Mechanisms of Regulation of Phospholipase D1
by Protein Kinase Cα

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**ABSTRACT**

It has been suggested that protein-protein interaction is important for protein kinase C (PKC) α to activate phospholipase D1 (PLD1). To determine the site(s) on PKCα that are involved in binding to PLD1, fragments containing the regulatory domain, catalytic domain and C1-C3 domain of PKCα were constructed and shown to be functional, but they all failed to bind and activate PLD1 in vivo and in vitro. A C-terminal 23 amino acid (aa) deletion mutant of PKCα was also found to be inactive. To define the binding/activation site(s) in the C-terminus of PKCα, 1-11 aa deletion mutants were made in this terminus. Deletion of up to 9 aa did not alter the ability of PKCα to bind and activate PLD1 while a 10 aa deletion was inactive. The residue at position 10 is Phe\(^{663}\). Mutations of this residue (F663D and F663A) caused loss of binding, activation and phosphorylation of PLD1, indicating that Phe\(^{663}\) is essential for these activities.

Time course experiments showed that the activation of PLD1 by PMA was much faster than its phosphorylation, and its activity decreased as phosphorylation increased with time. Staurosporine, a PKC inhibitor, completely inhibited PLD1 phosphorylation in response to PMA and also blocked the later decrease in PLD activity. The same results were found with the D481E mutant of PKCα which is unable to phosphorylate PLD1. These results indicate that neither the regulatory nor catalytic domains of PKCα alone can bind to or activate PLD1 and that a residue in the C-terminus of PKCα (Phe\(^{663}\)) is required for these effects. The initial activation of PLD1 by PMA is highly correlated with the binding of PKCα. Although PKCα can phosphorylate PLD1, this is a relatively slow process and is associated with inactivation of the enzyme.
INTRODUCTION

Phospholipase D (PLD)\(^1\) is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid (PA) and choline (1). PA can be metabolized to diacylglycerol (DAG) by PA phosphohydrolase. PA and DAG are involved in receptor-mediated intracellular signal transduction, secretion, cytoskeletal reorganization and the respiratory burst (2). To date two isoforms of mammalian PLD (PLD1 and PLD2) have been cloned. These isoforms share about 50% amino acid similarity, but exhibit quite different regulatory properties (2). PLD1 has a low basal activity and responds to protein kinase C (PKC) and to members of the Rho and Arf families of small G proteins (3-6), while PLD2 exhibits a high basal activity and shows little or no response to PKC, Rho or Arf in vitro (7-9). The intracellular localization of PLD1 remains ambiguous. Most reports indicate it is localized in the perinuclear region including the Golgi apparatus and some have reported its presence in caveolae (10-12).

PKC\(\alpha\) belongs to the group of conventional group of PKC isoforms which are regulated by Ca\(^{2+}\), DAG and phosphatidylserine (PS). It has a regulatory domain, which includes the pseudosubstrate, C1 (phorbol, DAG binding) domain and C2 (calcium, PS binding) domain and a catalytic domain, which includes the C3 (ATP binding) domain and C4 kinase domain. PKC\(\alpha\) is mainly located in the cytosol and can translocate to membrane fraction upon stimulation by phorbol esters or certain agonists (13). PKC\(\alpha\) is considered to play a major role in PLD1 activation (2). However, the mechanism(s) involved in PLD1 activation is still not clear. Some studies have shown that PKC\(\alpha\) activates PLD1 through a phosphorylation-independent mechanism, since PKC\(\alpha\) could activate this PLD isoform in vitro without ATP (4,12,14).
confirming earlier work (15,16). Furthermore, treatment of phosphorylated PLD1 with Ser/Thr phosphatase did not affect its activity (17). Instead of phosphorylation, protein-protein interaction has been considered the main mechanism for PKCα to activate PLD1. Association between PLD1 and PKCα has been observed in COS-7 cells (18) and in Rat1 fibroblasts (19). Utilizing proteolytic cleavage of PKCα to separate the regulatory and catalytic domains, it was suggested that the regulatory domain of PKCα was more important for PLD1 activation in vitro (16).

However, other groups have provided evidence that phosphorylation of PLD1 is needed for its activation. It was found that PMA-dependent PLD1 activation required ATP in cell-free systems from neutrophils and HL60 granulocytes (20). However, a possible role for ATP in PIP2 synthesis was not excluded. PLD1 was also found to be phosphorylated by PKCα at multiple sites during activation, and the phosphorylation occurred in caveolin-enriched microdomains within the plasma membrane (21,22). Mutation of three of the sites resulted in a partial reduction in the ability of PMA to activate the enzyme in vivo (21).

In this study, we determined the requirements for PKCα activation of PLD1. Different fragments of PKCα including the regulatory and catalytic domains and one that encompassed the C1-C3 domains were constructed to see which domain(s) was required for binding and activation of PLD1. In addition, several C-terminal deletion mutants of PKCα were constructed to define the role of the C-terminus. These studies indicted that both the regulatory and catalytic domains were required, and identified Phe$^{663}$ as a crucial residue in the binding and activation of PLD1. Studies of the binding of PKCα with PLD1 were performed and indicated a high correlation between binding and activation of the phospholipase. The role of phosphorylation was explored by determining the time courses of PLD1 activation and phosphorylation, and the effects of a
PKC inhibitor and a PKCα mutant deficient in kinase activity. The results indicated that phosphorylation was not required for activation of PLD1, but was associated with inactivation.
EXPERIMENTAL PROCEDURES

Materials - 4β-phorbol 12 myristate 13 acetate (PMA), staurosporine, bovine serum albumin, Nonidet P-40 (NP-40), phosphatidylinositol 4,5-bisphosphate (PIP₂) and horseradish peroxidase-conjugated secondary antibody were from Sigma. Dipalmitoylphosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylbutanol (PtdBut) standard were from Avanti Polar Lipids Corp. Dipalmitoylphosphatidylcholine [choline-methyl-³H] and [³H]myristic acid were from Perkin Elmer Life Science. Protein G-agarose beads, Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, fetal bovine serum, Tris-glycine SDS polyacrylamide gels, PcDNA3.1(+), PcDNA3.1(HisA,B,C) vectors, and anti-Xpress monoclonal antibody were from Invitrogen. The transfection reagent FuGENE6 and the protease inhibitor mixture were from Roche Molecular Biochemicals. COS-7 cells were from American Type Culture Collection (ATCC). Anti-PKCα monoclonal antibody was from BD Transduction Labs. Anti-PKCα (C-terminal) monoclonal antibody was from Upstate Biotechnology. Anti-phosphothreonine polyclonal antibody was from Zymed. Plasmid and PCR-product purification kits were from Qiagen. QuikChange mutagenesis kit and Pfu Turbo DNA polymerase were purchased from Stratagene. Anti-rabbit IgG, horseradish peroxidase, ECL reagent and film were from Amersham Pharmacia Biotech. All restriction enzymes and T4 DNA ligase were from New England Biolabs. The rat PKCα in PTB vector and the regulatory domain (1-311) in PCH3 vector were kindly provided by Dr. Susan Jaken (Eli Lilly).

Plasmid Construction – The rat PLD1 was cloned into PcDNA3.1(His) vector with the N-terminal Xpress tag. The rat PKCα and its regulatory domain were subcloned at the EcoRI site into PcDNA3.1(+) vector. Catalytic domain (312-672), C1-C3 (1-468), Δ 23 (1-649), Δ11 (1-
were generated by PCR with primers containing the 5’ and 3’ EcoRI sites. D481E, F663D and F663A mutants were generated using the QuikChange site directed mutagenesis kit from Stratagene. All constructs were sequenced to verify the coding regions and were well expressed in COS-7 cells.

**Cell Culture and Transfection** – COS-7 cells were maintained in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum in 10% CO₂. Six well plates were seeded with 2x10⁵ cells/well and 10 cm-dishes were seeded with 8x10⁵ cells 24 h before transfection with FuGENE6 according to the manufacturer’s instructions.

**In vivo PLD Assay** – After 5 h of transfection, cells in six well plates were serum-starved overnight (0.5% fetal bovine serum in DMEM) in the presence of 1 µCi/ml [³H]myristic acid. PLD activity was assayed by incubating the cells with 0.3% 1-butanol for 20 min and measuring the formation of [³H]PtdBut as a percentage of total labeled lipids as described before (23).

**Subcellular Fractionation** – After transfection and starvation overnight, 10 cm dishes of COS-7 cells were washed once with ice-cold phosphate-buffered saline (PBS) and then harvested using lysis buffer (25 mM Hepes, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and protease inhibitor mixture). After 10 s sonication for two times, the cell lysate was first centrifuged at 500 xg for 10 min to remove unbroken cells. The supernatant was then spun at 120,000 xg for 45 min at 4°C to separate the cytosolic and crude membrane fractions.

**In vitro PLD Assay** – For *in vitro* assay, the cells were either untransfected or separately transfected with PLD1 or PKCα or its mutants. The control supernatant or that containing
overexpressed PKCα or its mutants was used as the PKC fraction and the crude membranes containing PLD1 were resuspended in lysis buffer and used as PLD1 fraction. The PLD1 activity was measured by the formation of [³H]PtdBut in vitro as described (14). Briefly, phospholipid vesicles generated from phosphatidylethanolamine/PIP₂/PC(16:1:4:1) containing [palmitoyl-³H]PC (0.5 µCi/reaction) were used with 1-butanol (0.6%) as substrate. The reaction mixtures were incubated at 37°C for 30 min and stopped with chloroform/methanol/HCl (50:98:2). The lipids were extracted from the organic phase and resolved by thin layer chromatography. Bands co-migrating with a PtdBut standard were quantitated by liquid scintillation counting.

**Immunoprecipitation and Western Blotting** – COS-7 cells cultured in 10 cm plates were transfected and starved overnight as described above. The cells were washed once with ice-cold PBS and harvested using immunoprecipitation (IP) buffer containing 25 mM Hepes pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM KCl, 10 mM NaF, 10 mM Na₄P₂O₇, 1.2 mM Na₃VO₄, 1% NP-40 and protease inhibitors mixture. The cell suspensions were sonicated for 10 s and then spun at 120,000 xg for 45 min to pellet the detergent-insoluble fraction. The supernatant was then precleared by mixing it with 1 µg of affinity purified mouse IgG and 20 µl of a 1:1 slurry of protein G beads for 1h at 4°C. The mixture was then spun and the supernatant was incubated with 2 µl of anti X-press antibody and 20 µl of protein G beads overnight. The immunoprecipitates were washed four times with the IP buffer and then resuspended in SDS sample buffer. The samples were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The blots were then blocked with 1% BSA and incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody. The bands were detected using ECL.
RESULTS

Regulatory, Catalytic and C1-C3 Domains and a C-terminal Deletion Mutant of PKCα Fail to Stimulate PLD1. To see if some PKCα domains might be enough to activate PLD1, the regulatory domain (RD, 1-311, 37 kDa) catalytic domain (CATA, 312-672, 43 kDa), C1-C3 domain (1-468, 55 kDa) and a C-terminal 23 aa deletion mutant (Δ23, 1-649, 76 kDa) were constructed and their expression, binding and activation of PLD1 are shown in Fig. 1. Fig. 1A shows that none of the PKCα domains activated the endogenous PLD activity of COS-7 cells in the absence or presence of PMA. Co-expression of the regulatory and catalytic domains also did not restore the stimulation of PLD activity (data not shown). Similar results were obtained with overexpressed PLD1 in COS-7 cells (data not shown). Western blotting (Fig. 1B) shows that all the domains were well expressed and of the appropriate molecular mass. Some additional immunoreactive proteins were present in all samples, corresponding to endogenous PKCα and an unknown protein of 46 kDa. The catalytic domain was also well expressed (Fig. 1B). To demonstrate that the domains were functionally intact, PMA-induced membrane translocation of the wild type enzyme, regulatory domain and C1-C3 fragment was tested. Figure 1C shows that all these proteins translocated. As expected, the catalytic domain did not (data not shown). To test the functional intactness of the catalytic domain, the ability of this fragment and wild type PKCα to increase the Thr phosphorylation of proteins in COS-7 cell lysates was examined. Figure 1D illustrates that both PKCα and its catalytic domain induced marked phosphorylation of several proteins.

In vitro PLD1 assay results (Fig. 1E) were consistent with the in vivo PLD results i.e. none of the PKCα domains activated PLD1. Figs. 1F and G show the results of binding tests
between PLD1 and PKC\(\alpha\) or its domains. Before PMA stimulation, there was slight binding between PLD1 and PKC\(\alpha\), which was greatly increased by PMA. However, none of the domains was able to bind to PLD1 in the presence or absence of PMA. As shown in Fig. 1B, all the domains were well expressed, as was PLD1 (not shown).

**Definition of the C-terminal Residues of PKC\(\alpha\) Required for Activation and Binding of PLD1.** Fig. 1 demonstrated that a C-terminal 23 aa deletion of PKC\(\alpha\) resulted in a loss of its ability to bind and activate PLD1. To further define the residue(s) involved, C-terminal 1, 5, 6, 7, 8, 9, 10, 11 aa deletion mutants were made and shown to be well expressed in COS-7 cells (data not shown). Figure 2A shows the effects of the different PKC mutants on endogenous PLD activity in vivo. It is evident that, with deletion of 9 C-terminal residues (\(\Delta 9\)), PKC\(\alpha\) still retained its ability to activate PLD. However, a 10 aa deletion (\(\Delta 10\)) caused PKC\(\alpha\) to lose this. Similar results were found with overexpressed PLD1 in COS-7 cells (data not shown). To confirm these results, the \(\Delta 9\) and \(\Delta 10\) mutants were examined in an in vitro PLD1 assay. The results were consistent with the in vivo data in that \(\Delta 9\) mutant retained the ability to activate PLD1, while \(\Delta 10\) mutant lost it (Fig. 2B). Adding higher concentrations of PKC\(\alpha\) and the \(\Delta 9\) mutant increased the PLD activity while adding higher concentrations of the \(\Delta 10\) mutant did not, further supporting the conclusion that the \(\Delta 10\) mutant is inactive on PLD1 (data not shown). Figure 2C shows the binding between PLD1 and PKC\(\alpha\) or its C-terminal 9 and 10 aa deletion mutants. The \(\Delta 9\) mutant showed binding with PLD1 in the presence of PMA stimulation that was equivalent to that of the intact enzyme, whereas the \(\Delta 10\) mutant showed negligible binding. Importantly, both the \(\Delta 9\) and \(\Delta 10\) mutants still retained kinase activity as shown by the phosphorylation of proteins in the cell lysates (Fig. 1D).
Mutation of Phe\textsuperscript{663} causes PKC\(\alpha\) to lose its Ability to Activate and Bind PLD1. The above results indicate that Phe\textsuperscript{663}, which is 10 aa from the C-terminus is very important for PKC\(\alpha\) to activate PLD1. To prove this, two single amino acid mutants (F663D and F663A) were made to test their effects on PLD1. Both mutants were expressed very well in COS-7 cells and translocated from the cytosol to membrane fraction after PMA stimulation (data not shown). In vivo PLD assays showed that both mutants could not activate endogenous PLD activity in COS-7 cells in the presence or absence of PMA (Fig. 3A). Similar results were obtained when overexpressed PLD1 in vivo (data not shown). In vitro PLD assay results also showed that both mutants lost the ability to activate PLD1 (Fig. 3B). Similar results were obtained with higher concentrations of the PKC\(\alpha\) mutants were employed (data not shown). Figure 3C shows the binding between PLD1 and the two mutants. The results show that both mutants bound negligibly to PLD1 even in the presence of PMA stimulation. The phosphorylation of PLD1 by PKC\(\alpha\) was also studied using antibodies to phosphoSer, phosphoThr and phosphoTyr and the results showed that only Thr residues were detectably phosphorylated after PMA stimulation (data not shown). Figure 3D shows the results of PLD1 phosphorylation by PKC\(\alpha\) and its mutants. The data show that PKC\(\alpha\) greatly increased PLD1 phosphorylation upon PMA stimulation. The \(\Delta 9\) mutant could still phosphorylate PLD1 upon PMA stimulation while the \(\Delta 10\) mutant completely lost this function. The two Phe\textsuperscript{663} mutants also showed greatly decreased ability to phosphorylate PLD1. Expression of higher levels of the PKC\(\alpha\) mutants still showed greatly impaired PLD1 phosphorylation (data not shown). As expected from the findings with the deletion mutants, both the F663D and F663A mutants retained general phosphorylating ability (Fig. 1D). These results indicate that PLD1 is phosphorylated by PKC\(\alpha\) upon PMA stimulation and indicate that binding is required for this phosphorylation.
Time Course of PLD1 Activity and Thr Phosphorylation after PMA Stimulation.

The above results indicated that binding was required for PKCα to activate PLD1 since all the deletions and mutations that caused PKCα to lose its ability to bind to PLD1 also caused a loss of activation. However, since phosphorylation of PLD1 by PKCα must involve some association between the two enzymes, it was ambiguous whether or not PLD1 phosphorylation was required for its activation. To further study the role of phosphorylation in PLD1 activation, the time courses of PLD1 activation and phosphorylation upon PMA stimulation were studied. This required a modification of the usual protocol i.e. COS-7 cells were first treated with PMA for 1, 5, 15, 30 and 60 min and then 1-butanol was added and the cells were incubated for another 2 min. The results are shown in Figure 4. It is evident that PLD1 activity rose very rapidly after PMA stimulation, reaching a maximum in about 3-5 minutes. Thereafter the activity decreased to near basal (0.1% PtdBut) in 30 min. Figure 4B shows that PKCα rapidly translocated from the cytosol to membrane fraction within 1 min upon PMA stimulation and its membrane association increased over 30-60 min. Figure 4C shows the time course of PKCα binding with PLD1 after PMA treatment. The binding was also detectable at 1 min and increased during the 1 h experiment. Figure 4D shows that the PLD1 phosphorylation was not evident until 5 min, but then continuously increased during the 1 h incubation. The time course results indicated that the activation of PLD1 by PMA was much faster than its phosphorylation. This suggested that PLD1 activation was independent of its phosphorylation and raised the possibility that phosphorylation actually decreased the activity of the phospholipase.

The PKC Inhibitor Staurosporine and a Kinase-Deficient PKCα Mutant (D481E) both Eliminate PLD1 Phosphorylation by PMA while Blocking the Later Decline in Activity. To see if phosphorylation of PLD1 does decrease its activity, two kinds of approaches
were used: a PKC kinase inhibitor staurosporine and a kinase-deficient PKCα mutant (D481E) (24). Figure 5A shows that staurosporine strongly inhibited PLD1 phosphorylation induced by PKCα in the presence of PMA. The D481E mutant also showed barely detectable phosphorylation of PLD1 upon PMA stimulation (Fig. 5B), consistent with its lack of kinase activity. The time course experiments (Fig. 5C) showed that the inhibitor partially blocked the peak activation of PLD1 induced by PMA (0-10 min), and also diminished the later decline in activity (10-30 min). Similar results were seen in cells expressing endogenous PKC (data not shown). Compared with wild type PKCα, in cells expressing the D481E mutant, PMA induced a smaller initial activation of PLD and a slower later decline in PLD activity (Fig. 5C). In an effort to explain the differences in peak PLD activity, the effects of staurosporine and the D481E mutation on the binding of PKCα to PLD1 were tested. Staurosporine caused minimal effect on the binding between PKCα and PLD1 in the absence of PMA, but partially reduced the association in the presence of the phorbol ester (Fig. 5D). Binding to PLD1 was also less with the D481E mutant compared with wild type PKCα (Fig. 5E).
DISCUSSION

Binding between PKCα and PLD1 was first observed in COS-7 cells (17) and later in Swiss 3T3 fibroblasts (18). However, these studies did not examine the relationship between the binding and the activation of PLD1. In the present study, we provide much evidence that the activation of PLD1 by PMA is highly correlated with the binding of PKCα. This was demonstrated by the effects of deletions and mutations in PKCα on the two parameters (Figs. 2-4) and was supported by the observation that the initial time course of activation of PLD1 was correlated with its association with PKCα and not with its phosphorylation.

A major binding site for PKCα on PLD1 is located at the N-terminus (25-27) and it is likely that there is an additional site (26). However, the binding sites on PKCα for PLD1 are still not defined. In this study, we initially focussed on the binding sites for PLD1 in PKCα. A previous report using proteolysis and chromatography to separate the regulatory and catalytic domains of PKCα indicated that the regulatory domain might be the critical region for PLD1 activation in vitro (16)\(^2\), so we first studied the binding between PLD1 and this domain and showed no detectable binding. More PKCα fragments were made, including the catalytic domain and the C1-C3 fragment, but these fragments also failed to bind and activate PLD1. The fragments were shown to be functionally intact by either their membrane translocation in response to PMA or their retention of kinase activity. Thus these data indicated that PKCα with both the regulatory and catalytic domains intact was required for association and activation of PLD1.
Because PLD is membrane-associated, it is likely that the interaction between PKCα and PLD1 occurs at a membrane locus. Thus the C1 and C2 domains of the regulatory domain, which are required for membrane targeting and translocation of PKCα (29-31) would seem to be essential for activation of PLD1. By this reasoning, the catalytic domain, which stays in the cytosol irrespective of PMA stimulation (data not shown), should be unable to activate PLD1. The observation that the Δ23 mutant, which possesses the domains for membrane targeting, was unable to activate PLD1, indicates that PKCα also requires a residue(s) in the C-terminus in order to bind and activate PLD1.

Previous reports showed that with deletion of up to 11 aa in the C-terminus, PKCα retained kinase activity (32), so we made more C-terminal truncation mutants to study their effects on PLD1 activity. The results showed that the Δ9 mutant still bound to and activated PLD1 while the Δ10 mutant did not, and that both mutants retained kinase activity. In support of the conclusion that the C-terminal 10 aa position (Phe663) is important for PKCα to activate PLD1, two mutants of this residue (F663D and F663A) also failed to bind and activate PLD1. In agreement with a previous study (32), the F663D, F663A and Δ10 mutants retained kinase activity (Fig. 1D). Thus the inability of these mutants to phosphorylate PLD1 (Fig. 3D), reflects their inability to bind to the phospholipase (Fig. 2C and 3C).

The issue of whether or not phosphorylation is needed for PLD1 activation remains controversial. Several studies have shown that ATP is not needed for PKCα to activate PLD in vitro (4,12,14-16). Also, Ser/Thr phosphatase treatment dephosphorylates PLD1 in vitro but does not inhibit its activity (17). However, there is also a report showing that PLD1 is phosphorylated during activation by PKCα (22). Trypsin treatment of the phosphorylated

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enzyme immunoprecipitated from cells treated with PMA, followed by two-dimensional peptide
mapping revealed multiple P-peptides (22). Some of these overlapped with P-peptides generated
from the phosphorylation of PLD1 by PKCα \textit{in vitro}. These P-peptides were analyzed by mass
spectrometry to reveal phosphorylation of PLD1 at residues Ser\textsuperscript{2}, Thr\textsuperscript{147} and Ser\textsuperscript{561} (22).
Mutation of these to Ala resulted in a partial loss of PMA-stimulated PLD activity \textit{in vivo}.
However, many other P-peptides were not analyzed, raising the question of what
phosphorylation of these other residues would do to PLD1 activity. In addition, the effects of the
mutations on the activation of PLD1 by PKCα \textit{in vitro} were not tested.

Our results showed that PLD1 becomes Thr-phosphorylated during PMA treatment cells
expressing wild type PKCα, but not the Δ10, F663D and F663A mutants (Fig. 3D). However, to
answer the key question of whether or not this phosphorylation is required for PLD1 activation,
we carried out time course experiments to measure the real-time PLD1 activity after PMA
treatment (Fig. 4A) (32). Once PtdBut is formed in the PLD assay it is only slowly degraded, so
that the standard assay, in which 1-butanol is added first and then PMA is added for different
time lengths, only reflects the accumulation of PtdBut, not the real-time PLD1 activity. The real-
time results indicated that the increase in PLD1 activity was much faster than the
phosphorylation increase (Fig. 4A c.f. 4D). After the initial peak of PLD1 activation, there was a
slower decrease in activity, consistent with the frequently observed phenomenon that PtdBut
accumulation ceases after several minutes of treatment with PMA and some agonists when PLD
activity is measured using the conventional assay (for references, see 33). Thus, the initial
activity increase was not associated with detectable phosphorylation, whereas the subsequent
activity decrease was correlated with increased phosphorylation (Fig. 4A,D). In other words, the
results were consistent with PLD1 phosphorylation having an inhibitory effect on activity. PLD1
activity quickly reached its peak at 1 min, and membrane translocation and association of PKCα with PLD1 could be detected at that time (Fig. 4A, B, C). It therefore appears that the initial association of PKCα with PLD1 is sufficient for PLD1 to reach its full activation.

To further explore the relationship between PLD1 activation and phosphorylation, a PKC kinase inhibitor staurosporine and a kinase-deficient PKCα mutant (D481E) were used. As expected, staurosporine strongly inhibited PLD1 phosphorylation upon PMA stimulation (Fig. 5A). The D481E mutant also caused negligible phosphorylation of the enzyme (Fig. 5B) consistent with its lack of kinase activity. When the effects of these agents on the PLD1 activity time course in response to PMA were compared with wild type PKCα alone, both the inhibitor and the D481E mutation induced a lower peak of PLD1 activity, but then slowed the subsequent decline in activity (Fig. 5C). The latter results support the conclusion that phosphorylation of PLD1 inhibits its activity. However, the effects of both staurosporine and D481E on the PLD1 peak activity were not consistent with our proposed effect of phosphorylation. To study this, we explored the effects of these agents on the association between PLD1 and PKCα. Figure 5D shows that staurosporine partly decreased the association in the presence of PMA and Figure 5E shows that the D481E mutant bound PLD1 to a less extent than wild type PKCα. These changes in the association with PLD1 could explain the reduced initial activation of PLD. The reasons why staurosporine and the D481E mutation partly reduce the association of PKCα with PLD1 and, hence, decrease the initial activation of PLD1 are unclear. This is because the domains in PKCα that are involved in the binding/activation of PLD1 are unknown. Presumably the D481E mutation partially disrupts this interaction, as does the binding of staurosporine to the catalytic (ATP-binding) domain.
In summary, our results indicate that both the regulatory and catalytic domains of PKCα are required for activation of PLD1 and that there is a required residue (Phe\textsuperscript{663}) in the C-terminus. Surprisingly, our data indicate that phosphorylation is not required for the stimulatory action of PKCα on the enzyme \textit{in vivo} and suggest that phosphorylation is involved in the down regulation of PLD activation that is commonly seen at later times in cells treated with PMA and agonists.
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FOOTNOTES

1. The abbreviations used are: PLD, phospholipase D; PA, phosphatidic acid; DAG, diacylglycerol; PKC, protein kinase C; PS, phosphatidylserine; PIP₂, phosphatidylinositol 4,5-bisphosphate; PMA, 4β-phorbol 12 myristate 13 acetate; PC, phosphatidylcholine; DMEM, Dulbecco’s modified Eagle’s medium.

2. In a later study (28), the regulatory domain was also shown to activate PLD₁ \textit{in vitro}, but it was less potent and considerably less effective than intact PKCα.
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FIGURE LEGENDS

Figure 1. The effects of PKCα domains on binding and activation of PLD1 in COS-7 cells. 

(A) COS-7 cells were transfected with vector, PKCα wild type (WT), RD, CATA, C1-C3 and Δ23 respectively and in vivo PLD assay was carried out. (B) Cells were lysed using lysis buffer containing antiproteases EDTA and EGTA to limit protein degradation. The cell lysates were analyzed by SDS PAGE on 4-20% gels and Western blotted using anti-PKCα N-terminal antibody for WT, RD, C1-C3 and Δ23 and anti-PKCα C-terminal antibody for CATA respectively. (C) COS-7 cells expressing WT, RD, C1-C3 and Δ23 PKCα were fractionated into membranes and cytosol respectively as described in “Experimental Procedures”. Each fraction was analyzed by SDS PAGE and Western blotted using anti-PKCα antibody to test the translocation. “C”, cytosol, “M”, membranes. (D) COS-7 cells expressing WT, CATA, Δ9, Δ10, F663D and F663A PKCα were stimulated with PMA and lysed for Western blot analysis using anti-phosphoThr antibody. Vector transfected cells stimulated with PMA were used as control. (E) COS-7 cells respectively expressing PLD1 and WT, RD, CATA, C1-C3 or Δ23 PKCα were fractionated into membrane and cytosolic fractions for in vitro PLD assay. Membranes from PLD1 transfected cells and cytosol from nontransfected cells were used as control. (F,G) PLD1 was co-expressed in COS-7 cells with WT or RD, CATA, C1-C3 and Δ23 respectively. Cell lysates were immunoprecipitated using anti-Xpress antibody. Western blotting was carried out using both anti-Xpress and anti-PKCα N-terminal antibodies for WT, RD, C1-C3 and Δ23 (F), or both anti Xpress and anti-PKCα C-terminal antibodies for WT and CATA (G) respectively. Data are representative of at least 3 separate experiments.
Fig. 2. The effects of PKCα C-terminal truncation mutants on binding and activation of PLD1 in COS-7 cells. (A) COS-7 cells were transfected with vector, PKCα (WT), or Δ1, 5, 6, 7, 8, 9, 10 or 11 C-terminal aa mutants respectively for in vivo PLD assay. (B) Membranes from PLD1-transfected cells and cytosol from cells transfected with WT, Δ9 or Δ10 PKCα were obtained for in vitro assay. Cytosol from nontransfected cells was used as control. (C) PLD1 was co-expressed in COS-7 cells with PKCα, or its Δ9 and Δ10 mutants respectively. Cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using both anti-Xpress and anti-PKCα antibodies. Data are representative of at least 3 separate experiments.

Fig. 3. Effects of F663D and F663A mutants on binding and activation of PLD1 in COS-7 cells. (A) COS-7 cells were transfected with vector, PKCα (WT), F663D and F663A mutants respectively for in vivo PLD assay. (B) Membranes from PLD1-transfected cells and cytosol from cells transfected with PKCα, F663D or F663A were obtained for in vitro PLD assay. Membranes from PLD1-transfected cells and cytosol from nontransfected cells were used as control. (C) PLD1 was co-expressed in COS-7 cells with F663D or F663A. Cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using both anti-Xpress and anti-PKCα antibodies. (D) PLD1 was co-expressed in COS-7 cells with WT, Δ9, Δ10, F663D and F663A PKCα respectively. Cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using anti-PhosphoThr antibody (PThr PLD). Data are representative of at least 3 separate experiments.
Fig. 4. Time course of PKCα binding to and activation and phosphorylation of PLD1 upon PMA stimulation. COS-7 cells were co-transfected with PLD1 and PKCα. After transfection for 5 h, cells were starved for another 24 h. (A) For real-time PLD activity time course upon PMA stimulation, cells were treated with PMA for 1, 5, 15, 30 min and then 1-butanol was added and incubation continued for an additional 2 min. Cells without PMA treatment were incubated with 1-butanol for 2 min as 0 min control. (B) Cells were treated with PMA for 1, 5, 15, 30 and 60 min and then membrane and cytosol fractions were separated as described under “Experimental Procedures”. The samples were then analyzed using SDS-PAGE and Western blotting using anti-PKCα antibodies. (C,D) Cells were treated with PMA for 1, 5, 15, 30 and 60 min and then immunoprecipitated using anti-Xpress antibody. PLD1 and PKCα binding was detected by Western blotting using both anti-Xpress and anti-PKCα antibodies (C). Thr-phosphorylation of PLD1 was detected by Western blotting using anti-PhosphoThr antibody (P Thr PLD) (D). Data are representative of at least 3 separate experiments.

Fig. 5. Effects of staurosporine and D481E on PLD1 activation and phosphorylation, and binding to PKCα. (A) Cells were co-transfected with PLD1 and PKCα and treated with 20 and 100 nM staurosporine (SP) for 30 min before incubation for 30 min with 100 nM PMA. Cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using anti-PhosphoThr antibody (P Thr PLD). Cells with no SP treatment were used as control. (B) PLD1 was co-expressed in COS-7 cells with WT or D481E PKCα. After 30 min treatment with PMA (100 nM) cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using anti-PhosphoThr antibody. (C) Cells were co-transfected with PLD1 and WT PKCα (for
control and SP time course) or D481E PKCα (for D481E time course). Cells were treated with PMA for 1, 5, 15, 30 min (for SP time course, 100 nM SP was added 30 min before PMA stimulation). 1-butanol was then added and incubation was continued for additional 2 min. 1-butanol was also added to cells without PMA treatment for 2 min as 0 min control. Control, SP, and D481E time courses are shown as diamonds, squares and triangles respectively. (D) Cells were co-transfected with PLD1 and PKCα and treated with SP (20 nM and 100 nM) for 30 min before 30 min treatment with PMA (100 nM). Cell lysates were immunoprecipitated using anti-Xpress antibody and Western blotted using both anti-Xpress and anti-PKCα antibodies. (E) Cells were co-transfected with PLD1 and D481E and treated with PMA (100 nM) for 30 min. Cell lysates were immunoprecipitated with anti-Xpress antibody and Western blotted with anti-PKCα antibody. Data are representative of at least 3 separate experiments.
Fig. 1

A

PtdBut (% of total labelled lipids)

vector  WT  RD  CATA  C1-C3  Δ23

B

WT  RD  C1-C3  Δ23  kDa  CATA  kDa

81  64  50  37  64  50  37

vector  WT  RD  CATA  C1-C3  Δ23

Basal  PMA
Fig. 1
**E**

![Bar graph showing PtdBut (Counts) with control, cytosol, WT, RD, CATA, C1-C3, and Δ23 Conditions. The graph compares Basal and PMA states.]

**F**

![Western blot analysis showing protein bands for WT, RD, C1-C3, and Δ23 under Basal and PMA conditions. The bands indicate masses of 120 (PLD1), 82 (WT), 76 (Δ23), 55 (C1-C3), and 37 (RD).]

**Fig. 1**
Fig. 1

|        | WT | CATA | Mass (kDa) |
|--------|----|------|------------|
| PLD1   | 120|      | 120 (PLD1) |
| WT     | 82 |      | 82 (WT)    |
| CATA   | 43 |      | 43 (CATA)  |

PMA
−−−−+ + ++− − −−+ + ++
Fig. 2
Fig. 3
Fig. 3

|          | F663D | F663A |
|----------|-------|-------|
| PLD1     |       |       |
| PKC α    |       |       |
| PMA      | −     | +     | −     | +     |

|          | WT    | Δ9    | Δ10   | F663D | F663A |
|----------|-------|-------|-------|-------|-------|
| PThrPLD  |       |       |       |       |       |
| PMA      | −     | +     | −     | +     | −     | +     | −     | +     |

Fig. 3
A

PtdBut (% of total labeled lipids) vs PMA Incubation Time (min)

B

PMA Incubation Time (min)

| Time (min) | Membrane | Cytosol |
|-----------|----------|---------|
| 0         |          |         |
| 1         |          |         |
| 5         |          |         |
| 15        |          |         |
| 30        |          |         |
| 60        |          |         |

Fig. 4
**A**

PThrPLD →

| Treatment       | ++ | + | + | + | + |
|-----------------|----|---|---|---|---|
| SP(20 nM)       |    |   |   |   |   |
| SP(100 nM)      |    |   |   |   |   |
| PMA             | −− | + | − | + | − | + |

**B**

PThrPLD →

| Treatment | WT | D481E |
|-----------|----|-------|
| PMA       | −  | +     |

Fig. 5
Fig. 5

C

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D

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Fig. 5
