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Lactosylceramide Interacts with and Activates Cytosolic Phospholipase $A_2\alpha$*

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Background: LacCer is known to regulate PLA_2 activity in cells, but the precise mechanisms have not been elucidated.
Results: LacCer binds to $cPLA_2\alpha$ and increases its enzymatic activity.
Conclusion: LacCer is identified as a novel and direct activator of $cPLA_2\alpha$.
Significance: This research provides new insights into the regulatory mechanisms of $cPLA_2\alpha$ and the physiological functions of LacCer as a signaling molecule.

Lactosylceramide (LacCer) is a member of the glycosphingolipid family and is known to be a bioactive lipid in various cell physiological processes. However, the direct targets of LacCer and cellular events mediated by LacCer are largely unknown. In this study, we examined the effect of LacCer on the release of arachidonic acid (AA) and the activity of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$). In CHO-W11A cells, treatment with 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), an inhibitor of glucosylceramide synthase, reduced the glycosphingolipid level, and the release of AA induced by A23187 or platelet-activating factor was inhibited. The addition of LacCer reversed the PPMP effect on the stimulus-induced AA release. Exogenous LacCer stimulated the release of AA, which was decreased by treatment with an inhibitor of $cPLA_2\alpha$ or silencing of the enzyme. Treatment of CHO-W11A cells with LacCer induced the translocation of full-length $cPLA_2\alpha$ and its C2 domain from the cytosol to the Golgi apparatus. LacCer also induced the translocation of the D43N mutant of $cPLA_2\alpha$. Treatment of L929 cells with TNF-α induced LacCer generation and mediated the translocation of $cPLA_2\alpha$ and AA release, which was attenuated by treatment with PPMP. *In vitro* studies were then conducted to test whether LacCer interacts directly with $cPLA_2\alpha$. Phosphatidylcholine vesicles containing LacCer increased $cPLA_2\alpha$ activity. LacCer bound to $cPLA_2\alpha$ and its C2 domain in a Ca^{2+}-independent manner. Thus, we propose that LacCer is a direct activator of $cPLA_2\alpha$.

The production of the eicosanoids, proinflammatory lipid mediators that include prostaglandins, thromboxanes, and leukotrienes, is dependent on the availability of their precursor, free arachidonic acid (AA).[3] The release of AA from the *sn*-2 position of glycerophospholipids is a highly regulated process that occurs in response to a wide variety of stimuli, such as cytokines, growth factors, and neurotransmitters (1, 2). The 85-kDa group IVA cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) hydrolyzes glycerophospholipids at the *sn*-2 position to liberate AA. $cPLA_2\alpha$ is regulated mainly by binding of Ca^{2+}, phosphorylation at serine residues, and interaction with lipids. The binding of Ca^{2+} to the C2 domain of $cPLA_2\alpha$ triggers translocation of $cPLA_2\alpha$ from the cytosol to the perinuclear region, including the Golgi apparatus, endoplasmic reticulum, and nuclear envelope (3). The phosphorylation of Ser^{505} by MAPK appears to increase enzymatic activity (4). Anionic phospholipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2) and ceramide 1-phosphate (C1P), promote binding to lipid vesicles and increase the activity of $cPLA_2\alpha$ (1, 2). PIP_2 activates the enzyme by increasing catalytic efficiency through increased penetration of the membrane (5). C1P enhances the activity of $cPLA_2\alpha$ by increasing the resident time of the enzyme in the membrane through electrostatic interactions with cationic residues in the C2 domain (6).

Glycosphingolipids were largely known as components of the cell membrane. However, recently, these lipids have been accorded functional roles in multiple signal transduction pathways that lead to critical phenotypic changes in cells, such as cell proliferation, adhesion, and apoptosis (7–9). Lactosylceramide (LacCer) is a member of the glycosphingolipid family and plays pivotal roles as a precursor in the biosynthesis of complex glycosphingolipids. LacCer has also been shown to be a bioactive lipid involved in various cell signaling cascades. It has been reported that LacCer stimulates PECAM-1 (platelet endothelial cell adhesion molecule-1) in the adhesion and diapedesis of monocytes/lymphocytes via Ca^{2+}-independent PLA_2 and $cPLA_2\alpha$ (10). However, the role of LacCer in the activity of PLA_2 and AA release remains unclear.

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[3] The abbreviations used are: AA, arachidonic acid; $cPLA_2\alpha$, cytosolic phospholipase $A_2\alpha$; PIP_2, phosphatidylinositol 4,5-bisphosphate; C1P, ceramide 1-phosphate; LacCer, lactosylceramide; PAPC, 1-palmitoyl-2-[^{14}C]arachidonylphosphatidylcholine; PAF, platelet-activating factor; PPMP, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; GlcCer, glucosylceramide.

Previously, we examined the role of sphingolipids in the activity of cPLA$_2\alpha$ and the release of AA and demonstrated that sphingomyelin disturbs the binding to glycerophospholipids and so reduces the activity of cPLA$_2\alpha$ (11). We also found that C1P is a direct activator of cPLA$_2\alpha$ via the C2 domain (12). Sphingosine was shown to be a direct inhibitor of cPLA$_2\alpha$ (13). Thus, several sphingolipids regulate the activity of cPLA$_2\alpha$, but the role of glycosphingolipids in the activity of the enzyme is unknown. In this study, we investigated the mechanisms by which glycosphingolipids, especially LacCer, activate cPLA$_2\alpha$. LacCer was found to bind cPLA$_2\alpha$ directly and to be an inducer of cPLA$_2\alpha$ activation *in vitro* and in cells. Furthermore, we show that the activation of cPLA$_2\alpha$ in response to TNF-α was mediated through the generation of LacCer.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham Biosciences; 1-palmitoyl-2-[^{14}C]arachidonylphosphatidylcholine (PAPC; 48 mCi/mmol, 1776 MBq/mmol) from PerkinElmer Life Sciences; A23187 from Calbiochem; platelet-activating factor (PAF) and sphingosine 1-phosphate from Cayman Chemical (Ann Arbor, MI); U0126 from Promega; 1-phenyl-2-palmitoyl-amino-3-morpholino-1-propanol (PPMP), bovine glucosylceramide (GlcCer), bovine LacCer, and bovine GM1 from Matreya, LLC (Pleasant Gap, PA); C_{16}-C1P, 1-palmitoyl-2-oleoylphosphatidylinositol, 1-palmitoyl-2-oleoylphosphatidylcholine, and 1-palmitoyl-2-oleoylphosphatidylethanolamine from Avanti Polar Lipids (Alabaster, AL); BODIPY-C_5-LacCer and Oregon Green 488 BAPTA-1/AM from Invitrogen; and TNF-α from PeproTech (Rocky Hill, NJ). Pyrrophenone was generously provided by Dr. Kohji Hanasaki (Shionogi Co. Ltd., Osaka, Japan).

Cells and Cell Cultures—The CHO-W11A cell line stably expressing the guinea pig PAF receptor was cultured in Ham's F-12 medium supplemented with 10% FBS, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate at 37 °C and 5% CO_2. L929 mouse fibrosarcoma cells and a stable clone of L929 cells lacking cPLA$_2\alpha$ (L929-cPLA$_2\alpha$-shRNA, clone 49) established previously (14) were cultured in the same manner except for the use of DMEM containing 5% FBS instead of Ham's F-12 medium. The HEK293T cell line was cultured in DMEM containing 10% FBS.

AA Release Assay—Cells (2×10^4 cells) on 24-well plates were labeled by incubation for 18 h in 0.5 ml of medium containing 33 nCi of [^{3}H]AA and 0.1% BSA. The cells were washed and stimulated with reagents in medium containing 0.1% BSA and 10 mM HEPES (pH 7.4) for specific periods 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 is expressed as a percentage of the total amount of radioactivity incorporated.

Plasmid Construction, Transfection, and Confocal Microscopy—The plasmids for chimeric proteins containing GFP or DsRed at the N terminus of cPLA$_2\alpha$ (GFP-cPLA$_2\alpha$ and DsRed-cPLA$_2\alpha$), the C2 domain (GFP-C2), and the D43N mutant (GFP-D43N-cPLA$_2\alpha$) were kindly donated by Dr. Tetsuya Hirabayashi (The Tokyo Metropolitan Institute of Medical Science). For protein expression, cells were seeded at a density of 2×10^5 cells/60-mm dish and transiently transfected with 2 μg of expression vector with Lipofectamine PLUS (Invitrogen) 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) at a density of 1×10^4. After another 48 h of incubation, the culture medium was replaced, and the cells were washed with Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 0.1% BSA and stimulated with reagents in the same buffer. Fluorescent images were taken with a FluoView confocal laser scanning microscope system (Olympus).

PLA$_2$ Assay—HEK293T cells were transfected with an expression vector for human cPLA$_2\alpha$ (pcDNA4/HisMaxA-hcPLA$_2\alpha$) using Lipofectamine 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). PLA$_2$ activity was measured using mixed liposomes, each containing PAPC, LacCer, and Triton X-100 as a substrate. The mixed lipids in the solvent (1:1 chloroform/methanol) were dried under nitrogen. A solution of 0.00125% Triton X-100 was added, and the lipid was vortexed vigorously for 2 min and then sonicated for 5 min in a water bath. The assay buffer contained 100 mM HEPES (pH 7.4), 1 mg/ml BSA, 1 mM $CaCl_2$, and 10 mM dithiothreitol. The reaction was started by the addition of enzyme sources (12.5 μg), 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 Blotting—Cells were scraped and homogenized with ice-cold buffer containing 10 mM HEPES, 250 mM sucrose, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 1000 $\times$ g for 10 min. Soluble fractions were then centrifuged at 100,000 $\times$ *g* for 30 min, and equal amounts of proteins from membrane and cytosolic fractions were separated by 7.5% SDS-PAGE and electrotransferred onto polyvinyl difluoride membranes (Bio-Rad). cPLA$_2\alpha$ was detected using an anti-cPLA$_2\alpha$ monoclonal antibody (Santa Cruz Biotechnology) followed by an anti-mouse horseradish peroxidase antibody (Amersham Biosciences). The immunoreactive bands were visualized by enhanced chemiluminescence. Results were analyzed using a LAS-1000plus system equipped with Science Lab software (Fujifilm, Tokyo, Japan). The intensity of chemiluminescence was measured using NIH ImageJ software.

Measurement of Glycosphingolipids—CHO-W11A cells were washed three times with PBS. Lipids were extracted by the Bligh and Dyer method (15). The organic phase was then subjected to Iatrobead column chromatography. The mixture of glycosphingolipids was eluted with 9:1 acetone/methanol and dried under nitrogen. The lipids were spotted onto a Silica Gel 60 TLC plate. The plate was sprayed with 47% sulfuric acid and then heated at 150 °C on a hot plate and imaged using the LAS1000plus system.

RNA Interference—L929 cells were transfected with mouse GlcCer synthase siRNA (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX (Invitrogen) as recommended by the

manufacturer. Following 30 h of transfection, cells were labeled with [^{3}H]AA for 18 h and then washed and stimulated with TNF-α for 6 h.

Measurement of LacCer Synthesis—L929 cells were incubated in culture medium containing [^{14}C]serine for 18 h. The cells were washed and incubated with or without TNF-α for 2 h. The total lipid extract was then subjected to Iatrobead column chromatography. The mixture of glycosphingolipids was eluted with 9:1 acetone/methanol and dried under nitrogen. Glycosphingolipids were separated onto a Silica Gel 60 TLC plate using 100:42:6 chloroform/methanol/water. The TLC plate was dried, exposed to an imaging plate (BAS-IP MS 2025, Fujifilm), and visualized using a Typhoon FLA 9000 system (GE Healthcare).

Lipid-Protein Overlay Assay—Lipids were spotted onto a Hybond C membrane (Amersham Biosciences) and dried under nitrogen. The membrane was rewetted in water and blocked for 1 h in 2% BSA/TBS-T (TBS containing Tween 20). It was then exposed overnight at 4 °C to 0.5 μg/ml purified GST-cPLA$_2\alpha$ or GST-C2. The membrane was washed with TBS-T and exposed overnight at 4 °C to a 1:1000 dilution of anti-GST antibody in 2% BSA/TBS-T. It was washed with TBS-T and exposed to a 1:3000 dilution of horseradish peroxidase-conjugated anti-goat IgG antibody in 2% BSA/TBS-T for 1 h at room temperature. The immunoreactive spots were visualized by enhanced chemiluminescence. Purified recombinant GST did not bind any of the lipids using this protocol (data not shown).

Large Multilamellar Vesicle Binding Assay—Large multilamellar vesicles for LacCer were produced by drying 68.3 μl of a 1 mg/ml solution (100 μl) of 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl for each reaction, and the lipid was vortexed vigorously for 2 min. It was then mixed with 200 μl of buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 4 mM EGTA) and, for the calcium-containing reaction, 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) and 150 mM NaCl containing 0.5 μg of GST-cPLA$_2\alpha$. After 5 min at room temperature, the reaction was centrifuged at 10,000 $\times$ *g* for 10 min, and the supernatant was removed. 100 μl of 1$\times$ Laemmli buffer was added to the lipid pellet, and 50 μl of the lipid pellet was subjected to SDS-PAGE. Purified recombinant GST did not bind LacCer using this protocol (data not shown).

Calcium Imaging—CHO-W11A cells were seeded on glass-bottomed dishes and incubated for 48 h. The cells were washed and incubated with 5 μM Oregon Green 488 BAPTA-1/AM for 1 h at 37 °C. Cells were washed three times and then imaged using the FluoView confocal laser scanning microscope system.

Statistics—Values are the means $\pm$ S.E. for three to four independent experiments performed in triplicate. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance 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

LacCer Induces cPLA$_2\alpha$-dependent AA Release from Cells—To analyze the role of glycosphingolipids in cPLA$_2\alpha$-dependent AA release from cells, glycosphingolipid biosynthesis was inhibited by treatment with PPMP, a competitive inhibitor of GlcCer synthase. As shown in Fig. 1*A*, treatment of CHO-W11A cells with 1 μM PPMP for 48 h led to a significant decrease in cellular glycosphingolipid levels. We confirmed that the treatment of cells with PPMP at the concentrations used did not cause cytotoxicity and alteration of cPLA$_2\alpha$ expression levels during the test period (data not shown). Using this culture system, we determined whether the reduction in the cellular glycosphingolipid level affected the release of AA from cells. We previously reported that stimulation of CHO-W11A cells with A23187 or PAF induces cPLA$_2\alpha$-dependent AA release (11). As shown in Fig. 1*B*, the release of AA induced by 1 μM A23187 or 100 nM PAF was decreased in PPMP-treated cells. The reduced release of AA from PPMP-treated cells was rescued by supplementation of the culture medium with 30 μM GlcCer or LacCer. We next tested the effect of exogenous glycosphingolipids on the release of AA from CHO-W11A cells. As shown in Fig. 1*C*, treatment with LacCer increased the release of AA in a concentration-dependent manner. In addition, 30 μM LacCer induced the release of AA in a time-dependent manner, and the release at 30 min following stimulation was 2.5-fold of that upon treatment with vehicle (Fig. 1*D*). Other glycosphingolipids, such as GlcCer, galactosylceramide, and GM1, did not increase the release of AA from CHO-W11A cells (data not shown). Treatment of cells with 2 μM pyrrophenone, a selective inhibitor of cPLA$_2\alpha$, reduced the LacCer-induced release of AA (Fig. 1*E*). We next investigated the effect of LacCer on another cell type, murine L929 fibroblast cells. As shown in Fig. 1*F*, stimulation for 30 min with LacCer significantly increased the release of AA from L929 cells, which was inhibited by pyrrophenone. Furthermore, in stable cPLA$_2\alpha$ knockdown L929 cells (L929-cPLA$_2\alpha$-shRNA), LacCer did not induce the release of AA. These results suggest that glycosphingolipids, especially LacCer, play an important role in the regulation of cPLA$_2\alpha$-dependent release of AA in cells.

Effect of LacCer on cPLA$_2\alpha$ Translocation in Cells—To examine whether LacCer induces translocation of cPLA$_2\alpha$, we monitored the localization of GFP-cPLA$_2\alpha$ in living cells by confocal laser fluorescence microscopy. GFP-cPLA$_2\alpha$ was almost homogeneously present in the cytosol in CHO-W11A cells in the resting state. Treatment with LacCer triggered the translocation of GFP-cPLA$_2\alpha$ (but not GFP) to the membrane in a pattern consistent with the Golgi apparatus, endoplasmic reticulum, and nuclear envelope (Fig. 2*A*). To demonstrate that LacCer-activated cPLA$_2\alpha$ was translocated to Golgi membranes, the fluorescent signal of cPLA$_2\alpha$ was overlaid with a marker of the Golgi, red fluorescent protein-Golgi. As shown in Fig. 2*B*, GFP-cPLA$_2\alpha$ activated by LacCer was co-localized with red fluorescent protein-Golgi. The fluorescent LacCer BODIPY-C5-LacCer is internalized into endosomes and then transferred to the Golgi (16). Significant co-localization was observed between BODIPY-C5-LacCer and DsRed-cPLA$_2\alpha$ (Fig. 2*C*). Because the C2 domain of cPLA$_2\alpha$ is sufficient for translocation and can interact with lipids, we monitored the cellular localization of the C2 domain of cPLA$_2\alpha$ fused to GFP (GFP-C2). As shown in Fig. 2*D*, LacCer induced the translocation of GFP-C2 to the Golgi. These results suggest that cPLA$_2\alpha$ is translocated

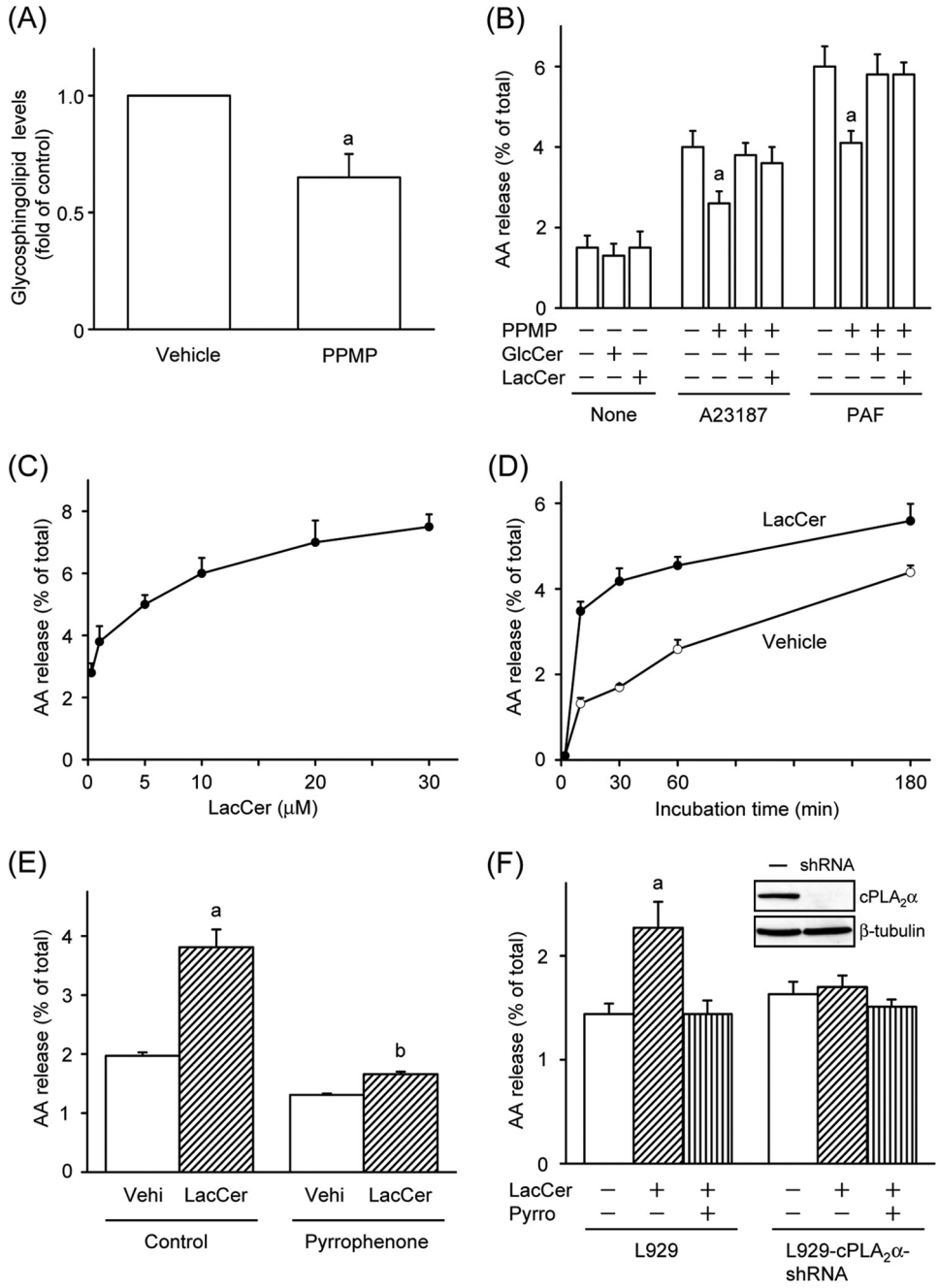

FIGURE 1. **LacCer induces cPLA$_2\alpha$-dependent AA release from cells.** *A*, CHO-W11A cells were cultured for 48 h in culture medium supplemented with vehicle or 1 μM PPMP. After the cells were washed, glycosphingolipids were extracted and spotted onto a TLC plate. *B*, CHO-W11A cells were cultured for 30 h in culture medium supplemented with or without 1 μM PPMP, 10 μM GlcCer, and/or 10 μM LacCer. The cells were then labeled by incubation for 18 h in 0.1% BSA- and [^{3}H]AA-containing medium supplemented with the same reagents. The labeled cells were washed and stimulated with 1 μM A23187 or 100 nM PAF for 30 min at 37 °C. ^{a}, $p < 0.05$, significantly different from the control without PPMP. *C*, [^{3}H]AA-labeled cells were stimulated with the indicated concentrations of LacCer for 30 min at 37 °C. *D*, [^{3}H]AA-labeled cells were stimulated with vehicle or 30 μM LacCer for the indicated time periods at 37 °C. ○, vehicle; ●, LacCer. *E*, the labeled cells were pretreated with vehicle (*Control*) or 2 μM pyrrophenone for 30 min prior to stimulation with vehicle (*Vehi*) or 30 μM LacCer for 30 min at 37 °C. ^{a}, $p < 0.05$, significantly different from the control without LacCer; ^{b}, $p < 0.05$, significantly different from the control without pyrrophenone. *F*, [^{3}H]AA-labeled L929 and L929-cPLA$_2\alpha$-shRNA cells were pretreated with 2 μM pyrrophenone (*Pyrro*) for 30 min and then stimulated with 30 μM LacCer for 30 min at 37 °C. The expression levels of cPLA$_2\alpha$ in both cells are shown. ^{a}, $p < 0.05$, significantly different from the control without LacCer. The data shown are the means ± S.E. for three experiments.

mainly to Golgi membranes through the C2 domain in response to LacCer.

LacCer Activates cPLA$_2\alpha$ without Intracellular Ca^{2+} Mobilization in Cells—To determine the Ca^{2+} requirement for LacCer-induced cPLA$_2\alpha$ translocation, the cellular localization of GFP-cPLA$_2\alpha$ in the presence of EGTA was examined. As shown in Fig. 3*A*, LacCer triggered the translocation of GFP-cPLA$_2\alpha$ in the presence of EGTA. The single substitution D43N in the C2 domain abrogates the Ca^{2+}-dependent translocation of cPLA$_2\alpha$ to membranes (17). The translocation of GFP-D43N-cPLA$_2\alpha$ was induced by treatment with LacCer, but not with A23187 (Fig. 3*B*). We next investigated whether

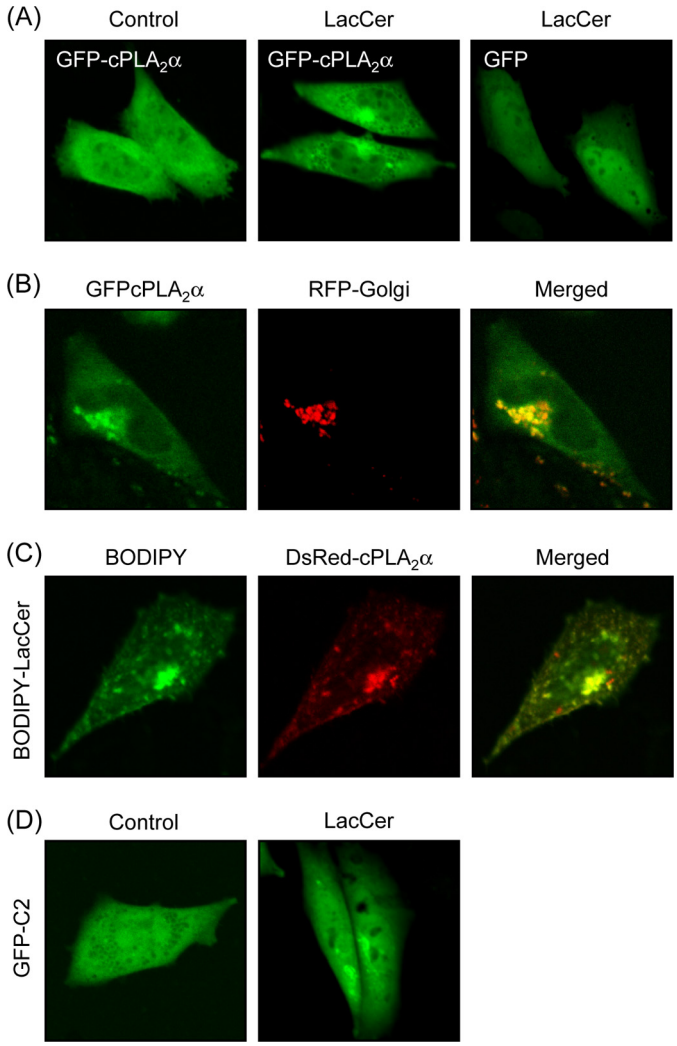

FIGURE 2. **LacCer induces cPLA$_2\alpha$ translocation in cells.** *A*, CHO-W11A cells transiently transfected with expression vectors for GFP-cPLA$_2\alpha$ and GFP were treated with vehicle (*Control*) or 30 μM LacCer for 1 h. *B*, CHO-W11A cells transiently transfected with expression vectors for GFP-cPLA$_2\alpha$ and red fluorescent protein (*RFP*)-Golgi were treated with 30 μM LacCer. *C*, DsRed-cPLA$_2\alpha$-expressing CHO-W11A cells were treated with 100 nM BODIPY-C5-LacCer for 1 h. *D*, GFP-C2-expressing CHO-W11A cells were treated with 30 μM LacCer for 1 h. In *A–D*, data are representative of three independent experiments.

LacCer would induce a rise in intracellular Ca^{2+} concentrations in CHO-W11A cells. Treatment with LacCer did not increase intracellular Ca^{2+} concentrations (Fig. 3*C*). Next, we determined the effect of EGTA on the release of AA induced by LacCer. As shown in Fig. 3*D*, LacCer induced the release of AA even in the presence of EGTA. Thus, LacCer triggers the translocation of cPLA$_2\alpha$ and then induces the release of AA without intracellular Ca^{2+} mobilization in CHO-W11A cells.

LacCer Directly Binds and Activates cPLA$_2\alpha$ in Vitro—We next determined whether LacCer would bind directly to cPLA$_2\alpha$ using a lipid-protein overlay assay. As shown in Fig. 4*A*, cPLA$_2\alpha$ bound as little as 80 nmol of LacCer. cPLA$_2\alpha$ was also found to bind to 1-palmitoyl-2-oleoylphosphatidylinositol, although to a lesser extent compared with LacCer (Fig. 4*B*). In contrast, cPLA$_2\alpha$ did not bind to 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylethanolamine, GM1, or sphingosine 1-phosphate when 100 nmol was bound to the membrane. The binding between LacCer and cPLA$_2\alpha$ could be detected even in the presence of EGTA (Fig. 4*C*), indicating that LacCer binds to cPLA$_2\alpha$ in a Ca^{2+}-independent manner. C1P is known to bind cPLA$_2\alpha$ in a calcium-dependent manner. As shown in Fig. 4*C*, C1P bound cPLA$_2\alpha$ in the presence of Ca^{2+}, which was inhibited in the presence of EGTA. The region of cPLA$_2\alpha$ that binds LacCer was also determined using the recombinantly expressed C2 domain of cPLA$_2\alpha$. As shown in Fig. 4*D*, LacCer bound to the C2 domain of cPLA$_2\alpha$. Next, we used a large multilamellar vesicle binding assay for cPLA$_2\alpha$. cPLA$_2\alpha$ bound to LacCer in both the presence and absence of Ca^{2+} (Fig. 4*E*). Thus, cPLA$_2\alpha$ interacts with LacCer via the C2 domain in a Ca^{2+}-independent manner.

To examine whether LacCer directly increases the enzymatic activity of cPLA$_2\alpha$, liposomes containing PAPC and LacCer were prepared and tested for activity *in vitro*. As shown in Fig. 5, the cPLA$_2\alpha$ activity in the presence of PAPC liposomes alone was ~1100 dpm. The cPLA$_2\alpha$ activity in the liposomes with PAPC and LacCer combined at a molar ratio of 95:5 was increased by 1.5-fold compared with that with PAPC alone. LacCer could not activate cPLA$_2\alpha$ in the presence of pyrrophenone or EGTA, indicating that the enzymatic activation of cPLA$_2\alpha$ by LacCer requires Ca^{2+}.

TNF-α Induces cPLA$_2\alpha$-dependent AA Release via LacCer Generation in L929 Cells—TNF-α is known to up-regulate the activity of cPLA$_2\alpha$ and the generation of LacCer (8, 18, 19). We next examined the role of LacCer generation in TNF-α-induced activation of cPLA$_2\alpha$. As shown in Fig. 6*A*, treatment of L929 cells with 10 nM TNF-α significantly increased intracellular LacCer levels. Treatment of cells with TNF-α induced the release of AA in a time-dependent manner for up to 6 h, and the release at 6 h following stimulation was 2-fold of that in the control (Fig. 6*B*). In cPLA$_2\alpha$ knockdown cells, TNF-α did not increase the release of AA (Fig. 6*C*). In addition, the TNF-α-induced release of AA from L929 cells was suppressed to near the control level by both treatment with PPMP (Fig. 6*C*) and silencing of GlcCer synthase (Fig. 6*D*). We next determined the effect of TNF-α on the translocation of cPLA$_2\alpha$ in L929 cells. When the cells were treated with TNF-α for 2 h, the expression level of cPLA$_2\alpha$ in membrane fractions was increased (Fig. 6*E*). In addition, TNF-α triggered the translocation of GFP-cPLA$_2\alpha$ in L929 cells, which was inhibited by pretreatment with PPMP (Fig. 6*F*). These results indicate that TNF-α increases the cellular LacCer levels, thereby activating cPLA$_2\alpha$.

DISCUSSION

In this study, we found that LacCer is a direct activator of cPLA$_2\alpha$. Although LacCer has been reported to be a bioactive lipid in various cell physiological processes, such as smooth muscle proliferation (20), expression of adhesion molecules (8), and β1-integrin clustering and endocytosis (21), the direct targets of LacCer and cellular events mediated by LacCer are largely unknown. We found that LacCer interacted with and activated cPLA$_2\alpha$ directly. In addition, we showed that depletion of glycosphingolipids by treatment with PPMP attenuated the induction of AA release in response to agonists. Although it is possible that the amounts of other sphingolipids were altered by treatment

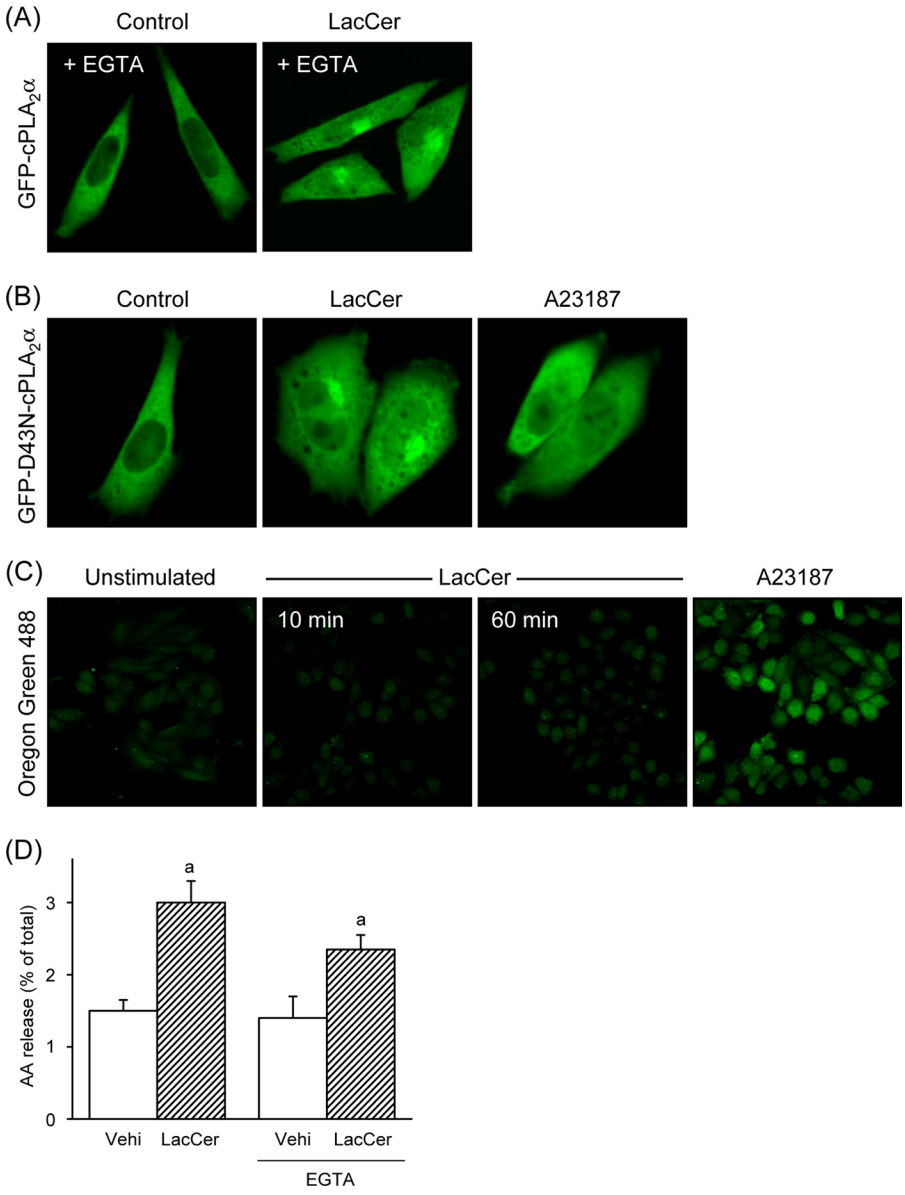

FIGURE 3. **LacCer activates cPLA$_2\alpha$ without intracellular Ca^{2+} mobilization.** *A*, CHO-W11A cells transiently transfected with expression vector for GFP-cPLA$_2\alpha$ were treated with vehicle (*Control*) or 30 μM LacCer for 1 h in the presence of 2 mM EGTA. *B*, GFP-D43N-cPLA$_2\alpha$-expressing CHO-W11A cells were treated with 30 μM LacCer or 5 μM A23187 for 1 h. *C*, CHO-W11A cells labeled with Oregon Green 488 were washed and then treated with 30 μM LacCer for 10 or 60 min or with 5 μM A23187 for 5 min. In *A–C*, data are representative of three independent experiments. *D*, [^{3}H]AA-labeled CHO-W11A cells were stimulated with vehicle (*Vehi*) or 30 μM LacCer for 30 min at 37 °C in the presence or absence of 2 mM EGTA. ^{a}, $p < 0.05$, significantly different from the control without LacCer.

with PPMP, the rescue of glycosphingolipids by exogenous addition of GlcCer and LacCer could overcome the effect of PPMP on the release of AA (Fig. 1*B*). Furthermore, exogenous LacCer increased the release of AA, and that induction was decreased by treatment with an inhibitor of cPLA$_2\alpha$ or silencing of the enzyme (Fig. 1, *E* and *F*). Thus, LacCer plays an important role in the activation of cPLA$_2\alpha$ and AA release.

Translocation of cPLA$_2\alpha$ from the cytosol to the membrane is necessary for its access to phospholipid substrate. In this study, depletion of glycosphingolipids by treatment with PPMP for 48 h did not inhibit the A23187-mediated translocation of cPLA$_2\alpha$ (data not shown). Thus, LacCer is required for induction of the release of AA, but not for translocation of cPLA$_2\alpha$ in response to A23187. LacCer may increase the retention time of cPLA$_2\alpha$ in the membrane, resulting in induction of cPLA$_2\alpha$ activity and AA release. cPLA$_2\alpha$ is translocated in response to Ca^{2+} binding, and its binding to Asp^{43} is critical for mediating the translocation (17). In this study, the translocation of GFP-D43N-cPLA$_2\alpha$ was induced by LacCer, but not by A23187 (Fig. 3*B*). In addition, treatment of CHO-W11A cells with LacCer, which did not mediate Ca^{2+} mobilization, induced the translocation of cPLA$_2\alpha$ even in the presence of EGTA (Fig. 3, *A* and *C*). These results suggest that the translocation of cPLA$_2\alpha$ induced by LacCer is Ca^{2+}-independent. However, it is also possible that exogenous LacCer accumulated in the membrane affects the Ca^{2+} dependence of cPLA$_2\alpha$.

It has been reported that the C2 domain of cPLA$_2\alpha$ binds to phosphatidylcholine and C1P in the presence of micromolar Ca^{2+} (22, 23). The domain also shows weak binding to phos-

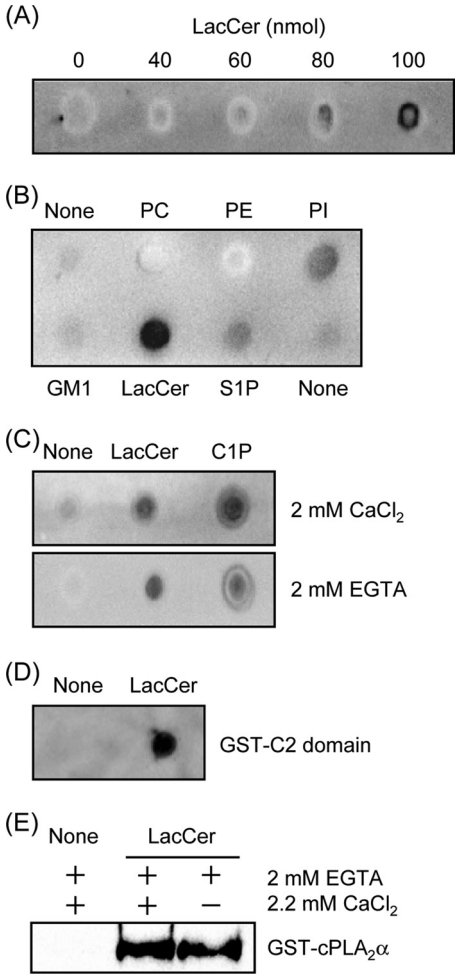

FIGURE 4. **cPLA$_2\alpha$ binds to LacCer in a calcium-independent manner.** *A*, the binding of cPLA$_2\alpha$ to LacCer was examined using the lipid-protein overlay assay as described under "Experimental Procedures." The indicated amounts of LacCer were spotted onto a Hybond C membrane. The membrane was exposed to purified GST-cPLA$_2\alpha$ overnight at 4 °C. *B*, the lipid-protein overlay assay was repeated with 100 nmol of lipids and GST-cPLA$_2\alpha$. *PC*, 1-palmitoyl-2-oleoylphosphatidylcholine; *PE*, 1-palmitoyl-2-oleoylphosphatidylethanolamine; *PI*, 1-palmitoyl-2-oleoylphosphatidylinositol; *S1P*, sphingosine 1-phosphate. *C*, the lipid-protein overlay assay was repeated with 100 nmol of LacCer, 20 nmol of C1P, and GST-cPLA$_2\alpha$ with or without 2 mM EGTA. *D*, the membrane spotted with 100 nmol of LacCer was exposed to the GST-tagged C2 domain of cPLA$_2\alpha$ overnight at 4 °C. *E*, the large multilamellar vesicle binding assay was performed as described under "Experimental Procedures." In *A–E*, data are representative of three independent experiments.

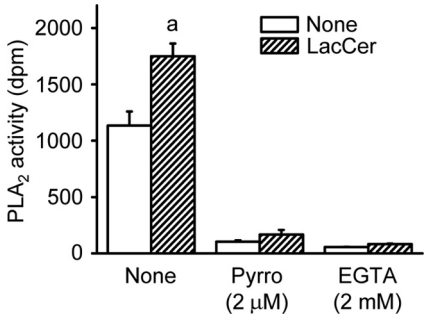

FIGURE 5. **LacCer activates cPLA$_2\alpha$ *in vitro*.** PLA$_2$ activity in the cytosolic fraction from HEK293T cells expressing human cPLA$_2\alpha$ was measured as described under "Experimental Procedures." PLA$_2$ activities toward PAPC and 95:5 PAPC/LacCer-mixed vesicles were measured in reaction buffer with or without 2 μM pyrrophenone (*Pyrro*) or 2 mM EGTA. The data shown are the means ± S.E. for three experiments. ^{a}, $p < 0.05$, significantly different from the control without LacCer.

phatidic acid vesicles in a Ca^{2+}-independent manner (22). Anionic phosphatidylmethanol binds to full-length cPLA$_2\alpha$ in the absence of Ca^{2+}, but Ca^{2+} is required for the binding of the C2 domain to this lipid (24). Thus, cPLA$_2\alpha$ displays both Ca^{2+}-dependent and Ca^{2+}-independent interfacial binding to vesicles. In this study, we showed that cPLA$_2\alpha$ bound to LacCer in a Ca^{2+}-independent manner (Fig. 4). However, Ca^{2+} was required for the activation of cPLA$_2\alpha$ by LacCer *in vitro* (Fig. 5). Thus, it may be possible that, even if cPLA$_2\alpha$ binds to LacCer, Ca^{2+} binding-mediated conformational changes of cPLA$_2\alpha$ are essential for promoting the penetration of the enzyme into the PAPC vesicles that were used for measuring the enzymatic activity. Treatment of cells with LacCer caused the translocation of the GFP-D43N-cPLA$_2\alpha$ mutant. Thus, this suggests that cPLA$_2\alpha$ can bind to the LacCer-containing cell membrane in a Ca^{2+}-independent manner, but Ca^{2+} is required for exerting its enzymatic activity.

C1P and PIP_2 bind to cPLA$_2\alpha$ and increase its activity. PIP_2 activates the enzyme by increasing catalytic efficiency through increased penetration of the membrane (5). The binding site of PIP_2 includes four lysine residues, which are located in the highly basic region of the catalytic domain on the side close to the membrane (25, 26). C1P enhances the activity of cPLA$_2\alpha$ by increasing the resident time of the enzyme in the membrane through electrostatic interactions with cationic residues in the C2 domain (6). In this study, we showed that LacCer binds to the C2 domain. The C2 domain is known to bind to various lipids by hydrophobic or electrostatic interactions. Because LacCer is a neutral glycosphingolipid, hydrophobic forces might be responsible for this interaction.

The liberation of AA from membrane phospholipids by activation of cPLA$_2\alpha$ is known to occur mainly in the perinuclear region, such as the Golgi and the endoplasmic reticulum. LacCer is located predominantly in the outer leaflet of the plasma membrane and the luminal leaflet of the Golgi, endosomes, and lysosomes. Several studies determined that the P-glycoprotein functions as a flippase in both the plasma membrane and the Golgi for phospholipids and simple glycosphingolipids, such as GlcCer, galactosylceramide, and LacCer (27, 28). Thus, the results from this study suggest that LacCer in the cytosolic leaflet of the Golgi promotes the binding of cPLA$_2\alpha$ to the membrane.

TNF-α regulates various physiological functions, such as cell proliferation, differentiation, adhesion, and death. It has been reported that various cell functions induced by TNF-α are mediated through the activation of cPLA$_2\alpha$ (18, 19). However, the cPLA$_2\alpha$-activating mechanisms of TNF-α have not been well elucidated. In this study, inhibition of synthesis of glycosphingolipids attenuated cPLA$_2\alpha$ translocation and AA release in response to TNF-α (Fig. 6). Thus, TNF-α activates cPLA$_2\alpha$ through the production of glycosphingolipids, especially LacCer. Previously, we reported that sphingomyelin disturbs the binding of cPLA$_2\alpha$ to glycerophospholipids and inhibits the release of AA (11). Numerous reports have shown that sphingomyelin is hydrolyzed to ceramide by sphingomyelinase in response to TNF-α (9, 29, 30). In addition, TNF-α induces the activation of β1,4-galactosyltransferase and the formation of LacCer, which is generated via the galactosylation of GlcCer

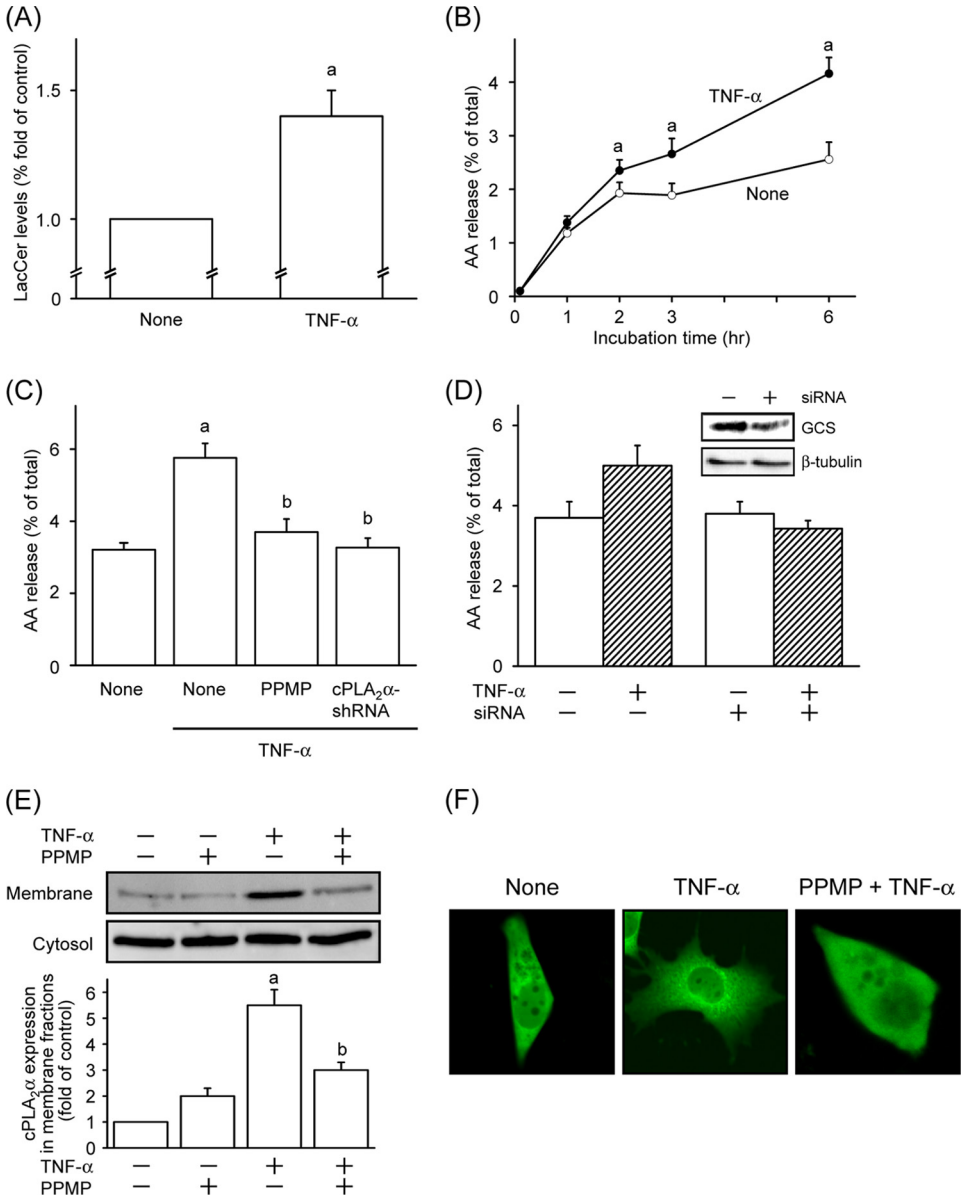

FIGURE 6. **TNF-α induces cPLA$_2\alpha$-dependent AA release via LacCer formation in L929 cells.** *A*, L929 cells labeled with [^{14}C]serine for 18 h were stimulated with or without 10 nM TNF-α for 2 h. After the cells were washed, lipids were extracted and separated by TLC as described under "Experimental Procedures." *B*, [^{3}H]AA-labeled L929 cells were stimulated with 10 nM TNF-α for the indicated time periods at 37 °C. ○, none; ●, TNF-α. *C*, L929 cells were pretreated with vehicle or 3 μM PPMP for 30 min, and then the cells and L929-cPLA$_2\alpha$-shRNA cells were stimulated with 10 nM TNF-α for 6 h at 37 °C. *D*, down-regulation of GlcCer synthase were performed as described under "Experimental Procedures." L929 cells transfected with or without GlcCer synthase siRNA were stimulated with 10 nM TNF-α for 6 h at 37 °C. The expression levels of GlcCer synthase (*GCS*) in cells are shown. *E*, L929 cells were pretreated with vehicle or 3 μM PPMP for 30 min and then stimulated with 10 nM TNF-α for 2 h at 37 °C. The cytosolic and membrane fractions were prepared and subjected to immunoblot analysis. *Upper panels*, immunoblotting with antibodies against cPLA$_2\alpha$. *Lower panel*, the histogram represents the expression levels of cPLA$_2\alpha$ in membrane fractions quantified using ImageJ. The data shown are the means ± S.E. for three experiments. ^{a}, $p < 0.05$, significantly different from the control without TNF-α; ^{b}, $p < 0.05$, significantly different from the control without PPMP or cPLA$_2\alpha$ knockdown. *F*, L929 cells transiently transfected with expression vector for GFP-cPLA$_2\alpha$ were treated with or without TNF-α for 2 h. PPMP were pretreated for 30 min before stimulation with TNF-α.

produced from ceramide (7). Thus, this suggests that, in response to TNF-α, the level of sphingomyelin as an inhibitor of cPLA$_2\alpha$ is decreased and that of LacCer as an activator is increased in the membrane, thereby inducing the catalytic ability of the enzyme.

In summary, we have shown that LacCer is a direct activator of cPLA$_2\alpha$. LacCer binds to the C2 domain of cPLA$_2\alpha$ and induces its translocation in a Ca^{2+}-independent manner. Furthermore, we have demonstrated the potential role of LacCer in TNF-α-induced cPLA$_2\alpha$ activation and AA release. These findings provide new insights into the regulatory mechanisms of cPLA$_2\alpha$ and the physiological functions of LacCer as a signaling molecule.

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