Intrinsic phosphatase activity of bovine brain calcineurin requires a tightly bound trace metal

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The divalent metal requirement of intrinsic phosphatase activity was investigated using native and trypsinized calcineurin. This was assessed by examining (1) the stimulation of the enzyme by various metals, (2) the inhibition of the enzyme activity by metal chelators (EDTA and EGTA), and (3) the restoration by various metals of the activity of the EDTA-inhibited calcineurin phosphatase. The results supported the view that a tightly bound trace metal is necessary for expression of the phosphatase activity of calcineurin and implicate Mn^{2+} as the tightly bound metal.

Calcineurin *Phosphatase* *Divalent cation*

1. INTRODUCTION

Calcineurin [1] was discovered by authors in [2] as an inhibitor of calmodulin-stimulated cyclic nucleotide phosphodiesterase. Subsequently, authors in [1] showed that it consists of two subunits (A, M_r 61 000; B, M_r 15 000) and those in [3,4] showed that it possesses a protein phosphatase activity, which is stimulated by calmodulin in the presence of Ca^{2+}. It is further shown that calmodulin binds to the A subunit whereas Ca^{2+} binds to the B subunit of the holoenzyme [1,5]. The A subunit appears to contain phosphatase activity, although the role of the B subunit in the regulation of phosphatase activity of the holoenzyme remains unclear [6,7].

The effects of various divalent metals on calcineurin phosphatase activity show differences depending on whether phosphatase is measured in the presence of calmodulin. In the absence of calmodulin, authors in [8] reported the highest phosphatase activity (with phosphotyrosine as substrate) in the presence of Mn^{2+} followed by Co^{2+} and Ca^{2+}. Authors in [9] assayed calcineurin phosphatase (with histone H_1 as substrate) with calmodulin and found Ni^{2+} to be the most potent activator. Authors in [3] have reported that Ca^{2+} supports calcineurin in phosphatase activity.

We have investigated the metal requirement of intrinsic phosphatase activity (e.g., activity in the absence of calmodulin and divalent metals) of bovine brain calcineurin by studying (1) the stimulation of phosphatase activity by divalent metals, (2) the inhibition of phosphatase by the divalent metal chelators, EDTA and EGTA, and (3) the restoration of the activity of 'chelator inhibited calcineurin' by divalent metals. In our work, we took advantage of the finding that controlled tryptic proteolysis of calcineurin markedly augmented the intrinsic phosphatase activity, which was calmodulin-insensitive. The results suggested that (1) calcineurin is a metalloprotein and its tightly bound metal is essential for phosphatase activity, (2) the metallic region of calcineurin is buried with its structure and thus is poorly accessible to metal chelators, (3) the metallic region is exposed following proteolysis such that EDTA chelates the tightly bound metal and inhibits phosphatase activity, and (4) the tightly bound metal is a trace metal likely to be Mn^{2+}.

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2. MATERIALS AND METHODS

2.1. Purification of calcineurin

Calcineurin was purified from bovine cerebral cortex extracts by sequential chromatography using Affigel blue, calmodulin affinity and DEAE–Sepharose or Sephacryl S-200 (in preparation). SDS-polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the preparation revealed that calcineurin comprised about 85% of the protein applied. The remainder (~10%) was accounted for by a protein of M_r 71 000; this protein must be a calmodulin-binding protein of unknown identity or function. Calcineurin showed equimolar amounts of the subunit A (M_r 61 000–63 000) and subunit B (M_r 15 000–17 000). The phosphatase activity ($\pm Ca^{2+}$–calmodulin) of our preparation was essentially similar (with myosin light chain as substrate) to that in [5]. Routinely, we used ^{32}P-labelled myelin basic protein (see below) in the phosphatase assay, since amongst the several protein substrates tested, it gave maximal phosphatase activity (in preparation).

2.2. Tryptic exposure to calcineurin

Calcineurin (100 μg/ml) was exposed to trypsin (0.2 μg/ml) for 20 min at 30°C in a medium containing 50 mM Tris–Cl (pH 7.4). The proteolysis was stopped by adding a 10-fold excess of trypsin inhibitor. Calcineurin incubated without trypsin under the same condition served as control. SDS–PAGE analysis revealed that tryptic exposure preferentially degraded the A subunit to two fragments of M_r 52 000 and 48 000; the B subunit region showed the presence of a doublet (M_r 16 000–14 000) and the M_r 71 000 protein of the preparation was not affected.

2.3. EDTA treatment

Native calcineurin or proteolyzed calcineurin (see above) were exposed to 10 mM EDTA for 20 min at 30°C. The aliquots were then assayed for intrinsic phosphatase. Additionally, following EDTA exposure, both types of calcineurins were dialyzed overnight against 4 l of Tris–Cl buffer to remove free EDTA and then assayed for intrinsic phosphatase activity.

2.4. Phosphatase assay

Intrinsic phosphatase activity is defined as the activity measured in the absence of divalent cation and calmodulin and was determined in medium containing 50 mM Tris–Cl (pH 7.0), 0.5 mM dithioerythritol, 50 mM NaCl, 50 μg bovine serum albumin, and ^{32}P-labeled myelin basic protein (equivalent to 25 or 100 pmol ^{32}P per assay). Calcineurin was added to start the reaction that was carried out for 5 min at 30°C. The phosphatase activity was linear under this condition. Various divalent metals and/or calmodulin were added to ascertain their effects on intrinsic phosphatase activity and are described in the appropriate legends. The ^{32}P released, after the reaction was stopped with 20% cold trichloracetic acid and the tubes centrifuged, was determined by liquid scintillation spectrometry. In each instance, the results shown were obtained from several (3–4) experiments carried out using the same calcineurin preparation.

2.5. Materials

Purified myelin basic protein (MBP) from porcine brain (kindly provided by Eli Lilly) was phosphorylated with [γ-^{32}P]ATP (New England Nuclear) in the presence of catalytic unit of cyclic AMP-dependent protein kinase (Sigma) and was dialyzed prior to its use in assay. Routinely, ^{32}P incorporation amounted to 0.25 mol/mol MBP. All other reagents were of analytical grade.

3. RESULTS

3.1. Effects of divalent metals on calcineurin phosphatase activity

3.1.1. In the absence of calmodulin

Intrinsic phosphatase activity was modulated differentially by various metals (each at 1 mM) (table 1). Mn^{2+} augmented (2–3-fold) phosphatase with half-maximal and maximal stimulation at 50 and 250 μM, respectively. Co^{2+} and Ni^{2+} inhibited phosphatase activity. Mg^{2+} and Ca^{2+} exerted little effect. Zn^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+} and Fe^{2+} markedly inhibited phosphatase activity (not shown).

3.1.2. In the presence of calmodulin

Calmodulin alone (without any divalent cation present) exerted little effect. Mn^{2+} and Ca^{2+} augmented phosphatase; Mg^{2+} showed no effect. Co^{2+} and Ni^{2+} inhibited phosphatase activity; this was also true for Zn^{2+}, Fe^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+}

Table 1

Divalent cation effects on phosphatase activity of calcineurin

Cation	Calmodulin	Phosphatase activity (nmol ^{32}P/min per mg) Native calcineurin	(%)	Trypsinized calcineurin	(%)
-	-	1.61	(100)	13.30	(100)
Mg^{2+}	-	1.58	(98)	12.00	(90)
Ca^{2+}	-	1.56	(97)	10.90	(82)
Mn^{2+}	-	2.94	(183)	32.45	(243)
Co^{2+}	-	0.18	(11)	0.51	(38)
Ni^{2+}	-	0.56	(35)	1.86	(7.5)
-	+	1.70	(100)	13.20	(100)
Mg^{2+}	+	1.80	(105)	13.90	(105)
Ca^{2+}	+	7.82	(460)	12.00	(91)
Mn^{2+}	+	13.28	(781)	33.20	(251)
Co^{2+}	+	0.27	(16)	0.80	(6)
Ni^{2+}	+	1.58	(93)	2.00	(15)

Phosphatase activity was determined as described in section 2. All cations were at 1 mM and calmodulin, 0.2 μg, when present

(not shown in table 1). The inhibitory effect of trace metals indicates a role of sulfhydryl groups in calcineurin phosphatase since *p*-chloromercuribenzoate also inhibits phosphatase (in preparation).

3.2. Divalent cation effects on phosphatase activity of proteolyzed calcineurin

Calcineurin was exposed to trypsin (calcineurin : trypsin = 500) for 20 min at 30°C and proteolysis was stopped by adding a 10-fold excess of trypsin inhibitor. Calcineurin incubated without trypsin served as control.

3.2.1. Phosphatase activity assayed without calmodulin

Intrinsic phosphatase activity of proteolyzed calcineurin was markedly (10-fold) higher relative to control, unproteolyzed calcineurin (table 1). Among the cations tested, only Mn^{2+} (1 mM) further augmented (2–3-fold) phosphatase activity of proteolyzed calcineurin. Mg^{2+} or Ca^{2+} showed little stimulatory effect; in fact, Ca^{2+} (1 mM) produced modest (20%) inhibition and Ni^{2+} and Co^{2+} inhibited phosphatase considerably.

3.2.2. Phosphatase activity assayed with calmodulin

In contrast to control calcineurin, phosphatase activity of proteolyzed calcineurin was poorly, if at all, stimulated by calmodulin determined in the absence and presence of Mn^{2+} or Ca^{2+}. The inhibitory effect of Ni^{2+}, Zn^{2+} and Co^{2+}, however, was present.

Table 2

Inhibition of intrinsic phosphatase activity by EDTA

	Phosphatase activity (nmol ^{32}P/min per mg) Native calcineurin	(%)	Proteolyzed calcineurin	(%)
Exp. A				
EDTA (mM)				
0	0.68	(100)	6.40	(100)
0.1	0.67	(100)	6.70	(105)
1	0.68	(100)	5.70	(89)
10	0.52	(76)	4.71	(73)
50	0.52	(76)	2.70	(42)
Exp. B				
EDTA (10 mM)				
treatment time (min)				
0	1.13	(100)	5.60	(100)
5	1.15	(101)	4.07	(73)
30	1.04	(92)	2.01	(36)
60	0.99	(88)	0.74	(13)

Native and proteolyzed calcineurin (see section 2) were assayed in the presence of varying concentrations of EDTA (exp. A) or native and proteolyzed calcineurin were exposed to 10 mM EDTA for varying times at 30°C prior to assay (exp. B).

3.3. *Effects of EDTA and EGTA on calcineurin phosphatase assayed without calmodulin*

3.3.1. Control calcineurin

A modest inhibition (up to 25%) of phosphatase was noted in the presence of higher concentrations of (10 mM or greater) EDTA or EGTA (not shown in table 2).

Table 3

Restoration of phosphatase activity of EDTA-inhibited calcineurin by divalent cations

	Cation	Phosphatase activity (nmol ^{32}P/min per mg)			
		Native calcineurin	(%)	Trypsinized calcineurin	(%)
Before EDTA treatment	–	0.27	(100)	3.36	(100)
	Mn^{2+}	0.87	(320)	8.70	(259)
	Ca^{2+}	0.25	(93)	2.80	(83)
	Mg^{2+}	0.27	(100)	3.80	(113)
	Co^{2+}	0.03	(11)	0.03	(1)
	Ni^{2+}	0.08	(30)	0.04	(1)
	Zn^{2+}	0.02	(7)	0.02	(1)
After EDTA treatment	–	0.18	(100)	0.15	(100)
	Mn^{2+}	0.97	(538)	8.20	(5460)
	Ca^{2+}	0.20	(111)	0.13	(87)
	Mg^{2+}	0.21	(116)	0.32	(213)
	Co^{2+}	0.04	(2)	0.07	(47)
	Ni^{2+}	0.04	(2)	0.08	(53)
	Zn^{2+}	0.05	(3)	0.03	(20)

Trypsinization of calcineurin was carried out as described in section 2. Control and trypsinized calcineurin were assayed immediately (before EDTA treatment) and then treated with 10 mM EDTA for 20 min at 30°C and dialyzed overnight against 4 l of 50 mM Tris–Cl buffer (pH 7.0) and assayed (after EDTA treatment). The assay medium contained 50 mM Tris–Cl (pH 7.0), 0.5 mM dithioerythritol, 50 mM NaCl, 50 μg bovine serum albumin, 0.2 μg calcineurin and 25 pmol ^{32}P-labeled myelin basic protein in a total volume of 50 μl. Divalent cations were at 1 mM as indicated. One nmol ^{32}P is equivalent to 10^6 cpm

3.3.2. Proteolyzed calcineurin

EDTA decreased (60–70%) the phosphatase activity (exp. A) in a concentration-dependent fashion. This inhibition also depended on the time of exposure of proetolyzed calcineurin to EDTA (exp. B). For example, 10 mM EDTA inhibited 90% of the phosphatase activity when treatment was carried out for 60 min at 30°C prior to assay. Incubation of proteolyzed calcineurin at 30°C for up to 60 min in buffer lacking EDTA showed no (inhibitory) effect on the phosphatase activity.

3.4. *Restoration of EDTA-inhibited phosphatase by divalent metals*

Proteolyzed calcineurin was first treated with 10 mM EDTA and then dialyzed overnight against large volumes (4 l) of Tris–Cl buffer (pH 7.0) to remove free EDTA. Control calcineurin was also treated with EDTA and subjected to dialysis. Phosphatase activities were then assayed in the presence of various metals. Note that phosphatase activity of proteolyzed calcineurin was decreased 95% following EDTA exposure (3.36 *vs* 0.15), and Mn^{2+} but not other divalent metals fully restored it to the level seen without EDTA exposure. In fact, Co^{2+}, Ni^{2+} and Zn^{2+} inhibited phosphatase activity. EDTA exposure of control calcineurin decreased phosphatase activity only modestly (~30%) and again Mn^{2+} but not other metals stimulated (i.e., fully restored) the enzyme activity; Co^{2+}, Zn^{2+} and Ni^{2+} were inhibitory.

4. DISCUSSION

The observation in [3] that calcineurin possesses protein phosphatase activity has now attracted considerable attention. It is well accepted that calcineurin phosphatase activity is stimulated by Ca^{2+}–calmodulin [3–9]. Authors in [3,5] have shown that calmodulin interacts with calcineurin A subunit and that Ca^{2+} binds to the calcineurin B subunit. Calcineurin A appears to possess catalytic (phosphatase) activity. Authors in [6] suggested that Ca^{2+} binding to the subunit B is necessary for the substrate binding to calcineurin A subunit, although their assays were carried out with Mn^{2+} rather than Ca^{2+}. In [7] it was considered that both subunits of calcineurin are necessary for optimal phosphatase activity of the holoenzyme. Mild proteolysis of the holoenzyme leads to fragmentation

of the A subunit with concomitant elevation of phosphatase activity and loss of stimulation by calmodulin [5].

The results of this study reveal additional interesting features of calcineurin phosphatase. The intrinsic phosphatase activity was markedly augmented by Mn^{2+} and only poorly, if at all, by other divalent cations. This is especially evident following controlled proetolysis of calcineurin. The facts that (a) EDTA or EGTA markedly inhibited the phosphatase of proteolyzed calcineurin and (b) Mn^{2+} but not other cations restored the activity of the EDTA inhibited calcineurin suggest that a tightly bound metal is necessary for enzyme activity and that this metal is likely to be Mn^{2+}. The modest inhibition of phosphatase activity of native calcineurin by EDTA but marked inhibition of proteolyzed calcineurin are indicative of the poor accessibility of the 'metallic region' of native calcineurin owing perhaps to being deeply buried within the structure of calcineurin. Mild proteolysis of calcineurin likely increases the accessibility of EDTA towards this metallic region such that the chelation of the tightly bound metal by EDTA markedly decreases this activity. Even in proteolyzed calcineurin, the maximal inhibitory effect of 10 mM EDTA required exposure of calcineurin to this chelator for 60 min. This also suggested that the metallic region is deeply buried in the 'hydrophobic core' of calcineurin. The lack of calmodulin stimulation of proteolyzed calcineurin suggests that the tryptic fragment is necessary for calmodulin binding to or effect on the phosphatase activity of the A subunit. The fact that the intrinsic phosphatase activity of proteolyzed calcineurin (although calmodulin-insensitive) is greater than that of the native calcineurin assayed with Ca^{2+}–calmodulin suggests that proteolysis has produced complex effects besides exposing the postulated metallic region of the holoenzyme. It seems that the catalytic reactivity is also markedly increased.

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