Ceramide Inhibition of Mammalian Phospholipase D1 and D2 Activities Is Antagonized by Phosphatidylinositol 4,5-Bisphosphate†

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ABSTRACT: Ceramides inhibit phospholipase D (PLD) activity in several mammalian cell types. These effects have been related to preventing activation by ARF1, RhoA, and protein kinase C-α and -β and therefore indicate that PLD1 is inhibited. In the present work, we investigated the effects of ceramides in inhibiting both PLD1 and PLD2 and the interaction with another activator, phosphatidylinositol 4,5-bisphosphate (PIP_2). PLD1 and PLD2 were overexpressed separately in Sf9 insect cells using baculovirus vectors. In our cell-free system, PLD1 activity was inhibited completely by C_2-ceramide at sub-optimum concentrations of PIP_2 (3 and 6 μM), whereas at supra-optimum PIP_2 concentrations (18 and 24 μM) C_2-ceramide did not inhibit PLD1 activity. Partially purified PLD2 exhibited an absolute requirement for PIP_2 when the activity was measured using Triton X-100 micelles. Ceramides inhibited PLD2 activity, and this inhibition was decreased as PIP_2 concentrations increased. However, C_2-ceramide also reversibly inhibited the activity of PLD1 and PLD2 mutants in which binding of PIP_2 was decreased, indicating that ceramides are interacting with the catalytic core of the mammalian PLDs. By contrast, C_2-ceramide failed to produce a significant inhibition of PLDs from bacteria and plants. Our results provide a novel demonstration that ceramides reversibly inhibit mammalian PLD2 as well as PLD1 activities and that both of these actions are more pronounced when PIP_2 concentrations are rate-limiting.

Phospholipase D (PLD)[1] is involved in such diverse physiological processes as membrane trafficking, mitogenesis, inflammation, and secretion (*1*−*4*). Many agonists, including growth factors, hormones, neurotransmitters, and cytokines, activate PLD to catalyze the hydrolysis of phosphatidylcholine (PC) to phosphatidate (PA) which can act as a signaling molecule. PA stimulates the respiratory burst in neutrophils, budding of coated vesicles in Golgi-enriched membranes, and activation of phosphatidylinositol 4-kinase, Ras, and Raf (for reviews, see refs *5* and *6*). In whole cells, PA is rapidly converted by lipid phosphate phosphatases (also known as phosphatidate phosphohydrolase) into DAG, which could activate some PKC isoforms depending upon its fatty acid composition (*6, 7*). PA can also be converted to lysoPA which is released by a variety of cells (*8*). Exogenous lysoPA acts as a potent growth factor through EDG (Endothelial cell Differentiation Gene) receptors including EDG-2, EDG4, and EDG7 (*9*−*15*).

Thus, there is great interest in how PLD activity is regulated and how this controls the formation of PA, lysoPA, and DAG. cDNAs for two mammalian PLDs have been cloned (*16*−*19*). PLD1 has low basal activity and can be activated by small GTP-binding proteins such as ARF and Rho and by PKC-α and -β (*16*). The PLD2 isoform has a high basal activity and is relatively insensitive to activation by ARF and Rho (*2, 3, 17*). Human PLD1 and PLD2 are also dependent on PIP_2 or phosphatidylinositol 3,4,5-trisphosphate for activity (*2, 3, 15*−*19*).

This laboratory demonstrated that ceramides inhibit PLD activity in intact rat fibroblasts by a variety of physiological stimulators (*20, 21*). Ceramides are lipid mediators produced following activation of sphingomyelinases by agonists such as tumor necrosis factor-α (*22*−*24*). The ceramide inhibition of PLD1 activity has been ascribed to a decreased translocation of ARF, Rho (*21*), and PKC-α and -β to membranes (*21, 25, 26*), and to the decreased activation of these PKCs (*27*). It was not known whether ceramides also inhibit PLD2. In the present study, we demonstrated that PLD2, in addition to PLD1, is reversibly inhibited by ceramides. These inhibitions were more potent at low PIP_2 concentrations. Both inhibitions should, therefore, be taken into account in whole

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[1] Abbreviations: ARF, ADP-ribosylation factor; PLD, phospholipase D; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C.

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cells where the physiological effects of ceramides on PLD activity are likely to be interactive with the availability of PIP_2.

EXPERIMENTAL PROCEDURES

Materials. HL60 and Sf9 insect cells were purchased from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium, Sf-900 II SFM complete Grace's medium, penicillin/streptomycin, and fetal bovine serum were obtained from GIBCO BRL, Life Technologies, Inc. (Burlington, ON, Canada). Molecular weight standards were purchased from Bio-Rad (Mississauga, ON, Canada). GTPγS and GDPβS were obtained from Boehringer Mannheim, Canada. PIP_2 triammonium salt was from Calbiochem, La Jolla, CA. C_2-ceramide, D-*threo*-dihydro-C_2-ceramide, *N*-palmitoyl-D-sphingosine (C_{16}-ceramide), *N*-palmitoyl-DL-dihydrosphingosine (dihydro-C_{16}-ceramide), and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO. PC, PE, and 1,2-dipalmitoyl-*sn*-glycerol/1,2-dioleoyl-*sn*-glycerol (DAG) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Rabbit polyclonal antibodies against ARF1 (*28*), PLD1 (*29*), and PLD2 (*30*) were prepared as described previously. L-3-Phosphatidyl[*N-methyl*-^{3}H]choline, horseradish peroxidase-conjugated anti-rabbit IgG, and the Enhanced Chemiluminescence Western blotting system were obtained from Amersham Life Science (Arlington, IL).

Expression and Purification of PLD1 and PLD2. PLD1 and PLD2 and several previously described mutants including PLD1 ΔN, PLD2 ΔN, PLD1 PIM87, miniPLD, and PLD2 R554G/R558G were expressed in insect cells using recombinant baculoviruses (*31*−*33*). 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 wild-type and mutant PLD proteins containing an N-terminal "glu-glu" tag. In this case, proteins were purified by chromatography on a resin containing immobilized monoclonal antibody and eluted by incubation of the resin with a competing peptide (*34*). 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.

For experiments in which the kinetics of PLD2 activity were determined with substrate presented in Triton X-100 micelles, membrane fractions from Sf9 cells that overexpressed PLD2 were solubilized with 1.0% Triton X-100 in 20 mM Tris buffer (pH 7.5), kept on ice for 30 min, and centrifuged at 99000*g* for 1 h at 4 °C. The resulting enzyme extract was loaded onto a 2 mL heparin-Sepharose (Pharmacia) column previously equilibrated with 20 mM Tris/HCl, pH 7.5, and 0.1% Triton X-100 (buffer A). The column was washed extensively with equilibration buffer. PLD2 was then eluted as a sharp peak by washing the column in the reverse direction with 1 M NaCl in buffer A. Fractions containing high PLD2 activity were pooled, dialyzed against buffer A, and stored at −70 °C until further use. This procedure only increased the specific activity of PLD2 by about 2-fold but effectively removed PIP_2 from the partially purified PLD2 preparation.

Cell Culture, Western-Blotting, and Analysis of PLD1 and PLD2. Differentiation of HL60 cells to granulocytes was induced with 1.25% DMSO (v/v) (*21*). Cell lysates from HL60 and Sf9 cells were added to boiling Laemmli sample buffer (*28*) and boiled for 7 min. PLD1 was immunoprecipitated (*29*), and proteins were separated on 8% SDS−PAGE gels and transferred to Immobilon PVDF membranes. Immunoblotting was performed using anti-PLD1 or anti-PLD2 antibodies, and the enzymes were detected by Enhanced Chemiluminescence (*29, 30*).

Measurement of PLD1 and PLD2 Activities. PLD1 activity was measured in cell fractions from HL60 cells by using liposomes of [^{3}H]PC (8.3 μM and about 100 000 dpm/assay), PE (138 μM), and PIP_2 (*35*). Liposomes were prepared in the presence of ceramides at the molar ratios indicated. A typical reaction mixture contained 5−10 μg of membrane or cytosolic protein, buffer B containing 162 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.5 mM EDTA, pH 7.4, recombinant ARF1 (5−10 μM), 30 μM GTPγS, 3 mM $MgCl_2$, and 2 mM $CaCl_2$ in a final volume of 100 μL. Incubations were performed for 60 min at 37 °C with gentle shaking. Reactions were stopped by the addition of chloroform/methanol followed by the separation of lipid and water-soluble components (*36*). [^{3}H]Choline from the upper aqueous phase was extracted into the organic phase with a scintillation cocktail consisting of 0.75 mL of 10 mM phosphate buffer, pH 7.5, 0.75 mL of tetraphenylboron 5 mg/mL in acetonitrile, and 3.5 mL of toluene containing 0.5 g/L 2,5-diphenyloxazole and 0.2 g/L POPOP *p*-bis[2-(5-phenyloxazolyl)]benzene, mixed by inverting about 52 times (*37*), and was determined by scintillation counting (*38*). This method efficiently separates [^{3}H]choline from [^{3}H]phosphocholine.

PLD2 activity was measured using mixed micelles of Triton X-100 with various bulk and surface concentrations of PIP_2 and [^{3}H]PC as indicated. Desired bulk concentrations of PIP_2 and PC were dried from chloroform solution and suspended in Triton X-100 so that the total lipid concentration in the Triton X-100 micelles did not exceed 18 mol % in order not to disrupt the micelle structure (*39*). The surface concentration of lipids in the mixed micelles was achieved by varying the amount of Triton X-100 added. The typical reaction mixture contained 0.1−0.3 μg of the partially purified PLD2, mixed micelles consisting of different mole percentags of [^{3}H]PC and PIP_2, 20 mM NaF, and buffer B (as described for PLD1 assay) in a total volume of 100 μL. Incubations were performed at 37 °C for 60 min, and [^{3}H]-choline was quantitated as described above.

Measurement of PLD Binding to Sucrose-Loaded Phospholipid Vesicles. The procedures used have been described previously (*33*). In brief, sucrose-loaded phospholipid vesicles of the indicated compositions were generated by extrusion and washed with and resuspended in isotonic KCl. Vesicles were incubated with PLD proteins on ice in siliconized micro-centrifuge tubes and then sedimented by ultracentrifugation. Binding of PLD enzymes to the vesicles was determined both by measurement of catalytic activity remaining in the supernatant and by detection and quantitation of vesicle-associated proteins after SDS−PAGE and western blotting.

RESULTS

Our first objective was to characterize the expression of PLD1 and PLD2 in HL60 cells and to express PLD1 and

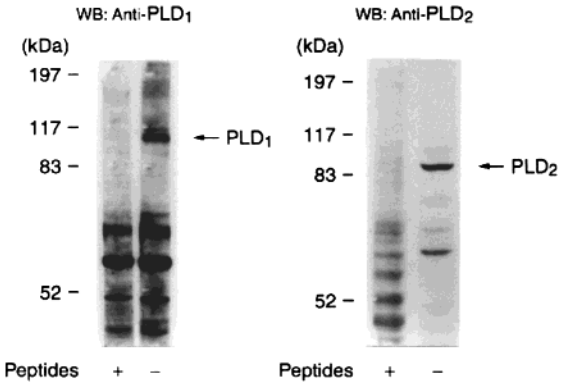

FIGURE 1: Detection of PLD1 and PLD2 in HL60 cells.The figure shows expression of PLD1 and PLD2 in DMSO-differentiated HL60 cells. Where indicated, the PLD1 or the PLD2 antibodies were incubated in the presence (+) and absence (−) of their respective immunizing peptides (10 μg/mL). The blots are from a single experiment, and similar results were obtained on three separate occasions.

PLD2 separately in Sf9 cells. Membranes from Sf9 cells that overexpressed PLD1 and PLD2 showed a strong immunoreactivity at 115 and 90 kDa, respectively (results not shown). The identities of these bands were verified since they were not observed when the antibodies were neutralized with the peptides used to prepare these respective antibodies. Overexpressed PLD2 was mainly confined to the membrane fraction of Sf9 cells (results not shown). In membrane extracts from HL60 cells, immunoreactive bands were revealed with anti-PLD1 at 115 and 117 kDa, suggesting the presence of two alternatively spliced variants of PLD1 (Figure 1). HL60 cells also expressed PLD2 with a major protein band around 90 kDa. The bands for PLD1 and PLD2 were also not detected when the antibodies were treated with the corresponding neutralizing peptides. These results demonstrate that the HL60 cells used previously to study PLD inhibition by ceramides (*21*) contained both PLD1 and PLD2 activities. Therefore, this previous work on the ceramide inhibition of PLD activity could have included effects on both PLD1 and PLD2, although the involvement of ARF and Rho would tend to implicate predominantly PLD1. We therefore investigated the effects of C_2-ceramide on both PLD1 and PLD2 activities and focused particularly on the interaction with PIP_2 concentrations.

Effect of C_2-ceramide on PLD1 Activity. PLD1 activity was measured by using liposomes of PC, PE, and PIP_2, essentially as described by Brown et al. (*35*). We could not employ Triton X-100 to disperse the lipids in defined micelles since detergents strongly inhibited PLD1 activity. Membranes from HL60 cells were incubated with a sub-optimum concentration (10 μg) of HL60 cytosolic protein (which contains ARF and Rho) and 30 μM GTPγS to stimulate PLD1. C_2-ceramide (100 μM) abolished PLD activity completely (Figure 2A) at sub-optimum concentrations (3 and 6 μM) of PIP_2. At supra-optimum PIP_2 concentrations (18 and 24 μM), C_2-ceramide increased rather than decreased PLD activity. To confirm that PLD1 rather than PLD2 activity was inhibited, we measured the effects of C_2-ceramide on membranes from Sf9 cells that overexpressed PLD1. A 50% inhibition of PLD1 activity was achieved in membranes that were incubated in the presence of 30 μM GTPγS, 10 μM ARF1, and 12 μM PIP_2 (Figure 2B). By contrast, the relatively inactive analogue of C_2-ceramide, dihydro-C_2-ceramide, did not inhibit PLD1 activity. C_2-ceramide (50 μM) also decreased the activity of recombinant PLD1 activity at concentrations up to 18 μM PIP_2, but there was no significant inhibition at 25 and 30 μM PIP_2 (Figure 2C).

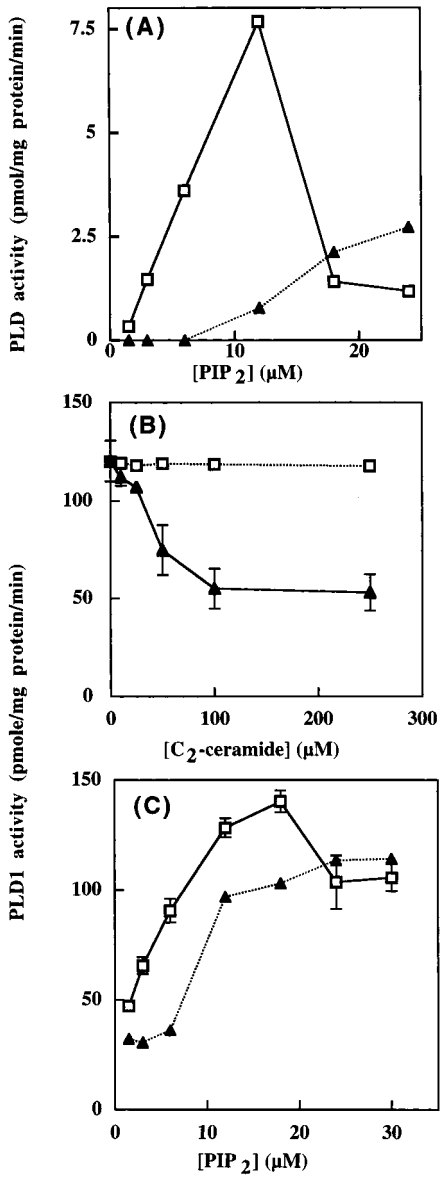

FIGURE 2: C_2-ceramide inhibition of PLD activity at low PIP_2 concentrations. Panel A shows the C_2-ceramide inhibition of PIP_2-dependent PLD activity in differentiated HL60 cells. Membrane protein (20 μg) from HL60 cells was incubated in the presence of sub-optimum (10 μg) cytosolic HL60 cell protein, 30 μM GTPγS, and various concentrations of PIP_2 as indicated. PLD activity was determined in the presence (▲) or absence of 100 μM C_2-ceramide (□). Panel B shows the effects of different concentrations of C_2-ceramide (▲) and dihydro-C_2-ceramide (□) on PLD1 activity. Panel C shows the overexpressed PLD1 activity in membranes from Sf9 cells at different PIP_2 concentrations and in the presence (▲) or absence of 100 μM C_2-ceramide (□). These latter assays contained 5−10 μg of membrane protein. Error bars (where large enough to be shown) are means ± SD of three measurements (panels A and C) or means ± ranges from the average of two experiments (B).

Effect of C_2-ceramide on PLD2 Activity. PLD2 activity was retained in Triton X-100. We therefore performed the assays under more controlled conditions by using PC in Triton micelles as a substrate rather than sonicated liposomes.

This enabled us to employ a surface dilution kinetic model (*39*) to study the ceramide inhibition. In this Triton X-100 system, lipid concentrations can be expressed either as bulk or as surface concentrations (mol %) in the Triton micelles. The concentration of lipids added to the Triton X-100 micelles did not exceed 18 mol % in order to ensure that the structure of the mixed micelles remained similar to that of pure Triton X-100. To verify the conclusions from the assays in the Triton micelle system, we also performed similar assays using the liposomal substrate as prepared for the assay of PLD1. There was no requirement for PE for PLD2 activity measured using the lipid liposome substrate, or in the Triton micelles (results not shown). Therefore, PE was omitted to simplify the assays. The activity of the recombinant PLD2 in both the liposome and Triton micelle assays was also independent of the presence of ARF and GTPγS, or GDPβS (results not shown).

The PIP_2 requirement for the activation of recombinant PLD2 in Sf9 membranes and partially purified PLD2 was measured using the Triton micelle assay in the presence of 100 μM PC (10.5 mol %). Partially purified PLD2 did not exhibit any activity in the absence of PIP_2 (Figure 3A). By contrast, there was significant PLD2 activity in the equivalent crude Sf9 membranes, at the level of 26 nmol (mg of protein)$^{-1}$ min^{-1}, in the absence of exogenously added PIP_2 (results not shown). This indicates that the partial purification of PLD2 in the presence of Triton X-100 had removed endogenous PIP_2. PLD2 activity increased with PIP_2 concentrations and reached an optimum at about 1.5 mol % (Figure 3A). A similar saturation profile was obtained with crude Sf9 membranes containing PLD2 (results not shown). The requirement of PIP_2 for PLD2 activity was examined further to determine the dependence either on the bulk concentration of PIP_2 or on its surface concentration in the micelle (Figure 3B). The reaction rate was relatively independent of the bulk concentrations of PIP_2 between 7.5 and 15 μM (Figure 3B). The requirement of PC for PLD2 activity is shown in Figure 3C. PLD2 activity was saturated at a surface PC concentration of between 7 and 12.5 mol %. The reaction rate was relatively independent of the bulk PC concentration when this was varied between 90 and 140 μM.

Having established the requirements for PC and PIP_2, we investigated if ceramides would inhibit PLD2 activity using the surface dilution kinetic model (*39*). First, we maintained PC at optimum concentrations between 7 and 12 mol % using a constant bulk concentration of 100 μM PC and examined the effects of C_2-ceramide at sub-optimum PIP_2 (0.5−1 mol %). Ceramide concentrations were varied between surface concentrations of 1−5 mol %, equivalent to bulk concentrations of 5−50 μM (Figure 4A). There was a different relationship between bulk and surface concentrations since the latter were achieved by varying Triton X-100 concentrations. C_2-ceramide produced a dose-dependent inhibition of PLD2 activity at 0.5 mol % of PIP_2, and this inhibition was almost complete at 4 and 5 mol % of C_2-ceramide (Figure 4A). The inhibition by C_2-ceramide decreased progressively in incubations where PIP_2 concentrations were increased to 0.75 and 1.0 mol %.

The C_2-ceramide inhibition of PLD2 was also tested at 1.0 mol % of PIP_2 and varying surface concentrations of PC. The degree of PLD2 inhibition was the same at 7, 8, and 9 mol % of PC (Figure 4B). The fact that C_2-ceramide inhibited PLD2 activity even at saturating concentrations of PC indicates that the inhibition is not competitive with respect to PC. We also verified that C_2-ceramide inhibited PLD2 activity in the liposome-based PLD2 assay. C_2-ceramide again inhibited PLD2 activity, and the effect was more striking at the lower PIP_2 concentrations (Figure 4C).

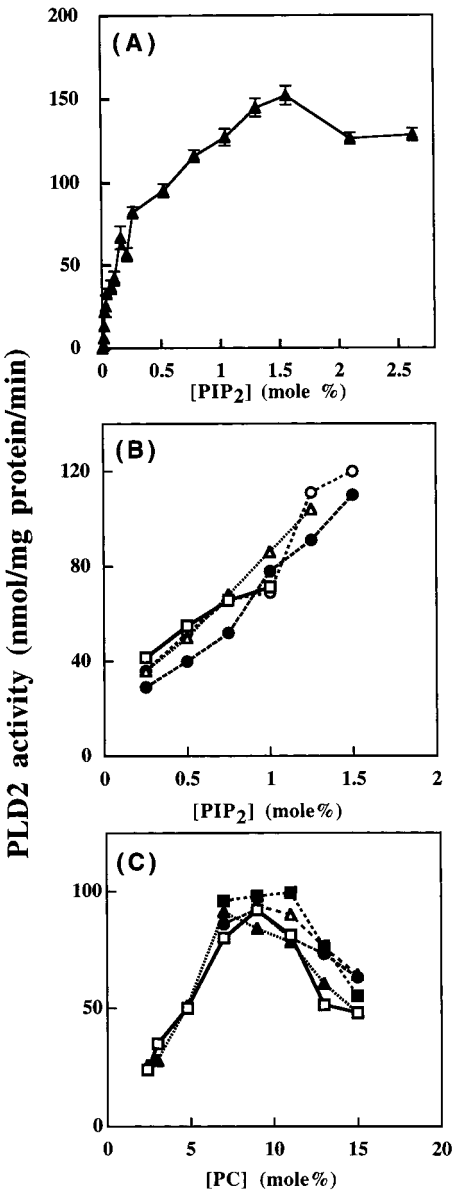

FIGURE 3: Requirement of PLD2 for PIP_2 and PC. Panel A illustrates the concentration-dependent activation of partially purified PLD2 by PIP_2 expressed as mol % in Triton micelles. Panel B shows the activation of PLD2 by different surface concentrations (mol %) of PIP_2 when incubated with bulk concentrations of PIP_2 of 7.5 (□), 10 (△), 12.5 (○), and 15 μM (●). PC was maintained at a bulk concentration of 110 μM and between 7 and 15 mol % in the Triton micelles. Panel C shows the effect of varying the surface concentrations of PC at bulk concentrations of PC of 90 (□), 100 (▲), 110 (△), 125 (●), and 140 μM (■). PIP_2 was maintained at a concentration of 1.0 mol %. Results for panel A are means ± SD (where large enough to be shown) of three to six different assays. Error bars for panels B and C have been omitted to avoid confusion.

Additional experiments were performed to determine the effects of a long-chain ceramide and diacylglycerol on PLD2 activity. Similar inhibitions to those observed with C_2-ceramide were obtained with naturally occurring long-chain C_{16}-ceramide which at 5 mol % decreased PLD2 activity by

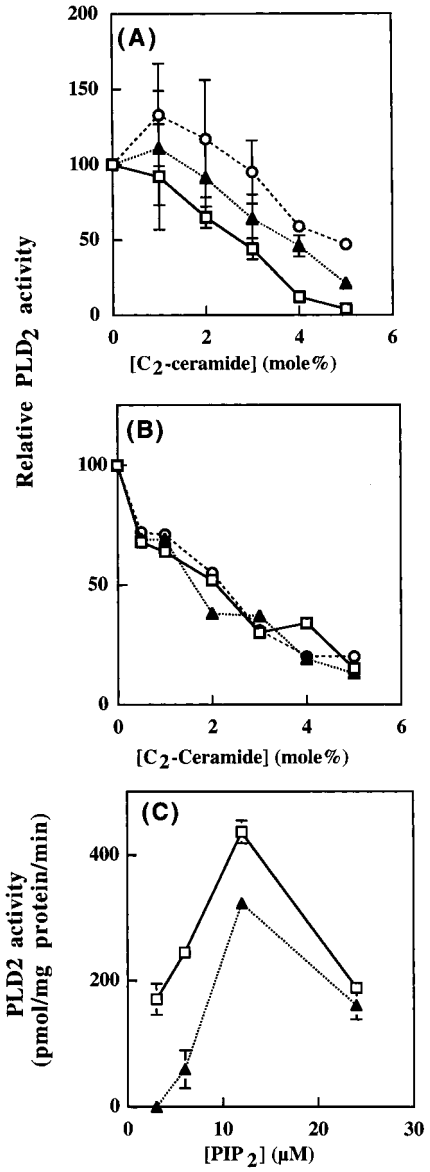

FIGURE 4: Inhibition of PLD2 by C_2-ceramide with respect to surface concentrations of PIP_2 and PC. Panel A describes the inhibition of partially purified PLD2 by various surface concentrations of C_2-ceramide with respect to surface concentrations of PIP_2 as 0.5 (□), 0.75 (▲), and 1.0 mol % PIP_2 (○). The bulk concentration of PIP_2 used was 7.5 μM, and the surface concentration of PC was varied between 7 and 12 mol %. Results are means ± SEM of triplicate determinations performed at least on three separate occasions. Panel B illustrates the inhibition of partially purified PLD2 by C_2-ceramide with surface concentrations of PC of 7 (□), 8 (▲), and 9 mol % (○). PIP_2 was maintained at 1.0 mol % whereas the bulk concentration of PC varied between 90 and 117 μM. Error bars have been omitted to avoid confusion. Panel C illustrates the activity of partially purified PLD2 in the absence (□) or presence of 25 μM C_2-ceramide (▲). C_2-ceramide was introduced into the liposome-based incubation system that contained PC, PE, and PIP_2. The bulk PC concentration was maintained at 100 μM. Error bars are means ± SD ($n = 3$).

46 and 68%, respectively (Table 1). The relatively inactive ceramide analogue, dihydro-C_{16}-ceramide, at 5 mol %, failed to inhibit PLD activity. Also, there was no inhibition with either dioleoyl-*sn*-diacylglycerol (Table 1) or dipalmitoyl-*sn*-glycerol (results not shown).

Ceramide Inhibition Reversibly Targets the PLD Catalytic Domain. To investigate the structural features of PLD1 and PLD2 required for inhibition by ceramide, we expressed a series of truncated PLD1 and PLD2 mutants in Sf9 cells and determined their susceptibility to inhibition by 20 and 50 μM C_2-ceramide. The substrate preparation used in these assays was that described previously except that the indicated concentrations of ceramides were included in liposomes that contained PE, PC, and PIP_2 (*31*−*34*). In the case of PLD1 mutants, these assays contained ARF1 preactivated with GTPγS. The data shown are activity measurements expressed as a percentage of PLD activity determined using vesicles containing no ceramide. All PLD1 and PLD2 mutants examined were inhibited by ceramides to a similar relative extent (Table 2). This included PLD1 and PLD2 mutants in which the N-termini of the proteins containing weak PH domain homology and the PX domain had been removed, PLD2 mutants displaying attenuated responsiveness to phosphoinositides (*33*), and a PLD1 mutant containing a small peptide insertion at amino acid 87 that is selectively unresponsive to PKC-α and -β (*32*). In particular, we found that miniPLD, which is an N-terminally truncated PLD1 mutant from which the variable catalytic domain "loop" insertion has also been deleted, was susceptible to inhibition by ceramides. MiniPLD is the smallest catalytically active PLD fragment that we have been able to make. These results, and particularly the susceptibility of miniPLD to inhibition by ceramides, suggest that ceramides interact with the catalytic core to inhibit PLD activity.

To investigate the reversibility of ceramide inhibition of PLD activity, we incubated Glu-Glu-tagged miniPLD with substrate containing liposomes with or without 50 μM C_2-

Table 1: Effects of C_{16}-ceramide, Dihydro-C_{16}-ceramides, and Diacylglycerol on PLD2 Activity[a]

additions	PLD2 activity [nmol min^{-1} (mg of protein)$^{-1}$] 0.5 mol % PIP_2	1 mol % PIP_2
none	68 ± 4	78 ± 5
C_{16}-ceramide	22 ± 5 (68)	42 ± 3 (46)
dihydro-C_{16}- ceramide	70 ± 6	72 ± 7
dioleoylglycerol	70 ± 8	80 ± 9

[a] The numbers in parentheses indicate the percentage of inhibition of PLD2 in the presence of 5 mol % of C_{16}-ceramide as indicated. Results are means ± SEM from three separate experiments and with each experiment performed with triplicate determinations.

Table 2: Inhibition of PLD1 and PLD2 Mutants by C_2-ceramide[a]

type of PLD	relative PLD activity (%) C_2-ceramide (20 μM)	C_2-ceramide (50 μM)
PLD1	48 ± 3	23 ± 2
PLD1 ΔN	47 ± 3	22 ± 1
PLD1 PIM87	47 ± 3	21 ± 1
miniPLD	47 ± 3	20 ± 2
PLD2	49 ± 2	21 ± 3
PLD2 ΔN	49 ± 2	22 ± 1
PLD2 R554/558G	48 ± 2	21 ± 2

[a] PLD proteins were expressed in Sf9 cells using baculovirus vectors. PLD activity was determined, and incubations for the PLD1 and its mutants contained 2 μM ARF1 preactivated with GTPγS. The data are means ± SD of triplicate determinations. PLD activity in the presence of C_2-ceramide is expressed relative to the rate obtained in the absence of ceramide. The experiment has been repeated once with similar results.

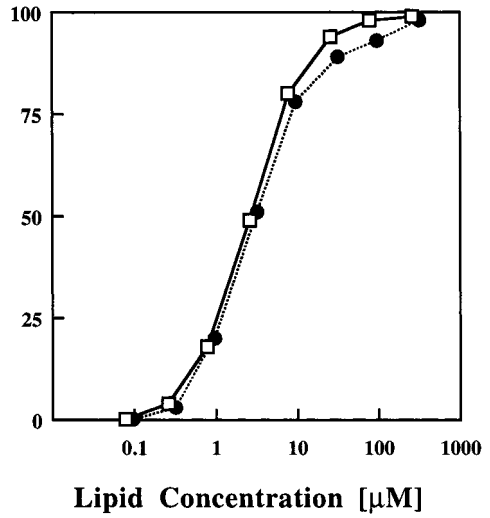

FIGURE 5: Binding of miniPLD to sucrose-loaded liposomes. Relative binding of miniPLD to vesicles containing 5 mol % PIP_2 and no ceramide (□) or 5 mol % PIP_2 and 20 mol % ceramide (●) is shown. Results are means of triplicate determinations, and the experiment was repeated once with similar results.

ceramide for 30 min at 37 °C. Incubations were terminated by dilution in lysis buffer, and the tagged PLD was purified by immunoaffinity chromatography. MiniPLD activity was unaltered by preexposure to ceramide, indicating that ceramides are acting as reversible inhibitors of these enzymes (not shown).

Ceramide Does Not Inhibit Binding of MiniPLD1 to PI-(4,5)P_2 in Sucrose-Loaded Bilayer Liposomes. Catalytic activity of PLD1 and PLD2 is stimulated by phosphoinositides containing a vicinal 4,5-phosphate pair, and this requires the integrity of a region of basic amino acids within the catalytic core. Integrity of this sequence motif is also required for binding of PLD1 and PLD2 to PIP_2 in bilayer liposomes. To investigate the possibility that ceramides inhibit PLD1 and PLD2 by inhibiting binding of the enzyme to PIP_2, we determined the effect of C_2-ceramide on binding of miniPLD to sucrose-loaded liposomes. MiniPLD was incubated with vesicles containing 5% PIP_2 with and without 20 mol % C_2-ceramide. MiniPLD binding to these vesicles was determined by monitoring PLD activity remaining in the supernatant after centrifugation. We have previously shown that, as reported for PLD2, inclusion of PIP_2 in these vesicles produces an approximately 10-fold increase in binding affinity of PLD1 and miniPLD to these vesicles. Binding of miniPLD to these vesicles was unaffected by ceramide (Figure 5).

Bacterial and Plant PLDs Are Not Inhibited Significantly by Ceramides. The following experiments determined if ceramides also inhibit PLD activity from plants and bacterial sources. These PLDs showed only a 40−50% increase in activity when PIP_2 was added to the liposomes in our assay system (Table 3). C_2-ceramide failed to produce significant inhibition in the activities of the bacterial and plant PLDs when measured in the presence or absence of PIP_2.

Table 3: Effect of PIP_2 and C_2-ceramide on Plant and Bacterial Phospholipase D Activities[a]

	phospholipase D activity (pmol/min)			
	cabbage PLD (0.5 unit)		PLD (0.75 unit) (*S. chromofuscus*)	
treatment	(−) PIP_2	(+) PIP_2	(−) PIP_2	(+) PIP_2
control	1.3 ± 0.03	2.0 ± 0.02	1.8 ± 0.5	2.6 ± 0.7
25 μM C_2-ceramide	1.2 ± 0.05	1.8 ± 0.2	1.8 ± 0.4	2.1 ± 0.7
50 μM C_2-ceramide	1.2 ± 0.1	1.8 ± 0.1	1.9 ± 1.0	2.2 ± 0.5

[a] PLD activity (from Sigma) was measured by using liposomes composed of PC, PE in the presence and absence of C_2-ceramide, or 12 μM PIP_2 as indicated. Values represent means ± ranges of two separate experiments performed with triplicate determinations.

DISCUSSION

The present work determined the characteristics of the inhibition of PLD1 and PLD2 by ceramides. So far, the mode of action of ceramides has concentrated on the inhibition of PLD1 and the prevention of PLD1 activation by protein kinase C-α and -β or by the small G-proteins, ARF, Rho, and Cdc42 (*21*). In the present studies, we concentrated on the interaction of the ceramides with the activation of both PLD1 and PLD2 by PIP_2. This was made possible by overexpressing the two PLD activities in Sf9 cells.

The results demonstrate for the first time that PLD2 is also inhibited by ceramides. For this study, we developed an assay based upon the use of Triton micelles so that defined concentrations of various lipids could be studied using a surface dilution model (*39*). We were unable to detect a stimulation of PLD2 activity by PE either in the Triton micelle assay or in the liposomal assay. Contrary to our results, Kodaki and Yamashita (*40*) reported a requirement of PE in addition to PIP_2 for the activity of rat brain PLD2. hPLD2 expressed in insect cells was also reported to be stimulated by ARF1 (*41*), but this stimulation of PLD2 was much less than that obtained with hPLD1. In our PLD2 assay systems, using liposomes or Triton micelles, there was no requirement for ARF1 or GTPγS. Similarly, PLD2 activity was not decreased by GDPβS. PLD2 had an absolute PIP_2 requirement for activity in the Triton micelle assay. PLD2 activity was independent of the bulk concentrations of PC and PIP_2 in the incubation and responded to changes in the surface concentration of the two lipids in the Triton micelles.

The C_2-ceramide inhibition also depended on the surface concentration of ceramide. This inhibition was decreased as the mole percent of PIP_2 in the micelles increased, indicating a competitive type of interaction. By contrast, inhibition of PLD2 by ceramides was not reversed by increasing the surface concentrations of PC. This result shows that the ceramide inhibition was not competitive with respect to the substrate, PC. PLD2 inhibition was also seen with C_{16}-ceramide but was not observed with dihydro-C_2-ceramide, dihydro-C_{16}-ceramide, or diacylglycerol. The ceramide inhibition was also observed with native PLD1 from HL60 cells and with recombinant wild type and various mutants of PLD1 and PLD2 expressed in Sf9 cells. The inhibition was not observed with sphingosine (*21*). These combined results demonstrate that the ceramide inhibition is specific and it is not restricted to the type of assay used to study the activities.

We had thought that the inhibition of PLD2 by ceramides might be caused by preventing its activation by PIP_2. However, this was not substantiated by the experiments using mutations of PLD1 and PLD2 in which interactions with PIP_2 were modified (*33*, *34*). Although, these mutant PLDs had low intrinsic activity, the relative inhibition by C_2-ceramide

was the same as for the wild-type enzyme. We had also postulated that the ceramide inhibition of PLD1 and PLD2 might result from a decreased interaction with PIP_2 since this could explain the competitive effect of these two lipids. However, the binding of miniPLD to PIP_2-containing vesicles was unaffected by ceramide. Our results, therefore, indicate that ceramides reversibly target the catalytic domains of mammalian PLDs and this effect is not observed with plant or bacterial PLDs.

A common characteristic of mammalian PLD1 and PLD2 is the need for PIP_2 for activity. It is significant that PIP_2 is probably a physiological activator for PLD (*2*−*4*, *35*), and the concentration of this lipid is likely to be limiting in cells for the expression of PLD activity. Ceramides are relatively potent at inhibiting PLD activity in cells compared to the concentrations needed for affecting other target systems where ceramides are known to act (*6*). In vitro, ceramides were required in relatively high concentrations to inhibit PLD1 activity in the presence of optimum concentrations of PIP_2 (*21*). The present results demonstrate that the inhibitory actions of ceramides on both PLD1 and PLD2 activities are more striking at sub-optimum PIP_2 concentrations. In fact, at supra-optimum concentrations of PIP_2, where PLD1 activity was inhibited, C_2-ceramide produced slight increases in activity.

In conclusion, the present work demonstrates for the first time that ceramides are potent inhibitors of PLD2 in addition to PLD1. This information should be taken into account when interpreting the biological implications of ceramide effects in whole cells that may contain both PLD1 and PLD2 activities. Our work also demonstrated that PIP_2 antagonizes the action of ceramides in inhibiting PLD1 and PLD2. This again has physiological implications in understanding the effect of phosphoinositides in activating mammalian PLD1 and PLD2 and how ceramides antagonize this activation. The inhibition of PLD1 activity by ceramides probably contributes to their effects in inhibiting vesicle trafficking (*42*−*44*).

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