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A Calcium-Dependent Potassium Current Is Increased by a Single-Gene Mutation in *Paramecium*

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Summary. The membrane currents of wild type *Paramecium tetraurelia* and the behavioral mutant *teaA* were analyzed under voltage clamp. The *teaA* mutant was shown to have a greatly increased outward current which was blocked completely by the combined use of internally delivered Cs^+ and external TEA^+. This, along with previous work (Satow, Y., Kung, C., 1976, *J. Exp. Biol.* **65**:51–63) identified this as a K^+ current. It was further found to be a calcium-activated K^+ current since this increased outward K^+ current cannot be elicited when the internal calcium is buffered with injected EGTA. The mutation *pwB*, which blocks the inward calcium current, also blocks this increased outward K^+ current in *teaA*. This shows that this mutant current is activated by calcium through the normal depolarization-sensitive calcium channel. While tail current decay kinetic analysis showed that the apparent inactivation rates for this calcium-dependent K^+ current are the same for mutant and wild type, the *teaA* current activates extremely rapidly. It is fully activated within 2 msec. This early activation of such a large outward current causes a characteristic reduction in the amplitude of the action potential of the *teaA* mutant. The *teaA* mutation had no effect on any of the other electrophysiological parameters examined. The phenotype of the *teaA* mutant is therefore a general decrease in responsiveness to depolarizing stimuli because of a rapidly activating calcium-dependent K^+ current which prematurely repolarizes the action potential.

Key Words calcium-dependent K^+ current · mutant · *Paramecium*

Introduction

Paramecium is a eukaryotic unicell with an excitable membrane. It has been used as a model system for a multidisciplinary study of membrane ion channels and the mechanisms involved in their control. Voltage-clamp studies of wild-type and various channel mutants have been used to demonstrate several voltage-activated, calcium-activated and touch-activated channels (Kung & Saimi, 1982; Richard, Saimi & Kung, 1986; Saimi, 1986). One of these types of channels is calcium-dependent K^+ channels.

Calcium-dependent K^+ channels are ubiquitous. They serve to regulate membrane potential and therefore other physiological processes such as secretion and contraction (*see,* for reviews Schwartz & Passow, 1983; Lattore & Miller, 1984; Petersen & Maruyama, 1984). The calcium-dependent K^+ current is generally involved in repolarizing the cell from a sustained depolarization and thereby maintaining a relatively stable membrane potential. Single-channel recordings have shown that there are at least three types of calcium-dependent and K^+-selective channels (Latorre & Miller, 1984; Petersen & Maruyama, 1984).

A calcium-dependent K^+ current has been described in *Paramecium* which has a reversal potential at the equilibrium potential of K^+, is absent when internal calcium concentration is kept low, activates slowly over a time course of seconds, and has an indicative slow tail current upon repolarization (Satow & Kung, 1980). A different, faster activating calcium-dependent K^+ current has recently been suggested (Richard et al., 1986) but this current has not yet been characterized in wild type.

Several behavioral mutants now exist that have alterations in their calcium-dependent K^+ currents and can be used to study the channels involved and their regulation. Mutants of the class called "pantophobiacs" have been isolated which lack most of the characterized slowly activating calcium-dependent K^+ current (Saimi et al., 1983). In such a mutant (*pntA*), the single-gene alteration which turns *down* this current has been traced to a molecular defect in calmodulin (Hinrichsen et al., 1985, 1986). The faster activating calcium-dependent K^+ current has been shown to be increased in the mutant called "restless." This mutant current differs from the

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slowly activating calcium-dependent K^+ current in that it is blocked by external TEA^+, activates within 100 msec, has a tail current that decays significantly faster, is most pronounced in the mutant in response to large hyperpolarizations, and appears to contribute to the resting membrane potential (Richard et al., 1986). A behavioral mutant of *Drosophila*, called "slopoke," has also been shown to have a decrease in one type of calcium-dependent K^+ current (Elkins, Ganetsky & Wu, 1986).

A different type of *Paramecium* behavioral mutant is called "TEA^+-insensitive," abbreviated as *teaA* here (Chang & Kung, 1976). Current injection experiments showed that *teaA* had an increase in an unidentified K^+ conductance. This caused the cell to respond less to depolarizing stimuli such as TEA^+ and Na^+ because the action potential was prematurely terminated (Satow & Kung, 1979). By using voltage-clamp techniques, we have found that the *teaA* defect is actually in a calcium-dependent K^+ current. In contrast to the *pantA* mutation, the *teaA* mutation turns *up* an outward-going, depolarization-induced calcium-dependent K^+ current.

Materials and Methods

Stocks and Cultures

We used wild-type *Paramecium tetraurelia* (51s) and the TEA^+-insensitive mutant (genotype *teaA/teaA*, d4-152, abbreviated as *teaA*), *pawn B* (*pwB/pwB*, d4-95, abbreviated as *pwB*), and the double mutant (*teaA/teaA pwB/pwB*, abbreviated *teaA-pwB*). All stocks also contained the mutation which prevented trichocyst discharge (*nd6/nd6*). Cells were grown at 21–23°C in Cerophyl medium innoculated with *Enterobacter aerogenes* 24 hr before introducing the paramecia (Sonneborn, 1970).

Solutions

The bath solutions were: (i) *Ca^{2+}-K^+ solution.* This contained 4 mM KCl, 1 mM $CaCl_2$, 1 mM MOPS (morpholinopropanesulphonic acid), 0.01 mM EDTA (ethylenediaminetetraacetic acid), and was buffered to pH 7.2 with Tris-base [Tris(hydroxymethyl) aminomethane]; (ii) *The Cs-TEA^+ solution.* This contained 4 mM CsCl, 10 mM TEA-Cl (tetraethylammonium chloride), 1 mM $CaCl_2$, 1 mM MOPS, 0.01 mM EDTA, and was buffered to pH 7.2 with Tris-base, and (iii) *The Na^+ solution.* This had 10 mM NaCl added to the Cs^+-TEA^+ solution. For the recordings of K^+ currents in the Ca^{2+}-K^+ solution, the electrodes contained 2 M KCl. To inject EGTA (ethylene-glycol-bis-aminoethylether-N,N-tetra acetic acid), the electrodes also contained (in addition to 2 M KCl) 400 mM EGTA, 1 mM HEPES (N-2-hydroxyethyl piperazine-N^1-2 ethane sulfonic acid), and Tris-base to buffer the solution to pH 7.1. When the Cs^+-TEA^+ solution or the Na^+ solution was used, the electrodes contained 2 M CsCl to further suppress the K^+ currents.

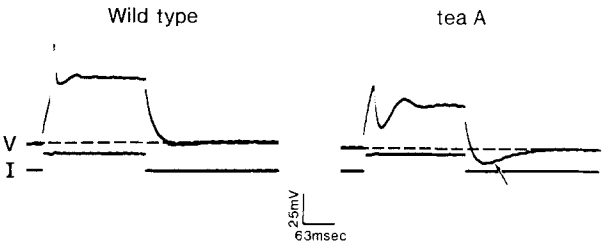

Fig. 1. Responses of the free-running membrane potential to outward current injected in wild type (left) and the mutant *teaA* (right). Cells were bathed in the Ca^{2+}-K^+ solution and recorded with electrodes filled with 500 mM KCl. The lower traces show the 250-msec, 1.0-nA outward current injected. The upper traces show the responses of the membrane potentials. Note that the mutant has a smaller action potential, a lower plateau depolarization during the current pulse and a prominent hyperpolarization after the current pulse (arrow)

Recording

The techniques for current injection and voltage-clamp analysis were similar to those described by Satow and Kung (1979). The microelectrodes used had resistances of 10–40 MΩ. In the voltage-clamp experiments the membrane potential was held at −40 mV and currents were recorded on a digital oscilloscope following step depolarizations or hyperpolarizations. Leakage currents, extrapolated from the currents measured near the holding level, were subtracted from all of the values plotted and were used to determine the membrane resistance, R_m. The R_m was also determined by current injection through unclamped membranes with higher resistance electrodes (500 mM KCl, tip resistances of 70–120 MΩ).

The method and the effectiveness of EGTA injection have been reported (Hennessey & Kung, 1985). The amplitudes of the tail currents are the zero-time values extrapolated from semilogarithmic plots. All experiments were performed at room temperature (21 to 25°C).

Results

Potential Responses to Injected Currents

The unclamped membrane of wild-type *Paramecium,* bathed in the Ca^{2+}-K^+ solution, can be excited by current injection. Injection of a constant outward current first triggers an action potential and then a plateau depolarization. The action potential is due to an influx of calcium followed by an efflux of K^+ (Naitoh & Eckert, 1974; Eckert, Naitoh & Machemer, 1976). The *teaA* mutation reduces the amplitude of the action potential and lowers the plateau (Fig. 1). These and other findings led Satow and Kung (1976) to conclude that the mutant has an increased K^+ conductance. Since the membrane currents were not directly monitored, it was not

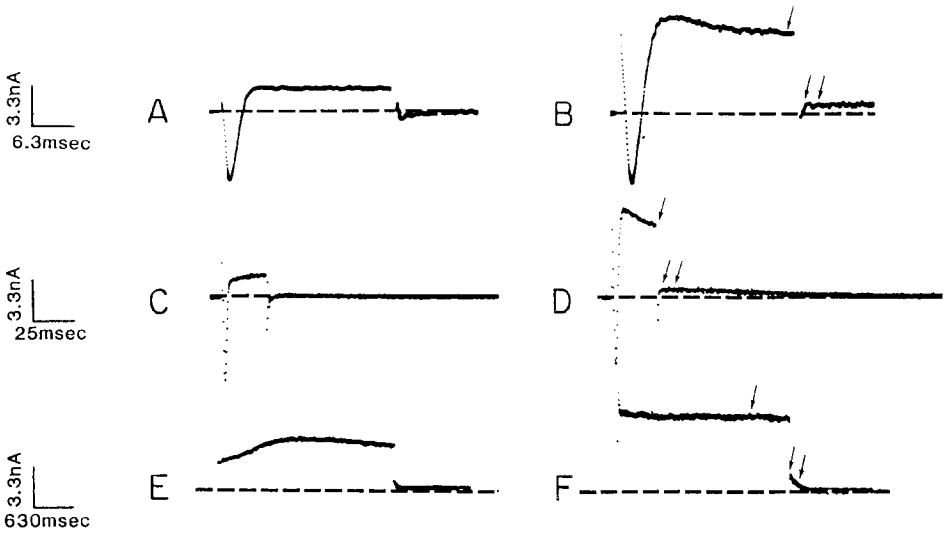

Fig. 2. The membrane currents of wild type (left) and *teaA* (right) upon step depolarizations under voltage clamp. The cells, bathed in the Ca^{2+}-K^+ solution, were first held at −40 mV, depolarized to −4 mV for 25 msec (*A–D*, early currents) or 2.5 sec (*E* and *F*, late current) and then returned to −40 mV. The −4 mV step depolarization was chosen because it induces the maximal inward Ca^{2+} transient. This transient is not significantly different between the wild type (*A*) and the mutant (*B*). The early outward current following the inward transient is, however, much larger in the mutant (arrows, *B* and *D*) than in the wild type (*A* and *C*). In the mutant, the repolarization at the end of the 25-msec pulse corresponds to a slow outward tail current (double arrows, *B* and *D*). This tail current was not observed in the wild type with the 25-msec short pulses (*A* and *C*). The late current (2.5 sec) of the wild type (*E*) which develops over 1 sec, is also smaller than the late current of the mutant (arrow, *F*). Note that the slow outward tail current was seen after the late outward current in the wild type (*E*). The tail is larger in the mutant and is seen after both the late (double arrows, *F*) and the early (double arrows, *B* and *D*) currents

possible to determine whether the conductance was activated by voltage or by internal calcium.

Voltage-clamp experiments have since revealed a slowly activating calcium-dependent K^+ current in wild-type *Paramecium*. The calcium that enters through the calcium channel upon a depolarization activates this K^+ outward current. Following repolarization, an indicative tail current decays with a time constant of approximately 100 msec (Satow & Kung, 1980; and *see* below). This is similar to the time course of the return of the large hyperpolarizing afterpotential of *teaA*, which is seen with current injection (Fig. 1, arrow).

Membrane Currents Upon Step Depolarizations

When the membrane potential of the wild type is held at −40 mV, the resting level of cells bathed in the Ca^{2+}-K^+ solution, a step depolarization from −40 to −4 mV activates a transient inward current which peaks at about 6 nA by 2 msec and then inactivates during the step depolarization. This inward transient is followed by an outward current which is sustained throughout the 25-msec depolarization. While there is no difference in the transient inward currents of *teaA* (5.9 ± 0.7 nA, mean ± SD, $n = 6$) and the wild type (6.4 ± 0.5 nA, $n = 6$) (Fig. 2*A–D*), the outward current is clearly larger in the mutant (Fig. 2 arrows) than in the wild type. The total outward current of the wild type is 1.6 ± 0.3 nA ($n = 6$) and that of the *teaA* is 5.5 ± 1.1 nA ($n = 6$). This greatly increased early outward current in the mutant (Fig. 2*B*, *D*, arrows) could either be the voltage-dependent K^+ current (Oertel, Schein & Kung, 1977; Satow & Kung, 1980) or a different outward current normally not activated at this early time.

The calcium-dependent K^+ current of the wild type develops slowly over half to one second during depolarizations large enough to trigger the calcium current (Satow & Kung, 1980; Saimi et al., 1983). Measured at 2.5 sec with a +36 mV depolarization, the total outward current of the wild type is 1.8 ± 0.4 nA ($n = 6$) (Fig. 2*E*) and that of *teaA* is 5.5 ± 0.9 nA ($n = 5$) (Fig. 2). Thus the increased outward current in *teaA* is seen in late as well as early times during the depolarization.

The slowly activating calcium-dependent K^+ current of the wild type relaxes in form of a tail current with a time constant of about 100 msec (Satow & Kung, 1980). Tail currents of this time course, following a 2.5 sec depolarization, are seen in both the wild type and the *teaA* mutant. The tail

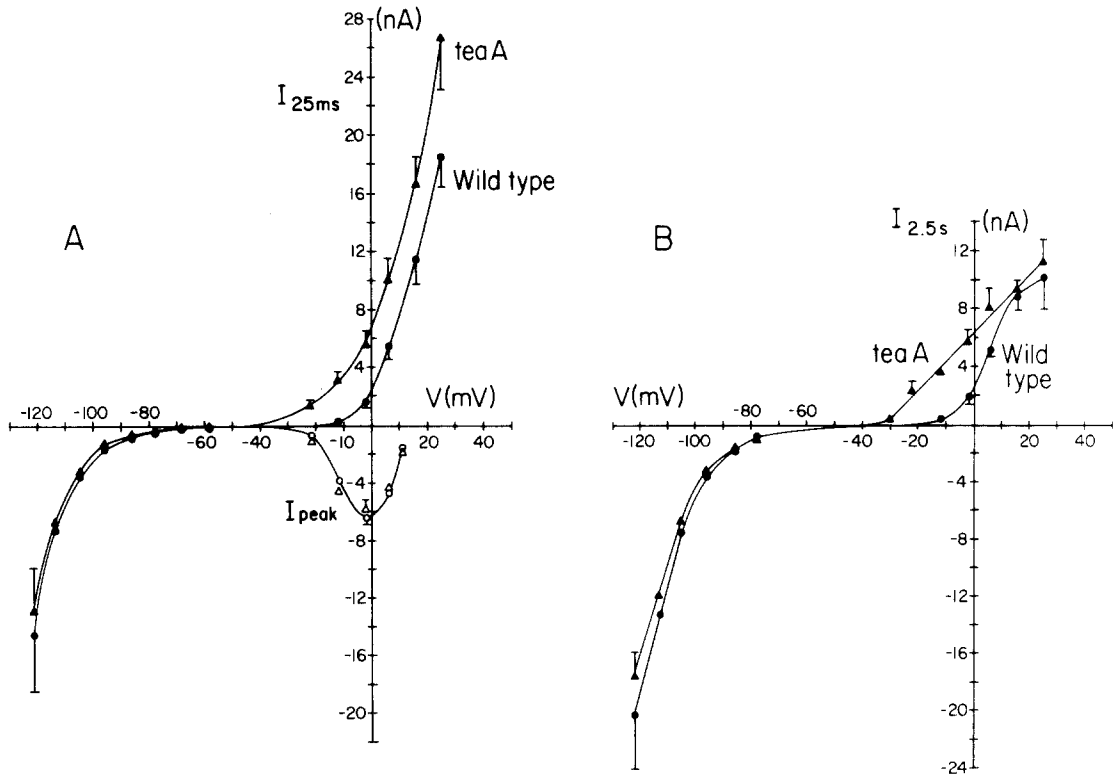

Fig. 3. Current-voltage plots of wild type and *teaA*. (*A*) *I-V* plots of early currents. Cells were bathed in the Ca^{2+}-K^+ solution and recorded with 3 M KCl electrodes. The membranes were held at −40 mV, stepped to the voltages indicated for 25 msec when their currents were measured and plotted. Note that the outward currents are significantly larger in the mutant (filled triangles) than in the wild type (filled circles) at all depolarization voltages but not the hyperpolarization voltages. The peak inward transients induced by depolarizing steps are also plotted. There is no significant difference between the inward transient of the wild type (open circles) and the mutant (open triangles). Leakage currents are subtracted. Mean ± SD, $n = 6$ each (SD are smaller than the width of the symbols when bars are not shown). (*B*) *I-V* plots of late currents. Recordings were as above except the voltage steps were 2.5 sec when the currents were measured and plotted. Note that the late outward currents of *teaA* (filled triangles) are larger than those of the wild type (filled circles) in the range of −22 to +6 mV. Leakage subtracted; mean ± SD, $n = 6$ each

is larger in the mutant (Fig. 2*F*, double arrows) than in the wild type (Fig. 2*E*). Surprisingly, this tail current is seen even after a 25-msec depolarization of the mutant (Fig. 2*B* and *D*, double arrows). Detailed analyses of the tail currents are given below.

Current-Voltage Relation

The above experiments were performed with a step to −4 mV, a step that induces the maximal inward calcium transient. Figure 3*A* (open symbols) plots the peak calcium inward transient against the step voltage. Note that the maximal inward transient of about −6 nA appears when the step is between −5 and 0 mV in both the wild type and the mutant. The early outward currents, measured at 25 msec, are also plotted (filled symbols). All depolarizations from the holding level of −40 mV induce larger outward currents in the mutant than in the wild type, but hyperpolarization-induced inward currents at 25 msec do not differ between the two strains.

The later outward currents, measured 2.5 sec after the step voltage changes, are plotted in Fig. 3*B*. As with the 25-msec currents, these depolarization-induced outward currents of the mutant are larger than those of the wild type. Beyond +10 mV the wild-type late currents are the same amplitude as those of the *teaA*. However, at these depolarized levels the space clamp restrictions prevent accurate voltage-clamp control and the Cs-TEA^+ method is not as effective.

The Larger Current in the Mutant is a K^+ Current

Virtually all the outward K^+ currents of *Paramecium* can be blocked by external TEA^+ and Cs^+ in combination with internal Cs^+ (Hennessey & Kung,

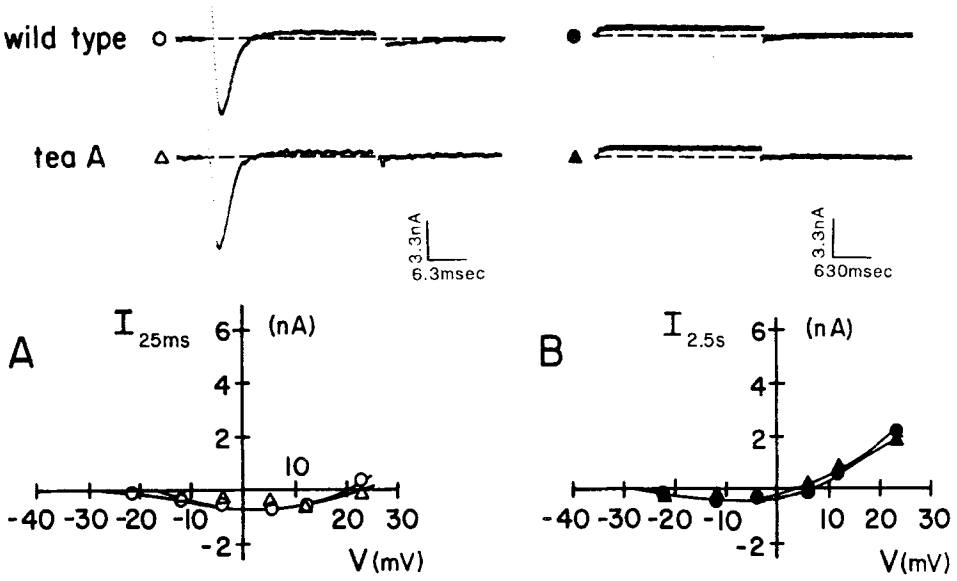

Fig. 4. Membrane currents of wild type and *teaA* mutant after blockage of K^+ current with Cs^+ and TEA^+. Cells were bathed in the Cs^+-TEA^+ solution and recorded with 2 M CsCl electrodes. They were held at 40 mV and stepped to different voltages. The left traces show the early currents upon 25-msec depolarizations from −40 to −4 mV; right ones show the late currents upon 2.5-msec steps of the same size. There is no significant difference between the currents of the wild type (top traces) and those of the mutant (lower traces). (*A*) *I-V* plots of early currents at 25 msec of wild type (open circles) and the mutant (open triangles). (*B*) *I-V* plots of the late currents at 2.5 sec of wild type (filled circles) and the mutant (filled triangles). No between-strain differences are seen. Leakage subtracted; means of four cells each. The SD are similar to those in Fig. 3

1984; Hinrichsen & Saimi, 1984). This procedure causes all of the depolarization-induced outward currents to be reduced to the leakage level (Fig. 4, traces), except when very high voltages (above +10 mV) are used with long durations (2.5 sec). The slow tail outward currents also disappeared. More importantly, the larger outward currents previously observed in the *teaA* are completely lost. The mutant currents are now identical to those of the wild type at early (25 msec, Fig. 4*A*) or late (2.5 sec, Fig. 4*B*) times. As with all of the other *I–V* curves, the currents are plotted after leakage subtraction. The small *inward* currents, shown in the plots of Fig. 4*A* and *B*, have previously been shown to be the residual calcium inward current after the early inactivation (Hinrichsen & Saimi, 1984). The loss of the mutation-related outward current upon Cs^+-TEA^+ treatment strongly indicates that it is a K^+ current. This larger K^+ current of the mutant, like the characterized calcium-dependent K^+ current, is not blocked by external TEA^+ alone (*data not shown*).

The transient inward currents, seen in Fig. 2*A* and *B*, are largely due to the well-characterized voltage-dependent inward calcium current. To test more rigorously whether there is a mutationally related difference in the calcium current, we measured it in isolation after the Cs^+-TEA^+ blockage of K^+ outward currents. The isolated transient inward calcium current of the mutant does not differ significantly from that of the wild type. The maximal inward transient is -5.7 ± 1.1 nA in the wild type and -6.9 ± 0.7 nA in the mutant (mean ± SD, $n = 4$, each). In both cases, the maxima occur at depolarization of −5 to 0 mV. The transient nature of the isolated inward calcium current is due to calcium-dependent calcium channel inactivation (Brehm & Eckert, 1978). The time course of this inactivation is normal in the *teaA* mutant. Thus, neither the calcium current, its inactivation, or its voltage dependence is affected by the *teaA* mutation.

Calcium Activates the Mutant Current

Since depolarizations induce an influx of calcium, we need to determine whether the excess outward K^+ current in the *teaA* mutant is induced directly by the depolarizations or indirectly by calcium. EGTA has been injected into paramecia to successfully block the calcium-activated currents (Saimi & Kung, 1980; Satow & Kung, 1980; Hennessey & Kung, 1985). We found that an injection of EGTA blocks most of the outward currents during depolarizations (Fig. 5). The blocked portion is larger when the outward K^+ current is measured at a later than at an earlier time (*compare* Fig. 2*A* and *B* with the

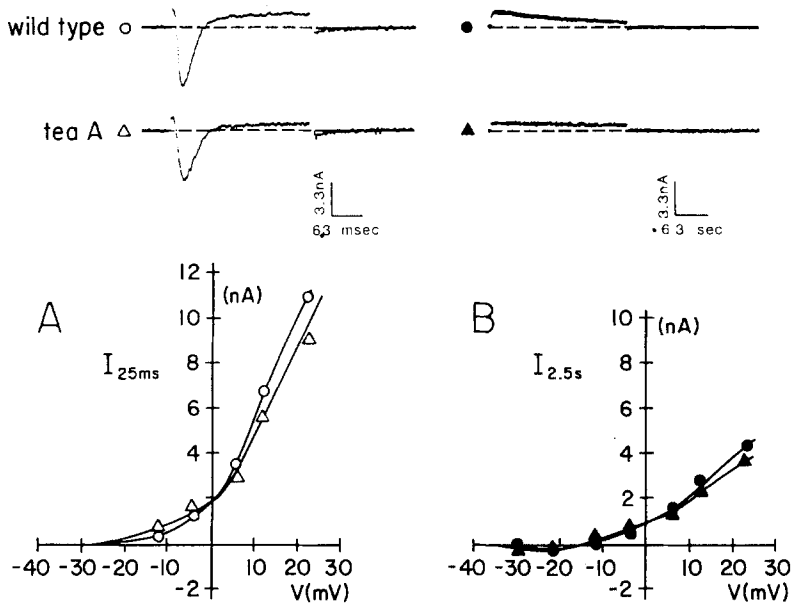

Fig. 5. Membrane currents of wild type and *teaA* mutant after internal application of EGTA. Cells were bathed in the Ca^{2+}-K^+ solution and recorded with electrodes of 2 M KCl and 400 mM EGTA (*see* Materials and Methods). The left traces show the early currents upon 25-msec depolarizations from −40 to −4 mV: the right traces show the late currents upon 2.5-msec steps of the same size. There is no between-strain difference. (*A*) *I-V* plots of early currents at 25 msec of wild type (open circles) and the mutant (open triangles). (*B*) *I-V* plots of the late currents at 2.5 sec of wild type (filled circles) and the mutant (filled triangles). No between-strain differences are seen. Leakage subtracted; means of five cells each. The SD are similar to those in Fig. 3

comparable traces in Fig. 5). The injection of EGTA also abolishes the slow tail current. These findings are consistent with the EGTA's blockage of the slowly developing calcium-dependent K^+ current. Important to our study of the *teaA* mutant, EGTA also abolishes the differences between the wild-type currents and the mutant currents. After EGTA injections, the currents from the two strains are indistinguishable at all voltages tested, both at the early (25 msec, Fig. 5*A*) and the late time (2.5 sec, Fig. 5*B*). The remaining outward currents, seen at 25 msec, are due primarily to the voltage dependent K^+ currents. These isolated voltage-dependent K^+ currents do not differ between wild type and *teaA*.

The Calcium Channel Controls the Mutant K^+ Current

The characterized calcium-dependent K^+ current is activated by the calcium which enters through the voltage-dependent calcium channel upon excitation (Satow & Kung, 1980). It is of interest to know whether the mutant current is also activated by the same calcium source.

The entry of calcium can be blocked by a different mutation which eliminates the calcium current. These types of mutations, called *pw* mutations (*pawns*), have been studied extensively (Oertel et al., 1977; Satow & Kung, 1980). We now examine the *teaA-pwB* double mutant to see whether the excess outward current associated with the *teaA* mutation remained in the absence of the depolarization-induced calcium influx. Figure 6 shows that the double mutant does not have an outward current larger than the *pwB* single mutant. No difference is detected at any of the voltages tested and at either the early (Fig. 6*A*) or late (Fig. 6*B*) time. The trajectories of the outward currents over these times are also indistinguishable between the two strains (Fig. 6, traces).

The relaxation of the outward voltage-dependent K^+ currents of both the wild type and the *teaA* mutant can be seen during the 2.5 sec depolarization with EGTA (Figs. 5 and 6, traces). This is consistent with the inactivation of the voltage-dependent K^+ current described previously (Satow & Kung, 1980).

Wild Type and teaA Calcium-Dependent K^+ Current Activation Kinetics

The calcium-dependent K^+ current of the wild type and that of the *teaA* mutant are compared in terms of their activation during the depolarization. An early activation of the mutant current is evident from the current traces immediately after the inward calcium current, as shown in Fig. 2. Because the outward current is the sum of several elements (*see* Discussion), its kinetics do not reflect the kinetics of the calcium-dependent K^+ current alone. Therefore, the amplitudes of the slow outward tail currents are used to measure that current. The amplitudes of these calcium-dependent K^+ tail currents are plotted as a function of the duration of the voltage steps to monitor the development of the calcium-dependent K^+ current during the depolariza-

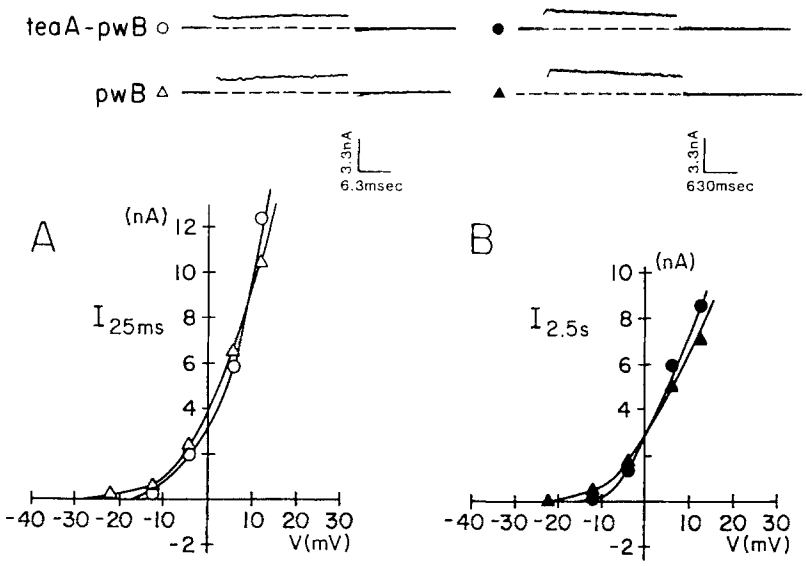

Fig. 6. Membrane currents of the *pwB* single mutant and the *teaA-pwB* double mutant. Cells were bathed in the Ca^{2+}-K^+ solution and recorded with 3 M KCl electrodes. The left traces show the early currents upon 25-msec depolarizations from −40 to −4 mV. Note the absence of transient inward currents (*compare* to Figs. 2, 4 and 5) due to the *pwB* mutation. The right traces show the late currents upon 2.5-sec steps of the same size. There is no between-strain difference. (*A*) *I-V* plots of early currents at 25 msec of the single mutant (open triangles) and the double mutant (open circles). (*B*) *I-V* plots of the late currents at 2.5 sec of the single (closed triangles) and the double mutant (closed circles). No between-strain differences are seen. Leakage subtracted; means of three cells each. The SD are similar to those in Fig. 3

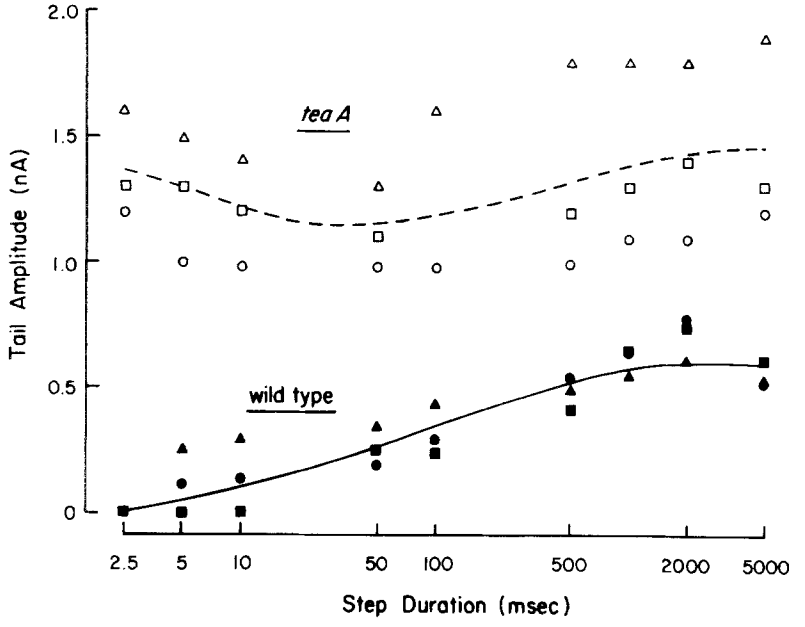

Fig. 7. The activation kinetics of the Ca^{2+}-dependent K^+ current as measured by the tail current amplitude in the wild type and the *teaA* mutant. Cells were bathed in the Ca^{2+}-K^+ solution, penetrated with 3 M KCl electrodes, held at −40 mV, stepped to −4 mV for various durations and then repolarized to −40 mV. The tail currents upon the repolarizations were individually plotted semilogarithmically and the current amplitude was linearly extrapolated to the onset of the repolarizations (*see* Fig. 8). These zero-time values are then replotted here against the step durations. Note that the wild-type current (filled symbols) develops slowly while the *teaA* current (open symbols) is already maximal as soon as we can measure it accurately at 2.5 msec. Different symbols represent different cells

tion. The voltage steps were +36 mV from the holding level of −40 mV. All of these tails can be approximated by single exponentials (*below*) and have time constants consistent with those previously described for calcium-dependent K^+ tail current decay rates (Satow & Kung, 1980). Figure 7 (filled symbols) shows that the wild-type currents grow over 1 sec while the *teaA* current already reaches its maximum by 2.5 msec (Fig. 7, open symbols). At 1 msec, the amplitude of the *teaA* tail current appears to be smaller. Because the voltage-clamp circuit has a time constant of 0.2 msec, the value at 1 msec can only be an underestimation of the true amplitude of the tail current after 1 msec of a square-pulse depolarization. The 1-msec time points are not shown in Fig. 7 due to this uncertainty.

The Relaxation Kinetics of the Calcium-Dependent K^+ Currents

The deactivation of the calcium-dependent K^+ current of the wild type has been established previously (Satow & Kung, 1980). We have compared the deactivation kinetics of that current in the wild

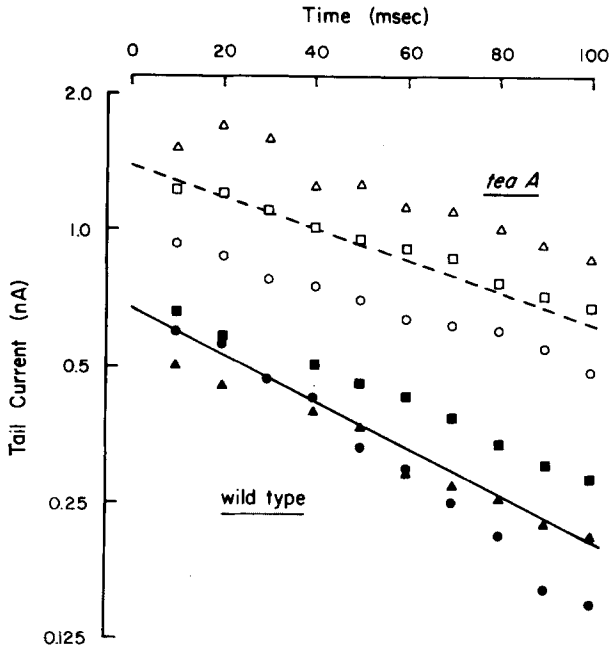

Fig. 8. The deactivation kinetics of the Ca^{2+}-dependent K^+ current. Cells were bathed in the Ca^{2+}-K^+ solution, penetrated with 3 M KCl electrodes, held at −40 mV, stepped to −4 mV for 2.5 sec and then repolarized to −40 mV. The tail currents at different times after the repolarization were measured and plotted against time. Lines show the least-squares linear fits. The decay rate of the wild type (continuous line) is similar to that of the mutant (broken line), although the mutant currents (open symbols) are larger than the wild-type currents (filled symbols) at all times. Different symbols represent different cells

type and in the *teaA* mutant. After depolarization from −40 to −4 mV for 2.5 sec, the tail currents are recorded when the membrane is repolarized. Both the wild-type and the mutant tails can be approximated by single exponentials. The time constants of the tails are 112, 114, and 60 msec in three individual wild-type cells (filled symbols, Fig. 8) and 105, 122 and 120 msec (open symbols, Fig. 8) in three *teaA* cells. These measurements do not differ ($t = 1.11$ $P > 0.10$, $df = 4$).

Other Electric Properties

Except for the calcium-dependent K^+ current, the other membrane properties of *teaA* are apparently normal. Resting membrane potentials were monitored in the Ca^{2+}-K^+ solution, and the resting membrane resistance was measured with small depolarizations and hyperpolarizations. These properties of the resting membranes show no difference between the *teaA* mutant and the wild type. Internal calcium also activates an inward Na^+ current in *Paramecium* (Saimi & Kung, 1980). Using a bath solution containing 10 mM Na^+ and the Cs-TEA^+ method to block the K^+ current, we have also measured this current and found no difference between the wild-type and the *teaA* current calcium-dependent Na^+ currents. The data and the statistics of these measurements are summarized in the Table.

Discussion

Paramecium exhibits "avoiding reactions," transient reversals of their ciliary beat direction, when stimulated. These reactions are the behavioral consequence of the calcium action potentials. Outward voltage-dependent K^+ currents, along with calcium-dependent calcium channel inactivation, are responsible for the downstroke and repolarization in wild-type. Wild-type *Paramecia* show avoiding reactions when they encounter high concentrations of external TEA^+ because it depolarizes the cell and the blockage of voltage-dependent K^+ currents favors the generation of action potentials. The *teaA* mutant was originally isolated based on its behaviorally poor response to TEA^+, hence the name "TEA^+-insensitive mutant" (Chang & Kung, 1976). Its responses to stimuli such as Na^+ are also weaker. Current injection experiments with unclamped membranes showed that this mutant had an increased K^+ conductance (Satow & Kung, 1980). The specific K^+ conductance was not identified, however.

Our work here supports these findings and extends the analysis of *teaA* to include specific ion currents by using voltage-clamp techniques. Such analysis of the membrane currents of *teaA* has identified the increased K^+ conductance as a calcium-dependent K^+ conductance. The evidence for this is: (i) Since the larger current of *teaA* is eliminated by the Cs-TEA^+ procedure, it is due to a K^+ current. This is supported by previous work which showed that this mutant has an increased K^+ conductance (Satow & Kung, 1976); (ii) EGTA injection eliminates the difference between wild type and the mutant; (iii) addition of the *pwB* mutation eliminates the difference between wild type and the double mutant; and (iv) no other membrane ion currents are altered in this mutant.

The calcium-dependent K^+ current activates very rapidly in *teaA* (Figs. 2 and 7). As early as 2 msec after the depolarization, the current has already reached its maximum. This is drastically different from the wild-type current which activates over hundreds of milliseconds (Fig. 7). Although the activation of the mutant current is too fast to measure accurately (Fig. 7, broken line), the current is absent at rest since it relaxes to the zero-

Table. Resting level, resting resistance and various membrane currents under voltage clamp in wild type or the *teaA* mutant[a]

Membrane property	Wild type	*teaA*	*n*
Resting potential[b]	−34.2 ± 2.9 mV	−38.0 ± 3.3 mV	6
Resting resistance[b]	38.5 ± 3.8 MΩ	43.7 ± 9.9 MΩ	6
Peak Ca^{2+} current (I_{Ca})[c]	−6.4 ± 0.5 nA	−5.9 ± 0.7 nA	6
Voltage for I_{Ca}[d]	~−2.0 mV	~−2.0 mV	6
Depolarization-activated K^+ current[b]	10.9 ± 3.3 nA	9.1 ± 4.9 nA	5
Hyperpolarization-activated K^+ current[b]	−14.5 ± 4.0 nA	−12.8 ± 3.0 nA	6
Ca-dependent Na current (I_{Na})[d]	−0.3, −1.0 nA	−0.8, −1.0 nA	2
Tail current of I_{Na}[d]	1.4, 0.9 nA	1.2, 1.3 nA	2

[a] Mean ± SD; *n* wild-type cells and *n* *teaA* cells. Individual data shown for $n = 2$.

[b] Measured in the Ca^{2+}-K^+ solutions. The resting properties were measured with 500 mM KCl electrodes; others with 3 M KCl electrodes. The depolarization-activated K^+ current was measured at +6 mV with a 25-msec step; the hyperpolarization-activated K^+ current at −122 mV with a 25-msec step.

[c] Measured with 2 M CsCl electrodes and the Cs^+-TEA^+ solution. I_{Ca} refers to the highest peak current inducible which occurs at a depolarization from −40 to about −2 mV.

[d] Measured with 2 M CsCl electrodes and the Na^+ solution. The small inward Na^+ current was induced by a step from −40 to −5 mV measured at 2.5 sec. The tail current was outward and was measured immediately upon the repolarization from −5 mV to the holding level of −40 mV.

current level after the depolarization, as does the wild-type current.

The total early outward currents of *teaA*, measured at 25 msec in the Ca^{2+}-K^+ solution with 2 M K^+ electrodes, consists of four components: (i) The leakage current. This was measured with small voltage steps. The resting membrane resistances, derived from these measurements, were the same for *teaA* and for the wild type (Table). The decreased membrane resistance values of *teaA* reported by Satow and Kung (1976) were apparently due to the increased calcium-dependent K^+ conductance activated during outward current injection experiments. (ii) the voltage-dependent outward K^+ current ("delayed rectifier"). This current was originally isolated using the pawn mutation *pwB* because this mutation reduces the calcium current (Oertel et al., 1977) and hence the calcium-dependent K^+ current as well (Satow & Kung, 1980). The outward current in *pwB* (or *teaA-pwB*), after background subtraction, is therefore the voltage-dependent K^+ current in virtual isolation. This current of *teaA* is not different from that of wild-type (Fig. 6). EGTA injection also isolates this current, showing no difference between wild type and *teaA* (Fig. 5). (iii) The sustained calcium current. The major part of the inward calcium current inactivates within the first few milliseconds (Brehm & Eckert, 1978; Hinrichsen & Saimi, 1984). However, a remnant of the current is sustained for up to 1 min (Hennessey & Kung, 1985). The Cs^+-TEA^+ method unveils this sustained calcium current by blocking all of the outward K^+ currents. The sustained inward calcium current, measured at 25 msec, was the same in *teaA* and wild type (Fig. 3*B*). Since EGTA increased this sustained calcium current by decreasing the calcium-dependent inactivation of the calcium channel (Hinrichsen & Saimi, 1984), it may contribute to the reduction of the outward current seen in Fig. 6*A* (compared to Figs. 3*A* and 6). (iv) The calcium-dependent K^+ current described and discussed in this paper. It is small at the early times in the wild type (*see* Fig. 7), but is very large in the *teaA* mutant.

The behavioral phenotype of *teaA* can now be explained as follows: Certain stimuli, such as TEA^+ or Na^+, cause depolarizations which open the voltage-dependent calcium channels as in the wild type. Calcium enters and activates the calcium-dependent K^+ channels (Satow & Kung, 1980). Since a calcium-dependent K^+ current is larger and activates faster in *teaA* than in wild type, it prematurely repolarizes the cell. The inward calcium current is reduced under free running membrane potential conditions because of this rapid repolarization. When measured under voltage-clamp conditions, the inward current is not reduced in the mutant because the voltage signal does not change. Therefore, the predominant effect of the increased calcium-dependent K^+ current of the mutant is to

cause a premature short circuiting of the voltage signal and not the inward current per se. In wild type, the brief action potential is normally not affected by the calcium-dependent K^+ current (Satow & Kung, 1980). The calcium-dependent K^+ current is usually involved in controlling slower events such as "Type II excitation" (Saimi et al., 1983). The membrane potential of the *teaA* mutant is depolarized by TEA^+ (Satow & Kung, 1976), but any further regenerative depolarization is short circuited by the large, fast activating, TEA^+-insensitive, calcium-dependent K^+ current.

Two calcium-dependent K^+ currents have been described in *Paramecium*. The best characterized is a slowly activating calcium-dependent K^+ current (Satow & Kung, 1980). The larger calcium-dependent K^+ current of *teaA* is similar to this current in that the tail current decay kinetics are the same and both are not completely blocked by external TEA^+, but they differ in that the *teaA* current activates much faster. A faster activating calcium-dependent K^+ current has been described in the "restless" mutant (Richard et al., 1986), but since it is effectively blocked by external TEA^+ it is most likely different than the large *teaA* calcium-dependent K^+ current. The restless and *teaA* mutants are also not allelic (Richard, Hinrichsen & Kung, 1985) and the *teaA* mutant shows no changes in the hyperpolarization-induced currents. Therefore, if the large *teaA* current involves a characterized channel type, it is most likely the slowly activating calcium-dependent K^+ channel. However, it is also possible that the *teaA* mutation brings out a new type of previously uncharacterized channels which may be similar to those involved in the fast activating, calcium-dependent K^+ current (I_{Acd}) of *Drosophila* (Salkoff, 1983; Elkins et al., 1986).

If the increased calcium-dependent K^+ current of *teaA* is due to the same species of channels as the slowly activating calcium-dependent K^+ channels of wild type, then the *teaA* mutation has changed either the amount of calcium available to open the channel or some property controlling the calcium-dependent K^+ channel itself. It is unlikely that the *teaA* mutant has an increase in internal calcium concentration because: (i) The duration of backward swimming in 20 mM K^+ is normal in *teaA*. This is a behavioral bioassay for internal calcium concentration during depolarization (Haga et al., 1984). (ii) Internal calcium also activates a channel which specifically conducts Na^+ (Saimi & Kung, 1980). We have re-examined the calcium-dependent Na^+ current in the wild type and the mutant and found no difference (Table). If internal calcium were higher in *teaA*, one would expect a higher Na^+ current in the mutant. (iii) The time constant of calcium inactivation of the calcium channel, tested in Cs^+-TEA^+ with twin 20-msec steps to V_{max}, is the same for *teaA* and wild type (time constant for *teaA* = 64.0 ± 6.2 sec, wild type = 59.5 ± 7.7 sec, n = 4–5). Brehm and Eckert (1978) showed that the time constant of this inactivation decreased when internal calcium concentration is increased. It is possible, however, that the spatial distribution of channels is altered in *teaA* such that the calcium-dependent K^+ channels are closer to the calcium channels and therefore detect the calcium sooner and to a greater extent than wild type.

To determine the exact nature of the *teaA* defect, and to examine the properties of the calcium-dependent K^+ channel itself, single channels should be analyzed by the patch-clamp technique (for review *see* Latorre & Miller, 1984; Petersen & Maruyama, 1984). Such experiments are currently underway in this laboratory (B. Martinac, Y. Saimi, *unpublished*). Calcium-dependent K^+ channels have been described in other systems which are voltage insensitive (opened only by calcium), openable by voltage even in the absence of calcium, and both voltage- and calcium-activated (Petersen & Maruyama, 1984). The binding of calcium to calcium-dependent K^+ channel may itself be voltage sensitive (Moczydlowski & Latorre, 1983). The question remains whether the mutated *teaA* calcium-dependent K^+ current is due to increased voltage sensitivity, increased calcium sensitivity, increases in unitary ion channel conductance, increases in the number of ion channels, alterations in the location of the channels, or to an increase in a currently uncharacterized type of calcium-dependent K^+ channel.

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