Ceramide 1-Phosphate Is a Direct Activator of Cytosolic Phospholipase A$_2^*$

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Recently, we demonstrated that ceramide kinase, and its product, ceramide 1-phosphate (Cer-1-P), were mediators of arachidonic acid released in cells in response to interleukin-1β and calcium ionophore (Pettus, B. J., Bielawska, A., Spiegel, S., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2003) J. Biol. Chem. 278, 38206–38213). In this study, we demonstrate that down-regulation of cytosolic phospholipase A$_2$ (cPLA$_2$) using RNA interference technology abolished the ability of Cer-1-P to induce arachidonic acid release in A549 cells, demonstrating that cPLA$_2$ is the key phospholipase A$_2$ downstream of Cer-1-P. Treatment of A549 cells with Cer-1-P (2.5 μM) induced the translocation of full-length cPLA$_2$ from the cytosol to the Golgi apparatus/perinuclear regions, which are known sites of translocation in response to agonists. Cer-1-P also induced the translocation of the CaLB/C2 domain of cPLA$_2$ in the same manner, suggesting that this domain is responsive to Cer-1-P either directly or indirectly. In vitro studies were then conducted to distinguish these two possibilities. In vitro binding studies disclosed that Cer-1-P interacts directly with full-length cPLA$_2$ and with the CaLB domain in a calcium- and lipid-specific manner with a $K_{Ca}$ of 1.54 μM. Furthermore, Cer-1-P induced a calcium-dependent increase in cPLA$_2$ enzymatic activity as well as lowering the $EC_{50}$ of calcium for the enzyme from 191 to 31 nM. This study identifies Cer-1-P as an anionic lipid that translocates and directly activates cPLA$_2$, demonstrating a role for this bioactive lipid in the mediation of inflammatory responses.

The production of arachidonic acid (AA)$^3$ is the initial rate-limiting step in eicosanoid biosynthesis, and the major phospholipase that regulates eicosanoid synthesis in response to agonists is group IVA cytosolic phospholipase A$_2$ (cPLA$_2$) (1, 2). Cell-specific and agonist-dependent events coordinate translocation of cPLA$_2$ to the nuclear envelope, endoplasmic reticulum, and Golgi apparatus (1–8). At these membranes, cPLA$_2$ hydrolyzes membrane phospholipids to produce arachidonic acid, initiating the eicosanoid synthetic pathways (1–8).

cPLA$_2$ was first characterized in platelets and macrophage cells and subsequently cloned from a macrophage cDNA library (1–4, 7–9). The cPLA$_2$ cDNA encodes for an 85-kDa protein, and the mRNA for cPLA$_2$ is widely expressed in the brain, lung, kidney, heart, and spleen (1–4, 7–9). In vitro, cPLA$_2$ is activated by Ca$^{2+}$; however, the addition of salt at physiologic concentrations will also induce enzyme activation, and thus, the catalytic activity of cPLA$_2$ is not dependent on Ca$^{2+}$ (1, 5, 6). On the other hand, the activation/translocation of cPLA$_2$ in cells requires the association of cPLA$_2$ with membranes in a Ca$^{2+}$-dependent manner (1–4). Near the N terminus of cPLA$_2$ is a stretch of ~120 amino acids that constitutes the Ca$^{2+}$-dependent lipid binding domain (CaLB domain) of the enzyme (1–5, 7). However, the specific membrane lipids that regulate the association of this domain with membranes and demonstrate interaction with cPLA$_2$ in low calcium (300 nM), as first shown by Knopf and co-workers (3), have yet to be defined. Furthermore, it is not known whether physiologic calcium is sufficient to activate cPLA$_2$, or whether activation also requires the generation of activating lipids.

Our laboratories recently reported that ceramide kinase and its product, ceramide 1-phosphate (Cer-1-P), are mediators of calcium ionophore- and interleukin-1β-induced AA release suggesting a role for the ceramide kinase/Cer-1-P pathway in the activation of a species of PLA$_2$ (10). Interestingly, ceramide kinase is a calcium-activated enzyme in a similar manner as cPLA$_2$, and these enzymes share similar patterns of tissue expression (7, 11). In this study, mechanisms by which ceramide 1-phosphate activates cPLA$_2$ were investigated. Cer-1-P was found to bind cPLA$_2$ directly and to be an inducer of cPLA$_2$ activation in vitro and in cells. Thus, for the first time, a specific intracellular target for this novel bioactive lipid has

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‡‡ The abbreviations used are: AA, arachidonic acid; PLA$_2$, phospholipase A$_2$; cPLA$_2$, cytosolic PLA$_2$; Cer-1-P, ceramide 1-phosphate; RNAi, RNA interference; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidyethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CaLB, Ca$^{2+}$-dependent lipid binding; GST, glutathione S-transferase; TBS, Tris-buffered saline; LMV, large multilamellar vesicle; GFP, green fluorescent protein; HEDTA, N-(2-hydroxyethyl)enediaminetetraacetic acid; FAF-BSA, fatty acid-free bovine serum albumin.

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brane-associated proteins as described previously for cPLA2 (12). Protein fractions (10 μg) from each sample were resolved on 15% SDS-PAGE under denaturing conditions and then transferred to 0.20-μm polyvinylidene difluoride membranes. After blocking overnight at 4°C with 5% nonfat milk in phosphate-buffered saline, 0.05% Tween 20 (M-PBS-T) and washing, the membranes were incubated with anti-cPLA2 (4-8C-3C) (Santa Cruz Biotechnology) in M-PBS-T for 2 h at room temperature. The membranes were washed extensively in phosphate-buffered saline, 0.05% Tween 20 (washing buffer). The bands were visualized using the appropriate horseradish peroxidase-conjugated anti-mouse IgG antibody and the ECL Western blotting detection system (Amersham Biosciences).

Lipid-Protein Overlay Assay—Various amounts of the indicated lipids (dioleoyl-PC, dioleoyl-PE, dioleoyl-PI, dioleoylcardi- lipin, dioleoyl-PS, α,ω-eposphingosine, α,ω-eposphingosine-1-phosphate, α,ω- C16-ceramide, α,ω-C20-ceramide 1-phosphate, and dioleoylglycerol) were spotted onto Hybond C membrane (Amersham Biosciences) and dried under vacuum. The membrane was re-wet in water and then blocked for 1 h in 3% fatty acid-free bovine serum albumin (FAP-BSA/TBS-T 0.1% Tween 20). The membrane was then exposed to 0.2 μg/ml cPLA2-GST in 3% FAP-BSA/TBS-T overnight at 4°C. The membrane was washed six times for 5 min with TBS-T and then exposed to a 1:2500 dilution of anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) in 3% FAP-BSA/TBS-T for 1 h at room temperature. The membrane was washed again six times for 5 min with TBS-T and then exposed to a 1:2500 dilution of horseradish peroxidase-conjugated anti-mouse IgG antibody (1.500X). The membranes were developed by enhanced chemiluminescence (ECL)/Western blot imaging (Amersham Biosciences). Purified recombinant GST did not bind any of the lipids using this protocol (data not shown).

Immunofluorescence—Cells were fixed and permeabilized in 3.7% formaldehyde, 0.1% Triton X-100, phosphate-buffered saline for 15 min. Cells were immunostained with 10 μg/ml anti-human cPLA2 antibody (C20) in phosphate-buffered saline, 5% human serum for 2 h at room temperature. After extensive washing with phosphate-buffered saline, cells were immunostained with 10 μg/ml anti-goat IgG-rhodamine-conjugated antibody in phosphate-buffered saline for 1 h at room temperature. After extensive washing with phosphate-buffered saline, cPLA2 localization was visualized by confocal microscopy at 568 nm using a 60X oil immersion lens with a 1.50× enhanced magnification.

TABLE I

| Time (h) | cPLA2 down-regulation | C-1-P-induced AA release | Inhibition of C-1-P-induced AA release | cPLA2 down-regulation |
|----------|----------------------|--------------------------|---------------------------------------|----------------------|
| 24       | 85.0                 | 57.0                     | 52.4                                  | 79.4                 |
| 48       | 85.0                 | 81.3                     | 79.4                                  | 79.4                 |

- Time from the end of the RNAi transfection to analysis of cPLA2 expression and the start of the assay for C-1-P-induced AA release.
- Mean percentage of the number of cells demonstrating intracellular fluorescence of RNAi (1 × 106 cells scored).
- Mean percentage of the down-regulation of immunoreactive cPLA2 normalized to total protein and α-tubulin as compared to scrambled (control) RNAi (100%).
- Mean percentage of the inhibition of C-1-P-induced AA release (2.5 μM C-1-P for 3 h) as compared to scrambled (control) RNAi (100%).
- Mean percentage of the inhibition of C-1-P-induced AA release (2.5 μM C-1-P for 3 h) as compared to scrambled (control) RNAi (100%) normalized to the percent of cPLA2 downregulation.

been demonstrated, establishing Cer-1-P as a signaling lipid in biological systems. Furthermore, this study also demonstrates a possible lipid “missing link” in the induction of eicosanoid synthesis in response to agonists.

MATERIALS AND METHODS

Cell Culture—A549 human lung adenocarcinoma cells (epithelial-derived) and J774.1 macrophage cells were grown in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 1-glutamine, 10% (v/v) fetal bovine serum (Invitrogen), 200 units/ml penicillin G sodium, and 200 μg/ml streptomycin sulfate. Cells were maintained under 80% confluency and standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37°C). For treatment, cells were plated in low glucose Dulbecco’s modified Eagle’s medium supplemented with 1-glutamine, 2% (v/v) fetal bovine serum, 200 μg/ml penicillin G sodium, and 200 μg/ml streptomycin sulfate and rested for 2 h prior to the addition of agonist.

Immunoblotting—Cells were fractionated into cytosol and membrane-associated proteins as described previously for cPLA2 (12). Protein fractions (10 μg) from each sample were resolved on 15% SDS-PAGE under denaturing conditions and then transferred to 0.20-μm polyvinylidene difluoride membranes. After blocking overnight at 4°C with 5% nonfat milk in phosphate-buffered saline, 0.05% Tween 20 (M-PBS-T) and washing, the membranes were incubated with anti-cPLA2 (4-8C-3C) (Santa Cruz Biotechnology) in M-PBS-T for 2 h at room temperature. The membranes were washed extensively in phosphate-buffered saline, 0.05% Tween 20 (washing buffer). The bands were visualized using the appropriate horseradish peroxidase-conjugated anti-mouse IgG antibody and the ECL Western blotting detection system (Amersham Biosciences).

Lipid-Protein Overlay Assay—Various amounts of the indicated lipids (dioleoyl-PC, dioleoyl-PE, dioleoyl-PI, dioleoylcardi- lipin, dioleoyl-PS, α,ω-eposphingosine, α,ω-eposphingosine-1-phosphate, α,ω-C16-ceramide, α,ω-C20-ceramide 1-phosphate, and dioleoylglycerol) were spotted onto Hybond C membrane (Amersham Biosciences) and dried under vacuum. The membrane was re-wet in water and then blocked for 1 h in 3% fatty acid-free bovine serum albumin (FAP-BSA/TBS-T 0.1% Tween 20). The membrane was then exposed to 0.2 μg/ml cPLA2-GST in 3% FAP-BSA/TBS-T overnight at 4°C. The membrane was washed six times for 5 min with TBS-T and then exposed to a 1:2500 dilution of anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) in 3% FAP-BSA/TBS-T for 1 h at room temperature. The membrane was washed again six times for 5 min with TBS-T and then exposed to a 1:2500 dilution of anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) in 3% FAP-BSA/TBS-T for 1 h at room temperature. The membrane was then washed 12 times for 5 min with TBS-T and then developed by ECL. Purified recombinant GST did not bind any of the lipids using this protocol (data not shown).

Immunofluorescence—Cells were fixed and permeabilized in 3.7% formaldehyde, 0.1% Triton X-100, phosphate-buffered saline for 15 min. Cells were immunostained with 10 μg/ml anti-human cPLA2 antibody (C20) in phosphate-buffered saline, 5% human serum for 2 h at room temperature. After extensive washing with phosphate-buffered saline, cells were immunostained with 10 μg/ml anti-goat IgG-rhodamine-conjugated antibody in phosphate-buffered saline for 1 h at room temperature. After extensive washing with phosphate-buffered saline, cPLA2 localization was visualized by confocal microscopy at 568 nm using a 60X oil immersion lens with a 1.5× enhanced magnification.

Inhibition of C-1-P-induced AA release was performed essentially as described under “Materials and Methods.” 48 h after post-transfection, the cells were treated with Cer-1-P (2.5 μM) for 3 h and assayed for AA release. Data are presented as the percent control of AA release controlled for equivalent number of cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are representative of three separate determinations on two separate occasions.

Immunolocalization of cPLA2—5 × 104 A549 cells were plated onto number 1 coverslips. 24 h later, the cells were starved overnight and then treated with either vehicle (0.1% dodecane:ethanol, 49:1) or 5 μM Cer-1-P. Cells were then fixed in 3.7% formalin in phosphate-buffered saline for 20 min at room temperature and then stained essentially as described (34). Primary antibodies were mouse anti-GM130 for the Golgi apparatus (BD Transduction Laboratories) and goat anti-cPLA2, and secondary antibodies were from Jackson ImmunoResearch. Images were collected on a Nikon TE-300 microscope at 60X using a CoolSnap (Roper Scientific) CCD camera driven by MetaMorph software (Universal Imaging Corp.).

cPLA2 Assay—cPLA2-GST (0.2 μg) was assayed as described (8). Briefly, a liposome substrate consisting of labeled PC (1-stearoyl-2-[14C]arachidonoyl (100,000 dpm/assay) (PerkinElmer Life Sciences)), unlabeled 1-stearoyl-2-arachidonoyl PC (Avanti) (30 μg/assay), and dioleoylglycerol (10 μg/assay) in 50 μm Tris-HCl, pH 7.4, was produced by sonication on ice for 3 min. α-C16-ceramide 1-phosphate vesicles were generated separately by sonication on ice three times for 30 s each in 50 μmol Tris-HCl, pH 7.4. cPLA2-GST (0.2 μg) was then assayed in a base buffer consisting of 50 μmol Tris-HCl, pH 7.4, and 150 μmol NaCl, the substrate liposome, 100 μM α-C16-ceramide 1-phosphate vesicles and varying concentrations of α-C16-ceramide 1-phosphate vesicles and varying concentrations of free calcium.

Quantification of the Release of Arachidonic Acid (and Metabolites)—A549 cells (5 × 104) were labeled overnight with 5 μCi/ml [3H]arachidonic acid (5 nCi). Cells were washed and placed in Dulbecco’s modified

![Fig. 1. cPLA2 is required for Cer-1-P-induced AA release.](http://www.jbc.org/doi-fig-viewer)
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Table II

| Time (h) | Transfection efficiency$^b$ | cPLA$_2$ down-regulation$^a$ | Inhibition of C-1-P-induced AA release$^a$ | Inhibition (normalized to cPLA$_2$ down-regulation) |
|---------|----------------------------|-----------------------------|----------------------------------------|--------------------------------------------------|
| 24      | ND                        | 36.0                        | 30                                     | 83.3                                             |
| 48      | ND                        | 61                          | 59                                     | 96.7                                             |

$^a$ Time from the end of the RNAi transfection to analysis of cPLA$_2$ expression and the start of the assay for C-1-P-induced AA release.

$^b$ Mean percentage of the number of cells demonstrating intracellular fluorescence of RNAi (1 × 10$^5$ cells scored).

$^c$ Mean percentage of the down-regulation of immunoreactive cPLA$_2$ normalized to total protein and α-tubulin as compared with scrambled (control) RNAi (100%).

$^d$ Percentage of the inhibition of C-1-P-induced AA release (2.5 μM C-1-P for 2 h) as compared with scrambled (control) RNAi (100%).

$^e$ Normalized to the percent of cPLA$_2$ down-regulation.

$^f$ ND, not determined.

Data are representative of three separate determinations on two separate occasions.

Eagle's medium supplemented with 2% fetal bovine serum for 2 h. Following treatment, medium was transferred to 1.5 ml polypropylene tubes, centrifuged at 10,000 × g, and 1/30 of the supernatant (10% of the total cPLA$_2$) was subjected to scintillation counting. Results were corrected for equivalent number of cells quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (13) and by verification of total AA labeling by scintillation counting.

Transfections—A549 cells (1 × 10$^5$) were transfected with 2 μg of cPLA$_2$-green fluorescent protein (GFP) or CalB/C2-GFP for 24 h using FuGENE6 reagent (Roche Applied Science) following the manufacturer's standard protocol. Following transfection, cells were treated with 2.5 μM D-ε-C18:1-ceramide 1-phosphate solubilized in 2% dodecane, 98% ETOH (final concentration in treatments was 0.002% dodecane, 0.098% ETOH) for 3 h. cPLA$_2$ or CalB/C2-GFP localization was visualized using an Olympus BX50WI confocal microscope at 488 nm (Fluoview detector) using a 40× liquid immersible lens with 1.5× enhanced magnification.

RNA Interference (RNAi)—Sequence-specific silencing of cPLA$_2$ was performed essentially as described using sequence-specific small interfering RNA reagents (10, 14, 15) using human cPLA$_2$ RNAi starting 300 nucleotides from the start codon (UGCUCGGCUAGUGCUGUUAdTdT and UAACACCUACCGUAAACcTdT) for A549 cells and mouse cPLA$_2$, RNAi starting 300 nucleotides from the start codon (ACCCUGAGCAGCCAUAAcTdT and AUGCAGGUGCCUGCCUGGGUdT) for J774.1 cells. All sequences were evaluated against the database using the NIH BLAST program to test for specificity (Xeragon). A549 or J774.1 cells (5 × 10$^4$) were transfected with the 21-nucleotide duplexes using OligofectAMINE (Invitrogen) as recommended by the manufacturer. Following the 4-h transfection, cells were labeled overnight with 5 μCi/ml [3H]arachidonic acid (5 nm). Cells were washed and placed in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum for 2 h. The optimal down-regulation time for cPLA$_2$ was 48 h post-transfection as judged by Western immunoblotting with an anti-cPLA$_2$ monoclonal antibody anti-cPLA$_2$ that recognizes both mouse and human cPLA$_2$. Total protein was normalized using post- Amido Black staining of polyvinylidene difluoride membranes and by simultaneous ECL development of α-tubulin using an anti-α-tubulin antibody (Santa Cruz Biotechnology).

Large Multilamellar Vesicle Binding Assay—Large multilamellar vesicles for Cer-1-P were produced by drying 18.3 μl of a 1 mg/ml d-e-C18:1-ceramide 1-phosphate under nitrogen for each reaction. A solution (100 μl) of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl solution for each reaction was added, and the lipid was vortexed vigorously for 2 min. Cer-1-P LMVs (100 μl) were mixed with buffer A (200 μl) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM EDTA), and for calcium-containing reactions Cer-1-P LMVs were mixed with 4.4 mM CaCl$_2$. The binding reaction was initiated by the addition of 100 μl of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 0.2 μg of cPLA$_2$-GST. After 5 min at room temperature, the reaction was centrifuged at 10,000 × g for 10 min, and the supernatant was transferred to a new 1.5-ml polypropylene tube. 200 μl of 1% Laemmli buffer was added to the lipid pellet, and 100 μl of 10% triton buffer was added to the 400 μl of supernatant. 35 μl of the lipid pellet was subjected to SDS-PAGE as described in Fig. 4A. Purified recombinant GST did not bind any of the lipids using this protocol (data not shown).

RESULTS

Ceramide 1-Phosphate Activates cPLA$_2$ in Cells—We have shown previously (10) that Cer-1-P specifically induces AA release in cells, and the generation of Cer-1-P is required for cytokine- and calcium ionophore-induced eicosanoid synthesis. These results suggested the activation of cPLA$_2$ in response to Cer-1-P. Therefore, at first, it became important to establish that cPLA$_2$ is the specific enzyme responsible for AA release in response to Cer-1-P. To determine whether cPLA$_2$ is downstream of Cer-1-P, RNAi technology was employed to knock down cPLA$_2$. OligofectAMINE transfection of fluoroein-tagged RNAi gave a transfection efficiency of 85% at 24 and 48 h in A549 cells (Table I). Using these conditions, cPLA$_2$ expression was down-regulated 81.3% with specific RNAi after 48 h post-transfection in A549 cells (Table I). This resulted in a 79.4% inhibition of Cer-1-P-induced (2.5 μM for 3 h) release of AA and metabolites in these cells as compared with scrambled RNAi and sham controls (Fig. 1). Normalization of these results to down-regulation of total cPLA$_2$ showed a nearly total abrogation of the Cer-1-P effects.

In another model of inflammation, J774.1 macrophages, cPLA$_2$ expression was down-regulated by 61% using specific RNAi after 48 h as judged by Western immunoblotting (Table II). As with the A549 cells, AA release elicited by treatment with Cer-1-P (2.5 μM for 2 h) was inhibited to a similar extent (59%) (Table II). Thus, normalization of these results to down-regulation of total cPLA$_2$ again showed a nearly total abrogation of the Cer-1-P effects (Table II). These data indicate that Cer-1-P induces AA release via activation of cPLA$_2$ in cells.

Because the above results implicated cPLA$_2$ as a downstream target for the action of Cer-1-P, studies were undertaken to determine whether Cer-1-P activates cPLA$_2$ in cells. To this end, we first examined whether Cer-1-P affects the association of cPLA$_2$ with cellular membranes because this is a requisite for cPLA$_2$ to act on its membrane phospholipid substrates. Treatment of A549 cells with Cer-1-P (2.5 μM) induced a significant and time-dependent increase in the membrane content of cPLA$_2$ (Fig. 2A). Cer-1-P did not significantly increase the total cellular content of immunoreactive cPLA$_2$. Thus, Cer-1-P induces the translocation of cPLA$_2$ to cell membranes without effects on cPLA$_2$ expression.

To verify that cPLA$_2$ translocated in intact cells, the effects of Cer-1-P treatment on the cellular localization of cPLA$_2$ were examined by immunofluorescence to detect endogenous cPLA$_2$ in fixed cells as well as using cPLA$_2$ fused to GFP to study the localization of the overexpressed protein in live cells (Fig. 2B). Treatment of A549 cells with Cer-1-P caused the translocation of cPLA$_2$ to membranes in a pattern consistent with the Golgi apparatus and perinuclear membranes (12, 16). This was shown for both the endogenous enzyme (Fig. 2B, IF) and the GFP fusion (Fig. 2B, GFP). To demonstrate that Cer-1-P treatment recapitulates the reported pattern of translocation of cPLA$_2$ to Golgi membranes in response to pro-inflammatory
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Fig. 2. The effect of ceramide 1-phosphate on cPLA₂ translocation in cells. A, the effect of natural ceramide 1-phosphate on the association of cPLA₂ with cellular membranes. A549 cells (2 × 10⁶) were treated with 2.5 μM de-C₁₆-ceramide 1-phosphate solubilized in 2% dodecane, 98% ETOH (final concentration in treatments was 0.002% dodecane, 0.098% ETOH) for the indicated times. The figure depicts cytosome- versus membrane-associated cPLA₂ by Western immunoblotting. Data are representative of three separate determinations on two separate occasions. B, the effect of natural ceramide 1-phosphate on the cellular localization of cPLA₂ in cells. A549 cells (1 × 10⁶) were treated with 2.5 μM de-C₁₆-ceramide 1-phosphate solubilized in 2% dodecane, 98% ETOH (final concentration in treatments was 0.002% dodecane, 0.098% ETOH) for 3 h. The subcellular localization of expressed cPLA₂ was examined by green fluorescent protein (GFP) technology in live cells, and endogenous cPLA₂ was examined by immunofluorescence (IF) in fixed cells. Data are representative of three separate determinations on two separate occasions. C, Cer-1-P induces translocation of endogenous cPLA₂ to the Golgi apparatus. A549 cells plated onto glass cover slips were treated with either 0.1% dodecane:ethanol (49:1) vehicle or 5 μM bovine brain Cer-1-P. 5 h later, cells were fixed and stained for cPLA₂ (red) and then stained with GM130 (green) as a marker for the Golgi apparatus. Cer-1-P-induced but not vehicle-induced translocation of cPLA₂ to the Golgi apparatus as indicated by co-localization with GM130 is shown (yellow staining in Overlay). Size bar: 20 μm. Data are representative of three separate determinations on three separate occasions. D, the effect of Cer-1-P on the cellular localization of the CaLB/C2 domain of cPLA₂ fused to green fluorescent protein in live cells. A549 cells (1 × 10⁶) were transfected with CaLB/C2-GFP and then treated with 2.5 μM de-C₁₆-ceramide 1-phosphate solubilized in 2% dodecane, 98% ETOH (final concentration in treatments was 0.002% dodecane, 0.098% ETOH) for 3 h. Data are representative of three separate determinations on two separate occasions.

Fig. 3. Natural ceramide 1-phosphate activates cPLA₂ in vitro. A, the effect of natural ceramide 1-phosphate on cPLA₂ enzymatic activity in vitro. cPLA₂-GST (0.2 μg) was assayed as described (10) in the presence of 100 μM free calcium and varying concentrations of de-C₁₆-ceramide 1-phosphate. Data are presented as cPLA₂ activity measured as disintegrations/min (DPM) of free [³H]arachidonic acid. Data are representative of three separate determinations on two separate occasions. B, the effect of natural ceramide 1-phosphate on the Ca²⁺ responsiveness of cPLA₂ in vitro. cPLA₂-GST (0.2 μg) was assayed as described (10) in the absence (●) or presence (○) of 50 μM de-C₁₆-ceramide 1-phosphate and varying concentrations of free calcium. Data are presented as cPLA₂ activity measured by corrected disintegrations/ min of free [³H]arachidonic acid. Data are representative of three separate determinations on two separate occasions.

CaLB/C2 domain of cPLA₂ fused to GFP (lacking the catalytic domain and, thus, the regulatory phosphorylation sites of cPLA₂) was examined in response to Cer-1-P treatment. Cer-1-P induced the translocation of the GFP-CaLB/C2 domain to Golgi and perinuclear membranes (Fig. 2D) similar to the translocation of endogenous cPLA₂ and GFP-cPLA₂ in response to Cer-1-P. Furthermore, Cer-1-P treatment had no effect on the phosphorylation of the cPLA₂ regulatory site, Ser-505, as judged by Western immunoblotting (data not shown). Thus, Cer-1-P likely induces the translocation of cPLA₂ through the CaLB/C2 domain, independent of increased phosphorylation of cPLA₂ on Ser-505 and independent of the catalytic activity of cPLA₂ or other functionalities outside the CaLB/C2 domain.

Ceramide 1-Phosphate Directly Binds and Activates cPLA₂ In Vitro—The CaLB/C2 domain of cPLA₂ has homology to the C2 domain of protein kinase C, which binds anionic phospholipids (3). Because Cer-1-P, an anionic phospholipid, induced the translocation of cPLA₂ through the CaLB/C2 domain, we wondered whether Cer-1-P directly activated cPLA₂ through this domain. To test this hypothesis, we first examined whether Cer-1-P increased the enzymatic activity and affected the Ca²⁺ affinity of cPLA₂ in vitro. In the presence of saturating Ca²⁺ and near physiological salt (150 mΜ NaCl), Cer-1-P increased the affinity of cPLA₂ in vitro. Therefore, ceramide 1-phosphate binds the CaLB/C2 domain of cPLA₂.
the activity of cPLA2 over 2-fold in a dose-dependent manner (Fig. 3A). Furthermore, the effect of Cer-1-P on cPLA2 enzymatic activity was calcium-dependent, because in the absence of calcium, Cer-1-P was unable to activate the enzyme (basal activity in Fig. 3B). The removal of salt from the assay had no effect on the ability of Cer-1-P to increase the activity of cPLA2 (data not shown). Using the concentration of Cer-1-P that induced maximal activation (50 μM) of cPLA2, the enzymatic activity of cPLA2 was examined with varying concentrations of Ca2+. In the presence of Cer-1-P, the EC50 of calcium was lowered from 191 to 31 nm (Fig. 3B). Thus, Cer-1-P can directly activate cPLA2 in a Ca2+-dependent manner, and Cer-1-P increases the affinity of cPLA2 for calcium analogous to the effect of phosphorylserine imparts on protein kinase C (20).

The second approach was to examine whether Cer-1-P bound directly to cPLA2 using a protein-lipid overlay assay. In the presence of Ca2+, cPLA2 was found to bind as little as 5 nmol of Cer-1-P (Fig. 4). cPLA2 was also found to bind as little as 5 nmol of dioleoyl-PI although to a lesser extent than Cer-1-P. On the other hand, cPLA2 did not bind to dioleoyl-PA, dioleoyl-PC, or dioleoyl-PS when 5 nmol was bound to the membrane (Fig. 4, left). Weak binding of cPLA2 to dioleoyl-PA, dioleoyl-PS, and dioleoyl-PC was observed at lipid concentrations greater than 25 nmol (data not shown). cPLA2 did not bind to diacylglycerol, sphingosine-1-phosphate, sphingosine, or ceramide at any lipid concentration (Fig. 4, right).

Next, we used a LMV assay described previously for cPLA2 (17). cPLA2 did not significantly bind Cer-1-P in the absence of Ca2+, but the binding of cPLA2 to Cer-1-P was dramatically increased in the presence of saturating Ca2+ (Fig. 5A). Using this assay and varying the free Ca2+ concentration, the KCa was determined to be 1.54 μM, which is almost a magnitude lower than the published KCa for PC (10–15 μM) and PS (14 μM) (Fig. 5B) (18). Thus, to date, Cer-1-P demonstrates the lowest KCa for cPLA2. Furthermore and importantly, the binding of cPLA2 to Cer-1-P was significantly enhanced in the presence of physiological calcium.

The region of cPLA2 that binds Cer-1-P was also determined by applying the LMV assay using recombinantly expressed CaLB/C2 and the catalytic domains of cPLA2. The CaLB/C2 domain of cPLA2 was found to bind Cer-1-P in a calcium-enhanced manner comparable with full-length cPLA2. On the other hand, cPLA2 did not bind Cer-1-P when the CaLB/C2 domain was removed (catalytic domain). Thus, cPLA2 interacts with Cer-1-P via the CaLB/C2 domain (Fig. 5C).

**DISCUSSION**

In this study, Cer-1-P was found to be a novel and direct activator of cPLA2; thus, this now suggests that Cer-1-P is a bioactive lipid in biological systems. Previously, Cer-1-P was reported to have effects on cell proliferation, DNA synthesis, and phagocytosis, but a direct target was not identified or hypothesized (21, 22). This study found that cPLA2 specifically interacted with Cer-1-P in a calcium-dependent manner and provided a mechanism for the previous observation that Cer-1-P was required for the induction of AA release in response to agonists that induce cPLA2 activation (10). Therefore, with the identification of a direct and specific intracellular target for Cer-1-P, this sphingolipid now fulfills the following criteria of a bioactive lipid joining the ranks of other bioactive lipids such as its close brethren, ceramide and sphingosine-1-phosphate. First, exogenous Cer-1-P induces a specific biochemical and cellular response (release of AA), and this action of Cer-1-P demonstrates lipid specificity in the induction of cPLA2 interaction/activation, AA release, and eicosanoid synthesis. Second, endogenous Cer-1-P reproduces these effects specifically, and Cer-1-P levels are regulated in response to agonists. Third, the generation of Cer-1-P is required for cPLA2 activation, AA release, and eicosanoid synthesis. Lastly, a direct target (cPLA2) that binds/interacts with and is activated by Cer-1-P has been identified. Therefore, the presented study has identified Cer-1-P as a possible new messenger lipid in biological systems.

This study also demonstrates that Cer-1-P is a direct activator of cPLA2 through interaction with the CaLB/C2 domain. This ability of the CaLB/C2 domain of cPLA2 to interact with an anionic lipid in a calcium-dependent manner is a somewhat unexpected finding in the cPLA2 field. The current “dogma” of lipid/CaLB domain interaction is one that proposes this interaction to be via hydrophobic forces alone and not by electrostatic interaction. It is currently accepted that the CaLB/C2 domain of cPLA2 binds to zwitterionic (e.g. PC) and neutral lipids in a calcium-dependent manner (17, 18). An exception to this was the report that a synthetic non-natural anionic lipid, phosphatidylmethanol, interacted with the CaLB/C2 domain of cPLA2 in a calcium-dependent and stereospecific manner (18,
Several reports have also alluded to the fact that the membrane binding of the CaLB/C2 domain of cPLA2 cannot be explained by hydrophobic interactions alone (25, 26), and the binding of cPLA2 to PC could not be analyzed by crystallography techniques probably because of the low affinity (26). The high $K_{\text{Ca}}$ (10 $\mu$M) of PC for the interaction with cPLA2 also argues against a physiologic role for this lipid in regulating the membrane binding of this enzyme in cells, because the first report on cPLA2/membrane interactions demonstrated that the enzyme associated with membranes in as little as 300 nM free calcium (3, 8, 16). In this study, a significant increase (2-fold) in the binding of Cer-1-P to cPLA2 was observed at 300 nM correlating with these early findings. Furthermore, physiologic salt induces the interaction of PC with cPLA2 without the necessity of free calcium, thus abolishing the calcium-dependence of the interaction (5, 6). On the other hand, physiologic salt had no effect on the ability of Cer-1-P to induce activation of cPLA2 or on the ability of cPLA2 to associate with Cer-1-P in a calcium-dependent manner. Thus, to date, Cer-1-P demonstrates the lowest $K_{\text{Ca}}$ and the greatest calcium dependence for interaction with cPLA2. The results from this study coupled with our previous findings that the ceramide kinase/Cer-1-P pathway is
required for PLA2 activation in response to calcium ionophore and cytokines suggest that Cer-1-P is a “missing link” in the regulation of eicosanoid pathways (10).

The demonstration that Cer-1-P is a direct activator of cPLA2 provides insight into the mechanism of cPLA2 translocation in response to agonists. Previously, it has been reported (27) that sphingomyelin inhibits or does not repulse cPLA2 from membranes. Numerous reports have established that sphingomyelin is hydrolyzed to ceramide by sphingomyelinase in response to inflammatory cytokines such as interleukin-1β and tumor necrosis factor α (28, 29). Because Cer-1-P is produced via the phosphorylation of ceramide, the production of ceramide in response to cPLA2-activating agonists is logical. Thus, one can hypothesize that in response to an inflammatory agonist (e.g., cytokines), sphingomyelin is hydrolyzed to ceramide followed by conversion of the ceramide to Cer-1-P by ceramide kinase. This possible mechanism would remove an inhibitor/repulsor from the membrane, reduce the rigidity of the membrane by producing ceramide, thereby increasing the penetration ability of the enzyme, and produce the activating lipid, Cer-1-P. This hypothesis correlates with our recent demonstration that Cer-1-P levels and ceramide kinase activity are increased in response to interleukin-1β. Furthermore, this hypothesis explains the reports that exogenous ceramide and sphingomyelin inhibits if not repulses cPLA2 from membranes. The possibility that ceramide kinase and Cer-1-P generation are upstream of cPLA2 activation raises the possibility of the development of a new generation of therapeutics for inflammatory disorders. Therapeutics based on ceramide kinase would have the benefit of blocking AA liberation as well as the unwanted formation of leukotrienes and COX-1-derived prostanooids (e.g., thromboxanes) possibly lowering the problems of side effects associated with both selective and non-selective COX inhibitors. Because non-steroidal anti-inflammatory drugs are also being used to treat cancer, this newly described pathway may have widespread applications.

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