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 (cam1) 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 cam1 (pariophobia) 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 Ca2+-based action potentials upon stimulation. The action potential is due to four specific ion channels, the voltage-dependent Ca2+ and K+ channels, and the Ca2+-dependent Na+ and K+ channels. The Ca2+ 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 Ca2+ channel activity inhibits the cells' ability to swim backwards, while the elimination of the Ca2+-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 cam1 Paramecium mutant (originally designated...
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**Materials and Methods**

**SYNCAM calmodulin** is iso-functional with vertebrate and plant calmodulins; it can be considered as 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 SYN-CAM 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 done as described (6, 16, 21). The new mutant calmodulins made as part of this study were verified by DNA sequence.

**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 |

**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 Ca2+-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 Ca2+-dependent K+ current when microinjected into cam1 Paramecium (Hinrichsen, R., unpublished observation).

Hinrichsen and co-workers (10) have demonstrated that the microinjection of wildtype Paramecium calmodulin can cause a temporary restoration of the Ca2+-dependent K+ current in cam1 Paramecium which lacks this current. This restoration also returns the cam1 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 Å, 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.

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
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 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 SPI-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.

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 intracellular 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 phenyalanine 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-
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.

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.

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

| Amino Acid Identified | 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 |
| 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 7 101 104 |
| Amino acid identified  | Phe Leu Ser Leu Met Ala Arg Lys Met Lys Asp Thr Asp Thr Glu 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.
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