Regulation of protein kinase C ζ by PI 3-kinase and PDK-1

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Background: Protein kinase C ζ (PKCζ) is a member of the PKC family of enzymes and is involved in a wide range of physiological processes including mitogenesis, protein synthesis, cell survival and transcriptional regulation. PKCζ has received considerable attention recently as a target of phosphoinositide 3-kinase (PI 3-kinase), although the mechanism of PKCζ activation is, as yet, unknown. Recent reports have also shown that the phosphoinositide-dependent protein kinase-1 (PDK-1), which binds with high affinity to the PI 3-kinase lipid product phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3), phosphorylates and potently activates two other PI 3-kinase targets, the protein kinases Akt/PKB and p70S6K. We therefore investigated whether PDK-1 is the kinase that activates PKCζ.

Results: *In vivo*, PI 3-kinase is both necessary and sufficient to activate PKCζ. PDK-1 phosphorylates and activates PKCζ *in vivo,* and we have shown that this is due to phosphorylation of threonine 410 in the PKCζ activation loop. *In vitro*, PDK-1 phosphorylates and activates PKCζ in a PtdIns-3,4,5-P_3-enhanced manner. PKCζ and PDK-1 are associated *in vivo*, and membrane targeting of PKCζ renders it constitutively active in cells.

Conclusions: Our results have identified PDK-1 as the kinase that phosphorylates and activates PKCζ in the PI 3-kinase signaling pathway. This phosphorylation and activation of PKCζ by PDK-1 is enhanced in the presence of PtdIns-3,4-5-P_3. Consistent with the notion that PKCs are enzymes that are regulated at the plasma membrane, a membrane-targeted PKCζ is constitutively active in the absence of agonist stimulation. The association between PKCζ and PDK-1 reveals extensive cross-talk between enzymes in the PI 3-kinase signaling pathway.

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Background

The protein kinase C (PKC) family comprises serine/threonine protein kinases found in mammalian cells; the 11 distinct isoforms have been implicated in a multitude of physiological processes [1]. PKC was originally described as an enzyme that could be activated *in vitro* and *in vivo* by the concerted actions of calcium and diacylglycerol (DAG) [2,3]. PKCs have been subdivided into three subfamilies according to their lipid-activation profiles: conventional PKCs (α, βI, βII and γ) are activated by both DAG and calcium; novel PKCs (δ, ε, η/*L* and θ) do not respond to calcium but require DAG; and atypical PKCs (ζ and ι/λ) are not activated by either DAG or calcium. The more recently discovered PKCμ, also known as PKD, may represent yet another subfamily. All PKCs require the lipid phosphatidylserine (PS) as a cofactor.

More recently, phosphorylation of PKC has been revealed as an important mechanism for regulating its activity [4–7]. Studies on PKCβII show that it is regulated by phosphorylation at three distinct residues [8–10], at least two of which are conserved in all other PKC isoforms. One critical residue for activation is the threonine in the activation loop (Thr500 in PKCβII, equivalent to Thr410 in PKCζ) [10–12]. This residue, and the motif surrounding it, is found in a large number of protein kinases of the AGC superfamily (protein kinases A, G and C) [13], and in some cases has been shown to be phosphorylated by an upstream kinase. Following phosphorylation of the activation-loop threonine, PKCs rapidly autophosphorylate on a threonine and a serine residue in the catalytic domain [10,12]. This leads to full activation of the enzyme and phosphorylation of substrates. An inhibitory sequence known as the pseudosubstrate domain is present in the amino terminus of all PKCs, and is thought to confer autoinhibition by binding to the substrate-binding site and masking one or more phosphorylation site(s) [14]. Upon lipid binding, a conformational change relieves this autoinhibition, allowing phosphorylation to occur. So far, no direct PKC-activating kinases have been described.

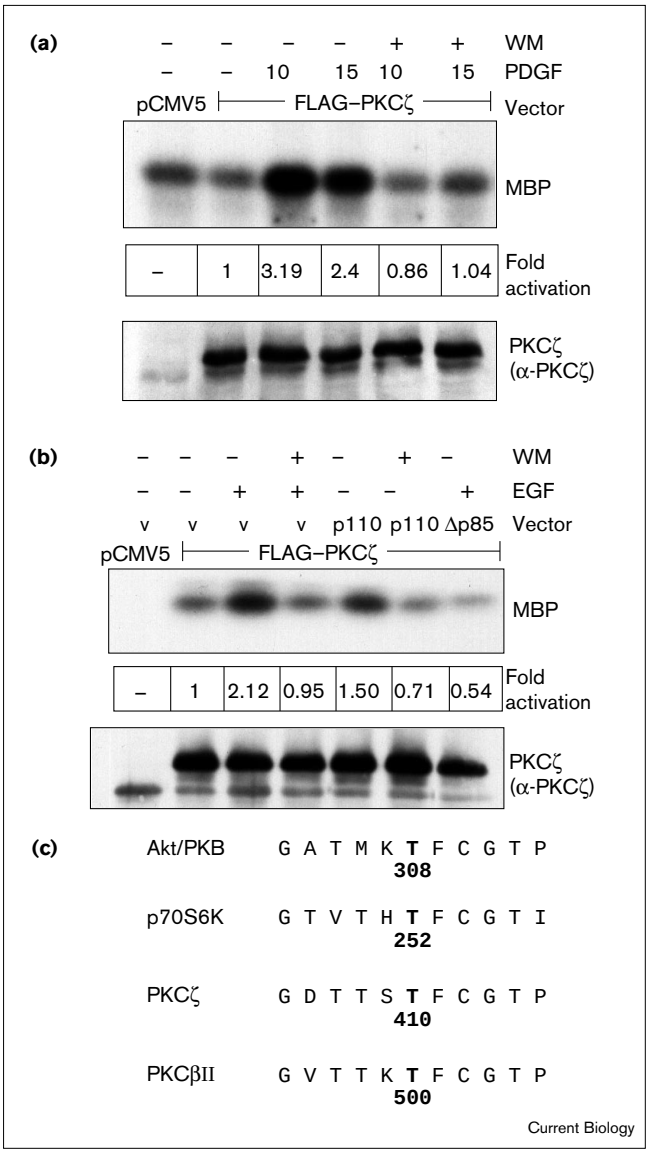


Figure 1

Activation of PKCζ downstream of PI 3-kinase. **(a)** NIH 3T3 fibroblasts were transiently transfected with vector alone (pCMV5) or the FLAG–PKCζ construct, and cells made quiescent in serum-free medium for 24 h. Cells were pretreated with 100 nM wortmannin (WM; +), or vehicle (DMSO) alone (–) for 20 min, then stimulated with PDGF BB (50 ng/ml) for the times (in min) indicated. PKCζ was immunoprecipitated from cell extracts and the washed immunoprecipitates assayed for activity. Protein kinase activity was monitored by phosphorylation of myelin basic protein (MBP), resolved by SDS–PAGE and quantitated on a Phosphorimager. Total cell lysates (10% of cell extracts) were resolved on 7.5% SDS–PAGE, transferred to nitrocellulose, and the levels of PKCζ expressed were detected by immunoblotting with a PKCζ-specific antibody (α-PKCζ). **(b)** 293 cells were co-transfected with vector alone (pCMV5), FLAG–PKCζ, Myc–p110.CAAX (p110) or GST–Δp85 (Δp85) in the combinations indicated, and cells serum starved and treated with wortmannin (WM) as in (a). Cells were stimulated with EGF (30 ng/ml) for 10 min (+), and cell extracts made. PKCζ was immunoprecipitated with the FLAG antibody, and an immune-complex kinase assay using MBP as exogenous substrate was carried out. Expression of PKCζ was detected with the PKCζ antibody (α-PKCζ), and Myc–p110.CAAX and GST–Δp85 were detected with Myc-specific and GST-specific antibodies (data not shown). The results in (a) and (b) are representative of three independent experiments. **(c)** Sequence alignment of the activation-loop threonine residues of mouse Akt/PKB (Thr308), human p70S6K (Thr252), rat PKCζ (Thr410) and rat PKCβII (Thr500).

PKCζ, an atypical family member, has received considerable attention recently as it has been implicated as a downstream target of phosphoinsitide 3-kinase (PI 3-kinase). The PI 3-kinase lipid product phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) activates PKCζ *in vitro* [15–17]. Stimulation of cells by insulin and insulin-like growth factor-1 (IGF-1) leads to activation of PKCζ which can be inhibited by chemical inhibitors of PI 3-kinase [17–19]. Similarly, activation of PI 3-kinase by lipopolysaccharide leads to activation of PKCζ which is sensitive to PI 3-kinase inhibitors and a dominant-negative PI 3-kinase mutation [16]. Activation of PKCζ as a result of expression of simian virus 40 (SV40) small T-antigen can also be blocked by inhibition of PI 3-kinase [20]. The closely related PKCλ isoform is activated in cells stimulated with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) by a PI 3-kinase dependent mechanism [21]. These findings strongly implicate PKCζ as an effector of PI 3-kinase in cells. However, the mechanism of regulation of PKCζ by PI 3-kinase remains unknown. Here, we have examined the mechanism of PKCζ regulation by phosphorylation and lipids *in vitro* and *in vivo*.

Results

Activation of PKCs following agonist stimulation of cells has typically been monitored by membrane translocation or by *in vivo* ^{32}P-labeling of the enzyme(s). Although conventional PKCs such as PKCα quantitatively translocate to the membrane following stimulation of cells by phorbol ester or growth factors, this is not generally true for other PKCs such as PKCζ. Similarly, these assays do not directly measure the protein kinase activity of PKCs. Therefore, to understand the regulation of PKCζ better, we have monitored its activation using immune-complex kinase assays. Similar assays are routinely used to measure the activation of the Akt/PKB protein kinase, a PI 3-kinase target that is also regulated by both lipid binding and phosphorylation.

PI 3-kinase is both necessary and sufficient to activate PKCζ *in vivo*

We have used a FLAG-epitope-tagged PKCζ (FLAG–PKCζ) construct to monitor PKCζ activation following mitogen stimulation. NIH 3T3 cells were transiently transfected with FLAG–PKCζ and its activity was measured in an anti-FLAG immune-complex protein kinase assay. PDGF stimulated a two- to threefold increase in the activity of PKCζ following serum starvation (Figure 1a). This activation was sensitive to the PI 3-kinase inhibitor wortmannin at concentrations that

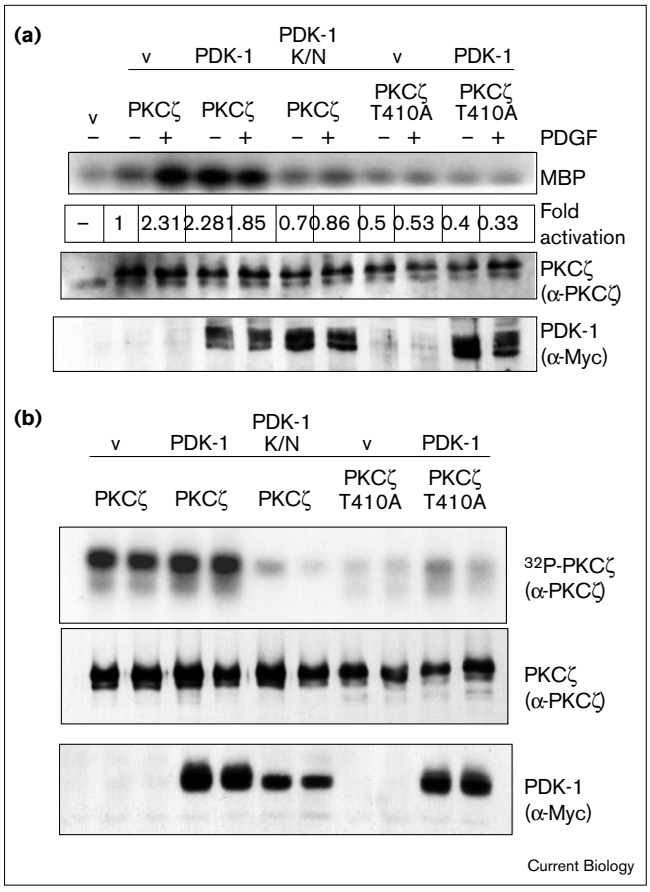
inhibit the accumulation of the PI 3-kinase lipid products PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. Similarly, PKCζ was activated following EGF stimulation of 293 cells in a wortmannin-sensitive manner (Figure 1b). In the same cells, a constitutively active, membrane-targeted mutant of PI 3-kinase (p110CAAX [22]) stimulated PKCζ activation in the absence of EGF, also in a wortmannin-sensitive manner. This activated PI 3-kinase mutant has also been shown to activate Akt/PKB and the kinase p70S6K in co-transfections [22]. Finally, a dominant-negative PI 3-kinase mutant (Δp85) inhibits EGF-stimulated activation of PKCζ in these cells. These data provide further evidence that in two cell types PKCζ is activated downstream of PI 3-kinase. Thus, PI 3-kinase, presumably acting through its lipid products PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, is both necessary and sufficient to activate PKCζ.

Recent work on the regulation of Akt/PKB has shown that a critical event in the activation of this enzyme is the phosphorylation of the activation-loop threonine (Thr308) by an upstream kinase (reviewed in [23]). The enzyme phosphoinositide-dependent protein kinase-1 (PDK-1) was purified by its ability to phosphorylate Thr308 in Akt/PKB [24–27]. The equivalent residue in p70S6K is also phosphorylated by PDK-1, leading to its activation [28,29]. There is considerable sequence similarity surrounding the activation-loop threonine of Akt/PKB, p70S6K and PKCζ (Figure 1c). For comparison, the activation-loop Thr500 residue of PKCβII is shown. Therefore, we next investigated the possibility that PDK-1 might be the PKCζ upstream kinase.

PDK-1 stimulates activation and phosphorylation of PKCζ *in vivo*

NIH 3T3 fibroblasts were co-transfected with FLAG–PKCζ and a Myc-epitope-tagged PDK-1 (Myc–PDK-1) construct (Figure 2a). The PDK-1 construct used here has previously been shown to activate Akt/PKB [27]. As shown in Figure 1a, PDGF stimulated the activation of PKCζ as measured in an immune-complex kinase assay (Figure 2a). Co-transfection with PDK-1 caused a constitutive activation of PKCζ in the absence of mitogen, and this was not enhanced upon PDGF stimulation. A kinase-inactive variant of PDK-1, in which the critical Lys110 residue is mutated to an Asn (PDK-1.K/N), blocked activation of PKCζ by PDGF. Next, we mutated the activation-loop Thr410 of PKCζ to an Ala (PKCζ.T410A; Figure 2a). This mutant possessed lower basal activity than the wild-type enzyme under serum-starved conditions and could not be activated by PDGF stimulation. Similarly, PDK-1 did not activate this mutant in either the presence or absence of PDGF. These data indicate that PDK-1 is capable of activating PKCζ in co-transfection experiments, and that this requires Thr410.

Figure 2



PDK-1 activates and phosphorylates PKCζ *in vivo*. **(a)** NIH 3T3 fibroblasts were transfected with either vector alone (v; pCMV5), or co-transfected with the indicated combinations of FLAG–PKCζ, FLAG–PKCζ.T410A, Myc–PDK-1, and Myc–PDK-1.K/N. Cells were serum starved for 24 h, then stimulated for 10 min with PDGF. PKCζ activity was assayed on a FLAG immunoprecipitate using MBP as a substrate. Equivalent levels of PKCζ and PDK-1 were expressed as detected by PKCζ (α-PKCζ) and Myc (α-Myc) immunoblotting, respectively. **(b)** 293 cells were co-transfected with PKCζ and PDK-1 constructs as in (a) and maintained in 10% fetal bovine serum (FBS) for 24 h. Cells were then labeled *in vivo* with ^{32}P. Cell extracts were made and PKCζ was immunoprecipitated with the FLAG antibody and resolved by SDS–PAGE, followed by autoradiography. Expression of PKCζ and PDK-1 was detected by immunoblotting 10% of total lysate with α-PKCζ and α-Myc antibodies.

To investigate the phosphorylation of PKCζ induced by PDK-1, 293 cells were co-transfected with PKCζ or PKCζ.T410A in the presence or absence of PDK-1, and cells labeled *in vivo* with ^{32}P (Figure 2b). In the absence of PDK-1, PKCζ is heavily phosphorylated, probably reflecting Thr410 phosphorylation and autophosphorylation. The overall phosphorylation of PKCζ is not increased upon co-transfection with wild-type PDK-1, but is dramatically reduced in the presence of PDK-1.K/N (Figure 2b). Little or no phosphate incorporation was observed in the PKCζ.T410A mutant, and phosphate incorporation was

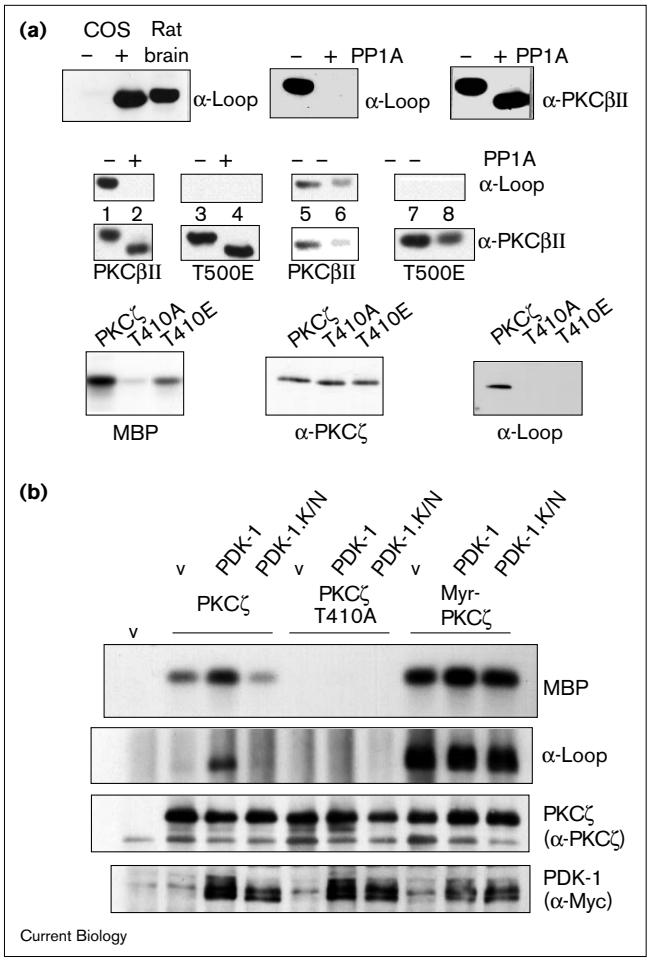
not enhanced upon co-transfection of PDK-1. This implies that the PDK-1.K/N mutant acts in a dominant-negative fashion to inhibit activation and phosphorylation of PKCζ. A kinase-inactive PDK-1 mutant has also been shown to inhibit insulin-stimulated p70S6K activation [29]. Eliminating phosphorylation of PKCζ Thr410 may therefore inhibit subsequent autophosphorylation of PKCζ. A similar model has been proposed for PKCβII, in which phosphorylation of Thr500 is a prerequisite for subsequent autophosphorylations [10].

PDK-1 stimulates phosphorylation of the PKCζ activation loop Thr410 *in vivo*

To demonstrate that PDK-1 activates PKCζ by phosphorylation of Thr410, and not another residue, we made use of a phospho-specific antibody raised against the phosphorylated activation-loop sequence of PKCβII. Figure 3 shows that this antibody (anti-loop) recognizes PKCβII from transfected COS cells or rat brain homogenates, but not PKCβII dephosphorylated by protein phosphatase 1A (PP1A) *in vitro* (Figure 3a, top panel). This antibody does not recognize other phosphorylated residues on PKCβII as it does not recognize a PKCβII.T500E mutant which is quantitatively phosphorylated at all other phosphorylation sites (Figure 3a, middle panel). This antibody also specifically recognizes PKCζ when phosphorylated at Thr410, but not the PKCζ.T410A mutant (Figure 3a, bottom panel). In addition, a PKCζ.T410E mutant which is active as judged by its phosphorylation of myelin basic protein is also not recognized by this antibody (Figure 3b, bottom panel). Thus, the anti-loop antibody is specific for the phosphorylated activation-loop Thr410 of PKCζ. We therefore used it to show definitively that PDK-1 regulates phosphorylation of this site in PKCζ *in vitro* and *in vivo*. 293 cells were co-transfected with PKCζ or PKCζ.T410A with either control vector, PDK-1 or PDK-1.K/N (Figure 3b). In serum-starved cells, the basal activity of PKCζ correlates with a small amount of phosphorylation of PKCζ on Thr410, as judged by immunoblotting with anti-loop antibody. Both the activity and phosphorylation of Thr410 are increased when PDK-1 is co-transfected. No increase in activity or phosphorylation was detected when PDK-1.K/N was used. PKCζ.T410A had little or no detectable protein kinase activity and was not recognized by the anti-loop antibody, either in the presence or absence of PDK-1 (Figure 3b).

As events leading to the activation of PKCζ are likely to occur at the plasma membrane, where both PI 3-kinase lipid products and PDK-1 are present, we reasoned that membrane targeting of PKCζ might lead to constitutive activation of the enzyme, as has been reported for Akt/PKB [30] and PDK-1 [31]. We fused the amino-terminal myristoylation sequence of p60 c-Src to the amino terminus of PKCζ, and transfected this mutant into cells (Figure 3b). In serum-starved cells, Myr–PKCζ has a fivefold higher

Figure 3



PDK-1 phosphorylates PKCζ Thr410 *in vivo*. **(a)** Top row, anti-loop (α-loop) immunoblot of total cell lysates from COS cells transfected with control vector alone (–) or cDNA encoding PKCβII (+), or the Triton X-100 soluble fraction of a rat brain homogenate. Cell lysates were treated with (+) or without (–) protein phosphatase 1A (PP1A, catalytic subunit) and immunoblotted with anti-loop (α-loop) or anti-PKCβII (α-PKCβII), as previously described [10]. Middle row, purified PKCβII or PKCβII.T500E (T500E) treated with or without PP1A and immunoblotted with anti-loop or anti-PKCβII, as above. The anti-PKCβII antibody does not discriminate between phosphorylated forms. Lanes 1,2, 20 ng PKCβII; lanes 3,4, 100 ng T500E; lane 5, 20 ng PKCβII control; lane 6, 20 ng PKCβII autophosphorylated; lane 7, 100 ng T500E control; lane 8, 100 ng T500E autophosphorylated. Bottom row, PKCζ, PKCζ.T410A and PKCζ.T410E expressed in 293 cells and immunoprecipitated with anti-FLAG antibody. PKCζ activity was determined by an MBP kinase assay. PKCζ was detected with the PKCζ-specific antibody, and Thr410 phosphorylation was detected with the anti-loop (α-loop) antibody. **(b)** 293 cells were co-transfected with FLAG–PKCζ, FLAG–PKCζ.T410A or Myr–PKCζ–FLAG and either vector alone (v; pCMV5), Myc–PDK-1 or Myc–PDK-1.K/N as indicated. Cell extracts were split into two, and one half was used to assay for PKCζ activity in an immune-complex kinase assay, as described in Figure 1a. The other half was immunoprecipitated with FLAG antibody and immunoblotted with the anti-loop antibody (α-loop). Levels of PKCζ and PDK-1 expression were detected by immunoblotting with α-PKCζ or α-Myc.

protein kinase activity compared with wild-type PKCζ, correlating with an increase in Thr410 phosphorylation. This could not be further increased by co-transfection of PDK-1 and, interestingly, could not be inhibited using the PDK-1.K/N mutant. One possible explanation for this is that in serum-starved cells, PDK-1 is presumably mostly cytosolic, whereas Myr–PKCζ is localized at the membrane, and is thus inaccessible to the kinase-inactive PDK-1.K/N. These data are consistent with the notion that PKCs are regulated at the plasma membrane. Therefore, PDK-1 functions as an activating kinase for PKCζ *in vivo* by inducing phosphorylation of the activation-loop Thr410.

Activation and phosphorylation of PKCζ by PDK-1 *in vitro*

Next, we tested the ability of PDK-1 to directly phosphorylate PKCζ *in vitro*. For these studies, we have used recombinant fusion proteins of PDK-1, Akt/PKB and PKCζ. For PDK-1, we generated wild-type and kinase-inactive (K110I) GST–PDK-1 fusion constructs. These were expressed in bacteria and purified on glutathione–Sepharose beads. Akt/PKB was expressed as a His-tagged fusion protein in baculovirus-infected insect cells. The recombinant protein was purified on a nickel-NTA column followed by Mono Q chromatography. The resulting Akt/PKB fusion protein had an apparent molecular mass of 70 kDa and was at least 90% pure (not shown). Finally, PKCζ was expressed as a recombinant wild-type protein in baculovirus-infected insect cells, and purified to over 95% purity by conventional chromatography.

To test whether our GST–PDK-1 is functional towards substrates, we incubated purified GST–PDK-1 with purified Akt/PKB. In the absence of Akt/PKB, GST–PDK-1 autophosphorylates independently of PtdIns-3,4,5-P_3 (Figure 4a). In the absence of PDK-1, Akt/PKB autophosphorylates at a low rate. When GST–PDK-1 is added, however, Akt/PKB is heavily phosphorylated only in the presence of PtdIns-3,4,5-P_3. The inactive GST–PDK-1.K/I does not phosphorylate Akt/PKB. These data are in complete agreement with previously reported findings concerning the phosphorylation of Akt/PKB by PDK-1 [24,25]. Therefore, we proceeded to examine the phosphorylation of PKCζ by PDK-1 under similar conditions.

Our purified recombinant PKCζ preparation autophosphorylates at a low rate. Incubation of PKCζ with phosphatidylethanolamine (PE)/PS mixed vesicles has little effect on the phosphorylation of PKCζ, either in the presence or absence of PtdIns-3,4,5-P_3 (Figure 4b). When PDK-1 is added, there is a fivefold increase in the phosphorylation of PKCζ with PE/PS, and this can be further augmented to 12-fold in the presence of PtdIns-3,4,5-P_3. This corresponds to an increase in Thr410 phosphorylation, as judged by immunoblotting with the anti-loop antibody (Figure 4b). GST–PDK-1.K/I does not phosphorylate PKCζ. Removal of the GST moiety from GST–PDK-1 by thrombin cleavage had no effect on the ability of PDK-1 to phosphorylate PKCζ, or the ability of PtdIns-3,4,5-P_3 to enhance this phosphorylation (not shown). These data are reminiscent of the phosphorylation of Akt/PKB by PDK-1 *in vitro*, which also requires PtdIns-3,4,5-P_3. In contrast, p70S6K does not require PtdIns-3,4,5-P_3 for PDK-1-mediated phosphorylation [28]. We found that PDK-1 phosphorylates PKCζ at a stoichiometry of 0.46 mol phosphate per mol PKCζ in the presence of PE/PS/PtdIns-3,4,5-P_3. This value agrees well with the stoichiometry of phosphorylation of Akt/PKB (0.6 mol/mol [24]) and p70S6K (0.3 mol/mol [28]) by PDK-1. In addition, we calculated a specific activity of PDK-1 towards PKCζ (66 units/mg) and Akt/PKB (126 units/mg). These values are in the same range as those previously reported for the specific activity of a bacterially expressed GST–PDK-1 fusion protein [26].

We also tested whether phosphorylation of PKCζ leads to activation *in vitro*. For this, we used a PKC substrate peptide designed on the basis of the optimal substrate recognition sequence of PKCs [32]. PDK-1 does not significantly phosphorylate this peptide in an *in vitro* filter-binding assay (Figure 4c). Purified PKCζ phosphorylates the peptide and phosphorylation is not significantly enhanced in the presence of either PS/PE or PS/PE/PtdIns-3,4,5-P_3 vesicles. PDK-1 caused a two- to threefold increase in the activity of PKCζ towards the peptide substrate which could be further enhanced to sixfold in the presence of PS/PE/PtdIns-3,4,5-P_3. The kinase-inactive PDK-1.K/I did not activate PKCζ in this assay. These data show that PDK-1 directly phosphorylates Thr410, and that this is sufficient to activate PKCζ *in vitro*.

Association of PKCζ with PDK-1 *in vivo*

As our data show that PKCζ is a PDK-1 substrate, we explored the possibility that PKCζ and PDK-1 are associated in cells. NIH 3T3 fibroblasts were co-transfected with FLAG–PKCζ and Myc–PDK-1 and, following serum starvation, cells were stimulated with PDGF. Association between PKCζ and PDK-1 was analyzed by anti-Myc immunoprecipitation. Figure 5 shows that PKCζ and PDK-1 can be co-immunoprecipitated *in vivo*. This association was specific to PDK-1 as PKCζ could not be immunoprecipitated with either non-immune serum or with irrelevant Myc-tagged proteins (Myc–Rac and Myc–Pak-1; Figure 5). In addition, this association appears to be constitutive, as it could be detected in unstimulated cells and was neither enhanced by PDGF nor inhibited by the PI 3-kinase inhibitor LY294002.

Discussion

Our data identify PDK-1 as the first known PKC-activating kinase. Our results clearly show that PDK-1 induces PKCζ phosphorylation *in vivo*, leading to its activation. Furthermore, PDK-1 directly phosphorylates PKCζ at the

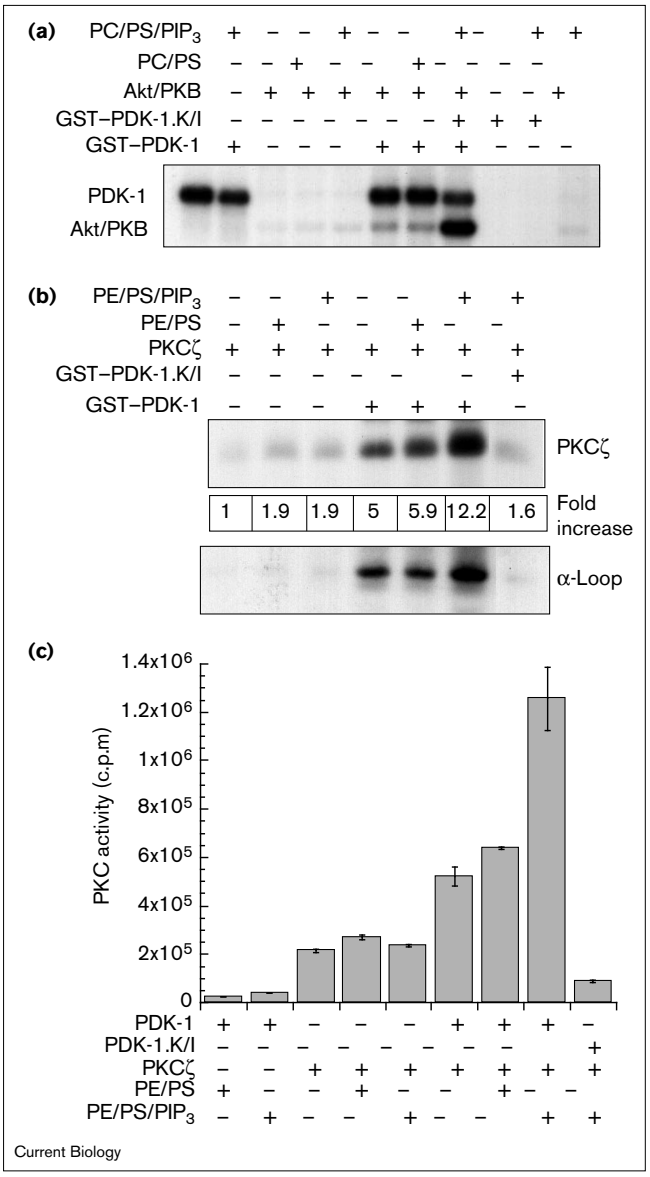


Figure 4

Phosphorylation and activation of Akt/PKB and PKCζ by PDK-1 *in vitro*. **(a)** Purified, recombinant GST–PDK-1, GST–PDK-1.K/I and His_6–Akt/PKB (0.2 μg of each protein) were incubated in an *in vitro* kinase assay in the presence or absence of mixed lipid vesicles. Phosphatidylcholine (PC)/PS mixed vesicles were added at a final concentration of 100 μM each. Where indicated, 10 μM PtdIns-3,4,5-P_3 (PIP_3: synthetic, dipalmitoyl C_{16} [38]) was added as PC/PS/PIP_3 mixed vesicles. The reactions were stopped by addition of SDS sample buffer and resolved by 10% SDS–PAGE, followed by autoradiography. **(b)** Purified PKCζ was incubated in an *in vitro* kinase assay with recombinant GST–PDK-1 and GST–PDK-1.K/I (0.2 μg of each protein), in the presence or absence of PE/PS or PE/PS/PIP_3 vesicles, as described in (a). The phosphorylated PKCζ was resolved by SDS–PAGE, transferred to nitrocellulose and quantitated on a Phosphorimager. The membrane was then immunoblotted with the anti-loop antibody (α-loop) to reveal phosphorylation of Thr410. The data in (a) and (b) are representative of four independent experiments. **(c)** Activation of PKCζ *in vitro* was assayed as described in (b), except that a PKCζ substrate peptide (RRRRRKGSFRRKK) was added to the reaction mix. A P81 phosphocellulose paper binding assay was used to measure incorporation of $\gamma^{32}P$ into peptide. The data shown are the means from three independent experiments.

activation-loop Thr410 residue *in vitro*. We have also demonstrated that PKCζ activation can be readily measured in an immune-complex kinase assay, following stimulation of cells by PDGF or EGF, or co-transfection with PDK-1. Because these assays are performed in the absence of PS and PtdIns-3,4-P_2/PtdIns-3,4,5-P_3, this indicates that once PKCζ is activated, it is covalently locked into an active conformation. This is reminiscent of the model proposed for Akt/PKB, in which both PtdIns-3,4,5-P_3 binding and phosphorylation by PDK-1 are required for activation [23]. However, once fully phosphorylated, Akt/PKB no longer requires PtdIns-3,4-5-P_3 and can be isolated as an active kinase. Recent studies have shown that insulin stimulation of rat adipocytes [17] and 32D cells [18] also leads to activation of PKCζ in a PI 3-kinase-dependent manner, as measured in immune-complex kinase assays. Similarly, lipopolysaccharide-treated human monocytes show increased PKCζ activity which can be blocked by chemical inhibitors of PI 3-kinase [16]. Whether similar assays can be used to detect activation of other PKC family members remains to be established.

As PKCζ phosphorylation/activation is almost completely blocked by co-expression of dominant-negative PDK-1 or by mutation of Thr410 (Figure 2b), it is likely that a pre-requisite for PKCζ activation is phosphorylation of Thr410. Analysis of PKCβII regulation has suggested a similar mechanism [10]. Phosphorylation of PKCβII Thr500 is required before the enzyme can autophosphorylate at two carboxy-terminal sites, Thr641 and Ser660. In PKCζ, Thr560 (analogous to Thr641 in PKCβII) is conserved and is therefore likely to be regulated by autophosphorylation. However, the second carboxy-terminal site in PKCζ (analogous to Ser660 in PKCβII) is replaced by a negatively charged Glu residue (Glu579). This substitution may confer the same net effect on the protein as phosphorylation, and may account for the high constitutive activity of PKCζ in certain cell types (Figure 1b), though this requires further investigation.

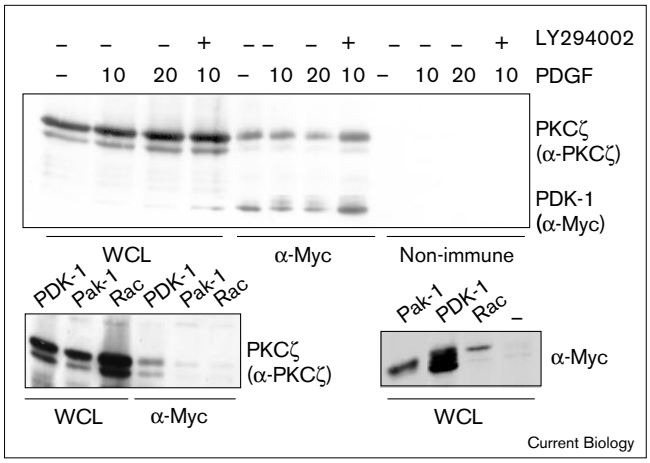
As PDK-1 is recruited to the membrane following agonist stimulation of cells [31], it is likely that some of its substrates that also have a lipid requirement are also translocated to the membrane. Consistent with this, we find that a membrane-targeted mutant of PKCζ is constitutively active in serum-starved cells, and this increase in activity correlates with an increase in Thr410 phosphorylation. Similarly, a membrane-targeted Akt/PKB mutant is also constitutively active *in vivo* [30]. It is possible that in unstimulated cells a low level of PtdIns-3,4,5-P_3 exists which may be sufficient to translocate a pool of PDK-1, but not Akt/PKB or PKCζ. In this scenario, the membrane-targeted Myr–Akt/PKB and Myr–PKCζ would then

be accessible to PDK-1, leading to phosphorylation and activation. This model is supported by the finding that PDK-1 has a very high affinity for both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [27]. Regardless of the precise mechanism, the finding that Myr–PKCζ is constitutively active is consistent with the notion that PKCs are enzymes that must translocate to the plasma membrane for activation.

Previous studies have suggested that PDK-1 is constitutively active in mammalian cells [26]. Our results, however, indicate that phosphorylation of PKCζ by PDK-1 is sensitive to PtdIns-3,4,5-P_3, as shown for Akt/PKB. In the case of Akt/PKB, several studies have indicated that the PtdIns-3,4,5-P_3 requirement is for Akt/PKB itself, rather than for PDK-1 [24,25]. It has been proposed that binding of PtdIns-3,4,5-P_3 to the pleckstrin homology (PH) domain of Akt/PKB induces a conformational change that renders Thr308 accessible to PDK-1. Consistent with this hypothesis, an Akt/PKB mutant lacking the PH domain no longer requires PtdIns-3,4,5-P_3 for PDK-1-mediated phosphorylation [25]. In the case of PKCζ, it is possible that PtdIns-3,4,5-P_3 also binds to the regulatory domain of the enzyme, relieving autoinhibition. Indeed, although our own results indicate that PtdIns-3,4,5-P_3 by itself is not sufficient to activate PKCζ significantly, others have presented evidence that both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 alone can stimulate the activation of PKCζ [15–17] and other PKCs [33–35]. It is likely that different protein preparations and/or assay conditions account for these disparate results and thus it is difficult to come to any conclusions concerning the reasons for the differences. Given that PDK-1 and PKCζ stably associate in cells (Figure 5), one possibility is that PDK-1 may have been present in what were presumed to be purified PKCζ preparations. Although it seems unlikely, we cannot rule out the fact that the PtdIns-3,4,5-P_3 requirement for PDK-1-mediated PKCζ phosphorylation lies with the PDK-1, rather than the PKC.

Together, our results identify PKCζ as the third *in vivo* substrate of PDK-1, along with Akt/PKB and p70S6K. There are both similarities and distinctions in the regulation of these three enzymes. First, the PtdIns-3,4,5-P_3 dependence of their phosphorylation by PDK-1 differs: whereas phosphorylation of p70S6K is PtdIns-3,4,5-P_3-independent, that of Akt/PKB is highly PtdIns-3,4,5-P_3-dependent. In contrast, measurable phosphorylation of PKCζ by PDK-1 is observed in the absence of lipids, and this is modestly enhanced by PtdIns-3,4,5-P_3. Second, while the co-localization between Akt/PKB and PDK-1 is PDGF-dependent [31], that between PKCζ and PDK-1 appears to be constitutive and PDGF-independent; association between p70S6K and PDK-1 has yet to be determined. Third, although both Akt/PKB and PKCζ can be rendered constitutively active by membrane-targeting, p70S6K cannot (M.M.C., unpublished observations).

Figure 5



Association of PKCζ with PDK-1 and Akt/PKB. NIH 3T3 fibroblasts were co-transfected with the indicated combinations of plasmid DNAs, then serum starved for 24 h. Cells were pretreated for 20 min with 30 μM LY294002 or with vehicle (DMSO) alone, then stimulated with PDGF (50 ng/ml) for the times indicated (in min). Anti-Myc immunoprecipitates (α-Myc) or control non-immune precipitations were resolved by SDS–PAGE followed by PKCζ (α-PKCζ) immunoblotting. PDK-1, Rac and Pak-1 were detected by Myc immunoblotting (α-Myc). Whole cell lysates (WCLs, 10% of that used for the immunoprecipitation) were also resolved.

The observation that PKCζ associates with PDK-1 *in vivo* suggests considerable cross-talk between effector molecules in the PI 3-kinase signaling pathway. Our data are consistent with a model in which a pre-existing complex of PDK-1 and PKCζ exists in unstimulated cells. PDK-1 cannot phosphorylate PKCζ, probably due to autoinhibitory mechanisms in PKCζ and/or PDK-1. Following agonist stimulation, this complex is presumably recruited to the plasma membrane at the site of PtdIns-3,4,5-P_3 synthesis. Here, PDK-1, and possibly PKCζ, bind to PtdIns-3,4,5-P_3 leading to phosphorylation of Thr410 and activation of PKCζ. It is possible that additional, as-yet-unidentified events at the plasma membrane may further contribute to the activation of PKCζ. At this point it is unclear whether the interaction between PKCζ and PDK-1 is direct, or whether one or more adaptor/scaffolding proteins are present. It also remains to be seen whether other PI 3-kinase effector molecules are also found in such a complex.

In summary, we have presented evidence that PDK-1 is the upstream activating kinase for PKCζ. Phosphorylation of PKCζ Thr410 by PDK-1 leads to activation of the enzyme, both *in vitro* and *in vivo*. Activation of PKCζ must therefore occur at the site of PtdIns-3,4,5-P_3 synthesis, where PDK-1 is localized. Consistent with this, membrane targeting of PKCζ renders the enzyme constitutively active. As the motif surrounding Thr410 is highly conserved in all other PKC family members, one might

predict that PDK-1, or a closely related isoform, also phosphorylates other PKCs.

Materials and methods

cDNA constructs

The following cDNA constructs were used in these studies. The FLAG-tagged PKCζ (FLAG–PKCζ) was generated by addition of the FLAG epitope (MDYKDDDDK) to the amino terminus of rat PKCζ (GenBank accession number P09217). The FLAG–PKCζ.T410A mutant was generated by mutating Thr410 to Ala. Both constructs were subcloned into the *Eco*RI sites of the pCMV5 mammalian expression vector [36]. The Myr–PKCζ–FLAG construct was generated by adding in-frame the first nine amino acids of the p60 c-Src myristoylation sequence (MGSNKSKPK) to the PKCζ amino terminus and the FLAG epitope to the carboxyl terminus. The Myc–PDK-1 construct has been previously described [27]. The kinase-inactive variant Myc–PDK-1.K/N was made by mutating the conserved critical Lys110 residue to Asn. Generation of the Myc–p110.CAAX [22] and GST–Δp85 [37] constructs has been described. The GST–PDK-1 and GST–PDK-1.K/I constructs were made by subcloning the appropriate PCR fragments of PDK-1 into the *Bam*HI and *Eco*RI sites of the pGEX 2T vector (Pharmacia). The PKCβII constructs have been described [11]. All constructs and mutants were made using PCR and checked by DNA sequencing.

Cell culture

Early passage NIH 3T3 cells (American Type Culture Collection) and 293 cells were maintained in Dulbecco's modified Eagle Medium (DMEM, Life Technologies) containing 10% calf serum at 37°C in a 5% CO_2 humidified atmosphere. 293 cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere.

Mammalian cell transfections

All transient transfections into NIH 3T3 and 293 cells were carried out using the Lipofectamine procedure (Life Technologies). Cells were seeded at a density of 1×10^5 per 35 mm dish 12 h before transfection. Each DNA construct was titrated against Lipofectamine to achieve uniform expression of the proteins, typically 1 μg of each DNA, combined with empty vector for a total of 2 μg per dish. The DNA–Lipofectamine mix was overlayed onto cells in transfection medium (Optimem, Life technologies) for 6 h, after which cells were washed and recovered in complete medium (DMEM + 10% heat-inactivated FBS) for 14 h. Cells were serum starved in DMEM–serum for 24 hr. For *in vivo* labeling studies, cells were transiently transfected and grown for 24 h in serum-containing medium then labeled for 2 h with 2 mCi/ml inorganic ^{32}P-orthophosphate (New England Nuclear) in phosphate-free and serum-free medium before stimulation.

Immunoprecipitations and immune-complex kinase assays

Following stimulation, cells were lysed in 1 ml buffer A (20 mM Tris pH 7.5, 10% glycerol, 1% NP-40, 10 mM EDTA, 150 mM NaCl, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C. Ten percent of the total lysate was immediately boiled in SDS sample buffer. Epitope-tagged proteins were immunoprecipitated with 1 μl/ml of the corresponding epitope antibodies, as follows: FLAG–PKCζ, M2 monoclonal (Kodak–IBI); Myc–PDK-1, 9E10 monoclonal (Boehringer Mannheim) and with 25 μl/ml of a 50% mix of protein A/G beads (Santa-Cruz). For immune-complex kinase assays, immunoprecipitates were washed stringently as follows: twice in buffer B (1× phosphate-buffered saline, 1% NP-40), 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). Immune-complex protein kinase assays were carried out in a total volume of 30 μl in the following reaction conditions: 10 mM $MgCl_2$ and 5 μg myelin basic protein (MBP), 10 μCi [γ-^{32}P]adenosine triphosphate (ATP, New England Nuclear) (25 μM) for 10 min at 25°C. Reactions were resolved by 12.5% SDS–PAGE and MBP phosphorylation quantitated on a Bio-Rad GS505 Molecular Imager System. For detecting association between PKCζ and PDK-1, immunoprecipitates were washed twice in buffer B and once in buffer D, then boiled in SDS sample buffer and resolved by 7.5% SDS–PAGE.

Recombinant protein expression and purification

GST–PDK-1 and GST–PDK-1.K/I were expressed in bacteria as follows. An overnight saturated culture of bacteria was diluted 10-fold into 1 liter of medium and cells grown at 37°C with shaking for 1 h. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) was then added and cells grown for a further 16 h at 15°C with constant shaking. Cells were harvested by centrifugation and pellets rapidly frozen and thawed at −70°C. Cells were resuspended in buffer E (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA and 1 mM PMSF), then lysed by addition of 1.5 mg/ml lysozyme (Sigma) and 1.5 mg/ml sodium deoxycholate. Bacterial DNA was digested with 10 μg/ml DNase I (Sigma) in the presence of 1 mM $MgSO_4$ (final concentration). Lysates were cleared by centrifugation at 15,000 rpm for 30 min at 4°C, and recombinant proteins coupled to 0.5 ml packed glutathione–Sepharose beads (Pharmacia) at 4°C for 1 h. Beads were washed three times in buffer F (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF), then three times in buffer D. Fusion proteins were eluted with 10 mM glutathione in buffer D, and stored at −20°C in 50% glycerol.

Recombinant Akt/PKB was expressed as a His_6 fusion protein in baculovirus-infected insect cells. *Spondoptera frugiperda* 9 (Sf9) cells (500 ml) were grown to a density of 1.5×10^6/ml and infected in a spinner flask with 50 ml high-titer (5×10^7pfu/ml) baculovirus directing the expression of a His_6–Akt/PKB fusion protein (Bac-to-Bac, Life Technologies). Infections were carried out for 3 days, after which cells were harvested by centrifugation and frozen at −70°C. Cells were lysed in buffer A (in the absence of EDTA) and cleared lysate applied to a 5 ml nickel–NTA column (Qiagen). Following extensive washing with buffer G (20 mM Tris pH 8.0, 500 mM KCl, 5 mM 2-mercaptoethanol (ME), 10% glycerol), His_6–Akt/PKB was eluted with 100 mM imidazole in buffer G. Fractions containing the protein peak were pooled, diluted in buffer H (20 mM Tris pH 8.0, 5 mM ME) and applied to a Mono Q column on FPLC (Pharmacia). Proteins were eluted with a 40 ml gradient of NaCl (0–0.5M) in buffer H. Fractions were resolved by SDS–PAGE and stained with Coomassie blue. The resulting purified Akt/PKB was stored in 50% glycerol at −20°C.

Recombinant PKCζ was expressed as a wild-type protein in Sf9 cells as described above for Akt/PKB. The recombinant protein as purified over four columns by FPLC, as previously described [33]. The purified protein was stored in 50% glycerol at −70°C.

In vitro *kinase assays*

Phosphorylation of Akt/PKB and PKCζ by GST–PDK-1 was assayed *in vitro* using 0.2 μg of each protein in a protein kinase assay comprising 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 25 μM [γ^{32}P]ATP (5 μCi per assay, 3000 Ci/mmol). Mixed lipid vesicles were prepared by drying a concentrated stock of lipid stored in chloroform:methanol (1:1) under a stream of nitrogen. Lipids were reconstituted by sonication into 25 mM Tris (pH 7.5), in an ice bath sonicator (Branson) at 4°C for 10 min at 50% output. Lipids were prepared fresh for each experiment. Activation of PKCζ by GST–PDK-1 was assayed as above, except that a synthetic peptide (RRRRRKGSFRRKK) was added to the reaction mix, at a final concentration of 100 μM. A P81 phosphocellulose paper binding assay was used to measure incorporation of γ^{32}P into peptide, as previously described [32]. Stoichiometry of phosphorylation of Akt/PKB and PKCζ was determined by a time-course of *in vitro* phosphorylation of PKCζ and Akt/PKB by PDK-1 (up to 120 min), separating the proteins by SDS–PAGE and excising the phosphorylated bands, followed by scintillation counting. One unit of PDK-1 activity is defined as that amount of enzyme required to transfer 1 nmol of phosphate/min/mg protein.

Supplementary material

Data demonstrating activation of PKCζ by PDK-1 with the GST moiety cleaved are published with this paper on the internet.

Acknowledgements

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Supplementary material

Regulation of protein kinase C ζ by PI 3-kinase and PDK-1

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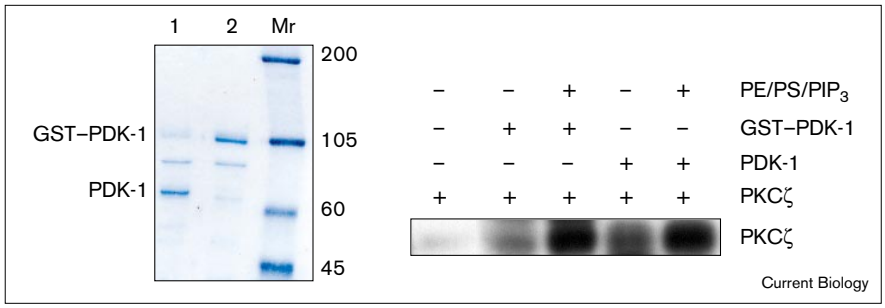
Figure S1



The GST tag does not affect the ability of PDK-1 to phosphorylate PKCζ. GST–PDK-1 was expressed in bacteria as described in the Materials and methods section of the paper, and coupled to glutathione beads. After washing in cleavage buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2.5 mM $CaCl_2$), 10 μg of protein was treated with thrombin (0.1 μg, 3800 Units/mg; lane 1, left panel) or buffer alone (lane 2), and incubated for 1 h at 4°C. 1 μg of each of PDK-1 and GST–PDK-1 (eluted from beads with 10 mM reduced glutathione) were analyzed by SDS–PAGE (left panel). The cleaved PDK-1 preparation had a molecular mass of approximately 65 kDa. 0.5 μg of each protein was used in an *in vitro* kinase assay with 0.5 μg purified PKCζ as described in the legend to Figure 4b. The results (right panel) show that, as presented in Figure 4b, PKCζ alone has a low intrinsic autokinase activity as detected by phosphorylation of the PKCζ band in the presence of [γ-^{32}P]ATP. Phosphorylation of PKCζ is increased when GST–PDK-1 is added, and further enhanced when mixed vesicles containing $PE/PS/PIP_3$ are added to the reaction mixture. The PDK-1 with the GST moiety removed is also capable of phosphorylating PKCζ to the same extent as is observed with GST–PDK-1. Thus, the GST tag neither interferes with nor enhances the ability of PDK-1 to phosphorylate PKCζ, or the ability of PtdIns-3,4-5-P_3 to enhance this reaction.