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Biochemical and Biophysical Research Communications 307 (2003) 64–68
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Zinc inhibits calcineurin activity in vitro by competing with nickel

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Received 27 May 2003

Abstract

Calcineurin (CN) is a Ca^{2+}/calmodulin (CaM)-dependent protein serine/threonine phosphatase that contains Zn^{2+} in its catalytic domain and can be stimulated by divalent ions such as Mn^{2+} and Ni^{2+}. In this study, the role of exogenous Zn^{2+} in the regulation of CN activity and its relevance to the role of Ni^{2+} was investigated. Zn^{2+} at a concentration range of 10 nM–10 μM inhibited Ni^{2+}-stimulated CN-activity in vitro in a dose-dependent manner and approximately 50% inhibition was attained with 0.25 μM Zn^{2+}. Kinetic analysis showed that Zn^{2+} inhibited the activity of CN by competing with Ni^{2+}. Interaction of CN and CaM was not inhibited with Zn^{2+} at 10 μM. Zn^{2+} never affected the activity of cAMP phosphodiesterase 1 or myosin light-chain kinase (CaM-dependent enzymes) and rather activated alkaline phosphatase. The present results indicate that Zn^{2+} should be a potent inhibitor for CN activity although this ion is essential for CN.

Keywords: Zinc; Protein phosphatase; Calcineurin

Calcineurin (CN), also known as protein phosphatase 2B, is a Ca^{2+}/calmodulin (CaM)-dependent protein serine/threonine phosphatase, which consists of a catalytic A-subunit (CN-A) and a regulatory B-subunit (CN-B) [1,2]. CN-A has a CaM-binding domain, a CN-B-binding domain, a C-terminal autoinhibitory domain, and a catalytic domain that contains a Fe^{2+}–Zn^{2+} binuclear center [3–6]. CN-B binds four Ca^{2+} in EF-hand motifs.

CN has been shown to play pivotal roles in a variety of cellular functions in both higher and lower eukaryotic organisms. For example, CN is involved in Ca^{2+}-induced nervous cell apoptosis [7] and the hyper-phosphorylation of the tau protein in Alzheimer's disease [8,9]. In T-cell activation, CN functions by de-phosphorylating the nuclear factor of activated T-cells (NF-AT) that enhance the expression of interleukin-2 (IL-2) [10]. In addition, the immunosuppressive drugs, cyclosporin A and FK506, have been found to inhibit CN via immunophilins [11]. CN has been shown to be involved in cytokinesis, cell shape, and ion homeostasis in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [12–14].

While the activity of CN is regulated by Ca^{2+} and CaM in vivo, it can be stimulated by divalent ions such as Mn^{2+} and Ni^{2+} in vitro [15–18]. However, the precise roles of some metal ions in the regulation of CN remain to be elucidated. In the case of Zn^{2+}, for example, it was reported that Zn^{2+} stimulated CN [19], but others reported that Zn^{2+} was not effective on CN [15,17].

On the other hand, Zn^{2+} is found in a variety of organisms and has been shown to be essential for many cellular functions, such as the actions of some metalloenzymes, the regulation of gene expression through zinc finger motifs of DNA-binding proteins, and the capsid formation of human immunodeficiency virus (HIV) [20–26].

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doi:10.1016/S0006-291X(03)01122-7

In this study, we investigated the effect of Zn^{2+} on the activity of CN in vitro and show here that Zn^{2+} at physiological concentrations inhibits the activity of CN by competing with Ni^{2+}.

Materials and methods

Reagents and purified proteins. Bovine brain calcineurin (CN), calmodulin (CaM), CaM-dependent cyclic nucleotide phosphodiesterase (PDE1), *p*-nitrophenylphosphate (pNPP), and anti-CN-A monoclonal antibody were obtained from Sigma (St. Louis, MO), and calf-intestinal alkaline phosphatase (AP) was from New England BioLabs (Beverly, MA). Smooth-muscle myosin and MLCK were purified from chicken gizzard as described previously [27,28]. It should be noted that the definitions of units for enzymatic activities used in this study are in accordance to the manufacturer's descriptions. One unit (U) of CN is approximately 6.5 pmol of CN and 1 U of CaM is approximately 14–15 pmol of CaM.

Assay for the activity of CN and AP. Unless otherwise stated, 3 U each of CN (~0.1 μM) and CaM (~0.2 μM) was incubated at 30 °C for 60 min in 200 μl of a reaction buffer (100 mM Hepes–NaOH, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$, various concentrations of Zn^{2+}, and 3 mM pNPP) in the presence or absence of 0.2 mM $NiCl_2$, and, in some experiments, the conditions varied as indicated. The reaction was stopped by adding 800 μl of 1 M Na_2CO_3. CN activity was quantified by measuring the absorbance (at 410 nm: A_{410}) of the reaction mixtures. To determine the relation between A_{410} and hydrolyzed pNPP, the A_{410} of the solutions of known concentrations of *p*-nitrophenol (NP), the product of hydrolyzed pNPP, was measured. For kinetic analysis, Lineweaver and Burk plot was used.

To further assess the activity of CN in the absence of Ni^{2+}, 10 U each of CN and CaM was incubated at 30 °C for 60 min in 100 μl of a reaction buffer (100 mM Hepes–NaOH, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 3 mM pNPP) in the presence or absence of 10 μM $ZnSO_4$, and the reaction was stopped by adding 100 μl of 1 M Na_2CO_3. CN activity was quantified by measuring the A_{410} of the reaction mixtures.

AP (26 mU) was incubated in 200 μl of an assay buffer (0.3 M glycine–NaOH, pH 10.5, 1 mM $MgCl_2$, and 6 mM pNPP) in the presence of various concentrations of $ZnSO_4$ at 37 °C for 5 min, and the reaction was stopped by adding 750 μl of 1 M Na_2CO_3 and 50 μl of 0.1 M EDTA. AP activity was quantified by measuring the A_{410} of the reaction mixtures.

Assay for PDE1 activity. According to the manufacturer's description, 1 U of PDE1 will hydrolyze 1 μmol of cAMP to 5′-AMP per minute at pH 7.5 at 30 °C. We have established a simple assay system for PDE1 activity where the amounts of 3′:5′-cyclic AMP (cAMP: substrate) are measured. PDE1 (10 mU) was incubated with various concentrations of $ZnSO_4$, 2.5 μM calmidazolium, or 50 μM W-7 in the presence of CaM (100 U) in 400 μl of an assay buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.4 μM cAMP) for 30 min at 30 °C, and the enzymatic reaction was stopped by the addition of 100 μl of 1 N HCl. Aliquots were neutralized and used for the assay for cAMP contents using a radio immunoassay (RIA) method [29], in which quadruplicate determination was performed for each sample. The apparent activity of PDE1 was calculated by subtracting the assessed cAMP contents from the starting amount of cAMP (0.4 μM) and was given with relative values.

Assay for myosin light-chain kinase. Purified myosin (3.7 μM) was phosphorylated with 0.086 μM myosin light-chain kinase (MLCK) and 0.3 μM CaM in a reaction buffer (20 mM Tris–HCl, pH 7.5, 2 mM ATP, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM dithiothreitol) in the presence or absence of 10 μM $ZnSO_4$ or calmidazolium at 25 °C for 20 min. After terminating the reaction by adding an equal volume of a sample buffer containing 6 M urea, 14 mM 2-mercaptoethanol, and 50 mM Tris–HCl (pH 6.8), samples were analyzed with urea–glycerol PAGE as described previously [30].

Assay for CN–CaM interaction. Approximately, 100 μl of CaM–Sepharose 4B (Amersham–Pharmacia, UK) was equilibrated in a reaction buffer (100 mM Hepes–NaOH, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$, 0.2 mM $NiCl_2$, and 0.01% (w/v) BSA) in the presence or absence of 10 μM $ZnSO_4$. CN (~0.1 μM) in 100 μl of the buffer containing 10 μM $ZnSO_4$ was applied to the CaM–Sepharose in an Eppendorf tube on ice and the mixture was incubated under agitation for 30 min. After quick centrifugation, the supernatant (100 μl) was collected, and the Sepharose was washed with 100 μl of the same buffer twice, while the supernatants were kept for SDS–PAGE. CN was then eluted with 100 μl of an elution buffer (100 mM Hepes–NaOH, pH 7.5, 0.1 M NaCl, 20 mM EGTA, 0.01% (w/v) BSA, and ±10 μM $ZnSO_4$) twice and the supernatants were kept. Eighteen microliter aliquots of all the collected supernatants were mixed with 12 μl of a 2× concentrated SDS-sample buffer, separated by SDS–PAGE (10% polyacrylamide gel), and transferred onto nitrocellulose membrane (NCM). NCM was incubated for 1 h with an anti-CN-A antibody (dilution, 1:500) in TBS–T (10 mM Tris–HCl, pH 7.5, 137 mM NaCl, and 0.1% (w/v) Tween 20) containing 5% (w/v) nonfat dried-milk powder. After washing with TBS–T, NCM was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody (dilution, 1:1000) in TBS–T containing 5% nonfat dried-milk powder. After washing with TBS–T, NCM was processed for visualization using the enhanced chemi-luminescence (ECL) kit according to the manufacturer's instructions (Amersham, UK) and exposed to Hyperfilm for ECL (Amersham, UK).

Results

Effects of Zn^{2+} on the activity of CN

We first established an in vitro assay system for CN activity using Ni^{2+} as a stimulator and examined the effects of Zn^{2+} on Ni^{2+}-stimulated CN-activity (Fig. 1A). Both $ZnCl_2$ and $ZnSO_4$ at a concentration range of 10 nM–10 μM inhibited Ni^{2+}-stimulated CN-activity in a dose-dependent manner despite the fact that Zn^{2+} is essential for CN as a component of the reaction center of this enzyme. Zn^{2+} at 10 μM inhibited Ni^{2+}-stimulated CN-activity almost perfectly and approximately 50% inhibition was attained with 0.25 μM Zn^{2+} (Fig. 1A). We also examined whether or not Zn^{2+} inhibits CN activity in the absence of Ni^{2+}. However, since CN activity was very low in the absence of Ni^{2+} ($A_{410} < 0.025$; Fig. 1A), it was difficult to evaluate the effect of Zn^{2+} on the activity (Fig. 1A). Thus, larger amounts of CN and CaM were used for the assay, where CN activity was inhibited dose-dependently with 1–100 μM Zn^{2+} and IC_{50} was 7 μM (Fig. 1B). These results indicate that Zn^{2+} should be a potent inhibitor of CN activity and also suggest that there is some relationship between Ni^{2+} and Zn^{2+} in the regulation of CN activity.

Kinetic analysis of the action of Zn^{2+}

To investigate the interaction among CN, Zn^{2+}, and pNPP or Ni^{2+}, the CN activity was measured in the presence of various concentrations of Zn^{2+} and pNPP (Fig. 2A) or Ni^{2+} (Fig. 2B). The data were plotted

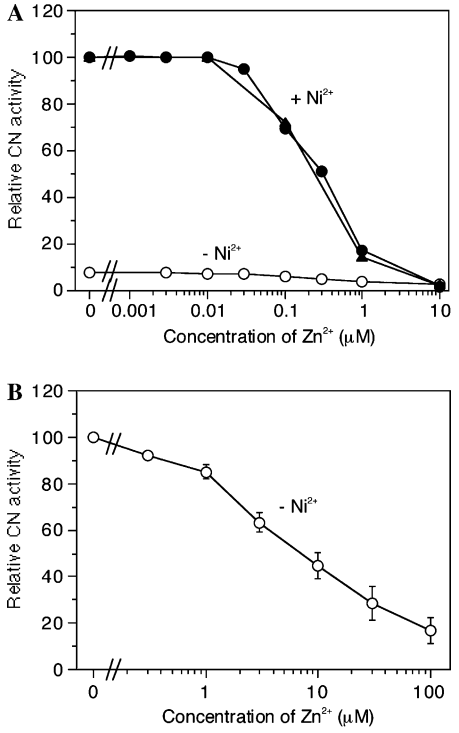

Fig. 1. Effect of Zn^{2+} on CN activity. (A) CN (3 U) and CaM (3 U) were incubated in a Hepes buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, and 3 mM pNPP) containing the indicated concentrations of $ZnCl_2$ (triangles) or $ZnSO_4$ (circles) in the presence (closed symbols) or absence (open circles) of 0.2 mM $NiCl_2$ at 30 °C for 60 min, and CN activity was determined by measuring the A_{410} of the reaction mixtures. Data are given with the relative activity versus control (= Ni^{2+}-induced CN activity in the absence of Zn^{2+} = 100). A_{410} of the controls were around 0.25. (B) CN (10 U) and CaM (10 U) were incubated in a Hepes buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, and 3 mM pNPP) containing the indicated concentrations of $ZnSO_4$ at 30 °C for 60 min, and CN activity was determined by measuring the A_{410} of the reaction mixtures. Data were transformed into the relative activity versus control (= CN activity in the absence of Zn^{2+} = 100) and are given with the mean values ± SD of three independent experiments. A_{410} of the controls were around 0.35.

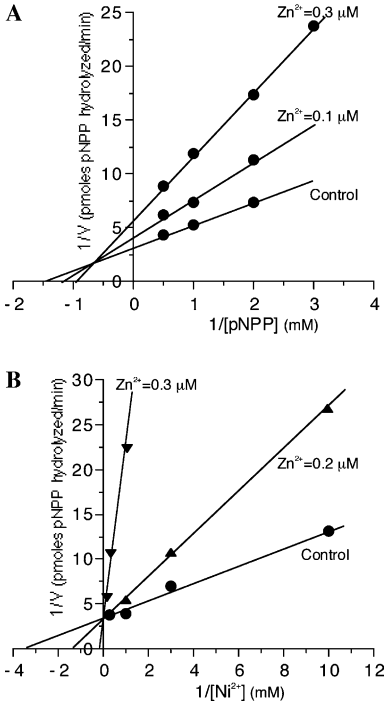

Fig. 2. Kinetic analysis of Zn^{2+}-induced inhibition of CN activity. CN (3 U) and CaM (3 U) were incubated in the presence of varying concentrations of pNPP and $ZnSO_4$ in a Hepes buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.2 mM $NiCl_2$) (A) or varying concentrations of $NiCl_2$ and $ZnSO_4$ in a Hepes buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, and 3 mM pNPP) (B) at 30 °C for 60 min, and CN activity was measured. The data were plotted as $1/v$ versus 1/[pNPP] (A) or 1/[Ni^{2+}] (B) and the fittest lines were determined by linear regression analysis. The value of the y-intercept represents the theoretical maximum velocity (V_{max}) obtained in the presence of pNPP (A) or Ni^{2+} (B). (A) Apparent V_{max} was calculated to be 0.31 nmol/tube/min and K_m was 0.64 mM in the absence of Zn^{2+}. $V_{max}I$ was 0.18 nmol/tube/min and K_mI was 1.08 mM in the presence of 0.3 μM Zn^{2+}. K_i was 0.16 μM and $K_i' = 0.42$ μM. The results indicate that Zn^{2+} is a non-competitive inhibitor toward the substrate. (B) Apparent V_{max} was calculated to be 0.28 nmol/tube/min in the absence of Zn^{2+}. The results indicate that Zn^{2+} is a competitive inhibitor toward Ni^{2+}.

according to the Lineweaver and Burk equation. As shown in Fig. 2A, the V_{max} values in the absence and presence of Zn^{2+} (0.1 or 0.3 μM) varied, and the apparent affinity of pNPP for CN decreased with increasing the concentrations of Zn^{2+}. The results indicate that Zn^{2+} can bind to both CN and a CN–pNPP complex with different K_i values and thereby inhibit CN activity, not competing with pNPP. On the other hand, Fig. 3B demonstrates that the V_{max} values in the absence and presence of Zn^{2+} at 0.2 or 0.3 μM were almost the same, while the apparent binding of Ni^{2+} to CN decreased with increasing the concentrations of Zn^{2+}. The results indicate that Zn^{2+} inhibits CN activation by competing with Ni^{2+}.

Effects of Zn^{2+} on the interaction of CN and CaM

Because CN is a CaM-dependent enzyme, the effect of Zn^{2+} on CN–CaM interaction was examined using

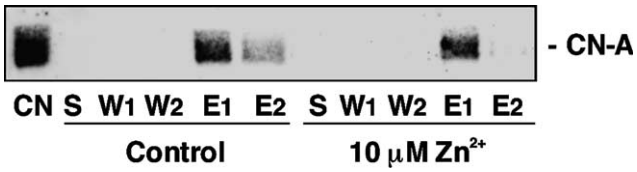

Fig. 3. Effect of Zn^{2+} on CN–CaM interaction. CN in a reaction buffer (lane CN) was mixed with CaM–Sepharose 4B in a tube in the absence (control) or presence of 10 μM $ZnSO_4$ (Zn^{2+}) for 30 min, and the supernatants were collected for Western blot (lane S). After being washed with the same buffer twice (lanes W1 and W2), CN was eluted with an elution buffer twice (lanes E1 and E2), and aliquots were analyzed by Western blot with an anti-CN-A antibody. A representative result out of three independent experiments is shown.

CaM–Sepharose. As shown in Fig. 3, CN bound well to CaM–Sepharose regardless of the presence of 10 μM Zn^{2+} and eluted with an EGTA-containing buffer,

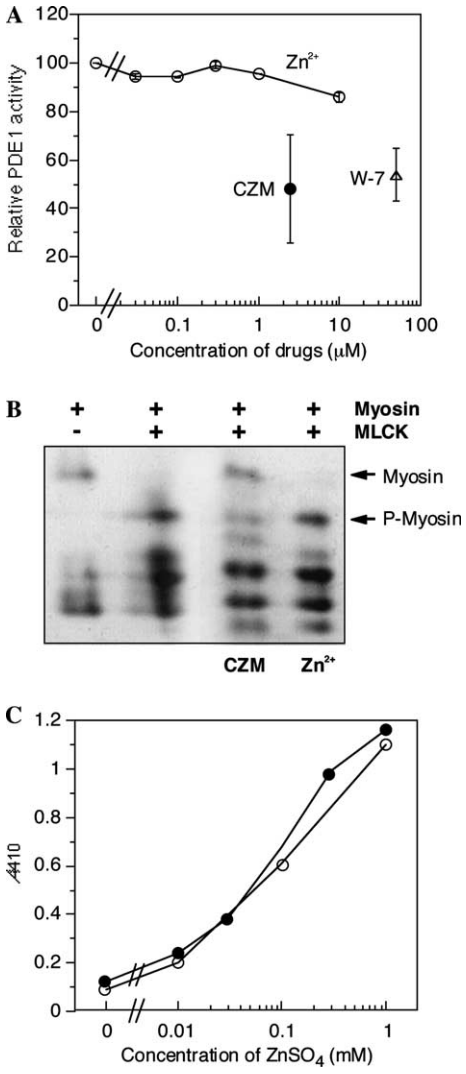

Fig. 4. Effect of Zn^{2+} on PDE1, MLCK, and AP. (A) PDE1 and CaM were incubated in an assay buffer containing the indicated concentrations of $ZnSO_4$, calmidazolium (CZM), or W-7 for 30 min at 30 °C, and aliquots were assayed for cAMP contents using an RIA method. The apparent activity of PDE1 was calculated by subtracting the assessed cAMP contents from the starting amount of cAMP (0.4 μM). Data are given with the mean values of relative activity (control = 100) ± SD of two independent experiments. The values of control activity were 10.70 and 10.75 pmol cAMP hydrolyzed/ml/min. (B) Myosin, CaM, and MLCK were incubated in an assay buffer in the presence or absence of 10 μM $ZnSO_4$ or 10 μM calmidazolium (CZM) at 25 °C for 20 min, and samples were analyzed by urea–glycerol PAGE. A representative result out of two independent experiments is shown. (C) AP was incubated in an assay buffer in the presence of the indicated concentrations of $ZnSO_4$ at 37 °C for 5 min and AP activity was assessed. The results of two independent experiments are shown.

indicating that Zn^{2+} never interferes with CN–CaM interaction.

Effects of Zn^{2+} on PDE1, MLCK, and AP

To assess whether or not Zn^{2+} inhibits some other CaM-dependent enzymes and/or phosphatase, we examined the effects of Zn^{2+} on CaM-dependent cyclic nucleotide phosphodiesterase (PDE1) (Fig. 4A), myosin light-chain kinase (MLCK) (Fig. 4B), and alkaline phosphatase (AP) (Fig. 4C). Zn^{2+} did not affect the activity of PDE1 and MLCK; instead, it activated AP, a Zn^{2+}-dependent enzyme.

Discussion

CN plays pivotal roles in many cellular functions in a variety of cells [7–14], including immune systems and brain systems, and can be activated in vitro by divalent ions such as Mn^{2+} and Ni^{2+} [15–18]. Ni^{2+} is a potent activator for CN, binding to the enzyme extremely tightly, and may thus serve as a physiological role [16,17]. On the other hand, Zn^{2+} is essential for many cellular functions [20–26] and CN also contains a Fe^{2+}-Zn^{2+} binuclear center in its catalytic domain [3–6]. However, the roles of metal ions including Zn^{2+} in the regulation of CN activity remained to be elucidated.

In the present study, we have investigated the role of Zn^{2+} in Ni^{2+}-stimulated CN-activity in vitro and found that Zn^{2+} at a concentration range of 0.01–10 μM inhibits the activity by competing with Ni^{2+} (Figs. 1A and 2) and also that Zn^{2+} never affects CN–CaM interaction (Fig. 3). It was also shown that Zn^{2+} can inhibit CN activity in the absence of Ni^{2+} (Fig. 1B). While both Zn^{2+} and Fe^{2+} are essential elements of the catalytic domain in CN-A, Zn^{2+} behaves as an inhibitor for CN, but Fe^{2+} does not (data not shown). It is of importance to note here that the effective concentrations of Zn^{2+} are under physiological concentrations of the ion (e.g., approximately 10–15 μM in adult human plasma) [31,32]. Therefore, although Zn^{2+} is needed for CN activity and thus for the immune system [33–35], it is possible that cells control (inhibit) CN activity in vivo by regulating cytosolic Zn^{2+} concentration.

Acknowledgment

This work was supported in part by grants (K.H. and Y.K.) from the Ministry of Education, Science, Sports and Culture of Japan.

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