Persistent activation of protein kinase D (PKD) via protein kinase C (PKC)-mediated signal transduction is accompanied by phosphorylation at Ser\(^{744}\) and Ser\(^{748}\) located in the catalytic domain activation loop, but whether PKC isoforms directly phosphorylate these residues, induce PKD autophosphorylation, or recruit intermediate upstream kinase(s) is unclear. Here, we explore the mechanism whereby PKC activates PKD in response to cellular stimuli. We first assessed in vitro PKC-PKD transphosphorylation and PKD activation. A PKD\(^{738-753}\) activation loop peptide was well phosphorylated by immunoprecipitated PKC isoforms, consistent with similarities between the loop and their known substrate specificities. A similar peptide with glutamic acid replacing Ser\(^{748}\) was preferentially phosphorylated by PKC\(\varepsilon\), suggesting that PKD containing phosphate at Ser\(^{748}\) is rapidly targeted by this isoform at Ser\(^{744}\). When incubated in the presence of phosphatidylinositol, phorbol 12,13-dibutyrate and ATP, intact PKD slowly autophosphorylated in the activation loop but only at Ser\(^{748}\). In contrast, addition of purified PKC\(\varepsilon\) to the incubation mixture induced rapid Ser\(^{744}\) and Ser\(^{748}\) phosphorylation, concomitant with persistent 2–3-fold increases in PKD activity, measured using reimmunoprecipitated PKD to phosphorylate an exogenous peptide, syntide-2. We also further examined pleckstrin homology domain-mediated PKD regulation to determine its relationship with activation loop phosphorylation. The high constitutive activity of the pleckstrin homology (PH) domain deletion mutant PKD-PHD was not abrogated by mutation of Ser\(^{744}\) and Ser\(^{748}\) to alanines, suggesting that one function of activation loop phosphorylation in the PKD activation mechanism is to relieve autoinhibition by the PH domain. These studies provide evidence of a direct PKC-PKD phosphorylation cascade and provide additional insight into the activation mechanism.
PKD Activation Loop Phosphorylation by PKCε Relieves Autoinhibition

(21–24) or lysophosphatidic acid (23, 25), activation of receptor tyrosine kinases such as the platelet-derived growth factor receptor (21), signaling via heterotrimeric and monomeric G proteins (26, 27), and oxidative stress (28) were demonstrated to induce PKD activation in a wide variety of cell types including Swiss 3T3 fibroblasts, small cell lung cancer and pancreatic cancer, normal epithelial and smooth muscle cell lines, cardiocytes, and lymphocytes (23, 25, 29–34).

Throughout all these studies, multiple lines of evidence have indicated that PKC activity is indispensable for PKD activation by cellular stimuli. Cell treatments with phorbol esters or other agents that bypass surface receptors and directly stimulate PKCs are potent triggers of cellular PKD activation (20, 35). Cotransfection of PKD, together with active mutant forms of PKCs or PKCζ, also dramatically activates PKD in the absence of cell stimulation (20, 36). Preincubation of cells with the specific PKC inhibitors GF 109203X (37) or Ro 31-8220 (38) that do not directly inhibit PKD (20) impairs PKD activation by all stimuli, reducing it by 70–80% when used at maximally effective concentrations (28). Furthermore, PKD interacts preferentially with PKCζ, forming complexes involving the PH domain (36). These findings imply the existence of PKC-PKD protein kinase cascade(s), and we have postulated that some PKC-dependent biological effects involve PKD acting either in parallel or as a downstream intermediate. In particular, PKD has been reported recently to mediate several important cellular activities and processes, including function and organization of the Golgi apparatus (39), metastatic tumor cell invasion (40), epidermal growth factor receptor signaling (41, 42), Na+/K+ antiporter (43), mitogen-activated protein kinase activation (44), proliferation (45), and adenomatous transformation (46). Moreover, critical downstream targets of PKD signaling are beginning to emerge (47–49). Therefore, the mechanism whereby PKC activates PKD has been attracting intense interest.

Our previous studies identified Ser744 and Ser748 in the PKD activation loop as phosphorylation sites critical for PKC-mediated PKD activation. Whereas a PKD mutant with Ser744 and Ser748 mutated to alanines (PKD-S744A/S748A) could not be activated by cellular stimuli, a mutant with these residues mutated to glutamic acid residues (PKD-S744E/S748E) possessed dramatically increased constitutive activity (50). We have shown that Ser744 and Ser748 are phosphorylated in vivo during PKD activation in response to phorbol ester stimulation, in a manner blocked by preincubation with GF 109203X (50, 51). Specifically, in two-dimensional 32P tryptic phosphopeptide maps, individual spots were selectively eliminated when PKD forms point-mutated at Ser744 or Ser748 were used in transfections (50). Because kinase-deficient PKD, which retains Ser744 and Ser748, also became phosphorylated during stimulation with phorbol ester, we concluded that transphosphorylation at these sites by an upstream kinase, rather than PKD, was a critical factor responsible for PKD activation. Where PKD forms at both Ser744 and Ser748 were used, supporting the view that PKC, but not PKD, activity induced phosphorylation of these residues. However, the precise mechanisms involved remain unclear (20, 36, 51–53). In the present study, we used peptides, full-length PKD, and mutant PKD proteins to examine whether PKC mediates direct PKD activation loop Ser744 and Ser748 phosphorylation and activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Stock cultures of COS-7 cells were maintained in 10-cm tissue culture plates by subculturing every 3–4 days in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. Confluent stock cells were reseeded at a density of 6 × 104 cells in 6-cm dishes, 8–18 h prior to transfections. All transfections and cotransfections were carried out with equivalent amounts of DNA (5 μg/dish for single transfections, 3 μg/dish of each DNA for cotransfections), using vector pcDNA3 as the control DNA added to single transfections. Transfections were carried out in Opti-MEM (Invitrogen) using Lipofectin (Invitrogen) at 10 μl/dish. DNA-Lipofectin complexes were formed according to the protocol provided by the manufacturer and then cell cultures were layered over with these DNA complexes in a final volume of 2.5 ml/dish in the absence of serum and incubated at 37 °C in a humidified atmosphere containing 5% CO2, for 5–6 h or overnight to allow uptake of complexes. Fetal bovine serum (10% final concentration) in Opti-MEM was then added to dishes to yield a final volume of 5 ml/dish. Cells were used for experiments after a further 72 h of incubation.

Plasmid Constructs and Fusion Proteins—Plasmid constructs encoding PKCs and wild-type and mutant PKD forms used in this study have been described previously (17, 18, 20, 36, 50). The construct pcDNA3-PKDΔPH/S744A/S748A was generated by standard subcloning procedures. Thus, a Bsu36I restriction fragment within pcDNA-PKDΔPH (nucleotides 2305–2827 of PKD) was replaced with a corresponding Bsu36I fragment excised from pcDNA3-PKD-S744A/S748A. The GST-Cat and GST-Cat/S744A/S748A fusion proteins were constructed in pGEX-4T by standard subcloning procedures. Thus, C-Terminal BmorHI fragments (corresponding to amino acids 559–918) of either wild-type PKD or PKD-S744A/ S748A were fused in-frame at the C-terminal of the sequence encoding glutathione S-transferase in the construct pGEX-4T (Amersham Biosciences). Fusion proteins were isolated by affinity chromatography using glutathione-Sepharose 4B (Roche Molecular Biochemicals) and eluted with 10 mM glutathione.

Immuno precipitation—COS-7 cells transfected either with wild-type or mutant PKD or PKC isoforms were treated as indicated in the figures and lysed in 1 ml of lysis buffer (1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride in 50 mM Tris-HCl, pH 7.4). Small amounts (typically 1/10) of these lysates were saved and combined with equal amounts of IP buffer and incubation with 1 μg of the appropriate antibody for 4 h at 4 °C. Immune complexes were washed twice with IP buffer containing 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 1 mM leupeptin, and 1 mM pepstatin A. Immunoprecipitates were dissolved in 2× SDS–PAGE sample buffer and run on a 10% or 12% gel, and transferred to nitrocellulose. Membrane-bound PKC or PKD immunoprecipitates, medium was removed, and lysis buffer was added to cells on ice. These lysates were cleared by centrifugation at 15,000 rpm for 10 min, and the cleared supernatants were combined with antibodies and protein A-agarose (30 μl) and placed on a rotating rotor at 4 °C for 3 h. Immune complexes were collected by brief spin, and washed thoroughly before subsequent kinase assays. Antibodies used for immunoprecipitations were either the isomer-specific anti sera from Santa Cruz Biotechnology (for PKCε, PKCζ, or PKCζ) or the previously described PA-1 antisemur (17) for PKD (1:100 dilution).

Peptide Phosphorylation Assays—For assays of peptide phosphorylation, PKD or PKCs, immune complexes were washed twice with lysis buffer and then twice with kinase buffer consisting of 30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 2 mM dithiothreitol. Substrate peptides (ε-peptide, syntide-2, PKD738–754, PKD-738–754/S744E, PKD-738–754/S748E, or the PKD C-terminal peptide EEREMKALSERVSIL) were added to the presence of [γ-32P]ATP (2 μCi/reaction diluted with cold ATP to give a final concentration of 10 μM) in kinase buffer (final reaction volume, 30 μl) and transferred to a water bath at 30 °C for 10 min. Reactions were terminated by adding 100 μl of 75 mM H3PO4, and 75 μl of the mixed supernatant was spotted to Whatman P-81 phosphocellulose paper. Papers were washed thoroughly in 75 μl H3PO4, and dried, and radioactivity incorporated into peptides was determined by detection of Cerenkov radiation in a scintillation counter.

PS/PDB phospholipid vesicles were used for assays of peptide or protein phosphorylation by PKD or PKCε were prepared by dehydrating 300 μg

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of PS under ethanol (200 μl) in a Speedvac dehydrator and then soni-
cating the dried lipid into kinase buffer (typically 300 μl) in the pres-
ence of added PDB and 0.05% nonionic detergent ( Triton X-100). For 
asays of syntide-2 phosphorylation by purified PKD or PKCe, fresh 
aliquots (typically 2 μg) of enzyme were thawed on ice, combined with 
PS/PDB vesicles (final concentrations, 200 μg/ml and 200 nm, respec-
tively, in kinase buffer), and aliquoted to tubes on ice containing kinase 
buffer with or without inhibitors as indicated in the figures. Reactions 
were initiated by adding a mixture containing syntide-2 (final concen-
tration, 2 mg/ml), together with 8 μM ATP (including [γ-32P]ATP at 2 
μCi/assay), and then transferring to a water bath at 30 °C for 7 min. 
Reactions were terminated by adding H3PO4 as above, spotted to P-81 
paper, and washed, and radioactivity was counted in a scintillation 
counter as above.

PKC-PKD Transphosphorylation/PKD Activation Assays—For 
transphosphorylation of full-length wild-type or kinase-defective PKD 
proteins by PKCs, we transfected COS-7 cells with PKD or PKD-
K618N. After 72 h, cells were lysed, and PKD protein was isolated in 
immune complexes using PA-1. In each experiment, parallel dishes 
were included to examine the persistence of PKD activation throughout 
immunoprecipitation, elution from immunocomplexes, and subsequent 
reimmunoprecipitation. PKD or PKD-K618N immunoprecipitates from 
unstimulated cells were washed twice with lysis buffer and then twice 
with kinase buffer. The PKD-PA-1 immune complexes were then incu-
bated with immunizing peptide (15 μg in 15 μl of kinase buffer) overnight on ice to elute PKD proteins. For transphosphorylation reac-
tions, the supernatant containing eluted PKD protein was transferred 
to fresh tubes on ice, adjusted to 20 μl, ATP (100 μM) was added on ice, 
and then reactions were initiated by mixing in purified PKCe (Calbio-
chem) for a final concentration of 5.8 μg/ml, combined with PS/PDB 
vesicles (either with or without 3 μM G60893), and transferred to a 
37 °C water bath. Reactions were terminated at 2.5 min by adding 1 μl of 
ice-cold lysis buffer and were transferred to ice. PKD antibody (C-20; 
Santa Cruz Biotechnology) was then added, and PKD was reimmu-
precipitated for 3 h, washed as before, and then subjected to syntide-
doubled assays in the presence of 3 μM G60893. Activation assays using purified PKCs to transphosphorylate purified PKD (Calbioch-
em) were conducted similarly, beginning with the addition of ATP to purified PKD in kinase buffer (20 μl).

For measurements of PKCe transphosphorylation/PKD autophos-
phorylation by [32P] incorporation, purified components (PKD (200 ng), 
orients with PKCe (70 ng) or PKCζ (70 ng) in the absence of PS/PDB 
vesicles, or PKD (250 ng), orients with PKCζ (150 ng), in the presence 
of PS/PDB vesicles and kinase buffer) were combined in microcentrifuge 
tubes (final volume, 20 μl) on ice. To initiate reactions, ATP at 100 μM 
with or without 1 μCi of [γ-32P]ATP/assay was added and incubated at 
30 °C for times indicated in the figures. Reactions were terminated by 
addition of 2× SDS-PAGE sample buffer and resolved by SDS-PAGE on 
8% gels. Protein phosphorylation was assessed by autoradiography of 
dried lipid into kinase buffer (typically 300 μl). For detection of PKCe transphosphorylation/PKD autophos-
phorylation at the PKD activation loop by Western blot analysis using 
phosphospecific antibodies, components (PKD, 275 ng/lane, with or 
without PKCe, 175 ng/lane, were combined in the presence of PS/PDB 
vesicles and incubated at 37 °C. At times indicated in the figures, 
aliquots were taken and combined with SDS-PAGE gel loading buffer 
for subsequent Western analysis.

Autophosphorylation or syntide-2 phosphorylation reactions to 
assess activity of the PKD-ΔPH-S744A/S748A mutant were conducted as 
described previously for PKD activity measurements (28). Briefly, 
COS-7 cells were transfected with the different plasmids. After 72 h, 
cells were left untreated or treated with PDB (200 nM for 10 min) and then 
lysed, and PKD or PKD mutant protein was immunoprecipitated 
from the lysates. Immunoprecipitates were washed twice with lysis 
buffer and twice with kinase buffer and subjected to autophosphoryla-
tion reactions or syntide-2 phosphorylation reactions as described (28).

Activity of the catalytic domain GST fusion proteins was measured by 
immobilization on glutathione-Sepharose beads and subsequent incuba-
tion with syntide-2 (15 μg/ml), in the absence of added PDB (200 nM) 
or PKCe, 175 ng/lane, were combined in the presence of PS/PDB 
vesicles and incubated at 37 °C. At times indicated in the figures, 
aliquots were taken and combined with SDS-PAGE gel loading buffer 
for subsequent Western analysis.

**Western blot analysis**—For Western blot analysis, samples or cell 
lysates were directly solubilized by boiling in SDS-PAGE sample buffer. 
After SDS-PAGE, proteins were transferred to Immobilon-P membranes 
(Millipore) as described previously (21). To block nonspecific protein binding, membranes were blocked by incubation with either 5% non-fat dry milk in PBS, pH 7.2 (for PKD C-20 or the 
antibody pS747 that recognizes PKD) (34), or 5% bovine serum albu-
min (0.1% Tween 20 in PBS) (for the antibody pS744 that recognizes PKD) (34) for 2–3 h at 20–25 °C or overnight at 4 °C. Membranes were 
then incubated at room temperature for 2–3 h with antisera, specifi-
cally recognizing PKD at a dilution of 1 μg/ml, the phospo-specific 
phosphoSer744 recognizing PKD phosphorylated at Ser744 (1:500) in 
PBS containing 3% non-fat dry milk, or the phospho-specific phos-
phoSer748 raised against a peptide containing phosphoSer744 and phos-
phoSer748, respectively, recognizing PKD phosphorylated at Ser748 (1:1000 
dilution in 5% bovine serum albumin/0.1% Tween 20 in PBS. Immuno-
reactive bands were visualized using horseradish peroxidase-conju-
gated anti-rabbit IgG and enhanced chemiluminescence (ECL reagents; 
Amer sham Biosciences) detection.

**Materials**—[γ-32P]ATP (6000 Ci/mmol) was from Amersham Bio-
sciences. Protein A-agarose and 4-(2-aminoethyl)-benzenesulfonyl fluo-
ride hydrochloride (Pefabloc) were from Roche Molecular Biochemicals. 
The anti-PKD antiserum (C-20) used in Western blot analysis was from 
Santa Cruz Biotechnologies, Palo Alto, CA. pS744 was from Cell Sig-
alling Technologies, Beverly, MA. Purified PKD (purity > 90% by 
SDS-PAGE), PKCζ (purity > 95%), and PKCe (purity > 95%), produced in 
cells from Spodoptera frugiperda, were from Calbiochem. Opti-MEM 
and Lipofectin were from Invitrogen. The PKC substrate peptide, pep-
tide ε, purified PKD-1, and its control substrate peptide, PKDκε, were 
produced from Alexis Biochemicals. Syntide-2 and PKCe autophos-
phorylation peptides were synthesized at the peptide synthesis core facility 
of CURE-DDRC at UCLA. The C-terminal PKD peptide, PKD CT 
and pS748, were synthesized by the central peptide synthesis and antibody 
production facility at Imperial Cancer Research Fund, London, United 
Kingdom. All other reagents were from standard suppliers as described in the text and were the highest grade commercially available.

**Results and Discussion**

**Comparison of the PKD Activation Loop Segment with the Known Substrate Recognition Sequences of PKC Isomers**—The initial 
membrane cloning and expression of PKD and its human homologue PKCμ was followed by the molecular cloning and expression 
of another related protein, termed PKD2, and two other clones thus far identified only at the cDNA level (human PKCκ/PKD3 and a Caenorhabditis elegans clone related to PKD2). A canonical region in the catalytic portion of protein kinases bridges two characteristic amino acid motifs in subdo-
 mains VII (DFG) and VIII (APE). This region typically forms an 
elongated loop positioned adjacent to the active site, referred to as 
an activation loop/segment because of targeting of this re-
 gion for regulatory control of enzyme activity by upstream 
protein kinases acting in a cascade fashion (54, 55). Interest-
ingly, the activation loop segment is 100% conserved among all 
members of the novel PKD protein kinase subfamily (Fig. 1A). 
We recently demonstrated that Ser744 and Ser748 are not phos-
phorylated significantly in resting cells and that phosphoryla-
tion of these residues is rapidly induced by multiple cellular 
stimuli in a PKC-dependent manner and is concomitant with 
persistent activation (34). Additional studies from this labora-
 tory have shown that phosphorylation at these sites is also 
closely linked with dynamic intracellular redistributions of 
PKD (56–59). Thus, PKD is regulated by PKC activity at multi-
ple levels, and direct phosphorylation of the activation loop 
may represent a convergence point for these events.

The amino acid residues in the vicinity of the serine or threonine 
residues targeted by different protein kinases comprise 
characteristic substrate recognition sequences that contrib-
tute to enzyme specificity. The substrate recognition sequences 
of various PKC isoforms and PKD/PKCε were analyzed by Cantley 
and co-workers (12) using directed peptide libraries. As shown in 
Fig. 1B, the substrate recognition sequences of PKCβ and PKCe 
possess similarities to the amino acid sequence surrounding 
Ser744 and Ser748 in the PKD activation loop, suggesting that 
these PKCs might directly phosphorylate these sites in vitro. In 
particular, the sequences clustered on both sides of Ser748, 
as well as Ser744, in PKD correspond to favored PKC recognition
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PKD mouse: 733
PKCζ human: 744
PKCε human: 748
PKD2 human: 759
PKD C. elegans: 733

PKD738–754: RIIGEKSFRRSVVGTPA
PKD738–754/S748E: RIIGEKSFRRSVVGTPA
PKD738–754/S744E: RIIGEKSFRRSVVGTPA

Fig. 1. Comparison of PKD family activation loop sequences. PKC isoform substrate specificities, and peptides used as substrates to assess PKC-mediated PKD activation loop phosphorylation. A, activation loop sequences spanning subdomains VII and VIII in the catalytic region of known PKD family members. Canonical DFG and APE motifs are in bold, and the serine residues corresponding to Ser744 and Ser748 in PKD are in bold and highlighted by grey boxes. B, the sequence specificity of PKCs, PKCζ, and PKCε emphasizing the region from −5 to +5 surrounding the targeted serine, and based on data of Nishikawa et al. (12). Residues are arranged vertically in decreasing order of preference; slash marks between residues indicate equivalent values. The residues identified as matches with the Ser744 site in PKD are circled, and the residues identified as matches with the Ser748 site in PKD are in boxes. C, peptides synthesized for this study based on the sequence of the amino acids 738–754 of PKD. PKD738–754, the peptide corresponding to the wild-type sequence in PKD; PKD738–754/S748E, the peptide corresponding to the sequence with Ser748 replaced by glutamic acid; PKD738–754/S744E, the peptide corresponding to the sequence with Ser744 replaced by glutamic acid. Amino acids corresponding to Ser744 and Ser748 are in bold.

sequences. Similarly, the amino acids in positions adjacent to and C-terminal to Ser744, or those N-terminal to Ser748, are strikingly similar to those forming PKCζ recognition sequences (Fig. 1B). However, the resemblance between the PKD activation segment and the substrate recognition sequence of atypical PKCζ is relatively limited (Fig. 1B), consistent with our previous findings that this isoform is unable to activate PKD in cotransfection experiments (20, 36, 60).

Phosphorylation of PKD Activation Loop Peptides by Different PKC Isoforms—To investigate whether PKCζ, PKCε, or PKCζ could recognize the PKD activation segment in vitro, we synthesized a peptide, RIIGEKSFRRSVVGTPA, comprising amino acids 738–754 of PKD (PKD738–754) (Fig. 1C) as a substrate for phosphorylation assays. The importance of dual activation loop phosphorylation in the PKD activation mechanism has been underscored by previous studies. Specifically, we demonstrated previously that mutation of both Ser744 and Ser748 to glutamic acid residues mimics dual phosphorylation in PKD produced a highly constitutively active enzyme (50). In contrast, single point mutation of Ser744 or Ser748 to glutamic acid residues introduce only minor alterations in PKD activity. Thus, second site phosphorylation of PKD singly phosphorylated at one of the activation loop sites is necessary for full activation. Therefore, to complement assays of PKD738–754 phosphorylation, we also synthesized peptides with individual glutamic acid changes at the positions corresponding to either Ser744 or Ser748 (PKD738–754/S744E and PKD738–754/S748E; shown in Fig. 1C) as substrates that mimic singly phosphorylated PKD activation loop segments in phosphorylation assays.

We first examined immunoprecipitated active PKD in assays of syntide-2, ε-peptide, or PKD738–754 phosphorylation, shown in Fig. 2A. Consistent with our previous studies, activated PKD phosphorylated syntide-2 strongly but ε-peptide to only a minimal extent (less than 5% of control), indicating that this peptide is a poor substrate for PKD (Fig. 2A). Interestingly, PKD738–754 was also poorly phosphorylated by PKD in these assays (Fig. 2A), suggesting a lack of intrinsic recognition of the activation loop peptide by PKD.

Next, specific PKC immunoprecipitates (wild-type PKCζ or PKCζ from transiently transfected COS-7 cells) or PKCζ (a mixture of endogenous wild-type and transfected active mutant enzyme expressed in COS-7 cells) were assayed for phosphorylation of peptide ε, syntide-2, or PKD738–754 (Fig. 2B). Peptide ε was identified previously as an excellent model substrate for all PKCs but not PKD (36, 61) and was therefore used as a normalization control to assess the relative degree of phosphorylation of PKD738–754 by PKCζ, PKCζ, or PKCζ. In comparison with control, and consistent with previous studies (62), syntide-2 was phosphorylated very well by the different PKC isoforms (Fig. 2B). The extent of syntide-2 phosphorylation ranged from −55% (for PKCζ and PKCζ) to 75% (for PKCζ) of peptide ε phosphorylation (Fig. 2B). Consistent with intrinsic recognition of the PKD activation loop by PKCζ and PKCζ, these isoforms phosphorylated PKD738–754 to an extent between −40% (PKCζ) and 50% (PKCζ) of those reached in peptide ε assays (Fig. 2B). In contrast, a peptide similar to PKD738–754 but with both Ser744 and Ser748 replaced with glutamic acid residues was not phosphorylated significantly in parallel assays (data not shown), indicating that the single threonine residue (corresponding to PKD Thr756) in these peptides was not phosphorylated by these PKCs.

As shown in Fig. 2B, PKCζ also phosphorylated PKD738–754 to an extent similar to those reached by PKCζ and PKCζ (−45% of the extent of peptide ε phosphorylation) suggesting that at least in vitro, this isoform also intrinsically recognizes the PKD activation loop as a possible substrate. However, previous studies from this laboratory indicated that PKCζ neither interacts with nor activates PKD significantly (20, 36, 60). Taken together, these results suggest that factors other than intrinsic substrate recognition (e.g., a failure to colocalize with PKD) contribute to the inability of PKCζ to activate PKD in vivo. Purified PDK-1, an upstream regulator that phosphorylates the activation loop of different PKC isoforms, as well as protein kinase B, did not phosphorylate PKD738–754 significantly (data not shown). Also shown in Fig. 2B, none of these PKCs significantly phosphorylated the C-terminal PKD peptide that contains a PKD autophosphorylation site (described under “Experimental Procedures”). These data were therefore included as a negative control for specific recognition by PKCs.

We then used the variant activation loop peptides described in Fig. 1A as substrates to examine whether PKCζ, PKCζ, or PKCζ act as second-site PKD activation loop kinases. Because

2 PKCζ favors negatively charged residue at position +4 to the targeted serine, which might be mimicked by negatively charged phosphoserine.

3 T. Iglesias, R. T. Waldron, and E. Rozengurt, unpublished observations.
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Fig. 2. Peptide phosphorylation assays of immunoprecipitated PKD and PKCs. A, PKD was immunoprecipitated from transiently transfected, PDB-stimulated COS-7 cells, and peptide phosphorylation assays were carried out as described under "Experimental Procedures" using syntide-2, peptide ε, and PKD738–754 as indicated. A representative Western blot analysis to detect immunoprecipitated PKD using anti-PKD antibody is shown in the inset. B, PKCγ, PKCe, or PKCi as indicated, were immunoprecipitated from COS-7 cells transiently transfected with PKC expression constructs, and peptide phosphorylation assays were carried out as described under "Experimental Procedures" using peptide ε, syntide-2, PKD738–754, or PKD CT, as indicated. Results are expressed as the percentage of the phosphorylation of peptide ε and represent the mean ± S.E. of at least five determinations. Representative Western blot analysis to detect immunoprecipitated PKC isoforms using specific anti-PKCe, PKCγ, or PKCi antibodies are shown in the inset. C, PKC isoforms were immunoprecipitated as in B, and peptide phosphorylation assays were carried out as described under "Experimental Procedures," using PKD738–754, PKD738–754/S744E (744E), or PKD738–754/S748E (748E), as indicated. Results are expressed as the percentage of the phosphorylation of PKD738–754 and represent the mean ± S.E. PKCγ phosphorylated 744E to a significantly greater extent than did PKCe, as assessed by Student's t test (p = 0.04, n = 9, shown with an asterisk). PKCe phosphorylated S748E to a significantly greater extent than S744E (p = 0.02, n ≥ 7, shown with a double asterisk). Phosphorylation of S748E by PKCe was significantly less than by PKCε (p = 0.04, n ≥ 7, shown with a double asterisk).

these PKCs all phosphorylated PKD738–754 to similar extents, we used this peptide as a normalization control to facilitate comparison of relative phosphorylation levels. Shown in Fig. 2C, both PKD738–754/S744E and PKD738–754/S748E were well phosphorylated by PKCγ, indicating that this isoform can act as a second-site kinase to phosphorylate PKD equally well after an initial phosphorylation at either Ser744 or Ser748. Interestingly, and in contrast with PKCγ, PKCe phosphorylated the two peptides differentially, demonstrating a strong preference for PKD738–754/S748E. These data indicated that this enzyme acts preferentially as a second-site kinase to phosphorylate Ser748. Interestingly, PKCε phosphorylated these peptides only modestly, suggesting that this isoform is relatively deficient as a second-site kinase for the PKD activation loop.

PKCe Increases PKD Autophosphorylation—To further examine whether PKC-mediated PKD activation was direct, we developed in vitro activation assays using purified components. We have shown previously that certain PKCs, notably PKCγ, form stable molecular complexes with the PKD PH domain when cotransfected, together with PKD in COS-7 cells (36). Therefore, for these studies we selected PKCe, which potently activates PKD but forms complexes with PKD relatively poorly. To establish the potential of PKCe to activate intact purified PKD in vitro by phosphorylation at the activation loop sites (i.e. corresponding to Ser744 and Ser748 in PKD), we carried out PKD autophosphorylation assays under conditions that either supported only autophosphorylation (i.e. purified PKD, together with [γ-32P]ATP) or a combination of transphosphorylation...
PKCε Rapidly and Directly Phosphorylates PKD in Both Activation Loop Sites, Whereas PKD Autophosphorylates at Ser748 Only—We next verified PKCε-mediated phosphorylation of the activation loop sites by Western analysis using the specific antibodies we characterized previously, which recognize the phosphorylated state of Ser744 and Ser748 in PKD (Fig. 4). In these experiments, we incubated PKD with PS/PDB and cold ATP, either alone or together with PKCε, and examined activation loop phosphorylation at 30-s intervals by Western analysis using the phosphospecific antibodies, pS748 and pS744.

Experiments using pS748 to monitor Ser748 phosphorylation over a 2.5-min time course, shown in Fig. 4, revealed the time-dependent appearance of a phosphorylated band corresponding to PKD. Thus, these data indicated that PKD could autophosphorylate at the activation loop after 1 min of incubation in the presence of activators. We also examined PKD phosphorylation in these reactions by Western analysis using the commercial antiserum that recognizes chiefly phosphoSer744 (and to a lesser extent phosphoSer748 (34)). Results shown in Fig. 4 illustrate that pS744 immunoreactivity in PKD increased very little over this time course. Because this antibody binds to a limited extent to phosphorylated Ser748, this low degree of immunoreactivity most likely reflects little or no autophosphorylation at Ser744 but rather corresponds to the increase in Ser748 phosphorylation observed using pS748.

Significantly, Ser748 autophosphorylation by isolated PKD occurred slowly, becoming detectable only after a lag of −1–1.5 min of the reaction. In contrast, PKD incubated with PKCε exhibited a rapid increase in pS748 immunoreactivity, detected within 30 s of the reaction (Fig. 4). Furthermore, when PKCε was included in reactions with PKD, pS744 immunoreactivity increased rapidly and produced a robust signal within 1 min, indicating that transphosphorylation of PKD Ser744 had occurred. These Western blots were also stripped and reprobed with C-20 to demonstrate that equal amounts of PKD protein were present, blots were stripped and reprobed with the anti-PKD antibody C-20 recognizing total protein. Western blots (upper panels) and corresponding band intensities depicted in the graph (below), as determined by scanning and densitometry, are representative of three experiments with similar results.

PKCε-mediated Activation Loop Phosphorylation Triggers Persistent PKD Activation in Vitro—Results in Fig. 4 verified that PKCε had phosphorylated the PKD activation loop sites in our in vitro assays using purified components. We next examined whether PKCε-mediated PKD activation loop phosphorylation was associated with stable increases in PKD activity by a two-stage assay. In our previous studies, we have assayed PKD in soluble form by eluting the enzyme from immunoprecipitates (50). Here, we transfected PKD or the kinase-deficient PKD mutant PKD-K618N into COS-7 cells, immunoprecipitated PKD from these cells, and then eluted the enzyme from immunoprecipitates as described under “Experimental Procedures.” We then incubated the soluble, eluted PKD either alone or with purified PKCε and unlabeled ATP in the first stage of the assay. After 2.5 min at 37 °C, the reactions were stopped and diluted with ice-cold buffer, and PKD was reimmunoprecipitated, and its catalytic activity was measured by syntide-2 phosphorylation in the second stage of the assay (Fig. 5B).

To substantiate further the role of PKC catalytic activity in the activation of PKD, we also used an inhibitor, G6983, of classic and novel PKCs including PKCε but not PKD (63). To determine the concentrations of G6983 required to selectively and completely eliminate PKCε activity in peptide phosphorylation and autophosphorylation (i.e. including purified PKCε), measuring the total extent of PKD phosphorylation over time. Shown in Fig. 3A, the time courses of PKD autophosphorylation with or without added PKCε were dramatically different when assays were carried out in the absence of PS/PDB vesicles. This was possible, because the preparation of PKCε used here possesses constitutive activity without allosteric effectors (not shown). Thus, purified PKD incubated alone autophosphorylated to only a very limited extent, reaching a maximum within the first 2.5 min of the assay and not increasing significantly thereafter. Similarly, inclusion of PKCε, a negative control for PKC-mediated PKD activation, promoted PKD autophosphorylation only very weakly over a 40-min time course, despite having a potent activity demonstrated by autophosphorylation (Fig. 3A), which was not enhanced in the presence of PS/PDB (not shown). In contrast, inclusion of PKCε promoted a continued strong increase in PKD phosphorylation (Fig. 3A). Collected data from multiple experiments, normalized to the initial PKD phosphorylation levels and shown in the graph in Fig. 3A, emphasized the selective enhancement of PKD autophosphorylation by PKCε. From these results, we conclude that catalytic activation of PKD triggered by PKCε transphosphorylation permits a dramatic overall increase in PKD autophosphorylation, consistent with our proposal that PKCε activates PKD by activation loop phosphorylation.

In the presence of PS/PDB vesicles that allosterically stimulate PKD enzyme activity without persistent activation, PKD autophosphorylation increased approximately linearly over a 40-min time course (Fig. 3B). Interestingly, even when PKD activity was directly stimulated, inclusion of PKCε in the assay increased the initial rate of PKD phosphorylation (Fig. 3B). These results are consistent with a modest initial acceleration of PKD autophosphorylation by PKCε-mediated activation and then continued autophosphorylation until all the possible sites approached saturation.

Fig. 4. PKD activation loop phosphorylation in the absence or presence of PKCε. Purified PKD, alone or together with purified PKCε (PKD + PKCε) were incubated with ATP in the presence of PS/PDB for the times indicated and then analyzed by SDS-PAGE and Western blot analysis using the phosphospecific antiserum pS744 or pS748 as described under “Experimental Procedures.” To demonstrate that equal amounts of PKD protein were present, blots were stripped and reprobed with the anti-PKD antibody C-20 recognizing total protein. Western blots (upper panels) and corresponding band intensities depicted in the graph (below), as determined by scanning and densitometry, are representative of three experiments with similar results.

Experimental Procedures.

To demonstrate PKCε-mediated activation and phosphorylation of PKD by transphosphorylation at 30-s intervals by Western analysis using the phosphospecific antibodies, pS748 and pS744 as described under “Experimental Procedures.” The time courses of PKD autophosphorylation at Ser744 but rather corresponds to the time-dependent appearance of a phosphorylated band corresponding to PKD. These data indicated that PKD could autophosphorylate at the activation loop after 1 min of incubation in the presence of activators. We also examined PKD phosphorylation in these reactions by Western analysis using the commercial antiserum that recognizes chiefly phosphoSer744 (and to a lesser extent phosphoSer748 (34)). Results shown in Fig. 4 illustrate that pS744 immunoreactivity in PKD increased very little over this time course. Because this antibody binds to a limited extent to phosphorylated Ser748, this low degree of immunoreactivity most likely reflects little or no autophosphorylation at Ser744 but rather corresponds to the increase in Ser748 phosphorylation observed using pS748.

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PKCε-mediated Activation Loop Phosphorylation Triggers Persistent PKD Activation in Vitro—Results in Fig. 4 verified that PKCε had phosphorylated the PKD activation loop sites in our in vitro assays using purified components. We next examined whether PKCε-mediated PKD activation loop phosphorylation was associated with stable increases in PKD activity by a two-stage assay. In our previous studies, we have assayed PKD in soluble form by eluting the enzyme from immunoprecipitates (50). Here, we transfected PKD or the kinase-deficient PKD mutant PKD-K618N into COS-7 cells, immunoprecipitated PKD from these cells, and then eluted the enzyme from immunoprecipitates as described under “Experimental Procedures.” We then incubated the soluble, eluted PKD either alone or with purified PKCε and unlabeled ATP in the first stage of the assay. After 2.5 min at 37 °C, the reactions were stopped and diluted with ice-cold buffer, and PKD was reimmunoprecipitated, and its catalytic activity was measured by syntide-2 phosphorylation in the second stage of the assay (Fig. 5B).

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PKD Activation Loop Phosphorylation by PKCε Relieves Autoinhibition

Results (Fig. 5B) demonstrated that PKD activated within cells retained its increased activity throughout all these procedures. Eluted PKD, incubated with ATP and PS/PDB and then immunoprecipitated and assayed for syntide-2 activity, had a low level of activity that was very similar to the control enzyme isolated from unstimulated cells (Fig. 5B). This clearly indicates that allosteric activators do not induce a persistent increase in PKD activity and that PKD must be phosphorylated at both serines in the activation loop to stabilize the active form of the enzyme. In contrast, eluted PKD incubated with PKCε, in the presence of ATP and PS/PDB, then immunoprecipitated and assayed, had dramatically increased activity, i.e. more than 4-fold that of the control enzyme (Fig. 5B). Consistent with PKCε activity in the initial incubation having been responsible for the observed increases in PKD activity, incubating PKD with PKCε in the presence of 3 μM G66983 reduced PKD activation by ~40% (Fig. 5B). Control Western blots, shown in Fig. 5B, demonstrated that equal amounts of PKD protein were isolated by the immunoprecipitation procedures.

To confirm that an increase in PKD activity, as opposed to that of a coimmunoprecipitated kinase, was responsible for the increased catalytic activity of the reimmunoprecipitated PKD, we incubated eluted kinase-deficient PKD mutant PKD-K618N (Fig. 5B) in parallel assays and then processed the protein as before for syntide-2 assays. Shown in Fig. 5B, these immunoprecipitates contained no significant activity.

We also carried out experiments similar to those in Fig. 5B but using the insect cell-expressed and purified PKD and PKCε in the initial transphosphorylations. Results, shown in Fig. 5C, demonstrate that incubation of PKD with PKCε increased the activity of the subsequently immunoprecipitated PKD by more than 2.5-fold in comparison with the same amount of PKD incubated by itself and then immunoprecipitated and assayed. Again, in all these syntide-2 assays, 3 μM G66983 was included to eliminate any possible traces of PKCε activity carried over into the PKD immunoprecipitates. Similar to results in Fig. 5B, this increase could be attenuated significantly by including 3 μM G66983 in the incubation mixture to inhibit PKCε activity. Taken together, the data in Fig. 5 demonstrate that PKCε-mediated direct phosphorylation of PKD promotes its conversion from an inactive to an active state.

Relief of Autoinhibition and Activation Loop Phosphorylation

Both Contribute to PKD Activation—Autoinhibition is a central feature of the regulation of protein kinase catalytic activity (64). Previous results from this laboratory indicated that PKD mutants lacking the pleckstrin homology domain or the cysteine-rich domain were highly active in the absence of stimulation (18, 19). These results suggest that in the intact kinase, the entire N-terminal regulatory domain, i.e. both the cysteine-rich and PH domains, help to maintain the enzyme in an inactive, autoinhibited state, and consequently, removal of either domain facilitates activation. Consistent with this interpretation, a maltose-binding protein-PKD catalytic domain fusion protein produced in bacteria was demonstrated previously to be catalytically active (13). Mutation of Ser744 and Ser748 in the activation loop to glutamic acid residues introduces negative charges that mimic phosphorylation, which generates a full-length kinase that is highly active in the absence of stimulation. Reciprocally, a PKD mutant in which Ser744 and Ser748 in the activation loop are replaced with unphosphorylatable alanine residues (PKD-S744A/S748A) is not activated within cells by stimuli that fully activate wild-type PKD (50). Thus, we hypothesize that the mechanism of PKD activation is based on phosphorylation.
a functional link between activation loop phosphorylation and relief of autoinhibition.

Further experiments were designed to assess the possible interdependence(s) between activation loop phosphorylation and relief of autoinhibition. We first examined the effect of activation loop phosphorylation on the catalytic activity of the isolated PKD catalytic domain using a wild-type catalytic domain fusion protein, GST-Cat, and a catalytic domain fusion protein with Ser744 and Ser748 mutated to unphosphorylatable alanines, PKD-GST-Cat/S744A/S748A. As shown in the inset in Fig. 6A, control GST had no catalytic activity. In contrast, and consistent with previous results using a different PKD catalytic domain fusion protein (MBP-CAT) (13), bacterially expressed GST-Cat vigorously phosphorylated syntide-2. Interestingly, an equivalent amount of GST-Cat/S744A/S748A phosphorylated syntide-2 to only a slightly lesser extent (~80% of that achieved by GST-Cat). Thus, even complete prevention of phosphorylation did not drastically affect the activity of the isolated catalytic domain, suggesting that activation loop phosphorylation is not needed for PKD activation in the truncated enzyme.

To assess the effect of preventing activation loop phosphorylation under circumstances in which PH domain-mediated regulation is removed, we generated a novel mutant derived from a PH domain deletion mutant (PKDΔPH) we described previously (18). Whereas PKDΔPH retains a wild-type activation loop sequence, the newly generated mutant has Ser744 and Ser748 mutated to alanine residues (PKDΔPH/S744A/S748A). We first compared the activity of this mutant, by both syntide-2 phosphorylation (Fig. 6A) and autophosphorylation assays (Fig. 6B), to that of wild-type PKD, PKD-S744A/S748A, and PKD-ΔPH, from stimulated or unstimulated cells. PKD from unstimulated cells had very low activity, and PDB stimulation of cells dramatically activated the enzyme by both types of assay. PKD-S744A/S748A was not active either constitutively or after cell stimulation with PDB. In contrast, PKDΔPH from unstimulated cells was dramatically active in comparison with the wild-type PKD, and PDB stimulation of cells did not further stimulate the activity of this mutant (Fig. 6, A and B). These results with PKD, PKD-S744A/S748A, and PKDΔPH were consistent with those we described previously (18, 50).

Interestingly, like PKDΔPH, PKDΔPH-S744A/S748A from unstimulated cells was also very active, indicating that activation loop mutation to alanines did not abrogate the function of the enzyme. Thus, this mutant displayed constitutive syntide-2 phosphorylation activity that was ~5-fold higher than wild-type PKD and about 60% of PKDΔPH (Fig. 6A). Results of analysis by autophosphorylation assays was similar (Fig. 6B). These results are consistent with the notion that removal of the PH domain releases PKD from autoinhibition. Although in these experiments, expression levels of PKDΔPH-S744A/S748A were, on average, slightly less than (0.75-fold) those of PKD-ΔPH (Fig. 6C), it was not clear whether this difference could fully account for the measured differences in activity between these two enzymes. Thus, it remains possible that activation loop phosphorylation could influence the overall activity in the absence of the PH domain.

We next analyzed the activation loop phosphorylation in the cell lysates from cells transfected with the enzymes assayed in Fig. 6, A and B, using the phosphospecific antibodies described above. As shown in Fig. 6C, PKD was unphosphorylated in unstimulated cells, and stimulation with PDB induced a dramatic increase in phosphorylation at Ser748 and Ser744, measured using pS748 and pS744, respectively. PKD-S744A/S748A could not be detected in either unstimulated or stimulated cells, as the phosphorylation sites responsible for antibody detection are altered by mutation.

Western analysis of PKDΔPH using pS748 and pS744 indicated that whereas the basal phosphorylation of these residues in unstimulated cells was slightly increased in comparison with wild-type enzyme, significant increases (in the case of pS748)
PKD Activation Loop Phosphorylation by PKC Relieves Autoinhibition

Recent additional studies elucidated dynamic movements of PKD in response to G protein-coupled receptor stimulation (56–59) by immunocytochemistry and real-time imaging. These studies demonstrated that PKD initially translocates to the plasma membrane, where the enzyme becomes activated, and then subsequently reverse translocates away from the plasma membrane, to gain access to targets elsewhere in the cell (56–58), including the nucleus (59). Interestingly, PKC-dependent phosphorylation of activation loop Ser744 and Ser748 was required for this dynamic behavior of activated PKD (58, 59).

Collectively, these results strongly suggest that the function of activation loop phosphorylation is to reverse an autoinhibitory effect of the PH domain.

CONCLUDING REMARKS

Previous studies from this laboratory demonstrated that pharmacological inhibition of PKC prior to cell stimulation prevents PKD activation, and cotransfection of PKCe or PKCζ, together with wild-type PKD, activates PKD in cells via dual phosphorylation of Ser744/Ser748. As these sites also become phosphorylated in kinase-deficient forms of PKD, we have proposed that PKD activation depends on PKC-mediated transphosphorylation rather than on PKD autophosphorylation (34, 50, 51).

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REFERENCES

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Berndige, M. J. (1993) Nature 361, 315–325
3. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
4. Mellor, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
5. Nob, D. Y., Shin, S. H., and Rhee, S. G. (1995) Biochem. Biophys. Res. Commun. 212, 158–163
6. Exton, J. H. (1997) Physiol. Rev. 77, 303–329
7. Nishizuka, Y. (1995) FASEB J. 9, 484–496
8. Nakasabata, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
9. Dempsey, E. C., Newton, A. C., Mohly-Rosen, D., Fields, A. P., Beyland, M. E., Insel, P. A., and Messing, R. O. (2000) Am. J. Physiol. Cell Mol. Physiol. 278, L429–L438
10. Mohly-Rosen, D., and Gordon, A. S. (1998) FASEB J. 12, 35–42
11. Jaken, S., and Parker, P. J. (2000) Bioessays 22, 245–254
12. Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1996) J. Biol. Chem. 271, 952–960
13. Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8572–8576
14. Shicon, F. J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. (1994) J. Biol. Chem. 269, 6140–6148
15. Hayashi, A., Seki, N., Hattori, A., Kozuma, S., and Saito, T. (1999) Biochim. Biophys. Acta 1450, 99–106
16. Sturany, S., Van Lint, J., Muller, W., Wilda, M., Hameister, H., Hocker, M., Brey, A., Gern, U., Vandenheede, J., Gress, T., Adler, G., and Seufertten, T. (2001) J. Biol. Chem. 276, 3310–3318
17. Van Lint, J. V., Sinnett-Smith, J., and Rozengurt, E. (1995) J. Biol. Chem. 270, 1455–1461
18. Igleias, T., and Rozengurt, E. (1998) J. Biol. Chem. 273, 410–416
19. Igleias, T., and Rozengurt, E. (1999) FEBS Lett. 451, 1–16
20. Zugaza, J. L., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1999) J. Biol. Chem. 274, 23952–23960
21. Chiu, T., and Rozengurt, E. (2001) FEBS Lett. 489, 101–106
22. Chiu, T., and Rozengurt, E. (2001) Am. J. Physiol. Cell Physiol. 280, C329–C342
23. Zhuo, F., Sinnett-Smith, J., Wang, H., Chiu, T., and Rozengurt, E. (2001) J. Cell. Physiol. 189, 291–305
24. Paulucci, L., Sinnett-Smith, J., and Rozengurt, E. (2000) Am. J. Physiol. 278, C33–C39
25. Yuan, J., Slice, L., Walsh, J. H., and Rozengurt, E. (2000) J. Biol. Chem. 275, 2157–2164
26. Yuan, J., Slice, L., and Rozengurt, E. (2001) J. Biol. Chem. 276, 38619–38627
27. Waldron, R. T., and Rozengurt, E. (2000) J. Biol. Chem. 275, 17114–17121
28. Paulucci, L., and Rozengurt, E. (1999) Cancer Res. 59, 572–577
29. Mehta, C., and Rozengurt, E. (1997) J. Biol. Chem. 272, 22737–22743
30. Rohrschneider, R., and Rozengurt, E. (1996) Mol. Cell. Endocrinol. 20, 247–253
31. Brooks, G., Goss, M. W., Rozengurt, E., and Galinane, M. (1997) J. Mol. Cell. Cardiol. 30, 211–223
32. Harrow, R. S., Goss, M. W., Rozengurt, E., and Akvakan, M. (2000) J. Mol. Cell. Cardiol. 32, 1073–1079
33. Matthews, S. A., Rozengurt, E., and Cantrell, D. (2000) J. Exp. Med. 191, 2075–2082
34. Waldron, R. T., Rey, O., Igleias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) J. Biol. Chem. 276, 45261–45265
35. Matthews, S. A., Petit, G. R., and Rozengurt, E. (1997) J. Biol. Chem. 272, 20245–20250
36. Waldron, R. T., Igleias, T., and Rozengurt, E. (1999) J. Biol. Chem. 274, 9224–9230
37. Toullier, D., Pannetti, P., Coste, H., Belleveruge, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boisson, P., Bourrier, E., Lorilolle, F., et al. (1991) J. Biol. Chem. 266, 1571–1578
38. Yeo, J. J., Provost, J. J., and Exton, J. H. (1997) Biochim. Biophys. Acta 1356, 308–320

or even very dramatic increases (in the case of pS744) in immunoreactivity were produced in the protein upon PDB stimulation of cells. These results were interesting taken in the light of those in Fig. 6, A and B, as they clearly indicate that the high constitutive activity of PKDΔPH can be dissociated from quantitative phosphorylation at the activation loop, as this event was dramatically induced during cell stimulation but was not associated with a corresponding increase in enzyme activity. Taken together, these results strongly suggest that the function of activation loop phosphorylation is to reverse an autoinhibitory effect of the PH domain.
Protein Kinase C Phosphorylates Protein Kinase D Activation Loop Ser^{744} and Ser^{748} and Releases Autoinhibition by the Pleckstrin Homology Domain
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