Modulation of the activity of cytosolic phospholipase A2α (cPLA2α) by cellular sphingolipids and inhibition of cPLA2α by sphingomyelin

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Abstract We examined the effect of the cellular sphingolipid level on the release of arachidonic acid (AA) and activity of cytosolic phospholipase A2α (cPLA2α) using two Chinese hamster ovary (CHO)-K1-derived mutants deficient in sphingolipid synthesis: LY-B cells defective in the LCB1 subunit of serine palmitoyltransferase for de novo synthesis of sphingolipid species, and LY-A cells defective in the ceramide transfer protein CERT for SM synthesis. When LY-B and LY-A cells were cultured in Nutridoma medium and the sphingolipid level was reduced, the release of AA stimulated by the Ca^{2+} ionophore A23187 increased 2-fold and 1.7-fold, respectively, compared with that from control cells. The enhancement in LY-B cells was decreased by adding sphingosine and treatment with the cPLA2α inhibitor. When CHO cells were treated with an acid sphingomyelinase inhibitor to increase the cellular SM level, the release of AA induced by A23187 or PAF was decreased. In vitro studies were then conducted to test whether SM interacts directly with cPLA2α. Phosphatidylcholine vesicles containing SM reduced cPLA2α activity. Furthermore, SM disturbed the binding of cPLA2α to glycerophospholipids. These results suggest that SM at the biomembrane plays important roles in regulating the cPLA2α-dependent release of AA by inhibiting the binding of cPLA2α to glycerophospholipids.—Nakamura, H., S. Wakita, A. Suganami, Y. Tamura, K. Hanada, and T. Murayama. **Modulation of the activity of cytosolic phospholipase A2α (cPLA2α) by cellular sphingolipids and inhibition of cPLA2α by sphingomyelin. *J. Lipid Res.* 2010.** 51: **720–728.**

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Arachidonic acid (AA) is a precursor of eicosanoids, including prostaglandins, thromboxanes, and leukotrienes, playing an important role in several physiological functions (1). The biosynthesis of these AA metabolites occurs mainly through the activation of phospholipase A2 (PLA2) in response to a wide variety of stimuli such as cytokines, growth factors, and neurotransmitters (2). PLA2 catalyzes hydrolysis of the sn-2 position of glycerophospholipids to release free AA. Mammalian cells have structurally diverse forms of PLA2 including secretory PLA2, Ca^{2+}-independent PLA2, and cytosolic PLA2 (cPLA2) (3, 4). Among these PLA2s, the 85 kDa cPLA2, specifically cPLA2α, is highly selective for glycerophospholipids containing AA. cPLA2α is regulated mainly by an increase in the intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) and by the phosphorylation on serine residues by mitogen-activated protein kinase (MAPK) (3, 4). The binding of Ca^{2+} to the C2 domain of cPLA2α triggers translocation of cPLA2α from the cytosol to the perinuclear region including the Golgi apparatus, endoplasmic reticulum (ER), and nuclear envelope. cPLA2α can be phosphorylated at Ser^{505}, Ser^{515}, and Ser^{727}, which increases its intrinsic enzymatic activity 2- to 3-fold in vitro (5–8).

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Abbreviations: AA, arachidonic acid; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; CHO, Chinese hamster ovary; cPLA2α, cytosolic PLA2α; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; G_{M3}, *N*-acetylneuraminyl lactosylceramide; MAPK, mitogen-activated protein kinase; PAF, platelet-activating factor; PAPC, 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP2, phosphatidylinositol-4,5-bisphosphate; PLA2, phospholipase A2; PS, phosphatidylserine; SMase, sphingomyelinase.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table, one figure, and a Methods section.

Recent studies have revealed that the activity of cPLA2α is modulated by sphingolipids such as ceramide, ceramide-1-phosphate, and sphingosine. Although ceramide is reported to activate cPLA2α by interacting with the C2 domain in vitro (9), the effects of ceramides including cell-permeable ceramides on cPLA2α activity and the release of AA in cells are controversial, with both stimulation and inhibition reported (10, 11). Ceramide is metabolized by various enzymes including ceramidase producing sphingosine, ceramide kinase producing ceramide-1-phosphate (C1P), and SM synthase producing SM, etc (12, 13). Our previous report showed that sphingosine, which inhibits the release of AA in cells, is a direct inhibitor of cPLA2α in vitro (14). We also found that C1P is a direct activator of cPLA2α via the C2 domain (15), as have several other reports (16, 17). Although, in previous studies, the mechanisms regulating cPLA2α activity and AA release were investigated by exogenous adding sphingolipids and in vitro analyses, little is known about the role of endogenous sphingolipids in these regulatory functions.

Here, we examined changes in the release of AA and activity of cPLA2α in sphingolipid-deficient cells, the chinese hamster ovary (CHO)-K1-derived mutant cell lines LY-B and LY-A. The LY-B strain has a defect in the LCB1 subunit of serine palmitoyltransferase and is therefore incapable of de novo synthesis of any sphingolipid species (18). The LY-A strain has a missense mutation in the ceramide transfer protein CERT and is defective in de novo synthesis of SM (19). We found that cellular levels of sphingolipids, especially SM, regulate the cPLA2α-dependent release of AA. In addition, this study showed for the first time that SM disturbs the binding to glycerophospholipids, and so reduces the enzymatic activity of cPLA2α.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK); 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine (48 mCi/mmol, 1776 MBq/mmol) from Perkin Elmer (Boston, MA); bovine SM, D-*erythro*-sphingosine, desipramine, imipramine, and amitriptyline from Sigma (St. Louis, MO); A23187 from Calbiochem (La Jolla, CA); platelet-activating factor (PAF) and phosphatidylinositol-4,5-bisphosphate (PIP2) from Cayman (Ann Arbor, MI); U0126 from Promega (Woods Hallow, WI); Nutridoma-SP from Roche (Basel, Swizerland); bovine phosphatidylserine (PS) from Matreya (Pleasant Gap, PA); and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC) and 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE) from Avanti Polar Lipids (Alabaster, AL). Pyrrophenone was generously provided by Dr. K. Hanasaki (Shionogi. Co. Ltd, Osaka, Japan).

Cells and cell cultures

The CHO-K1-derived mutant cell lines, LY-A and LY-B, and their complemented derivatives, LY-A/hCERT and LY-B/cLCB1, were established in Dr. K. Hanada's laboratory. Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate was used as a normal culture medium (Normal medium). Nutridoma medium (F-12 medium containing 1% Nutridoma-SP and 0.1% FBS) was used as a sphingolipid-deficient culture medium. The CHO-W11A cell line stably expresses the guinea pig PAF receptor (20). All CHO cells were maintained in Normal medium at 37°C and 5% CO2. The human embryonic kidney (HEK) 293T cell line was cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO2.

AA release assay

The cells were seeded onto 24-well culture plates at a density of 1×10^4 cells / well in Normal medium. For the depletion of sphingolipids, the medium was removed and the cells were cultured in Nutridoma medium for 30 h. The cells were then labeled by incubation for 18 h in 0.5 ml of Ham's F12 medium containing 33 nCi [^{3}H]AA and 0.1% fatty acid-free BSA. For the accumulation of SM, the cells were cultured in Normal medium containing acid sphingomyelinase (SMase) inhibitor for 30 h, then labeled by incubation for 18 h in 0.5 ml of Normal medium containing [^{3}H]AA and acid SMase inhibitor. The cells were washed and stimulated with reagents in DMEM containing 0.1% BSA and 10 mM HEPES (pH7.4) at 37°C. The radioactivity of supernatants and cell lysates (in 1% Triton X-100) was measured by liquid scintillation counting. The amount of radioactivity released into the supernatant was expressed as a percentage of the total amount of radioactivity incorporated.

Plasmid construction, transfection, and confocal microscopy

The plasmid for a chimeric protein containing enhanced green fluorescent protein (GFP) at the N-terminus of cPLA2α (GFP-cPLA2α) was prepared as described previously (20). For GFP-cPLA2α expression, cells were seeded at a density of 2×10^5 cells / 60-mm dish and transiently transfected with 2 μg of the expression vector with LipofectAMINE PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 3 h of incubation, transfected cells were seeded on coverslips (12 mm in diameter) of glass-bottomed dishes (IWAKI, Japan) at a density of 1×10^4. The cells were then cultured in Normal medium for 18 h. After another 48 h of incubation in Nutridoma medium, the culture medium was replaced and the cells were washed with HBSS buffer containing 10 mM HEPES (pH7.4) and 0.1% BSA and stimulated with reagents in the same buffer. Fluorescence images were taken with a FLUOVIEW confocal laser scanning microscope system (Olympus, Japan).

PLA2 assay

HEK293T cells were transfected with an expression vector for human cPLA2α (pcDNA4/HisMax A-human cPLA2α) using LiopofectAMINE PLUS. Following transfection, the cells were homogenized with a Potter homogenizer in lysis buffer (0.34 M sucrose, 100 μM dithiothreitol, 10 mM HEPES (pH 7.4), 0.2% CHAPS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride). PLA2 activity was measured using mixed micelles each containing 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine (PAPC), PS, PE, SM, and Triton X-100 as a substrate. The mixed lipids in the solvent (chloroform / methanol = 1:1) were dried under nitrogen. A solution of 0.00125% TritonX-100 was added and the lipid was vortexed vigorously for 2 min, then it was sonicated for 5 min in the water bath. When the liposome was generated separately, every lipid was sonicated separately. The assay buffer contained 100 mM HEPES (pH7.4), 1 mg/ml BSA, 4 mM CaCl2, and 10 mM dithiothreitol. The reaction was started by the addition of enzyme sources, and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated with Dole's reagent, and silica gel powder was used to recover free fatty acid in an n-heptane layer. Radioactivity was measured with a liquid scintillation counter.

Western blot analyses

Cells were scraped and sonicated with ice-cold buffer containing 20 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride. Soluble and insoluble fractions were then separated by centrifugation at 17,400 *g* for 30 min at 4°C. Protein concentrations were determined with the Bio-Rad Protein Assay. Laemmli electrophoresis sample buffer (5×) was added to the soluble fractions, and SDS-PAGE was performed using 30 μg of lysate. After electrophoresis, proteins were electroblotted onto polyvinyldifluoride membranes. cPLA2α and β-tubulin were detected using an anti-cPLA2α monoclonal antibody (Santa Cruz Biotechnology) and an anti-β-tubulin antibody (Sigma), respectively, followed by an anti-mouse horseradish peroxidase antibody (Amersham). Phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and ERK1/2 were detected using an anti-phospho-Thr^{202}/Thr^{204}-ERK1/2 antibody (Cell Signaling) and a mixture of anti-ERK-1 and anti-ERK-2 antibodies (C-16 and C14, Santa Cruz Biotech), respectively, followed an anti-rabbit IgG horseradish peroxidase antibody (Amersham). The immunoreactive bands were visualized by enhanced chemiluminescence.

Lipid extraction and TLC

Cells were rinsed three times with PBS buffer. Lipids were extracted by the Bligh and Dyer method (21). The organic phase was dried under nitrogen. Dried samples were dissolved in 10 μl of chloroform: methanol (1:1) and analyzed on Silica Gel 60 TLC plates (Merck) using chloroform: methanol: water (65:25:4). The plates were dried and sprayed with 47% sulfuric acid. They were then heated at 150°C on a hot plate and imaged using Fuji film LAS1000.

Lipid-protein overlay assay

Lipids were spotted onto a Hybond C membrane (Amersham Biosciences) and dried under nitrogen. The membrane was rewet in water and blocked for 1 h in 2% BSA/TBS-T. It was then exposed overnight at 4°C to lysate (0.5 μg/μl protein) from HEK293T cells transiently transfected with the expression vector for cPLA2α. The membrane was washed with TBS-T and exposed to a 1:1000 dilution of anti-cPLA2α monoclonal antibody in 2% BSA/TBS-T for 1 h at room temperature. It was washed with TBS-T and exposed to a 1:3000 dilution of anti-mouse IgG horseradish peroxidase antibody in 2% BSA/TBS-T for 1 h at room temperature. The immunoreactive spots were visualized by enhanced chemiluminescence.

Statistics

Values are the means ± SEM for three to four independent experiments performed in triplicate. In some cases, data are shown as the means ± SD of two or three determinations in a typical representative experiment. In the case of multiple comparisons, the significance of differences was determined using a one-way ANOVA by Dunnett's or Tukey's test. For pairwise comparisons, Student's two-tailed *t*-test was used. P values < 0.05 were considered to be significant.

RESULTS

Enhancement of cPLA2α-dependent AA release in sphingolipid-deficient cells

Strain LY-B, a CHO-K1 cell mutant defective in the LCB1 subunit of serine palmitoyltransferase, is unable to synthesize any sphingolipid species de novo. As shown in Fig. 1A, when LY-B cells were cultured in a sphingolipid-deficient medium (Nutridoma medium) for 30 h and then in Ham's F-12 medium containing 0.1% BSA for 18 h, the SM level was ∼30% of the level in wild-type CHO-K1 cells as previously reported (22). Also, when LY-B cells were cultured in Normal medium instead of Nutridoma medium, the SM levels was ∼85% of the level in CHO-K1 cells (data not shown). The reduced contents of SM in LY-B cells were reversed to the wild-type level by genetic complementation of the LY-B strain with hamster LCB1 cDNA (LY-B/cLCB1 strain). We confirmed that the cultivation of these cells in the sphingolipid-deficient culture conditions did not cause cytotoxicity during the test period (data not shown). Using this culture system, we determined whether the reduction in the cellular sphingolipid level affected the release of AA from cells. Because the cPLA2α-dependent release of AA from cells was enhanced by an increase in $[Ca^{2+}]i$, we used the calcium ionophore,

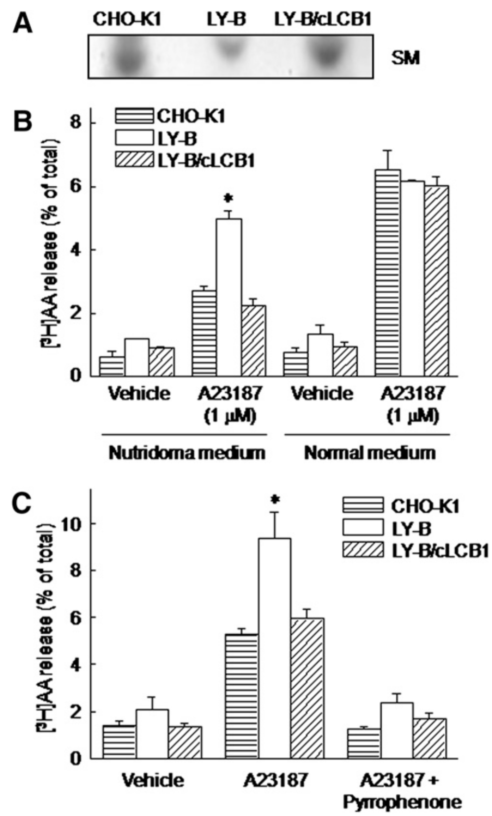

Fig. 1. Enhancement of cPLA2α-dependent AA release in sphingolipid-deficient cells. Cells were cultured in Nutridoma medium at 37°C for 30 h. They were then incubated for 18 h in Ham's F-12 medium containing 0.1% BSA. A: After the cells were washed, lipids were extracted and separated by TLC. B: Cells were cultured in Normal medium or Nutridoma medium at 37°C for 30 h. They were then labeled by incubation for 18 h in Ham's F-12 medium containing $[^3H]$AA and 0.1% BSA. The labeled cells were stimulated with 1 μM A23187 for 30 min at 37°C. C: Cells were prepared as above. $[^3H]$AA was further added with 0.1% BSA for 18 h. The labeled cells were pretreated for 30 min with or without 2 μM pyrrophenone. The cells were washed and then stimulated with vehicle, 1 μM A23187, and 2 μM pyrrophenone for 30 min at 37°C. The data shown are the mean ± SEM. for three experiments. *$p < 0.05$, significantly different from the values in LY-B/cLCB1 cells.

A23187, as a stimulant. The A23187-induced release of AA from LY-B cells cultured in Nutridoma medium was 2-fold the wild-type level, whereas there was no significant difference in the release of AA between CHO-K1 and LY-B/cLCB1 cells (Fig. 1B). When cells were cultured in Normal medium that contained 10% FBS, there was no appreciable difference in the release of AA induced by A23187 among LY-B, LY-B/cLCB1, and CHO-K1 cells. In addition, the enhanced release from sphingolipid-deficient cells was almost completely inhibited by treatment with pyrrophenone, a selective inhibitor of cPLA2α (Fig. 1C), indicating that sphingolipid deficiency enhances the cPLA2α-dependent release of AA in cells.

Enhancement of AA release is restored by adding exogenous sphingosine in LY-B cells

The enhanced release of AA from LY-B cells in response to A23187 may be accompanied by: *1*) an elevation of the level of cPLA2α, *2*) induction of cPLA2α translocation, *3*) phosphorylation, or *4*) the interaction of lipids with cPLA2α. These possibilities were tested in LY-B and LY-B/cLCB1 cells. **Figure 2A** shows that there is no difference in the expression of cPLA2α between LY-B and LY-B/cLCB1 cells. To examine the A23187-induced translocation of cPLA2α, we monitored the localization of GFP-cPLA2α in living cells by confocal laser fluorescence microscopy. GFP-cPLA2α was almost homogeneously present in the cytosol in LY-B and LY-B/cLCB1 cells transiently expressing GFP-cPLA2α, in the resting state. Stimulation of these cells with A23187 triggered the translocation of cPLA2α to the perinuclear region within 1 min (Fig. 2B), and the GFP-cPLA2α fluorescence was retained for over 10 min (data not shown). Thus, the behavior of GFP-cPLA2α in response to A23187 in these cells was similar. We next determined the effect of A23187 on the phosphorylation of ERK1/2, which phosphorylate and activate cPLA2α, by Western blotting. Treatment with A23187 caused the phosphorylation of ERK1/2 within 20 min in LY-B and LY-B/cLCB1 cells, and the responses were similar between these cells (Fig. 2C). Treatment with U0126, an inhibitor of the ERK pathway, reduced the A23187-induced release of AA from both LY-B and LY-B/cLCB1 cells in a similar degree. However, the release from LY-B cells remained significant compared with that from LY-B/cLCB1 cells (Fig. 2D).

It has been reported that the addition of D-*erythro*-sphingosine restored the amounts of SM and *N*-acetylneuraminyl lactosylceramide (G_{M3}) in LY-B cells to wild-type levels without affecting other lipids such as ceramide and glucosylceramide (22). We confirmed that the amount of SM in LY-B cells was restored by supplementation of the culture medium with 1 μM D-*erythro*-sphingosine (**Fig. 3A**). The enhanced release of AA from LY-B cells was suppressed to the LY-B/cLCB1 level when cells were cultured with D-*erythro*-sphingosine (Fig. 3B), indicating that a deficiency of SM or G_{M3} may contribute to regulation of the cPLA2α-dependent release of AA in LY-B cells.

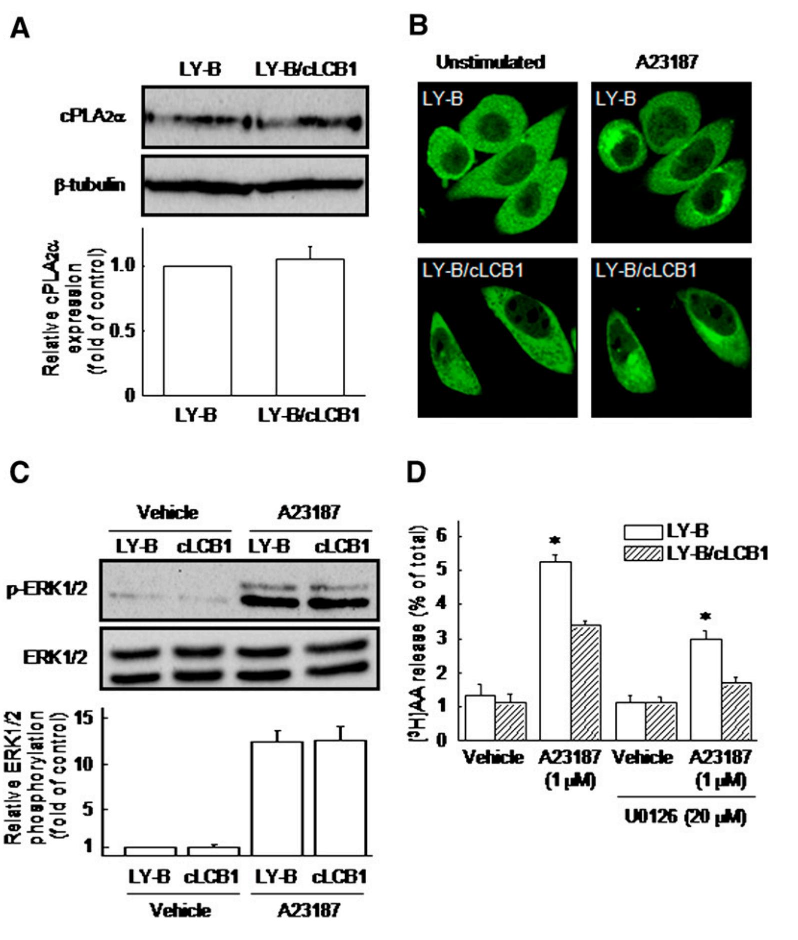

Fig. 2. Effect of sphingolipid-deficiency on the protein levels, translocation and phosphorylation of cPLA2α in LY-B cells. Cells were cultured in Nutridoma medium at 37°C for 30 h, then incubated in Ham's F-12 medium containing 0.1% BSA for 18 h. A: The protein levels of cPLA2α in cell lysates were determined using anti-cPLA2α antibody. Upper panels, immunoblotting with antibodies against cPLA2α and β-tubulin. The histograms represent ratio of cPLA2α to β-tubulin as assessed with pooled densitometric data (mean ± SD) from three independent experiments. Data were normalized to ratio of cPLA2α to β-tubulin of LY-B cells. B: Cells transiently transfected with an expression vector for GFP-cPLA2α were stimulated with 1 μM A23187 for 2 min. C: Cells were prepared as above. The cells were stimulated with 1 μM A23187 for 20 min at 37°C and were subjected to immunoblot analysis. Upper panels, immunoblotting with antibodies against phospho-ERK1/2 (p-ERK1/2) and ERK1/2. The histograms represent ratio of p-ERK1/2 to total ERK1/2 as assessed with pooled densitometric data (mean ± SD) from three independent experiments. Data were normalized to ratio of p-ERK1/2 to total ERK1/2 of vehicle-treated LY-B cells. D: Cells were prepared as above. [^{3}H]AA was further added with 0.1% BSA for 18 h. The labeled cells were incubated for 30 min with or without 20 μM U0126 and stimulated with 1 μM A23187 for 30 min at 37°C. The data shown are the mean ± SEM for three experiments. *$p < 0.05$, significantly different from the values in the absence of A23187. In A–C, data are representative of three independent experiments.

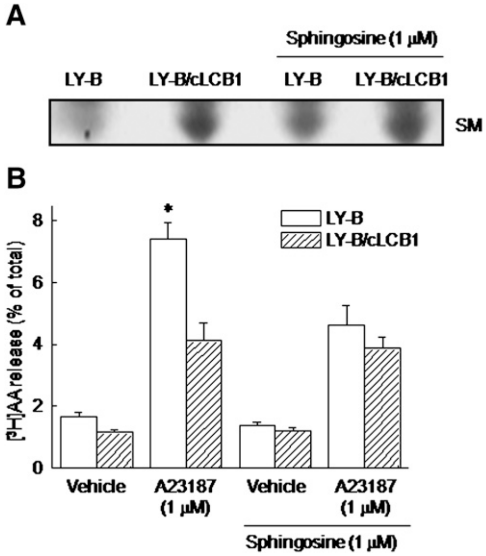

Fig. 3. Enhancement of AA release is reversed by adding sphingosine in LY-B cells. Cells were cultured in Nutridoma medium with or without 1 μM D-*erythro*-sphingosine at 37°C for 30 h, followed by Ham's F-12 medium containing 0.1% BSA with or without 1 μM D-*erythro*-sphingosine for 18 h. A: After the cells were washed, lipids were extracted and separated by TLC. B: Cells were prepared as above. [^{3}H]AA was further added for 18 h. After the cells were washed, the amount of AA released from the cells stimulated with 1 μM A23187 was measured. The data shown are the mean ± SEM for three experiments. *$p < 0.05$, significantly different from the values in LY-B/cLCB1 cells.

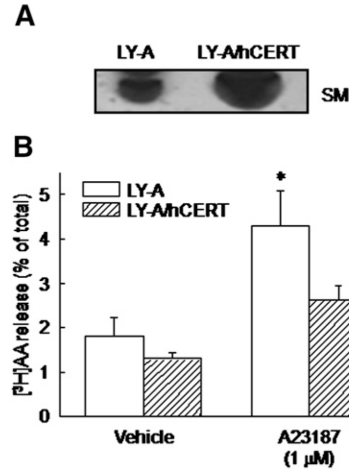

Fig. 4. Enhancement of AA release in LY-A cells. Cells were cultured in Nutridoma medium at 37°C for 30 h, then incubated in Ham's F-12 medium containing 0.1% BSA for 18 h. A: After the cells were washed, lipids were extracted and separated by TLC. B: Cells were prepared as above. [^{3}H]AA was further added with 0.1% BSA for 18 h. The labeled cells were washed and stimulated with 1 μM A23187 for 30 min at 37°C. The data shown are the mean ± SEM for three experiments. *$p < 0.05$, significantly different from the values in LY-A/hCERT cells.

SM affects cPLA2α-dependent AA release in cells

To examine the effect of cellular SM content on the cPLA2α-dependent release of AA, we used a CHO-K1 cell mutant, strain LY-A, defective in the de novo synthesis of SM because of a mutation in the ceramide transfer protein CERT, as well as LY-A cells stably transformed with human CERT cDNA (LY-A/hCERT strain). Indeed, when cells were cultured in Nutridoma medium for 30 h and then in Ham's F-12 medium containing 0.1% BSA for 18 h, the SM level was lower in LY-A cells than in LY-A/hCERT cells (**Fig. 4A**). Using this culture system, we determined whether a reduction in the cellular SM level affected the release of AA in response to A23187. As shown in Fig. 4B, the amount of AA released by A23187 was significantly greater from LY-A cells than LY-A/hCERT cells.

We next examined the effects of acid SMase inhibitors acting to increase the cellular SM level on the release of AA using CHO cell lines stably expressing the PAF receptor (CHO-W11A cells). Stimulation of the receptor results in the activation of MAPK and an increase in [Ca^{2+}]i, thereby activating cPLA2α (20). We determined the level of SM in CHO-W11A cells cultured for 48 h in Normal medium containing an acid SMase inhibitor, desipramine, imipramine, or amitriptyline. **Figure 5A** and B show that levels of SM were much higher in CHO-W11A cells treated with desipramine or amitriptyline than in the control cells. While that response was weak in the imipramine-treated cells. Treatment with desipramine or amitriptyline decreased the A23187- or PAF-induced release of AA significantly, whereas treatment with imipramine decreased the release slightly but significantly (Fig. 5C). Interestingly, there was a strong inverse correlation between the cellular content of SM and the A23187- or PAF-induced release of AA in CHO-W11A cells (Fig. 5D). There was no difference in the expression of cPLA2α between the inhibitor-treated cells and the untreated cells (data not shown). In addition, the phosphorylation of cPLA2α and ERK1/2, and the translocation of cPLA2α, in response to PAF, were also similar in each of the cells (data not shown). These results suggest that cellular SM affects the cPLA2α-dependent release of AA in cells.

SM disturbs the binding of cPLA2α to glycerophospholipids and reduces the enzymatic activity in vitro

To examine whether SM directly reduced the enzymatic activity of cPLA2α, liposomes containing PAPC and SM were prepared and tested for cPLA2α activity in vitro. The cPLA2α activity in the presence of PAPC liposomes alone was about 2000 dpm (**Fig. 6A**). The cPLA2α activity in the liposomes, which combined PAPC with SM at a molar ratio of 1:1, was markedly attenuated. The activity in the PAPC liposomes with PS was slightly lower but that in the liposomes with PE was the same the control level. Next, we examined whether SM decreased the activity of cPLA2α by binding to the enzyme. The SM vesicles generated separately from PAPC vesicles did not reduce cPLA2α activity, suggesting a possibility that SM does not bind to cPLA2α (Fig. 6B).

We determined whether SM bound directly to cPLA2α using a lipid-protein overlay assay. cPLA2α was found not bind to SM when 100 nmol was bound to the membrane (**Fig. 7A**). On the other hand, cPLA2α was found to bind

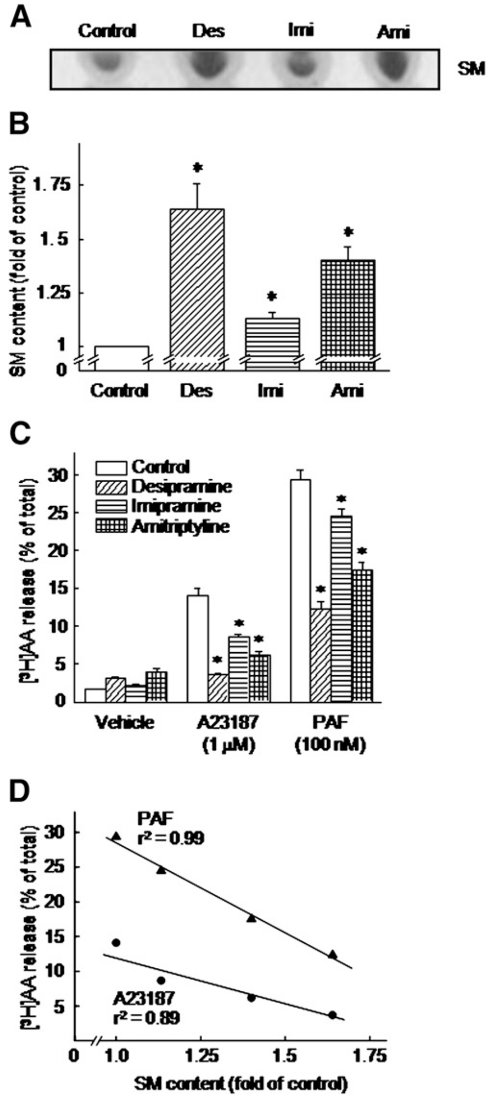

Fig. 5. Effect of SM accumulation on AA release in CHO-W11A cells. Cells were cultured for 30 h in Normal medium with or without acid SMase inhibitors, 30 μM desipramine, 10 μM imipramine, or 30 μM amitriptiline. A: The cells were further incubated for 18 h in Normal medium with or without acid SMase inhibitors. After the cells were washed, lipids were extracted and separated by TLC. B: The amounts of SM shown in A were quantified using software for densitometric analyses. C: Cells were prepared as above. They were then labeled through incubation for 18 h in Normal medium containing [^{3}H]AA supplemented with or without acid SMase inhibitors. The labeled cells were washed and stimulated with 1 μM A23187 or 100 nM PAF for 30 min at 37°C. The data shown are the mean ± SEM for three experiments. *$p < 0.05$, significantly different from the values in the absence of inhibitors. D: The amount of SM increased by the acid SMase inhibitor and the release of AA induced by A23187 or PAF are shown on the *x* axis and *y* axis, respectively. Data on the amount of SM are presented as percentages of the control value. Data from three independent experiments were used for the analysis.

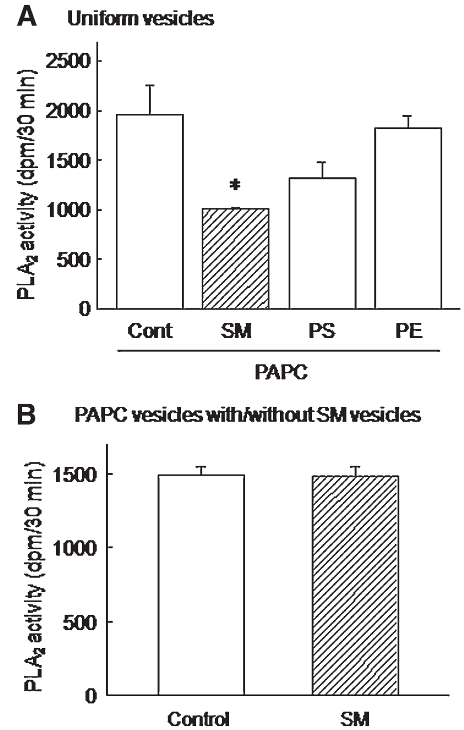

Fig. 6. SM reduces the activity of cPLA2α in vitro. PLA2 activity in the cytosolic fraction from HEK293T cells expressing human cPLA2α was measured as described in Materials and Methods. A: Liposomes containing 2 μM of labeled PAPC and phospholipids, SM, PS or PE respectively, at a molar ratio of 1:1 were prepared by sonication. B: The liposomes containing 2 μM of labeled PAPC and the 2 μM SM vesicles were generated separately by sonication. Then, sources of enzyme were added and incubated for 30 min at 37°C. The data are the mean ± SEM for three experiments.

to as little as 100 nmol of PS, and a binding of cPLA2α to PIP2 was observed at 5 nmol. cPLA2α did not bind to PC or PE at 100 nmol under the conditions. We next examined whether SM disturbs the binding of cPLA2α to glycerophospholipids. As shown in Fig. 7B, cPLA2α did not bind to PS or PIP2 when SM was bound to the membrane. Thus, SM disturbs the binding to glycerophospholipids and reduces the enzymatic activity of cPLA2α.

Then, we predicted the possible interaction mode of cPLA2α with SM, PIP2, PS, PC, and PE by using cPLA2α/2-(N-morpholino) ethanesulfonic acid complex (PDB; 1CJY) as a template structure. According to the result of molecular modeling as shown in supplementary Fig. I, cPLA2α interacts with SM, PIP2, PS, PC or PE. As shown in supplementary Table I, the interaction energy (IE) of cPLA2α with SM, PIP2, PS, PC and PE were consistent with the results of binding assay (Fig. 7).

DISCUSSION

The activity of cPLA2α is known to be regulated by sphingolipids such as ceramide, C1P, and sphingosine (9, 14–17, 23). Although previous reports including one from our laboratory have shown by adding exogenous sphingolipids and in vitro analyses that sphingolipids modify the activity of cPLA2α and release of AA, little is known about whether altered levels of endogenous sphingolipids affect cPLA2α-dependent responses. In the present study, we found that a deficiency in sphingolipids enhanced the cPLA2α-dependent release of AA in LY-B cells (Fig. 1).

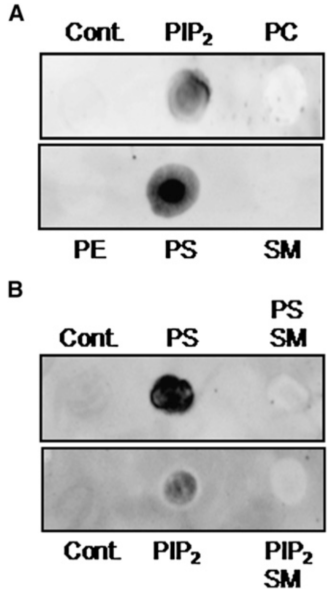

Fig. 7. SM disturbs the binding of cPLA2α to glycerophospholipids. The binding of cPLA2α to various glycerophospholipids was examined using the lipid-protein overlay assay as described in Materials and Methods. A: The binding of cPLA2α to glycerophospholipids. Several lipids, 100 nmol PC, 100 nmol PS, 100 nmol PE, 5 nmol PIP2, and 100 nmol SM, were spotted onto a Hybond C membrane. B: The effect of SM on the binding of cPLA2α to PS or PIP2. The mixed lipids in the solvent (chloroform / methanol = 1:1) were spotted onto a Hybond C membrane. The membrane was exposed to enzyme sources overnight at 4°C. Three independent experiments gave similar results.

This alteration was restored by cultivating in the 10% FBS-containing medium (Fig. 1B) or by adding sphingosine (Fig. 3), indicating that the cellular sphingolipid level is a critical modulator of cPLA2α-dependent AA release. Although sphingosine is metabolized producing several sphingolipids, cellular levels of SM and G_{M3} are rescued by the addition of sphingosine to the culture medium in LY-B cells (22). In LY-A cells having a lower level of SM, the amount of AA released by A23187 was much greater than that released from LY-A/hCERT cells (Fig. 4). In addition, when CHO-W11A cells were cultured in medium containing an acid SMase inhibitor and the SM level was increased, there was a strong inverse correlation between the level of SM and the release of AA (Fig. 5). Thus, the level of cellular SM plays an important role in the regulation of the cPLA2α-dependent release of AA.

Stimulation with A23187 or PAF activates cPLA2α via Ca^{2+} and phosphorylation signals. The Ca^{2+}-induced translocation of cPLA2α from the cytosol to the perinuclear region and subsequent constitutive binding to the membrane are important steps in regulating AA release. Although the treatment of LY-B cells with A23187 triggered the translocation of GFP-cPLA2α, there was no appreciable difference in response between LY-B and LY-B/cLCB1 cells (Fig. 2B). Phosphorylation of cPLA2α on Ser^{505} by ERK1/2 increases its intrinsic enzymatic activity. It has been reported that decreased sphingolipid levels in LY-B cells caused the phosphorylation of ERK1/2 via unknown mechanisms (24). In the present study, however, phosphorylated ERK1/2 was not observed in resting LY-B cells (Fig. 2C). In addition, there was no difference in the A23187-induced phosphorylation of ERK1/2 between LY-B and LY-B/cLCB1 cells. This discrepancy is probably due to culture in our use of a serum-free medium containing 0.1% BSA for 18 h before the stimulation of cells with vehicle or A23187. When the acid SMase inhibitor-treated CHO-W11A cells and the untreated cells were stimulated with PAF, levels of the translocation of GFP-cPLA2α and phosphorylation of cPLA2α on Ser^{505} were similar between the cell types (data not shown). In addition, there was no difference in the expression of cPLA2α between acid SMase-treated and untreated cells (data not shown). These results suggest that SM modifies the release of AA by interacting with cPLA2α, not by inducing the translocation and phosphorylation of the enzyme.

Various investigators have demonstrated that SM inhibits several phospholipases. Subbaiah and Liu (25) reported that lecithin-cholesterol acyltransferase, a specialized phospholipase A responsible for the esterification of cholesterol in plasma, was inhibited by SM. The activities of lipoprotein lipase and secretory PLA2 have also been shown to be inhibited by SM in vitro (26–30). Dawson et al. (31) reported that diacylglycerol-stimulated intracellular phospholipases were strongly inhibited by SM. Although there are several reports of the inhibition of various phospholipases by SM, the exact mechanisms involved are unknown in most cases. The present study also found that the incorporation of SM into PC liposomes induced inhibition of cPLA2α activity in vitro (Fig. 6A). However, the SM vesicles generated separately from the PC vesicles did not affect the activity of cPLA2α (Fig. 6B), indicating that SM did not inhibit the activity by binding to the enzyme. cPLA2α preferentially binds to PC in the presence of micromolar concentrations of Ca^{2+} (32). The structure of SM bears a strong resemblance to that of PC. Both have a phosphocholine head group and two long-chain hydrophobic residues. The close structural similarity with PC enables SM not only to be an integral part of the bilayer, but also to interact strongly with membrane PC. Unlike PC, however, SM does not have the easily hydrolysable acyl ester linkage. Because SM is a nonhydrolysable structural analog of PC, it may competitively inhibit cPLA2α. Also, because SM decreases the fluidity of membranes and increases packing density in the hydrophobic core and changes the water structure at the interface (33), it may decrease cPLA2α binding.

The liberation of AA from membrane phospholipids by activation of cPLA2α is known to occur mainly in the perinuclear region such as the Golgi and the ER. SM is predominantly present in the outer leaflet of the plasma membrane and the inner leaflet of the Golgi and endosomes but not in the ER (34). Although it is known that about 15% of the total phospholipids are SM in the Golgi (34), the ratio of the SM contents between the inner leaflet and the cytosolic leaflet of the Golgi is unknown. Our present study may suggest that SM in the cytosolic leaflet of the Golgi interferes with the binding of cPLA2α to the membrane.

Anionic phospholipids, particularly C1P, PIP2, and phosphatidylmethanol, promote the binding to lipid vesicles and increase the activity of cPLA2α (3, 4). C1P enhances the activity of cPLA2α by increasing the resident time of the enzyme to the membrane through electrostatic interactions with cationic residues in the C2 domain (17). PIP2 activates the enzyme by increasing catalytic efficiency through increased penetration of the membrane (35). The binding site of PIP2 includes four lysine residues, which are located in the highly basic region of the catalytic domain on the side close to the membrane (36, 37). In the present study, we found that SM disturbed the binding of cPLA2α to PIP2 (Fig. 7B). Thus, SM may decrease the activity of cPLA2α by inhibiting its binding to PIP2, thereby protecting PC and other glycerophospholipids from excessive hydrolysis. The effect of SM on the interaction between cPLA2α and other anionic phospholipids such as C1P and PE should be studied. However, we could not detect a marked binding of cPLA2α with C1P, like SM, under our conditions used in the present study (data not shown). Thus, we could not exclude the possibility about direct binding with SM, which resulting an inhibition of the enzyme binding with PS and PIP2. The interaction between cPLA2α and the lipids including C1P, PE and SM should be studied by using more optimized conditions and/or other methods in future.

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