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Electrophysiological Evidence Suggests a Defective Ca^{2+} Control Mechanism in a New *Paramecium* Mutant

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Summary. A new mutant of *Paramecium tetraurelia*, k-shyA, was characterized behaviorally and electrophysiologically. The mutant cell exhibited prolonged backward swimming episodes in response to depolarizing conditions. Electrophysiological comparison of k-shyA with wild type cells under voltage clamp revealed that the properties of three Ca^{2+}-regulated currents were altered in the mutant. (i) The voltage-dependent Ca^{2+} current recovered from Ca^{2+}-dependent inactivation two- to 10-fold more slowly than wild type. Ca^{2+} current amplitudes were also reduced in the mutant, but could be restored by EGTA injection. (ii) The decay of the Ca^{2+}-dependent K^+ tail current was slower in the mutant. (iii) The decay of the Ca^{2+}-dependent Na^+ tail current was also slower in the mutant. All other membrane properties studied, including the resting membrane potential and resistance and the voltage-sensitive K^+ currents, were normal in k-shyA. Considered together, these observations are consistent with a defect in the ability of k-shyA to reduce the free intracellular Ca^{2+} concentration following stimulation. The possible targets of the genetic lesion and alternative explanations are discussed. The k-shy mutants may provide a useful tool for molecular and physiological analyses of the regulation of Ca^{2+} metabolism in *Paramecium*.

Key Words *Paramecium* · calcium · cilia · mutants · Ca^{2+} pump · Ca^{2+} buffering · ion channels

Introduction

Calcium ion has been identified as an important intracellular messenger that regulates a wide variety of cellular functions in many diverse organisms (Campbell, 1983). In most, if not all, cells, cytoplasmic Ca^{2+} concentrations are kept low (<1 μM), and small changes in Ca^{2+} flux can therefore lead to relatively large changes in intracellular Ca^{2+} concentration. Many of the responses triggered by Ca^{2+} are transient; they begin when the Ca^{2+} concentration rises above micromolar levels and they cease, within seconds or milliseconds, as Ca^{2+} returns to the prestimulus level. Mechanisms that regulate and maintain Ca^{2+} concentrations in cells are therefore crucial to normal cellular function.

In the ciliated protozoan *Paramecium*, internal free Ca^{2+} is a key regulator of ciliary-based motility (Eckert, Naitoh & Machemer, 1976; Kung & Saimi, 1982). Swimming behavior is controlled by the activity of voltage-dependent Ca^{2+} channels that are localized in the ciliary membrane. Depolarization of the cell membrane, elicited by ionic, mechanical, or electrical stimuli, open Ca^{2+} channels, leading to Ca^{2+} influx down a steep electrochemical gradient. Ca^{2+} then triggers reversal of the ciliary beat direction, resulting in transient backward swimming of the cell. In addition, Ca^{2+} modifies swimming behavior indirectly by regulating ion currents which influence membrane potential and Ca^{2+}-channel activity (*see below*). Considering the multiple roles of Ca^{2+} in measurable physiological responses together with the ability to perform genetic and biochemical analyses, *Paramecium* provides a useful model system for studying the regulation of Ca^{2+} and its role as a second messenger.

Previous work has implicated several membrane phenomena as important regulators of the Ca^{2+} signal in *Paramecium* (Kung & Saimi, 1982; Hinrichsen et al., 1985). The Ca^{2+} current is activated by depolarizing voltages and is transient, showing both "fast" Ca^{2+}-dependent inactivation (Brehm & Eckert, 1978) and "slow" voltage-dependent inactivation (Hennessey & Kung, 1985). In addition, several K^+ conductances including a voltage-dependent K^+ current (Oertel, Schein & Kung, 1977) and a Ca^{2+}-activated K^+ current (Satow & Kung, 1980*a*) regulate the Ca^{2+} current by rectification of the membrane potential. A Ca^{2+}-dependent Na^+ current, important for regulating membrane

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potential in Na^+-containing solutions, has also been described (Saimi & Kung, 1980).

Although it has been proposed that mechanisms for reducing intracellular Ca^{2+} (e.g., Ca^{2+} pumps, Ca^{2+} sequestration systems) are also important to the control of *Paramecium* behavior (Eckert, 1972; Browning & Nelson, 1976; Martinac & Hildebrand, 1981), very little is known of these mechanisms. We have isolated a novel class of mutants that give exaggerated swimming responses to various stimuli. These mutations fall into two complementation groups, are recessive, and segregate as single gene loci in standard genetic crosses (T.C. Evans and D.L. Nelson, *in preparation*). We describe the membrane properties of one complementation group, k-shyA, which suggest that this mutant fails to regulate intracellular Ca^{2+} levels normally, and hence may be defective in a Ca^{2+} removal mechanism. The k-shy mutants may be useful in the analysis of Ca^{2+} extrusion or buffering mechanisms and their role(s) in the regulation of swimming behavior.

Materials and Methods

Strains and Culture Conditions

For convenience in the electrophysiological studies, we used the mutant *nd6*, which cannot discharge its trichocysts (Sonneborn, 1975). The behavioral and electrophysiological properties of *nd6* are identical with those of the parent 51s strain, and *nd6* is referred to here as wild type. All mutant strains used also carried the *nd6* mutation. The mutant k-shyA[1] (*ksA*[1], d4-644) and wild type stocks were cultured at 23–25°C in bacterized "minimal medium" containing 5 mM MOPS (3-[N-morpholino]propanesulfonic acid), 4 mM KCl, 1 mM $CaCl_2$, 0.44 mM sodium citrate, 0.3 mg/liter casamino acids, 80 μM $MgSO_4$, and trace metals, at pH 7.0. The minimal medium was inoculated with *Enterobacter aerogenes* one day before inoculation with *Paramecium*. For some experiments, cells were cultured in bacterized Cerophyl medium (Sonneborn, 1970). The phenotypes of the mutants and wild type were similar under both growth conditions.

Genetics and Behavior

Wild type cells, either 51S or *nd6*, were mutagenized as described (Kung, 1971; Hinrichsen & Kung, 1984), and the k-shy mutants were selected on the basis of prolonged backward swimming in depolarizing solutions. The details of the selection procedures and genetic characterization of the k-shy mutants will be described elsewhere (T.C. Evans and D.L. Nelson, *in preparation*). The *ksA*[1] (d4-644) allele of k-shyA was derived from an *nd6* parent, and was used for all of the experiments presented here unless otherwise noted.

For behavioral analysis, cells were first transferred to a "resting solution" (1 mM KCl, 1 mM $CaCl_2$, 1 mM MOPS, 0.01 mM EDTA, pH 7.2) for at least 30 min. One to three cells were then transferred to one of the following test solutions: (i) 10–50 mM KCl, (ii) 2–20 mM NaCl, (iii) 10 mM TEA-Cl (tetraethylammonium chloride), or (iv) 2–8 mM $BaCl_2$. All test solutions contained 1 mM $CaCl_2$, 1 mM MOPS, 0.01 mM EDTA, pH 7.2 and at least 1 mM KCl. The transient backward jerks (ciliary reversal) and the continuous backward swimming episodes were observed under a dissecting microscope. The duration of backward swimming was defined as the time in seconds required for a cell to resume forward swimming following ciliary reversal. Measurements were made from 5–10 cells and the means and standard deviations (SD) were determined. Each experiment was repeated at least three times.

Electrophysiological Recordings

Several different recording solutions were used. (i) *K^+-Ca^{2+} solution:* The bath solution contained 1 mM KCl and 1 mM $CaCl_2$ and the glass electrodes were filled with 3 M KCl (for recording the total currents), 3 M KCl with 0.5 M EGTA (for buffering internal free Ca^{2+}), or 0.4 M KCl (for current injection experiments). (ii) *Cs-TEA solution:* The bath consisted of 4 mM CsCl, 10 mM TEA-Cl, and 1 mM $CaCl_2$, and the recording electrodes contained 2 M CsCl (to block K^+ currents and isolate the voltage-dependent inward Ca^{2+} current, I_{Ca}), (iii) *Na^+ solution:* The bath contained 10 mM NaCl, 4 mM CsCl, 10 mM TEA-Cl and 1 mM $CaCl_2$ (to block K^+ currents and isolate the Ca^{2+}-dependent Na^+ current). Electrodes contained 2 M CsCl. All solutions also contained 1 mM MOPS and were brought to pH 7.2 with Tris base. The 2 and 3 M electrodes had resistances of 10–20 MΩ, while the 0.4 M KCl electrodes were 70–100 MΩ. When EGTA was included in the electrodes the pH was adjusted to 7.0 with Tris base.

The techniques for two-electrode voltage clamp and current injection analysis were the same as those initially described by Satow and Kung (1979) and modified by Hennessey and Kung (1985). In the voltage-clamp experiments the membrane potential was held at −40 mV and currents were recorded on a Nicolet digital oscilloscope. The voltage-step durations were either 25 msec or 2.5 sec, and the amplitudes were in 10-mV increments from −150 to +50 mV. The resting membrane resistance, R_m, was extrapolated linearly from the currents measured near the holding level in response to + or −5 and 10 mV steps. These passive background currents were subtracted from all of the current measurements. The R_m was also determined by measuring the voltage changes in response to current injections of 0.1 to 1.0 nA and determining the slope of the linear portion of a plot of voltage *vs.* current. The recording temperature was 23–25°C and the data are expressed in all cases as the mean ±SD.

Tail Current Measurements

All tail currents were plotted as the log of the measured current as a function of time. Since these decay rates were single exponentials, the maximal tail current amplitudes were derived by extrapolating to zero time. The time constants (τ values) were defined as the time necessary for the tail current to decline to 63% of its maximal amplitude and were determined for tail currents elicited after a 2.0-sec voltage step from the holding level of −40 to −6 mV.

Recovery from I_{Ca} Inactivation

The rates of return from Ca^{2+}-dependent Ca^{2+} current inactivation ("fast" inactivation) were determined by the twin-pulse paradigm described by Brehm, Eckert, and Tillotson (1980). The Cs-TEA procedure was used to isolate the I_{Ca}. Pairs of identical voltage steps (25 msec duration and +34 mV amplitude) were presented with each pair separated by a different interval (50 msec–1 min). Each pair of pulses elicited a pair of Ca^{2+} currents, I_1 and I_2. With short intervals (less than 100 msec), I_2 amplitudes were reduced relative to I_1 due to inactivation of Ca^{2+} channels by the residual Ca^{2+} that entered during I_1 (*see* Fig. 3). As the intervals were lengthened, the I_2 amplitudes increased and eventually equalled I_1. To determine the rate of recovery from Ca^{2+}-dependent Ca^{2+} channel inactivation, the logarithm of I_2/I_1 for several pairs of pulses was plotted as a function of time between pulses. This produced linear plots and the time constants (τ values) were determined to be the time necessary for I_2/I_1 to reach 0.63.

The onset of "slow," voltage-dependent inactivation was assayed as described in Hennessey and Kung (1985). The Ca^{2+} currents (I_{Ca}) were isolated with the Cs-TEA procedure, and EGTA was injected to eliminate the "fast" inactivation. Briefly, the peak I_{Ca} seen in response to a 36-mV step of variable duration (1–60 sec) (I_1) was compared with the second I_{Ca} (I_2) induced 50 msec later. The log of I_2/I_1 was plotted against duration of the first pulse and the time constants were determined.

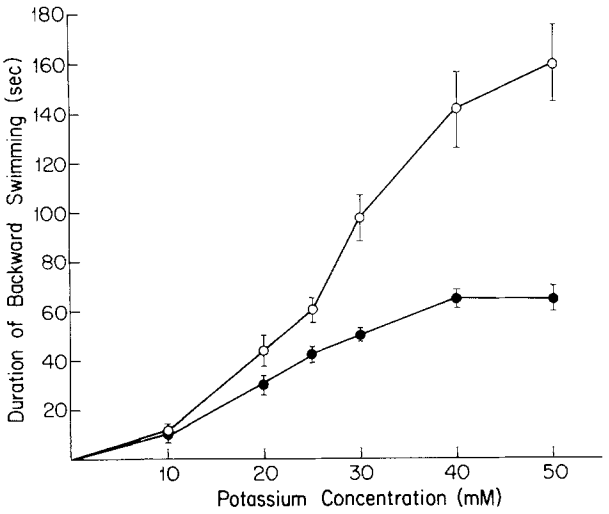

Fig. 1. The duration of backward swimming induced by transfer to K^+ test solutions. Backward swimming periods for k-shyA (○—○) and wild type (●—●) were measured as described in Materials and Methods after transfer from resting solution to test solutions containing various K^+ concentrations. All values represent the mean of six cells ± SD. All solutions were room temperature

Results

Behavioral Phenotype of k-shyA

The swimming behavior of k-shyA was distinguishable from that of wild type in all test solutions. In the "minimal medium," wild type cells swam forward with occasional brief backward jerks (ciliary reversals) of less than one body length. In contrast, k-shyA cells underwent bursts of prolonged and continuous ciliary reversal, lasting several body lengths, from which they recovered sluggishly. The difference between mutant and wild type was best seen by measuring the duration of backward swimming in response to the K^+ test solution. When transferred from the resting solution to test solutions containing 20–50 mM K^+, wild type cells swam backward continuously for tens of seconds and then gradually returned to slow forward swimming (Fig. 1). The k-shyA cells displayed longer backward swimming responses, lasting up to 3 min in 50 mM K^+ at 25°C.

Test solutions containing Ba^{2+}, Na^+, or tetraethylammonium ions (TEA^+) (4–10 mM) also elicited brief and repetitive backward swimming responses in wild type and k-shyA cells. The k-shyA responses in each of these solutions were qualitatively distinct from wild type, as the mutant ciliary reversals showed slower transitions to forward swimming. However, the k-shyA responses to Ba^{2+} and TEA^+ solutions were complicated in that the ciliary reversal episodes arose less frequently in mutant cells than in wild type (*see* Discussion). Despite this complexity, the exaggerated swimming behavior of k-shyA in K^+ test solutions and in growth media clearly suggested a hyperexcitable phenotype. Therefore, we next analyzed several membrane properties relevant to Ca^{2+} regulation.

Voltage-Dependent Ca^{2+} Current

For measurements of the voltage-dependent Ca^{2+} current (I_{Ca}), the CsCl-TEA recording solutions were used. This current is activated by depolarizing voltages and both activates and inactivates in milliseconds (Satow & Kung, 1979; *see* Fig. 3). The fast inactivation of the current has been shown to be mediated by Ca^{2+} (Brehm & Eckert, 1978). Following 20-msec step depolarizations, transient inward Ca^{2+} currents were observed in both wild type and k-shyA cells (*see* Fig. 3). Although no differences were detected in the activation kinetics or voltage sensitivities of the I_{Ca}, current amplitudes were significantly reduced in the k-shyA mutant at all voltage steps tested (Fig. 2*A*). This result seemed to conflict with what might be expected from the behavioral phenotype of k-shyA since the analyses of the pawn and *cnr*C mutants of *Paramecium* have suggested that reduced I_{Ca} amplitude leads to pro-

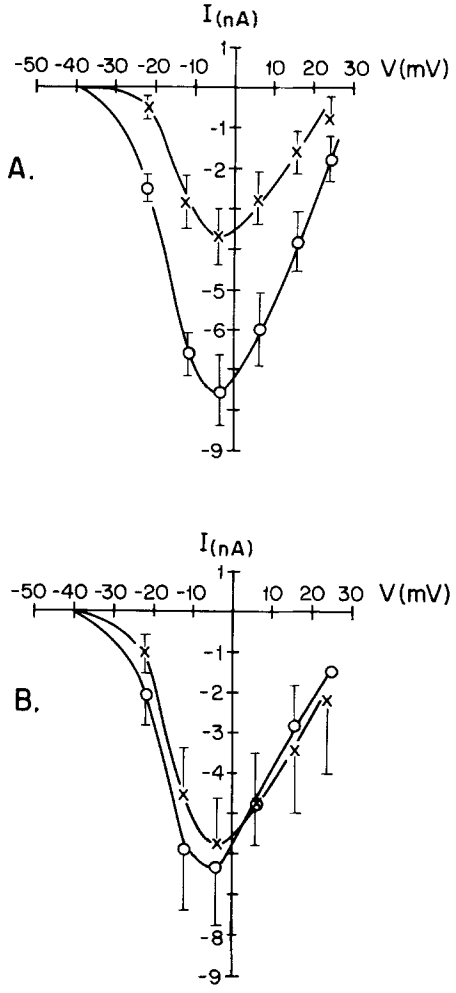

Fig. 2. Ca^{2+} current-voltage relationships for k-shyA and wild type. (*A*) I_{Ca} amplitudes for k-shyA (×—×) and wild type (O—O) were measured in the Cs-TEA solution at various voltage steps. Each step was 25 msec in duration. (*B*) Same as for *A* except that cells were injected with EGTA as described in Materials and Methods before the measurements of I_{Ca}. As in *A*, each point represents the mean ± SD of three cells

portionally reduced backward swimming responses (Satow & Kung, 1980*b*; Haga et al., 1984). To test whether the reduced I_{Ca} amplitudes in k-shyA were due to higher internal Ca^{2+} concentrations and consequent I_{Ca} inactivation, mutant and wild type cells were injected with EGTA and the I_{Ca} was measured. The EGTA injections not only inhibited I_{Ca} fast inactivation, they also restored the current amplitudes of k-shyA to wild type levels (Fig. 2*B*). In contrast, the injection of EGTA into pawnC (d4-131), a mutant with I_{Ca} reduced to a similar extent as k-shyA, had no effect on current amplitude (*data not shown*). These results suggested that k-shyA has higher resting Ca^{2+} levels or faster Ca^{2+} current inactivation (*see* Discussion).

Table 1. Summary of Ca^{2+}-sensitive current parameters: wild type *vs.* k-shyA

	Wild type	k-shyA	*n*
I_{Ca} amplitude (nA)	−7.5 ± 0.95	−3.7 ± 0.71	4
I_{Ca} amplitude with EGTA	−6.3 ± 1.5	−5.8 ± 1.2	5
τ for I_{Ca} inactivation recovery (msec)	39.3 ± 7.1	82.3 ± 4.5	4
τ for I_K^{Ca} tail (msec)	41.5 ± 8.5	233.0 ± 45.0	4
τ for I_{Na}^{Ca} tail (msec)	110.0 ± 20.0	351.0 ± 43.0	5

Conditions for current measurements, EGTA injection and time constant (τ) determinations are described in Materials and Methods. The Ca^{2+} current (I_{Ca}) values were measured with voltage steps of +34 mV and 25 msec duration. Comparison of paired I_{Ca} pulses as a function of time between pulses (*see* Materials and Methods) was used to determine time constants (τ) for recovery from I_{Ca} inactivation. The tail currents of the Ca^{2+}-dependent K current (I_K^{Ca}) and the Ca^{2+}-dependent Na^+-current (I_{Na}^{Ca}) were recorded following the termination of voltage steps of +34 mV and 2.5 sec duration. All measurements are expressed as the mean ± SD of *n* cells.

Recovery from "Fast" I_{Ca} Inactivation

To test whether the Ca^{2+}-dependent reduction of I_{Ca} in k-shyA might be due to an impaired ability to reduce internal free Ca^{2+}, we next determined the rates of recovery from fast I_{Ca} inactivation. The rate at which the Ca^{2+} channels recover from inactivation by Ca^{2+} should be at least partly dependent upon the rate at which the Ca^{2+} is removed from the vicinity of the inactivation mechanism. To determine the inactivation recovery rate, we used the twin-pulse protocol of Brehm et al. (1980), which measures the recovery of I_{Ca} amplitude from the Ca^{2+} delivered by a previous I_{Ca} pulse (*see* Materials and Methods). We found that the time constants for the return of I_{Ca} following fast inactivation were consistently longer for k-shyA than for wild type (Table 1). This difference is demonstrated by the two pairs of I_{Ca} pulses shown in Fig. 3. For both wild type and mutant, the time between pulses was too short to allow complete recovery of Ca^{2+} channels from inactivation. While the wild type I_2 was reduced by 40% in this experiment, I_2 in k-shyA was reduced by 67%, a result of the slower rate of return from inactivation in k-shyA. Increasing the duration of the first pulse to 2 sec caused an even more dramatic difference in recovery rates; the time constant for recovery from fast I_{Ca} inactivation was 59.0 ± 3.0 msec for wild type and 815 ± 161 msec for k-shyA (n = 4). The slow recovery rate of k-shyA was not due to permanent damage to the Ca^{2+} current function, since I_2 eventually reached 80 to 90% of I_1 regardless of the duration of the first

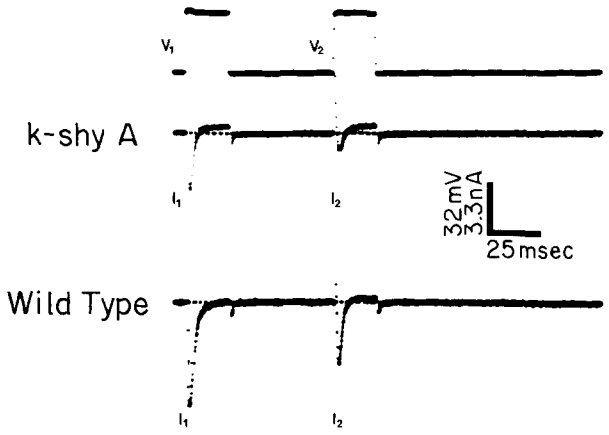

Fig. 3. Recovery from the Ca^{2+}-dependent "fast" inactivation of the Ca^{2+} current. For both wild type and k-shyA the twin voltage steps (V_1 and V_2) (Materials and Methods) were 25 msec in duration, +34 mV in amplitude, and separated by 50 msec. I_1 and I_2 refer to the two I_{Ca} produced by the voltage steps. The Cs-TEA procedure (Materials and Methods) was used to isolate the I_{Ca} for this analysis

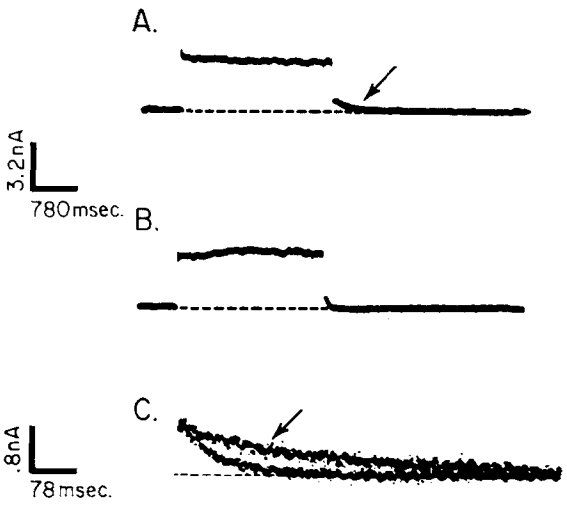

Fig. 4. Tail currents of the Ca^{2+}-dependent K^+ current. The Ca^{2+}-dependent K^+ current and its indicative tail (*see* arrow) are shown for the k-shyA mutant (*A*) and for wild type (*B*) with a 2.5-sec voltage step to +34 mV. The tail currents are expanded and superimposed in *C*. The arrow marks the k-shyA tail current. The K^+-Ca^{2+} solution and KCl electrodes were used

pulse. Nor was this difference caused by the reduction of I_{Ca} amplitude of k-shyA since the rate of inactivation recovery of pawnC (τ = 39.0 ± 7 msec) was indistinguishable from the wild type rate. Furthermore, the reduction of the k-shyA recovery rate was dependent on internal Ca^{2+} since injection of EGTA eliminated the differences between mutant and wild type (*data not shown, also see below*). These results are consistent with the hypothesis that, following excitation, there is a prolonged elevation of the free Ca^{2+} concentration in k-shyA.

"Slow" I_{Ca} Inactivation

A second type of Ca^{2+} current inactivation ("slow" inactivation) has recently been described (Hennessey & Kung, 1985). It is distinguished from fast inactivation by its very slow kinetics of onset and recovery (tens of seconds) and its insensitivity to Ca^{2+}. Because it may be an important regulator of ciliary reversal, and to be sure that it did not influence the measured kinetics of recovery from fast inactivation, we examined the properties of slow inactivation in k-shyA. EGTA was used with the CsCl electrodes to block fast inactivation (*see* Materials and Methods). The mean time constant for onset of slow inactivation was 43.7 ± 19.1 sec for k-shyA and 44.7 ± 11.4 sec for wild type (n = 3), values that are not significantly different. The mutant behavioral phenotype is therefore not due to an altered slow inactivation mechanism. Furthermore, the onset of slow inactivation could not significantly influence the effect of k-shyA on recovery from fast inactivation; under the conditions for measuring slow inactivation, I_2 was reduced by only 20% after a 2-sec depolarization, and the ratio of I_2/I_1 for k-shyA was the same as for wild type at all pulse durations.

Ca^{2+}-Dependent K^+ and Na^+ Currents

Two Ca^{2+}-activated currents, the Ca^{2+}-dependent K^+ current (Satow & Kung, 1980*a*) and the Ca^{2+}-dependent Na^+ current (Saimi & Kung, 1980; Saimi, 1986), have been characterized in *Paramecium*. Both currents activate slowly (hundreds of milliseconds) after step depolarizations, and display distinct tail currents following repolarization to resting potential. However, these currents differ in kinetics and ion selectivities, can be separated genetically (*see* Hinrichsen et al., 1985), and thus clearly represent distinct entities. If k-shyA were indeed defective in the regulation of internal Ca^{2+} levels, then the mutation may affect the properties of these Ca^{2+}-sensitive conductances.

The Ca^{2+}-dependent K^+ current, measured in the K^+-Ca^{2+} solution (Materials and Methods) is best represented by the outward tail current seen following termination of a 2-sec voltage step (*see* Fig. 4, arrow). Although both the characteristic slow development and amplitude of the Ca^{2+}-dependent K^+ current were similar in k-shyA and wild type (*data not shown*), the simple exponential decay of the tail current was much slower in k-shyA

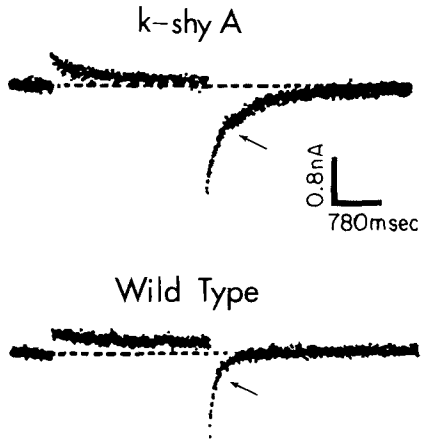

Fig. 5. Tail currents of the Ca^{2+}-dependent Na^+ current in k-shyA and wild type. The Na^+ Cs-TEA solution was used to block all K^+ currents and each tail current (shown by arrows) was recorded after a 2-sec depolarization from −40 to −6 mV. In contrast to the Ca^{2+}-dependent K^+ current tail (Fig. 4), this tail current is inward

(Table 1 and Fig. 4*C*). The decay kinetics in pawnC, however, were indistinguishable from the kinetics in wild type (*data not shown*), again indicating that a small I_{Ca} alone cannot produce the differences seen in k-shyA.

The Ca^{2+}-dependent Na^+-current was isolated by using the Na^+ solution which includes Cs^+ and TEA^+ to block all of the K^+ currents (Materials and Methods). The indicative inward tail current, seen after a 2-sec depolarization from the holding level of −40 mV to −6 mV, decayed more slowly in k-shyA than in wild type (Fig. 5); the mean time constant for the decay was threefold larger for the mutant (Table 1). These results and the effect of the mutation on the Ca^{2+}-dependent K^+ tail current kinetics are also both consistent with a prolonged elevation of internal Ca^{2+} in k-shyA cells following depolarization.

Other Membrane Properties

Further voltage-clamp measurements showed that the voltage sensitivities and current amplitudes of the voltage-dependent K^+ current (delayed rectifier) and the hyperpolarization-induced current (anomalous rectifier) (Oertel et al., 1977, 1978) did not differ between k-shyA and wild type (*data not shown*). The membrane resistance, determined by linear extrapolation of the currents induced by small (5 and 10 mV) voltage steps, was also the same for wild type and k-shyA (Table 2).

We also examined the active and passive membrane voltage responses to injected current in cells

Table 2. Passive and active membrane potential responses to injected current

	Wild type	k-shyA	*n*
Action potential peak (mV)	52.0 ± 5.5	39.0 ± 2.5	4
Afterhyperpolarization (mV)	−0.7 ± 1.4	−5.7 ± 1.6	4
R_m (MΩ)	45.0 ± 5.8	41.4 ± 8.3	4
V_m (mV)	−43.3 ± 2.0	−42.8 ± 1.0	4

The current injected was 0.95 nA and 200 msec in duration. The resting membrane resistance (R_m) was determined as described in Materials and Methods. V_m is the resting membrane potential in 1 mM KCl, 1 mM $CaCl_2$, 1 mM MOPS. All values are expressed as means of *n* cells ± SD.

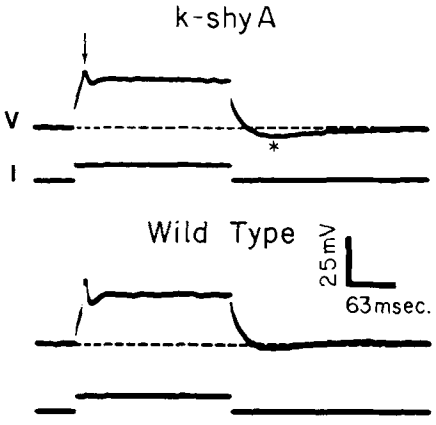

Fig. 6. Action potentials induced by injected current in wild type and k-shyA. The current injected (*I*) was 0.95 nA and 200 msec in duration. In k-shyA, the amplitude of the action potential (arrows on voltage traces, *V*) is reduced and the extent of the afterhyperpolarization (*) is increased. Conditions for recording are described in Materials and Methods

bathed in KCl solution. The resting membrane potentials of k-shyA cells were not different from those of wild type (Table 2), and the resting membrane resistances were also indistinguishable. Upon injection of sufficient current, action potentials were induced in both wild type and k-shyA. The action potentials of k-shyA differed from those of wild type in two respects: (i) the peak height of the action potential was reduced in the mutant, and (ii) a larger afterhyperpolarization was seen in k-shyA following termination of the injected current (Fig. 6, Table 2). Since the height of this Ca^{2+}-based action potential reflects the size of the inward Ca^{2+} current, the reduction of the amplitude of the action potential in k-shyA can be explained by the reduction of I_{Ca} seen under voltage clamp. The larger afterhyperpolarization in the mutant may be related to the prolonged activation of the Ca^{2+}-dependent

K^+ current, since repolarization is affected by this current (Saimi et al., 1983).

Discussion

The behavioral and electrophysiological properties of k-shyA show it to be a new type of *Paramecium* mutant which responds to depolarizing stimuli with prolonged backward swimming. The effects of the mutation on four separable Ca^{2+}-sensitive phenomena, considered together, suggest that k-shyA has a prolonged elevation of intracellular Ca^{2+} following excitation. First, the rate of decay of the Ca^{2+}-dependent K^+ tail current in k-shyA was five to six times slower than in wild type. Second, the decay of the Ca^{2+}-dependent Na^+ tail current was also slower. Third, the rate of recovery from Ca^{2+}-dependent Ca^{2+} channel inactivation was two- to 10-fold slower than in wild type, depending on the duration of the Ca^{2+} pulse. Finally, k-shyA cells recovered more slowly than wild type from the depolarization-induced backward swimming which is known to result from elevated intraciliary Ca^{2+}.

We also found that the amplitude of the inward Ca^{2+} current was reduced in the mutant, but could be restored to the wild type level by the injection of EGTA. This restoration by EGTA might mean that the resting level of internal Ca^{2+} is high in the mutant. Alternatively, it is possible that slow removal of Ca^{2+} in the mutant results in more rapid accumulation of Ca^{2+} during the inward Ca^{2+} current, causing faster inactivation of Ca^{2+} channels and a smaller peak Ca^{2+} current. We have not rigorously examined I_{Ca} inactivation rates in k-shyA. In either case, the reduction of I_{Ca} in the mutant is the result of Ca^{2+}-dependent inactivation of Ca^{2+} channels. It is also clear that the phenotype of k-shyA is not simply due to the decreased inward Ca^{2+} current since another mutant (pawnC) with a similarly reduced I_{Ca} does not show any of the other defects seen in k-shyA.

The various k-shyA phenotypes are restricted to Ca^{2+}-sensitive functions. The voltage-dependent K^+ currents remain unaltered in the mutant. Furthermore, the mutant's resting membrane potential and resting resistance were not different from those of wild type. In fact, after intracellular injection of EGTA, we could detect no differences between mutant and wild type in any membrane electrical parameter. Thus a general defect in the membrane properties is not likely to exist.

The slow decrease in k-shyA Ca^{2+} levels after excitation that is implied by these observations could result either from an impaired ability to remove Ca^{2+} or from an increase in Ca^{2+} influx. The latter seems unlikely. The transient inward Ca^{2+} current is clearly reduced in the mutant, since the Ca^{2+} current amplitude is small and current inactivation (both "fast" and "slow") is either normal or more efficient than in the wild type. Similarly, the loss of one of the known rectifying K^+ currents cannot explain the phenotype since these currents are normal or more active in k-shyA. It could be argued that the k-shyA mutation increases a novel Ca^{2+} conductance, different from the voltage-dependent Ca^{2+} channel. However, we have examined membrane currents under conditions that suppress K^+ and Na^+ currents (in Cs-TEA solution) at various voltages and time scales and have detected no additional inward currents specific to k-shyA cells. Membrane potential and resistance measured under these conditions were also not different from those of wild type (*data not shown*).

Alternatively, the k-shyA mutant could conceivably be altered in some gene product that is involved in a regulatory mechanism that responds to Ca^{2+}. Ca^{2+} levels might be normal, but the responding pathway more active or more sensitive to Ca^{2+}. For this scenario to explain the phenotype, however, the altered gene product must regulate all four of the Ca^{2+}-sensitive functions affected by k-shyA. Clearly we cannot exclude this interesting possibility, although there is no evidence as yet for a regulatory system with these pleiotropic properties in *Paramecium*. One possible candidate is a pathway involving the Ca^{2+} regulatory protein calmodulin. Recent evidence strongly implicates a role for calmodulin in controlling the Ca^{2+}-dependent K^+ current (Hinrichsen et al., 1986). The effects of calmodulin inhibitors on permeabilized cells support the possibility that calmodulin may also regulate the ciliary reversal machinery (Otter, Satir & Satir, 1984; Izumi & Nakaoka, 1987). However, the roles, if any, for calmodulin in the regulation of the other functions altered in k-shyA are not known.

The simplest hypothesis is that k-shyA is defective in reducing the intracellular Ca^{2+} levels that become elevated by depolarization and/or other physiological processes. The target of the mutation could be the structure, regulation, or localization of an energy-dependent Ca^{2+} pump or Ca^{2+} exchanger in the plasma membrane. Alternatively, some component of an intracellular Ca^{2+}-sequestering organelle or cytoplasmic buffering system might be altered. Since the k-shyA behavioral phenotype is more strongly expressed immediately after a shift up in temperature (T.C. Evans and D.L. Nelson, *in preparation*), the wild type gene product may directly mediate or regulate the defective function, rather than control temporally distant functions

(e.g., protein synthesis). Qualitatively, k-shyA grows, mates, and undergoes autogamy as well as wild type (T.C. Evans, *unpublished observations*), thus severe defects in energy metabolism or other essential functions are not likely to be involved. Clearly, direct measurements of Ca^{2+} levels and fluxes in cells and in systems *in vitro* will be needed in the future to test this hypothesis and to resolve the possible targets of mutation.

Previous studies have made it clear that the regulation of Ca^{2+}-induced swimming behavior in *Paramecium* is complex (Kung & Saimi, 1982; Hinrichsen et al., 1985). Ca^{2+} enters through ciliary Ca^{2+} channels and is the trigger for ciliary reversal. Ca^{2+} also acts as a negative regulator that limits the duration and extent of its own entry by inactivation of the Ca^{2+} channels and by activation of a Ca^{2+}-dependent K^+ conductance. Adding to these regulatory mechanisms are the voltage-dependent K^+ current and slow inactivation of the Ca^{2+} current. The behavioral and electrophysiological phenotypes described here are distinct from the phenotypes of all other *Paramecium* mutants described to date, and the k-shyA mutation may therefore define yet another mechanism involved in the regulation of the Ca^{2+} signal.

Considering the multiple roles of Ca^{2+} in regulating ciliary function, one might predict that a mutation in a Ca^{2+} removal mechanism, possibly like k-shyA, would have pleiotropic and counteracting effects. Indeed, the reduced Ca^{2+} current, prolonged Ca^{2+}-dependent K^+ current, and resulting "weak" action potential (*see* Fig. 6) of k-shyA all predict a loss of excitability. Yet the mutant exhibits the swimming behavior of a hyperexcitable cell in growth media and K^+ solutions. The apparent decrease in frequency of ciliary reversals in Ba^{2+} and TEA^+ solutions may be explained by these electrophysiological changes that tend to suppress excitability.

Little is known about the mechanisms for Ca^{2+} extrusion or sequestration in *Paramecium*. Several membrane-associated Ca^{2+}-dependent ATPases have been described, but their functions are unknown (Riddle, Rauh & Nelson, 1982; Tiggerman & Plattner, 1982; Doughty & Kaneshiro, 1983; Travis & Nelson, 1986). Browning and Nelson (1976) measured the efflux of ^{45}Ca from prelabeled cells and found that metabolic poisons that decreased ATP levels did not inhibit Ca^{2+} efflux. Martinac and Hildebrand (1981) measured Ca^{2+} influx and efflux by flow dialysis and predicted the presence of a highly efficient Ca^{2+} pump. Muto and Nozowa (1984) have described an ATP-dependent Ca^{2+} uptake system in microsomes from the ciliated protozoan *Tetrahymena*. In none of these cases was the relevance to motility control certain. In higher organisms, ATP-dependent Ca^{2+} pumps from plasma and sarcoplasmic membranes have been purified and extensively characterized and a variety of Na^+/Ca^{2+} exchangers have been described (Schatzman, 1985; Ikemoto, 1982). However, the precise physiological roles and regulation of such extrusion mechanisms is not fully understood. Should the k-shy mutants prove to be defective in Ca^{2+} removal, they may be useful in the molecular identification and analysis of a behaviorally relevant mechanism of Ca^{2+} regulation in *Paramecium*, and provide insight into the regulation of Ca^{2+} levels of other excitable cells. They may also provide a means of experimentally manipulating Ca^{2+} levels in cells as an aid to the study of the mechanisms of Ca^{2+} action in *Paramecium*.

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