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Protein kinase C isoforms in bovine aortic endothelial cells: role in regulation of P_{2Y}- and P_{2U}-purinoceptor-stimulated prostacyclin release

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1 Enhanced synthesis of prostacyclin (PGI_2) and inositol polyphosphates in bovine aortic endothelial cells in response to ATP and ADP is mediated by co-existing P_{2Y}- and P_{2U}-purinoceptors. Here we examine the regulation of these responses by isoforms of protein kinase C (PKC).

2 Immunoblots with antisera specific for 8 different PKC isoforms revealed the presence of α, ε and ζ, while no immunoreactivity was found for β, γ, δ, η and θ isoforms. PKC-α was largely cytosolic in unstimulated cells and almost all translocated to the membrane (Triton X-100 soluble) after a 1 min treatment with the PKC activating phorbol myristate acetate (PMA); PKC-ε was always in a Triton X-100 insoluble membrane fraction, while PKC-ζ was found in both soluble and membrane bound (Triton X-100 soluble) forms in the unstimulated cells and was unaffected by PMA.

3 Treatment with PMA for 6 h led to a 90% downregulation of PKC-α, while the immunoreactivity to the ε and ζ isoforms remained largely unchanged.

4 After either 10 min or 6 h exposure to PMA the PGI_2 response to activation of both receptors was enhanced, while the inositol 1,4,5-trisphosphate response to P_{2Y}-purinoceptor activation was substantially attenuated and the P_{2U}-purinoceptor response was unchanged. Thus the PGI_2 response to PMA under conditions when 90% of the PKC-α was lost resembles that seen on acute stimulation of PKC by PMA, and the PGI_2 response does not correlate with the phospholipase C response.

5 Inhibition of PKC with the isoform non-selective inhibitors, Ro 31-8220 and Go 6850 abolished the PGI_2 response to both P_{2U}- and P_{2Y}-purinoceptor stimulation. However, Go 6976, which preferentially inhibits Ca^{2+} sensitive isoforms (such as PKC-α) and not Ca^{2+} insensitive isoforms (such as PKC-ε), had no effect on the PGI_2 response.

6 The results show that there is a requirement for PKC in the stimulation of PGI_2 production by endothelial P_{2Y}- and P_{2U}-purinoceptors. Both downregulation and inhibition studies show that PKC-α is not responsible for the regulation of the response to P_2-purinergic stimulation, and imply that the response is mediated by PKC-ε (PKC-ζ is unresponsive to PMA), or an as yet uncharacterized PKC isoform.

Keywords: P_{2Y}-purinoceptors; P_{2U}-purinoceptors; protein kinase C isoforms; prostacyclin (PGI_2); endothelial cells

Introduction

The early report of ATP stimulated release of protaglandins from the vasculature (Needleman *et al.*, 1974), and subsequently the recognition that this contributes to endothelium-dependent vasorelaxation in the form of release of prostacyclin (PGI_2) from endothelial cells (Pearson *et al.*, 1983), established an important role for P_2-purinoceptors in endothelial function. Later studies attempted to investigate the receptor types and intracellular mechanisms involved in this response. The demonstration of a response to UTP as well as ATP (Needham *et al.*, 1987) and the presence of 'atypical' endothelial purinoceptors (Allsup & Boarder, 1990) led to the recognition of multiple G protein-coupled ATP receptors regulating vascular endothelial cells. Extracellular ATP and ADP control the stimulation of PGI_2 release from endothelial cells by acting on G protein-coupled P_2-purinoceptors (Boarder *et al.*, 1995). In bovine culutured aortic endothelial cells (BAECs) this is by action on two co-existing receptor types, the P_{2Y}-purinoceptors and the P_{2U}-purinoceptors (Motte *et al.*, 1993; Wilkinson *et al.*, 1993; 1994a). These receptors are each linked to phospholipase C, formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), and diacylglycerol, and raised intracellular Ca^{2+} and PKC activity. The control of endothelial PGI_2 synthesis in response to receptor activation has been interpreted as Ca^{2+} driven activation of PLA_2 modulated by PKC (Pearson *et al.*, 1983; Needham *et al.*, 1987; Martin & Wysolmerski, 1987; Carter *et al.*, 1988; 1989; Zavoico *et al.*, 1990; Lustig *et al.*, 1992; Lin *et al.*, 1992). However, it is also accepted that PLA_2 is subject to other receptor-linked regulatory influences, such as interaction with heterotrimeric G proteins (Jeselma & Axelrod, 1987; Silk *et al.*, 1989), and modulation by protein phosphorylations. Phosphorylating activities shown to be significant for PLA_2 stimulation and PGI_2 release are tyrosine kinases (e.g. Kast *et al.*, 1993; Bowden *et al.*, 1995), mitogen activated protein kinases (MAPK) (Lin *et al.*, 1993; Sa *et al.*, 1995), and protein kinase C (PKC) (Carter *et al.*, 1988; Zavoico *et al.*, 1990).

While both the BAEC P_{2Y}-purinoceptors are coupled to phospholipase C activation, there are differences in the nature of the response. For example, the P_{2Y}-purinoceptor $Ins(1,4,5)P_3$ response is attenuated by stimulation of PKC with phorbol myristate acetate (PMA), and enhanced by PKC inhibition with Ro 31-8220, but the P_{2U}-purinoceptor response is unaffected by these manipulations of PKC activity (Purkiss *et al.*, 1994). This illustrates the differential control of the two purinoceptor responses by PKC. To understand further the mechanisms and consequences of this differential control, in

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this paper we have extended the investigations into the isoforms of PKC in these cultured endothelial cells, and the influence of PKC on the regulation of PGI_2 release by the P_{2U}- and P_{2Y}-purinoceptors.

Methods

Cell culture

Fresh bovine aortae were collected and BAECs prepared as described by Booyse *et al.* (1975). Von Willebrand factor immunopositive cells were cultured in Minimal Essential Medium D-valine (MEM D-Val) with 10% foetal calf serum (FCS), 25 i.u. ml^{-1} penicillin, 25 $\mu g\ ml^{-1}$ streptomycin, and 27 $mg\ ml^{-1}$ glutamine and used just as they reached confluence. Cells were in 24 well multiwells for the 6-keto $PGF_{1\alpha}$ and inositol trisphosphate experiments, and in 80 cm^2 flasks for the PKC isoform studies, and were used after 24 h in medium with no serum.

Immunodetection of PKC

Cultured endothelial cells were washed with Earle's balanced salt solution (EBSS) containing (in mM): Nacl 130, KCl 5.3, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5.6, HEPES 25, $NaHPO_4$ 1 and phenol red 0.03 (pH 7.4) and maintained in culture for a further 24 h period in serum-free MEM D-Val. The cells were then incubated with or without the phorbol ester for the time periods indicated in the figurer legends. For the extraction of total (cytosolic and membrane-bound) PKC the cells were subsequently washed with ice-cold PBS and scraped into ice-cold homogenization buffer containing 25 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 25 $\mu g\ ml^{-1}$ leupeptin, 1 mM phenylmethylsulphonyl fluoride and 0.1% Triton X-100 (pH 7.5). All subsequent steps were carried out at 4°C. The cells were lysed in a Teflon pestle tissue grinder and centrifuged for 10 min at 14 000 *g*, after which sodium dodecyl sulphate (SDS) sample buffer was added to the supernatant. The sample was sonicated, boiled and finally stored at −20°C before being subjected to SDS-polyacrylamide gel electrophoresis (SDS/PAGE).

To obtain subcellular (cytosolic or membrane) fractions the cells were washed with ice-cold PBS and scraped into ice-cold homogenization buffer without Triton X-100, after which the cells were lysed and centrifuged as above. The supernatants were used as a source for the cytosolic fraction of PKC. The pellets were sonicated in Triton X-100- containing homogenization buffer and centrifuged for 10 min at 14 000 *g*, thus yielding the membrane (solubilized particulate) fraction. The presence of immunoreactivity in the membrane pellet in a form which was Triton insoluble was also investigated, in which case the membrane was solubilised directly in the SDS/PAGE sample buffer.

The PKC fractions were subjected to SDS/PAGE as described by Laemmli (1970). The separated proteins were then transferred on to mitrocellulose paper using a Bio-Rad (Hercules, CA). Transblot apparatus and a blotting buffer containing 48 mM Tris, 39 mM glycine, 0.03% SDS and 20% (v/v) methanol. After transfer the nitrocellulose membranes were treated overnight with 10% (w/v) non-fat dried milk, which was used as the blocking agent. The membranes were then incubated for 4 h at room temperature with polyclonal antibodies raised against the different PKC isoforms and further incubated for 1 h with donkey horseradish peroxidase-conjugated second antibodies. In some cases the specificity of the blot was tested by inclusion of excess of the peptide against which the antiserum was raised. In this case the antiserum was preincubated with the peptide for 1 h prior to use. Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL) detection system (Amersham). The intensities of the autoradiographs were scanned with an UltraScan apparatus from BioRad.

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

The cells were washed twice at 37°C in balanced salt solution (BSS) NaCl 125, KCl 5.4, $NaHCO_3$ 16.1, $MgSO_4$ 0.8, glucose 5.5, HEPES 30, NaH_2PO_4, $CaCl_2$ 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. 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 ligand was by adsorption onto dextran coated charcoal.

Ins(1,4,5)P_3 measurement

Cells were washed twice, followed by preincubation with PMA as indicated. The incubate was removed and replaced with BSS containing agonist and PMA as indicated. After the 5 s incubation the reaction was stopped with trichloroacetic acid, and the Ins(1,4,5)P_3 in the cells was extracted and measured by a protein binding assay as described in Purkiss *et al.* (1994).

Materials

Rabbit polyclonal antibodies raised against PKC isoforms β, γ, δ, η and ζ were a kind gift from Dr P.J. Parker, ICRF, London U.K. They were raised against peptides corresponding to the predicted COOH-sequence as previously described (Schaap & Parker, 1990; Marais & Parker, 1991; Olivier & Parker, 1991; Ways *et al.*, 1992; Dekker *et al.*, 1992). Rabbit polyclonal antibody against PKC-ε was purchased from Boehringer Mannheim Biochemicals, Germany, and mouse monoclonal antibody against PKC-α was purchased from UBI (TCS, Buckingham, UK). Secondary horseradish peroxidase-labelled antibodies, reagents for enhanced chemiluminesence detection, and 6-keto[5,6,9,11,12,14,15-^{3}H]-$PGF_{1\alpha}$, were from Amersham International, Buckinghamshire, U.K. The 6-keto $PGF_{1\alpha}$ antiserum was from Sigma, Poole, Dorset, U.K. Ro 31-8220 (see compound 3 in Davis *et al.* (1989) for structure) was a kind gift of Dr G. Lawton (Roche Products Ltd, Welwyn, Herts, U.K.) while Go 6976 and Go 6850 (see Martiny-Baron *et al.* (1993) for structure) were purchased from Calbiochem, Nottingham, U.K. Cell culture supplies were from GIBCO Life Technologies (Paisley, Scotland), 2MeSATP was from Research Biochemicals (Semat, Herts, U.K.) while other biochemicals were from Sigma, Poole, Dorset, U.K., or Fisons, Loughborough, U.K.

Data analysis and presentation

Data are expressed as mean ± s.e.mean for the indicated number of experiments, with significance determined by Student's *t* test.

Results

PKC isoforms: immunoreactivity in whole cell extracts

The presence of PKC isoforms in extracts of BAE cells was evaluated by Western blotting using antisera specific for 8 different isoforms. Positive immunoblots, using whole cell extracts, were obtained for α, ε and ζ isoforms, while no immunoreactivity was obtained for β, γ, δ η and θ isoforms. Each antiserum was tested in this procedure on a rat brain extract; Figure 1 shows a typical immunoblot for the threee positive antisera. The results shown include confirmation of the specificity of the immunoblot for PKC-ε and PKC-ζ using the

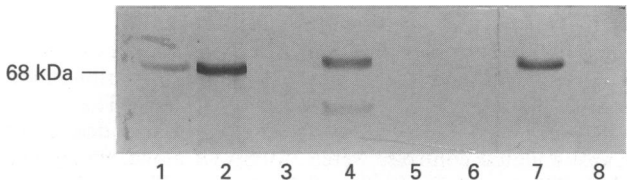

Figure 1 Identification of PKC isoforms in endothelial cell extracts by Western blotting with isoenzyme specific antisera. The six lanes on the left are analyses of whole endothelial cell extracts; the two lanes on the right are of rat brain extract (processed on the same gel) as positive controls for the detection of PKC-β. For the ε, ζ and β polyclonal antisera controls with the antigen peptide are shown (indicated as +P); the α antiserum was monoclonal. Lane 1, α; lane 2, ε; lane 3, ε+P; lane 4 ζ; lane 5 ζ+P; lane 6, β; lane 7, β (rat brain); lane 8, β+P (rat brain). A representative example of several similar experiments.

peptides against which the polyclonal antisera were raised (PKC-α antiserum was monoclonal). Figure 1 also shows a negative result for the PKC-β immunoblot of whole cell BAE extract, contrasted with a positive result for PKC-β with a rat brain extract which was simultaneously analysed. The PKC-β isoform was chosen to illustrate a negative result since it has previously been reported that this isoform is present in vascular endothelial cells (Hecker *et al.*, 1993). The PKC-β antiserum was non-selective for the $\beta_{1/2}$ variants, and though based on the human sequence reacts effectively with both rat and bovine PKC-$\beta_{1/2}$ (Marais & Parker, 1989).

PKC isoforms: distribution in membrane and cytosolic fractions, and translocation in response to phorbol ester

The distribution of the individual PKC isozymes between cytosolic and membrane fractions was investigated in untreated cells maintained serum-free for 24 h. Both PKC-α and PKC-ζ could be found in membrane bound and cytosolic form in the quiescent cells; the immunoreactivity in the membrane pellet could be solubilized by treatment with 0.1% Triton X-100. PKC-α was predominantly cytosolic (Figure 2a), while PKC-ζ showed more immunoreactivity in the membrane bound form than in the cytosol (Figure 2c). Contrasting with this, PKC-ε immunoreactivity was all membrane bound: the membrane bound nature of PKC-ε differed from that of the other two PKC isoforms present in that it was all membrane bound, but

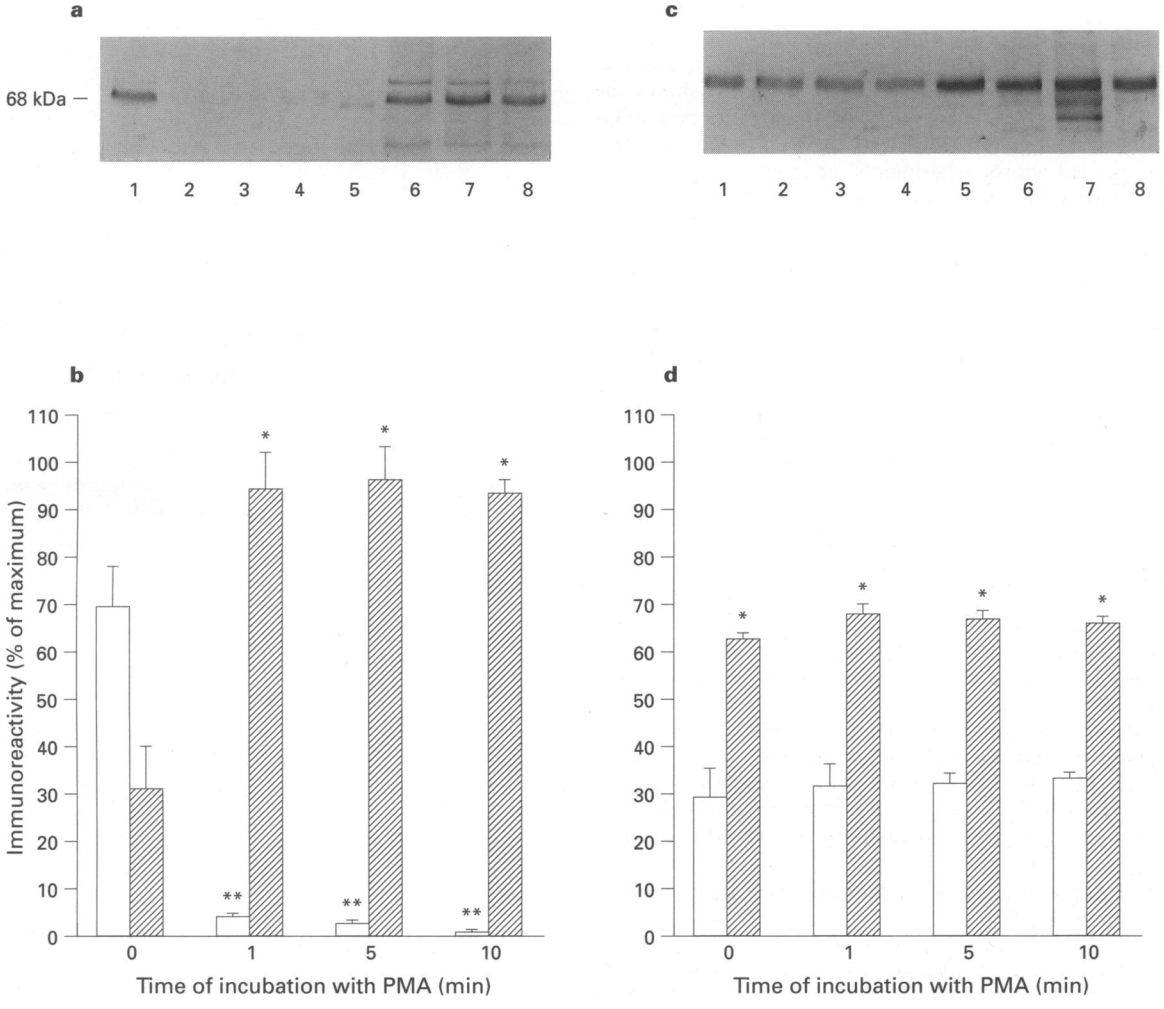

Figure 2 Distribution of PKC isoforms and translocation in response to PMA. The upper panels (a and c) are examples of autoradiographs from representative experiments; the histograms below (b and d) are results of pooled data, collected by laser densitometry of autoradiographs from 3 separate experiments. Cells were exposed to 1 μM PMA for 0, 1, 5 or 10 min, membrane and cytosolic fractions separated, and analysed by Western blot using antisera specific for PKC-α (a and b) and PKC-ζ (c and d). In (a) and (b) lanes are: 1–4, cytosolic, 5–8, membrane; 1 and 5, control with no PMA treatment. The remaining lanes are from experiments with exposure to PMA for 1 min (2 and 6), 5 min (3 and 7), and 10 min (4 and 8). In (b) and (d) the open columns are for the cytosolic fraction, and the hatched columns are the membrane fraction. Significantly different from no PMA treatment: $^{*}P<0.01$; $^{**}P<0.002$.

could not be solubilized by treatment with 0.1% Triton X-100 (data not shown).

The responsiveness of the PKC isoforms to the PKC activating phorbol ester PMA was evaluated. The differential translocation of PKC isoforms from the cytosolic to the membrane fraction in response to treatment with PMA has been widely reported. Figure 2 shows the effect of treatment with 1 μM PMA for up to 10 min on the distribution of PKC-α (Figure 2a and b) and PKC-ζ (Figure 2c and d). In each case a single autoradiography result is shown (upper panels) beside the pooled results from scanning of autoradiographs from repetitive experiments (lower panels): the loss from the cytoplasm was expressed as % of the level found in controls (no PMA treatment), and the gain in the membranes expressed as fold stimulation over the level seen in the controls. The results show that upon treatment with PMA, PKC-α was quickly translocated from the soluble to the particulate (Triton X-100 soluble) fraction. Essentially total translocation was achieved within 1 min of stimulation. However, PMA did not induce translocation of PKC-ζ to the particulate fraction or change the content of this isoform. Parallel analyses were carried out for PKC-ε immunoreactivity. The distribution did not change as a result of incubation with PMA for up to 10 min, PKC-ε remaining bound to the membrane in a Triton insoluble manner.

PKC isoforms: downregulation by phorbol ester

The effect of longer term treatment of BAECs with PMA on the levels of each individual isoform was studied, in order to establish the pattern of downregulation. Figure 3 shows the effects of 6–24 h treatment with PMA. In each case a typical autoradiograph is shown and data collected by scanning, and pooled across experiments, are presented. It can be seen in Figure 3a that within the minimum period investigated of 6 h, PKC-α showed a loss of most of the immunoreactivity (up to 95% loss) in these whole cell extracts. Almost 100% loss of reactivity was achieved by 24 h. Equivalent analysis for PKC-ε (Figure 3b) showed that treatment of PMA for 6 h caused a loss of approximately 25–30% in immunoreactivity in the Triton X-100 insoluble fraction. Longer treatment with PMA significantly reduced immunoreactivity by 70%. PKC-ζ unexpectedly showed a variable downregulation response, with a loss in some experiments but not others giving a result which was not consistently significant between 6 and 24 h, when pooled across experiments (data not shown). It was also characteristic of these experiments that forms of PKC-ζ immunoreactivity with a lower molecular weight appeared after certain times of treatment with PMA. Apparent downregulation in some experiments, and appearance of a lower molecular weight species, could be explained by partial proteolysis, as reported before (Ono *et al.*, 1989; Ways *et al.*, 1992). The differential effect of PMA between these isoforms was most clear at 6 h exposure, when PKC-α was substantially downregulated (amount remaining after PMA exposure was $13.51 \pm 1.5\%$ of control, $n=3$ experiments) but with PKC-ε and PKC-ζ there was a much smaller reduction, with the majority of immunoreactivity still present.

P_{2Y} and P_{2U}-purinoceptor stimulated PGI_2 production: modulation by PKC

Pretreatment of cells for 10 min with 1 μM PMA potentiated both the 2MeSATP and UTP stimulated release of 6-keto $PGF_{1\alpha}$ (Figure 4). PMA in the absence of agonist had no effect ($115 \pm 19\%$ of no PMA controls, $n=6$). Pretreatment of cells with a relatively specific PKC inhibitor, Ro 31-8220 (compound 3 in Davis *et al.*, 1989) abolished both UTP and 2MeSATP stimulation of 6-keto $PGF_{1\alpha}$ accumulation. The effect of Ro 31-8220 in the absence of agonist was a small fall in the level of accumulated 6-keto $PGF_{1\alpha}$ ($79 \pm 4.2\%$, $n=6$). Table 1 shows results with two further PKC inhibitors. Go 6850 is, like Ro 31-8220, a nonselective PKC inhibitor, while Go 6976 is selective for the Ca^{2+}-sensitive PKC isoforms (Martiny-Baron *et al.*, 1993). Table 1 shows that Go 6850, like Ro 31-8220, completely inhibited the response to both P_2-purinoceptor agonists. However, there was no effect of Go 6976. The results of all 3 such experiments undertaken, normalized by deduction of unstimulated controls, were: 2MeSATP alone, 96.2 ± 7.6; 2MeSATP with Go 6850, 22.4 ± 9.8**; 2MeSATP with Go 6976, 90.8 ± 12.4; UTP alone, 104.9 ± 10.3; UTP with Go 6850, 27.8 ± 13.2*; UTP with Go 6976, 101.2 ± 10.9 (experiments were as for Table 1, expressed as mean $\pm$ s.e.mean of 6-keto $PGF_{1\alpha}$, where significance of difference from no PKC inhibitor was $^{**}P<0.01$, and $^{*}P<0.02$). These results show that stimulation of PGI_2 release by both P_{2Y}- and P_{2U}-purinoceptors was PKC-dependent. The results further suggest

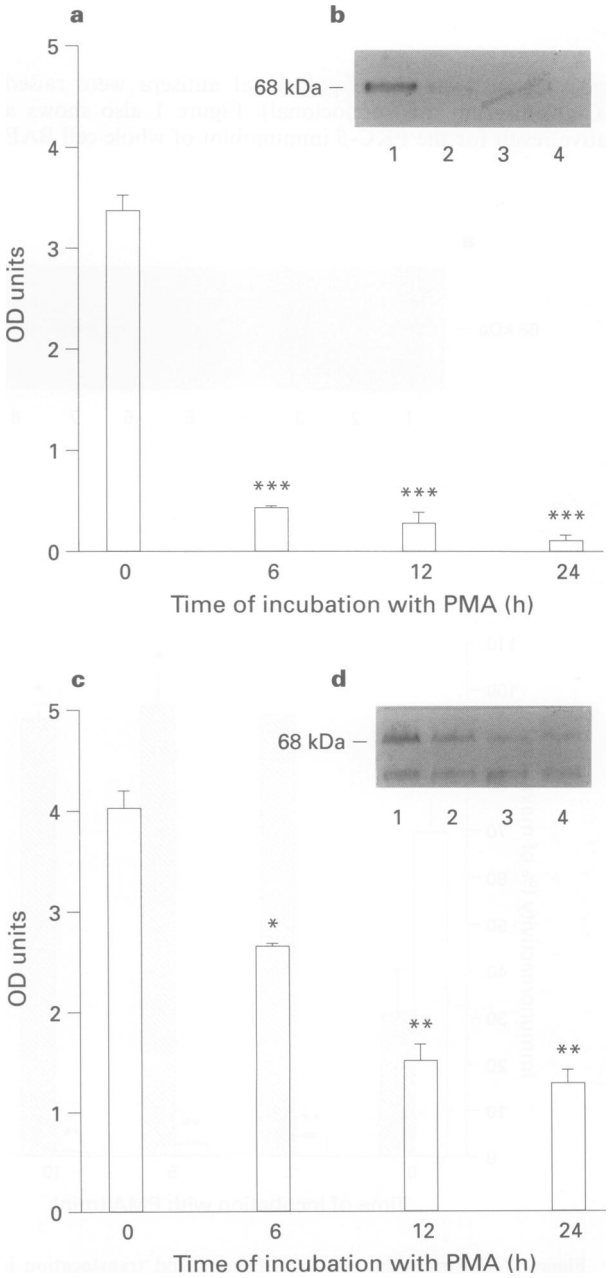

Figure 3 Downregulation of PKC isoforms in whole cell extracts in response to prolonged exposure to 1 μM PMA. Histograms are of data pooled from 3 separate experiments: (a) PKC-α; (c) PKC-ε. Insets are each a representative Western blot autoradiograph: (b) PKC-α; (d) PKC-ε. Lane 1, control; lane 2, 6 h PMA; lane 3, 12 h PMA; lane 4, 24 h PMA.

that this PKC-dependency was not due to a Ca^{2+}-sensitive PKC isoform, such as PKC-α.

Effect of 6 h PMA pretreatment on P_{2Y} and P_{2U} stimulated 6-keto $PGF_{1\alpha}$ and Ins(1,4,5)P_3 accumulation

Treatment with PMA for 6 h had similar effects to that caused by treatment for 10 min (Figure 4). PMA alone for either time had no effect on levels of PGI_2 release. This is shown above for 10 min pretreatment, while for 6 h pretreatment with PMA and subsequent accumulation during a 10 min incubation in the absence of agonist the level of 6-keto $PGF_{1\alpha}$ was 97.0±3.5% of the no PMA control ($n=4$). The period of 6 h was chosen to give the largest differential effect on the PKC isoforms: at this time 90% of PKC-α was lost, while the other two isoforms detected were only downregulated to a small degree. Yet these two PMA treatments had the same influence on enhancing 6-keto $PGF_{1\alpha}$ accumulation stimulated by either 2MeSATP or UTP (Figure 4). The effect of 24 h pretreatment with PMA was quite different (Table 2). There was a significant fall with 24 h PMA treatment in the unstimulated controls. There was no significant effect of 24 h PMA on responses to 2MeSATP or UTP when data were pooled across experiments, although in each individual experiment 24 h PMA gave a reduction in the responses. These results show that 6 h pretreatment with PMA had the same influence as 10 min pretreatment with PMA: the loss of 90% of PKC-α does not result in a situation which mimics the inhibition of PKC by Ro 31-8220. However, by 24 h PMA, the loss of PKC-ε immunoreactivity was also substantial (Figure 3), and at this time the ability of PMA to enhance 6-keto $PGF_{1\alpha}$ accumulation was also lost.

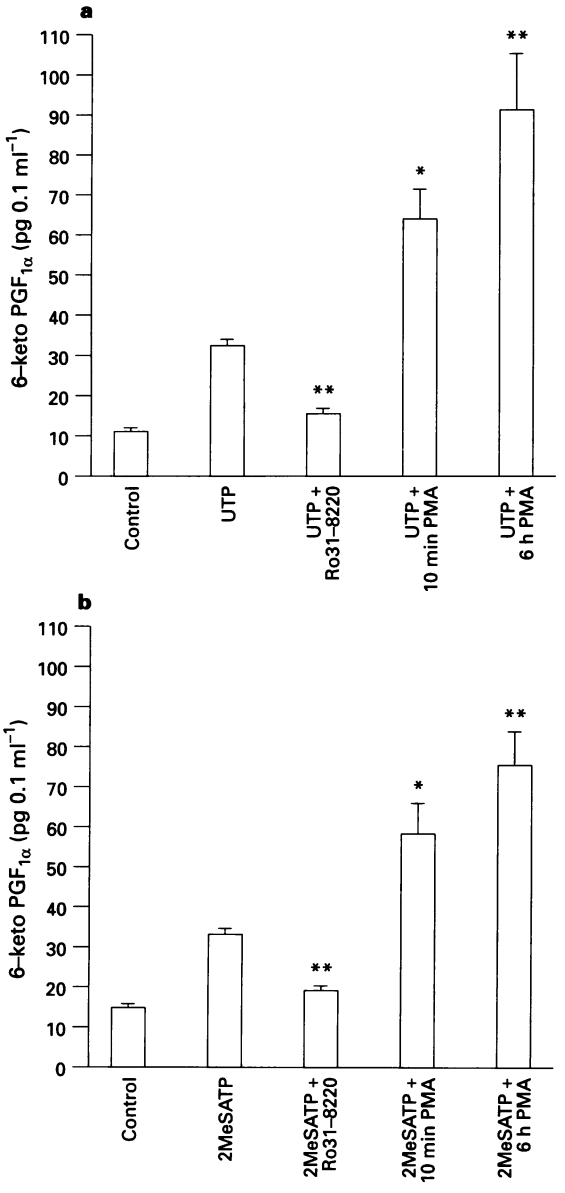

Figure 4 Accumulation of 6-keto $PGF_{1\alpha}$ in supernatants of cells stimulated with 300 μM UTP (a) or 30 μM 2MeSATP (b): effect of preincubation with 10 μM Ro31-8220 (for 10 min) or 1 μM PMA (for 10 min or 6 h). Preincubations in the presence of Ro 31-8220 or PMA were for the times shown; these compounds were also present during the 5 min stimulation period. Results are pooled across 3 separate experiments, each in quadruplicate. Significantly different from agonist alone: $*P<0.05$; $**P<0.02$.

To understand further the effect of selective downregulation of PKC isoforms on the agonist-stimulated production of PGI_2, we looked at the PLC response to the two agonists, measuring the accumulation of Ins(1,4,5)P_3. We have previously shown that the UTP response was unaffected by either stimulation or inhibition of PKC, while the 2MeSATP response was reduced by 10 min pretreatment with PMA and enhanced by 10 min pretreatment with Ro 31-8220 (Purkiss *et al.*, 1994). This effect of 10 min PMA on the phospholipase C response is confirmed in the present study (Table 3). We also found (Table 3) that 6 h pretreatment with PMA attenuated the Ins(1,4,5)P_3 response to 2MeSATP, while the response to UTP was unaffected. This again shows that 6 h pretreatment with PMA has the same effect as 10 min PMA; the effect of loss of 90% of PKC-α is clearly different from the effect of inhibition of PKC.

Discussion

In this paper we have investigated the role of PKC in the response of vascular endothelial cells to stimulation of both

Table 1 The effects of pretreatment with Go 6850 and Go 6976 on agonist stimulated 6-keto $PGF_{1\alpha}$accumulation

	Control	*Go 6850*	*Go 6976*
Control	104.5±12.0	83.8±6.9	85.1±1.8
2MeSATP	214.6±22.4	86.8±8.4	199.9±10.6
Control	94.3±9.3	82.5±12.1	91.8±9.3
UTP	219.2±5.2	84.2±7.1	212.0±3.2

Go 6850 and Go 6976 were present at a concentration of 10 μM for a 10 min preincubation and 5 min incubation in the presence of the agonists, 2MeSATP at 30 μM and UTP at 300 μM. Data (pg 6-keto $PGF_{1\alpha}$ 0.1 ml^{-1}, mean± s.e.mean) are from a single representative experiment undertaken with quadruplicate determinations; data pooled across experiments are presented in the text.

Table 2 The effects of 24 h PMA pretreatment on 6-keto $PGF_{1\alpha}$ accumulation

	Control	*24 h PMA*
Control	23.8±2.0	12.9±2.3
2MeSATP	65.0±5.4	43.8±3.3
UTP	64.5±3.8	449±2.6

PMA was present at 1 μM for 24 h before and during the 5 min incubation with the agonists 2MeSATP at 30 μM and UTP at 300 μM. Data (pg 6-keto $PGF_{1\alpha}$ 0.1 ml^{-1}, mean± s.e.mean) are from 3 separate experiments, each in quadruplicate.

Table 3 The effects of 10 min and 6 h PMA pretreatment on the 2MeSATP and UTP stimulated accumulation of $Ins(1,4,5)P_3$

		PMA	
	0	*10 min*	*6 h*
Control	5.4±0.6	4.0±0.5	5.1±0.5
2MeSATP	11.8±0.2	5.8±0.5**	7.6±0.4*
Control	6.9±0.9	6.8±1.1	5.4±1.4
UTP	12.9±3.8	12.1±1.7	12.1±1.9

Pretreatment was with 1 μM PMA for the time indicated. When present in the pretreatment, PMA was also present during the 5 s stimulation, in the presence of agonists as indicated (2MeSATP at 30 μM and UTP at 300 μM). Data (pmol/well) are mean±s.e.mean from 3 separate experiments each in triplicate. There is no significant effect of PMA except where indicated: *$P<0.002$, **$P<0.001$.

P_{2Y}- and P_{2U}-purinoceptors and have explored the possible contribution of individual isoforms of PKC. We show that inhibition of PKC by 2 isoform non-selective agents, Ro 31-8220 and Go 6850, eliminates the response to PKC. A third PKC inhibitor, Go 6976, has been shown to be very effective as an inhibitor of Ca^{2+}-dependent PKC isoforms α and β, but ineffective as an inhibitor of the Ca^{2+}-independent isoforms δ and ε (Martiny-Baron *et al.*, 1993). Here we show that Go 6976 does not affect the production of PGI_2 in response to P_2-purinoceptor stimulation, in stark contrast to the effect of Go 6850 and Ro 31-8220. The results indicate that PKC is necessary for the PGI_2 response, and suggest that the isoform responsible is not of the Ca^{2+}-sensitive classical PKC (cPKC) category. Using Western blots in a survey employing 8 isoform specific antisera, we have shown that these cultured endothelial cells contain one member of the cPKC group (PKC-α), one of the Ca^{2+}-insensitive novel, nPKC group (PKC-ε), and one of the atypical, aPKC, group (ζ-PKC). PKC-β was not detectable in our cells, in contrast to an earlier report by Hecker *et al.* (1993). The PKC-ε isoform has the unusual characteristic in these cells of being membrane-bound in the absence of stimulation of the cells; the nature of the association with the membrane is also unusual, in that it is not solubilized by treatment with Triton which is effective for the other isoforms following their translocation in response to PMA. Regardless of these curious aspects of PKC-ε, the results with the selective PKC inhibitor, Go 6976, suggest that PKC-α is not the isoform required for the P_2-purinoceptor PGI_2 response. It has been widely reported that the PKC isoforms show different patterns of loss (downregulation) on prolonged exposure to PMA (e.g. Huwiler *et al.*, 1991; Olivier & Parker, 1992; Puceat *et al.*, 1994), and we have utilised this differential downregulation in an attempt to relate the regulation of P_{2U}- and P_{2Y}-purinoceptor responses to individual isoforms. The most frequent observation is that the cPKC isoforms are downregulated first, followed by the nPKC isoforms, while the aPKC isoforms are not seen to downregulate at all. We found this to be the case with the endothelial cells: the α isoform downregulated first, the ε isoform was lost more slowly, while the ζ isoform failed to downregulate. In some individual experiments 95% of the PKC-α was lost following 6 h PMA treatment, and yet the PGI_2 release in response to P_2-purinoceptor stimulation was not attenuated. This failure of 6 h PMA to attenuate the PGI_2 response would alone suggest that it is unlikely to be modulated by PKC-α. In this paper we observe that the response is increased. This is the same effect as with acute (10 min) PMA, despite the fact that at 6 h most of the PKC-α is lost. Taken together with the studies described with the selective PKC inhibitor, these results strongly suggest that PKC-α is not the isoform responsible for the mediation of the PGI_2 response to P_2-purinoceptor stimulation. Of the remaining 2 isoforms we have detected, PKC-ζ is unresponsive to PMA, in the present report as in many published studies. The evaluation of a role for PKC-ε is made more difficult by its membrane-bound nature, but in this paper (downregulation studies) as elsewhere it is seen to be responsive to PMA. The combined downregulation and inhibitor studies therefore point to PKC-ε as the isoform responsible for the mediation of the PGI_2 response to both P_{2Y}- and P_{2U}-purinoceptor stimulation. The 6 h downregulation studies described here also implicate the involvement of PKC-ε in the regulation of the phospholipase C response to P_{2Y}-purinoceptor stimulation. However this conclusion is less secure since it is not underpinned by data obtained with selective PKC inhibition.

Our observations show that PMA alone for 10 min or 6 h, had no effect, but for 24 h reduced, the accumulation of 6-keto $PGF_{1\alpha}$, over a subsequent 5 min period. The lack of effect at 6 h is surprising in view of the report by Carter *et al.* (1989), who show an accumulation of 6-keto $PGF_{1\alpha}$ in the medium of human umbilical vein endothelial cells in response to treatment with 100 nM PMA for between 30 min and 3 h.

In this paper we have shown that stimulation of the P_{2Y}-purinoceptor with 2MeSATP elevates PGI_2 production in a manner that is enhanced by the presence of PMA, even though the $Ins(1,4,5)P_3$ accumulation is profoundly reduced. This indicates an apparent dissociation between the accumulation of Ca^{2+} mobilizing $Ins(1,4,5)P_3$ and the stimulation of PGI_2 release. One possible explanation for this is the sensitization of PLA_2 by PKC, resulting in an increased activation of PLA_2 by Ca^{2+}, despite the reduction in the size of a Ca^{2+} response. This is unlikely to explain the present results, since the effect of PMA on the PGI_2 response is the same for UTP. In this case the $Ins(1,4,5)P_3$ response is unaffected. If PMA caused a sensitization of the PGI_2 biosynthetic machinery to Ca^{2+} then the PGI_2 response to UTP would be enhanced to a greater degree than the response to 2MeSATP, where an attenuation of the phospholipase C response is also occurring. Similarly the effects of PKC inhibition by Ro 31-8220 (eliminating the PGI_2 response to both receptor types but selectively enhancing the elevation of $Ins(1,4,5)P_3$ by 2MeSATP) provide a further example of dissociation of the $Ins(1,4,5)P_3$ response and the PGI_2 response. These observations show that the control of PGI_2 release by these endothelial receptors is not determined by the level of $Ins(1,4,5)P_3$ (and by implication, elevations in cytosolic Ca^{2+}), and provide evidence for an obligate and central role for PKC in the response. This requires a re-evaluation of the relationship between $Ins(1,4,5)P_3$ and PGI_2 production, and the role of PKC, in endothelial cells. Similar conclusions have been reached in earlier studies with other receptors and cell types (e.g. Burch & Axelrod, 1987; Felder *et al.*, 1990).

Despite the refractory nature of the P_{2U}-purinoceptor phospholipase C response to modulation by PKC, it is unlikely that both receptor types activate PKC since both responses are inhibited by Ro 31-8220. This conclusion, that stimulation of P_{2U}-purinoceptors causes activation of PKC, is consistent with our previous observation that homologous desensitization of the UTP response is affected by PKC inhibition (Wilkinson *et al.*, 1994b). The differential downregulation results shown here are consistent with the possibility that both receptor types activate the same PKC isoforms in the generation of the PGI_2 response.

In a related study we have shown that stimulation of these P_2-purinoceptors enhances tyrosine phosphorylation and provided evidence that this is required for agonist-stimulated PGI_2 production (Bowden *et al.*, 1995). These observations, taken together with those in the present report, require re-evaluation of the position that endothelial PGI_2 production is essentially controlled by elevated cytosolic Ca^{2+} and only modulated by phosphorylating events. Wijkander & Sundler (1992) have shown that stimulation of macrophages with PMA leads to phosphorylation of cytosolic PLA_2 and a consequent increase in activity which is independent of the stimulation of PLA_2 by Ca^{2+}. Reports that there is a correlation between agonist stimulated Ca^{2+} and the PGI_2 response (Carter *et al.*, 1989;

1990), despite providing much support for the Ca^{2+} model for endothelial PLA_2 regulation, cannot demonstrate a causal role between the two. Many other intracellular events would also be expected to correlate with PGI_2 production following agonist stimulation. The observations reported here and elsewhere (Bowden *et al.*, 1995) showing a dissociation between the Ins(1,4,5)P_3 response and the PGI_2 response, and an obligate role for both PKC and tyrosine kinases, give some indication of which of these events may be important in this endothelial response. Despite this, a causal role for Ca^{2+} in the endothelial PGI_2 response does seem likely in view of observations that depletion and buffering of intracellular Ca^{2+} attenuates the stimulation of 6-keto $PGF_{1\alpha}$ accumulation (Hallam *et al.*, 1988). We propose, therefore, that both protein kinase pathways and elevated cytosolic Ca^{2+} are required for the PGI_2 response to P_2-purinoceptor agonists.

It is of interest to note that the PMA-stimulated phosphorylation of PLA_2 described by Wijkander & Sundler (1992), while downstream of PKC stimulation, may be due to phosphorylation of the phospholipase by another kinase, such as PKC stimulated mitogen activated protein kinase (MAPK) activity (Lin *et al.*, 1993; Sa *et al.*, 1995). It has been widely reported, in other systems, that stimulation of PKC by PMA leads to activation of MAPK. We have recently shown (Patel V. & Boarder M.R., in preparation) that stimulation of either P_{2Y}- or P_{2U}-purinoceptors on cultured endothelial cells leads to MAPK activation. It is interesting to speculate, therefore, that the observations on PKC described here, and those on endothelial P_2-purinoceptor stimulated tyrosine phosphorylations reported independently (Bowden *et al.*, 1995), may converge at the level of MAPK and may represent different ways of controlling the MAPK regulation of PLA_2 activity and thus endothelial PGI_2 production.

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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.

BOARDER, M.R., WEISMAN, G.A., TURNERE, J.T. & WILKINSON, G.F. (1995). G protein-coupled P_2-purinoceptors: from molecular biology to functional responses. *Trends Pharmacol. Sci.*, **16**, 133–139.

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

BOWDEN, A., PATEL, V., BROWN, C. & BOARDER, M.R. (1995). Evidence for requirement of tyrosine phosphorylation in the endothelial P_{2Y}- and P_{2U}-purinoceptor stimulation of prostacyclin release. *Br. J. Pharmacol.*, **116**, 2563–2569.

BURCH, R.M. & AXELROD, J. (1987). Dissociation of bradykinin induced prostaglandin formation from phosphatidyinositol turnover in Swiss 3T3 fibroblasts: evidence for G-protein regulation of phospholipase A_2. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6374–6378.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2Y}-purinoceptor-mediated prostacyclin relelase 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 intracellular Ca^{2+}. *Biochem. J.*, **262**, 431–437.

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIOXON, 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.

DEKKER, L.V., PARKER, P.J. & MCINTYRE, P. (1992). Biochemical properties of rat protein kinase C-η expressed in COS cells. *FEBS Lett.*, **312**, 195–199.

FELDER, C.C., DIETER, P., KINSELLA, J., TAMURA, K., KANTERMAN, R.Y. & AXELROD, J. (1990). Transfectred m_5 muscarinic acetylcholine receptor stimulates phospholipase A_2 by inducing both calcium influx and activation of protein kinase C. *J. Pharmacol. Exp. Therap.*, **255**, 1140–1147.

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

HECKER, M., LUCKHOFF, A. & BUSSE, R. (1993). Modulation of endothelial autocoid release by protein kinase C: feedback inhibition or non-specific attenuation of receptor-dependent cell activation? *J. Cell. Physiol.*, **156**, 571–578.

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

JESELMA, C. & AXELROD, J. (1987). Stimulation of phospholipase A_2 activity in bovine rod outer segments by the βγ subunits of transducin and its inhibition by the α subunit. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3623–3627.

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.

LAEMMLI, U.K. (1970). Cleavage of proteins during the assembly of the head of bacteriophage T_4. *Nature*, **227**, 680–685.

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.

MARAIS & PARKER. (1989). Purification and characterisation of bovine brain protein kinase C isotypes α, β and γ. *Eur. J. Biochem.*, **182**, 129–137.

MARAIS, R.M. & PARKER, P.J. (1991). Properties of protein kinase C isotypes from bovine brain. In *Methods in Enzymology*, ed. Hunter T & Sefton B.M. Orlando: Academic Press. pp234–241.

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.

MARTINY-BARON, G., KAZANIETZ, M.G., MISCHAK, H., BLUMBERG, P.M., KOCHS, G., HUG, H., MARME, D. & SCHACHTELE, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go6976. *J. Biol. Chem.*, **268**, 9194–9197.

MOTTE, S., PIROTTON, S. & BOYNAEMS, J.M. (1993). Heterogeneity of ATP receptors in aortic endothelial cells. *Cir. 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.

NEDDLEMEAN, P., MINKES, M.S. & DOUGLAS, J.R. (1974). Stimulation of prostaglandin biosynthesis by adenine nucleotides; profile of prostaglandin release by perfused organs. *Circ. Res.*, **34**, 455–461.

OLIVIER, A.R. & PARKER, P.J. (1991). Expression and characterisation of PKC-δ. *Eur. J. Biochem.*, **200,** 1116–1120.

OLIVIER, A.R. & PARKER, P.J. (1992). Identification of multiple PKC isoforms in Swiss 3T3 cells: differential downregulation by phorbol ester. *J. Cell. Physiol.*, **152,** 240–244.

ONO, Y., FUJI, T., OGITA, K., KIKKAWA, U., IGARASHI, K. & NISHIZUKA, Y. (1989). Protein kinase C ζ subspecies from rat brain: Its structure, expression, and properties. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 3099–3103.

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.

PUCEAT, M., HILALDANDAN, R., STRULOVICI, B., BRUNTON, L.L. & BROWN, J.H. (1994). Differential regulation of protien kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J. Biol. Chem.*, **269,** 16938–16944.

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.*, **111,** 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.

SCHAAP, D. & PARKER, P.J. (1990). Expression, purification and characterisation of PKC-ε. *J. Biol. Chem.*, **265,** 7301–7303.

SILK, S.T., CLEJAN, S. & WITKOM, K. (1989). Evidence of GTP-binding protein regulation of phospholipase A_2 activity in isolated human platelet membranes. *J. Biol. Chem.*, **264,** 21466–21469.

WAYS, D.K., COOK, P.P., WEBSTER, C. & PARKER, P.J. (1992). Effect of phorbol esters on protein kinase C-ζ. *J. Biol. Chem.*, **267,** 4799–4805.

WIJKANDER, J. & SUNDLER, R. (1992). Regulation of arachidonate-mobilising phospholipase A_2 by phosphorylation via protein kinase C in macrophages. *FEBS Lett.*, **311,** 299–301.

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

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., PURKISS, J.R. & BOARDER, M.R. (1994b). Differential heterologous and homologous desensitisation of two receptors for ATP (P_{2Y} purinoceptors and nucleotide receptors) coexisting on endothelial cells. *Mol. Pharm.*, **45,** 731–736.

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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