Effects of cations on ceramide-activated protein phosphatase 2A

Sehamuddin Galadari^{1,2}, Abdulkadir Hago¹
and Mahendra Patel¹

¹Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates

²Corresponding author: Tel, +9713-7039-507;
Fax, +9713-7672033; E-mail, sehamuddin@uaeu.ac.ae

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Abstract

Characterization of ceramide-effector(s), which includes protein phosphatase 2A (PP2A) is an important prelude to understanding the molecular basis of sphingolipid-mediated biological effects such as cell growth, differentiation and apoptosis. Recently, the existence of a metal-dependent form of PP2A has been reported (Cai *et al.*, 1995). In this study, we investigated the effects of metal ions and chelators on ceramide-activated PP2A (CAPP). Our study demonstrates that at 0.5 mM concentration, Mg²⁺ appears to have no significant effect on either basal or ceramide-stimulated phosphatase activities, whereas Ca²⁺ stimulated the basal phosphatase activity, but was inhibitory towards CAPP. Moreover, the divalent cations Cr²⁺, Mn²⁺, Fe²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ were tested and all were found to be inhibitory towards both CAPP and basal phosphatase activities. By contrast, Cs⁺ and Li⁺ had almost no effect on CAPP, although both stimulated basal phosphatase activity. The effects of EDTA and EGTA were tested and it was observed that EDTA decreased CAPP activity in a dose-dependent fashion, but had no effect upon basal phosphatase activity. These results suggest that CAPP is a metal-dependent protein, but, because Ca²⁺ inhibited CAPP and EGTA was much less potent than EDTA in inhibiting CAPP, Ca²⁺ is unlikely to be its metal cofactor.

Keywords: cations, ceramide, phosphatases

Introduction

Sphingolipids constitute a diverse and ubiquitous group of membrane lipids with diverse molecular structures and multiple biological effects (Bell *et al.*, 1993). These lipids have also been shown to be important for the understanding of a number of biological processes such as cell growth, differentiation, stress and apoptosis

(Hannun and Bell, 1989; Hannun, 1996; Kolesnick and Kronke, 1998). Several studies have shown that the action of a number of agonists leads to the activation of sphingomyelinases, and sphingomyelin hydrolysis leads to ceramide generation (Merrill and Jones, 1990; Zhang *et al.*, 1997). It has been reported that once generated, ceramide acts through a ceramide-activated protein phosphatase 2A (CAPP) to elicit some of its downstream effects (Dobrowsky and Hannun, 1992; Galadari *et al.*, 1998). The identification of a phosphatase as a molecular target for the action of ceramide defines a novel intracellular signaling pathway with potential roles in the regulation of cell proliferation and differentiation. Although CAPP is a prime candidate for mediating ceramide signaling in mammalian cells, its mechanism of regulation remains elusive. Biochemical characterization of CAPP is, therefore, a prelude to a better understanding of the mechanism by which ceramide causes its downstream effects upon its effector molecules. Recent studies indicate that protein phosphatase 2A (PP 2A), like protein phosphatase 1 (PP 1), can exist in a metal ion-dependent form (Cai *et al.*, 1995). This may represent a new mechanism for the regulation of PP2A. In this study, a quick one-step column method has been introduced for the partial purification of CAPP, allowing characterization of the role of metal cations on partially purified CAPP.

Materials and Methods

Materials

Lipids were purchased from Matreya (Pleasant Gap, USA), [γ -³²P]ATP was obtained from NEN (Boston, USA). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, USA). All cell culture material, such as media and fetal calf serum, were from Life Technologies Inc. (Paisley, Scotland), Chromatography columns were from Pharmacia Upjohn Biotech (Uppsala, Sweden).

Cell culture

Molt-4 cells (a kind gift from Dr. Y Hannun, Medical University of South Carolina, USA) were maintained in RPMI-1640 in the presence of 10% fetal calf serum and grown in an atmosphere of 5% CO₂ at 37°C.

Partial purification of CAPP

Confluent cultures of Molt-4 cells were harvested by centrifugation and washed three times with phosphate buffered saline, prior to resuspension in homogenization

buffer (50 mM Tris-HCl pH 7.4, containing 1 mM benzamidin, 1 mM dithiothreitol, 1 mM PMSF, 2.5 µg/ml leupeptin, and 2 µg/ml pepstatin). The cells were then subjected to an atmosphere of 350 psi under oxygen-free nitrogen in a Parr Nitrogen Cavitation vessel (Parr Instruments Inc. USA) and left to stand on ice for 20 min. After that the vessel was carefully brought to normal atmosphere, and the cell suspension was very carefully collected on ice. The suspension was centrifuged at 100,000 *g* and the supernatant was collected for chromatography and further study. Partial purification of CAPP was achieved by Mono-Q ion-exchange chromatography. The Mono-Q (HR 5/5) column was equilibrated in the equilibration buffer A (50 mM Tris-HCl pH 7.4, containing 1 mM benzamidin, 1 mM dithiothreitol, and 10% glycerol). The cell supernatant was loaded on to the Mono-Q column and the protein was eluted by a linear salt gradient.

Preparation of phosphatase substrate

Phosphorylated myelin basic protein (MBP) was prepared as previously described (Galadari *et al.*, 1998). Briefly, MBP-phosphorylation was carried out by using the catalytic subunit of cAMP-dependent kinase in an assay reaction containing 50 mM Tris-HCl, 0.5 mM dithiothreitol, 10 mM β-mercaptoethanol, 10 mM MgCl₂, 0.1 mM [γ-³²P]ATP (2-7) × 10³ cpm/mol, 1 mg of MBP, and 250 units/ml of catalytic subunit of cAMP-dependent protein kinase. The reaction was carried out at 37°C for 2 h after which it was stopped by the addition of 0.17 ml of ice cold 100% TCA. Immediately, the tubes were vortexed and left to stand on ice for at least 30 min. After that the tubes were centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was discarded. The pellet was washed twice with 1 ml aliquots of ice-cold acetone kept at -20°C, and finally it was resuspended in 1 ml of 50 mM Tris-HCl, pH 7.4. The concentration of the labeled substrate was based on the specific activity of the [γ-³²P]ATP. Typical preparations of [³²P]-MBP contained 3-9 nmol of P/mg. Assays were performed so that hydrolysis did not exceed 10% of total substrate added.

Myelin basic protein-phosphatase assay

This assay was performed as previously described (Dobrowsky *et al.*, 1993). Briefly, the assay was performed in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5-2 ng of protein and the indicated concentration of radiolabeled substrate in a final assay volume of 0.1 ml. Ceramide solution was prepared fresh in 100% ethanol. The final ethanol concentration in the assay was always kept at 1%. Reactions were run for 15 min at 30°C, and they were terminated by addition of 0.1 ml of 1 mM KH₂PO₄ in 1 N H₂SO₄. The assays were done in triplicate. The released [³²P]-phosphate was sequestered by the addi-

tion of 0.3 ml of 2% ammonium molybdate, and the phosphomolybdate complex was extracted with the addition of 1 ml of toluene : isobutanol (1:1, v/v). Quantitation was done by liquid scintillation spectrometry of an aliquot of the extracted phosphomolybdate complex. CAPP activity was quantitated as the difference in the protein phosphatase activity in the presence and absence of ceramide.

Results

Partial purification of CAPP on ion-exchange chromatography

Cytosolic extracts from Molt-4 cells were loaded on a pre-equilibrated Mono-Q column (HR 5/5) and CAPP was eluted by a linear salt gradient, as can be seen in Figure 1. One ml fractions were collected and assayed for MBP-phosphatase activity in the presence and absence of C₂ ceramide. Figure 1 illustrates a typical chromatographic profile for the partial purification of CAPP, obtained from Molt-4 cells. Those fractions containing the maximum CAPP activity were pooled and used for further studies.

Effects of metal ions on phosphatase activities

Partially purified CAPP was subjected to extensive dialysis in the presence of 10 mM, and then 0.1 mM EDTA, prior to treatment with cations. Figure 2 summarizes the effects of cations at 0.5 mM concentration on basal, as

Figure 1. Partial purification of CAPP. The high speed supernatant of Molt-4 cells was applied to Mono-Q (5HR) column that was pre-equilibrated with buffer A (50 mM Tris-HCl pH 7.4, containing 1 mM benzamidin, 1 mM dithiothreitol, and 10% glycerol). The protein was eluted with buffer A containing 1 M NaCl, and 1 ml fractions were collected. MBP-Phosphatase assays were carried out as described under Materials and Methods.

Figure 2. The effect of metal cations on basal and CAPP phosphatase activities. The pooled enzymatically active fractions were dialysed in the presence of 10 mM, and then 0.1 mM EDTA, prior to treatment with cations in the presence (CAPP activity) and absence (basal phosphatase activity) of 20 mM C_2 ceramide.

well as CAPP phosphatase activities. As shown in Figure 2, both Li^+ and Cs^+ stimulated basal phosphatase activity, while having a rather moderate inhibitory effect on CAPP activity. Interestingly, Mg^{2+} had no significant effect on either basal or CAPP activities. On the other hand, Ca^{2+} stimulated the basal phosphatase activity by about 40% while inhibiting CAPP activity by more than 20%. Moreover, Cr^{2+} , Fe^{2+} , Cu^{2+} , and Zn^{2+} all nearly totally inhibited CAPP activity and Cr^{2+} reduced the basal activity by about 60%, while Fe^{2+} , Cu^{2+} and Zn^{2+} reduced it by 90%. Both Mn^{2+} and Ni^{2+} reduced the basal and the CAPP activities to almost the same degree.

Figures 3A and B summarize the effects of divalent metal cations on MBP-phosphatase activity following an extensive dialysis with EDTA. At 1 mM concentration, both Mg^{2+} and Ca^{2+} slightly stimulated the basal phosphatase activity, while having almost no effect upon CAPP activity. But, at the same concentration, Mn^{2+} and Zn^{2+} inhibited both activities partially and totally, respectively. At 10 mM concentration, both Mg^{2+} and Ca^{2+} slightly inhibited the basal (Figure 3A) and the ceramide-activated (Figure 3B) phosphatase activities and Mn^{2+} almost totally inhibited both activities.

Effect of metal chelators on partially purified CAPP

In an attempt to investigate the possibility of metal dependence of ceramide-activated PP2A, the basal and CAPP activities of partially purified phosphatase was measured in the presence of the metal chelators EDTA and EGTA. As shown in Figure 4A, EDTA inhibited CAPP in a dose dependent fashion. Indeed, at 10 mM concentration, EDTA inhibited CAPP by almost 50%,

Figure 3. Effect of divalent metal cations on basal (A) and CAPP (B) MBP-phosphatase activity. Partially purified phosphatase was dialyzed extensively against EDTA at 10 mM, followed by 0.1 mM EDTA prior to preincubation with the indicated concentrations of cations. The activity was measured in the presence and absence of 20 mM C_2 ceramide as described under Materials and Methods.

while having no effect upon the basal phosphatase activity. At 100 mM concentration, EDTA almost totally inhibited CAPP activity while showing only a minor inhibitory effect upon the basal phosphatase activity.

Figure 4B illustrates the effect of EGTA on phosphatase activity. Unlike EDTA, EGTA at 5 mM did not inhibit CAPP activity at all and only at 50 mM concentration did EGTA inhibit CAPP by 37%. Again, the basal phosphatase activity was inhibited only slightly at 50 mM EGTA.

Discussion

Recently, the existence of a metal-dependent form of PP2A has been reported (Cai *et al.*, 1995). In this study, we investigated the effects of metal ions and chelators on metal ion-depleted CAPP. The present study clearly shows that monovalent cations of the alkali metals,

Figure 4. The effect of EDTA (A) and EGTA (B) upon both basal-phosphatase and CAPP activities. Partially purified enzyme was preincubated with the indicated concentrations of the chelators prior to stimulation with (CAPP activity) and without (basal phosphatase activity) 20 mM C_2 ceramide.

namely Li^+ and Cs^+ stimulated basal phosphatase activity, but had no major effect upon CAPP activity. The effect of Ca^{2+} on basal activity was similar to Li^+ and Cs^+ , except that Ca^{2+} also slightly inhibited CAPP activity while Mg^{2+} had a little effect on either activity.

It has long been known that divalent cations, in particular Mn^{2+} and Mg^{2+} , activate certain phosphatase preparations (Kato *et al.*, 1975). Indeed, it has been shown that some, such as Mn^{2+} , enhances intrinsic calcineurin phosphatase activity, while others, like Ni^{2+} , Fe^{2+} , Zn^{2+} , and Cu^{2+} , are strongly inhibitory (Gupta *et al.*, 1990). Using a recombinant PP1 catalytic subunit, a stable metalloenzyme form of PP1 has been generated (Chu *et al.*, 1996). It has been suggested that at least two metal binding sites exist on the enzyme and that PP1 may be an Fe^{2+}/Zn^{2+} metalloenzyme *in vivo* (Chu *et al.*, 1996). In the present study, Zn^{2+} , Fe^{2+} and Cu^{2+} completely inhibited and Ni^{2+} and Mn^{2+} partially inhibited CAPP. In addition it was shown that CAPP activity was totally inhibited by Cr^{2+} , but this cation appeared to be

less effective in inhibiting the basal phosphatase activity. In view of the absence of any significant stimulation of CAPP activity by the transition metal cations, the effect of metal ion sequestration upon CAPP activity was studied. Indeed, at 10 mM EDTA concentration, almost half of the CAPP activity was inhibited, yet no effect on the basal phosphatase activity was seen. This indicates that CAPP activity is more responsive to EDTA than is the basal phosphatase activity, which is in agreement with previous reports on the effect of chelators on calcineurin phosphatase activity (Gupta *et al.*, 1990). Furthermore, from this study it can be seen that at concentrations up to 5 mM EGTA has no effect upon CAPP activity and only slightly inhibited basal phosphatase activity. In fact, major inhibitory effects are only seen at 50 mM EGTA. It is clear, therefore, that EGTA is less effective than EDTA in the inhibition of CAPP activity. This is in contradiction to previous reports, in which EDTA and EGTA were shown to have equal effects on calcineurin (Gupta *et al.*, 1984, 1990). Therefore, from these findings it seems probable that CAPP is a metal dependent enzyme. Although, it is unlikely that the metal cofactor is Ca^{2+} since EGTA was a less effective inhibitor than EDTA. However, since the CAPP preparation used in this study was only partially purified, an indirect effect by the cations or the chelators tested can not be ruled out. Therefore, further studies will be required to identify the metal cation that is essential for CAPP activity.

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References

- Bell RM, Merrill AH, Jr., Hannun YA. (Eds.) Sphingolipids, Part A: Functions and breakdown products. In *Advances in Lipid Research*, Vol. 25, Academic Press, San Diego, California. 1993
- Cai L, Chu Y, Wilson SE, Schlender KK. A metal-dependent form of protein phosphatase 2A. *Biochem Biophys Res Commun* 1995;208(1):274-79
- Chu Y, Lee EYC, Schlender KK. Activation of protein phosphatase 1. *J Biol Chem* 1996;271(5):2574-77
- Dobrowsky RT, Hannun YA. Ceramide stimulates a cytosolic protein phosphatase. *J Biol Chem* 1992;267(8):5048-51
- Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. *J Biol Chem* 1993;268(21):15523-30

Galadari S, Kishikawa K, Kamibayashi C, Mumby MC, Hannun YA. Purification and characterization of ceramide-activated protein phosphatases. *Biochemistry* 1998;37(32):11232-38

Gupta RC, Khandelwal RL, Sulakhe PV. Divalent cation effects on calcineurin phosphatase: differential involvement of hydrophobic and metal binding domains in the regulation of the enzyme activity. *Mol Cell Biochem* 1990;97:53-66

Gupta RC, Khandelwal RL, Sulakhe PV. Intrinsic phosphatase activity of bovine brain calcineurin requires a tightly bound trace metal. *FEBS Lett* 1984;169(2):251-55

Hannun YA, Bell RM. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* 1989;243:500-507

Hannun YA. Functions of ceramide in coordinating cellular responses to stress. *Science* 1996;274:1855-59

Kato K, Kobayashi M, Sato S. Inactivation and reactivation of phosphoprotein phosphatase of rabbit skeletal muscle. *J Biochem* 1975;77:811-15

Kolesnick RN, Kronke M. Regulation of ceramide production and apoptosis. *Annu Rev Physiol* 1998;60:643-65

Merrill AH. Jr., Jones D. An update of the enzymology and regulation of sphingomyelin metabolism. *Biochimica et Biophysica Acta* 1990;1044:1-12

Zhang P, Liu B, Jenkins GM, Hannun YA, Obeid LM. Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. *J Biol Chem* 1997;272(15):9609-12