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Phosphorylation and inhibition of ceramide kinase by protein kinase C-β: Their changes by serine residue mutations

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A B S T R A C T

Ceramide kinase (CerK) phosphorylates ceramide to ceramide-1-phosphate (C1P), and various roles for the CerK/C1P pathway in the regulation of cellular/biological functions have been demonstrated. CerK is constitutively phosphorylated at several serine (Ser, S) residues, however, the roles of Ser residues, including their phosphorylation, in CerK activity, have not yet been elucidated in detail. Therefore, we conducted the present study to investigate this issue. In A549 cells expressing wild-type CerK, a treatment with phorbol 12-myristate 13-acetate (PMA) decreased the formation of C1P in a protein kinase C (PKC)-βI/II-mediated manner. In the Phos-tag SDS-PAGE analysis, CerK existed in its phosphorylated form and was further phosphorylated by the PMA treatment in a PKC-βI/II-mediated manner. We examined the effects of the displacement of Ser residues (72/300/340/403/408/427) in CerK by alanine (Ala, A) on its activity and phosphorylation. Triple mutations (S340/408/427A), but not a single or double mutations (S340/408A), in CerK significantly decreased the formation of C1P. PMA-induced phosphorylation levels in S340/408A- and S340/408/427A-CerK were significantly and maximally reduced, respectively, but were similar in CerK with a single mutation and wild-type CerK. Ser residue mutations tested, including six mutations, did not affect PMA-induced decreases in C1P formation more than expected. Treatments with the protein phosphatase inhibitors, okadaic acid and cyclosporine A, decreased the formation of C1P. These results demonstrated that the activity of CerK was regulated in a phosphorylation-dependent manner in cells.

1. Introduction

Ceramide and its metabolites, alternatively called sphingolipid metabolites, play crucial roles in cell fate/functions and human health/diseases [1,2]. Ceramide kinase (CerK), which mainly exists in membrane compartments, including the inner plasma membrane, phosphorylates ceramide to ceramide-1-phosphate (C1P). The intracellular C1P formed appears to be transported between membranes via vesicular as well as C1P-transfer protein-mediated trafficking [3], and C1P is released into extracellular spaces and/or the blood [4,5]. C1P has been established as a bioactive lipid that functions intracellularly and extracellularly. C1P binds with and regulates the activities of α-type cytosolic phospholipase A_2 (group IVA phospholipase A_2) [6,7] and tumor necrosis factor α-converting enzyme [8], and the extracellular application of C1P has been shown to modulate cellular responses, such as cell migration, proliferation, and apoptosis, directly and/or indirectly [2,9,10].

The cloning and functional characterization of CerK were achieved by Drs. Kohama and Spiegel's group [11] based on its sequence homology to sphingosine kinase type-1 (SphK1). CerK is ubiquitously expressed in various tissues, including the thymus, leukocytes, the brain, and heart [11,12]. CerK-depleted ($CerK^{(-/-)}$) mice were

Abbreviations: CerK, Ceramide kinase; C1P, ceramide-1-phosphate; SphK, sphingosine kinase; $CerK^{(-/-)}$, CerK depletion; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; GF109203x, bisindolylmaleimide I Gö697612-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; HBDDE, 2,2′,3,3′4,4′-hexahydroxy-1,1′-biphenyl-6,6′-demethanol dimethyl ether; LY333531, ruboxistaurin; SB203580, 4-(4-fluorophenyl)-2-(4-(methylsulfonyl)phenyl)-1H-imidazol-5-yl)pyridin; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; U0126, 2,3-bis[amino[(2-aminophenyl)thio]methylene]-butanedinitrile; SP600125, anthrax[1,9-*cd*]pyrazol-6(2H)-one; FR180204, 5-(2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-1H-pyrazolo[3,4-*c*]pyridazin-3-amine; ERK1/2, extracellular signal-related kinase-1/2; NOR4, *N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridinecarboxamide; HA, hemagglutinin; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPKs, mitogen-activated protein kinases; PP, protein phosphatase

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healthy, including their life span [13,14]; however, a slight alteration in emotional behavior, including ambulatory movement [13], and a defect in neutrophil/leukocyte/lymphocyte homeostasis in the blood and spleen [15] were observed in native CerK$^{(-/-)}$ mice. CerK$^{(-/-)}$ mice developed a more severe/lethal response in models of fulminant pneumonia [15] and ulcerative colitis [14]. A relationship was reported between strong CerK expression and cancer cells from the estrogen receptor-negative subgroup of breast cancer patients, and the subgroup of patients with strong CerK expression had a poorer prognosis [16]. C1P was shown to act as an autocrine/paracrine proliferative signal in endothelial colony-forming cells from Kaposi sarcoma patients [17]. Thus, the CerK/C1P pathway appears to be a therapeutic target for immune-related diseases, neuronal diseases, and cancer.

The transcriptional regulation of CerK has been studied in detail [9,18,19], while the post-translational regulation of CerK activity, including its stability and direct modifications to CerK, remains unclear. The activity of CerK, including its recombinant enzyme, is dependent on divalent cations, particularly Ca^{2+} ions [4,11]; however, divalent cations may be essential factors for a kinase reaction of CerK [20]. In previous studies including ours, based on cellular assays, treatments with the Ca^{2+} ionophore A23187 increased CerK activity and/or the formation of C1P in various types of cells [4,6,21], and calmodulin was found to act as a Ca^{2+} sensor for CerK [22]. CerK activity was also shown to be sensitive to oxidation via a cluster of cysteine residues in CerK [9,23]. Bornancin's group revealed that CerK is a phosphoprotein, and the phosphorylation of several Ser residues affected the stability of the active enzyme conformation [9,24]. We previously reported that treatment with orthovanadate [25] and the overexpression of c-Src [26] increased the formation of C1P via CerK in A549 cells. However, the mechanisms responsible for the phosphorylation-mediated regulation of CerK activity have not yet been elucidated in detail. In the present study, we showed that the treatment of A549 cells with phorbol 12-myristate 13-acetate (PMA) reduced the formation of C1P via the protein kinase C (PKC) pathway. Furthermore, treatments with protein phosphatase inhibitors decreased the formation of C1P. The effects of the displacement of several serine (Ser, S) residues to alanine (Ala, A) in CerK on the activity and phosphorylation of the enzyme were also examined.

2. Materials and methods

2.1. Materials

4-Nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-ceramide), which has an NBD-bound C6-*N*-acyl chain and C18-sphingosine (a native alkyl chain length), was purchased from Molecular Probes (Eugene, OR, USA) and was also prepared by Prof. Nishida (Chiba University, Japan). Ceramide-NBD, which has a C6-*N*-acyl chain and NBD-bound C14-sphingosine, was prepared by Prof. Nishida. Other reagents were as follows: PMA, bisindolylmaleimide I (GF109203x) and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976, Calbiochem, La Jolla, CA); 2,2′,3,3′4,4′-hexahydroxy-1,1′-biphenyl-6,6′-demethanol dimethyl ether (HBDDE, Santa Cruz Biotech, Santa Cruz, CA); ruboxistaurin (LY333531), rottlerin, forskolin, and okadaic acid (Sigma-Aldrich, St. Louis, MO); cyclosporine A (Enzo Life Science, Farmingdale, NY); 4-(4-(4-fluorophenyl)-2-(4-(methylsulfonyl)phenyl)-1H-imidazol-5-yl)pyridine (SB203580, SYN Kinase, San Diego, CF); 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 2,3-bis[amino[(2-aminophenyl)thio]methylene]-butanedinitrile (U0126), anthrax[1,9-*cd*]pyrazol-6(2H)-one (SP600125), 5-(2-phenylpyrazolo[1,5-*a*]pyridine-3-yl)-1H-pyrazolo[3,4-*c*]pyridazin-3-amine (FR180204, extracellular signal-related kinase (ERK) Inhibitor II) and prostaglandin E_2 (Cayman, Ann Arbor, MI); epidermal growth factor and interleukin-1β (recombinant, R&D Systems, Minneapolis, MN); angiotensin II (human, Peptide Institute, Osaka); transforming growth factor-β1 and tumor necrosis factor-α (Pepro Tech, Rocky Hill, NJ); D-*erythro*-sphingosine-1-phosphate (Alexis, Australia); *N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridinecarboxamide (NOR4, DOJINDO, Kumamoto, Japan). The concentrations of receptor agonists, including epidermal growth factor, were similar to those in previous studies using A549 cells, and those of the inhibitors of kinases/phosphatases, including GF109203x and okadaic acid, were similar to those in previous studies [27–33].

2.2. Plasmids

pMXs-Puro, pMXs-Neo, and pMXs-U6-Puro retroviral vectors, and the pCMV-VSV-G envelope vector were gifts from Dr. Kitamura (University of Tokyo, Japan). The knockdown of CerK was performed with shRNA to silence CerK (shCerK; target sequence GGACAAGGCA AGCGGATAT). The nucleotides for shRNA were annealed and subcloned into the *Bam*HI/*Eco*RI sites of the pMXs-U6-Puro retroviral vector. To construct hemagglutinin (HA)-tagged CerK (CerK-HA), cDNA encoding human CerK (a gift from Dr. Kohama, Daiichi-Sankyo, Co., Ltd., Tokyo, Japan) was amplified by PCR using a forward primer 5'-TAAAAGATCTCGGAGATGGGGGCGA-3′, and a reverse primer 5'-TAT AGGAATTCCGCTGTGTGAGTCTGGC-3′, and this amplification product was cloned into the pHA-N1 vector at *Bgl*II/*Eco*RI sites. The pHA-N1 vector was created by replacing enhanced green fluorescent protein (GFP) with HA in the pEGFP-N1 vector. In retroviral infection, CerK-HA was amplified by PCR using a forward primer 5'-TATATTAATTAACG GAGATGGGGGCGACGG-3′, and a reverse primer 5'-AGTAGCGGCCGC TTTAAGCGTAATCTGGAA-3′, and this amplification product was cloned into the pMXs-Puro and pMXs-Neo retroviral vectors at *Pac*I/*Not*I sites. Several mutants of CerK-HA were created by PCR using pHA-N1-CerK-HA or pMXs-Neo-CerK-HA as a template with the primers shown in Supplemental Table 1.

2.3. Cells and transfection

Previously we reported the regulation of CerK activity in A549 human lung adenocarcinoma cells [25,26], although CerK activity was low compared with those of other ceramide metabolic enzymes (Fig. 1A). In the present study, we selected A549 cells for expression of wild-type and mutants CerK. A549 cells and the Plat-GP packaging cell line (a gift from Dr. Kitamura) were cultured in Dulbecco's modified Eagle's medium (D'MEM, Nacalai, Kyoto, Japan) containing 10% fetal bovine serum. Plat-GP cells were transfected with retroviral vectors and the pCMV-VSV-G envelope vector using a Lipofectamine reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Retrovirus-containing supernatants were collected 48 h after transfection. In order to establish a stable CerK-knockdown cell line (shCerK), A549 cells were cultured in retrovirus-containing supernatants with 8 μg/mL polybrene for 8 h and were selected in 1 μg/mL puromycin. In order to establish stable CerK-HA-expressing cell lines, A549 or shCerK cells were cultured in retrovirus-containing supernatants with 8 μg/mL polybrene for 8 h and were selected in 1 μg/mL puromycin and/or 1 μg/mL G418. The expression of CerK-HA was confirmed by measuring the formation of NBD-C1P in NBD-ceramide-treated cells and by a Western blotting analysis with an anti-HA antibody (Fig. 1A and B). In order to generate A549 cells transiently expressing CerK-GFP, wild-type CerK-HA, and mutant CerK-HA, A549 cells at the 70–80% confluent stage were transfected with the respective vectors using Lipofectamine 2000 (Invitrogen) for 3 h in Opti-MEM medium (Invitrogen) without serum. Cells were then cultured for 24 h in D'MEM containing 5% serum. The vectors for PKC-βI and PKC-βII were gifts from Prof. Saito (Kobe University). PKC-βI siRNA and PKC-βII siRNA (sc-29,450 and sc-39,170, respectively) were purchased from Santa Cruz Biotech. In the present study, two types of A549 cells expressing CerK-HA, stably expressing cells and transiently expressing cells, were used. Cells stably expressing CerK and the mutants were used in the assay on the formation of NBD-

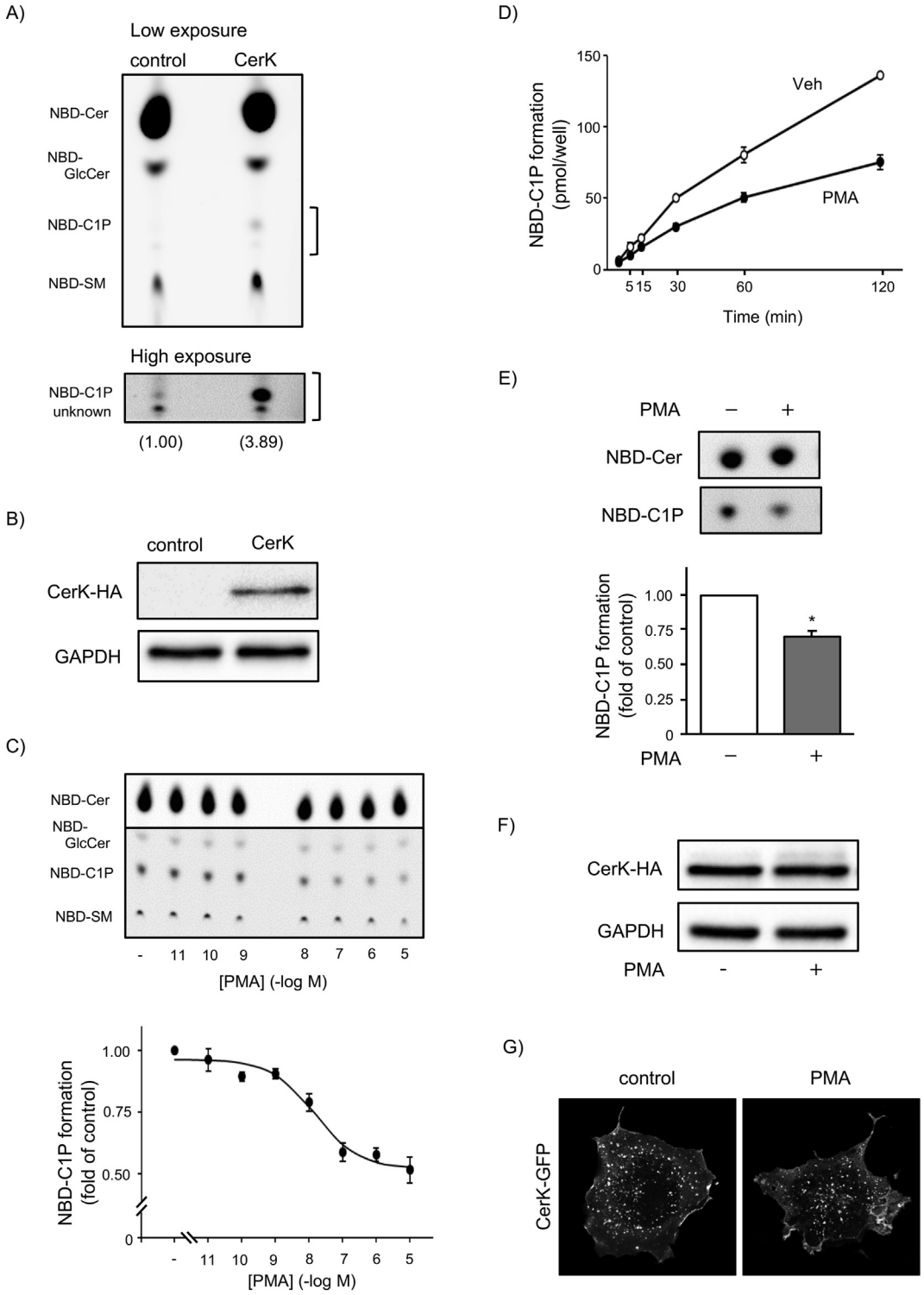

Fig. 1. Decrease in NBD-C1P formation by the PMA treatment in A549 cells stably expressing CerK-HA. In A, A549 cells stably expressing CerK-HA and control cells were incubated with 10 μM NBD-ceramide for 30 min, and lipids, including NBD-C1P, were extracted and separated by the TLC method. Typical images of a TLC plate were shown in the upper panel. In the lower panel, images of NBD-C1P were enhanced with longer exposure time. Bands showing low mobility, of which levels were not affected by CerK expression, were unknown product(s). In B, the expression of CerK-HA was confirmed by Western blotting with an anti-HA antibody. In A and B, data were from typical experiments from 2 to 3 representative experiments. In C and D, A549 cells expressing CerK-HA were treated with the indicated concentrations of PMA for 30 min (C) or were treated with 100 nM PMA for the indicated periods (D) in the presence of 10 μM NBD-ceramide. In E, homogenate fractions prepared from cells treated with 100 nM PMA for 30 min were incubated with 10 μM NBD-ceramide for 30 min in vitro. In C and E, NBD-C1P levels were expressed as fold changes from the control, and data are the means ± S.D. of three independent experiments. * $P < .05$, significantly different from the control. In F, CerK-HA levels were examined before and after the PMA treatment (100 nM, 30 min). In G, the cellular localization of CerK-GFP in COS-7 cells was analyzed before and after the PMA treatment using confocal microscopy. In F and G, typical images were from two representative experiments.

C1P, and cells transiently expressing CerK were mainly used to measure phosphorylated levels of CerK. In a transient expression system, the treatment with PMA did not inhibit the formation of NBD-C1P possibly because the large amounts of CerK proteins than those in the stable expression system mask and/or disturb PMA responses.

2.4. Measurement of NBD-C1P formation in cells and in vitro

NBD-ceramide has been used to study ceramide metabolism and as a marker of the Golgi complex [4,25]. In order to measure the formation of C1P in cells and the activity of CerK in vitro, NBD-ceramide was used as described previously [20,26] with minor modifications. Briefly, A549 cells expressing the wild-type and each CerK mutant on 12-well plates were incubated with 10 μM NBD-ceramide at 37 °C for 30 min in modified Hanks' balanced salt solution containing 0.1% albumin. Reagents, such as PMA and the chemicals tested as receptor agonists, were then added. In some cases, cells were pretreated with the indicated kinase/phosphatase inhibitors for 30 min before the incubation with NBD-ceramide. Lipids including NBD-C1P in wells (both cells and buffer) were extracted using chloroform-methanol. CerK activities in cell homogenate fractions were measured in cardiolipin/Triton X-100 buffer. Briefly, A549 cells were treated with vehicle or PMA, and cells after washing were scraped and homogenized with ice-cold lysis buffer (10 mM HEPES, 2 mM EGTA, 40 mM KCl, and protease inhibitors, pH 7.4). After centrifugation (1000 *g*, 10 min), equal amounts of protein (100–150 μg/tube, 100 μL) in homogenate fractions were used as enzyme preparations. The mixture of NBD-ceramide and cardiolipin was dried with N_2 gas, and then mixed micelles were prepared by sonicating (3 min) in reaction buffer (40 mM HEPES, 100 mM KCl, 3 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP, pH 7.4) containing Triton X-100. Micelles were diluted five-fold with reaction buffer. In the assay, 100 μL of the reaction buffer containing NBD-ceramide and 100 μL of enzyme preparations were mixed and incubated at 37 °C for 30 min. The final concentrations of the reagents in the assay mixture were as follows: 36 μM cardiolipin, 0.0125% Triton X-100, and 10 μM NBD-ceramide. Lipids including NBD-C1P were extracted and analyzed on TLC silica gel-60 plates (#195724, Merk, Darmstadt, Germany). In quantitative analyses, each amount (pmol) of NBD-ceramide was spotted on the upper area of the plate after TLC, and NBD fluorescence intensities were measured using LAS1000-Plus (Fuji Film, Tokyo, Japan; 470 nm excitation and 515 nm emission) and ImageJ software. In several experiments (Figs. 2B and 6), ceramide-NBD, which has a C6-*N*-acyl chain and NBD-bound C14-sphingosine, was used to monitor ceramide metabolism instead of NBD-ceramide. The metabolic characteristics of ceramide-NBD were similar to those of NBD-ceramide (Suppl. Fig. 1). Thus, we evaluated the results obtained using NBD-ceramide and ceramide-NBD as being similar.

2.5. Measurement of C1P phosphatase activity in cells

A549 cells stably expressing CerK on 12-well plates were incubated with 1 μM NBD-C1P (Echelon, Salt Lake, UT) at 37 °C for 1 h in modified Hanks' balanced salt solution containing 0.1% albumin. Lipids including NBD-C1P were extracted and analyzed on TLC silica gel-60 plates.

2.6. Fluorescence microscopy of living cells

COS-7 cells were used to monitor the cellular localization of CerK-GFP because they were suitable for confocal microscopy analysis. COS-7 cells transiently expressing CerK-GFP were seeded on cover-slips (12 mm in diameter) on glass-bottomed dishes (Iwaki, Tokyo) and were cultured for 24–48 h. Fluorescent images of cells treated with vehicle and 100 nM PMA for 30 min were obtained with a FluoView-FV500 confocal laser scanning microscope system (Olympus, Tokyo).

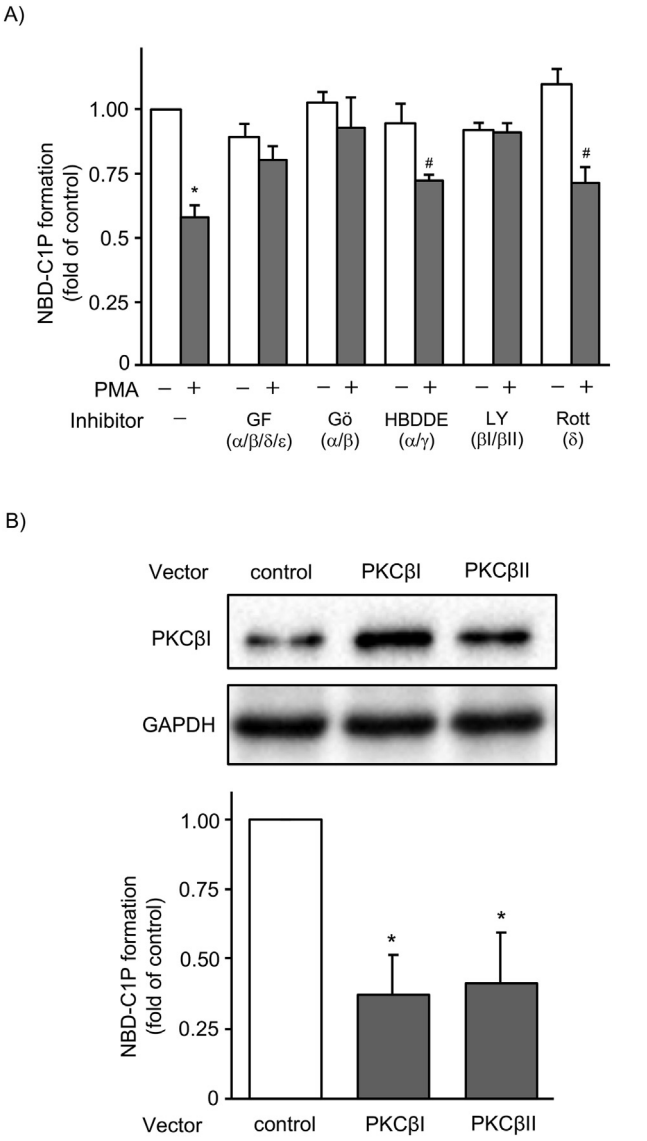

Fig. 2. The involvement of PKC-βI/II in PMA-induced decreases in the formation of NBD-C1P. In A, A549 cells stably expressing CerK-HA were pretreated with vehicle, 10 μM GF109203x (GF), 1 μM Gö6976 (Gö), 50 μM HDBBE (HB), 200 nM LY333531 (LY), and 3 μM rottlerin (Rott) for 30 min before the PMA treatment. Cells were incubated with 10 μM NBD-ceramide with vehicle and 100 nM PMA for 30 min. In B, cells were transiently transfected with vectors for PKC-βI and PKC-βII and were then incubated with 10 μM ceramide-NBD for 30 min. PKC-βI levels were measured by Western blotting with an anti-PKC-βI antibody. In A and B, NBD-C1P levels were expressed as fold changes from the control, and data are the means ± S.D. of three independent experiments. * $P < .05$, significantly different from the control in A and B. $^{\#}P < .05$, significantly different from the respective control without PMA in A.

2.7. Protein extraction and Western blot analyses with normal and Phos-tag SDS-PAGE

Immunoblotting to detect proteins, such as CerK-HA and PKC-βI, was performed as described previously [20,26] with minor modifications. Cells on 12-well plates were scraped after washing and homogenized with Laemmli buffer. Samples were sonicated (probe type) and boiled, and then equal amounts of the proteins (10–20 μg/lane) of lysates were separated by normal SDS-PAGE or Phos-tag SDS-PAGE and electrotransferred onto PVDF membranes (Bio-Rad, Hercules, CA). A resolving gel containing 5.5% acrylamide, 15 μM Phos-tag (WAKO Chemicals, Osaka, Japan), and 30 μM $MnCl_2$ and lysates from A549

cells transiently expressing CerK were used in the Phos-tag SDS-PAGE analysis. The following antibodies (1:500–1:1000) were used: an anti-HA antibody (3F10, Roche, Basel, Switzerland); anti-PKC-βI antibody (sc-209, Santa Cruz Biotech); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (5A12, Wako, Osaka, Japan); and anti-rabbit and anti-mouse IgG horseradish peroxidase antibodies (Amersham, Buckinghamshire, UK). The ratio of the respective protein to GAPDH was calculated, and data were expressed as fold changes from the control value.

2.8. Data presentation and statistical analysis

Values are the mean ± standard deviation (S.D.) for the indicated number (n) of independent experiments. An ANOVA with Dunnett's or Tukey's test and the Student's two-tailed t-test were used for multiple comparisons and pair-wise comparisons, respectively. A p value < .05 was considered to be significant.

3. Results

3.1. PMA-induced inhibition of CerK activity in A549 cells stably expressing wild-type CerK-HA

We established a stable A549 cell line expressing wild-type CerK-HA. These cells showed the enhanced formation of NBD-C1P (3.9-fold of the control) after incubation with 10 μM NBD-ceramide for 30 min (Fig. 1A). The expression of CerK-HA was confirmed by Western blotting using the anti-HA antibody (Fig. 1B). The treatment with PMA decreased the formation of NBD-C1P in a concentration-dependent manner (Fig. 1C), and the inhibitory effects of 100 nM PMA were detected immediately after the PMA treatment (Fig. 1D). The decrease in CerK activity induced by the PMA treatment was observed in the homogenate fractions of cells; the levels of NBD-C1P that formed in fractions from PMA-treated cells were significantly less than those from control cells (Fig. 1E). CerK-HA protein levels in homogenate fractions were not affected by the PMA treatment (Fig. 1F). In Fig. 1G, COS-7 cells were transiently transfected with a vector for CerK-GFP, and the cellular localization of CerK-GFP was examined using confocal microscopy. CerK-GFP mainly existed in the plasma membrane and possibly in endosomes showing vesicular specks, and its localization was not affected before or after the PMA treatment. C1P has been reported to be dephosphorylated by lipid phosphate phosphatases [34]. We then investigated the effects of the PMA treatment on the metabolism of C1P, including the dephosphorylation of C1P. In cells treated with 10 μM NBD-C1P for 30 min, NBD-SM levels were approximately 2–4%, and those of NBD-ceramide and NBD-GlcCer were < 1% of NBD-C1P levels, suggesting the formation of NBD-ceramide from NBD-C1P and the resulting NBD-ceramide metabolites. The values of NBD-C1P and metabolites were not affected in cells pretreated with 100 nM PMA for 30 min before the application of NBD-C1P; these values were between 0.95- and 1.07-fold of control values without PMA. Thus, the PMA treatment did not appear to modify the metabolism of C1P, including its dephosphorylation.

The existence and functions of various receptors on A549 cells were previously reported [27,29,31–33]. In order to clarify the involvement of receptor-mediated pathways in the regulation of CerK, the effects of following receptor agonists on the formation of NBD-C1P for 30 min were assayed. The values (fold of the control) were 1.04 ± 0.18-fold with 100 ng/mL of epidermal growth factor, 1.02 ± 0.09 with 10 μM prostaglandin E_2, 0.94 ± 0.06 with 100 nM angiotensin II, 1.13 ± 0.09 with100 nM sphingosine-1-phosphate, 1.15 ± 0.08 with 10 ng/mL of transforming growth factor-β1, 1.07 ± 0.07 with 10 nM tumor necrosis factor-α, and 1.11 ± 0.12 with 2.5 ng/mL of interleukin-1β (n = 3–4, respectively).

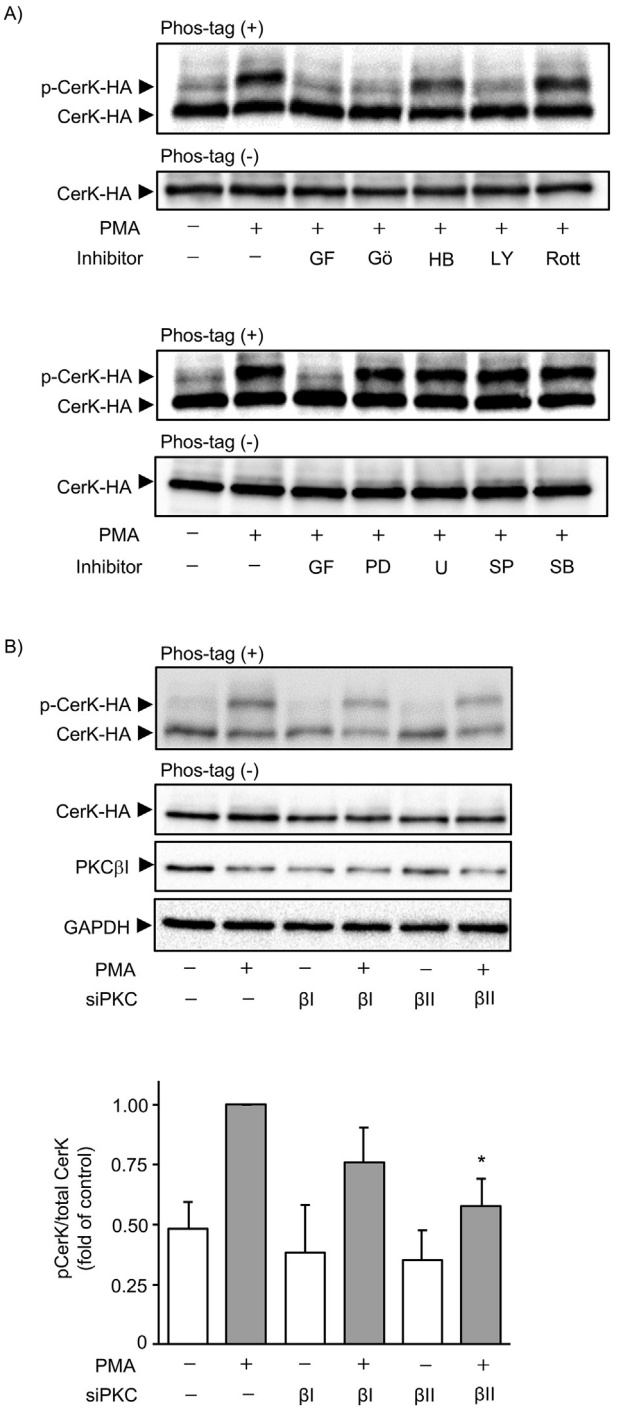

Fig. 3. Effects of kinase inhibitors on the PMA-induced phosphorylation of CerK. In A, A549 cells transiently expressing CerK-HA were pretreated with vehicle or 10 μM GF109203x (GF), 1 μM Gö6976 (Gö), 50 μM HDBBE (HB), 200 nM LY333531 (LY), 3 μM rottlerin (Rott), 10 μM PD98059 (PD), 10 μM U0126 (U), 10 μM SP600125 (SP), and 10 μM SB203580 (SB) for 30 min. Images were from typical experiments from two representative experiments. In B, A549 cells were co-transfected with the vector for CerK-HA and the siRNAs for PKC-βI or PKC-βII. Cells were then treated with 100 nM PMA for 30 min and phosphorylated CerK levels were detected by Western Blotting with the anti-HA antibody after separation by normal and Phos-tag SDS-PAGE. Quantitative data were shown in the lower panel. * P < .05, significantly different from the control with PMA.

3.2. Involvement of PKC-βI/II on PMA-induced decreases in C1P formation

The roles of PKCs on PMA-induced decreases in NBD-C1P formation were examined (Fig. 2). A549 cells express various types of PKC, including PKC-β [35,36]. We used the following inhibitors of PKCs at the concentrations indicated: 10 μM GF109203x for the main inhibition of PKC-α/β/δ/ε, 1 μM Gö6976 for PKC-α/β, 50 μM HBDDE for PKC-α/γ, 200 nM LY333531 for PKC-βI/βII, and 3 μM rottlerin for PKC-δ. The pretreatments with GF109203x, Gö6976, and LY333531, which commonly inhibit PKC-β, abolished PMA-induced inhibitory effects. In contrast, pretreatments with HBDDE and rottlerin did not affect PMA responses. Van Overloop et al. [37] previously reported that GF109203x, HBDDE, and rottlerin at 0.5 mM directly inhibited recombinant CerK activity, whereas the treatment of A549 cells with these reagents at μM concentrations did not affect the formation of NBD-C1P. We then investigated whether PKC-β is involved in the PMA-induced inhibition of C1P formation using molecular techniques. We examined the effects of the knockdown of PKC-βI/II on PMA responses. The treatment with 100 nM PMA still inhibited the formation of NBD-C1P in A549 cells treated with PKC-βI/II siRNAs possibly because the down-regulation of PKC-βI (Fig. 3B) and PKC-βII (data not shown) was partial, approximately 50% of the control, under our conditions. The effects of the overexpression of PKC-βI/II were then examined (Fig. 2B). The transfection of vectors for not only PKC-βI but also PKC-βII significantly decreased the formation of C1P. The transfection of the vector for PKC-βI, but not PKC-βII, increased PKC-βI protein levels. Thus, PKC-βI and -βII appeared to inhibit CerK activity in A549 cells expressing CerK-HA independently. The PMA treatment and/or PKC activation stimulate multiple signaling pathways, including mitogen-activated protein kinases (MAPKs), in cells, including A549 cells [28,36]. Pretreatments with inhibitors of MAPKs, FR180204 (for ERK1/2), SP600125 (for c-Jun N-terminal kinase), and SB203580 (for p38 kinase), at 10 μM and pretreatments with activators of protein kinases A and G, 10 μM forskolin (a direct activator of adenylyl cyclase) and 0.3 mM NOR4 (a donor of nitric oxide that activates guanylyl cyclase), respectively, did not affect the formation of NBD-C1P with and without the PMA treatment. The values for the formation of NBD-C1P in cells pretreated with these reagents ranged between 0.9- and 1.1-fold (of the control) and between 0.6- and 0.7-fold in the absence and presence of 100 nM PMA, respectively.

3.3. Involvement of PKC-βI/II in the PMA-induced phosphorylation of CerK

CerK was shown to be constitutively phosphorylated in mammalian COS-1 cells at Ser^{340}/Ser^{408} residues using massspectrometry techniques [24]. The phosphorylation levels of CerK-HA in A549 cells transiently transfected with the vector for CerK-HA were analyzed using Phos-tag SDS-PAGE (Fig. 3A). Under our conditions, there were two bands that reacted with the anti-HA antibody in control cells, and the treatment with 100 nM PMA for 30 min increased the intensity of the upper band. In standard SDS-PAGE, a single reactive band to the antibody was detected with and without the PMA treatment. The results from our mutagenesis study, as described below, and previous studies [38,39] also supported the upper band being the phosphorylated form (s) of CerK. In order to establish whether protein kinases are involved in the PMA-induced phosphorylation of CerK, the effects of the inhibitors of PKCs and MAPKs were examined. The pretreatments with 10 μM GF109203x, 1 μM Gö6976, and 200 nM LY333531, which inhibit PKC-βI/II, markedly reduced phosphorylated CerK-HA levels in PMA-treated cells. The other inhibitors tested, such as rottlerin and SP600125, were ineffective. Phosphorylated CerK-HA levels in control cells without the PMA treatment were low and varied depending on the experiments, and the levels with inhibitors, including GF109203x, were similar to those in control (data not shown). We then investigated the effects of the molecular inhibition of PKC-βI and -βII on phosphorylated CerK-HA levels (Fig. 3B). The treatment with the PKC-βI siRNA decreased PKC-βI levels in cells treated with and without PMA, and slightly reduced phosphorylated CerK-HA levels in PMA-treated cells. The treatment with the PKC-βII siRNA significantly reduced phosphorylated CerK-HA levels in PMA-treated cells and did not affect PKC-βI levels in cells before the PMA treatment. In PMA-treated cells, PKC-βI levels appeared to decrease, possibly reflecting the proteolytic activation of PKC-βI. These results showed that the PMA-induced phosphorylation of CerK-HA was mediated by PKC-βI/βII activation in cells.

3.4. Effects of the substitution of Ser residues to Ala on CerK-HA phosphorylation

In the present study, we constructed vectors for CerK with mutation (s), the substitution of six Ser residues to Ala, and examined their effects on the phosphorylation of CerK and formation of C1P. Six Ser residues, Ser^{72}, Ser^{300}, Ser^{340}, Ser^{403}, Ser^{408}, and Ser^{427}, in CerK were selected based on previous findings [11,24] and predicted information (NetPhos 2.0 Server). A549 cells were transiently transfected with the respective vectors, and the phosphorylation levels of CerK-HA in cells were examined 30 min after the PMA treatment. A single mutation in the Ser residue (S340A or S408A) of CerK did not affect the levels of phosphorylated CerK levels in PMA-treated cells (Fig. 4A), and other mutations in different Ser residues also did not affect PMA responses (Suppl. Table 2). We then examined the effects of multiple mutations in Ser residues, CerK-S340/408A-HA, CerK-S340/408/427A-HA, CerK-S300/340/408/427A-HA, and CerK-S72/300/340/403/408/427A-HA, on the PMA-induced phosphorylation of CerK (Fig. 4B and Table 1). PMA-induced phosphorylation levels in CerK-S340/408A-HA were significantly lower, while those in CerK-S340/408/427A-HA were

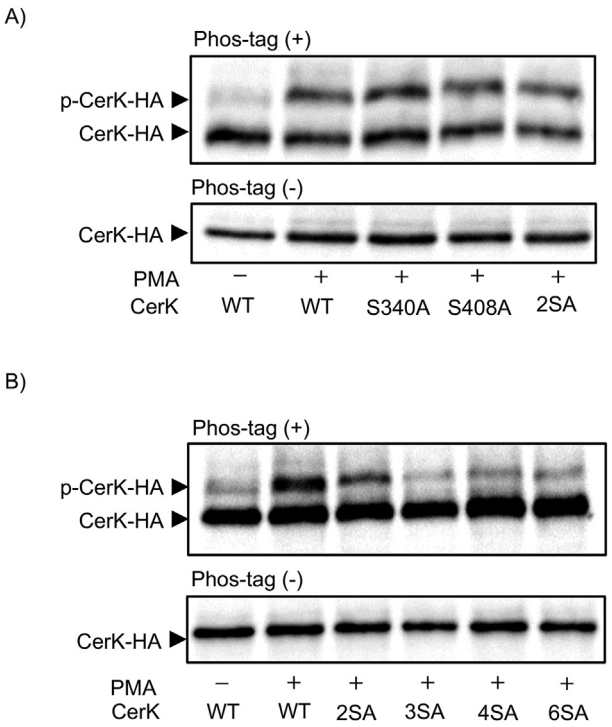

Fig. 4. Phosphorylation levels of CerK in cells expressing wild-type and mutant CerKs. A549 cells transiently expressing the wild-type (WT) and each mutant of CerK-HA. Phosphorylated CerK levels were examined after the treatment with 100 nM PMA for 30 min. In A, typical images of cells expressing CerK-S340A-HA, CerK-S408A-HA, and CerK-S340/408A(2SA)-HA, were shown. In B, typical images of cells expressing CerK with multiple mutations, S340/408A (2SA), S340/408/427A (3SA), S300/340/408/427A (4SA), and S72/300/340/403/408/427A (6SA), were shown. Quantitative data from CerK with a single mutation and multiple mutations in Ser residues were shown in Supplementary Table 1 and Table 1, respectively.

Table 1
Roles of Ser residues in CerK on the PMA-induced phosphorylation of CerK and inhibition of NBD-C1P formation.

	Phosphorylated CerK (fold of the PMA response)		NBD-C1P (fold of the control)
	None	PMA	None
WT (5)	0.42 ± 0.09	1	1
S340/408A (3)	N.D.	0.80 ± 0.07[a]	1.13 ± 0.16
S340/408/427A (3)	N.D.	0.56 ± 0.04[a]	0.55 ± 0.08[a]
S300/340/408/427A (3)	N.D.	0.60 ± 0.11[a]	Not tested
S72/300/340/403/408/427A (3)	N.D.	0.68 ± 0.04[a]	0.68 ± 0.09[a]

A549 cells were transiently transfected with the respective CerK-HA mutant vectors. In measurements of phosphorylated CerK levels, cells were incubated with vehicle or 100 nM PMA for 30 min, and levels were analyzed by Phos-tag SDS-PAGE. Phosphorylated CerK levels were expressed as fold changes from the value in PMA-treated cells expressing wild-type CerK (WT). Phosphorylated CerK levels in cells without PMA were low and varied because of the different expression levels of CerK proteins. Thus, we could not determine (N.D.) In measurements of NBD-C1P, cells were incubated with 10 μM NBD-ceramide for 30 min. The formation of NBD-C1P was normalized by CerK-HA levels in the respective mutant cells, and data were expressed as fold changes from the value in control cells expressing wild-type CerK-HA. Data are the means ± S.D. of the indicated number of independent experiments. * $P < .05$, significantly different from the control.

markedly lower than phosphorylation levels in wild-type CerK. Additional mutations (Ser^{72}/Ser^{300}/Ser^{403}) did not appear to regulate PMA-induced phosphorylation levels in CerK. The phosphorylation levels of CerK-S72/300/340/403/408/427A-HA by the PMA treatment were approximately 0.6-fold of the PMA response in wild-type CerK, which appeared to be greater than the endogenous phosphorylation levels of wild-type CerK-HA without the PMA treatment (0.4-fold). These results suggest that the PMA treatment phosphorylates Ser^{340}/Ser^{408}/Ser^{427} residues in CerK in a co-operative manner and that other amino acid residues, except for the Ser residues tested, exist as targets for the PMA/PKC-β pathway. The effects of Ser/Ala mutations on basal phosphorylation levels in CerK were not elucidated because phosphorylation levels were low and varied depending on experiments.

3.5. Effects of the substitution of Ser residues to Ala on the formation of NBD-C1P

We investigated the effects of mutations in Ser residues on CerK activity by measuring the formation of NBD-C1P in A549 cells transiently expressing CerK-HA. The formation of NBD-C1P was not significantly affected by the respective single mutations (Suppl. Table 2), whereas its formation in cells expressing CerK-S427A-HA was slightly less than that in cells expressing wild-type CerK-HA. The formation of NBD-C1P in cells expressing CerK-S340/408/427A-HA, but not CerK-S340/408A-HA, was significantly less than that in control cells (0.55 ± 0.08-fold) (Table 1). The mutation-induced response was not enhanced by six mutations, CerK-S72/300/340/403/408/427A-HA. Similar results were obtained in A549 cells stably expressing CerK*, the shCerK-resistant form of CerK. In these experiments, A549 cells stably expressing CerK*-HA were prepared by the transfection of the respective mutant vectors of CerK*-HA to A549-shCerK cells (Fig. 5). Since the treatment with PMA significantly reduced the formation of NBD-C1P in A549 cells expressing wild-type CerK*, this cell system appeared to maintain the activity of CerK in a PKC-sensitive manner. Cells expressing CerK*s with a single mutation, including CerK*-S427A, formed a similar amount of NBD-C1P to that in cells expressing wild-type CerK*-HA (Suppl. Table 3), and the six mutations, CerK*-S72/300/340/403/408/427A-HA, significantly reduced its formation (Fig. 5A). These results suggest that the Ser^{427} residue in addition to the Ser^{340} and Ser^{408} residues are critical for the basal activity of CerK*. The effects of the PMA treatment on C1P formation were then examined in cells expressing CerK* with mutations. In contrast to our expectations, the PMA treatment significantly inhibited the formation of NBD-C1P in cells expressing CerK* with six mutations, CerK*-S72/300/340/403/408/427A-HA, to a similar degree to that in cells expressing wild-type CerK* (Fig. 5B).

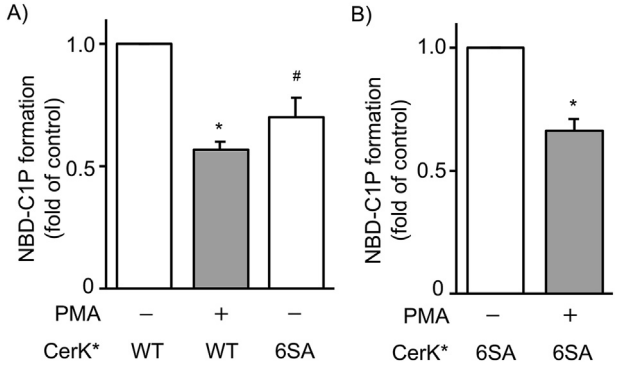

Fig. 5. The formation of NBD-C1P in A549 cells stably expressing CerK with multiple mutations. In these experiments, A549-shCerK cells were transfected with the respective vectors for CerK*, the shCerK-resistant form of CerK. CerK* and CerK both have the same amino acid sequence. A549 cells expressing CerK-S72/300/340/403/408/427A-HA (CerK-6SA) and control cells expressing wild-type CerK (WT) were incubated with 10 μM NBD-ceramide for 30 min. In A, 100 nM PMA was added to control cells. In B, 100 nM PMA and vehicle were added to A549 cells expressing CerK-6SA-HA. The formation of NBD-C1P was normalized by CerK-HA protein levels in the respective cells, and data were expressed as fold changes from the control. * $P < .05$, significantly different from values without the PMA treatment. $^{\#}P < .05$, significantly different from control cells.

3.6. Effects of protein phosphatase inhibitors on the formation of C1P

In order to further clarify the phosphorylation-mediated cellular regulation of CerK, the effects of protein Ser/Thr phosphatase inhibitors on the formation of NBD-C1P were examined. A549 cells stably expressing wild-type CerK-HA were pretreated for the indicated periods with the respective inhibitors, and then incubated with ceramide-NBD for 30 min in the presence of inhibitors. Okadaic acid for the inhibition of PP2A, cyclosporine A for the inhibition of PP2B, and hydrogen peroxide for the wide-spectrum inhibition of protein phosphatases [40,41] were used in the present study (Fig. 6). The treatments with 10 nM and 100 nM okadaic acid for 6 h inhibited the formation of NBD-C1P. The treatment with 100 nM okadaic acid did not affect CerK-HA protein levels (1.02-fold of the control). The pretreatment with 10 nM okadaic acid did not affect NBD-SM or NBD-glucosylceramide levels, while that with 100 nM okadaic acid slightly, approximately 15–20%, decreased and increased the levels of NBD-SM and NBD-glucosylceramide, respectively. The pretreatments with cyclosporine A and hydrogen peroxide for 30 min decreased the formation of NBD-C1P (Fig. 6), without affecting the levels of other ceramide metabolites and the CerK-HA protein (data not shown). These results suggest that the inhibition of protein Ser/Thr phosphatases, possibly resulting in the activation of phosphorylation signaling, decreased the formation of C1P in cells.

4. Discussion

4.1. CerK as a phosphoprotein

In the present study, we propose that phosphorylation signaling(s) to CerK reduced the activity of CerK. Chen et al. [24] revealed that human CerK was produced as a phosphoprotein in COS-1 cells by

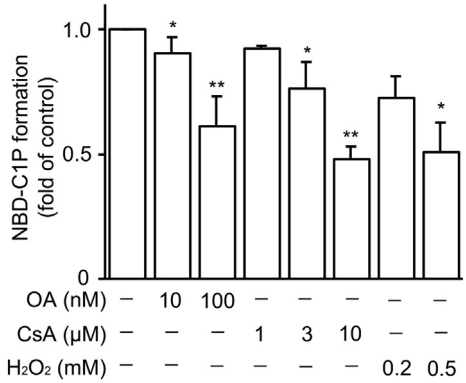

Fig. 6. Effects of inhibitors of protein phosphatases on the formation of NBD-C1P. A549 cells stably expressing CerK-HA were pretreated with vehicle and 10 nM and 100 nM okadaic acid (OA) for 6 h. In other experiments, cells were pretreated with the indicated concentrations of cyclosporine A (CsA) and hydrogen peroxide (H_2O_2) for 30 min. Cells were then incubated with 10 μM ceramide-NBD for 30 min in the presence of the respective reagents. NBD-C1P levels were expressed as fold changes from the control, and data are the means ± S.D. of 3–4 independent experiments. * $P < .05$, significantly different from the control.

cultivation with ^{32}P-orthophosphate, and identified two phosphorylated Ser residues, Ser^{340}/Ser^{408}, in CerK. In the present study, we confirmed that human CerK existed as a phosphoprotein in A549 cells using Phos-tag SDS-PAGE. Under our conditions, approximately 30–50% (% of the levels in PMA-treated cells) of CerK was phosphorylated in cells without the PMA treatment. It has been proposed that all members of the diacylglycerol kinase family, including SphK1/2 and CerK, have an equivalent Ser phosphosite [42], and that Ser^{340} in CerK is a counterpart to Ser^{225} (in the human SphK1 sequence) in SphK1 [24]. The enzyme activity of SphK1 was dependent on the phosphorylation of Ser^{225}, and the displacement of Ser^{225} to Ala decreased its activity to 70% of wild-type SphK1 [42–44]. However, the displacement of Ser^{340} to Ala in CerK did not affect the formation of C1P without the PMA treatment or the two PMA-induced responses, namely, a decrease in the formation of C1P and the phosphorylation of CerK (Suppl. Tables 2 and 3). The invalidity of the single mutant, CerK-S340A-HA, on the formation of C1P was consistent with previous findings from COS-1 cells [24]. In terms of features, the Ser^{340} residue in CerK did not appear to be responsible for the Ser^{225} residue in SphK1. The Ser^{408} residue in CerK was proposed to be a putative phosphoregulatory site of the enzyme [24], whereas the formation of C1P and PMA-induced phosphorylation of CerK in cells expressing CerK-S408A-HA were similar to those in cells expressing wild-type CerK-HA. Although Ser^{427} in CerK is speculated to be phosphorylated by protein kinases A and G based on the consensus motif [24], this kinase-mediated regulation of CerK activity may be excluded because of the invalidity of forskolin and NOR4 on the formation of NBD-C1P. In addition, CerK-S427A-HA did not affect the formation of C1P (Suppl. Tables 2 and 3). Double (S340/408A) and triple mutations (S340/408/427A) in CerK reduced phosphorylated CerK levels to 0.8- and 0.56-fold of that in wild-type CerK, respectively (Fig. 4B and Table 1). Our results suggest that i) CerK in cells existed as a phosphoprotein, ii) CerK activity was regulated by mutations in three Ser residues, Ser^{340}/Ser^{408}/Ser^{427}, and iii) CerK, at the Ser^{340}/Ser^{408} /Ser^{427} residues, but not at Ser^{72}/Ser^{300}/Ser^{403}, may be phosphorylated by the PMA treatment. Previous studies showed the stoichiometry and/or quantification of phosphorylated protein levels using a Phos-tag SDS-PAGE analysis [38,39]; however, we detected a single phosphorylated band of CerK-HA, in which phosphorylated levels were modified by mutations in Ser residues in CerK (Figs. 3 and 4). These differences may be due to technical differences, such as the composition of the resolving gel and the conditions of electrophoresis in the Phos-tag SDS-PAGE analysis. This issue needs to be resolved in order to clarify the number of phosphorylated residues in CerK.

4.2. PKC-β-mediated inhibition of CerK activity in PMA-treated A549 cells

The activity of CerK is translationally regulated by various mechanisms and/or factors, such as lipids, interactive proteins, and oxidation [9,20,23,45]. The regulation of CerK activity by phosphorylation has not yet been elucidated in detail; however, we previously reported an increase in CerK activity by tyrosine phosphorylation-related stimuli, a treatment with orthovanadate [25], and the expression of Src kinase [26]. In the present study, the treatment with PMA concentration- and time-dependently inhibited the formation of C1P in A549 cells expressing wild-type CerK-HA within 30 min, and PMA responses were reproducible in homogenate fractions prepared from PMA-treated cells (Fig. 1). The PMA treatment did not affect the subcellular localization of CerK or its protein levels. Thus, the PMA treatment appeared to inhibit the enzyme activity of CerK. The results from the pharmacological inhibition and molecular expression of PKC-βI/II (Fig. 2) suggested roles for the PKC-β pathway in PMA-induced decreases in C1P formation. To the best of our knowledge, the present study is the first to show that the PMA treatment decreased the formation of C1P via the PKC-βI/II-mediated pathway in cells. The PMA treatment has been reported to up-regulate the expression of CerK [11]. Thus, the PMA treatment appears to exert dual effects on CerK activity; the acute inhibition of CerK within 30 min (this study) and an increase in CerK expression.

4.3. Roles of Ser residues tested on the activity and phosphorylation of CerK

Since CerK is a phosphoprotein (Figs. 3 and 4), we investigated the relationship between activity and phosphorylation status, including the distinct roles of Ser residues in CerK. In the transient expression system of CerK, CerK-S427A-HA slightly (approximately 20%) decreased the formation of NBD-C1P (Suppl. Table 2), and the mutant, in cooperation with mutations (S340/408A), significantly decreased its formation (Table 1). In the stable expression system of CerK, the mutant CerK-HA with six mutations, including S427A, also markedly decreased the formation of NBD-C1P (Fig. 5). Chen et al. [24] examined the stability of the active CerK conformation by measuring the resistance of CerK to Triton X-100; the order of instability was CerK-S427A > CerK-S427D (aspartic acid (D) substitution to mimic the phosphorylated state) > wild-type CerK, and a similar order was observed for the Ser^{340} residue. Thus, the Ser^{427} residue of CerK, in cooperation with other Ser residues (possibly $Ser^{340/408}$), appeared to regulate its enzyme activity in cells without stimuli, and the Ser^{427} residue (and $Ser^{340/408}$ residues) may be endogenously phosphorylated, resulting in the maintenance of CerK activity/stability. However, the involvement of PKC-β in the possible phosphorylation of Ser residues appeared to be excluded because the inhibition of the PKC-βI/II pathway did not affect the phosphorylation levels of CerK-HA or the formation of NBD-C1P in control cells. Further studies are needed in order to clarify the role(s) of the Ser^{427} residue in CerK in the regulation of its basal activity.

As described above, the inhibition of PKC-βI/II decreased two PMA-induced responses; a decrease in the formation of NBD-C1P (Fig. 2) and increase in phosphorylated CerK levels (Fig. 3). In the case of SphK1, the displacement of Ser^{225} to Ala blocked the ability of PKC activation to increase SphK1 activity [42,43]. Furthermore, the displacement of Ser^{45} or Ser^{46} to Ala in yeast Dgk1 diacylglycerol kinase reduced phosphorylation and resulted in the activation of the enzyme by casein kinase II [46]. Since the Ser^{340}/Ser^{408}/Ser^{427} residues in CerK appeared to be phosphorylated by the PMA/PKC-β pathway, the displacement of Ser residues to Ala in CerK may affect the PMA-induced inhibition of C1P formation. Contrary to expectations, the combination of the six mutations of Ser residues, in addition to a single mutation (Suppl. Table 3), did not affect PMA-induced decreases in C1P formation (Fig. 5B). We revealed cooperation between Ser^{427} and other Ser

residues (specifically Ser^{340}/Ser^{408}) to maintain the activity of CerK (Table 1 and Fig. 5). Cooperation was observed between the Ser^{340}, Ser^{408}, and Ser^{427} residues in the PMA-induced phosphorylation of CerK (Table 1). The cooperation of amino acids for CerK functions, activity, and cellular localization was also reported between the Arg^{96} and Arg^{98} residues in the pleckstrin homology domain of CerK [45]. The amino acids responsible for the PKC-β-mediated inhibition of CerK have yet to be identified, and it currently remains unclear whether the PMA/PKC-β pathway regulates CerK directly and indirectly.

4.4. Summary and future issues

In the present study, we showed that the treatment with PMA increased phosphorylated CerK levels and inhibited its enzyme activity via the PKC-βI/II-mediated pathway, and treatments with inhibitors of protein Ser/Thr phosphatases also reduced the formation of C1P in cells. We previously suggested the regulation of CerK by tyrosine phosphorylation-related stimuli, a treatment with orthovanadate [25], and the overexpression of c-Src [26]. Our results indicate the diverse regulation of CerK activity, negatively and positively, by Ser and tyrosine phosphorylation pathways, respectively. The physiological and/or pathological cellular conditions and/or regulatory factors coupled with the PKC- and protein phosphatase-mediated regulation of CerK activity have not yet been identified.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

HT and HA carried out all assays including introducing mutations to CerK vectors, measurement of ceramide metabolites, western blotting, and cell imaging. TM participated in the coordination of the experiments and draft manuscript writing. HN conceived, coordinated, designed, and analyzed the experiments as well as manuscript writing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.11.008.

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