Phosphorylation and activation of p42 and p44 mitogen-activated protein kinase are required for the P2 purinoceptor stimulation of endothelial prostacyclin production

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Extracellular ATP and ADP, released from platelets and other sites stimulate the endothelial production of prostacyclin (PGI₂) by acting on G-protein-coupled P2Y₁ and P2Y₄ purinoceptors, contributing to the maintenance of a non-thrombogenic surface. The mechanism, widely described as being dependent on elevated cytosolic [Ca⁺²], also requires protein tyrosine phosphorylation. Here we show that activation of both these P2 receptor types leads to the tyrosine phosphorylation and activation of both the p42 and p44 forms of mitogen-activated protein kinase (MAPK).

2-Methylthio-ATP and UTP, selectively activating P2Y₁ and P2Y₄ purinoceptors respectively, and ATP, a non-selective agonist at these two receptors, stimulate the tyrosine phosphorylation of both p42²⁴⁴⁺⁺ and p44²⁴⁴⁺⁺, as revealed by Western blots with an antiserum specific for the tyrosine-phosphorylated forms of the enzymes. By using separation on Resource Q columns, peptide kinase activity associated with the phosphorylated MAPK enzymes distributes into two peaks, one mainly p42²⁴⁴⁺⁺ and one mainly p44²⁴⁴⁺⁺, both of which are stimulated by ATP with respect to kinase activity and phospho-MAPK immunoreactivity. Stimulation of P2Y₁ or P2Y₄ purinoceptors leads to a severalfold increase in PGI₂ efflux; this was blocked in a dose-dependent manner by the selective MAPK kinase inhibitor PD98059. This drug also blocked the agonist-stimulated increase in phospho-MAPK immunoreactivity for both p42²⁴⁴⁺⁺ and p44²⁴⁴⁺⁺ but left the phospholipase C response to P2 agonists essentially unchanged. Olomoucine has been reported to inhibit p44²⁴⁴⁺⁺ activity. Here we show that in the same concentration range olomoucine inhibits activity in both peaks from the Resource Q column and also the agonist stimulation of 6-keto-PGF₁α but has no effect on agonist-stimulated phospho-MAPK immunoreactivity. These results provide direct evidence for the involvement of p42 and p44 MAPK in the PGI₂ response of intact endothelial cells; we have shown that both the endothelial P2Y purinoceptors are linked to activation of MAPK, and that activation of this pathway is a requirement for the stimulation by ATP/ADP of endothelial PGI₂ production.

INTRODUCTION

The tyrosine kinase/mitogen-activated protein kinase (MAPK) cascade elucidated in the response of single transmembrane growth factor receptors is now known to be widely activated by cell surface receptors of the heterotrimeric G-protein-coupled seven-transmembrane superfamily [1–4]. Activation of the p42 and p44 forms of MAPK by G-protein-coupled receptors might be by a variety of pathways in different receptor-cell combinations, commonly involving tyrosine kinase activity [5,6]. The role of heterotrimeric G-protein-coupled MAPK activation is poorly understood; it is widely discussed in terms of the control of mitogenesis and differentiation, relatively slow and sustained events that also occur in response to activation of growth-factor-like receptors and are known to require the MAPK pathway. The possibility that more rapid and transient responses to G-protein-coupled receptors might also be dependent on MAPK activation has received little attention, although a recent study has provided intriguing indications of the interaction of the tyrosine kinase/MAPK pathway in the regulation of transient cellular events [5]. Here we investigate this possibility in the control of vascular endothelial cells by extracellular ATP.

Extracellular ATP and ADP are widespread in cell–cell communication, acting on cell-surface receptors designated P2 purinoceptors [7]. A salient example of this is the control of endothelium by ATP and ADP, released principally from platelets but also from other local sites, acting on G-protein-coupled P2 purinoceptors (P2Y purinoceptors) and stimulating the production of prostacyclin (PGI₂), an anti-thrombotic agent [8]. In cultured bovine aortic endothelial cells (BAECs) there are two co-existing receptors that respond to ATP and ADP of endothelial PGI₂, an anti-thrombotic agent [8]. In cultured bovine aortic endothelial cells (BAECs) there are two co-existing receptors that respond to ATP and ADP of endothelial PGI₂ production.

Abbreviations used: BAECs, bovine aortic endothelial cells; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase or MAPK/extracellular-signal-related protein kinase; 2MeSATP, 2-methylthio-ATP; PGI₂, prostacyclin; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; PLC, phospholipase C.

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endothelial cell homogenates the stimulation of cytosolic PLA₂ by growth factor is via an MAPK-dependent pathway. However, there has been no demonstration of a role for MAPK in the control of intact endothelial cells by ATP and ADP. ATP has been shown to stimulate MAPK in a study on the proliferation of renal mesangial cells [26]. Here we show that both the P2Y₁ and the P2Y₁₄ purinoceptors on cultured endothelial cells are linked to MAPK activation, and using a novel inhibitor of MAPK kinase (MEK; PD98059) [27] and the p44MAPK inhibitor olomoucine [28] we provide evidence that MAPK activation is required for the enhancement of PGI₄ production in response to stimulation of these receptors.

EXPERIMENTAL

Materials

The antibody specific for phosphorylated MAPK was raised in rabbits against a peptide sequence corresponding to residues 196–209 of human p44 MAPK in which the tyrosine residue at position 204 was phosphorylated; the antibody for the unphosphorylated MAPK was raised against residues 191–211 of human p44 MAPK; both were purchased from New England Biolabs. 6-Keto[5,6,9,11,12,14,15-H³]PGF₁α, [γ-32P]ATP and enhanced chemiluminescence reagents for developing Western blots were from Amersham. The 6-kg-PGF₁α antisera was from Sigma. Olomoucine was from LC Laboratories (Alexis Trading, Nottingham, Notts., U.K.). PD98059 was a gift from Dr. A. Saltiel (Parke-Davis, Ann Arbor, MI, U.S.A.). Cell culture supplies were from Gibco, and other reagents were purchased from Sigma or Fisons.

Cell Culture

Fresh bovine aortae were collected and BAECs prepared as described [29], von Willebrand factor-immunopositive cells were cultured in minimal essential medium D-valine with 10% (v/v) fetal calf serum, 25 i.u./ml penicillin, 25 µg/ml streptomycin and 27 µg/ml glutamine, and used just as they reached confluence. Cells were in 175 cm² flasks for the Resource Q analyses, 80 cm² flasks for the Western blots and the in-gel assay, and 24-well plates for the 6-keto-PGF₁α analyses. In each case cells were maintained serum-free for 24 h before use.

Immunoblotting

After cells had been kept for 24 h in 10 ml of serum-free medium, agonists were added in 1 µl at 11-fold final concentration. After 5 min at 37 °C the medium was gently aspirated and liquid nitrogen added directly to the flask, followed by extraction into homogenizing buffer [20 mM Tris (pH 7.4)/2 mM EDTA/10 µg/ml leupeptin/20 µM E-64/2 µg/ml aprotinin/1 µM pepstatin/50 mM sodium fluoride/2.5 mM sodium orthovanadate/62.5 mM β-glycerophosphate/1 mM PMSF/0.1% Triton X-100], and heating in SDS buffer. Proteins were separated on 10% (w/v) polyacrylamide gels and blotted with a Bio-Rad transblot apparatus on to nitrocellulose membranes. Antisera were used at 1:500 dilution after blocking overnight in 10% (w/v) dried milk, followed by a second antibody conjugated to horseradish peroxidase, and detected with the Amersham enhanced chemiluminescence procedure. In some cases blots were scanned with a Bio-Rad densitometer. Our results, from Western blots of bacterially expressed p44 MAPK in both phosphorylated and unphosphorylated forms, and Western blots of extracts of endothelial cells and vascular smooth muscle cells, showed that the phospho-MAPK antisera recognizes both p42 and p44 forms of phospho-MAPK. Unphosphorylated proteins were not recognized, as determined by lack of immunoreactivity to the negative control unphosphorylated recombinant protein, and lack of immunoreactivity in cell extracts from unstimulated samples that showed clear immunoreactive p42 and p44 bands when using the antisera against the unphosphorylated forms of MAPK. In contrast the antisera for the unphosphorylated MAPK recognized p42 MAPK in both its phosphorylated and unphosphorylated forms, whereas p44 MAPK was preferentially recognized in the unphosphorylated form. The use of the phospho-MAPK specific antisera in these immunoblots provided an alternative to the use of immunoprecipitation with anti-MAPK antisera followed by detection with anti-phosphotyrosine antisera, with the advantages of speed and accuracy deriving from the elimination of the immunoprecipitation step.

Resource Q chromatography

Cells from 175 cm² flasks were extracted into 1.2 ml of homogenizing buffer, and 1 ml of supernatant was loaded on a Resource Q column and eluted at 1 ml/min in a linear gradient of 0–0.5 M NaCl in 20 mM Tris buffer (pH 7.4)/10 mM β-glycerophosphate/1 mM EDTA/1 mM sodium orthovanadate. Aliquots of fractions were assayed immediately for nonapeptide kinase activity, and the remainder was precipitated with 0.5 M trichloroacetic acid. After centrifugation for 5 min at 14000 g, the pellets were used for Western blots.

Peptide kinase assay

A 10 µl aliquot of each column fraction was incubated for 20 min at 30 °C in the presence of 15 µl of assay system comprising 25 mM MgCl₂, 1 mM substrate peptide APRTPGGR [30] and 50 µM ATP/[γ-32P]ATP (1 µCi per tube). The reaction was terminated by the addition of 20% (w/v) trichloroacetic acid (40 µl per sample); each sample was washed on P81 paper with 75 mM phosphoric acid, and counted for ³²P.

Assay of 6-keto-PGF₁α accumulation

The accumulation of 6-keto-PGF₁α in the medium was used as an index of PGI₃ formation. Cells in 24-well plates were gently washed and preincubated in 1 ml of medium, with or without PD98059 or olomoucine. After 30 min, 730 µl was gently removed and replaced with 30 µl of agonists as indicated at 10-fold final concentration. After a further 5 min the medium was removed and assayed for 6-keto-PGF₁α as described previously [22].

Total [³H] inositol (poly)phosphates

Cells were labelled with [³H]inositol and stimulated for a 15 min incubation period in the presence of 10 mM Li⁺, followed by extraction and scintillation counting of total [³H]inositol (poly)phosphates essentially as described [9].

RESULTS AND DISCUSSION

We have previously shown that cultured BAECs contain co-existing P2Y₁ and P2Y₁₄ purinoceptors, and that under the conditions used here 2 methylthio-ATP (2MeSATP) selectively stimulates PLC by acting on the P2Y₁ receptor and UTP by acting on the P2Y₁₄ receptor, whereas ATP stimulates PLC by acting on both receptor types [9,13]. Furthermore both these receptors are linked to vasorelaxant responses [10] and the generation of PGI₃ from the cultured cells [22]. Under conditions
of little detectable MAPK phosphorylation in the absence of added agonist, we found that 2MeSATP, UTP and ATP each elicited a substantial phosphorylation of bands corresponding to the position of p42 MAPK, collected by laser scanning, showing means ± S.E.M. of absorbance in the p42 band (hatched bars) and the p44 band (open bars); (B) a representative blot from those generating the data in (A), probed with an antiserum specific for the tyrosine-phosphorylated form of MAPK; (C) the same blot stripped of the first antiserum and reprobed with antiserum for the unphosphorylated form of MAPK. In (B) and (C), lane 1, control; lane 2, ATP; lane 3, UTP; lane 4, 2MeSATP.

To investigate whether these increases in protein phosphorylation were related to a stimulation of enzyme activity, we fractionated cell extracts on Resource Q anion-exchange columns, and combined assay of the fractions for kinase activity, with immunoblotting fractions for phospho-MAPK band. The fractions from unstimulated samples had no detectable phospho-MAPK immunoreactivity (results not shown). The first peak of kinase activity principally corresponded to the p42 phospho-MAPK band, and the second peak to the p44 phospho-MAPK band.

Taken together, these results show that both of the co-existing G-protein-coupled P2 purinoceptors located on cultured endothelial cells are linked to activation of p42MAPK and p44MAPK. We have previously shown that whereas the P2Y1 purinoceptor (stimulated selectively by 2MeSATP) is linked to PLC in a pertussis-toxin-insensitive manner, the P2Y2 purinoceptor response (selectively stimulated by UTP) is pertussis-toxin-sensitive [13], indicating differential coupling to G-proteins. The observation that that they are both linked to MAPK activation might indicate that ATP can regulate the MAPK cascade by two separate pathways in these endothelial cells, corresponding to pertussis-toxin-sensitive and -insensitive pathways for the P2Y1 and P2Y2 receptors respectively. In this context it is of interest to note recent studies that have shown that both α[subscript GTP] and βγ heterotrimeric G-protein subunits might contribute to stimulation of pathways to MAPK activation (see, for example, [5,6,31–33]).

Activation of MAPK requires its phosphorylation at both threonine and tyrosine residues by a dual kinase, MEK [34,35]. To determine whether the MAPK cascade is involved in the stimulation of endothelial PGI[subscript 2] release by P2 purinergic agonists we used a recently described MEK inhibitor, PD98059. This compound has been used as a selective inhibitor of MEK activation [36] to show the requirement of the MAPK pathway in the differentiation of PC12 cells induced by nerve growth factor [27] and the stimulation of 2-deoxyglucose uptake by
activity incorporated into total ATP of MAPK in PC12 cells with an IC\textsubscript{50} consistent with the inhibition of nerve growth factor stimulation stimulated and control values declining to the same level. This is At 10\(\mu\)M PD98059 the response was completely lost, with stimulated and control values declining to the same level. This is consistent with the inhibition of nerve growth factor stimulation of MAPK in PC12 cells with an IC\textsubscript{50} of 1\(\mu\)M. At 10\(\mu\)M PD98059 the response was completely lost, with stimulated and control values declining to the same level. This is consistent with the inhibition of nerve growth factor stimulation of MAPK in PC12 cells with an IC\textsubscript{50} of 2\(\mu\)M [27], in the absence of effects on related nerve growth-stimulated events. We established (Figure 4A) that activation of both Resource Q peaks was attenuated by 10\(\mu\)M PD98059 and in Figure 4B that this pretreatment inhibits the ATP-stimulated tyrosine phosphorylation of p42\textsuperscript{mapk} and p44\textsuperscript{mapk}, directly demonstrating the effectiveness of PD98059 in preventing the activation of MAPK by MEK in the cultured endothelial cells. The residual stimulated activity in the kinase assay (Figure 4A), but complete loss of the 6-keto-PGF\textsubscript{1\alpha} response (Figure 3), might indicate that a threshold of MAPK activity must be reached before it can support a PGI\textsubscript{2} response. Alternatively it might be that different pools of MAPK are activated by pathways showing differential sensitivities to PD98059, and that these are not equally involved in the PGI\textsubscript{2} response. The latter explanation is encouraged by previous studies, which show differential sensitivities to PD98059 of alternative routes to MEK activation, while reporting the selective action of this agent as an MEK inhibitor [27,35,36].

In the present series of experiments we determined that 10\(\mu\)M PD98059 has no effect on the PLC response to P2 purinergic stimulation in these cells: unstimulated control, 2372\(\pm\)14 d.p.m.; control plus PD98059, 2078\(\pm\)276 d.p.m.; ATP, 12624\(\pm\)615 d.p.m.; ATP + PD98059, 9474\(\pm\)518 d.p.m. Results are means\(\pm\)S.E.M. (\(n=3\) from a single representative experiment) of the radioactivity incorporated into total \(^{3}H\)inositol (poly)phosphates accumulating with a 15 min stimulation period, with 100\(\mu\)M ATP and 10\(\mu\)M PD98059.

To investigate further a possible role for MAPK in the ATP-stimulated 6-keto-PGF\textsubscript{1\alpha} response, we used olomoucine, a cyclin-dependent kinase inhibitor also reported to inhibit p44\textsuperscript{mapk} [28]. When ATP-stimulated cells were extracted and separated into two peaks of peptide kinase activity on Resource Q columns, the addition of olomoucine inhibited the activity in both these peaks (Figure 5A) with a potency similar to that reported for inhibition of p44\textsuperscript{mapk} [28]. Olomoucine was therefore effective at inhibiting both forms of MAPK stimulated by ATP in endothelial cells. If MAPK is required for the stimulation by ATP of PGI\textsubscript{2} production, then this should be inhibited by olomoucine. Consistent with this, when added to cells before and during treatment with ATP, olomoucine inhibited the stimulation of 6-keto-PGF\textsubscript{1\alpha} accumulation (Figure 5B).

However, it is possible that olomoucine was attenuating other components of the pathway from the receptor to PGI\textsubscript{2} production. To establish whether olomoucine was having an effect on any of the steps upstream of MAPK we examined the agonist stimulation of PLC. There was no effect of olomoucine on the ATP-stimulated formation of total \(^{3}H\)inositol (poly)phosphates: control, 902\(\pm\)46 d.p.m.; ATP, 4721\(\pm\)273 d.p.m.; ATP plus 100\(\mu\)M olomoucine, 4501\(\pm\)128 d.p.m.; ATP plus 300\(\mu\)M olomoucine, 3932\(\pm\)354 d.p.m. Results are means\(\pm\)S.E.M. (\(n=3\) from a single representative experiment) of the radioactivity incorporated into total \(^{3}H\)inositol (poly)phosphates accumulating with a 15 min stimulation period with 100\(\mu\)M ATP. This shows that olomoucine had no effect on the processes between agonist binding and accumulation of inositol phosphates. Furthermore we found that olomoucine did not affect the agonist stimulation of phospho-MAPK immunoreactivity.
This work is complementary to our earlier demonstration of a requirement for tyrosine phosphorylation [22] and protein kinase C [20] in the pathway to stimulation of PGI₃ production in P2Y-stimulated endothelial cells, and to a recent report showing that endothelial P2 receptors are linked to stimulation of MAPK activity [38]. The role of elevated [Ca²⁺] in the endothelial PGI₃ response has been well established (see, for example, [16]). We do not know how different components of the signalling pathway, [Ca²⁺], PKC and MAPK, interact in the control of this aspect of endothelial function. It is relevant that recent studies on cells transiently transfected with cPLA₂ suggest that in intact cells the activation of cPLA₂ requires phosphorylation by MAPK, followed by translocation to the site of the lipid substrate caused by an increase in cytosolic [Ca²⁺] [39, 40]. Activation (by phosphorylation) and translocation (by Ca²⁺) are independent events, both required for stimulation of the release of arachidonic acid. In this paper we have provided evidence for a role for MAPK in the P₂-purinergic stimulation of endothelial PGI₃ production, but the hypothesis that this occurs by the phosphorylation of cPLA₂ at Ser-505 requires further investigation.

Despite a large number of recent studies of the pathways whereby MAPK might be activated by heterotrimeric G-protein-coupled receptors, there has been little indication of the diversity of responses that this might mediate. It has widely been interpreted that the ability of G-protein-coupled receptors to activate MAPK is a reflection of their ability to modulate cellular proliferation and differentiation (see, for example, [4]). Here we show that in cultured endothelial cells the stimulation of P2 purinoceptors leads to the phosphorylation and activation of MAPK, and provide striking evidence that this is an absolute requirement for the PGI₃ response of these cells to ATP. This is consistent with a report, published while this paper was in preparation [41], showing that the PGI₃ response to thrombin stimulation of human umbilical vein endothelial cells is dependent on p42 MAPK activation. Taken together, these reports provide novel insights into the mechanisms of regulation of vascular endothelial function, and provide an example, in a native system, of a role for MAPK in short-term responses to G-protein-coupled receptor activation.

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REFERENCES


Figure 5 Effects of olomoucine on ATP-stimulated MAPK activity and the 6-keto-PGF₁α response

(A) Cells were stimulated with ATP and extracts separated by Resource Q chromatography into two peaks, each of which was then assayed for peptide kinase activity (mean ± S.E.M., n = 3) in the presence of the concentrations of olomoucine shown. Symbols: ○, first peak (p42MAPK); ●, second peak (p44MAPK). (B) Cells were stimulated with 300 µM ATP for 5 min in the presence of 300 µM olomoucine and the 6-keto-PGF₁α response was measured as described (means ± S.E.M., n = 3). (C) Phospho-MAPK Western blot. Stimulation with 300 µM ATP in the presence and absence of olomoucine (300 µM) shows that olomoucine (results not shown).

(Figure 5C), showing that olomoucine did not affect the activation of the pathway from the occupation of the P2 receptors to phosphorylation of MAPK by MEK. We have further shown that the 6-keto-PGF₁α assay itself was unaffected by the presence of olomoucine (results not shown).

It has previously been reported that MAPK activity was required for the stimulation of cPLA₂ by basic fibroblast growth factor [25] by using immunodepletion of MAPK, and subsequent stimulation with growth factor, in a broken-cell preparation. This protocol eliminates many regulatory mechanisms operational within an intact cell, including changes in [Ca²⁺] and protein kinase C, in response to receptor activation. Here we provide direct evidence that MAPK is part of the pathway to stimulation of PGI₃ production in intact endothelial cells.
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