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Long Chain Ceramides Activate Protein Phosphatase-1 and Protein Phosphatase-2A

ACTIVATION IS STEREOSPECIFIC AND REGULATED BY PHOSPHATIDIC ACID*

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The search for potential targets for ceramide action led to the identification of ceramide-activated protein phosphatases, which include protein phosphatase-2A (PP2A) and protein phosphatase-1 (PP1) with roles in regulating apoptosis and cell growth. Thus far, *in vitro* studies on ceramide-activated protein phosphatases have been restricted to the use of short chain ceramides, limiting the extent of mechanistic insight. In this study, we show that the long chain D-*erythro*-C_{18}-ceramide activated PP2A (AB′C trimer), PP2Ac (catalytic subunit of PP2A), and PP1γc and -αc (catalytic subunits of PP1γ and -1α isoforms, respectively) 2–6-fold in the presence of dodecane, a lipid-solubilizing agent, with 50% maximal activation achieved at approximately 10 μM D-*erythro*-C_{18}-ceramide. The diastereoisomers of D-*erythro*-C_{18}-ceramide, D-*threo*-, and L-*threo*-C_{18}-ceramide, as well as the enantiomeric L-*erythro*-C_{18}-ceramide, did not activate PP1 or PP2A, but they inhibited PP1 and PP2A activity. The addition of phosphatidic acid decreased the basal activity of PP1c but also increased the stimulation by D-*erythro*-C_{18}-ceramide from 1.8- to 2.8-fold and decreased the EC_{50} of D-*erythro*-C_{18}-ceramide to 4.45 μM. The addition of 150 mM KCl decreased the basal activity of PP1 and the dose of D-*erythro*-C_{18}-ceramide necessary to activate PP1c (EC_{50} = 6.25 μM) and increased the ceramide responsiveness up to 10–17-fold. These studies disclose stereospecific activation of PP1 and PP2A by long chain natural ceramides under near physiologic ionic strengths *in vitro*. The implications of these studies for mechanisms of ceramide action are discussed.

Several lines of evidence have suggested ceramide as an important regulator of various stress responses and growth mechanisms. First, formation of ceramide from the hydrolysis of sphingomyelin or from *de novo* pathways is observed in response to inducers of stress such as tumor necrosis factor-α, γ-interferon, 1,α-25-dihydroxyvitamin D_3, interleukin-1, ultraviolet light, heat, chemotherapeutic agents, FAS antigen, and nerve growth factor (1–9). Second, the addition of exogenous ceramide or the enhancement of cellular levels of ceramide induces cell differentiation, cell cycle arrest, apoptosis, or cell senescence in various cell types (4, 10, 11). Third, the action of ceramide relates mechanistically to key regulators of growth such as the retinoblastoma gene product (Rb),[1] caspases, Bcl-2, and p53 (12–18). Fourth, studies in yeast have demonstrated an essential role for sphingolipids in many stress responses where ceramide may function in the adaptation to heat (19, 20). Finally, studies with knock-out mice lacking acid sphingomyelinase or with fumonisin B1, an inhibitor of ceramide synthesis, have disclosed necessary roles for ceramide in several pathways of growth regulation (21, 22).

These emerging roles of ceramide necessitate a mechanistic understanding of ceramide action, This goal has led to the identification of several candidate ceramide-regulated enzymes, including ceramide-activated protein kinase and ceramide-activated protein phosphatase (CAPP) (23, 24). CAPP was first identified as a member of the 2A class of serine/threonine phosphatases (PP2A) (22–24). Recently, we have also demonstrated that protein phophatase-1 (PP1) is a target for ceramide.[2] The specificity for CAPP activation *in vitro* closely resembled the specificity for various cellular activities of ceramide such as apoptosis (23, 24).

Possible direct downstream targets for these CAPP enzymes include c-Jun, protein kinase Cα, and Rb, which has been shown to function in ceramide-dependent cell cycle arrest pathways (3, 5, 13). Recent studies have demonstrated Rb as a specific substrate for PP1 *in vitro* and as an *in vivo* target for CAPP with dephosphorylation of Rb resulting from ceramide treatment in MOLT-4 cells.[2] Also in Molt-4 cells, protein kinase Cα has been demonstrated to be regulated by PP2A and not PP1 in response to ceramide, demonstrating substrate specificities between CAPP enzymes (25). Galarreta and co-workers (26) demonstrated *in vivo* and *in vitro* that ceramide leads to dephosphorylation of c-Jun, and okadaic acid inhibited this effect in A431 cells. Therefore, c-Jun, protein kinase Cα, and Rb are likely candidates for direct substrates of CAPP in mediating ceramide effects.

Although short chain ceramides have clearly been demonstrated to activate the PP2A trimer (AB′C), PP2A catalytic subunit (PP2Ac), and PP1 catalytic subunit (PP1c) *in vitro*,[2] a major problem has been the inability to utilize more natural

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[1] The abbreviations used are: Rb, retinoblastoma gene product; CAPP, ceramide-activated protein phosphatase; PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; PP1Ac, catalytic subunit of PP-1A; PP2Ac, catalytic subunit of PP-2A; PP1αc, catalytic subunit of PP1α isoform; PP1γc, catalytic subunit of PP1γ isoform; MBP, myelin basic protein; PA, phosphatidic acid; EC_{50}, concentration giving 50% effectiveness.

[2] K. Kishikawa, C. E. Chalfant, J. Y. Lee, A. Bielawska, S. H. Galadari, L. M. Obeid, and Y. A. Hannun, manuscript submitted for publication.

long chain ceramides. In the present study, we overcome this problem of delivering long chain ceramides to both of the CAPP enzymes, PP1 and PP2A. We also characterize the enzymes under various physiological environments related to ceramide responsiveness.

EXPERIMENTAL PROCEDURES

Materials—Myelin basic protein (MBP) purified from bovine brain, ATP, protein kinase A purified from bovine heart, and β-mercaptoethanol were purchased from Sigma. Dithiothreitol was obtained from Bachem, and dodecane was obtained from Aldrich. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products. C_{18}-ceramides were synthesized as described (27). Protein phosphatase 2A (PP2A) trimer (AB′C) and catalytic subunit PP2Ac were purified from bovine heart as described (28). Recombinant human protein phosphatase-1γ catalytic subunit (PP1γc) and rabbit protein phosphatase-1α catalytic subunit (PP1αc) were purchased from Calbiochem. PP1αc is supplied preincubated with Mn^{2+}.

Preparation of ^{32}P-Phosphorylated MBP—Myelin basic protein was labeled in a 0.5-ml reaction containing 1 mg of MBP, 50 mM Tris-HCl, pH 7.4, 90 mM $MgCl_2$, 0.1 mM cold ATP, 5 mM dithiothreitol, 10 mM β-mercaptoethanol, 40 μCi of [γ-^{32}P]ATP (0.2 mM), and 125 units of protein kinase A. After the components were mixed, the reaction was incubated at 37 °C for 2 h. 170 μl of a 100% trichloroacetic acid solution was added, and labeled MBP was precipitated on ice for 20 min. The precipitated substrate was washed twice with acetone at −20 °C, air-dried, and reconstituted in 1 ml of 50 mM Tris-HCl, pH 7.4. The specific activity of ^{32}P-labeled MBP was ~22 μCi/mg.

Solubilization of Long Chain Ceramides and Phosphatidic Acid—C_{18}-ceramide was brought up in a 2% dodecane-EtOH solution. The solution was dissolved by incubation at 37 °C. After solubilization, the ceramide solution was kept at 37 °C until addition to reaction tube. Egg yolk phosphatidic acid in chloroform was dried under nitrogen and resuspended in 50 mM Tris-HCl, pH 7.4, by ultrasonication.

Phosphatase Assays—Reactions were carried out in 13 × 100-mm borosilicate glass tubes. Solubilized ceramides were added to tubes containing PP1αc in Buffer A (50 mM Tris-HCl, pH 7.4) with the EtOH concentration not exceeding 1%. Stock enzyme was diluted to 1units/ml in Buffer A, and 10 milliunits was added to each tube. Components were preincubated for 5 min at 30 °C. Reaction were initiated with 0.005 ml of ^{32}P-labeled myelin basic protein (1 mg/ml) in Buffer A. After 20 min at 30 °C, the assay was terminated by the addition of 0.1 ml of 1 mM KH_2PO_4 in 1 N H_2SO_4 followed by the addition of 0.3 ml 2% ammonium molybdate. After 10 min, 1 ml of isobutanol:toluene (1:1) was added, and each reaction was vortexed for 10 s. The reactions were centrifuged at 1000 × *g* for 10 min, and an aliquot of the upper organic phase was removed, mixed with scintillant, and counted. PP1γc, PP2A trimer, and PP2Ac were assayed as described for PP1αc. For PP1γc and PP1αc, 1 unit of activity is defined as the amount of enzyme that will hydrolyze 1.0 nmol of *p*-nitrophenyl phosphate/min at 30 °C, pH 7.0. For PP2A trimer and the catalytic subunit, 1 unit of activity is defined as the amount of enzyme that will hydrolyze 1.0 nmol of phosphorylase/min at 30 °C, pH 7.0.

RESULTS

Long Chain Ceramide Activates PP1 and PP2A in Vitro—Cell-permeable short chain ceramides have been shown to activate protein phosphatase-1 and -2A *in vitro* (22–24).[2] The difficulty in delivering long chain ceramides *in vitro* and *in vivo* stems from solubility problems, because long chain neutral lipids are poorly soluble in aqueous environments. To overcome this problem of delivery *in vitro*, we mixed long chain ceramides with 2% dodecane, resulting in a final reaction concentration of 0.02% dodecane and variable concentrations of ceramide (29). Dodecane alone did not have any effect on phosphatase activity, but solubilized D-*erythro*-C_{18}-ceramide activated PP1αc (240% of control, EC_{50} = 8.75 μM), PP1γc (190% of control, EC_{50} = 11.5 μM), PP2Ac (179% of control, EC_{50} = 11.25 μM), and PP2A trimer (AB′C) (580% of control, EC_{50} = 10.6 μM) in a dose-dependent manner (Fig. 1). Initial activation was not observed until D-*erythro*-C_{18}-ceramide concentrations reached 7.5 μM with saturation occurring at 12.5–15.0 μM D-*erythro*-C_{18}-ceramide for 10 milliunits of enzyme (Fig. 1). A nonspecific inhibition of both PP1 and PP2A enzymes was observed at lower doses (3 and 5 μM) of long chain ceramides (see below) (Fig. 1).

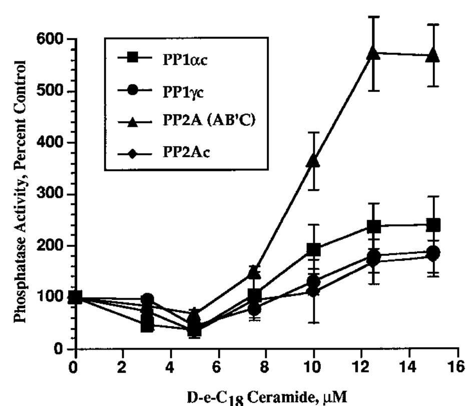

FIG. 1. **Activation of PP1αc, PP1γc, PP2Ac, and PP2A (AB′C) by long chain ceramides *in vitro*.** Solubilized D-*erythro*-C_{18}-ceramide was added to the assay reactions at concentrations of 0, 3, 5, 7.5, 10, 12.5, and 15 μM. Each phosphatase was assayed as described under "Experimental Procedures." Results are expressed as percent of initial activity in the absence of D-*erythro*-C_{18}-ceramide (*D-e-C$_{18}$ Ceramide*). Data are the mean ± S.E. of at least triplicate experiments reproduced on at least three separate occasions. The different phosphatases are designated as follows: ▲ = PP2A, ■ = PP1αc, ♦ = PP2Ac, and ● = PP1γc.

Activation of PP1 and PP2A Is Stereospecific—To examine the stereospecificity of the phosphatase activation by long chain ceramides, we tested stereoisomers of C_{18}-ceramide solubilized in dodecane (Fig. 2, *A–D*). D-*Erythro*-C_{18}-ceramide (2*S*,3*R*) is the naturally occurring ceramide, with L-*erythro*-C_{18}-ceramide (2*R*,3*S*) being its mirror image. The diastereoisomers, the *threo* conformations, differ from *erythro* conformations in that the C-2 configuration relative to C-3 is in a *cis*-conformation. In the *erythro* conformation, the groups are in a *trans*-conformation. We found that, similar to D-*erythro*-C_{18}-ceramide, each stereoisomer inhibited the phosphatases at low doses (3–5 μM), but only D-*erythro*-C_{18}-ceramide activated each phosphatase at 7.5–15 μM (Fig. 2, *A–D*).

Phosphatidic Acid Inhibits PP1 Enzyme Activity but Decreases the Dose of D-erythro-C$_{18}$-Ceramide Needed to Activate PP1—In other studies, we have shown that PA inhibits protein phosphatase-1 but not PP2A activity *in vitro*.[3] Thus, we examined whether PA could regulate ceramide effects on PP1. Fig. 3 depicts PP1γc activity in the presence of 500 nM phosphatidic acid and increasing doses of D-*erythro*-C_{18}-ceramide. The addition of PA to the reaction lowered the basal activity of protein phosphatase-1 as expected, with 500 nM PA causing 95% inhibition of PP1γc (Fig. 3). The inhibition of PP1γc by low concentrations of long chain ceramides was relieved in the presence of PA, and the maximum stimulation by ceramide was enhanced from 374.3 to 581.0 fmol of P_i released/min (Fig. 3), such that in the presence of PA, the fold stimulation by ceramide increased up to 2.8-fold. Importantly, lower doses of D-*erythro*-C_{18}-ceramide were able to activate PP1γc with an EC_{50} of 4.45 μM. PP1αc behaved in a manner similar to PP1γc, with an increase in maximal stimulation from 472.8 to 738.8 fmol of P_i released/min and a decrease in the EC_{50} to 5.5 μM (data not shown). Stereospecificity was still retained for both PP1αc and PP1γc under these conditions, further demonstrating the specificity of D-*erythro*-C_{18}-ceramide (data not shown).

Near Physiological Ionic Strength Relieves the Inhibition by Unnatural Ceramides and Increases D-Erythro-C$_{18}$-ceramide Responsiveness—Acidic phospholipids have been demonstrated

[3] Kishikawa, K., Chalfant, C. E., Perry, D. K., Bielawska, A., and Hannun, Y. A. (1999) *J. Biol. Chem.*, in press.

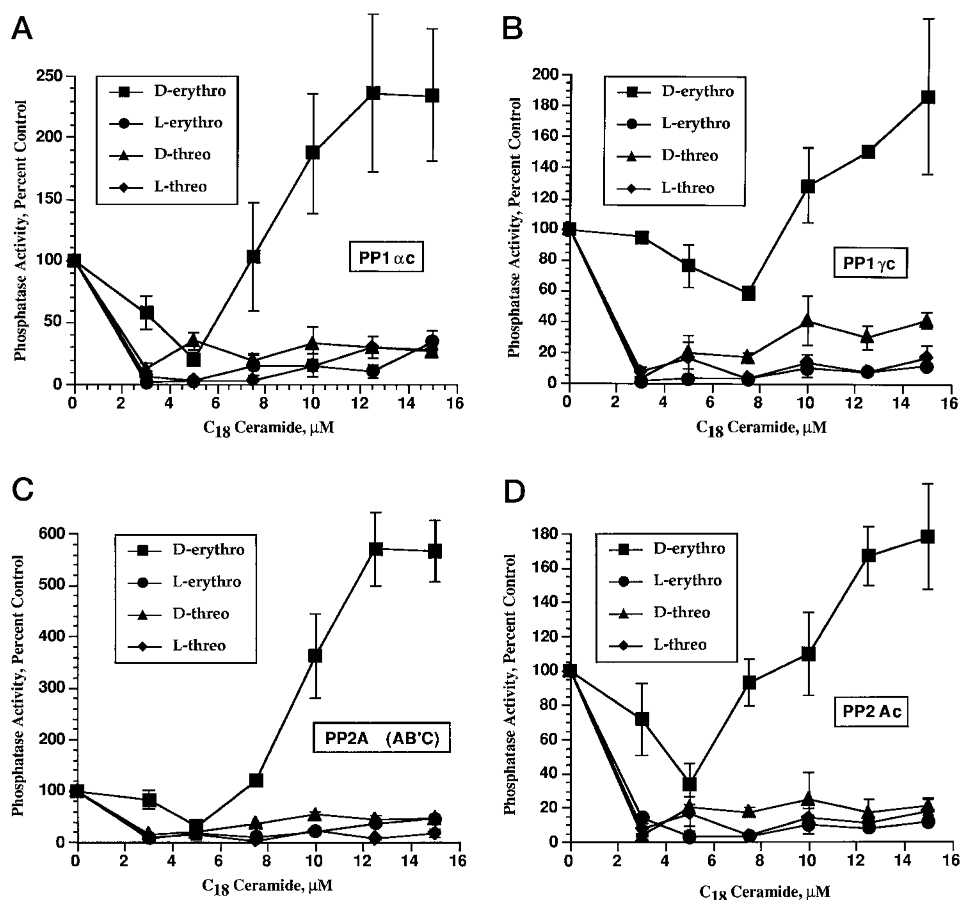

FIG. 2. **Effects of ceramide stereoisomers on protein phosphatases.** Activation of PP1αc (*A*), PP1γc (*B*), PP2A (AB′C) (*C*), and PP2Ac (*D*) by long chain ceramides is stereospecific. Solubilized C_{18}-ceramide stereoisomers were added to the assay reactions at concentrations of 0, 3, 5, 7.5, 10, 12.5, and 15 μM. Phosphatases were assayed as described under "Experimental Procedures." Results are expressed as percent of initial activity in the absence of C_{18}-ceramide. Data are mean ± S.E. of at least triplicate experiments reproduced on at least three separate occasions. Stereoisomers are designated as follows: ▲ = D-*threo*-C_{18}-ceramide, ■ = D-*erythro*-C_{18}-ceramide, ♦ = L-*threo*-C_{18}-ceramide, and ● = L-*erythro*-C_{18}-ceramide.

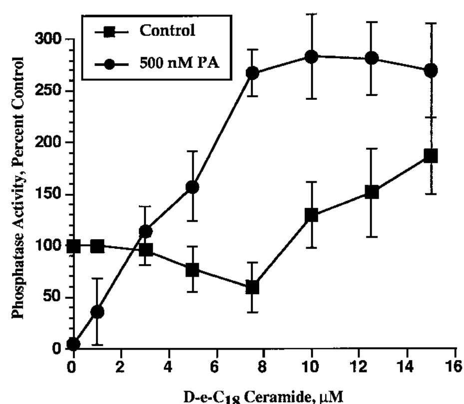

FIG. 3. **Effects of phosphatidic acid on long chain ceramide activation of protein phosphatase-1.** 500 nM PA and solubilized D-*erythro*-C_{18}-ceramide at concentrations of 0, 3, 5, 7.5, 10, 12.5, and 15 μM were added to the assay reactions. PP1γc was assayed as described under "Experimental Procedures." Results are expressed as the percent of initial activity of PP1γc in the presence of 500 nM PA (*PA*) and the absence of D-*erythro*-C_{18}-ceramide (*D-e-C_{18} Ceramide*). Data are the mean ± S.E. of at least triplicate experiments reproduced on at least three separate occasions. The different phosphatases are designated as follows: ● = PP1γc in the presence of 500 nM PA, and ■ = PP1γc in the absence of PA.

to bind PP1 subunits under near physiological ionic strengths (30). Therefore, we examined whether 150 mM KCl affected ceramide responsiveness and PA inhibition. The addition of KCl lowered the dose of D-*erythro*-C_{18}-ceramide necessary for activation of each phosphatase and increased the maximal stimulation of PP1γc from 374.3 to 1753.3 fmol of P_i released/min (EC_{50} = 6.25 μM) (Fig. 4*A*). Similarly, activation of PP1αc was increased from 472.8 to 4137 fmol of P_i released/min (EC_{50} = 5.55 μM), PP2Ac from 352.6 to 1319.9 fmol of P_i released/min (EC_{50} = 6.75 μM), and PP2A (AB′C) trimer from 1152.5 to 2403.4 fmol of P_i released/min (EC_{50} = 6.25 μM) (data not shown). The addition of salt also decreased the basal activity of each phosphatase by 50%, and therefore the fold stimulation was even higher. For example, ceramide increased the fold stimulation of PP1γc from 1.9- to 8.9-fold in the presence of 150 mM KCl when compared with control activity in the absence of 150 mM KCl. The actual fold stimulation increased to 17.8-fold when compared with control activity in the presence of 150 mM KCl. Even low concentrations of KCl were able to increase ceramide responsiveness, as the addition of 15 mM KCl increased maximal stimulation of PP1γc to 1103.3 fmol of P_i released/min and decreased the EC_{50} to 9.0 μM (Fig. 4*A*).

Ceramide stereospecificity for each phosphatase was completely retained (Fig. 4*B*). Furthermore, L-*erythro*-C_{18}-ceramide and PA retained an inhibitory effect, but the IC_{50} increased from approximately 100 nM to 1 μM in the presence of 150 mM KCl (data not shown). Inhibition of CAPPs by D- and L-*threo*-C_{18}-ceramides was relieved under these conditions of 150 mM KCl (Fig. 4*B*). Also, the addition of 150 mM NaCl had the same effects as 150 mM KCl by both increasing D-*erythro*-C_{18}-ceramide responsivenes and relieving nonspecific inhibi-

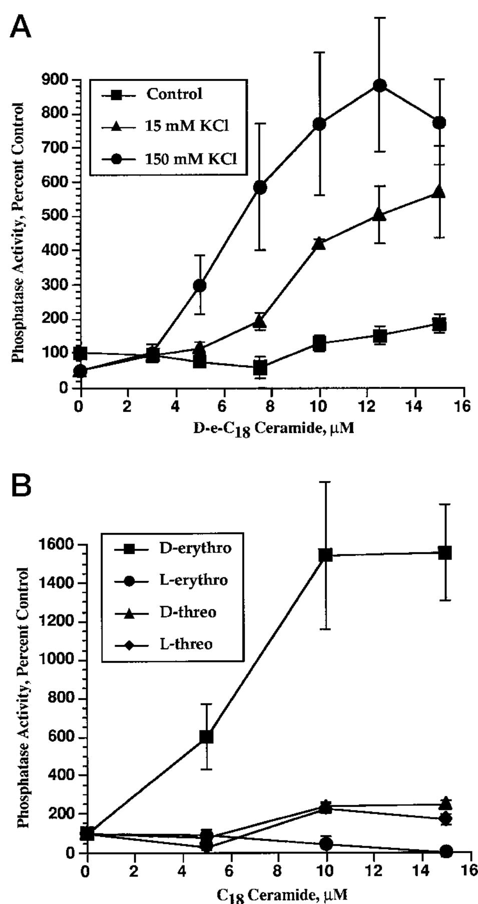

Fig. 4. **Effect of near physiological ionic strength on ceramide-activated protein phosphatase.** *A*, long chain ceramide effects on PP1γc were assayed in near physiological KCl buffer (50 mM Tris-HCl, pH 7.4, 0, 15, or 150 mM KCl, and 0.05 mM dithiothreitol). Solubilized D-*erythro*-C_{18}-ceramide (*D-e-C_{18} Ceramide*) was added to the assay reactions at concentrations of 0, 5, 10, and 15 μM. PP1γc was assayed as described under "Experimental Procedures." Results are expressed as the percent of initial activity in the absence of D-*erythro*-C_{18}-ceramide and KCl. Data are the mean ± S.E. of at least triplicate experiments reproduced on at least three separate occasions. *Closed triangles* designate the presence of 15 mM KCl, *closed squares* designate the absence of KCl, and *closed circles* designate the presence of 150 mM KCl. *B*, the stereospecificity of C_{18}-ceramide was examined in the presence of near physiological ionic strength. PP1γc was assayed as described above in 150 mM KCl. Data are presented as the percent control in the presence of 150 mM KCl with *closed triangles* designating D-*threo*-C_{18}-ceramide, *closed squares* designating D-*erythro*-C_{18}-ceramide, *closed diamonds* designating L-*threo*-C_{18}-ceramide, and *closed circles* designating L-*erythro*-C_{18}-ceramide.

tions by the other stereoisomers (data not shown).

Effects of Cations on PP1 and PP2A Activity and Ceramide Responsiveness—To examine whether the activation of PP1 and PP2A by long chain ceramides was dependent on or affected by cations, we preincubated the phosphatases with the different cations thought to be bound to the phosphatase metal binding site, Zn^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, and Mn^{2+} (31). Fig. 5 depicts the effects of these cations on PP1γc activity. Unlike PP1αc, PP1γc is supplied without preincubation with Mn^{2+} and was therefore used for these studies. Similar to other laboratories, we found that Mn^{2+} increased PP1γc activity by 200% (Fig. 5) (31). PP2A trimer and PP2Ac behaved in a similar manner, demonstrating a 175% increase in activity when preincubated with Mn^{2+} (data not shown). Pre-binding PP1γc with Mn^{2+} increased the V_{max} with ceramide from 1753.3 to 3201.3 fmol of P_i/min but had no effect on the overall fold stimulation by ceramide (Fig. 5). Mn^{2+} also had no effect on

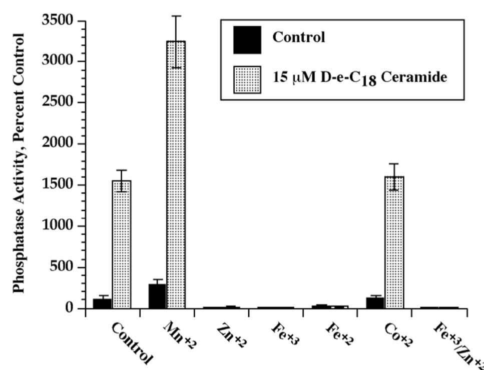

Fig. 5. **Effects of cations on ceramide-activated protein phosphatases.** Solubilized D-*erythro*-C_{18}-ceramide (*D-e-C_{18} Ceramide*) was added to the assay reactions at concentrations of 0 and 15 μM. PP1γc preincubated with the designated cation at 1 mM for 20 min was assayed as described under "Experimental Procedures." Results are expressed as the percent of initial activity in the absence of D-*erythro*-C_{18}-ceramide and cations. Data are the mean ± S.E. of at least triplicate experiments reproduced on at least three separate occasions.

inhibition by PA (data not shown). Incubation with Co^{+2} acted to increase basal activity by 25%, but it had no effect on ceramide responsiveness (Fig. 5). Incubation with $Fe^{+2,}$ Fe^{+3}, or Zn^{+2} completely inhibited PP1 activity and ceramide activation (Fig. 5). Preincubation of the enzyme with Mn^{2+} could not rescue the Zn^{2+} and Fe^{3+} inhibition (data not shown).

DISCUSSION

Ceramide-activated protein phosphatase was initially identified as a phosphatase-activated by cell-permeable D-*erythro*-C_2-ceramide, and at least two phosphatases, PP1 and PP2A, are now known to respond to short chain ceramides (23, 24–26, 33). Prior to this study, naturally occurring ceramides had not been shown to activate protein phosphatases. In this study, we demonstrate that, once solubilized with dodecane, D-*erythro*-C_{18}-ceramide activates two CAPP enzymes, PP1 and PP2A. This activation was very specific, as the unnatural stereoisomers of D-*erythro*-C_{18}-ceramide did not activate PP1 nor PP2A. These observations are important for several reasons. First, specificity for ceramide is now clearly demonstrated for the long chain ceramide, and this stereochemical specificity is better defined with the long chain isomers than with the short chain ones. Second, a reproducible assay has now been developed that allows the study of CAPPs under more physiologic conditions (natural ceramides and near physiological ionic strength). Third, a specific ceramide-binding/interaction site is now inferred to be present on the catalytic subunit of at least PP1 and PP2A.

Our system demonstrates strict stereospecificity for D-*erythro*-C_{18}-ceramide, because neither its enantiomer, L-*erythro*-C_{18}-ceramide, nor its diastereomers, D- and L-*threo*-C_{18}-ceramide, increased CAPP activity. The fact that even the mirror image of naturally occurring D-*erythro*-C_{18}-ceramide, L-*erythro*-C_{18}-ceramide, did not activate the phosphatases shows that the interaction is very specific and not an environmental effect on the enzymes.

In studying the optimal environment for delivery of long chain ceramides to CAPP, we initially found, as did Hirabayashi and co-workers (29) in cell studies, that dodecane acted as a useful vehicle for solubilizing long chain ceramides. We also found that the addition of near physiological levels of KCl to the reaction reduced inhibition of PP1 by both PA and long chain ceramides while enhancing ceramide activation of each phosphatase studied. Only PA and L-*erythro*-C_{18}-ceramide remained inhibitory under these conditions, although the dose necessary for inhibition of PP1 was increased approximately

10-fold. Importantly, stereospecificity was still retained such that D-*erythro*-C_{18}-ceramide was very effective, whereas its enantiomer, L-*erythro*-C_{18}-ceramide, lacked any activity. At this point, it is not clear why increasing the salt decreases the inhibition of PP1 by PA and low doses of long chain ceramides while enhancing the responsiveness of PP1 and PP2A to D-*erythro*-C_{18}-ceramide. It is assumed that the enzyme's conformation is affected under physiological salt conditions such that with no salt, there is easier access to many lipids nonspecifically, whereas under near physiological ionic strength, interactions are more specific.

These findings also suggest that a specific binding site for D-*erythro*-C_{18}-ceramide is present on both the PP1 and PP2A catalytic subunits. The stereospecificity of this activation suggests a direct and specific interaction of ceramide with the catalytic subunits of these phosphatases. Because the enantiomer and diastereomers of D-*erythro*-C_{18}-ceramide do not activate the phosphatases, the orientation of the C-2 and C-3 carbons relative to the sphingolipid backbone is suggested to be important for proper interaction and binding of ceramide to CAPP.

Another intriguing observation emerged with the finding that the PP2A heterotrimeric complex was activated to a greater extent than the catalytic subunit alone. This finding suggests that the A and B subunits of trimeric PP2A, which are important for compartmentalization and substrate specificity (38, 39), may also impart a conformation that allows for greater ceramide stimulation. Alternatively, ceramide may also interact with the A and B subunits to impart greater phosphatase activity. A third possibility is that A and B preferentially interact with a form of C that is more responsive to ceramide. These possibilities are currently under investigation.

The dual action of PA and D-*erythro*-C_{18}-ceramide on PP1 carries important implications for the physiological environment in which PP1 resides. CAPP phosphatases such as PP1 and PP2A have roles in apoptosis and cell cycle arrest. Therefore, a role for PA, a product of phospholipase D, may be hypothesized in the suppression of phosphatase activity until the proper stimuli (*e.g.* ceramide) are generated to protect the cell from undergoing premature apoptosis or inhibition of growth.

Establishing the conditions necessary for delivery of long chain ceramides to PP1 and PP2A allowed us to examine the influence of cations on PP1 activity and ceramide responsiveness. We demonstrated, as had others, that preincubation with Mn^{2+} increased PP1c basal activity (31). We also found that Mn^{2+} increased PP2Ac and PP2A trimer basal activity. Mn^{2+} did not, however, affect ceramide responsiveness. We also examined other cations of which Zn^{2+}, Fe^{3+}, and Fe^{2+} potently inhibited PP1 and PP2A activity *in vitro*; ceramide was not able to overcome this inhibition of PP1 and PP2A. The inhibition may be the result of allosteric effects as suggested by Schlender and co-workers (31). The influence of Zn^{+2} inhibiting apotosis is well known (36, 37), and our observations suggest yet another target for this anti-apoptotic cation.

In this study, we have demonstrated that both PP1 and PP2A are activated by long chain ceramides and that this activation is stereospecific. We have also demonstrated that PA and salt have important effects on this activation by lowering the ceramide concentration necessary for achieving phosphatase activation. We also show that $Fe^{2+/3+}$ and Zn^{2+} inhibit PP1 and PP2A activity, with ceramide not able to overcome cation inhibition. Clearly, this study demonstrates several new avenues of CAPP regulation and that physiologic environments can enable these enzymes to respond to natural ceramide rapidly and effectively.

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