Analysis of the Molecular Basis of Calmodulin Defects That Affect Ion Channel-mediated Cellular Responses: Site-specific Mutagenesis and Microinjection

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Abstract. The ability of microinjected calmodulin to temporarily restore an ion channel-mediated behavioral phenotype of a calmodulin mutant in *Paramecium tetraurelia* (*caml*) is dependent on the amino acid side chain that is present at residue 101, even when there is extensive variation in the rest of the amino acid sequence. Analysis of conservation of serine-101 in calmodulin suggests that the ability of calmodulin to regulate this ion channel-associated cell function may be a biological role of calmodulin that is widely distributed phylogenetically. A series of mutant calmodulins that differ only at residue-101 were produced by in vitro site-specific mutagenesis and expression in *Escherichia coli*, purified to chemical homogeneity, and tested for their ability to temporarily restore a wild-type behavioral phenotype to *caml* (*pantophobiacA*[1]) *Paramecium*. Calmodulins with glycine-101 or tyrosine-101 had minimal activity; calmodulins with phenylalanine-101 or alanine-101 had no detectable activity. However, as a standard of comparison, all of the calmodulins were able to activate a calmodulin-regulated enzyme, myosin light chain kinase, that is sensitive to point mutations elsewhere in the calmodulin molecule. Overall, these results support the hypothesis that the structural features of calmodulin required for the transduction of calcium signals varies with the particular pathway that is being regulated and provide insight into why inherited mutations of calmodulin at residue 101 are nonlethal and selective in their phenotypic effects.

THE stimulus-response transduction pathways mediated by a calcium signal appear to involve a class of intracellular calcium-binding proteins that includes calmodulin (CaM)[1] (29). Calmodulin is an integral subunit of several enzymes (4) and is found associated with cytoskeletal proteins and membrane structures (5, 19, 27, 28). Although there is increasing knowledge about the molecular mechanisms of some calmodulin-regulated activities, little is known about the role of calmodulin in the regulation of ion channels.

The evidence for calmodulin involvement in the regulation of ion channels comes from electrophysiological investigations (26), biochemical reconstitution studies (13), and analysis of mutant organisms (10, 17, 22). Data implicating calmodulin involvement in the regulation of a widely distributed low conductance, calcium-dependent potassium channel come mainly from studies of the pantophobic mutants of *Paramecium*, which have defects in calcium-dependent cation conductances (10, 12, 17, 22).

Paramecium tetraurelia has an excitable membrane and generates Ca^{2+}-based action potentials upon stimulation. The action potential is due to four specific ion channels, the voltage-dependent Ca^{2+} and K^+ channels, and the Ca^{2+}-dependent Na^+ and K^+ channels. The Ca^{2+} and Na^+ channels allow an influx of ions, which depolarizes the cell, whereas the two K^+ channels allow an efflux of K^+ from the cell, which leads to cellular repolarization. An action potential leads to an increase in intracellular calcium, a transient reversal in the direction of the ciliary beat, and a subsequent change in the direction of swimming (from forward to backward swimming); the stronger the action potential, the longer the backward swimming response. With these swimming phenotypes in mind, one can estimate the electrical properties of a cell by observing its swimming behavior.

It has been shown that genetic mutations that alter the properties of each specific ion channel have distinct effects on the behavior of cells. For example, the elimination of Ca^{2+} channel activity inhibits the cells' ability to swim backwards, while the elimination of the Ca^{2+}-dependent K^+ channel causes the cells to swim backward for much longer periods of time because of the difficulty in repolarizing the membrane. Several of these behavioral mutants in *Paramecium* have been characterized at the molecular level (12, 17, 22). In each case, a defect has been localized to calmodulin. In the *caml Paramecium* mutant (originally designated

1. *Abbreviations used in this paper*: CaM, calmodulin; MLCK, myosin light chain kinase.

pntA[1]) (10), the serine at residue 101 of calmodulin is replaced by a phenylalanine (S101→F101) (22). The behavioral phenotype of the *caml Paramecium* mutant can be temporarily restored to the wild-type *Paramecium* phenotype, including the enhancement of calcium-dependent K^+ conductance, by the microinjection of calmodulin purified from wild-type *Paramecium* (10).

The results with the pantophobiac mutant *Paramecium* system have demonstrated that it is possible for a eukaryotic cell to have nonlethal mutations of calmodulin in more than one structural domain, and have provided evidence for calmodulin involvement in the regulation of a signal transduction system, ion channels. However, we must increase our knowledge about the molecular basis of these in vivo effects with *Paramecium* to gain a full understanding of the mechanisms involved in calmodulin regulation of ion channel activity. This knowledge would aid attempts to extend studies of calmodulin regulation of ion channels to other phylogenetic species. In this report, we summarize an initial set of comparative studies that use site-specific mutagenesis of CaM and analysis of the in vivo and in vitro activity of a set of recombinant-encoded CaMs. In addition, we report the results of reinvestigation of the phylogenetic conservation of CaM's amino acid sequence that was prompted by the results of the structure–function studies.

Materials and Methods

SYNCAM calmodulin is isofunctional with vertebrate and plant calmodulin; it can be considered a standard of comparison calmodulin that is less susceptible in its phylogenetic variation in activity than most naturally occurring CaMs. As described in detail by Weber et al. (31), a model for SYNCAM calmodulin (also referred to as S101 in the paper) was obtained by using the alpha carbon trace of rat testes calmodulin (Brookhaven Protein Data Bank release 39, 1/87) as an initial template and sequentially fitting the SYNCAM sequence to this model. The S101F mutant calmodulin model was obtained by replacing the serine-101 side chain with a phenylalanine side chain. This structure and that of SYNCAM calmodulin were refined by energy minimization techniques (DISCOVER Program, Biosym Technologies, San Diego, CA).

Cassette-based site-specific mutagenesis and protein production were done as described (6, 16, 21). The new mutant calmodulins made as part of this study differ only at amino acid residue 101 (see Table I for the different calmodulins used). The sequences of all clones used subsequently for protein production were verified by automated DNA sequence analysis (33). These sequence data are available from EMBL/GenBank/DDBJ under accession number M11334. As with previous SYNCAM calmodulin mutants (6, 9, 11, 21, 31), the amino acid composition of each purified calmodulin was consistent, within experimental error, with that expected from the DNA sequence.

Calmodulin from *Tetrahymena pyriformis* was purified essentially as described previously (23, 30). Briefly, the protocol included precipitation with TCA, extraction with Tris/EGTA (pH 7.5), precipitation with ammonium sulfate (80% saturated), isoelectric precipitation (pH 4.0), heat treatment (95°C, 5 min) in the presence of Ca^{2+}, and phenyl Sepharose chromatography. The approach and protocols used for the determination of the amino acid sequence of *Tetrahymena pyriformis* calmodulin were as described previously for *Paramecium* calmodulin (22, 23). The repetitive yield on all Edman degradations was >90%. All peptide recoveries from digests were in the range of 20–60%.

Table I. Changes in the Calmodulin Molecule at Amino Acid 101

Calmodulin name	Residue 101	Side group
S101	Serine	Hydroxyl group
S101F	Phenylalanine	Nonpolar aromatic ring
S101A	Alanine	Nonpolar methyl group
S101G	Glycine	Hydrogen side chain
S101Y	Tyrosine	Phenolic hydroxyl group

The ability of chemically homogeneous calmodulins to temporarily restore wild-type behavior to the *caml* mutant of *Paramecium* has been demonstrated previously (10). The injected cells were tested at various times in a testing solution of 10 mM NaCl, 5 mM TEA^+, 1 mM KCl, 1 mM $CaCl_2$, and 1 mM Hepes (pH 7.5); this solution causes an ~10-s backward swimming response in wild-type cells and a 80-s response with *caml*. The behavioral response was quantitated by determining the relative wild-type swimming phenotype. The relative wild-type swimming phenotype is determined by the following equation:

$$\text{relative wt phenotype} = (1-(X-wt/cam^1-wt)$$

where X = the response (seconds of backward swimming) of the injected cell, wt = the response of the wild-type controls, and *caml* = the response of the *caml* controls. All injected cells were tested in this manner and the data were averaged. Only cells that divided (this would cause the injected calmodulin to be diluted), were eliminated.

The ability of the different calmodulins to quantitatively activate chicken gizzard myosin light chain kinase (15, 20), was determined as previously described. Briefly, the assay included 50 mM Hepes (pH 7.5), 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM DTT, 0.2 mM [gamma-^{32}P]-ATP + ATP, and 50 μM peptide substrate (KKRPQRATSNVFAM). As discussed previously (6, 31), this screening assay is designed to detect differences in kinetic constants of CaM:myosin light chain kinase (MLCK) complexes brought about by CaM mutations. More detailed kinetic analyses of all of the mutant CaMs used in this study were done exactly as described previously (24).

Results

Inspection of energy-minimized models for SYNCAM calmodulin and the S101F mutant calmodulin (Fig. 1) did not reveal major differences in structure and indicated that the side chain at residue 101 has significant surface exposure (see the yellow side chains for amino acid 101 in Fig. 1). These results raise the possibility that this position in the CaM structure can accommodate a variety of amino acid side chains with a maintenance of overall structural integrity for the protein, and suggest that functional differences between the mutant calmodulins are not due to major perturbations in the structure of the calmodulin molecule.

We then examined the effect of various amino acids at residue 101 on the regulation of the behavioral response in *Paramecium* (which is a consequence of the Ca^{2+}-dependent K^+ conductance). A series of calmodulins that differ only at residue 101 (Table I, Fig. 2) were produced by site-specific mutagenesis and expression in *E. coli* (see Materials and Methods). A threonine substitution was not made because calmodulin from *Tetrahymena pyriformis*, a closely related unicellular ciliated organism, was reported (32) to have a threonine at residue 101, and *Tetrahymena thermophila* calmodulin was found to restore the activity of the Ca^{2+}-dependent K^+ current when microinjected into *caml Paramecium* (Hinrichsen, R., unpublished observation).

Hinrichsen and co-workers (10) have demonstrated that the microinjection of wildtype *Paramecium* CaM can cause a temporary restoration of the Ca^{2+}-dependent K^+ current in *caml Paramecium* which lacks this current. This restoration also returns the *caml* cells to a wild-type behavioral phenotype in terms of its backward swimming response when stimulated by various ionic solutions (10). Based on these results, the ability of the chemically homogeneous engineered calmodulins to restore the wild-type behavioral

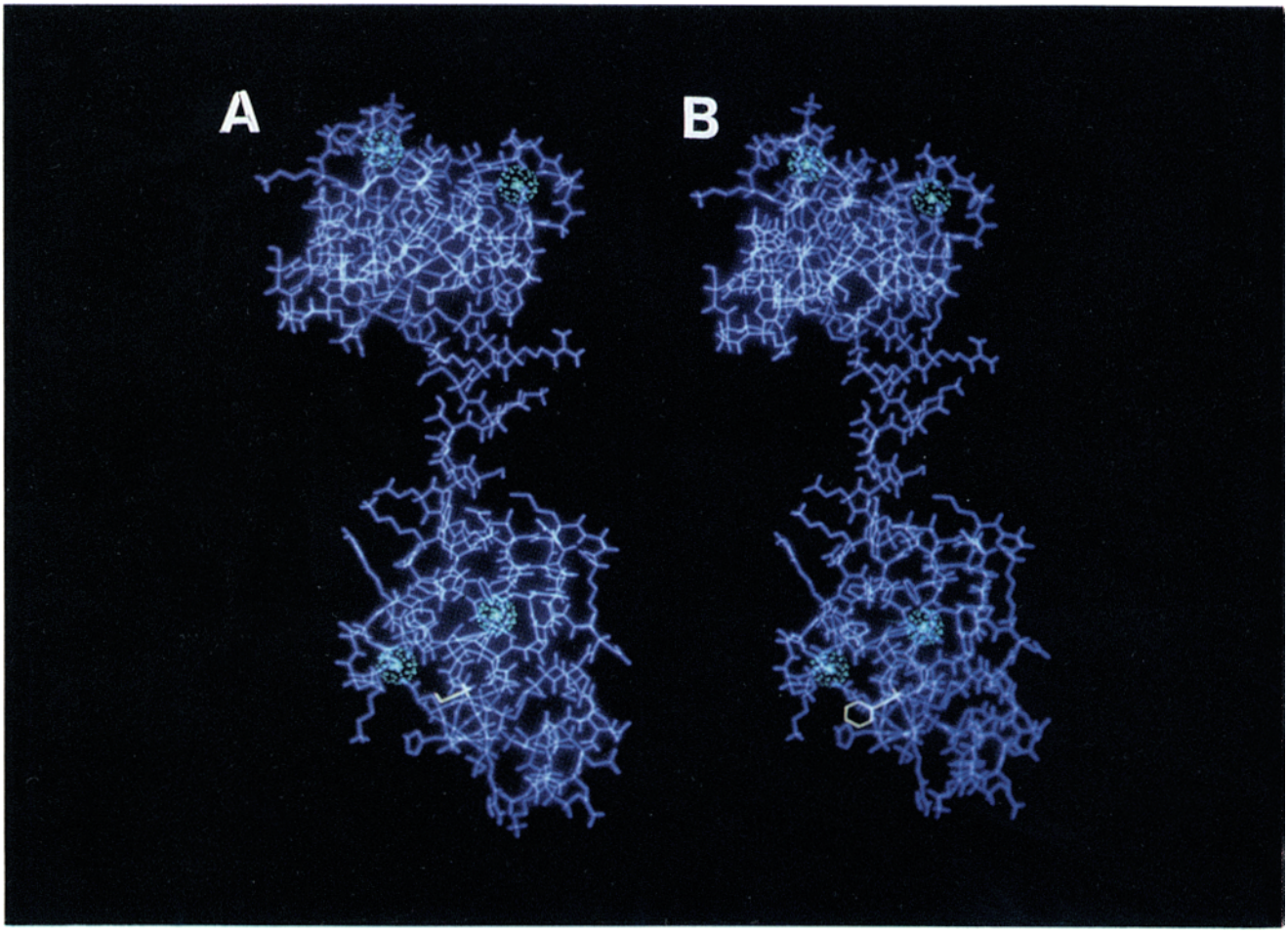

Figure 1. Displays of computed calmodulin models that differ only at residue 101. The backbone atoms and one possible position of side chain atoms are shown in blue, except for residue 101, which is highlighted in yellow. The green spheres represent the Ca^{2+} atoms in the Ca^{2+}-binding domains of the calmodulin molecule. (*A*) A display of an energy-minimized model for SYNCAM calmodulin that has been described previously (31). The model was constructed based on the initial alpha-carbon trace for vertebrate calmodulin (2). The root mean square difference between the refined vertebrate calmodulin data (2) and the SYNCAM calmodulin is <0.9 A, suggesting very similar structures. The amino acid sequences for vertebrate and SYNCAM calmodulins are given in Fig. 2. (*B*) A display of an energy-minimized model of the S101F mutant calmodulin. The only amino acid sequence difference between the SYNCAM and S101F calmodulins is the serine to phenylalanine change at residue 101. No gross structural changes were detected when the models of SYNCAM and S101F were compared. Models of vertebrate (2), SYNCAM (31), and S101F mutant calmodulins are all consistent with significant surface exposure of the side chain at residue 101.

phenotype was tested after injection into *caml* cells (Fig. 3). SYNCAM calmodulin is able to restore temporarily the wild-type behavioral phenotype, whereas the S101 F mutant calmodulin completely lacks this restorative activity. These results are similar to the differences found between the calmodulin of wildtype and *caml* mutant *Paramecium* (10), and suggest, therefore, that the Ca^{2+}-dependent K^+ current is also restored. Although the S101G and S101Y mutants showed some restorative activity (Fig. 3), the activity was at least an order of magnitude reduced as compared to that found with calmodulins containing serine at residue 101. The S101A mutant, on the other hand, had no restorative ability whatsoever. It is important to note that the injection of SYNCAM modified at other amino acids (e.g., amino acid 115 and 33) completely restores the *caml* mutant to the wild-type behavioral phenotype (Hinrichsen, R., unpublished data). Thus, the reduction in activity with the calmodulins modified at amino acid 101 is specific to this amino acid residue.

The functional selectivity of these mutations was demonstrated (Fig. 4) by the ability of these mutant calmodulins to activate MLCK, an extensively characterized CaM-regulated enzyme. Previous studies (6, 31) have shown that MLCK is sensitive to mutations in some surface-exposed, hydrophilic amino acids of calmodulin. As shown in Fig. 4, all of the calmodulins mutated at residue 101 activate MLCK to the same extent. Comparative kinetic analysis of wild-type and S101F CaM:MLCK complexes demonstrated that they were nearly identical in kinetic properties. The S101F-CaM:MLCK complex gave an apparent peptide substrate K_m of 10.8 ± 1.2 μM, compared with 10.6 ± 2.5 μM for wild-type CaM (24). Similarly, the S101F-CaM:MLCK complex gave an apparent V_{max} of 1.91 ± 0.05 μmol/min per mg compared to 2.07 ± 0.04 μmol/min per mg for the wild-type

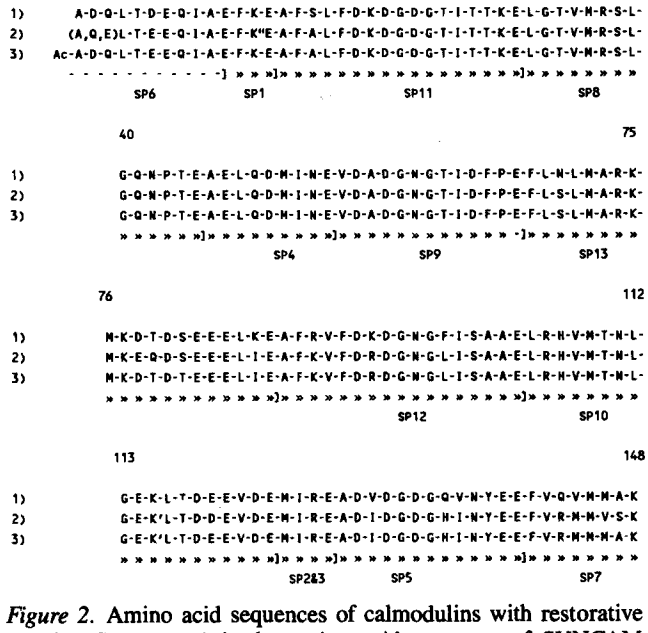

Figure 2. Amino acid sequences of calmodulins with restorative activity. Sequence 1 is the amino acid sequence of SYNCAM calmodulin (21). The amino acid sequence of the mutant calmodulins produced as part of this study differ from the SYNCAM calmodulin sequence only at residue 101 (see Table I). Sequence 2 is *Paramecium* calmodulin (23). Sequence 3 is the amino acid sequence of *Tetrahymena* calmodulin determined as part of the studies reported here, and differs from that previously reported (32) at residues 81, 101, and 146. The sequence strategy is as follows:] indicates boundaries of peptides isolated from V8 protease digests (these peptides are labeled SP1-13): ≫ indicates residues placed by Edman degradation; -, residues placed by amino acid composition and phylogenetic similarity. These sequence data are available from EMBL/GenBank/DDBJ under accession number M11334.

CaM:MLCK complex. Because the calmodulin titration assays (Fig. 4) of MLCK activation by the Ser-101 mutants are similar to wild-type, these results are consistent with little or no change in MLCK kinetic parameters. Studies of other mutant CaM:MLCK complexes have demonstrated that alterations in MLCK activity observed in the CaM titration assays are well correlated with differences in MLCK:CaM complex kinetics because a trend of increased substrate K_m values is observed (24). Altogether, the comparative studies with MLCK confirm the selectivity of the functional effects that are seen with changes at residue 101 of CaM, and are consistent with results obtained from computational chemistry that suggest the maintenance of the overall structural features of calmodulin in these mutants (i.e., a gross and overall change in the structural features of CaM would be expected to affect a number of distinct CaM-regulated activities).

Since the above results showed a strong preference for serine-101 in regards to the activation of the Ca^{2+}-dependent K^+ channel, we decided to reexamine the *Tetrahymena* calmodulin sequence which was reported to have a threonine at residue 101 (32). As summarized in Table II and Figure 2, the reinvestigation of the sequence demonstrated that *Tetrahymena pyriformis* calmodulin has a serine at residue 101. In addition, there were differences from the reported sequence seen at residues 81 (THR instead of SER) and 146 (MET instead of ALA). All other amino acids were as reported previously.

Discussion

The results presented here establish that a cellular phenotype, which involves regulation of a calcium-dependent K^+ conductance, is dependent on the presence of a serine-101 in CaM. Although glycine or tyrosine can partially substitute for serine, there is a clear preference by *Paramecium* (and possibly other organisms not yet studied) for a serine in the context of divergent CaM sequences. This preference is reflected in the intraorder phylogenetic conservation of serine, and raises the question of whether or not there has been coevolution of calmodulin and the macromolecular targets of calmodulin functioning in this cellular regulatory role. Clearly, the insights and reagents produced as a result of this initial study provide a starting point in attempts to address the question of phylogenetic distribution, as well as the molecular mechanisms, of calmodulin regulation of cellular phenotypes through calcium-dependent K^+ conductances.

It was not possible to predict with a high degree of confidence that the functional effects of the point mutations of calmodulin found in the pantophobiac *Paramecium* (22, 23) could be mimicked if they occurred outside of the *Paramecium* amino acid sequence. *Paramecium* calmodulin has 18 amino acid sequence differences from SYNCAM calmodulin, has 16 differences from vertebrate CaM, and is unique at 4 positions compared with all known calmodulin sequences. In addition, the limited phylogenetic distribution of some CaM-regulated activities (e.g., myosin light chain kinase activity) raises the possibility of some phylogenetic specificity of CaM function, analogous to the species specificity seen with growth hormone action. The demonstration (14) that yeast CaM cannot efficiently activate vertebrate MLCK is one example. The results reported here demonstrate that a serine to phenylalanine mutation in CaM can have the same qualitative effect on a cellular phenotype when found in the context of multiple CaM structures. These results indicate that the phylogenetic differences among CaM sequences do not act as intragenic suppressors (i.e., are not functioning as second-site revertants) of the phenotypic effects of the serine-101 mutations, and suggest a broader phylogenetic signifi-

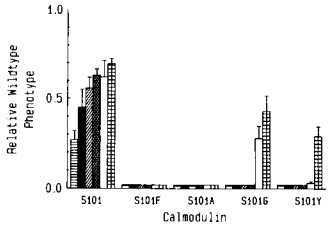

Figure 3. Ability of microinjected calmodulins to restore wild-type phenotype to *caml Paramecium*. The relative ability of each calmodulin to temporarily restore a population of mutant *Paramecium* to a wild-type phenotype in a dose-dependent manner was tested by injecting chemically homogeneous samples of each calmodulin into cells and monitoring motility responses (backward swimming after an ionic stimulus) as previously described (10) and summarized in Materials and Methods. The ordinate indicates relative wild-type phenotype and the abscissa indicates the calmodulin injected into an individual cell. Each set of bars indicates the dose-dependent restoration of wt activity by a particular CaM (error bars denote the standard deviation in the response of the injected cells). The doses of the calmodulins are 0.5 pg/cell (*horizontal*), 1.0 pg/cell (*solid*), 2.5 pg/cell (*diagonals*), 5.0 pg/cell (*cross-hatched*), 7.5 pg/cell (*open*), and 10.0 pg/cell (*checkered*). Each bar represents a minimum of eight data points.

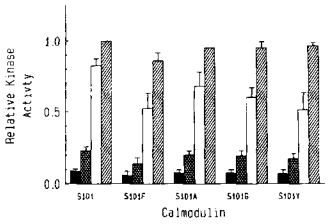

Figure 4. All calmodulins differing at residue 101 activate a protein kinase. The ordinate gives the activity of the myosin light chain kinase on a scale of 0–1, where 1 is the maximum activity obtained with MLCK and fully active SYNCAM CaM at 100 nM. The values are an average of duplicates at each concentration of calmodulin (error bars are the standard deviation). The concentration of CaMs are 1 nM (*solid*), 5 nM (*cross-hatched*), 50 nM (*open*), and 100 nM (*diagonals*). As discussed in the text, kinetic analyses also demonstrate that these mutant CaMs are indistinguishable in their ability to function as a regulatory subunit of MLCK.

cance to the results obtained with *Paramecium* mutants that have altered regulation of calcium-dependent ion conductances.

Although the molecular mechanisms whereby point mutations at residue 101 of calmodulin alter the regulation of ion channels and the cellular phenotype are unknown, our current state of knowledge of how calmodulin structure is related to in vivo and in vitro function provides several reasonable hypotheses that can be tested. Among the plausible explanations are alterations in the macroscopic binding constants of CaM and Ca^{2+}, selective alterations of protein: protein interactions, or a combination of both. A hypothesis (2) based on the crystal structure of vertebrate calmodulin is that the side chain oxygen atom of Ser 101 is involved in the binding of calcium via hydrogen bonding to water. However, it is not possible to predict at this time how a perturbation of this structural feature would alter ion binding characteristics of calmodulin. Recent ion binding studies of the S101F mutant calmodulin (Haiech, J., M.-C. Kilhoffer, T. J. Lukas, and D. M. Watterson, unpublished results) have shown that the S101F CaM has four calcium-selective binding sites that are characteristic of calmodulin (9, 11), although there may be an alteration in the microscopic Ca^{2+}-binding constants. Further, previous studies (18) of calcium binding to CaM when it is bound to an enzyme have shown that there is a significant enhancement of calmodulin's affinity for calcium when it is part of a protein complex. Thus, a simplistic explanation in which the phenotype is altered solely due to the loss of a calcium-binding site is lacking in its appeal, but cannot be completely rejected at this time.

The serine-101 alteration could result from abnormal interactions between the calmodulin and the proteins to which it binds. Initial studies (Wilson, W., and D. M. Watterson, unpublished observation; Hinrichsen, R., and Pollock, M., unpublished observations) using calmodulin binding techniques to examine extracts of membrane fractions from *Paramecium* and rabbit kidneys (both of which contain a low-conductance, calcium-regulated potassium efflux activity) demonstrated a diminished interaction of S101F mutant calmodulins with the calmodulin-binding proteins. One of these proteins has been purified (Wilson, W., and D. M. Watterson, unpublished) and shown to be structurally related to the β-subunit of the G-protein family (1, 7). However, the interaction of this protein with calmodulin is weak compared with that between calmodulin and MLCK, and there are other proteins in the membrane fractions whose interaction with calmodulin is diminished by the S101F mutation. That calmodulins with serine-101 can temporarily restore the phenotypic defects of a *Paramecium* with a S101F calmodulin mutation, yet microinjected S101F calmodulin cannot cause the defective phenotype in wild-type *Paramecia*, is consistent with an altered interaction of S101F calmodulin with endogenous structures. However, more extensive studies are required before the various mechanistic possibilities, including altered turnover of CaM, can be addressed with any confidence.

Although the biological response studied here involves *Paramecium*, the insight and reagents produced as a result of this study may help investigation into the possible involvement of calmodulin in the regulation of ion channels in other organisms. For example, these results raise the possibility of using calmodulins differing by a single amino acid to develop differential calmodulin-Sepharose chromatography procedures for purification (8), or differential ligand screening of cellular extracts (3, 29) and expression libraries (25, 27), in attempts to isolate and characterize proteins involved in regulation of calcium-regulated ion channels. Clearly, these are logical next steps based on the results presented here, and their successful completion should enhance our knowledge about the proteins involved in the regulation of calcium-modulated ion channels.

We thank Paul Matrisian and Augustine Smith (Vanderbilt University) for

Table II. Automated Edman Degradation Data for Regions of Revised Amino Acid Sequence of Tetrahymena Calmodulin*

	Sequence Data																		
	141					146		148											
Amino acid identified‡	Phe	Val	Arg	Met	Met	Met	Ala	Lys											
Amount (*pmol*)‡	66	56	69	38	49	44	39	16											
	88													101			104		
Amino acid identified‡	Ala	Phe	Lys	Val	Phe	Asp	Arg	Asp	Gly	Asn	Gly	Leu	Ile	Ser	Ala	Ala	Glu		
Amount (*pmol*)‡	162	192	123	171	142	75	105	62	68	55	46	82	50	12	37	61	7		
	68													81					87
Amino acid identified‡	Phe	Leu	Ser	Leu	Met	Ala	Arg	Lys	Met	Lys	Asp	Thr	Asp	Thr	Glu	Glu	Glu	Leu	Ile	Glu
Amount (*pmol*)‡	653	596	157	496	379	290	256	225	237	183	207	144	107	162	239	241	166	90	64	20

* Shaded residues are those different from Yazawa et al. (32). The intact calmodulin has a blocked amino terminus. Therefore, the amino acid sequence was determined by automated Edman degradation of peptides purified by HPLC from protease digests of the calmodulin.
‡ The identity and amount of phenylthiohydantoin-amino acid derivative identified at each cycle of the automated Edman degradation reaction are given.

their expert technical assistance, and Janis Elsner and Cindy Reeder (Vanderbilt University) for their help with the preparation of this manuscript.

This work was supported in part by National Science Foundation grant DCB-8706681 (R. D. Hinrichsen), National Institutes of Health grant GM-30861 (D. M. Watterson), a grant from the Deutsche Forschungsgemeinschaft (J. Schultz) and grant G196 from the Cystic Fibrosis Foundation (D. M. Watterson). E. Wilson was a trainee on National Cancer Institute training grant CA09582.

Received for publication 1 June 1990 and in revised form 27 August 1990.

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