Effects of ceramide-1-phosphate on cultured cells: dependence on dodecane in the vehicle[S]

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Abstract Ceramide-1-phosphate (C1P), the product of ceramide kinase, is a sphingophospholipid with recently recognized signaling properties. In particular, it was reported to be mitogenic and capable of direct stimulation of cytosolic phospholipase $A_{2\alpha}$. Much of the present knowledge has relied on the use of C1P of various acyl chain lengths, together with diverse protocols to deliver it to cultured cells. A mixture of ethanol (or methanol) with dodecane, as the vehicle, has become popular. However, the contribution of this solvent to the observed effects of C1P has not been documented. Here, we show that addition of C1P in ethanol-dodecane to culture medium leads to irreversible cytotoxic effects. These culminate in mitochondrial swelling, vacuole formation, and cell death. Not only the toxicity of C1P, but also its ability to trigger prostaglandin E_2 release, is fully dependent upon addition of a premade C1P-dodecane mixture. Furthermore, we show that these effects are not restricted to C1P. They result from the capacity of dodecane to interact with phospholipids; hence, they go undetected with a vehicle control. This study should raise awareness about the use of dodecane for phospholipid delivery and, in turn, help in unraveling C1P signaling, which is still poorly understood.—Tauzin, L., C. Graf, M. Sun, P. Rovina, N. Bouveyron, M. Jaritz, A. Winiski, N. Hartmann, F. Staedtler, A. Billich, T. Baumruker, M. Zhang, and F. Bornancin. **Effects of ceramide-1-phosphate on cultured cells: dependence on dodecane in the vehicle. *J. Lipid Res.* 2007.** 48: **66–76.**

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Ceramide-1-phosphate (C1P) was first described in brain synaptic vesicles, HL60 cells, and neutrophils (1–3). Recent insight since the cloning of ceramide kinase (4), the only known enzyme capable of generating C1P from ceramide, has provided evidence that C1P is endowed with signaling functions. C1P was reported to participate in eicosanoid synthesis (5), in which it synergizes with sphingosine-1-phosphate (6). This led to proposal of the cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) as the first molecular target of C1P (7). Other studies have suggested a role of ceramide kinase/C1P in mast cell degranulation (8), regulation of acidic sphingomyelinase and control of apoptosis in bone marrow-derived macrophages (9), calcium mobilization in GH4C1 pituitary and Jurkat T-cells (10, 11), and dopamine release in PC12 cells (12). A role for C1P in fibroblast proliferation was reported a decade ago (13, 14). More recently, C1P was shown to activate phosphatidylinositol 3-kinase and proposed to have a role in macrophage survival (15).

Whether C1P is present in the bloodstream or other extracellular fluids and can be taken up by cells under physiological conditions is currently unknown. Whether C1P interacts with cell surface receptors or needs to be transported across the plasma membrane is equally unknown. Nevertheless, many laboratories, including ours, have tried to elucidate ceramide kinase signaling pathways by adding exogenous C1P to cells in culture. Although reports are still not numerous, there is already complexity attributable to variability in the choice of the acyl chain length of the C1P used, or in the choice of the organic solvent used, such as DMSO (10, 11), methanol/chloroform (16), or ethanol (or methanol)/dodecane (5–7, 14); others have prepared fine dispersions of C1P by sonication in aqueous medium (13, 15). The ethanol (or methanol)/dodecane mixture, in particular, used at 98:2, was first reported 10 years ago (14) and proposed to be useful to elucidate the biological functions and mechanisms of

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Abbreviations: ATCC, American Type Culture Collection; C1P, ceramide-1-phosphate; $cPLA_{2\alpha}$, cytosolic phospholipase $A_{2\alpha}$; ER, endoplasmic reticulum; LAMP2, lysosome-associated membrane protein 2; NBD-Cer, nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide; PA, phosphatidic acid; PG, phosphatidylglycerol; PGE_2, prostaglandin E_2; YFP, yellow fluorescent protein.

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[S] The online version of this article (available at http://www.jlr.org) contains supplemental data in the form of 2 figures and 1 table.

action of C1P. Since then, it has become increasingly popular. In particular, two described processes, the mitogenicity of C1P (14) and its ability to stimulate $cPLA_2$ (5–7), have relied on the use of dodecane.

When addressing the capacity of C1P to act as a mitogenic signal in fibroblasts, we observed, unexpectedly, that this phospholipid was toxic in the conditions used. The study reported here was undertaken to understand the molecular and cellular basis of these toxic effects.

EXPERIMENTAL PROCEDURES

Materials

Absolute ethanol and methanol were from Merck, and dodecane was from Aldrich. C8-ceramide and C8-C1P were from Cayman, C18-ceramide was from Avanti, and natural C1P (a mixture of C18-C1P and C24-C1P isolated from bovine brain) as well as C8-phosphatidic acid (C8-PA) and phosphatidic glycerol were from Sigma. Nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-Cer) was from Molecular Probes. HP-TLC plates were from Merck. Lipoprotein-free FBS and LDL were from Sigma.

Plasmids

Human lysosome-associated membrane protein 2 (LAMP2) cDNA was generated by PCR from a human multiple tissue cDNA library (Clontech) using primer A (5′TCCGCTCGAGGTCGCCGCCTGCTCTGCGGGGTC) and primer B (5′GCGCGGATCCTGGTGGTGGTGGTGGTGCAGAGTCTGATATCCAGCATAACTTTT). For mammalian expression, a plasmid containing LAMP2 fused to yellow fluorescent protein (YFP), was constructed by in-frame insertion of the generated LAMP2 cDNA into the pEYFP-N1 vector, between XhoI and BamHI sites. LAMP2 was fused to the N terminus of YFP with a 6-histidine linker in between. The plasmid was verified by DNA sequencing on the entire fusion insert. Golgi-YFP plasmid was obtained from Clontech.

Cell culture and treatments

COS-1 cells (used in Figs. 2 and 5–7 below), obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, were cultured in DMEM with 10% FBS at 37°C and 5% CO_2 in a humidified atmosphere. Cells were seeded on 24-well plates at 0.25×10^4 cells/cm^2 (0.06×10^4 cells/cm^2 for Fig. 2). Treatments were performed in 1% FBS-containing DMEM for 24 h, unless stated otherwise, and residual viability was measured using Alamar Blue (Serotec). A549 cells (used in Figs. 1 and 9 below), obtained from the American Type Culture Collection (ATCC), were grown in low-glucose DMEM with 10% FBS. For experiments, cells were seeded at 0.15×10^5 cells/cm^2. The FBS content was reduced to 2% at 2 h before treatments. After 2–5 h of treatment, prostaglandin E_2 (PGE_2) release was assayed in culture supernatants (see below). Primary fibroblasts (used in Figs. 2–4 below) were isolated from Balb/c mouse kidneys, grown in DMEM plus 10% FBS, and used within 7–10 days of isolation under the same conditions as for A549 cells, with seeding densities between 0.08 and 0.15×10^5 cells/cm^2. Wild-type CHO cells (used in Fig. 8 below and supplementary Fig. II), obtained from the ATCC, were maintained in Ham's F-12 medium supplemented with 10% FBS (HyClone, Logan, UT). CV-1, a monkey kidney fibroblast cell line (used in Fig. 8 below), was obtained from the ATCC and grown in medium containing 90% Eagle's MEM and 10% FBS. For cell treatment, FBS content was reduced to 2%. C8-C1P was used throughout the study.

Assays

For viability assays, we used resazurin (Alamar Blue; Serotec), a redox indicator whose reduction into resofurin is dependent on cellular reducing equivalents provided by the mitochondrial respiratory chain (17). Assays were performed after 18–24 h treatment periods, except for those shown in Fig. 3 below, which immediately followed the removal of conditioned medium for PGE_2 measurement. Cells were incubated with 10% (v/v) Alamar Blue at 37°C for 1.5–2 h in 1% serum-containing medium (COS-1 cells) or 10% serum-containing medium (A549 and primary fibroblasts). Fluorescence was recorded (excitation, 530 nm; emission, 600 nm; cutoff, 590 nm) in a Spectramax Gemini XS Fluorescence Microplate reader (Molecular Devices). Viability was assessed with the LIVE/DEAD system (Molecular Probes), which relies on two dyes, calcein AM and ethidium homodimer. Lactate dehydrogenase release was assayed with CytoTox-ONE (Promega).

PGE_2 measurements were performed using a commercially available enzyme immunoassay (Cayman 514010); after a short centrifugation, cell culture supernatants were diluted with assay buffer 1:2 to 1:5, and the assay was performed according to the manufacturer's instructions.

Fluorescence microscopy

COS-1 cells were seeded into four chambered coverslips (Lab-TEK II; Nalge Nunc) at 2×10^4 cells per chamber. Twenty-four hours after seeding, cells were switched to 1% FBS-containing medium and treated. Live-cell fluorescence microscopy was done on an Axiovert 200 M inverted microscope equipped with an AxioCam MRc high-resolution microscopy camera (Zeiss) and Plan-Neofluar 40×/1.30 oil differential interference contrast and Plan-Apochromat 63×/1.40 oil differential interference contrast objectives.

LAMP2-YFP, Golgi-YFP, or MitoTracker Green fluorescence in living CHO and CV1 cells was captured using charge-coupled device imaging systems that include standard filters for YFP or FITC, objectives of 100× magnification, a Nikon TE2000 microscope, and a CoolSnap HQ Monochrome charge-coupled device camera (Photometrics). The CoolSnap HQ camera was controlled with MetaMorph software (Universal Imaging Corp.). To visualize mitochondria with phase-contrast time-lapse imaging, cells were cultured in Lab-TEK chambered coverslips and imaged with a Hamamatsu electron microscopy camera and a Nikon TE300 microscope with 100× objective. Cells were kept in the LiveCell incubation system mounted on the Hamamatsu/Nikon TE300 imaging system, with 5% CO_2 supply at 37°C, during the imaging intermission to keep them growing normally.

Preparation of NBD-C1P

NBD-C1P was prepared from NBD-Cer, using recombinant *Escherichia coli* diacylglycerol kinase (Calbiochem) and a cardiolipin/octylglucosyl-based micellar assay, according to published procedures (18). NBD-C1P was separated from NBD-Cer by preparative HP-TLC using butanol-acetic acid-water (3:1:1) as the developer. NBD-C1P was extracted two times with $CHCl_3$/methanol/HCl (100:100:1) with vortexing followed by sonication, concentrated in a SpeedVac, and washed twice with ethanol. The obtained NBD-C1P, pure to 97%, was quantified with a spectrophotometer, based on the spectral characteristics of the NBD moiety, and dried down until use.

DNA microarray analysis

A549 cells were grown in 75 cm^2 flasks (1.1×10^6/flask; day 1) in low-glucose DMEM with 10% FBS. On day 3, FBS was reduced to 2%. After a 2 h preincubation, cells were stimulated in quin-

Supplemental Material can be found at:
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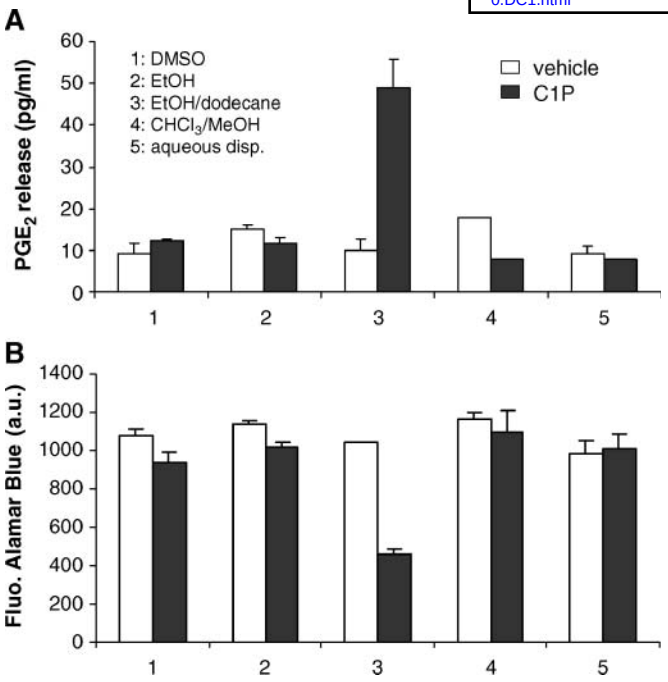


Fig. 1. Dodecane is needed for ceramide-1-phosphate (C1P)-induced prostaglandin E_2 (PGE_2) release. A549 cells were grown and treated as described in Experimental Procedures. After 2 h of treatment with 10 μM C8-C1P in DMSO (1), ethanol (EtOH) (2), ethanol-dodecane (98:2) (3), chloroform-methanol ($CHCl_3$/MeOH; 50:50) (4), and sonication in medium (5), the cell culture supernatant was processed for PGE_2 release measurement (A), and the cell monolayer was further incubated for 20 h before viability was assessed with Alamar Blue (B). White bars, vehicle alone; black bars, C8-C1P (10 μM). Data shown are means ± SD. a.u., arbitrary units.

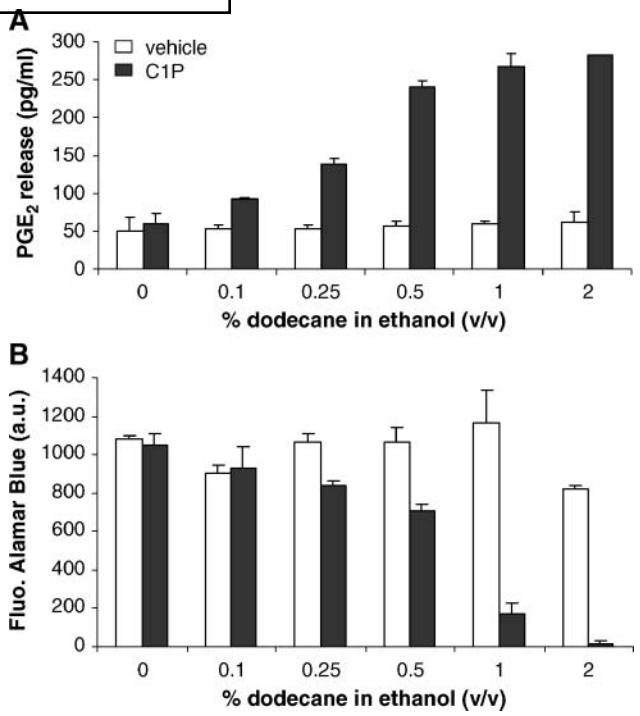


Fig. 2. PGE_2 release and decreased viability as a function of dodecane concentration. A: Primary fibroblasts were incubated for 2 h with 10 μM C8-C1P prepared from a 10 mM ethanolic solution with various dodecane concentrations (0.1–2%), and PGE_2 was measured in cell culture supernatants. B: COS-1 cells were treated for 24 h with the same solutions as in A, and residual viability was assessed with Alamar Blue. White bars, vehicle alone [ethanol-dodecane (98:2); 0.1%]; black bars, C8-C1P (10 μM). Data shown are means ± SD. a.u., arbitrary units.

tuplicate with medium only (1 h), vehicle only [0.1% ethanol-dodecane (98:2)] (1 and 3 h), or 10 μM C1P in ethanol-dodecane (1 and 3 h). Cells were harvested with trypsin and spun. Cells were lysed for total RNA extraction using Stratagene's Absolutely RNA Miniprep (400800), and the RNA was stored at −70°C until target labeling. RNA integrity was confirmed using denaturing agarose gel electrophoresis with 1% SeaKem Agarose LE (Cambrex) prepared in 1% formaldehyde/MOPS buffer (Ambion) as well as with the RNA 6000 Nano LabChip system on Bioanalyzer 2100 (Agilent Technologies). The experiment was conducted on a GeneChip® Human Genome 133 2.0 Plus Expression Array (Affymetrix). The oligonucleotide probes were 25-mers, and 11 probe pairs per sequence were used. Double-stranded cDNA synthesis was carried out with 5 μg of total RNA (SuperScript double-stranded cDNA synthesis kit; Invitrogen) according to the manufacturer's instructions. After purification (QIAquick; Qiagen), the cDNA was transcribed in vitro in the presence of biotinylated ribonucleotides (Bioarray high-yield T7 DNA transcription kit; ENZO Life Sciences, Inc.). The labeled copy RNA was purified on an affinity resin (RNeasy; Qiagen), quantified, and fragmented into strands of ~50–200 nucleotides in length. Hybridization was carried out at 45°C for ~18 h. The arrays were stained on a GeneChip Fluidics Workstation 450 and scanned on GeneArray Scanner 3000 according to the manufacturer's technical manual (Affymetrix). The scanned images were converted into numerical values of signal intensity and into categorical expression-level measurements using Micro Array Analysis Suite version 5.0 software (MAS5; Affymetrix). The signal intensities were uploaded into GeneSpring (Agilent Technologies) for filtering and statistical analysis. First, the signal intensities of each sample were centered on 1 through division by the individual sample mean. Then, the treatment samples (C1P) were normalized toward the control samples (vehicle). The probe was subsequently filtered for present or marginal flags, MAS5 norm values > 50, and GeneSpring Error Model-derived control values > 30.34. On average, a fraction of ~38% of all probe sets passed these filters. Finally, we filtered for Benjamini and Hochberg false-discovery rate-adjusted *t*-test *P* values < 0.01, which should reflect significant changes from 1.

RESULTS

Dodecane is needed for C1P-induced PGE_2 release

The effects of exogenously added C1P in cells that have been reported in the literature are based on the use of C1P either dispersed in aqueous medium (13, 15) or dissolved in DMSO (10, 11), in chloroform-methanol (16), or in a mixture of ethanol (or methanol) and dodecane (5–7, 14). To evaluate and compare these vehicles for C1P delivery, we used an already described functional readout, such as the capacity of C1P to activate $cPLA_{2\alpha}$ and trigger PGE_2 production (5). We treated A549 cells with 10 μM C8-C1P, dissolved in DMSO, ethanol, ethanol-dodecane (98:2), or chloroform-methanol (50:50), or dispersed in medium after sonication. As seen in **Fig. 1**, only when solubilized in

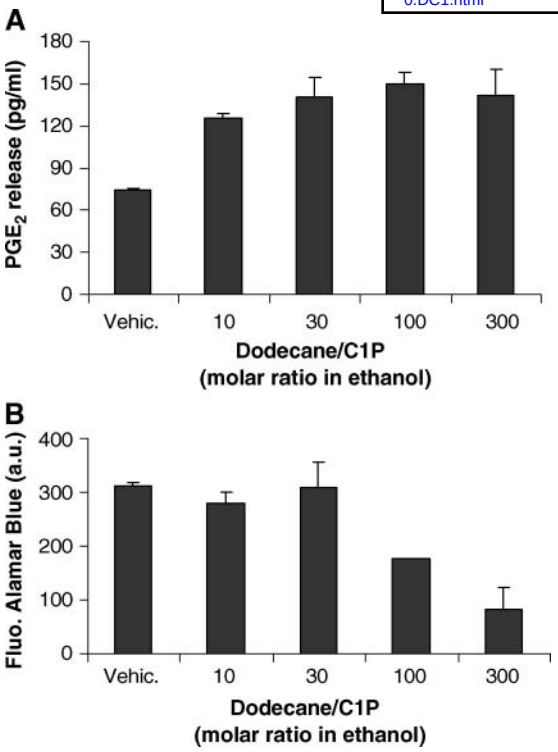


Fig. 3. The C1P-dodecane mixture. Primary fibroblasts were incubated for 5 h with 1 μM C8-C1P prepared from various stock solutions: 10, 3, 1, and 0.3 mM in ethanol-dodecane (98:2), leading to the following respective dodecane/C1P molar ratios: 10, 30, 100, and 300. Final vehicle concentration was adjusted to 0.3% for all conditions, by separate injection of ethanol-dodecane (98:2). Cell culture supernatants were collected for PGE_2 measurements (A), and cell monolayers were immediately incubated in the presence of Alamar Blue to record cell viability (B). Data shown are means ± SD. a.u., arbitrary units.

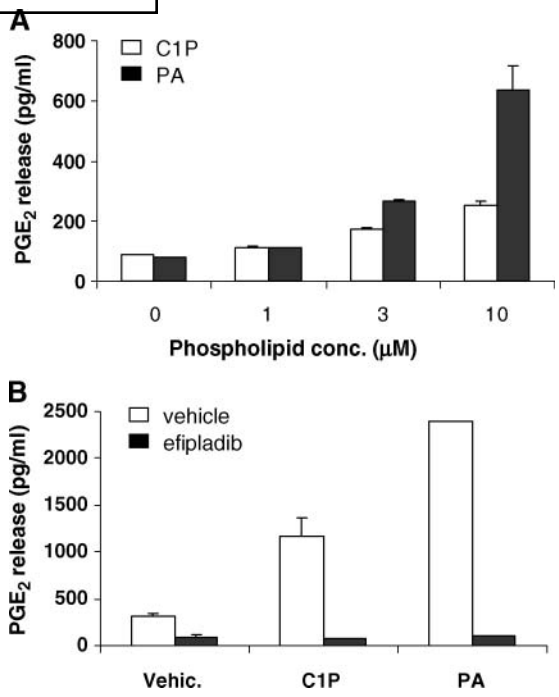


Fig. 4. Phosphatidic acid (PA) in ethanol-dodecane can trigger cytosolic phospholipase A_2 ($cPLA_2$)-dependent PGE_2 release. A: Primary fibroblasts were treated for 2 h in the presence of various concentrations of C8-C1P (white bars) or C8-PA (black bars) made from 10 mM stock solutions in ethanol-dodecane (98:2). Cell culture supernatants were collected for PGE_2 measurement. B: Fibroblasts were treated as in A with 10 μM lipid either in the absence (white bars) or presence (black bars) of 50 nM 4-[3-[5-chloro-2-[2-[[[(3,4-dichlorophenyl)methyl]sulfonyl]amino]ethyl]1-(diphenylmethyl)-1H-indol-3-yl]propyl]benzoic acid (efipladib), and cell culture supernatants were collected for PGE_2 measurements. Data shown are means ± SD.

ethanol-dodecane could C1P stimulate PGE_2 release after 2 h of incubation. Ethanol alone did not enable C1P to trigger PGE_2 release. Of note, when viability was assayed subsequently, a significant decrease was observed, but only with cells that had been treated with C1P in ethanol-dodecane. Neither on PGE_2 release nor on viability did the vehicle itself (ethanol-dodecane) exhibit any effect of its own.

PGE_2 release and decreased viability as a function of dodecane concentration

In the experiment described above, we used a 2% dodecane-containing ethanolic solution to vehicle C1P, as described in the original study (14). We then examined the proportion of dodecane needed to support C1P-mediated effects. The lowest concentration of dodecane tested [0.1% (v/v) in ethanol] was already effective, resulting in some PGE_2 release (**Fig. 2**). PGE_2 release increased as a function of dodecane concentration, reaching a plateau between 0.5% and 1% dodecane. Strikingly, induction of PGE_2 release and cell death occurred in parallel with the concentrations of dodecane in ethanol used to solubilize C1P (Fig. 2).

The dodecane/C1P mixture

The dependence of C1P-mediated effects on the concentration of dodecane, although the vehicle alone had no effect, led us to consider whether C1P was able to interact with dodecane. Therefore, we used a low final concentration of C1P, 1 μM, which when arising from an original preparation made at 10 mM in ethanol-dodecane (98:2) is poorly effective on PGE_2 release. In this ethanolic stock preparation, the molecular ratio of dodecane to C1P is ∼10. We compared the effects of C1P at 1 μM arising for such a stock preparation with the effects of C1P at 1 μM but arising from 3, 1, or 0.3 mM stock preparations in ethanol-dodecane (98:2) (i.e., with respective dodecane/C1P molar ratios of 30, 100, or 300). By separate injection of ethanol-dodecane (98:2), the final concentration of ethanol-dodecane was adjusted to be identical everywhere at 0.3%. C1P at 1 μM, in all conditions, was able to induce PGE_2 release (**Fig. 3**, upper panel). The resulting toxicity, however, was highly dependent upon the molar ratio of dodecane/C1P in the original preparations (Fig. 3, lower panel). The higher this molar ratio, the more toxic 1 μM C1P was. If one takes the toxicity into ac-

count to correct the potential of C1P to stimulate PGE_2 release (5), this would imply that the higher the molar ratio of dodecane to C1P, the more effective C1P appears to be. This experiment suggests that dodecane in the vehicle interacts with C1P in a way that cannot be recapitulated by an independent injection of vehicle at the time of cell treatment. Therefore, how the stock solution is prepared affects the results and the interpretations thereof. In the remainder of this study, except when stated otherwise, we used 10 μM C1P arising from 10 mM stock solutions made in ethanol-dodecane (98:2) (i.e., dodecane/C1P ≈ 10).

Other phospholipids in ethanol-dodecane can induce PGE_2 release: dependence on $cPLA_2$

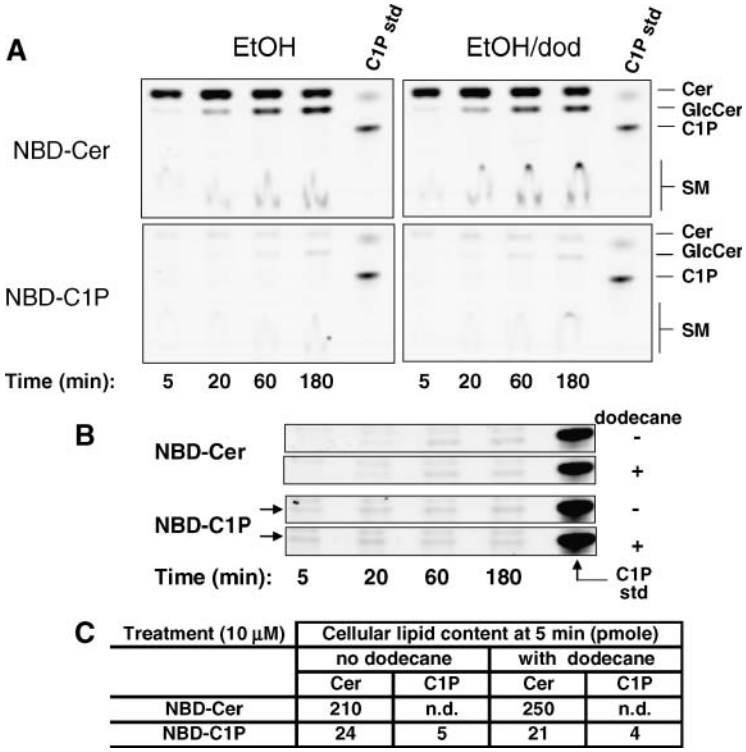
Evidence was provided both in vitro and in cell-based assays that $cPLA_{2\alpha}$ is a direct target of C1P (5–7). Furthermore, the selectivity toward C1P was established using lipid vesicles of various composition (7). Given the evidence reported above for a combination of C1P and dodecane as a basis for the induction of PGE_2 release, we wanted to examine the case of other phospholipids. As shown in **Fig. 4A**, PA was also able to promote PGE_2 release in fibroblasts in a concentration-dependent manner. That this is dependent upon $cPLA_2$ activity was established using the selective $cPLA_{2\alpha}$ inhibitor, efipladib (19) (Fig. 4B). Therefore, it appears that the capacity of phospholipids to stimulate a $cPLA_2$-dependent PGE_2 release when presented to cells in ethanol-dodecane is not restricted to C1P. Phosphatidylglycerol (PG) was also tested and showed enhanced PGE_2 release as well (see supplementary Fig. I). As noted above for Fig. 3, the final amplitude of the measured PGE_2 release seemed to inversely correlate with the induced toxicity of the respective phospholipid (Fig. 4 and supplementary Fig. I).

Dodecane does not improve the uptake of fluorescent C1P

The first study reporting the use of dodecane with C1P (14) mentioned its benefit to enhance C1P penetration into cells. We synthesized NBD-C1P from NBD-Cer and studied the cellular uptake of both lipids in the presence and absence of dodecane. As seen in **Fig. 5A**, NBD-Cer itself was readily absorbed and metabolized into glucosylceramide and sphingomyelin, regardless of the presence of dodecane. In sharp contrast, very little fluorescence was associated with cells treated with NBD-C1P (Fig. 5A). Most of it likely arose from the dephosphorylation of NBD-C1P and subsequent conversion to glucosylceramide and sphingomyelin. Only minute amounts of NBD-C1P were found associated with the cells after 5 min of incubation, which dodecane did not significantly increase (Fig. 5B, C). Therefore, the effects of dodecane may not result from increased delivery of C1P to cells.

C8-C1P with dodecane, but not C8-Cer with dodecane, is toxic to cells: protection by serum LDL

We compared the influence of dodecane on cell viability when used as a vehicle for Cer and C1P. **Figure 6A** shows that concentrations of either C8-C1P or C8-Cer < 0.5 μM are well tolerated and might even slightly increase viability compared with vehicle alone. However, at >0.5 μM, C8-C1P was found to be very toxic, with an EC_{50} of ~3 μM. This EC_{50} value matches the concentrations that have been used to date to characterize C1P effects and reported



C

Treatment (10 μM)	Cellular lipid content at 5 min (pmole)			
	no dodecane		with dodecane	
	Cer	C1P	Cer	C1P
NBD-Cer	210	n.d.	250	n.d.
NBD-C1P	24	5	21	4

Fig. 5. Dodecane does not improve the cellular uptake of fluorescent C1P. A: COS-1 cells were incubated in 1% FBS-containing medium with 1 μM nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-Cer) or 1 μM NBD-C1P, prepared as described in Experimental Procedures. Fluorescent lipid stock solutions were prepared in ethanol (EtOH) or in ethanol-dodecane (EtOH/dod; 98:2). At the indicated times, cells were harvested and their lipid contents analyzed by thin-layer chromatography. Prepared fluorescent C1P (130 pmol) was loaded in parallel as a control. Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin. B: An overexposed TLC plate area, allowing for detection of C1P. The arrows indicate NBD-C1P levels that can be detected in NBD-C1P-treated cells at early time points; dodecane did not influence those levels. C: In NBD-C1P-treated cells, the level of NBD-Cer formed upon dephosphorylation was the major cell-associated fluorescent lipid species after 5 min. It reached ~10% of that of NBD-Cer-treated cells. At 5 min, the level of NBD-C1P that associated with cells in NBD-C1P-treated cells was no more than 20% of the level of NBD-Cer that occurred through dephosphorylation of NBD-C1P. This proportion decreased with time. n.d., not detectable.

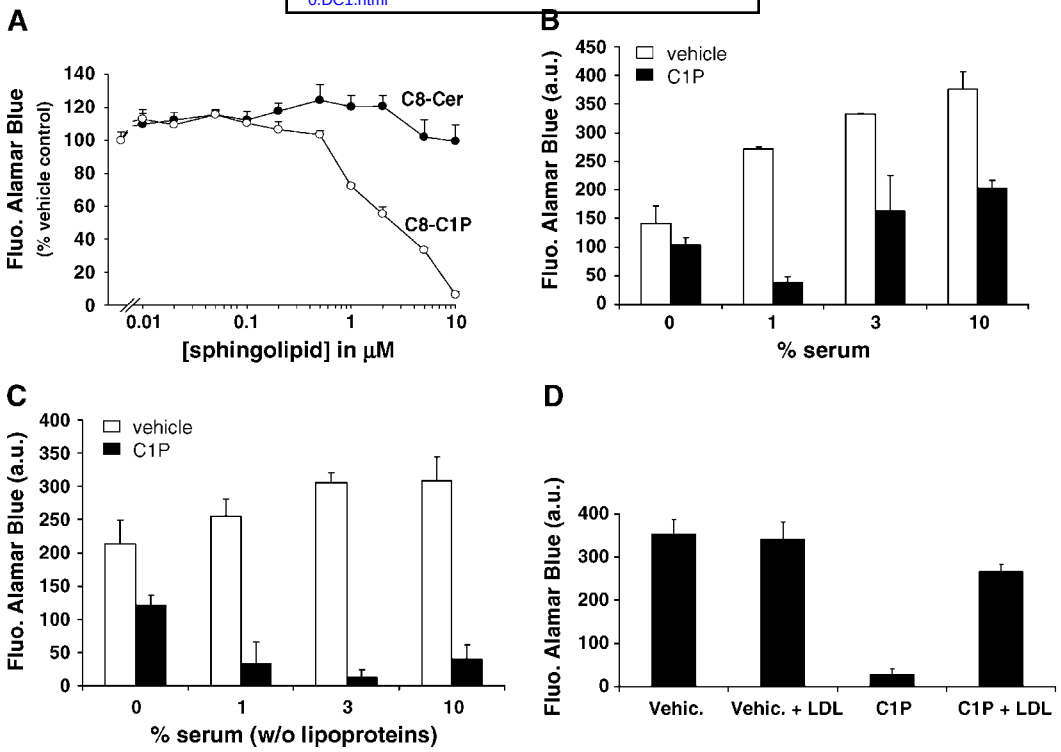


Fig. 6. C8-C1P-dodecane is more toxic to cells than C8-Cer-dodecane: protection by serum LDL. A: COS-1 cells were incubated in 1% serum-containing medium, with a concentration range of either C8-Cer (black circles) or C8-C1P (white circles), established from a 10 mM stock solution for each lipid, prepared in ethanol-dodecane (98:2). Twenty-four hours later, residual viability was measured with Alamar Blue. B: COS-1 cells were adapted to various serum concentrations (0, 1, 3, or 10%) for 10 min before medium was renewed that contained vehicle or 10 μM C8-C1P from a 10 mM stock solution prepared as in A, with the same respective serum amounts. Treatments were allowed to proceed for 24 h before residual viability was measured. C: Same as in B but using serum deprived of lipoproteins. D: COS-1 cells were treated as in A with vehicle or 10 μM C8-C1P in 1% LDL-deprived serum either without or after addition of 0.15 mg/ml LDL. Residual viability was measured after 24 h. Data shown are means ± SD. a.u., arbitrary units.

in the literature. Remarkably, C8-Cer, even at concentrations as high as 10 μM, was barely toxic. Similar observations were made when using natural C1P (a mixture of C18-C1P and C24-C1P; $EC_{50} \approx 3$ μM) and C18-Cer ($EC_{50} \approx 10$ μM) (data not shown). The toxicity of C1P in ethanol-dodecane was most obvious in the presence of low serum amounts but also occurred in the absence of serum and with increased serum levels (Fig. 6B). The partial protection afforded by increased concentrations of serum was lost when the serum used had been depleted of lipoproteins beforehand (Fig. 6C). Consistently, the addition of LDL to 1% serum-containing medium was sufficient to protect cells from C1P-mediated cell death (Fig. 6D). Epidermal growth factor could not substitute for LDL to prevent C1P/dodecane toxicity, implying that the protection afforded by LDL was not the result of an effect on cell proliferation (data not shown).

C1P-dodecane-mediated cell death happens after drastic mitochondria vacuolation

Addition of C1P in ethanol-dodecane (hereafter referred to as C1P-dodecane) rapidly affected cell viability (**Fig. 7**, left). Before cells eventually detached from the substratum (usually after a few hours; see below), cell viability had decreased dramatically, to ~30% of untreated levels. Moreover, the toxic effects of C1P-dodecane were found to be irreversible: changing the medium after various incubation times did not rescue the treated cells (Fig. 7, left). After 3 h of incubation in the presence of C1P-dodecane, cells had flattened and an intense production of vacuoles was observed in cells labeled with fluorescent calcein AM (Fig. 7, right). At later time points, cell extensions became visible, and from 5 h onward, cells massively detached from the substratum. Fluorescent calcein AM was also used to probe the viability of cells treated with C1P-dodecane. Combined with the ethidium homodimer-1 fluorescent dye, it failed to reveal any damage of the plasma membrane. This was further probed by measuring the extracellular release of lactate dehydrogenase, which did not increase upon C1P-dodecane treatment (data not shown). Therefore, although a contribution of the plasma membrane could not be ruled out, these observations suggested that the toxicity of C1P-dodecane probably affected intracellular organelles more than the plasma membrane itself.

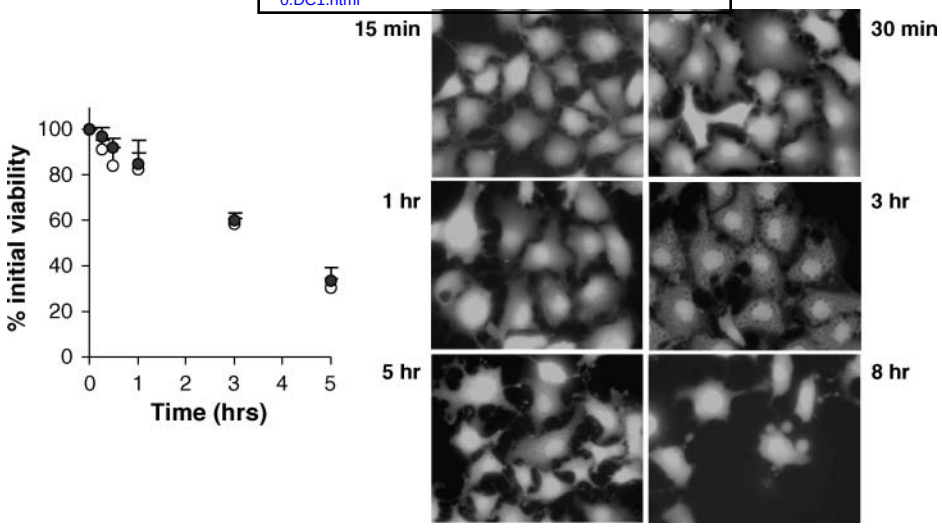


Fig. 7. Kinetics of C1P-dodecane-mediated cell death. Left: COS-1 cells were treated in 1% FBS with 10 μM C8-C1P made from a 10 mM stock in ethanol-dodecane (98:2). For the direct kinetics, residual viability was measured at the indicated times (black circles). For the indirect kinetics, the medium was removed and replaced by fresh medium without C1P, and the incubation was continued until 24 h was reached for all samples, at which point residual viability was measured (white circles). Data shown are means ± SD. Right: Cells treated in the same way were stained with 2 μM calcein AM and visualized with fluorescence microscopy.

To identify these organelles, we used fluorescent probes such as the LAMP2-YFP, which localizes to late endosomes and lysosomes, and Golgi-YFP. LAMP2-YFP was expressed as tubulovesicular structures in cells under vehicle control (see supplementary Fig. IIA–C). C1P-dodecane treatment did not significantly alter the distribution and morphology of the late endocytic compartment. Most of the vacuoles generated under C1P-dodecane treatment did not colocalize with the LAMP2 compartment (see supplementary Fig. IID–F). C1P-dodecane-induced vacuoles also did not colocalize with the Golgi-YFP-labeled Golgi apparatus (see supplementary Fig. IIG–I), even in cells rounded up under these conditions (see supplementary Fig. IIJ–L). Therefore, both probes indicate that the late endosomal and lysosomal compartments as well as the Golgi complex probably did not contribute significantly to the formation of vesicles that occurred upon C1P-dodecane treatment. In contrast, by tracking the mitochondria with the MitoTracker Green stain, we observed that C1P-dodecane-induced vacuoles are in fact swollen mitochondria (**Fig. 8**). Even more, there might be a possibility of fusion of mitochondria with the endoplasmic reticulum (ER), as indicated by the following: *i*) enlarged mitochondria appeared to be associated with ER fibers; *ii*) the MitoTracker stain was able to stain both mitochondria and ER in cells treated with C1P but not with vehicle (Fig. 8G, H); and *iii*) at the end of the swelling, large vacuoles appeared close to the nuclear envelope, a region that is part of the ER system (Fig. 8D–H).

DNA microarray analysis of C1P-dodecane-induced cellular effects

We performed a DNA microarray analysis of A549 cells treated either with or without vehicle or C1P at 10 μM for 1 or 3 h. This study indicated that C1P-dodecane can statistically significantly affect the expression of ~2,200 genes, as measured by 2,823 probe sets, most evident by 3 h (**Fig. 9A**; see supplementary Table I). Such a large set of regulated genes was unexpected. However, this may be consistent with the cellular stress induced by C1P-dodecane underscored in this study. According to MetaCore (GeneGo, Inc.), >300 gene ontology processes would statistically significantly associate with these 2,200 genes ($P \leq 0.01$). The top gene ontology processes were "transcription," "regulation of transcription, DNA-dependent," and "regulation of progression through cell cycle." MetaCore also listed >80 statistically significantly associated known pathways (maps). Here, "MAPK cascade," "IL-6," and "IL-1" maps ranked top. Surprisingly, cyclooxygenase-2 mRNA was one of the most strongly upregulated genes upon C1P-dodecane treatment, whereas $cPLA_{2\alpha}$ mRNA levels were not modified (Fig. 9B).

DISCUSSION

When testing various solvents reportedly used for C1P cell delivery and monitoring PGE_2 release as a functional readout, we observed that ethanol, only when it contained dodecane, enabled the capacity of C1P to induce PGE_2 release (Fig. 1). The impact of dodecane increased in a concentration-dependent manner, reaching a plateau at 1% (Fig. 2). It is likely, therefore, that colloidal dispersions of C1P-dodecane are being produced via the injection of ethanolic solutions in culture medium (20, 21). To verify whether dodecane might have facilitated the cellular uptake of added C1P, we prepared fluorescent C1P. In

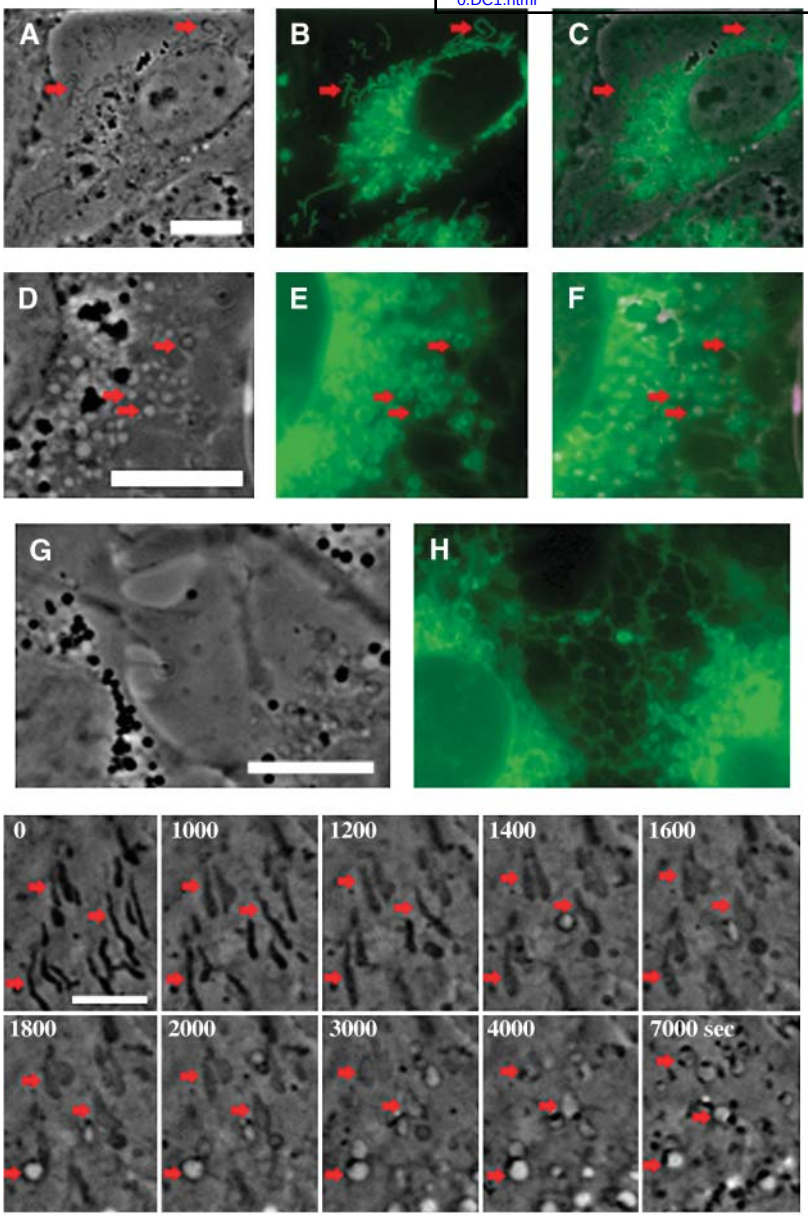


Fig. 8. C1P-dodecane induces mitochondrial swelling. Top: CHO cells were treated for 4 h in vehicle (A–C) or in the presence of 10 μM C1P made from 10 mM stock solutions in ethanol-dodecane (98:2) (D–F). MitoTracker Green FM (final concentration, 50 ng/ml) was added in the medium to label mitochondria compartments. Phase-contrast and fluorescent images were captured from the same live cells. Normal mitochondria can be observed in the phase-contrast image (A) or with MitoTracker Green FM staining (B), as also shown by the complete colocalization of the two (C). In C1P-treated cells, normal mitochondria morphology disappears and numerous vacuoles can be visualized (D–F). Bars = 10 μm. Middle: CV-1 cells were treated with 10 μM C1P made from 10 mM stock solutions in ethanol-dodecane (98:2). Mitochondria were then labeled with MitoTracker Green FM in live cells as described above for CHO cells. In 50–70% of cells treated with C1P, besides vacuoles, MitoTracker also labels a network of fiber-like structures, including the nuclear envelope, which is reminiscent of endoplasmic reticulum (ER). Both swollen mitochondria and ER structures appear to associate with each other, indicating that C1P-dodecane may cause mitochondria and ER membrane fusion (G, H). Bar = 10 μm. Bottom: Time-lapse analysis. CV-1 cells were treated for 0–5 h in the presence of 20 μM C1P made from 10 mM stock solutions in ethanol-dodecane (98:2). Time-lapse images were captured using phase-contrast to visualize mitochondria morphology under C1P-dodecane treatment. Mitochondria enlargement and vacuolation can be clearly followed (red arrows). The vacuolation started at ~20 min after treatment in such conditions. Most of the mitochondria became vacuolated in the cells.

agreement with a recent report (22), the presence of dodecane did not enhance the uptake and metabolism of fluorescent NBD-Cer, which was followed in parallel (Fig. 5). We show now that dodecane did not increase the cellular uptake of NBD-C1P either (Fig. 5). This appears to contrast with the findings of Gomez-Munoz and coworkers (14), who reported an increased uptake of ^{32}P-labeled C1P in the presence of methanol-dodecane. However, in this study, methanol-dodecane, as a vehicle for C1P, was not compared with methanol alone but rather with aqueous sonication of C1P. Collectively, therefore, it can be hypothesized that the permissive effect of dodecane on C1P-induced PGE_2 release is not a consequence of an increased cellular uptake of C1P. Moreover, dodecane also supported PA and PG stimulation of PGE_2 release (Fig. 4; see supplementary Fig. I), leading us to consider a possible direct role of dodecane itself in these observed effects.

A recent study showed that dodecane is able to interact with phospholipid bilayers, leading to vesicle aggregation, perturbation of the gel-fluid transition and lamellar-hexagonal transitions, and promotion of flip-flop movements (23). This study further showed that alkane-to-phospholipid interaction ratios can exceed 1,000. We provide in Fig. 3 the first biological evidence suggesting that dodecane may also interact with C1P. Indeed, we found that C1P at 1 μM is far more toxic when the dodecane/C1P molar ratio is 300 in the ethanolic preparation, compared with when it is 30, even if the final concentration of dodecane in the culture medium is adjusted in both samples (by separate injection of vehicle alone). Therefore, it is the molar ratio

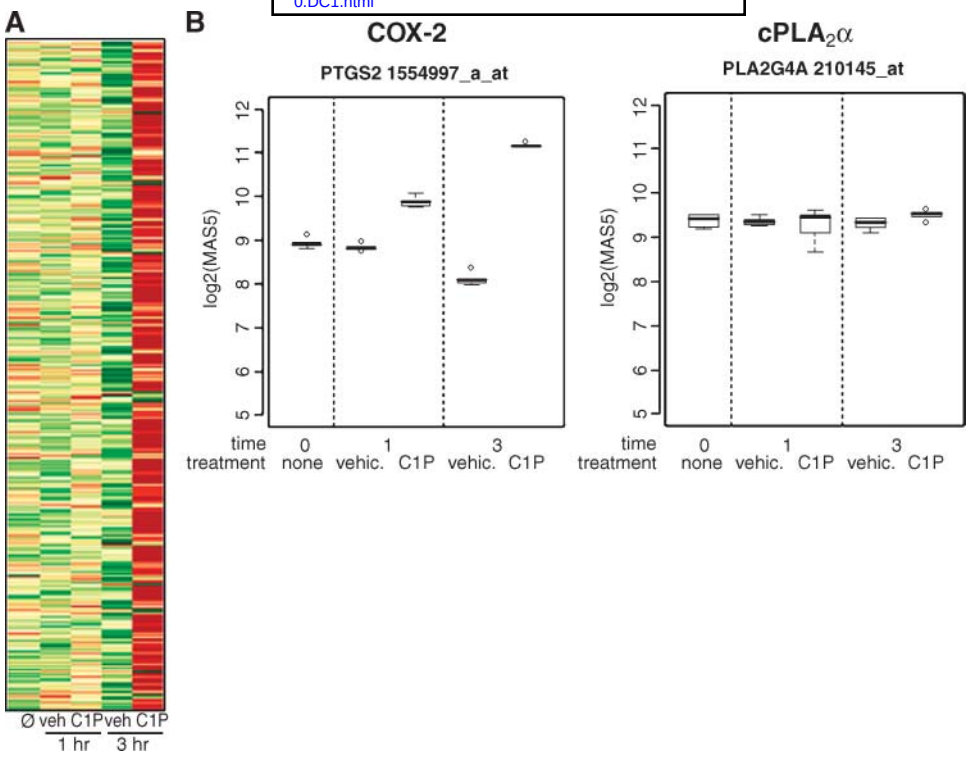


Fig. 9. DNA microarray analysis of C1P-dodecane-treated cells. A549 cells were treated for 1 or 3 h with vehicle only (veh) or in the presence of 10 μM C1P made from 10 mM stock solutions in ethanol-dodecane (98:2) (C1P). mRNA was extracted and processed for microarray analysis as described in Experimental Procedures. A: The heat map displays the log_2-normalized signal intensities for different treatment conditions (no treatment/vehicle/C1P) and time points (1 and 3 h) for all probe sets that changed statistically significantly between control (vehicle) and treatment (C1P). Data are shown as means of five replicates ± SD. The colors reflect upregulation (red) and downregulation (green). Intensities for 2,823 probe sets are displayed. B: Log_2 (MAS5) signal intensities, proportional to the original mRNA levels, are indicated for PTGS2 [cyclooxygenase-2 (COX-2)] and $cPLA_{2\alpha}$. Data are shown as means of five replicates ± SD. For PTGS2, a second probe set (No. 204748), representing the same gene, was available on the chip, and it gave identical results (see supplementary Table I).

of C1P to dodecane in the original preparation that is important. In this respect, the phospholipid (here C1P) acts to emulsify the alkane (here dodecane), the final emulsion being responsible for the observed cellular effects.

This work suggests that a combination of phospholipids, such as C1P or PA, with dodecane leads to an exquisite activation of the $cPLA_2/PGE_2$ cascade. Indeed, efipladib, a selective $cPLA_{2\alpha}$ inhibitor, completely abolished stimulation (Fig. 4). Remarkably, microarray experiments (Fig. 9) showed increased cyclooxygenase-2 mRNA expression upon C1P-dodecane treatment. Therefore, if $cPLA_{2\alpha}$ is indeed a target for C1P, working with C1P in ethanolic dodecane adds an unspecific level of stimulation of the arachidonic cascade that may explain the high levels of PGE_2 release achievable. This unspecific activation may also explain why PA, which was reported to be incapable of $cPLA_{2\alpha}$ activation (24), was found here to strongly induce PGE_2 release. Of note, the concentration-dependence of C1P-dodecane-induced cell death paralleled that required for PGE_2 production (Fig. 2). Nevertheless, these two events are not linked, because toxicity of C1P-dodecane also occurred in the presence of efipladib (data not shown).

C8-C1P-dodecane was shown to be more toxic than C8-Cer-dodecane (Figs. 6, 7). These findings may not account for the biological effect of the respective sphingolipids but rather may result from their distinct ability to interact with dodecane (23). C1P-dodecane-mediated toxicity was most evident in low-serum-containing medium, but remarkably, it was detectable at all serum concentrations, and even in its absence (Fig. 6). We showed that the lower toxicity observed at high serum levels results from the presence of increased levels of lipoproteins. Lipoproteins are enriched in phospholipids and therefore may be able to act as a sink. The original report on the use of C1P-dodecane (14) mentioned its positive effect on the proliferation of NIH 3T3 fibroblasts, constitutively overexpressing the epidermal growth factor receptor. In light of the toxicity of C1P-dodecane identified and characterized here, it is difficult to understand how this combination might have enhanced cell proliferation. Because recent

literature also reports on lack of proliferative effects of C1P (25), how C1P may act as a genuine mitogenic agent remains to be clarified.

The mechanism by which C1P-dodecane exerts detrimental effects was investigated, and this pointed to mitochondria. Why mitochondria in particular are targeted by C1P-dodecane (Fig. 8), whether directly or indirectly, is unknown. Very recently, a synthetic cationic ceramide analog, LCL-30, was shown to accumulate in the mitochondria of cancer cells, causing vacuolation and cell death (26). Thus, under certain circumstances, there might be a tropism for exogenously added ionic sphingolipids toward mitochondria. However, the parallel is not complete, because LCL-30 was found not to be toxic to primary hepatocytes (26), whereas C1P-dodecane, to some extent, was toxic also to the primary cells we examined (Fig. 3; see supplementary Fig. I; data not shown). In addition, we provide evidence for the fusion of mitochondria with the ER. This might be the reason why C1P-dodecane stimulates PGE_2 formation, which is known to take place in the perinuclear region. We observed that PA-dodecane was also able to stimulate PGE_2 release and to give rise to vesicles, such as C1P-dodecane; PG-dodecane was less effective on PGE_2 release, probably as a result of higher toxicity (see supplementary Fig. I). Together, the molar ratio of the anionic phospholipid versus the alkane, rather than the identity of the phospholipid and the alkane, is likely to be key. In this context, the biophysical studies of Urbina et al. (23) showed that shorter alkanes, such as octane and decane, have lipid bilayer-modifying properties as well.

In summary, cellular activity of C1P, as reported here, is seen only when C1P and dodecane are added to the culture medium from a premade mixture in ethanol. The cellular effects, including major mitochondrial toxicity, are difficult to ascribe solely to C1P or dodecane, because they only occur in the presence of the C1P-dodecane combination. A decade ago, ethanol-dodecane was advertised as a delivery vehicle to elucidate the biological function of ceramide (27), and this was applied soon afterward to C1P (14). Since that time, many studies have been performed using this solvent mixture as a vehicle. However, results from this report indicate that interpretation of the data generated in these previous studies is not straightforward. For future work, attempts to modulate endogenous C1P levels, rather than exogenous administration, may prove more beneficial to understand the "true" cellular effects of C1P.

NOTE ADDED IN PROOF

The authors further checked by LC-MS, and found there was no change in C8-C1P cellular uptake after 1 hr, be the vehicle ethanol-dodecane or ethanol alone.

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REFERENCES

1. Bajjalieh, S. M., T. F. Martin, and E. Floor. 1989. Synaptic vesicle ceramide kinase. *J. Biol. Chem.* **264:** 14354–14360.
2. Kolesnick, R. N., and M. R. Hemer. 1990. Characterization of a ceramide kinase activity from human leukemia (HL60) cells. *J. Biol. Chem.* **265:** 18803–18808.
3. Hinkovska-Galcheva, V. T., L. A. Boxer, P. J. Mansfield, D. Harsh, A. Blackwood, and J. A. Shayman. 1998. The formation of ceramide-1-phosphate during neutrophil phagocytosis and its role in liposome fusion. *J. Biol. Chem.* **273:** 33203–33209.
4. Sugiura, M., K. Kono, H. Liu, T. Shimizugawa, H. Minekura, S. Spiegel, and T. Kohama. 2002. Ceramide kinase, a novel lipid kinase. *J. Biol. Chem.* **277:** 23294–23300.
5. Pettus, B. J., A. Bielawska, S. Spiegel, P. Roddy, Y. A. Hannun, and C. E. Chalfant. 2003. Ceramide kinase mediates cytokine-and calcium ionophore-induced arachidonic acid release. *J. Biol. Chem.* **278:** 38206–38213.
6. Pettus, B. J., K. Kitatani, C. E. Chalfant, T. A. Taha, T. Kawamori, J. Bielawski, L. M. Obeid, and Y. A. Hannun. 2005. The coordination of prostaglandin E2 production by sphingosine-1-phosphate and ceramide-1-phosphate. *Mol. Pharmacol.* **68:** 330–335.
7. Pettus, B. J., A. Bielawska, P. Subramanian, D. S. Wijesinghe, M. Maceyka, C. C. Leslie, J. H. Evans, J. Freiberg, P. Roddy, Y. A. Hannun, et al. 2004. Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J. Biol. Chem.* **279:** 11320–11326.
8. Mitsutake, S., T. J. Kim, Y. Inagaki, M. Kato, T. Yamashita, and Y. Igarashi. 2004. Ceramide kinase is a mediator of calcium-dependent degranulation in mast cells. *J. Biol. Chem.* **279:** 17570–17577.
9. Gomez-Munoz, A., J. Y. Kong, B. Salh, and U. P. Steinbrecher. 2004. Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. *J. Lipid Res.* **45:** 99–105.
10. Tornquist, K., T. Blom, R. Shariatmadari, and M. Pasternack. 2004. Ceramide 1-phosphate enhances calcium entry through voltage-operated calcium channels by a protein kinase C-dependent mechanism in GH4C1 rat pituitary cells. *Biochem. J.* **380:** 661–668.
11. Colina, C., A. Flores, C. Castillo, M. d. R. Garrido, A. Israel, R. DiPolo, and G. Benaim. 2005. Ceramide-1-P induces Ca^{2+} mobilization in Jurkat T-cells by elevation of Ins(1,4,5)-P3 and activation of a store-operated calcium channel. *Biochem. Biophys. Res. Commun.* **336:** 54–60.
12. Jeon, H. J., D. H. Lee, M. S. Kang, M. O. Lee, K. M. Jung, S. Y. Jung, and D. K. Kim. 2005. Dopamine release in PC12 cells is mediated by Ca^{2+}-dependent production of ceramide via sphingomyelin pathway. *J. Neurochem.* **95:** 811–820.
13. Gomez-Munoz, A., P. A. Duffy, A. Martin, L. O'Brien, H. S. Byun, R. Bittman, and D. N. Brindley. 1995. Short-chain ceramide-1-phosphates are novel stimulators of DNA synthesis and cell division: antagonism by cell-permeable ceramides. *Mol. Pharmacol.* **47:** 833–839.
14. Gomez-Munoz, A., L. M. Frago, L. Alvarez, and I. Varela-Nieto. 1997. Stimulation of DNA synthesis by natural ceramide 1-phosphate. *Biochem. J.* **325:** 435–440.
15. Gomez-Munoz, A., J. Y. Kong, K. Parhar, S. W. Wang, P. Gangoiti, M. Gonzalez, S. Eivemark, B. Salh, V. Duronio, and U. P. Steinbrecher. 2005. Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway. *FEBS Lett.* **579:** 3744–3750.
16. Nakamura, H., T. Hirabayashi, M. Shimizu, and T. Murayama. 2006. Ceramide-1-phosphate activates cytosolic phospholipase A2alpha directly and by PKC pathway. *Biochem. Pharmacol.* **71:** 850–857.
17. O'Brien, J., I. Wilson, T. Orton, and F. Pognan. 2000. Investigation of the Alamar Blue (Resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **267:** 5421–5426.
18. Bajjalieh, S., and R. Batchelor. 1999. Ceramide kinase. *Methods Enzymol.* **311:** 207–215.
19. McKew, J. C., S. Y. Tam, J. D. Clark, K. L. Lee, L. Chen, P. Thakker, F-W. Sum, M. L. Behnke, and B. Hu. 2003. Preparation of indolyl containing sulfonamides as inhibitors of cytosolic phospholipase A2. Patent WO 2003048122.
20. Batzri, S., and E. D. Korn. 1973. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta.* **298:** 1015–1019.

21. Kremer, J. M. H., M. W. Van der Esker, C. Pathmamanoharan, and P. H. Wiersema. 1977. Vesicles of variable diameter prepared by a modified injection method. *Biochemistry.* **16:** 3932–3935.
22. Tserng, K-Y., and R. L. Griffin. 2004. Ceramide metabolite, not intact ceramide molecule, may be responsible for cellular toxicity. *Biochem. J.* **380:** 715–722.
23. Urbina, P., A. Alonso, F. X. Contreras, F. M. Goni, D. J. Lopez, L. R. Montes, and J. Sot. 2006. Alkanes are not innocuous vehicles for hydrophobic reagents in membrane studies. *Chem. Phys. Lipids.* **139:** 107–114.
24. Subramanian, P., R. V. Stahelin, Z. Szulc, A. Bielawska, W. Cho, and C. E. Chalfant. 2005. Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2 alpha and enhances the interaction of the enzyme with phosphatidylcholine. *J. Biol. Chem.* **280:** 17601–17607.
25. Hogback, S., P. Leppimaki, B. Rudnas, S. Bjorklund, J. P. Slotte, and K. Tornquist. 2003. Ceramide 1-phosphate increases intracellular free calcium concentrations in thyroid FRTL-5 cells. *Biochem. J.* **370:** 111–119.
26. Dindo, D., F. Dahm, Z. Szulc, A. Bielawska, L. M. Obeid, Y. A. Hannun, R. Graf, and P-A. Clavien. 2006. Cationic long-chain ceramide LCL-30 induces cell death by mitochondrial targeting in SW403 cells. *Mol. Cancer Ther.* **5:** 1520–1529.
27. Li, J., G. Zhang, S. Uematsu, Y. Akahori, and Y. Hirabayashi. 1995. Induction of apoptotic DNA fragmentation and cell death by natural ceramide. *FEBS Lett.* **358:** 211–214.