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Direct comparison of Ca^{2+} requirements for calmodulin interaction with and activation of protein phosphatase

(calcineurin/dansyl-calmodulin/complex formation/enzyme mechanism)

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ABSTRACT **The mechanism of Ca^{2+}-dependent protein–protein interaction and enzyme activation by calmodulin was investigated with the phosphoprotein phosphatase, calcineurin. Dimethylaminonaphthalene (dansyl)-calmodulin, a fluorescent derivative used to monitor complex formation, produced similar maximal activation (10- to 12-fold) with a Ca^{2+} dependence (K_a = 17 μM) identical to that of native calmodulin. The Ca^{2+}-dependent increase in fluorescence intensity of dansyl-calmodulin was enhanced 100–150% by calcineurin, indicating complex formation; the concentration of Ca^{2+} required for a half-maximal increase in fluorescence was the same ($K_{1/2} \approx 7$ μM) with and without calcineurin. Since the Ca^{2+} concentration required for activation appeared to differ from that necessary for protein–protein interaction, a method was devised to measure both the formation of complexes between dansyl-calmodulin and calcineurin and enzyme activity in the same samples. Direct comparison of interaction (measured by polarization of fluorescence) and enzyme activity demonstrated different Ca^{2+} requirements for the two events. Whereas dansyl-calmodulin–calcineurin interaction, measured in the presence of phosphoprotein substrate, exhibited very little cooperativity (Hill coefficient = 1.2, Ca^{2+} concentration required for the half-maximal increase in fluorescence, $K_{1/2}$, $\approx$ 6 μM), phosphatase activation was highly cooperative (Hill coefficient = 3.5) and required 3 times higher Ca^{2+} concentration for half-maximal stimulation. Equivalent results were obtained with *p*-nitrophenyl phosphate as substrate. These data are consistent with a sequential mechanism for interaction and activation wherein filling of perhaps two Ca^{2+} sites permits calmodulin interaction with the phosphatase; this complex is inactive, requiring further binding of Ca^{2+} for activation. Such a scheme would provide a sensitive switch for control of enzyme activity within a narrow range of free Ca^{2+} concentration.**

Calmodulin, a highly conserved Ca^{2+}-binding protein (1), has four Ca^{2+}-binding sites that apparently differ in affinity (2, 3). At physiologic ionic strength and Mg^{2+} concentration, the two higher affinity sites are reported to have dissociation constants of 3–8 μM while the other sites have estimated binding constants of 20–150 μM (2–5). In the Ca^{2+}-bound state, calmodulin can interact with and activate a number of enzymes (for reviews see refs. 6 and 7). A question of primary importance in understanding the function of this signalling system relates to the Ca^{2+} requirement for the effect of calmodulin; that is, must all Ca^{2+} sites be filled to produce activation or must only specific sites be filled? In this regard, the activation by calmodulin of several of its target enzymes, cyclic nucleotide phosphodiesterase (4, 8), phosphorylase *b* kinase (9), Ca^{2+}-dependent, Mg^{2+}-activated ATPase (10), and myosin light chain kinase (11), has been examined using kinetic analysis of enzyme stimulation. These studies focused on the Ca^{2+} binding to calmodulin that is required to produce activation and, in each case, it was concluded that occupancy of three or four sites was necessary for activation. In several of the studies, enzyme activation and interaction with calmodulin were assumed to be equivalent; certainly, it was not possible to discriminate between the two events since, in most of these, only enzyme activity was monitored. It is, of course, feasible that interaction of calmodulin with a target enzyme is not sufficient for activation; i.e., an enzymatically inactive complex might be formed at subsaturating amounts of Ca^{2+}, requiring further Ca^{2+} for activation.

To investigate this possibility, we employed a fluorescent derivative of calmodulin, dimethylaminonaphthalene (dansyl)-calmodulin, that has been used to monitor the Ca^{2+}-dependent interaction with several calmodulin-binding proteins (12, 13) or model peptides (14) and the calmodulin-activated protein phosphatase calcineurin (15). By measuring the amount of calmodulin–calcineurin complex in the presence of enzyme substrate, and quantifying product formation in that same sample, interaction and activation at different Ca^{2+} concentrations were directly compared.

METHODS

Purification and Preparation of Proteins. Calmodulin and calcineurin, purified from bovine brain as described (12, 16), were stored at −20°C either lyophilized or, in the case of calcineurin, in 40% (vol/vol) glycerol. Both proteins were essentially homogenous by analytical ultracentrifugation and by $NaDodSO_4$ gel electrophoresis. Phosphorylated myosin light chains from turkey gizzard (1 mol of phosphate/mol of peptide) prepared as reported (17) were the generous gift of James R. Sellers. Dansyl-calmodulin (0.65 mol of dansyl/mol of protein) prepared as described (12) was, after exhaustive dialysis against 20 mM Tris·HCl buffer, pH 7.8, containing 100 mM NaCl and 0.1 mM EGTA (buffer A), distributed in portions and stored frozen at −20°C. Prior to use, all proteins were desalted in buffer A on small gel filtration columns (P-6, Bio-Rad).

Assay of Phosphatase Activity. The *p*-nitrophenyl phosphatase activity of 0.6 μM calcineurin (18) was assayed with 20 mM Tris·HCl, pH 7.8, containing ovalbumin (0.2 mg/ml), 0.1 mM EGTA, 5 mM $MgCl_2$, and 20 mM ditris *p*-nitrophenyl phosphate (Sigma) in a total volume of 200 μl. For assays of calmodulin-stimulated activity, 0.2 mM Ca^{2+} and 1 μM calmodulin were included in the incubation. After 3–10 min at 30°C, reactions were terminated by the addition of 0.8 ml of 1 M sodium carbonate, pH 10, and absorbance at 412 nm was measured. Enzyme activity was calculated by using a molar extinction coefficient of 18,000 for *p*-nitrophenol. Phosphoprotein phosphatase activity was assayed in the same system except that 1 μM [^{32}P]myosin light chains replaced *p*-nitrophenyl phosphate. Reaction was terminated by addition of 0.5 ml of 20% (wt/vol) trichloroacetic acid. After centrifugation for 2 min in a microcentrifuge (Beck-

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man), a sample of supernatant was taken for radioassay. In protein phosphatase assays, less than 30% of substrate was hydrolyzed.

Measurement of Fluorescence. Fluorescence measurements were made by using a Perkin–Elmer spectrophotofluorimeter, model 650-40, equipped with microprocessor for automatic calculation of fluorescence polarization. All particulars were as described (12), except that measurements were carried out at 26°C.

Materials. A 0.5 M solution of Ultrapure Tris·HCl (Schwarz/Mann), pH 7.8, was exhaustively treated with Chelex (Bio-Rad) to remove contaminating metals. $CaCl_2$ was obtained as a 0.1 M analytical standard from Orion (Cambridge, MA). $MgCl_2$ was Puratronic grade from Ventron (Danvers, MA). EGTA (Eastman Kodak) was routinely recrystallized from distilled water (80°C), dried to constant weight, and stored as the free acid; this step removed fluorescent impurities observed in some batches. Stock solutions of $CaCl_2$ and EGTA were stored frozen to minimize evaporation, since even a 5% change can greatly affect calculated free Ca^{2+} concentration. Chelex-treated distilled water (<0.5 μM Ca^{2+}) was used throughout.

Calculations of Free Ca^{2+} Concentration. Calculations of free Ca^{2+} in the presence of 100 μM EGTA were made by using the stability constant of 4.4×10^6 liter/mol, as recommended by Bartfai (19) for Tris·HCl buffers of ≈pH 7.6. No corrections have been made for binding of Ca^{2+} to calmodulin or calcineurin.

RESULTS

Dansyl-calmodulin was shown previously to activate cyclic nucleotide phosphodiesterase activity with essentially the same affinity and efficacy as native calmodulin (12). On equilibrium sedimentation and gel filtration, the dansylated protein showed no apparent heterogeneity and displayed hydrodynamic properties identical to those of the native molecule. However, dansylation can occur at lysine, tyrosine, and histidine residues (20), and the presence of differently dansylated molecules may present problems in analysis of results. Dansyl amino acids were identified by thin-layer chromatography (21) after acid hydrolysis of dansyl-calmodulin. When dansyl-calmodulin was prepared in the presence of Ca^{2+}, virtually all fluorescence migrated with monodansyllysine, whereas preparation in the presence of EGTA yielded ≈30% of the fluorescence as dansyl-tyrosine (R.L.K., unpublished data). Without added Ca^{2+} or EGTA, essentially only dansyl-lysine was observed, consistent with partial saturation of Ca^{2+} sites under this condition. Since the lyophilized calmodulin used for dansylation contained ≈1.5 mol of Ca^{2+}/mol of protein when analyzed by atomic absorption spectroscopy, it would appear that occupancy of these sites effectively prevents tyrosine modification. After treatment with CNBr and high performance anion-exchange chromatography, >85% of dansyl fluorescence was associated with a single, acidic peptide (Fig. 1); a second peak of fluorescence eluted at or near the unretained volume of the column. In agreement with the data from acid hydrolysis, the major fluorescent peak did not exhibit tyrosine fluorescence. Thus, the fluorescence of dansyl-calmodulin is localized predominantly to a single domain in the protein.

Maximal phosphatase activation was obtained with equimolar native calmodulin and calcineurin, whereas ≈20–25% more dansyl-calmodulin was needed for comparable maximal stimulation (Fig. 2). Similarly, maximal interaction between dansyl-calmodulin and phosphatase required a molar ratio of 1.15:1 (*Inset*, Fig. 2). These data suggested that a small percentage of dansyl-calmodulin molecules did not interact with the phosphatase; however, by far the majority of the preparation (≈80%) interacted with and activated the phosphatase. Furthermore, the Ca^{2+} dependence of activation by native and dansyl-calmodulin appeared virtually identical, with half-maximal activation at ≈17 μM free Ca^{2+} (Fig. 3). Activation was highly cooperative with stimulation increasing from 10% of the maximum to 90% with only a 3-fold change in Ca^{2+} concentration; the calculated Hill coefficient was ≈3.5 (*Inset*, Fig. 3). Activity

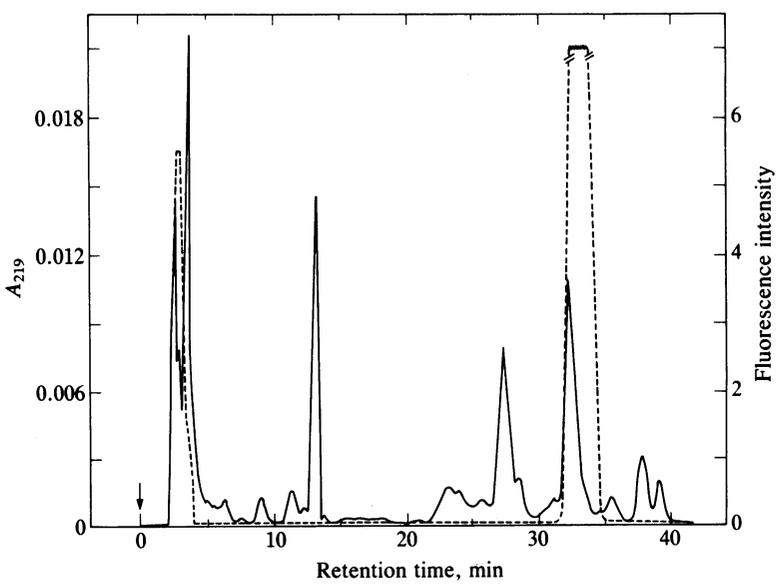

FIG. 1. Anion-exchange high performance liquid chromatography of peptides produced by CNBr cleavage of dansyl-calmodulin. Lyophilized dansyl-calmodulin (2.5 mg, 0.15 μmol) was dissolved in 165 μl of 77% (vol/vol) formic acid, added to 70 μl of 86 mM CNBr (6 μmol), and incubated for 28 hr at room temperature (22). After lyophilization, the peptides were taken up in 2.0 ml of distilled water and 25 μl was injected onto a DuPont Zorbax-SAX column (25 × 0.46 cm) equilibrated in 50 mM potassium phosphate, pH 4.1. The column was then eluted with a gradient of 50–300 mM potassium phosphate, pH 4.1, and the effluent continuously monitored for UV absorption at 219 nm (—) and dansyl fluorescence (---) (excitation 340 nm, emission 520 nm). Flow rate was 1 ml/min and temperature 26°C. The optical density tracing was corrected for increasing absorbance due to the phosphate gradient. For the experiment shown in this figure, two peaks of tyrosine fluorescence (excitation 280 nm, emission 340 nm) also were observed; one eluted at ≈2.8 min, the second at ≈27.8 min. When the two peaks of dansyl fluorescence were compared, ≈90% of the total fluorescence intensity (emission 490 nm) was found in the second peak.

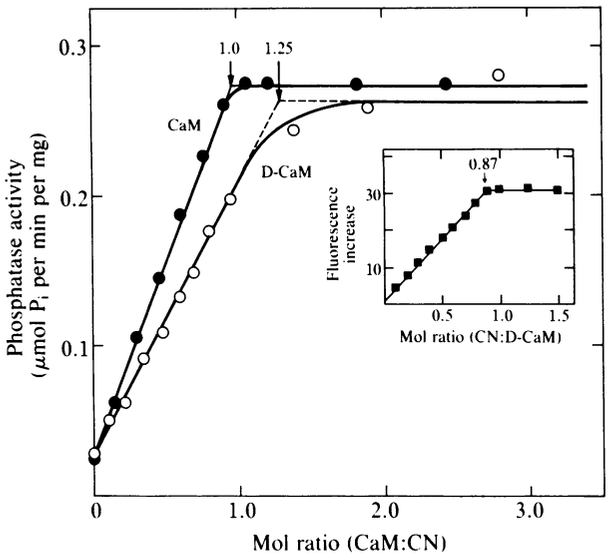

FIG. 2. Stoichiometry of phosphatase activation by native and dansyl-calmodulin. The phosphatase activity of 0.6 μM calcineurin (CN) was measured with 20 mM *p*-nitrophenyl phosphate and 5 mM $MgCl_2$, at the indicated mol ratios of native calmodulin (CaM) (●) or dansyl-calmodulin (D-CaM) (○) to calcineurin. (*Inset*) Stoichiometry of interaction between calcineurin and dansyl-calmodulin. The increase in fluorescence of 0.25 μM dansyl-calmodulin (■), in the presence of 100 μM free Ca^{2+}, was measured after successive additions of calcineurin to give the indicated mol ratios. Note that the ratio of 0.87 corresponds to a CaM:CN ratio of ≈1.15. The maximum change in fluorescence intensity (ordinate) produced by addition of calcineurin was 135% in this experiment. Arrows indicate the extrapolated equivalence points for stoichiometric effect.

decreased ≈25–30% with increasing Ca^{2+} concentration above 100 μM (Fig. 3). The same effects of Ca^{2+} concentration were observed in assays with 2 or 5 mM *p*-nitrophenyl phosphate (data not shown).

Dansyl-calmodulin undergoes a Ca^{2+}-dependent shift in emission maximum and an increase in fluorescence intensity that is further enhanced in the presence of binding proteins (12). When measured at pH 7.6 in the presence of 5 mM $MgCl_2$, the half-maximal change was observed at 6–7 μM (6.8 ± 0.8 μM, $n = 4$). The presence of calcineurin dramatically increased this intensity but did not noticeably alter the Ca^{2+} dependence of the effect (Fig. 4). Thus, it appeared that the Ca^{2+} requirement for complex formation might be less than that for activation. To examine this directly, interaction with dansyl-calmodulin and phosphatase activity (with phosphorylated myosin light chains as substrate) were measured in the same sample (Fig. 5). In all experiments, the Ca^{2+} required for half-maximal activation (≈20 μM) was ≈3 times that for interaction (≈6 μM); these values were in good agreement with those obtained in the absence of substrate (Fig. 4). The calculated Hill coefficient for interaction was ≈1.2, whereas that for activation was 3.3, suggesting strong cooperativity for activation in contrast to that for interaction. Identical data were obtained using the chromogenic substrate, *p*-nitrophenyl phosphate, indicating that the effect was independent of the enzyme substrate (data not shown).

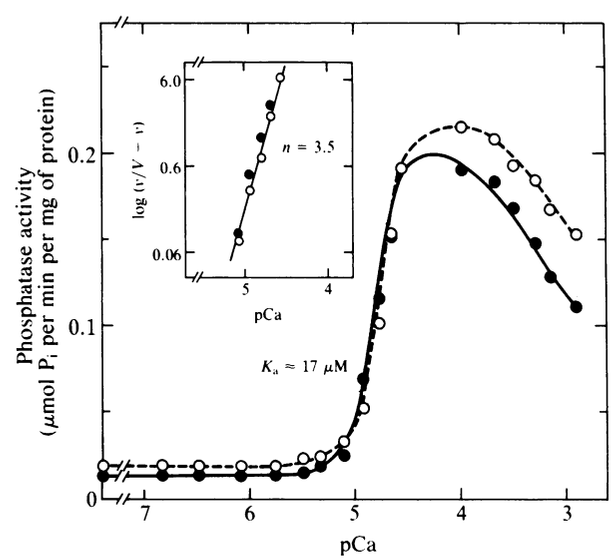

FIG. 3. Ca^{2+} dependence of phosphatase activation by native and dansyl-calmodulin. The phosphatase activity of 0.6 μM calcineurin (CN) was measured with 20 mM *p*-nitrophenyl phosphate and 5 mM $MgCl_2$ at the indicated concentrations of free Ca^{2+} in the presence of 0.75 μM dansyl-calmodulin (○) or native calmodulin (●). (*Inset*) Fractional activation of phosphatase ($v/V - v$) is plotted at different free Ca^{2+} concentrations where v is the velocity at a given Ca^{2+} concentration and V is the maximal velocity. The slope gives the Hill coefficient (n).

DISCUSSION

The ability of calmodulin to bind Ca^{2+} at multiple, nonidentical sites provides a number of possibilities for activation mechanism. In the two simplest extreme cases, calmodulin might interact with its binding protein in the absence of Ca^{2+} but require partial or complete occupancy of its Ca^{2+}-binding sites to produce activation. Alternatively, calmodulin might require complete saturation to interact with and activate a specific enzyme. The spectrum of possibilities intermediate between these extremes might permit specificity in activation of particular enzymes, an idea suggested by Haeich *et al.* (23). To date, the Ca^{2+}-independent association of calmodulin with soluble enzymes has not been reported, although such an interaction with a membrane protein has been

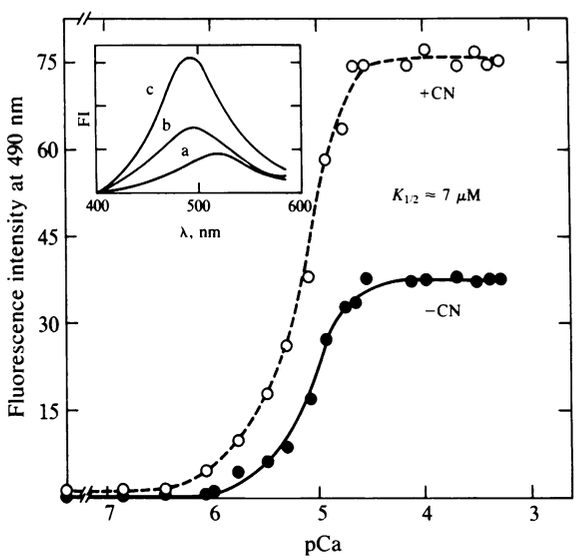

FIG. 4. Ca^{2+} dependence of dansyl-calmodulin fluorescence in the presence and absence of calcineurin. The fluorescence intensity of 0.6 μM dansyl-calmodulin was measured at the indicated concentrations of free Ca^{2+} and 5 mM $MgCl_2$ in the presence (○) or absence of 0.6 μM calcineurin (CN) (●). Basal fluorescence (that in the absence of added Ca^{2+}) has been subtracted from each data point. (*Inset*) Fluorescence emission spectra of dansyl-calmodulin in the presence of 0.1 mM EGTA (curve a), after addition of 0.2 mM Ca^{2+} (curve b), and in the presence of Ca^{2+} plus calcineurin (curve c). The emission maximum in curve a was 525 nm and in curves b and c was ≈490 nm. FI, fluorescence intensity.

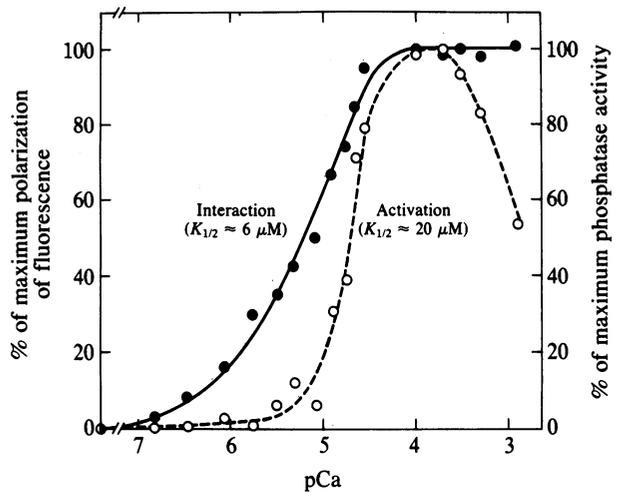

FIG. 5. Comparison of the Ca^{2+} dependence of phosphatase activation and interaction with dansyl-calmodulin in the same samples. Assay mixtures containing 0.6 μM calcineurin (CN), 0.75 μM dansyl-calmodulin, the indicated concentration of Ca^{2+} and 5 mM $MgCl_2$, but lacking substrate were prepared in 1.5-ml plastic microcentrifuge tubes. Reaction was initiated by the addition of 1 μM ^{32}P-labeled myosin light chains. The sample was transferred to a quartz microcuvette and five polarization measurements were made over a 2.5-min period. The mean is plotted (●). Sample was then returned to the centrifuge tube and, at exactly 3 min, 0.5 ml of 20% (wt/vol) trichloroacetic acid was added to terminate the reaction. After centrifugation, supernatant radioactivity was measured. The data are expressed as the percent of maximum change (at 100 μM Ca^{2+}) from the basal (no added Ca^{2+}) condition (○). Basal and maximum values for polarization were 0.096 and 0.155, respectively, and for enzyme activity 0.17 and 2 nmol of P_i released per min per mg of calcineurin.

described (24). However, it is widely assumed that interaction of calmodulin with its target enzymes is Ca^{2+}-dependent and that formation of the protein complex is sufficient for activation, provided other necessary cofactors are present.

A number of kinetic studies have concluded that occupancy of either three or four Ca^{2+} sites on calmodulin is required for enzyme activation and, by inference, interaction (4, 8–11). Paradoxically, physical studies of calmodulin using intrinsic tyrosine fluorescence (25), circular dichroism (26), nuclear magnetic resonance (27), and fluorescence of derivatized calmodulin (13) indicate that a major conformational event is complete upon saturation of only two Ca^{2+} sites. In this regard, Dedman *et al.* (28) also noted that the major conformational transition occurred at lower Ca^{2+} concentrations than did activation of phosphodiesterase. This would appear to suggest several independent Ca^{2+}-induced conformational states, some of which are not capable of interaction with a calmodulin-binding protein. One might envision, alternatively, a sequential process of Ca^{2+} binding to calmodulin in which interaction precedes activation of a target enzyme. Such a view is consistent with ordered binding of Ca^{2+} to nonidentical sites (23, 29) and also with the finding that protein–protein interaction appeared to require less Ca^{2+} than did activation of phosphodiesterase under similar, but not identical, experimental conditions (13). Interpretation of the latter experiments was complicated by the inability to assay enzyme activity at protein concentrations of ≈1 μM, needed for measurement of complex formation, and by the lack of enzyme substrate during measurement of interaction.

We describe here an approach that allows quantification of dansyl-calmodulin–calcineurin complexes as well as phosphatase activity in the same experimental samples. Other than dansyl-calmodulin, calcineurin, and phosphatase substrate, the only components in the reaction mixture were Tris·HCl at pH 7.6, $MgCl_2$ as enzyme cofactor, crystalline ovalbumin (5 μM) to minimize nonspecific protein interactions, and an EGTA/Ca^{2+} buffer to permit calculation of free Ca^{2+} concentrations.* Thus, all elements in the system were well defined. Dansyl-calmodulin appears to be an appropriate analogue of the native molecule with respect to its ability to activate, *per se*, and the Ca^{2+} dependency of activation was indistinguishable from that observed with native calmodulin. The 10- to 12-fold stimulation observed for calmodulin-activated dephosphorylation of myosin light chains is similar to that reported by others (30, 31).

Activation appeared to be a positively cooperative process (Hill coefficient of 3.3–3.5), suggesting perhaps a linkage between several Ca^{2+}-binding sites involved in interaction/activation. By contrast, the interaction of dansyl-calmodulin with calcineurin showed little cooperativity (Hill coefficient of 1.1–1.3) and required only 6–7 μM Ca^{2+} for half-maximal complex formation.† The data indicate that when ≈50% of the enzyme molecules exist in complex with calmodulin, enzyme activation is less than 10% of maximal, consistent with the conclusion that complex formation alone is not sufficient for enzyme activity. At this point, only a slight

*In our assays, the concentrations of Ca total and EGTA total that gave half-maximal enzyme stimulation were 112–115 μM and 100 μM, respectively. For half-maximal complex formation, Ca total was 101–102 μM at 100 μM EGTA.

†We have examined interaction/activation in the same samples using dansyl-calmodulin and cyclic nucleotide phosphodiesterase; the results are quantitatively the same as observed with the protein phosphatase, indicating that this relationship can be extended to other calmodulin-dependent enzymes (R.L.K., C. Coulson, M.V., unpublished data).

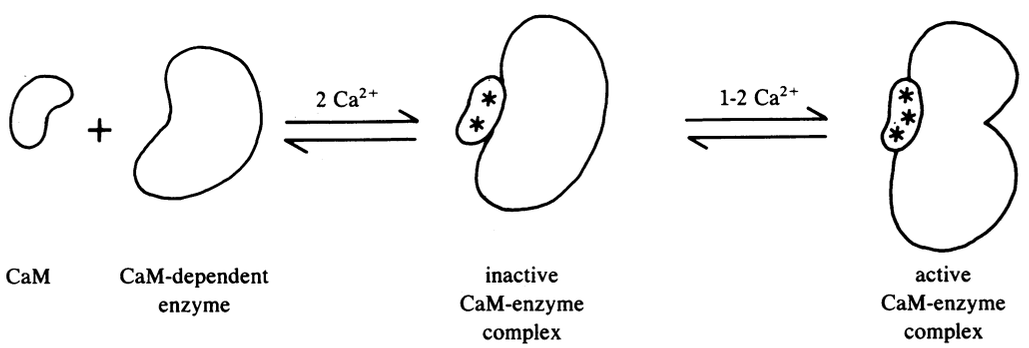

FIG. 6. Hypothetical scheme for sequential Ca^{2+}-dependent states of interaction and enzyme activation. A mechanism consistent with the experimental results shows interaction of calmodulin (CaM) with a target enzyme upon saturation of the two higher affinity Ca^{2+}-binding sites. This intermediate calmodulin–enzyme complex remains inactive until additional Ca^{2+} (1–2 sites) binding occurs, resulting in an activated enzyme complex.

increase in Ca^{2+} concentration is required for a large increase in activation. When 80% of enzyme exists in complex, ≈50% of maximal activity is observed. Such highly cooperative enzyme activation following complex formation is suggestive of an increase in the affinity of calmodulin for Ca^{2+} at sites other than those directly involved in protein–protein interaction. However, it must be cautioned that cooperative binding of ligand does not necessarily lead to cooperative activation nor can one infer from activity that changes in the state of cooperativity (32) have occurred.

A simple hypothesis to explain these findings might be that Ca^{2+} binding at two high affinity sites induces a conformation of calmodulin that allows interaction with its target enzyme (12); this interaction, though not sufficient for activation, greatly increases the affinity of other sites for Ca^{2+}, binding of which triggers a second conformational event that activates the enzyme (Fig. 6) (13). Indeed, mathematical analysis of "energy coupling" between calmodulin and troponin I has suggested a selective increase in the affinity for Ca^{2+} at lower affinity sites (3). Although our data clearly distinguish between the Ca^{2+} requirement for interaction and that for activation, the exact number of Ca^{2+} sites involved in these events has not been directly determined. The coincidence of the increase in fluorescence intensity, which requires occupancy of two Ca^{2+} sites, with changes in polarization is strongly suggestive that Ca^{2+} binding at those two sites is sufficient for protein interaction, although cryptic binding of a third Ca^{2+} ion in the presence of binding protein cannot be excluded (13). Based upon the kinetic and Ca^{2+}-binding data of others, as well as the strong cooperativity of activation, one would predict that binding of the third, or perhaps third and fourth, Ca^{2+} ions would lead to enzyme activation.

Such a cooperative switching mechanism is especially appealing in a regulatory system. It would allow complexes to exist in a ready state, capable of activation/inactivation by brief increases/decreases in cytosolic Ca^{2+}. Such a system could respond to rapid oscillations of Ca^{2+} better than one requiring protein–protein interaction (or dissociation) to initiate or terminate activation, since diffusion of Ca^{2+} would be rate limiting. In the central nervous system, such fine tuning of response might be particularly critical, e.g., for summation of multiple inputs. In addition, for those calmodulin-regulated enzymes that may be associated with cytoskeletal elements, the ability of calmodulin to exist in complex with binding proteins at Ca^{2+} concentrations lower than those required for activation could effectively result in functional (as well as physical) compartmentalization of calmodulin involved in specific cellular processes.

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1. Jamieson, G. A., Bronson, D. D., Schachat, F. H. & Vanaman, T. C. (1980) *Ann. N.Y. Acad. Sci.* **356,** 1–13.
2. Crouch, T. H. & Klee, C. B. (1980) *Biochemistry* **19,** 3692–3698.
3. Keller, C. H., Olwin, B. B., La Porte, D. C. & Storm, D. R. (1982) *Biochemistry* **21,** 156–162.
4. Huang, C. H., Chau, V., Chock, P. B., Wang, J. H. & Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 871–879.
5. Burger, D., Cox, J. A., Comte, M. & Stein, E. A. (1984) *Biochemistry* **23,** 1966–1971.
6. Cheung, W. Y. (1980) *Science* **207,** 19–26.
7. Klee, C. B. & Vanaman, T. C. (1982) *Adv. Protein Chem.* **35,** 213–321.
8. Cox, J. A., Malnöe, A. & Stein, E. A. (1981) *J. Biol. Chem.* **256,** 3218–3222.
9. Burger, D., Stein, E. A. & Cox, J. A. (1983) *J. Biol. Chem.* **258,** 14733–14739.
10. Cox, J. A., Comte, M. & Stein, E. A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4265–4269.
11. Blumenthal, D. K. & Stull, J. T. (1982) *Biochemistry* **21,** 2386–2391.
12. Kincaid, R. L., Vaughan, M., Osborne, J. C., Jr., & Tkachuk, V. A. (1982) *J. Biol. Chem.* **257,** 10638–10643.
13. Kincaid, R. L., Danello, M. A. T., Osborne, J. C., Jr., Tkachuk, V. A. & Vaughan, M. (1984) *Adv. Cycl. Nucleotide Protein Phospho. Res.* **16,** 77–87.
14. Malencick, D. A. & Anderson, S. R. (1982) *Biochemistry* **21,** 3480–3486.
15. Klee, C. B., Crouch, T. H. & Krinks, M. H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6270–6273.
16. Kincaid, R. L., Manganiello, V. C., Odya, C. E., Osborne, J. C., Stith-Coleman, I. E. & Vaughan, M. (1984) *J. Biol. Chem.* **259,** 5158–5166.
17. Pato, M. D. & Adelstein, R. S. (1981) *J. Biol. Chem.* **255,** 6535–6538.
18. Pallen, C. & Wang, J. H. (1984) *J. Biol. Chem.* **258,** 8550–8553.
19. Bartfai, T. (1979) *Adv. Cyclic Nucleotide Res.* **10,** 219–242.
20. Hartley, B. S. & Massey, V. (1956) *Biochim. Biophys. Acta* **21,** 58–65.
21. Bruton, C. J. & Hartley, B. S. (1970) *J. Mol. Biol.* **52,** 165–178.
22. Vanaman, T. C., Wakil, S. J. & Hill, R. L. (1968) *J. Biol. Chem.* **243,** 6409–6419.
23. Haiech, J., Klee, C. B. & Demaille, J. G. (1981) *Biochemistry* **20,** 3890–3897.
24. Andreasen, T. J., Leutje, C. W., Heideman, W. & Storm, D. R. (1984) *Biochemistry* **22,** 4615–4618.
25. Kilhoffer, M.-C., Demaille, J. G. & Gerard, D. (1981) *Biochemistry* **20,** 4407–4414.
26. Klee, C. B. (1977) *Biochemistry* **16,** 1017–1024.
27. Seamon, K. B. (1980) *Biochemistry* **19,** 207–215.
28. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252,** 8415–8422.
29. Wallace, R. W., Tallant, E. A., Dockter, M. E. & Cheung, W. Y. (1982) *J. Biol. Chem.* **257,** 1845–1854.
30. Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B. & Cohen, P. (1982) *FEBS Lett.* **137,** 80–84.
31. Manalan, A. S. & Klee, C. B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4291–4295.
32. Tanford, C., Reynolds, J. A. & Johnson, E. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4688–4692.