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Restoration of the Calcium Binding Activity of Mutant Calmodulins toward Normal by the Presence of a Calmodulin Binding Structure*

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The altered calcium binding activity of calmodulins (CaM) with point mutations can be restored toward that of wild type CaMs by the formation of a complex between CaM and a CaM binding sequence. Three different site-specific mutations resulted in selective effects on the apparent stoichiometry and affinity of CaM for calcium, with maintenance of the ability to activate myosin light chain kinase. The effects on calcium binding, however, were suppressed when the mutant CaMs were complexed with RS20, a peptide analog of a myosin light chain kinase CaM binding site. The mutations included: 1) a Glu→Ala mutation at two phylogenetically conserved calcium ligands in the second (E67A-CaM) and fourth (E140A-CaM) sites; and 2) a Ser→Phe mutation at residue 101 (S101F-CaM) which affects ion channel regulation. The mutant CaMs bind 4 calciums in the absence of magnesium, but two sites have approximately 60- to 300-fold weaker binding than wild-type CaM (SYNCAM CaM). E67A-CaM and E140A-CaM bound only two calciums and S101F-CaM bound 4 calciums in the presence of magnesium. E67A-CaM and E140A-CaM recovered the ability to bind 4 calcium ions in the presence of the RS20 CaM binding peptide. These results are consistent with models in which the calcium binding activity of CaM within a supramolecular complex is different from purified CaM and raise the possibility that the selective functional effects of *in vivo* mutations in the calcium binding sites of CaM might be partially due to the ability of some CaM binding proteins to select and utilize CaM conformations with calcium ligation structures different from the so-called canonical EF-hand.

Calmodulin (CaM)[1] belongs to a class of proteins that binds calcium ions, under conditions approximating physiological, with relatively high affinity and selectivity (*i.e.* the relative affinity for calcium is greater than that for magnesium). This class of calcium binding proteins is characterized by the presence of amino acid sequence motifs which have the potential of forming characteristic helix-loop-helix structures, often referred to as EF-hands (for a recent review, see Strynadka and James, 1989). In the case of CaM and closely related proteins such as troponin C, the helix-loop-helix motifs are found in sets of interacting pairs. Each calcium binding structural motif is composed of a β-structure that is flanked on each side by α-helices. Six residues in the center of the calcium binding structure are involved in calcium ligation through the use of oxygen-containing amino acid side chains or α-carbonyl oxygens from the the polypeptide backbone. The 6 calcium ligation residues have a spacing in the amino acid sequence of 1, 3, 5, 7, 9, and 12. The residue at position 12 is a phylogenetically conserved glutamic acid.

Recent detailed models (Boguta and Bierzynski, 1988) predict that mutation of the conserved glutamic acid at position 12 to a nonacidic residue would abolish calcium binding. However, little information is available concerning site-specific mutagenesis of residue 12 or other conserved residues involved in calcium ligation by CaM. Therefore, as a first step in probing how the sequence motifs of the CaM family of proteins are related to calcium-selective binding, we have mutated the conserved glutamic acid residue at position 12 in each of two calcium binding structures (one in each half of CaM) and examined the effect on calcium binding activity. For comparative purposes, we analyzed the calcium binding activity of a serine to a phenylalanine mutation at amino acid residue 101 of CaM (position 9 of the calcium ligation structure).

In addition to analyzing the effects of mutations at the conserved positions 9 and 12, we also examined how the calcium binding properties of the mutant CaMs were changed by the presence of a CaM binding structure. The reason for this type of study is severalfold. First, the calcium binding activity of wild-type CaMs can be increased when CaM is bound to a CaM-regulated enzyme (Olwin *et al.*, 1984). Second, multiple CaM conformations exist in solution, and CaM binding peptides appear to be able to select and stabilize preferentially some of these conformational states (Chabbert *et al.*, 1990). Third, there is debate over how well the crystal structure of CaM as an isolated protein reflects the more physiological state of CaM complexed with a CaM binding structure.

We report here that the mutant CaMs are changed in their relative affinity for, or stoichiometry of, binding of calcium when the purified CaMs were analyzed. In addition, we report that the relative affinity and the stoichiometry were restored toward the wild type when the calcium binding studies were done in the presence of the CaM binding peptide RS20.

MATERIALS AND METHODS

Ultrapure water (Milli-Q apparatus, Millipore Inc.) was used for all experiments. Buffers used in Ca^{2+} binding studies were stored in

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[1] The abbreviations used are: CaM, calmodulin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

plastic containers to minimize Ca^{2+} contamination. Protein concentrations were determined by ultraviolet spectroscopy calibrated to a calmodulin standard (Kilhoffer *et al.*, 1988) or by hydrolysis in 6 N HCl followed by amino acid analysis as described previously (Craig *et al.*, 1987). Site-specific mutagenesis and production of recombinant DNA-encoded calmodulins was done using previously described procedures (Roberts *et al.*, 1985; Craig *et al.*, 1987). All mutant gene constructs were verified by DNA sequence analysis, and the purified proteins gave the amino acid compositions expected, based on translation of the DNA sequence.

Calcium binding to calmodulins was determined by the flow dialysis method described previously (Haiech *et al.*, 1981). All experiments were done in 50 mM HEPES buffer, pH 7.5. Experiments were also done with Mg^{2+} added as required. A typical experiment contained 10–50 μM calmodulin, 0.4–1 μCi of $^{45}Ca^{2+}$ followed by sequential additions of unlabeled Ca^{2+} to the flow cell. Blank experiments without added proteins were done to correct for nonspecific binding to the dialysis membrane which becomes significant at concentrations $>10^{-4}$ M Ca^{2+}. Calcium binding data for most calmodulins in the absence of Mg^{2+} were fitted to a multiple site equation (Haiech *et al.*, 1981) to obtain macroscopic binding constants. Calcium binding data for calmodulins in the presence of Mg^{2+} were determined from the binding isotherm described previously (Kilhoffer *et al.*, 1988) to obtain stoichiometry, while $K_{0.5}$ values were calculated from the midpoint of the titration curve. Binding data for calmodulin-peptide complexes were also determined from the titration curves to obtain $K_{0.5}$ values, stoichiometry, and Hill coefficients.

Assessment of myosin light chain kinase activation and 3′,5′ cyclic nucleotide phosphodiesterase activation were done essentially as described previously (Craig *et al.*, 1987; Weber *et al.*, 1989). Briefly, myosin light chain kinase activity was measured in 50 μl of buffer containing 50 mM HEPES, 0.1 mM Ca^{2+}, 5 mM Mg^{2+}, 1 mM dithiothreitol, 0.2 mM ATP-[^{32}P]ATP (final specific activity 200–400 cpm/pmol), 50 μM peptide substrate (KKRPQRATSNVFAM), 0.1 mg/ml bovine serum albumin, 1.4 nM chicken gizzard myosin light chain kinase, and 0.5–50 nM concentrations of the desired calmodulin. Assays were initiated by the addition of the kinase and run for 20 min at 25 °C. To stop the reaction, an aliquot of the mixture was applied to 1-cm squares of phosphocellulose papers (Whatman P-81) and immersed into 75 mM phosphoric acid. Filters were washed three times in 75 mM phosphoric acid (total wash time 15–30 min), once in 95% ethanol, and air-dried. Radioactivity was determined by liquid scintillation counting of the filters in Ecoscint-O (National Diagnostics). 3′,5′ Cyclic nucleotide phosphodiesterase activity was determined as described in Craig *et al.* (1987) except that the buffer was 50 mM HEPES, pH 7.5, containing 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, 1 mM dithiothreitol, and reactions were carried out at 25 °C for 20 min.

RESULTS

Using point mutations, we have studied the relative importance of three different residues on the ion selectivity and the enzyme regulatory activity of calmodulin. The locations of the mutations in the calmodulins made in this study are shown in Fig. 1. SYNCAM or VU-1 calmodulin (Roberts *et al.*, 1985) is the standard of comparison and has calcium binding properties similar to other wild-type calmodulins (Kilhoffer *et al.*, 1988). E67A-CaM, E140A-CaM, and S101F-CaM are the point mutations of SYNCAM where Glu-67, Glu-140, and Ser-101 have been changed to Ala-67, Ala-140, and Phe-101, respectively (Fig. 1). Glu-67 and Glu-140 occupy position 12 in calcium binding loops 2 and 4 (Fig. 1). The Ser to Phe mutation at residue 101 is position 9 of loop 3 (Fig. 1) and was first observed in a calmodulin mutant from *Paramecium* which exhibits an altered potassium efflux phenotype (Schaefer *et al.*, 1987).

Loop1	Asp	Lys	Asp	Gly	Asp	Gly	Thr	Ile	Thr	Thr	Lys	Glu (31)
Loop2	Asp	Ala	Asp	Gly	Asn	Gly	Thr	Ile	Asp	Phe	Pro	Glu (67) Ala
Loop3	Asp	Lys	Asp	Gly	Asn	Gly	Phe	Ile	Ser Phe	Ala	Ala	Glu (104)
Loop4	Asp	Val	Asp	Gly	Asp	Gly	Gln	Val	Asn	Tyr	Glu	Glu (140) Ala

FIG. 1. **Amino acid sequences of the 12-residue calcium ligation structures of CaM and location of mutations.** The amino acid sequence of the calcium binding loops of a wild-type standard calmodulin (SYNCAM or VU-1 CaM) is shown on each line. The amino acid changes in the calcium binding mutants (E67A-CaM, S101F-CaM, and E140A-CaM) are noted. The *numerals* at the *end* of each *line* denote the residue number in CaM for the last amino acid in each line.

Comparative Calcium Binding of Calmodulin Mutants—Under the experimental conditions (50 mM HEPES, pH 7.5) where Ca^{2+} is the only added divalent cation, we found four calcium binding sites for each of these mutants as shown in Fig. 2. E67A-CaM, E140A-CaM, and S101F-CaM exhibit two classes of sites: higher affinity sites with calcium binding affinity similar to SYNCAM (the reference synthetic calmodulin) and lower affinity sites with affinities up to 60- to 300-fold lower than the previous ones. These results are consistent with mutation of one calcium binding site (either in the amino-terminal lobe or in the carboxyl-terminal lobe) altering calcium binding activity in two sites, most probably in the contiguous site of the same half. In theory, the mutated residues (E67A, E140A, or S101F) could be directly involved in the coupling between two sites of a lobe or could modify the structure of the loop in such a way that interaction between sites is altered. Regardless, none of the single site mutations completely eliminates Ca^{2+} binding from any site under these conditions.

Calcium binding to the lower affinity sites could be fitted to a Scatchard model with an association constant K_3 showing values between 0.011×10^6 M^{-1} and 0.0024×10^6 M^{-1} depending on the mutant (Table I). On the other hand, calcium binding to the higher affinity sites showed cooperativity and could not be fitted to a Scatchard model. K_1 and K_2 (Table I) correspond to the macroscopic association constants of these two sites and were determined using a general Adair-Klotz equation as indicated under "Materials and Methods."

Modulation of Calcium Binding by Magnesium Ion and a Calmodulin Binding Peptide—Under intracellular physiological conditions, CaMs are found in the presence of other divalent cations, especially magnesium that is often present in greater abundance than calcium. Therefore, the calcium binding activity in the presence of relatively high magnesium concentrations was examined. Although the three mutant

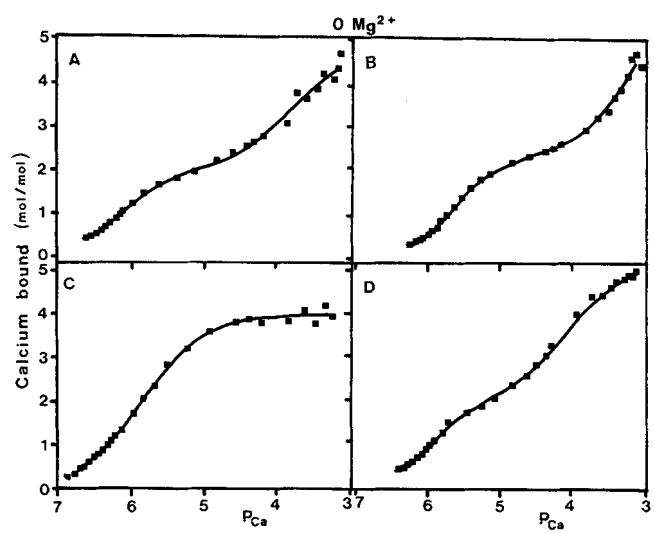

FIG. 2. **Calcium binding by calmodulins in the absence of magnesium.** Calcium binding data were obtained by flow dialysis experiments as described under "Materials and Methods." The *four panels* correspond to the binding curves of E67A-CaM (*A*), E140A-CaM (*B*), wild-type/SYNCAM CaM (*C*), and S101F-CaM (*D*).

TABLE I

Comparative Ca^{2+} activity constants of mutants and wild-type calmodulins in the absence of magnesium ion

Calcium binding parameters were determined from the Ca^{2+} binding equation:

$$\nu = \frac{K_1\alpha + 2K_1K_2\alpha^2}{1 + K_1\alpha + K_1K_2\alpha^2} + \frac{n_3K_3\alpha}{1 + K_3\alpha}$$

where ν is bound Ca^{2+}, α is the free Ca^{2+}, and K_1,K_2 are the macroscopic association constants for the higher affinity calcium binding sites, while K_3 is an association constant for n_3 lower affinity sites.

Protein	K_1	K_2	K_3
		$\times 10^{-6}\ M^{-1}$	
SYNCAM[a]	1.4	0.35	0.7
E67A-CaM	0.19	1.61	0.0024
E140A-CaM	1.70	1.03	0.0063
S101F-CaM	0.95	0.77	0.011

[a] Under the data analysis conditions used, calcium binding to the wild-type standard SYNCAM can yield four sites of similar affinity, although more detailed analyses (Kilhoffer *et al.*, 1988; Haiech *et al.*, 1990) have shown the four sites are not functionally equivalent.

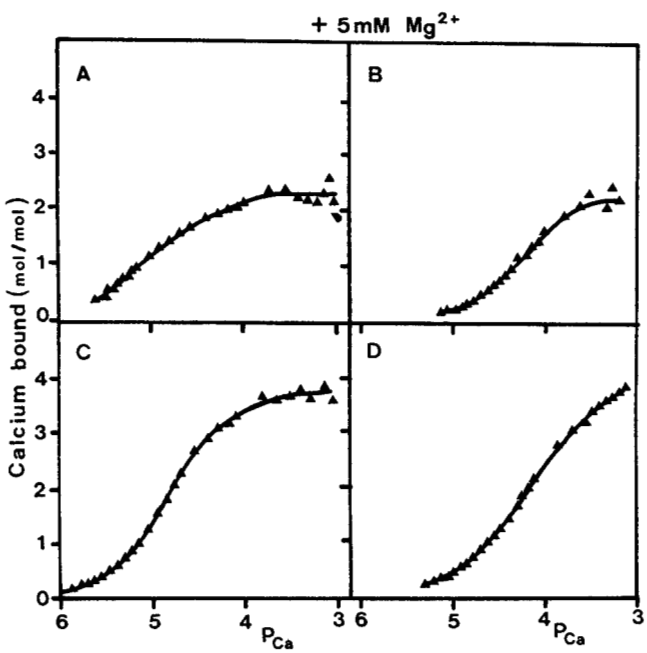

FIG. 3. **Calcium binding by calmodulins in the presence of 5 mM magnesium.** Calcium binding was determined as described under "Materials and Methods." As in Fig. 2, the *four panels* correspond to the binding curves of E67A-CaM (*A*), E140A-CaM (*B*), SYNCAM (*C*), and S101F-CaM (*D*).

CaMs apparently have four calcium binding sites in the absence of magnesium, E67A-CaM and E140A-CaM exhibit two calcium sites in the presence of 5 mM magnesium, whereas S101F-CaM has four calcium binding sites (Fig. 3, *A–D*, and Table II). Significant differences in the apparent 50% binding values ($K_{0.5}$, Table II) for E140A-CaM and S101F-CaM compared to SYNCAM are also apparent. These results suggest that Mg^{2+} competes with Ca^{2+} in all sites of E67A-CaM and E140A-CaM resulting in the loss of calcium from two sites. On the other hand, 5 mM magnesium ion only competes well at one class of sites in S101F-CaM, which appears to be the higher affinity Ca^{2+} sites. These data imply that calcium/magnesium selectivity under these experimental conditions is modified to various degrees in all three of the mutants. Whether other ions are capable of similar Ca^{2+} competition was not investigated in this work.

TABLE II

Comparison of Ca^{2+} activity of mutant and wild-type calmodulins in the presence or absence of a CaM binding peptide

All binding experiments were done in the presence of 5 mM Mg^{2+} ion. $K_{0.5}$ is the calculated midpoint of the binding curves (Fig. 4) for CaM or CaM-peptide complexes. N_s is the calculated Ca^{2+} binding stoichiometry. n is the Hill coefficient for Ca^{2+} binding to the CaM-peptide complex.

Protein	CaM only		CaM·RS20 complex		
	$K_{0.5\mu M}$	N_s	$K_{0.5\mu M}$	N_s	n
Wild-type CaM	14	3.8	1.0	4.1	1.3
E67A-CaM	11	2.3	1.4	3.8	0.6
E140A-CaM	63	2.3	5.5	3.9	0.8
S101F-CaM	63	4.1	1.9	4.1	1.4

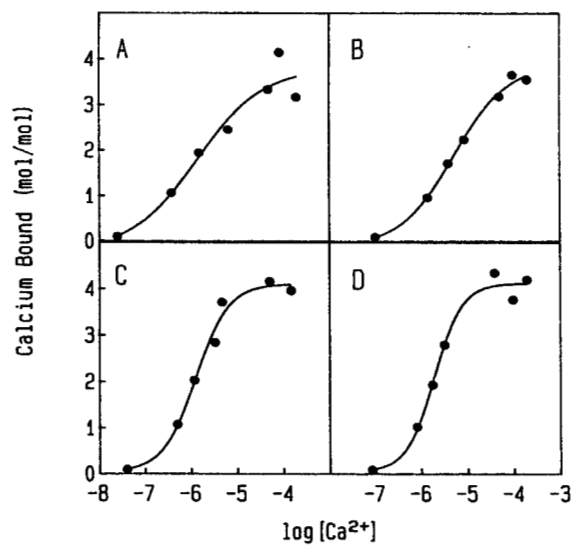

FIG. 4. **Calcium binding by calmodulins in the presence of 5 mM magnesium and calmodulin binding peptide RS20.** Shown here are the Ca^{2+} binding curves for E67A-CaM·RS20 complex (*A*), E140A-CaM·RS20 complex (*B*), SYNCAM·RS20 complex (*C*), and S101F-CaM·RS20 complex (*D*). *Curves* were calculated by fitting the data to a sigmoidal titration curve using the Graph Pad program version 2.0 (copyright H. Motulsky, distributed by ISI Inc.)

Ca^{2+} binding to calmodulin is known to be influenced by the formation of calmodulin-myosin light chain kinase complexes (Olwin *et al.*, 1984; Olwin and Storm, 1985). Therefore, we investigated calcium binding to the wild-type and mutant calmodulins in the presence of 1.4 molar eq of the peptide RS20, a synthetic analog of a myosin light chain kinase CaM binding site (Lukas *et al.*, 1986; Shoemaker *et al.*, 1990; Lukas *et al.*, 1989). As seen in Fig. 4 and Table II, the stoichiometry of Ca^{2+} binding to E67A-CaM in the presence of RS20 and Mg^{2+} increases such that the binding stoichiometry is indistinguishable from that obtained with wild-type calmodulin in the presence of the peptide, *i.e.* 4 mol/mol. Similar results were obtained with E140A-CaM (Fig. 4 and Table II). As a control, S101F-CaM does not undergo a change in Ca^{2+} binding stoichiometry in the presence of RS20 (Table II). It is also evident from the data that the relative affinity of the calmodulins for Ca^{2+} increases in the presence of RS20, consistent with previous studies of CaM-myosin light chain kinase complexes (Olwin *et al.*, 1984). Although RS20 quantitatively restores the Ca^{2+} binding ability of the mutants, the difference in the overall shape of the calcium binding isotherm of Fig. 4 suggests a difference in the calcium binding mechanisms of the three calmodulin mutants-RS20 complexes. Nevertheless, the results show that formation of CaM-peptide

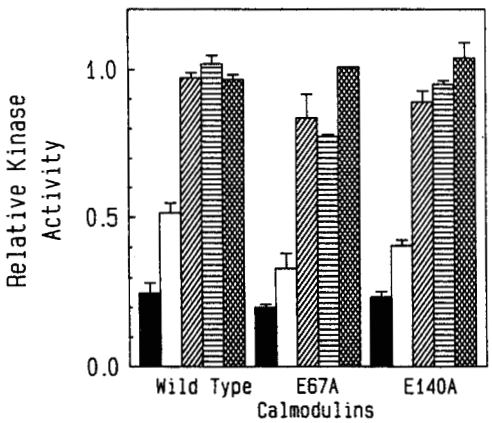

FIG. 5. **Activation of myosin light chain kinase by calmodulins.** Total calmodulin concentrations are represented in the *bar graph* as follows: 0.5 nM (*solid bars*), 1.0 nM (*open bars*), 5.0 nM (*slanted bars*), 10.0 nM (*horizontal bars*), and 50 nM (*criss-crossed bars*). Activity data were normalized to the maximum obtained with SYNCAM CaM at a concentration of 50 nM. *Error bars* represent the standard error for duplicate points.

complexes can modify both the Ca^{2+} binding stoichiometry and affinities of calmodulin mutants which have lost Ca^{2+} selectivity in the presence of Mg^{2+} ion.

Activation of Myosin Light Chain Kinase by Calcium Binding Site Mutants of Calmodulin—In light of the modulation of the calcium binding to calmodulin by the calmodulin binding peptide, RS20, it was of interest to examine the ability of the calcium binding mutants to activate myosin light chain kinase. Under the standard conditions which have 5 mM Mg^{2+} and 0.1 mM Ca^{2+}, all the mutants quantitatively activate myosin light chain kinase (Fig. 5). In experiments done in the presence of excess calcium chelator (2 mM EGTA), E67A-CaM and E140A-CaM activated myosin light chain kinase to less than 1% of the maximum obtained in the presence of 0.1 mM Ca^{2+} ion, indicating that the activation remains Ca^{2+} dependent. Similar experiments done with 3′,5′ cyclic nucleotide phosphodiesterase indicate that all of the mutants fully activate this enzyme in a calcium-dependent manner (data not shown). However, the concentration of E140A-CaM needed to activate phosphodiesterase to 50% of maximum was about 10-fold greater than that of SYNCAM.

DISCUSSION

As discussed in more detail below, the results of this and previous studies allow at least six conclusions or forecasts about the potential effects of calcium binding site mutations in calmodulin on calcium binding activity. First, a single mutation in a calcium binding site can give quantitatively different effects on calcium binding activity depending upon the magnesium concentration, the presence of a calmodulin binding structure, and whether or not the mutation alters coupling between sites. For example, magnesium tends to antagonize calcium binding (with respect to stoichiometry in certain mutants), but the presence of a calmodulin binding structure reverses significantly the antagonistic effects of magnesium. Second, mutation of the conserved glutamic acid at position 12 of a calcium binding site does not necessarily abolish calcium binding. Third, a point mutation in a calcium binding loop can alter ion binding in other sites (mutational evidence in support of coupling between sites). Fourth, modification of position 12 in a calcium ligation structure (E67A-CaM and E140A-CaM mutants) does not appear to alter drastically the magnesium binding properties of the site, whereas position 9 alterations (S101F-CaM mutant) do appear to affect magnesium binding. In other words, modification of position 9 in a calcium ligation site may result in a detectable effect on both calcium and magnesium binding of calmodulin. However, direct magnesium binding studies need to be done to confirm this hypothesis. Fifth, the differences in calcium binding properties of mutations in similar domain positions (E67A-CaM and E140A-CaM) and the previously noted (Haiech *et al.*, 1981; Haiech and Watterson, 1988) different classes of calcium binding sites in wild-type calmodulin are consistent with the proposed (Haiech *et al.*, 1990) sequential/ordered mechanism of calcium binding. However, it should be noted that the binding data alone do not reveal the order of sites. Sixth, some CaM binding proteins are probably able either to alter the structure of certain mutant CaMs or select a CaM conformation such that the CaM-enzyme signal transduction complex is able to compensate for the CaM mutation. In addition, the results with the E67A-CaM and the E140A-CaM mutations (a decrease in calcium binding activity with removal of a canonical EF-hand carboxylate ligand, retention of the ability to activate enzyme in a calcium-dependent manner, and restoration of calcium binding toward normal with the formation of a calcium-CaM-CaM-binding structure complex) raise the possibility that CaM-regulated proteins can function in the presence of a helix-loop-helix structure that is a modification of a canonical EF-hand structure. Clearly, the ability of the CaM binding peptide to restore calcium-selective binding to mutations in each half of CaM demonstrates that the functional effects of peptide binding are not limited to one-half of the molecule, in agreement with structural studies using NMR (Seeholzer and Wand, 1989) and fluorescence spectroscopy (Chabbert *et al.*, 1990), and provide one possible mechanistic explanation for how inherited mutations of CaM could selectively alter one CaM-regulated pathway but not another, *e.g.* ion channel regulation but not protein kinase regulation (Heinrichsen *et al.*, 1990).

The calcium binding data for these mutant CaMs are consistent with coupling between pairs of sites in each half of calmodulin and residue 12 of the calcium ligation structure being key to this coupling. For example, modifying one of the calcium binding sites in E140A-CaM and E67A-CaM resulted in the loss of calcium-selective binding in two sites. In addition, the analysis of the data in light of previous reports (Haiech *et al.*, 1990; Kilhoffer *et al.*, 1988) allows for a tentative assignment of calcium-selective/high affinity binding activity in these two mutant CaMs to specific sets of sites in the structure. The first two sites in calmodulin occupied by Ca^{2+} have been identified by the use of fluorescence (Kilhoffer *et al.*, 1988) and NMR studies (Seamon, 1980; Klevit *et al.*, 1984; Sutoo *et al.*, 1989) as loops 3 and 4 (Fig. 1). Therefore, it is reasonable to assign the high affinity calcium binding sites of E67A-CaM to the carboxyl-terminal sites for the following reasons: 1) the mutation is in the amino-terminal set of sites; 2) the apparent binding constants for the high affinity calcium binding by the mutant CaM are similar to SYNCAM for the two first sites occupied. Based on a similar argument, the high affinity sites of E140A-CaM appear to be the amino-terminal pair of sites.

Mg^{2+} ions seem to compete with all calcium binding sites of CaM (dissociation constants for Mg^{2+} ranges in the absence of salt from 0.7 to 2.7×10^{-4} M (Haiech *et al.*, 1981) and in the presence of salt from 0.5 to 3.3×10^{-3} M (Tsai *et al.*, 1987)). Therefore, in the presence of 5 mM Mg^{2+}, Ca^{2+} binding sites must present an affinity decrease by a factor of 10 to 100. Given the fact that E67A-CaM and E140A-CaM present two sites in the presence of Mg^{2+} and S101F-CaM has four

sites and that the three mutants exhibit both high and low affinity sites in the absence of Mg^{2+}, one would logically conclude that: 1) Mg^{2+} competes with all sites on both SYNCAM and mutants E67A-CaM and E140A-CaM and 2) Mg^{2+} competes well only on the high affinity sites of S101F-CaM as Mg^{2+} does not suppress the two low affinity sites of this mutant. Therefore, modifying position 12 of the loop affects the Ca^{2+} binding, but apparently does not alter Mg^{2+} binding. On the other hand, the replacement of Ser in position 9 of the third loop affects both Ca^{2+} and Mg^{2+} binding.

The ability of RS20 CaM binding sequence to restore the calcium binding activity of the mutant CaMs toward that of SYNCAM CaMs provides a second example of how the functional effects of CaM mutations can be suppressed by a CaM binding protein. Previously, Shoemaker *et al.* (1990) showed that a mutation in the RS20 region of myosin light chain kinase could restore the catalytic activity of a mutant CaM-myosin light chain kinase complex toward normal; *i.e.* a mutation in the CaM binding region of myosin light chain kinase could suppress the effects of CaM mutations on the protein kinase activity of the CaM-myosin light chain kinase complex. Although the ability of the CaM binding RS20 peptide to restore toward normal the ion-selective binding activity of CaMs mutated in calcium binding sites is also a suppressor effect, the functional effect is apparently through a different step in the molecular mechanism of enzyme activation. If calcium binding experiments done in the presence of the peptide analog (RS20) of the myosin light chain kinase CaM binding site are representative of what occurs in the CaM-myosin light chain kinase complex, then CaM in the CaM-myosin light chain kinase complex would retain the ability to bind 4 mol of Ca^{2+} per mol of CaM.

Altogether, these results and those of Shoemaker *et al.* (1990) indicate that any deleterious phenotypic effects of mutations of either CaM or CaM binding proteins have the potential of being suppressed at the level of the CaM·CaM binding protein complex. This, in turn, suggests the need to increase our knowledge about CaM structure and function when CaM is a component of a supramolecular complex. In addition, these results and those of Shoemaker *et al.* (1990) suggest that inherited mutations of CaM or CaM binding proteins may not always manifest themselves phenotypically under "resting" physiological conditions (*i.e.* they might be asymptomatic in the absence of stress or physiological changes). Although the results suggest that the effect of inherited CaM mutations (Schaefer *et al.*, 1987; Lukas *et al.*, 1989) might be selective and nonlethal due to the ability of some CaM binding proteins to suppress selectively the phenotypic effects of certain CaM mutations, additional studies of mutant CaMs and other CaM-regulated enzymes are required before it will be possible to predict which types of CaM mutations can be suppressed by a given CaM binding protein.

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