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Effect of metal ions on the activity of the catalytic domain of calcineurin

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Abstract

Calcineurin (CN) is a heterodimer, composed of a catalytic subunit (CNA) and a regulatory subunit (CNB). There are four functional domains present in CNA, which are catalytic domain (CNa), CNB-binding domain (BBH), CaM-binding domain (CBH) and autoinhibitory domain (AI). It has been shown previously that the *in vitro* activity of calcineurin is relied primarily on the binding of metal ions. Mn^{2+} and Ni^{2+} are the most crucial cation-activators for this enzyme. In order to determine which domain(s) in CN is functionally regulated by metal ions, the rat CNA α subunit and its catalytic domain (CNa) were cloned and expressed in *E. coli*. The effects of Mn^{2+}, Ni^{2+} and Mg^{2+} on the catalytic activity of these purified proteins were examined. Our results demonstrate that all the metal ions tested in this study activated either CNA or CNa. However, the activation degree of CNa by the metal ions was much higher than that of CNA. In term of different metal ions, the activating extents to CNA and CNa were different. To CNA, the activating order from high to low was $Mg^{2+}>>Ni^{2+}>Mn^{2+}$, but $Mn^{2+}>Ni^{2+}>>Mg^{2+}$ to CNa. No effect of CaM/Ca^{2+} and CNB/Ca^{2+} on the activity of CNa was observed in our experiments. Moreover, a weak interaction (or untight coordination binding) between metal ions and the enzyme molecule was also identified. These results suggest that the activation of these enzymes by the exogenous metal ions might be via both regulating fragment of CNA (including BBH, CBH and AI) and catalytic domain (CNa), and mainly via regulating fragment to CNA and mainly via catalytic domain to CNa. The activating extents of metal ions via catalytic domain were higher than that via regulating fragment. The results obtained in this study should be very useful for understanding the molecular mechanism underlying the interaction between calcineurin and metal ions, especially Mn^{2+}, Ni^{2+} and Mg^{2+}.

Abbreviations: CaM – calmodulin, CN – calcineurin; CNA – calcineurin A subunit; pNPP – p-Nitrophenyl phosphate

Introduction

Calcineurin (CN) was a phosphatase ubiquitously expressed in human tissues, with the highest level in brain and immune system (Rusnak *et al*. 2000). CN is multifunctional in cell. The function of the protein is mainly exerted by regulating protein dephosphorylation. Abnormal levels of calcineurin in the cell cause many diseases, such as retentivity decline (Thomas *et al.* 2001), Alzhimer disease (Landner *et al.* 1996), cardiac hypertrophy (Valerie *et al.* 2002), and lesions in immune system (Leslic *et al.* 2001).

Calcineurin is a 80 Kda protein, with a holoenzyme structure of a 1:1 ratio of calcineurin A (CNA, 61 Kda) and calcineurin B (CNB, 19 Kda) by a non-covalent bond. The protein has an isoelectric point of 4.5 (Klee *et al.* 1983). CNA is composed of catalytic domain (CNa), CNB-binding domain (BBH) (Sikkink *et al.* 1995), CaM-binding domain (CBH) and autoinhibitory domain (Hubbard *et al*. 1989). Both CN and CNA can be bound and activated by calmodulin. CNB bound by Calcium binds to the CNB-binding domain of CNA and regulates the activity of CNA (Rusnak *et al*. 2000).

CN is a metalloproteophosphatase that can bind Zn and Fe. The binding of CN to metal ions plays a central role in the activation of this enzyme, which is believed to participate in many dephosphorylation processes (Rusnak *et al.* 2000). The *in vitro* activation of natural CN, recombinant CNA and CNa all depends on the exogenous metal ions, such as Mn^{2+}, Ni^{2+}, VO^{2+}, and so on, of which Mn^{2+} and Ni^{2+} are the most important activators to CN, CNA and CNa. The high concentration of Mn^{2+} in brain is related to the highest level of calcineurin in brain. Based upon their effects on phosphatase activity, five categories of the cations have been classified (Pallen *et al.*, 1984). The first category includes the transition metal ions, including Ni^{2+}, Mn^{2+} and Co^{2+}, which are the best activators of calcineurin. The second category is Mg^{2+}, which is a very good activator of calcineurin under alkaline condition (pH 8.6). However, several features of Mg^{2+} activation differ sufficiently from the transition-metal-ion activation. The third category includes Ca^{2+}, Ba^{2+} and Sr^{2+}, which have little effect on calcineurin in the presence of calmodulin but less or no detectable effect in the absence of calmodulin (Li *et al.* 1984). The fourth category is composed of the cations that do not stimulate calcineurin activity in the presence or absence of calmodulin. These cations include Al^{3+}, Be^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+} (Chernoff *et al.* 1984). The fifth category includes Zn^{2+} and Cd^{2+}, which have been reported to inhibit Ca^{2+}/calmodulin-, Ni^{2+}-, and Mn^{2+}-stimulated activation of the phosphatase. Mn^{2+} and Ni^{2+} are the best activators of calcineurin and thus have been studied extensively. Pallen et al (Pallen *et al.* 1986) have demonstrated by direct metal ion binding studies that Mn^{2+} and Ni^{2+} regulated the calcineurin activity *via* binding a high affinity Mn^{2+}-binding site or a distinct Ni^{2+}-binding site on CN. Other studies have showed that the metal ions could selectively affect the interaction between enzyme and substrate by having an effect upon the conformation changes from native state to hydrolysis state of enzyme-ion-substrate complex (Martin *et al.* 1999). The studies of the effect of ions on calcineurin by replacing ions complex $Co(NH_3)_6{}^{3+}$ with $Mn(H_2O)_6{}^{2+}$ have showed that metal ions activate calcineurin through an external encirclement coordination mechanism (Martin *et al.* 1999). In the current study, we cloned and expressed the catalytic subunit (CNA) of calcineurin and the catalytic domain (CNa) of CNA. Assays of their enzyme activity show that the activity of CNa is ten- to twenty-fold higher than that of CN and/or CNA. Moreover, the activity of CNa is not regulated by CaM and CNB but cation-dependent with some exogenous metal ions stronger than CN and CNA. In addition, the extent of Mn^{2+}, Ni^{2+} and Mg^{2+} activating CN, CNA and CNa is different. The activating order for CN is $Mg^{2+}>>Ni^{2+}>Mn^{2+}$ (Pallen *et al.* 1984), $Mg^{2+}>>Ni^{2+}>Mn^{2+}$ for CNA, and $Mn^{2+}>Ni^{2+}>>Mg^{2+}$ for CNa. Calcium is important for the activity of CN and CNA but has no effect on the activity of CNa. The results of pre-incubating Mn^{2+} with CNa showed that the coordinating bind between exogenous metal ions, such as Mn^{2+} and Ni^{2+}, and the enzyme molecule was not as stable as the bind between endogenous metal ion Fe^{2+}/Zn^{2+} and the enzyme molecule. The activation of the enzyme by Mn^{2+} and Ni^{2+} was not exerted by replacing the endogenous metal ions, but by slightly changing the conformation of the regulating fragment and catalytic fragment.

Materials and methods

Bacterial strains, culture medium and vector

The strains HMS174(λ DE3) and BL21(λ DE3) were conserved by our laboratory. They were all precultured in the LB medium and then expressed in the TM medium induced by IPTG (16 uM to HMS174(λ DE3) and 100 uM to BL21(λDE3)). The vector pET-21a(+) was also conserved by our laboratory. The rat cDNA library of CNA-α (gifted by Dr Perrino, Vollum Institute, Portland, Oregon)

Vector construction

According to the sequence of cDNA of CNA, the primers of CNA and CNa were designed respectively as below:

CNA:
5′-AGGAGATATACATATGTCCGAGCCCAAGGC
3′-CGCGAAGCTTTCACTGAATATTGCT
CNa:
5′-AGGAGATATACATATGTCCGAGCCCAAGGC
3′-CGCGAAGCTTCACATGAAATTTGGGAGCC

Then by using PCR and molecular cloning methods from the CNA-α cDNA library, we construct the expressing vector pET21a/CNA and pET21a/CNa.

Preparation of enzyme

CNA: Expression of CNA was previously described (Wei *et al.* 1997). It's modified and briefly as below: the cell *E. coli* HMS174 (DE3) containing expressing vector was grown at 37 °C in terrific media in 1.5 l cultures (OD_{600} was about 0.6∼0.8) and then induced with 16 μM IPTG followed by growth for an additional 5 h at 37 °C. The cells were harvested and resuspended in buffer A (20 mM Mops, 1 mM EGTA, 1% β-ME, 0.4 mM PMSF, pH 7.6). The cells were disrupted by supersonic. The lysate was then centrifuged at 48 000 × g for 30 min at 4 °C. Adding ammonium sulfate to 45% saturation precipitated the supernatant. After centrifugation at 48 000 g for 20 min at 4 °C, the pellets were resuspended in buffer B (20 mM Mops, 1% β-ME,0.4 mM PMSF, 0.5 mM $CaCl_2$, pH 7.4), and then mixed with CaM-sephorose 4B that was pre-equilibrated with buffer B (adding $CaCl_2$ to 5 mM). The mixture was gently mixed by rotation for 1 h at 4 °C and then poured into a column, washed with the equilibration buffer. The aim protein was eluted with buffer C (10 mM Mops, 1 mM EGTA, 1%β-ME, 0.4 mM PMSF, pH 7.4).

CNa: The expressing cells were harvested by centrifugation at 4000 g, at 4 °C for 20 min and resuspended in homogenizing buffer (50 mM Mops, 100 mM NaCl, 25 mM sucrose, 2 mM EDTA, 2 mM EGTA, 5% Glycerol, 1% β-ME, 0.4 mM PMSF, pH 7.4), and disrupted by supersonic. The lysate was then centrifuged at 48,000 g for 30 min at 4 °C. The supernatant was precipitated by addition of ammonium sulfate to 45% saturation. After centrifugation for 20 min (48,000 g, 4 °C), the pellets were resuspended in buffer D (20 mM Mops, 20 mM NaCl, 2 mM EDTA, 5% Glycerol, 1% β-ME, 0.4 mM PMSF, pH 7.4) and then desalted in SephadexG-25 column with buffer D. The fractions were applied to DEAE column pre-equilibrated with buffer D. Then the column was gradient eluted with buffer E (D in addition to 2 M NaCl). The active fractions were collected and concentrated with 45% ammonium sulfate. The precipitate was resuspended in butter F (20 mM Mops, 50 mM NaCl, 0.2 mE EDTA, 1% β-ME, 0.4 mM PMSF, pH 7.0) and then applied to a superdex-75 column, eluted with buffer F. The active fractions were collected.

Enzyme assay

The activity of the CNA subunit was assayed using p-nitrophenyl phosphate (pNPP) as the substrate. The enzyme CNA solution (20 μl, containing about 1.16 mg/ml CNA) was mixed with 180 μl assaying buffer (20 mM pNPP, 50 mM Tris-HCl, 0.2 mg/ml BSA, 1 mM $CaCl_2$, 2 μM CaM, 2 μM CNB, 1 mM DTT and exogenous metal ions, such as Mn^{2+}, Ni^{2+}, or Mg^{2+}, pH 7.4) in 30 °C bath for 20 min. The activity of the CNa was assayed also using pNPP as the substrate. The enzyme CNa solution was 10 μl (containing about 0.150 mg/ml CNa) and added 180 μl assaying buffer (20 mM pNPP, 50 mM Tris-HCl, 0.2 mg/ml BSA and exogenous metal ions as above, pH 7.4) and additional 10 μl ddH_2O to the reaction volume 200 μl. The reaction was terminated by the addition of 1.8 ml terminus buffer (0.5 M Na_2CO_3, 20 mM EDTA) and the absorbance at 410 nm was measured using a lacking-enzyme control.

Effect of metal ions on CNA and CNa

Assays of CNA or CNa activity include different concentration of metal ion in assaying buffer (Mg^{2+} in assaying buffer at pH 8.6).

Activity assay of CNa after pre-incubating with Mn^{2+}.

The CNa solution was pre-incubated with different concentration of Mn^{2+} and then the activity of CNa is assayed with three kinds of assaying buffer, without Mn^{2+}, with 1 mM Mn^{2+}, and with different concentration of EGTA, respectively.

Results

Expression and purification of aim proteins

The coding sequences for CNA and CNa were amplified by PCR and cloned into pET-21a(+) vector. The expression vectors pET-21a (+)/CNA and pET-21a(+)/CNa were confirmed by restriction enzyme digestion and sequence analysis (Figure 1A and data not shown). The target proteins, CNA and CNa, were expressed in *E. coli* strain BL-21(λ DE3). The purified proteins were assayed by SDS-PAGE (Figure 1B).

Effect of Ca^{2+} on CNa

It has been shown previously that the concentrations of Ca^{2+} are very important for the activation of CNA in the presence of CaM and CNB. Surprisingly almost no effect of Ca^{2+} on the activity of CNa was detected in the high concentration of Ca^{2+} (10 mM) (Figure 2).

As shown in Figure 2, the activity of CNa was not changed obviously with the increasing of [Ca^{2+}]. Our

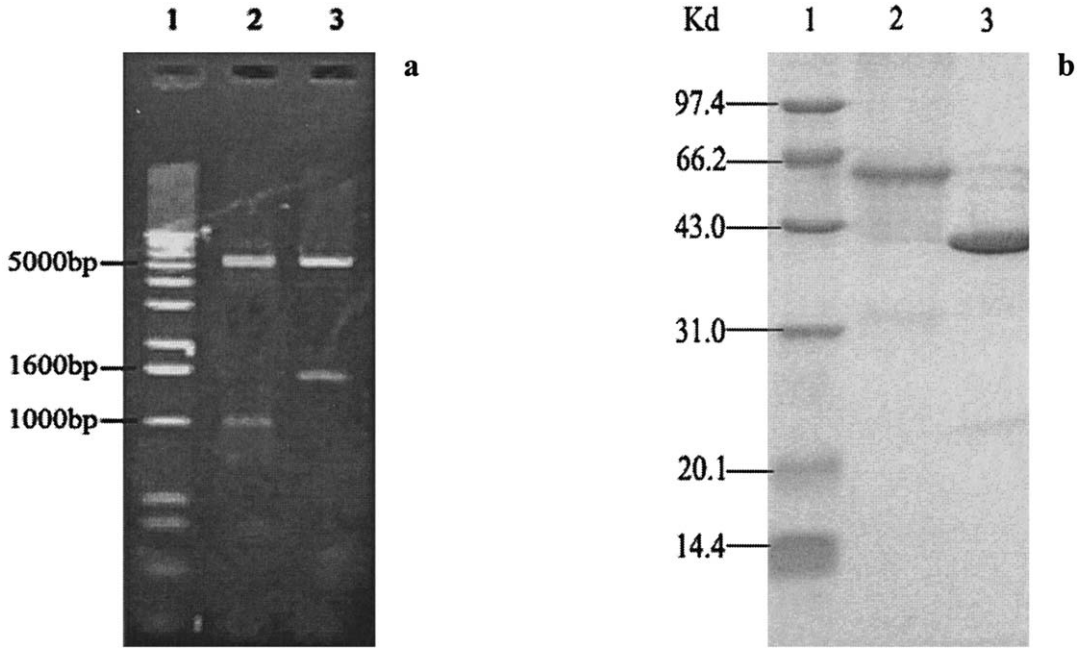

Fig. 1. (a) cDNA of CNA and CNa. lane1 DNA Marker; lane2 Vector and cDNA of CNa; lane3 Vector and cDNA of CNA. (b) Purified CNA and CNa. lane1 Protein Marker; lane2 Purified CNA; lane3 Purified CNa.

further analysis indicates that the activity of CNa is not regulated by CaM and CNB (data not shown).

Effect of Mn^{2+}, Ni^{2+} and Mg^{2+} on CNA

The effects of Mn^{2+}, Ni^{2+} and Mg^{2+} on CNA were illustrated in Figure 3. As being seen in this Figure, the effect of Mn^{2+} was higher than Ni^{2+} in stimulating the CNA activity with low concentrations. However, the maximum activating degree (355 U/mg) of CNA by Mn^{2+} (about 1∼5 mM) was lower than that (386 U/mg) by Ni^{2+} (∼5 mM). The effect of Mg^{2+} is much higher than Mn^{2+} and Ni^{2+} in the maximum activating degree while assayed at pH 8.6. The activity of CNA was peaked at 684 U/mg with 10 mM Mg^{2+}. When the concentrations of Mn^{2+} and Ni^{2+} exceeded those required for the maximum activation of CNA, such as Mn^{2+} 1∼5 mM and Ni^{2+} ∼5 mM, the CNA activity was gradually decreased. However, the CNA activity was further increased by Mg^{2+} even at 10 mM (the maximal activating concentration of Mg^{2+} is about 80 mM, data not shown).

Thus, the order of maximum extent of metal ions in activating CNA enzyme activity is $Mg^{2+}>>Ni^{2+}>Mn^{2+}$. This result was consistent with that of CN (Pallen *et al.* 1984). According to the highest sensitivity of enzyme to Mn^{2+} in the lower concentration, we guess that Mn^{2+} may be the best metal ion activator of calcineurin *in vivo*.

Effect of Mn^{2+}, Ni^{2+} and Mg^{2+} on CNa

The concentration of Mn^{2+} (about 5 mM) with maximum activation of CNa was lower than that of Ni^{2+} (about 40 mM) and the maximum activation concentration of Mg^{2+} exceeded 10 mM (about 80 mM). Ni^{2+} was about 75% as effective as Mn^{2+} in maximum stimulation of CNa. The maximum activation of Mg^{2+} to CNa was much lower than Mn^{2+} and Ni^{2+}. Mn^{2+} could stimulate the activity of CNa from 0 to 6613 U/mg, and Ni^{2+} from 0 to 5836 U/mg (40 mM). However, the activity of CNa only reached 1645 U/mg with 10 mM Mg^{2+} (only about 3716 U/mg even if at 80 mM). In addition, the extents of Mn^{2+} and Ni^{2+} and Mg^{2+} in stimulating CNa activity were all extremely higher than that of CNA (Figures 3 and 4). The activating extent of Mn^{2+} to CNa was about 18.6-fold to CNA, Ni^{2+} about 15.1-fold, and Mg^{2+} about 2.4-fold at 10 mM (2.8-fold at 80 mM).

Therefore, the extent of metal ions to CNa enzyme activity was $Mn^{2+}>Ni^{2+}>>Mg^{2+}$ (Figure 4). The extent of exogenous metal ions in activating CNa was greatly higher than that in activating CNA and CN.

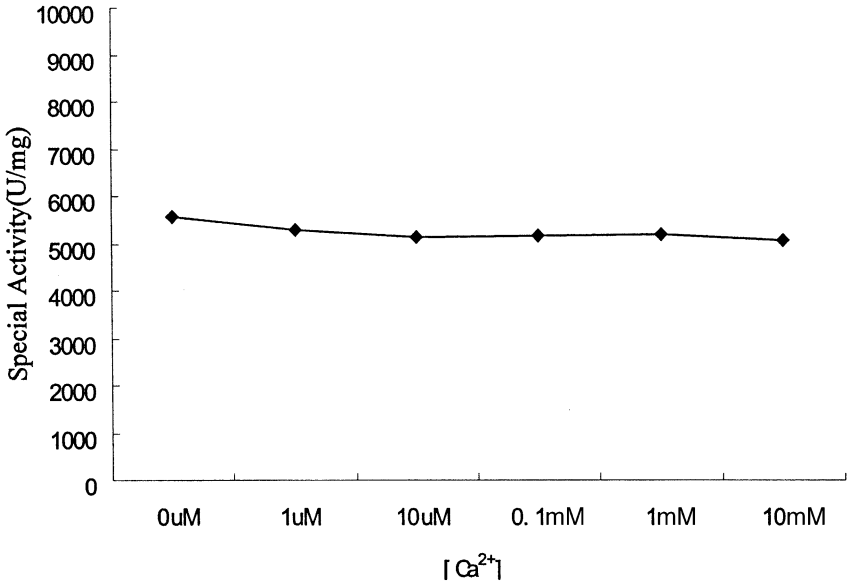

Fig. 2. The effect of $[Ca^{2+}]$ on enzyme CNa. Note: 1 U/mg = 1 nmol mg^{-1} min^{-1}; the same hereinafter.

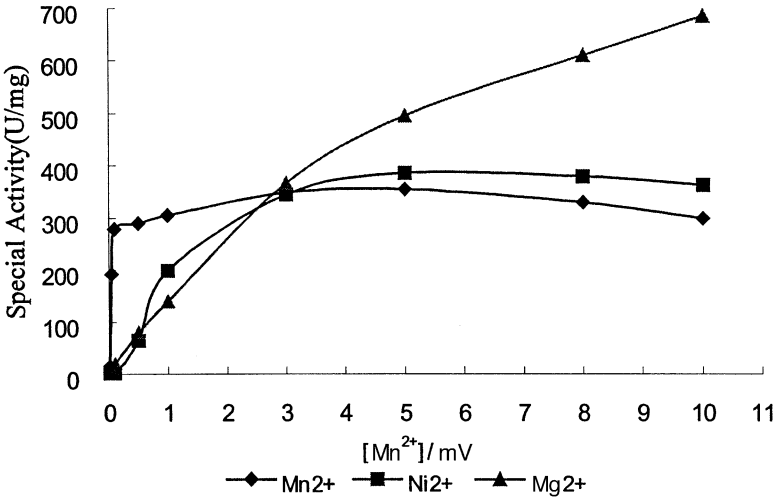

Fig. 3. Effects of Mn^{2+}, Ni^{2+}, and Mg^{2+} on CNA activity.

Table 1. The values of special activities assayed with assaying buffer containing 1 mM Mn^{2+} in different terminus time.

	1 min	5 min	10 min	15 min	20 min	25 min	30 min
0.5 mM	520	2803	4755	6095	7973	9595	10728
1.0 mM	639	2966	4735	6639	8153	9350	10636
2.0 mM	582	3034	5037	6735	8156	9323	10503
3.0 mM	629	2894	4891	6711	7888	9605	10344

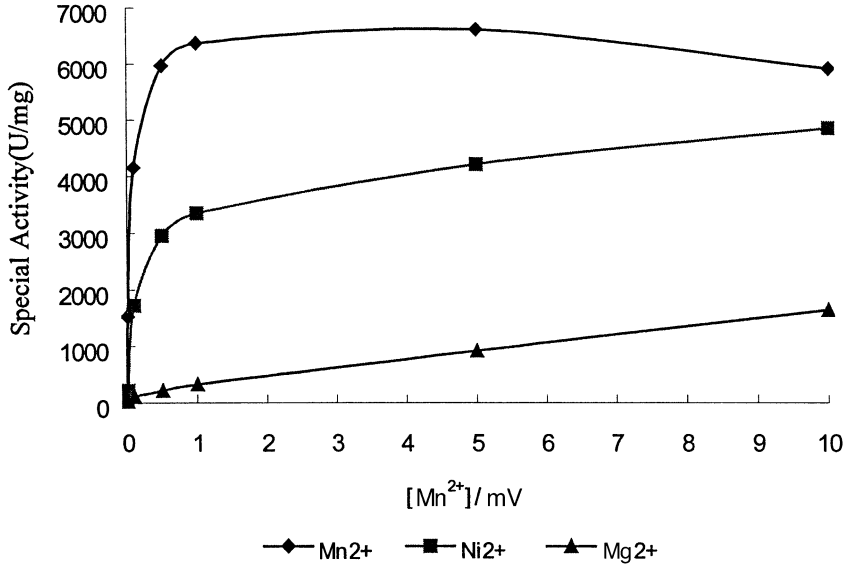

Fig. 4. Effects of Mn^{2+}, Ni^{2+}, and Mg^{2+} on CNa activity.

Assay of CNa incubated with Mn^{2+}

After pre-incubated with different concentrations of Mn^{2+} for sufficient time, the enzyme activity of CNa was assayed with three different kinds of assaying buffers containing 1 mM Mn^{2+}, Mn^{2+} free, and different concentrations of EGTA. Results are shown in Figure 5 and Table 1.

As can be seen in Figure 5A, the CNa activity was not affected by the pre-incubating time (0 min, 10 min, 20 min, 30 min in Figure 5a), but strongly associated with the concentrations of Mn^{2+} (1 mM, 2 mM, 3 mM, 5 mM, 10 mM, 20 mM and 50 mM in Figure 5a). These results suggest that the interaction between metal ion and enzyme molecule was an instant process.

The CNa activity decreased with the increased concentrations of EGTA in the assay buffer (Figure 5B). This result demonstrated that Mn^{2+} had a higher affinity for EGTA than that for the enzyme molecule and CNa cannot be activated by the EGTA-compound Mn^{2+}.

The result shown in Table 1 was the activity assay of CNa pre-incubated with various concentrations of Mn^{2+} (0.5 mM, 1.0 mM, 2.0 mM and 3.0 mM) and terminated at the different time point. The assaying buffer contained 1 mM Mn^{2+}.

As indicated in the Table, the activity of CNa was not related to the concentrations of Mn^{2+} pre-incubated with CNa and to the reacting system. These findings suggest that Mn^{2+} bound to the enzyme through a weak coordination bond.

Furthermore, compared to the CNa activity when pre-incubated with Mn^{2+} in the assay buffer, the activating extent of CNa pre-incubated with Mn^{2+} was much lower than that with Mn^{2+} in the assay buffer where the concentration of Mn^{2+} was converted into the final concentration of Mn^{2+} in the 0.2 ml reaction system (Figure 6). This result indicates that dilution of Mn^{2+} (to pre-incubated condition) could decrease the activation of Mn^{2+} suggesting that Mn^{2+} loosely bound the activating sites of enzyme molecule.

Discussion

The effect of metal ions on the activity of calcineurin is a dynamic area that has been studied extensively (Rusnak *et al.* 2000). The molecular mechanism of the interaction between Mn^{2+} or Ni^{2+} and calcineurin is not clear because of its complexity. Martin and Jurado used the metal ion Tb^{3+} as a possible probe to research the interaction between Mn^{2+} and calcineurin. They thought Mn^{2+} may act with the active-site of enzyme. It was reported that subunit A purified from bovine brain calcineurin with denatured and renatured methods was activited with Mn^{2+}; Co^{2+} and Ni^{2+} partially substituted for Mn^{2+}; but Ca^{2+}, Mg^{2+} and Zn^{2+} were ineffective (Merat *et al.* 1984). But in our experiments, we expressed and purified the key fragment-catalytic

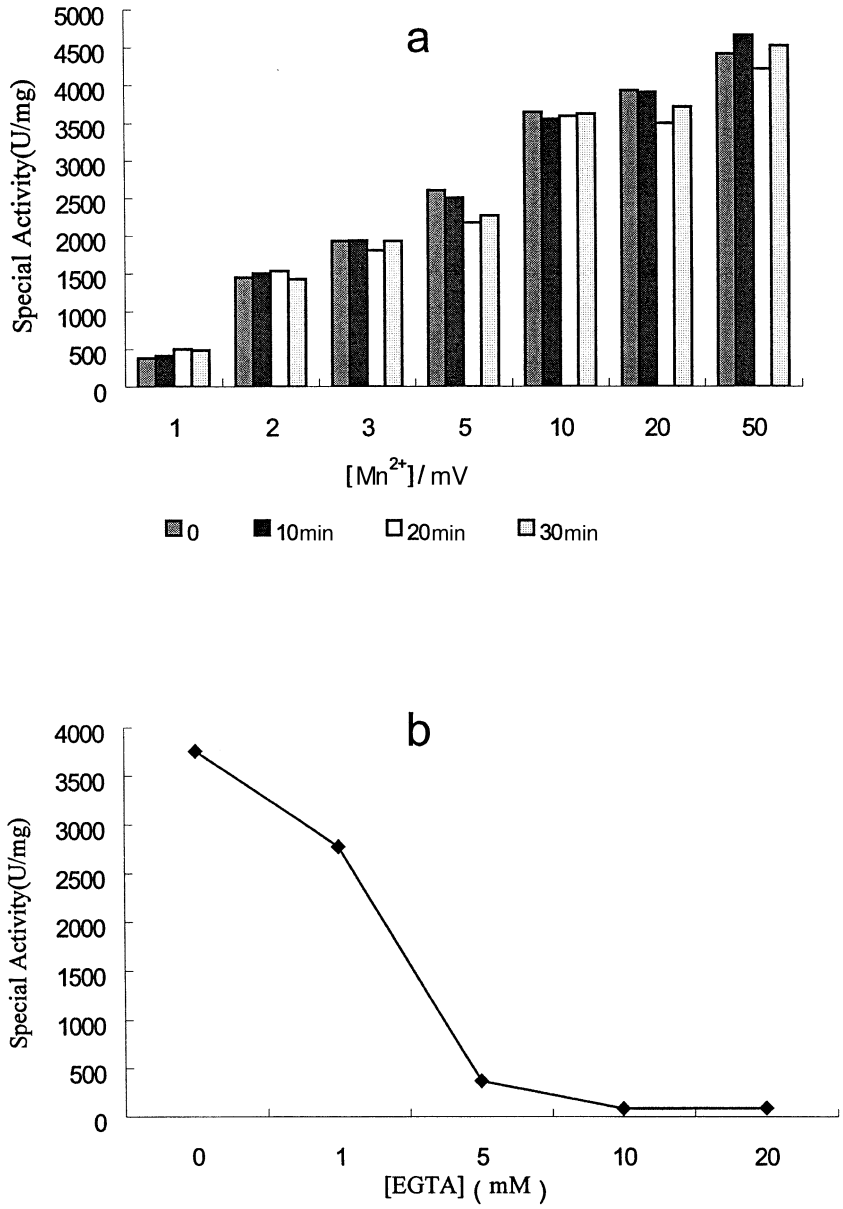

Fig. 5. Activity assay of CNa after pre-incubating with different concentration of Mn^{2+} in different assaying buffer. (a) Activation assay of CNa which were incubated with different concentrations Mn^{2+} by Mn^{2+}-free assay buffer. (b) Activation assay of CNa which was incubated with 20 mM Mn^{2+} by assay buffer contained different concentrations of EGTA.

domain of CNA and could directly study the effects of metal ions on its activity. From the results of our experiments, some conclusions and hypotheses that metal ions affect on calcineurin activity were obtained by several studies that measured the calcineurin A subunit activity and catalytic domain activity of A subunit. Our results demonstrated that the exogenous metal ions do not only truly activate the CNA and CNa, but they all act with the active-site of enzyme. The results also indicate that the effect of Ca^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+} on the activity of CNa is different from that on CNA.

A current model is that Ca^{2+} has little or no stimulation of the activity of calcineurin in the absence of calmodulin. In the presence of calmodulin, the stimulatory effect of Ca^{2+} on the activation of the

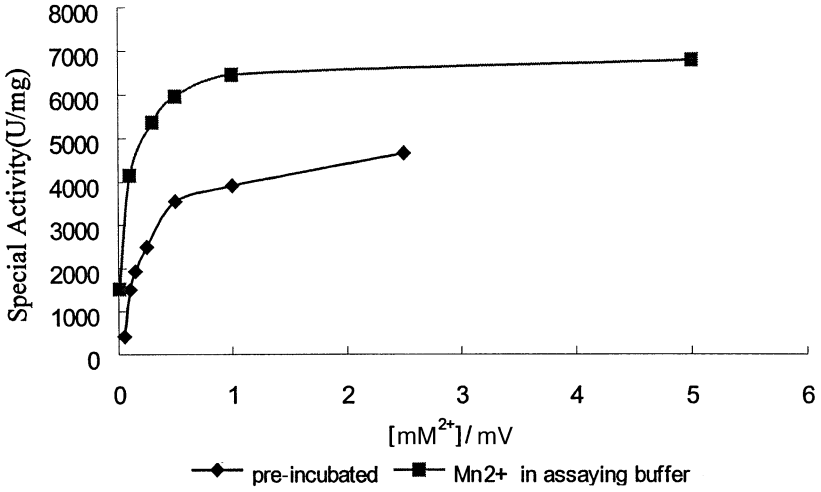

Fig. 6. the activating cases of Mn^{2+} in pre-incubated with enzyme and in assay buffer when all converting into final concentration of Mn^{2+} in reaction system.

phosphatase has been reported previously, which is on the average eight- to ten-fold lower than the transition metal ion-stimulated activity (Li *et al.* 1984; Pallen *et al.* 1984). A conclusion has been made that Ca^{2+} exerts its effects on activity of calcineurin in two ways: (1) *via* its interaction with calmodulin, and (2) *via* its interaction with CNA and CNB. Following the binding of Ca^{2+}, conformation of CNB is altered, which allows CNB to bind to BBH of CNA and regulate the activity of the whole enzyme. Our data indicate that no effect of Ca^{2+} on the activity of CNa even in the presence of CaM and CNB. Thus, this finding suggests that the effect of Ca^{2+} on the CN is not mediated through the catalytic domain (it's not different with the apparent loss of Ca^{2+} sensitivity of the bovine brain enzyme in molecular mechanism. It's due to that CN could be degraded in the purification process).

The molecular mechanism of Mn^{2+} and Ni^{2+}-mediated activation of calcineurin is not as clear as that of Ca^{2+} although it is commonly accepted that the enzyme activity is Mn^{2+} or Ni^{2+} -dependent. Previous studies showed that the interactions of Mn^{2+} and Ni^{2+} with whole enzyme-calcineurin molecules existed (Pallen *et al.* 1984; Rusnak *et al.* 2000). However, which domain was the activating interaction surface of metal ions? From our results, we found that metal ions not only interacted with catalytic subunit A of CN, but also especially interacted with catalytic domain (CNa). As suggested previously by others (Pallen & Wang 1986), we propose that the high affinity metal-ion binding site should be in catalytic domain if it existed. In our opinion, the activating signal could transfer to catalytic center via regulating fragment (including BBH, CBH and AI domains) in CNA and in CN. But in CNa, metal ions directly acted with catalytic domain and activated CNa greatly.

The activating extent of metal ions – Mn^{2+}, Ni^{2+}, and Mg^{2+} – to enzyme CNA and CNa was different, and that was much higher to CNa than that to CNA. This difference was resulted from the absence or presence of regulating fragment of CNA. Thus, it's obvious that the regulatory fragment plays a crucial role in activating enzyme by metal ions. In the presence of regulatory fragment, metal ions activate enzyme via this fragment. The activating signal of metal ions was transferred to catalytic site by the regulatory fragment. But in CNa, metal ions directly acted with catalytic domain and activated the enzyme. So the activating extent of this kind of activation was much stronger than that of former indirect manner. In addition, there were some stronger activating sites of metal ions exposed when cutting the regulating fragment and resulted in our experiment results (Figures 3 and 4).

Mg^{2+}-stimulated activities of CN (Li *et al.* 1984) and CNA were obviously higher than those by Mn^{2+}- and Ni^{2+}-stimulated. In our experiment, Mg^{2+}-stimulated CNa activity was much lower than Mn^{2+}- and Ni^{2+}-stimulated activity, that is, $Mg^{2+} >> Ni^{2+} > Mn^{2+}$ in CNA and $Mn^{2+} > Ni^{2+} >> Mg^{2+}$ in CNa. The results demonstrated that the molecular mechanism of these metal ions in activating enzyme was different from each other. Magnesium is a primary element and manganese and nickel are all transient elements. The preferential atoms coordinated

with the three metal ions and their formed coordinating compounds were quite different. The steric structures of these compounds were also different. Therefore, the different changes in space conformation of enzyme (such as CN/CNA and CNa) would occur after these metal ions bound to enzyme molecule by coordination bonds. The different dimensional changes in enzyme conformation may result in the different activating extents by the three metal ions.

In pre-incubating experiments, the activation of CNa is measured in the assay buffer with 1 mM Mn^{2+}, without Mn^{2+} or with EGTA after the CNa has incubated with different concentrations of Mn^{2+} including 1 mM, 2 mM, 3 mM, 5 mM, 10 mM, 20 mM and 50 mM. As shown in Figures 5 and 6, the CNa activity was determined by the final concentration of Mn^{2+} in the assaying mixture and no relationship with the pre-incubating Mn^{2+} concentration and the incubation time. Dilution and EGTA could all decrease the activating extent of Mn^{2+} on the CNa although the lowest Mn^{2+} concentration (1 mM) in incubation solution could get to 125-fold of enzyme concentration (0.008 mM). These findings indicate that binding of Mn^{2+} with CNa was a kind of loose coordination and undone by dilution and EGTA.

In summary, our data suggest that the binding of metal ions to CN/CNA and CNa was directly responsible for enzyme activation. When metal ions bound to these specific regions in these proteins, according to the crystal structure of CN (Goldberg *et al.* 1995; Griffith *et al.* 1995), the conformations of enzyme molecule change slightly and result in the steric hindrance which prevented substrate accessing catalytic center reducing and enzyme activity increasing. Here we designed and primarily studied the effects of metal ions on the catalytic domain. All these results should be efficacious for further studies on the detailed molecular mechanism of interaction between metal ion and enzyme molecule.

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