methods

Use of high performance liquid chromatography-electrospray ionization-tandem mass spectrometry for the analysis of ceramide-1-phosphate levels[S]

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Abstract **Ceramide-1-phosphate (C1P) is a bioactive sphingolipid with roles in several biological processes. Currently, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC ESI-MS/MS) offers the most efficient method of quantifying C1P. However, the published protocols have several drawbacks causing overestimations and carryovers. Here, the reported overestimation of C1P was shown to be due to incomplete neutralization of base hydrolyzed lipid extracts leading to the hydrolysis of SM to C1P. Actual quantity of C1P in cells (6 pmols/10^6 cells) was much lower than previously reported. Also, the major species of C1P produced by ceramide kinase (CERK) was found to be $d_{18:1/16:0}$ with a minority of $d_{18:1/24:1}$ and $d_{18:1/24:0}$. The artifactual production of C1P from SM was used for generating C1Ps as retention time markers. Elimination of carryovers between samples and a 2-fold enhancement in the signal strength was achieved by heating the chromatographic column to 60°C. The role of ceramide transport protein (CERT) in supplying substrate to CERK was also revalidated using this new assay. Finally, our results demonstrate the presence of additional pathway(s) for generation of the C1P subspecies, $d_{18:1/18:0}$ C1P, as well as a significant portion of $d_{18:1/16:0}$, $d_{18:1/24:1}$, and $d_{18:1/24:0}$. In conclusion, this study introduces a much improved and validated method for detection of C1P by mass spectrometry and demonstrates specific changes in the C1P subspecies profiles upon downregulation of CERK and CERT.**—Wijesinghe, D. S., J. C. Allegood, L. B. Gentile, T. E. Fox, M. Kester, and C. E. Chalfant. **Use of high performance liquid chromatography-electrospray ionization-tandem mass spectrometry for the analysis of ceramide-1-phosphate levels.** ***J. Lipid Res.*** **2010.** 51: **641–651.**

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Ceramide-1-phosphate (C1P) is a phospho-sphingolipid that is gaining increasing recognition as an important bioactive lipid molecule. The first report of a biological effect for C1P was by Gomez-Muñoz et al. (1), who demonstrated that short chain C1P induced DNA synthesis in Rat-1 fibroblasts (1). Later, treatment with long chain C1P was shown to have a similar effect on T17 fibroblasts (2). A more recent report along the same line of research demonstrated that C1P prevented programmed cell death in bone marrow-derived macrophages after withdrawal of macrophage colony-stimulating factor (3) and that treatment of these cells with C1P effectively blocked the activation of caspases and prevented DNA fragmentation upon serum removal. The same laboratory group also demonstrated that this effect was due to a decrease of ceramide levels via inhibition of acid sphingomyelinase by C1P (3). Following a different line of research, Hinkovska-Galcheva et al. (4) demonstrated that ceramide kinase (CERK) is activated in the context of phagocytosis in neutrophils after challenging the cells with formyl peptide and antibody-coated erythrocytes. Furthermore, C1P was shown to play a distinct role in membrane fusion, possibly explaining the early finding that high levels of C1P are found in synaptic vesicles (5).

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Abbreviations: AA, arachidonic acid; C1P, ceramide-1-phosphate; CERK, ceramide kinase; CERT, ceramide transport protein; IL-1β, interleukin-1β; MRM, multiple reaction monitoring; PC, phosphatidylcholine; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; RP, reverse phase; TNFα, tumor necrosis factor α.

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

Published findings from our laboratory have also demonstrated a biological function for C1P as a direct activator of $cPLA_2\alpha$ through interaction with the C2/CaLB domain (6). These results, coupled with the previous findings that CERK/C1P pathway is required for $cPLA_2\alpha$ activation in response to calcium ionophore and inflammatory cytokines (7), demonstrated that C1P was a major regulator of the eicosanoid synthetic pathway. A role for CERK and its product, C1P, in a separate pathway of allergic/inflammatory signaling as Mitsutake et al. (8) demonstrated that treatment of RBL-2H3 cells or overexpression of CERK in mast cells enhanced the degranulation induced by A23187. Recent biophysical studies have shown that C1P has the ability to affect biological processes by either directly binding enzymes (10, 11) or via membrane deformation (9).

Multiple species of C1P are present in cells (10) differing from each other in their acyl chain length, which varies from 14 carbons to 26 carbons. Until recently, only total cellular levels of C1P could be quantified via radiolabeling techniques such as steady-state labeling with ^{3}H palmitate or pulse labeling using ^{32}P orthophosphate followed by chromatographic separation (TLC). Current developments in high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC ESI-MS/MS) has provided a very powerful tool for analysis of C1P at the subspecies levels. Ability to identify analytes with three levels of confidence (retention time, analyte specific precursor ion, and species specific product ion) coupled with sub-picomolar levels of detection and quantitation results in unsurpassed accuracy and sensitivity. To date, several HPLC ESI-MS/MS protocols have been reported for quantitation of cellular C1P levels (10–12). Nearly all of the reported methods utilize separation on a reverse phase column followed by mass spectrometric analysis via multiple reaction monitoring (MRM) in either positive ($[M+H]^+/264.4$) or negative ion ($[M-H]^-/78.9$) mode. Although the positive ion mode provides more structurally informative data, the signal strength for the MRM transition is low. The reverse is true for the detection of C1P by the negative ion mode. Nearly all of these published protocols have certain drawbacks when used to analyze cellular levels of C1P, and to our knowledge, none have been completely validated as to quantitation using radioactive labeling. Some reported HPLC ESI-MS/MS methods encountered problems in overestimating the cellular levels of C1P when compared with data from steady-state labeling studies. In addition, these methods demonstrated significant sample-to-sample carryover issues. Recently, a slightly modified protocol was reported by Graf et al. (13), but this mass spectrometric protocol for C1P was only able to quantitate $d_{18:1/16:0}$ C1P. Lastly, the absence of commercially available internal standards for the different chain lengths of C1P has been another drawback making unambiguous peak assignment difficult.

In this article, we introduce a modified and validated method for quantifying cellular levels of C1P by reverse phase HPLC ESI-MS/MS based on the previously reported protocol by Merrill et al. (12). The method improves on the existing protocols for C1P analysis in having little to no carryover and a 2-fold increase in signal strength allowing for the quantification of smaller changes in the C1P profiles. Furthermore, the quantification of the C1P levels in cells was validated by radiolabeling techniques and the problem in the overestimation of C1P levels identified. A rapid method for generating multiple chain lengths of C1P is also outlined for use as retention time markers. Using this method, we demonstrate changes in the C1P profiles upon treatment of cells with CERK small interfering (si)RNA, CERT siRNA, and a pharmacological inhibitor of ceramide transport protein (CERT) (HPA-12). Specifically, all three treatments altered the levels of $d_{18:1/16:0}$ C1P with minor effects on $d_{18:1/24:1}$ and $d_{18:1/24:0}$ C1P levels. These data indicate that CERK is only responsible for a certain subset of C1P in cells. In addition, the CERK-derived pool of C1P was shown to be dependent on CERT, thereby revalidating our earlier findings that CERK utilizes a substrate ceramide transported via CERT.

EXPERIMENTAL PROCEDURES

Cell culture

All cultured cells were obtained from the American Type Culture Collection. A549 lung adenocarcinoma cells were grown in 50% DMEM (BioWhittaker) and 50% RPMI (BioWhittaker) supplemented with 10% fetal bovine serum (Invitrogen) and 2% penicillin/streptomycin (BioWhittaker). Cells were maintained under 5% CO2 at 37°C by routine passage every 3 days. A_{549} cells were grown in DMEM supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin under the same conditions. For treatments, the medium was replaced 2 h before the addition of the agonist by DMEM containing 2% fetal bovine serum and 2% penicillin/streptomycin.

RNA interference

Sequence-specific silencing of CERK and CERT was performed using sequence-specific siRNA as described previously (14). The human CERK RNA interference sequence starts at 142 nucleotides from the start codon (UGCCUGCUCUGUGCCUGUAdTdT and UACAGGCACAGAGCAGGCAdTdT) (9). The siRNA for human CERT was from Dharmacon (catalog No. M-012101-00). The sequence targets accession numbers are NM_005713 and NM_031361. siRNA (100 nM) were transfected into A549 cells using Dharmafect (Dharmacon) according to the manufacturer's instructions. After incubation for 48 h, cells were analyzed by Western immunoblotting using specific antibodies against CERK and CERT as well as qPCR for CERK mRNA levels. After incubation for 48 h, cells were analyzed for C1P levels by TLC or MS.

Treatment with CERT inhibitors

A549 cells (1×10^6) were plated on 10 cm plates in the appropriate medium and grown under standard incubator conditions (SIC) overnight, which produced a cell confluency of 80% the following day. The media was changed to 2% serum and the cells were rested for 3 h. Stock solutions of the HPA-12 inhibitor of CERT and its inactive racemic control mixture were made at 2 mM and 10 mM in DMSO and were added to cells at dilutions of 1:1000 and 1:5000 to obtain final concentrations of 2 μM. The final DMSO concentrations were 14 mM and 2.82 mM, respectively. DMSO by itself was used as a sham control at the same final concentrations.

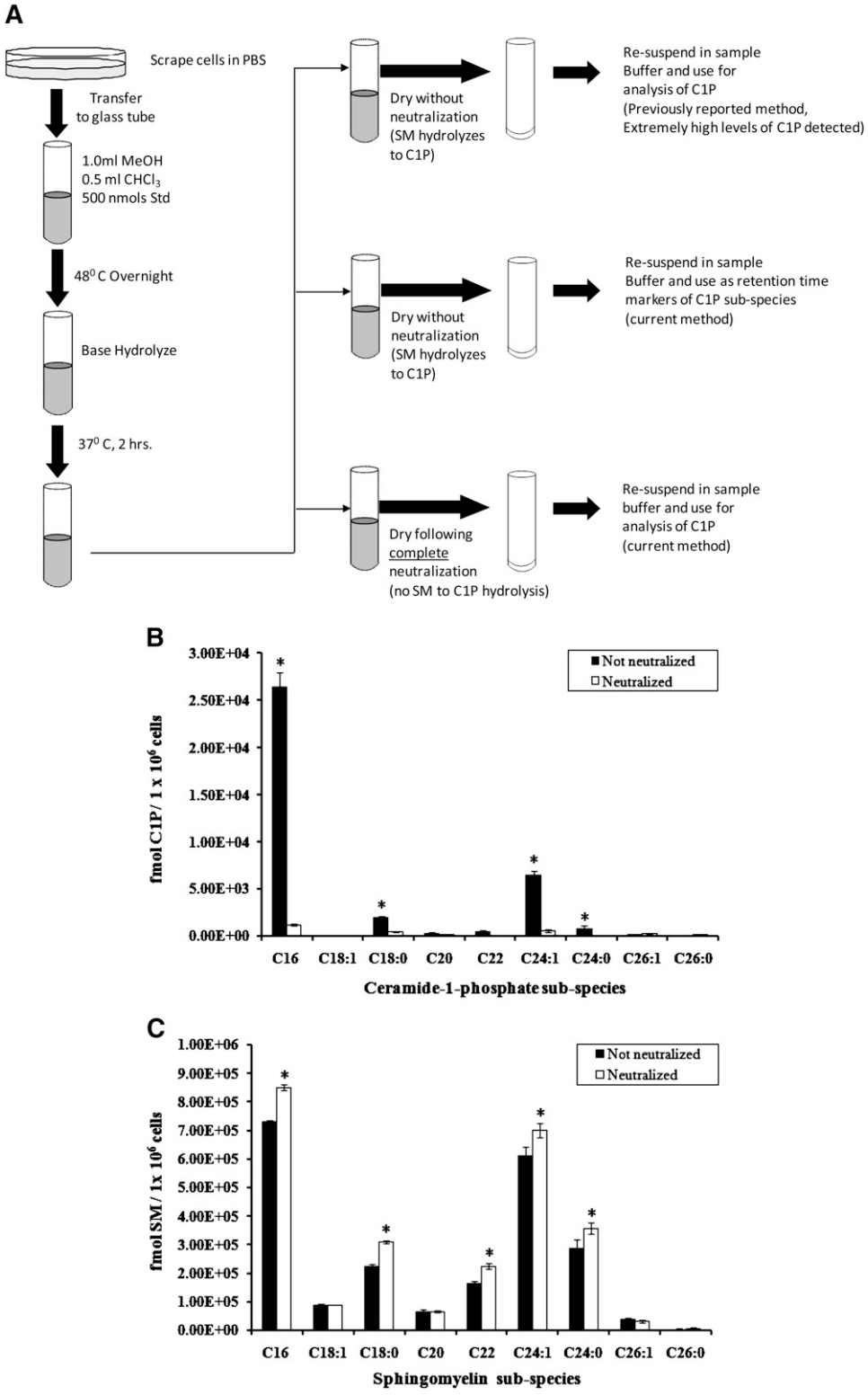

Fig. 1. Incomplete neutralization leads to increased amounts of C1P via the hydrolysis of sphingomyelin. A549 cells (1×10^6) were plated on 10 cm plates in the appropriate medium and grown under standard incubator conditions (SIC) overnight. The following day, cells were harvested and lipids were extracted as detailed in the Materials and Methods section. Following base hydrolysis, one half of the lipid extracts was dried without neutralization (black bars) while the remainder was dried following complete neutralization (white bars). All samples were resolubilized in reverse-phase sample buffer and subjected to analysis by reverse-phase HPLC-ESI MS/MS for (B) levels of C1P and (C) for levels of SM as described in Materials and Methods. The data are a mean of three samples ± SE. (* $P < 0.001$).

Mass spectrometric analysis

A549 cells (1×10^6) were plated on 10 cm plates in the appropriate medium and grown overnight under SIC, which produced a cell confluency of 80% the following day. The next day, cells were subjected to the relevant treatment, the plates were placed on ice, washed once with ice cold PBS, and harvested by scraping in 200 µl of PBS. An aliquot of cells was taken for standardization (total protein). Lipids were extracted from 250 µl of the remaining cells as described by Merrill et al. (12) with the slight modifications outlined in this paper. In summary, to the remaining cells, 1 ml of methanol was added and sonicated to obtain a homogeneous mix followed by the addion of 500 µl of chloroform and 500 pmols of internal standards (Avanti). Internal standards used were $d_{18:1/12:0}$ ceramide-1-phosphate sphingomyelin, ceramide, glucosylceramide, lactosylceramide and $d_{17:1}$-sphingosine, sphinganine, sphingosine-1-phosphate, sphinganine-1-phosphate. The mixture was sonicated and incubated overnight at 48°C. Extracts were then subjected to base hydrolysis for 2 h at 37°C and neutralized by the addition of glacial acetic acid. The neutralization was confirmed with pH paper. Half of the extract was dried down and brought up in reverse phase sample buffer (60%A:40%B). To the remainder of the extract, 1 ml of chloroform and 2 ml of water were added, and the lower phase was transferred to another tube, dried down, and brought up in normal phase sample buffer (98%A:2%B). C1P, SM, and ceramide were quantified using HPLC ESI-MS/MS as described by Merrill et al. (12) and using the modifications described in this article. C1P species were separated using a Discovery C18 column on a Shimadzu HPLC and subjected to mass spectrometric analysis using a 4000 Q-Trap (Applied Biosystems). MRM transiton monitored were: 562.4/264.4 ($d_{18:1/12:0}$), 590.4/264.4 ($d_{18:1/14:0}$), 618.4/264.4 ($d_{18:1/16:0}$), 620.4/264.4 ($d_{18:0/16:0}$), 644.4/264.4 ($d_{18:1/18:1}$), 646.4/264.4 ($d_{18:1/18:0}$), 674.4/264.4 ($d_{18:1/20:0}$), 702.4/264.4 ($d_{18:1/22:0}$), 728.4/264.4 ($d_{18:1/24:1}$), 730.4/264.4 ($d_{18:1/24:0}$), 756.4/264.4 ($d_{18:1/26:1}$), 758.4/264.4 ($d_{18:1/26:0}$) used at collision energies +41, +43.5, +43.5, +46.0, +46.0, +48.5, +51.0, +53.5, +56.0, +56.0, +58.5, +58.5 V, respectively. The chromatography apparatus for C1P was a 2.1mm × 50 mm Discovery C18 HPLC column. The solvents for reverse phase separation for C1P were: solvent A, 58:41:1 CH3OH/water/HCOOH and solvent B, 99:1 CH3OH/HCOOH. Both solvents contained 5 mM ammonium formate (12). The solvents for normal phase separation of ceramides and SMs were: solvent A, CH_3CN: H_2O:CH_3COOH 97:2:1, and solvent B, CH_3OH: H_2O: $CH_3(CH_2)_2CH_2OH$: CH_3COOH 64:15:20:1. Both contained 5 mM ammonium acetate.

RESULTS

The reported high levels of C1P in mammalian cells are artifactual due to incomplete neutralization and SM hydrolysis

Previously, C1P has been demonstrated to be present in cells in significant quantities (150–300 pmols /10^6 cells) (14). This reported concentration of C1P in cells was not in agreement with data from steady-steady state labeling experiments (30 pmols/10^6) (15). Careful scrutiny of the established mass spectrometric protocol for measuring C1P levels revealed that base hydrolyzed lipid extracts were being dried without neutralizing (**Fig. 1A**). This observation led to the hypothesis that the high levels of C1P previously reported were artifactual and possibly due to hydrolysis of SM (Fig. 1A). Therefore, we examined the levels of C1P in lipid extracts with and without neutralization prior to drying. The completely neutralized lipid extracts had >75% lower C1P levels compared with lipid extracts that were not neutralized prior to drying (Fig. 1B). Upon further investigation, a corresponding decrease was observed in the SM levels of nonneutralized lipid extracts (Fig. 1C). To further demonstrate that the drying of improperly neutralized samples leads to breakdown of SM to C1P, a pure solution of SM was subjected to lipid extraction with and without neutralization. The mass spectrometric analysis revealed a decrease in the levels of SM following dry down without neutralization (supplementary

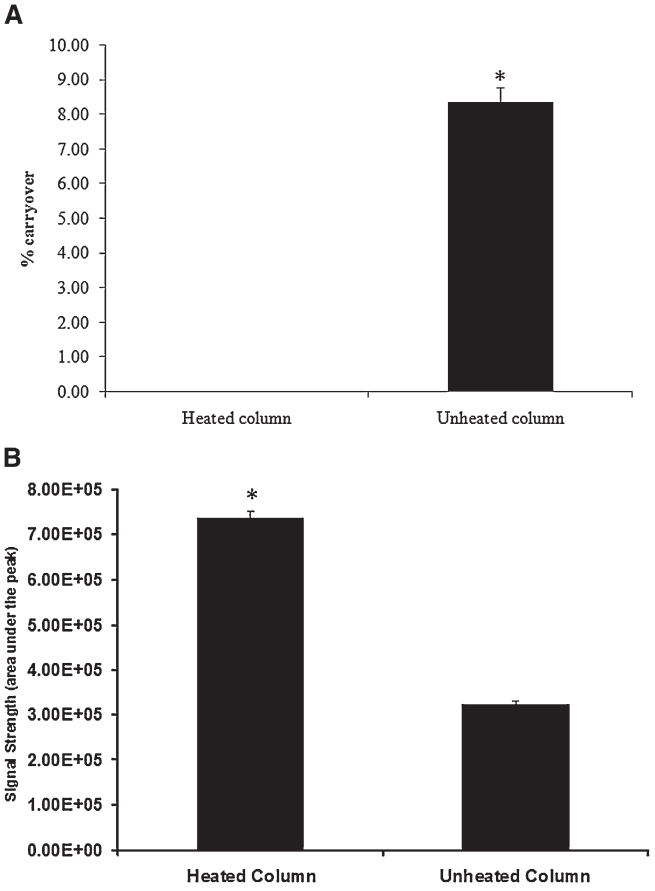

Fig. 2. Heating of the C18 reverse phase column to 60°C eliminates C1P carryover and results in a 2-fold increase in the signal strength of C1P. A: D-e-C_{12} C1P (8 pmols) was analyzed using a C18 reverse-phase column as described in the Materials and Methods following chromatographic separation on a C18 reverse-phase column that is either heated at 60°C or maintained at room temperature. The data are an average of 10 injections ± SD. (* $P < 0.01$). B: A549 cells were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, cells were harvested and lipids were extracted as detailed in the Materials and Methods section. Following base hydrolysis, the lipid sample was dried with and without neutralization, resolubilized in reverse-phase sample buffer, and subjected to C1P analysis by reverse-phase HPLC-ESI MS/MS using the modified method described in the Materials and Methods section. The data is presented as the percent carryover observed for C1P in a blank sample following 10 consecutive infusions of 100 µl each of the C1P retention time standards following separation in an unheated or heated C18 column. Data are the average of three separate experiments.

Fig. IA). Investigation for possible breakdown products revealed that although the greatest increase is observed in C1P levels (supplementary Fig. IB), significant increases were also observed in S1P (supplementary Fig. IC), sphingophosphoryl choline (supplementary Fig. ID), and ceramide (supplementary Fig. IE) levels. Therefore, the process of drying lipid samples under basic conditions induces the partial hydrolysis of SM to C1P and other component molecules resulting in their overestimation during mass spectrometric analysis.

Elimination of sample-to-sample carryover and a 2-fold increase in the signal response for C1P was achieved by heating the C18 column to 60°C

Another concern in the protocol for analysis of C1P by MS was the high rate of carryover between samples. This observed carryover has been problematic for analysis of $d_{18:1/16:0}$ C1P, and these carryovers sometimes exceeded 10%, making accurate quantitation of small changes in C1P levels difficult. Upon investigating multiple conditions, prevention of carryover was accomplished by heating the C18 column to 60°C. Following 10 sample injections, no carryover was observed with the heated column method (**Fig. 2A**) whereas an approximately 10% carryover was observed when using an unheated column (Fig. 2A). In addition, low amounts of C1P in a given sample resulted in a decreased signal intensity, making the detection of the low abundant C1P species difficult. We hypothesized that removal of carryover by preheating the C18 column would also enhance the detection of low abundance subspecies of C1P. To investigate whether preheating the elution solvent enhances the signal response, 8 pmols of $d_{18:1/12:0}$ C1P was analyzed with and without heating the column to 60°C. Prewarming of the eluting solvent

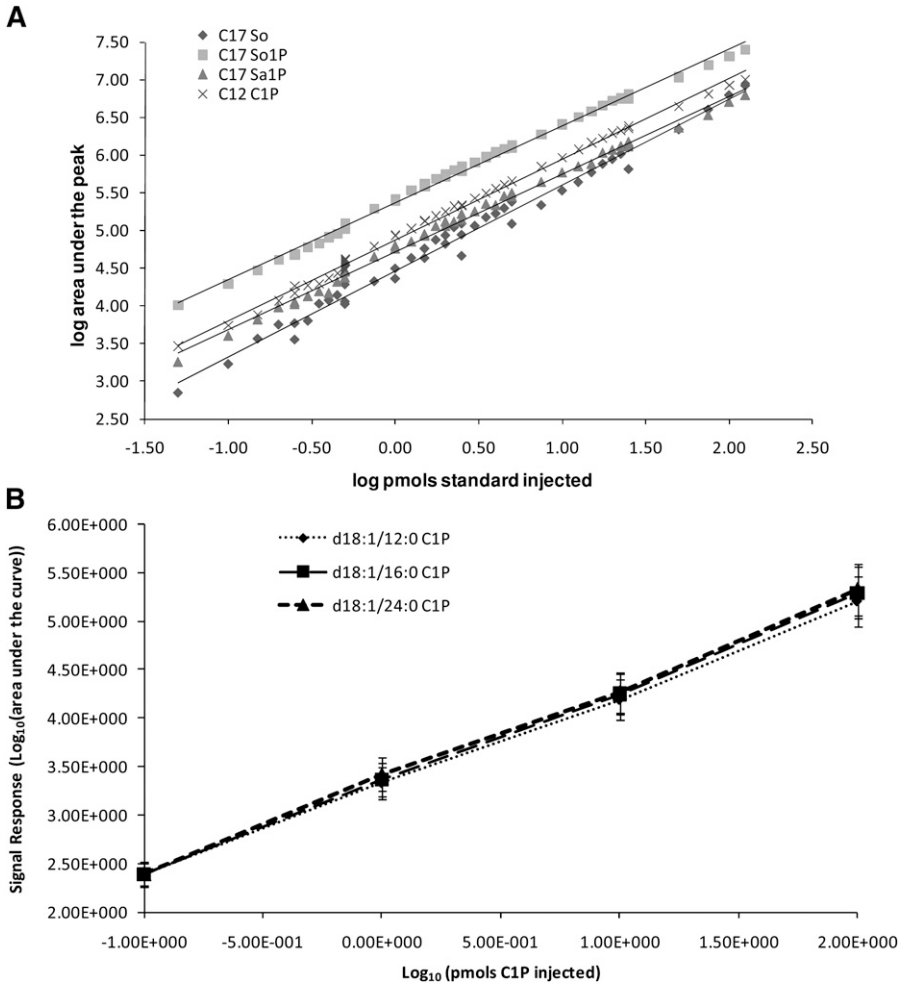

Fig. 3. The modified method for detection of C1P shows a linear response in the range of 5 fmols to 125 pmols for C1P and LCB standards. A: An internal standard mix containing $d_{17:1}$ sphingosine (C17 So), sphingosine-1-phosphate (C17 So1P), sphinganine-1-phosphate (C17 Sa1P), and $d_{18:1/12:0}$ ceramide-1-phosphate (C12 C1P) was prepared varying in concentration from 5 to 125 pmols. These standard solutions in LCB buffer were analyzed using the method modified to overcome the carryover problem. The signal response as measured by the area under the peak versus the amount analyzed in pmols were plotted as a log-log plot. B: 0.1, 1, 10, and 100 pmols of $d_{18:1/12:0}$, $d_{18:1/16:0}$, and $d_{18:1/24:0}$ C1P were analyzed using the method modified to overcome the carryover problem. The signal response was measured by the area under the peak versus the amount analyzed in pmols were plotted as a log-log plot. Data are the average of three separate sample injections ± SE.

enhanced the signal response for C1P by a factor of 2 (Fig. 2B). However, we did not see a highly significant change in resolution between the two methods, indicating that the increased signal intensity is not due to an increase in resolution (data not shown). Thus, the application of heat to the reverse phase C18 HPLC column when using a methanol:water/methanol system completely eliminates the problem of carryover while enhancing the signal strength of various C1P subspecies.

Signal response for C1P was linear over three orders of magnitude

As the levels of C1P in cells were found to be low in cells, the question arises whether the C1P signal response with the modified method was linear within the physiologic range to accurately quantify changes by HPLC ESI-MS/MS. To investigate the linearity of our HPLC ESI-MS/MS method, increasing amounts of C1P ranging from 50 fmols to 150 pmols in a standard mixture containing $d_{18:1/12:0}$ C1P, $d_{17:1}$ sphingosine, $d_{17:1}$ sphinganine, $d_{17:1}$ sphingosine-1-phosphate, $d_{17:1}$ sphinganine-1-phosphate were analyzed using this newly modified method. The signal response as measured by the area under the peak was found to be linear in the range analyzed (over three orders of magnitude) (**Fig. 3A**). The dependence of the signal response on the acyl chain length of C1P was investigated using a dilution series ranging from 0.1 to 100 pmols of $d_{18:1/12:0}$, $d_{18:1/18:.0}$ and $d_{18:1/24:0}$ C1P (Fig. 3B). No significant difference in signal response was observed between the different chain lengths. This data is in agreement with that of Shaner et al. (10) and further strengthen their findings that $d_{18:1/12:0}$ C1P can be used as an internal standard for quantifying the longer acyl chain lengths of C1P. The limit of quantitation was well below 50 fmols (data not shown). Therefore, this modified method is applicable in the quantitation of small changes of a particular C1P subspecies.

Retention time markers are essential for unambiguous peak assignment for low abundant species of C1P

Although complete neutralization of samples prior to analysis corrected SM hydrolysis issues, a drastic reduction of the signal for C1P was observed. The decreased signal resulted in the intensities of the peaks corresponding to C1P becoming comparable to those from non-C1P species with the same MRM transitions. The absence of a single prominent peak for C1P led to difficulty in the identification of peaks corresponding to a particular C1P transition (**Fig. 4**). This issue is very problematic when attempting to quantify low abundant subspecies of C1P, which tended to be hidden among nonspecific peaks (Fig. 4 inset). The availability of retention time markers for C1P became necessary for peak identification. Because base hydrolysis of SM results in the formation of multiple C1P subspecies in high picomolar quantities, the possibility of using C1P derived from base hydrolysis of cellular SM as retention time markers was investigated. A base hydrolyzed lipid extract

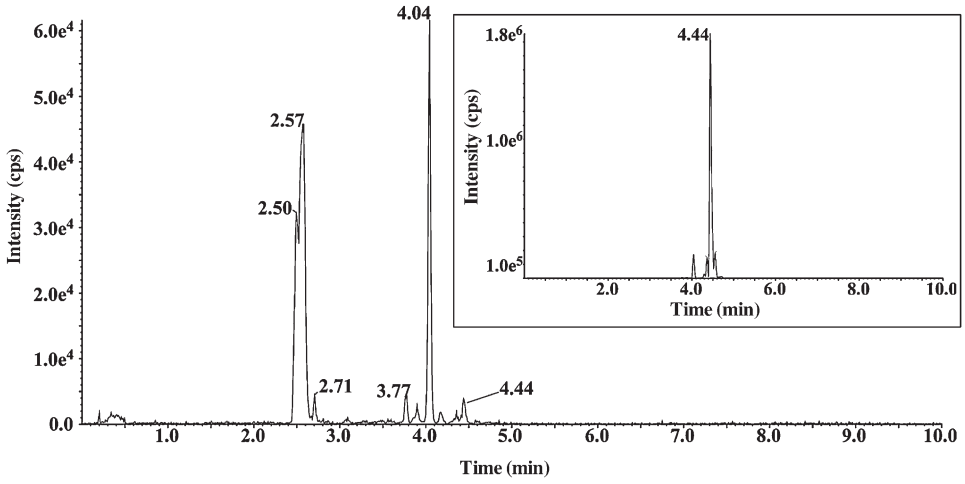

Fig. 4. Retention time standards are required for the unambiguous peak assignment in the quantitation of naturally occurring levels of C1P. MRM transition for a particular C1P species gives rise to multiple peaks in the excited ion chromatogram. A549 cells (1×10^6) were plated on 10 cm plates in the appropriate medium and grown overnight under SIC. The cells were then harvested and lipids were extracted as detailed in Materials and Methods section. Following base hydrolysis, the lipid sample was completely neutralized, dried, resolubilized in reverse-phase sample buffer, and subjected to C1P analysis by reverse-phase HPLC-ESI MS/MS using the modified method described in the Materials and Methods section. The signal trace corresponding to $d_{18:0/24:1}$ C1P is presented as retention time versus peak intensity. Inset: Retention time markers generated via base hydrolysis of cellular sphingomyelin aid in the identification of the C1P specific peaks. A549 cells were plated on 10 cm plates in the appropriate medium and grown under standard incubator conditions overnight. The following day, cells were harvested and lipids were extracted as detailed in Materials and Methods. Following base hydrolysis, the lipid sample was dried without neutralization, resolubilized in reverse-phase sample buffer, and subjected to C1P analysis by reverse-phase HPLC-ESI MS/MS using the modified method described in the Materials and Methods. The signal trace corresponding to $d_{18:0/24:1}$ C1P is presented as retention time versus peak intensity.

from A549 cells was dried without neutralization and subjected to LC-MS/MS analysis under the same conditions for C1P analysis. The appearances of prominent and clearly distinguishable peaks for C1P (Fig. 4B) allowed for identification of retention times for each C1P subspecies within each batch of samples analyzed. This greatly aided in the identification of chromatographic peaks resulting from a C1P specific MRM transition. Therefore, although the hydrolysis of SM to C1P due to a lack of neutralization caused an overestimation of C1P in cells, this same hydrolysis issue was also fortuitous and necessary in producing inexpensive retention time markers for C1P for identification of C1P-specific peaks.

CERK is only responsible for a subset of C1P in cells

Recent publications from Bornancin et al. (13) have demonstrated that significant quantities of C1P were still present in CERK null mice. These mice demonstrated a decrease in the $d_{18:1/16:0}$ C1P, but the mass spectrometric method utilized in the report could only analyze this one subspecies of C1P. In order to investigate whether CERK was responsible for multiple subspecies of C1P in cells, C1P was quantified in A_{549} cells following downregulation of CERK by siRNA treatment. CERK siRNA downregulated the enzyme >80% at both the mRNA and protein level as previously described by our laboratory (14) (data not shown). In accord with CERK knockout data reported by Graf et al. (13), $d_{18:1/16:0}$ C1P was found to be decreased by greater than 50% (**Fig. 5A**). However, significant changes were also observed in $d_{18:1/24:0}$ C1P ($P < 0.001$) and $d_{18:1/24:1}$ C1P ($P < 0.05$) as well (Fig. 5A). To confirm these findings as well as the HPLC ESI-MS/MS quantification, A_{549} cells were again treated with CERK siRNA, steady-state labeled with ^{32}P orthophosphate, subjected to lipid extraction separated by TLC, and quantified by scintillation counting. Quantification of C1P levels by steady-state labeling demonstrated that total C1P levels were decreased by approximately 50% (Fig. 5B) in accord with our HPLC ESI-MS/MS method. These data demonstrate that CERK is mainly responsible for the generation of a subset of $d_{18:1/16:0}$ C1P in cells, but also plays a significant role in the generation of the $d_{18:1/24:0}$ subspecies of C1P and a minor role in the generation of $d_{18:1/24:1}$ C1P. In addition, the total C1P as quantified by steady-state labeling (6.6 pmols). Our data demonstrate that C1P measured by steady-state labeling with ^{32}P orthophosphate was within 10% of the total C1P measured by the described mass spectrometric method (5.7 pmols) Thus, these data demonstrate that the HPLC ESI-MS/MS method is an accurate method for quantifying C1P levels and examining changes in the levels of C1P.

Ceramide transported by CERT is utilized by CERK in the production of $d_{18:1/16:0}$ C1P

A previous report by our laboratory has demonstrated that CERK utilized ceramide transported by CERT as a substrate. However, a recent publication by Boath et al. (16) cast doubt on this aspect of C1P anabolism and in light of the issue with C1P overestimations in cells, we re-evaluated the role of CERT in this mechanism. In this regard, A_{549} cells were treated with siRNA to specifically downregulate CERT, and the changes to the C1P profile were compared against those of control siRNA treated cells using the new mass spectrometric method (Fig. 5A). Similar to the downregulation of CERK, the downregulation of CERT also resulted in a 50% decrease in $d_{18:1/16:0}$

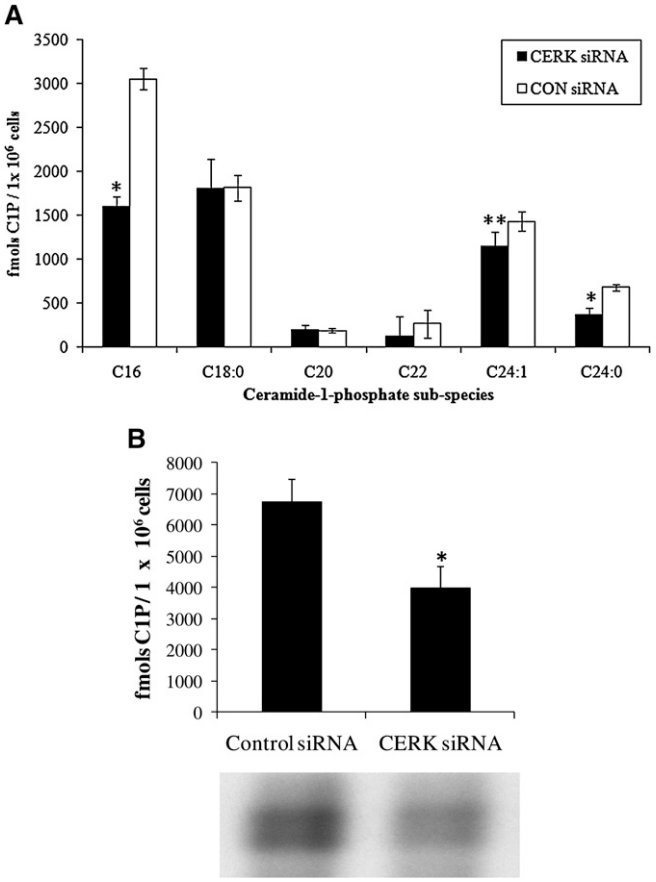

Fig. 5. CERK is responsible for the production of a specific C1P subspecies. A: A549 cells (5×10^5) were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, the cells were treated with either nontargeting siRNA or siRNA against CERK as described in the Materials and Methods section. 24 h post siRNA treatment, cells were harvested and subjected to lipid analysis using the modified method described in the Materials and Methods section. Black bars, amount C1P detected in A549 cells following siRNA treatment against CERK. White bars, amount of C1P detected in A549 cells following treatment with a nontargeting pool of siRNA. B: A549 cells (5×10^5) were plated on 10 cm plates in the appropriate medium and grown overnight under standard incubator conditions. The following day, the cells were treated with either nontargeting siRNA or siRNA against CERK as described in the Materials and Methods section. Twenty-four h post-siRNA treatment, phospholipids were labeled by incubating in media containing 30 μCi/ml ^{32}P orthophosphate. The lipids were harvested and subjected to lipid analysis using TLC and autoradiography using the modified method described in the Materials and Methods section. The spots on the TLC plate corresponding to C1P as denoted by the standards were scraped and counted by scintillation counting. Black bars, amount of C1P detected in A549 cells following siRNA treatment against CERK. White bars, amount of C1P detected in A549 cells following treatment with a nontargeting pool of siRNA. The data are a mean of three samples ± SE. (* $P < 0.001$, ** $P < 0.01$).

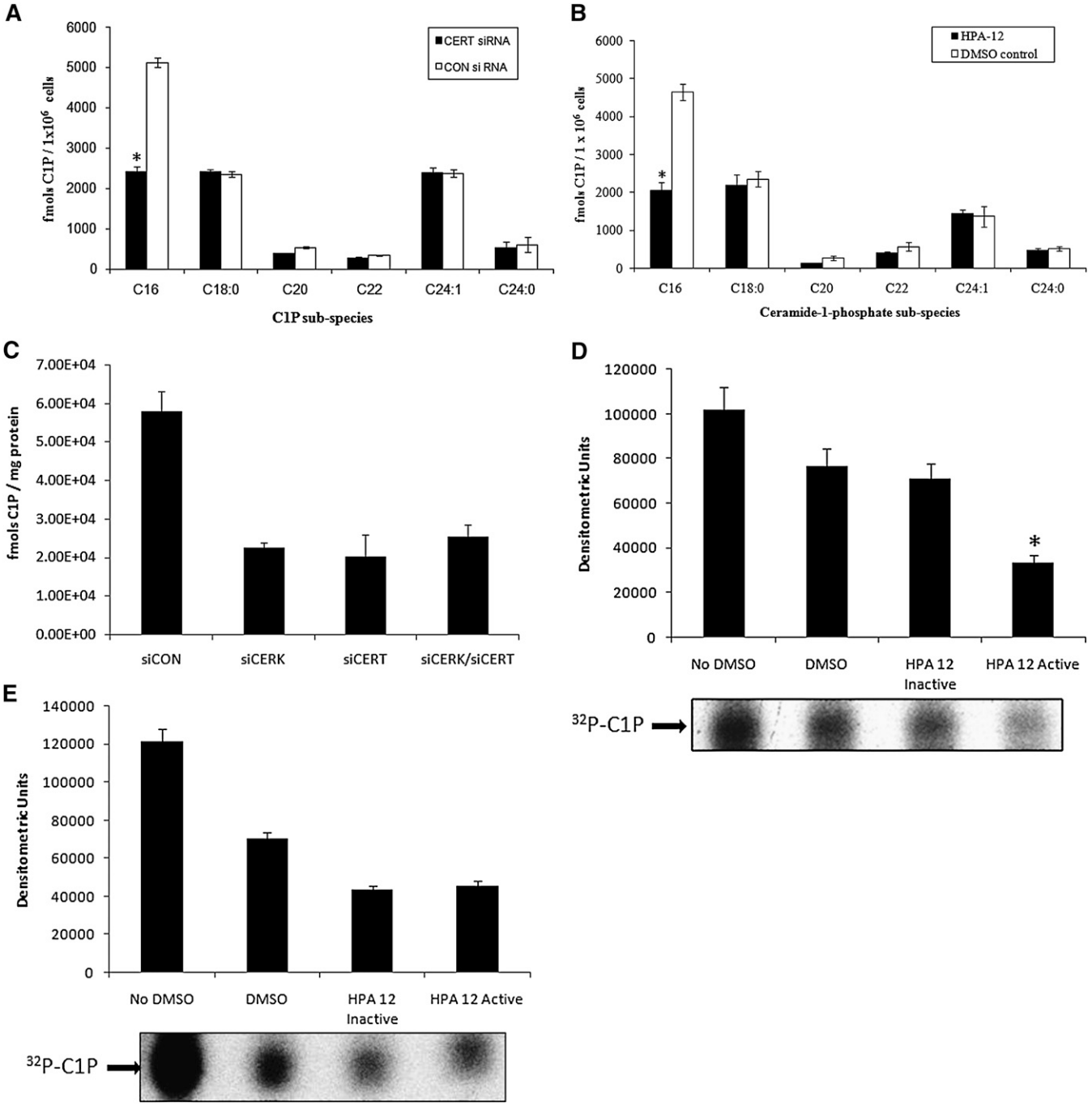

Fig. 6. Ceramide transported via the CERT pathway is specifically used for the generation of $d_{18:1/16:0}$ C1P. A: Downregulation of CERT by siRNA leads to a 50% decrease in $d_{18:1/16:0}$ C1P but does not affect any other chain lengths. A549 cells (5×10^5) were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, the cells were treated with nontargeting siRNA and siRNA against CERT as described in the Materials and Methods section. Forty-eight h post siRNA treatment, cells were harvested as described in the Materials and Methods section and subjected to lipid analysis using the modified method described in the Materials and Methods section. Black bars, amount C1P detected in A549 cells following siRNA treatment against CERT. White bars, amount of C1P detected in A549 cells following treatment with a nontargeting pool of siRNA. B: Inhibition of CERT using the specific pharmacological inhibitor HPA-12 results in a 50% decrease in $d_{18:1/16:0}$ C1P but demonstrates no affect on the other chain lengths. A549 cells (1×10^5) were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, the cells were treated with the CERT inhibitor HPA-12 or its inactive racemic mix at a concentration of 10μM and at a dilution of 1:5000. The cells were grown in SIC overnight and lipids were extracted as described in the Materials and Methods section. The lipids were analyzed according to the modified method described in the Materials and Methods section. Black bars, amount C1P detected in A549 cells following treatment with CERT inhibitor HPA-12. White bars, mount of C1P detected in A549 cells following treatment with inactive racemic mix of the CERT inhibitor HPA-12. C: Cotreatment of A_{549} cells with siRNA against CERK and CERT does not demonstrate a synergistic decrease. A_{549} cells (5×10^5) were plated on 10 cm plates in the appropriate medium and grown under standard incubator conditions overnight. The following day, the cells were treated with nontargeting siRNA and siRNA against CERT, siRNA against CERT, and siRNA against both CERK and CERT as described in the Materials and Methods section. Twenty-four h post-siRNA treatment, cells were harvested as described in the Materials and Methods section and subjected

C1P levels with minimal effects on the other chain lengths (**Fig. 6A**). To further verify this observation, cells were also treated with HPA-12, a pharmacological inhibitor of CERT. Similar to downregulation of CERT, HPA-12 treatment also demonstrated a 50% decrease in $d_{18:1/16:0}$ C1P with minimal changes in the other chain lengths of C1P (Fig. 6B). Cotreatment of A_{549} cells with siRNA against both CERK and CERT did not demonstrate an additional decrease in $d_{18:1/16:0}$ C1P (Fig. 6C). To verify the results obtained by MS, A_{549} cells were pretreated for 1 h with HPA-12 followed by a λ-^{32}P ATP pulse chase of three h. At the end of three hours, the lipids were extracted as described in Materials and Methods and subjected to TLC analysis. Densitometric analysis of the autoradiogram revealed a 50% reduction in the levels of C1P in HPA-12 treated cells compared with cells treated with DMSO or the inactive control compound (Fig. 6D). These data indicate that CERK is utilizing ceramide transported via CERT to produce $d_{18:1/16:0}$ C1P and that a CERT-independent pathway is responsible for supplying the substrate for other CERK derived C1P subspecies (e.g., $d_{18:1:24:0}$ and $d_{18:1/24:1}$ C1P).

DISCUSSION

The presence of C1P in cells and its role in many cellular biologies have been under investigation for almost two decades. The means of quantitatively analyzing their changes became practical only with the invention of the appropriate mass spectrometric techniques. The pioneering work by Merrill et al. (12) made mass spectrometric analysis of C1P possible. However, there were a few drawbacks in these early methods that questioned their accuracy in quantifying C1P, and this study introduces an improved and validated method for quantitation of various subspecies of this important lipid. C1P is a challenging molecule to investigate by MS in a practical sense due to several reasons. The greatest drawback is that the ionization efficiency of the molecule is quite low compared with similar anionic lipid species. One would assume that C1P ionizes effectively (operating pH range of 8.0–2.0) in the negative mode due to its anionic nature. However, in the gas phase, C1P acts more like a base than an acid whereby it gains a proton more readily than losing one. This low ionization efficiency results in low signal strength, which makes quantitation difficult. Quantitation is especially difficult for the low abundance species of C1P such as $d_{18:1/24:0}$ C1P. Thus, a low flow rate method where ionization efficiency is much greater is a better choice for quantitative analysis of C1P. Furthermore, as the data presented in this study demonstrate, quantitative analysis of C1P by HPLC ESI-MS/MS can achieve a very practical limit of quantitation in the low fmol range.

A major drawback in the mass spectrometric analysis of C1P was the lack of proper internal standards. The ideal internal standards for HPLC ESI-MS/MS analysis of C1P are C1P subspecies labeled with a stable isotope. Inclusion of a known quantity of such standards would not only allow for the retention times of each subspecies to be identified, but also for more accurate quantitation in an RP method by accounting for possible ion suppression for each individual subspecies of C1P analyzed. The investigations carried out by Shaner et al. (10), have demonstrated the validity of using a single uncommon C1P species ($d_{18:1/12:0}$ C1P) toward the quantitation of all subspecies of C1P; however, with very low levels of C1P present in the sample and the low ionization efficiency of C1P, retention time markers become essential for C1P analysis by HPLC ESI-MS/MS (**Table 1**). The observation that C1P can be easily synthesized from SM through base hydrolysis of biological samples is an asset allowing for quick generation of all subspecies of C1P. This method of producing C1P is faster, cheaper, and less labor intensive than enzymatic conversion of ceramide by CERK. This method is also safer than enzymatic conversion from SM via bacterial or spider venom sphingomyelinase D. The possibility of using this technique for the commercial production of C1P as opposed to organic synthesis is an interesting aspect that is worthwhile investigating. However, it should be noted that when measuring absolute quantities of C1P using the described method, a correction needs to be made to account for the loss in signal due to the presence of ^{13}C isotope in the analyte as described by Han and Gros (17, 18). Although this correction is required when comparing C1P chain length distribution between cell types, it is less relevant when comparing the changes between the same chain length of C1P from different experiments or comparing the relative ratios of the chain lengths of C1P from different experiments.

Although CERK is the only known enzyme to produce C1P, evidence has been accumulating for the presence of alternative pathways. The most notable of these is the presence of significant quantities of C1P in CERK$^{-/-}$ mice (13,

to lipid analysis using the modified method described in the Materials and Methods section. D: HPA 12 specifically decrease C1P when treated at a low DMSO concentration. A549 cells (1×10^5) were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, the cells were pretreated for 6 h with DMSO alone, CERT inhibitor HPA-12, or its inactive racemic mix at a concentration of 10μM in DMSO and at a dilution of 1:5000. The cells were then subjected to a pulse treatment with 30 μCi/ml ^{32}P orthophosphate followed by a 2 h chase. The lipids were extracted, separated by TLC, and subjected to autoradiographic detection as described in the Materials and Methods section. The amounts of ^{32}P labeled $d_{18:1/16:0}$ C1P between the different groups of treated cells were compared by densitometric analysis. E: Effect of HPA 12 on C1P generation is masked by a nonspecific effect by DMSO. A549 cells (1×10^5) were plated on 10 cm plates in the appropriate medium and grown under SIC overnight. The following day, the cells were pretreated for 6 h with DMSO alone, CERT inhibitor HPA-12, or its inactive racemic mix at a concentration of 10μM in DMSO and at a dilution of 1:1000. The cells were then subjected to a pulse treatment with 30 μCi/ml ^{32}P orthophosphate followed by a 2 h chase. The lipids were extracted, separated by TLC, and subjected to autoradiographic detection as described in the Materials and Methods section. The amounts of ^{32}P labeled $d_{18:1/16:0}$ C1P between the different groups of treated cells were compared by densitometric analysis. Data are the average of three different experiments ± SEM.

TABLE 1. AB 4000 Mass spectrometer settings and retention times for reverse chromatographic separation under the described conditions for long chain bases, long chain base phosphates, and C1P

	Precursor Ion *m/z*	Product Ion *m/z*	DP	CE	CXP	Retention Time
$d_{17:1}$ So	286.40	268.30	135.00	15.00	15.00	0.569
$d_{17:1}$ Sa	288.40	260.20	50.00	21.00	15.00	0.688
$d_{18:1}$ So	300.50	282.30	50.00	21.00	16.00	0.587
$d_{18:1}$ So	302.50	284.30	50.00	23.00	16.00	0.725
$d_{17:1}$ So1P	366.40	250.40	50.00	23.00	16.00	0.706
$d_{17:1}$ Sa1P	368.40	252.40	50.00	25.00	16.00	0.844
$d_{18:1}$ So1P	380.40	264.40	81.00	25.00	16.00	0.954
$d_{18:1}$ Sa1P	382.40	266.40	81.00	25.00	16.00	0.697
$d_{18:1/2:0}$ C1P	422.30	264.40	81.00	27.50	16.00	1.21
$d_{18:1/12:0}$ C1P	562.40	264.40	81.00	41.00	16.00	3.24
$d_{18:1/14:0}$ C1P	590.40	264.40	81.00	43.50	16.00	3.44
$d_{18:1/16:0}$ C1P	618.50	264.40	81.00	46.00	16.00	3.82
$d_{18:0/16:0}$ C1P	620.50	266.40	81.00	46.00	16.00	3.95
$d_{18:1/18:1}$ C1P	644.50	264.40	81.00	48.50	16.00	3.85
$d_{18:1/18:0}$ C1P	646.50	264.40	81.00	48.50	16.00	4.05
$d_{18:1/20:0}$ C1P	674.60	264.40	81.00	51.00	16.00	4.15
$d_{18:1/22:0}$ C1P	702.70	264.40	81.00	53.50	16.00	4.38
$d_{18:1/24:1}$ C1P	728.60	264.40	81.00	56.00	16.00	4.58
$d_{18:1/24:0}$ C1P	730.60	264.40	81.00	56.00	16.00	4.66
$d_{18:1/26:1}$ C1P	756.70	264.40	81.00	58.50	16.00	4.68
$d_{18:1/26:0}$ C1P	758.70	264.40	81.00	58.50	16.00	4.71

16, 19). Bornancin et al. (13) noted that these mice are deficient in a significant portion of their $d_{18:1/16:0}$ C1P levels compared with the wild-type mice. However, no mention was made as to the effects on the other chain lengths of C1P. The data presented here confirm these initial findings by Graf et al. (13) while also demonstrating that CERK produces at least one other subspecies. Thus, it is evident that one or more pathways exist that are responsible for the CERK-independent portion of $d_{18:1/16:0}$ and $d_{18:1/24:0}$ C1P as well as the other chain lengths of C1P. Importantly for biological mechanisms regulated by C1P, the siRNA mediated downregulation of CERK, and the resulting 50% downregulation of $d_{18:1/16:0}$ C1P, caused nearly a 80% downregulation of A23187 mediated arachidonic acid release, indicating that it is likely not the total C1P, but the CERK-derived pool that is important in the activation of $cPLA_2\alpha$. Inasmuch as it has been demonstrated that all biologically available forms of C1P can activate $cPLA_2\alpha$ (20), the fact that the CERK-derived pool is playing such an important role can be attributed to the fact that synthesis of C1P by CERK is occurring in a specific location in the cell. More specifically, CERK is generating C1P in the *trans*–Golgi where $cPLA_2\alpha$ has been demonstrated to localize to upon stimulation. Thus, the location of C1P production is the overriding factor of importance in terms of C1P-mediated activation of $cPLA_2\alpha$, and not the total amount of C1P in the cell.

The activation of $cPLA_2\alpha$ by CERK-derived C1P previously led to our laboratory discovering that CERK utilizes ceramide transported to the *trans*-Golgi by CERT (14). In contrast, Boath et al. (16) reported that a CERT specific inhibitor (HPA-12) showed no effect on the levels C1P in COS cells. However, the treatment of A_{549} cells with the same inhibitor as well as downregulation of CERT by a specific siRNA clearly demonstrated a 50% decrease in $d_{18:1/16:0}$ C1P analogus to the effect of CERK siRNA. Furthermore, simultaneous downregulation of CERT and CERK did not further lower the levels of $d_{18:1/16:0}$ C1P, demonstrating these two enzymes are in the same anabolic pathway for this C1P species. The disparity between these two studies may be due to the different cell types used, but our experiments during the course of the study suggest that the concentration of DMSO (14 mM) utilized in the previous study may have produced an artifactual suppression of C1P, leading to the disparity in reported results and a lack of HPA-12 effect on C1P in cells (Fig. 6E). Decreasing the final concentration of DMSO by 5-fold led to the contrasting finding that HPA-12 significantly decreased the levels of $d_{18:1/16:0}$ C1P by HPA-12 (Fig. 6D) when compared with the inactive racemic mixture and the DMSO control. Thus, commonly utilized levels of DMSO (14 mM, 1:1000 dilution) have distinct effects on sphingolipid metabolism, specifically C1P levels. Another possibility for the different reports is that low abundance of the C1P as a noticeable effect will not be observed until the CERT dependent ceramide pool is depleted to a such an extent that substrate ceramide becomes limiting for CERK. This may explain why an effect is observed with both siRNA treatment and HPA-12 treatment over a longer period of time (3 h), but not when treated with HPA-12 for a shorter period of time (30 min) (16). Furthermore, there is relatively nothing known about the turnover of C1P in cells, and a slow rate of catabolism could also account for the disparate findings. Regardless, utilizing both siRNA and small molecule inhibitor methodologies, the study clearly demonstrates that CERT provides the substrate $d_{18:1/16:0}$ ceramide to CERK, which also makes logical sense in the context of $cPLA_2\alpha$ activation.

Overall, the current study demonstrates the validity of an improved method developed for the quantitative analysis of C1P by HPLC ESI-MS/MS. Using this method, we demonstrate for the first time the accurate changes on the C1P chain length profile upon downregulation of CERK and CERT. Our data demonstrate that CERK is only re-

sponsible for the production of a subset of C1P subspecies, and the CERK derived $d_{18:1/16:0}$ C1P pool is dependent on substrate ceramide transported by CERT as previously reported.

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