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Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1)

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Background: Phosphorylation critically regulates the catalytic function of most members of the protein kinase superfamily. One such member, protein kinase C (PKC), contains two phosphorylation switches: a site on the activation loop that is phosphorylated by another kinase, and two autophosphorylation sites in the carboxyl terminus. For conventional PKC isozymes, the mature enzyme, which is present in the detergent-soluble fraction of cells, is quantitatively phosphorylated at the carboxy-terminal sites but only partially phosphorylated on the activation loop.

Results: This study identifies the recently discovered phosphoinositide-dependent kinase 1, PDK-1, as a regulator of the activation loop of conventional PKC isozymes. First, studies *in vivo* revealed that PDK-1 controls the amount of mature (carboxy-terminally phosphorylated) conventional PKC. More specifically, co-expression of the conventional PKC isoform PKC βII with a catalytically inactive form of PDK-1 in COS-7 cells resulted in both the accumulation of non-phosphorylated PKC and a corresponding decrease in PKC activity. Second, studies *in vitro* using purified proteins established that PDK-1 specifically phosphorylates the activation loop of PKC α and βII. The phosphorylation of the mature PKC enzyme did not modulate its basal activity or its maximal cofactor-dependent activity. Rather, the phosphorylation of non-phosphorylated enzyme by PDK-1 triggered carboxy-terminal phosphorylation of PKC, thus providing the first step in the generation of catalytically competent (mature) enzyme.

Conclusions: We have shown that PDK-1 controls the phosphorylation of conventional PKC isozymes *in vivo*. Studies performed *in vitro* establish that PDK-1 directly phosphorylates PKC on the activation loop, thereby allowing carboxy-terminal phosphorylation of PKC. These data suggest that phosphorylation of the activation loop by PDK-1 provides the first step in the processing of conventional PKC isozymes by phosphorylation.

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Background

The elucidation of the first crystal structure of a kinase, that of protein kinase A, unveiled a key regulatory switch in kinase function: phosphorylation of the activation loop (reviewed in [1]). Ionic interactions between a critically positioned phosphate group, located on a conserved loop near the entrance to the active site, and residues within the active site were shown to correctly align residues for catalysis. This phosphorylation switch is found throughout the kinase superfamily, being shared by both tyrosine kinases and serine/threonine kinases [2]. For some kinases, such as protein kinase A, phosphorylation of the activation loop occurs during the maturation of the enzyme, with activation of the kinase resulting from subsequent interactions with second messengers [3]. For others, such as the cyclin-dependent kinase Cdk2 and the mitogen-activated protein (MAP) kinases, phosphorylation is triggered by specific stimuli and serves as the direct switch to activate the enzymes [4,5]. One class of kinases that is regulated by phosphorylation at the activation loop is the protein kinase C (PKC) family.

PKC isozymes transduce the myriad of signals promoting phospholipid hydrolysis [6]. This family of enzymes comprises three subclasses: conventional (α, βI, βII and γ), novel (δ, ε, θ and η/L) and atypical (ζ and λ/ι). For conventional and novel isozymes, the generation of diacylglycerol by phospholipase C or D recruits cytosolic PKC to the membrane. Binding of PKC to the membrane is mediated by two membrane-targeting domains, C1 and C2, which, upon interaction with their ligands diacylglycerol and phosphatidylserine, provide the energy to release an autoinhibitory (pseudosubstrate) domain from the active site [7]. The regulation of atypical PKC isozymes differs in that diacylglycerol does not regulate the function of the enzyme. Rather, mounting evidence has implicated

phosphoinositide (PI)3-kinase as providing the stimulus to activate these PKC isozymes.

Before PKC is competent to respond to second messengers, it must first be phosphorylated at three conserved positions: the activation loop and two positions at the carboxyl terminus of the protein. These positions are Thr500, Thr641 and Ser660 in PKC βII [8,9]. Hydroxyl-containing residues are found at corresponding positions throughout the PKC family, with the exception of the atypical PKC isozymes, which have a glutamic acid residue at the position corresponding to Ser660 [9].

Biochemical studies indicate that the phosphorylation of the activation loop is a transphosphorylation event mediated by another kinase [9,10]. In contrast, the two carboxy-terminal phosphorylations have been shown to be autophosphorylation events *in vitro* [9,11]. The phosphorylation of the activation loop appears to occur first, leading to the hypothesis that transphosphorylation at this position alters the conformation of the kinase, so as to allow for the two autophosphorylations at the carboxyl terminus. Once phosphorylated at the two carboxy-terminal positions, the conventional PKC isozymess no longer require phosphate at the activation loop to be catalytically competent: dephosphorylation of mature (that is, carboxy-terminally phosphorylated) PKC βII at Thr500 does not affect the maximal activity of the enzyme [12].

The recently discovered PI-dependent protein kinases (PDKs) are ideal candidates for catalyzing the phosphorylation of the activation loop of PKC: PDK-1 phosphorylates the activation loop of protein kinase B (PKB or Akt) on a sequence that has a high similarity to that found in the activation loop of PKC [13]. PDK-1 also phosphorylates the related kinases p70S6 kinase and protein kinase A [3,14,15]. The phosphorylation of PKB is triggered by the generation of phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) through PI3-kinase; this lipid binds to the pleckstrin homology (PH) domain of PKB, causing a conformational change that exposes the phosphorylatable threonine residue on the activation loop [16,17]. Although PDK-1 itself contains a PH domain, ligand-binding to this domain does not appear to be essential for catalysis [16]. Rather, the PH domain of PDK-1 may serve to localize PDK-1 near its membrane-bound substrates [17,18]. The studies with PKB suggest that, at least for one kinase, phosphorylation at the activation loop by PDK-1 is regulated by the substrate conformation.

This study explores the phosphorylation of the conventional PKC isozymes by PDK-1. More specifically, we have shown that PDK-1 regulates the phosphorylation state of PKC in COS-7 cells and catalyzes the direct phosphorylation of the activation loop of PKC *in vitro*. Thus, PDK-1 provides the trigger to initiate the processing of conventional PKC isozymes by phosphorylation.

Results

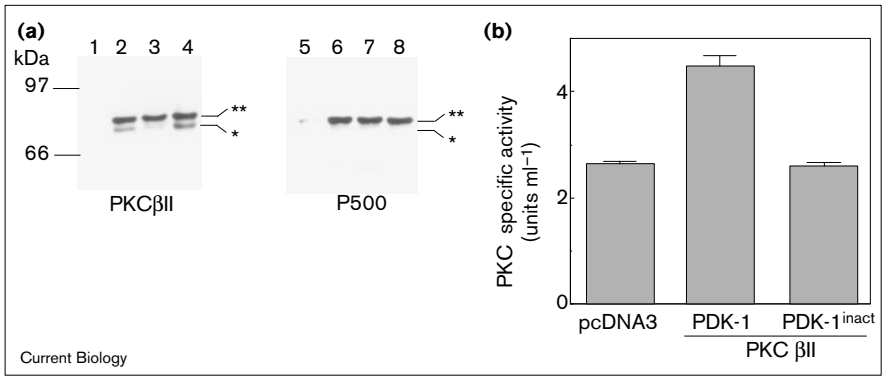
Regulation of PKC by PDK-1 *in vivo*

To test whether PDK-1 regulates the function of the conventional PKC isozymes *in vivo*, we examined whether the phosphorylation of PKC βII was different in COS-7 cells co-expressing either wild-type PDK-1 or the kinase-inactive PDK-1^{inact}. Cells were transfected with vector alone, PKC βII, PKC βII and PDK-1, or PKC βII and inactive PDK-1^{inact}; cell lysates were then subjected to SDS–PAGE and western blotting and probed either with an antibody that does not discriminate between phosphorylation states or with an antibody (P500) that specifically recognizes the phosphorylated activation loop [19].

Figure 1a shows that when PKC βII is expressed in COS-7 cells, it migrates as a doublet in SDS–PAGE (lane 2). We have previously shown that the upper band represents protein that is quantitatively phosphorylated at the two carboxy-terminal positions and partially phosphorylated at the activation loop; this species, referred to as mature PKC, partitions exclusively in the detergent-soluble cell fraction [9]. The lower band represents protein that has not been phosphorylated at either of the carboxy-terminal sites (an intermediate band, not visible in Figure 1a, contains protein that is phosphorylated at Thr641 but not Ser660) [9,20]). Co-expression of PDK-1 resulted in a decrease in the amount of faster-migrating PKC βII (Figure 1a, lane 3). Conversely, co-expression of PDK-1^{inact} resulted in a marked increase in the amount of faster-migrating PKC βII (Figure 1a, lane 4). Quantitative analysis revealed that the lower band represented 23 ± 3% of the total PKC in the absence of co-expressed PDK-1, 12.6 ± 0.6 % in the presence of PDK-1, and 38 ± 9% in the presence of PDK-1^{inact} (Figure 1a, lanes 2,3,4, respectively). Thus, overexpression of PDK-1 resulted in the generation of a larger fraction of mature PKC, whereas overexpression of the kinase-inactive PDK-1^{inact} resulted in the accumulation of non-phosphorylated PKC. In all cases, the lower band partitioned in the detergent-insoluble cell fraction (data not shown). The expression of the PDK-1 constructs was confirmed by reprobing blots with anti-Myc antibodies (data not shown).

Figure 1a also shows the result of probing the blot of COS-7 cell lysates with the antibody P500. When normalized to the immunoreactivity obtained with the non-discriminatory antibody, no significant differences were noted between the phosphorylation state of the activation loop of the various samples. In other words, overexpression of wild-type PDK-1 or the kinase-inactive PDK-1^{inact} had no readily detectable effect on the degree of phosphorylation of the activation loop of mature PKC (Figure 1a, slower-migrating band) under the transfection conditions of these experiments. Thus, PDK-1 affects the fraction of PKC βII that is processed to the mature form, but does

Figure 1



Co-expression of PDK-1 or PDK-1^{inact} with PKC βII in COS-7 cells affects the fraction of mature PKC and the specific activity of PKC. **(a)** Western blots of lysates of COS-7 cells transfected with the pcDNA3 vector DNA alone (lanes 1,5), or with vectors carrying PKC βII (lanes 2,6), PKC βII and PDK-1 (lanes 3,7), or PKC βII and PDK-1^{inact} (lanes 4,8). The blots were probed with a non-discriminatory antibody against PKC βII (PKCβII, Calbiochem; lanes 1–4) or with an antibody that specifically labels the phosphorylated activation loop (P500; lanes 5–8). A double asterisk indicates the position at which mature PKC migrates; a single asterisk denotes the position at which non-phosphorylated PKC migrates. The results are representative of five independent transfections. **(b)** PKC activity was measured in the presence or absence of phosphatidylserine, diacylglycerol and Ca^{2+}, in lysates of COS-7 cells transfected with vector alone (pcDNA3), PKC βII and PDK-1, or PKC βII and PDK-1^{inact}. The data are presented as the mean ± SEM of sextuplicate assays from the transfections in (a).

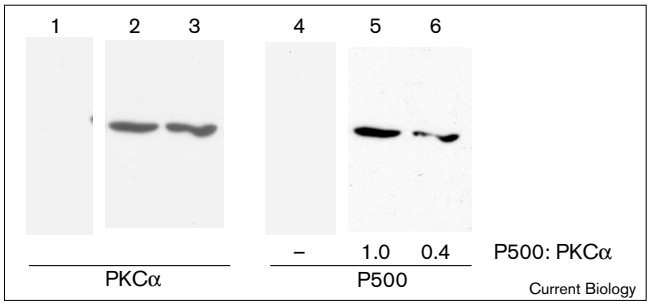
not detectably affect the fraction of mature form that is phosphorylated on the activation loop.

The effect of overexpressing active or inactive PDK-1 on the PKC activity in COS-7 cells overexpressing PKC βII is presented in Figure 1b. Co-expression of PDK-1 and PKC βII resulted in a twofold increase in the total kinase activity relative to cells transfected with vector alone; this increase represents that resulting from PKC βII expression and was not significantly affected by co-expression of PDK-1 (data not shown). Co-expression of PDK-1^{inact} resulted in a marked reduction in the total PKC activity. This reduction probably reflects a decrease in the activity of both the transfected PKC βII and the endogenous PKC α. Thus, PDK-1^{inact} serves as a dominant-negative inhibitor of PKC activity.

We also explored the effect of co-expressing PDK-1 or PDK-1^{inact} with PKC α. Figure 2 shows that co-expression of PDK-1^{inact} resulted in an approximately 60% reduction in the fraction of mature PKC α that was phosphorylated on the activation loop relative to that present in cells co-expressing wild-type PDK-1.

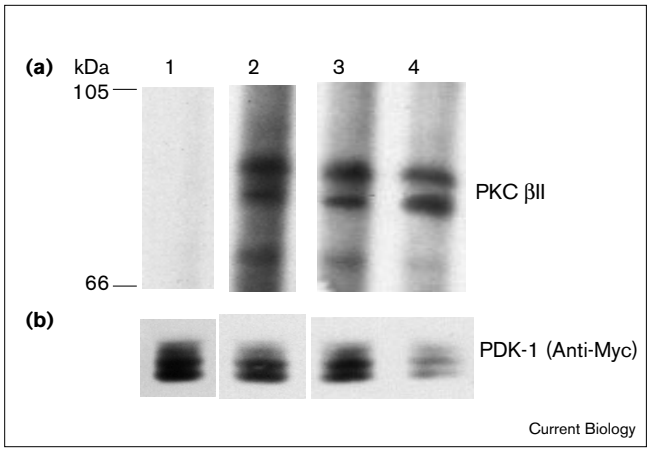
We next addressed whether PDK-1 forms a complex with PKC βII *in vivo*. 293 cells were co-transfected with vectors encoding Myc-tagged PDK-1 and PKC βII. The PDK-1 was subsequently immunoprecipitated and the resulting immunoprecipitates analyzed for the presence of PKC βII. Figure 3a shows that PKC βII was present in immunoprecipitates from both serum-starved (lane 2), or serum-treated (lane 3) cells. The amount of PKC βII in the immunoprecipitates was insensitive to the presence of wortmannin, an inhibitor of PI3-kinase (lane 4). Note that the PKC βII migrated as a doublet, probably reflecting the presence of both the phosphorylated and dephosphorylated species of the enzyme; both forms were present in western blots of whole cell lysates (data not shown; the large fraction of faster-migrating species typically arises as a result of serum-starvation, which promotes the dephosphorylation of PKC; A.S. Edwards and A.C.N., unpublished observations). No PKC βII was detected in cells transfected with PDK-1 alone (Figure 3a, lane 1). Figure 3b shows the presence of Myc-tagged PDK-1 in whole cell lysates from all transfections. Western blot analysis of whole cell lysates also demonstrated that comparable levels of PKC βII were expressed in all transfections (data not shown). These data reveal that PDK-1 and PKC βII associate in cells and that this association is not modulated by serum starvation or inhibitors of PI3-kinase.

Figure 2



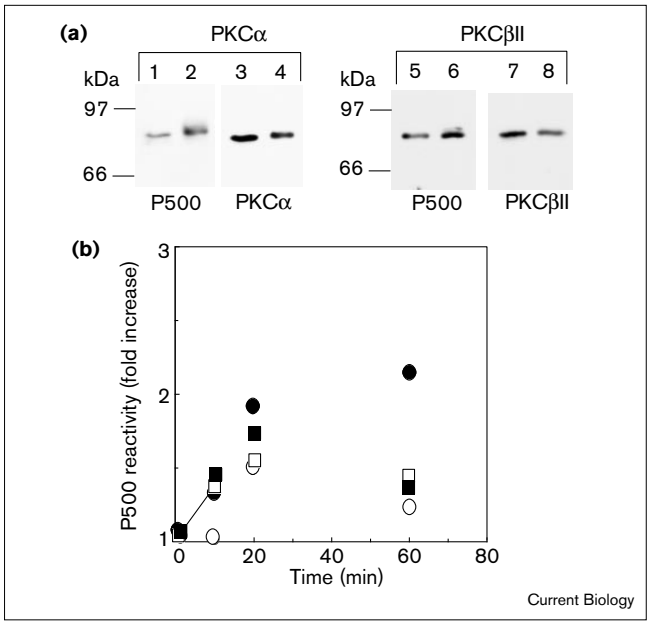
Co-expression of PDK-1 or PDK-1^{inact} with PKC α affects the fraction of mature PKC that is phosphorylated on the activation loop. Western blots of lysates of COS-7 cells transfected with pcDNA3 alone (lanes 1,4), or with vectors carrying PKC α and PDK-1 (lanes 2,5), or PKC α and PDK-1^{inact} (lanes 3,6). The blots were probed using two antibodies: a non-discriminatory antibody against PKC α (PKCα; Transduction Laboratories) and the P500 antibody. The ratio of P500 immunoreactivity to PKC α immunoreactivity, which was obtained by densitometric analysis of the data, is shown in arbitrary units under lanes 4–6. The data are representative of three independent transfections.

Figure 3



PKC βII associates with PDK-1 *in vivo*. 293 cells were transfected with the cDNA for Myc-tagged PDK-1 (lanes 1–4) and PKC βII (lanes 2–4). The transfected cells were serum-starved for 24 h, and then treated with 10% foetal bovine serum (FBS) for 20 min (lane 3), or with 10% FBS and 100 nM wortmannin for 10 min (lane 4). **(a)** PDK-1 was immunoprecipitated from lysates using an anti-Myc monoclonal antibody, and western blots of precipitated protein were probed for PKC βII (Santa Cruz antibody). **(b)** Blot of whole cell lysate from the transfected cells probed with an anti-Myc antibody to detect PDK-1 expression.

Figure 4



PDK-1 phosphorylates the activation loop of conventional PKC isozymes *in vitro*. **(a)** Western blots of mature PKC α (lanes 1–4) and PKC βII (lanes 5–8) after incubation with Mg^{2+}–ATP and either wild-type PDK-1 (lanes 2,4,6,8) or PDK-1^{inact} (lanes 1,3,5,7); PKC and PDK-1 concentrations were 3 nM and 40 nM, respectively. The blot was probed with P500 antibody (lanes 1,2,5,6), or with non-discriminatory antibodies against PKC α (Transduction Laboratories; lanes 3,4) or PKC βII (Santa Cruz; lanes 7,8). **(b)** PKC (3 nM) was incubated with PDK-1 (40 nM) and Mg^{2+}–ATP for the indicated times in the absence (open circles) or presence of sonicated phosphatidylcholine vesicles containing 40 mol% phosphatidylserine and 5 mol% diacylglycerol (filled circles), 10 mol% PI(3,4,5)P_3 (open squares), or 40% phosphatidylserine, 5 mol% diacylglycerol and 10 mol% PI(3,4,5)P_3 (filled squares); where mol% refers to the percentage of each lipid species in the vesicle membrane on a molar basis. The reaction mixture contained 200 μM $CaCl_2$.

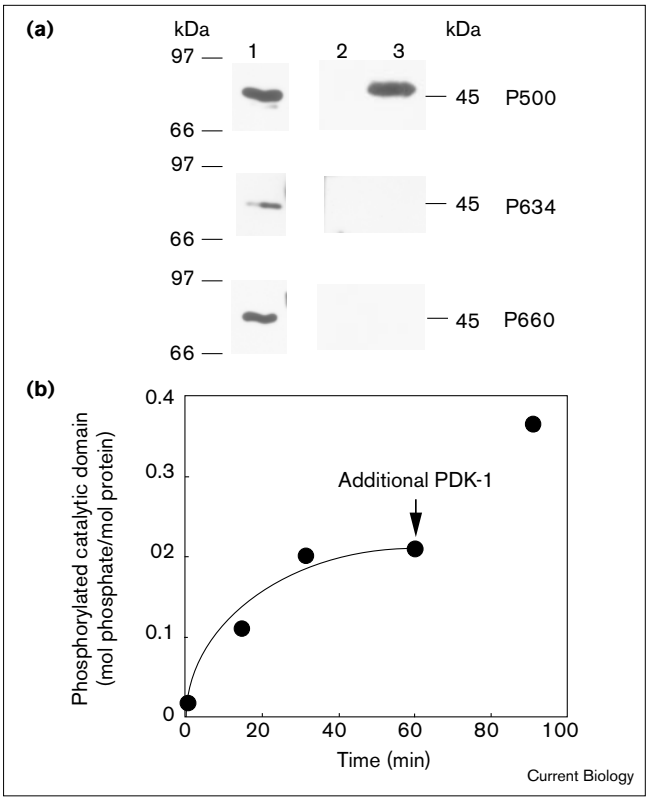
Phosphorylation of conventional PKC isozymes by PDK-1 *in vitro*

The above experiments reveal that PDK-1 alters the fraction of mature PKC βII and forms a complex with this isozyme *in vivo*. This led us to ask whether PDK-1 directly phosphorylates the activation loop of conventional PKC isozymes. To this end, we incubated baculovirally expressed PKC α or βII with either pure wild-type PDK-1 or PDK-1^{inact}, in the presence of Mg^{2+}–ATP. We used two forms of PKC: protein that was quantitatively phosphorylated at the two carboxy-terminal positions and partially dephosphorylated on the activation loop (mature PKC), or dephosphorylated protein. The former represents native PKC isolated from the detergent-soluble fraction of insect cells — mass spectrometric analysis previously established that approximately 60% of this PKC is phosphorylated on the activation loop and quantitatively phosphorylated on the two carboxy-terminal positions [9]. The latter form of PKC was generated by treating native PKC with the catalytic subunit of protein phosphatase 1 [12]. Phosphorylation on the activation loop was detected using the P500 antibody.

Figure 4a shows that incubation with wild-type PDK-1 resulted in a significant increase in the phosphorylation of the activation loop of both mature PKC α (lane 2) and PKC βII (lane 6) relative to the basal phosphorylation state observed upon incubation with no kinase (data not shown) or with PDK-1^{inact} (lanes 1,5). Immunoreactivity increased by approximately 40% upon incubation with wild-type PDK-1, which is consistent with almost complete phosphorylation of the activation loop. This increased immunoreactivity did not result from changes in the amount of PKC, as assessed by probing blots with antibodies to each isozyme that do not discriminate between the different phosphorylation states of PKC (Figure 4a, lanes 3,4,7,8).

Figure 4b shows that the phosphorylation of mature PKC βII by PDK-1 did not depend on the PKC activators phosphatidylserine and Ca^{2+}, or on the presence of PI(3,4,5)P_3. The initial rate of phosphorylation was the same (approximately 0.01 pmol phosphate incorporated per minute) in the absence of vesicles (open circles), or in the presence of phosphatidylcholine vesicles containing phosphatidylserine and diacylglycerol (filled circles), PI(3,4,5)P_3 (open squares), or phosphatidylserine, diacylglycerol and

Figure 5



PDK-1 specifically phosphorylates the activation loop of the bacterially expressed kinase domain of PKC βII. **(a)** Western blots of lysates from cells transfected with a construct carrying the kinase domain of PKC βII (50 nM) following incubation with either PDK-1 (30 nM; lane 3) or PDK-1^{inact} (lane 2) in the presence of Mg^{2+}–ATP. The blots were probed with P500 antibody, or with an antibody that recognizes phosphorylated Thr643 and, to a much lower extent, phosphorylated Thr641 (P634), or with an antibody that recognizes phosphorylated Ser660 (P660). Lane 1 contains mature, full-length PKC from the same blots. **(b)** The time course of phosphorylation by PDK-1 of the bacterially expressed kinase domain of PKC βII. The purified catalytic domain (15 nM) was incubated with PDK-1 (80 nM) in the presence of Mg^{2+}–ATP for the indicated times. The arrow denotes when an additional aliquot of PDK-1 was added to the reaction mixture.

$PI(3,4,5)P_3$ (filled squares). Thus, the rate of phosphorylation of PKC was not sensitive to whether it was membrane associated, nor did it require the active conformation of the enzyme.

To explore whether the phosphorylation by PDK-1 was limited to the activation loop, we examined the phosphorylation of a bacterially expressed kinase domain (this construct is inactive and therefore does not autophosphorylate). The western blot in Figure 5a shows that a construct comprising residues 286–673 of PKC βII was phosphorylated on the activation loop by PDK-1 (lane 3; P500), but was not phosphorylated on the carboxy-terminal phosphorylation sites Thr634 or Thr641 (lane 3; P634) or Ser660 (lane 3; P660). Antibodies that selectively recognize phosphate on these latter positions recognized similar amounts of mature PKC βII (Figure 5a, lane 1) but did not recognize the kinase domain constructs (Figure 5a, lane 2). Consistent with the specific phosphorylation of the activation loop, a construct in which the activation loop threonine (Thr500) and two adjacent threonine residues (Thr497 and Thr498) were replaced with alanine residues was not radiolabeled following incubation with PDK-1 and [^{32}P]ATP (data not shown), whereas the wild-type kinase construct was effectively labelled with ^{32}P (Figure 5b). These data indicate that Thr500 is the sole substrate in the kinase domain for PDK-1.

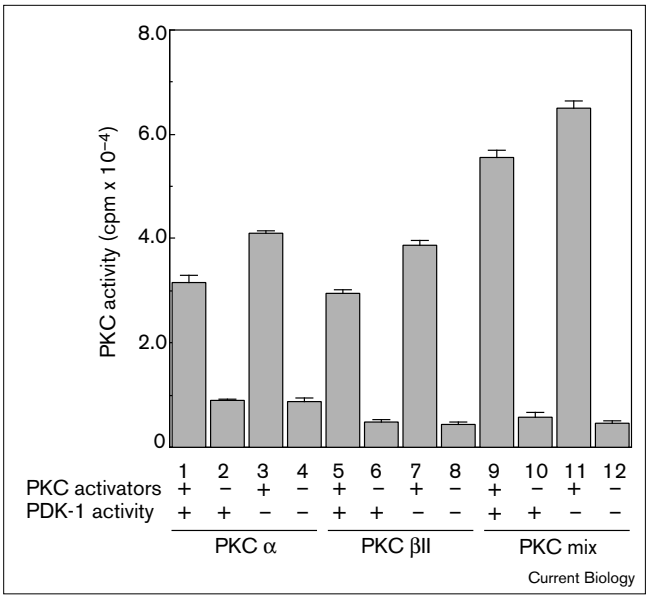
Figure 5b presents a time course for the phosphorylation of the catalytic domain of PKC following incubation with PDK-1. After 60 minutes, 0.2 mol phosphate were incorporated per mol PKC. This stoichiometry increased to 0.4 upon the renewed addition of PDK-1, indicating that the substoichiometric phosphorylation was due to PDK-1 losing activity rather than to saturation of substrate sites.

The effect of phosphorylation by PDK-1 on PKC activity

We next addressed whether the phosphorylation by PDK-1 affected either the basal or the cofactor-dependent activity of mature PKC. As described for the experiments in Figure 4, PKC was stoichiometrically phosphorylated by PDK-1. Figure 6 shows that the basal (cofactor-independent) activity of PKC α, βII or a mixture of rat brain isozymes was similar following phosphorylation by PDK-1 (columns 2, 6 and 10, respectively) or following incubation with PDK-1^{inact} (columns 4, 8 and 12, respectively). Thus, phosphorylation of the activation loop of mature enzyme does not activate the conventional PKC isozymes. Nor did phosphorylation by PDK-1 increase the maximal rate of catalysis observed in the presence of saturating levels of the cofactors Ca^{2+}, phosphatidylserine and diacylglycerol (compare columns 1 and 3, 5 and 7, and 9 and 11). Curiously, we noted that the activity of PKC following incubation with PDK-1^{inact} was typically slightly elevated relative to that following incubation with wild-type PDK-1 or with no PDK-1 (data not shown). The finding that phosphorylation by PDK-1 does not significantly affect the maximal catalytic rate of mature PKC is consistent with previous findings showing that dephosphorylation of Thr500 and Ser660 of mature PKC βII does not affect the enzyme's V_{max} [9,12].

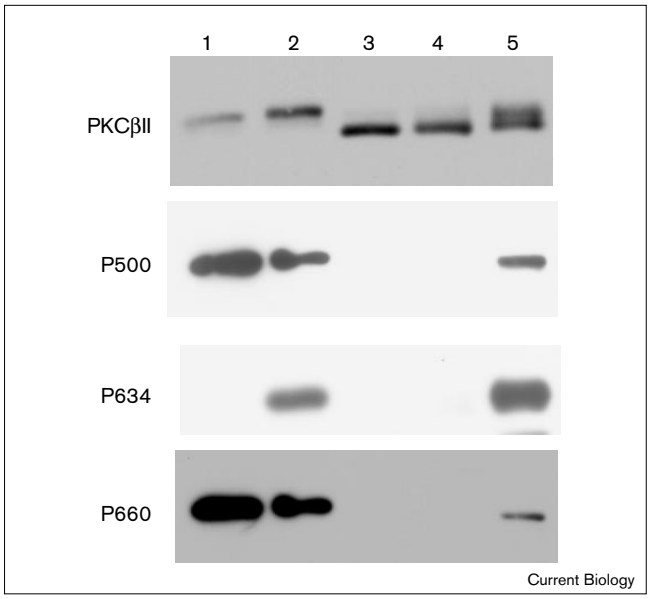
The above data revealed that PDK-1 does not regulate the activity of mature PKC. This led us to ask the question: does PDK-1 regulate the activity of non-phosphorylated enzyme? Pure PKC βII was treated with protein phosphatase 1 under conditions that resulted in the quantitative dephosphorylation of the enzyme and a complete loss of enzymatic activity [9,12]. The dephosphorylated protein was then incubated with PDK-1 and Mg^{2+}–ATP in the presence of PKC activators, and the phosphorylation

Figure 6



Phosphorylation by PDK-1 does not affect the activity of conventional PKC isozymes. PKC α, βII and a mixture of isozymes (PKC mix) were incubated with purified PDK-1 or PDK-1^{inact} in the presence of Mg^{2+}–ATP. Following this incubation, PKC activity was measured in the presence or absence of PKC activators (phosphatidylserine, diacylglycerol and Ca^{2+}), as described in Materials and methods. The data represent the mean ± SEM of triplicate assays.

Figure 7



The phosphorylation of dephosphorylated PKC on its activation loop triggers carboxy-terminal phosphorylation. PKC βII (3 nM) was treated with the catalytic subunit of protein phosphatase 1 to effect quantitative dephosphorylation. The dephosphorylated enzyme was then incubated with PDK-1^{inact} or PDK-1 (30 nM) and Mg^{2+}–ATP. Western blots show wild-type PKC (lane 1), wild-type enzyme that was autophosphorylated *in vitro* (lane 2), wild-type enzyme treated with protein phosphatase 1 (lane 3) and then incubated with PDK-1^{inact} (lane 4) or wild-type PDK-1 (lane 5). The blots were probed with a non-discriminatory antibody against PKC βII (PKCβII; Santa Cruz), P500 antibody, and antibodies that recognize phospho-Thr634 (P634) or phospho-Ser660 (P660).

state of the protein was subsequently examined using antibodies that specifically recognize the activation loop, the *in vitro* autophosphorylation site Thr634 [21], or the *in vivo* autophosphorylation site Ser660.

Figure 7 shows that the treatment of mature PKC with protein phosphatase 1 resulted in the loss of immunoreactivity to the P500 and P660 antibodies and an increase in electrophoretic mobility (lane 3) relative to the untreated enzyme (lane 1). The untreated enzyme was not significantly labeled by the P634 antibody (this antibody weakly recognizes P641 [21]). The autophosphorylation of PKC *in vitro* (Figure 7, lane 2), however, resulted in a dramatic increase in reactivity to the P634 antibody, due to intramolecular autophosphorylation on Thr634 [11]. The incubation of quantitatively dephosphorylated PKC βII with active PDK-1 (Figure 7, lane 5), but not with PDK-1^{inact} (Figure 7, lane 4), resulted in phosphorylation of the activation loop. Thus, PDK-1 phosphorylates the activation loop of PKC βII independently of whether or not the carboxyl terminus is phosphorylated. Importantly, phosphorylation of the activation loop was accompanied by phosphate incorporation into Ser660 (Figure 7, lane 5, P660 panel). In addition, the PDK-1-treated enzyme was heavily phosphorylated on the *in vitro* autophosphorylation site, Thr634: the degree of phosphorylation of the phosphatase-treated, re-phosphorylated sample (Figure 7, lane 5) was similar to that of native enzyme allowed to autophosphorylate *in vitro* (Figure 7, lane 2). Note that approximately half of the PKC in the PDK-1-treated sample had a decreased electrophoretic mobility, indicating that half of the protein had been phosphorylated at the carboxyl terminus. These data suggest that PDK-1 phosphorylation at the activation loop triggers the autophosphorylation activity of PKC.

Discussion

Data from studies *in vivo* and *in vitro* suggest that PDK-1 controls the activation-loop phosphorylation switch of the conventional PKC isozymes. More specifically, the data presented here establish that PDK-1 phosphorylates the activation loop of conventional PKC isozymes, and that this phosphorylation controls the maturation of PKC but does not actually modulate the activity of mature (carboxy-terminally phosphorylated) enzyme.

PDK-1 phosphorylates the activation loop of conventional PKC isozymes

Experiments performed *in vitro* reveal that PDK-1 directly and specifically phosphorylates the activation loop of the

conventional PKC isozymes. This phosphorylation is independent of the phosphorylation state of the carboxyl terminus: mature PKC that is quantitatively phosphorylated at the carboxy-terminal sites, the bacterially expressed kinase domain that has never been phosphorylated, or mature PKC that has been dephosphorylated *in vitro* all serve as effective substrates for PDK-1. Neither does this phosphorylation appear to require the binding of cofactor to either kinase: PDK-1 phosphorylated mature PKC at the same rate in the absence or presence of PKC's cofactors or $PI(3,4,5)P_3$. Curiously, molecular modeling suggests that occupation of the active site by the pseudosubstrate effectively masks Thr500 [22]. Consistent with this proposal, the activation-loop phosphate group is relatively resistant to phosphatases when the pseudosubstrate occupies the active site; efficient dephosphorylation requires the active conformation of PKC [12]. It is thus surprising that for the reverse reaction, access of PDK-1 to Thr500 is not dependent upon pseudosubstrate removal. The elucidation of the crystal structures of a number of kinases with or without activation loop phosphates reveals dramatic changes in the conformation of this loop, however [1,2,4]. Thus, one possibility is that the non-phosphorylated activation loop of PKC is unstructured and much more accessible than the phosphorylated activation loop. If this is the case, phosphorylation would not require pseudosubstrate exposure, whereas dephosphorylation would require the active conformation of the enzyme.

Activation-loop phosphorylation does not regulate the maximal activity of mature PKC

In vitro kinase assays revealed that phosphorylation of the activation loop of mature conventional PKC isozymes has no detectable effect on their maximal catalytic activity. Phosphorylation by PDK-1 had no significant effect on either the basal activity or lipid–Ca^{2+}-stimulated activity of PKC α, βII or a mixture of rat brain PKC isozymes. Analysis of phosphorylation-site mutants previously led to the suggestion that negative charge on the activation loop is an essential prerequisite to forming catalytically competent PKC but that, once the mature enzyme is formed, the phosphate group on the activation loop is not necessary for catalysis. First, mutation of Thr500 to valine in PKC βII, or of the corresponding Thr497 to alanine in PKC α, results in a form of kinase that cannot achieve catalytic competence: it cannot be activated by cofactors [23,24] and it is not phosphorylated at the carboxyl terminus (A.S. Edwards and A.C.N., unpublished observations). Second, dephosphorylation studies have revealed that once PKC has been processed by the two carboxy-terminal phosphorylations, negative charge at the activation loop is no longer required for catalysis: treatment of mature PKC with protein phosphatase 2A dephosphorylates the activation loop and Ser660, producing a kinase that has the same V_{max} as the mature PKC [9,12]. The lack of effect of activation-loop phosphorylation on the maximal catalytic activity of conventional PKC isozymes noted in this study confirms that phosphate on the activation loop does not affect the catalytic rate of mature (carboxy-terminally phosphorylated) PKC. Thus, phosphorylation of the activation loop is required to prime the conventional PKC isozymes to permit the two rapid (auto)phosphorylations at the carboxyl terminus. The phosphorylation of substrate by PKC is dependent on cofactor binding, however.

Phosphorylation of the activation loop triggers carboxy-terminal phosphorylation of unphosphorylated enzyme

When mature PKC is quantitatively dephosphorylated by treatment with protein phosphatase 1, the enzyme is inactivated and incapable of re-autophosphorylation [12]. Here, we show that phosphorylation of the activation loop by PDK-1 results in the re-activation of PKC, as assessed by the re-incorporation of phosphate on the *in vivo* autophosphorylation site Ser660 and the *in vitro* autophosphorylation site, Thr634. The bacterially expressed kinase domain of PKC is phosphorylated by PDK-1 only on Thr500 and not on the carboxy-terminal sites, suggesting that the carboxy-terminal sites are not substrates for PDK-1 (the bacterially expressed PKC is catalytically inactive, even when a mutant form of PKC containing a glutamic acid residue instead of threonine at position 500 is expressed, suggesting that inactivity results from misfolding).

PDK-1 regulates the fraction of mature PKC βII *in vivo*

Co-expression studies revealed that PDK-1 regulates the fraction of mature PKC βII in COS-7 cells. Under our cell culture conditions, typically about 20% of the total expressed PKC βII is not phosphorylated, as assessed by its increased mobility on SDS–PAGE ([9] and Figure 4). This pool of PKC partitions with the detergent-insoluble fraction; phosphorylation at the two carboxy-terminal sites coincides with the release of the enzyme into the detergent-soluble fraction [9,20]. In this study, we show that co-expression with PDK-1 resulted in a twofold reduction in the amount of faster-migrating, non-phosphorylated PKC. In contrast, co-expression with PDK-1^{inact} resulted in an almost twofold increase in the amount of faster-migrating, non-phosphorylated PKC βII. These results suggest that phosphorylation by PDK-1 provides the first, and possibly rate-limiting, step in the processing of PKC by phosphorylation: this processing is accelerated when PDK-1 is overexpressed, and is inhibited in the presence of the dominant–negative PDK-1^{inact}.

Results from this study also showed that the fraction of mature PKC βII that is phosphorylated on the activation loop was not detectably affected by overexpression of the kinase-active or kinase-inactive PDK-1 constructs. In contrast, co-expression of PDK-1^{inact} caused an approximately twofold reduction in the fraction of mature PKC α overexpressed in COS-7 cells relative to co-expression of the wild-type PDK-1. Mass-spectrometric analysis has previously

established that approximately 60% of the PKC present in the detergent-soluble fraction of mammalian tissue (bovine retinas) or insect cells is phosphorylated on the activation loop [9]. If phosphorylation of the activation loop is required to process mature PKC, as mutagenesis data suggest, then phosphatases must regulate the phosphorylation state of the activation loop of mature PKC. The current data would thus indicate that overexpression of PDK-1 can alter the equilibrium between the dephosphorylated and phosphorylated mature PKC; this effect was more evident for PKC α than for PKC βII.

PDK-1 associates with PKC βII *in vivo*

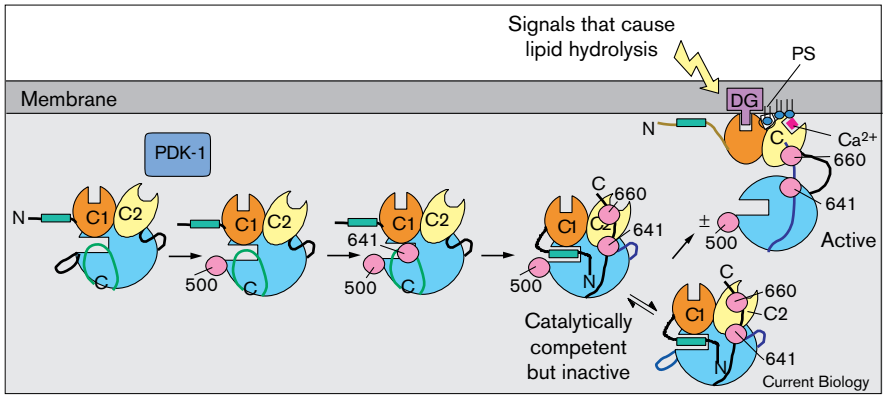
Co-immunoprecipitation experiments revealed that PKC βII associates with PDK-1 *in vivo*. This association was independent of modulation by PI3-kinase: similar amounts of PKC βII associated with PDK-1 in serum-starved, serum-treated or wortmannin-treated 293 cells. These data suggest that PDK-1 associates constitutively with conventional PKC isozymes.

Contrasting roles of activation-loop phosphorylation in conventional and atypical PKC isozymes

For conventional PKC isozymes, phosphorylation of the activation loop is required to process mature PKC [10,23,24]. This phosphorylation does not, however, regulate the maximal catalytic activity of the mature enzyme. Activation results from the specific interaction of its regulatory domains with the second messenger diacylglycerol and with phosphatidylserine; these interactions provide the energy to release the autoinhibitory pseudosubstrate domain from the active site. A similar role for the phosphorylation of the activation loop has been ascribed to protein kinase A, in which phosphorylation of the activation loop is required for the maturation of this kinase. As with PKC, this phosphorylation event does not activate protein kinase A, but rather, cAMP binding to the regulatory subunit is required to release an autoinhibitory domain from the active site [25]. Thus, the conventional PKC isozymes and protein kinase A have two regulatory switches: phosphorylation of the activation loop, which renders the enzyme catalytically competent (that is, capable of becoming activated), and cofactor binding, which removes the autoinhibitory domain from the active site.

The regulation of conventional PKC isozymes by PDK contrasts strikingly with its regulation of the atypical PKC isozymes. Recently, PKC ζ has been shown to be phosphorylated by PDK-1 on its activation loop, an event which serves as a molecular switch to turn on the catalytic activity of the kinase [19,26]. This phosphorylation-dependent activation occurs in the absence of cofactors, although it is stimulated by the presence of phospholipid mixtures containing $PI(3,4,5)P_3$. Thus, this phosphorylation event is similar to that of PKB, which is stimulated by $PI(3,4,5)P_3$, and contrasts with that of the conventional PKC isozymes, which is independent of $PI(3,4,5)P_3$ and thus more similar to that of p70S6 kinase. The direct activation of PKC ζ resulting from phosphorylation of the activation loop suggests that the atypical PKC isozymes are regulated by a mechanism that is similar to that of Cdk2: phosphorylation of the activation loop serves as a direct 'on–off' switch for catalysis [4].

Figure 8



Model showing the proposed role of PDK-1 in the processing of conventional PKC isozymes. Newly synthesized PKC is shown on the far left in a conformation in which the activation loop is disordered. The loop lies to the left of the kinase domain (blue circle with a cleft representing active site); the carboxyl terminus is shown as occupying the active site (based on finding that autophosphorylation here proceeds by an intramolecular reaction [11,30]) and the pseudosubstrate (green rectangle) is shown displaced from the active site (based on finding that the pseudosubstrate in precursor PKC is proteolytically labile; E.M.D. and A.C.N., unpublished observations). Phosphorylation by PDK-1 on the activation loop (Thr500 in PKC βII) is the first event in the processing of PKC; this phosphorylation does not appear to be regulated by cofactors for either kinase. It appears to be the first step in the maturation of PKC, as overexpression of a kinase-inactive PDK-1 results in accumulation of unphosphorylated, precursor PKC. The transphosphorylation is followed by two rapid autophosphorylations at the carboxyl terminus (Thr641 and Ser660 in PKC βII) that lock PKC in a catalytically competent conformation [19,31]). Phosphates are shown in pink. The mature enzyme phosphorylated at three positions localizes to the cytosol where it is competent to respond to second messengers. This species of PKC is subject to a specific dephosphorylation at the activation loop (lower right), an event that does not affect the enzyme's cofactor-dependent activation. The generation of diacylglycerol (DG) causes mature PKC to translocate to the membrane (upper right), where the interaction of its two membrane-targeting domains, C1 (orange oval) and C2 (yellow oval), with diacylglycerol and phosphatidylserine provides the energy to release the pseudosubstrate from the active site.

Conclusions

The above data reveal that PDK-1 phosphorylates the activation loop of conventional PKC isozymes, and that this phosphorylation is a step required for the maturation of conventional PKC isozymes. This phosphorylation does not activate PKC, but rather, it serves to trigger the two carboxy-terminal (auto)phosphorylations that are required to lock PKC in a catalytically competent conformation. Once this mature conformation is achieved, the phosphate group on the activation loop does not regulate the activity of the enzyme. The mature enzyme is activated by cofactor binding, which releases the pseudosubstrate from the active site (Figure 8). Dual regulation by phosphorylation and cofactors affords exquisite sensitivity in the molecular mechanisms regulating kinase function.

Materials and methods

Antibodies and constructs

Bovine brain L-α-phosphatidylserine and *sn*-1,2-dioleoylglycerol were obtained from Avanti Polar Lipids. PI(3,4,5)P_3 was obtained from Matreya. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore. Triton X-100, microcystin, and peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Calbiochem. The chemiluminescence SuperSignal kit was purchased from Pierce and wortmannin from Sigma. A PKC-selective peptide (Ac-FKKSFKL-NH_2) [27] was synthesized in the laboratory of E. Komives at the University of California at San Diego. PKC βII was purified from the baculovirus expression system, as previously described [28]. Baculovirally expressed PKC α was partially purified by Q Sepharose chromatography. PKC was stored at −20°C in 10 mM Tris buffer pH 7.5 (4°C), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 150 mM KCl and 50% glycerol. The high-speed supernatant following homogenization of rat brain in buffer containing 1% Triton X-100, 20 mM Tris buffer pH 7.5 (4°C), 1 mM EDTA, 1 mM EGTA, 1 mM DTT was stored in an equal volume of glycerol at −20°C. GST-tagged PDK-1 and a catalytically-inactive mutant of PDK-1 (containing point mutation K110N) were expressed in *Escherichia coli*, purified on glutathione-conjugated agarose (Pharmacia), and stored at −20°C in 12 mM Tris buffer pH 7.5 (4°C), 6 mM glutathione and 40% glycerol [19]. Histidine-tagged constructs of the catalytic domain of PKC βII (residues 286–673; Histidine tag at the amino terminus) and the T447A/T448A/T500A triple mutant (J. Johnson, unpublished data) were expressed in *E. coli* and purified on Probond nickel resin (Invitrogen) and stored at −20°C in 50 mM Tris buffer pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 150 mM imidizole. Polyclonal antibodies against the carboxyl terminus of PKC βII were purchased from Santa Cruz Biotechnology and Calbiochem. Monoclonal antibodies against PKC α or Myc were obtained from Transduction Laboratories or Boehringer, respectively. A polyclonal antibody (P500) directed against the phosphorylated activation loop of PKC βII was generated by immunizing rabbits with a phosphopeptide based on the sequence: $_{494}$DGVTTK<u>pT</u>FCGTPD$_{506}$ (<u>pT</u> refers to phospho-Thr, corresponding to Thr500). The specificity for the activation loop phosphate has been previously described [19]: P500 specifically labels the phosphorylated activation loop of conventional, novel, and atypical PKC isozymes but not the unphosphorylated proteins (based on the detection limit of our assay, there is a >100-fold increase in immunoreactivity for phosphorylated species compared with unphosphorylated species; this antibody does not label the carboxy-terminal sites nor additional *in vitro* autophosphorylation sites [19]). An antibody directed against phosphorylated Thr634 (strong reactivity) and Thr641 (weak reactivity) [21] was provided by David Sweatt (Baylor College of Medicine), and one directed against phosphorylated Ser660 was provided by Michael Comb (New England Biolabs). The cDNA for rat PKC βII [29] was a gift from Daniel E. Koshland, Jr. (University of California, Berkeley). The catalytic subunit of protein phosphatase 1 was a gift from Anna DePaoli-Roach (Indiana University).

PDK-1 phosphorylation of PKC

Full-length PKC βII or α (approximately 3 nM) was incubated with purified PDK-1 or PDK-1^{inact} (40 nM) in 10 mM HEPES pH 7.5, 500 μM ATP, 50 μM $MgCl_2$ and 1 mM DTT at 30°C for 1 h, unless otherwise stated. In some experiments, 200 μM $CaCl_2$ and sonicated vesicles (100 μM total lipid) composed of diacylglycerol : phosphatidylserine : phosphatidylcholine (5:40:55 mol fraction), PI(3,4,5)P_3 : phosphatidylcholine (10:90 mol fraction), or diacylglycerol : PI(3,4,5)P_3 : phosphatidylserine : phosphatidylcholine (5:10:40:45 mol fraction) were included in the phosphorylation mixture. Two kinase-domain constructs (30 nm), wild-type PKC βII catalytic domain or T447A/T448A/T500A catalytic domain, were incubated with purified PDK-1 or PDK-1^{inact} (40 nM) in 10 mM HEPES buffer pH 7.5, 500 μM ATP, 50 μM $MgCl_2$ at 30°C for 1 h, unless otherwise stated. Dephosphorylated PKC was obtained by incubating pure PKC βII (6 nM) with the catalytic subunit of protein phosphatase 1 (15 nM) in the presence of 10 mM HEPES buffer pH 7.5, 200 μM $MnCl_2$, 1 mM DTT, 200 μM $CaCl_2$, and multilammellar suspensions of 140 μM phosphatidylserine, 4 μM diacylglycerol at 22°C for 30 min, as described [12]. The dephosphorylation reaction was stopped by the addition of 1 μM microcystin. The dephosphorylated protein was then incubated, in the same reaction mixture, with PDK-1 or PDK-1^{inact} (30 nM) in the presence of 500 μM ATP and 50 μM $MgCl_2$, as described above, for 1 h at 30°C. Reactions were quenched by the addition of 1/5 volume of SDS–PAGE sample buffer [28]. Proteins were separated by SDS–PAGE (7% polyacrylamide) and electrophoretically transferred to PVDF membranes. Western blots were performed by probing PVDF membranes with the indicated primary antibodies, incubating with peroxidase-conjugated secondary antibodies, and detecting labeling with chemiluminescence. In some cases, western blots were analyzed using a BioRad Molecular Imager System in order to quantify antibody labeling.

Determination of catalytic domain stoichiometry

Purified PKC βII catalytic domain (15 nM) was incubated with PDK-1 or PDK-1^{inact} (80 nM) at 30°C in the presence of 10 mM HEPES buffer pH 7.5, 500 μM [γ-^{32}P]ATP, 50 μM $MgCl_2$ for 1–60 min. At 60 min, an additional 70 nM PDK-1 or PDK-1^{inact} was added and the reaction was allowed to continue for an additional 30 min. Phosphorylation reactions were quenched by the addition of 1/5 volume SDS–PAGE sample buffer; proteins were separated by SDS–PAGE and stained with Coomassie blue. Bands containing the catalytic domain were excised from the gel and ^{32}P incorporation was determined by liquid scintillation counting in 5 ml scintillation fluid (Biosafe II, Research International Corp.).

PKC activity after phosphorylation by PDK-1

PKC α, βII, or rat brain Triton X-100 soluble supernatant (all containing approximately 0.3 nmol PKC) were incubated with PDK-1 or PDK-1^{inact} (20 nM) in 10 mM HEPES, 1 mM DTT and 100 μM [γ-^{32}P]ATP for 30 min at 30°C. PKC activity was measured by adding 150 nM selective peptide in the presence or absence of lipid (140 μM phosphatidylserine and 4 μM diacylglycerol) and 125 μM $CaCl_2$ in a final volume of 80 μl for 5–7 min at 30°C. Reactions were quenched by the addition of 25 μl of a solution containing 0.1 M ATP and 0.1 M EDTA (pH 8–9). Aliquots (85 μl) were spotted onto P81 ion-exchange chromatography paper and washed four times with 0.4% (v/v) phosphoric acid and once with 95% ethanol; ^{32}P incorporation was then detected by liquid scintillation counting in 5 ml scintillation fluid.

Co-expression of PKC and PDK-1 in COS-7 cells

COS-7 cells were transfected with 0.5 μg vector DNA (pcDNA3), 0.5 μg PKC βII or PKC α DNA (in pcDNA3), or 0.5 μg PKC βII or PKC α DNA (in pcDNA3) and 2 μg Myc-tagged PDK-1 or PDK-1^{inact} DNA [19] using the Superfect transfection method (Qiagen). DNA was added to 150 μl serum-free DMEM and 15 μl Superfect reagent and incubated at 22°C for 5 min. The DNA-containing mixture was added to cells plated at 70% confluency on 6-well tissue culture dishes. Cells

were incubated overnight at 37°C, and then washed with DMEM containing 10% FBS. Cells were harvested 48 h after transfection in 250 µl lysis buffer containing 20 mM HEPES buffer pH 7.5, 1% Triton X-100, 1 mM DTT, 300 µM PMSF, 200 µM benzamidine, 40 µg ml^{-1} leupeptin and 100 nM microcystin. Proteins in cell lysates were separated by SDS–PAGE (7% polyacrylamide) and electrophoretically transferred to PVDF membranes. PKC was detected by western blot analysis, as described above. The PKC activity in 5 µl COS-7 cell lysate was obtained by measuring the Ca^{2+}–lipid-dependent phosphorylation of a PKC-selective peptide, as described [22].

Immunoprecipitation

293 cells were transfected with Myc-tagged PDK-1 cDNA (0.4 µg) alone or with PKC βII cDNA (0.6 µg) using the Lipofectamine (Life Technologies) procedure, incubated for 12 h in DMEM plus 10% heat-inactivated FBS, and then serum-starved by incubation in DMEM (no serum) for 24 h, as described [19]. Cells were then treated with DMEM containing 10% FBS for 20 min and then subjected to lysis as described [19]. In some cases, cells were treated with 100 nM wortmannin for 20 min prior to incubation with FBS. PDK-1 was immunoprecipitated with the anti-Myc monoclonal antibody 9E10 (Boehringer Mannheim), samples analyzed on 7.5% polyacrylamide gels, and blots probed with antibodies to PKC βII (Santa Cruz). PDK-1 and PKC βII expression was confirmed by western blot analysis of whole cell lysates using antibodies to the Myc tag or PKC βII, respectively.

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