Phospholipase D1 in Caveolae: Regulation by Protein Kinase Cα and Caveolin-1[†]

Jae Ho Kim, Jung Min Han, Sukmook Lee, Yong Kim, Taehoon G. Lee, Jong Bae Park, Sang Do Lee, Pann-Ghill Suh, and Sung Ho Ryu*

Department of Life Science and School of Environmental Engineering, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea

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ABSTRACT: Caveolae are small plasma membrane invaginations that have been implicated in cell signaling, and caveolin is a principal structural component of the caveolar membrane. Previously we have demonstrated that protein kinase Cα (PKCα) directly interacts with phospholipase D1 (PLD1), activating the enzymatic activity of PLD1 in the presence of phorbol 12-myristate 13-acetate (PMA) [Lee, T. G., et al. (1997) *Biochim. Biophys. Acta 1347*, 199−204]. In this study, using a detergent-free procedure for the purification of a caveolin-enriched membrane fraction (CEM) and immunoblot analysis, we show that PLD1 is enriched in the CEMs of 3Y1 rat fibroblasts. Purified PLD1 directly bound to a glutathione *S*-transferase−caveolin-1 fusion protein in in vitro binding assays. The association of PLD1 with caveolin-1 could be completely eliminated by preincubation of PLD1 with an oligopeptide corresponding to the scaffolding domain (amino acids 82−101) of caveolin-1, indicating that caveolin-1 interacts with PLD1 through the scaffolding domain. The peptide also inhibited PKCα-stimulated PLD1 activity and the interaction between PLD1 and PKCα with an IC_{50} of 0.5 μM. PMA elicits translocation of PKCα to the CEMs, inducing PLD activation through the interaction of PKCα with PLD1 in the CEMs. Caveolin-1 also coimmunoprecipitated with PLD1 in the absence of PMA, and the amounts of coimmunoprecipitated caveolin-1 decreased in response to treatment with PMA. Taken together, our results suggest a new mechanism for the regulation of the PKCα-dependent PLD activity through the molecular interaction between PLD1, PKCα, and caveolin-1 in caveolae.

Phospholipase D (PLD)[1] catalyzes the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline. PA itself can then be converted to diacylglycerol or lysophosphatidic acid, second messengers which activate various downstream signaling events (*1*−*4*). In mammalian cells, the molecular identities of the two isozymes of PLD, PLD1 and PLD2, have been elucidated (*5*). Several factors have been reported to stimulate PLD1 activity in vitro, including PIP_2, small G proteins such as ARF and RhoA, and PKC (*6*). We have recently reported that PKCα directly interacts with PLD1 when activating PLD activity in the presence of PMA in NIH3T3 cells (*7*). Despite various reports on the regulation of the PLD activity, the intracellular localization of PLD1 and the PKCα-dependent PLD activation remained ambiguous.

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* To whom correspondence should be addressed at the Department of Life Science, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea. Telephone: 82-562-279-2292. Fax: 82-562-279-2199. E-mail: sungho@postech.ac.kr.

[1] Abbreviations: PKCα, protein kinase Cα; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; CEMs, caveolin-enriched membrane fractions; small G proteins, low molecular weight GTP-binding proteins; ARF, ADP-ribosylation factor; EGF, epidermal growth factor; dipalmitoyl-PC, dipalmitoylphosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; Cav-1 peptide, oligopeptide corresponding to caveolin-1 scaffolding domain; Cav-X peptide, scrambled Cav-1 peptide; PLC, phospholipase C; GST, glutathione *S*-transferase; PEt, phosphatidylethanol.

It has been reported that a variety of signaling molecules including PIP_2, G proteins, G protein-coupled receptors, eNOS, PKC, and receptor or nonreceptor protein-tyrosine kinases are enriched in a specialized region, the caveolae, within cells (*8*−*11*). Caveolin, the principal structural protein of caveolae, directly interacts with diverse signaling molecules such as G protein, eNOS, Src kinase, receptor for epidermal growth factor (EGF), and PKCα and appears to serve as structural "scaffolding" within the caveolae (*8*). It has been demonstrated that a short stretch in the membrane proximal region of the cytosolic amino-terminal caveolin domain (caveolin scaffolding domain) mediates these interactions. Synthetic oligopeptides corresponding to this caveolin scaffolding domain (Cav-1 peptide) inhibit the enzymatic activities of several signaling proteins, including Src kinase, PKCα, and receptor for EGF (*8*). The inhibitory action of the scaffolding domain suggests that the caveolin scaffolding domain mediates both association with and inhibition by caveolin of diverse proteins. Our data demonstrate the localization of PLD1 and the PKCα-dependent PLD activation in CEMs. Furthermore, we extended this paradigm to the analysis of the interaction between caveolin and PLD1. We also explored caveolin's role in modulation of the interaction between PLD1 and PKCα.

MATERIALS AND METHODS

Materials. Dipalmitoyl-PC, dioleoylphosphatidylethanolamine, and PIP_2 were purchased from Sigma (St. Louis,

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MO). Dipalmitoylphosphatidyl[*methyl*-^{3}H]choline came from Amersham International (Buckinghamshire, United Kingdom). Phosphatidylethanol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The Cav-1 peptide (DGIWKASFTTFTVTKYWFYR, amino acid residues 82−101) and the scrambled Cav-1 peptide (WGIDKAFFTTSTVTYKWFRY, termed Cav-X peptide) were made by the Peptide Library Support Facility (Pohang, Korea). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-caveolin-1 antibody was from Transduction Laboratories (Lexington, KY). The rabbit antisera against the synthetic oligopeptides corresponding to amino acid residues 1063−1072 of the sequence of human PLD1 were raised as described previously (*7*). To recognize PLD1a, an alternatively spliced form of PLD1, we raised rabbit antisera against the synthetic oligopeptides corresponding to amino acid residues 608−620 of the sequence of human PLD1.

Cell Culture. 3Y1 and COS-7 cells were maintained in low-glucose DMEM and DMEM, respectively, supplemented with 10% (v/v) bovine calf serum (HyClone), 100 units of penicillin/mL, and 100 μg of streptomycin/mL at 37 °C in a humidified, CO_2-controlled (5%) incubator. PLD1b was transiently overexpressed in COS-7 cells using DEAE-Dextran as reported previously (*12*).

Construction and Purification of Glutathione S-Transferase−Caveolin Fusion Proteins. The cDNA for caveolin-1 was obtained from a human testis cDNA library by polymerase chain reaction. The N-terminal amplimer (CGGGATCCATGTCTGGGGGCAAATACGTAG) and C-terminal amplimer (CGGAATTCCGTTGCTGTATTAGCAACTTGGAAC) were designed to anneal to the caveolin-1 sequence and subcloned into the multicloning site (*Bam*HI and *Eco*RI) of the pGEX 4T-1 vector (Pharmacia Biotech, Inc.). After expression in *Escherichia coli* (BL21 strain; Novagen, Inc.), the glutathione *S*-transferase (GST)−caveolin-1 fusion protein was purified by affinity chromatography on glutathione−agarose beads as described (*13*).

Purification of PLD1 and PKCα *from Rat Brain.* PLD1 was purified from rat brain using anti-PLD1 antibody coupled to protein A−Sepharose resin as described (*7*). PKCα from rat brain cytosol was purified to homogeneity with the help of DEAE-Sepharose, hydroxyapatite, phenyl-Sepharose, and HiTrap heparin as also previously described (*7*).

Assay of PLD Activity. PLD activity was measured by choline release from phosphatidylcholine essentially as described (*14*) with a slight modification. In brief, aliquots of the purified PLD1 preparation were added to 125 μL of a standard assay mixture consisting of buffer A (50 mM Tris-HCl, pH 7.3, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM EGTA, 80 mM KCl, and 1 mM dithiothreitol) and 25 μL of phospholipid vesicles composed of dioleoylphosphatidylethanolamine, PIP_2, and dipalmitoyl-PC in a molar ratio of 16:1.4:1 and dipalmitoyl[*methyl*-^{3}H]choline (total 200 000 cpm/assay). The final concentration of PC was 3.3 μM.

Binding of PLD1 to Immobilized GST−Caveolin-1 Fusion Protein. Aliquots (0.1 μg) of PLD1 purified from rat brain were incubated with 2 μg of immobilized GST or GST−caveolin-1 fusion protein in the absence or presence of the Cav-1 peptide or the Cav-X peptide for 1 h at 4 °C. The resins were washed 3 times with 1 mL of buffer B [20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 150 mM NaCl, 0.1% Triton X-100] and subjected to SDS−PAGE followed by blotting with anti-PLD1 antibody.

Detergent-Free Purification of Caveolin-Rich Membrane Fractions. 3Y1 cells grown to confluence in 150-mm dishes were used to prepare the caveolin-enriched membrane fractions (CEMs) as previously described using sodium carbonate (15). In brief, 3Y1 cells were washed 2 times in phosphate-buffered saline and scraped into 2 mL of 500 mM sodium carbonate, pH 11.0. The cell suspension was homogenized using a loosely fitting Dounce homogenizer (10 strokes), sheared in a Polytron tissue grinder (three 10-s bursts; Kinematica GmbH, Brinkmann Instruments, Westbury, NY), and subjected to sonication using an ultrasonicator (three 20-s bursts; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). The homogenate was then adjusted to 45% sucrose by addition of 2 mL of 90% sucrose prepared in MBS buffer [25 mM MES−NaOH (pH 6.5) and 150 mM NaCl] and placed at the bottom of an ultracentrifuge tube. A 5−35% discontinuous sucrose gradient was layered on and the sample centrifuged at 39 000 rpm for 16 h in an SW41 rotor (Beckman Instruments). A light-scattering band formed at the 5/35% sucrose interface. From the top of each gradient, 1 mL gradient fractions were collected, yielding a total of 12 fractions. As shown previously (15), caveolin-1 migrates mainly to fraction 5 in these sucrose gradients. The gradient fractions were analyzed by SDS−PAGE in an 8−16% gradient polyacrylamide gel.

Coimmunoprecipitation of PKCα *with PLD1 from CEMs.* The membranes from the CEMs were isolated by centrifugation at 100000*g* for 1 h at 4 °C. The isolated membranes were treated with IP buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% Nonidet P-40, 0.4% deoxycholate, 60 mM β-octylglucoside, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin and aprotinin), and the insoluble pellet was removed by centrifugation at 15000*g* for 15 min. The supernatant was incubated with anti-PLD1 antibody or preimmune serum precoupled to protein A−agarose resin for 1 h at 4 °C. The immune complexes were sedimented by centrifugation at 15 000 rpm in a microfuge and washed 3 times with 1 mL of ice-cold IP buffer. The bound proteins were solubilized in sample buffer, the amounts of PLD1, caveolin-1, and PKCα were analyzed by immunoblotting, and the intensity of the bands detected by immunoblotting was quantified by a Bio-Rad Model GS-700 imaging densitomer.

Measurement of Phosphatidylethanol (PEt) Formation. 3Y1 cells grown to subconfluence in 150-mm dishes were serum-starved in low-glucose DMEM supplemented with 0.5% dialyzed fetal bovine serum (HyClone). The cells were then incubated with 15 mL of serum-free low-glucose DMEM containing 3 μCi/mL [^{3}H]myristic acid for 3 h. After washing the cell monolayer twice with phosphate-buffered saline, the cells were treated with fresh serum-free low-glucose DMEM with (or without for the control) PMA in the presence of 1.5% ethanol for 5 min at 37 °C, and the CEMs were obtained using sodium carbonate. Lipids were extracted from 1 mL of CEMs (fraction 5) by addition of 1 mL of chloroform and 1 mL of methanol. The organic phase was harvested, dried, and spotted onto a Silica Gel 60 TLC plate which was then developed with chloroform/methanol/

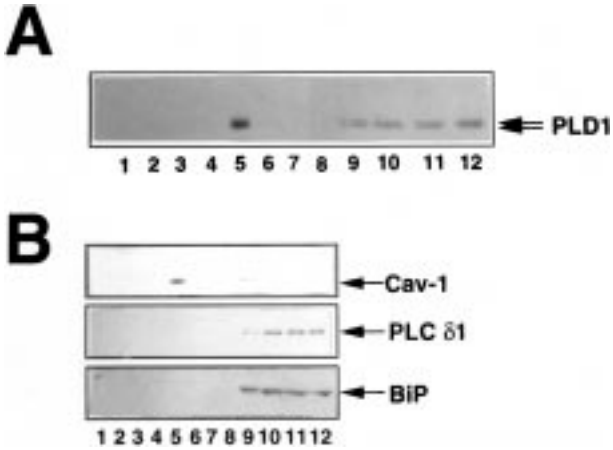

FIGURE 1: Enrichment of PLD1 in CEMs of 3Y1 cells. Sucrose gradient fractions were collected and subjected to immunoblot analysis with specific antibodies directed against PLD1, caveolin-1 (Cav-1), PLCδ1, and BiP. Data are representative of two separate experiments.

acetic acid (90:10:10 by volume). The plate was then exposed to iodine vapor to stain samples of the lipid standard (10 μg of PEt). The amount of radioactivity associated with PEt was determined by liquid-scintillation counting after scraping the samples from the TLC plate.

Western Blotting. After electrophoresis, the proteins were transferred to nitrocellulose. Blocking was performed in TTBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder. The membranes were then incubated with primary antibodies at the concentration recommended by the manufacturer for 2 h at room temperature. The immunoblots were subsequently washed and incubated with horseradish peroxidase-linked secondary antibodies (Kirkegaad and Perry Laboratories, Inc.) for 1 h at room temperature, rinsed 4 times in TTBS buffer, and developed with horseradish peroxidase-dependent chemiluminescence (Amersham Corp.).

RESULTS

Subcellular Distribution of PLD1. To isolate caveolin-enriched membrane fractions from 3Y1 cells, we used a detergent-free purification method based on the resistance to extraction of the caveolin complexes in the presence of sodium carbonate as described under Materials and Methods. Aliquots of the individual gradient fractions collected were separated by SDS−PAGE, transferred onto nitrocellulose membranes, and immunoprobed with anti-caveolin-1 or anti-PLD1 antibodies, respectively. As seen in Figure 1, caveolin-1 was enriched in fraction 5, which corresponds to 5−35% sucrose equilibrium density. PLD1 was detected in the same fraction as caveolin-1, but also in fractions 9−12. In contrast, cytoplasmic phospholipase C-δ1 and an endoplasmic reticulum chaperon protein (BiP) were excluded from the CEMs, demonstrating the specific localization of PLD1 in the CEMs. Intriguingly, the anti-PLD1 antibody recognized two protein bands of approximately 120 kDa but with recognizably different molecular masses. Hammond et al. reported recently the existence of two splice-variants of PLD1, PLD1a and PLD1b, with similar regulatory properties (*6*). Using an antibody that specifically recognized PLD1a, we found that the upper band of the two bands is PLD1a

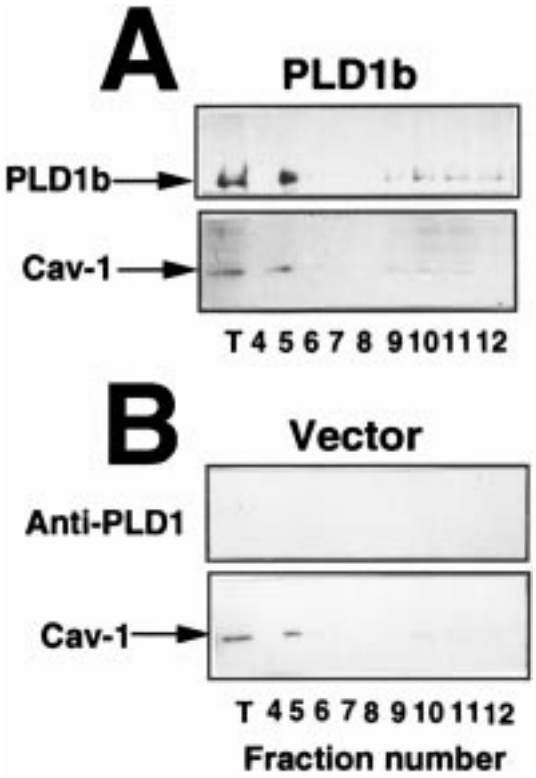

FIGURE 2: Recombinant PLD1b expressed in COS-7 cells is targeted to CEMs. COS-7 cells were transfected with plasmids bearing the PLD1b gene (A) and with control plasmid (B), respectively. CEMs were purified by the sucrose density gradient method. Aliquots (25 μL) of lysate (T) and of the gradient fractions were subjected to SDS−PAGE, followed by immunoblot analysis with specific antibodies against PLD1 and caveolin-1 (Cav-1). Data are representative of two separate experiments.

(data not shown). We therefore assume that the two bands are PLD1a and PLD1b, respectively.

Localization of Transiently Expressed PLD1b in Caveolin-Enriched Membranes of COS-7 Cells. To confirm the localization of PLD1 in CEMs, we transiently expressed PLD1b in COS-7 cells and then monitored the localization of PLD1b in CEMs by immunoblot probed with anti-PLD1 antibody. As shown in Figure 2, COS-7 cells contain only a small amount of PLD1, whereas the transiently expressed PLD1b shows up as a distinctive protein band with an apparent molecular mass of 120 kDa and is recognized by anti-PLD1 antibody, indicating that the protein band consists of PLD1b. The expressed PLD1b is enriched in CEMs (fraction 5) of the COS-7 cells as shown in Figure 2A. In contrast to PLD1b, the amount of caveolin-1 was not affected by the transient expression of PLD1b. Consistent with the localization of PLD1 in the CEMs of 3Y1 cells, this result confirms the localization of PLD1 in the CEMs.

Direct Interaction of PLD1 with Caveolin-1. Caveolin-1 has been reported to interact with several signaling molecules, i.e., Gα subunit, PKCα, c-Src, H-Ras, receptor for EGF, through a common membrane-proximal region in its NH_2-terminal cytoplasmic domain, the caveolin scaffolding domain (*8*, *19*−*22*). These observations, together with the results described above, led us to investigate whether PLD1 directly interacts with caveolin-1. Figure 3 shows that PLD1 purified from rat brain specifically bound to the immobilized GST−caveolin-1 fusion protein and not to immobilized GST protein by itself. Moreover, the interaction between PLD1

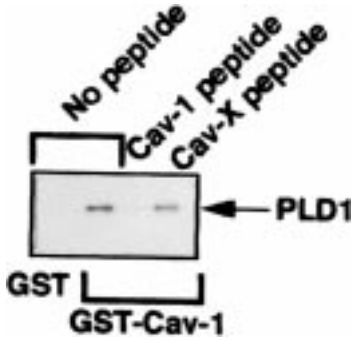

FIGURE 3: Interaction between caveolin-1 and PLD1 through the caveolin-1 scaffolding domain. PLD1 purified from rat brain was incubated with immobilized GST or the GST−caveolin-1 fusion protein in the absence or presence of 10 μM caveolin scaffolding domain peptide (Cav-1 peptide) from caveolin-1 or the scrambled peptide (Cav-X peptide) as described under Materials and Methods. The resins were washed and subjected to SDS−PAGE followed by blotting and immunoprobing with anti-PLD1 antibody. Data are representative of two separate experiments.

and caveolin-1 was dramatically reduced by addition of 10 μM synthetic oligopeptide corresponding to the caveolin-1 scaffolding domain (Cav-1 peptide) but not of the scrambled version (termed Cav-X peptide) which was synthesized as previously described (*22*). Various reports about the interaction between caveolin and its interacting proteins have demonstrated specific interaction with caveolin-1 (*19*−*21*). Taken together, these result indicate that the caveolin-1 scaffolding domain is implicated in the interaction of PLD1 with caveolin-1.

*Effect of the Caveolin Scaffolding Domain on PKC*α*-Dependent PLD Activity.* Because caveolin negatively regulates the functions of caveolin-interacting molecules, such as PKCα, c-Src, receptor for EGF, eNOS, and Gα subunit (*19*−*22*), we next examined whether caveolin functionally interacts with PLD1 as well. PLD1 can be activated by PKCα in a PMA-dependent manner (*16*−*18*). We, therefore, evaluated the effect of caveolin-1 on the PKCα-dependent PLD activity using Cav-1 peptide, because purified caveolin tends to aggregate at high concentrations (23) and would, therefore, not be usable in our in vitro assays. The PLD activities stimulated by 10 nM PKCα and 100 nM PMA are inhibited by the Cav-1 peptide with an IC_{50} of about 0.5 μM as shown in Figure 4A, whereas the Cav-X peptide had only a modest inhibitory effect at higher peptide concentrations. To explore whether the inhibition of PKCα-stimulated PLD activity by the Cav-1 peptide is caused from the direct interaction between PLD1 and Cav-1 peptide, we examined the effect of Cav-1 peptide on the basal PLD activity of PLD1. As shown in Figure 4B, Cav-1 peptide specifically inhibited the basal PLD activity as well as the PKCα-dependent PLD activity, indicating that the Cav-1 peptide inhibits the PLD activity through specific interaction with PLD1. Recently, Oka et al. (*19*) reported that the Cav-1 peptide inhibits the PMA-dependent PKCα activation. Consistent with this result, we found that the inhibitory effect of Cav-1 peptide on the PKCα-stimulated PLD activity was not fully recovered by the addition of 50 nM PKCα, which is an excess concentration to stimulate PLD activity (Figure 4C), suggesting the partial implication of the PKCα inhibition by Cav-1 peptide on the supression of PKCα-stimulated PLD activity. Therefore, the results suggest that caveolin-1 plays

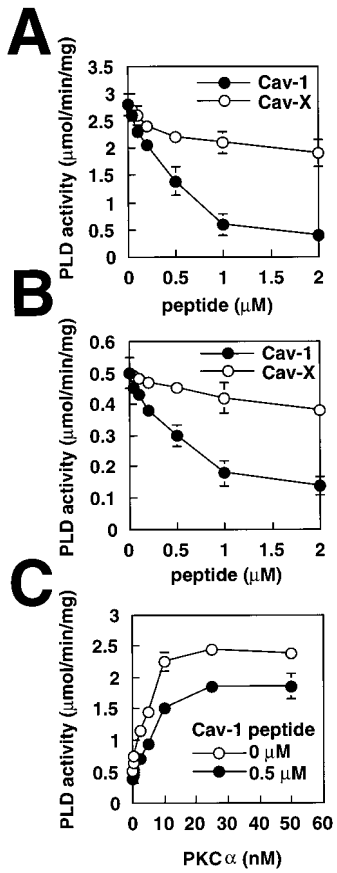

FIGURE 4: Inhibition of PLD activity by the caveolin-1 scaffolding domain peptide. (A) The Cav-1 and Cav-X peptides were evaluated for their dose-dependent effect on PKCα-dependent choline-releasing activity of 0.5 nM PLD1 in the presence of 100 nM PMA. (B) The Cav-1 and Cav-X peptides were evaluated for their dose-dependent effect on basal PLD activity of 0.5 nM PLD1. (C) Dose-dependent effect of PKCα on PLD activity was measured in the absence (open circles) or presence (closed circles) of 0.5 μM Cav-1 peptide. Means ± SE from three independent assays are shown.

a role in negatively regulating PKCα-dependent PLD activity through direct interaction with PLD1.

*Function of the Caveolin Scaffolding Domain in the Interaction of PKC*α *with PLD1.* Next, we examined the effect of the Cav-1 peptide on the interaction of PKCα with PLD1. As shown in Figure 5, PKCα directly bound to PLD1 coupled to immunoaffinity resin in response to PMA. Intriguingly, this association of PKCα with PLD1 could be inhibited by addition of 2 μM Cav-1 peptide but not of the Cav-X peptide. This result demonstrates that the scaffolding peptide interferes with the interaction between PKCα and PLD1 through direct association with the two proteins and is thus implicated in the negative regulation of the PKCα-dependent PLD1 activity.

*Decreased Interaction of PLD1 with Caveolin-1 in Response to PMA-Dependent Association with PKC*α *in CEMs.* To find out whether the interaction between PLD1 and PKCα occurs in the CEMs, we examined the localization of PKCα in CEMs of 3Y1 cells in response to PMA treatment. Recently, Hope et al. reported the redistribution of PKCα to the CEMs after treatment of A431 cells with PMA (*10*). As expected, PKCα was only weakly detectable in CEMs before the PMA treatment; however, PKCα clearly translocated to the CEMs after treatment of the 3Y1 cells with PMA

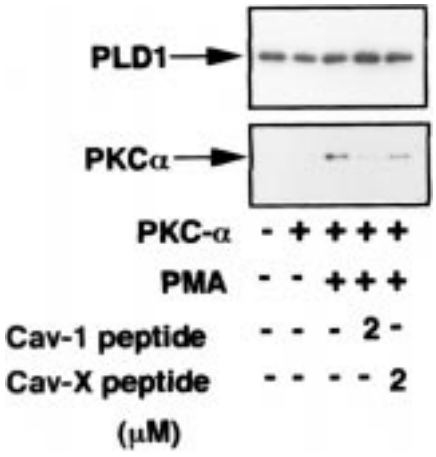

FIGURE 5: Inhibition of the interaction between PKCα and PLD1 by the caveolin-1 scaffolding domain peptide. Aliquots (0.1 μg) of rat brain PLD1 were incubated with anti-PLD1 antibody (2 μg) coupled to protein A−agarose. The immune complexes were then incubated with 20 nM PKCα and Cav-1 or Cav-X peptides of the indicated concentrations in the absence or the presence of 100 nM PMA at 37 °C for 15 min under standard assay conditions. The resulting immune complexes were washed 3 times with buffer B and subjected to SDS−PAGE and immunoblot analysis with antibodies against PLD1 and PKCα. Data are representative of two separate experiments.

for 5 min (Figure 6A). In contrast to PKCα, the distribution of PLD1 did not change over the time course, suggesting that PKCα translocates to the CEMs in a PMA-dependent manner, while PLD1 preexists in that location. In agreement with Hope et al. (*10*), the distribution of caveolin-1 was not affected by the PMA treatment (data not shown). Next, we examined whether the translocation of PKCα induces the interaction of PLD1 with PKCα. As shown in Figure 6B, PKCα and caveolin-1 coimmunoprecipitated with PLD1 from CEMs of 3Y1 cells, and the amounts of PLD1-associated PKCα increased after treatment of the cells with PMA, whereas the amounts of caveolin-1 in immune complexes decreased in response to the treatment with PMA. A horseradish peroxidase-dependent chemiluminescence system was used for detection, and the developed bands were quantified by a densitometer (Figure 6C). These results suggest that the interaction of PLD1 with caveolin-1 may be relieved by the PMA-induced association of PKCα with PLD1. To clarify whether PLD1 is activated by the interaction with PKCα in CEMs, we measured the production of PEt which is formed by PLD when ethanol is used as a nucleophile instead of water to cleave PC, in the CEMs. As shown in Figure 6D, the treatment of 3Y1 cells with PMA increased the amount of PEt accumulated in the CEMs. The PEt accumulated in CEMs is about 30% of the total PEt produced in PMA-treated 3Y1 cells (data not shown), indicating the enrichment of PEt as well as PLD1 in CEMs. Taken together, these results suggest that PKCα translocates to CEMs in response to PMA treatment and then directly interacts with PLD1 and, thus, may activate the enzymatic activity of PLD1.

DISCUSSION

It has been demonstrated that PLD activation can occur along several distinct signaling pathways, including PKC, small G proteins of the Ras superfamily, and protein tyrosine kinases. CEMs have been shown to harbor a variety of signaling molecules, including PKC, phospholipase C (PLC) γ1, receptors for EGF and platelet-derived growth factor, and PIP_2 (*19*−*26*). Recent reports suggest CEMs as the location of receptor tyrosine kinase-induced signaling, including EGF-dependent PLC activation and platelet-derived growth factor-induced tyrosine phosphorylation (*26*). We have previously demonstrated that PLCγ1-induced diacylglycerol generation and subsequent PKC activation elicit the activation of PLD in NIH3T3 cells (*27*). Recent data showing that the scaffolding domain of caveolin-1 is sufficient for the binding of PKC, Ha-Ras, G protein α subunit, and protein tyrosine kinases suggest that in addition to its role as a structural protein for CEMs, specific protein−protein interactions between caveolin and other resident proteins can regulate signal transduction (*8*, *28*). Therefore, CEMs may play a role in agonist-induced PLD activation by accumulating PLD-activating factors such as receptor tyrosine kinase, PLC, PIP_2, and PKCα.

The interaction of PLD1 with PKCα appears to serve as the key determinant of enzyme activity, and this interaction may be dramatically regulated by PMA. In this study, we demonstrated the localization of PLD1 in CEMs and PMA-dependent association of PKCα with PLD1 in CEMs. Consistent with our results, the existence of RACK, a receptor for PKCα, in caveolae and translocation of PKCα to CEMs have been reported (*29*). Moreover, caveolin-1 has been reported to inhibit the kinase activity of PKCα with an IC_{50} of about 4 μM through direct interaction with PKCα (*19*). Here, we showed how PLD activity is regulated by the caveolin scaffolding peptide through direct interaction. Caveolin-1 interferes with the interaction between PLD1 and PKCα and inhibits the PKCα-stimulated PLD1 activity through its scaffolding domain in vitro. The Cav-1 peptide inhibited not only the PKCα-dependent PLD activity (Figure 4A) but also the basal PLD activity of PLD1 with an IC_{50} of about 0.5 μM (Figure 4B), suggesting that the inhibitory effect of the peptide on PLD activity is mainly derived from direct interaction with PLD1. Based on these findings, it is possible to speculate that PLD1 is kept "less active" by binding to caveolin-1. In response to PMA, PKCα is recruited to the CEMs to lift the inhibition imposed by caveolin-1 binding. We found that caveolin-1 coimmunoprecipitated with PLD1 from CEMs and that the amount of caveolin-1 associated with the immune complexes decreases in response to treatment with PMA (Figure 6B), and these results supports the latter concept.

Enriched PLD activity has been isolated from almost every region of the cell, including plasma membrane, cytoplasm, nucleus, and 'endomembranes' consisting of the Golgi, endoplasmic reticulum, and endosomes (*5*, *30*−*33*). Colley et al. reported the subcellular localization of influenza-epitope-tagged PLD1 and PLD2 in the endomembranes and the plasma membrane of REF-52 rat embryo fibroblasts, respectively (*5*), whereas we found PLD1- and PKCα-dependent PLD activity localized in the plasma membrane of 3Y1 cells (*34*). Intriguingly, caveolin-1 has been reported to be highly enriched inside *trans*-Golgi network-derived exocytic vesicles and plasma membrane invaginations called caveolae (*35*−*37*), suggesting a possible colocalization of PLD1 and caveolin-1 in these subcellular compartments.

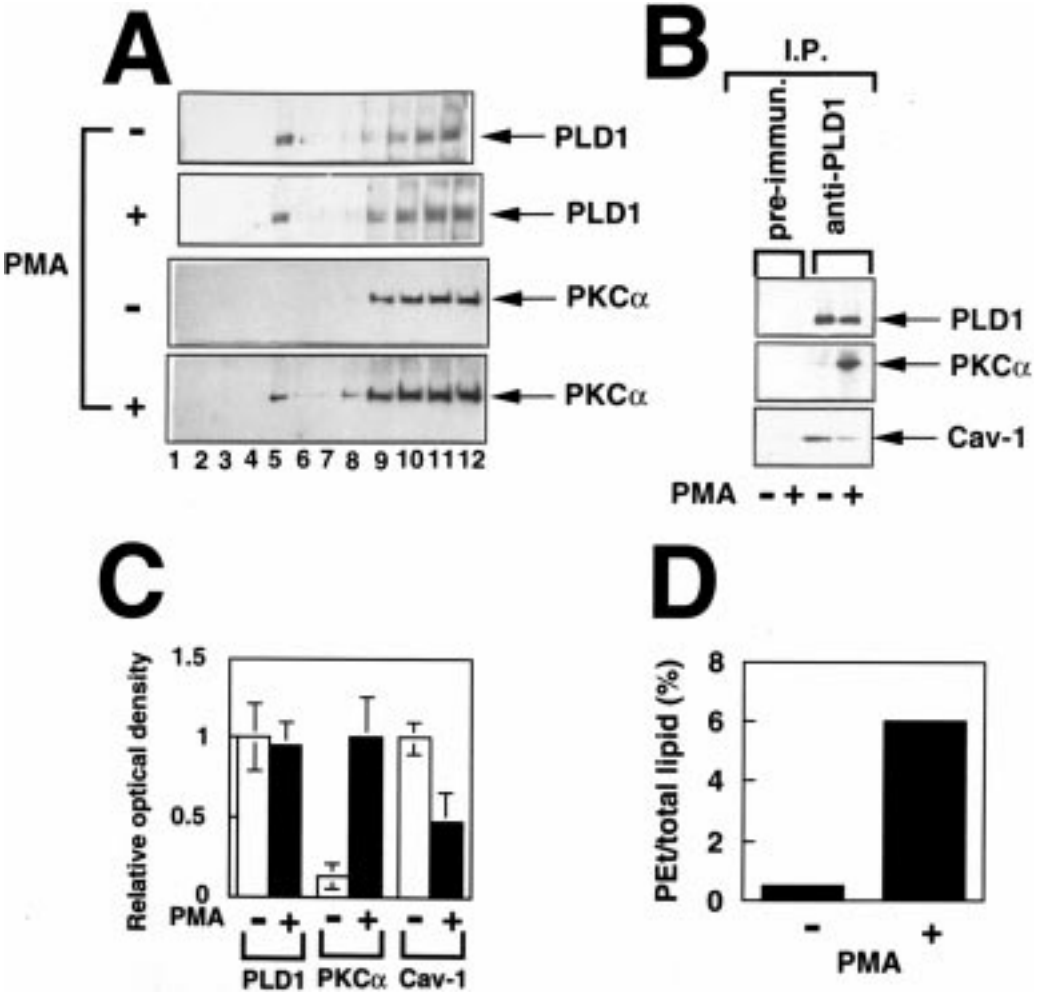

FIGURE 6: Translocation of PKCα to CEMs and PKCα-dependent PLD1 activation in response to PMA. (A) Two 15-cm dishes of 3Y1 cells were serum-starved for 24 h and treated with 0.1% DMSO (−) or with 100 nM PMA (+) for 5 min at 37 °C. The cells were then subjected to carbonate-based homogenization, after which the homogenate was adjusted to 45% sucrose. 4 mL of homogenate was fractionated on a discontinuous sucrose gradient by ultracentrifugation, and membranes within aliquots (25 μL) of the gradient fractions were isolated by centrifugation at 300000*g* for 20 min. Isolated membranes were subjected to SDS−PAGE and immunoblot analysis using anti-PKCα antibody. (B) Coimmunoprecipitation of PKCα and caveolin-1 with PLD1 from CEMs. Membrane fractions were isolated from the CEMs (0.8 mL) and extracted as described under Materials and Methods. The extracts were immunoprecipitated with control antibody or anti-PLD1 antibody, and the immune complexes were subjected to SDS−PAGE followed by immunoblot analysis with antibodies against PLD1, caveolin-1, and PKCα. (C) Quantitation of relative amounts of PLD1, caveolin-1 (Cav-1), and PKCα was performed by scanning the spots on the film with a densitometer. Data represent the mean ± SD from three different experiments. (D) Quiescent 3Y1 cells were labeled with [^{3}H]myristic acid, treated with (or without for the control) 100 nM PMA in the presence of 1.5% ethanol for 5 min at 37 °C. PEt of the CEMs was resolved and measured as described under Materials and Methods. These data are representative of three separate experiments.

Caveolae have been implicated in exocytosis and endocytic pathways, such as transcytosis and potocytosis (*38*−*40*). Recent reports provide evidence of a role for PKCα in the regulation of potocytosis and caveolar invagination (*41*−*43*). Interestingly, RhoA, which is an activator of PLD1, has been reported to be localized in caveolae (*44*) and to be implicated in caveolae formation (*45*). Although it is still unknown whether PLD1 is involved in the caveolae-mediated exo- and endocytosis, it is possible that increased PA levels, generated as a consequence of PLD activity, could function in these processes by being hydrolyzed to diacylglycerol which would further trigger activation of PKC. In conclusion, our studies have identified the site of PLD1- and PKCα-dependent PLD activation in CEMs, where caveolin-1 has a negative regulatory function in the interaction between PLD1 and PKCα. We suggest a possible role for PLD1 in caveolae-mediated physiological responses.

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