Phospholipase D1 Is Phosphorylated and Activated by Protein Kinase C in Caveolin-enriched Microdomains within the Plasma Membrane*

Received for publication, January 13, 2000

Yong Kim, Jung Min Han, Byung Ryul Han, Kyung-Ah Lee, Jae Ho Kim, Byoung Dae Lee, Il-Ho Jang, Pann-Ghill Suh, and Sung Ho Ryu‡

From the Department of Life Science and Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea

Activities of phospholipase D (PLD) in diverse subcellular organelles have been identified but the details of regulatory mechanisms in such locations are unknown. Protein kinase C (PKC) is a major regulator of PLD. Serine 2, threonine 147, and serine 561 residues of phospholipase D1 (PLD1) were determined as sites of phosphorylation by PKC (Kim, Y., Han, J. M., Park, J. B., Lee, S. D., Oh, Y. S., Chung, C., Lee, T. G., Kim, J. H., Park, S. K., Yoo, J. S., Suh, P. G., Ryu, S. H. (1999) Biochemistry 38, 10344–10351). In our present study, a triple mutation of these phosphorylation sites diminished markedly phorbol 12-myristate 13-acetate (PMA)-induced PLD1 activity in COS-7 cells. We looked at the location of the PLD1 phosphorylation by PKC by observing PMA-induced band shifts and by use of anti-phospho-PLD1 monoclonal antibody. The shifted PMA-induced proteins and the immunoreactivity of the anti-phospho-PLD1 antibody were mainly found in the caveolin-enriched membrane (CEM) fraction. Depletion of cellular cholesterol led to a loss of this compartmentalization of phosphorylated PLD1 in the CEM. Replacement of the cellular cholesterol led to the restoration of phosphorylated PLD1 in the CEM. Immunocytochemical studies of COS-7 cells revealed that PLD1 was localized in the plasma membrane as well as in the vesicular structures in the cytoplasm, but the phosphorylation of PLD1 occurred only in the plasma membrane. Our results, therefore, show that phosphorylation, and thereby activation, of PLD1 by PKC occurs in the caveolin and cholesterol-enriched low density domain of the plasma membrane in COS-7 cells.

A variety of ligands activate phospholipase D (PLD) in many cell types (1). PLD hydrolyzes phosphatidylcholine generating phosphatidic acid (PA) and choline (2). PA can be converted into diacylglycerol by PA phosphohydrolase (3). As second messengers, PA or diacylglycerol can mediate receptor-mediated intracellular signaling (1, 2). PLD activity is regulated by protein kinases, small molecular weight G-proteins and Ca^{2+} (1). Protein kinase C (PKC) has been suggested as a major upstream mediator of PLD stimulation by various agonists in many different cells (1, 4–7). In most cases PLD activation induced by a variety of ligands is blocked by PKC inhibitors (1, 4–7). Reconstitution of membrane suspensions as the source of PLD with cytosolic fractions as sources of activators revealed that conventional PKCs, especially of the α- and β-type, were phorbol 12-myristate 13-acetate (PMA)-dependent stimulators of PLD (8).

The first mammalian PLD, PLD1, has been cloned, expressed, and purified to homogeneity (9, 10). The purified PLD1 interacted with PKCα directly and was activated PLD1 in the absence of ATP, that is, via phosphorylation-independent mechanism (10–12). In cells, PKCα was also found associated with PLD1 after PMA stimulation (12). PLD2, another isozyme of PLD, showed high basal activity in vitro but was only poorly activated by PKCα (13). Transient expression of PLD1 or PLD2 in COS-7 cells revealed that only PLD1 was stimulated by PMA (13). Therefore, PLD1 is regarded as the isozyme of PLD under PKC regulation. Although PKC activates PLD1 without a phosphorylation process in vitro, phosphorylations at multiple residues of PLD1 occur in cells upon PMA-stimulation (14). Serine 2, threonine 147, and serine 561 of PLD1 have been determined as the sites being directly phosphorylated by PKC (14). Mutations of each of these phosphorylation sites revealed that the phosphorylation at these sites was required for the activation of PLD1 transiently expressed in COS-7 cells (14).

PLD is a membrane-associated enzyme, and its product, PA, which is a functionally important product, is a lipid. Therefore, learning more about the localization of the enzyme and its product PA is important to the understanding of its function. PLD activities have been reported for diverse subcellular organelles including plasma membrane, Golgi vesicles, endoplasmic reticulum, secretory vesicles, and the nuclear membrane (15–20). There are consequently the following questions to be answered: which isozyme of PLD localizes to a certain organelle, how is the isozyme differentially regulated, and what is its role? The ADP-riboseylation factor (ARF), which is a key regulatory molecule in membrane traffic, was identified as an activator of PLD1 (9). It has also been reported that PLD1 is localized in endoplasmic reticulum-Golgi intermediate compartment, secretory granules, and lysosome, where PLD1 may play a role with ARF in vesicle transport and exocytosis (13, 21–23). However, more intense investigations are required to reveal details of molecular mechanism of regulation and the roles of any particular PLD isozyme in each subcellular location. Here we report the results of our study of the location of the PLD1 phosphorylation process using immunocytochemical
were transfected with 0.05, 0.2, or 1 μg DNA of vector (pcDNA3.1), wild

method as well as biochemical fractionation. For the first
time, we show experimental evidence for the specific location
of PLD1 phosphorylation and the phosphorylation-dependent
activation of PLD1 executed directly by PKC.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA, methyl-β-cyclodextrin (M-β-CD), cholesterol, so-
dium cholate, glutaraldehyde, and paraformaldehyde were purchased
from Sigma. Catalytic subunit of protein phosphatase 1, phenylmeth-
ylsulfonyl fluoride, leupeptin, and aprotinin were obtained from Roche
Biochemicals. [3H]myristic acid and a chemiluminescence kit
from Pierce. Protein A, rhodamine-conjugated anti-mouse antibody and fluorescein
isothiocyanate-conjugated goat anti-rabbit antibody were purchased
from Amersham Pharmacia Biotech (Buck-
inhamshire, United Kingdom). Silica gel 60 TLC plates and Triton
X-100 were acquired from EM Science (Gibbstown, NJ). Immobilized
Protein A, rhodamine-conjugated anti-mouse antibody and fluorescein
isothiocyanate-conjugated goat anti-rabbit antibody were purchased
from Pierce. Dulbecco’s modified Eagle’s medium and LipofectAMiNE
were purchased from Life Technologies, Inc. Bovine calf serum was
obtained from HyClone (Logan, UT). Keyhole limpet hemocyanin was
from Calbiochem (San Diego, CA). Horseradish peroxidase-conjugated
goat anti-rabbit IgG or anti-mouse IgG, IgM, and IgA came from
Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). An anti-
tibody against the C-terminal region of PLD1 was made and purified as
described (12). Anti-PKCα polyclonal antibody was obtained from Dr.
Yusuf A. Hannun (Medical University of South Carolina, Charleston,
SC). Anti-caveolin-1 monoclonal antibody was purchased from Trans-
duction Laboratories (Lexington, KY). Anti-Grp 78 (BiP) polyclonal
antibody was from StressGen Biotechnologies Corp. (Collegeville, PA).

**Isolation of Caveolin-enriched Membranes (CEM)—CEM fractions
were prepared as described previously (24) with some modification.
In brief, 2.5 × 10^5 COS-7 cells/100-mm dish were transfected with 5 μg
of pcDNA3.1 expression vector containing rPLD1b cDNA. 24 h after
transfection, the cells were starved for 24 h and then treated with
PMA for 15 min. The COS-7 cells were then washed twice with serum-free medium and then treated with
PMA for 15 min. The COS-7 cells were then washed with phosphate-
buffered saline and scraped into 2 ml of 500 mM sodium carbonate, pH
11.0. The cell suspension was homogenized using a Dounce homoge-
nizer and Polytron tissue grinder and subjected to sonication. The
homogenate was adjusted to 40% sucrose by addition of 80% sucrose
in 1-ml fractions were collected from the top, yielding a total of 12
fractions.

**Preparation of Sterol-M-β-CD Complexes**—Sterol-M-β-CD complexes
were prepared as described by Pike et al. (25). Briefly, 6 mg of sterol was
dissolved in 90 μl of isopropyl alcohol/chloroform (2:1). M-β-CD (200
mg) was dissolved in 2.2 ml of water and heated to 80 °C with stirring
in a water bath. The sterol was then added in small aliquots and the
solution stirred until clear. This solution now contained 6.8 mM sterol.

**Anti-phospho-PLD1 Monoclonal Antibody**—A PLD1 phosphopeptide
including threonine 147 (TKRHpTFRRQN, corresponding to rat PLD1
residues 143–152) was coupled to carrier protein keyhole limpet
hemocyanin with glutaraldehyde. Hybridoma monoclones producing anti-
phospho-PLD1 antibody were obtained by fusion of Sp2/O-Ag14 mouse
myeloma cells with spleen cells derived from BALB/c mice immunized
with the peptide-hemocyanin conjugate. Standard fusion, screening,
and cloning procedures were followed (26).

**In Vivo Phosphorylation of Purified Recombinant (His)_6-PLD1b by
Recombinant PKCa—Recombinant (His)_6-PLD1b and PKCs were ex-

![Image 73x516 to 273x729]

**Fig. 1.** PMA-induced PLD1 activity is reduced in cells carrying a triple
mutation of phosphorylation sites. A, 5 × 10^5 COS-7 cells were transfectioned with 0.05, 0.2, or 1 μg DNA of vector (pcDNA3.1), wild
type PLD1b (WT), or triple mutant PLD1b (S2A/T147A/S561A). 24 h after
transfection, the cells were starved for 24 h prior to incubation with 100 nM PMA for 15 min. 40 μg of wild type lysate and 4 μg of triple mutant lysate were directly subjected to SDS-PAGE in a 6% standard long gel (18 × 16 cm) followed by immunoblot analysis with anti-C-terminal PLD1 antibody. B, 2.5 × 10^5 COS-7 cells were transfected with 5 μg of wild

type PLD1b. Starved cells were then incubated with 100 nM PMA or no
stimulation for 15 min. PLD1b was immunoprecipitated from the lysates
with anti-C-terminal PLD1 antibody. The immune complexes were
then subjected to SDS-PAGE in a 6% standard long gel (18 × 16 cm), followed by immunoblot analysis with anti-C-terminal PLD1 antibody. The data are representative of three separate experiments.

**Fig. 2.** Multiple bands of PLD1 are generated by a phospho-
ylation process. A, 5 × 10^5 COS-7 cells were transfected with 1 μg of
wild type PLD1b (WT) or triple mutant (TM) DNA. 24 h after transfection,
the cells were starved for 24 h and then treated with 100 nM PMA
for 15 min. 40 μg of wild type lysate and 4 μg of triple mutant lysate were directly subjected to SDS-PAGE in a 6% standard long gel (18 × 16 cm) followed by immunoblot analysis with anti-C-terminal PLD1 antibody. B, 2.5 × 10^5 COS-7 cells were transfected with 5 μg of wild
type PLD1b. Starved cells were then incubated with 100 nM PMA or no
stimulation for 15 min. PLD1b was immunoprecipitated from the lysates
with anti-C-terminal PLD1 antibody. The data are representative of three
separate experiments.
Supernatant of hybridoma cells secreting anti-phospho-PLD1, 0.4 blotting and analysis was done as described previously (27). Culture and 1% sodium cholate) containing protease inhibitors (0.5 mM phen-
mM ATP under stimulation with 1 μM PMA. The proteins were separated by 8% SDS-PAGE in a small gel (10 × 10 cm) and subjected to immunoblot analysis with anti-C-terminal PLD1 antibody or anti-phospho-PLD1 antibody. After transfection with PLD1b and serum starvation, COS-7 cells were stimulated with 100 nM PMA for 15 min. PLD1 was immunoprecipitated from 2 mg of cell lysate. The proteins were separated by 6% SDS-PAGE in a long gel and subjected to immunoblot analysis with anti-C-terminal PLD1 antibody or anti-phospho-PLD1 antibody. These data are the representative of three independent experiments.

Immunoprecipitation and Immunoblot Analysis of PLD1—Immunoprecipitation was done as described previously (27). Briefly, the cells were washed with cold PBS and lysed in 1 ml of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, and 10 mM NaCl, 1% Triton X-100, and 1% sodium cholate) containing protease inhibitors (0.5 mM phen-
mM ATP under stimulation with 1 μM PMA. The proteins were separated by 8% SDS-PAGE in a small gel (10 × 10 cm) and subjected to immunoblot analysis with anti-C-terminal PLD1 antibody or anti-phospho-PLD1 antibody. After transfection with PLD1b and serum starvation, COS-7 cells were stimulated with 100 nM PMA for 15 min. PLD1 was immunoprecipitated from 2 mg of cell lysate. The proteins were separated by 6% SDS-PAGE in a long gel and subjected to immunoblot analysis with anti-C-terminal PLD1 antibody or anti-phospho-PLD1 antibody. These data are the representative of three independent experiments.

**Results**

Phosphorylation of PLD1 Is Required for PMA-induced PLD1 Activation—Previously we had studied the PKC-dependent regulatory mechanism of PLD1. Treatment with PMA, an activator of PKC, resulted in phosphorylation at multiple residues of PLD1. Among them, serine 2, threonine 147, and serine 561 were identified as direct phosphorylation sites by PKC (14). Single mutations of each of those sites partially attenuated PMA-induced PLD1 activity (14). We now constructed a triple mutant (S2A/T147A/S561A) of those phosphorylation sites and investigated the PMA-induced PLD1 activity. Under similar expression conditions (compare transfection with 1 μg of wild type DNA and with 0.2 μg of triplet mutant DNA in Fig. 1B), only a negligible amount of activity was seen for the triple mutant as compared with wild type PLD1 (Fig. 1). This suggests that phosphorylation by PKC is a major activation mechanism for PMA-dependent PLD1 activity. However, more expression of the triple mutant protein (more than 2-fold) raised the activity to about half that of wild type (Fig. 1). Other unidentified PKC phosphorylation site on PLD1 or indirect involvement of PKC may contribute to the residual activity by the triple mutant.

**PMA Induces Band Shifts of PLD1**—Phosphorylation of some proteins can result in changes in the proteins mobility (band shift) in SDS-polyacrylamide gels. Because PLD1 is phosphorylated on multiple residues, we examined band shifting of PLD1 after PMA stimulation. After transfection of PLD1 into COS-7 cells, the immunoblot analysis of the expressed proteins showed smeared but distinctly several bands of PLD1. To better analyze these multiple bands, we separated the PLD1 bands in a long 6% SDS-polyacrylamide gel. In the lysates of resting cells, we observed two major bands of PLD1 (Fig. 2A).
When we incubated the cells with inorganic $^{32}$P and immunoprecipitated the PLD1, only the upper band was labeled with $^{32}$P (data not shown). This indicates that phosphorylation of PLD1 occurs also under resting condition. Treatment of the cells with PMA caused band shifts indicating changes in the mobility of PLD1 in SDS-PAGE. More than two shifted bands in the middle of the region between the two basal bands appeared (Fig. 2A). The triple phosphorylation mutant showed a reduced upper band and increased lower band under both resting and stimulated conditions, indicating that some of those shifted bands of wild type PLD1 were generated by PKC phosphorylation. All of the smeared upper bands of immunoprecipitated PLD1 could be abolished by the treatment of the immunoprecipitate with the catalytic subunit of protein phosphatase 1 (PP1), which is one of the major types of protein serine/threonine phosphatase (Fig. 2B). This suggested that the band shifts were caused by phosphorylation of PLD1 at multiple sites of serine or threonine.

**Anti-phospho-PLD1 Monoclonal Antibody Specifically Recognizes PKC-phosphorylated PLD1**—The band shifts of PLD1 involve complex phosphorylations mediated by other kinases as well as PKC. Therefore, band shifts alone are inadequate markers of PLD1 phosphorylation by PKC. To generate specific antibodies against the PKC phosphorylation site of PLD1, we raised antibodies against three phosphopeptides, which each included either serine 2, threonine 147, or serine 561. From among these peptides, we successfully got a monoclonal antibody (phospho-PLD1 antibody) only against the peptide phosphorylated at threonine 147. This phospho-PLD1 antibody recognized specifically PKC-phosphorylated PLD1 in vitro, while it did not react with unphosphorylated PLD1 (Fig. 3A). The antibody also recognized in vivo phosphorylated PLD1, which was induced by treatment with PMA (Fig. 3B). A smear and two distinct bands were recognized by the phospho-PLD1 antibody only after treatment with PMA, suggesting that the PKC-phosphorylated PLD1 existed in multiple states of phosphorylation.

**Phosphorylated PLD1 Is Localized in CEM**—Previously PLD1 was thought to exist at multiple subcellular locations including the plasma membrane and vesicular structures such as trans-Golgi reticulum, Golgi, endosome, secretory vesicles, and/or lysosomes (13, 22, 23, 27). We tried to pinpoint the subcellular location where PLD1 was activated by PKC. We have shown earlier that some portion of expressed PLD1 existed in the plasma membrane and in particular in the caveolin-enriched low density membranes to which PKCα translocated after stimulation with PMA (24, 27). As shown in Fig. 1, direct phosphorylation of PLD1 by PKC is a critical step in PMA-induced PLD1 activation. We next transiently expressed PLD1 in COS-7 cells, resolved the CEM fraction, and checked the location of PLD1 phosphorylation. Caveolin was highly enriched in the CEM fraction, and the endoplasmic reticulum chaperon protein (BiP) was excluded from CEM fraction, indicating good preparation. Although PLD1 is present in both CEM and non-CEM, phosphorylation of PLD1 by PKC was detectable only in the CEM fraction to which PKCα translocated (Fig. 4A). When we resolved the proteins of the CEM fraction and those of a representative sample of the non-CEM fractions (pooling fractions from 8 to 12), band shifts also occurred only in the CEM fraction (Fig. 4B). These results indicated that the direct phosphorylation of PLD1 by PKC occurred in the CEM portion.

**The Partitioning of PLD1 Phosphorylation to the CEM Fraction Is Cholesterol-dependent**—The CEM fraction is composed of membrane domains that are enriched in cholesterol and glycosphingolipid content (29, 30). Cholesterol depletion of the CEM fraction leads to a loss of compartmentalization of caveolin and other signal-related molecules in the low density fractions (25). We, therefore, examined what effect cholesterol depletion would have on the compartmentalization of phosphorylated PLD1. M-β-CD is a cholesterol-binding agent that removes cholesterol from intact cells (25). In preliminary experiments, 5 mM M-β-CD had no effect on the compartmen-
Localization of cavelin for up to 90 min (data not shown). However, when we treated COS-7 cells with 5 mM M-β-CD for 2 h, more than half of the cavelin moved to the non-CEM fractions (Fig. 5). The treatment for 2 h was not toxic to the cells, as determined by trypan blue exclusion (data not shown). Under the same conditions, the amount of PLD1 and translocated PKCα was also reduced in the CEM fraction and was quantitatively recovered in non-CEM fractions. While phosphorylated PLD1 was reasonably reduced in the CEM fraction, it was, surprisingly, increased in non-CEM fractions. Whereas M-β-CD can remove cholesterol from cell membranes, the soluble complex of cholesterol and M-β-CD can mediate the incorporation of cholesterol into membranes. Treatment of cells with the cholesterol complex reversed the cyclicodextrin-induced loss of compartmentalization of PLD1, phosphorylated PLD1, and translocated PKCα (Fig. 5). Measurement of the cholesterol levels in the CEM fraction after treatment with the cholesterol-M-β-CD complex revealed repletion of cholesterol in the cell membrane (data not shown). These results confirm that PLD1, especially phosphorylated PLD1, localizes to the cholesterol-enriched low density membrane domain. To determine whether the compartmentalization of PLD1 and translocated PKCα are important for PLD1 activation, we measured PMA-stimulated PLD1 activity in COS-7 cells after treatment with M-β-CD. Treatment with M-β-CD or add-back of cholesterol did not affect PMA-induced PLD1 activity (Fig. 6). This indicates that the compartmentalization to the low density membranes is not the only way to reveal PMA-dependent PLD1 activity.

**PMA-induced Phosphorylation of PLD1 Mainly Occurs in the Plasma Membrane of Intact Cells**—Previously, we had observed that GFP-PLD1 localized to the plasma membrane as well as to vesicular structures in the cytoplasm of fibroblastic 3Y1 cells (27). After treatment with PMA, GFP-PKCα translocated from the cytosol to the plasma membrane. Therefore, it has been hypothesized that the plasma membrane was the main site of PLD1 activation by PKCα (27). To test this hypothesis, we tried to stain the phosphorylated PLD1 in intact cells with phosphospecific antibody. First, to test the quality of the antibody for immunocytochemistry, we stained the cells overexpressing GFP or GFP-PLD1 in the presence or absence of PMA. As shown in Fig. 7, the phospho-PLD1 antibody specifically stained PLD1 only after stimulation with PMA indicating that the antibody was adequate for immunostaining. GFP-PLD1 overexpressed in COS-7 cells was present in the plasma membrane and vesicular structures of the COS-7 cells (Fig. 8A) just as in the 3Y1 cells (27), but after treatment with PMA, phosphorylated PLD1 was mainly detected in the plasma membrane. Overexpression of PLD1 without GFP fusion and staining of PLD1 with anti-C-terminal PLD1 antibody or phosphorylated PLD1 antibody gave the same results, indicating that the localization is due to PLD1 itself (Fig. 8B). These results prove that phosphorylation and activation of PLD1 mediated directly by PKC occurs in the plasma membrane despite the fact that PLD1 exists at multiple locations in the cells.

**DISCUSSION**

PKC has been considered a major activator of PLD in most cells, but the activation mechanism has not yet been fully studied in detail yet. Because PLD1 has been found expressed at various subcellular locations, different molecular mechanisms may be involved in the regulation of PLD1 at each such location. Our group had observed that PLD1 and PMA-stimulated PKCα co-immunoprecipitated and co-fractionated with a marker for the plasma membrane, in particular CEM (12, 24, 27). Recently, we identified three residues in PLD1 that are phosphorylated by PKC. About half of the PLD1 activity was abolished by a single point mutation in any of these residues, a substitution with alanine as compared with the wild type (14). We prepared triple mutant containing all three mutated phosphorylation sites and showed that almost all of the PMA-stimulated PLD1 activity had disappeared in the mutant (Fig. 1). These results allowed us to conclude that the phosphorylated PLD1 was the active form of PLD1 in intact cells. Although we previously suggested that the CEM in the plasma membrane might be the major site of PLD1 regulation by PKCα, there was no direct evidence proving this hypothesis. In this report, for the first time, we present experimental evidence proving the compartmentalization of PLD1 phosphorylation by PKC in the CEMs within the plasma membrane.

PLD1 at different locations may be differentially regulated by different activators. One of a well known activator of PLD1 is the ARF, a small molecular weight G-protein that is required for many vesicular processes between Golgi, ER, and plasma membranes (21). Previously, it has been reported that PLD1 localizes to dispersed small vesicles that overlap with the location of markers for the endoplasmic reticulum-Golgi intermediate compartment, secretory granules, and lysosomes where PLD1 may play a role in vesicle transport and exocytosis (13, 21–23). On the other hand, PKC, as another important stimulator, phosphorylates PLD1 mainly in the CEM. Thus, PKC-regulated PLD1 may operate in a different function as well as location than ARF-activated PLD1.

Phosphorylation at certain residues of a protein induces mobility shifts in SDS-PAGE. Treatment of cells with PMA induced band shifts of PLD1, which mainly occurred in the CEM.
fraction (Fig. 4B). In the resting state, PLD1 is basally phosphorylated by unknown kinase(s). As a consequence, phosphorylated (upper) and unphosphorylated (lower) bands were detected as shown in Fig. 2A. Upon treatment with PMA, PKC-phosphorylated PLD1 bands appeared between the upper and lower bands (Fig. 3B). In the triple mutant of PKC-phosphorylation sites, those shifted bands were significantly reduced but not completely abolished. This suggests involvement of PKC phosphorylation and other yet undetermined phosphorylation events that would explain the band shifts (Fig. 2A). In addition, we observed a reduction in the upper PLD1 band after PMA stimulation as shown in Fig. 3B. It is therefore probable that some dephosphorylation of PLD1 may coincide with the phosphorylation process induced by PMA stimulation. An unknown protein phosphatase stimulated by PKC might cause dephosphorylation of the upper band. However, after PMA stimulation, we did not detect any spots suggesting the dephosphorylation in two-dimensional phosphopeptide mapping. Therefore, the mobility shifts of PLD1 may reveal a more complex pattern. They may be caused by a complex mechanism involving multiple phosphorylations mediated directly or indirectly by PKC and probably also dephosphorylation events. Based on this reasoning, we concluded that the PMA-induced mobility shifts do not just imply direct phosphorylation of PLD1 by PKC. We, therefore, developed a monoclonal antibody, which successfully recognized specifically PKC-phosphorylated PLD1.

Although PLD1 fractionated into the CEM fraction as well as the non-CEM fraction, PKC-phosphorylated PLD1 was located only in the CEM (Fig. 4). CEM is a cholesterol-enriched membrane domain. Cholesterol depletion from the CEM by using M-β-CD led to the movement of PLD1 from low density to high density membranes. After PMA stimulation, surprisingly, these dislocated PLD1 molecules seemed to be phosphorylated, while the enzyme residing originally in the high density membrane part had remained unphosphorylated (Fig. 5). This result could be explained by a hypothetical existence of a certain PLD1-binding protein, which is located in the CEM and which mediates the interaction between PKC and PLD1. If PKC can phosphorylate PLD1 only through the binding protein and the binding protein moves to the non-CEM together with PLD1 by depletion of cholesterol, then only newly moved PLD1 and not originally resident PLD1 should be phosphorylated in the non-CEM. Consequently, the total amount of phosphorylated PLD1 would not be changed by M-β-CD. In addition, the PMA-stimulated accumulation of phosphatidylethanol in total lipid extract was not affected by the treatment with M-β-CD as seen in Fig. 6. Depletion of cholesterol may cause the reduction of PLD1 activity in the CEM, but the increase in the non-CEM because of the dislocated phosphorylation of PLD1. These results suggest that the compartmentalization of the PLD1 phosphorylation may be the result of the presence of an unknown protein mediating the interaction between PKC and PLD1 in cholesterol-enriched CEMs.

Caveolae, flask-shaped invaginations of the membrane, are considered a specific form of rafts, i.e. glycosphingolipid- and cholesterol-enriched domains. Caveolin is the major protein component of caveolae. Phosphorylation of PLD1 is compartmentalized to the caveolin-enriched and cholesterol-enriched microdomain fraction. Immunostaining of phospho-PLD1 revealed that the plasma membrane is the major site for the direct activation of PLD1 by PKC. We know that PLD1 in the plasma membrane is co-localized with caveolin 1 in intact cells (data not shown). Caveolae have been implicated in membrane signaling and membrane trafficking such as polarized sorting of apical membrane proteins and endocytosis (31). In addition,
caveolae are clustered in regions of the cell surface rich in actin filaments, and the reorganization of the cytoskeleton begins at such sites (32, 33). However, the role of PA formation in the CEM brought about PLD1 phosphorylated by PKC is still unknown. Further investigations are required to establish the role of the PLD1 phosphorylation and activation by PKC in the CEM.

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J. Biol. Chem. 2000, 275:13621-13627.
doi: 10.1074/jbc.275.18.13621

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