Potent Direct Inhibition of Mammalian Phospholipase D Isoenzymes by Calphostin-c†

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ABSTRACT: Calphostin-c inhibits protein kinase C (PKC) isoenzymes by covalent modification of the lipid binding regulatory domain. Exposure of cells to calphostin-c elicits PKC independent effects including disruption of intracellular transport, growth inhibition, and stimulation of apoptosis suggesting actions at additional targets. Phospholipase D (PLD) enzymes are targets for activation by PKC. We have investigated the PKC isoenzyme selectivity for activation of two mammalian PLD enzymes, PLD1 and PLD2, by PKC. We examined the sensitivity of this process to widely used PKC inhibitors and report the surprising finding that calphostin-c is a potent direct inhibitor of PLD1 and PLD2. In vitro, calphostin-c inhibits activity of both PLD1 and PLD2 with an IC_{50} of approximately 100 nM. Inhibition is not overcome by protein and lipid activators of these enzymes and does not involve blockade of phosphatidylinositol 4,5-bisphosphate-dependent PLD binding to substrate containing liposomes. Studies using a series of deletion and point mutants of the enzymes suggest that calphostin-c targets the PLD catalytic domain. Inhibition of PLD by calphostin-c in vitro involves stable and apparently irreversible modification of the enzyme. Activity of both PLD1 and PLD2 can be inhibited by calphostin-c treatment of intact cells in a manner that is independent of upstream actions of PKC. Our results suggest that inhibition of PLD1 and PLD2 may explain some of the PKC-independent effects of calphostin-c observed when the compound is applied to intact cells.

Calphostin-c is a perylenequinone metabolite of the fungus *Cladosporium caldosporoides* that inhibits kinase activity of protein kinase C (PKC)[1] isoenzymes (Figure 1). Inhibition of PKC by calphostin-c is highly potent (IC_{50} 50 nM) irreversible and light-dependent. Because calphostin-c treatment of PKC is accompanied by blockade of phorbol ester binding to the enzyme the regulatory domain of these enzymes has been presumed to be the major site targeted by the inhibitor. The inhibitory mechanism has been proposed to involve covalent attachment of the calphostin molecule to PKC with localized damage to the protein subsequently caused by light-dependent free radical formation (*1*, *2*). However, the precise site of interaction of calphostin-c with PKC remains undefined. Although more specific than many of the widely used catalytic site-directed PKC inhibitors such as staurosporine and indolemaleimides, calphostin-c clearly exerts a number of PKC-independent effects when applied to intact cells. At 50−100 nM, calphostin-c causes a rapid disruption of intracellular membrane structures including the ER and Golgi (*3*). Similar concentrations of calphostin-c inhibit cell growth and promote apoptosis (*4*, *5*). Both of these phenomena cannot be mimicked by PKC inhibitors that target the catalytic site or by PKC down regulation. These findings suggest that additional targets for calphostin-c exist.

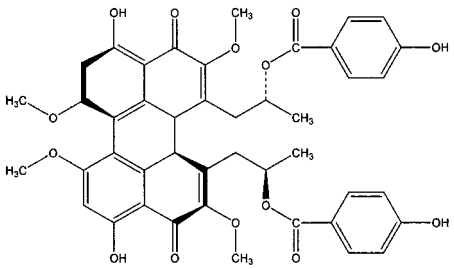

FIGURE 1: Structure of calphostin-c.

Activation of many cell surface receptors stimulates hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) producing phosphatidic acid (PA) and choline. PA and its metabolites, diacylglycerol (DG) and *lyso*PA, have a number of biological activities that include effects on cell surface receptors, protein kinases, phosphatases, regulation of small GTP-binding proteins. PA may also regulate protein−membrane interactions through changes in membrane charge and surface properties. PLD has been implicated in pathways of cell regulation that control membrane trafficking, cell morphology, and mitogenesis (*6*, *7*). Mammalian cells contain phosphoinositide and fatty acid responsive PLD activities. PLD1 and PLD2 constitute the phosphoinositide-dependent class of PLD enzymes. In vitro,

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[1] Abbreviations: PC: phosphatidylcholine; PS: phosphatidylserine; PE: phosphatidylethanolamine; PA: phosphatidic acid; DG: diacylglycerol; PtdBuOH: phosphatidylbutanol; PLD: phospholipase D; PLC: inositol lipid-specific phospholipase C; PKC: protein kinase C; ARF: ADP-ribosylation factor; $PI(4,5)P_2$: phosphatidylinositol 4,5-bisphosphate; GTPgS: guanosine 5′-*o*-thiotrisphosphate; PMSF: phenylmethylsulphonylfluoride; PMA: phorbol myristate acetate.

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PLD1 activity is stimulated by ARF and Rho family GTP-binding proteins and the conventional PKC isoforms -α and -β (*8*−*13*). Purified murine PLD2 is insensitive to the GTP binding protein activators of PLD1 although very modest stimulation by ARF has been reported for the human homologue of this enzyme (*14*−*16*).

PKC is clearly an important upstream regulator of PLD activity, but the mechanisms involved appear complex. Activation of PLD1 by PKC in vitro can be observed in the absence of ATP, and this effect is completely independent of protein kinase activity (*10*, *11*, *13*). However, work using both intact and broken cell systems, including the use of inhibitors of PKC catalytic activity, suggest that protein kinase activity is required for PLD activation by PKC in some settings (*17*−*19*). PLD1 is a substrate for phosphorylation by PKC (*12*). This modification does not directly regulate catalysis but controls stimulus dependent relocalization of the enzyme within the cell (*20*). Recent work using a PLD1 mutant that is a PKC substrate but selectively unresponsive to direct activation by PKC suggests that this mode of regulation accounts for rapid increases in PLD activity in response to agonists, while another mechanism, possibly involving phosphorylation, may account for slower more sustained increases in PLD activity (*21*).

During the course of investigations of the mechanism and specificity with which PKC isoenzymes regulate PLD1 and PLD2, we made the surprising finding that calphostin-c is a potent direct and irreversible inhibitor of both PLD enzymes. Our results suggest that PLD1 and PLD are relevant targets for calphostin-c and that inhibition of PLD may explain some of the PKC-independent actions of this inhibitor on cell growth and organelle function in target cells.

EXPERIMENTAL PROCEDURES

General Reagents and Methods. Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). PI(4,5)-P_2 was purified from bovine brain (*22*). Bisindolylmaleimide I hydrochloride and calphostin-c were from Calbiochem (San Diego, CA) and Sigma (St. Louis, MO). Cabbage PLD was from Sigma (St. Louis, MO). Human ARF1 and human RhoA were expressed and purified as described (*11*).

SDS−*PAGE, Western Blotting, and Autoradiography.* SDS−PAGE and Western blotting were performed as described previously (*11*, *23*). HA epitope tagged proteins were detected with the 12CA5 monoclonal antibody Boehringer Mannheim (Indianapolis, IN).

Expression and Purification of PLD1 and PLD2. PLD1 and PLD2 and a number of previously described mutants including PLD1 N, PLD2ΔN, PLD1 PIM87 miniPLD and PLD2R554GR558G were expressed in insect cells using recombinant baculoviruses (*24*, *25*). In some cases, insect cell lysates or membranes were used as sources of PLD activity. In other cases, the PLD enzymes were purified by immunoaffinity chromatography. We also generated recombinant baculoviruses for expression of some of the wild-type and mutant PLD proteins containing an N-terminal "glu-glu" tag. In this case, proteins were purified by chromatography on a resin-immobilized monoclonal antibody and eluted by incubation of the resin with a competing peptide (*11*). For experiments using miniPLD, the eluting peptide was removed by gel filtration chromatography on Sephadex G25, and the protein preparation was concentrated to approximately 1 mg/mL by pressure filtration.

Expression and Purification of PKC Isoforms. Recombinant baculoviruses for expression of the human PKC isoforms used in this study were a kind gift of Dr. Larry Ballas, Sphinx Pharmaceuticals Inc. (Durham, NC). The PKC proteins were purified by minor adaptations of methods previously described (*11*, *26*, *27*). Five hundred milliliters suspension cultures of SF-9 cells were infected with viruses at a multiplicity of infection of 10 and cultured for 48 h at 27 °C with constant stirring. The cells were harvested by gentle centrifugation, the pellets were washed once in phosphate buffered saline (PBS) and then resuspended in 10 mL of PBS before disruption by nitrogen cavitation (Parr cell bomb, 1300 psi for 30 min). The cell extract was discharged into buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM benzamidine, and 0.1 mM PMSF and centrifuged at 50000*g* for 1 h. Starting with the supernatant obtained, the purification procedure employed sequential chromatography on Source 15Q, (Pharmacia, Piscataway, NJ), threonine sepharose 4B CL, and Source 15 Phenyl (Pharmacia, Piscataway, NJ). Fractions were analyzed by determination of histone kinase activity and immunoblotting with appropriate PKC isoenzyme-selective antibodies (Santa Cruz Biotech, Santa Cruz, CA). The purity of the PKC preparations used in these experiments was greater than 90%, and the specific activity of the purified enzymes was comparable to that reported by others. These PKC preparations used contained no detectable PLD activity measured as described below.

*Expression and Purification of the Regulatory Domain of PKC-*α. cDNA encoding the PKC-α regultory domain was inserted into PET 28a from Novagen (Madison, WI) and transformed into the *Escherichia coli* strain BL-21DE3. Expression and purification followed manufacturer's procedures. Briefly, an overnight culture was diluted to 600 mL and grown to $OD_{600} = 0.6$, induced with 1 mM IPTG, and cultured for an additional 3 h. Bacteria were pelleted, washed in PBS, and lysed in 12 mL binding buffer (20 mM Tris, pH 7.9, 5 mM imidazole, 500 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, and 10 μg/mL leupeptin) containing 1 mg/mL lysozyme and 2% Triton X-100. After sonication and centrifugation, the lysate was batch loaded onto 0.5 mL NiNTA sepharose (Invitrogen Inc, Carlsbad, CA), washed in binding buffer containing 60 mM imidazole, and eluted with 0.5 mL of binding buffer containing 1 M imidazole. Protein was exchanged into buffer containing 20 mM Tris, pH 7.9, 500 mM NaCl, 1 mM EGTA, 1 mM DTT by gel filtration chromatography on Sephadex G-25.

Determination of PLD Activity in Vitro. PLD1 activity was measured using [^{3}H]-PC presented in sonicated lipid dispersions. When inhibitors were used, they were preincubated with the assay mixture for 10 min at 37 °C, and the assays were initiated by the addition of substrate (*11*).

Measurement of PLD Binding to Sucrose Loaded Phospholipid Vesicles. The procedures used have been described previously (*23*). In brief, sucrose-loaded phospholipid vesicles of the indicated compositions were generated by extrusion and washed with and resuspended in isotonic KCl. The vesicles were incubated with PLD proteins on ice in siliconized microcentrifuge tubes and then sedimented by ultracentrifugation. Binding of PLD enzymes to the vesicles

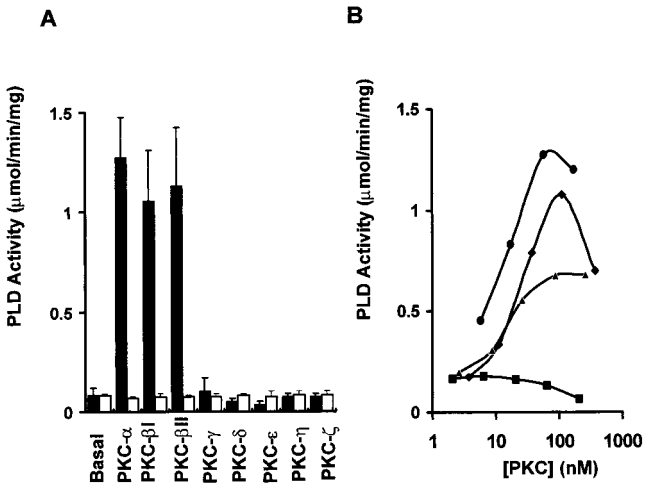

FIGURE 2: Activation of PLD1 and PLD2 by purified PKC isoforms. (A) Activity of purified PLD1 and PLD2 was determined in the presence of purified PKCs-α, -βI, -βII, -γ, -δ, -ϵ, -η, and -ζ at final concentrations of 50 nM. Incubations 100 nM PMA. The data shown for PLD1 (solid bars) and PLD2 (white bars) are means ± SEM of three separate experiments. (B) Activity of PLD1 was determined as the concentrations of PKCs-α (●), βI (◆), βII (▲), and γ (■) were increased as shown. Incubations contained 100 nM PMA, but no ATP. The data shown are means ± SEM of three separate experiments.

was determined by both measurement of catalytic activity remaining in the supernatant and by detection and quantitation of vesicle-associated proteins after SDS−PAGE and Western blotting.

Transient Expression of PLD1PIM87 and PLD2 in HEK-293 Cells. Cells were grown on poly-L lysine-coated 12-well culture plates and transfected with pCGNPLD1PIM87, RhoA Val14, PLD2 or a vector control using lipofectamine and OPTI-MEM (Life Technologies, Gaithersburg, MD) (*23*). Each transfection contained a total of 2 μg of DNA. Twenty four hours post-transfection, the cells were labeled with [^{3}H]-palmitic acid or [^{32}P]-PO_4^{2-} for 12 h, serum starved for 1 h, and then stimulated with 100 nM PMA or 19 μM A23187 in the presence of 0.3% *n*-butanol. Lipids were extracted from the cells and phosphatidylbutanol (PtdBuOH) isolated by thin-layer chromatography. Expression of the PLD isoenzymes was monitored in parallel by extraction of transfected cells with SDS−PAGE sample buffer and Western blotting.

RESULTS

Selective Activation of PLD1 by PKCs-α, -βI, and -βII. Activity of purified PLD1 and PLD2 was determined in the presence of PKCs-α, -βI, -βII, -γ, -δ, -ϵ, -η, and -ζ. The purified recombinant PKC isoenzymes were included in the assays at final concentrations of 50 nM which is a maximally effective concentration of PKC-α for stimulation of PLD1. Incubations contained 100 nM PMA. PKC-α, -βI, and -βII all increased PLD1 activity substantially (−15, −13, and −14-fold respectively), while PKC-γ and PKCs -δ, -ϵ, -η, and -ζ had no effect on activity of this enzyme. PLD2 activity was insensitive to stimulation by all of the PKC isoforms tested (Figure 2, panel A). Activation of PLD1 by PKC-α, -βI, and -βII was dependent on the concentration of PKC enzymes. PKC-α was somewhat more potent than PKCs-βI and -βII. The effects of PKC on PLD1 activity appeared saturable although some inhibition was seen at higher PKC concentrations. By contrast, PKC-γ had no effect on PLD1 activity even at concentrations that were 50−100 fold higher than those at which PKCs-α and -β increased activity of this PLD enzyme (Figure 2, panel B). The activation of PLD1 by PKC-α and -β was completely independent of ATP. Inclusion of either ATP or APPNHP in the incubations did not increase the potency or efficacy with which these PKC enzymes activated PLD1 (Table 1). ARF, Rho, and PKC can synergize with each other to increase PLD activity. We measured PLD1 activity in the presence of specific PKC isoforms, in combination with ARF and Rho. Activation of PLD1 by both PKC-α and -β was synergistic with ARF1 and RhoA. In contrast, effects on PKC-α and -β on PLD1 activity were additive at submaximal concentrations but not synergistic with each other. For example, using the PLD1 preparation described in Figure 2, PLD1 activities measured in the presence of 100 nM PKC-α or PKC-βI or a combination of the two isoenzymes were 1.3 ± 0.2, 1.1 ± 0.2, and 1.2 ± 0.2 mmol min^{-1} mg^{-1}, respectively. PKC-γ, which does not activate PLD1, had no effect on regulation of PLD1 activity by PKCs-α and -β or by these GTP-binding proteins. These results suggest that PKC-α and -β interact with the same site on PLD1.

Activation of PLD1 by PKC-α Is Independent of PKC Catalytic Activity. Conventional PKC isoforms are comprised of two modular domains. The N-terminal regulatory domain contains conserved regions for Ca^{2+} and lipid binding, while the C-terminal domain contains sites for substrate binding and catalysis. Since phosphorylation is not needed for activation of PLD1, it is possible that the regulatory domain contains the structural determinants required for PLD1 regulation. We subjected purified PKC-α to limited proteolysis and resolved the catalytic and regulatory domains by chromatography on a Source 15 phenyl column. The PKC-α catalytic domain prepared in this manner had no effect on PLD1 activity at concentrations that were at least 100-fold in excess of those at which the intact enzyme stimulated PLD1 activity (data not shown). Isolation of the regulatory domain by this procedure was complicated by the sensitivity of this fragment of the enzyme to further proteolysis. We expressed the 38-kDa regulatory domain of PKC-α (PKC-αR) in *E. coli* with an appended C-terminal His6 tag and purified the protein by metal ion affinity chromatography (Figure 3, panel A). This protein and a minor species of molecular mass 28 Da were detected by immunoblotting with the PKC-α selective monoclonal antibody MC5 (Figure 3, panel B). This isolated PKC-α regulatory domain increased PLD1 activity in a concentration-dependent manner. In comparison to the holoenzyme, the PKC-α regulatory domain was both a somewhat less potent ($K_{0.5}$ 30 as compared to 3 nM) and considerably less effective activator of PLD1. As observed with intact PKC-α, activation of PLD1 by the PKC-α regulatory domain was synergistic with ARF1. (Figure 3, panels C and D). Activation of PLD1 by PKC-α was modestly stimulated by Ca^{2+}, DG, PS, or PMA although the magnitude of these effects was reduced in comparison to their well-characterized stimulation of the histone kinase activity of PKC. This is likely due to the direct stimulatory effect of PI(4,5)P_2 on PKC (*27*) because effects of Ca^{2+}, DG, PS, or PMA on the histone kinase activity of PKC-α were also reduced when measured in the presence of the concentrations of PI(4,5)P_2

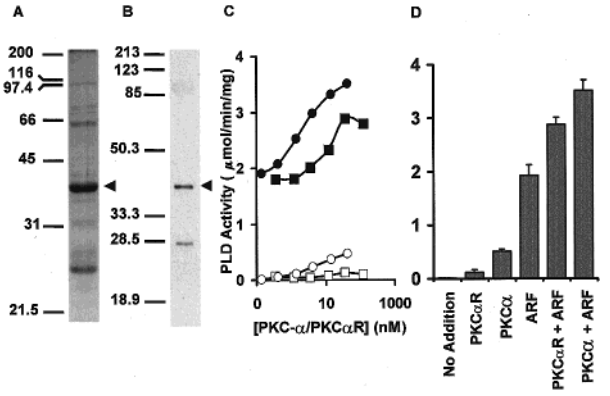

FIGURE 3: Activation of PLD1 by regulatory domain of PKC-α. Two micrograms of the purified bacterially expressed PKC-α regulatory domain preparation was separated by SDS−PAGE on a 10% gel, and proteins were visualized by staining with Coomassie blue. Positions of molecular weight markers are shown. (B) 400 ng of the PKC-α regulatory domain preparation was separated by SDS−PAGE and analyzed by Western blotting using a PKC-α selective monoclonal antibody MC5. (C) PLD1 was assayed in the presence of increasing concentrations of PKC α (○, ●) or PKC-α regulatory domain (□, ■) in the presence (closed symbols) or absence (open symbols) of 870 nM GTPγS loaded ARF1. The data are means of triplicate determinations from a single experiment. (D) The effect of PKC-α (172 nM) and the PKC-α regulatory domain (280 nM) alone or in the presence of 750 nM GTPγS-loaded ARF1 on activity of purified PLD1 was determined. The data shown are means ± SEM from three separate experiments.

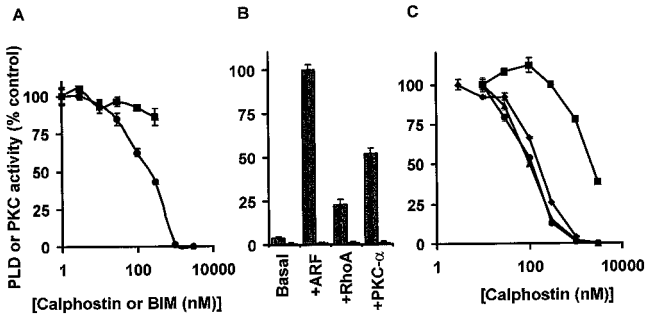

FIGURE 4: Effects of PKC inhibitors on PLD1 and PLD2 activity. (A) Purified PLD1b was assayed in the presence of 40 nM PKC-α, 30 nM PMA, and increasing concentrations of bisindolylmaleimide I (BIM) (■) or calphostin-c (●). The data shown are means ± SEM of three separate experiments. (B) Purified PLD1 activity was determined in the presence of 870 nM GTPγS-loaded ARF, 187 nM GTPγS-loaded RhoA, or 42 nM PKC-α, without (gray bars) or with (white bars) 3 μM calphostin-c. Incubations with PKC-α contained 100 nM PMA. The data shown are means ± sem of three independent experiments. (C) PLD1 (▲), PLD2 (♦), and cabbage PLD (■) activity was measured in the presence of increasing concentrations of calphostin-c. PLD1 assays contained 870 nM GTPγS-activated ARF1. The protein kinase activity of PKC-α was measured using histone as substrate (●). The data shown are means ± SEM of three independent experiments.

used in the PLD assays. By contrast, activation of PLD1 by the PKC-α regulatory domain was unaffected by Ca^{2+}, DG, PS, or PMA (data not shown).

Effects of PKC Inhibitors on Activation of PLD1 by PKC-α. Two major classes of inhibitors of PKC have been described. One class competes for binding of ATP to the catalytic site and is typified by the Bisindolylmaleimides. The second class, of which calphostin-c is the most widely used, block the interaction of lipid activators with the PKC regulatory domain. PLD activity was determined in the presence of a maximally effective concentration of PKC-α and increasing concentrations of bisindolylmaleimide and calphostin-c were included in the incubations. Bisindolylmaleimide had no effect on PLD1 activity, even at concentrations that are 100-fold greater than its IC_{50} for inhibition of PKC catalytic activity. By contrast, calphostin-c was found to be a potent inhibitor of PKC-α stimulated PLD1 activity (IC_{50} 200 nM) (Figure 4, panel A).

Calphostin-c Is a Potent Direct Inhibitor of PLD1 and PLD2. Calphostin-c inhibited the basal catalytic activity of PLD by greater than 90% suggesting a PKC-independent action. Calphostin-c also inhibited PLD1 activity when measured in the presence of supramaximally effective concentrations of GTP-activated ARF1 or Rho A (Figure 4, panel B). We examined the concentration dependence with which calphostin-c inhibited ARF activated PLD1, PLD2, cabbage PLD, and histone kinase activity of PKC-α. The IC_{50} for inhibition of PKCα, PLD1, and PLD2 by calphostin-c were all in the range of 100−200 nM. Cabbage PLD activity was significantly less susceptible to inhibition by calphostin-c (Figure 4, panel C). Effects of calphostin-c on PKC have been reported to be light-dependent. Our experiments were performed under fluorescent light. Exposure of the samples to long wavelength UV light did not increase the potency of inhibition of PLD activity, and we also observed potent inhibition of PLD activity by calphostin-c in the dark (not shown).

Calphostin-c Irreversibly Inhibits the PLD Catalytic Domain. To further probe the mechanism of interaction between calphostin-c and the PLD enzymes, we examined the susceptibility of a series of PLD1 and PLD2 mutants to inhibition by calphostin-c. These included mutants in which the N-terminal domain of the enzymes had been removed (PLD1ΔN and PLD2 ΔN), a form of PLD1 in which both the N-terminus and the "loop" region within the catalytic domain had been removed (mini-PLD1) as well as an insertional mutant of PLD1 that is selectively unresponsive to stimulation by PKC (PLD1PIM87). Sf9 cell lysates were used as sources of PLD activity. In comparison to material obtained from sf9 cells expressing an irrelevant control protein, expression of each of these PLD variants was accompanied by substantial increases in PLD activity measured in the presence of $PI(4,5)P_2$ and maximal concentrations of ARF1. Activity of all of the PLD1 and PLD2 variants examined was dramatically inhibited by 5 μM calphostin-c (Figure 5, panel A). To examine the mechanism of inhibition of PLD by calphostin-c, we used an epitope tagged form of miniPLD1 which can be rapidly purified by immunoaffinity chromatography. The purified miniPLD was exposed to 5 μM calphostin-c or vehicle for 30 min and then reisolated (Figure 5, panel B). Calphostin-c has a high intrinsic fluorescence. Fluorescence measurements indicated that the inhibitor was effectively removed from the miniPLD preparation by this procedure (not shown). Activity of the reisolated miniPLD was inhibited by calphostin-c pretreatment. Activity of the inhibited enzyme could not be overcome by increasing substrate concentration (not shown). These results suggest that calphostin-c binds tightly or irreversibly to effect inhibition of PLD activity (Figure 5, panel C).

Calphostin-c Does Not Inhibit PLD Binding to PI(4,5)P2 Containing Liposomes. Activity of PLD1 and PLD2 is strongly dependent on $PI(4,5)P_2$. Activity of miniPLD that had been treated with calphostin-c and reisolated could not

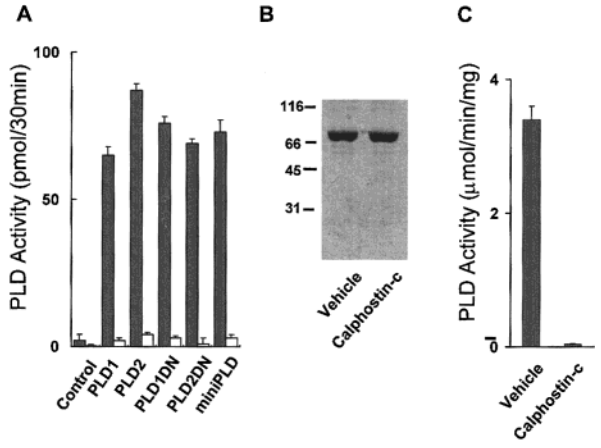

FIGURE 5: Calphostin-c irreversibly inhibits the PLD catalytic domain. (A) PLD activity was determined in extracts from sf9 cells expressing an irrelevant control protein or the indicated PLD enzymes and mutants. Assays contained 1 μM GTPγS activated ARF1. (B) Vehicle or calphostin-c treated miniPLD was analyzed by SDS PAGE and proteins visualized by staining with comassie blue. (C) Activity of vehicle or calphostin-c treated miniPLD was determined following reisolation by immunoaffinity chromatography. Data are means ± SD of triplicate determinations.

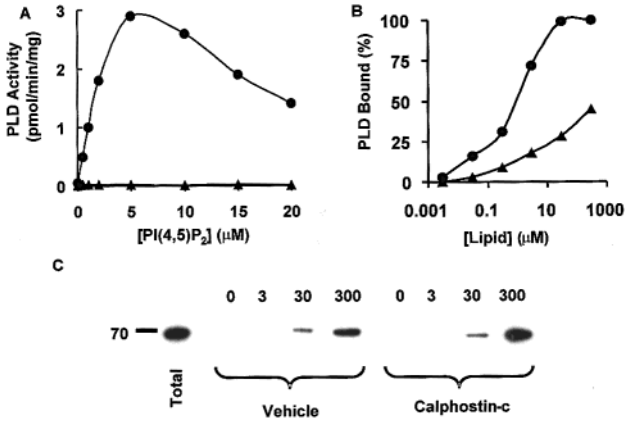

FIGURE 6: Calphostin-c does not inhibit binding of PLD to PI-(4,5)P2. (A) Activity of vehicle (●) or calphostin-c treated (▲) miniPLD was determined in the presence of increasing concentrations of PI(4,5)P_2. The data are means of duplicate assays. (B) Binding of miniPLD to PI(4,5)P_2-containing sucrose loaded phospholipid composed of 1:1:1 PC/PS/PE containing 5 mol % PI(4,5)-P_2 (●) or 7 mol % PI (▲) was quantitated by measuring PLD activity remaining in the supernatant after sedimentation of the vesicles. (C) Binding of vehicle or calphostin-c treated miniPLD to vesicles composed of 1:1:1 PC/PS/PE containing 5 mol % PI-(4,5)P_2 was analyzed by Western blot analysis of vesicle bound proteins following ultracentrifugation.

be overcome by increased concentrations of PI(4,5)P_2 (Figure 6, panel A). Calphostin-c also inhibited basal catalytic activity of a purified PLD2 mutant, PLD2R554G/R558G with reduced responsiveness to PI(4,5)P_2 (not shown). We previously reported that a region of basically charged amino acid residues conserved among all of the phosphoinositide responsive PLD enzymes mediates binding of PLD2 to PI-(4,5)P_2 (*28*). The affinity of binding of miniPLD1 to sucrose-loaded liposomes composed of 1:1:1 PC:PS:PE was increased approximately 10-fold when 5% PI(4,5)P_2 was included in the liposomes (Figure 6, panel B). miniPLD that had been inhibited by calphostin-c pretreatment bound to the liposomes with a similar affinity to that of the untreated enzyme (Figure 6, panel C).

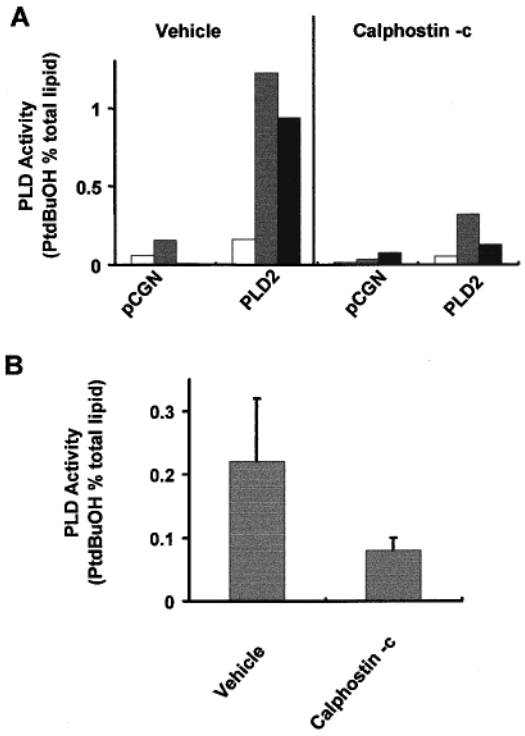

FIGURE 7: Calphostin-c inhibits PLD1 and PLD2 in HEK293 cells. (A) PLD activity was determined in HEK293 cells transfected with vector or PLD2 and treated with vehicle or 1 μM calphostin-c under basal conditions or after stimulation with PMA or A23187 (white, gray, and black bars, respectively). The data are means of duplicate determinations. (B) HEK293 cells were cotransfected with RhoA Val14 and PLD1PIM87 and PLD activity determined following treatment with vehicle or 1 μM calphostin-c. PLD activity in vector transfected cells has been subtracted and the data shown are means ± SD of triplicate assays.

Inhibition of PLD1 and PLD2 by Calphostin-c Treatment of Intact Cells. We examined the susceptibility of PLD2 to inhibition by calphostin-c when expressed in HEK293 cells. Both endogenous HEK293 cell PLD activity and PLD2 activity were increased substantially by stimulation with PMA and Ca^{2+} ionophore. Cells treated with 5 μM calphostin-c exhibited basal, PMA- and ionophore-stimulated PLD activities that were 36, 34, and 36% of those measured in vehicle treated cells (Figure 7, panel A). Since it is likely that PKC plays an indirect role in regulation of PLD2 activity we also examined the effects of calphostin-c treatment on activity of a PLD1 mutant, PLD1 PIM87 that is selectively unresponsive to activation by PKC in vitro and uncoupled from rapid activation by PKC when expressed in cells (*21*). Activity of PLD1 PIM87 can be increased by coexpression with activated RhoA. We found that 5 μM calphostin-c produced a 91% inhibition of the activity of Rho activated PLD1 PIM87 (Figure 7, panel B). Taken together, our results suggest that inhibition of PLD1 and PLD2 activity by calphostin-c treatment of intact cells are likely to result, at least in part, from direct effects on PLD catalytic activity rather than through inhibition of upstream actions of PKC.

DISCUSSION

Selective Activation of PLD1 by PKCs-α *and -*β. The work reported here definitively demonstrates that PLD1 is selectively activated by PKCs-α, -βI, and -βII. A variety of other members of the conventional, novel and atypical PKC classes

did not activate PLD1, and none of the PKC enzymes tested increased activity of PLD2. PKC-α was a somewhat more potent activator of PLD1 than PKC-*β* and the maximal stimulation observed with PKC-α was greater than that seen with PKC-*β*I and -*β*II. The ineffectiveness of PKC-*γ* is notable since this enzyme is highly homologous to the other conventional PKC isoenzymes, particularly in the regulatory domain. PLD1 is activated by three classes of proteins: ARF, Rho, and PKC. Our results suggest that three separate regulatory protein interaction sites exist on PLD1, for these activators. The finding that activation of PLD1 by PKC-α and -*β* is not synergistic suggests that both isoforms interact with the same site. The PKC isoenzyme selectivity for activation of human PLD1 we report is comparable to that observed in reconstitution experiments using PLD1 containing preparations from pig brain, HL60 cells, and a recombinant rat brain enzyme (*12*, *13*, *28*).

Role of the PKC-α Regulatory Domain in Activation of PLD1. Activation of PLD1 by PKC-α can be observed in the absence of ATP but is promoted by phorbol esters. When subjected to proteolysis, the regulatory domain but not the catalytic domain of a brain PKC preparation increased PLD activity (*13*). We compared stimulation of PLD1 by PKC-α and the PKC-α regulatory domain. Although the regulatory domain could activate PLD1, it was both less potent and less effective than the holoenzyme. Stimulation of PLD may involve both the catalytic and the regulatory domains of the PKC enzymes or alternatively the recombinant PKC-α regulatory domain may simply be improperly folded. Prolonged activation of PKC-α results in generation of the regulatory domain by proteolytic cleavage of the hinge region of the enzyme (*29*). The regulatory domain generated in this manner may have a physiological role in sustained activation of PLD1.

Direct Inhibition of PLD1 and PLD2 by Calphostin-c. Whereas activation of PLD1 by PKC-α was completely insensitive to an active site-directed inhibitor, bisindolemaleimide, we found that calphostin-c was an effective inhibitor of both PLD1 and PLD2 activity. Calphostin-c inhibited PLD1, PLD2, and the kinase activity of PKC-α with similar potencies. Calphostin-c appeared to target the PLD catalytic site because N-terminal regulatory and "loop" regions of PLD1 and PLD2 were dispensable for inhibition. Using miniPLD which is a PLD1 fragment that can be expressed at high level and purified using an epitope tag, we found that inhibition of PLD activity by calphostin-c arises from either tight binding or covalent interaction of the inhibitor with PLD. We were unable to definitively demonstrate covalent modification of miniPLD by calphostin-c using mass spectroscopy analysis following proteolysis. However, since calphostin-c has been shown to inhibit PKC by covalent interaction, it seems likely that a similar mechanism is operating here. Calphostin-c treatment blocks diacylglycerol/phorbol ester binding to the PKC regulatory domain. Calphostin-c also inhibits the activity of certain DG kinase isoforms (*30*). Members of the "HKD" domain class of PLD enzymes can catalyze transphosphatidylation reactions that effectively use primary alcohols and glycerols, and DG is a substrate for PLD1 and PLD2 (*31*). It is therefore possible that the PLD enzymes contain a selective binding site for these substrates which may be targeted by calphostin-c. However, we were unable to block inhibition of PLD by calphostin-c using DG or alcohols so if these agents are competing the affinity for calphotin-c must be appreciably higher than that of the DG or alcohol substrates. Inactivation of PKC by calphostin-c has been reported to have a light sensitive component. We found that inactivation of PLD1 by calphostin-c could be observed in the dark, under ambient light conditions or under fluorescent light, and there was no significant difference in the potency or magnitude of the inhibition observed. For PKC, the initial reaction of calphostin-c with the protein is not light-dependent but that maximal inhibition is promoted by UV illumination that may involve localized damage to the enzyme through light-dependent free radical formation. In the case of PLD, it seems likely that covalent interaction with calphostin-c is sufficient to produce effective inhibition of catalysis. However, in experiments with intact cells, we did observe a modest potentiation of the inhibitory effects of calphostin-c by exposure of the cells to UV light. It is possible that additional aspects of PLD regulation in intact cells, for example, interactions with other regulatory proteins are more susceptible to inhibition by light-dependent mechanisms that inhibition of PLD catalytic activity measured in vitro.

Does Inhibition of PLD Account for Non-PKC Mediated Effects of Calphostin-c? Our experiments using transfected HEK293 cells, particularly those using a PLD1 mutant (PLD1PIM87) that is insensitive to direct activation by PKC, suggest that calphostin-c also directly inhibits PLD1 and PLD2 activity in intact cells. Calphostin-c has been reported to inhibit receptor-regulated PLD activity in a number of cell types reviewed in ref *7*. These results have generally been interpreted to suggest an upstream role for PKC in regulation of PLD activity although one study concluded that inhibition of PLD activity by calphostin-c was PKC-independent but possibly mediated though effects on Rho GTP binding proteins (*32*). Our results suggest that direct inhibition of PLD1 and PLD2 activity may account for inhibition of PLD activity observed when cells are treated with calphostin-c. Calphostin-c produces a number of effects on target cells that cannot be mimicked by PKC inhibitors that target the catalytic site. One such dramatic effect of calphostin-c is rapid disruption of the endoplasmic reticulum and Golgi apparatus (*3*). PLD1 has been proposed to play a central role in ARF-regulated vesicular transport both between the ER and the Golgi and as well as between Golgi cisternae so it is possible that inhibition of PLD1 by calphostin-c may produce these effects (*33*). Calphostin-c also inhibits cell growth and promotes apoptosis in an apparently PKC-independent manner (*4*, *34*). PLD can be activated by growth hormone receptors so inhibition of PLD activity may block an important component of the mitogenic signal (*7*). PLD2 plays a role in regulation of changes in cell morphology and motility through regulation of phosphoinositide-dependent effects on the actin cytoskeleton. Inhibition of PLD2 activity by calphostin-c might lead to disruption of cell anchorage resulting in apoptosis (*35*). A lack of pharmacological inhibitors has impeded progress in understanding the role of PLD in mammalian cells. Further understanding of the mechanism by which calphostin-c inhibits PLD may provide a springboard for development of selective PLD inhibitors that would be valuable tools for probing PLD function.

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