Dual Calcium Ion Regulation of Calcineurin by Calmodulin and Calcineurin B

Paul M. Stemmer*,‡ and Claude B. Klee*

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received December 23, 1993; Revised Manuscript Received March 29, 1994®

ABSTRACT: The dependence of calcineurin on Ca^{2+} for activity is the result of the concerted action of calmodulin, which increases the turnover rate of the enzyme and modulates its response to Ca^{2+} transients, and of calcineurin B, which decreases the K_m of the enzyme for its substrate. The calmodulin-stimulated protein phosphatase calcineurin is under the control of two functionally distinct, but structurally similar, Ca^{2+}-regulated proteins, calmodulin and calcineurin B. The Ca^{2+}-dependent activation of calcineurin by calmodulin is highly cooperative (Hill coefficient of 2.8–3), and the concentration of Ca^{2+} needed for half-maximum activation decreases from 1.3 to 0.6 μM when the concentration of calmodulin is increased from 0.03 to 20 μM. Conversely, the affinity of calmodulin for Ca^{2+} is increased by more than 2 orders of magnitude in the presence of a peptide corresponding to the calmodulin-binding domain of calcineurin A. Calmodulin increases the V_{max} without changing the K_m value of the enzyme. Unlike calmodulin, calcineurin B interacts with calcineurin A in the presence of EGTA, and Ca^{2+} binding to calcineurin B stimulates native calcineurin up to only 10% of the maximum activity achieved with calmodulin. The Ca^{2+}-dependent activation of a proteolyzed derivative of calcineurin, calcineurin-45, which lacks the regulatory domain, was used to study the role of calcineurin B. Removal of the regulatory domain increases the V_{max} of calcineurin, as does binding of calmodulin, but it also increases the affinity of calcineurin for Ca^{2+}. Ca^{2+} binding to calcineurin B decreases the K_m value of calcineurin without changing its V_{max}. Like native calcineurin, calcineurin-45 contains two high-affinity Ca^{2+} sites ($K_d < 0.07$ μM) and one or two low-affinity sites ($K_d > 0.07$ μM).

The calmodulin-stimulated protein phosphatase calcineurin is the only calmodulin-regulated enzyme subject to a dual Ca^{2+} control mediated by two different Ca^{2+}-regulated proteins, calmodulin and calcineurin B (Klee et al., 1987). Like calmodulin, calcineurin B is a Ca^{2+}-binding protein with four "EF-hand" Ca^{2+}-binding sites (Aitken et al., 1982); its secondary structure, determined by multidimensional NMR, is similar to that of calmodulin (Anglister et al., 1993). Despite their structural similarity, the two proteins are not functionally interchangeable. Calcineurin B cannot replace calmodulin in the activation of the enzyme, and calmodulin cannot substitute for calcineurin B in the reconstitution of calcineurin (Merat et al., 1985; Klee et al., 1985). The interaction of calmodulin with calcineurin is Ca^{2+}-dependent and accompanied by a large increase in protein phosphatase activity. Calcineurin B is tightly bound to the enzyme even at low Ca^{2+} concentration, and Ca^{2+} binding to calcineurin B stimulates the enzyme to less than 10% of the activity achieved with calmodulin (Stewart et al., 1982).

The dual Ca^{2+} regulation is a highly conserved property of calcineurin from yeast to man. All the isolated calcineurins, regardless of source, have the same two-subunit structure (Klee et al., 1987). The organization of the functional domains of calcineurin A is also conserved and consists of a catalytic domain in the amino-terminal half and a regulatory domain in the carboxyl-terminal half. The regulatory domain contains calmodulin- and calcineurin B-binding subdomains and an autoinhibitory region (Hubbard & Klee, 1989). The enzyme is likely to be stimulated by calmodulin by a mechanism which involves the displacement of the autoinhibitory region upon calmodulin binding. The amino acid sequence of calcineurin B and that of its binding subdomain[1] on calcineurin A are the most highly conserved features of calcineurin. Human, mouse, and rat calcineurin B have identical amino acid sequences (Guerini et al., 1989; Ueki et al., 1992), and there is 85 and 56% identity between *Drosophila melanogaster* and yeast calcineurin B and the vertebrate proteins (Guerini et al., 1992; Cyert et al., 1992; Kuno et al., 1991). The calmodulin-binding subdomains of human, rat, mouse, *Neurospora crassa*, and *D. melanogaster* calcineurin A are highly conserved, and the calcineurin B-binding subdomains of these species are identical with the exception of two amino acid substitutions in *Neurospora* and the two yeast isoforms (Kincaid et al., 1988, 1991; Guerini & Klee, 1989; Kuno et al., 1989; Ito et al., 1989; Higuchi et al., 1991; Guerini et al., 1992; Cyert et al., 1991; Liu et al., 1991; Ye & Bretscher, 1992).

The essential role of calcineurin B for calcineurin activity was demonstrated by Merat et al. (1985). With the exception of *Neurospora* calcineurin A, which is active in the absence of calcineurin B (Higuchi et al., 1991), the calmodulin stimulation of all recombinant calcineurins A depends on the addition of calcineurin B (Perrino et al., 1992; Ueki & Kincaid, 1993). Calcineurin B is also needed for the inhibition of calcineurin by the immunosuppressive drugs cyclosporin A and FK506 complexed with their respective immunophilins (Haddy et al., 1992). This requirement is consistent with the recent report that calcineurin B itself may be the target of the immunosuppressants (Li & Handschumacher, 1993). Despite these important roles of calcineurin B, little is known about its mechanism of action. In this paper we compare the Ca^{2+} activation of calcineurin mediated by calmodulin and calcineurin B and demonstrate that calmodulin and calcineurin B activate the phosphatase activity of calcineurin by different

‡ Present address: Department of Pediatrics, COP Room 1012, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198-6055.

® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] P. M. Stemmer, M. H. Krinks, M.-P. Strub, and C. B. Klee, manuscript in preparation.

and complementary mechanisms. The concerted action of these two regulatory proteins is responsible for the requirement of the enzyme for the levels of Ca^{2+} found in stimulated cells.

MATERIALS AND METHODS

Materials

Bovine brain calcineurin and ram testis calmodulin were isolated as previously described (Klee et al., 1983; Newton et al., 1984). Calcineurin-45, obtained by limited proteolysis of calcineurin with clostripain in the absence of calmodulin, was separated from small peptides by gel filtration on Sephadex-G100.[1] A synthetic peptide (ARKEVIRNKIRAIGKMAR-VFSVLR) corresponding to the calmodulin-binding domain of calcineurin A, as predicted by Kincaid et al. (1988), was purchased from Peptide Technologies, Washington, DC, and purified by HPLC[2] on a μBondapak C18 column (10-μm resin diameter; 3.9 mm × 30 cm) eluted with a 0 to 40% linear gradient of acetonitrile in 0.01% trifluoroacetic acid. The peptide-containing fractions were dried by flash evaporation, and the peptide was dissolved in deionized water at a final concentration of 0.6 mM. The synthetic calcineurin peptide substrate DLDVPIPGRFDRRVSVAAE (Blumenthal et al., 1986), purchased from Peninsula Laboratories, was purified by HPLC and phosphorylated with the catalytic subunit of cAMP-dependent protein kinase (a generous gift of Dr. E. Fischer, University of Washington, Seattle) as previously described (Hubbard & Klee, 1991). Standard solutions of $CaCl_2$ were obtained from Radiometer, and standard solutions of EDTA and $TbCl_3$ were from Aldrich.

Methods

Removal of Contaminating Metals. Plasticware, dialysis membranes, and stock solutions used for the flow dialysis experiments were treated as described by Crouch and Klee (1980). Calmodulin solutions were freed of contaminating EGTA by two successive gel filtrations over Sephadex G-25 PD-10 columns (Pharmacia) equilibrated in buffer A (10 mM Hepes–KOH, pH 7.6, containing 100 mM KCl). Contaminating metals were removed by passage through a column of Bio-Rad Chelex 100 [0.5 mL/(mg of calmodulin)] equilibrated with buffer A (Klee 1977). The stock solution of calmodulin-binding peptide was used without prior decalcification. Calcineurin was partially decalcified by passage through Chelex [1 mL/(mg of protein)] or parvalbumin–Sepharose columns. One to two moles of Ca^{2+} remained bound per mole of calcineurin. The parvalbumin–Sepharose used for these experiments was prepared by coupling 1 mg of frog parvalbumin (a gift of J. Haiech, University of Marseille, France) to 1 mL of CNBr-activated Sepharose 4-B according to the manufacturer's instructions. It had a capacity of 140 nmol of Ca^{2+}/mL after decalcification by treatment with 100 mM KCl adjusted to pH 4 with acetic acid.

Ca^{2+} Concentration Measurements. Total Ca^{2+} was measured by atomic absorption with a Perkin-Elmer 5000 atomic absorption spectrophotometer equipped with an autosampler and a graphite furnace (Haiech et al., 1981). Standard curves measured in the presence of the samples to be tested were used to correct for the effect of solvents and proteins on the Ca^{2+} measurements. Hepes and KCl decreased the signal, while Mg^{2+} increased it. No significant effect was observed at protein concentrations up to 1 mg/mL.

Flow Dialysis Experiments. Ca^{2+} binding to calmodulin and to equimolar mixtures of calmodulin and calmodulin-binding peptide was measured by the method of Colowick and Womack (1969) with the flow dialysis chamber of Feldman (1978) as described by Haiech et al. (1981) with the following modifications. The calmodulin concentration was varied from 10 to 25 μM when Ca^{2+} binding was examined in the absence of peptide and from 3 to 5 μM in the presence of an equimolar amount of calmodulin-binding peptide. The flow rate of the dialysate was decreased from 3 to 1 mL/min when the calmodulin-binding peptide was included in the experiment. The volume of the protein solutions was increased to 2 mL to minimize the effects of Ca^{2+} loss. The contents of the flow cell were weighed at the end of the experiment to verify that the volume remained constant. All Ca^{2+} additions were made with Hamilton syringes. The concentration of $^{45}Ca^{2+}$ at the start and the end of the experiment was measured as the average of five independent determinations. Addition of 1 mM carrier Ca^{2+} to the starting material, prior to sampling for Ca^{2+} determinations, was required to prevent loss of radioactive ligand by adsorption to the walls of the pipet tips at low Ca^{2+} concentration. The molar concentration of total Ca^{2+} at the start of the experiment was less than 10% of the calmodulin concentration.

Calculations of Bound Ligand and Correction for Loss of Ligand during Dialysis. The calculations of bound Ca^{2+} measured by flow dialysis experiments are based on the assumption that no significant loss of unlabeled or radioactive ligand occurs during the course of the experiments. A consistent loss of 12–17% of the radioactive ligand observed during the 2.5–3-h dialysis experiments prompted us to examine the effect of this loss on the quantitative analysis of the Ca^{2+}-binding data. After each addition of Ca^{2+}, the content of radioactive ligand in the flow well (L^*) was corrected for $^{45}Ca^{2+}$ lost in the effluent collected prior to the next addition of Ca^{2+} (ΔL^*). The amount of radioactive ligand remaining after n successive additions of Ca^{2+} was

$$L^*_n = L^*_i - (\Delta L^*_1 + \ldots + \Delta L^*_n - 1) \quad (1)$$

where ΔL^* is the sum of the counts (cpm) in the fractions of effluent collected during the equilibration and the plateau following an addition of Ca^{2+} to the well, L^*_i is the initial amount of radioactive ligand, and $\Delta L^*_{1\ldots n}$ is the loss of L^* at steps 1–n. The loss of unlabeled ligand after each addition ($\Delta L_{1\ldots n}$) was calculated on the basis of the specific activity of Ca^{2+} after the Ca^{2+} addition corrected for Ca^{2+} and $^{45}Ca^{2+}$ lost at each step.

$$\Delta L_n = \Delta L^*_n (L_{n-1}/L^*_{n-1}) \quad (2)$$

The amount of Ca^{2+} remaining after n successive additions of Ca^{2+} is

$$L_n = L_T - (\Delta L_1 + \ldots + \Delta L_{n-1}) \quad (3)$$

L_T being the total Ca^{2+} added to the well at step n, and $L_{1\ldots n}$, the Ca^{2+} in the well corrected for the loss of Ca^{2+} up to step $1\ldots n$. The free Ca^{2+} (Ca^{2+}_f) was calculated as described by Haiech et al. (1981) using the ratio of (counts per minute)/milliliter in the effluent after each Ca^{2+} addition ($c^*_{1\ldots n-1}$) to the (counts per minute)/milliliter in the effluent recovered after addition of a large excess of unlabeled ligand (c_n) at the end of the experiments after correction for the dilution and the loss of labeled ligand at each step.

[2] Abbreviations: HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; CaM, calmodulin.

Table 1: Effect of the Correction for Loss of Ligand during Flow Dialysis on the Determination of Binding Parameters[a]

parameter	without correction	with correction
K_1[b]	16 ± 7	11.7 ± 2.4
K_2	2 ± 1	3.2 ± 0.8
K_3	86 ± 28	112.0 ± 23
K_4	39 ± 17	46.0 ± 14
j	0.02 ± 0.002	0.014 ± 0.002
N	0.96 ± 0.01	1.02 ± 0.01
rsd[c]	0.054	0.040

[a] The experimental conditions are described in Methods. Data are from a representative experiment in which calmodulin was 20 μM and $MgCl_2$ was 6 mM. [b] Macroscopic dissociation constants K_1, K_2, K_3, and K_4 (10^{-6} M) were determined by fitting the data to the Adair–Klotz equation (Edsall & Wymann, 1958):

$$y = y^* \frac{N(x/K_1 + 2x^2/K_1K_2 + 3x^3/K_1K_2K_3 + 4x^4/K_1K_2K_3K_4)}{4(1 + x/K_1 + x^2/K_1K_2 + x^3/K_1K_2K_3 + x^4/K_1K_2K_3K_4)} + jx$$

where y is the moles of bound Ca^{2+} determined experimentally, j is the slope term for nonspecific binding,[3] x is the concentration of free Ca^{2+}, and y^* is the estimated value for the maximum amount of bound Ca^{2+}. The factor N is the ratio of the value of y^* derived from fitting the data to that calculated on the basis of the experimental protein determination. $y^* = 4m/MW$, where m is the amount of protein (mg/mL) and MW is the molecular weight of calmodulin (16 500). Standard errors are shown. [c] Residual standard deviation.

$$Ca^{2+}_f = (c^*_{1\ldots n-1}/c^*_n)L_{1\ldots n-1} \quad (4)$$

As shown in Table 1, correction for the loss of ligand during the flow dialysis experiment significantly improves the computer fit of the data with a standard deviation of the residuals of 0.04, as opposed to 0.054, and consistently lower standard errors in the determinations of the macroscopic constants. As expected, the extent of the correction depends on the extent of Ca^{2+} loss and the duration of the experiment.

Protein Determinations. The protein concentrations were determined spectroscopically using the following extinction coefficients: ϵ_{276nm} = 3300 for decalcified calmodulin (Klee & Vanaman, 1982), ϵ_{278nm} = 57 940 for calcineurin, ϵ_{278nm} = 52 000 for calcineurin-45[1], and ϵ_{258nm} = 195 for the synthetic peptide[4] corresponding to the calmodulin-binding domain of calcineurin A.

Enzymatic Assays. Phosphatase activity was assayed as previously described (Hubbard & Klee, 1991). The incubation mixture (60 μL) was 40 mM Tris-HCl, pH 7.5, containing 100 mM KCl; 6 mM $MgCl_2$; 0.1 mM dithiothreitol; 0.1 mg/mL bovine serum albumin; EGTA, $CaCl_2$, and calmodulin as described for individual experiments; and enzyme at a final concentration of 3–30 nM. The reaction was routinely started by addition of substrate to a final concentration of 1 μM (2–280 μM for the determination of the kinetic constants). The substrate concentration was based on the specific activity of ATP used for the phosphorylation reaction. Initial rates were calculated as described earlier (Klee et al., 1983). The concentration of free Ca^{2+} in the EGTA-buffered solutions was calculated with a computer program (Jean & Klee, 1986) using the dissociation constants of EGTA for Ca^{2+}, Mg^{2+}, and K^+ reported by Fabiato and Fabiato (1979). A mean K_d value of 23 μM, determined in this study, was used for the dissociation constant of the four Ca^{2+} sites of calmodulin in the presence of 6 mM Mg^{2+}. The concentration of the stock EGTA solution was determined by titration of a standard solution of Tb^{3+} (Woyski & Harris, 1963). The data were corrected for a decrease in the pH from 7.5 to 7.3 at 30 °C but were not corrected for the drop in pH from 7.1 to 6.8 when the incubation mixtures containing 5 mM EGTA were brought from 10^{-7} to 5 × 10^{-6} M free Ca^{2+}.

[3] The value of the j term is independent of calmodulin concentration (0.04 ± 0.005 and 0.035 ± 0.005 with 5 and 25 μM calmodulin, respectively), indicating that it reflects nonspecific binding of Ca^{2+} to the membranes rather than Ca^{2+} binding to low-affinity sites on calmodulin.

[4] The extinction coefficient of the calmodulin-binding peptide is based on the absorbance of its single phenylalanyl residue at 256 nm.

Table 2: Ca^{2+}-Dependent Activation of Native and Proteolyzed Calcineurin[a]

Ca^{2+}[b] (μM)	CaM (μM)	K_{act}[c] (μM)	Hill coefficient	max. act.[d] [nmol/(min·mg)]
		Native Calcineurin		
≤1				0.14 ± 0.02 (10)
10			n.d.[e]	1.10 ± 0.02 (3)
10	3			10.9 ± 0.6 (10)
100	0.03	1.34	3.1	10.3[f]
30	0.3	0.79	3.1	9.9[f]
10	3.0	0.62	2.9	10.9[f]
5.5	20.0	0.56	2.8	12.4[f]
		Calcineurin-45		
≤1				0.8 ± 0.09 (7)
10		0.06	1.8	10.0 ± 0.6 (7)
10	3.0			10.8 ± 0.6 (3)

[a] The phosphatase activity was assayed in the presence of the indicated concentrations of calmodulin and Ca^{2+} as illustrated in Figures 1 and 4. The enzyme concentration was 2–10 nM. [b] The Ca^{2+} concentrations were adjusted with 2 mM (native calcineurin) and 5 mM (calcineurin-45) EGTA as described in Methods and the caption to Figure 1. [c] Concentration of Ca^{2+} required for half-maximal activity. [d] The initial rates were measured, in the presence of 1 μM substrate, as described in Methods, and the maximum activities are the mean ± the standard error of 3–10 such determinations. The number of independent determinations is shown in parentheses. [e] The biphasic character of the calmodulin-independent activation of calcineurin by Ca^{2+} prevented the accurate determination of a Hill coefficient. [f] Maximum activities of the enzyme in the experiments illustrated in Figure 1.

RESULTS

Calmodulin-Dependent Stimulation of Calcineurin by Ca^{2+}. In the absence of Ca^{2+} the enzymatic activity of calcineurin is very low [0.14 nmol/(min·mg)]. Addition of Ca^{2+} results in an almost 10-fold stimulation, whereas a 100-fold stimulation is observed upon the combined addition of Ca^{2+} and calmodulin (Table 2). The calmodulin-mediated Ca^{2+}-dependent stimulation of calcineurin is strongly cooperative with a Hill coefficient of 2.8–3.1, indicating that binding to at least three Ca^{2+} sites is required for activity (Figure 1 and Table 2). Consistent with the fact that calmodulin stimulation of calcineurin is the result of the Ca^{2+}-dependent binding of calmodulin to calcineurin, the concentration of Ca^{2+} needed for activation decreases with increasing concentrations of calmodulin (Figure 1).

The apparent affinity of calmodulin for Ca^{2+} is expected to increase in the presence of calcineurin. In order to test this expectation, we measured the binding of Ca^{2+} to an equimolar mixture of calmodulin and a synthetic peptide whose sequence corresponds to the calmodulin-binding domain of calcineurin. This peptide prevents the activation of calcineurin by calmodulin with a k_i of 1 nM but does not inhibit the calmodulin-independent phosphatase activity of calcineurin-45 (M. H. Krinks and C. B. Klee, unpublished observations). The binding of Ca^{2+} to calmodulin was measured in the absence or presence of a physiological concentration of Mg^{2+} (1 mM) and in the presence of the concentration of Mg^{2+} (6 mM) used for the protein phosphatase measurements. As shown by the data in Figure 2, the affinity of calmodulin for Ca^{2+} is increased by 1–2 orders of magnitude in the presence of the peptide. The

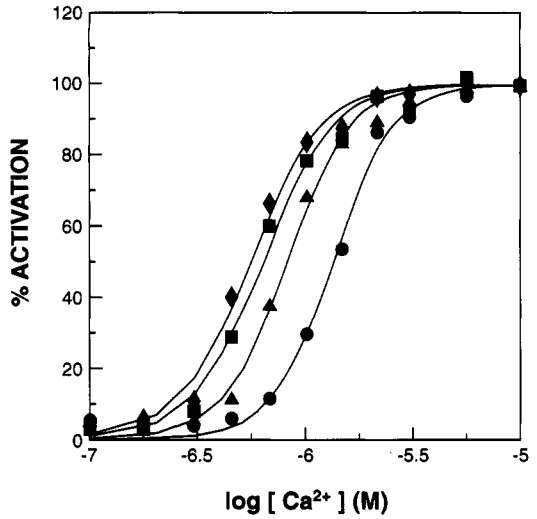

FIGURE 1: Activation of calcineurin protein phosphatase activity as a function of free Ca^{2+} concentration at different levels of calmodulin. The calmodulin concentrations are 0.03 (●), 0.3 (▲), 3 (■), and 20 μM (♦). The data are corrected for calmodulin-independent Ca^{2+}-stimulated activity. The free Ca^{2+} concentrations were calculated as described in Methods. All buffers were treated with Chelex, EGTA was 5 mM, and added Ca^{2+} was in the millimolar range to minimize the contribution of contaminating Ca^{2+}.

macroscopic dissociation constants derived from such binding experiments are listed in Table 3. In the absence of Mg^{2+} the affinity of the first site to be occupied is low (K_1=17.8 μM), and that of the second site is high (K_2 = 0.4 μM), suggesting positive cooperativity between these two sites. The relatively low affinities of the remaining two sites (K_3 = 14.7 and K_4 = 11.2 μM) are not significantly different, in agreement with the report of Minowa and Yagi (1984) showing that cooperativity is less marked for the low-affinity than for the high-affinity sites. Addition of 1 mM Mg^{2+} has a complex effect on Ca^{2+} binding to calmodulin. It greatly increases the affinity of the first site and slightly decreases the affinities of the second and the third, while it moderately increases the affinity of the fourth. Higher concentrations of Mg^{2+} significantly decrease the affinities, probably by competing with Ca^{2+}, at all four sites. This effect is particularly significant on those sites whose affinities was increased by addition of 1 mM Mg^{2+}. Addition of the calmodulin-binding peptide greatly increases the affinity of calmodulin for Ca^{2+} (Figure 2) but also exerts different effects on the different sites (Table 3). A 270-fold increase in the affinity of the third site and a 10–15-fold increase in the affinities of the first, second, and fourth sites are observed. In the presence of 6 mM Mg^{2+} similar effects are observed, but in this case the affinity of the first site is also increased substantially (75-fold) upon addition of the peptide.

These data do not determine how many Ca^{2+} sites on calmodulin need to be filled to support the activation of calcineurin. A comparison of the probability of the number of Ca^{2+} sites occupied, calculated on the basis of the Ca^{2+} binding measured in the presence of the calmodulin-binding peptide (Table 3), and the stimulation of the phosphatase activity, measured in the presence of 0.03 μM calcineurin and 3 μM calmodulin, is not valid. The number of sites could be overestimated since the concentration of calcineurin used for the activity measurements is lower than the concentration of peptide in the binding experiments. Alternatively, the higher affinity of calcineurin for calmodulin (K_d = 0.1 nM) than for the peptide (K_d = 1 nM) (Hubbard & Klee, 1987) can lead to an underestimation of the affinity of the calmodulin-calcineurin complex for Ca^{2+} (Hubbard & Klee, 1987).

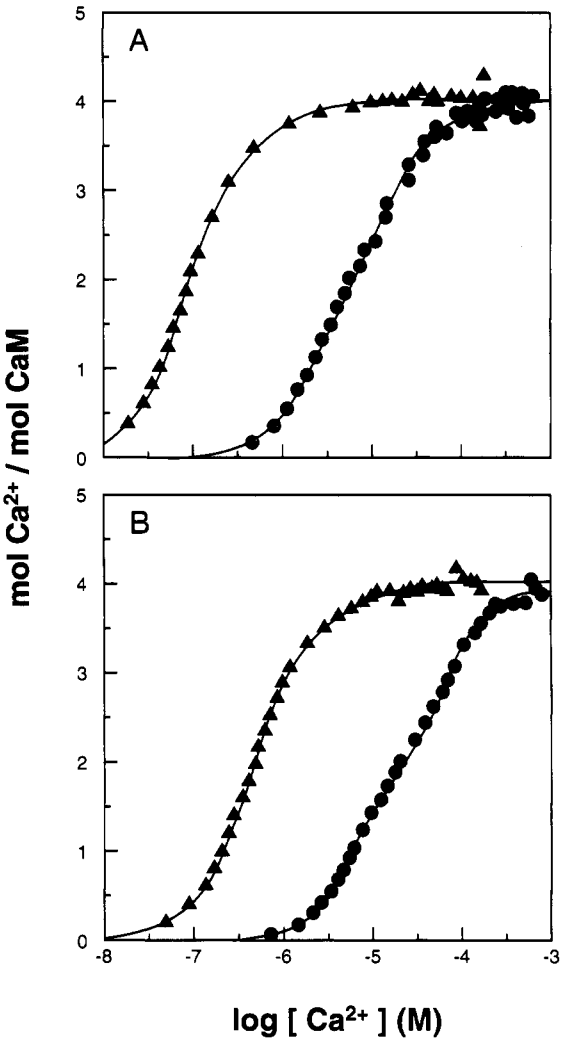

FIGURE 2: Effect of the calmodulin-binding domain of calcineurin on Ca^{2+} binding to calmodulin: panel A, in the presence of 1 mM Mg^{2+}; panel B, in the presence of 6 mM Mg^{2+}. The calmodulin concentration was 25 μM in the absence of peptide (●) and 5 μM in the presence of 5 μM peptide (▲). The data are corrected for binding to the membrane [j values were 0.025 (A) and 0.018 (B) in the absence of peptide and 0.03 (A) and 0.04 (B) in its presence] and for calmodulin concentration (<±6%) using the N values determined by the computer fitting of the data, as described in Table 3.

Table 3: Macroscopic Ca^{2+} Dissociation Constants for Calmodulin in the Presence and Absence of the Calmodulin-Binding Domain of Calcineurin[a]

additions		macroscopic dissociation constants (μM)[b]				
Mg^{2+} (mM)	peptide (μM)	K_1	K_2	K_3	K_4	n[c]
0	0	17.8 (5.1)	0.4 (0.2)	14.7 (2.0)	11.2 (3.1)	3
1	0	0.9 (0.2)	1.1 (0.04)	21.5 (0.5)	2.8 (0.5)	3
1	3–5	0.06 (0.01)	0.1 (0.01)	0.08 (0.01)	0.3 (0.04)	4
6	0	14.6 (5.9)	3.3 (0.03)	123.4 (31.1)	45.7 (0.02)	2
6	3–5	0.2 (0.06)	0.7 (0.4)	0.3 (0.2)	3.1 (2.7)	2

[a] The experimental conditions are described in Methods. The calmodulin concentration was 20–25 μM in the absence of peptide and 3–5 μM in the presence of an equimolar concentration of peptide. [b] Macroscopic dissociation constants K_1, K_2, K_3, and K_4 were determined as described in footnote b of Table 1. The value of factor N varied from 0.95 to 1.05. Standard errors of the mean for n = 3 or 4 and differences in values for n = 2 are indicated in parentheses. [c] Number of determinations.

Ca^{2+} Binding to Calcineurin B Is Required for Calmodulin Stimulation. The following rate equation was derived by Huang et al. (1981) to analyze the Ca^{2+}-dependent activation

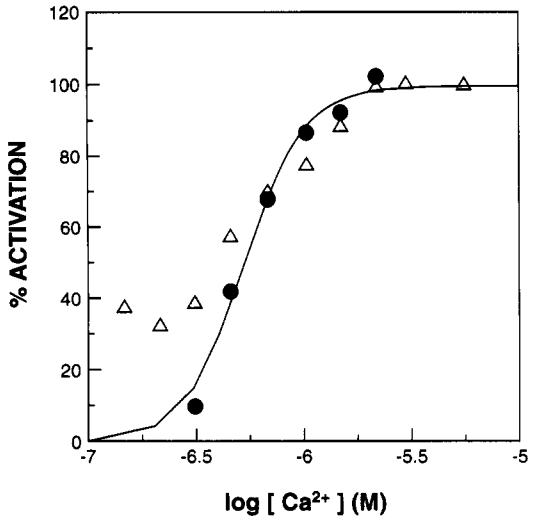

FIGURE 3: Ca^{2+} dependence of the activation of calcineurin in the absence and presence of saturating concentrations of calmodulin. Calcineurin activity was measured in the absence of calmodulin (△). The y intercepts of double-reciprocal plots of the % activation as a function of calmodulin concentration (data illustrated in Figure 1) were used to calculate the % activation at an infinite concentration of calmodulin (v) as a function of Ca^{2+} concentration (●). The line was generated by fitting the data to $v = (100[Ca^{2+}]^n/k)/(1 + [Ca^{2+}]^n/k)$.

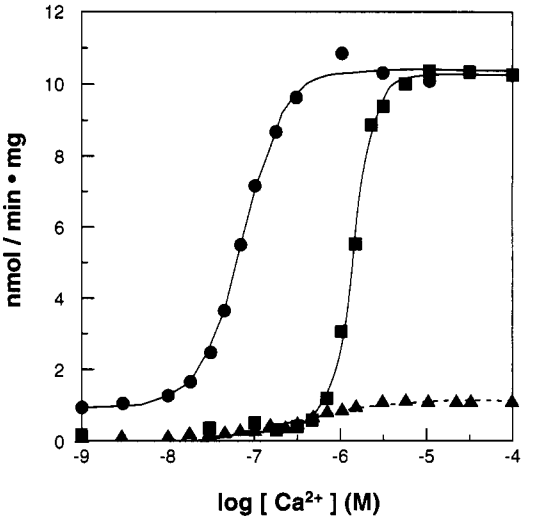

FIGURE 4: Ca^{2+}-dependent activation of the proteolyzed derivative of calcineurin (calcineurin-45). The calmodulin-independent protein phosphatase activities of calcineurin-45 (●) and native calcineurin without (▲) and with calmodulin (■) were measured in the presence of 1 μM substrate. EGTA was 5 mM.

of cyclic nucleotide phosphodiesterase in the presence of excess calmodulin:

$$\frac{1}{\Delta v} = \frac{1}{\Delta k e_t}\left(\frac{\Phi_1}{\Phi_2} + \frac{\Phi_3 K_e}{\Phi_2[\mathrm{CaM}]_t}\right)$$

In this equation, Δv is the calmodulin-stimulated activity minus the basal activity; Δk is the difference between the catalytic rate constants for the activated and nonactivated enzyme species; e_t is the calcineurin concentration; $[\mathrm{CaM}]_t$ is the total calmodulin concentration; and Φ_1, Φ_2, and Φ_3 are defined as follows:

$$\Phi_1 = 1 + C/K'_1 + C^2/K'_1K'_2 + C^3/K'_1K'_2K'_3 + C^4/K'_1K'_2K'_3K'_4$$

$$\Phi_2 = C^4/K'_1K'_2K'_3K'_4$$

$$\Phi_3 = 1 + C/K_1 + C^2/K_1K_2 + C^3/K_1K_2K_3 + C^4/K_1K_2K_3K_4$$

in which C is Ca^{2+} concentration; K_1, K_2, K_3, and K_4 are the macroscopic dissociation constants of calmodulin; and K'_1, K'_2, K'_3, and K'_4 are the corresponding macroscopic constants of the enzyme·calmodulin complex.

When the calmodulin concentration ($[\mathrm{CaM}]_t$) was in excess of calcineurin concentration (e_t)—0.3, 3, and 20 μM calmodulin, as opposed to 0.03 μM calcineurin (Figure 1)—as was reported for the activation of cyclic nucleotide phosphodiesterase, plots of $1/\Delta v$ as a function of $1/[\mathrm{CaM}]_t$ at different Ca^{2+} concentrations were linear (data not shown). Unlike the plots of the cyclic nucleotide phosphodiesterase (Huang et al., 1981), the calcineurin plots did not intercept on the ordinate. A plot of the extrapolated values of the Ca^{2+}-dependent phosphatase activity at an infinite concentration of calmodulin (derived from the y intercepts), expressed as the percent of maximum activity, is illustrated in Figure 3. At an infinite concentration of calmodulin, the Ca^{2+} dependence of the calmodulin stimulation of calcineurin closely resembles the Ca^{2+} dependence of the calcineurin B-mediated activation of calcineurin measured in the absence of calmodulin.[5] Thus, Ca^{2+} binding to calcineurin B appears to be a prerequisite for the calmodulin stimulation of calcineurin.

Calmodulin-Independent Activation of Calcineurin by Ca^{2+} Binding to Calcineurin B. In the absence of calmodulin, Ca^{2+} has only a small stimulatory effect on the phosphatase activity of calcineurin which is difficult to analyze quantitatively. Furthermore, the Ca^{2+}-dependent activation of native calcineurin is biphasic with a small, but reproducible, activation between 0.01 and 0.1 μM Ca^{2+}, which is probably the result of a 2–3% contamination of native calcineurin by calcineurin-45 (see below).

We selected the proteolyzed derivative of calcineurin, calcineurin-45, to characterize the calmodulin-independent, calcineurin B-mediated activation of calcineurin. As shown in Figure 4, in the absence of calmodulin, calcineurin-45 exhibits a low, but significant, level of activity at Ca^{2+} concentrations below 10 nM, and it is stimulated more than 10-fold by increasing Ca^{2+} concentration with a K_{act} of 0.06 μM. Native calcineurin, assayed in the absence of calmodulin, is inactive at less than 10 nM Ca^{2+} and is maximally activated with a K_{act} of 0.5 μM. The activation of calcineurin-45 is less cooperative than the calmodulin-dependent activation of calcineurin with a Hill coefficient of 1.8 (Table 2). The high affinity of calcineurin-45 for Ca^{2+} accounts for the difficulties encountered in the decalcification of calcineurin-45. In order to characterize the Ca^{2+}-binding properties of calcineurin-45, the protein was first equilibrated with a saturating concentration of $^{45}Ca^{2+}$ and the exchange of the protein-bound $^{45}Ca^{2+}$ with unlabeled Ca^{2+} was monitored by gel filtration on Sephadex G-25 columns equilibrated with decreasing concentrations of Ca^{2+}, as shown in Figure 5. During the 40 min required for the elution of the protein from the column, more than 95% of the bound $^{45}Ca^{2+}$ exchanges with unlabeled Ca^{2+} when the concentration of free Ca^{2+} is 2.1 μM and only 5% of $^{45}Ca^{2+}$ remains bound to calcineurin-45 (Figure 5A). When the concentration of Ca^{2+} is decreased to 0.06 μM, the amount of unlabeled free Ca^{2+} (a total of 0.6 nmol in the first 10 fractions of the column as opposed to 6 nmol of $^{45}Ca^{2+}$ in the protein sample) is insufficient to significantly decrease

[5] The high basal activity below 0.1 μM Ca^{2+} (Figure 4) is believed to be due to the presence of partially degraded calcineurin.

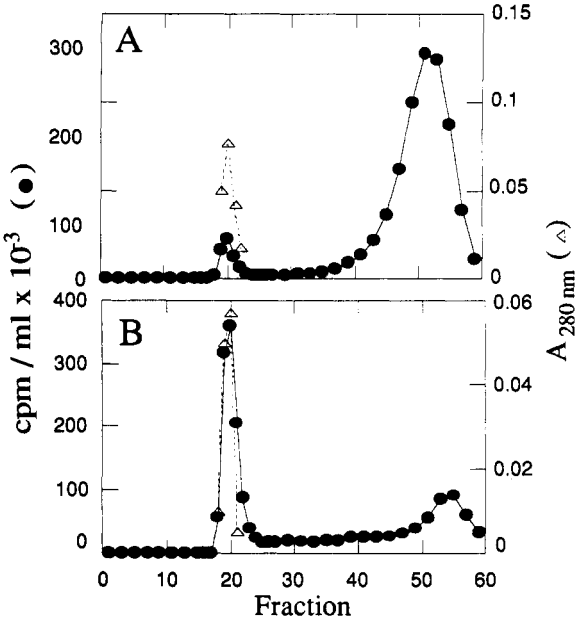

FIGURE 5: Displacement of $^{45}Ca^{2+}$ bound to calcineurin-45 by gel filtration in the presence of unlabeled Ca^{2+}. Calcineurin-45 (4.2 μM) was dialyzed for 48 h at 0–4 °C against buffer E (10 mM Tris Cl, pH 7.5, containing 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM dithiothreitol) made 0.7 μM $^{45}Ca^{2+}$ to a constant specific activity of 190 ± 20 cpm/pmol. Aliquots of 0.5 mL containing 9 nmol of Ca^{2+}, determined by atomic absorption, were applied to 22-mL (1 × 29 cm) Sephadex G-25 columns equilibrated at 22 °C with buffer E made 2.1 (panel A) or 0.06 (panel B) μM Ca^{2+}. The columns were monitored for radioactivity (●). Absorbance at 280 nm (△) was used to determine the protein concentration. Total Ca^{2+} in buffer and protein-containing fractions was determined by atomic absorption spectrophotometry.

Table 4: Ca^{2+} Binding to Calcineurin-45[a]

free Ca^{2+} (μM)	bound Ca^{2+} [b] (mol/mol)
0.07	2.3 ± 0.06
0.17	2.5 ± 0.31
0.33	2.7 ± 0.1
0.7	2.8
2.2	2.7 ± 0.22

[a] The experimental conditions are described in the caption to Figure 5. [b] ±Standard deviation from the mean of three determinations.

the specific activity of bound Ca^{2+}. Thus, most of the $^{45}Ca^{2+}$ remained associated with calcineurin-45 (Figure 5B), confirming the high affinity of the protein for Ca^{2+}. As summarized in Table 4, more than 2 mol of Ca^{2+} is bound per mole of calcineurin-45 at 0.07 μM Ca^{2+}, a concentration of Ca^{2+} sufficient to support 50% of the maximum activity. No significant increase in Ca^{2+} binding is observed above 0.3 μM Ca^{2+} where maximum activation is achieved. The Ca^{2+}-dependent activation of calcineurin-45 (Figure 4) appears to coincide with the occupancy of one or two "low-affinity" sites, whereas calcineurin-45 is inactive when one or two "high-affinity" sites are occupied below 0.03 μM Ca^{2+}. A Hill coefficient of 1.8 would be consistent with the involvement of two low-affinity Ca^{2+} sites in the activation process.[6]

Effect on Kinetic Parameters. A large increase in V_{max} with little effect on the K_m value accompanies the Ca^{2+}-dependent binding of calmodulin to calcineurin (Table 5). A moderate increase in V_{max} accompanies Ca^{2+} binding to calcineurin B with apparently no effect on the K_m value.

[6] Underestimation of the total number of Ca^{2+} sites may be due to overestimation of the protein concentration due to light scattering.

Table 5: Effect of Ca^{2+} on the Kinetic Parameters of Calcineurin-Mediated Peptide Dephosphorylation[a]

enzyme[b]	Ca^{2+} [c]	EGTA (mM)	K_m[d]	V_{max} [nmol/(min·mg)]
calcineurin (2)	0	5	52 ± 19	50 ± 9
calcineurin (3)	0.01	0	100 ± 13	320 ± 18
calcineurin/CaM (3)	0.01	0	94 ± 11	2012 ± 95
calcineurin-45 (2)	0	5	395 ± 63	1016 ± 113
calcineurin-45 (2)	0.01	0	68 ● 10	1079 ± 77

[a] The assay conditions are described in Methods. [b] The numbers in parentheses indicate the number of experiments. [c] Added Ca^{2+} was not corrected for contaminating Ca^{2+}. [d] ±Standard errors of the mean for $n = 3$ and differences in values for $n = 2$.

Removal of the regulatory domain of calcineurin, including both calmodulin-binding and autoinhibitory domains, not only has a large effect on the V_{max}, similar to that of the putative displacement of this regulatory domain induced by calmodulin binding to the calmodulin-binding domain, but also increases the K_m value by 8-fold. Reversal of this change in the K_m value requires the binding of Ca^{2+} to calcineurin B. Thus, Ca^{2+} binding to the two regulatory proteins, calmodulin and calcineurin B, has different effects on the kinetic parameters of the phosphatase activity of calcineurin.

DISCUSSION

The activation of calmodulin-stimulated enzymes by Ca^{2+} is the result of the Ca^{2+}-dependent interaction of calmodulin with these enzymes. Thus, the Ca^{2+} concentration required for activation of cyclic nucleotide phosphodiesterase and myosin light chain kinase was shown to decrease as the concentration of calmodulin is increased (Huang et al., 1981; Blumenthal & Stull, 1980; Olwin & Storm, 1985). In the presence of micromolar concentrations of calmodulin, all these enzymes are activated at levels of Ca^{2+} found in stimulated cells (0.5–1 μM). Likewise, the Ca^{2+} concentration required for half-maximal activation of calcineurin by calmodulin decreases from 1.3 to 0.6 μM as the calmodulin concentration is increased from 0.03 to 20 μM. It is not clear why these values of the K_{act} are lower than the value of 20 μM reported by others (Kincaid & Vaughan, 1986). As for most calmodulin-regulated enzymes, the activation of calcineurin is highly cooperative, allowing the enzyme to respond to very narrow Ca^{2+} thresholds (Stewart et al., 1982). Hill coefficients of 2.8–3.1 indicate that at least three Ca^{2+} sites are involved in the activation process. Since calcineurin is itself a Ca^{2+}-binding protein, these sites could be located on calcineurin B as well as on calmodulin.

The affinity of calmodulin for Ca^{2+} should increase in the presence of calcineurin. The lack of effect of calcineurin on the affinity of calmodulin for Ca^{2+} previously reported (Kincaid & Vaughan, 1986) is therefore unexpected. This conclusion was not based on direct Ca^{2+} binding to calmodulin but on the Ca^{2+} dependence of a conformational change affecting the environment of dansylated residues in calmodulin monitored in the presence and absence of calcineurin. Although increased fluorescence was observed at lower Ca^{2+} concentrations in the presence of calcineurin than in its absence, the concentrations of Ca^{2+} needed for the half-maximal increase in the fluorescence were similar in both cases. The preparation of dansylated calmodulin used in these studies contained inactive protein, which was possibly unable to interact with calcineurin. Ca^{2+} binding to this inactive derivative would not be affected by calcineurin, thereby obscuring the contribution of calcineurin to the affinity of the calmodulin·calcineurin complex for Ca^{2+}.

Direct measurement of the affinity of calmodulin for Ca^{2+} in the presence of calcineurin by flow dialysis is difficult because calcineurin B also contains four Ca^{2+}-binding sites. To avoid this complexity, we measured the affinity of calmodulin for Ca^{2+} in the presence of the calmodulin-binding peptide of calcineurin. In the absence of the peptide the four Ca^{2+} sites of calmodulin have relatively low affinities for Ca^{2+}, and the macroscopic constants are consistent with the generally accepted sequential and cooperative binding of Ca^{2+} to the two high-affinity carboxyl-terminal sites followed by occupancy of the two low-affinity amino-terminal sites [as reviewed by Forsen (1986)]. In the presence of 6 mM Mg^{2+}, calmodulin still binds four Ca^{2+}, although with decreased affinity. In the presence of the calmodulin-binding peptide, the affinities of all four sites are increased with a particularly large, 300–400-fold increase of the affinity of the third site. This peptide-dependent increase of the affinity of calmodulin for Ca^{2+} indicates that Ca^{2+} binding to calmodulin and not to calcineurin B is responsible for the large Ca^{2+}-dependent increase of the affinity of calcineurin for calmodulin (from $<10^{-5}$M to 10^{-10} M).[7] A similar increase in the affinity of calmodulin for Ca^{2+} in the presence of calmodulin-binding peptides (Maulet & Cox, 1983; Yasawa et al., 1987) or calmodulin-binding proteins (Olwin & Storm, 1985) has been reported. With the exception of the calmodulin-binding domain of the Ca^{2+} ATPase of the plasma membrane (Yasawa et al., 1992), these increases in affinity are less pronounced than in the case of calcineurin. The sequential binding of Ca^{2+} to calmodulin is altered differently by different targets. A large decrease in K_4 is observed with myosin light chain kinase (Olwin et al., 1984), whereas the calmodulin-binding peptide of the ATPase exerts its largest effect on K_2 (Yasawa et al., 1992). These differences could reflect different modes of interaction of calmodulin with different targets (Newton et al., 1984; Ni & Klee, 1985). Occupancy of the four Ca^{2+} sites is required for activation of myosin light chain kinase, whereas the ATPase, which is activated by the carboxyl-terminal fragment of calmodulin (Guerini et al., 1984), may affect primarily the filling of the two carboxyl-terminal sites.

It is not possible on the basis of the Ca^{2+}-binding data alone to identify the sequence in which the sites are successively filled. It was proposed that occupancy of the two carboxyl-terminal Ca^{2+} sites, which is sufficient to promote calcineurin binding to calmodulin, although with low affinity (Ni & Klee, 1985), also increases the flexibility of the central helix (Mackall & Klee, 1991). The structure of the complex of calmodulin with a calmodulin-binding peptide indicates that distortion of the central helix is required to allow interaction of the amino-terminal half of calmodulin with the calmodulin-binding peptide bound to the carboxyl-terminal half of calmodulin (Ikura et al., 1992; Meador et al., 1992). Since peptide binding to either of the two halves of calmodulin depends on the Ca^{2+}-induced formation of a hydrophobic patch, the proximity of the peptide to the amino-terminal domains of calmodulin would facilitate interaction and thereby increase the affinity of a third site postulated to be on the amino-terminal half of calmodulin. The two cooperative sites predicted by the Hill plots could be located on opposite halves of calmodulin as opposed to adjacent sites as is the case in the absence of the target peptide. A similar model has been proposed by Yasawa et al. (1987) to explain the positive cooperative binding of Ca^{2+} to the two halves of calmodulin in the presence of mastoporan.

Despite the important role of calcineurin B in the regulation of the phosphatase activity of calcineurin, little is known about its mechanism of action. Calcineurin B is required to reconstitute the phosphatase activity of calcineurin from its isolated subunits (Merat et al., 1985). It is also required for the function of calcineurin in promoting adaptation of yeast cells to pheromone treatment (Cyert et al., 1992). Kinetic analysis of the Ca^{2+}-calmodulin-dependent activation of calcineurin using the model proposed by Huang et al. (1981) suggests that calmodulin stimulation requires Ca^{2+} binding to calcineurin B, in agreement with the reports that calcineurin B is required for the reconstitution of a calmodulin-stimulated protein phosphatase from calcineurin A expressed in *Escherichia coli* or SF-9 cells (Perrino et al., 1992; Ueki & Kincaid, 1993). The affinity of two of the Ca^{2+} sites of calcineurin B is very high ($K_d < 10^{-8}$ M). These sites, occupied at resting Ca^{2+} levels, may serve a structural role by ensuring the tight interaction of the two subunits, while occupancy of the remaining sites would be responsible for the calmodulin-independent as well as the calmodulin-dependent activation. Like calcineurin-45, native calcineurin has at least two high-affinity sites and one or two low-affinity sites whose occupancy is required for the calcineurin B-mediated activation.[8]

Removal of the regulatory domain of calcineurin A by proteolysis results in a large increase in the V_{max} similar to that resulting from the proposed displacement of the autoinhibitory domain induced by the Ca^{2+}-dependent binding of calmodulin. The modified enzyme remains Ca^{2+}-dependent and can therefore be used to study the role of calcineurin B in the Ca^{2+}-dependent activation of calcineurin. In contrast to the native enzyme whose V_{max} is dependent on Ca^{2+} and calmodulin, the V_{max} of the proteolyzed enzyme is calmodulin-independent. Removal of the regulatory domain is also accompanied by a 5–6-fold increase of the K_m value, and the Ca^{2+} dependence of the activity of calcineurin-45 is due to a Ca^{2+}-induced decrease of the K_m value of the enzyme. The Ca^{2+}-induced decrease of the K_m appears to reflect an increase in the affinity of calcineurin for its substrate since the k_2 terms in the expression of the K_m [$K_m = (k_{-1}/k_1) + (k_2/k_1)$] are apparently identical in the presence and absence of Ca^{2+}. Likewise, Ca^{2+} binding to calcineurin B is responsible for the increased affinity of native calcineurin for its substrate, which allowed Tonks and Cohen (1983) to purify calcineurin by affinity chromatography on phosphorylated myosin light chain coupled to Sepharose.

The data presented in this paper indicate that the two regulatory proteins, calcineurin B and calmodulin, play different roles in the Ca^{2+} stimulation of calcineurin. Ca^{2+} binding to calmodulin displaces the autoinhibitory domain and increases V_{max}, whereas Ca^{2+} binding to calcineurin B increases the affinity of calcineurin for its substrate. The absolute requirement of Ca^{2+} binding to calcineurin B ensures that the enzyme is dependent on stimulated levels of Ca^{2+} for activity and controls the Ca^{2+}-independent activation of the enzyme by proteolysis occurring *in vivo*.

[7] Binding of nitrocalmodulin to calcineurin was not detected at concentrations of nitrocalmodulin and calcineurin up to 10 μM (P. Richman and C. B. Klee, unpublished observations).

[8] Calcineurin and calcineurin-45 retain equivalent amounts of Ca^{2+} after treatment with Bio-Rad Chelex, indicating that the high-affinity Ca^{2+}-binding sites are not affected by proteolysis but that the "activation sites" have higher affinity for Ca^{2+} in calcineurin-45 as suggested by the low K_{act} of calcineurin-45 compared to the K_{act} of native calcineurin (P. M. Stemmer, unpublished observations).

REFERENCES

Aitken, A., Cohen, P., Santikarn, S., Williams, D. H., Calder, A. G., & Klee, C. B. (1982) *FEBS Lett. 150*, 314–317.

Anglister, J., Grzesiek, S., Wang, A. C., Ren, H., Klee, C. B., & Bax, A. (1994) *Biochemistry 33*, 3540–3547.
Blumenthal, D. K., & Stull, J. T. (1980) *Biochemistry 19*, 5608–5614.
Blumenthal, D. K., Takio, K., Hansen, R. S., & Krebs, E. G. (1986) *J. Biol. Chem. 261*, 8140–8145.
Colowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.
Crouch, T. H., & Klee, C. B. (1980) *Biochemistry 19*, 3692–3698.
Cyert, M. S., & Thorner, J. (1992) *Mol. Cell. Biol. 12*, 3460–3469.
Cyert, M. S., Kunisawa, R., Kaim, D., & Thorner, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7376–7380.
Edsall, J. T., & Wymann, J. (1958) in *Biophysical Chemistry* Vol. 1, pp 591–662, Academic Press, New York.
Fabiato, A., & Fabiato, F. (1979) *J. Physiol. (Paris) 75*, 463–505.
Feldman, K. (1978) *Anal. Biochem. 88*, 225–235.
Forsen, S., Vogel, H. J., & Drackenberg, T. (1986) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 6, pp 113–157, Academic Press, New York.
Guerini, D., & Klee, C. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9183–9187.
Guerini, D., Krebs, J., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15172–15177.
Guerini, D., Krinks, M. H., Sikela, J. M., Hahn, W. E., & Klee, C. B. (1989) *DNA 8*, 675–682.
Guerini, D., Montell, C., & Klee, C. B. (1992) *J. Biol. Chem. 267*, 22542–22549.
Haddy, A., Swanson, S. K.-H., Born, T. L., & Rusnak, F. (1992) *FEBS Lett. 314*, 37–40.
Haiech, J., Klee, C. B., & Demaille, J. G. (1981) *Biochemistry 20*, 3890–3897.
Higuchi, S., Tamura, J., Giri, P. R., Polli, J. W., & Kincaid, R. L. (1991) *J. Biol. Chem. 266*, 18104–18112.
Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., & Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 871–874.
Hubbard, M. J., & Klee, C. B. (1987) *J. Biol. Chem. 262*, 15062–15070.
Hubbard, M. J., & Klee, C. B. (1989) *Biochemistry 28*, 1868–1874.
Hubbard, M. J., & Klee, C. B. (1991) in *Molecular Neurobiology, A Practical Approach* (Chad, J., & Wheal, H., Eds.) pp 135–157, IRL Press, Oxford.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632–638.
Ito, A., Hashimoto, T., Hirai, M., Takeda, T., Shuntoh, H., Kuno, T., & Tanaka, C. (1989) *Biochem. Biophys. Res. Commun. 163*, 1492–1497.
Jean, T., & Klee, C. B. (1986) *J. Biol. Chem. 261*, 16414–16420.
Kincaid, R. L., & Vaughan, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1193–1197.
Kincaid, R. L., Giri, P. R., Higuchi, S., Tamura, J., Dixon, S. C., Marietta, C. A., Amorese, D. C., & Martin, B. M. (1991) *J. Biol. Chem. 265* 11312–11319.
Kincaid, R. L., Nightingale, M. S., & Martin, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8983–8987.
Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.
Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 213–321.
Klee, C. B., Draetta, G., & Hubbard, M. J. (1987) *Adv. Enzymol. 61*, 149–200.
Klee, C. B., Krinks, M. H., Manalan, A. S., Cohen, P., & Stewart, A. A. (1983) *Methods Enzymol. 102*, 227–244.
Klee, C. B., Krinks, M. H., Manalan, A. S., Draetta, G. F., & Newton, D. L. (1985) *Adv. Protein Phosphatases 1*, 135–146.
Kuno, T., Tanaka, H., Mukai, H., Chang, C.-D., Hiraga, K., Miyakawa, T., & Tanaka, C. (1991) *Biochem. Biophys. Res. Commun. 180*, 1159–1163.
Kuno, T., Takeda, T., Hirai, M., Ito, A., Mukai, H., & Tanaka, C. (1989) *Biochem. Biophys. Res. Commun. 165*, 1352–1358.
Li, W., & Handschumacher, R. E. (1993) *J. Biol. Chem. 268*, 14040–14044.
Liu, Y., Ishii, S., Tokai, M., Tsutsumi, H., Ohki, O., Akada, R., Tanaka, K., Tsuchiya, E., Fukui, S., & Miyakawa, T. (1991) *MGG, Mol. Gen. Genet. 227*, 52–59.
Mackall, J., & Klee, C. B. (1991) *Biochemistry 30*, 7242–7247.
Maulet, Y., & Cox, J. A. (1983) *Biochemistry 22*, 5680–5686.
Meador, W. E., Means, A. R., & Quiocho, F. A. (1992) *Science 257*, 1251–1255.
Merat, D. H., Hu, Z. Y., Carter, T. E., & Cheung, W. Y. (1985) *J. Biol. Chem. 260*, 11053–11059.
Minowa, O., & Yagi, K. (1984) *J. Biochem. (Tokyo) 56*, 1175–1182.
Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) *J. Biol. Chem. 259*, 4419–4426.
Newton, D. L., Krinks, M. H., Kaufman, J. B., Shiloach, J., & Klee, C. B. (1988) *Prep. Biochem. 18*, 247–259.
Ni, W-C., & Klee, C. B. (1985) *J. Biol. Chem. 260*, 6974–6981.
Olwin, B. B., & Storm, D. R. (1985) *Biochemistry 24*, 8081–8086.
Olwin, B. B., Edelman, A. M., Krebs, E. G., & Storm, D. R. (1984) *J. Biol. Chem. 259*, 10949–10955.
Perrino, B. A., Fong, Y.-L., Brickey, D. A., Saitoh, Y., Ushio, Y., Fukanaga, K., Miyamoto, E., & Soderling, T. R. (1992) *J. Biol. Chem. 267*, 15965–15969.
Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B., & Cohen, P. (1982) *FEBS Lett. 137*, 80–84.
Tonks, N. H., & Cohen, P. (1983) *Biochim. Biophys. Acta 747*, 191–193.
Ueki, K., & Kincaid, R. L. (1993) *J. Biol. Chem. 268*, 6554–6559.
Ueki, K., Muramatsu, T., & Kincaid, R. L. (1992) *Biochem. Biophys. Res. Commun. 187*, 537–543.
Woyski, M. M., & Harris, R. E. (1963) in *Treatise on Analytical Chemistry, Part II*, Vol. 8, p 57, Interscience, New York.
Yazawa, M., Ikura, M., Hikichi, K., Ying, L., & Yagi, K. (1987) *J. Biol. Chem. 262*, 10951–10954.
Yazawa, M., Vorherr, T., James, P., Carafoli, E., & Yagi, K. (1992) *Biochemistry 31*, 3171–3176.
Ye, R. R., & Bretscher, A. (1992) *Eur. J. Biochem. 204*, 713–723.