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 *cam1* in *Paramecium tetraurelia*. The *cam1* 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 *cam1* 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 *cam1*, 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 *Paramecium* calmodulin, as does the parental *cam1* calmodulin, and a different mobility on acid-urea gels relative to both wild-type and *cam1* 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*^{1}) specifically inhibits the Ca^{2+}-dependent K^+ channel (HINRICHSEN *et al.* 1986), while the *cam2* mutant (formerly designated *pntA*^{2}) affects both the Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+} channel mutant *pawnA* (d4-94), the trichocyst nondischarge mutant *nd6* and the calmodulin mutant *cam1* (d4-622, formally designated *pntA*^{1}). Cells were cultured at 28°C in wheat grass-enriched media with stigmasterol (5 mg/ml), buffered with sodium phosphate and Tris-Cl 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 F_2 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 $CaCl_2$, 1 mM HEPES and 10^{-5} 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: Suppressors of the *cam1* mutant were isolated using a modification of the previously described Ba^{2+} lethality assay (HINRICHSEN, SAIMI and KUNG 1984). Cells were placed in resting solution for at least 5 min and then transferred to 6 mM $BaCl_2$ and left for a designated period of time at room temperature. While the wild-type cells eventually died in this solution due to the Ba^{2+} which enters through the Ca^{2+} 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 calmodulin 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 (HENNESSEY and KUNG 1987).

Biochemical techniques: The wild-type, *cam1* and *cam101* calmodulins were run on mini acid-urea gels (10% acrylamide and 2.5 M urea) for 6 hr at 4°. 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 [^{3}H] 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 Ca^{2+}-EGTA buffering system. Free calcium concentrations were calculated using a computer program based on published data for Ca^{2+}:EGTA dissociation constants (CALDWELL 1970).

The radioimmunoassay (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 ^{125}I (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 [^{125}I]calmodulin. Separation of the bound and free [^{125}I] 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 ^{125}I in the pellet was determined using a Cobra Auto-Gamma gamma counter. To ascertain the relationship of the tested calmodulin to that of

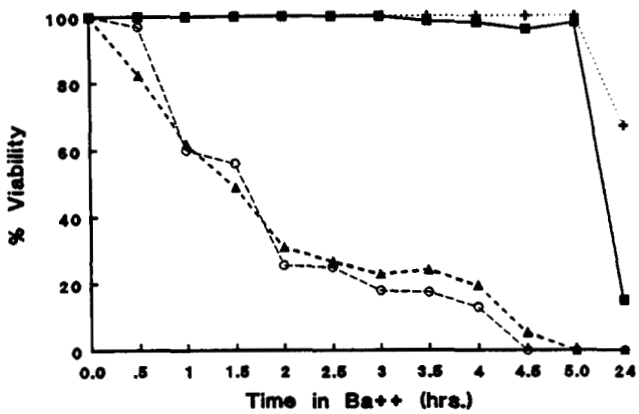

FIGURE 1.—The Ba^{2+} lethality assay. Approximately 10^5 wild-type (■——■), *cam1* (▲- - -▲), *pawn A* (+···+) or *cam1/pawn A* (○- -○) cells were placed in 20 ml of 6 mM $BaCl_2$ in resting solution (1 mM KCl, 1 mM HEPES, 1 mM $CaCl_2$ and 10^{-5} M EDTA, pH 7.2) and left at 23°. At designated periods of time, an aliquot of cells was removed and the percentage of viable cells was scored. Cells were judged to be viable if they were still actively swimming, nonswimming cells were considered dead or dying.

wild type, varying amounts of cold calmodulin from wild type, *cam1* or *cam101* were added to the reaction mixture, along with a constant amount of ^{125}I-labeled wild-type calmodulin and the amount of antibody that would bring down 50% of the ^{125}I. The greater the affinity of the cold calmodulin for the antibody (in other words, the more similar it was to the labeled wild-type calmodulin), the better it competed with the labeled wild-type calmodulin.

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 10^5 cpm of ^{125}I-labeled wild-type, *cam1* or *cam101* calmodulin in the presence of 15 μM 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* (HINRICHSEN, 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/pawnA* double mutant, which has the behavioral phenotype of the *pwnA* mutant (HINRICHSEN *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

TABLE 1

Behavioral responses of *cam1* suppressor

Cell line	Solution tested[a]				
	Ba^{2+}	10 Na^+	10 Na^+/5 TEA	10 TEA	40 K^+
51s	AR[b]	AR	19.6 (4.2)[c]	AR	35.0 (2.8)
cam1	20.0 (4.6)	16.8 (4.8)	88.4 (11)	15.0 (4.2)	34.8 (2.9)
cam101	4.5 (1.3)	4.7 (0.8)	19.8 (4.0)	AR	25.9 (4.4)

[a] All ions tested were chloride salts and in a resting solution of 1 mM KCl, 1 mM $CaCl_2$, 1 mM HEPES, and 10^{-5} EDTA, pH 7.2.

[b] AR (avoiding response): this is a less than 1-sec backward swimming response to a stimulus.

[c] 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.

(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 Ba^{2+} 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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-dependent K^+ current (SAIMI *et al.* 1983). When the *cam101* cells were tested under the same conditions, the Ca^{2+}-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 Ca^{2+}-dependent K^+ channel. Moreover, the hyperpolarization-induced Ca^{2+}-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 Ca^{2+}-dependent K^+ current,

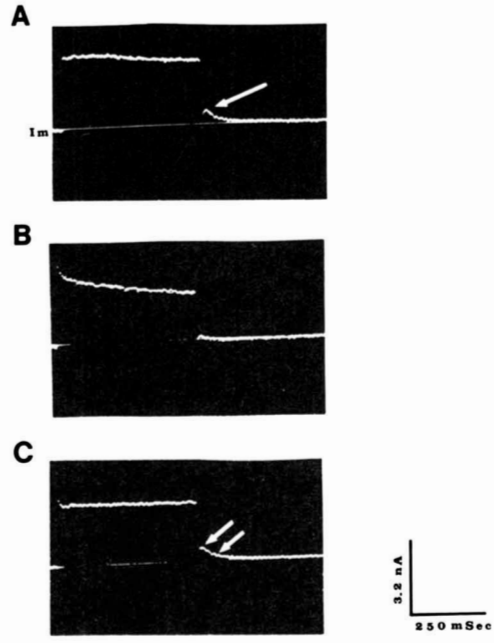

FIGURE 2.—Late outward currents in wild type (A), *cam1* (B) and *cam101* (C). The late outward currents were induced by a voltage step from −40 mV to 0 mV for 500 msec under voltage clamp. The wild-type cells showed a steady outward current during the depolarization step and an outward tail current (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 $CaCl_2$, 1 mM HEPES and 0.01 mM EDTA, pH 7.2.

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 F_1 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 F_1 cells then were carried into the F_2 by autogamy. There was a

TABLE 2

Genetics of the *cam1* suppressor

Cross	F_1 phenotype[a]	F_2 segregation	P^b
1. *cam101* × *nd6*	Wild type 100%	$+^{nd}$/*cam101*:*nd6*/*cam101*:$+^{nd}$/cam^+:*nd6*/cam^+ 221 263 236 252	0.25
2. *nd6,cam101* × $+^{nd}$,*cam1*	*cam101* 100%	*cam101*:*cam1*:wild type 373 431 0	0.11

[a] This phenotype was determined by testing the cells in the same solutions listed in Table 1.
[b] The χ^2 values were calculated with an expected ratio of 1:1:1:1 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 Ba^{2+} solutions), but no *cam1* cells were observed. If *cam101* were an intergenic suppressor of *cam1*, there would have been a class of F_2 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 F_1 cells would be heterozygotic for both *cam1* and the suppressor, and should display the wild-type phenotype. But if they are within the same gene, the heterozygous F_1 should be the *cam1* or *cam101* phenotype (depending on which calmodulin type was dominant). The F_1 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 F_2, 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 (HINRICHSEN *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.

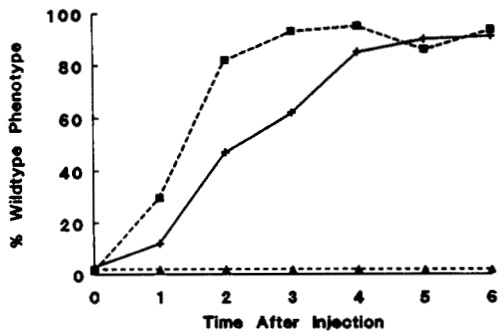

FIGURE 3.—The microinjection of wild-type (■– – –■), *cam1* (▲– – –▲) and *cam101* (+——+) calmodulin into *cam1* cells. The *cam1* cells were injected with 2 pg of the calmodulin and immediately placed in a resting solution that did not permit cell growth. The behavioral response (time of backward swimming) was then tested at designated times in a solution containing 10 mM NaCl and 5 mM TEA^+ in resting buffer. The percentage of the wild-type response was calculated according to the following equation:

$$\text{Relative wt phenotype} = (1 - (X - \text{wt}/\text{cam1} - \text{wt}) \times 100$$

where X = the response (seconds of backward swimming) of the injected cell, wt = the response of the wild-type controls, and *cam1* = the response of the *cam1* controls. All injected cells were tested in this manner and the data were averaged. Each time point represents a minimum of five injected cells. Any injected cell that divided was discarded, to eliminate the problem of dilution of the injected material.

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

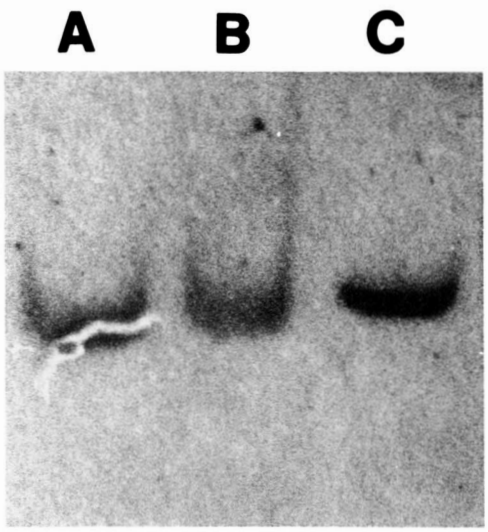

FIGURE 4.—An acid-urea gel to test the mobility of the different calmodulin types. (A) *cam1*, (B) *cam101* and (C) wild-type calmodulin were run for 6 hr and stained with Coomassie blue. As can be seen, the *cam1* calmodulin has a faster mobility compared to the wild-type calmodulin, while the *cam101* calmodulin ran in-between the other two.

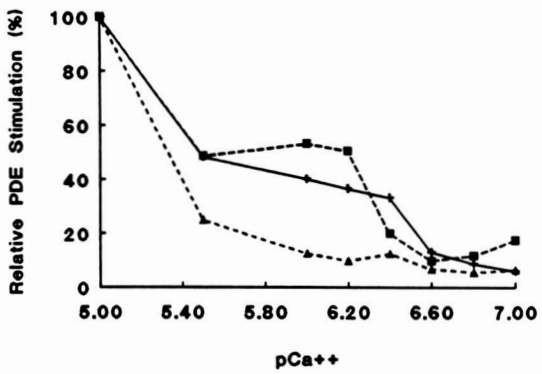

FIGURE 5.—A PDE assay using different calmodulin types and various free calcium concentrations. The wild type (■– – –■), *cam1* (▲– – –▲) and *cam101* (+——+) calmodulin was used to stimulate bovine PDE. The same amount of calmodulin (1 μg) was used in each assay, with the free Ca^{2+} concentration varied using a Ca^{2+}-EGTA buffering system. The data were calculated as a percentage of the stimulation of wild-type calmodulin in the presence of 10^{-3} M Ca^{2+} (pCa^{2+} of 3). There was full stimulation by all calmodulin types at a pCa^{2+} of 5, but the *cam1* calmodulin activity dropped off rapidly as compared to the wild-type and *cam101* calmodulin. At free Ca^{2+} concentrations of less than pCa^{2+} 6.8, there was very little activity in any of the three calmodulin types.

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

The *cam1* 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 *cam1* calmodulin becomes a less effective activator of PDE than the wild-type calmodulin. The *cam101* 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 μM, all three calmodulin types gave

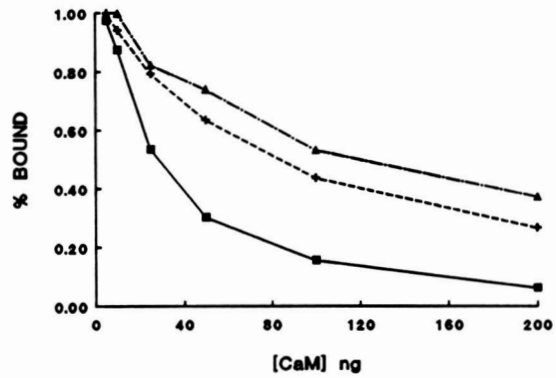

FIGURE 6.—The calmodulin radioimmunoassay. This assay used cold wild-type (■——■), *cam1* (▲·–·▲) and *cam101* (+– – –+) calmodulin to compete with the labeled wild-type calmodulin. Wild-type calmodulin was labeled with ^{125}I and used to bind to the anti-*P. tetraurelia* calmodulin antibody. Different concentrations of cold calmodulin were added to the reaction, which would compete with the labeled calmodulin. The greater the decline in the amount of wild-type ^{125}I-labeled calmodulin bound (taken into the pellet) by the antibody, the more similar the calmodulin was to the wild-type calmodulin. The *cam1* does not bind to the antibody with the same affinity as the wild-type calmodulin. The *cam101* also has a reduced affinity for the antibody.

maximal stimulation. As the calcium concentration was lowered, however, the effectiveness of the *cam1* calmodulin was decreased as compared to the wild-type and *cam101* calmodulin. The difference between wild type and *cam1* at lower free calcium concentrations suggests that the *cam1* calmodulin does not bind calcium as well as wild-type calmodulin (the *cam1* calmodulin is altered in the third calcium binding domain). The results in Figure 5 suggest that the *cam101* 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 *cam1* 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 *cam1* 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 *cam101* calmodulin behaves in a manner similar to the *cam1* calmodulin, much more cold *cam101* 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 *cam1* calmodulin, and has a reduced

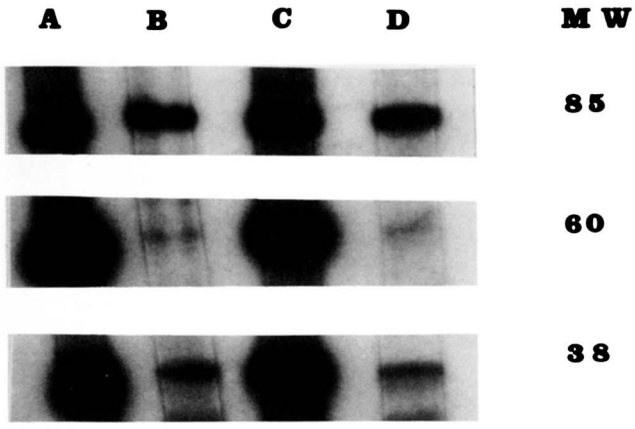

FIGURE 7.—A calmodulin-binding protein assay. ^{125}I-labeled wild-type (A), *cam1* (B), wild-type (C) and *cam101* (D) calmodulin was used to label Triton X-100 extracted membrane proteins from wild-type cells. Three calmodulin-binding proteins are shown, with molecular masses of 85, 60 and 38 kD. As can be seen, the wild-type calmodulin can bind to all three proteins with a high affinity, whereas the *cam1* and *cam101* calmodulins only bind to the 85-kD protein with only a slightly reduced affinity as compared to that seen with wild-type calmodulin.

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 ^{125}I-labeled wild-type calmodulin to a much greater extent than ^{125}I-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 Ba^{2+} 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 I), (2) the activity of the Ca^{2+}-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 (HINRICHSEN *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 Ca^{2+}-dependent K^+ current in Paramecium (HINRICHSEN *et al.* 1990). Therefore, the conserved nature of the SER at residue 101 argues for its importance in the regulation of Ca^{2+}-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. HINRICHSEN, unpublished data).

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

sequencing the *cam1* and *cal101* 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 (HENNESSEY 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, *pwnA* (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 *pwnA* 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 intergenic 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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