Chain length specificity for activation of cPLA$_2\alpha$ by C1P: use of the dodecane delivery system to determine lipid-specific effects

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Abstract Previously, our laboratory demonstrated that ceramide-1-phosphate (C1P) specifically activated group IVA cytosolic phospholipase A$_2$ (cPLA$_2\alpha$) in vitro. In this study, we investigated the chain length specificity of this interaction. C1P with an acyl-chain of ≥6 carbons efficiently activated cPLA$_2\alpha$ in vitro, whereas C$_2$-C1P, was unable to do so. Delivery of C1P to cells via the newly characterized ethanol/dodecane system demonstrated a lipid-specific activation of cPLA$_2\alpha$, AA release, and PGE$_2$ synthesis (EC$_{50}$ = 400 nM) when compared to structurally similar lipids. C1P delivered as vesicles in water also induced a lipid-specific increase in AA release. Mass spectrometric analysis demonstrated that C1P delivered via ethanol/dodecane induced a 3-fold increase in endogenous C1P with little metabolism to ceramide. C1P was also more efficiently delivered (>3-fold) to internal membranes by ethanol/dodecane as compared to vesiculated C1P. Using this now established delivery method for lipids, C$_2$-C1P was shown to be ineffective in the induction of AA release as compared with C$_6$-C1P, C$_{16}$-C1P, and C$_{18:1}$ C1P. Here, we demonstrate that C1P requires ≥6 carbon acyl-chain to activate cPLA$_2\alpha$. Thus, published reports on the biological activity of C$_2$-C1P are not via eicosanoid synthesis. Furthermore, this study demonstrates that the alcohol/dodecane system can be used to efficiently deliver exogenous phospholipids to cells for the examination of specific biological effects.—Wijesinghe, D. S., P. Subramanian, N. F. Lamour, L. B. Gentile, M. H. Granado, A. Bielawska, Z. Szulc, A. Gomez-Munoz, and C. E. Chalfant. **Chain length specificity for activation of cPLA$_2\alpha$ by C1P: use of the dodecane delivery system to determine lipid-specific effects. *J. Lipid Res.* 2009.** 50: **1986–1995.**

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The first report of a biological effect for ceramide-1-phosphate (C1P) was by Gomez-Munoz et al. (1), which demonstrated that short chain (not naturally found in cells) C1P induced DNA synthesis in Rat-1 fibroblasts. Later, treatment of T17 fibroblasts with long chain, natural C1P was also demonstrated to induce a potent increase in DNA synthesis (2) and the levels of proliferating nuclear antigen. Following this line of research, a recent report demonstrated that C1P prevented programmed cell death in bone marrow-derived macrophages after withdrawal of macrophage colony-stimulating factor (3). Treatment of these cells with C1P effectively blocked the activation of caspases and prevented DNA fragmentation upon serum removal.

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Abbreviations: AA, arachidonic acid; CERK, ceramide kinase; C1P, ceramide-1-phosphate; cPLA$_2\alpha$, group IVA cytosolic phospholipase A$_2$; EtOH, ethanol; GFP, green fluorescent protein; PA, phosphatidic acid; PAPC, 1-palmitoyl-2-arachidonoylphosphotidylcholine; PC, phosphatidylcholine; PDI, protein disulfide isomerase; PGE$_2$, prostaglandin E$_2$; 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 a figure.

In the same study, it was demonstrated that C1P treatment inhibited ceramide generation from acid sphingomyelinase (A-SMase). Finally, A-SMase was shown to be a direct target of C1P inducing inhibition of this enzyme (4).

In the last few years, a number of reports have continued to demonstrate distinct biological mechanisms regulated by the sphingolipid, C1P, and the enzyme responsible for its synthesis, ceramide kinase (CERK). For example, Hinkovska-Galcheva et al. (5) demonstrated that CERK was activated in the context of phagocytosis in neutrophils after challenging the cells with formyl peptide and antibody-coated erythrocytes (FMLP/EIgG). Thus, these data demonstrated that C1P may play a distinct role in membrane fusion, possibly explaining the early finding that high levels of C1P are found in synaptic vesicles (6). Our laboratory has also demonstrated a biological function for C1P as a direct activator of cPLA$_2\alpha$ through interaction with the C2/CaLB domain (7). These results, coupled with the previous findings that the CERK/C1P pathway is required for cPLA$_2\alpha$ activation in response to calcium ionophore and cytokines (8), demonstrated that C1P was a "missing link" in the eicosanoid synthetic pathway. A role for CERK and its product, C1P, in a separate pathway of allergic/inflammatory signaling has also been reported in mast cells. Mitsutake et al. (9) demonstrated that treatment of RBL-2H3 cells or overexpression of CERK in these cells enhanced the degranulation induced by A23187.

Although there is a growing list of biological functions attributed to C1P, it is unclear whether an effect observed for different chain lengths of C1P can be extrapolated to all biological observations. In this regard, many chain lengths of C1P have been utilized exogenously to examine biological effects. For example, short chain C1Ps are ideal candidates for studying the biology of C1P as their higher solubility allows for relatively easy delivery to target cells. In this regard, Högback et al. (10) and Tornquist et al. (11) showed that C_2-C1P induced an increase in the intracellular Ca^{+2} levels in $FRTL_5$ cells and GH_4C_1 rat pituitary cells. Using the same lipid, Graf et al. (12) showed a correlation between apoptosis and enhanced C_2-C1P formation upon C_2-ceramide treatment of CERK overexpressing COS cells. C_2, C_8, and long chain C1P have also been demonstrated to cause ^{3}H thymidine incorporation into DNA (1, 2). Our laboratory, using the naturally-occurring $C_{16:0}$ and $C_{18:1}$ C1P, showed the lipid is a cofactor in the activation of cPLA$_2\alpha$ and synthesis of eicosanoids (7, 8). Because eicosanoids pathways have roles in calcium homeostasis (13, 14), cell survival (15), apoptosis (16), and cell growth (16), the question remains whether these biologies reported for C1P can be attributed simply to cPLA$_2\alpha$ activation. In addition, a recent paper by Tauzin et al. (17) demonstrated that using dodecane to deliver phospholipids induced eicosanoid synthesis and loss of cell viability in a nonspecific manner, casting doubts on the validity of this well-established method of lipid delivery.

In this paper, we show that, although the ethanol (EtOH)/dodecane system successfully delivers all chain lengths of C1P to cells, not all chain lengths activate cPLA$_2\alpha$ in vitro and in cells. Thus, certain biologies attributed to C_2-C1P are not via activation of cPLA$_2\alpha$. In addition, we also demonstrate that, at low concentrations, C1P exerts biological effects that are specific, nontoxic, and distinct from other structurally similar lipid molecules as well as the delivery medium. Therefore, this study shows that the alcohol/dodecane delivery system can be utilized to study lipid-specific effects if proper controls and appropriate lipid concentrations are utilized. Furthermore, the study demonstrates that certain reported biologies (e.g., calcium release) for C1P are not via activation of eicosanoid biosynthesis.

MATERIALS AND METHODS

Materials

All cultured cells were obtained from American Type Culture Collection. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). D-e-$C_{18:1}$ ceramide-1-phosphate, D-e-$C_{16:0}$ ceramide-1-phosphate, D-e-$C_{6:0}$ ceramide-1-phosphate, and D-e-$C_{2:0}$ ceramide-1-phosphate for the treatment of A549 were purchased from Avanti, produced via large scale phosphorylation of ceramide, or by base hydrolysis of the relevant sphingomyelin (SM). D-e-$C_{18:1}$ dimethyl ester of C1P was custom synthesized by the Medical University of South Carolina lipidomics core facility. C1P used in the treatment of NR8383 cells, EtOH, and dodecane are from Sigma-Aldrich. Ceramide and phosphatidic acid (PA) were purchased from Avanti Polar Lipids. DMEM, RPMI, FBS, and penicillin/streptomycin (100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate) were obtained from Invitrogen Life Technologies, Carlsbad, CA.

Cell culture

A549 lung adenocarcinoma cells were grown in 50% RPMI 1640 and 50% DMEM supplemented with L-glutamine, 10% (v/v) FBS, and 2% (v/v) penicillin/streptomycin. Cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, 37°C). RAW macrophages were grown in DMEM supplemented with L-glutamine, 10% (v/v) FBS, and 2% (v/v) penicillin/streptomycin. For treatments, the medium was replaced 2 h prior to the addition of the agonist by DMEM containing 2% FBS and 2% penicillin/streptomycin. Alveolar macrophage cells (NR8383) were cultured in Ham's F12 medium supplemented with 15% FBS, 2.74 mg/ml of glucose, and 2% (v/v) penicillin/streptomycin. Floating and adherent cells were collected after centrifugation, resuspended in fresh medium, and seeded in new culture dishes. They were then cultured in a humidified atmosphere containing 5%CO_2 at 37°C.

Dispersion of C1P in aqueous solution

C1P was dissolved in EtOH to make a 10 mM solution. The required amount of C1P from this stock solution was dried down under N_2 gas. Water was added to the dried ceramide to the desired concentration. The solution was then sonicated on ice until a clear solution was obtained and used soon thereafter.

Dispersion of C1P in EtOH/dodecane

EtOH and dodecane were mixed at a ratio of 98:2, followed by vortexing and prewarming to 37°C. Meanwhile, C1P was dissolved in chloroform-methanol 1:1. The required volume was then dried down under N_2 gas. The prewarmed EtOH-dodecane mixture was added to the dried C1P such that the final concentration was

2.5 mM (a stock solution up to 10 mM can be made). This mixture was thoroughly vortexed and incubated at 37°C for a further 20 min followed by further vortexing. The stock solution, thus prepared, was diluted to the required concentration using EtOH/dodecane and was used to treat the cells.

Treatment of cells with phospholipids

The stock solution of C1P in delivery medium was incubated at 37°C followed by vortexing. C1P was diluted to the appropriate concentration in EtOH/dodecane solution, and added to cells at a dilution of 1:1000. This concentration was used to prevent adverse effects on cells by the delivery medium itself. PA and ceramide similarly prepared in EtOH/dodecane was included as a sham control at the same concentration as the treatment.

Mixed micelle assay for cPLA$_2\alpha$

cPLA$_2\alpha$ activity was measured in a phosphatidylcholine (PC)-mixed micelle assay in a standard buffer composed of 80 mM HEPES, pH 7.5, 150 mM NaCl, 10 μM free Ca^{2+}, and 1 mM dithiothreitol. The assay also contained 0.3 mM 1-palmitoyl-2-arachidonoylphosphotidylcholine (PAPC) with 250,000 dpm of [^{14}C] PAPC, 2 mM Triton X-100, 26% glycerol, and 500 ng of purified cPLA$_2\alpha$ protein in a total volume of 200 μl. To prepare the substrate, an appropriate volume of cold PAPC in chloroform, indicated phospholipids, and [^{14}C] PAPC in toluene/EtOH 1:1 solution were evaporated under nitrogen. Triton X-100 was added to the dried lipid to give a 4-fold concentrated substrate solution (1.2 mM PAPC). The solution was probe sonicated on ice (1min on, 1 min off for 3 min). The reaction was initiated by adding 500 ng of the enzyme and was stopped by the addition of 2.5 ml of Dole reagent (2-propanol, heptane, 0.5 M H_2SO_4; 400:100:20, v/v/v). The amount of [^{14}C] arachidonic acid (AA) produced was determined using the Dole procedure as previously described (18). All of the assays were conducted for 45 min at 37°C. Statistical and kinetic analyses were performed using Sigma-Plot Enzyme Kinetics software, version 1.1, from SYSSTAT Software, Inc.

Quantification of AA release

A549 cells (5×10^4) were labeled overnight with 5 μCi/ml [^{3}H] AA (5 nM). Cells were washed and placed in DMEM supplemented with 2% FBS for 2 h. Following treatment, medium was transferred to 1.5-ml polypropylene tubes, centrifuged at 10,000 *g*, and [^{3}H] AA (and metabolites) cpm were determined by scintillation counting. Results were controlled for equivalent number of cells quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (7) and by verification of total AA labeling by scintillation counting. In AA release experiments of NR 8383 cells, both adherent and floating cells were collected and seeded in 35 mm plates at a density of 1 x10^6 cells/ml. [3H]AA (0.20 μCi/ml) was added and cells incubated overnight. The cells were then washed twice with Ham's F12 medium supplemented with 0.2% BSA, and experiments were performed in Ham's F12 medium supplemented with 0.1% BSA. Six h after addition of the appropriate agonist, the medium was collected and centrifuged at 10000 *g* for 5 min. The radioactivity in the supernatants was measured using a liquid scintillation counter Packard Tri-Carb2700TR (Meriden, CT). An aliquot of the supernatant was used in some experiments for analysis of radiolabeled compounds, confirming that most of the radioactivity was in arachidonate.

PGE$_2$ assay

The ELISA plate (Cayman Chemical), coated with goat anti-mouse IgG was loaded at 50 μl per well of standard/sample, where the sample was diluted at 1 in 40 in 1× EIA buffer, 50 μl of prostaglandin E_2 (PGE_2) EIA AChE tracer (Cayman Chemical), and 50 μl of PGE_2 monoclonal antibody (Cayman Chemical). The control wells received 50 μl of 1× EIA buffer along with 50 μl of PGE_2 EIA AChE tracer and 50 μl of PGE_2 monoclonal antibody. The plate was covered and kept at 4°C for 16 h. The plate was then washed and 200 μl of Ellman's reagent (Cayman Chemical) was added to each well and was allowed to develop in the dark with low shaking at room temperature for 90 min. Following the developing step, absorbance in each well at 405 nM was read using a microplate spectrophotometer (BMG Labtech FLUOStar Optima). This assay was normalized by WST-1 assay (Roche Diagnostics) following the manufacturer's instructions. WST-1 reagent (10% of the total volume) was added to the cells and the plate was incubated at 37°C for 30 min. The optical density was then measured (at 450 nM vs. a reference of 630 nM) using a microplate spectrophotometer (BIO-TEK KC Junior).

Confocal microscopy

A549 cells were seeded onto 22 × 22 mm coverslips (Fisher) in 35 mm diameter plates in their appropriate media and incubated at 37°C under 5% CO_2 overnight. The following day, cells were transfected with adenovirus containing green fluorescent protein (GFP)-cPLA$_2$ at 10 multiplicity of infection (MOI). After 48 h incubation, the cells were treated with C1P or PA (1μM) solubilized in EtOH-dodecane (98:2). Cells were washed twice with PBS to remove the excess protein and then fixed on the coverslips with 100% cold methanol for 10 min at −20°C. Coverslips were mounted in 10 mM n-propagalate in gycerol, and were viewed using an Olympus BX50WI confocal microscope at 488 nM (Fluoview detector) using a 40× liquid immersable lens with a 1.5×-enhanced magnification microscopy.

Lipid uptake analysis by radiolabeled C1P

D-e-$C_{18:1}$ ceramide was subjected to enzymatic conversion to C1P in the presence of λ^{32}P labeled ATP as previously described (19, 20) and purified as previously described (19, 20). A549 cells were seeded onto 10 cm dishes at a density of 5×10^4 and incubated overnight under standard incubation conditions. On the day of treatment, the cells were washed in PBS and transferred to media containing 2% serum and incubated under standard incubation conditions for 2 h. Lipids were prepared by mixing radiolabeled and unlabeled C1P such that the final lipid concentration was 1 mM. These lipids were solubilized either in EtOH/dodecane as described elsewhere or by sonicating in water for 5 min. The resulting 1 mM lipid solutions were added to the cells at a dilution of 1:1000 and incubated for 2 h. At the end of the incubation period the cells were washed three times in ice cold PBS prior to fractionation.

Subcellular fractionation of plasma membrane versus internal membranes

The plasma membranes of the harvested cells were disrupted by four consecutive freeze-thaw cycles. The internal membranes were separated from the plasma membrane by centrifugation at 10,000 g for 5 min. The two fractions were counted separately using a Beckman LS 6500 scintillation counter.

Subcellular fractionation of different organelle membrane fractions

A549 cells in 10 cm plates treated for lipid uptake were suspended in buffer containing 20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose, and protease inhibitor cocktail (Sigma). The plasma membranes of the harvested cells were disrupted by four consecutive freeze thaw cycles

followed by homogenization by passing through a 23G needle 10 times. Subcellular fractionation was performed by differential centrifugation using a modification of the technique described by Maceyka et al. (21). The postnuclear supernatants were centrifuged at 5,000 *g* for 10 min to generate the heavy membrane (mitochondria- and *trans*-Golgi-enriched fraction). The supernatants were then centrifuged at 17,000 *g* for 15 min to obtain the light membrane fraction (endoplasmic reticulum- and *cis*-Golgi-enriched fraction). The remaining supernatants were centrifuged at 100,000 *g* for 1 h to obtain the plasma membrane and the cytosol. One hundred μl of each fraction was counted using a Beckman LS 6500 scintillation counter.

Mass spectrometric analysis

(1×10^6) A549 cells were seeded onto 10 cm dishes and incubated overnight under standard incubator conditions. The following day, cells were washed with PBS and treated with 1μM solution of D-erythro-$C_{18:1}$ C1P or ceramide for 2 h in 2% medium. Thereafter, the cells were washed and harvested in cold PBS as described (22). The cell pellets were stored at −80°C until extraction and analyzed by mass spectrometry. An aliquot of cells was taken for standardization (Total DNA). The lipids were extracted as described by Merrill et al. (22) and quantified using liquid chromatography electrospray ionization tandem mass spectrometry using a Shimadzu HPLC system coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems) as described (22).

Statistical analysis

Statistical differences between treatment groups were determined by a 2-tailed, unpaired Student *t*-test when appropriate. *P* values less than or equal to 0.05 were considered significant.

RESULTS

C1P activates cPLA$_2$α in a chain length-specific manner

C1Ps ranging from acyl chain lengths of 14 to 26 carbons are present in mammalian cells with $C_{16:0}$, $C_{18:0}$, and $C_{24:1}$ generally being the more abundant. However, there are reports as to the existence of chain lengths as short as two carbons (23) and many studies have utilized C_2-C1P as an exogenous agonist (1, 11, 24). In this study, the ability of various chain lengths of C1P to activate cPLA$_2$α was examined. To this end, we utilized C_2, C_6, C_{16}, and $C_{18:1}$ C1P and an established in vitro assay for cPLA$_2$α. All chain lengths of C1P except C_2-C1P substantially activated cPLA$_2$α (**Fig. 1**). C_2-C1P induced an insignificant increase in cPLA$_2$α activity similar to that of our previous reports of S1P, LPA, and PA (Fig. 1). Thus, C1P requires an acyl chain length of >2 carbons for significant activation of cPLA$_2$α.

Natural C1P is a lipid-specific inducer of AA release, cPLA$_2$α activation, and eicosanoid synthesis

The recent report by Tauzin et al. (17) elegantly and comprehensively demonstrated that the biological effects on cells, in particular PGE_2 synthesis and cell death, were not lipid specific when: *1*) high doses of lipids were used (10 μM), and *2*) the lipids were delivered using EtOH/dodecane. In 2003, our laboratory demonstrated that a lipid-specific effect on the activation of cPLA$_2$α, induction

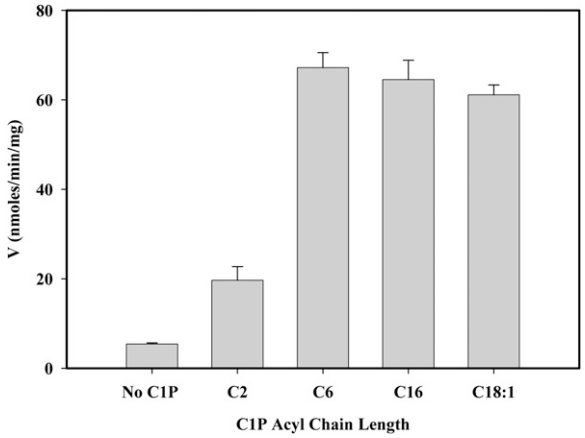

Fig. 1. Activation of cPLA$_2$α by C1P is dependent on the acyl chain length. cPLA$_2$α activity was measured as a function of PC molar concentration in the absence and presence of 4 mol % D-*erythro* C_2, C_6, C_{16}, and $C_{18:1}$ C1P for 45 min at 37°C as described in Materials and Methods. The PC mole fraction was held constant at 0.137. Data are presented as cPLA$_2$α activity measured as nanomoles of arachidonic acid produced/minute/milligram of recombinant cPLA$_2$α ± SE.

of AA release, and eicosanoid synthesis was observed for C1P when low doses of lipids (≤1 μM) were used via the EtOH/dodecane delivery system (7, 8). Furthermore, a collaborative study with Spiegel and coworkers (25) demonstrated no loss of cell viability when <5 μM of C1P was delivered to cells via EtOH/dodecane. Therefore, we hypothesized that the dose of lipids used when delivered via EtOH/dodecane was the reason for these contrasting observations, and chose to validate this lipid delivery system before proceeding to examine the chain length specificity of C1P activation of cPLA$_2$α in cells. Therefore, we first examined the effects of related/similar lipids delivered via EtOH/dodecane (98:2 v/v) on cPLA$_2$α translocation, AA release, and eicosanoid synthesis. Treatment of A549 cells with D-erythro-$C_{18:1}$ ceramide-1-phosphate, a naturally occurring sphingolipid, rapidly induced an increase in AA release (**Fig. 2A**) with concomitant increase in PGE_2 synthesis (Fig. 2B). This effect was dose-dependent with an EC_{50} of 400 nM C1P at 2 h with 200 nM C1P inducing a significant increase in AA release and PGE_2 synthesis. Therefore, treatment of cells with C1P induces activation of a PLA_2 species and induces a dose-dependent increase in AA release, which subsequently leads to eicosanoid production. As previously reported by our laboratory, C1P at ≤1 μM had no effect on cell viability ((7, 25), data not shown).

To demonstrate that the effect of C1P on AA and PGE_2 release was lipid-specific, A549 cells were also treated in the same experiments with various doses of the closely related lipid, PA, and a direct metabolite of C1P, and D-erythro $C_{18:1}$ ceramide (Cer) (Fig. 2A). Both ceramide and PA had only marginal effects on AA release (approximately 2-fold) in the submicromolar range as compared with treatment of A549 cells with the vehicle control requiring at least 750 nM for the effect. PGE_2 synthesis followed a similar pattern of induction (Fig. 2B). Higher doses of PA

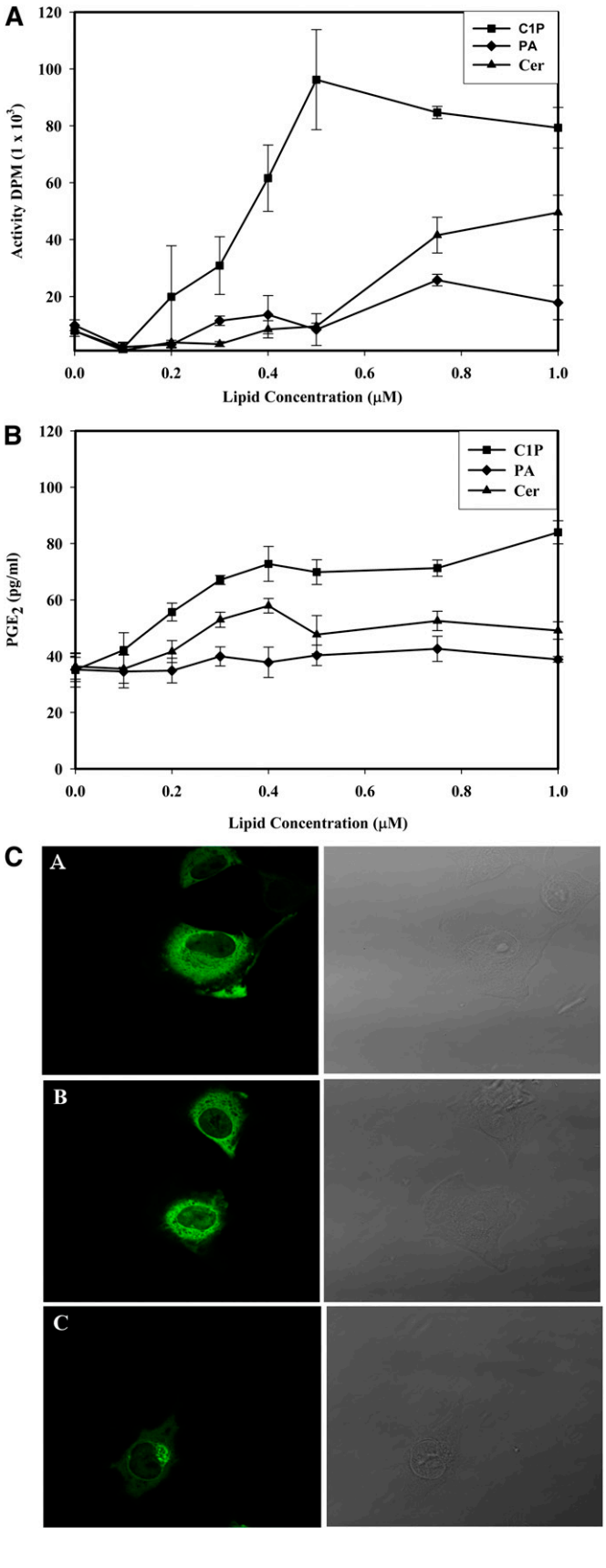

Fig. 2. The effects of natural C1P on AA release and PGE_2 synthesis. A: The effects of ceramide-1-phosphate on AA release is lipid-specific at low doses. A549 cells (5×10^4) were labeled overnight with 5 μCi/ml [^{3}H]AA (5 nM). Cells were washed and placed in DMEM supplemented with 2% fetal bovine serum for 2 h, followed by treatment with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 μM of D-e-$C_{18:1}$ C1P (■), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA) (◆), or D-e-$C_{18:1}$ ceramide (▲) solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) for 2 h. For quantification of AA release, media was transferred to 1.5 ml polypropylene tubes, centrifuged 10,000 *g*, and ^{3}H AA determined by scintillation counting. The results are presented as DPM of ^{3}H-AA per ml of media controlled for equivalent number of cells by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Data are representative of 15 separate determinations on 5 separate occasions. B: Natural C1P, but not the structurally similar PA nor ceramide, is capable of inducing PGE_2 synthesis. A549 cells (5×10^4) were washed and placed in DMEM supplemented with 2% FBS for 2 h. Cells were then treated with 0.1, 0.2, 0.4, 0.5, 0.75, and 1 μM D-e-$C_{18:1}$ C1P (■), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA) (◆), or D-e-$C_{18:1}$ ceramide (▲) solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) for 2 h. For measurement of PGE_2 levels, media were assayed according to manufacturer's instructions using the Prostaglandin E_2 monoclonal EIA Kit from Cayman Chemical (Ann Arbor, MI, Catalog No. 514010). Briefly, media containing PGE_2 competes with PGE_2 acetylcholinestaerase conjugate for a limited amount of PGE_2 monoclonal antibody. The antibody-PGE_2 conjugate binds to a goat-anti-mouse antibody previously attached to the wells. The plate is washed to remove any unbound reagents and then the substrate to acetylcholinesterase is provided. The concentration of PGE_2 in a sample is inversely proportional to the yellow color produced. The results are presented as picograms of PGE_2 per ml of media controlled for equivalent number of cells by MTT assay. Data are representative of six separate determinations on two separate occasions. C: $cPLA_2\alpha$ translocates specifically in response to C1P. A549 cells (1×10^5) were infected at 10 MOI with an adenoviral construct containing $cPLA_2\alpha$-GFP. 48 h postinfection, cells were treated with 500 nM D-e-$C_{18:1}$ Ceramide (A), 500 nM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA) (B), and 500 nM D-e-$C_{18:1}$ C1P (C), all solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) for 2 h. $cPLA_2\alpha$ localization was visualized using an Olympus BX50WI confocal microscope at 488 nM (Fluoview detector) using a 40× liquid immersable lens with a 1.5×-enhanced magnification. Data are representative of three separate determinations on two separate occasions.

(≥1 μM) did induce dramatic AA release (62% as effective as C1P on AA release) in accord with the findings of Tauzin et al. (17) (data not shown). Thus, C1P is a potent and specific effector of AA and PGE_2 release in cells when submicromolar doses of lipids were added exogenously in EtOH/dodecane.

We then examined the lipid specificity of $cPLA_2\alpha$ activation. Upon activation, $cPLA_2\alpha$ is translocated from the cytosol to associate with the Golgi and perinuclear membranes in cells (7). Therefore, to determine the lipid specificity of $cPLA_2\alpha$ activation/translocation in cells, we examined whether C1P versus PA affects the association of $cPLA_2\alpha$ with cellular membranes using cPLA2α fused to GFP. Treatment of A549 cells with 500 nM C1P for 2 h induced a significant increase of $cPLA_2\alpha$ in the Golgi and perinuclear membranes (Fig. 2C). The same doses of PA or the delivery medium alone had no effect on the translocation of $cPLA_2\alpha$ (Fig. 2C).

To determine whether the activation of $cPLA_2\alpha$ by C1P was dependent on delivery of C1P by EtOH/dodecane, naturally occurring C1P was directly sonicated in water

and delivered to NR8383 macrophages. A significant increase in AA release at 15 μM C1P was observed (**Fig. 3**). In addition, the stimulation of AA release in the macrophages was specific for C1P as other related phospholipids, S1P (data not shown) and PA, had no effect (Fig. 3). Although higher doses are required, C1P induces activation of the eicosanoid cascade irrespective of delivery vehicle and in a lipid-specific manner. The higher dose of C1P required to elicit a response, compared with delivery by EtOH/dodecane, is likely due to the lower efficiency of the delivery of C1P as vesicles.

Exogenous C1P is slowly metabolized to ceramide

Tauzin et al. (17) reported that C1P delivered by EtOH/dodecane was rapidly metabolized and its uptake was not enhanced by this delivery system. We hypothesized that this observation on uptake and metabolism may be due to the toxicity induced by using 10 μM C1P delivered with EtOH/dodecane, or the use of the unnatural analog, NBD-C1P. To determine the kinetics of C1P metabolism and examine cellular uptake, A549 cells were treated with natural (D-e-$C_{18:1}$) ceramide and C1P and the levels of these lipids were analyzed by mass spectrometry (**Fig. 4**). C1P exogenously delivered by EtOH/dodecane demonstrated a rapid increase (within 2 h) in the 18:1 acyl chain length group of endogenous C1P in A549 cells (Fig. 4). The data also demonstrate that C1P was slowly metabolized to ceramide in A549 cells (Fig. 4, inset) with only a small increase in $C_{18:1}$ ceramide upon addition of D-e-$C_{18:1}$ C1P to cells. This translated into only a 2.8% increase in total ceramide in accord with the recent collaborative publication with Mitra et al. (25). Thus, natural C1P delivered in low doses to A549 cells is not metabolized rapidly to ceramide at submicromolar concentrations.

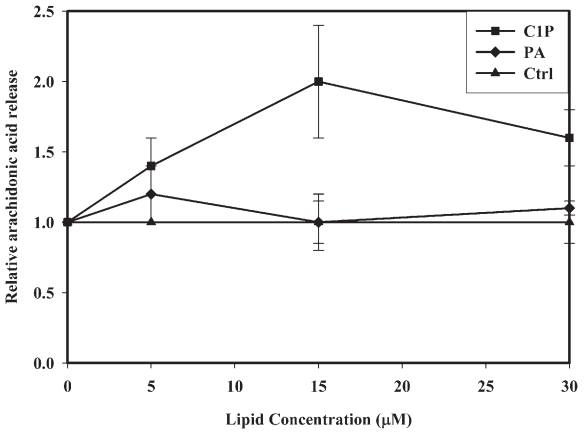

Fig. 3. Activation of cPLA$_2$α by C1P is independent of its delivery medium. [^{3}H]AA (0.20 μCi/ml) labeled NR8383 cells (1×10^6 cells/ml) were treated with C1P (◆), PA (■), and Ctrl (H_2O) (▲) sonicated in water at 5, 15, and 30 μM for 6 h. For quantification, the media was collected and centrifuged at 10000 *g* for 5 min and the amount of [^{3}H] labeled AA in the media was quantified by scintillation counting. The results are presented as DPM (dissociations per minute) of radioactivity per ml of media.

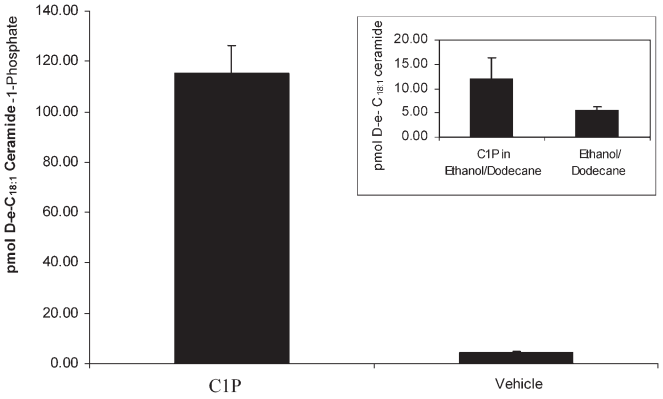

Fig. 4. C1P is efficiently delivered to cells via EtOH/dodecane and is slowly metabolized to ceramide. A549 cells (1×10^6) were treated with 500nM D-e-$C_{18:1}$ C1P for 2 h. The cells were then harvested and lipids extracted followed by analysis by mass spectroscopy. Data are presented as pmols of lipid. Inset: Exogenously added C1P is slowly metabolized to ceramide. A549 cells (1×10^6) were treated with 500nM D-e-$C_{18:1}$ C1P for 2 h. The cells were then harvested and lipids extracted followed by analysis by mass spectroscopy for C1P and ceramide (inset). Data is presented as pmols lipids. Error bars indicate SD.

C1P is delivered more efficiently to internal membranes with EtOH/dodecane

Currently, the delivery of C1P as vesicles is considered nontoxic to cells. However, the relative efficiency of this method for delivering C1P to internal membranes when compared with the EtOH/dodecane system is not known. To investigate the uptake efficiency of C1P, we used ^{32}P-labeled C1P at low doses to examine the uptake of C1P delivered via EtOH/dodecane versus vesicle-based delivery (**Fig. 5**). Following treatment (500 nM C1P for 2 h), the concentration of radiolabeled C1P in the total internal membranes was found to increase >2-fold when delivered via EtOH/dodecane versus vesicles (Fig. 5A). Thus, C1P delivered via the EtOH/dodecane method is more efficient in delivering C1P to internal membranes.

In order to further compare the intracellular membranes for lipid delivery by EtOH/dodecane versus vesicles, radiolabeled C1P (^{32}P) was delivered using both EtOH/dodecane and sonicated vesicles followed by subcellular fractionation as previously described by our laboratory. The EtOH/dodecane delivery system was more efficient at delivering C1P to all internal membranes, including the nucleus, mitochondria, *trans*-Golgi, the endoplasmic reticulum (ER), and the *cis*-Golgi when compared with the vesicular delivery system (Fig. 5B). Importantly, C1P was delivered >3-fold by EtOH/dodecane to the site of cPLA$_2$α translocation (*trans*-Golgi enriched fraction) compared with sonicated vesicles. Western analysis using antibodies against standard organelle markers confirmed the purity of organelles in each fraction (Fig. 5C) as previously described (26). Antibodies against lamin, purified mitochondria, TGN46, protein disulfide isomerase (PDI), and caveolin were used to identify nuclear, mitochondrial, *trans*-golgi, ER, and plasma membrane fractions, respectively. These markers are specific for the respective organelles

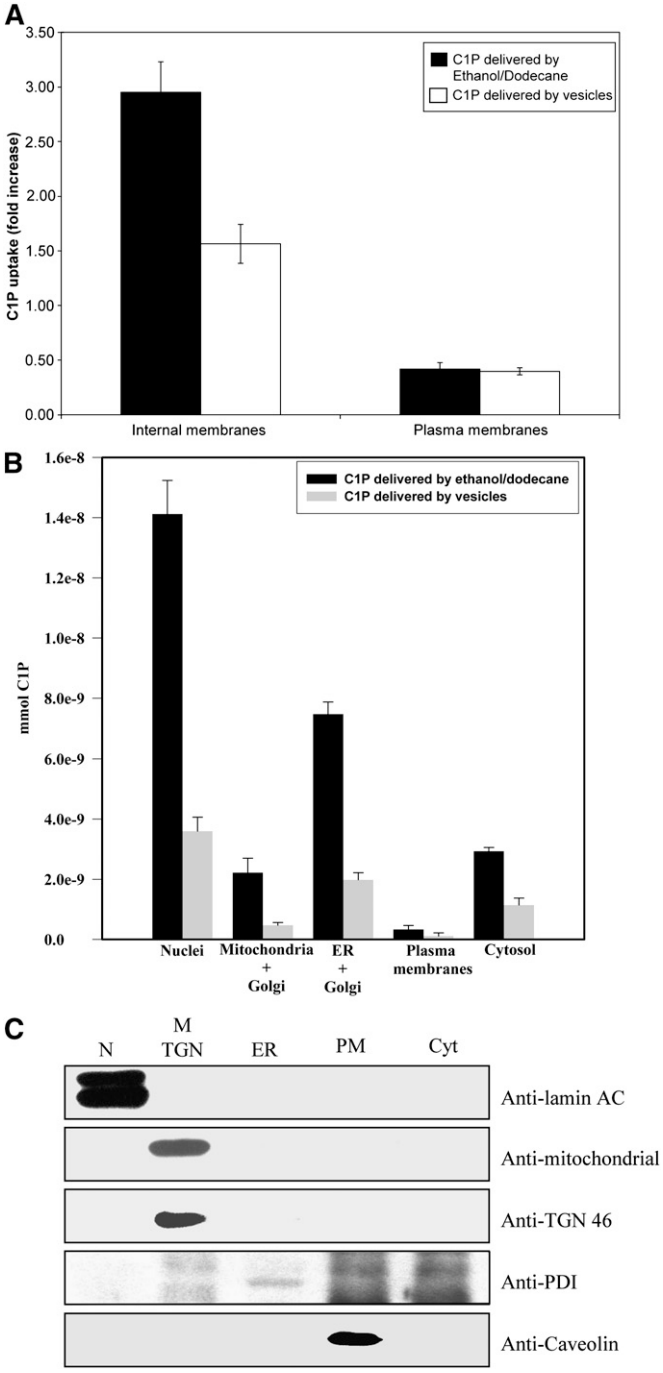

Fig. 5. C1P is efficiently taken up by cells into internal membranes when delivered via EtOH/dodecane. A: A549 cells (1×10^6) were treated for 2 h with radiolabeled 500nM D-e-$C_{18:1}$ C1P solubilized in either EtOH/dodecane or by sonication. The cells were then harvested and lysed by freeze thawing. The plasma membranes were separated from the internal membranes and the amounts of radiolabeled lipids in the different fractions measured by scintillation counting. The results are presented as the fold increase of the levels of C1P over background in each fraction for the different methods of delivery. B: C1P delivered via EtOH/dodecane system reaches specific internal membranes with higher efficiency. A549 cells (1×10^6) were treated for 2 h with radiolabeled, 500nM D-e-$C_{18:1}$ C1P solubilized in either EtOH/dodecane or by sonication. The cells were then harvested and lysed by freeze thawing and homogenized. The resultant mixture was subjected to subcellular fractionation via differential centrifugation as previously described (26). All fractions were confirmed as previously described by our laboratory (26). The results are presented as a comparison of the levels of C1P in each fraction for the different methods of delivery of C1P. C: Differential centrifugation allows the separation of the different organelles into different subcellular fractions. A_{549} cells were treated and subjected to subcellular fractionation as in 5B to obtain nuclear (N), mitochondrial and *trans*-Golgi (M,TGN), endoplasmic reticulum (ER), plasma membrane (PM) and cytosolic (Cyt) fractions. All fractions were probed with organelle specific markers to assay for purity of the fractions. Anti-lamin AC (Santa Cruz 1:1000) for nuclear, anti-mitochondrial (AbCam 1:1000), anti-TGN46 for trans-Golgi (AbCam 1:1000), anti-protein disulfide isomerase (PDI) for ER (AbCam 1:1000), and anti-caveolin 1 for plasma membrane (Santa Cruz 1:1000) were used as organelle markers. Note: only the ER fraction demonstrated the proper PDI signal. The chemiluminescence signals observed in the other fractions are nonspecific, and are not present when immunoblotting using purified PDI.

and are not detected on others. Antibodies against these markers are routinely used to confirm the purity of subcellular organelle preparations.

Long chain C1P specifically induces AA release by cPLA$_2\alpha$

Once the EtOH/dodecane delivery system was established to deliver C1P to the proper organelles and produce a lipid specific activation of cPLA$_2\alpha$, we investigated the effects of C_2, C_6, C_{16}, and $C_{18:1}$ C1P treatment on cells. Although the cellular concentration of C_2-C1P in internal membranes increased to the same extent as the $C_{18:1}$-C1P when delivered by the EtOH/dodecane delivery system (**Fig. 6A**, inset), induction of AA release was not observed (Fig. 6A). Thus, intracellular activation of a PLA$_2$ species by C1P is also chain length specific.

To investigate, whether C_2-C1P could activate cPLA$_2\alpha$ in cells, we again delivered short chain (C_2) and long chain ($C_{18:1}$) C1P to A549 cells using the EtOH/dodecane system. The levels of membrane associated and cytosolic cPLA$_2\alpha$ following C1P treatment was measured. Western blot analysis revealed a significant increase of cPLA$_2\alpha$ in the membrane fraction of the $C_{18:1}$ treated cells. However, no increase in cPLA$_2\alpha$ in the membrane fraction was observed in C_2-C1P treated cells (Fig. 6C).

To further demonstrate that activation of cPLA$_2\alpha$ is limited to naturally occurring C1P, we compared the ability of dimethyl ester of D-e-$C_{18:1}$ C1P, dihydro D-e-C_{16} C1P, and the naturally occurring D-e-$C_{18:1}$ C1P (Supplementary Fig. IA) to activate cPLA$_2\alpha$ in vitro and in cells. Compared with the natural counterpart, dimethyl C1P was not able to activate cPLA$_2\alpha$ in vitro (Supplementary Fig. IB) and was a very poor inducer of AA release from cells (Supplementary Fig. IC). Dihydro D-e-C_{16} C1P, however, activated cPLA$_2\alpha$ to the same extent as the naturally occurring C1P. This indicates that the 4,5 double bond of C1P is not required for interaction with cPLA$_2\alpha$ (data not shown).

Recently, our laboratory has identified the C1P binding site on cPLA$_2\alpha$, which allowed us to generate a mutant cPLA$_2\alpha$ (R57A/K58A/R59A) that significantly reduced binding to C1P yet has no effects on structure, basal activity,

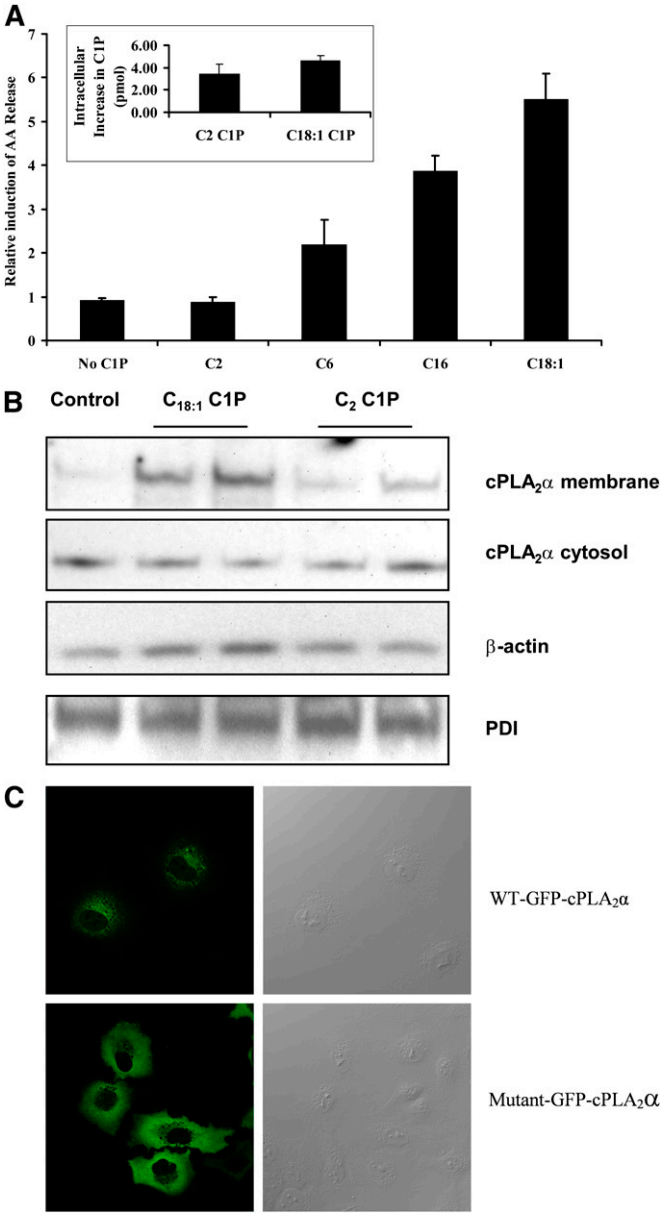

Fig. 6. Naturally occurring C1P are the best activators of cPLA$_2\alpha$ in vivo. A: A549 cells (5×10^4) were labeled overnight with 5 μCi/ml [^{3}H]AA (5 nM). Cells were washed and placed in DMEM supplemented with 2% FBS for 2 h. Cells were then treated with either EtOH/dodecane alone, media (No C1P) or 500 nM D-*erythro* C_2, C_6, C_{16}, and $C_{18:1}$ C1P solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) as previously described for C1P(7) for 2 h. For quantification of AA release, media was transferred to 1.5 ml polypropylene tubes, centrifuged at 10,000 *g*, and ^{3}H cpm (counts per minute) determined by scintillation counting. Results were controlled for equivalent number of cells quantified by WST assay. The results are presented as fold increase in AA release when compared with EtOH/dodecane treatment. The results are an average of three experiments ± SD. A, inset: EtOH/dodacane system successfully delivers both the long and short chain C1Ps to cells. A549 cells (1×10^6) were treated for 2 h with 1μM D-e-C_2 C1P or D-e-$C_{18:1}$C1P. The cells were then harvested and analyzed by mass spectrometry as previously described. Data are expressed as increase in pmol quantity of the lipid over that of the controls. B: cPLA$_2\alpha$ translocates to the membrane in response to natural long chain C1P but not the short C_2-C1P. A549 cells (1×10^5) were infected at 10 MOI with an adenoviral construct containing cPLA$_2\alpha$-GFP. 48 h postinfection, cells were treated with 500 nM D-e-$C_{18:1}$ C1P or C_2-C1P solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) for 2 h. The cells were subsequently lysed and centrifuged at 100,000 *g* to separate membranes from the cytosol. Equal total protein from each fraction was subjected to western analysis and probed for the indicated proteins. Data are representative of six separate determinations on two separate occasions. C: Translocation of cPLA$_2\alpha$ in response to C1P is due to a direct interaction with C1P. A549 cells (1×10^5) were infected at 10 MOI with an adenoviral constructs containing wild-type and mutant (R57A/K58A/R59A) cPLA2α-GFP. 48 h postinfection, cells were treated with 1μM D-e-$C_{18:1}$ C1P solubilized in 2% dodecane/98% EtOH (final concentration in treatments was 0.002% dodecane/0.098% EtOH) for 3 h. cPLA$_2\alpha$ localization was visualized using an Olympus BX50WI confocal microscope at 488 nM (Fluoview detector) using a 40× liquid immersable lens with a 1.5×-enhanced magnification. Data are representative of three separate determinations on two separate occasions.

or the calcium response (27). Using GFP-tagged wild-type and mutant cPLA$_2\alpha$, we investigated their translocation in response to $C_{18:1}$-C1P delivered by EtOH/dodecane in A_{549} cells. Confocal analysis revealed >3-fold translocation of the wild-type cPLA$_2\alpha$ compared with the mutant in response to $C_{18:1}$-C1P (Fig. 6D). As all other functionalities of the mutant cPLA$_2\alpha$ are the same as the wild-type, we conclude that activation of cPLA$_2\alpha$ by C1P is via a direct interaction and not through an indirect effect of other nonspecific biologies associated with the lipid.

DISCUSSION

Our laboratory reported in 2003 that C1P delivered in EtOH/dodecane stimulated cPLA$_2\alpha$ in A549 cells in a lipid-specific manner (7). However, the acyl chain length specificity of C1P for this interaction was not examined. In both in vitro and cellular studies, C_2-C1P was not able to significantly increase AA release over control. Therefore, this study demonstrates that an acyl chain length of more than two carbons was necessary for activation of cPLA$_2\alpha$. Furthermore, data from cellular studies in which C1P was delivered in EtOH/dodecane indicate that the naturally occurring C1Ps are the most potent activators of cPLA$_2\alpha$, with C_{16} and $C_{18:1}$ C1P giving over a 4-fold increase in AA release over C_2-C1P. The demonstration that the dimethyl analog of D-e-$C_{18:1}$ C1P was unable to activate cPLA$_2\alpha$ further strengthens the argument that activation of the enzyme occurs only in the presence of naturally occurring analogs of C1P.

These findings have relevance to the reported biological mechanisms attributed to exogenous C1P treatment. For example, several studies using C_2 C1P have shown intracellular increases in Ca^{+2} (10, 11). As cPLA$_2\alpha$ is a calcium-stimulated enzyme, it may be argued that the activation of cPLA$_2\alpha$ by C1P is due to an increase in intracellular calcium. The current data clearly demonstrate that C_2 C1P does not cause activation of cPLA$_2\alpha$ in vitro nor release AA through the activation of cPLA$_2\alpha$ when delivered to cells. Thus, the activation of cPLA$_2\alpha$ by C1P is not via an increase in the intracellular Ca^{+2} levels, corroborating our earlier

findings that ceramide kinase is required for activation of cPLA$_2\alpha$ by calcium ionophores. Furthermore, induction of calcium release observed in response to C_2-C1P is not via the reverse mechanism of activation of cPLA$_2\alpha$ and subsequent generation of PGE_2, a known inducer of Ca^{+2} release.

Thus, the current study also highlights the usefulness of C_2-C1P in studying the biological effects of C1P that are independent of the activation of cPLA$_2\alpha$. For example, the stimulation of DNA synthesis and cell division observed by exogenous treatment with C_2 C1P (1) is not due to any downstream effects of the activation of cPLA$_2\alpha$ and increases in eicosanoids. Therefore, C_2-C1P is now a "tool" to examine noneicosanoids biologies regulated by C1P. Furthermore, C_2-C1P may also be used to differentiate between direct targets of cPLA$_2\alpha$ and A-SMase.

This study also provides additional proof that translocation of cPLA$_2\alpha$ to membranes in response to C1P is via a direct interaction, as C1P binding site mutants showed significantly reduced translocation (Fig. 6C). This mutant can now be used as a tool to investigate C1P independent translocation of cPLA$_2\alpha$. As the mechanisms and triggers behind the generation of C1P is not currently understood, this is an important tool to differentiate between agonists causing C1P-independent translocation and those causing translocation via a direct increase in C1P.

The observation that cPLA$_2\alpha$ is activated by long chain naturally occurring C1P is in agreement with our previous work (19) with regards to ceramide kinase, the only known mammalian enzyme to date to produce C1P. Substrate preference of CERK is for ceramides containing acyl chains of at least 12 carbons (19). Confocal studies demonstrate that, in A_{549} cells, CERK localizes to the Golgi apparatus, the site of AA release by cPLA$_2\alpha$. Thus, it is quite clear that ceramide phosphorylation by CERK is geared toward producing C1P causing maximal activation of cPLA$_2\alpha$.

This study also addresses the recent publication by Tauzin et al. (17), which raised doubts as to the lipid specificity of the activation of cPLA$_2\alpha$ by C1P when delivered via the well-established EtOH/dodecane system. We specifically show that the stimulation of AA release in the macrophages and A549 cells was specific for C1P as other related phospholipids such as ceramide and PA failed to do so. The lipid-specific effect required the use of low doses of C1P ($\leq$1 μM) as previously reported by our laboratory. C1P at concentrations $\leq$500 nM demonstrated complete lipid specificity in the induction of AA release from A549 cells. In accord with the recent report by Tauzin et al. (17), lipid specificity was lost as concentrations of phospholipids increased above 1 μM. The loss of lipid specificity correlated with the loss of cell viability as recently reported by Mitra et al. (25) and Tauzin et al. (17). Thus, our study demonstrates that the contrasting findings between the two laboratories was the difference in concentration of C1P utilized. C1P is indeed a specific activator of AA release and cPLA$_2\alpha$ activation when low concentrations are utilized and no loss of cell viability is observed.

In agreement with the lipid-specific effect of C1P on AA release, the presented data also shows that C1P dispersed in water interacts readily with NR8383 macrophages to induce AA release. This was also found in A549 cells but higher concentrations were required than with the use of the EtOH/dodecane system (data not shown). The observation that C1P can activate cPLA$_2\alpha$ in the absence of dodecane, or any other organic solvent, is also relevant because it discards any possible nonspecific interaction of the phospholipid with the organic compounds used for its delivery to cells in culture. In this regard, it should be emphasized that the stimulatory effect of C1P on proliferation of rat-1 fibroblasts and the inhibition of apoptosis in bone marrow-derived macrophages were all observed using C1P dispersed in water in the absence of any organic solvent (1, 3, 4). Therefore, ideally, lipids should be delivered in aqueous solutions so as to avoid any side effects that might be generated when organic solvents are added to biological tissues or cells in culture. Unfortunately, high concentrations of the phospholipids are required and in the case of some cell types (e.g., A549 cells), vesiculated phospholipids are not as efficiently transported to certain internal membranes (e.g., *trans*-Golgi). In these cases, the dodecane/alcohol delivery system is an alternative for the enhancement of lipid uptake.

In conclusion, the presented study answers the contrasting observations from several laboratories on the biological effects of lipids delivered by EtOH/dodecane. This study also demonstrates that the alcohol/dodecane delivery system can be used to examine biological effects by specific lipids as long as certain controls are observed. In particular, researchers need to use low doses of lipids, less than 1 μM. Doses higher than 1 μM have effects on cell stress and viability, which can cause misinterpretation of results. The metabolism of the lipid is also of key importance as well as uptake; thus, closely-related lipids, as well as direct metabolites of similar solubilities, should be used as specificity controls. Importantly, all of these parameters need to be established for each specific cell type as viability may be affected at lower concentrations. Furthermore, monitoring of efficient uptake of the lipid should also be undertaken. With these measures observed, this study demonstrates that the dodecane delivery system for lipids can be used to study specific biological effects, especially when coupled to genetic, cell biology, and enzymology approaches. Finally, this study also demonstrates that the C1P-cPLA$_2\alpha$ interaction is structurally specific with proper acyl chain length being an essential criterion for activation of the enzyme. Furthermore, cellular biologies observed from treatment of cells with C_2-C1P cannot be attributed to cPLA$_2\alpha$ activation and subsequent eicosanoids synthesis.

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