, and no differences were obtained following down regulation of PKC using TPA. Following removal of extracellular Ca^{2+}, initial activation of MAP kinase by UTP was reduced but not abolished, suggesting the involvement of both Ca^{2+}-dependent and -independent isoforms of PKC in the regulation of MAP kinase by UTP. In rat renal mesangial cells the activation of MAP kinase by UTP was not inhibited by the PKC inhibitor, CGP 41251, which is more selective for Ca^{2+}-dependent isoforms (Huwiler & Pfeilschifter, 1994). This suggests that PKCε rather than PKCα may function as the upstream activator of raf-1 in response to UTP stimulation within renal mesangial cells. It is unlikely to be the exclusive PKC isoform involved in MAP kinase activation in EAhy 926 cells since removal of extracellular Ca^{2+} partially inhibited the MAP kinase signal and this became more apparent at later times. Thus PKCα may play a role in the regulation of MAP kinase and this possibility is currently being addressed in our laboratory. It is also possible that removal of extracellular calcium affects other signalling events leading to a reduction of MAP kinase activation but the lack of stimulation of MAP kinase in response to A23187 suggests that increased intracellular calcium alone is not sufficient to account for the activation of MAP kinase observed in these cells.

In cells pretreated with pertussis toxin we found no inhibition of UTP-stimulated MAP kinase activation, suggesting no involvement of a G_i protein in the transduction of the receptor signal. However, it has been shown that the generation of second messenger signals by P_{2U}-purinoceptor activation, such as $InsP_3$ formation, is significantly reduced following pertussis toxin pretreatment in endothelial cells (Boeynaems & Pearson, 1990), suggesting the possible involvement of βγ subunits in the regulation of MAP kinase with possibly the PLC isoform PLCβ2 being important (Katz *et al.*, 1992). We have previously found significant inhibition of LPA-mediated activation of MAP kinase in EAhy 926 cells following pretreatment with pertussis toxin (McLees *et al.*, 1995), suggesting that the signalling components of the system are intact in this cell line. Thus, these results indicate that either the P_{2U}-purinoceptor is unlikely to be coupling to the same signalling pathways as in primary endothelial cells or that the activation of MAP kinase may be through an undefined mechanism.

In summary the results presented in this paper show that the nucleotides ATP and UTP stimulate MAP kinase in a transient manner in EAhy 926 endothelial cells through activation of P_{2U}-purinoceptor. The upstream pathway of MAP kinase activation appears to be totally dependent on PKC activation possibly involving the PKCε and PKCα isoforms.

We wish to thank Dr D.A. Cohen and L. Cowell (Amersham International) for the kind gift of the BIOTRAK MAP kinase assay kit used in this study. Dr I.M. Varndell for PKC antibodies, Dr G. Lawton for Ro-31 8220 and Dr A. Crilley for the gift of the EAhy 926 cell line. This work was funded by grants to R.P. from the British Heart Foundation and the Medical Research Council.

References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2-purinergic receptors of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype and of inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

ANDERSON, N.G., MALLER, J.L., TONKS, N.K. & STURGILL, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature*, **343**, 651–653.

BOEYNAEMS, J.M. & PEARSON, J.D. (1990). P_2-purinoceptors on vascular endothelial cells: physiological significance and transduction mechanisms. *Trends Pharmacol. Sci.*, **11**, 34–37.

BOUTHERIN-FALSON, O., REUSE, S., DUMONT, J.E. & BOEUNAEMS, J.M. (1990). Increased levels of c-fos and c-myc mRNA in ATP-stimulated endothelial cells. *Biochem. Biophys. Res. Commun.*, **172**, 306–312.

BRINER, V.A. & KERN, F. (1994). ATP stimulates Ca^{2+} mobilization by a nucleotide receptor in glomerular endothelial cells. *Am. J. Physiol.*, **266**, F210–F217.

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

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS. Lett.*, **259**, 61–63.

DAVIS, R.J. (1993). The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.*, **268**, 14553–14556.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1980). Simultaneous analysis of a family of sigmoidal curves application to bioassay and physiological dose response curves. *Am. J. Physiol.*, **235**, E97–E102.

DEMOLLE, D., LECOMTE, M. & BOWYNAEMS, J.M. (1988). Pattern of protein phosphorylation in aortic endothelial cells: modulation by adenine nucleotides and bradykinin. *J. Biol. Chem.*, **263**, 18459–18465.

EDGELL, C.-J.S., MCDONALD, C.C. & GRAHAM, J.B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridisation. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 3734–3737.

FLEMING, I., FISSLTHALER, B. & BUSSE, R. (1995). Calcium signalling in endothelial cells involves activation of tyrosine kinases and leads to activation of mitogen-activated protein kinases. *Circ. Res.*, **76**, 522–529.

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

GRAHAM, A., MCLEES, A., KENNEDY, C. & PLEVIN, R. (1995). Uridine 5′ triphosphate stimulates mitogen-activated protein kinase in EAhy 926 endothelial cells. *Br. J. Pharmacol.*, **115**, 6P.

HUWILER, A. & PFEILSCHIFTER, J. (1994). Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. *Br. J. Pharmacol.*, **113**, 1455–1463.

KATZ, A., WU, D. & SIMON, M.I. (1992). Subunits βγ of heterotrimeric G protein activate β2 isoform of phospholipase C. *Nature*, **360**, 686–689.

KELM, M., FEELISCH, M., SPAHR, R., PIPER, H.M., NOACK, E. & SCHRADER, J. (1988). Quantitative and kinetic characterization of nitric oxide and EDRF released from endothelial cells. *Biochem. Biophys. Res. Commun.*, **154**, 236–244.

KENNEDY, C. (1990). P_1- and P_2-Purinoceptor subtypes- an update. *Arch. Pharmacol. Ther.*, **303**, 30–50.

KITAZONO, T., TAKESHIGE, K., CRAGOE Jr, E.J. & MINAKAMI, S. (1988). Intracellular pH changes of cultured bovine aortic endothelial cells in response to ATP addition. *Biochem. Biophys. Res. Commun.*, **152**, 1304–1309.

KOLCH, W., HEIDECKER, G., KOCHS, G., HUMMEL, R., VAHIDI, H., MISCHAK, H., FINKENZELLER, G., MARME, D. & RAPP, U. (1993). Protein kinase Cα activates RAF-1 by direct phosphorylation. *Nature*, **364**, 249–252.

MALARKEY, K., BELHAM, C.M., PAUL, A., GRAHAM, A., McLEES, A., SCOTT, P.H. & PLEVIN, R. (1995). The regulation of tyrosine kinase signalling pathways in response to growth factor and G-protein coupled receptors. *Biochem. J.*, **309**, 361–375.

MANNIX, R.J., MOATTER, T., KELLEY, K.A. & GERRITSEN, M.E. (1993). Cellular signalling responses mediated by a novel nucleotide receptor in rabbit microvessel endothelium. *Am. J. Physiol.*, **265**, H675–H680.

MARTIN, W., CUSACK, N.J., CARLETON, J.S. & GORDON, J.L. (1985). Specificity of P2-purinoceptor that mediates endothelium dependent relaxation of the pig aorta. *Eur. J. Pharmacol.*, **108**, 295–299.

MOTTE, S., PIROTTON, S. & BOEYNAEM, J.M. (1993). Heterogeneity of ATP receptors in aortic endothelial cells. *Circ. Res.*, **72**, 504–510.

McLEES, A., GRAHAM, A., MALARKEY, K., GOULD, G.W. & PLEVIN, R. (1995). The regulation of pp42 MAP kinase by protein kinase C-dependent and pertussis toxin sensitive mechanisms in the EAhy 926 endothelial cell line. *Biochem. J.*, **307**, 743–748.

NEARY, J.T., ZHU, Q. & NORENBERG, M.D. (1994). ATP signalling in astrocytes: activation of MAP kinase. *Drug Dev. Res.*, **31**, 302.

NEEDHAM, L., CUSACK, N.J., PEARSON, J.D. & GORDON, J.L. (1987). Characteristics of the P2 purinoceptor that mediates prostacyclin production by pig aortic endothelial cells. *Eur. J. Pharmacol.*, **134**, 199–209.

O'CONNOR, S.E., DAINTY, I.A. & LEFF. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

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

PFEILSCHIFTER, J. & MERRIWEATHER, C. (1993). Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinase C-ε in rat renal mesangial cells. *Br. J. Pharmacol.*, **110**, 847–853.

PURKISS, J.R., WILKINSON, G.F. & BOARDER, M.R. (1993). Evidence for a nucelotide receptor on adrenal medullary endothelial cells linked to phospholipase C and phospholipase D. *Br. J. Pharmacol.*, **108**, 1031–1037.

SAVILLE, M.K., GRAHAM, A., MALARKEY, K., PATERSON, A., GOULD, G.W. & PLEVIN, R. (1994). Regulation of endothelin-1 and lysophosphatidic acid-stimulated tyrosine phosphorylation of focal adhesion kinase ($pp125^{fak}$) in rat-1 fibroblasts. *Biochem. J.*, **301**, 407–414.

THOMAS, G. (1992). MAP kinase by any other name smells just as sweet. *Cell*, **68**, 3–6.

VAN DAELE, P., VAN COEVORDEN, A., ROGER, P.P. & BOEYNAEMS, J.-M. (1992). Effects of adenine nucleotides on the proliferation of aortic endothelial cells. *Circ. Res.*, **70**, 82–90.

(Received April 20, 1995
Revised November 13, 1995
Accepted November 27, 1995)