Ceramide Kinase Is a Mediator of Calcium-dependent Degranulation in Mast Cells*

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Ceramide kinase (CERK) catalyzes the conversion of ceramide to ceramide 1-phosphate (C1P) and is known to be activated by calcium. Although several groups have examined the functions of CERK and its product C1P, the functions of C1P and CERK are not understood. We studied the RBL-2H3 cell line, a widely used model for mast cells, and found that CERK and C1P are required for activation of the degranulation process in mast cells. We found that C1P formation was enhanced during activation induced by IgE/antigen or by Ca2+ ionophore A23187. The formation of C1P required the intracellular elevation of Ca2+. We generated RBL-2H3 cells that stably express CERK, and when these cells were treated with A23187, a concomitant C1P formation was observed and degranulation increased 4-fold, compared with mock transfectants. The cell-permeable N-acetylsphingosine (Cer-enzyme), a poor substrate of CERK, inhibited both the formation of C1P and degranulation, indicating that C1P formation was necessary for degranulation. Exogenous introduction of CERK into permeabilized RBL-2H3 cells caused degranulation. We identified a cytosolic localization of CERK that provides exposure to cytosolic Ca2+. Taken together, these results indicate that C1P formation is a necessary step in the degranulation pathway in RBL-2H3 cells.

Mast cells mediate inflammation and hypersensitivity by releasing bioactive molecules stored in their cytoplasmic granules. Stimulation through the cross-linking of IgE, bound to its mast cell receptor FcεR1, triggers a series of biochemical events, including an increase in cytosolic Ca2+ and activation of both nonreceptor protein-tyrosine kinases and phospholipases (1). These cellular events culminate in the exocytosis of granular content. Mast cell degranulation has been the subject of many studies, however, mainly focused on the early signaling events, including an increase in cytosolic Ca2+ and activation of both nonreceptor protein-tyrosine kinases and phospholipases (1). However, known inhibitors or antagonists for calmodulin and protein kinase II completely inhibit degranulation (3, 5), suggesting unknown mechanisms in Ca2+-dependent degranulation. In this study, we provide the first evidence that ceramide kinase (CERK) is involved in mast cell degranulation.

Sphingolipids are ubiquitous constituents of eukaryotic cells with essential roles in cell growth, survival, and death (6, 7). Sphingolipid metabolites include second messengers sphingosine (Sph), sphingosine 1-phosphate (S1P), ceramide (Cer), and ceramide 1-phosphate (C1P), all of which are involved in common signaling pathways controlling cell development, differentiation, activation, proliferation, and function (8–10). Mast cell activation is regulated by the balance of Sph and S1P (11), and S1P alone is thought to be involved in Ca2+ signaling following antigen stimulation (12–14).

Cer is the precursor for all sphingolipids and functions as a second messenger in a variety of cellular events including apoptosis and cell differentiation (9), and its intracellular level is tightly controlled. Many bioactive agents stimulate neutral sphingomyelinase, which degrades sphingomyelin to Cer. The two enzymes that metabolize ceramide are ceramidase that converts Cer to Sph and a fatty acid. Since Sph is not produced de novo synthesis (15), ceramidase is crucial for its generation and that of its catabolite S1P, which can regulate cell growth (16) and motility (17, 18).

The second enzyme is CERK, which phosphorylates Cer to produce C1P, which was initially described as a Ca2+-stimulated lipid kinase that copurified with brain synaptic vesicles (19). The CERK activity has also been reported in HL60 cells (20) and neutrophils (21). However, there are no reports at present ascribing the biological function of CERK in mast cells. Based on its sequence homology to sphingosine kinase type 1, CERK was cloned in 2002 (22). The functions of CERK and C1P have been examined by several groups. CERK is involved in phagolysosome formation in polymorphonuclear leukocytes and also in liposome fusion (23). C1P has been reported to have mitogenic effects (24), although when exogenously added it was hydrolyzed rapidly by phosphatases (25). More recently, C1P was found to induce arachidonic acid release and prostanoid synthesis (26), although many of these experiments were based mediated phospholipase D activation in rat basophilic leukemia (RBL-2H3 cells) (3), and Ca2+/calmodulin-dependent protein kinase II reportedly plays an important role in exocytosis (4). However, known inhibitors or antagonists for calmodulin and protein kinase II completely inhibit degranulation (3, 5), suggesting unknown mechanisms in Ca2+-dependent degranulation. In this study, we provide the first evidence that ceramide kinase (CERK) is involved in mast cell degranulation.

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1 The abbreviations used are: CERK, ceramide kinase; C1P, ceramide 1-phosphate; PLD, phospholipase D; Cer, ceramide; Sph, sphingosine; S1P, sphingosine 1-phosphate; PNP/GlcNAc, β-nitrophophyl N-acetyl-β-D-glucosaminidase; HPTLC, high performance TLC; PIPES, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin; DNP, 2,4-dinitrophenol; CHO, Chinese hamster ovary.
Ceramide Kinase in Mast Cell Degranulation

EXPERIMENTAL PROCEDURES

Materials—Ceramide (C18:0, d18:1), N-acetylphosphoglycerine (C2-Cer), cardiolipin, G418, p-nitrophenyl N-acetyl-β-D-glucosaminide (PNP-GlcNAc), streptolysin O, 1,4-diazobicyclo[2.2.2]octane, A23187, anti-FLAG monoclonal antibody (M2), and anti-FLAG polyclonal antibody were all purchased from Sigma. [32P]ATP and [3H]Sph were from PerkinElmer Life Sciences and American Radiolabeled Chemicals, respectively. All reagents were of the highest purity available.

Cell Culture and Degranulation Assay—Rat basophilic leukemia (RBL-2H3) cells and transfectants were cultured as monolayers in Eagle's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For degranulation assays, cells were harvested and washed with Tyrode's buffer (137 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM NaHCO3, 0.1% bovine serum albumin). The cells were centrifuged at 800 g, resuspended in HEPES-buffered Tyrode's buffer (10 mM HEPES, 137 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 0.1% bovine serum albumin), and washed twice with Tyrode's buffer. After incubation, the cells were harvested and washed twice with Tyrode's buffer. Subsequently, the cells were stimulated at 37 °C with the Ca2+ ionophore A23187 for the indicated time. Reactions were terminated by adding 7 volumes of chloroform/methanol (1:1, v/v). Two phases were generated by adding 1.8 volumes of 1 M KCl. The organic phase was dried and subjected to a minialkaline treatment to remove glycerophospholipids as described previously (20). Lipids were re-extracted by the method of Bligh and Dyer (31).

PLD Assays—PLD activity was measured as described in Ref. 30. Metabolic Labeling of Cells—Cells (106) were incubated with 2 μCi of carrier-free [32P]orthophosphoric acid (PerkinElmer Life Sciences) at 37 °C in Eagle's minimal essential medium supplemented with 10% fetal bovine serum for 90 min. After incubation, the cells were harvested and washed twice with Tyrode's buffer. Subsequently, the cells were stimulated at 37 °C with the Ca2+ ionophore A23187 for the indicated time. Reactions were terminated by adding 7 volumes of chloroform/methanol (1:1, v/v). Two phases were generated by adding 1.8 volumes of 1 M KCl. The organic phase was dried and subjected to a minialkaline treatment to remove glycerophospholipids as described above. Lipids were re-extracted by the method of Bligh and Dyer (31).

on exogenously added C1P. In the present study, we investigated cellular CERK/C1P functions by increasing the intracellular level of C1P through the expression of the CERK gene. By using this approach we revealed a previously unknown function for CERK in mast cell degranulation.

Materials—Ceramide (C18:0, d18:1), N-acetylphosphoglycerine (C2-Cer), cardiolipin, G418, p-nitrophenyl N-acetyl-β-D-glucosaminide (PNP-GlcNAc), streptolysin O, 1,4-diazobicyclo[2.2.2]octane, A23187, anti-FLAG monoclonal antibody (M2), and anti-FLAG polyclonal antibody were all purchased from Sigma. [32P]ATP and [3H]Sph were from PerkinElmer Life Sciences and American Radiolabeled Chemicals, respectively. All reagents were of the highest purity available.

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SDS-PAGE. From a rabbit immunized with the purified recombinant CERK, antiserum was obtained and purified using a GST-CERK-coupled to N-hydroxysuccinimide-activated HiTrap column (Amersham Biosciences), according to the manufacturer’s instructions.

Localization of Intracellular CERK—The open reading frame of the CERK gene was amplified and cloned in-frame into the pcDNA3 (Invitrogen) plasmids and transfected to RBL-2H3 cells using LipofectAMINETM2000. After overnight incubation, cells were washed with phosphate-buffered saline, fixed, and permeabilized for 10 min in phosphate-buffered saline containing 3.7% formaldehyde and 0.2% Triton X-100. Anti-CERK antibody was incubated with the cells overnight at 4°C. The cells were washed and incubated with Alexa 488-conjugated anti-rabbit IgG (Molecular Probes). For colocalization studies, the cells were incubated with anti-GM130 (Transduction Laboratories), anti-KDEL (StressGen Biotechnologies), or anti-serotonin (Dako Cytomation) antibodies overnight at 4°C, followed by incubation with Alexa 594-conjugated anti-mouse IgG (Molecular Probes) or Alexa-594-conjugated phalloidin (Molecular Probes) for 2 h at room temperature. The coverslips were mounted using 90% (w/v) glycerol containing 25% (w/v) 1,4-diazabicyclo[2.2.2]octane, and the cells were visualized by confocal fluorescence microscopy (Zeiss, LSM510).

RESULTS

Ca2+-induced Mast Cell Activation Increases C1P—We examined the expression of CERK in RBL-2H3 cells, and we detected CERK mRNA expression using reverse transcriptase-PCR (Fig. 1A). Furthermore, we were able to detect CERK activity using an in vitro kinase assay with [32P]ATP (Fig. 1B). These results established that CERK is present in RBL-2H3 cells.

![Fig. 2. Ca2+-dependent formation of C1P and degranulation in RBL-2H3 cells. A and B, RBL-2H3 cells (10^6) were harvested and incubated with 1 μCi of [3H]Sph for 30 min (A) or 2 μCi of [32P]orthophosphoric acid for 90 min (B) and then washed twice with Tyrode’s buffer. Labeled cells were stimulated at 37°C with 0.1 μM A23187 or Me6SO (vehicle) in the presence or absence of 2 mM EGTA for 30 min. Lipid extraction, alkaline treatment, and thin layer chromatography were performed as described under “Experimental Procedures.” A, the bands corresponding to [3H]C1P were quantified by NIH Image version 1.62, and no treatment with ionophore and EGTA was expressed as 1.0. We confirmed that this [3H]C1P and [32P]C1P were resistant to alkaline treatment and converted to [3H]S1P and [32P]S1P, respectively, after acid hydrolysis as described previously (20). B, RBL-2H3 cells were treated with or without 0.1 μM A23187 for 30 min in the presence or absence of 2 mM EGTA. Degranulation was monitored by β-hexosaminidase assays of culture supernatants using PNP-GlcNAc as described under “Experimental Procedures.” The results are the mean values ± S.E. from three independent experiments.

![Fig. 3. Expression and activity of stable CERK transfectants. Plasmids carrying the CERK gene were transfected to RBL-2H3 cells, and stable transfectants were selected as described under “Experimental Procedures.” We obtained six independent clones that we named RBL-CK1–6. Of these clones, RBL-CK3 and RBL-CK4 showed the highest expression of CERK. A, CERK protein expression in RBL-CK3 and RBL-CK4 transfectants was determined by Western blotting using an anti-FLAG polyclonal antibody after immunoprecipitation with an anti-FLAG monoclonal antibody (M2). Asterisk indicates nonspecific bands. B, CERK activity in RBL-CK3 and RBL-CK4 clones was measured by an in vitro kinase assay as described under “Experimental Procedures” and compared with the activity in RBL-2H3 parent cells (labeled as 1).]
We next examined whether the amount of C1P changed when RBL-2H3 cells were activated by IgE cross-linking. The cells were incubated with $[^{32}P]$orthophosphoric acid and DNP-specific IgE for 90 min and then stimulated with antigen (DNP-H9280). The amount of C1P increased by $\sim$2-fold in response to IgE/antigen stimulation (Fig. 1C).

CERK is known to be a Ca$^{2+}$-activated lipid kinase. This raised the possibility that the C1P increases might be because of Ca$^{2+}$-mediated activation of CERK. Antigen-mediated cross-linking of the IgE receptor FceRI causes the rapid elevation of intracellular Ca$^{2+}$, which is indispensable in the degranulation of mast cells. The Ca$^{2+}$ ionophore A23187 is a well known inducer of degranulation in mast cells that acts by elevating the intracellular concentrations of Ca$^{2+}$, mimicking the effect observed in activated mast cells (36). By using this reagent, we can exclude any complicated pathways that exist between FceRI and Ca$^{2+}$ release and can focus downstream of the Ca$^{2+}$ signaling.

Next, we examined whether the amount of C1P changed upon mast cell activation induced by A23187. RBL-2H3 cells were labeled with $[^{3}H]$Sph for 10 min and then treated with A23187 for 30 min. C1P levels in A23187-treated cells increased as compared with those of untreated cells (Fig. 2A). To eliminate the possibility that Sph uptake was enhanced by Ca$^{2+}$, $[^{32}P]$orthophosphoric acid labeling was also performed. A23187 caused a 3-fold increase in C1P formation (Fig. 2B). Degranulation in the A23187-treated RBL-2H3 cells was confirmed by $\beta$-hexosaminidase assay of the culture supernatants (Fig. 2C) and was inhibited by the chelator EGTA. C1P formation was also blocked in the presence of EGTA (Fig. 2A). These results support the contention that the C1P formation was dependent on an intracellular elevation of Ca$^{2+}$.

Because CERK has been shown to be activated by Ca$^{2+}$ (19, 20, 22), and the degranulation of mast cells requires elevated intracellular Ca$^{2+}$ levels, it was reasonable to consider a role for CERK in the Ca$^{2+}$-dependent degranulation pathway in mast cells.

**Ca$^{2+}$-dependent Activation of CERK in Mast Cells**—To investigate the role of CERK in degranulation in mast cells, RBL-2H3 cells stably expressing FLAG-tagged CERK were generated. Six independent clones were selected. Of these, RBL-CK3 and RBL-CK4 expressed the highest levels of CERK (Fig. 3A). CERK activity was measured using an in vitro kinase assay. The CERK activity in the RBL-CK3 clone was 50 times higher and in the RBL-CK4 clone was 250 times higher and in the RBL-CK4 clone was 50 times higher than that in the parent cells (Fig. 3B).

In a previous report, CERK was shown to be activated by Ca$^{2+}$ using a partially purified enzyme (19) and a lysate from CERK-overexpressing cells (22). In our experiment, immunoprecipitated CERK was directly activated by Ca$^{2+}$ without addition of cytosol (Fig. 4A). Next, we examined Ca$^{2+}$-dependent degranulation in the newly established clones. RBL-2H3 cells and mock and CERK transfectants were treated with 0.1 $\mu$M A23187 for 30 min. A23187 induced degranulation in RBL-2H3 cells, and this degranulation was completely blocked by the addition of EGTA (Fig. 4B). In RBL-CK3 and -CK4 clones,
the extent of degranulation increased after treatment with A23187 compared with that observed in either parent or mock-transfected cells. Nevertheless, this increased degranulation was completely blocked by 2 mM EGTA indicating that the increased degranulation remained fully Ca\textsuperscript{2+}-dependent (Fig. 4B). Overexpression of CERK apparently enhanced Ca\textsuperscript{2+} sensitivity, resulting in higher extent of degranulation. The extent of degranulation was higher for RBL-CK3 (4.5 times over parent cells) than for RBL-CK4 (2.9 times), correlating with their CERK activities (Fig. 3B). These results strongly support the involvement of CERK in Ca\textsuperscript{2+}-dependent mast cell degranulation.

Next, we determined whether CERK overexpression leads to elevated C1P level. RBL-CK3 cells were labeled with \[^{3}H\]Sph for 30 min and incubated with or without 0.1 \(\mu M\) A23187 and 2 mM EGTA. The addition of A23187 caused an increase in \[^{3}H\]C1P formation, and this increase was blocked by the addition of EGTA (Fig. 5A), the same tendency observed in parental RBL-2H3 cells (Fig. 2A). To compare the C1P formation in CERK-transfected cells to that in parent or mock-transfected cells, the clones were incubated with carrier-free \[^{32}P\]orthophosphoric acid for 90 min at 37 °C and incubated with 0.1 \(\mu M\) A23187 for various times. Lipids were extracted, and glycerophospholipids were removed by mild alkaline hydrolysis. The \[^{32}P\]C1P bands seemed likely to contain several bands. Because acid hydrolysis of these lipids produced a single \(^{32}P\)-S1P band (not shown), several bands of \[^{32}P\]C1P were due to its heterogeneity in the fatty acid moiety of ceramide. After a 30-min activation, the \[^{32}P\]C1P levels in RBL-CK3 and RBL-CK4 were 12.0 and 9.4 times higher, respectively, than that of wild type RBL-2H3 cells (Fig. 5B). These results indicate that A23187-induced degranulation is accompanied by increased C1P formation in these clones.

**Inhibition of C1P Formation Causes Decreased Degranulation in Mast Cells**—The cell-permeable \(N\)-acetylphosphosine (\(C_{2-}\)Cer) is commonly used in studying Cer signaling. PLD plays an important role in the degranulation pathway induced by IgE/antigen, and its inhibition causes a decrease in the degranulation pathway induced by IgE/antigen (37). In mast cells, \(C_{2-}\)Cer is known to inhibit IgE-induced degranulation by inhibiting PLD (30, 38), likely via a protein kinase C isozyme and adenosine 5’-diphosphate ribosylation factor (39). However, although \(C_{2-}\)Cer inhibits PLD activation induced by IgE/antigen, Ca\textsuperscript{2+} ionophore-induced PLD activation is not inhibited by \(C_{2-}\)Cer (30). Thus, the inhibitory effect of \(C_{2-}\)Cer on PLD activation may depend on molecules that act upstream of the Ca\textsuperscript{2+} release. With this in mind, we examined the effect of \(C_{2-}\)Cer on PLD activation in RBL-2H3 cells induced by A23187, but we found no inhibition (Fig. 6A). Therefore, the inhibitory effect of \(C_{2-}\)Cer on PLD is not involved in A23187-induced degranulation.

We then examined the effect of \(C_{2-}\)Cer in degranulation induced by A23187 in RBL-2H3 and RBL-CK3 cells, and we found that 50 \(\mu M\) of \(C_{2-}\)Cer inhibited this degranulation (Fig. 6B, hatched bars). We also examined the C1P formation and found that preincubation with 50 \(\mu M\) \(C_{2-}\)Cer dramatically decreased the formation of \[^{32}P\]C1P (Fig. 6C). The inhibition of \(C_{2-}\)Cer on \(\beta\)-hexosaminidase release and C1P formation occurred concomitantly in all four clones. These results suggest that inhibition of C1P formation leads to a decrease in degranulation. Although \(C_{2-}\)Cer has been shown to be a poor substrate for CERK (22), \(C_{2-}\)Cer 1-phosphate production was also detected (not shown). These results indicate that inhibition of endogenous generation of C1P is accompanied by a decrease in degranulation. They further suggest that CERK is one of the targets of \(C_{2-}\)Cer leading to inhibition of degranulation.

**Introduction of Exogenous CERK Protein Causes Degranulation in RBL-2H3 Cells**—Phospholipases have a critical role in the formation of \[^{3}H\]phosphatidylethanol was quantified by liquid scintillation counting. Black bar, no treatment; white bar, 0.1 \(\mu M\) A23187 for 10 min; hatched bar, preincubation with 50 \(\mu M\) \(C_{2-}\)Cer followed by treatment with 0.1 \(\mu M\) A23187 for 10 min. B, cells were preincubated with Me\textsubscript{3}SO (white bar) or 20 (gray bar), 50 (hatched bar), or 100 \(\mu M\) (dotted bar) \(C_{2-}\)Cer for 10 min and then treated with 0.1 \(\mu M\) A23187 for 30 min. Inhibition of C1P formation is accompanied by a decrease in degranulation. These results indicate that A23187-induced degranulation is accompanied by increased C1P formation in these clones.

![Fig. 6. Effect of \(C_{2-}\)Cer on PLD activity, degranulation, and C1P formation induced by A23187.](http://www.jbc.org/Downloadedfrom)
degranulation, so the addition of exogenous PLD or phospholipase C is sufficient to trigger degranulation in SLO-permeabilized RBL-2H3 cells (40). We introduced exogenous CERK into SLO-permeabilized cells. The enzyme and a control protein were prepared from CERK- or mock-transfected CHO cells by immunoprecipitation, and the CERK activity of each sample was confirmed by in vitro kinase assays (Fig. 7A). The activity of the immunoprecipitated enzyme was destroyed by boiling in some samples (Fig. 7A, lane 3). β-Hexosaminidase release was enhanced only in those cells that were exposed to active CERK (Fig. 7B, lane 2) but not in those treated with the boiled enzyme (lane 3). Furthermore, because a control sample prepared from mock-transfected cells failed to enhance β-hexosaminidase release (lane 1), the degranulation caused by the immunoprecipitated CERK-treated cells was not due to other proteins present in the sample. These results strongly suggest that the enzymatic activity of CERK elicits degranulation in these cells.

Cytoplasmic Localization of CERK in RBL-2H3—Although the gene encoding CERK was identified recently, the cellular localization of this enzyme has not been characterized. To elucidate the subcellular distribution of CERK, we raised an anti-CERK antibody (IgG) in rabbits using recombinant CERK expressed in E. coli. The specificity of our anti-CERK antibody was examined by Western blotting (Fig. 8A). Bands consistent with the molecular weight of CERK were clearly seen in the lysate from CERK-transfected CHO cells (lane 1), but not in the lysate from mock-transfected (lane 2). Thus, this antibody was confirmed to bind to CERK specifically.

By using this antibody, we examined the intracellular localization of CERK. CERK was stained with the anti-CERK antibody, and the staining pattern was compared with that of other antigens commonly used as markers of specific intracellular compartments. Specific antibodies were used in separate samples to stain the endoplasmic reticulum (KDEL), Golgi apparatus (GM130), secretory granules (serotonin), or filamentous actin (phalloidin). Some areas of colocalization were observed with the endoplasmic reticulum, as shown in the merged images (Fig. 8B-a). Little colocalization of CERK was observed with Golgi apparatus (Fig. 8B-b), secretory granules (Fig. 8B-c), or plasma membrane (Fig. 8B-d). However, in some cells we found strong CERK expressed in the plasma membrane (Fig. 8B-e). According to our data base searches, no distinct signal sequence or organelle retention signals could be found in the amino acid sequence of CERK. In a previous report, the major activity of CERK was recovered in the membrane fraction (22). However, in RBL-2H3 cells, CERK was predominantly localized in the cytosol along with more than 70% of the CERK activity (data not shown). Because some cells expressed this enzyme in the plasma membrane, the localization of CERK may be regulated by some stimuli.

DISCUSSION

Many early studies of C1P function relied on the use of exogenous C1P. Recently, the cDNA encoding CERK was cloned, providing new tools for the elucidation of the intracellular functions of CERK/C1P signaling axis. In the present study, we generated RBL-2H3 clones, which stably express CERK (RBL-CK3 and RBL-CK4). These clones exhibited a high extent of degranulation when it was induced by a Ca²⁺ ionophore. The elevated degranulation in the CERK transfecants paralleled the C1P formation in a Ca²⁺-dependent manner. Additionally, both were inhibited by cell-permeable C₂-Cer, a synthetic Cer analog. Introduction of CERK protein into permeabilized cells was enough to induce degranulation of mast cells. These results strongly indicate the involvement of CERK in degranulation of mast cells. Although C₂-Cer is known to be a poor substrate of CERK (22), some formation of C₂-C1P was observed (data not shown). These observations suggest that the conversion of endogenous Cer to C1P influences the properties of the membrane during degranulation.

An elegant study by Shayman and co-workers (23) demonstrated that CERK plays an important role in phagolysosomal formation in neutrophils. They also provided evidence that the addition of exogenous C1P promotes liposome fusion. Their data strongly suggest that the conversion of Cer to C1P alters membrane fusogenicity resulting in enhanced vesicle fusion. In the present study, we found that Ca²⁺-dependent C1P formation was involved in the degranulation pathway of mast cells. The degranulation process includes the fusion of the plasma membrane with the secretory granules. Our findings can be explained by an alteration in membrane fusogenicity elicited by an increase in C1P.

Most of the biological effects of C₂-Cer are structurally specific and cannot be reproduced by C₂-dihydro-Cer; this includes the inhibitory effect on degranulation. Most interesting, the inhibitory effect of C₂-Cer on C1P formation also could not be reproduced by either C₂-dihydro-Cer or Sph (data not shown). In this regard, the inhibition of degranulation by C₂-Cer com-
completely coincides with its inhibitory effect on C1P formation and is consistent with the inhibition of CERK. C2-Cer is commonly used in studying intracellular Cer effects, and there are several presumed targets for C2-Cer including protein kinase C isozymes, PLD (39), and phosphatidylinositol 3-kinase (41). Here we provided the first demonstration that CERK might be a target of C2-Cer during inhibition of mast cell degranulation. C1P may alternatively act as a lipid second messenger. A recent study (26) revealed that C1P induced arachidonic acid release and activated prostanoïd synthesis. C1P was also shown to stimulate DNA synthesis in fibroblasts (24). In our experiments, C1P played an essential role in the downstream signaling of Ca2+. Recently, soluble N-ethylmaleimide attachment protein receptors and associated regulators have been shown to control the Ca2+-dependent exocytosis in RBL-2H3 cells (42). Therefore, it is of interest whether C1P may in some fashion control these soluble N-ethylmaleimide attachment protein receptor proteins or the small GTPase protein Rab.

In conclusion, we have demonstrated antibodies against CERK and demonstrated the cytosolic distribution of CERK in mast cells. However, in a previous study, CERK was found in the membrane fraction in HEK293 cells (22). We also observed CERK to be associated with the plasma membrane in HEK293 cells (data not shown). In RBL-2H3 cells, some cells expressed CERK in the plasma membrane (Fig. 8B-c). CERK contains a PH domain in its N terminus, and we have already confirmed its specific binding to the phosphatidylinositol phosphate species. Thus, the cytosolic distribution of CERK may not only depend on the cell type and conditions but also on local lipid composition.

In conclusion, we have demonstrated a novel function for the lipid kinase CERK in mast cells. The target molecules of C1P, the CERK product, as well as the reasons why the intracellular distribution of CERK differs among cell types are topics of future studies.

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**FIG. 8.** Subcellular localization of CERK in RBL-2H3 cells. A, the specificity of anti-CERK antibody was examined by Western blotting. The CERK- or mock-transfected CHO cells were lysed and separated by SDS-PAGE. Western blotting was then performed using anti-CERK antibody. Lane 1, the lysate from CERK-transfected CHO cells; lane 2, the lysate from mock-transfected CHO cells. B, vectors expressing CERK were transiently transfected in RBL-2H3 cells. Twenty four hours after transfection, cells were fixed and stained with anti-CERK antibody and Alexa 488 goat anti-rabbit IgG and then visualized by immunofluorescence microscopy. For colocalization studies, the cells were incubated with anti-KDEL (endoplasmic reticulum, a), anti-GM130 (Golgi apparatus, b), or anti-serotonin (secretory granules, c) overnight at 4 °C, followed by incubation with Alexa 594-conjugated anti-mouse IgG or with Alexa 594-conjugated phalloidin (plasma membrane, d and e) for 2 h at room temperature. Merged images are shown in the right panels.
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