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Evidence for requirement of tyrosine phosphorylation in endothelial P_{2Y}- and P_{2U}- purinoceptor stimulation of prostacyclin release

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1 The release of prostacylin (PGI_2) from vascular endothelial cells is stimulated by ATP acting at G protein-coupled P_2-purinoceptors. Here we investigate the hypothesis that tyrosine protein phosphorylations are involved in this response.

2 The use of Western blots with anti-phosphotyrosine antibodies showed that 30 μM 2MeSATP (selective for P_{2Y}-purinoceptors), 300 μM UTP (selective for P_{2U}-purinoceptors) and 300 μM ATP (effective at both these purinoceptors), each stimulate the tyrosine phosphorylation of proteins in bovine cultured aortic endothelial cells. Each of these agonists also stimulates 6-keto $PGF_{1\alpha}$ accumulation in the medium (an index of PGI_2 release) in these cells in the same period.

3 The tyrosine kinase inhibitor, genistein, inhibits the 6-keto $PGF_{1\alpha}$ response with the same concentration-dependency (1-100 μM) as the tyrosine phosphorylation response.

4 Tyrphostin, a structurally and functionally distinct tyrosine kinase inhibitor, is also a potent inhibitor (0.1-10 μM) of the 6-keto $PGF_{1\alpha}$ response.

5 Neither tyrphostin nor genistein inhibit the phospholipase C response to P_2-purinoceptor stimulation. Furthermore, these inhibitors do not affect the 6-keto $PGF_{1\alpha}$ response to ionomycin.

6 These results show that the regulation of vascular endothelial cells by ATP acting at both P_{2Y}- and P_{2U}-purinoceptors involves the stimulation of tyrosine phosphorylation, and suggest that this is a necessary event for the purinoceptor-mediated stimulation of PGI_2 production.

Keywords: P_2-purinoceptors; prostacyclin; phospholipase A_2; endothelium; tyrosine kinases

Introduction

Two widespread classes of cell surface receptor are those for growth factors, with a single transmembrane span and intrinsic tryosine kinase activity, and those receptors which have seven transmembrane domains and are coupled to effector mechanisms by G proteins. The recognition that tryosine kinase activity is intrinsically involved in the function of growth factor receptors has been followed by more recent observations that regulation of tyrosine protein phosphorylations may be involved in the receptor effector cascade following activation of G protein-coupled receptors (e.g. Force *et al.*, 1991; Marrero *et al.*, 1994; Chen *et al.*, 1994; Duff *et al.*, 1994). This tyrosine phosphorylation response is generally reported for G protein-coupled receptors which also activate phospholipase C (PLC), and has been considered as downstream of the consequent elevation in cystolic Ca^{2+} and activation of protein kinase C (PKC) (Force *et al.*, 1991; Huckle *et al.*, 1992; Offermanns *et al.*, 1993). Recently PLC-independent mechanisms have been proposed for the G protein activation of tyrosine kinases (e.g. Tsukada *et al.*, 1994; Marrero *et al.*, 1995). G protein-coupled receptors have also been shown to be coupled to the activation of mitogen activated protein kinase (MAPK). This is a response which is known to require tyrosine phosphorylation (jointly with threonine phosphorylation) and has been variously described as downstream of PLC or independent of PLC, depending on the receptor-cell combination investigated (e.g Winitz *et al.*, 1993; Granot *et al.*, 1993; Crespo *et al.*, 1994).

The cytosolic form of phospholipase A_2 (PLA_2) is the rate-limiting enzyme in the receptor regulated pathway for eicosanoid biosynthesis in diverse cell types. Particular significance has been attached to the activation of PLA_2 in vascular endothelial cells, stimulating the release of PGI_2 and consequent influence over both platelet and smooth muscle function. Endothelial production of PGI_2 is regulated by cell surface receptors, including the G protein-coupled receptors for ATP and ADP (the P_2-purinoceptors) (Pearson *et al.*, 1983; Needham *et al.*, 1987; Lustig *et al.*, 1992). This response involves the elevation of cytosolic Ca^{2+} and the activation of the cytosolic form of PLA_2 (Martin *et al.*, 1987; Carter *et al.*, 1988; Lin *et al.*, 1992; Lustig *et al.*, 1992), a response known to be modulated by PKC (Carter *et al.*, 1989). In bovine aortic endothelial cells, ATP acts on co-existing P_{2Y} and P_{2U}-purinoceptors, both linked to vasorelaxant responses by G proteins and PLC (Motte *et al.*, 1993; Wilkinson *et al.*, 1993; 1994).

It is known that the p42 form of MAPK is capable of phosphorylating and activating PLA_2 in recombinant systems (Lin *et al.*, 1993), and that basic fibroblast growth factor, acting on its intrinsic tyrosine kinase receptor, stimulates endothelial PLA_2 by MAPK-dependent phosphorylation (Sa *et al.*, 1995). These results implicate tyrosine kinase pathways in the control of endothelial PLA_2. In renal mesangial cells it has recently been shown that tyrosine phosphorylation and MAPK activity are regulated by G protein-linked P_2-purinoceptors (Huwiler & Pfeilshifter, 1994), However, nothing is known about a possible role for tyrosine kinase activities in the regulation of endothelial cells by G protein-coupled receptors. In this paper we provide the first demonstration of tyrosine protein kinases activation by G protein-coupled endothelial P_2-purinoceptors, and present evidence that activation of tyrosine kinases is required for the stimulation of PGI_2 release by purinoceptor agonists such as ATP.

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Methods

Cell culture

Bovine aortic endothelial cells (BAECs) were prepared by the enzymic digestion of fresh aortae, as described in Booyse *et al.* (1975), cultured in Minimal Essential Medium D-valine with 10% foetal calf serum, 10% newborn calf serum, 25 i.u. ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 10 mg ml^{-1} gentamycin, 250 μg ml^{-1} fungizone and 27 mg ml^{-1} glutamine, and used after passage as a just confluent monolayer in 24 well multiwells.

Measurement of 6-keto $PGF_{1\alpha}$ release

The accumulation of 6-keto $PGF_{1\alpha}$ in the supernatant of cells stimulated in wells of 24 well multiwells was used as an index of PGI_2 release. In preliminary studies the incubation period was initiated by aspiration of well contents and replacement with the incubate. It was found that this change of medium alone generated a rapid and substantial increase in 6-keto $PGF_{1\alpha}$ accumulation which masked early effects of inclusion of agonists. We therefore designed a modified protocol which gave a stable level in the control unstimulated cells at each of the time points used. The cells were first washed twice in warm balanced salt solution (BSS, composition mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.1, $MgSO_4$ 0.8, glucose 5.5, HEPES 30, NaH_2PO_4 1, $CaCl_2$ 1.8; gassed with 5% CO_2 and adjusted to pH 7.4 with NaOH); 1 ml of BSS was then added to begin a 10 min preincubation, at the end of which 730 μl of the preincubate was removed and 30 μl of the agonist, at 10 fold final concentration, was added, to begin the incubation period. The incubation period was terminated by the removal of the supernatants to ice and storage at −20°C prior to assay. Where tyrosine kinase inhibitors were used they were present for a 30 min preincubation, as well as during the incubation. In these cases the cells were preincubated for 20 min with the inhibitor, followed by a second preincubation of 10 min duration with 1 ml of the inhibitor, at the end of which the incubation was started by removal of 730 μl and addition of 30 μl of 10 fold concentration of agonist. The radioimmunoassay for 6-keto $PGF_{1\alpha}$ used 25 μl (9000) d.p.m. of 6-keto [5,6,9,11,12,14,15-^{3}H]-prostaglandin $F_{1\alpha}$, 25 μl of polyclonal anti-6-keto $PGF_{1\alpha}$ at a 1:10 dilution, and 50 μl of sample or standard. After incubation overnight at 4°C, separation of bound from free was by adsorption onto dextran coated charcoal.

Phosphotyrosine Western blots

BAECs were cultured to 90% confluence, when they were maintained in serum-free medium for 24 h prior to use. The flask was then placed in a 37°C water bath, the medium was aspirated and the cells washed twice with 10 ml BSS. After a 2 min period the medium was carefully removed and replaced with 2 ml BSS containing 250 μM sodium pervanadate and agonists as indicated. After a 5 min incubation the BSS was decanted and liquid N_2 added to the flask to cover the monolayer, and the flask transferred to ice. As the last of the liquid N_2 boiled away, 300 μl of homogenizing buffer (20 mM Tris-HCl pH 7.4; 2 mM ethylenediamine tetraacetic acid (EDTA), 10 μg ml^{-1} leupeptin, 20 μM E-64, 2 μg ml^{-1} aprotinin, 1 μM pepstatin A, 50 mM sodium fluoride, 2.5 mM sodium orthovanadate, 62.5 mM β-gylcerophosphate, 1 mM phenymethysulphonyfluoride, 0.1% Triton X-100) was added. The flask was then sonicated in iced water for 12 min, cells scrape harvested and centrifuged at 12,000 r.p.m. at 4°C for 12 min. An aliquot of the supernatant was reserved for protein determination, while the remainder was added to an equal volume of double concentration of Leammli's sample buffer (final: 0.0625 M Tris HCl pH 6.8, 2% SDS, 10% gylcerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue). These extracts were then subject to 10% SDS–PAGE, the proteins electrophoretically transferred to nitrocellulose membrane, immunoblotted with anti-phosphotyrosine monoclonal antibodies, and developed with anti-mouse Ig peroxidase-linked antibody and the Amersham enhanced chemiluminescence (ECL) system. Visualisation with autoradiography was followed by laser densitometry.

Assay of [^{3}H]-inositol (polyphosphates)

Cells were labelled with [^{3}H]-*myo*-inositol for 48 h, stimulated and total [^{3}H]-inositol (poly)phosphates extracted and measured essentially as described in Wilkinson *et al.* (1993).

Materials

The monoclonal anti-phosphotyrosine antibodies and polyclonal anti-6-keto $PGF_{1\alpha}$antiserum were purchased from Sigma, Poole, Dorset. The 6-keto [5,6,9,11,12,14,15-^{3}H]-$PGF_{1\alpha}$, unlabelled 6-keto$PGF_{1\alpha}$, and ECL reagents were from Amersham (Bucks), genistein and tyrphostin A25 from Gibco Life Technologies (Paisley, Scotland), 2MeSATP from Research Biochemicals (Semat, Herts), while other biochemicals were from Sigma or Fisons (Loughborough, Leicestershire).

Results

Stimulation of PGI_2 production and tyrosine phosphorylations by P_{2Y}- and P_{2U}-purinoceptor agonists

Three agonists were used in this study: 2MeSATP (30 μM) and UTP (300 μM) maximally and selectively stimulated the P_{2Y}- and P_{2U}-purinoceptors respectively, while ATP at 300 μM maximally stimulated both receptor subtypes. Each of these agonists were seen to stimulate the release of PGI_2, as indicated by the accumulation of 6-keto $PGF_{1\alpha}$ in the medium. The time course of accumulation of 6-keto $PGF_{1\alpha}$, shown in Figure 1a, was maximal at 3.5–5 min for all 3 agonists. Characteristically the response to ATP was approximately the equivalent of the sum of the responses to UTP and 2MeSATP. While in Figure 1 the response to UTP was smaller than that to 2MeSATP, the relative size of these two responses varied between experiments and this difference was not reliable (ratio of responses to 2MeSATP/UTP pooled across experiments was 1.71 ± 0.55). Examples of concentration-response curves for responses to UTP and 2MeSATP are shown in Figures 2a and b respectvely. The EC_{50} pooled across 4 experiments (each in triplicate) was 28.0 μM (log $EC_{50} = 1.45 \pm 0.084$ μM) for UTP and 7.08 μM (log $EC_{50} = 0.85 \pm 0.125$ μM) for 2MeSATP.

To determine whether vascular endothelial G-protein P_2-purinoceptors are linked to activation of tyrosine kinase in the endothelial cells, we stimulated for 5 min with the 3 agonists in the presence of pervanadate to inhibit protein tyrosine phosphatases. Figure 1b shows that each agonist resulted in an enhanced level of tyrosine phosphoproteins in multiple bands on the Western blot, indicating that activation of both P_{2Y}- and P_{2U}-purinoceptors involves tyrosine kinase activation as part of the receptor mechanism. The data from laser scanning of the autoradiographs and pooling across 9 separate experiments showed that, expressed as fold over unstimulated control levels, the response was 5.8 ± 0.63 for 300 μM ATP, 5.0 ± 0.19 for 30 μM 2MeSATP, and 2.8 ± 0.61 for 300 μM UTP.

Effects of tyrosine kinase inhibitors on responses to P_2-purinoceptor agonists

Figure 2a and b show the concentration-response curves to UTP and 2MeSATP respectively in the absence and the presence of 100 μM genistein. Genistein was present for a 30 min preincubation as well as during the 5 min incubation. Genistein completely abolished the response to each of these agonists throughout the concentration-response range. Figure 3a

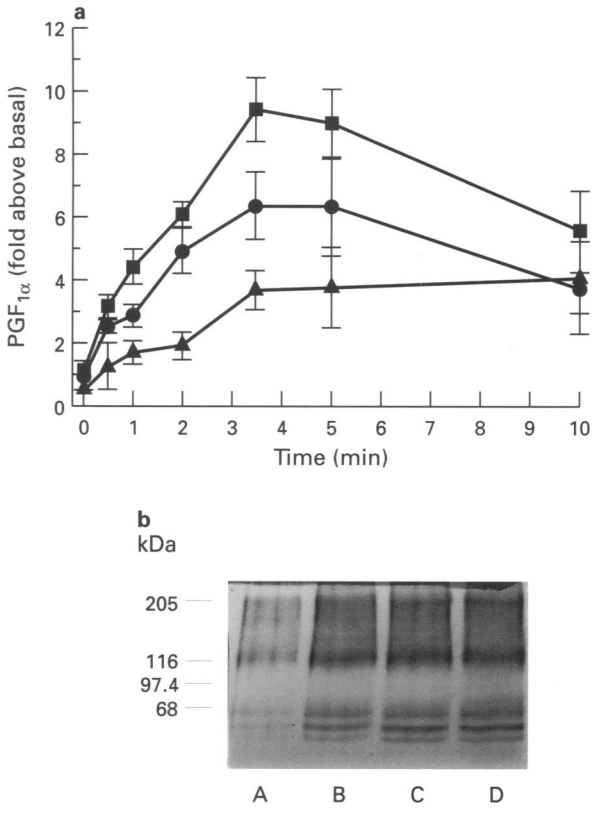

Figure 1 Stimulation of BAECs by ATP, UTP and 2MeSATP. (a) Time course of stimulation of 6-keto $PGF_{1\alpha}$ accumulation in response to 300 μM ATP (■), 300 μM UTP (▲), 30 μM 2MeSATP (●). Data are expressed as fold over basal (incubation for the same time in the absence of agonist) and pooled to give mean ± s.e.mean, $n=3$. (b) Tyrosine phosphoprotein immunoblots showing effect of 5 min incubation with: (A) control with no agonists; (B) UTP (300 μM); (C) 2MeSATP (30 μM); (D) ATP (300 μM). One of 3 similar experiments.

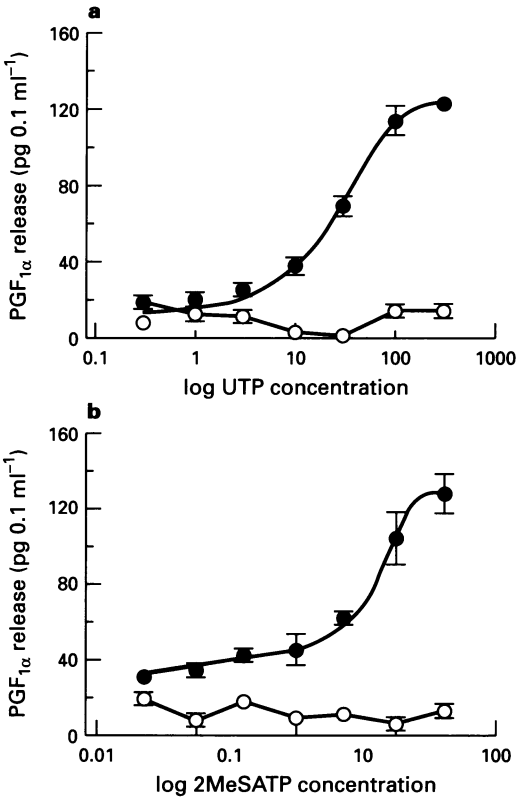

Figure 2 Effect of 100 μM genistein (○, or control with no genistein, ●) on the accumulation of 6-keto $PGF_{1\alpha}$ in response to increasing concentrations of (a) UTP; (b) 2MeSATP. Data are mean ± s.e.mean, $n=3$, from one experiment representative of 3.

and b shows the response to 30 μM 2MeSATP and 300 μM UTP with increasing concentrations of genistein. The effect of genistein on the levels of 6-keto $PGF_{1\alpha}$ in the medium from unstimulated control wells is also shown. The results show an inhibitory effect of genistein on unstimulated release of PGI_2 (seen with most, but not all, experiments). Pooled across 6 experiments each in triplicate, the IC_{50} for the inhibition by genistein on the control release was 39.5 μM (log $EC_{50} = -4.40 \pm 1.14$ μM). When stimulated release was determined by deduction of the control for each concentration of genistein, the IC_{50} for the 2MeSATP stimulation was 20.4 μM (log $EC_{50} = -4.69 \pm 0.49$ μM), and the IC_{50} for UTP stimulation was 16.2 μM (log $EC_{50} = -4.79 \pm 0.48$ μM). These results show that 6-keto $PGF_{1\alpha}$ accumulation in response to both P_{2Y}- and P_{2U}-purinoceptor stimulation was sensitive to the tyrosine kinase inhibitor genistein with a similar concentration-dependency.

Tyrphostin is another tyrosine kinase inihibtor, based on a different structure. Figure 4 shows that tyrphostin was a potent inhibitor of both the UTP and the 2MeSATP stimulation of 6-keto $PGF_{1\alpha}$. The IC_{50} of the inhibition by tyrphostin of the stimulation by 2MeSATP and UTP of 6-keto $PGF_{1\alpha}$ accumulation was 0.91 (log -6.04 ± 0.39 μM) and 0.51 (log -6.29 ± 0.25 μM) respectively (3 separate experiments, each in triplicate).

To test the hypothesis further that the inhibitory effect of genistein was due to tyrosine kinase inhibition, the effect of different concentrations of genistein on the P_2-purinoceptor stimulation of tyrosine phosphoprotein abundance on Western blots was examined. Figure 5a shows a sample blot in which the effect of 3–100 μM genistein on ATP-stimulated tyrosine phosphorylation is shown. In Figure 5b the results from sev-

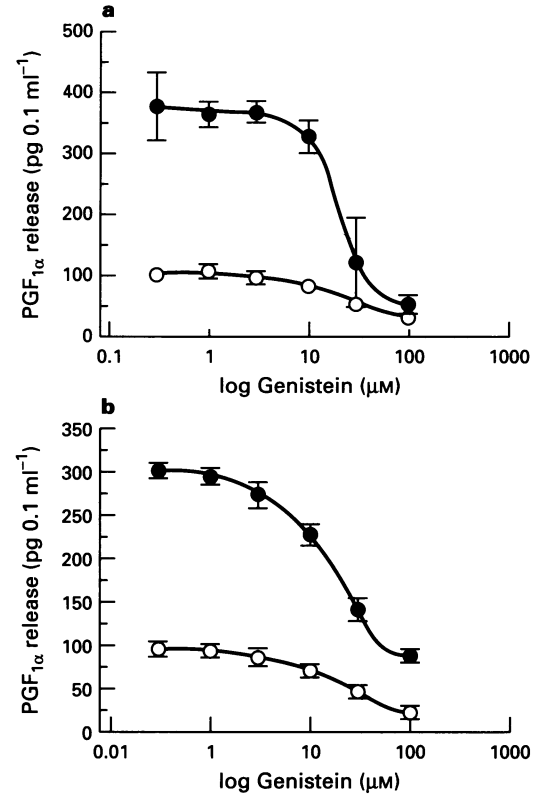

Figure 3 Effect of increasing concentrations of genistein on stimulation of 6-keto $PGF_{1\alpha}$ by (a) 30 μM 2MeSATP, and (b) 300 μM UTP (●) or on unstimulated controls (○). Data are mean ± s.e.mean, $n=3$, from one experiment representative of 3.

eral experiments, scanned and pooled, is used to show that genistein produces a profound inhibition of ATP stimulated tyrosine phosphorylation with an EC_{50} of 14.1 (log -4.85 ± 0.03 μM). This is essentially the same concentration-dependency as that seen with agonist-stimulated 6-keto $PGF_{1\alpha}$ stimulation.

To investigate whether the two tyrosine kinase inhibitors interfere with any part of the process from receptor occupation by the agonist to the activation of PLC, we looked at the generation of total [^{3}H]-inositol (poly)phosphates in response to 20 min stimulation by ATP [^{3}H]-inositol labelled cells in the presence of increasing concentrations of either tyrphostin or genistein. We found that these inhibitors had no effect on the stimulation of PLC (Table 1).

Stimulation with ionomycin: effect of tyrosine kinase inhibitors

Ionomycin is a calcium ionophore which stimulates PGI_2 production by elevating cytosolic Ca^{2+}. Across 3 separate experiments, 5 μM ionomycin stimulated 6-keto $PGF_{1\alpha}$ by 7.8 ± 0.88 fold over basal. As shown in Figure 6, there was no effect on this stimulation of either genistein or tyrphostin, at concentrations up to 100 μM and 10 μM respectively.

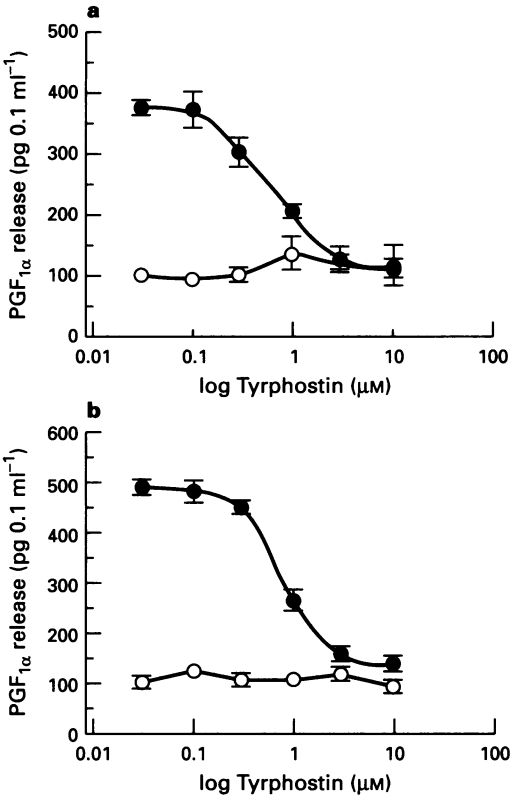

Figure 4 Effect of increasing concentrations of tyrphostin on stimulation of 6-keto $PGF_{1\alpha}$ by (a) 30 μM 2MeSATP, and (b) 300 μM UTP (●) or on unstimulated controls (○). Data are mean ± s.e.mean, $n = 3$, from one experiment representative of 3.

Discussion

The importance of vascular endothelial cells in controlling local aspects of circulatory function is well established. This is largely accomplished by the release of mediators such as endothelin, nitric oxide and PGI_2, under the regulatory influence of cell surface receptors. An important example of this reg-

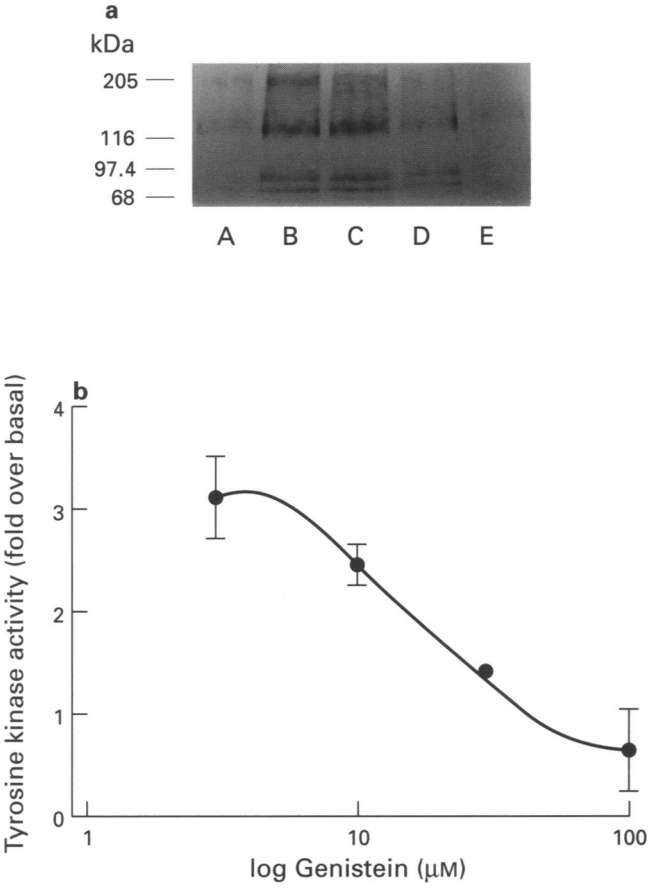

Figure 5 Effect of genistein on the ATP stimulated accumulation of tyrosine phosphoproteins. (a) A sample anti-phosphotyrosine immunoblot: lane (A) unstimulated controls; lanes (B-E) stimulated with 300 μM ATP with genistein at 3 μM (lane B), 10 μM (lane C), 30 μM (lane D) and 100 μM (lane E). (b) Quantification of immunoblots by laser scanning and data pooled across 3 experiments, mean ± s.e.mean.

Table 1 Stimulation of phospholipase C by ATP in the presence of genistein and tyrphostin

	Genistein (μM)				
	0	*3*	*0*	*30*	*100*
ATP (100 μM)	4899 ± 544	6049 ± 1407	5372 ± 472	5402 ± 766	7250 ± 974
	Tyrphostin (μM)				
	0	*0.3*	*1*	*3*	*10*
ATP (100 μM)	3322 ± 272	4199 ± 445	3661 ± 201	3708 ± 89	3658 ± 175

Data are d.p.m of total [^{3}H]-inositol (poly)phosphates in extracts of cells stimulated with ATP (100 μM) for 20 min, presented as mean ± s.e.mean ($n = 3$) from single experiments, representative of 3 experiments. The unstimulated controls for the genistein experiment were 377 ± 48 d.p.m. and for the tyrphostin, 406 ± 69 d.p.m.

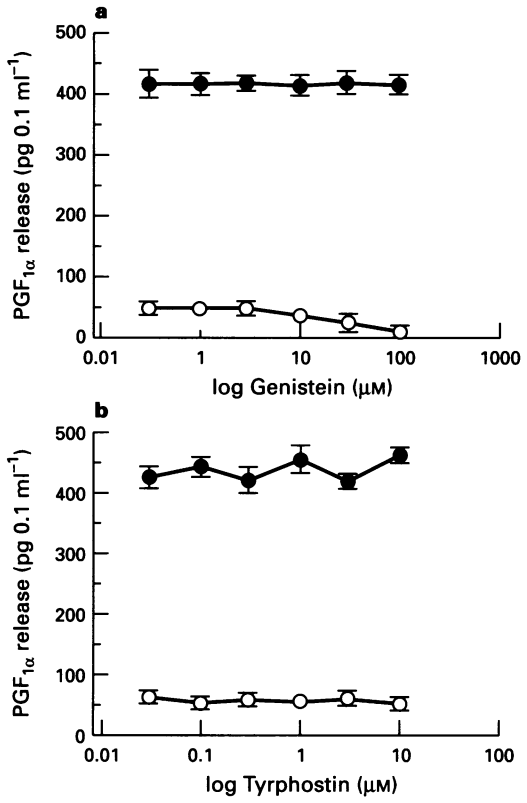

Figure 6 Effect of increasing concentrations of genistein (a) or tyrphostin (b) on stimulation of 6-keto $PGF_{1\alpha}$ by 5 μM ionomycin (●) or on unstimulated controls (○). Data are mean ± s.e.mean, $n=3$, from one experiment representative of 3.

ulation is the influence of the receptors for ATP, the P_2-purinoceptors, which have long been known to control the release of mediators, including PGI_2 (e.g. Pearson *et al.*, 1983; Needham *et al.*, 1987). In BAECs these receptors comprise co-existing P_{2U}- and P_{2Y}-purinoceptors (Wilkinson *et al.*, 1993), and in this study we have asked whether tyrosine phosphorylations are involved in the mechanism whereby these receptors control the release of PGI_2 from cultured endothelial cells.

We have confirmed that stimulation of both the P_{2Y} - and P_{2U}-purinoceptors present on BAECs leads to the enhanced release of PGI_2, with time courses and concentration-response relationships which are consistent with the previously characterized involvement of PLC (Wilkinson *et al.*, 1993; Purkiss *et al.*, 1994). In unpublished work we have shown that these receptors stimulate [^{3}H]-*lyso*-phosphatidylcholine production in [^{3}H]-choline labelled cells and the liberation of [^{3}H]-arachidonic acid from [^{3}H]-arachidonate-labelled cells, confirming the expectations from the literature that the receptors regulate PGI_2 production by the activation of PLA_2. In addition we show in the present paper that these two receptors stimulate the accumulation of tyrosine phosphoproteins under conditions in which protein phosphatases are inhibited. This establishes that endothelial P_2-purinoceptors are linked to the activation of tyrosine kinases.

To determine whether the activation of tyrosine kinases was involved in the P_{2U}- and P_{2Y}- purinoceptor stimulation of PGI_2 release, two tyrosine kinase inhibitors with different modes of action were used. Genistein inhibits the action of tyrosine kinases by competing with ATP for a binding site in the tyrosine kinase, resulting in the formation of a non-productive enzyme-substrate complex (Akiyama *et al.*, 1987). Tyrphostin is a competitive inhibitor of substrate binding (Gazit *et al.*, 1989). In our experiments genistein, but not tyrphostin, inhibited the basal release of PGI_2, while both tyrosine kinase inhibitors were able to abolish agonist-stimulated PGI_2 release. Neither inhibitor affected release in response to the Ca^{2+} ionophore, ionomycin.

Genistein had no influence on standard curves for 6-keto $PGF_{1\alpha}$ radioimmunoassay: this was shown by adding the inhibitor direct to the assay tubes (unpublished) and by the lack of effect on ionomycin stimulation described here. Neither genistein nor tyrphostin, in the concentration-range in which they inhibited tyrosine phosphorylation and PGI_2 production, had any affect on the P_2-purinoceptor stimulation of PLC in these cells. In further unpublished work we have shown: (i) inhibition of basal release by 20 min incubation with genistein recovers after a 5 min wash; (ii) on culture of cells for 3 days after a 20 min exposure to genistein there was no effect on growth or appearance of cells. These various observations show that genistein, and to some extent tyrphostin, are not influencing the results by interference with the radioimmunoassay, attenuation of binding to the receptors, or interference with the signal transduction mechanism from receptor to phospholipase C. The latter point is particularly important in view of the reported effect of genistein on attenuating Ca^{2+} mobilisation and phosphoinositide turnover reported by Ozaki *et al.* (1993).

The EC_{50} values found in the present study for inhibition of agonist-stimulated PGI_2 release are similar to those reported in other studies for inhibition of tyrosine phosphorylation (e.g. Akiyama *et al.*, 1987; Gazit *et al.*, 1989). In confirmation of this we showed inhibition of agonist-stimulated tyrosine phosphorylation within the same concentration-range as inhibition of stimulated PGI_2 release. Taken with the use of two inhibitors with differing structures and differing site of action, it is likely that the effects of these two compounds on PGI_2 is due to their inhibition of tyrosine kinase activities. We conclude from these experiments that agonist activation of tyrosine kinases is necessary for the P_2-purinoceptor stimulation of PGI_2 release.

Previous work has shown that stimulation of PGI_2 release in this system is driven by the elevation of cytosolic Ca^{2+} (Carter *et al.*, 1988), and that this response to Ca^{2+} is enhanced by activation of PKC (Carter *et al.*, 1989; Zavoico *et al.*, 1990). The results presented here show that, while tyrosine phosphorylation is necessary for the agonist-stimulated PGI_2 release, it is not required when release is stimulated by the high levels of cytosolic Ca^{2+} achieved by ionomycin. As an hypothesis to explain these results we propose that tyrosine phosphorylation enhances the sensitivity of the PGI_2 response to Ca^{2+}. In this model, the levels of cytosolic Ca^{2+} achieved by agonist stimulation are only effective when there is concurrent tyrosine phosphorylation (reducing the threshold for Ca^{2+} activation of PLA_2), while the higher Ca^{2+} levels achieved by ionomycin are sufficient to cause maximal activation in the absence of tyrosine phosphorylation. A further important question, the level of involvement of the tyrosine phosphorylation, is a matter for speculation. It may be upstream of the PLA_2 activity, perhaps involving MAPK (Lin *et al.*, 1993; Sa *et al.*, 1995), or it may be at the level of PLA_2 involving the direct tyrosine phosphorylation of this enzyme (Kast *et al.*, 1993).

In conclusion we have shown that both P_{2Y}- and P_{2U}-endothelial purinoceptors are coupled to tyrosine phosphorylation and have provided compelling evidence that tyrosine kinase activation is required for the stimulation by ATP of PGI_2 release from endothelial cells.

References

AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, H., WATANABE, S., ITOH, N., SHIBUYA, M. & FUKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262**, 5592–5595.

BOOYSE, F.M., SEDLACK, B.J. & RAFELSON, M.E. (1975). Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells. *Trombos. Diath. Haemorrh.*, **34**, 825–839.

CARTER, T.D., HALLAM, T,J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2Y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95,** 1181–1190.

CARTER, T.D., HALLAM. T.J., & PEARSON, J.D. (1989). Protein kinase C activation alters the sensitivity of agonist stimulated endothelial cell prostacyclin production to intracelluar Ca^{2+}. *Biochem. J.*, **262,** 431–437.

CHEN, Y., POUYSSEGUR, J., COURTNEIDGE, S.A. & OBBERGHEN-SCHILLING, E.V. (1994). Activation of Src family kinase activity by the G protein-coupled thrombin receptor in growth-responsive fibroblasts. *J. Biol. Chem.*, **269,** 27372–27377.

CRESPO, P., XU, N., SIMONDS, W.F. & GUTKIND, J.S. (1994). Ras-dependent activation of MAP kinase pathway mediated by G protein β,γ subunits. *Nature*, **369,** 418–420.

DUFF, J.L., MARRERO, M.B., PAXTON, W.G., CHARLES, C.H., LAU, L.F., BERNSTEIN, K.E. & BERK, B.C. (1994) Angiotensin II induces JCH134, a protein tyrosine phosphatase, in vascular smooth muscle cells. *J. Biol. Chem.*, **268,** 26037–26040.

FORCE, T., KYRIAKIS, J.M., AVRUCH, J. & BONVENTRE, J.B. (1991). Endothelin, vasopressin and angiotensin II enhance tyrosine phosphorylation by protein kinase C dependent and independent pathways in glomerular mesangial cells. *J. Biol. Chem.*, **266,** 10754–10759.

GAZIT, A., YAISH, P., GILON, C. & LEVITSKI, A. (1989). Tyrphostins I: synthesis and biological activity of protein kinase inhibitors. *J. Med. Chem.*, **32,** 2344–2352.

GRANOT, Y., ERIKSON, E., FIRDMAN, H., PUTTEN, V.V., WILLIAMS, B., SCHRIER, R.W. & MALLER, J.W. (1993). Direct evidence for tyrosine and threonine phosphorylation and activation of mitogen-activated protein kinase by vasopressin in cultured rat vascular smooth muscle cells. *J. Biol. Chem.*, **268,** 9564–9569.

HUCKLE, W.R., DY, R. & EARP, H.S. (1992). Calcium-dependent increase in tyrosine kinase activity stimulated by angiotensin II. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 8837–8841.

HUWILER, A., FABBRIO, D. & PFEILSHIFTER, J. (1991). Possible regulatory functions of protein kinase C-α and ε isozymes in rat mesangial cells. Stimulation of prostaglandin synthesis and feedback inhibition of angiotensin II-stimulated phosphoinositide hydrolysis. *Biochem. J.*, **279,** 441–445.

KAST, R., FURSTENBURGER, G. & MARKS, F. (1993). Activation of cytosolic phospholipase A_2 by transforming growth factor-α in HEL-30 keratinocytes. *J. Biol. Chem.*, **268,** 16795–16802.

LIN, L.L., LIN, A.Y. & KNOPF, J.L. (1992). Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 6147–6151.

LIN, L.L., WARTMANN, M., LIN, A.Y., KNOPF, J.L., SETH, A. & DAVIS, R.J. (1993). $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell.*, **72,** 269–278.

LUSTIG, K.D., SPORTIELLO, M.G., ERB, L. & WEISMAN, G.A. (1992). A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionised forms of ATP and UTP. *Biochem. J.*, **284,** 733–739.

MARRERO, M.B., PAXTON, W.G., DUFF, J.L., BERJ, B.C. & BERNSTEIN, K.E. (1994). Angiotensin II stimulates tyrosine phosphorylation of phosphoilipase C-γl in vascular smooth muscle cells. *J. Biol. Chem.*, **269,** 10935–10939.

MARRERO, M.B., SCHIEFFER, B., PAXTON, W., HEERDT, L., BERK, B., DELAFONTAINE, P. & BERNSTEIN, K.E. (1995). Direct stimulation of Jak/STAT pathway by the angiotensin II AT_1 receptor. *Nature.*, **375,** 247–250.

MARTIN, T.W. & WYSOLMERSKI, R.B. (1987). Ca^{2+}-dependent and Ca^{2+}-independent pathways for the release of arachidonic acid from phosphatidylinositol in endothelial cells. *J. Biol. Chem.*, **262,** 13086–13092.

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

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.

OFFERMANNS, S., BOMBIEN, E. & SCHULTZ, G. (1993). Thrombin Ca^{2+} dependently stimulates protein tyrosine phosphorylation in BC_3Hl muscle cells. *Biochem. J.*, **290,** 27–32.

OZAKI, Y., YATOMI, Y. JINNAI, Y. & KUME, S. (1993). Effects of genistein, a tyrosine kinase inhibitor, on platelet functions. *Biochem. Pharmacol.*, **46,** 395–403.

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

PURKISS, J.R., WILKINSON, G.F. & BOARDER, M.R. (1994). Differential regulation of inositol 1,4,5-trisphosphate by co-existing P_{2Y}-purinergic and nucleotide receptors on bovine aorta endothelial cells. *Br. J. Pharmacol.*, **11,** 723–728.

SA, G., MURUGESAN, G., JAYE, M., IVASHCHENKO, Y. & FOX, P.L. (1995). Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via a p42 mitogen-activated protein kinase dependent phosphorylation pathway in endothelial cells. *J. Biol. Chem.*, **270,** 2360–2366.

TSUKADA, S., SIMON, M.I., WITTE, O.N., & KATZ, A. (1994). Binding of $\beta\gamma$ subunits of heterotrimeric G proteins to the PH domain of Brutons tyrosine kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 11256–11260.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1993). The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2Y}- purinoceptors and nucleotide receptors linked to phospholipase C. *Br. J. Pharmacol.* **108,** 689–693.

WILKINSON, G.F., MCKECHNIE, K., DAINTY, I. & BOARDER, M.R. (1994). P_{2Y}-purinoceptor and nucleotide receptor induced relaxation of precontracted bovine aortic collaterol artery rings: differential sensitivity to suramin and indomethacin. *J. Pharmacol. Exp. Ther.*, **268,** 881–887.

WINITZ, S., RUSSELL, M., QIAN, N.-X., GARDNER, A., DWYER, L. & JOHNSON, G.L. (1993). Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.*, **268,** 19196–19199.

ZAVOICO, G.H., HRBOLICH, J.K., GIMBRONE, M.A. & SCHAFER, A. (1990). Enhancement of thrombin-and ionomycin-stimulating prostacyclin and platelet activating factor production in cultured endothelial cells by a tumor promoting phorbol ester. *J. Cell. Physiol.*, **143,** 596–605.

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