Ceramide kinase (CerK) and ceramide-1-phosphate (C1P) are involved in various cellular functions, while regulation of the enzyme activity has not been well elucidated. We herein investigated the effects of several glycerophospholipids on human recombinant CerK activity with CaCl₂ and MgCl₂ by measuring the formation of fluorescent labeled C1P in vitro. CerK activities were 44.1±11.4 (pmol/g/min) with vehicle, 137±29 with 2 mM CaCl₂, and 144±32 with 2 mM MgCl₂ in the glycerol/albumin buffer. The addition of glycerophospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid had no effect on CerK activity with CaCl₂, although Pl(4,5)P₂ and phosphatidic acid bound to CerK in the lipid–protein overlay assay. The addition of cardiolipin (diphasphatidylglycerol) at concentrations up to 0.1 µM increased, whereas those more than 1 µM decreased CerK activity with CaCl₂/MgCl₂. In the lipid–protein overlay assay, cardiolipin bound to CerK and CerK lacking pleckstrin homology (PH) domain, but not PH domain of CerK, in CaCl₂-independent manner. Cardiolipin also bound to CerK in the multilamellar vesicle binding assay. A deviation from the normal range of cellular cardiolipin, both the decrease by phospholipase D6 expression and increase by an exogenous addition of the lipid, negatively regulated C1P formation in intact HepG2 cells. Our results revealed that cardiolipin bound to CerK and regulated the formation of C1P in vitro and in cells.

Key words  ceramide kinase; cardiolipin; glycerophospholipid

MATERIALS AND METHODS

Materials  The materials used in this study and their sources were as follows. A fluorescent ceramide, 4-nitrobenzene-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-ceramide) was purchased from Molecular Probes (Eugene, OR, U.S.A.); cardiolipin (from bovine heart), glycerol, Triton X-100, and bovine serum albumin (fatty acid-free) were from Sigma-Aldrich (St. Louis, MO, U.S.A.); phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and PI were from Matreya LLC (Pleasant Gap, PA, U.S.A.); Pl(4,5)P₂ (1,2-dipalmitoyl) was from Cayman (Ann Arbor, MI, U.S.A.). Stock solutions of the respective lipids were as follows: cardiolipin (ethanol), glycerophospholipids such as phosphatidylcholine and phosphatidylethanolamine (chloroform–methanol,
2:1, v/v), Pi(4,5)P_{2} (distilled water), and NBD-ceramide (dimethyl sulfoxide (DMSO)). In some experiments, cells were cultured with cardiolipin micelles in medium with serum. The cardiolipin micelles were prepared by sonication of medium with cardiolipin for 5 min in a bath-type sonicator until the mixture becomes a homogenous dispersion. After being cultured for the indicated times, cells were washed with cardiolipin-free medium and then used to measure the formation of NBD-C1P and cellular levels of cardiolipin.

**Construction of Plasmids for Recombinant Human CerK and Green Fluorescent Protein (GFP)-Tagged Phospholipase D6 (PLD6, Mitochondrial PLD) and Preparation of CerK** cDNA encoding human CerK (a gift from Dr. Kohama, Daiichi-Sankyo, Co., Ltd., Tokyo, Japan) was amplified by PCR using a forward primer 5'-TAA AGG ATCCAT GGGGGC GAC GGGG-3', and a reverse primer 5'-TAT AGC TCG AGT CAG CTG TGT GAG TCT GG-3'. This amplification product (full-length, 1–537) was cloned into BamHI and XhoI of pGEX-6P-1 vector to generate the glutathione S-transferase (GST) fusion construct. Likewise, two deletion mutants: PH domain of CerK (1–124, PH-CerK), and CerK lacking PH domain (125–537, ΔPH-CerK) were created using a forward primer 5'-TAA AGG ATCCAT GGGGGC GAC GGGG-3', and a reverse primer 5'-TAT AGC TCG AGT CACTCC AGT CACCCTCG-3' for PH-CerK, and a forward primer 5'-TAA AGG ATC CA AGCTGAGCAGTG CAGA 3', and a reverse primer 5'-TAT AGC TCG AGT CACTCC AGT CACCCTCG-3' for ΔPH-CerK, and a forward primer 5'-TAA AGG ATC CA AGCTGAGCAGTG CAGA 3', and a reverse primer 5'-TAT AGC TCG AGT CACTCC AGT CACCCTCG-3'.

**CerK Activity Assay** The activity of recombinant CerK in vitro with 10 μM NBD-ceramide was measured in the two buffers, as described previously,[20,22] with modifications. The glycerol/albumin buffer was used to examine the effects of glycerophospholipids including cardiolipin on CerK activity. As described in the Results, supplementation of glycerophospholipids including cardiolipin did not change CerK activity in the cardiolipin/Triton X-100 buffer. To study the CaCl_{2}/MgCl_{2} responsibility on CerK activity, both the glycerol/albumin buffer and cardiolipin/Triton X-100 buffer were used. The assays using the glycerol/albumin buffer were performed as follows. NBD-ceramide and cardiolipin were directly added to the reaction buffer (90 μL). CerK (30 ng/tube, 10 μM) was added to the assay mixtures (total volume 100 μL), and incubated at 37°C for 15 min. The assay mixture consisted of 10% glycerol, 0.02% albumin, 100 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 40 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), CaCl_{2}, and/or MgCl_{2} at the indicated concentrations. The assays using the cardiolipin/Triton X-100 buffer were performed as follows. Mixes containing cardiolipin, Triton X-100, and NBD-ceramide were prepared by drying lipids from stock solutions with N_{2} gas and then sonicating (3 min) in reaction buffer (100 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 40 mM HEPES, pH 7.4). The mixtures were diluted five-fold with reaction buffer, and 100-μL reactions were initiated by the addition of CerK (30 ng), CaCl_{2}, and/or MgCl_{2}. The final concentrations of the reagents in the mixed micelles were: 36 μM cardiolipin, 0.0125% Triton X-100, and 10 μM NBD-ceramide. In the two assays, concentration of glutathione derived from CerK preparation was 0.046 μM.

In both buffers, the reaction was stopped by the addition of 125 μL of chloroform and 250 μL of methanol after the incubation. In order to extract C1P to the organic phase, the pH of the mixture was adjusted to approximately 2–3 by the addition of 5 nL HCl (1 μL), and the mixtures were vigorously vortexed and incubated at 4°C for 10 min. After adding 125 μL of chloroform and 125 μL of water, the samples were then divided into organic and aqueous phases using centrifugation (3000 rpm, 10 min). NBD-C1P extracted in the organic phase (100 μL) was dried under nitrogen. Dried samples were dissolved in 10 μL of chloroform–methanol (1:1) and analyzed on silica gel-60 TLC plate (Merk, Germany). The formation of NBD-C1P was measured for 15 min at 37°C, and CerK activity (pmol NBD-C1P/μg CerK protein/min) was examined. In the glycerol/albumin and cardiolipin/Triton X-100 buffers with and without supplements, such as CaCl_{2}–MgCl_{2} and the indicated lipids, the formation of NBD-C1P was linear for 30 min depending on the concentration of CerK from 10 to 30 ng per assay tube.

**Lipid–Protein Binding Assays: Overlay Assay and Large Multilamellar Vesicle Binding Assay** Assays were performed as previously described[20] with minor modifications. Briefly, tested lipids were dissolved in the indicated solvents and spotted onto a Hydrophobic C membrane (Amersham) in the lipid–protein overlay assay. After drying, the membrane was rewetted and blocked for 1 h in the TBS/Tween 20 buffer containing 2% albumin, and then exposed overnight at 4°C to respective preparations of GST-tagged recombinant human CerK proteins containing 0.1 mM CaCl_{2}. In some cases, the CerK preparations containing 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA) was used. The washed membranes were treated with an anti-GST antibody (27457701V, GE Health Care, Buckinghamshire, U.K.), and the immunoreactive spots were visualized using a second antibody. In the vesicle binding assay, vesicles of cardiolipin or phosphatidylycholine were prepared by vigorously vortexing the lipids in the buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 mM CaCl_{2}). The binding reaction was initiated by the adding of lipid vesicle solutions and GST-CerK. After 5 min at room temperature, the reaction mixture was centrifuged at 17000×g for 10 min, and the supernatant was removed. After repeated washing and centrifugation, the pellet was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the immunoreactive...
bands to an anti-GST antibody were visualized using a second antibody. In the two assays, purified recombinant GST did not bind to any of the lipids.

Transfection of HepG2 Cells with the PLD6-GFP Vector, and Measurement of PLD6-GFP Expression Regarding protein expression, cells were seeded at a density of 4.5×10⁴ cells/well (12-well plate), cultured for 2 d in Dulbecco’s modified Eagle’s medium (DMEM) with serum, and transiently transfected with 2 µg of expression vectors for Lipofectamine PLUS (Invitrogen) in Opti-MEM without serum, according to the manufacturer’s protocol. After a 3-h incubation, transfected cells were further cultured with DMEM with serum for 24 h, and then used in various assays such as the expression of PLD6-GFP, measurement of intracellular cardiolipin levels, and formation of C1P. The expression of PLD6-GFP was confirmed by Western blotting using an anti-GFP antibody (sc-8334, Santa Cruz Biotech. Dallas, TX, U.S.A.). In some cases, transfected cells were seeded on the coverslips (12 mm in diameter) of glass-bottomed dishes (Iwaki, Tokyo, Japan). After being cultured for 40–48 h, the cells were washed with buffer and GFP-derived fluorescent images were taken with a Fluoview-FV500 confocal laser scanning microscope system (Olympus, Tokyo).

Measurement of Intracellular Cardiolipin Levels Phospholipids including cardiolipin in HepG2 cells expressing PLD6-GFP and cultured with cardiolipin were extracted in chloroform–methanol–HCl solution as described previously with minor modifications. The organic fraction after evaporation was separated on silica gel-60 HPTLC plate. The positions of phospholipids such as cardiolipin and PI on the plate after visualization using iodine staining were determined by matching the migration distance to standards and previously reported Rf values. The intensity of iodine staining was measured using NIH ImageJ software.

Measurement of NBD-C1P Formation in HepG2 Cells Cells were incubated with 10 µM NBD-ceramide for 30 min or 1 h, and lipids including NBD-labeled ceramide metabolites in wells (both cells and medium) were extracted by chloroform–methanol–HCl solution as described previously with minor modifications. The organic fraction after evaporation was separated on silica gel-60 HPTLC plate. The positions of phospholipids such as cardiolipin and PI on the plate after visualization using iodine staining were determined by matching the migration distance to standards and previously reported Rf values. The intensity of iodine staining was measured using NIH ImageJ software.

RESULTS

Roles of CaCl₂ and MgCl₂ on CerK Activity First, activities of human recombinant CerK were examined with and without CaCl₂ and MgCl₂. In the glycerol/albumin buffer containing the substrates (1 mM ATP and 10 µM NBD-ceramide), our preparations of recombinant CerK exhibited a marked activity in vitro (Figs. 1A, B, open circles). The activity was 44.1±11.4 pmol/µg/min (n=7, Fig. 1C). CerK activity was increased by the addition of CaCl₂ and MgCl₂, and the addition of CaCl₂ and MgCl₂ at 0.1 mM led to almost maximal stimulatory effects on CerK activity (Fig. 1C). CaCl₂ and MgCl₂ at 2 mM both increased this activity approximately 3-fold over that of control activity. CerK activity was previously reported to increase from 0.1 to 10 µM Ca²⁺ thus, we investigated the roles of CaCl₂ on CerK activity in detail. The activities of CerK with and without 0.1 mM CaCl₂ were almost completely abolished in the presence of 2 mM EGTA, while those with 2.5 mM CaCl₂ and 0.1 mM MgCl₂ were not modified by EGTA (Fig. 1D). The addition of not only 2 mM EGTA, but also 2 mM ethylenediaminetetraacetic acid (EDTA) to the assay mixtures markedly decreased CerK activity (Table 1). The addition of CaCl₂ at 10 µM did not increase CerK activity (40–50 pmol/µg/min), whereas 50 µM CaCl₂ markedly increased CerK activity to approximately half of that with 0.1 mM. The combination of CaCl₂ and MgCl₂ at 1 mM did not enhance CerK activity; the value with CaCl₂/MgCl₂ was 0.97±0.12-fold of that with MgCl₂ alone (n=3).

Effects of Glycerophospholipids on CerK Activity Next, we investigated the effects of various glycerophospholipids on CerK activity in the glycerol/albumin buffer. Phosphatidylcholine, phosphatidylethanolamine, PI, and phosphatidylserine at 0.1 µM had no significant effect on CerK activity (Table 2). Phosphatidic acid, an acidic glycerophospholipid, at 0.1 µM did not change CerK activity. These lipids including phosphatidic acid at 1 µM did not change CerK activity; the values of CerK activity with the lipids were from 0.9- to 1.1-fold and from 3.1- to 3.4-fold of control in the absence and presence of 0.1 mM CaCl₂, respectively, which were similar as those without the lipids. PI[4,5]P₂ at 0.1 µM had no effect, whereas the lipid at 1 µM slightly inhibited CerK activity with 0.1 mM CaCl₂ (Table 2): a relative CerK activity with 1 µM PI[4,5]P₂ was 82±8% (% of the control without the lipid, n=3). Addition of 0.1 µM cardiolipin (diphasphatidylglycerol) slightly stimulated CerK activity in the absence of CaCl₂, but the responses were not significant (Figs. 2A, B). In the presence of 0.1 mM CaCl₂, cardiolipin showed a bell-shaped response that depended on the lipid concentrations: cardiolipin stimulated CerK activity from 0.05 to 0.1 µM and inhibited it at concentrations greater than 0.5 µM. The activities of CerK with 1 and 5 µM cardiolipin were significantly less than that with 0.1 µM. Cardiolipin at 0.1 µM increased CerK activity with 0.1 mM MgCl₂ (Figs. 2C, D). In the presence of 0.1 mM MgCl₂, the activity of CerK
with 1 µM cardiolipin was 1.6- and 1.8-fold in two independent experiments, which were markedly less than that with 0.1 µM. Thus, cardiolipin appeared to show dual effects, stimulation with 0.1 µM and inhibition with 1 µM, on CerK activity in the presence of MgCl₂. We examined the effect of 0.1 µM cardiolipin on the concentration-dependency of CaCl₂ or MgCl₂ from 0.01 to 0.1 mM, and the lipid did not affect the sensitivity of CerK activity to CaCl₂ and MgCl₂ in a typical experiment (data not shown). These results demonstrated that cardiolipin, not other glycerophospholipids tested, directly affected CerK activity in vitro in the glycerol/albumin buffer containing CaCl₂ or MgCl₂.

Next, we examined CerK activity in the cardiolipin/Triton X-100 buffer, and the basal activity and the CaCl₂-MgCl₂ responsibility in the buffer were compared with those in the glycerol/albumin buffer (Fig. 2E, Table 3). CerK activity was 26.7±2.9 pmol/µg/min (n=7) without the exogenous addition of CaCl₂/MgCl₂ which was less than that in the glycerol/albumin buffer, 44.1±11.4 pmol/µg/min. When CerK activity of a same recombinant CerK preparation was measured in the two buffers in an experiment, CerK activity in the cardiolipin/Triton X-100 buffer was 40.1±9.6% of that in the glycerol/albumin buffer (p<0.05, n=3). CerK activity in the cardiolipin/Triton X-100 buffer was increased by the addition of CaCl₂ and MgCl₂. Interestingly, the CerK activity with 1 mM MgCl₂ in the cardiolipin/Triton X-100 buffer was two-fold of that in the glycerol/albumin buffer. The addition of CaCl₂ at 10 µM did not increase CerK activity (20–30 pmol/µg/min), and the activity with 50 µM CaCl₂ was approximately half of that with 0.1 mM in the cardiolipin/Triton X-100 buffer. The CerK activity with CaCl₂/MgCl₂ at both 1 mM was 0.8±0.2-fold of that with MgCl₂ alone (n=3). Similar to the response in the glycerol/albumin buffer, CerK activity was markedly decreased in the presence of 2 mM EGTA and EDTA in the cardiolipin/Triton X-100 buffer (Table 1). In the cardiolipin/Triton X-100 buffer, supplementation of glycerophospholipids.
such as PI(4,5)P2 and cardiolipin at concentrations from 0.1 to 1 µM did not change CerK activity (data not shown). The obtained data, regulation of CerK activity by cardiolipin in the glycerol/albumin buffer and a greater CerK activity in the cardiolipin/Triton X-100 buffer with MgCl2 showed that cardiolipin affected the activity of human recombinant CerK.

Binding of Cardiolipin with CerK  We employed another method to show the direct effect of cardiolipin on CerK: lipid–protein binding assays in two manners. In the lipid–protein overlay assay, cardiolipin bound to GST-CerK in a lipid concentration-dependent manner in the presence of 0.1 mM CaCl2 (Figs. 3A, B). PI(4,5)P2 and phosphatidic acid at 2 nmol also bound to GST-CerK, as described previously.16,17) Other glycerolipids tested did not bind to GST-CerK. Cardiolipin bound to GST-ΔPH-CerK (Fig. 3C), but not GST-PH-CerK (Fig. 3D) or the GST protein (Fig. 3E), under our conditions. Since divalent cations including Ca2+ may interact with the negatively charged head-group of cardiolipin, we examined the effects of CaCl2 on cardiolipin binding to CerK. The direct binding of cardiolipin to GST-CerK and GST-ΔPH-CerK was observed in the presence of 1 mM EGTA without CaCl2: the bindings of both proteins were approximately 80–90% of those with 0.1 mM CaCl2. The direct binding of cardiolipin to GST-CerK was examined in another lipid–protein binding assay, the large multilamellar vesicle binding assay (Fig. 3F). CerK bound to vesicles containing cardiolipin, but not phosphatidylcholine.

Table 3. CerK Activity in the Glycerol/Albumin Buffer and the Cardiolipin/Triton X-100 Buffer

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Glycerol/Albumin</th>
<th>Cardiolipin/Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CerK activity (pmol/µg/min)</td>
<td>CerK activity (pmol/µg/min)</td>
</tr>
<tr>
<td>None</td>
<td>44.1±11.4</td>
<td>26.7±2.9</td>
</tr>
<tr>
<td>1 mM CaCl2</td>
<td>110±25</td>
<td>101±12</td>
</tr>
<tr>
<td>1 mM MgCl2</td>
<td>118±26</td>
<td>240±11</td>
</tr>
<tr>
<td>CaCl2 and MgCl2</td>
<td>CerK activity (fold of the value with MgCl2)</td>
<td>0.97±0.12</td>
</tr>
</tbody>
</table>

Some data shown in Fig. 1C (the glycerol/albumin buffer) and Fig. 2E (cardiolipin/Triton X-100 buffer) were summarized. CerK activity was measured with vehicle, 1 mM CaCl2, 1 mM MgCl2, and the combination of CaCl2/MgCl2. The effects of combination of CaCl2/MgCl2 were expressed as a percentage of activity with 1 mM MgCl2 in the respective buffer, and are the mean±S.D. of three independent experiments.

Possible Role of Cardiolipin on C1P Formation in Cells Choi et al. reported that over-expression of mitochondrial PLD6 caused a marked decrease in cardiolipin levels and that PLD6-GFP localized in mitochondria in NIH3T3 cells.25 Therefore, we attempted to regulate cardiolipin levels in HepG2 cells by transient transfection with the vector for PLD6-GFP (Fig. 4). The expression of PLD6-GFP was confirmed at the protein level (Fig. 4A). Choi et al. showed the localization of PLD6-GFP in the mitochondria in NIH3T3...
cells. A similar intracellular localization of PLD6-GFP was observed in HepG2 cells (Fig. 4B). The cellular amounts of cardiolipin, but not those of other glycerophospholipids such as phosphatidylcholine and PI, were decreased in cells expressing PLD6-GFP (Fig. 4C). In cells expressing PLD6-GFP, cardiolipin level was 71% (% of the control), and levels of PE, PI, PS, and PC were 98, 99, 102, and 103%, respectively, in a typical experiment. Control HepG2 cells and HepG2 cells expressing PLD6-GFP were incubated with 10 µM NBD-ceramide for 30 min, and the levels of NBD-fluorescence in the extracted cellular lipids were analyzed. The formation of NBD-C1P was significantly less in HepG2 cells expressing PLD6-GFP than in the control cells (Figs. 4D, E). The formation of NBD-sphingomyelin was slightly but significantly less in HepG2 cells expressing PLD6-GFP than in control cells. Cellular levels of NBD-glucosylceramide, NBD-ceramide, and the endogenous CerK protein (data not shown) were almost the same in the two cells. These results suggest a probable stimulatory effect of endogenous cardiolipin on C1P formation. In contrast, cultivation of HepG2 cells with cardiolipin at greater concentrations over 10 µM for 24 h showed an inhibitory effect on the formation of NBD-C1P (Fig. 5). The cardiolipin treatment did not appear to change amounts of NBD-ceramide taken up and other ceramide metabolites: for instance, the values of NBD-ceramide, NBD-glucosylceramide and NBD-sphingomyelin in 50 µM cardiolipin-treated cells were 96–104% of the respective values in control cells. In a typical experiment, the levels of cardiolipin including the lipid attached to the cell membrane in HepG2 cells treated for 24 h with 50 µM cardiolipin were 120–130% those of control cells without cardiolipin. The levels of other phospholipids such as phosphatidylcholine and PI were not changed by the cultivation of cells with cardiolipin (data not shown). We could not detect the stimulatory effects of cardiolipin exogenously added under our experimental conditions: the levels of NBD-C1P in HepG2 cells incubated for 1 h with NBD-ceramide in the presence of 0.1–10 µM cardiolipin were approximately 90% that of control cells without cardiolipin. The cultivation of HepG2 cells expressing PLD6-GFP for 24 h with 0.1–10 µM cardiolipin did not reverse the decreased formation of C1P, and we could not examine the effects of cardiolipin at greater concentrations over 10 µM on the cells because the cultivation caused cell detachment (data not shown). Over-expression of PLD6-GFP by itself and application of cardiolipin alone from 10 to 50 µM for 24 h did not cause cell rounding and cell detachment (data not shown).

DISCUSSION

In the present study, we demonstrated that cardiolipin bound to CerK in vitro and showed diverse effects on CerK activity in vitro and in intact cells (a probable stimulation by endogenous cardiolipin and inhibition by exogenously added cardiolipin).

**Binding of Cardiolipin to CerK in Vitro** Cardiolipin is used as a molecule-stabilizing reagent in various assay systems in vitro, and has been shown to interact with various proteins possibly through the physical properties of the anionic lipid. 28–30 Also, activity of CerK was measured in cardiolipin-containing buffers in many previous reports.1,19,20,22,23 However, direct evidence to show the physical association between cardiolipin and CerK as well as activation of this enzyme by the lipid does not currently exist. In the present study, we showed for the first time that cardiolipin directly bound to recombinant CerK in vitro using two lipid–protein binding assays, an overlay assay and large multilamellar vesicle binding assay (Fig. 3). In the lipid–protein overlay assay, cardiolipin bound to the full length of CerK and ΔPH-CerK, but not PH-CerK. Previous studies reported that ΔPH-CerK has the capacity to bind to lipids. Carré et al. reported that...
ΔPH-CerK exhibited slight, but distinct binding to liposomes of phospholipids containing phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in a sucrose-loaded liposome assay; however, PH-CerK strongly bound to the liposomes.\(^\text{15}\) Kim et al. reported that PI(4,5)P\(_2\) mainly bound to PH-CerK, but other poly-phosphorylated PIs including PI3-phosphate bound to CerK via the non-PH domain of CerK.\(^\text{16}\) Rovina et al. reported that PI(4,5)P\(_2\) bound to the C-terminally FLAG-tagged CerK, but not to the C-terminally FLAG-tagged PH domain of CerK, in a similar lipid–protein overlay assay.\(^\text{17}\) In addition to PH domain, poly-phosphorylated forms of PI including PI(4,5)P\(_2\) have been shown to bind to various domains having basic motifs in proteins.\(^\text{31}\) These results including ours suggest that CerK may have multiple binding domains of the lipids such as cardiolipin and PI(4,5)P\(_2\). The exact domains and/or amino acids of CerK responsible for cardiolipin binding have yet to be identified.

**Regulation of CerK Activity by Cardiolipin in Vitro**

Both PI(4,5)P\(_2\) and phosphatidic acid were shown to bind to CerK.\(^\text{16,17}\) It was reported that PI(4,5)P\(_2\) did not change CerK activity,\(^\text{16}\) while the effect of phosphatidic acid on the activity has not yet been demonstrated. We confirmed the binding of CerK to PI(4,5)P\(_2\) and phosphatidic acid (Fig. 3A). Under our conditions, supplementation with PI(4,5)P\(_2\) and phosphatidic acid at 0.1 µM (Fig. 3D) and (E), cells expressing GFP and PLD6-GFP were incubated with 10 µM NBD-ceramide for 30 min, and ceramide metabolites including NBD-C1P in wells (cells and medium) were extracted. Ceramide metabolites were separated by a TLC method. Typical images of NBD-ceramide and the metabolites on TLC plates were shown in (D), and quantitative data were shown in (E). NBD-ceramide (NBD-Cer, Cer), NBD-glucosylceramide (NBD-GlcCer, GlcCer), NBD-C1P (C1P), NBD-sphingomyelin (NBD-SM, SM). Data were expressed as percentages of respective NBD-labeled molecules in control cells expressing GFP alone. Data are the mean±S.D. of three independent experiments. **p<0.01, significantly different from the control.

Fig. 4. Decreases in Cardiolipin Levels and NBD-C1P Formation in HepG2 Cells Over-Expressing PLD6

HepG2 cells were transiently transfected with expression vectors for GFP and GFP-tagged PLD6, and cultured for 24 h. (A) The expression of GFP and PLD6-GFP proteins was confirmed by Western blotting. (B) Green-fluorescence derived from the PLD6-GFP protein in cells was confirmed by confocal laser microscopy. (C) The phospholipids in cells were extracted, and separated on a TLC plate. Cardiolipin (CL), phosphatidylethanolamine (PE), PI, phosphatidylcholine (PS), and phosphatidylycholine (PC). Data in (A), (B), and (C) are representative of two independent experiments. In (D) and (E), cells expressing GFP and PLD6-GFP were incubated with 10 µM NBD-ceramide for 30 min, and ceramide metabolites including NBD-C1P in wells (cells and medium) were extracted. Ceramide metabolites were separated by a TLC method. Typical images of NBD-ceramide and the metabolites on TLC plates were shown in (D), and quantitative data were shown in (E). NBD-ceramide (NBD-Cer, Cer), NBD-glucosylceramide (NBD-GlcCer, GlcCer), NBD-C1P (C1P), NBD-sphingomyelin (NBD-SM, SM). Data were expressed as percentages of respective NBD-labeled molecules in control cells expressing GFP alone. Data are the mean±S.D. of three independent experiments. **p<0.01, significantly different from the control.
cardiolipin appeared to be the first case for the direct regulation of CerK by glycerophospholipids. The concentration dependency of CaCl$_2$ and MgCl$_2$ in the cardiolipin/Triton X-100 buffer (Fig. 2E) was almost the same as that in the glycerol/albumin buffer (Fig. 1C), and the addition of cardiolipin did not appear to change this dependency in the glycerol/albumin buffer. CerK activity without CaCl$_2$/MgCl$_2$ in the cardiolipin/Triton X-100 buffer was significantly less than that in the glycerol/albumin buffer at 0.1 mM CaCl$_2$ and/or C1P formation by cardiolipin including specificity of molecular forms of cardiolipin remain to be elucidated.

**Regulation of CerK Activity by Cardiolipin in Cells**

In the present study, a deviation from the normal range of cardiolipin, both the decrease by PLD6 expression and increase by an exogenous addition of the lipid, negatively regulated C1P formation in intact cells, although we could not have a direct evidence showing a stimulatory effect of cardiolipin. CerK was mainly detected in the plasma membranes, Golgi complex, and endosomes in cells, although the localization of CerK in the cytosol was reported in rat basophilic leukemia (RBL-CK3) cells. Cardiolipin appeared to be attached to the cytosolic face of the cellular organelles including the plasma membranes, and the dynamic cellular movement of cardiolipin has been proposed between mitochondria and other organelles including endosomes in cells. Enzymes for ceramide-related pathways including sphingosine kinases 1/2 were activated by cardiolipin in vitro, and many enzymes of the pathway exist in non-mitochondrial compartments such as the plasma membrane and endosomes. A probable intracellular movement of cardiolipin and/or a transient localization of cardiolipin may explain the interaction of ceramide metabolic enzymes including CerK and cardiolipin in the non-mitochondrial compartments in cells. Identification of intracellular compartments for cardiolipin–CerK interaction remains to be elucidated. Alterations of cardiolipin in cells have been shown to be involved in various pathological conditions including ischemia, hypothyroidism, Barth syndrome, and aging, etc. Also, CerK and C1P appear to be involved in ischemia and neurodegenerative diseases. Possible interactions between cardiolipin and CerK/C1P should be determined physiologically and pathologically.

**Effects of Ca$^{2+}$ and Mg$^{2+}$ on CerK Activity**

In the present study, the addition of CaCl$_2$ increased CerK activity in both the glycerol/albumin and cardiolipin/Triton X-100 buffers (Figs. 1, 2). The addition of 2 mM EGTA markedly decreased CerK activity with 0.1 mM CaCl$_2$ without changing its activity with 0.1 mM MgCl$_2$ in the glycerol/albumin buffer (Fig. 1D). These results suggested the selective trapping of Ca$^{2+}$ by EGTA and were consistent with previous findings showing
that CerK was a Ca\(^{2+}\)-dependent enzyme.\(^1\)–\(^3\) Under our conditions, the addition of Ca\(_{\text{Cl}_2}\) up to 10 \(\mu\)M did not increase CerK activity in either buffer. Similar findings were reported in immunoprecipitated CerK activity from rat basophilic leukemia cells.\(^9\) The addition of Mg\(_{\text{Cl}_2}\) increased CerK activity without Ca\(_{\text{Cl}_2}\) in both buffers (Figs. 1C, 2E). Since the addition of 0.1 mM Mg\(_{\text{Cl}_2}\) increased CerK activity in the presence of EGTA, Mg\(_{\text{Cl}_2}\) appeared to have stimulatory effects on CerK activity in a Ca\(^{2+}\)-independent manner. The results obtained in vitro suggested that either Ca\(_{\text{Cl}_2}\) or Mg\(_{\text{Cl}_2}\), possibly the divalent cations, Ca\(^{2+}\) or Mg\(^{2+}\), was essential for CerK activity. In the cardiolipin/Triton X-100 buffer, the addition of 1 mM Mg\(_{\text{Cl}_2}\) alone increased CerK activity by 8-fold, which was much greater than that by 1 mM Ca\(_{\text{Cl}_2}\), and the co-addition of Ca\(_{\text{Cl}_2}\)–Mg\(_{\text{Cl}_2}\) did not show additive and/or synergic effects. The results were consistent with those in a previous report by Van Overloop et al.\(^\text{10}\) In kinase reactions, divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) interact and reduce negative charges in the phosphate groups of ATP, and may generally enhance the transfer of phosphate groups to substrates and the association between enzymes and ATP. Previous findings and the present results suggest that Ca\(^{2+}\) is not a direct activator of CerK, while divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) appear to be essential factors for a kinase reaction of CerK. Mitsutake and Igarashi reported that calmodulin bound to CerK, which resulted in the Ca\(^{2+}\)-dependent activation of CerK.\(^\text{10}\) The regulatory mechanisms of Ca\(^{2+}\) on CerK including roles of Ca\(^{2+}\)-binding proteins such as calmodulin and calcineurin on the enzyme have yet to be established.

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Conflict of Interest The authors declare no conflict of interest.

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REFERENCES


