An Intragenic Suppressor of a Calmodulin Mutation in Paramecium: Genetic and Biochemical Characterization

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ABSTRACT

We describe a suppressor of the calmodulin mutant *caml* in *Paramecium tetraurelia*. The *caml* mutant, which has a SER → PHE change at residue 101 of the third calcium-binding domain, inhibits the activity of the Ca\(^{2+}\)-dependent K\(^{+}\) current and causes exaggerated behavioral responses to most stimuli. An enrichment scheme, based on an increased sensitivity to Ba\(^{2+}\) in *caml* cells, was used to isolate suppressors. One such suppressor, designated *cam101*, restores both the activity of the Ca\(^{2+}\)-dependent K\(^{+}\) current and behavioral responses of the cells. We show that the *cam101* mutant is an intragenic suppressor of *caml*, based on genetic and microinjection data. The *cam101* calmodulin is shown to be similar to wild-type calmodulin in terms of its ability to stimulate calmodulin-dependent phosphodiesterase at low concentrations of free calcium. However, the *cam101* calmodulin has a reduced affinity for a monoclonal antibody to wild-type calmodulin, and a different mobility on acid-urea gels relative to both wild-type and *caml* calmodulin. We have been able to demonstrate that the isolation of intragenic suppressors of a calmodulin mutation is possible, which allows for the further genetic analysis of structure-function relationships in the calmodulin molecule.

CALCIUM is known to play a role as a second messenger in numerous intracellular pathways (RASMUSSEN 1981). A number of calcium-modulated proteins have been discovered that, in combination with increased intracellular calcium, control various cellular processes. One such calcium-modulated protein is calmodulin, a small, acidic protein found in all eukaryotic organisms studied (KLEE and VANAMAN 1982; MANALAN and KLEE 1984). Calcium-calmodulin complexes play a key role in the regulation of a number of cellular processes, including the control of cyclic nucleotide levels, phospholipase activity, phosphorylation and dephosphorylation, microtubular function (MANALAN and KLEE 1984) and the modulation of ion channels (HINRICHSEN et al. 1986; LUKAS et al. 1989; KINK et al. 1990).

Calmodulin has four high affinity calcium-binding domains, with two domains on each side of the molecule separated by a long α-helical linker (KLEE and VANAMAN 1982; STRYNADKA and JAMES 1989). There is a cooperative effect in calcium binding between the first two and last two binding domains. The binding of calcium to calmodulin causes a conformational change of the molecule which results in the exposure of hydrophobic domains (STRYNADKA and JAMES 1989). These hydrophobic domains are able to interact with various calmodulin-binding proteins in the cell. Much effort has gone into the elucidation of the biochemical and structural features of the calmodulin molecule (BABU, BUGG and COOK 1988; ROBERTS, LUKAS and WATTERSON 1986). Nevertheless, many questions remain unresolved in terms of the relationship of structure and function in calmodulin.

Genetic engineering has been recently utilized to probe the structure-function relationships of the calmodulin molecule (CRAIG et al. 1987; PERSECHINI et al. 1989; GEORGE et al. 1990). With this approach an altered calmodulin is expressed in a bacterial expression system, purified and then tested using in vitro enzyme assays. However, the observed effects do not necessarily apply to an in vivo situation. *Paramecium tetraurelia* presents a means of exploring calmodulin structure and function in vivo, utilizing nonlethal calmodulin mutants (HINRICHSEN et al. 1986; LUKAS et al. 1989; KINK et al. 1990). Calmodulin mutants in Paramecium affect the function of specific ion channels and are proving useful for the further elucidation of calmodulin structure and its function in the cell.

Paramecium undergoes defined behavioral responses (changes in the direction of swimming) that are initiated by the generation of action potentials (for a review, see HINRICHSEN and SCHULTZ 1988). The action potential generated by *P. tetraurelia* is controlled by four separate ion channels, the voltage-dependent Ca\(^{2+}\) and K\(^{+}\) channels and the calcium-dependent K\(^{+}\) and Na\(^{+}\) channels (SAIMI and KUNG 1987; SCHULTZ, KLUMPP and HINRICHSEN 1990). Both of the calcium-dependent channels are con-
trolled by calmodulin (Preston et al. 1990, 1991). Several of the mutations that affect these channels reside within the calmodulin gene (Hinrichsen et al. 1986; Lukas et al. 1989; Kink et al. 1990). The cam1 mutant (formerly designated pntA') specifically inhibits the Ca²⁺-dependent K⁺ channel (Hinrichsen et al. 1986), while the cam2 mutant (formerly designated pntA²) affects both the Ca²⁺-dependent K⁺ and Na⁺ channels (Lukas et al. 1989). Recently, Kung and colleagues (Kink et al. 1990) found that several previously described behavioral mutants are also calmodulin mutants that affect the Ca²⁺-dependent Na⁺ current. The mechanism by which calmodulin modulates ion channels in Paramecium is presently unknown. Regardless, the ability to generate viable calmodulin mutations that affect a specific cellular function in vivo makes it a very attractive system.

The goal of the research presented in this paper was to uncover suppressor mutations of the cam1 mutant. We have discovered one such mutant, designated cam101. The behavioral phenotype of this mutant is similar to wild type, and the Ca²⁺-dependent K⁺ channel activity has returned. We have discovered that the cam101 calmodulin gene still retained the original cam1 mutation (SER → PHE at amino acid 101), but there were two new mutations, ASP → TYR at amino acid 80 and a ARG → LYS at amino acid 106. A separate paper describes the cloning of the wild-type, cam1 and suppressor calmodulin genes from P. tetraurelia (C. Russell and R. D. Hinrichsen, manuscript in preparation). The present paper examines the genetic and biochemical properties of the cam101 mutant as compared to wild type and cam1.

MATERIALS AND METHODS

**Stocks and culture conditions:** We used P. tetraurelia 51s (wild type), the Ca²⁺ channel mutant pawnA (d4-94), the trichocyst nondischarge mutant nd6 and the calmodulin mutant cam1 (d4-622, formally designated pntA'). Cells were cultured at 28°C in wheat grass-enriched media with stigmasterol (5 mg/ml), buffered with sodium phosphate and Tris-CI and bacterized with Enterobacter aerogenes [see Sonneborn (1970) for details of the culturing methods].

**Genetic techniques and mutagenesis:** Standard methods were employed in all crosses using the self-fertilization process, called autogamy, to generate F₂ cells (see Hinrichsen and Kung 1984, for details of the genetic techniques used for Paramecium). The trichocyst nondischarge mutant nd6 was used as a genetic marker in all crosses. Mutagenesis was performed by treating cam1 cells with N-methyl-N'-nitro-N-nitrosoguanidine (2 mg/50 ml) for 1.5 hr as described previously (Hinrichsen, Saimi and Kung 1984). The cells utilized for mutagenesis had grown at least 20 fissions from the last nuclear reorganization, which allows the cells to undergo autogamy when starved. Once mutagenized, the cells were allowed to undergo autogamy in order to make cells homozygotic at all loci and then grown for 6–8 fissions to overcome phenomic lag before being screened for suppressors of the cam1 mutation.

**Behavioral assays:** The methods employed to evaluate the behavioral responses of the cells were the same as those used previously (Saimi et al. 1983) with slight modifications. The cells were incubated in a resting solution (1 mM KCl, 1 mM CaCl₂, 1 mM HEPES and 10⁻⁶ M EDTA, pH 7.5) for 10 min before they were tested in the same resting buffer to which the appropriate ion was added. The time of backward swimming in the test solutions was determined for the different cell types. All tests were conducted at room temperature using cells in the log phase of growth.

**Screening for suppressors:** Suppressor of the cam1 mutant were isolated using a modification of the previously described Ba²⁺ lethality assay (Hinrichsen, Saimi and Kung 1984). Cells were placed in resting solution for at least 5 min and then transferred to 6 mM BaCl₂ and left for a designated period of time at room temperature. While the wild-type cells eventually died in this solution due to the Ba²⁺ which enters through the Ca²⁺ channels, the cam1 mutant died much sooner. Consequently, viable cells were collected as presumptive cam1 suppressors, cloned and tested for their behavioral responses.

**Microinjection and electrophysiological techniques:** Cells were microinjected with calmodulin as described previously (Hinrichsen et al. 1986). Approximately 20 pl (10% of the total cell volume) of calmodulin (2 pg total) was injected into each cell. The cells were tested at various intervals after the injections in a testing solution (10 mM NaCl and 5 mM TEA⁺ in resting solution) that easily distinguished the wild-type from cam1 cells. The cam1 mutant used for these injections was purified as described previously (Burgess-Cassler et al. 1987). The homogeneity of the preparation was judged using silver-stained SDS gels.

The electrophysiological techniques of cell penetration, intracellular recording and voltage clamp were the same as those described previously (Hennessy and Kung 1987).

**Biochemical techniques:** The wild-type, cam1 and cam101 calmodulins were run on mini acid-urea gels (10% acrylamide and 2.5 mM urea) for 6 hr at 4°C. A similar system has been used to distinguish wild type and cam1 calmodulin (Burgess-Cassler et al. 1987).

Phosphodiesterase (PDE) assays were performed in accordance with published procedures (Slaughter, Chafouleas and Means 1985), monitoring the cleavage of [³²P]cAMP. The activity of the calmodulin-dependent bovine PDE (Sigma) was judged by its ability to cleave the cAMP in the presence or absence of calmodulin. To determine the calcium dependence of the different calmodulins, the free calcium concentration in the reaction was varied using a Ca²⁺:EGTA buffering system. Free calcium concentrations were calculated using a computer program based on published data for Ca⁺⁺:EGTA dissociation constants (Caldwell 1970).

The radiolmmunoassay (RIA) for the Paramecium calmodulin was performed using a modification of a previously described procedure (Chafouleas, Dedman and Means 1982). Paramecium wild-type calmodulin was labeled with [¹²⁵I] Bolton and Hunter 1973); 10–15,000 cpm of the labeled calmodulin was used for each experiment. A monoclonal antibody specific to P. tetraurelia calmodulin, which has a reduced affinity for the cam1 calmodulin (Burgess-Cassler et al. 1987) was used. Initial experiments determined the amount of antibody that brought down 50% of the [¹²⁵I] calmodulin. Separation of the bound and free [¹²⁵I] calmodulin was accomplished using Amerlex-M (Amersham) containing sheep anti-mouse IgG attached to a magnetic separation reagent; the bound calmodulin was spun down into the pellet. The amount of [¹²⁵I] in the pellet was determined using a Cobra Auto-Gamma gamma counter. To ascertain the relationship of the tested calmodulin to that of
assumed that increased Ba\(^{2+}\) would enter the variety of ionic and environmental stimuli. It was compared to wild-type cells during the extended action potentials, as compared to wild-type cells (SAIMI results in an overreactive behavioral response to a presence of Ba\(^{2+}\) than wild-type cells (Figure 1). Wild-type cells did not die in the 6 mM Ba\(^{2+}\) solution for it was discovered that Suppressors of the "KUNG 1984)."

"petro"s were considered dead or dying. The proteins to Immobilon-P transfer membrane (Millipore), contain several calmodulin-binding proteins) were extracted and left at (1 0 -0)

"calcium. The calmodulin-binding protein assays were performed using previously described techniques for Paramecium (Evans and Nelson 1989). Cilia from wild-type cells (which contain several calmodulin-binding proteins) were extracted with 1% Triton X-100 and the extracted membrane proteins were run on 12% SDS gels. After electrotransfer of the proteins to Immobilon-P transfer membrane (Millipore), the gels were probed with 10\(^{6}\) cpm of \(^{125}\)I-labeled wild-type, cam1 or cam101 calmodulin in the presence of 15 \(\mu\)M free calcium.

RESULTS

Enriching for suppressor mutants: Ba\(^{2+}\) ions enter through the Ca\(^{2+}\) channels of Paramecium with nearly the same efficiency as Ca\(^{2+}\) ions (SAIMI and KUNG 1982), but the Ba\(^{2+}\) eventually leads to cell death. Suppressors of the cam1 mutant were uncovered after it was discovered that cam1 cells die sooner in the presence of Ba\(^{2+}\) than wild-type cells (Figure 1). Wild-type cells did not die in the 6 mM Ba\(^{2+}\) solution for 12–24 hr, whereas the cam1 cells were all dead within 5 hr. A similar result has been observed with another overreactive mutant, Dancer (HINRICHSSEN, SAIMI and KUNG 1984).

The cam1 cells display extended action potentials compared to wild-type cells (SAIMI et al. 1983), which results in an overreactive behavioral response to a variety of ionic and environmental stimuli. It was assumed that increased Ba\(^{2+}\) would enter the cam1 cells during the extended action potentials, as compared to wild-type cells, and cause the increased lethality. However, this simplistic explanation was shown not to be the case when a double mutant, cam1 and pwnA (a mutation that inhibits the Ca\(^{2+}\) channel), was tested in the Ba\(^{2+}\) solutions. The single pwnA mutant was able to live longer in Ba\(^{2+}\) than wild-type cells; greater than 70% of the pwnA cells were viable after 24 hr, compared to less than 20% of the wild-type cells (Figure 1). This was not unexpected since the inactive ciliary Ca\(^{2+}\) channels of pwnA will not pass Ba\(^{2+}\) ions; the cells probably die from Ba\(^{2+}\) that enters through Ca\(^{2+}\) channels on the cell body (they are not affected by the pwnA mutation). However, the cam1/pwnA double mutant, which has the behavioral phenotype of the pwnA mutant (HINRICHSSEN et al. 1985), died in the Ba\(^{2+}\) solution at the same rate as the cam1 mutant (Figure 1). This would suggest that the cam1 cells died prematurely in the Ba\(^{2+}\) solution for reasons other than the fact that more Ba\(^{2+}\) entered the cells as compared to wild type. It may be that the cam1 calmodulin has a lethal effect when combined with Ba\(^{2+}\), and the small amount that enters through the cell body Ca\(^{2+}\) channels is enough to cause lethality. If this is the case, the Ba\(^{2+}\) lethality assay may be suitable for the isolation of intragenic suppressors of the calmodulin mutation (which would overcome the effect with Ba\(^{2+}\)), but not for most intergenic suppressors (since the mutant calmodulin would still be present).

To isolate intragenic suppressors, cam1 cells mutagenized with nitrosoguanidine were placed in a 6 mM BaCl\(_2\) solution and left for 10 hr at 22°. Viable cells were isolated, cloned and tested for their behavioral phenotype. Of the 163 cells isolated, 90% of these cells still displayed the cam1 phenotype. The majority of these were unaltered cam1 cells that made it through the screening process (i.e., they still died prematurely in Ba\(^{2+}\)). Of the remaining 10%, a number were less sensitive to BaCl\(_2\) killing, but still displayed the cam1 behavioral phenotype. However, 5 of the 163 isolated cells displayed a nearly wild-type phenotype. Several were true revertants to the wild-type sequence. One cell line, that regained wild-type behavior but is not a true revertant, designated cam101, is the subject of this paper.

Behavioral and electrophysiological analysis of cam101: The cam101 cells were scored for their behavioral responses in comparison to wild-type and cam1 cells. They were tested in different solutions known to elicit specific responses in the form of backward swimming. The cam1 cells display much more exaggerated behavioral responses than wild type in all solutions tested with the exception of 40 mM KCl (Table 1). These behavioral differences have been demonstrated previously and are accounted for by the lack of a Ca\(^{2+}\)-dependent K\(^{+}\) current in the cam1 cells.
(SAIMI et al. 1983) which results in an extended action potential and longer backward swimming. When the putative cam1 suppressor, cam101, was tested in these ionic solutions, their behavioral responses were similar to those of the wild-type cells (Table 1). The cam101 cells slightly overreacted in the Ba2+ and Na+ solutions as compared to wild type, but not nearly to the extent seen with the cam1 cells. Thus, cam101 cells behave in a manner similar to, but distinguishable from, wild-type cells.

We examined the cam101 cells electrophysiologically in order to ascertain if the Ca2+-dependent K+ current was present. As mentioned earlier, the original cam1 cells are lacking this current, which leads to the observed behavioral defects. The late outward currents (of which the Ca2+-dependent K+ current is predominant) were examined under voltage clamp. The outward currents were induced using a voltage step from -40 mV to 0 mV for 500 msec in a solution with no Na+ present (preventing contaminating Ca2+-dependent Na+ inward currents). The wild-type cells show a steadily rising outward current and a pronounced tail current when the voltage is stepped back down to -40 mV (Figure 2A). This tail current is indicative of the Ca2+-dependent K+ current in Paramecium (SAIMI et al. 1983). The cam1 cells, on the other hand, showed a steady decline in the late outward currents and no tail current (Figure 2B), indicative of the loss of the Ca2+-dependent K+ current (SAIMI et al. 1983). When the cam101 cells were tested under the same conditions, the Ca2+-dependent K+ current and a typical tail current were observed (Figure 2C, double arrows). The tail current of the wild-type cells was 0.55 nA (± 0.09), cam1 was 0.17 (± 0.07) and cam101 was 0.58 nA (± 0.14). This demonstrates that the cam101 suppressor mutation has restored the activity of the Ca2+-dependent K+ channel. Moreover, the hyperpolarization-induced Ca2+-dependent K+ current, which is also lacking in the cam1 cells (PRESTON, SAIMI and KUNG 1990), has returned in the cam101 cells (data not shown).

Besides the lack of a Ca2+-dependent K+ current, the cam1 cells are also defective in their ability to mate with cells of the opposite mating type (HINRICHSEN et al. 1985). cam1 cells mating with wild-type cells are able to form the anterior holdfast union, but not a complete cell union. Complete fusion of two cells is required for proper transfer of the meiotic nuclei and cross-fertilization. This defective mating phenotype in cam1 is somewhat leaky so that crosses with cam1 can be performed. When the cam101 cells were tested for their ability to mate, they formed mating pairs as well as the wild-type cells. Therefore, the suppression of the cam1 phenotype in the cam101 suppressor is not restricted to the behavioral (swimming) phenotype, but is also seen in their ability to mate.

**The genetics of cam101:** To determine if the cam101 mutation was an intra- or intergenic suppressor of cam1, we crossed the cam101 mutant to wild-type cells that carried the trichocyst nondischarge mutation (nd6) as a genetic marker. The F1 behavioral phenotype of the cells was similar to wild type (Table 2 (cross 1)). The cells were tested for their behavioral responses to the different solutions listed in Table 1, and there was no indication of the phenotype displayed by either cam1 or cam101. The F1 cells then were carried into the F2 by autogamy. There was a

### Table 1

**Behavioral responses of cam1 suppressor**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ba2+</th>
<th>10 Na+</th>
<th>10 Na+/5 TEA</th>
<th>10 TEA</th>
<th>10 K+</th>
</tr>
</thead>
<tbody>
<tr>
<td>51s</td>
<td>AR^b</td>
<td>AR</td>
<td>19.6 (4.2)</td>
<td>AR</td>
<td>35.0 (2.8)</td>
</tr>
<tr>
<td>cam1</td>
<td>20.0 (4.6)</td>
<td>16.8 (4.8)</td>
<td>88.4 (11)</td>
<td>15.0 (4.2)</td>
<td>34.8 (2.9)</td>
</tr>
<tr>
<td>cam101</td>
<td>4.5 (1.3)</td>
<td>4.7 (0.8)</td>
<td>19.8 (4.0)</td>
<td>AR</td>
<td>25.9 (4.4)</td>
</tr>
</tbody>
</table>

* All ions tested were chloride salts and in a resting solution of 1 mM KCl, 1 mM CaCl2, 1 mM HEPES, and 10^-6 EDTA, pH 7.2.
* AR (avoiding response): this is a less than 1-sec backward swimming response to a stimulus.
* Time in seconds of the backward swimming response. The parentheses indicate the standard deviation. At least 15 cells were tested in each different ionic solution.

**Figure 2.** — Late outward currents in wild type (A), cam1 (B) and cam101 (C). The late outward currents were induced by a voltage step from -40 mV to 0 mV for 500 msec under voltage clamp. The wild-type cells showed a steady outward current during the depolarization step and an outward tail current (arrow) upon repolarization to -40 mV. In the cam1 cells (B), there is a steadily declining outward current during the depolarization step and no outward tail current. This is in accordance with previous work with cam1 (SAIMI et al. 1983). In the cam101 suppressor cells (C), the outward current and tail current (double arrow) are present, much like the wild-type cells. All cells were bathed in 10 mM TEA chloride, 1 mM CaCl2, 1 mM HEPES and 0.01 mM EDTA, pH 7.2.
Suppressor of a Calmodulin Mutation

TABLE 2

Genetics of the cam1 suppressor

<table>
<thead>
<tr>
<th>Cross</th>
<th>F1 phenotype</th>
<th>F2 segregation</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. cam101 x nd6</td>
<td>Wild type</td>
<td>+/+/cam101:nd6/cam101:+/+/cam101:nd6/cam101</td>
<td>0.25</td>
</tr>
<tr>
<td>2. nd6,cam101 x +/+,cam1</td>
<td>cam101</td>
<td>cam101:cam1:wild type</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* This phenotype was determined by testing the cells in the same solutions listed in Table 1. 
* The x² values were calculated with an expected ratio of 1:1:1 for cross 1 and 1:1:0 for cross 2.

1:1 segregation of the cam101 and wild-type phenotypes (the cam101 cells were distinguished by a slight overreaction to Na⁺ and Ba²⁺ solutions), but no cam1 cells were observed. If cam101 were an intergenic suppressor of cam1, there would have been a class of F2 cells that displayed the cam1 phenotype.

To determine if cam101 was a closely linked, intergenic suppressor, we crossed the cam101 suppressor cells to cam1. If they were very closely linked, but separate loci, the F1 cells would be homozygotic for both cam1 and the suppressor, and should display the wild-type phenotype. But if they are within the same gene, the heterozygous F1 should be the cam1 or cam101 phenotype (depending on which calmodulin type was dominant). The F1 phenotype of the cells in this cross was the same as cam101 (Table 2, cross 2). There was an approximate 1:1 segregation of cam1 and cam101 in the F2, with no cells showing the wild-type phenotype. The data presented in Table 2 (cross 2) represents several crosses that were combined, and wild-type cells, as a result of recombination, were never observed. From these two crosses, we conclude that the cam101 mutant is most probably an intragenic suppressor of the cam1 mutation.

As a test of this conclusion, we injected purified cam101 calmodulin into the cam1 cells. The cam1 cells are temporarily restored to the wild-type phenotype upon the injection of wild-type calmodulin (HINRICHS et al. 1986). Within 2–4 hr of injecting the wild-type calmodulin, the behavioral phenotype of the cam1 cells is restored to the wild-type phenotype; after 30–36 hr the cells return to the mutant phenotype. As little as 0.6 pg of calmodulin will bring about this restoration, while the injection of as much as 10 pg of cam1 calmodulin into cam1 has no effect. If the cam101 mutation is actually within the calmodulin gene, one would expect purified cam101 calmodulin would restore the cam1 cells. However, the calmodulin from an intergenic suppressor would still be cam1-like and not have such an effect. 2 pg of the cam101 calmodulin was able to restore the cam1 cells to nearly the same extent as the wild-type calmodulin, whereas the cam1 calmodulin had absolutely no effect (Figure 3). A small, but significant, difference between the wild-type and cam101 calmodulin was observed when less than 1 pg of calmodulin was injected (data not shown). This would appear to conclusively demonstrate that the cam101 mutation is within the calmodulin gene and somehow alters the defect caused by the original mutation.

Biochemical characterization of the cam101 calmodulin: There are several known biochemical differences between wild type and cam1 calmodulin (BURGESS et al. 1987). Therefore, the cam101 calmodulin was biochemically analyzed and compared to that of cam1 and wild type.

First, we measured the mobility of the different calmodulins on acid-urea gels. The cam1 calmodulin has a faster mobility on acid-urea gels than wild type (BURGESS-CASSLER et al. 1987). Figure 4 shows that the cam101 calmodulin runs at a level between that of wild type and cam1. This result is easily reproduced.
maximal stimulation. As the calcium concentration was lowered, however, the effectiveness of the cam 1 calmodulin was decreased as compared to the wild-type and cam 101 calmodulin. The difference between wild type and cam 1 at lower free calcium concentrations suggests that the cam 1 calmodulin does not bind calcium as well as wild-type calmodulin (the cam 1 calmodulin is altered in the third calcium binding domain). The results in Figure 5 suggest that the cam 101 calmodulin is nearly as effective at binding calcium as wild-type calmodulin. Binding studies of the Ca\(^{2+}\) to the various calmodulin species will enable us to directly test this possibility.

A monoclonal antibody specific to Paramecium calmodulin has been shown to bind the cam 1 calmodulin with a lower affinity than wild type (Burgess-Cassler et al. 1987). The epitope of the antibody overlaps that area of the calmodulin molecule where the cam 1 mutation resides (A. Burgess-Cassler, personal communication). We have devised a rapid RIA to study the relationships of the different calmodulins using \(^{125}\)I-labeled wild-type Paramecium calmodulin and the monoclonal antibody. The \(^{125}\)I-labeled wild-type calmodulin was added to the monoclonal antibody, along with varying amounts of cold calmodulin. The greater the decline in binding of the labeled calmodulin, the more similar the test calmodulin is to wild-type Paramecium calmodulin. As can be seen in Figure 6, the cam 101 calmodulin behaves in a manner similar to the cam 1 calmodulin, much more cold cam 101 calmodulin is required to reduce the amount of labeled wild-type calmodulin in the pellet. This would indicate that the suppressor calmodulin still has the same (or similar) epitope as the cam 1 calmodulin, and has a reduced

The cam 1 and wild-type calmodulins activate the calmodulin-dependent enzyme PDE to the same extent when an excess of calcium is present. However, as the free Ca\(^{2+}\) concentration is lowered, the cam 1 calmodulin becomes a less effective activator of PDE than the wild-type calmodulin. The cam 101 calmodulin was found to be nearly as effective an activator of PDE as wild-type calmodulin at lower concentrations of free calcium (Figure 5). At a free Ca\(^{2+}\) concentration of 10 \(\mu\)M, all three calmodulin types gave

and has been used as a convenient means of comparing different types of calmodulin.

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affinity for the antibody as compared to the wild-type calmodulin.

Finally, we evaluated the ability of the cam101 calmodulin to bind to two specific calmodulin-binding proteins for which cam1 calmodulin is known to have a decreased affinity (R. D. HINRICHSEN, T. EVANS and M. POLLOCK, manuscript in preparation). These proteins, with molecular masses of 38 and 60 kD, are located in the ciliary membrane of P. tetraurelia, and bind 125I-labeled wild-type calmodulin to a much greater extent than 125I-labeled cam1 calmodulin. We tested the ability of the cam101 calmodulin to label the 38- and 60-kD proteins, to ascertain if it acted like the wild-type or cam1 calmodulin. As can be seen from Figure 7, the wild-type calmodulin was able to bind to both the 38- and 60-kD proteins with a high affinity, but both the cam1 and cam101 calmodulin were greatly reduced in their ability to bind to either protein. However, they were able to bind to another calmodulin-binding protein, with a molecular mass of 85 kD, with only a slightly reduced affinity as compared to the wild-type calmodulin. Therefore, the cam101 suppressor, even though it has restored some of the biochemical phenotype of the cam1 calmodulin, is unable to bind to certain calmodulin-binding proteins with the affinity of wild-type calmodulin.

DISCUSSION

We have described a technique that can be employed to isolate intragenic suppressors of a calmodulin mutation. The Ba2+ lethality assay allowed us to isolate and characterize an intragenic suppressor of a calmodulin mutant from P. tetraurelia which we designated cam101. The calmodulin of cam101 still had several of the properties of the original cam1 calmodulin: (1) a reduced affinity for a monoclonal antibody to wild-type calmodulin (Figure 6) (2) inability to bind with high affinity to two specific calmodulin-binding proteins (Figure 7). On the other hand, the cam101 mutation rectifies several aspects of the cam1 mutation: (1) the behavioral phenotype of the cam1 cells when tested in a variety of ionic solutions (Table 1), (2) the activity of the Ca2+-dependent K+ current (Fig. 2), (3) the cam1 cells ability to mate properly and (4) the calmodulin stimulation of the calmodulin-dependent enzyme phosphodiesterase in the presence of physiological concentrations of calcium.

The calmodulin of the original cam1 mutant has been shown to possess a single amino acid change as compared to wild type. Within the third calcium-binding domain of the calmodulin molecule, the SER at residue 101 is changed to PHE (SCHAEFER et al. 1987). The side chain oxygen atom of SER at amino acid 101 has been implicated in the binding of calcium via hydrogen bonding to water, and the SER 101 appears to initiate the existing helix from this calcium-binding loop (STRYNADKA and JAMES 1989). We have recently demonstrated that the SER at residue 101 is highly conserved among the various species of calmodulin (HINRICHSSEN et al. 1990); only the calmodulin of Schizosaccharomyces pombe is known to have a different amino acid at this position, a change of SER → THR (TAKEDA and YAMAMOTO 1989). We have also discovered that the replacement of SER with TYR, GLY or ALA at amino acid residue 101 allows for little or no restoration of the Ca2+-dependent K+ current in Paramecium (HINRICHSSEN et al. 1990). Therefore, the conserved nature of the SER at residue 101 argues for its importance in the regulation of Ca2+-dependent ion conductances in Paramecium.

The evidence presented in this paper that indicates cam101 is an intragenic suppressor of the original SER → PHE mutation is based on a combination of genetic data and the injection of cam101 calmodulin into the cam1 cells. The cam101 mutation did not segregate from the cam1 mutation, and a heterozygote of cam1/cam101 showed the cam101 phenotype (Table 2). Furthermore, when the cam101 calmodulin was injected into the cam1 cells, the cells were temporarily restored to the wild-type phenotype (Figure 3). This is most easily explained if a new change in the calmodulin molecule itself is responsible for its ability to restore nearly wild-type behavior. Conceivably, a second-site suppressor could alter a post-translational modification of the cam1 calmodulin, but there is no evidence of differences in post-translational modifications between wild-type and cam1 calmodulin (R. D. HINRICHSSEN, unpublished data).

We have recently confirmed that the cam101 mutant is indeed an intragenic suppressor of cam1 by

![Figure 7. -A calmodulin-binding protein assay. 125I-labeled wild-type (A), cam1 (B), wild-type (C) and cam101 (D) calmodulin was used to label Triton X-100 extracted membrane proteins from wild-type cells. Three calmodulin-binding proteins are shown, with molecular masses of 85, 60 and 38 kD. As can be seen, the wild-type calmodulin can bind to all three proteins with a high affinity, whereas the cam1 and cam101 calmodulin only bind to the 85-kD protein with only a slightly reduced affinity as compared to that seen with wild-type calmodulin.](image-url)
sequencing the cam1 and call101 calmodulin genes (C. Russell and R. Hinrichsen, manuscript in preparation). The cam101 calmodulin, besides possessing the original SER → PHE at amino acid 101, has two other changes, a ASP → TYR change at amino acid 80 and ARG → LYS at amino acid 106. It is not certain if both of these changes, or only one of them, is required for the suppressor phenotype. However, this does establish that the effects of the original mutation in the P. tetraurelia calmodulin can be reversed by intragenic mutations.

It has been shown previously that the cam1 mutant cells have a pronounced difficulty undergoing successful mating. This is true of a second mutation within the calmodulin gene, cam2, which also eliminates the Ca^{2+}-dependent K^+ current in P. tetraurelia (C. Kung, personal communication). We have established previously that this defect in mating is most likely not a consequence of the eliminated Ca^{2+}-dependent K^+ current, but rather the altered calmodulin. The behavioral mutant TeaA has an exaggerated Ca^{2+}-dependent K^+ current (Hennessy and Kung 1987). In the double mutant, TeaA and cam1, the Ca^{2+}-dependent K^+ current returns (the TeaA mutation is dominant over the cam1 phenotype) while the cam1 calmodulin is still present; but it still has difficulty mating (Hinrichsen et al. 1985). This would imply that the mating defect is not a consequence of a decreased Ca^{2+}-dependent K^+ current, but rather the cam1 calmodulin. The restoration of normal mating in the cam101 cells implies that the change(s) in the calmodulin molecule of the cam101 cells not only restores its ability to modulate the Ca^{2+}-dependent K^+ current, but also of its ability to regulate the sequence of events which take place during mating in P. tetraurelia. Precisely how calmodulin controls these events is unclear although proper fusion of the cells requires ventral deciliation, a process that appears abnormal in cam1 cells (R. D. Hinrichsen, unpublished data). Without proper deciliation, the cells cannot fuse, and thus cannot exchange pronuclei. The function of calmodulin in this process is unknown.

While this paper demonstrates that it is possible to isolate intragenic suppressors mutations of the calmodulin molecule, we did not detect intergenic suppressors from several screens which we have conducted. This may not be surprising in light of the fact that the double mutant, pawnA (which is significantly decreased in Ca^{2+} channel activity (Saimi and Kung 1987)) and cam1 was shown to suppress the cam1 behavioral phenotype (Hinrichsen et al. 1985), but still dies quickly in Ba^{2+} solutions (Figure 1). The pawnA mutant by itself survives longer in Ba^{2+} than wild-type cells (Figure 1). This suggests that the reason the cam1 cells die sooner in the Ba^{2+} solutions is not solely due to excess Ba^{2+} entering the cells, but also because the combination of Ba^{2+} and the cam1 mutant calmodulin is lethal. The reason for this increased lethality is unknown.Suppressor mutations have been isolated from another overreactive behavioral mutant of Paramecium, the Ca^{2+} channel mutant Dancer. Dancer has wild-type calmodulin and exhibits early lethality in Ba^{2+} solutions (Hinrichsen, Saimi and Kung 1984). Approximately one-half of the intergenic suppressors isolated were found to be pawn-like mutations (R. D. Hinrichsen, unpublished data). This demonstrates that the Ba^{2+} lethality screen employed in this paper can be utilized to isolate intragenic suppressors of certain overreactive behavioral mutations of P. tetraurelia. However, the use of this enrichment technique for the isolation of intergenic suppressors of cam1 does not appear to be feasible. We are in the process of employing different behavioral screens to isolate such mutants from cam1 cells. Furthermore, we are also isolating more intragenic suppressors of cam1 in order to better understand the structure-function relationships of calmodulin for the regulation of the Ca^{2+}-dependent K^+ channel and mating phenomena in P. tetraurelia.

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LITERATURE CITED


Hinrichsen, R., and C. Kung, 1984  Genetic analysis of axonemal


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