Ceramide Inhibits Phospholipase D in a Cell-free System*

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Recent evidence in whole cells has implicated ceramide in the regulation of phospholipase D (PLD). In intact HL-60 cells, phorbol myristate acetate (PMA) activated PLD as measured by \(^{3}H\)palmitate-labeled phosphatidylcholine conversion to phosphatidylethanol in the presence of 2% ethanol. \(C_{6}\)-Ceramide completely inhibited PLD activation after 4 h of treatment and was maximally active at 10 \(\mu\)M. The activity was structurally specific in that the structural analogs 4,5-dihydro-\(C_{6}\)-ceramide and dioctanoylglycerol were inactive. Although ceramide inhibited PMA-induced activation of PLD, it did not inhibit translocation of protein kinase C (PKC) to the membrane in response to PMA.

In a cell-free system, we confirmed that PLD is activated by guanosine 5'-O-(3-thiotriphosphate (GTP\(^{\gamma}\))S); however, ceramide had no effect on this activity under a variety of conditions. Activation of PLD by GTP\(^{\gamma}\)S was synergistically enhanced by the addition of PKC activators. This upstream effect was inhibited rapidly and specifically by ceramide (30 \(\mu\)M). Recombinant ARF plus PKC\(\alpha\) substituted for crude cytosol in the activation of PLD, and this activity was inhibited by \(C_{6}\)-ceramide. Taken together, these data show that ceramide interferes with PKC-mediated activation of PLD.

**EXPERIMENTAL PROCEDURES**

**Materials**—Myristoylated recombinant ARF (5.7%) was a generous gift of Dr. Richard Kahn (Emory University). Recombinant PKC\(\alpha\) was a generous gift of Dr. Doriano Fabbro (Ciba Geigy, Basel, Switzerland). Silica Gel 60 thin-layer chromatography plates were from Whatman, and solvents were supplied by Fisher Scientific. Dipalmitylophosphatidylcholine (5\(\mu\)M) was from DuPont NEN. Solvents were from Mallinckrodt Chemical Works (analytical grade). Fetal bovine serum and RPMI was from Life Technologies, Inc. Phosphatidylethanol and dioleoylglycerol were from Avanti Polar Lipids, Inc. ATP was from Pharmacia Biotech Inc. \(\alpha\)-Erythro-\(N\)-hexanoyl-sphinganine and \(\alpha\)-erythro-\(N\)-(\(3\)-thiotriphosphate; \(GTP^{\gamma}\))S were synthesized as described (14). Other reagents were purchased from Sigma.

**Cell Culture**—HL-60 cells (American Type Culture Collection) were grown in RPMI (Life Technologies, Inc.) supplemented with 5% NaHCO\(_3\) and 10% fetal bovine serum. Cells were maintained at 37 °C in 5% CO\(_2\) and subcultured twice times per week. Routine tests verified that the cells were free of Mycoplasma.

**PLD**

PLD has been extensively investigated for the last several years. It has been implicated in the regulation of inflammatory and immune responses, cellular trafficking, and cell growth (1). This broad range of effects has complicated the elucidation of the mechanism of activation of PLD but has also led to the belief that there is a family of enzymes that probably possess distinct activation pathways (1). The best supported mechanisms of activation involve PKC or the small soluble GTP-binding proteins Rho and ARF (1–4). We have previously demonstrated a role for PLD in fibroblast mitogenesis and immunoresponses, cellular trafficking, and cell growth (1).

**PKC Translocation**—HL-60 cells (2.5 \(\times\) 10\(^7\) cells/ml) were treated with \(C_{6}\)-ceramide or ethanol (0.1%) for 4 h and then with 100 \(n\)M PMA for 20 min at 37 °C. Cells were harvested as described (16) and analyzed by Western blot analysis. Protein was estimated by Bradford analysis (17). Western Blots—Samples (100 \(\mu\)g protein from HL60 cells) were run on 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose at 4 °C overnight (8). Blots were then washed with 5% nonfat dry milk in PBS for 1 h at 20 °C and incubated with PKC isoenzyme-specific antisera (18) at a dilution of 1:500 at 1:1000 with or without competing peptide (20–40 \(\mu\)g) for 2 h at 20 °C. The blots were washed three times with 5% nonfat dry milk in PBS for 15 min at 20 °C and then once with PBS. The blots were incubated with secondary antiserum (goat anti-rabbit linked to horseradish peroxidase) for 2 h at 20 °C and then washed three times with PBS.
developed using ECL under conditions described by the manufacturer (Amer sham Corp.).

Whole-cell PLD Assay—Cells (2.5 × 10^5 in 1 ml) were seeded in 24-well plates. Cells were rested and radiolabeled with [3H]palmitic acid (3 μCi, added in 3 μl ethanol) in serum-free media for 24 h, which resulted in approximately 95% being incorporated into the phosphatidylcholine fraction. Cells were also pretreated with 10 μM C6-ceramide for the indicated time. Cells were stimulated in 2% ethanol with 100 nM PMA for the indicated times. Incubations were stopped by the addition of 1 ml methanol on ice. Cells were transferred to a glass tube and extracted (15). Lipids were analyzed by thin layer chromatography using solvent A for phosphatidylcholine analysis, chloroform:methanol:acetic acid:H2O (50:25:8:4), or solvent B for phosphatidylethanol analysis, the upper phase of a mixture of ethyl acetate:iso-octane:acetic acid:H2O (80:50:20:100) (19).

Cell-free PLD Assay—Cells were treated as described (3). Briefly, HL-60 cells (5–8 × 10^6) were pelleted and then washed in PBS. The cell pellet was suspended in approximately 2 ml of homogenization buffer (2 ml) using a 19-gauge needle and was recentrifuged, as was the supernatant. This pellet was resuspended in 500 μl. Proteins were stable for at least 2 weeks when diluted with an equal volume of 100% glycerol, frozen in a dry ice/ethanol bath, and stored at −90 °C. Assays were performed essentially as described previously (3) except that 3.4 μg of membrane protein and 7.1 μg of cytosol protein were used, and the radiospecific activity of the substrate dipalmitoylphosphatidylcholine was increased such that at the same molar concentration, 120,000 dpm radiolabel were added per tube. C6-Ceramide was added in ethanol (1 μl) to protein components prior to activators. An additional 33 μM CaCl2 was added to the reactions as well as 15 μM GTPγS, 50 nM PMA, 4 μM ATP, and 16 mM MgCl2 where indicated. Incubations expressed in Figs. 9 and 10 were done in 120 μl final volume.

RESULTS

Previous studies from our laboratory and others demonstrated that ceramide inhibits PLD activation in fibroblasts (8, 11, 12) and neutrophils (10). To define the target for ceramide, we used a cell-free system from HL-60 cells to investigate the action of ceramide on PLD. In intact HL-60 cells, PLD was inhibited by ceramide. Fig. 1 demonstrates that ceramide inhibited PMA-induced PLD activity in a dose- and time-dependent manner such that PLD activity decreased as early as 1 h after ceramide treatment and was at base-line unstimulated levels by 4 h. C6-Ceramide was effective at concentrations as low as 2 μM, and maximal activity was reached by 10 μM C6-ceramide.

To measure how quickly ceramide was reaching its putative internal target and whether a metabolite might be the active species, we studied the uptake and metabolism of [14C]C6-ceramide. We considered that ceramide may exert its effect upstream of

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**Fig. 1. PLD activation by PMA and inhibition by ceramide.** Cells (2.5 × 10^5 in 1 ml) were seeded in 24-well plates. Cells were rested and radiolabeled with [3H]palmitic acid (3 μCi, added in 3 μl ethanol) in serum-free media for 24 h. Cells were also pretreated with 10 μM C6-ceramide for the indicated time (A) or treated for 24 h with the indicated concentration of C6-ceramide (B). Cells were stimulated in 2% ethanol with 100 nM PMA for the 20 min. The cells were placed on ice, and then 1 ml methanol was added to stop the reaction. Wells were washed with an additional 1 ml of methanol, and lipids were extracted (15). Lipids were separated by thin-layer chromatography (solvent B), and phosphatidylethanol (PDE) was quantitated by liquid scintillation spectrometry. These experiments were performed in duplicate (mean ± S.E. (bars)) and are each representative of three experiments.
the GTPγS-activable protein. Since PKC-induced activation of PLD in intact cells was inhibited by ceramide (Fig. 1), we tested the effect of PKC activators on the cell-free PLD activity. The addition of PKC activators to membrane and cytosol fractions in the absence of GTPγS had no effect on PLD activity (Fig. 5). When these were added in combination with GTPγS, however, we observed a synergistic increase in activity by 5-fold over background (Fig. 5). This effect of PKC activation was ATP-dependent, indicating that it was mediated by phosphorylation. These results suggested that PKC activation of PLD may be an important control site in this two-step activation of PLD in HL60 cells.

To test the hypothesis that ceramide-mediated inhibition of PLD was through inhibition of the PKC-activable component, we optimized and characterized the PKC-dependent, cell-free system. This was initially performed by studying the protein dependence of the PKC-activable PLD. Varying the membrane protein concentration while keeping the cytosol protein fixed at 7.1 μg showed that optimal PKC activation of PLD was obtained in the presence of 3.4 μg of membrane protein (Fig. 6A). Varying the cytosol protein concentration while keeping the membrane protein concentration constant at 3.4 μg showed that optimal PKC-activable PLD was seen with 7.1 μg of cytosol protein (Fig. 6B). These optimal protein concentrations were then used to survey other requirements and potential activators/inhibitors of PKC-activable PLD (Fig. 7). The Ca2+ requirement was found to have a narrow optimum at 4 mM total Ca2+ (data not shown). The activity was completely inhibited by high levels of Zn2+, Mn2+, and Ca2+. PLD activity is thought to be regulated by tyrosine kinases (20, 21). We wanted to determine if this regulation involved the PKC-activable component of PLD. The tyrosine kinase inhibitor genistein did not inhibit PKC-activable PLD, but curiously, the phosphotyrosine phosphatase inhibitor orthovanadate was totally inhibitory (Fig. 7). Also, the ceramide-activated protein phosphatase/protein phosphatase 2A inhibitor okadaic acid was without effect (Fig. 7).

Once the PKC activation of PLD was established and its primary components and requirements characterized, we proceeded to test the possibility that C8-ceramide would act at this level. C8-Ceramide was added to the protein components first, mixed, and then the activators Ca2+, GTPγS, PMA, and MgATP were added, and the incubations were performed as above. Our results showed that ceramide inhibited the PKC-dependent activation of GTP-binding protein-dependent PLD activity in a dose-dependent fashion such that as little as 1 μM of C8-ceramide was effective at inhibiting PKC-activable PLD with maximal effects seen with 30 μM of C8-ceramides (Fig. 8). This inhibition was not through sequestration of PMA because PMA concentrations from 50 nM to 3 μM were used, and ceramide inhibition was unchanged (data not shown). These data demonstrate that the mechanism of inhibition of PLD by ceramide is via inhibiting the PKC-activable component. Coupled with the lack of inhibition of PKC translocation by ceramide and its inability to inhibit PKC directly (Ref. 22 and data not shown), these data localize the ceramide target between PKC and the GTP-binding protein in the activation of PLD in HL60 cells.

In HL60 cells, the GTPγS-responsive element involved in PLD activation was shown to be cytosolic and was identified as ARF (3, 4); however, in neutrophils the GTP-binding protein component was shown to be in the membrane and was identified as Rho (2). In our HL60 cells, the PLD activity appears to be in the membrane (Ref. 3 and data not shown). PLD activation by GTPγS, however, requires a cytosolic component in that the addition of GTPγS to membrane in the absence of cytosol only slightly stimulated PLD activity from 1195 ± 476 to
FIG. 6. Protein dependence of PKC/GTP-binding protein activation of PLD. Assays were performed as described under “Experimental Procedures.” Incubations contained indicated amounts of membrane (A) and cytosol (B) plus 7.1 μg cytosol protein (A) or 3.4 μg membrane protein (B) in addition to 33 μM CaCl₂ and, where indicated, GTPγS (15 μM), PMA (50 nM), and ATP (4 μM). Data are the mean ± range (bars, S.E.) of duplicate measurements. GTPγS, GTPγS.

1399 ± 269 dpm phosphorylcholine, and Western blot analysis showed that ARF was greater than 95% cytosolic (data not shown). Therefore, we studied the role of ARF in PLD activation and its inhibition by ceramide.

Our first goal was to reconstitute membrane PLD activation using defined cytosolic components, i.e., ARF and PKC. Substitution of recombinant 5.7% myristoylated ARF1 for cytosol reconstituted GTPγS activation (Fig. 9A). The addition of baculovirus-expressed PKCa also stimulated PLD activity. This was in contrast to what was seen using cytosol as the source of PKC, where cytosol and PKC activators alone (i.e., in the absence of GTPγS) did not activate PLD. The addition of rARF1 and PKCa stimulated high levels of PLD activity (Fig. 9A), thereby completely reconstituting the activation pattern seen with crude cytosol.

Next we defined the requirements for the activators (GTPγS, PMA, and ATP). The substitution of rARF for cytosol substantially activated PLD in the presence of GTPγS (Fig. 9B). Significantly greater activation was achieved by the addition of PMA and ATP (Fig. 9B), which may be due to a small amount of PKC that is membrane-associated in this preparation. The substitution of rPKCa for cytosol greatly stimulated PLD in washed membranes (Fig. 9C). This required only the PKC activators PMA and ATP, whereas no further activation by GTPγS was observed. Complete reconstitution of the two-step activation pathway with rARF1 and rPKCa required both GTP-binding protein and PKC activation (Fig. 9D) because it showed progressively greater activity with the addition of GTPγS, PMA plus ATP, and GTPγS plus PMA plus ATP.

We next evaluated the inhibitory effect of C₆-ceramide on this well-defined system (Fig. 10). C₆-Ceramide was unable to inhibit ARF-activable PLD; however, C₆-ceramide completely abolished the synergistic activation by PKC. Higher concentrations of C₆-ceramide were required to maintain the lipid:protein ratio the same as that used in the crude system. These results with defined components recapitulate the observations in the crude system. They also define the least system required to preserve the effects of PKC and the action of ceramide. Importantly, they suggest, in inhibiting PLD, the target for ceramide resides in the membrane.

FIG. 7. Characteristics of PKC/GTP-binding protein activated PLD. Assays were performed as described under “Experimental Procedures.” Inhibitors were added to the protein components just prior to the enzyme activators CaCl₂ (33 μM), GTPγS (GTPγS; 15 μM), PMA (50 nM), and ATP (4 μM) and the substrate. Data are the mean ± range (bars, S.E.) of duplicate measurements. Ok Acid, okadaic acid; Genest., genestein; Vn, vanadate.

FIG. 8. PKC activation of PLD is inhibited by ceramide. Extracts from 5 × 10⁶ cells were prepared, and PLD assays were performed as described under “Experimental Procedures.” Data are representative of four experiments; bars, S.E. GTPγS, GTPγS.
constitution of PLD activation with washed membranes plus two recombinant cytosolic proteins, ARF and PKCa, demonstrates that other cytosolic proteins are not required for activation. A role for membrane-associated Rho is also possible (2), although we were not able to rule that out in these studies.

Our finding that ceramide does not directly inhibit phosphatidylinositol bisphosphate-dependent PLD as illustrated by Reinhold et al. (29), Massenburg et al. (30), Tyagi et al. (31), and Song and Foster (32) (see also Ref. 1). One PLD is activated by fatty acids, whereas another requires PKC. The small molecular mass GTP-binding proteins ARF and Rho each activate a PLD (1), possibly working together (33, 34) and on the same enzyme.

Some studies have shown a synergistic activation of PLD by protein kinases and the small GTP-binding proteins (35, 36). We also found this synergy, where activation of the GTP-binding proteins alone stimulates PLD significantly and the addition of PKC substantially augments GTP-binding protein activation of PLD. This activation by PKC required ATP and PMA in our studies; however, ATPγS and AMP-PNP were equally effective as ATP in the rARF plus rPKC assay system (data not shown; see also Ref. 37). Olson et al. (38) reported that PKC activation of PLD is ATP-dependent. On the other hand, Conricode et al.

The discovery that ceramide inhibits PLD activation in this more defined system indicates that ceramide might inhibit the interaction of PKC with ARF/PLD without affecting PKC translocation per se. In addition to our finding that PKC translocation is not inhibited by ceramide, other studies show that ceramide does not inhibit PKC phosphorylation activity in vitro (16, 22). Jones and Murray (11) also found inhibition of bradykinin stimulation of PLD activity by C2-ceramide in the intact mouse epidermal cell line HEL-37. In that study, the authors demonstrate only slight inhibition of translocation of PKCa in response to C2-ceramide. Blobe et al. (25) have shown that PKCa interacts specifically with actin filaments. It is possible that this interaction is affected by ceramide. In any case, our reconstituted system and the results of others (26–28) implicate a novel role for PKC in signal transduction that is independent of classical PKC action i.e. translocation and phosphorylation, and that it is this action that is inhibited by ceramide. Although the regulatory pathways for phospholipase(s) D remain far from clear, a considerable amount of information has emerged recently. It is apparent that within a certain cell type, there are more than one PLD, as illustrated by Reinhold et al. (29), Massenburg et al. (30), Tyagi et al. (31), and Song and Foster (32) (see also Ref. 1). One PLD is activated by fatty acids, whereas another requires PKC. The small molecular mass GTP-binding proteins ARF and Rho each activate a PLD (1), possibly working together (33, 34) and on the same enzyme.
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