The Divalent Cation Dependence of Bovine Brain Calmodulin-dependent Phosphatase*

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The divalent cation dependence of a calmodulin-stimulated phosphatase from bovine brain has been characterized kinetically using phosphorylated myelin basic protein and casein as substrates. At saturating concentrations of calmodulin, dephosphorylation of both myelin basic protein and casein was catalyzed 8- to 10-fold more rapidly at saturating concentrations of Mn\textsuperscript{2+} than at saturating concentrations of Ca\textsuperscript{2+}. Half-maximal rates of dephosphorylation of both substrates occurred at either 15 mM Mn\textsuperscript{2+} or 1 mM Ca\textsuperscript{2+}, and the $K_{a}$ for each ion was not influenced appreciably by the presence of calmodulin. Half-maximal rates of dephosphorylation were observed at concentrations of calmodulin ranging from $3 \times 10^{-8}$ to $10^{-6}$ M at saturating concentrations of divalent cations depending on the substrate used and the particular cation chosen. Trypsin treatment of the phosphatase activated the enzyme several-fold, eliminated its calmodulin dependence, but did not alter the Mn\textsuperscript{2+} concentration dependence of the activity. Ca\textsuperscript{2+} (10 mM) increased dephosphorylation rates without altering the Mn\textsuperscript{2+} concentration dependence of the phosphatase activity regardless of the presence of calmodulin. Mg\textsuperscript{2+} at millimolar concentrations did not alter the Ca\textsuperscript{2+} or Mn\textsuperscript{2+} concentration dependence of the activity. As measured without calmodulin, Ca\textsuperscript{2+} (90 mM) or Mn\textsuperscript{2+} (200 mM) produced nearly identical alterations of the far ultraviolet circular dichroic spectrum of the phosphatase.

Variation of intracellular calcium ion concentration is now widely recognized to mediate alterations of cellular function in diverse systems in response to a multiplicity of stimuli (1). During the past decade a growing body of evidence has established that many of the regulatory actions of Ca\textsuperscript{2+} are mediated intracellularly by interactions of Ca\textsuperscript{2+} with a ubiquitous multifunctional regulatory protein termed calmodulin (2, 3). Examination of calmodulin-binding proteins prepared by affinity chromatographic procedures in several laboratories (4-6) led to the identification, purification, and characterization of a heat-labile inhibitor of calmodulin-stimulated cyclic nucleotide phosphodiesterase. The protein was found to be a heterodimer, composed of a Mr 61,000 calmodulin-binding A subunit and a Mr 15,000 B subunit (5). The protein was designated calcineurin because it was found at high concentrations in brain tissue and bound 4 mol of Ca\textsuperscript{2+} at micromolar concentrations (5). The affinity of the binding sites of calci

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neurin for Ca\textsuperscript{2+}, potential cooperativity among those sites, and their ability to discriminate among diverse divalent cations, however, remains unknown. Using immunological and immunohistochemical procedures calcineurin has been found to be located predominantly, but not exclusively, in brain (6, 7), at neuronal sites (8) associated with postsynaptic density (9) and structures.

Recently, Stewart et al. (11) reported the purification of a calmodulin-stimulated phosphoprotein phosphatase from rabbit skeletal muscle. The striking similarity of its subunit structure to that reported previously for calcineurin led to the recognition that bovine brain calcineurin is a calmodulin-stimulated phosphoprotein phosphatase (11, 12). The calmodulin-dependent phosphatase from rabbit skeletal muscle has been characterized in some detail kinetically (13) with respect to its in vitro substrate specificity, Ca\textsuperscript{2+}, and calmodulin dependence. The present report characterizes in detail the interaction of bovine brain calcineurin with diverse divalent cations by examining divalent cation-dependent alterations of its kinetic properties and far ultraviolet circular dichroic spectrum.

EXPERIMENTAL PROCEDURES

Materials

Tris base, soybean trypsin inhibitor, and ammonium sulfate were obtained from Sigma. Casein was purchased from Matheson, Coleman and Bell, East Rutherford, N.J. Spectrographically standardized magnesium sulfate, manganese sulfate, calcium carbonate, and potassium chloride were obtained from Johnson, Mathey Chemicals, Ltd., London, England. Nickel chloride, cobalt chloride, strontium chloride, barium chloride, zinc sulfate (all ACS reagent grade), and atomic absorption standard solutions of CaCl\textsubscript{2}, MgCl\textsubscript{2}, and MnCl\textsubscript{2} were purchased from Fisher. Trypsin (toxophenylalanyl chloromethyl ketone-treated) was obtained from Worthington; Chelex 100 and Affigel 15 were obtained from Bio-Rad; Ultrogel AcA-34 was obtained from LKB Instruments, Rockville, MD. DEAE-cellulose (DE-32) was obtained from Whatman, and phenyl-Sepharose was from Pharmacia Fine Chemicals. $\gamma$-(\textsuperscript{32}P)ATP was obtained from ICN Pharmaceuticals, Irvine, CA.

Calmodulin was prepared by chromatography on phenyl-Sepharose of heat-treated proteins obtained by isoelectric precipitation of ammonium sulfate-fractionated bovine brain extract as described by Gopalakrishna and Anderson (14). Myelin basic protein was obtained from bovine spinal cord using the phosphocellulose chromatographic procedure of Oshiro and Eylar (15). The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from bovine skeletal muscle by the procedure of Beavo et al. (16).

Preparation of Phosphorylated Substrate

Myelin basic protein or casein (10 mg/ml) in 100 mM Tris, pH 7.5, 5 mM MgCl\textsubscript{2}, 100 mM NaCl, 1 mM ATP, 0.9 mCi of $\gamma$-(\textsuperscript{32}P)ATP, and 50 units of cyclic AMP-dependent protein kinase C subunit were incubated in a final reaction volume of 5 ml for 2 h at 30 °C. Casein was dephosphorylated prior to incubation by the procedure of Reimann (17). The phosphorylated proteins were harvested by adjust-
ment to 20% trichloroacetic acid followed by centrifugation at 4 °C at 10,000 × g for 15 min. The supernatants were discarded, and the pellets were redissolved in 4 ml of 500 mM Tris, pH 7.5, 6 M urea and dialyzed against two changes in 10 mM acetic acid (1000-fold volume excess) followed by one change (1000-fold excess) of 10 mM Tris, pH 7.4, which had been freed of divalent cations by passage over a column of Chelex 100. The preparations of phosphorylated proteins were routinely found to contain less than 20 nM Ca2+ and undetectable Mn2+ by atomic absorption spectrophotometry. Myelin basic protein phosphorylated by this procedure commonly incorporated approximately 0.7 mol of phosphate/mole, while casein was apparently far less efficiently phosphorylated, incorporating only 0.02 mol of phosphate/mole.

Incubation of the phosphorylated myelin basic protein with excess calcineurin indicated that 80, 95, and 99.5% of total incorporated radioactive phosphate were released following incubations of 0.5, 4, and 19 h, respectively. Similarly, incubation of phosphorylated casein with excess calcineurin revealed that 67, 94, and 85% of total incorporated radioactive phosphate were released from substrate following incubations of 0.5, 4-, and 19-h duration, respectively. The concentrations of substrate present in the incubations as indicated in the figure and table legends represent the total protein concentration present in molar terms based upon a biuret (18) assay of protein and the published molecular weights of each protein. Since myelin basic protein (without enzyme) and phosphorylcalmodulin-APF-myelin basic protein kinase at both serine 110 and threonine 34 (19), while casein contained approximately 1 pM Ca2+ and undetectable Mn2+ based upon calibration by atomic absorption spectrophotometry. Phosphorylations less than 0.1 pM were consistently achieved as evidenced by phosphopeptide substrate are proportionately less than the total molar concentration of protein by an unspecified value.

Preparation of Divalent Cation-free Reagents

Incubations were constructed from a stock buffer of 1 M Tris, pH 7.5, that had been depleted of divalent cations by passage over a column of Chelex 100. Samples of stock buffer were routinely found to contain approximately 1 μM Ca2+ and undetectable Mn2+ based upon calibration by atomic absorption spectrophotometry. Phosphorylated myelin basic protein, phosphorylated casein, and calmodulin were freed of divalent cations by dialysis against acetic acid and Chelex-treated Tris as described above, while calcineurin was dialyzed only against Chelex-treated Tris buffer. Since these reagents were diluted approximately 40-fold during enzyme assays, Ca2+ concentrations less than 0.1 μM were consistently achieved as evidenced by calibration by atomic absorption spectrophotometry. The sources of potassium, calcium, manganese, and magnesium ions used were spectrophotometrically standardized reagents containing negligible (1 ppm) contamination with foreign divalent cations.

Phosphatase Assay Procedure

Phosphatase activity was measured as described previously by Wolff et al. (21). Standard incubations contained 25 mM Tris, pH 7.5, 100 mM KCl, and concentrations of substrate, calcineurin, calmodulin, and divalent cation as indicated in the figure and table legends. The release of 32P from phosphorylated substrates was linear with time and enzyme concentration for up to 15% release of total incorporated radioactivity. Further, examination of the time dependence of calcineurin myelin basic protein phosphatase activity with or without calmodulin at limiting or saturating concentrations of either Ca2+ or Mn2+ was found to be linear for at least 30 min in assays initiated with calcineurin as long as substrate consumption was maintained at less than 15%. Similarly, despite the substoichiometric phosphorylation of casein, the time dependence of casein dephosphorylation by calcineurin was linear with either Ca2+ or Mn2+ as the divalent cation source as long as substrate consumption was maintained at less than 15%. Accordingly, calcineurin was diluted appropriately to maintain these linear conditions. Activities are expressed as the moles of phosphate released per min·mg of added calcineurin as calculated from counts released after adjustment for the reaction blank (without enzyme) and the specific activity of the substrate. Reaction blanks with either substrate routinely contained from 0.2 to 0.5% of total added counts. The specific activity of the substrate was assumed to be identical to that of the [γ-32P]ATP utilized for their phosphorylation. The specific activity of the ATP was assessed by determining the concentration of ATP spectrophotometrically.

Circular Dichroic Spectrophotometry

Circular dichroic spectrophotometric measurements were made at 25°C on a Cary model 61 circular dichrometer. A quartz cell of 5-mm path length was used for all measurements. The instrument was calibrated with a 0.1% aqueous solution of D-10-camphorsulfonic acid. Mean residue ellipticities (θM) were calculated from the relationship θM = θM/2° M/100 IC, where θM is the observed ellipticity, M is the mean residue molecular weight (assumed to be 117), I is the cell path length in centimeters, and C is the concentration in molar. θM is measured in degree cm2/dmol. The helical content of calcineurin was estimated by the procedure of Greenfield and Fasman (22).

Samples of calcineurin for far ultraviolet CD analysis were examined at 5 mM Tris, pH 7.4, 150 mM KCl in a 5-mm cell at a 0.2 full scale setting at a protein concentration of 0.18 mg/ml.

Preparation of Calcineurin

Step 1. Homogenization and Preparation of Crude Extract—Sixteen bovine brains (6.18 kg) were obtained from a local supplier. The brains were homogenized in 3 volumes of 25 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EGTA, 0.5 mM diethiothreitol, 0.2 mM dithiopyrophosphate, and 2 μM/ml leupeptin with a Waring Blender at high speed for 1 min. The homogenate was centrifuged at 14,000 × g for 1 h to sediment nuclei and mitochondria. The supernatant was dialyzed against a 5 mM Tris, pH 7.5, 7.4, 7.0, and 6.8, which had been depleted of divalent cations by passage over a column of calmodulin-Affi-Gel15 equilibrated in Buffer A containing 1 mM NaCl, 0.2 mM diisopropyl fluorophosphate, 0.1 mM dithiothreitol, 1 μM/ml leupeptin (Buffer A) containing 0.3 mM EGTA, and dialyzed against two changes of 16 liters of dissolution buffer. The dialysate was centrifuged in a Ti 45 rotor at 100,000 × g for 1 h to clarify it for following dialysis.

Step 2. Ammonium Sulfate Fractionation—The crude extract was adjusted to 65% saturation by the addition of solid ammonium sulfate, and the suspension was allowed to settle for 30 min. The suspension was centrifuged at 14,000 × g for 40 min. The pellet was dissolved in a final volume of 1 liter of Buffer B containing 25 mM Tris, pH 7.5, 125 mM NaCl, 0.2 mM diisopropyl fluorophosphate, 0.1 mM dithiothreitol, 1 μM/ml leupeptin (Buffer B) containing 0.3 mM EGTA, and dialyzed against two changes of 16 liters of dissolution buffer. The dialysate was centrifuged in a Ti 45 rotor at 100,000 × g for 1 h to clarify it for following dialysis.

Step 3. DEAE-cellulose Chromatography—The clarified sample was applied to a 6 × 60-cm column of DEAE-cellulose equilibrated with Buffer A containing 0.3 mM EGTA. The column was developed with equilibration buffer until eluted fractions exhibited an absorbance at 280 nm not less than 0.1. These fractions when pooled were found to contain less than 1% of added calmodulin when boiled samples were assayed by recombiant activation of cyclic nucleotide phosphodies-

Step 4. Calmodulin Affinity Chromatography—Calmodulin (300 μM) from bovine brain prepared by a modification of the procedure of Gopalakrishna and Anderson (34) was eluted to 150 ml of Affi-Gel 15 in 0.1 M NaHCO3, pH 8.0, by following the manufacturer’s instructions. The calmodulin-depleted extract obtained from DEAE-cellulose chromatography was adjusted to 200-mU aliquots to contain 1 mM total added Ca2+ and was applied to a 2 × 60-cm column of Chelex-100 equilibrated with reconstitu-

Step 5. Gel Filtration on Aca-34—The lyophilized sample was reconstituted in 4 ml of 10 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM diethiothreitol, 0.05 mM EGTA, and 5 μM/ml leupeptin and was applied to a 2 × 60-cm column of Aca-34 equilibrated with reconstitu-

In an attempt to identify the possibility that forms of phosphatase

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1 The abbreviation used is: EGTA, ethylene glycol bism(3-aminoethyl ether)-N,N,N',N"-tetracetic acid.
other than calcineurin might be found among proteins eluted from immobilized calmodulin by EGTA, fractions from across the Aca-34 gel filtration elution profile were examined for phosphatase activity using four substrates phosphorylated with two different protein kinases. Calf thymus histone 2b, rabbit skeletal muscle myosin light chain, bovine myelin basic protein phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase, and phosphorylase A phosphorylated with rabbit skeletal muscle phosphorylase kinase were examined in incubations containing 1 mM MgCl2 and either 1 mM EGTA, 0.25 mM CaCl2, or 1 mM MnCl2 in the presence of 10 μg/ml calmodulin (data not shown). A single peak of H2b, myosin light chain, and myelin basic protein phosphatase was observed and corresponded in elution volume to calcineurin as revealed by stained sodium dodecyl sulfate-polyacrylamide gel electrophoregrams. No phosphorylase A phosphatase activity was detected regardless of the condition of assay. We thus believe that our phosphatase preparation was not contaminated by phosphatase forms 1, 2A, or 2C.

RESULTS

The Effect of Divalent Cations and Calmodulin on Calcineurin Phosphatase Activity—The myelin basic protein concentration dependence of calcineurin-catalyzed dephosphorylation was measured at either 200 μM Mn2+ or 100 μM Ca2+ in the presence or absence of calmodulin (Table I). As measured with 200 μM Mn2+ without calmodulin an apparent Kₘ of 8 μM for substrate was obtained with a Vₘₐₓ of 98 nmol of phosphate released per min·mg of calmodulin. The presence of calmodulin increased maximal velocity 4-fold and appeared to reduce the apparent Kₘ for myelin basic protein slightly. Substitution of Ca2+ (100 μM) for Mn2+ increased the apparent Kₘ for myelin basic protein approximately 2-fold and reduced maximal velocity more than 6-fold. Similar effects of Ca2+, Mn2+, and calmodulin on calcineurin-catalyzed dephosphorylation of casein were observed; however, maximal rates of dephosphorylation of myelin basic protein were more than 200-fold higher than those observed for casein.

The Ca2+ concentration dependence of calmodulin-stimulated phosphodiesterase (24) and calmodulin-stimulated myosin light chain kinase (25) has been shown to vary as a function of calmodulin concentrations, such that when calmodulin is present in stoichiometric excess relative to stimulated enzymes, the Kₘ for Ca2+ is shifted to lower concentrations, and the pattern of activation exhibits progressively increased degrees of positive cooperativity. Thus it was deemed important to establish the calmodulin concentration dependence of calcineurin phosphatase activity in order that the divergent cation dependence of activity could be determined subsequently at a range of limiting through saturating concentrations of calmodulin. These measurements were made using both myelin basic protein and casein as substrate and were conducted with both Mn2+ and Ca2+ as activating cation. Calcineurin was found previously to complex with and alter the susceptibility of various phosphorylated histones to dephosphorylation by calmodulin-independent phosphatases (21). Such complexes were dissociated at high ionic strength. This observation prompted an examination of the concentration dependence of calmodulin effects to assure that its actions were directed to enzyme rather than substrate.

The calmodulin concentration dependence of calcineurin-catalyzed dephosphorylation of both myelin basic protein and casein was measured with either Ca2+ or Mn2+ as the activating divalent cation (Fig. 1). Half-maximal activation of either myelin basic protein dephosphorylation or casein dephosphorylation occurred at 1–2 × 10⁻⁷ M calmodulin when Ca2+ was the source of activating ion. With Mn2+ as activating ion, the Kₘ for calmodulin was reduced to 3 × 10⁻⁸ M when measured with casein as substrate and increased to 10⁻⁹ M with myelin basic protein as substrate. The underlying basis for these observations is unclear but may reflect formation of complexes between calmodulin and myelin basic protein which are enhanced by the presence of Mn2+. It should be emphasized, however, that while a portion of calmodulin present may have complexed to myelin basic protein, its effects on activity were not substrate directed since calmodulin was always present at concentrations one to three orders of magnitude lower than the substrate.

The Mn2+ concentration dependence of calcineurin in myelin basic protein phosphatase activity was determined in incubations without calmodulin or at 0.08, 0.8, or 8 μM calmodulin (Fig. 2). The Kₘ determined for total added Mn2+ were 16, 16, 20, and 30 μM, respectively. Replots of data (not shown) according to the Hill equation provided Hill coefficients for Mn2+ of 0.87, 0.96, 1.00, and 1.94 in incubations.
calcineurin casein phosphatase activity was determined in various concentrations. The Ca\(^{2+}\) concentration dependence of calcineurin activation, and no apparent positive cooperativity was observed for Mn\(^{2+}\) even at the highest calmodulin concentration. The Ca\(^{2+}\) concentration dependence of calcineurin by calmodulin was approximately 8-fold at optimal Mn\(^{2+}\). The Ca\(^{2+}\) concentration dependence of calcineurin myelin basic protein phosphatase activity was similarly determined without calmodulin or at 0.08, 0.8, or 8 \(\mu\)M calmodulin (Fig. 3). The \(K_{\text{act}}\) values determined for Ca\(^{2+}\) were 0.8, 1.1, and 2.4 \(\mu\)M, respectively. Once again, at the highest concentration of calmodulin (8 \(\mu\)M), activation by Ca\(^{2+}\) exhibited positive cooperativity with a Hill coefficient of 3.4 and occurred at higher concentrations of total added Ca\(^{2+}\) than was observed when measured at the lower concentrations of calmodulin. The degree of activation of calcineurin by calmodulin was approximately 20-fold at optimal Ca\(^{2+}\). At optimal calmodulin, myelin basic protein phosphatase activity was 8-fold higher with Mn\(^{2+}\) than with Ca\(^{2+}\) as activating divalent cation.

In order to evaluate the possibility that the divalent cation dependence of calcineurin activity is altered by potential interactions of divalent cations with substrate, measurements of the Mn\(^{2+}\) and Ca\(^{2+}\) concentration dependence of activity were repeated at various concentrations of calmodulin using casein as substrate. The Mn\(^{2+}\) concentration dependence of calcineurin casein phosphatase activity was determined in incubations without calmodulin or at 0.012, 0.12, or 1.2 \(\mu\)M CaM (Fig. 4, panel A). The \(K_{\text{act}}\) values determined for Mn\(^{2+}\) were found to be 13, 16, 14, and 14 \(\mu\)M, respectively. These \(K_{\text{act}}\) values are very similar to those found using myelin basic protein substrate in incubations without calmodulin or in incubations containing less than 0.8 \(\mu\)M calmodulin. Calculated Hill coefficients for Mn\(^{2+}\)-dependent casein phosphatase activity were 1.03, 1.06, 1.03, and 1.06 at 0, 0.012, 0.12, and 1.2 \(\mu\)M calmodulin. Thus, calmodulin did not alter the concentrations of Mn\(^{2+}\) required for calcineurin activation, and no apparent positive cooperativity was observed for Mn\(^{2+}\) even at the highest calmodulin concentration. The Ca\(^{2+}\) concentration dependence of calcineurin casein phosphatase activity was measured without calmodulin or at 0.036, 0.36, or 3.6 \(\mu\)M calmodulin. The \(K_{\text{act}}\) values determined for Ca\(^{2+}\) were 1.6, 1.2, 1.6, and 1.2 \(\mu\)M, respectively, and were apparently not influenced by calmodulin. The activation of calcineurin by Ca\(^{2+}\) as measured at 0.36 and 3.6 \(\mu\)M calmodulin exhibited positive cooperativity with Hill coefficients of 2.2 and 2.3, respectively. In the absence of calmodulin...
Divalent Cation Dependence of Calcineurin Phosphatase

Calcineurin was routinely observed to display approximately 10-fold higher activities when Mn²⁺ rather than Ca²⁺ was used as the source of divalent cation. If Ca²⁺ and Mn²⁺ exerted their effects on activity by binding to common sites, it was predicted that Ca²⁺ at concentrations above its $K_a$ would exert a competitive inhibition on Mn²⁺ concentration-dependent activity. To examine this possibility, the effect of Ca²⁺ (1 or 10 nM) on the Mn²⁺ concentration dependence of activity was determined in the presence (Fig. 6, panel B) or absence (Fig. 6, panel A) of calmodulin. At each condition of measurement Ca²⁺ provided a small increase of activity without altering the Mn²⁺ concentration dependence of activity.

The Mg²⁺ concentration dependence of calcineurin myelin basic protein phosphatase activity was measured without or with 6 µM calmodulin (Fig. 7). The $K_a$ for Mg²⁺ was found to be 3 mM irrespective of the presence of calmodulin. Calmodulin provided no activation of calcineurin phosphatase with Mg²⁺ as the sole source of divalent cation. Activities measured at 1 mM added EDTA were no different from those measured without added divalent cation. Optimal Mg²⁺ provided a 75% stimulation of activity relative to divalent cation-free conditions and elicited phosphatase activities 50-fold lower than those observed at optimal Mn²⁺ and calmodulin.

The Ca²⁺ concentration dependence of calmodulin-stimulated phosphodiesterase activity (24) and calmodulin-stimulated myosin light kinase activity (25) is inhibited competitively by Mg²⁺. This observation is compatible with the hypothesis that the dependence of these enzymes on Ca²⁺ is mediated by interaction at the divalent cation-binding sites of calmodulin, which have been shown in several laboratories to bind Mg²⁺ competitively (27-31). To explore further the possibility that the Ca²⁺ dependence of calcineurin phosphatase activity was mediated through interactions with the

or at low calmodulin (0.036 µM), calcineurin exhibited poor Ca²⁺ dependence. The values for Ca²⁺ dependence measured with casein were quite similar to those determined using myelin basic protein as substrate.

We have treated calcineurin with trypsin (Fig. 5) under conditions identical to those described by Manalan and Klee (26). Under these conditions subunit A was completely degraded to a M₄, 45,000 product while the B subunit remained unaltered as revealed by stained sodium dodecyl sulfate-polyacrylamide gel electrophoretograms (data not shown). This confirmed the observation of Manalan and Klee (26), who had further shown that the $M_4$, 45,000 subunit A fragment had lost its calmodulin-binding domain as revealed by failure to bind iodinated calmodulin measured by a gel overlay procedure. Kinetic measurements of the myelin basic protein phosphatase activity of our trypsin-treated calcineurin (Fig. 5, panel A) revealed a preparation with increased activity as measured without calmodulin and which was no longer responsive to stimulation by calmodulin unless concentrations of calmodulin approached stoichiometric equivalence to substrate. Untreated enzyme was stimulated more than 4-fold by calmodulin. Determination of the Mn²⁺ concentration dependence of trypsin-treated calcineurin revealed a $K_a$ of 15 µM with phosphorylated myelin basic phosphatase as substrate. This value is very similar to that measured for untreated calcineurin in the absence of or at low concentrations of calmodulin.

Calcineurin was used as the source of divalent cation. If Ca²⁺ and Mn²⁺ mediated by interaction at the divalent cation-binding sites of calmodulin, which have been shown in several laboratories to bind Mg²⁺ competitively (27-31). To explore further the possibility that the Ca²⁺ dependence of calcineurin phosphatase activity was mediated through interactions with the...
divalent cation-binding sites of calmodulin, the effect of Mg$^{2+}$ on the Ca$^{2+}$ concentration dependence of myelin basic protein phosphatase activity was determined (Fig. 8). A $K_{ac}$ of 1.2 $\mu$M Ca$^{2+}$ was determined as measured without added Mg$^{2+}$ or in the presence of either 1 or 10 mM Mg$^{2+}$. Thus no competition between Mg$^{2+}$ and Ca$^{2+}$ was observed. Similarly, Mg$^{2+}$ did not alter the Mn$^{2+}$ concentration dependence of activity (data not shown).

In order to evaluate the relative efficiency of various divalent cations in supporting calcineurin phosphatase activity, an array of different cations was examined at a concentration of 200 $\mu$M in the absence or presence of calmodulin. Decreasing degrees of activity were observed in the order Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Sr$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Zn$^{2+}$, Mg$^{2+}$. With the exception of Mg$^{2+}$, all cations showed substantially greater activities when measured in the presence of calmodulin than when measured in its absence.

The Effect of Alternate Activators and Calmodulin Homologs on Activity—Phospholipids with detergent-like properties, as well as anionic detergents, have been shown (32) to substitute for calmodulin in activating calmodulin-dependent phosphodiesterase. Lyso phosphatidylcholine (3 to 330 $\mu$g/ml) and sodium dodecyl sulfate (30 to 100 $\mu$M) at concentrations known to stimulate phosphodiesterase exerted no effect on phosphatase activity as measured at limiting or saturating concentrations of Mn$^{2+}$ or in incubations with no added divalent cation. Similarly several high affinity Ca$^{2+}$-binding proteins, homologous to calmodulin (32), that is rabbit skeletal muscle troponin C, rabbit skeletal muscle parvalbumin, and bovine brain S-100b, exerted no effects on phosphatase activity with either Ca$^{2+}$ or Mn$^{2+}$ as activating ion when present at protein concentrations of 1 $\mu$M. Thus calcineurin would appear to recognize calmodulin quite specifically.

The Effect of Ca$^{2+}$ and Mn$^{2+}$ on the Far Ultraviolet Circular Dichroic Spectrum of Calcineurin—The far ultraviolet circular dichroic spectrum of calcineurin was examined in 5 mM Tris, pH 7.4, 150 mM KCl without additions, or with 90 $\mu$M Ca$^{2+}$, 200 $\mu$M Mn$^{2+}$, or 200 $\mu$M EDTA (Fig. 9). At each condition of measurement the spectrum revealed negative maxima at 222 and 208 nm as is commonly observed in polypeptides with appreciable helical content (22); however, the values of the molar ellipticities at 208 nm (4000-5000 degree cm$^2$/dmol) are compatible with a low helical content and appreciable degrees of random coil structure. Adjustment of the solution of calcineurin to contain either Ca$^{2+}$ or Mn$^{2+}$ reduced the negative maxima at 222 and 208 nm to virtually identical degrees, suggesting that interaction of calcineurin with either divalent cation produced comparable alterations of secondary structure of calcineurin. Addition of EDTA to the calcineurin did not alter the circular dichroic spectrum consistent with the assertion that the preparation of calcineurin was divalent cation free.

DISCUSSION

The data presented in this report establish that calmodulin exerts its stimulatory effect on calcineurin phosphatase activity by increasing the turnover number of the enzyme without appreciably altering the $K_{ac}$ of the enzyme for substrate (Table I). Mn$^{2+}$ (Figs. 2 and 4A), or Ca$^{2+}$ (Figs. 3 and 4B). The maximal velocity of calcineurin was found to be approximately 10-fold higher with Mn$^{2+}$ as the activating ion than with Ca$^{2+}$ as the activating ion (Tables I and II, Figs. 2 and 3); however, the $K_{ac}$ for divalent ion was approximately 10-fold lower when Ca$^{2+}$ (1 $\mu$M) than when Mn$^{2+}$ (15 $\mu$M) was the activating species.

The effects of Ca$^{2+}$ and Mn$^{2+}$ on calcineurin phosphatase activity are exerted by interaction with site(s) on the phosphatase rather than calmodulin. The site of these interactions is uncertain but may occur on the B subunit which has been reported to be homologous in primary structure to calmodulin (34), a well-characterized divalent cation-binding protein. Ob-
servations in accord with this hypothesis include the following. Ca$^{2+}$ and Mn$^{2+}$ exerted effects on activity in the absence of added calmodulin, with activation exhibiting $K_{act}$ values identical to those observed at submicromolar concentrations of activating calmodulin (Figs. 2, 3, and 4). Treatment of calcineurin with trypsin generated an activated calmodulin-unresponsive form, with an unaltered Mn$^{2+}$ concentration dependence. Finally, both Ca$^{2+}$ and Mn$^{2+}$ were found to affect the secondary structure of calcineurin in the absence of calmodulin as evidenced by their ability to produce alterations in the far ultraviolet circular dichroic spectrum of calcineurin (Fig. 9).

The apparent $K_{act}$ values determined for Ca$^{2+}$ and Mn$^{2+}$ would appear to be largely substrate independent. A $K_{act}$ for Mn$^{2+}$ of approximately 15 $\mu$M was determined for the enzyme at submicromolar concentrations of calmodulin, regardless of whether casein (Fig. 4A) or myelin basic protein (Fig. 2) was the substrate, and the phosphatase exhibited cation concentration dependencies which appeared to obey Michaelis-Menten kinetics. Only when measured at high concentrations of calmodulin (8 $\mu$M, Fig. 2) was apparent positive cooperativity observed for Mn$^{2+}$ dependence. Since calmodulin is known to bind 4 mol of Mn$^{2+}$ at concentrations in the micromolar range (31), we believe that at high calmodulin concentrations a significant portion of the total Mn$^{2+}$ present is calmodulin bound, rather than freely available to interact with calcineurin. In incubations containing submicromolar concentrations of activating calmodulin, the fraction of calmodulin-bound Mn$^{2+}$ would represent only a minor portion of the total. In such incubations, protein components are present only in nanomolar concentrations; thus the free concentration of divalent cation and total concentration as measured by atomic absorption spectrophotometry are nearly identical values. Similarly, a $K_{act}$ for Ca$^{2+}$ of approximately 1 $\mu$M was observed with myelin basic protein as substrate (Fig. 3), and a value of 1.5 $\mu$M was determined with casein (Fig. 4B) as substrate, as long as submicromolar activating concentrations of calmodulin were used. These activating concentrations of Ca$^{2+}$ very closely resemble those reported previously from other laboratories (11, 13).

The sites on calcineurin with which Ca$^{2+}$ and Mn$^{2+}$ interact to exert their effects on activity appear to be different. Since the maximal velocity of calcineurin is approximately 10-fold higher with Mn$^{2+}$ as opposed to Ca$^{2+}$ as the activating ion, one would predict that a common site of interaction would allow high Ca$^{2+}$ concentrations to displace Mn$^{2+}$ resulting in a 10-fold reduction of activity. This inhibition would, in turn, be reversible by elevated concentrations of Mn$^{2+}$. Further, in the presence of concentrations of Ca$^{2+}$ at or above its $K_{act}$, the concentration dependence for Mn$^{2+}$ should be shifted to higher values in close accord with the equation $K_{act(Mn)} = K_{act}(1 + [I/K])$ (where Mn$^{2+}$ is the activating and Ca$^{2+}$ the inhibitory species) (35). However, Ca$^{2+}$ at 10 $\mu$M concentration, a value 10-fold higher than its $K_{act}$, did not inhibit Mn$^{2+}$-dependent activity at all but provided increased degrees of enzyme activity (Fig. 6). Further, no alteration of the Mn$^{2+}$ dependence of activity was observed. These additive effects of Ca$^{2+}$ and Mn$^{2+}$ were observed regardless of whether calmodulin was present and indicate interaction with different sites or, alternatively, interaction of each metal with different enzyme forms. The presence of distinct enzyme forms seems the less likely possibility since interaction of saturating concentrations of either Ca$^{2+}$ or Mn$^{2+}$ with calcineurin produced identical alterations in the far ultraviolet circular dichroic spectrum of the enzyme (Fig. 8). If, for example, Ca$^{2+}$ were interacting with a minor form or species of enzyme to elicit its effect on activity, one would expect that small, almost undetectable, effects on secondary structure would be obtained with Ca$^{2+}$ relative to those observed for Mn$^{2+}$. However, almost identical spectral changes were seen.

The physiological and regulatory significance of the apparently greater activity of the phosphatase in the presence of Mn$^{2+}$ rather than Ca$^{2+}$ is obscure, since it is doubtful that cellular concentrations of manganese undergo alteration in response to stimuli or are found intracellularly at concentrations of 15 $\mu$M.

Since intracellular concentrations of Mg$^{2+}$ are normally in the millimolar range (36) studies were undertaken to determine if the presence of Mg$^{2+}$ might alter the interaction of calcineurin with Ca$^{2+}$ or Mn$^{2+}$ to provide divalent cation-interacting properties more closely resembling those observed for other calmodulin-regulated enzymes. Magnesium as the sole source of divalent cation was found to have very little effect on activity, relative to the divalent cation-free condition (Table II, Fig. 7), and did not support a calmodulin-dependent stimulation of activity. Further, magnesium did not alter the Ca$^{2+}$ (Fig. 8) or Mn$^{2+}$ (not shown) concentration dependence of activity to an appreciable extent. Failure of Mg$^{2+}$ to compete with Ca$^{2+}$ or Mn$^{2+}$ for effects on activity provides additional evidence that the Mn$^{2+}$ and Ca$^{2+}$ effects which limit activity are not exerted through calmodulin.

A calcineurin-dependent protein phosphatase, purified from rabbit skeletal muscle by Stewart et al. (13) using procedures including chromatography on immobilized calmodulin, has been shown to dephosphorylate inhibitor 1, phosphorylase kinase, and myosin light chains at rates ranging from approximately 800 to 2000 nmol/min·mg. These rates are only slightly higher than those noted for myelin basic protein, which was chosen because it can be prepared readily in a homogeneous state in gram quantities (15) and is phosphorylated readily by the catalytic subunit of cyclic AMP-dependent protein kinase. It was noted by Stewart et al. that calcineurin phosphatase activity, as monitored during purification by catalysis of inhibitor 1 dephosphorylation, showed identical degrees of calmodulin-dependent activity with either Ca$^{2+}$ or Mn$^{2+}$ as the divalent cation source, until the final step of purification, which employed chromatography on calmodulin-Sepharose. Following affinity chromatography, activities measured with Mn$^{2+}$ were 12-fold higher than when measured with Ca$^{2+}$. The basis of this alteration of properties is unknown. Further, it was noted that following affinity chromatography the phosphatase was no longer readily susceptible to inhibition by trifluoperazine. Our preparation of calcineurin from bovine brain similarly employs affinity chromatography on immobilized calmodulin, and the enzyme obtained shows a greater than 10-fold Mn$^{2+}$/Ca$^{2+}$ activity ratio and is not readily susceptible to inhibition by trifluoperazine (apparent $K_i = 140$ $\mu$M, data not shown). Attempts to measure calmodulin-dependent phosphatase activity prior to affinity chromatography were unsuccessful because using myelin basic protein as substrate the vast majority of phosphatase activity prior to affinity chromatography is calmodulin independent. It is possible that a tightly bound ion is removed from association with calcineurin when calmodulin-bound calcineurin is eluted with chelators during affinity chromatography. It would be valuable to compare the content of and regulation by divalent cations, of a preparation of homogeneous phosphatase generated without calmodulin affinity chromatography. As yet, no such procedure has been described.

Preincubation of calcineurin with Ni$^{2+}$ has been reported to produce both a nonlinear time-dependent enzyme activation and a quenching of tryptophan fluorescence (37, 38).
These effects were calmodulin independent and irreversible by high concentrations of chelator. We have examined the time dependency of our preparation of calcineurin in catalyzing myelin basic protein dephosphorylation in incubations initiated with ion. Whether measured with limiting or saturating concentrations of Mn$^{2+}$ or Ca$^{2+}$, with or without calmodulin, linear dependencies of activity were seen (data not shown). The stimulations of activity by divalent cations were reversible within 1 min by addition of chelator (data not shown). Similar behavior with Mn$^{2+}$ has been noted by others (39). We have not yet conducted similar studies of the time dependency and reversibility of calcineurin-catalyzed myelin basic protein dephosphorylation with Ni$^{2+}$ as the activating ion. However, when phosphatase activity was measured continuously and spectrophotometrically using hydrolysis of p-nitrophenyl phosphate (37), a slow time-dependent reactivation was seen with Ni$^{2+}$, which was rapidly accelerated by calmodulin (data not shown). With Mn$^{2+}$ as activating ion, the nonlinear reactivation occurred within 30 s and maintained linear rates thereafter (data not shown). More detailed kinetic studies will be required to understand this reactivation event.

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