

## Regulation of novel protein kinase C $\epsilon$ by phosphorylation

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The activity and intracellular localization of protein kinase C (PKC) family members are controlled by phosphorylation at three highly conserved sites in the catalytic kinase domain. In the case of the novel PKC $\epsilon$  isoform, these are Thr<sup>566</sup> in the activation loop, Thr<sup>710</sup> in the turn motif and Ser<sup>729</sup> in the C-terminal hydrophobic motif. In the present study, we analysed the contribution of the phosphoinositide-dependent kinase 1 (PDK-1) and PKC $\epsilon$  kinase activity in controlling the phosphorylation of Thr<sup>566</sup> and Ser<sup>729</sup>. In NIH 3T3 fibroblasts, PKC $\epsilon$  migrated as a single band, and stimulation with platelet-derived growth factor resulted in the appearance of a second band with a slower electrophoretic mobility, concomitant with an increase in phosphorylation of Thr<sup>566</sup> and Ser<sup>729</sup>. Cells transfected with an active PDK-1 allele also resulted in increased PKC $\epsilon$  Thr<sup>566</sup> and Ser<sup>729</sup> phosphorylation, whereas an active myristoylated PKC $\epsilon$  mutant was constitutively phosphorylated at these sites. Protein kinase-inactive mutants of PKC $\epsilon$  were not phosphorylated at

Ser<sup>729</sup> in cells, and phosphorylation of this site leads to dephosphorylation of the activation-loop Thr<sup>566</sup>, an effect which can be reversed with either okadaic acid or co-transfection with active PDK-1. *In vitro*, PDK-1 catalysed the phosphorylation of purified PKC $\epsilon$  in the presence of mixed micelles containing either diacylglycerol or PtdIns(3,4,5)P<sub>3</sub>, concomitant with an increase in Ser<sup>729</sup> phosphorylation. These studies reveal that the mechanism of phosphorylation of a novel PKC is the same as that for conventional PKCs: PDK-1 phosphorylation of the activation loop triggers autophosphorylation of the hydrophobic motif. However, the regulation of this phosphorylation is different for novel and conventional PKCs. Specifically, the phosphorylation of novel PKCs is regulated rather than constitutive.

**Key words:** phosphoinositide-dependent kinase 1, phosphoinositide 3-kinase, PKC, second messengers, signal transduction.

### INTRODUCTION

Protein kinase C (PKC) comprises a family of 12 distinct phospholipid-dependent serine/threonine protein kinases which are members of the extended AGC (protein kinase A, G and C) superfamily [1]. PKC isoforms have been subdivided into three separate subfamilies according to lipid and cofactor requirements [2,3]. Conventional PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II and PKC $\gamma$  are activated by Ca<sup>2+</sup> and diacylglycerol (DAG), whereas novel PKC $\delta$ , PKC $\epsilon$ , PKC $\eta$  and PKC $\theta$  are Ca<sup>2+</sup>-insensitive because they lack a conventional, Ca<sup>2+</sup>-responsive C2 domain. The atypical PKC subfamily comprises the  $\zeta$  and  $\iota/\lambda$  isoforms, which are Ca<sup>2+</sup>- and DAG-insensitive. All PKCs also require binding of the cofactor phosphatidylserine (PtdSer), as this promotes the release of the autoinhibitory pseudosubstrate domain from the substrate-binding cavity [4]. A separate family of kinases include PKC $\mu$ /protein kinase D1 and protein kinase D2, as well as the more recently cloned PKC $\nu$ . PKCs have been directly implicated in mediating a wide range of physiological processes ranging from cell growth and proliferation, differentiation, apoptosis and protection from apoptosis, cell motility and cytoskeletal rearrangements [5]. Likely explanations for the diversity of such responses attributed to one enzyme family include discrete subcellular and tissue distribution as well as phosphorylation of distinct substrates.

As with other members of the AGC kinase family, PKCs are phosphorylated at three distinct sites in the catalytic kinase core,

and the sequences surrounding these serine/threonine residues are highly conserved among PKCs and other AGC kinases [6,7]. Phosphorylation of these sites is required for the enzyme to bind to, and be activated by, DAG [6,8,9]. The first and rate-limiting phosphorylation occurs in the activation-loop sequence, and phosphorylation of the activation loop is required to correctly re-align residues in the active site. Evidence has mounted recently that phosphoinositide-dependent kinase 1 (PDK-1) is the upstream kinase which directly phosphorylates the activation loop of many, if not all, PKCs [10]. Phosphorylation by PDK-1 provides a link with the phosphoinositide 3-kinase (PI 3-kinase) pathway, as the PI 3-kinase lipid products PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> bind and recruit PDK-1 to membranes, leading to phosphorylation of downstream substrates such as PKCs as well as the first described target of PDK-1, the proto-oncogene Akt/protein kinase B (PKB) [11]. In the case of conventional PKC $\beta$ II, phosphorylation by PDK-1 at the activation-loop Thr<sup>500</sup> does not directly activate the PKC; rather, it promotes the autophosphorylation of two additional residues in the C-terminus to produce a mature and fully phosphorylated enzyme that is competent to respond to lipid second messengers [12]. The first of these has been termed the 'turn motif', as the serine/threonine residue is typically flanked by a proline residue and, on the basis of modelling studies with the structure of the related protein kinase A catalytic domain, is found at the top of the upper lobe of the kinase domain. The very-C-terminal site is referred to as the 'hydrophobic motif' because the phospho-acceptor residue is

Abbreviations used: A.T.C.C., American Type Culture Collection; BIM I, bisindolylmaleimide I; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FLAG, MDYKDDDDK [methionine (M) was added by PCR as it represents the initiator residue]; GST, glutathione S-transferase; HEK293, human embryonic kidney 293 cells; MBP, myelin basic protein; mTOR, mammalian target of rapamycin; OA, okadaic acid; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PDK-1, phosphoinositide-dependent kinase 1; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; T566A (etc.), Thr<sup>566</sup>→Ala mutant; TBS, Tris-buffered saline; pThr (etc.), phosphothreonine (etc.).

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typically flanked by hydrophobic residues. With atypical PKCs such as PKC $\zeta$ , phosphorylation by PDK-1 occurs in a PI 3-kinase-dependent manner and results in activation of the PKC [13,14], such that phosphorylation of the activation loop acts as a switch to directly modulate kinase activity, as is the case with Akt/PKB. Interestingly, in the atypical PKC $\zeta$  and PKC $\iota$  isoforms the hydrophobic acceptor serine/threonine residue is replaced by a glutamic acid residue. Thus, although both conventional and atypical PKCs are phosphorylated by PDK-1, the consequence for PKC activity differs between various isoforms.

The novel PKC $\epsilon$  isoform is also phosphorylated by PDK-1 *in vitro* and in cells [14], and this explains previous reports indicating a PI 3-kinase requirement for PKC $\epsilon$  activation by platelet-derived growth factor (PDGF) and other growth factors [15]. PDK-1 phosphorylates PKC $\epsilon$  at the activation-loop residue Thr<sup>566</sup>, and there is evidence that PI 3-kinase- and PDK-1-dependent phosphorylation of PKC $\epsilon$  leads to activation [16,17]. The regulation of the C-terminal turn motif (Thr<sup>710</sup>) and hydrophobic site (Ser<sup>729</sup>) of PKC $\epsilon$  is less well understood. One report suggested that a protein complex including atypical PKC $\zeta$  is responsible for the heterologous phosphorylation of PKC $\delta$  and PKC $\epsilon$  at Ser<sup>729</sup> [16]. Moreover, studies using reportedly 'specific' PKC inhibitors have suggested that PKC $\epsilon$  Ser<sup>729</sup> phosphorylation is mediated either by a heterologous kinase [17] or by PKC itself [18]. In addition, regulation of PKC $\delta$  and PKC $\epsilon$  hydrophobic-site phosphorylation is antagonized by rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) and p70<sup>S6</sup>-kinase pathway [17], suggesting that phosphorylation of these PKCs is downstream of mTOR and in an amino-acid-sensing pathway. These studies have made a clear case for the regulated phosphorylation and dephosphorylation of PKC $\epsilon$  at Ser<sup>729</sup>, which has implications for the proper intracellular localization and function of the kinase. What has remained elusive, however, is the precise mechanism by which Ser<sup>729</sup> is phosphorylated. In the present study we addressed the mechanism of regulation of PKC $\epsilon$  by phosphorylation using a mutational strategy combined with phosphospecific antibodies against Thr<sup>566</sup> and Ser<sup>729</sup>. We show that transfection of cells with PDK-1 leads to phosphorylation of PKC $\epsilon$  at Thr<sup>566</sup> as well as Ser<sup>729</sup>. This latter phosphorylation depends on the intrinsic catalytic activity of PKC, suggesting that it is mediated by autophosphorylation and not by an upstream kinase. Consistent with this, pure PKC $\epsilon$  autophosphorylates at Ser<sup>729</sup> *in vitro*. These results suggest that, at least under these conditions, phosphorylation of PKC $\epsilon$  at Ser<sup>729</sup> is mediated primarily by autophosphorylation, such that PDK-1 is the only *in vivo* upstream kinase for this PKC.

## EXPERIMENTAL

### Materials

Human embryonic 293 (HEK 293) cells and early passage (127) NIH 3T3 fibroblasts were obtained from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.). Cell-culture medium was from Life Technologies (Carlsbad, CA, U.S.A.), and fetal-bovine serum (FBS) was from Irvine Scientific (Santa Ana, CA, U.S.A.). [ $\gamma$ -<sup>32</sup>P]ATP was from New England Nuclear (Boston, MA, U.S.A.). Myelin basic protein (MBP) was from Life Technologies. Bovine brain PtdSer, L- $\alpha$ -phosphatidylcholine (PtdCho) and *sn*-1,2-dioleoylglycerol were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA, U.S.A.). Okadaic acid (OA), microcystin and bisindolylmaleimide I (BIM I GF 109203X) were obtained from Calbiochem (San Diego, CA, U.S.A.). PDGF-BB homodimer was obtained from Life

Technologies. All other chemicals were from Sigma–Aldrich (St Louis, MO, U.S.A.).

### Antibodies

An affinity-purified polyclonal antibody specific for PKC $\epsilon$  was obtained from Santa Cruz (Santa Cruz, CA, U.S.A.). An antibody against the Myc epitope (EQKLISEEDL) was purified from the 9E10 hybridoma in-house on a Hi-TRAP Protein-G column (Amersham–Pharmacia, Piscataway, NJ, U.S.A.). A phosphospecific antibody against the activation loop of PKC $\zeta$  (Thr<sup>410</sup>), and which cross-reacts with the activation-loop Thr<sup>566</sup> of PKC $\epsilon$ , was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A phosphospecific antibody (pan-PKC antibody) against the C-terminal-hydrophobic-site Ser<sup>660</sup> of PKC $\beta$ II, and which cross-reacts with all PKCs, including PKC $\epsilon$ , was obtained from New England Biolabs (Beverly, MA, U.S.A.). A phosphospecific antibody against the C-terminal-turn-motif Thr<sup>638</sup> of PKC $\alpha$ , and which cross-reacts with PKC $\epsilon$ , was obtained from New England Biolabs. The M2 monoclonal anti-FLAG antibody raised against the FLAG sequence (MDYKDDDDK) was obtained from Sigma–Aldrich.

### cDNA constructs

The human cDNA for PKC $\epsilon$  was used for all constructs (GenBank<sup>®</sup> accession number Q02156), and the FLAG epitope was added to the C-terminus by PCR. The T566A (Thr<sup>566</sup>  $\rightarrow$  Ala), T566E, T710E, K437W, T566G/K437W, T710G/K437T and S729A PKC $\epsilon$  mutants were made by PCR using the QuikChange strategy (Stratagene, La Jolla, CA, U.S.A.). All constructs were subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). The Myc-tagged PDK-1 and Myc.PDK-1.Lys110Asn, also in the pcDNA3 vector, have been described [13]. The Myr.PKC $\epsilon$  construct was made by adding in-frame the first nine amino acids of the p60 c-Src myristoylation sequence (MGSNKSCKPK) to the PKC $\epsilon$  N-terminus and the FLAG sequence to the C-terminus. All constructs and mutants were verified by DNA sequencing.

### Tissue culture and transfection

NIH 3T3 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% (v/v) heat-inactivated FBS at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. All transient transfections into HEK293 cells were carried out using the LIPOFECTAMINE<sup>™</sup> procedure (Life Technologies). Cells were seeded at a density of  $1 \times 10^5$  per 35-mm-diameter dish 12 h before transfection. Each DNA construct was titrated against LIPOFECTAMINE<sup>™</sup> to achieve uniform expression of the proteins, typically 1  $\mu$ g of each DNA, combined with empty vector for a total of 2  $\mu$ g per dish. The DNA–LIPOFECTAMINE<sup>™</sup> complex mix was overlaid on cells in transfection medium (Optimem; Life Technologies) for 6 h, after which cells were washed and recovered in complete medium (DMEM + 10% FBS) for 14 h. Cells were either left in complete medium, or serum-starved in DMEM/serum for 12 h as noted.

### Purification of recombinant PKC $\epsilon$ and PDK-1

Recombinant GST (glutathione S-transferase)-tagged PKC $\epsilon$  and His<sub>6</sub>-tagged PDK-1 were expressed in baculovirus-infected insect cells. *Spondoptera frugiperda* 9 (Sf9) cells (500 ml) were grown to a density of  $1.5 \times 10^6$ /ml and infected in a spinner flask with 50 ml of high-titre ( $5 \times 10^7$  plaque-forming units/ml) baculovirus directing the expression of a GST–PKC $\epsilon$  and His<sub>6</sub>–PDK-1 fusion

protein. Infections were carried out for 3 days, after which cells were harvested by centrifugation and frozen at  $-70^{\circ}\text{C}$ . Cells were lysed in buffer A (in the absence of EDTA) and cleared lysate was applied to a 5 ml  $\text{Ni}^{2+}$ -nitrilotriacetate (Qiagen, Valencia, CA, U.S.A.) column for His<sub>6</sub>-PDK-1, and a 5 ml GSH-Sepharose CL-4B column (Amersham-Pharmacia) for GST-PKC $\epsilon$ . Following extensive washing with buffer G [20 mM Tris (pH 8.0)/500 mM KCl/5 mM 2-mercaptoethanol/10% glycerol], the proteins were eluted with either 100 mM imidazole (His<sub>6</sub>-PDK-1) or 10 mM GSH in buffer G. Fractions containing the protein peaks were pooled, diluted in buffer H [20 mM Tris (pH 8.0)/5 mM 2-mercaptoethanol] and applied to a Mono Q FPLC column (Amersham-Pharmacia). Proteins were eluted with a 40 ml linear gradient of NaCl (0–0.5 M) in buffer H. Fractions were resolved by SDS/PAGE and stained with Coomassie Blue. The GST tag on GST-PKC $\epsilon$  was removed by thrombin cleavage, as previously described [13]. The resulting proteins were over 95% pure and stored in 50% (v/v) glycerol at  $-20^{\circ}\text{C}$ .

### Immunoprecipitation and immune-complex kinase assays

Following stimulation, cells were lysed in 1 ml of buffer A [20 mM Tris (pH 7.5)/10% glycerol/1% Nonidet P40/10 mM EDTA/150 mM NaCl/20 mM NaF/5 mM sodium pyrophosphate/1 mM sodium vanadate/1  $\mu\text{g}/\text{ml}$  leupeptin/1  $\mu\text{g}/\text{ml}$  pepstatin A/1 mM PMSF] at  $4^{\circ}\text{C}$ . Approx. 10% of the total lysate was immediately boiled in SDS sample buffer. Epitope-tagged proteins were immunoprecipitated with 1  $\mu\text{g}/\text{ml}$  of the corresponding epitope antibodies, as follows: FLAG-PKC $\epsilon$  and all PKC $\epsilon$  mutants, M2 monoclonal antibody and endogenous PKC $\epsilon$ , anti-PKC $\epsilon$  antibody, and with a 25  $\mu\text{l}/\text{ml}$  of a 50% mix of Protein A/G beads (Santa Cruz).

Immunoprecipitates were washed stringently as follows: twice in buffer B (1  $\times$  PBS/1% Nonidet P40), twice in buffer C [10 mM Tris (pH 7.5)/0.5 M LiCl] and twice in buffer D [10 mM Tris (pH 7.5)/100 mM NaCl/1 mM EDTA]. Immunoprecipitates were either boiled in SDS sample buffer and resolved by SDS/7.5% (w/v)-PAGE, or used for immune-complex kinase assays carried out in a total volume of 30  $\mu\text{l}$  under the following reaction conditions: 10 mM  $\text{MgCl}_2$ , 5  $\mu\text{g}$  of MBP and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (80  $\mu\text{M}$ ) for 20 min at  $25^{\circ}\text{C}$ . Reaction mixtures were boiled in SDS sample buffer and resolved by SDS/12.5% PAGE; MBP phosphorylation was detected by autoradiography.

### In vitro phosphorylation

Phosphorylation of purified PKC $\epsilon$  was assayed *in vitro* using 0.2  $\mu\text{g}$  of PKC $\epsilon$  and PDK-1 in a protein kinase assay mixture comprising 20 mM Tris, pH 7.5, 25 mM  $\text{MgCl}_2$ , 80  $\mu\text{M}$  ATP, with or without 5  $\mu\text{Ci}/\text{assay}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci/mmol), with or without the indicated lipid vesicles, for 20 min at  $25^{\circ}\text{C}$ . Mixed lipid vesicles were prepared by drying a concentrated stock of lipid [either DAG or PtdIns(3,4,5) $P_3$  in a PtdCho/PtdSer background, as noted] stored in chloroform/methanol (1:1, v/v) under a stream of nitrogen. Lipids were reconstituted by sonication into 25 mM Tris, pH 7.5, in an ice-bath sonicator at  $4^{\circ}\text{C}$  for 10 min at 50% output. Lipids were prepared fresh for each experiment. Reactions were terminated by addition of SDS sample buffer. For autophosphorylation assays, pure PKC $\epsilon$  (1  $\mu\text{M}$ ) was incubated with pure protein phosphatase 2Ac in the presence of 20 mM Hepes, 1 mM dithiothreitol, 140  $\mu\text{M}$  PtdSer and 4  $\mu\text{M}$  DAG for 10 min at  $22^{\circ}\text{C}$ , essentially as described in [12]. The dephosphorylation reaction was quenched by addition of 4  $\mu\text{M}$  microcystin and re-autophosphorylation

was initiated by the addition of 100  $\mu\text{M}$  ATP/5 mM  $\text{MgCl}_2$  and continued incubation for 5 min at  $22^{\circ}\text{C}$ . Aliquots before and after dephosphorylation/rephosphorylation were quenched in sample buffer containing 10 mM EGTA. Reaction mixtures were separated by SDS/7.5% PAGE and either immunoblotted with the indicated antibodies, or exposed to autoradiography, as noted.

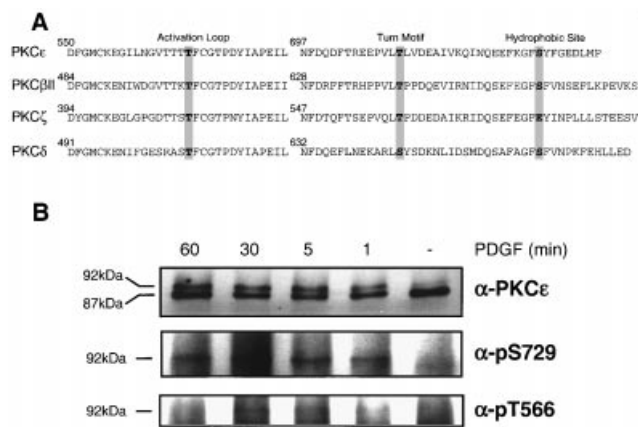
### Western blotting

Immunoprecipitates of PKC $\epsilon$ , immune-complex kinase assays and total lysates were resolved by either SDS/7.5% or SDS/12.5% PAGE, and transferred to nitrocellulose membranes, blocked in 2% BSA in 1  $\times$  Tris-buffered saline (TBS; 10 mM Tris/HCl/150 mM NaCl, pH 8.0), and incubated with the following dilutions of antibody in 1  $\times$  TBS + 0.2% Tween-20 for 12 h at  $4^{\circ}\text{C}$ : anti-Myc, 1:5000; anti-pThr410 [to detect phosphoThr<sup>566</sup> (pThr<sup>566</sup>)], 1:1000; anti-pan-PKC (to detect pSer<sup>729</sup>), 1:1000; anti-PKC $\epsilon$ , 1:1000. Goat anti-mouse or anti-rabbit antibodies (Boehringer Mannheim, Indianapolis, IN, U.S.A.) were used at 1:10000, and proteins were revealed using a Western-blot chemiluminescence reagent (Renaissance system; New England Nuclear).

## RESULTS

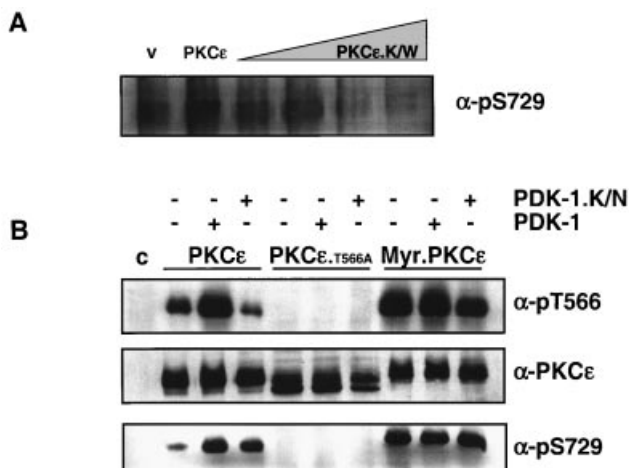
### Mitogen stimulation of PKC $\epsilon$ phosphorylation

The amino acid sequences surrounding the activation loop, turn motif and hydrophobic sites in PKCs are highly conserved. Figure 1(A) shows an alignment of the sequence surrounding these motifs in the catalytic kinase domains of PKC $\epsilon$ , PKC $\beta$ II, PKC $\delta$  and PKC $\zeta$ . In human PKC $\epsilon$ , these phosphorylation sites correspond to Thr<sup>566</sup>, Thr<sup>710</sup> and Ser<sup>729</sup>. PKC $\epsilon$  has often been reported to migrate as a 'doublet' under various cell growth conditions [19]. By analogy with conventional PKC isoforms such as PKC $\beta$ II, this is likely to represent different phosphorylated species of the kinase, as serum stimulation of various cells leads to phosphorylation of PKC $\epsilon$  at Thr<sup>566</sup> and Ser<sup>729</sup> [17]. In an initial attempt to investigate the regulation of PKC $\epsilon$  phosphorylation, early-passage NIH 3T3 fibroblasts were serum-starved and stimulated with saturating doses of PDGF over a period of 60 min. Endogenous PKC $\epsilon$  was immunoprecipitated with a specific antibody, and immunoblotted with antibodies against PKC $\epsilon$ , pThr<sup>566</sup> and pSer<sup>729</sup>. As shown in Figure 1(B), PKC $\epsilon$  from quiescent, serum-starved cells resolved primarily as a fast-migrating single band on SDS/PAGE, with a molecular mass of 87 kDa. PDGF stimulation of cells as early as 1 min resulted in the appearance of a second, slower-migrating species of 92 kDa, and this persisted for the time course of the experiment, up to 60 min of stimulation with growth factor. Immunoblotting with pThr<sup>566</sup> and pSer<sup>729</sup> antibodies revealed that PKC $\epsilon$  from quiescent cells had a low level of phosphorylation at these two positions, which was increased upon PDGF stimulation in a time-dependent manner. Thus there was a correlation between the appearance of the slower-migrating species of PKC $\epsilon$  (92 kDa) and the phosphorylation of Thr<sup>566</sup> and Ser<sup>729</sup>, suggesting that mitogen stimulation of cells activated signalling pathway(s) responsible for mediating the phosphorylation of these sites. In a separate experiment, NIH 3T3 fibroblasts were transfected with either a FLAG-tagged wild-type human PKC $\epsilon$  construct, or increasing amounts of a PKC $\epsilon$  mutant allele in which the critical lysine residue in the ATP-binding pocket was replaced with a tryptophan residue (PKC $\epsilon$ .K/W), rendering the kinase inactive towards substrates. Cells were then stimulated with PDGF, and PKC $\epsilon$  immunoprecipitated and immunoblotted with the pSer<sup>729</sup>



**Figure 1** PKC $\epsilon$  is phosphorylated in PDGF-stimulated NIH 3T3 fibroblasts

(A) Amino acid sequence alignment of the motifs surrounding the activation loop, turn motif and hydrophobic motifs in the catalytic domains of human PKC $\epsilon$ , PKC $\beta$ II, PKC $\delta$  and PKC $\zeta$ . The phospho-acceptor serine/threonine residues are shaded. The number of the first amino acid residue in each sequence also is shown. (B) Quiescent, early-passage NIH 3T3 fibroblasts were serum-starved for 24 h in serum-free media and stimulated with 50 ng/ml PDGF-BB for the times indicated, and PKC $\epsilon$  immunoprecipitated with anti-PKC $\epsilon$ . PKC $\epsilon$  was detected by immunoblotting with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ) and with phospho-specific antibodies against Thr<sup>566</sup> ( $\alpha$ -pT566) and Ser<sup>729</sup> ( $\alpha$ -pS729). The position of the two arrows indicates the slow- and fast-migrating species of PKC $\epsilon$  (92 and 87 kDa respectively).



**Figure 2** PDK-1 mediates phosphorylation of Thr<sup>566</sup> in PKC $\epsilon$

(A) Quiescent, early-passage NIH 3T3 fibroblasts were transfected with either vector alone (v), wild-type FLAG-tagged PKC $\epsilon$  (PKC $\epsilon$ ) or increasing concentrations (1–5  $\mu$ g) of kinase-inactive FLAG-PKC $\epsilon$  (PKC $\epsilon$ .K/W) as indicated. Cells were stimulated with 50 ng/ml PDGF-BB and PKC $\epsilon$  was immunoprecipitated with anti-PKC $\epsilon$ . PKC $\epsilon$  was detected by immunoblotting with a the phospho-specific Ser<sup>729</sup> antibody ( $\alpha$ -pS729). (B) HEK293 cells were transfected with the indicated PKC $\epsilon$  cDNAs, either alone or in combination with vector alone (pcDNA3), PDK-1 or kinase inactive PDK-1 (PDK-1.K/N). Cells were serum-starved for 12 h, and the transfected PKC $\epsilon$  immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with either anti-pThr<sup>566</sup> ( $\alpha$ -pT566) or anti-pSer<sup>729</sup> ( $\alpha$ -pS729). Equivalent expression of PKC $\epsilon$  was monitored by immunoblotting total lysates with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ). c, control, untransfected cells. The results are representative of three independent experiments carried out under the same conditions.

antibody (Figure 2A). Consistent with the result in Figure 1(B), wild-type PKC $\epsilon$  was phosphorylated at Ser<sup>729</sup>, but this was not observed with the kinase-inactive allele (Figure 2A), suggesting

that PKC $\epsilon$  activity is required for the phosphorylation of this site in mitogen-stimulated cells.

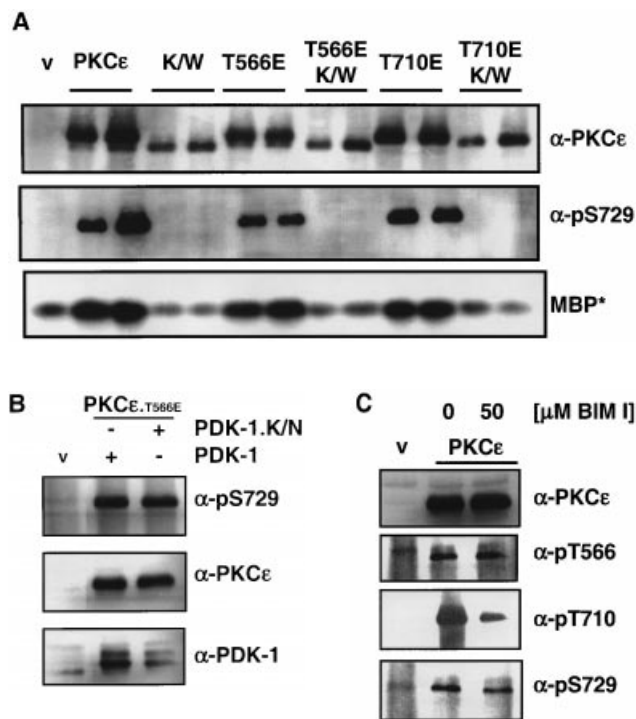
### Phosphorylation of PKC $\epsilon$ by PDK-1 *in vivo*

To investigate the role of PDK-1 in the phosphorylation of PKC $\epsilon$ , HEK293 cells were transfected with PKC $\epsilon$  either alone or in combination with wild-type or kinase-inactive alleles of PDK-1. Phosphorylation of PKC $\epsilon$  was monitored by immunoblotting anti-FLAG immunoprecipitates with pThr<sup>566</sup> and pSer<sup>729</sup> antibodies. The results in Figure 2(B) demonstrate that the basal level of PKC $\epsilon$  phosphorylation at Thr<sup>566</sup> was significantly increased by co-transfection with wild-type PDK-1. Conversely, co-transfection with a kinase-inactive mutant of PDK-1 led to a slight decrease in phosphorylation of Thr<sup>566</sup>. As expected, a PKC $\epsilon$  mutant in which Thr<sup>566</sup> was replaced by an alanine residue (PKC $\epsilon$ .T566A) was not phosphorylated at the activation loop in either the presence or absence of PDK-1, confirming that this antibody is specific for pThr<sup>566</sup>. The pattern of phosphorylation of Ser<sup>729</sup> mirrored that of Thr<sup>566</sup>; basal phosphorylation was increased with wild-type PDK-1, although curiously the PDK-1.K/N mutant did not cause a decrease in basal Ser<sup>729</sup> phosphorylation, as was observed with Thr<sup>566</sup> (Figure 2B). The small increase in PKC $\epsilon$  Ser<sup>729</sup> phosphorylation in the presence of kinase-inactive PDK-1 was not reproducible over the course of several experiments. Importantly, the PKC $\epsilon$ .T566A mutant was also not phosphorylated at Ser<sup>729</sup> in either the presence or absence of PDK-1. One interpretation of this result is that phosphorylation of the C-terminal hydrophobic Ser<sup>729</sup> residue can only occur when Thr<sup>566</sup> is phosphorylated.

For many AGC kinases, artificial membrane targeting using myristoylation or farnesylation sequences added to the N- or C-termini of the protein results in a constitutively active kinase *in vivo*. We recently used such a strategy for the atypical PKC $\zeta$  isotype and showed that it was constitutively phosphorylated at the activation-loop Thr<sup>410</sup> residue [13]. Addition of the pp60<sup>src</sup> myristoylation sequence to the N-terminus of PKC $\epsilon$  also resulted in constitutive phosphorylation at both Thr<sup>566</sup> and Ser<sup>729</sup>, and this could not be further increased by co-transfecting PDK-1, or decreased with kinase-inactive PDK-1.K/N (Figure 2B). The Myr.PKC $\epsilon$  mutant was also constitutively active as judged by immune-complex kinase assays (results not shown). In all cases, equivalent expression of the exogenous PKC $\epsilon$  was monitored by immunoblotting with the anti-PKC $\epsilon$  antibody. Thus PDK-1 is able to mediate phosphorylation of PKC $\epsilon$  at Thr<sup>566</sup>, with a concomitant increase in phosphorylation of Ser<sup>729</sup>.

### Regulation of Ser<sup>729</sup> phosphorylation

Next, we addressed the mechanism of phosphorylation of PKC $\epsilon$  at Ser<sup>729</sup>. To do this, a series of PKC $\epsilon$  kinase-inactive mutants were transfected into HEK293 cells, immunoprecipitated with anti-FLAG and either immunoblotted with anti-pSer<sup>729</sup>, or assayed for MBP kinase activity in an immune-complex kinase assay. Figure 3(A) shows that, when wild-type PKC $\epsilon$  was transfected into cells, it had a high MBP kinase activity and was also phosphorylated on Ser<sup>729</sup> in cells grown in the presence of 10% FBS. Conversely, the PKC $\epsilon$ .K/W mutant had no appreciable MBP kinase activity, comparable with vector-alone-transfected cells, and, importantly, was not phosphorylated on Ser<sup>729</sup>. PKC $\epsilon$ .K/W was consistently expressed at lower levels compared with the wild-type protein, but, over the course of many experiments, we were never able to detect phosphorylation of Ser<sup>729</sup>, even when the levels of the two proteins were normalized. One trivial explanation for the lack of phosphorylation of PKC $\epsilon$ .K/W could be that, even in growing cells, this



**Figure 3** Kinase-inactive mutants of PKC $\epsilon$  are not phosphorylated at Ser<sup>729</sup>

(A) The indicated PKC $\epsilon$  constructs were transiently transfected into HEK293 cells, in duplicate, and cells left growing in the presence of 10% FBS in complete medium. The lysates were split into two equal fractions, and the transfected PKC $\epsilon$  immunoprecipitated with anti-FLAG antibody. One immunoprecipitate was immunoblotted with anti-pSer<sup>729</sup> ( $\alpha$ -pS729), and the other used in an immune-complex MBP kinase assay to measure PKC $\epsilon$  activity (MBP\*). A fraction of the lysate (10%) was also immunoblotted with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ). The immunoblots are representative of four independent experiments. (B) HEK293 cells were transfected with either vector alone (v), or co-transfected with PKC $\epsilon$ .T566E and either wild-type PDK-1 (PDK-1) or kinase inactive PDK-1 (PDK-1.K/N) as indicated. PKC $\epsilon$  was immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-pSer<sup>729</sup> ( $\alpha$ -pS729). A fraction of the lysate (10%) was also immunoblotted with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ) and anti-PDK-1 ( $\alpha$ -PDK-1). The immunoblots are representative of three independent experiments. (C) HEK293 cells were transfected with either vector alone (v) or wild-type PKC $\epsilon$  as indicated. Cells growing in 10% serum were left untreated (0) or treated for 30 min with 50  $\mu$ M BIM I. The lysates were split into three equal fractions, and the transfected PKC $\epsilon$  immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were immunoblotted with either anti-pSer<sup>729</sup> ( $\alpha$ -pS729), anti-pThr<sup>566</sup> ( $\alpha$ -pT566) or anti-pThr<sup>710</sup> ( $\alpha$ -pT710) as indicated. A fraction of the lysate (10%) was also immunoblotted with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ).

mutant may not be phosphorylated at Thr<sup>566</sup> and, as shown above, a PKC $\epsilon$ .T566A mutant is not phosphorylated at Ser<sup>729</sup> (Figure 2B). To eliminate this possibility, a mutant PKC $\epsilon$  was made in which Thr<sup>566</sup> was mutated to an acidic glutamic acid residue, effectively mimicking the negative charge imparted by the addition of a phosphate group on serine/threonine residues. A PKC $\epsilon$ .T566E mutant was effectively phosphorylated at Ser<sup>729</sup>, and was also able to phosphorylate MBP similarly to the wild-type enzyme. However, introduction of the K/W mutation in the T566E background inactivated the kinase, and this mutant was also not phosphorylated at Ser<sup>729</sup>. Therefore the lack of a negative charge at the activation loop is not the reason why the PKC $\epsilon$ .K/W mutation lacks phosphate at Ser<sup>729</sup>. Finally, co-transfection of cells with PKC $\epsilon$ .T566E with either wild-type or kinase-inactive PDK-1 had no effect on the constitutive phosphorylation of Ser<sup>729</sup> (Figure 3B), further supporting the notion that PDK-1 is not able to directly phosphorylate the hydrophobic site.

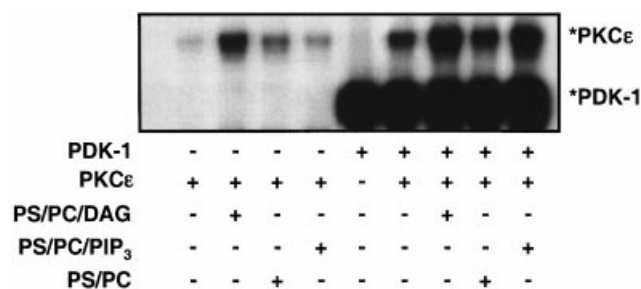
In conventional PKCs, phosphorylation of the turn motif is required to lock the enzyme into a stable and catalytically competent conformation, and, at least in the case of PKC $\beta$ /II, this is mediated by autophosphorylation [20]. Mutation of this residue to an alanine residue results in a catalytically inactive enzyme. Thus another simple explanation for the lack of Ser<sup>729</sup> phosphorylation in PKC $\epsilon$ .K/W could be lack of phosphorylation at the turn motif, which, in the case of PKC $\epsilon$ , is Thr<sup>710</sup>. However, this was not the case, because a PKC $\epsilon$  mutant with a negative charge at Thr<sup>710</sup> in the kinase-inactive background (PKC $\epsilon$ .T710E/K/W) was also not phosphorylated at Ser<sup>729</sup>, whereas the corresponding PKC $\epsilon$ .T710E was both phosphorylated and kinase-active (Figure 3A). Similarly, a double mutant of PKC $\epsilon$  with negative charge at both Thr<sup>566</sup> and Thr<sup>710</sup> in the kinase-inactive background was also not phosphorylated at Ser<sup>729</sup>. Thus, in all cases in which PKC $\epsilon$  was inactive as a protein kinase, there was no phosphorylation of Ser<sup>729</sup>, indicating that PKC $\epsilon$  may be the kinase which phosphorylates this residue *in vivo*.

The PKC inhibitor BIM I has been shown in previous studies to block phosphorylation of the PKC $\delta$  and PKC $\epsilon$  turn motif sites (Ser<sup>463</sup> and Thr<sup>710</sup>) without affecting the phosphorylation of either of the activation-loop hydrophobic residues [17]. Under similar experimental conditions, HEK293 cells transfected with a wild-type PKC $\epsilon$  allele were pre-treated with BIM I in the presence of 10% serum, and the phosphorylation state of PKC $\epsilon$  was examined (Figure 3C). As expected, neither PKC $\epsilon$  protein levels nor phosphorylation of activation-loop Thr<sup>566</sup> was affected by this treatment. Similarly, phosphorylation of the turn motif Thr<sup>710</sup> was partially blocked by BIM I. In contrast with the previous report, this inhibitor also partially blocked hydrophobic Ser<sup>729</sup> phosphorylation. This result is distinct from the previous report, because Parekh and co-workers [17] reported a complete inhibition of PKC $\delta$  and PKC $\epsilon$  hydrophobic-site phosphorylation. The difference between the two studies probably reflects the experimental conditions used, including the presence of serum in the culture conditions in the present study, as well as the phosphospecific antibodies used. As discussed below, it is also possible that BIM I may be affecting a protein phosphatase which regulates PKC $\epsilon$  dephosphorylation.

#### *In vitro* phosphorylation of PKC $\epsilon$ by PDK-1

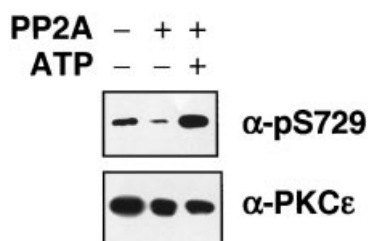
The above experiments predict that PKC $\epsilon$  should auto-phosphorylate at Ser<sup>729</sup> *in vitro*, and that this requires prior phosphorylation of Thr<sup>566</sup> by PDK-1. To determine this, His<sub>6</sub>-tagged PDK-1 and GST-tagged PKC $\epsilon$  were expressed and purified from baculovirus-infected insect cells, and used in an *in vitro* kinase assay in the presence or absence of DAG or PtdIns(3,4,5)P<sub>3</sub> presented in PtdCho/PtdSer mixed micelles. The autoradiograph in Figure 4 shows that as predicted, purified PKC $\epsilon$  in the presence of PtdSer/PtdCho/DAG auto-phosphorylates at a low rate in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, and that this is increased upon addition of purified PDK-1 to the assay. Maximal PKC $\epsilon$  phosphorylation is observed in the presence of PtdSer/PtdCho/DAG, presumably reflecting Thr<sup>566</sup> phosphorylation, which would then permit Thr<sup>710</sup> and Ser<sup>729</sup> autophosphorylation. Interestingly, the same level of PKC $\epsilon$  phosphorylation was observed when DAG was replaced with PtdIns(3,4,5)P<sub>3</sub>, and this effect was not observed when PDK-1 was omitted from the mixture.

To conclusively demonstrate that, *in vitro*, PKC $\epsilon$  can auto-phosphorylate at Ser<sup>729</sup>, highly purified recombinant PKC $\epsilon$  was first dephosphorylated with the protein phosphatase PP2A, treated with microcystin to inactivate the phosphatase, then



**Figure 4** *In vitro* phosphorylation of PKC $\epsilon$  by PDK-1 occurs in the presence of DAG or PtdIns(3,4,5) P<sub>3</sub>

Purified recombinant PKC $\epsilon$  was incubated either alone (—) or in the presence (+) of purified recombinant PDK-1 and/or mixed lipid vesicles containing PtdSer/PtdCho (PS/PC), PtdSer/PtdCho/PtdIns(3,4,5)P<sub>3</sub> (PS/PC/PIP<sub>3</sub>) or PtdSer/PtdCho/DAG (PS/PC/DAG), as indicated. The reaction was initiated by addition of [ $\gamma$ -<sup>32</sup>P]ATP and allowed to proceed for 20 min. Phosphorylation of PKC $\epsilon$  (\*PKC $\epsilon$ ) and PDK-1 (\*PDK-1) was detected by autoradiography. The experiment is representative of three independent experiments.



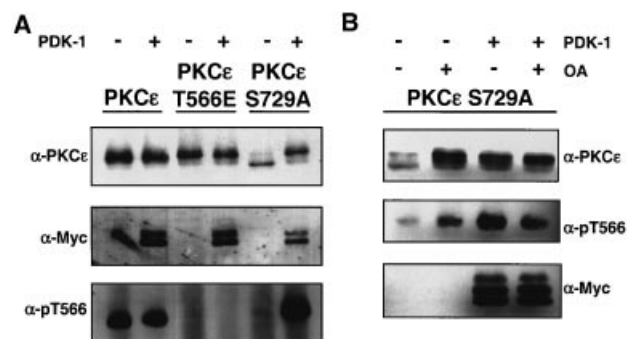
**Figure 5** Autophosphorylation of PKC $\epsilon$  at Ser<sup>729</sup>

Purified PKC $\epsilon$  was dephosphorylated at Ser<sup>729</sup> by treatment with the catalytic subunit of phosphatase PP2A as described in the Experimental section. Following addition of microcystin, the protein was incubated with Mg<sup>2+</sup>/ATP for 5 min. Phosphorylation of Ser<sup>729</sup> was detected by probing with an antibody specific for the phosphorylated hydrophobic motif ( $\alpha$ -pS729; upper panel) and total PKC $\epsilon$  was detected by probing with a phosphorylation-insensitive polyclonal antibody ( $\alpha$ -PKC $\epsilon$ ; lower panel). Lane 1, starting material; lane 2, PP2A-treated sample; lane 3, PP2A-treated sample that was subsequently re-autophosphorylated. Each lane contains 2  $\mu$ g of PKC.

incubated with non-radioactively labelled Mg<sup>2+</sup>/ATP to allow re-autophosphorylation. PKC $\epsilon$  was then detected by immunoblotting with either anti-PKC $\epsilon$  or anti-pSer<sup>729</sup>. The results in Figure 5 show that treatment with phosphatase PP2A results in a significant loss of phosphate at Ser<sup>729</sup>, essentially as previously described for PKC $\beta$ II [21]. Subsequent incubation with ATP caused a significant increase in phosphorylation of Ser<sup>729</sup>, indicating that the kinase is able to re-autophosphorylate at this site *in vitro*.

#### Phosphatase-sensitivity of activation-loop Thr<sup>566</sup>

Previous studies have shown that loss of phosphate at the PKC activation loop does not lead to any appreciable loss in protein kinase activity [8,9]. To investigate the phosphatase-sensitivity of the Thr<sup>566</sup>, and the role played by Ser<sup>729</sup> in this effect, the phosphatase inhibitor OA was used in combination with a PKC $\epsilon$  mutant which shows no appreciable phosphorylation at Thr<sup>566</sup> in growing cells. HEK293 cells were co-transfected with PKC $\epsilon$ , PKC $\epsilon$ .T566E and PKC $\epsilon$ .S729A with either PDK-1 or vector alone, and grown in the presence of 10% FBS. Under these conditions, PKC $\epsilon$  was phosphorylated at Thr<sup>566</sup> even in the



**Figure 6** Lack of negative charge at Ser<sup>729</sup> accelerates dephosphorylation of Thr<sup>566</sup>

(A) The indicated PKC $\epsilon$  constructs were transiently transfected into HEK293 cells, either with PDK-1 or vector alone (—), and cells left growing in the presence of 10% FBS in complete medium. PKC $\epsilon$  was immunoprecipitated with anti-FLAG and immunoblotted with anti-pThr<sup>566</sup> ( $\alpha$ -pT566). A fraction of the lysate (10%) was used to monitor for expression of PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ) or PDK-1 ( $\alpha$ -Myc). (B) The PKC $\epsilon$ .S729A mutant was transiently transfected into HEK293 cells as in (A), and cells treated with 1  $\mu$ M OA for 30 min (+) or not (—). PKC $\epsilon$ .S729A was immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-pThr<sup>566</sup> ( $\alpha$ -pT566). Total lysates were immunoblotted with anti-PKC $\epsilon$  ( $\alpha$ -PKC $\epsilon$ ) or anti-Myc ( $\alpha$ -Myc). The results are representative of three independent experiments carried out under identical conditions.

absence of PDK-1, as seen in Figure 3(A), due to the action of the endogenous PDK-1. As expected, there was no phosphorylation of the control T566E mutant. Unlike wild-type PKC $\epsilon$ , the S729A mutant was not phosphorylated at Thr<sup>566</sup>, presumably because the protective effect of phosphate at Ser<sup>729</sup> is lacking. In this mutant, phosphorylation of Thr<sup>566</sup> could be induced by overexpression of active PDK-1. This was also concomitant with a marked shift in the mobility of PKC $\epsilon$  to the slower-migrating 92 kDa band, probably due to phosphorylation of Thr<sup>710</sup>.

To characterize further the phosphatase-sensitivity of Thr<sup>566</sup>, cells were co-transfected with PKC $\epsilon$ .S729A and PDK-1, and treated with OA for 30 min. As with PDK-1 overexpression, OA treatment resulted in an increase in PKC $\epsilon$ .S729A phosphorylation (Figure 6B). There was no additive effect with PDK-1 overexpression in combination with OA treatment. These results show that lack of negative charge at the hydrophobic Ser<sup>729</sup> residue accelerates dephosphorylation of pThr<sup>566</sup>, which can be reversed by overexpression of PDK-1 or by inhibition of the phosphatase(s) which remove phosphate at the activation loop.

#### DISCUSSION

Numerous studies have highlighted the importance of phosphorylation in controlling the activation and function of PKC in signal-transduction pathways. Before PKC is able to bind to, and be activated by, DAG, it must first be phosphorylated at three distinct residues in the catalytic kinase domain [6]. The first and rate-limiting phosphorylation is at a highly conserved threonine residue in the activation loop, and several reports have provided clear evidence that, in both conventional (e.g. PKC $\beta$ II [12]), novel (e.g. PKC $\delta$  [14]) and atypical (e.g. PKC $\zeta$  [13,14]) PKCs, this reaction is carried out by a heterologous upstream kinase, the PDK-1 enzyme. However, there appear to be differences in the net effect of activation-loop phosphorylation of PKC, depending on the isotype. In the case of conventional PKC $\beta$ II, phosphorylation of Thr<sup>500</sup> does not lead to activation of the kinase; rather, it allows the subsequent autophosphorylation of the turn motif Thr<sup>641</sup> and hydrophobic-site Ser<sup>660</sup> [12,20]. These phosphorylation reactions precede the

activation of conventional PKCs, which occurs by binding to membranes containing PtdSer and DAG in a  $\text{Ca}^{2+}$ -dependent manner. This leads to release of the pseudosubstrate domain from the substrate-binding cavity. Unlike PKC $\beta$ II, phosphorylation of atypical PKC $\zeta$  by PDK-1 leads to a direct activation of the kinase in a PtdIns(3,4,5) $P_3$  (and thus PI 3-kinase)-dependent manner [14,22], in a mechanism reminiscent of the activation of the Akt/PKB kinase [23]. Phosphorylation of PKC $\zeta$  at the activation-loop Thr<sup>410</sup> also leads to autophosphorylation of the turn-motif Thr<sup>560</sup> [24]. PDK-1 has also been shown to phosphorylate novel PKCs, most notably PKC $\delta$ , and this also leads to activation of the kinase [14]. The notion that PDK-1 is the upstream kinase for all PKC isotypes has been reinforced by the findings that, in cells lacking PDK-1, there is no phosphorylation of PKC $\zeta$  at Thr<sup>410</sup> [25], and that PDK-1 directly interacts with PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II, PKC $\delta$ , PKC $\epsilon$ , PKC $\zeta$  and PKC $\iota$  through the kinase domain of each enzyme [12,14].

In the present study we further explored the regulation of PKC by phosphorylation and focused on the novel PKC $\epsilon$  isotype. Our results show that mitogen stimulation of cells leads to a decrease in the electrophoretic mobility of PKC $\epsilon$  which resolves into a distinct doublet when cells are stimulated with PDGF. Under these conditions, PKC $\epsilon$  is also phosphorylated at Thr<sup>566</sup> and Ser<sup>729</sup>. These results are consistent with previous observations showing phosphorylation of PKC $\epsilon$  at Thr<sup>566</sup> and Ser<sup>729</sup> in response to serum-stimulation of HEK293 cells, which was concomitant with an increase in PKC $\epsilon$  activity [17]. A separate study also mapped Thr<sup>566</sup> and Ser<sup>729</sup> as phosphorylation sites in PKC $\epsilon$  isolated from confluent NIH 3T3 fibroblasts, and suggested that phosphorylation of Ser<sup>729</sup> was primarily responsible for appearance of the slower-migrating 92 kDa species [19], results consistent with those obtained in studies on PKC $\beta$ II [6]. Our studies also confirm that overexpression of active PDK-1 in cells leads to phosphorylation of Thr<sup>566</sup>, whereas a kinase-inactive PDK-1 mutant decreased basal Thr<sup>566</sup> phosphorylation, suggesting either that PDK-1.K/N acts in dominant-negative fashion towards endogenous PDK-1, or that it somehow accelerates dephosphorylation of the activation loop, which is known to be highly susceptible to phosphatases. We favour the latter explanation, as we have failed to see a dominant-negative effect of PDK-1.K/N on endogenous PDK-1 activity and because the basal level of Ser<sup>729</sup> phosphorylation is not decreased by this PDK-1 mutant. However, the two explanations are not mutually exclusive. We also show that a PKC $\epsilon$  mutant which cannot be phosphorylated at the hydrophobic site (PKC $\epsilon$ .S729A) is not phosphorylated at the activation loop in growing cells, unlike wild-type PKC $\epsilon$ . Increased phosphorylation at Thr<sup>566</sup> in PKC $\epsilon$ .S729A can be induced by overexpression of PDK-1 or by treatment with the phosphatase inhibitor OA. These results are consistent with the notion that negative charge at the hydrophobic motif has a protective effect on activation-loop dephosphorylation. Indeed, it has been proposed that a reciprocal effect of activation-loop phosphorylation protects hydrophobic-site dephosphorylation [8,9]. At any rate, the results clearly show that, in cells expressing PDK-1, phosphorylation of Thr<sup>566</sup> as well as Ser<sup>729</sup> is increased.

Unlike regulation of PKC $\epsilon$  activation-loop phosphorylation, that of the hydrophobic site is less well understood. Our mutagenesis studies reveal that, unlike wild-type PKC $\epsilon$ , a catalytically inactive variant of PKC $\epsilon$  is not phosphorylated at Ser<sup>729</sup> in growing HEK293 cells or PDGF-stimulated fibroblasts. Replacing a negative charge at the activation loop (T566E.K/W) or turn motif (T710E.K/W) in the kinase-inactive background did not rescue phosphorylation at the hydrophobic site, and therefore the implication is that regulation of PKC $\epsilon$  at Ser<sup>729</sup> is

mediated by autophosphorylation by PKC $\epsilon$  itself. To our knowledge, this is the first use of kinase-inactive mutants of PKC $\epsilon$  to probe for Ser<sup>729</sup> phosphorylation. Negative charge at the PKC $\epsilon$  activation loop (T566E) induced constitutive phosphorylation at Ser<sup>729</sup> (Figures 3A and 3B) and this was not enhanced by co-expression of active PDK-1. Co-expression of PDK-1 with kinase inactive PKC $\epsilon$  was also not able to induce Ser<sup>729</sup> phosphorylation (V. Cenni and A. Toker, unpublished work). The notion that PKC $\epsilon$  is responsible for Ser<sup>729</sup> autophosphorylation is consistent with the finding that purified PKC $\epsilon$  could be phosphorylated *in vitro* by PDK-1, with an increase in phosphorylation of Thr<sup>566</sup> as well as Ser<sup>729</sup>. During the course of these *in vitro* experiments, we also noticed that maximal PKC $\epsilon$  phosphorylation could be achieved not only in the presence of PDK-1 with PtdSer/PtdCho/DAG mixed micelles, but also in the presence of PtdIns(3,4,5) $P_3$ . This is reminiscent of the *in vitro* phosphorylation of Akt/PKB by PDK-1, which is also dependent on PtdIns(3,4,5) $P_3$  [11,23]. However, here the PtdIns(3,4,5) $P_3$  requirement lies with the pleckstrin homology (PH) domain of Akt/PKB, which undergoes a conformational change upon PtdIns(3,4,5) $P_3$  binding to expose the activation-loop Thr<sup>308</sup>. PKC $\epsilon$ , on the other hand, does not have a PH domain, suggesting that the PtdIns(3,4,5) $P_3$  effect shown here lies with the PDK-1. Although PDK-1 is reportedly constitutively active, requiring only PtdIns(3,4,5) $P_3$  binding for proper intracellular localization, as opposed to increasing catalytic activity, there are reports which contrast with this view. Most notably, PI 3-kinase is required for PDK-1 to efficiently phosphorylate PKC $\zeta$  and PKC $\delta$ , an effect which is lost when the PH domain of PDK-1 is deleted [14], although one report has provided evidence for a PtdIns(3,4,5) $P_3$ -dependency on PKC $\zeta$ , specifically through the pseudosubstrate domain [24].

The present study shows that, *in vitro* and *in vivo*, PKC $\epsilon$  is responsible for autophosphorylation of Ser<sup>729</sup>. On the one hand, this is analogous to the model presented for conventional PKC $\beta$ II [10]; kinase-inactive mutants are not phosphorylated at the hydrophobic site, and *in vitro*-purified enzyme can autophosphorylate at the hydrophobic and turn motifs [21]. On the other hand, this autophosphorylation model is in stark contrast with those of other studies, which have proposed that regulation of PKC $\delta$  and PKC $\epsilon$  hydrophobic-site phosphorylation is mediated by a putative heterologous kinase [16,17]. Specifically, in a study by Parekh and co-workers, the PKC inhibitor BIM I blocked phosphorylation of the PKC $\delta/\epsilon$  turn motifs (Ser<sup>643</sup> and Thr<sup>710</sup> respectively), with no effect on activation-loop or hydrophobic-site phosphorylation [17]. In the present study we found that BIM I pre-treatment of cells led to a small, but reproducible, decrease in the phosphorylation of both Thr<sup>710</sup> and Ser<sup>729</sup>, with no effect on activation-loop phosphorylation (Thr<sup>566</sup>) or PKC $\epsilon$  protein levels (Figure 3C). The discrepancy between the two studies is likely due to different cell-culture conditions and experimental approaches, and therefore the present study does not entirely exclude the possibility that heterologous Ser<sup>729</sup> kinase functions under some conditions. It is also important to note that, although pharmacological approaches such as these are useful in evaluating the regulation of phosphorylation events, they can also be potentially misleading. Reportedly 'specific' inhibitors may not only inhibit one or more PKCs, but, importantly, may be affecting a protein phosphatase for the activation loop, turn motif and/or hydrophobic sites, effectively uncoupling these events. In this regard, it is also noteworthy that a distinct PKC inhibitor, Ro-31-8220, blocks phosphorylation of Ser<sup>729</sup> [18], consistent with an autophosphorylation model, although again a role for a PKC-dependent phosphatase cannot be excluded.

Using a similar pharmacological approach it was also reported that novel PKC $\delta$  as well as PKC $\epsilon$  hydrophobic-site phosphorylation is mediated via an mTOR-dependent pathway, comprising a complex which includes atypical PKC $\zeta$ . The mTOR antagonist rapamycin blocks phosphorylation of Ser<sup>729</sup> (and Thr<sup>662</sup> in PKC $\delta$ ), but not activation-loop phosphorylation of both PKCs [17], and this appears to be through the activation of an mTOR-sensitive phosphatase which controls dephosphorylation of the hydrophobic site [18]. Ziegler and co-workers isolated a protein complex which was capable of phosphorylating PKC $\delta$  at the hydrophobic Ser<sup>662</sup> residue in a rapamycin- and PI 3-kinase-sensitive manner [16]. The atypical PKC $\zeta$  isotype was found to be a component of this complex, suggesting that this PKC might be the Ser<sup>662</sup> kinase, although it should be stressed that wild-type immunopurified PKC $\zeta$  could not mimic the effect of this complex on PKC $\delta$  phosphorylation. In addition, the regulation of PKC $\epsilon$  hydrophobic-site phosphorylation by this PKC $\zeta$ -containing complex has not been reported.

Thus, although it is widely agreed that PDK-1 is the universal PKC upstream kinase for the activation loop, there appear to be two contrasting theories for the regulation of hydrophobic-site phosphorylation. For both PKC $\beta$ II [21] and PKC $\epsilon$  (the present study), kinase-inactive mutants of both PKCs are not phosphorylated at their respective C-termini, and *in vitro* the enzymes can autophosphorylate at Ser<sup>660</sup> and Ser<sup>729</sup> respectively. However, at least in the case of PKC $\delta$ , a protein complex which is able to heterologously phosphorylate this enzyme at the hydrophobic site has been identified, although the precise nature of the 'activating' kinase is not known. One possibility is that PKC $\delta$  is unique in this regard, whereas other PKCs, such as PKC $\beta$ II and PKC $\epsilon$ , are primarily regulated by autophosphorylation *in vivo*. It is also noteworthy that, despite extensive efforts, no defined protein kinase activity capable of phosphorylating the hydrophobic site in PKCs has been described or cloned. This is reminiscent of the controversy surrounding the regulation of the equivalent hydrophobic site of Akt/PKB, Ser<sup>473</sup>, which has been proposed by some to be regulated by a putative heterologous upstream kinase termed 'PDK-2' [26]. Although originally thought to be a modified form of PDK-1, it is now clear that PDK-1 cannot directly mediate phosphorylation of Akt/PKB at Ser<sup>473</sup> [27]. Our own studies have shown that regulation of this site in Akt/PKB can occur through autophosphorylation [28]. Regardless of the mechanism of action of chemical inhibitors, the cumulative data thus far suggest that a heterologous upstream kinase regulates PKC $\epsilon$  hydrophobic-site phosphorylation under some conditions, whereas the present studies point to autophosphorylation as an alternative regulatory mechanism.

Understanding the precise mechanisms which govern PKC $\epsilon$  phosphorylation and activation is important because of the critical role played by this kinase in cell physiology. PKC $\epsilon$  is the only PKC which confers an oncogenic phenotype when over-expressed [29], and appears to do so by activating the mitogen-activated-protein-kinase pathway [30,31]. PKC $\epsilon$  has also been directly implicated in regulating the actin cytoskeleton and has been shown to directly bind to actin through a specific motif [32]. Consistent with this, PKC $\epsilon$  is also important for the attachment of many cells to the substratum [33]. In particular,  $\beta$ 1-integrin-dependent attachment of fibroblasts to collagen was shown to require both PI 3-kinase and PKC $\epsilon$ , and the activated PKC $\epsilon$  allele described here (Myr.PKC $\epsilon$ ) was able to increase cell spreading independently of upstream signals such as PI 3-kinase [34].

In summary, we have provided evidence that regulation of PKC $\epsilon$  occurs via PDK-1-mediated phosphorylation of the

activation-loop Thr<sup>566</sup>, and that autophosphorylation is responsible for regulation of Ser<sup>729</sup>. Whether this occurs in an intramolecular (*cis*) manner, or whether one PKC $\epsilon$  molecule can phosphorylate an adjacent one at Ser<sup>729</sup> (*in trans*) is not known, but, by analogy with PKC $\beta$ II, the former is likely to be the case for PKC $\epsilon$  as well. Whether a heterologous upstream kinase for PKC $\epsilon$ , or indeed any other PKC, exists, still remains to be determined, but any model describing the regulation of PKC $\epsilon$  by phosphorylation should take into account autophosphorylation of the hydrophobic site as one such mechanism.

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