Vascular endothelial growth factor-induced prostacyclin production is mediated by a protein kinase C (PKC)-dependent activation of extracellular signal-regulated protein kinases 1 and 2 involving PKC-δ and by mobilization of intracellular Ca^{2+}

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We reported previously that vascular endothelial growth factor (VEGF) stimulates prostacyclin (PGI_2) production via activation of the extracellular signal-regulated kinase (ERK) cascade. In this paper, we examined the role of protein kinase C (PKC) in this pathway. VEGF-induced PGI_2 generation and arachidonic acid release in human umbilical vein endothelial cells were inhibited by the PKC inhibitors GF109203X and calphostin C. VEGF increased PKC activity and immunoreactivity of the PKCδ, α and ϵ isoforms in particulate fractions of cells. PKC inhibitors blocked VEGF-induced activation of ERK, MEK (mitogen-activated protein kinase kinase) and the cytosolic phospholipase A_2, but had little effect on ERK activation induced by basic fibroblast growth factor. GF109203X, calphostin C and the PKCδ-selective inhibitor, rottlerin, did not inhibit activation of the KDR receptor for VEGF. Inhibition of Ca^{2+} fluxes using BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester)] blocked VEGF-induced PGI_2 production but did not inhibit ERK activation. Neither activation nor inhibition of the NO/cGMP pathway had any effect on VEGF induction of ERK activity and PGI_2 synthesis. Wortmannin partially inhibited VEGF stimulation of PGI_2 production, but did not inhibit VEGF-induced ERK activity. VEGF-induced ERK activation and PGI_2 production were blocked by rottlerin, and VEGF increased association of PKCδ with Raf-1, the upstream activator of MEK. The PKC-selective inhibitor Go6976 did not inhibit ERK activation and had only a partial effect on PGI_2 production. These findings indicate that activation of PKC plays a crucial role in VEGF signalling via the ERK cascade leading to PGI_2 synthesis and suggest that the PKCδ isoform may be a key mediator of VEGF-induced activation of the ERK pathway via increased association with Raf-1.

Key words: calcium, endothelium, MAP kinase, nitric oxide, phospholipase A_2.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a secreted polypeptide factor, also known as vascular permeability factor, which is essential for embryonic vasculogenesis and plays a major role in angiogenesis in a variety of disease states [1–3]. VEGF expression is up-regulated by hypoxia and several cytokines and growth factors in diverse cell types [4–6]. VEGF elicits an array of biological activities *in vivo* and *in vitro* including the proliferation and migration of endothelial cells [7,8], increased vascular permeability [9] and endothelial cell survival [10]. Because of its abilities to accelerate re-endothelialization after vascular injury, suppress neointimal hyperplasia in vascular smooth-muscle cells and improve blood flow in ischaemic tissues through the stimulation of collateral blood vessel formation [11–14], VEGF has recently become the focus of interest as a potential therapeutic agent for ischaemic heart disease [13,14].

Two distinct protein tyrosine kinase receptors for VEGF have so far been identified, the *fms*-like tyrosine kinase, Flt-1, and KDR/Flk-1 [15]. Though expression of both receptors occurs in endothelial cells, KDR/Flk-1 is thought to mediate angiogenesis and most of the endothelial biological effects of VEGF [8,15], and it is at present unclear what functional endothelial responses are mediated by Flt-1. Previous studies show that VEGF activates several early signalling cascades in endothelial cells mediated via KDR, including p42/p44 extracellular signal-regulated kinases (ERKs) 1 and 2, protein kinase C (PKC) and tyrosine phosphorylation of several components including phospholipase C-γ, p125 focal adhesion kinase and paxillin [16–19]. The roles of these signalling events in mediating the biologically important effects of VEGF in endothelial cells are incompletely understood, however.

Recent findings have implicated increased production of the vasodilatory factors NO and prostacyclin (PGI_2) in mediating some of the diverse biological actions of VEGF, including angiogenesis, cell migration, increased permeability, vasodilatation and hypotension, and inhibition of vascular-smooth-muscle-cell proliferation [12,18,20–23]. PGI_2 in particular has several biological effects, including inhibition of vascular-smooth-muscle-cell proliferation and neointima formation [24,25], and inhibition of platelet aggregation [26], which are likely to play a key role in mediating some of the functions of VEGF *in vivo*.

Abbreviations used: bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; PGI_2, prostacyclin; $cPLA_2$, cytosolic phospholipase A_2; PKC, protein kinase C; VEGF, vascular endothelial growth factor; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; PI3-kinase, phosphoinositide 3-kinase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; ODQ, 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one.

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Synthesis of PGI_2 results from enzymic phospholipase A_2-mediated release of arachidonic acid from membrane phospholipids and the subsequent conversion of arachidonic acid into prostaglandin H2 via cyclo-oxygenase. Agonist-driven arachidonic acid release in several cell types is mediated by the Ca^{2+}-sensitive 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) [27,28]. The rapid mobilization of Ca^{2+} from intracellular stores is thought to play an important role in endothelial PGI_2 release stimulated by agonists for G-protein-coupled receptors [29,30]. Activation of $cPLA_2$ also occurs independently of increased intracellular [Ca^{2+}] [31] and phosphorylation of $cPLA_2$ by members of the mitogen-activated protein (MAP) kinase family, including p42/p44 ERKs [32,33] and p38 MAP kinase [34], plays a major role in mediating activation in some cell types. We reported that VEGF stimulates PGI_2 synthesis and $cPLA_2$ phosphorylation in human umbilical vein endothelial cells (HUVECs) via an ERK-dependent pathway [18]. The relationship between the VEGF-stimulated PGI_2 and NO pathways is unclear, although it was reported recently that VEGF-induced activation of the ERK cascade may be mediated via the NO/cGMP pathway [35].

Here, we investigated the signal-transduction mechanisms involved in VEGF stimulation of ERK-dependent PGI_2 generation and examined the relationship between the ERK/PGI_2 and NO/cGMP pathways in VEGF signalling. These findings identify a PKC-mediated pathway for VEGF-induced ERK activation and PGI_2 generation that is independent of NO. The results suggest further that PKCδ is involved in mediating VEGF stimulation of ERK-dependent endothelial PGI_2 generation.

MATERIALS AND METHODS

Cell culture

HUVECs were obtained from umbilical cords by collagenase digestion and cultured on 1% gelatin-coated plates in EBM (BioWhittaker) supplemented with 10% fetal calf serum, 10 μg/ml hEGF, 50 μg/ml gentamicin sulphate, 50 μg/ml amphotericin B and bovine brain extract. Confluent monolayers of cells were used at passage 2 plated in either 90 mm dishes or 6 well plates.

PGI_2 and $cPLA_2$ assays

Cells were washed twice in serum-free EBM medium and, after treatments, the PGI_2 content of cell supernatants was quantified by enzyme immunoassay of 6-keto $PGF_{1\alpha}$, the stable breakdown product of PGI_2.

$cPLA_2$ activation was determined by mobility shift in SDS/PAGE as described previously [18].

Arachidonic acid release

Arachidonic acid release was determined as described in [18]. Confluent HUVEC cultures were incubated for 24 h with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (1 μCi/ml, 211 Ci/mmol). The cells were then washed twice with medium 199 and incubated in 1 ml of this medium supplemented with additions as indicated. After the times of treatment indicated, the medium was removed, centrifuged at 16000 ***g*** for 5 min, and the radioactivity in the supernatant was determined using a scintillation counter.

cGMP measurement

Intracellular cGMP production was measured by enzyme immunoassay (Amersham) as described in the manufacturer's instructions.

Western blotting

After treatments, cells were washed twice in ice-cold PBS and extracted in SDS/PAGE sample buffer [2% (w/v) SDS/63.5 mM Tris/HCl, pH 6.8/10% glycerol/200 μM Na_3VO_4/5 μg/ml leupeptin/0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF)]. The lysates were clarified by centrifugation (14000 ***g***, 20 min at 4 °C), heated to 95–100 °C for 5 min, run on SDS/PAGE gels and transferred on to Immobilon-P (Millipore). The membranes were blocked with 7% non-fat milk in 20 mM Tris/HCl, pH 7.5/150 mM NaCl/0.1% Tween 20 and incubated with primary antibody diluted in the blocking solution. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL® plus, Amersham). Bands were quantified by scanning densitometry and calculating the density of individual bands using QuantiScan software.

Immunoprecipitation

Cells were washed with ice-cold PBS and lysed with 1 × lysis buffer (1% Triton X-100/150 mM NaCl/10 mM Tris/HCl, pH 7.4/1 mM EGTA/1 mM EDTA/0.2 mM sodium vanadate/0.2 mM AEBSF/0.5% Nonidet P-40). The lysate was incubated at 4 °C for 20 min with constant agitation and insoluble material was removed by centrifugation (14000 ***g***, 20 min at 4 °C). The clarified lysate was incubated with 5 μg/ml agarose-conjugated primary antibody for 3 h with constant agitation at 4 °C. The immunoprecipitates were then washed three times with 1 × lysis buffer, resuspended in 2 × SDS/PAGE sample buffer and analysed by SDS/PAGE.

PKC assay and PKC translocation

Total PKC activity was measured in cell extracts using a PKC assay kit (Calbiochem) according to the manufacturer's instructions.

After treatments with factors in serum-free medium at 37 °C for the indicated times, cells were collected in ice-cold homogenization buffer [20 mM Tris/HCl, pH 7.5/5 mM EDTA/10 mM EGTA/0.3% (v/v) β-mercaptoethanol/0.1 mM AEBSF/10 mM benzamidine] and sonicated on ice (three times for 5 s at 20 Hz). The homogenate was centrifuged at 100000 ***g*** for 60 min at 4 °C. The supernatant was collected and used as the cytosolic fraction. The pellet was resuspended in homogenization buffer containing 0.5% Brij 58, incubated for 30 min at 4 °C with constant agitation and centrifuged at 100000 ***g*** for 60 min. The supernatant was saved and used as the particulate fraction. Both fractions were run on SDS/PAGE gels with 8% acrylamide and then immunoblotted.

ERK assay

Cell extracts were prepared in SDS/PAGE sample buffer, collected by scraping, heated to 95 °C for 10 min, run on 12.5% acrylamide SDS/PAGE gels and immunoblotted with an antibody that specifically recognizes p42 and p44 MAP kinases (ERK-1 and ERK-2) activated by phosphorylation at Tyr-204.

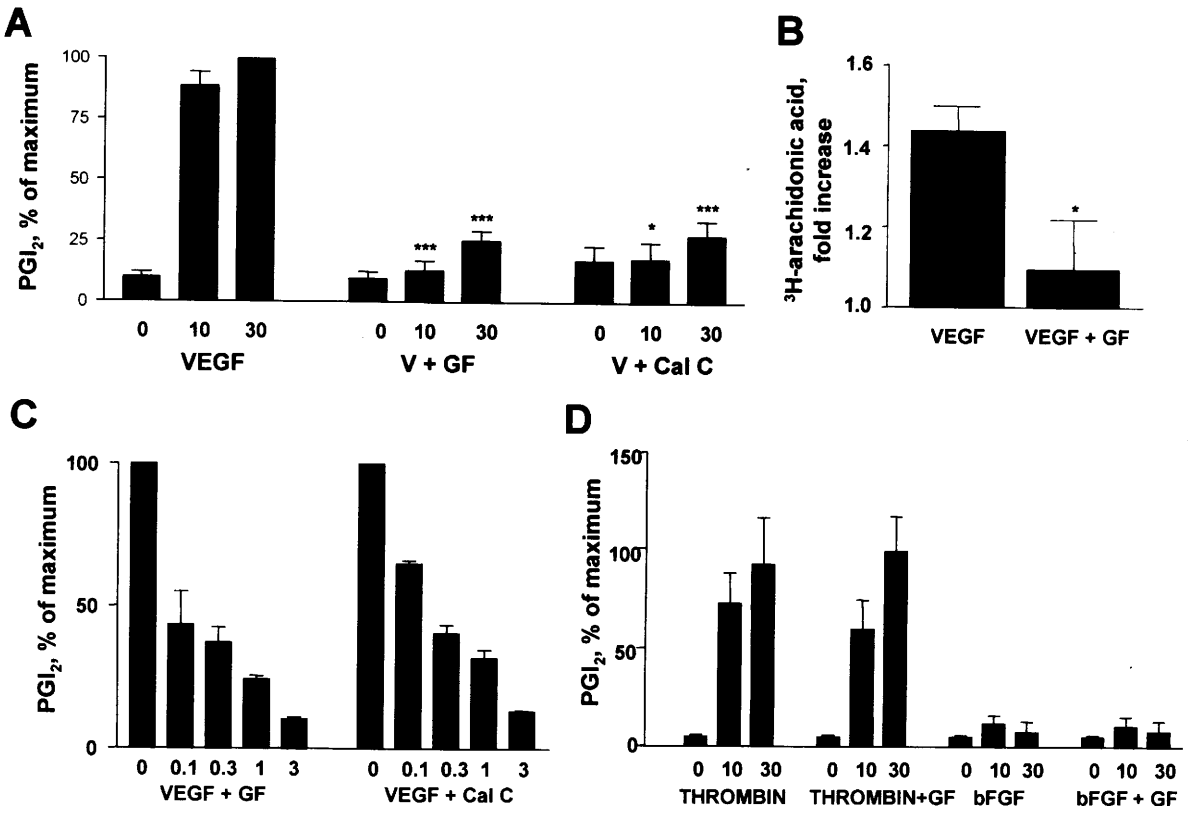

Figure 1 Inhibition of VEGF-induced PGI_2 production and arachidonic acid release by PKC inhibitors

(**A**) Confluent HUVECs were washed twice with serum-free medium and incubated for the indicated times (in min) with 25 ng/ml VEGF (V), in the absence or presence of 3 μM GF109203X (+GF) or 3 μM calphostin C (+Cal C). Values for PGI_2 released into the medium are expressed as a mean percentage ± S.E.M. of the maximum VEGF-stimulated production of 6-keto-$PGF_{1\alpha}$ and represent results from 13 (GF109203X) and four (calphostin C) experiments. $^{***}P < 0.0001$ and $^{*}P < 0.02$ for VEGF + inhibitor versus VEGF. In parallel samples, the mean values for basal and VEGF-stimulated PGI_2 release (6-keto $PGF_{1\alpha}$ production) after 10 and 30 min were, respectively, 3.1 ± 0.5, 29 ± 5 and 31 ± 6 pg/10^4 cells. In these and all subsequent experiments with inhibitors, cells were pretreated for 30 min with or without the inhibitor prior to addition of VEGF or other physiological agonists and all cells were incubated with an equivalent volume of DMSO. (**B**) Confluent HUVECs were incubated for 10 min with 25 ng/ml VEGF in the absence or presence of 3 μM GF109203X (+GF) and arachidonic acid released into the medium was determined, expressed as mean fold increase ± S.E.M. above the control unstimulated release in the absence of VEGF. $^{*}P$ values for control versus VEGF at 10 min, and VEGF at 10 min versus VEGF + GF109203X were both < 0.01. (**C**) Concentration-dependence of the effects of GF109203X and calphostin C on VEGF-stimulated PGI_2 production. Cells were incubated for 30 min with 25 ng/ml VEGF in the presence of the indicated concentrations (μM) of either GF109203X or calphostin C. PGI_2 release is expressed as mean ± S.E.M. of the maximum VEGF-stimulated production of 6-keto-$PGF_{1\alpha}$. (**D**) HUVECs were incubated for the indicated times (in min) with either 1 unit/ml thrombin or 25 ng/ml basic fibroblast growth factor in the absence or presence of 3 μM GF109203X (+GF). Values for PGI_2 released into the medium are expressed as a mean percentage ± S.E.M. of the maximum VEGF-stimulated production of 6-keto-$PGF_{1\alpha}$ and represent results from three independent experiments.

Ca^{2+} mobilization

HUVECs cultured in 48-well plates were washed and incubated with 1 μM Fluo-3 for 60 min at room temperature. Cells pre-loaded with Fluo-3 were washed three times with a buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 0.1 % BSA and 10 mM Hepes, pH 7.4. Ca^{2+} was measured continuously in a Fluostar 403 fluorometer (BMG Labtechnologies) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Radiolabelled ligand binding

Binding of ^{125}I-$VEGF_{165}$ (the 165-amino acid isoform of human VEGF) to high-affinity binding sites in HUVECs was performed essentially as described in [8]. Cells were pretreated for 30 min at 37 °C with or without GF109203X, and then transferred to Dulbecco's modified Eagle's medium/Hepes, pH 7.2, containing either 0.03 or 0.1 nM ^{125}I-$VEGF_{165}$ (1200–1800 Ci/mmol) and incubated for 2 h at either 4 or 37 °C. Cells were lysed in 0.25 M NaOH/0.5 % SDS and counted in a gamma counter. Non-specific binding was determined by incubation with ^{125}I-$VEGF_{165}$ in the presence of a 100-fold excess of unlabelled $VEGF_{165}$.

VEGF-receptor activation

Following treatments, cells were lysed at 4 °C in 1 ml of a solution containing 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM di-sodium pyrophosphate, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM AEBSF, 0.5 % Nonidet P-40 and 1 % Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 15000 ***g*** for 10 min and incubated with 1 μg/ml of a KDR polyclonal antibody directed against residues 1158–1345 for 3 h at 4 °C. Immunocomplexes were collected by incubating lysates with Protein A/G–agarose beads for a further 1 h. Immunoprecipitates were washed three times with lysis buffer and then either immunoblotted with 4G10 anti-phosphotyrosine antibody or were washed twice with kinase assay buffer comprising 50 mM Hepes, pH 7.4/10 mM $MnCl_2$/1 mM dithiothreitol and used for assay of kinase activity. Assay of KDR kinase activity was performed in 20 μl of kinase assay buffer

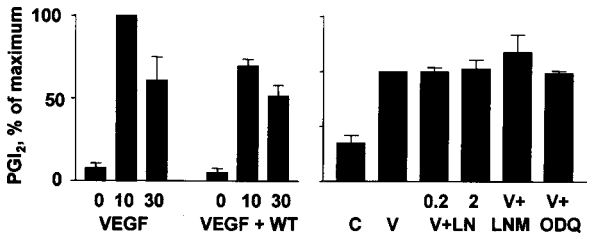

Figure 2 Effects of inhibitors of PI3-kinase and NO pathways on VEGF-induced PGI_2 production

HUVECs were treated with 25 ng/ml VEGF for the times indicated (in min) or, where not stated, for 30 min, in the absence (VEGF, V) or presence of 100 nM wortmannin (+WT), 0.2 or 2 mM L-NAME (+LN), 200 μM L-NMMA (+LNM) or 10 μM ODQ (1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one). PGI_2 release was then measured as described in the Materials and methods section. Values for production of 6-keto-$PGF_{1\alpha}$ are expressed as in the legend to Figure 1 and were obtained from three (WT), four (L-NAME), and three (L-NMMA) experiments.

containing 10 μCi of [γ-^{32}P]ATP (3000–5000 Ci/mmol) for 15 min at 30 °C as described in [8].

Statistical analysis

Statistical analysis of the differences between means obtained from multiple experiments was performed using one-way ANOVA. $P < 0.05$ was considered to be significant.

Materials

Recombinant VEGF was obtained from R & D Systems. Antibody to the activated phosphorylated form of p42/p44 ERKs was purchased from New England Biolabs. $cPLA_2$ antiserum was a gift of Dr Ruth Kramer (Eli Lilly, Indianapolis, IN, U.S.A.). Raf-1 agarose conjugate, and antibodies to KDR, and α, δ, ϵ and ζ PKC isoforms were from Santa Cruz Biotechnology and used at a concentration of 3 μg/ml. 4G10 was obtained from Upstate Biotechnology. PKC inhibitors, SU5614, wortmannin and BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester)] were from Calbiochem. Fluo-3 was from Molecular Probes. ECL reagents, horseradish peroxidase-conjugated anti-mouse IgG, [γ-^{32}P]ATP, ^{125}I-VEGF, cGMP and PGI_2 assay kits were from Amersham (Little Chalfont, Bucks., U.K.). All other reagents used were of the purest grade available.

RESULTS

Signalling pathways mediating VEGF-induced PGI_2 production

The role of PKC in the signalling mechanisms that lie proximal to ERK in the stimulation of PGI_2 generation was examined using selective PKC inhibitors. The bis-indolylmaleimide inhibitor, GF109203X, and the structurally unrelated and mechanistically distinct PKC inhibitor, calphostin C [36–38], both blocked PGI_2 synthesis in response to VEGF (Figure 1).

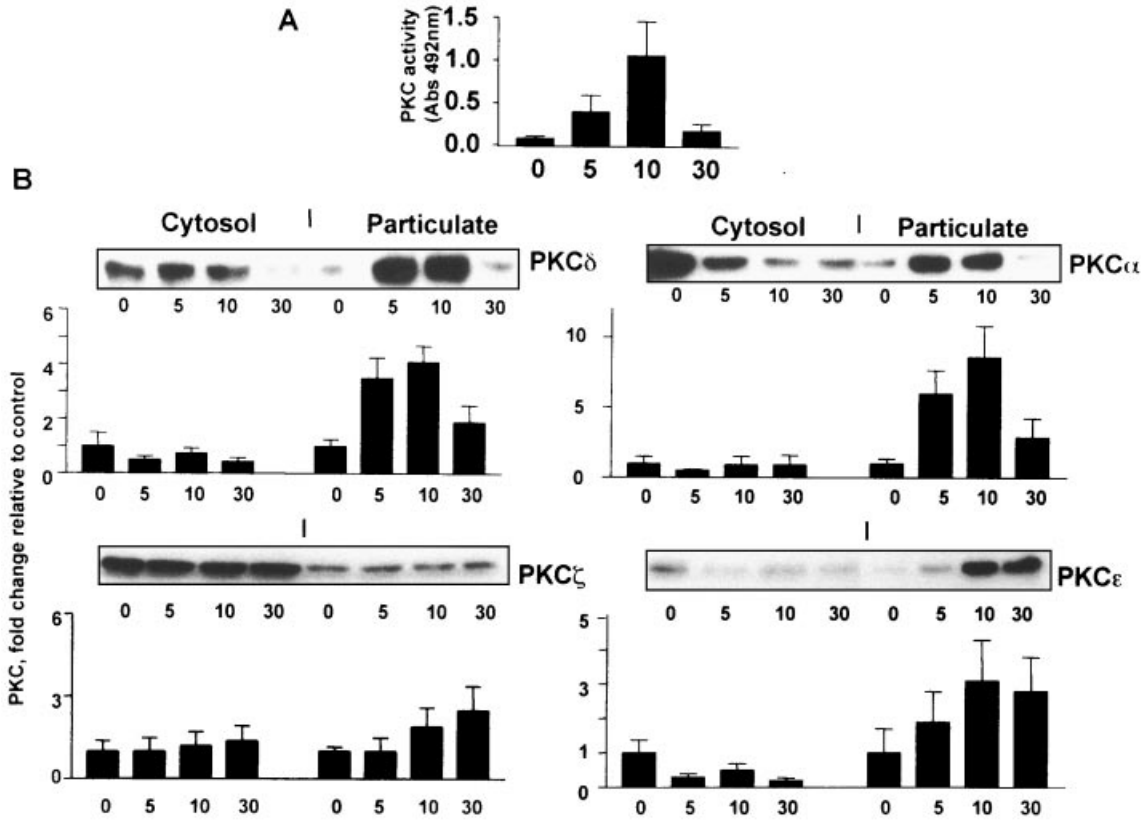

Figure 3 VEGF activates PKC and induces translocation of PKCδ, ε and α to a particulate fraction

(**A**) HUVECs were treated with 25 ng/ml VEGF for the indicated times (in min) and total PKC activity was measured in cell extracts as described in the Materials and methods section. Results are expressed as means ± S.E.M. ($n = 4$). (**B**) Particulate and cytosolic fractions were prepared from cells treated for different times with 25 ng/ml VEGF and equal amounts of protein were immunoblotted with antibody to different PKC isoforms as indicated. The autoradiograms shown are representative of nine (PKCδ), three (PKCϵ), eight (PKCα) and five (PKCζ) independent experiments. Quantification of immunoreactivity was performed by scanning densitometry using QuantiScan software and is presented as fold change (mean ± S.E.M.) relative to the control level.

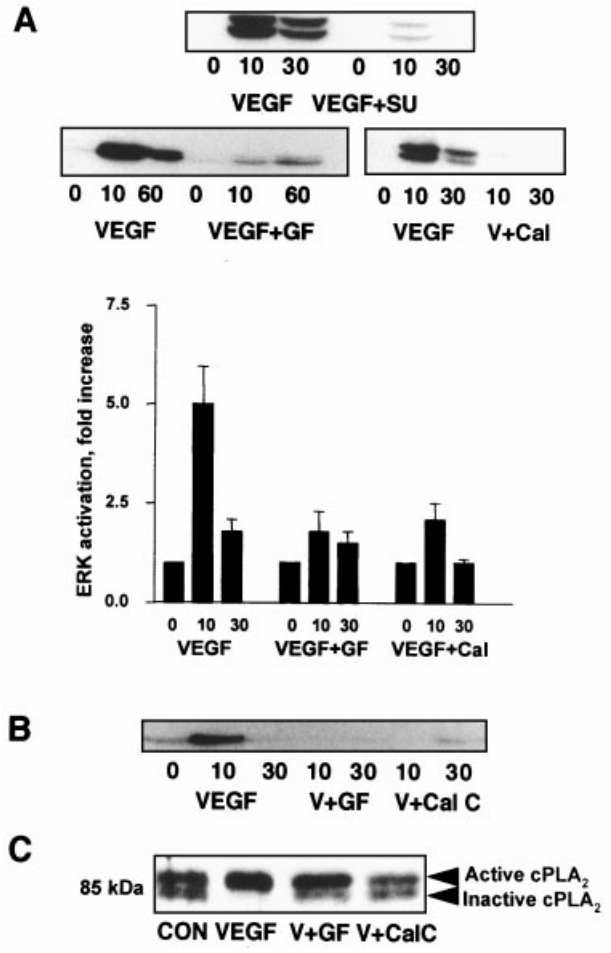

Figure 4 VEGF-induced activation of ERK1/2, MEK and cPLA$_2$ is inhibited by PKC inhibitors

(**A**) HUVECs were treated with 25 ng/ml VEGF for the indicated times (in min) in the absence (VEGF, V) or presence of 5 μM SU5614 (+SU), 3 μM GF109203X (+GF) or 1 μM calphostin C (+Cal). The results shown are representative of two (SU5614), 10 (GF109203X) and seven (calphostin C) independent experiments. Quantification of p42 ERK activity was performed by scanning densitometry (lower panel) and the areas of the bands integrated using QuantiScan software for effects of GF109203X and calphostin C and is presented as means $\pm$ S.E.M. Very similar results were obtained for p44 ERK (not shown). (**B**) Cells were incubated with 25 ng/ml VEGF for the indicated times (in min) in the absence or presence of either 3 μM GF109203X or 1 μM calphostin C and cell extracts were immunoblotted with antibody to activated MEK-1. Results are representative of three independent experiments. (**C**) Effect of PKC inhibitors on cPLA$_2$ phosphorylation. HUVECs were incubated for 10 min with vehicle (control, CON), or with 25 ng/ml VEGF with or without 3 μM GF109203X or 1 μM calphostin C. Cell extracts were prepared and immunoblotted with a specific antibody for the 85 kDa cPLA$_2$. The positions of active and inactive forms of cPLA$_2$ are indicated by arrowheads. The results are representative of four independent experiments.

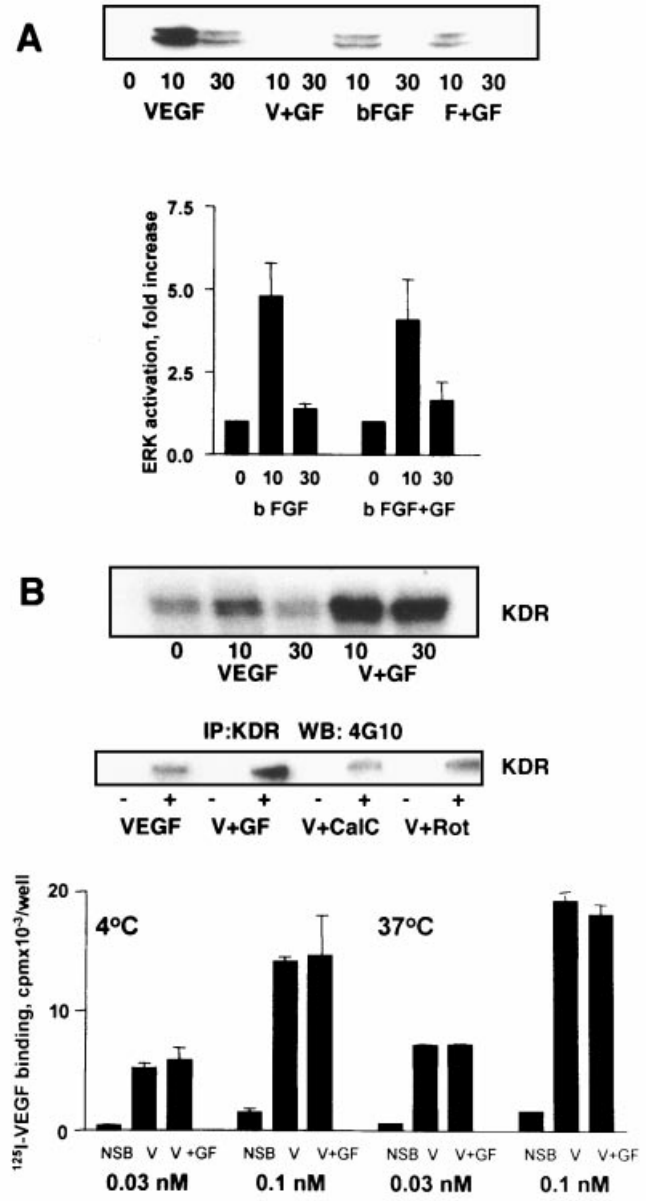

Figure 5 Inhibition of VEGF-induced ERK1/2 activation by PKC inhibitors is selective and not due to KDR inhibition

(**A**) HUVECs were treated for the indicated times (in min) with VEGF (25 ng/ml) or bFGF (F, 25 ng/ml), in the absence or presence (+GF) of 3 μM GF109203X. Results for bFGF are representative of four separate experiments and quantification of p42 ERK activity is presented as means $\pm$ S.E.M. (**B**; upper panel) Cells were pretreated for 30 min in the absence (VEGF) or presence of 3 μM GF109203X (V+GF), and treated for the times indicated (in min) with 25 ng/ml VEGF. KDR immunoprecipitates were then prepared and used for determination of kinase activity. (Middle panel) In other experiments, cells were pretreated with 3 μM GF109203X (V+GF), 1 μM calphostin C (V+CalC) or 1 μM rottlerin (V+Rot) for 30 min and treated for a further 10 min in the absence (−) or presence (+) of 25 ng/ml VEGF. KDR immunoprecipitates (IP) were then prepared and used for immunoblotting (WB) with 4G10 anti-phosphotyrosine antibody as described in the Materials and methods section. Bands corresponding to KDR (approx. 205 kDa) are shown in both autoradiograms. (Lower panel) Specific binding of 0.03 or 0.1 nM ^{125}I-VEGF$_{165}$ to intact HUVECs was determined at 4 or 37 °C after pretreatment in the presence (V+GF) or absence (V) of 3 μM GF109203X. Results are given as mean $\pm$ S.E.M. ($n = 3$). Non-specific ^{125}I-VEGF$_{165}$ binding (NSB) is shown for comparison.

GF109203X and calphostin C reduced PGI_2 synthesis, which had been induced by a 10 min treatment with VEGF, to $12 \pm 4\%$ (mean $\pm$ S.E.M., $n = 13$) and $17 \pm 7\%$ ($n = 4$) of the stimulated level, respectively (Figure 1A), compared with a basal level of PGI_2 release that was 10% of the VEGF-stimulated level. The respective values for inhibition of VEGF-stimulated production after 30 min were $25 \pm 4\%$ ($n = 13$) and $27 \pm 6\%$ ($n = 4$). A second bis-indolylmaleimide inhibitor, Ro-813220, similarly caused a marked inhibition of VEGF-induced PGI_2 production (results not shown). GF109203X also inhibited VEGF-stimulated arachidonic acid release (Figure 1B). Dose–response data for the effects of GF109203X and calphostin C on VEGF-stimulated PGI_2 generation showed that half-maximal inhibition occurred at approx. 0.1 μM and 0.1–0.3 μM, respectively (Figure 1C). The concentration of GF109203X that inhibited VEGF-induced PGI_2

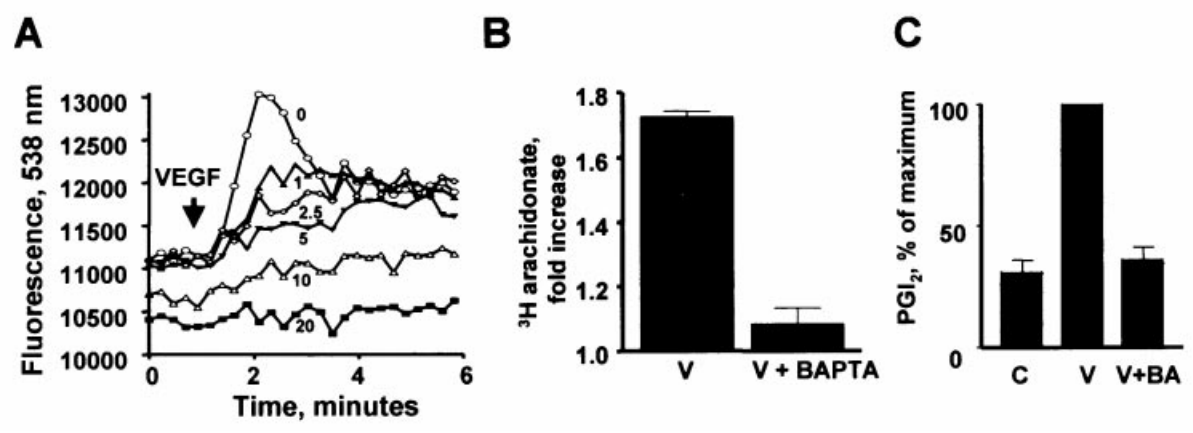

Figure 6 VEGF-induced PGI_2 production is blocked by an inhibitor of intracellular Ca^{2+} mobilization

(**A**) HUVECs were loaded with Fluo-3 and intracellular Ca^{2+} was monitored continuously as described in the Materials and methods section. BAPTA/AM was added to cells at 20 (■), 10 (△), 5 (▼), 2.5 (◇), 1.25 (▲) or 0 μg/ml (○) for 10 min and then 25 ng/ml VEGF was added at the time indicated by the arrow. (**B** and **C**) HUVECs were pretreated for 30 min with 5 μg/ml BAPTA/AM (+BA) and 25 ng/ml VEGF was added for a further 10 min. [^{3}H]Arachidonic acid release (**B**) and PGI_2 release (**C**) were determined as in the Materials and methods section and results are presented as described in the legend to Figure 1. C, control.

production was very similar to that reported to effectively inhibit PKC in other intact cells without affecting other signalling pathways [36]. The striking inhibitory effect of PKC inhibitors was selective for the PGI_2 response to VEGF. Thus PGI_2 generation induced by thrombin was not significantly reduced by pretreatment with GF109203X (Figure 1D). Basic fibroblast growth factor (bFGF) induced a weak PGI_2 response which was also unaffected by GF109203X, and neither GF109203X nor calphostin C inhibited basal production of PGI_2.

The phosphoinositide 3-kinase (PI3-kinase) inhibitor, wortmannin, had a partial inhibitory effect on PGI_2 generation induced by VEGF after 10 min, but had no significant effect on release induced by a 30 min treatment (Figure 2). Neither NO donors, nor the endothelial NO synthase inhibitors L-NAME (N^G-nitro-L-arginine methyl ester) and L-NMMA (N^G-monomethyl-L-arginine), caused any significant change in the PGI_2 synthetic response to VEGF (Figure 2). Some differences in the effects of VEGF on PGI_2 production after 30 min were noted between experiments (compare Figures 1A and 2). This may reflect variation in the time course of $cPLA_2$ activation between experiments and/or different batches of HUVECs.

VEGF-induced PKC activation

The marked inhibition of VEGF-stimulated PGI_2 synthesis by inhibitors of PKC activation suggested that PKC was important for upstream signalling leading to the stimulation of PGI_2 biosynthesis. VEGF induced a time-dependent increase in PKC activity measured in extracts of HUVECs that was detectable at 5 min, sustained at 10 min and declined after 30 min (Figure 3A). It was next determined whether VEGF induced the translocation to a particulate fraction of specific PKC isoforms. Consistent with previous findings [39], HUVECs contained detectable levels of α, δ, ϵ and ζ PKC isoforms, and other isoforms were not detected by Western blotting (results not shown). Immunoblot analysis showed that VEGF induced a marked increase in the immunoreactivity of PKCδ and PKCα in the particulate fraction which was evident after 5 min, sustained after 10 min and declined after 30 min (Figure 3B). The small decrease in cytosolic PKCδ observed in these experiments may reflect the translocation to the particulate fraction of only a small pool of total PKCδ. Indeed, PKCδ immunoblots of whole-cell lysates indicated that PKCδ present in both cytosolic and particulate fractions accounted for only a proportion of total PKCδ immunoreactivity in HUVECs (results not shown). VEGF also promoted translocation of PKCϵ to the particulate fraction with a different time course, showing a somewhat slower increase that was sustained for up to 30 min (Figure 3B). VEGF caused a modest increase in PKCζ immunoreactivity in the particulate fraction, but this effect was not significant (Figure 3B).

VEGF activates ERKs 1 and 2 via a PKC-dependent pathway

The marked inhibition of VEGF-induced PGI_2 production by PKC inhibitors raised the possibility that these compounds might be acting at a point in the signalling pathway proximal to ERK activation. VEGF induced a striking and transient increase in activity of ERKs 1 and 2 which was maximal at 10 min, declined after 30 min and was inhibited by the selective KDR inhibitor SU5614 (Figure 4). VEGF activated ERKs 1 and 2 (p42 and p44, respectively) to an approximately equivalent extent. GF109203X and calphostin C both markedly inhibited VEGF-induced activation of ERKs 1 and 2 (Figure 4). VEGF activated MAP kinase kinase (MEK) transiently with an increase after 5–10 min which declined to the basal level after 30 min; VEGF-induced MEK activation after 10 min was strikingly inhibited by GF109203X and calphostin C (Figure 4B). GF109203X and calphostin C also inhibited the VEGF-induced shift in mobility of the 85 kDa $cPLA_2$ from a more rapidly migrating and less phosphorylated (inactive) form to a more slowly migrating phosphorylated (active) form (Figure 4C).

Specificity of PKC inhibitors

It was next examined whether the effects of the PKC inhibitors on VEGF stimulation of ERK activity were selective and due to non-specific inhibition of VEGF-receptor binding or activation.

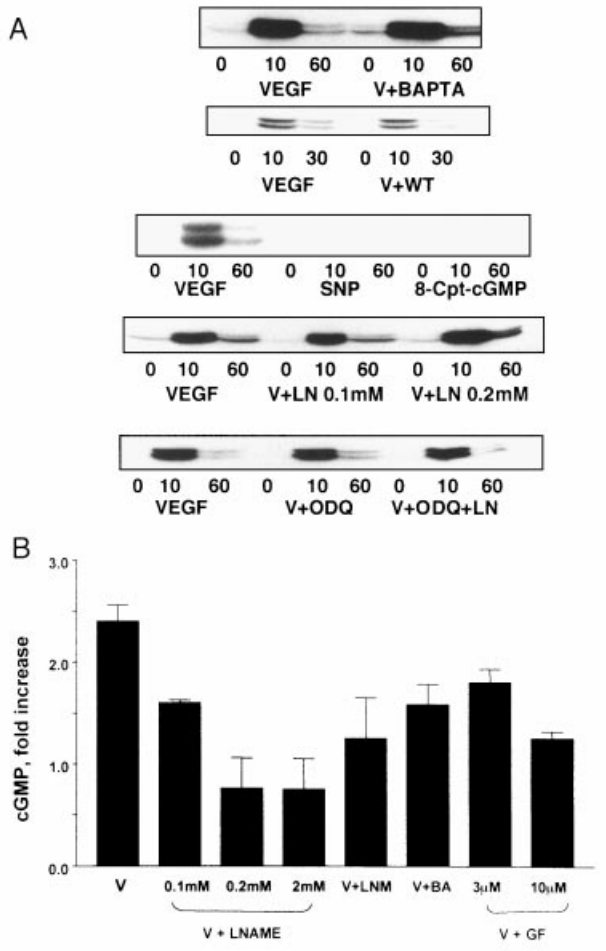

Figure 7 VEGF-induced ERK1/2 activation is independent of Ca^{2+}, PI3-kinase and NO production

(**A**) Cells were pretreated for 30 min with 10 μg/ml BAPTA/AM (+BAPTA), 100 nM wortmannin (+WT), 100 or 200 μM L-NAME (+LN), 10 μM ODQ or 10 μM ODQ + 200 μM L-NAME (+ODQ+LN) as indicated, and then incubated with 25 ng/ml VEGF (VEGF, V) for the times shown (in min). Some cells were treated with either 100 μM sodium nitroprusside (SNP) or 10 μM 8-Cpt-cGMP for the times shown. ERK1/2 activation was then determined. The results shown are representative of three to five separate experiments. (**B**) Cells were pretreated for 30 min with L-NAME, L-NMMA (LNM, 200 μM), BAPTA/AM (BA, 5 μg/ml) or GF109203X (GF) at the concentrations indicated, and then treated for 30 min with 25 ng/ml VEGF in the presence of 100 μM isobutylmethylxanthine. cGMP was extracted and measured as described in the Materials and methods section. Results are presented as the fold increase (means ± S.E.M., $n = 3$) above control basal production.

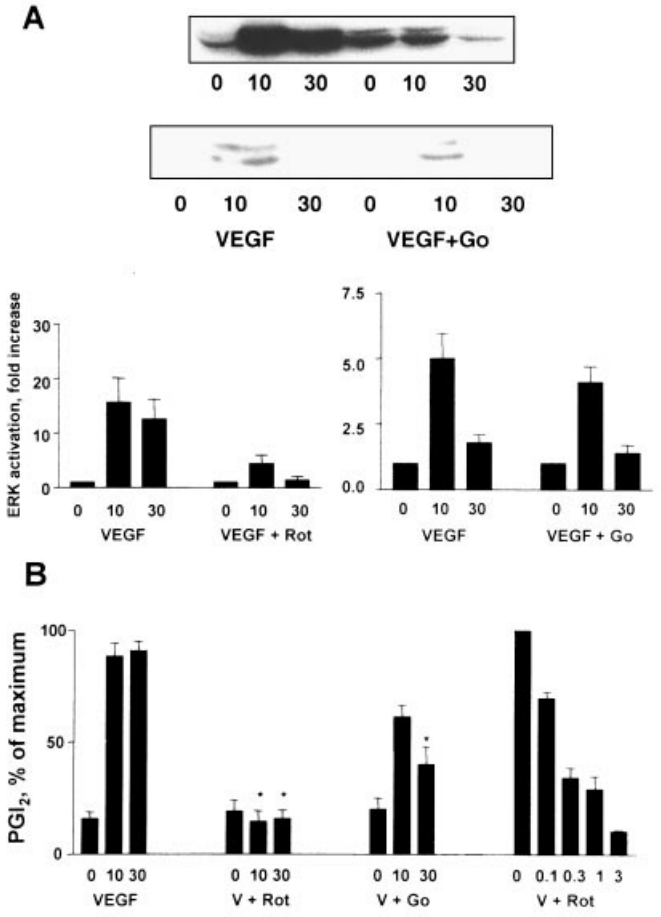

Figure 8 Involvement of PKCδ in VEGF-induced ERK1/2 activation and PGI_2 production

(**A**) Cells were pretreated for 30 min in the absence (VEGF) or presence of either 1 μM rottlerin (VEGF + Rot) or 1 μM Go6976 (VEGF + Go) and then incubated with 25 ng/ml VEGF for the times indicated (in min). ERK activation was determined as before. (Lower panel) Results for ERK activation were quantified as before and are presented as fold increases (means ± S.E.M., $n = 4$). (**B**) PGI_2 production was determined after VEGF treatments in the same cells as used for determination of ERK activation in (**A**). Results for PGI_2 are the means ± S.E.M. ($n = 4$). $^*P < 0.01$ for VEGF + rottlerin versus VEGF after 10 and 30 min and VEGF + Go6976 versus VEGF after 30 min.

GF109203X had no significant effect on bFGF-induced ERK activation (Figure 5A). Thrombin-induced ERK activation was much weaker than that of either VEGF or bFGF and was also not inhibited by GF109203X (results not shown). GF109203X did not inhibit receptor activation, as judged by assay of either kinase activity *in vitro* or KDR tyrosine phosphorylation determined in KDR immunoprecipitates, and had no effect on the binding of ^{125}I-VEGF to high-affinity sites in intact cells (Figure 5B). Calphostin C also had no inhibitory effect on KDR activation (Figure 5B). It was noted consistently that GF109203X caused some enhancement of KDR receptor activity. The reason for this effect is unclear but it may be due to inhibition of a kinase involved in negative regulation of KDR activation.

VEGF-induced ERK activation is independent of intracellular Ca^{2+}, PI3-kinase and NO pathways

The cell-permeant Ca^{2+}-chelating agent BAPTA/AM markedly reduced VEGF-induced mobilization of intracellular Ca^{2+} at 5 μg/ml without lowering the resting intracellular [Ca^{2+}] (Figure 6A). At the same concentration BAPTA/AM blocked VEGF-induced generation of PGI_2 and arachidonic acid (Figures 6B and 6C), but had no inhibitory effect on VEGF-induced ERK activation (Figure 7). Wortmannin also caused no inhibition of VEGF-dependent ERK activity (Figure 7). Modulators both of NO production, including L-NAME, L-NMMA and sodium nitroprusside, and of the cGMP pathway, including the guanylate cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ), and the protein kinase G activator 8-Cpt-cGMP, had no effect on either basal or VEGF-stimulated ERK activation (Figure 7). It was verified that endothelial NO synthase inhibitors blocked the VEGF-induced increase in intracellular cGMP

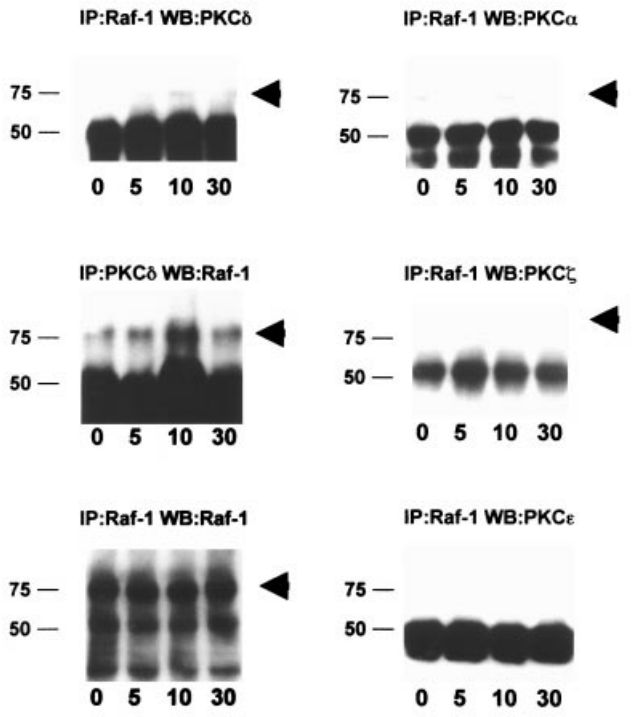

Figure 9 VEGF stimulates association between PKCδ and Raf-1

Cells were treated with 25 ng/ml VEGF for the times indicated (in min), Raf-1 immunoprecipitates (IP) were prepared, and after SDS/PAGE were immunoblotted with antibodies to PKCδ, PKCα, PKCζ, PKCϵ or Raf-1, as indicated. PKCδ immunoprecipitates were also prepared and blotted (WB) with anti-Raf-1 antibody. The arrowheads indicate the positions of the specific immunoreactive bands recognized in immunoblots. Results are representative of three experiments. The positions of molecular-mass markers ($\times 10^{-3}$) are indicated on the left.

(Figure 7). In addition, pretreatment with either BAPTA/AM or GF109203X also decreased the VEGF-stimulated accumulation of cGMP (Figure 7).

Role of PKCδ in VEGF-induced ERK activation

To investigate the role of PKCδ in VEGF signalling through the ERK cascade, we examined the effect of the PKCδ-selective inhibitor rottlerin [40]. As shown in Figure 8(A), rottlerin markedly inhibited early VEGF-induced ERK activation and PGI_2 production at 1 μM, a concentration similar to the previously reported IC_{50} (3–6 μM) for rottlerin-induced inhibition of PKCδ and which had no inhibitory effect on VEGF-induced KDR tyrosine phosphorylation (Figure 5B). Rottlerin also prevented VEGF-induced activation of $cPLA_2$, similar to the effect of GF109203X and calphostin C (results not shown). Inhibition of PGI_2 synthesis was dose-dependent with a half-maximum effect between 0.1 and 0.3 μM rottlerin. In contrast to the results obtained with rottlerin, Go6976, a selective inhibitor of PKCα [41], had no inhibitory effect on VEGF-induced ERK1/2 activation (Figure 8A). Go6976 also had no detectable inhibitory effect on MEK activity (results not shown). However, Go6976 did partially inhibit VEGF-induced PGI_2 production at 10 min and caused a more marked inhibition after 30 min (Figure 8B).

It was next determined whether VEGF regulated PKCδ association with Raf-1, an upstream activator of MEK. Immunoprecipitates of Raf-1 were prepared from VEGF-treated HUVECs and subsequently immunoblotted with PKCδ antibody. The results show that PKCδ was present at a low or barely detectable level in Raf-1 immunoprecipitates prepared from control untreated cells, and increased in immunoprecipitates from cells treated with VEGF for 5 and 10 min, and declined after 30 min. Similarly, when PKCδ immunoprecipitates were prepared from VEGF-treated cells and blotted with Raf-1 antibody, the results indicated that Raf-1 increased markedly in PKCδ immunoprecipitates after 10 min of VEGF treatment (Figure 9). Raf-1 immunoblots of Raf-1 immunoprecipitates showed that Raf-1 was present at similar levels after all times of VEGF treatment. VEGF did not increase Raf-1 association of PKCα, PKCϵ or PKCζ but was found to decrease a constitutive association between PKCα and Raf-1 (Figure 9).

DISCUSSION

We reported previously that VEGF-stimulated activation of $cPLA_2$ activation and production of PGI_2 occurs via the ERK pathway [18]. The signal-transduction mechanisms through which VEGF induces ERK activation and stimulates downstream biological functions in endothelial cells remain largely undefined, however. In this paper, our aim was to determine the contribution of PKC in the signalling pathways which mediate VEGF-induced ERK activation and PGI_2 production.

Our findings indicate that VEGF activation of the ERK cascade and ERK-dependent PGI_2 biosynthesis occur via a PKC-dependent pathway. This conclusion is based on the following results. GF109203X and calphostin C, two structurally unrelated PKC inhibitors that inhibit PKCs through distinct mechanisms, blocked VEGF-induced PGI_2 generation. GF109203X also inhibited release of arachidonic acid, the major precursor for prostanoid biosynthesis, and prevented VEGF-stimulated $cPLA_2$ phosphorylation. A salient feature of our results was that GF109203X and calphostin C strongly inhibited the early increase in both ERK and MEK activation induced by VEGF. The effects of GF109203X were not the result of a non-specific decrease in KDR ligand binding or activation, since, at concentrations of GF109203X that prevented VEGF-stimulated PGI_2 production and ERK activation, the inhibitor had no effect either on VEGF radiolabelled ligand binding to high-affinity sites or on KDR-associated kinase activity measured in immune-complex assays. GF109203X did not inhibit thrombin-stimulated PGI_2 production, indicating that components of the PGI_2 biosynthetic pathway distal to $cPLA_2$ activation were not affected by this compound. In addition, since ERK activity stimulated by bFGF in HUVECs was not decreased markedly by the inhibitor, and GF109203X enhanced ERK activation in response to thrombin, our findings suggest that the effect of GF109203X on VEGF-induced ERK activation may be selective for signalling downstream of VEGF receptors. This receptor is most likely to be KDR, given that a KDR-selective inhibitor blocked VEGF-induced ERK activation, and that neither Flt-1 nor Flt-1-specific ligands mediate ERK activation [15,18].

VEGF increased PKC activity in HUVECs and increased the immunoreactivity of δ, ϵ and α PKC isoforms in the particulate fraction but had only a very modest effect on PKCζ. The time courses for PKC activity and PKCδ translocation correlated, both showing a transient increase evident from 5–10 min, and decreasing to near the control level after 30 min. Increased PKCϵ translocation to the membrane rose more slowly, however, and was sustained for 30 min. The finding that the PKCδ-selective inhibitor, rottlerin, markedly inhibited VEGF-induced ERK activation and PGI_2 production indicates that VEGF signalling through the ERK pathway is mediated at least in part by PKCδ.

This conclusion is further supported by the fact that VEGF increased the association of PKCδ with Raf-1, as determined by co-immunoprecipitation with a very similar time course to that for PKC activity and PKCδ translocation. In the absence of a PKCϵ-specific inhibitor, we do not preclude a role for this isoform in mediating VEGF-induced ERK activation or PGI_2 production. The partial inhibition of VEGF-stimulated PGI_2 production by the PKCα-selective inhibitor Go6976 suggests that PKCα also plays a role in mediating this biological response. However, since VEGF did not increase Raf-1 association with PKCα and Go6976 had no inhibitory effect on either ERK or MEK activation, we conclude that this isoform is unlikely to be involved in VEGF-induced ERK activity and ERK-dependent PGI_2 synthesis.

Previous findings have implicated PKC in signalling pathways leading to PGI_2 production and release in response to other agonists [42]. It has been unclear how PKC regulates this response, but some findings have suggested that PKC acts in concert with a rise in intracellular Ca^{2+} to increase the sensitivity of agonist-stimulated PGI_2 release [30]. Our findings therefore suggest a novel role for PKC in the mediation of ERK activation and subsequent stimulation of PGI_2 generation. The conclusion that PKC is important for biologically relevant VEGF signalling is supported further by GF109203X-dependent inhibition of VEGF-induced intracellular cGMP accumulation, and is consistent with recent reports that PKC is activated by VEGF and that PKCα and ζ isoforms play a role in VEGF-stimulated proliferation of HUVECs [19,43,44]. Differences between these findings and those in the present paper may reflect the involvement of distinct PKC isoforms in different biological functions.

Ca^{2+} is thought to play a major role in the rapid release of PGI_2 and activation of $cPLA_2$ [29,30]. Our findings show that inhibition of VEGF-stimulated Ca^{2+} mobilization [45] also blocked VEGF-induced PGI_2 production without inhibiting ERK activation. These findings are most consistent with the conclusion that a rise in intracellular Ca^{2+} is important for the release of PGI_2 in response to VEGF, but does not mediate the biosynthetic pathway dependent on ERK activation. Other pathways may also play a role in VEGF-receptor signalling leading to PGI_2 release. The PI3-kinase inhibitor wortmannin partially inhibited VEGF-induced PGI_2 production after 10 min without affecting ERK activation, suggesting that PI3-kinase may contribute to early ERK-independent PGI_2 production.

The relationship between the NO and PGI_2 production pathways in the response to VEGF in HUVECs was also investigated. In contrast to two previous reports suggesting that the NO pathway mediates VEGF-induced activation of the ERK cascade [35], we found that neither stimulation nor inhibition of NO production had any effect on ERK activity or PGI_2 production. Consistent with this conclusion, a recent report also found that VEGF stimulation of NO production and PGI_2 release occurred via independent pathways that bifurcated distal to Src [46].

The major pathway through which protein tyrosine kinase receptors activate ERKs involves tyrosine phosphorylation and receptor association of the adapter protein, GRB-2, subsequent stimulation of the guanine-nucleotide-exchange protein SOS and activation of Ras, which in turn activates Raf-1 and the distal ERK cascade. VEGF has been reported to stimulate tyrosine phosphorylation of several SH2-domain-containing signalling proteins [16,17,47]. In particular, VEGF was shown recently to stimulate tyrosine phosphorylation of Shc and promote formation of a complex between Shc and GRB-2 in porcine aortic endothelial cells overexpressing KDR [47]. So far, however, neither tyrosine phosphorylation of Shc nor VEGF-stimulated association between Shc and GRB-2 have been shown to lead to activation of Ras or the ERK pathway. In addition, while this manuscript was in preparation, and in accord with our results, it was reported that VEGF induces PKC-dependent and Ras-independent induction of the Raf-MEK-ERK pathway in sinusoidal endothelial cells [48]. The finding that VEGF stimulates ERK-mediated PGI_2 production and cGMP accumulation via PKC and promotes association between PKCδ and Raf-1 indicates a novel pathway for activation of the ERK cascade by a protein tyrosine kinase receptor for a polypeptide growth factor. Whereas our findings do not preclude a role for the GRB-2-SOS-Ras pathway in VEGF stimulation of the ERK cascade, they suggest that PKC plays a major role in this signalling mechanism. Delineating the molecular sequelae of events leading from the KDR receptor to ERK activation will be a major goal of further studies. Since the PGI_2 pathway is increasingly implicated in biological actions of VEGF *in vivo*, defining the signalling mechanisms mediating this response should also contribute towards an improved understanding of the molecular basis for role(s) of VEGF in health and disease.

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