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Interactions Between Mutants with Defects in Two Ca^{2+}-Dependent K^+ Currents of *Paramecium tetraurelia*

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Summary. *Paramecium tetraurelia* possesses two Ca^{2+}-dependent K^+ currents, activated upon depolarization $I_{K(Ca,d)}$, or upon hyperpolarization $I_{K(Ca,h)}$. The two currents are mediated by pharmacologically distinct ion channel populations. Three mutations of *P. tetraurelia* affect these currents. Pantophobiac A mutations (*pntA*) cause calmodulin sequence defects, resulting in the loss of both Ca^{2+}-dependent K^+ currents. A second mutation, TEA-insensitive A (*teaA*), greatly enhances $I_{K(Ca,d)}$ but has no affect on $I_{K(Ca,h)}$. A third mutation, *restless* (*rst*), also increases $I_{K(Ca,d)}$ slightly, but its principle effect is in causing an early activation of $I_{K(Ca,h)}$. Interactions between the products of these three genes were investigated by constructing three double mutants. Both *teaA* and *rst* restore $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ in pantophobiac A^1, but the phenotypes of *teaA* and *rst* are not corrected by a second mutation. These observations may indicate a role for the gene products of *teaA* and *rst* in regulating the activity of $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$, respectively.

Key Words calmodulin · Ca^{2+}-dependent K^+ channels · ion channel regulation · mutations · *Paramecium*

Introduction

Paramecium tetraurelia possesses two Ca^{2+}-dependent K^+ currents. The first of these activates slowly upon depolarization ($I_{K(Ca,d)}$) and helps repolarize the cell during periods of prolonged membrane excitation (Satow & Kung, 1980; Saimi et al., 1983). Ca^{2+}-dependent K^+ conductances similarly stabilize membrane potential in a variety of cells (Meech, 1978; Petersen & Maruyama, 1984). Two behavioral mutants of *P. tetraurelia* have defects in $I_{K(Ca,d)}$. Pantophobiac lacks $I_{K(Ca,d)}$, so that this mutant responds to appropriate stimuli with a greatly prolonged action potential (Saimi et al., 1983). Conversely, the TEA-insensitive A (*teaA*) mutation causes $I_{K(Ca,d)}$ to activate faster and more strongly than normal, thereby foreshortening the action potential (Satow & Kung, 1976; Hennessey & Kung, 1987).

The second Ca^{2+}-dependent K^+ current is activated upon membrane hyperpolarization ($I_{K(Ca,h)}$) (Richard, Saimi & Kung, 1986; Preston, Saimi & Kung, 1990*a*). The function of this inward rectifier is less clear than of $I_{K(Ca,d)}$, but it may also act as a membrane potential damping mechanism. When $I_{K(Ca,h)}$ is enhanced by mutation (the restless mutation, *rst*), the cell's membrane potential becomes set at more negative levels compared with the wild type, and behaves as a pure K^+ electrode in response to changes in $[K^+]_o$ (Richard, Hinrichsen & Kung, 1985). In contrast, $I_{K(Ca,h)}$ is missing in pantophobiac A^1, the mutant that also lacks $I_{K(Ca,d)}$ (Preston et al., 1990*b*).

The observation that a single gene mutation (*pntA*^{1}) disrupts the function of both $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ is curious, because Richard et al. (1986) suggested that the two conductances may be mediated by separate molecular entities. It was of interest, therefore, to establish more firmly whether there are indeed two distinct Ca^{2+}-dependent K^+ permeabilities in *Paramecium*. In the present study, the Ca^{2+}-dependent K^+ conductances of the three behavioral mutants (pantophobiac A^1, TEA-insensitive A, and restless) are examined. In attempts to discern possible functional and regulatory interactions between the gene products of these three mutants, three double mutants ("*pntA*^{1}, *teaA*", "*pntA*^{1}, *rst*", and "*rst, teaA*") were constructed and analyzed electrophysiologically. This genetic technique has been used with considerable success in attempts to unravel the intricacies of membrane excitation in *Drosophila* (*see* review by Ganetzky & Wu, 1986).

Materials and Methods

Cell Stocks and Culture Conditions

The following strains of *Paramecium tetraurelia*, derived from stock 51s, were used: d4-622 pantophobiac A^1 (*pntA*^{1}/*pntA*^{1}), d4-647 restless (*rst*/*rst*), and d4-152 TEA-insensitive A (*teaA*/*teaA*). "*pntA*^{1}, *rst*" denotes the double mutant "*pntA*^{1}/*pntA*^{1}, *rst*/*rst*", constructed from a cross of the two single mutant parents.

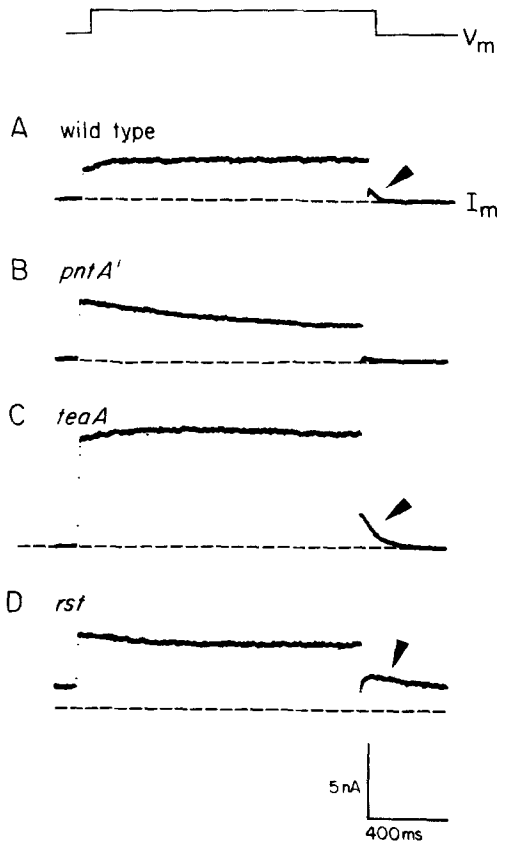

Fig. 1. Ca^{2+}-dependent K^+ currents activated upon depolarization. Ca^{2+}-dependent K^+ currents were activated by depolarization for 1500 msec to −10 mV. Arrowheads indicate the tail current of $I_{K(Ca,d)}$ upon returning to −40 mV. The broken line in this and subsequent figures represents zero current level. (*A*) In the wild type, $I_{K(Ca,d)}$ is apparent as a slowly activating outward current, and as an outward tail current (arrowhead). (*B*) Depolarization of pantophobiac A^1 elicits $I_{Ca(d)}$ and $I_{K(d)}$ in the absence of a Ca^{2+}-dependent component. (*C*) The Ca^{2+}-dependent K^+ current elicited upon depolarization of TEA-insensitive A is greatly enhanced compared with that of the wild type; note the strong tail current. (*D*) Depolarization of restless elicits a Ca^{2+}-dependent K^+ current that is only slightly increased in amplitude compared with that of the wild type

"p*ntA*^{1}, *teaA*" and "*rst, teaA*" similarly denote double mutants from crosses. All three mutations are genetically unlinked. All strains also contained the trichocyst nondischarge mutation nd-6 (*nd6/nd6*). Single and double mutants were obtained, and their genetic identity verified, using standard techniques (Sonneborn, 1970; Hinrichsen et al., 1985). Cells were maintained in culture as described in the accompanying report (Preston et al., 1990*a*).

Solutions, Electrophysiological Recording Techniques, and Data Analysis

The techniques and conditions used to record and voltage clamp the membrane potential of *Paramecium* are described in the accompanying report, as are methods used in data analysis (Preston et al., 1990*a*). K^+ currents were recorded using 3 M KCl microelectrodes from cells bathed in K^+-solution. K^+-solution comprises (in mM): 4 KCl, 1 Ca^{2+}, 0.01 EDTA, 1 HEPES buffer, pH 7.2. Ca^{2+} currents were recorded using 4 M CsCl microelectrodes. CsCl electrodes were used in combination with TEA^+-solution to inhibit K^+ currents. TEA^+-solution contains (in mM): 10 TEA^+, 1 Ca^{2+}, 0.01 EDTA, and 1 HEPES buffer, pH 7.2. A holding potential of −40 mV was used throughout. Membrane currents were filtered at 1–2 kHz and are presented without leakage correction. All data are presented as means ± SD, whereas levels of statistical significance between means were determined using a Student's *t* test. *P* values of <0.05 were considered significant.

Table 1. Ca^{2+}-dependent K^+ current activated by depolarization

Cell strain	I_{1500} (nA)	I_{tail} (nA)	*n*
Wild type	2.6 ± 0.6	0.5 ± 0.1	12
p*ntA*^{1}	2.0 ± 0.5	0.1 ± 0.1	8
teaA	8.5 ± 2.9	2.1 ± 0.9	5
rst	3.3 ± 1.1	0.6 ± 0.3	9
p*ntA*^{1}, *teaA*	5.8 ± 1.8	1.5 ± 0.4	9
p*ntA*^{1}, *rst*	2.9 ± 0.6	0.4 ± 0.3	6
rst, teaA	8.4 ± 1.7	2.0 ± 0.6	7

Wild type and mutant cells in K^+-solution were depolarized to −10 mV for 1500 msec. I_{1500} refers to the amplitude of the evoked outward current 1500 msec into the voltage step. Termination of the voltage step by returning to −40 mV elicits an outward tail current, the amplitude of which was measured 20 msec after returning to holding potential (I_{tail}). Data are means ± SD of *n* cells.

Results

Ca^{2+}-Dependent K^+ Currents of the Wild Type

The Ca^{2+}-dependent K^+ currents of wild-type *P. tetraurelia* are detailed elsewhere (Satow & Kung, 1980); a brief description of these currents follows so that they may be compared with the currents of mutant strains.

Depolarization of the wild type (Fig. 1*A*) elicits a rapid inward Ca^{2+} transient (not resolved in the figure), followed by a delayed, voltage-dependent K^+ current. The Ca^{2+}-dependent K^+ conductance ($I_{K(Ca,d)}$) activates slowly over a period of 1–2 sec; a slow outward tail current that reflects deactivation of $I_{K(Ca,d)}$ is seen upon returning to holding level. This tail is variable in form. Although its decaying phase was previously fitted with an exponent (Satow & Kung, 1980), strains with an enhanced $I_{K(Ca,d)}$ exhibit tail currents whose decay may be preceded by a plateau. In order to avoid artificially high estimates of current amplitudes at the instance of membrane repolarization, comparisons of tail-current amplitudes were made at 20 msec after returning to holding potential (Table 1).

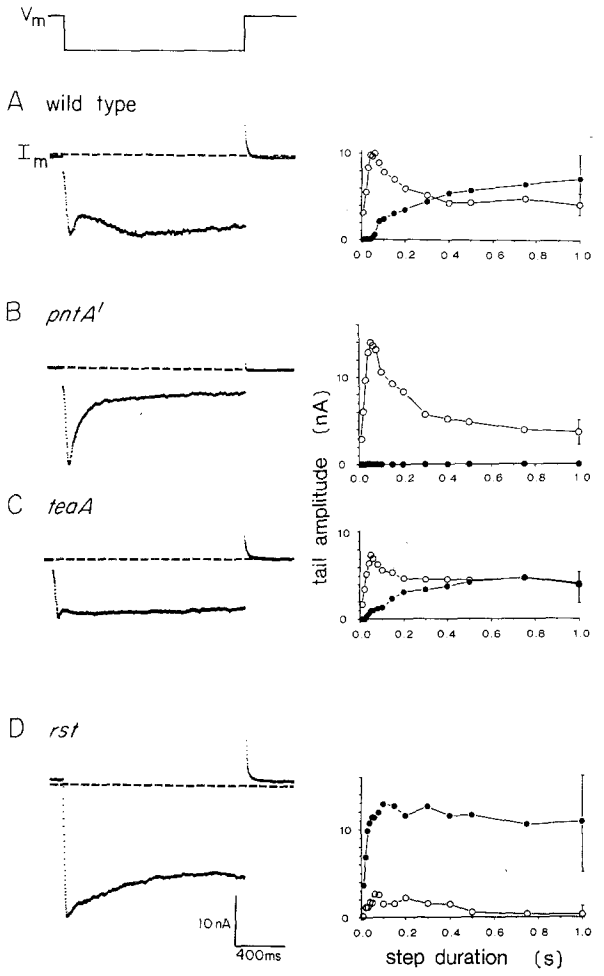

Fig. 2. Currents activated upon hyperpolarization of the wild type and single mutants. The traces to the left in each case show currents evoked by 1500-msec hyperpolarizations of (*A*) the wild type, (*B*) pantophobiac A^1, (*C*) TEA-insensitive A, and (*D*) restless to −110 mV. These voltage steps activate a Ca^{2+} current $I_{Ca(h)}$, a voltage-dependent K^+ current ($I_{K(h)}$) and, (with the exception of pantophobiac A^1) a Ca^{2+}-dependent K^+ current ($I_{K(Ca,h)}$). The tail current elicited by a return to −40 mV contains both a fast-decaying, voltage-dependent K^+ component, and a slow-decaying, Ca^{2+}-dependent component. Graphs to the right show the dependence of the amplitudes of each of these components on the duration of hyperpolarization. $I_{K(h)}$ is represented by the open circles, whereas the filled circles represent $I_{K(Ca,h)}$. For clarity, SD ranges are given for only one of the means; other values were similarly variable. (*A*) In the wild type, $I_{K(h)}$ activates rapidly upon hyperpolarization and then inactivates, whereas $I_{K(Ca,h)}$ activates slowly and is sustained ($n = 20$). (*B*) Hyperpolarization of pantophobiac A^1 activates $I_{K(h)}$ in the absence of a Ca^{2+}-dependent component. $I_{K(h)}$ activates and inactivates with a time course that is similar to that of the wild type ($n = 7$). (*C*) The tail current elicited by hyperpolarization of TEA-insensitive A comprises both fast-decaying and slow-decaying components, similar to that of the wild type ($n = 7$). (*D*) Restless responds to even short (10 msec) hyperpolarizations with activation of both $I_{K(h)}$ and $I_{K(Ca,h)}$. $I_{K(Ca,h)}$ is generally increased in amplitude compared with the wild type, whereas $I_{K(h)}$ is generally decreased in amplitude ($n = 9$)

Table 2. Ca^{2+}-dependent K^+ current activated upon hyperpolarization

Cell strain	I_{500} (nA)	I_{tail} (nA)	*n*
Wild type	−13.1 ± 2.1	3.9 ± 1.7	15
p*ntA*^{1}	−12.9 ± 3.4	0.0 ± 0.0	6
teaA	−10.6 ± 2.1	4.2 ± 2.0	7
rst	−17.7 ± 5.8	11.6 ± 4.5	10
p*ntA*^{1}, *teaA*	−5.8 ± 1.7	0.4 ± 0.4	8
p*ntA*^{1}, *rst*	−12.2 ± 4.2	8.2 ± 6.3	9
rst, *teaA*	−17.2 ± 3.6	11.1 ± 3.9	10

Wild type and mutant cells in K^+-solution were hyperpolarized to −110 mV for 500 msec. I_{500} is the amplitude of the resultant current 500 msec into the voltage step. A return to holding potential elicits an outward tail current, comprising both voltage-dependent ($I_{K(h)}$) and Ca^{2+}-dependent ($I_{K(Ca,h)}$) components. I_{tail} refers to the amplitude of the Ca^{2+}-dependent K^+ component of the tail current. Data are means ± SD of *n* cells.

Hyperpolarization of *P. tetraurelia* elicits an inward current composite (Fig. 2*A*, left). The outward tail current that follows a return to −40 mV represents the sum of two exponentially decaying components (Preston et al., 1990*a*). A fast-decaying component ($\tau \approx 3.5$ msec) reflects deactivation of the voltage-dependent K^+ current ($I_{K(h)}$), whereas a slow-decaying component ($\tau \approx 20$ msec) represents the tail of a Ca^{2+}-dependent K^+ current ($I_{K(Ca,h)}$). Analysis of tail currents resulting from steps to −110 mV of 10- to 1000-msec duration (Fig. 2*A*, right) suggests that $I_{K(h)}$ activates rapidly upon hyperpolarization, peaks at ca. 50 msec, and then inactivates to a new sustained level. In contrast, $I_{K(Ca,h)}$ activates slowly during hyperpolarizations of ≥50 msec, reaching a plateau after 750–1000 msec.

Pantophobiac, TEA-Insensitive, and Restless

The membrane currents of pantophobiac A^1, TEA-insensitive A, and restless have been described previously (Saimi et al., 1983; Richard et al., 1986; Hennessey & Kung, 1987; Preston et al., 1990*b*).

Pantophobiac lacks a Ca^{2+}-dependent K^+ current activated upon either depolarization or hyperpolarization. The loss of $I_{K(Ca,d)}$ is apparent in both the amplitude of the outward current activated upon depolarization, and in the near loss of an associated tail current (Fig. 1*B*; Table 1). Hyperpolarization of pantophobiac A^1 elicits $I_{K(h)}$ and its tail current alone, in the absence of any Ca^{2+}-activated component (Fig. 2*B*; Table 2).

TEA-insensitive A has been characterized as a strain with an enhanced, fast-activating $I_{K(Ca,d)}$. Thus, whereas this current activates slowly during

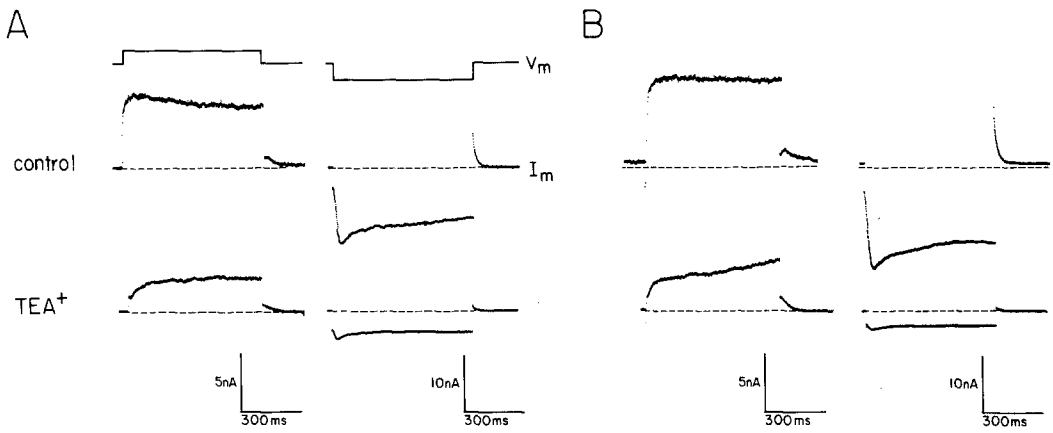

Fig. 3. Effects of TEA^+ on Ca^{2+}-dependent K^+ currents. (*A*) Upper traces show the responses of a wild-type cell to a 750-msec depolarization to −10 mV and a 750-msec hyperpolarization to −110 mV in K^+-solution containing 10 mM choline$^+$. Lower traces show responses to the same voltage steps when 10 mM TEA^+ replaces choline. TEA^+ has no affect on $I_{K(Ca,d)}$ or its tail current, but inhibits both $I_{K(h)}$ and $I_{K(Ca,h)}$. (*B*) Responses of restless in the absence and presence of TEA^+. Solutions and stimuli are the same as used in *A* above. As in the wild type, $I_{K(Ca,d)}$ of restless is insensitive to TEA^+ (left), whereas $I_{K(Ca,h)}$ is inhibited (right). Note that there is an approximate 1 nA shift in holding current upon addition of TEA^+, supporting the notion that an enhanced $I_{K(Ca,h)}$ is responsible for the increased 'resting' membrane potential of this mutant (Richard, Hinrichsen & Kung, 1985)

depolarization of the wild type, even short (≥2 msec) depolarizations of TEA-insensitive A elicit large outward currents and strong outward tail currents upon repolarization (Fig. 1*C*). The mutation appears specific for $I_{K(Ca,d)}$, since the currents activated by hyperpolarization of TEA-insensitive A are comparable with those of the wild type (Fig. 2*C*; Table 2).

The *rst* mutation affects both Ca^{2+}-dependent K^+ currents. Depolarization of restless elicits outward currents and associated tail currents that are increased in amplitude and activate earlier than those of the wild type (Fig. 1*D*), but these alterations in $I_{K(Ca,d)}$ are not as extensive as those caused by *teaA*. The effects of the *rst* mutation on $I_{K(Ca,h)}$ are more severe (Fig. 2*D*). In the wild type, hyperpolarizations of ≥50 msec are necessary to activate $I_{K(Ca,h)}$ (Fig. 2*A*), but this current activates during 10-msec steps in restless (Fig. 2*D*). The tail current of $I_{K(Ca,h)}$ increases rapidly in amplitude upon hyperpolarization, peaks at 50–100 msec, and declines slightly thereafter. Although $I_{K(h)}$ activates in restless with a similar time course to that of the wild type, this current is generally reduced in amplitude in the mutant. The extent of this reduction is highly variable from cell to cell, however. The time constants of the fast and slow components of restless tail currents are not significantly different from those of the wild type (*not shown*).

Evidence for Two Distinct Ca^{2+}-Dependent K^+ Channels in *Paramecium*

Since the two Ca^{2+}-dependent K^+ currents of *Paramecium* are eliminated by a single mutation (*pntA*[1]), it could be argued that the two currents are mediated by a single Ca^{2+}-dependent channel population. This possibility was tested by examining the relative sensitivities of the two currents to external TEA^+. Adding 10 mM TEA^+ to K^+-solution inhibits >80% of the hyperpolarization-activated inward current, and nearly eliminates both the fast-decaying and slow-decaying components of the tail current (Fig. 3*A*). In contrast, TEA^+ has little effect on the slow, depolarization-activated outward current or its tail current (Fig. 3*A*; the reduced outward current amplitude in the presence of TEA^+ reflects suppression of $I_{K(d)}$ by this drug). Since the efficacy of TEA^+ in blocking K^+ channels is voltage-dependent, (*see* Stanfield, 1983), the inhibition by TEA^+ of $I_{K(Ca,h)}$ but not $I_{K(Ca,d)}$ is insufficient evidence for the existence of two separate channel populations. Fortunately, the availability of the *rst* mutation makes it possible to activate $I_{K(Ca,h)}$ within the decay of $I_{K(Ca,d)}$ and thereby show that the two currents are differentially inhibited by TEA^+. Both Ca^{2+}-dependent K^+ currents activate slowly in the wild type, so this simultaneous activation is normally not possible. First, the differential sensitivity of $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ to TEA^+ in restless was confirmed; Fig. 3*B* shows that whereas $I_{K(Ca,h)}$ is inhibited completely by 10 mM $[TEA^+]_o$, TEA^+ has little effect on $I_{K(Ca,d)}$. Simultaneous activation of $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ in restless was achieved as follows. Cells in K^+-solution were depolarized for 600 msec to elicit $I_{K(Ca,d)}$ and its tail current. Ten msec into the decay of this tail current, the cell was stepped to −110 mV for 20 msec to elicit $I_{K(Ca,h)}$ and its associated outward tail (Fig. 4). The decay of $I_{K(Ca,h)}$ can be observed superimposed over the decay of $I_{K(Ca,d)}$. This

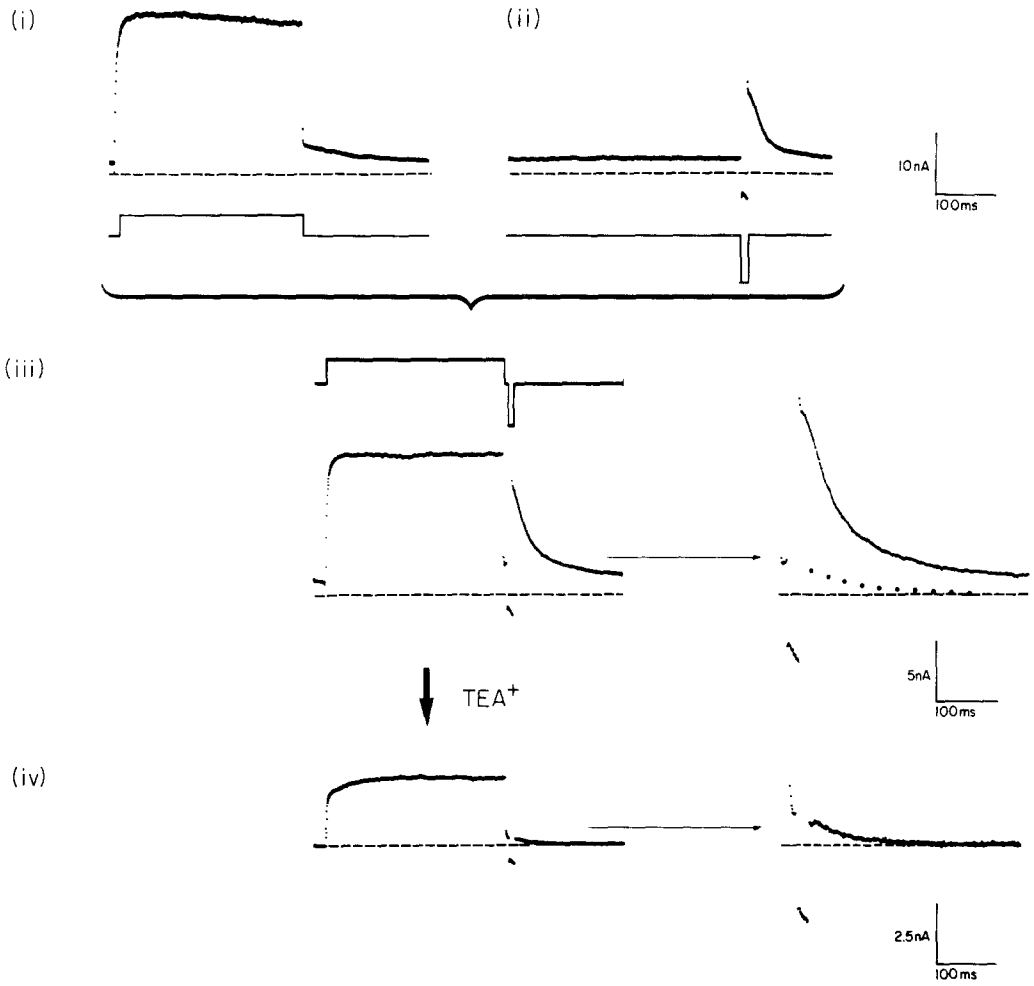

Fig. 4. Differential inhibition of the Ca^{2+}-dependent K^+ currents by TEA^+. (*i*) A restless cell in K^+-solution containing 10 mM choline$^+$ was depolarized for 600 msec to 0 mV to elicit $I_{K(Ca,d)}$ and its associated outward tail current. (*ii*) A 20-msec hyperpolarization to −110 mV elicits $I_{K(Ca,h)}$ and its associated tail current. (*iii*) Combining these stimuli causes the activation of $I_{K(Ca,h)}$ within the decay of $I_{K(Ca,d)}$. The tail current that follows this dual voltage step comprises both depolarization- and hyperpolarization-activated components. The presumed time course of decay of the former within the composite is indicated by a dotted line in the expanded view of the current (right: the dashed line in this and the trace below shows holding current level). (*iv*) When 10 mM TEA^+ replaces choline, $I_{K(Ca,h)}$ is inhibited; a dual depolarization/hyperpolarization elicits only $I_{K(Ca,d)}$ and its tail current

paired depolarization-hyperpolarization was then repeated in the presence of 10 mM TEA^+. TEA^+ does not prevent the activation of $I_{K(Ca,d)}$ or its tail current, but a subsequent 20-msec hyperpolarization forces the drug into the channel that mediates $I_{K(Ca,h)}$, thereby inhibiting both the inward K^+ current and its outward K^+ tail. If $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ flowed through the same channel, this voltage-induced inhibition would have foreshortened the decay $I_{K(Ca,d)}$. The fact that the time course of decay is unaltered by the hyperpolarization (Fig. 4) suggests that the two conductances are mediated by pharmacologically distinct ion channels.

The discovery of two pharmacologically distinct Ca^{2+}-dependent K^+ conductances in *Paramecium*, and the existence of mutants with specific defects in these currents, lead to a genetic investigation of any possible functional or regulatory interactions between the two channels. Three double mutants were constructed and characterized under voltage clamp: "p*ntA*^{1}, *teaA*," "p*ntA*^{1}, *rst*," and "*rst, teaA*."

Double Mutants

a) $I_{K(Ca,d)}$: Both the *teaA* and *rst* mutations restore $I_{K(Ca,d)}$ in pantophobiac A^1 (Fig. 5*A*). Depolarization of the "p*ntA*^{1}, *teaA*" double mutant elicits both a strong outward current and a pronounced outward tail current that is characteristic of TEA-insensitive A (Fig. 5*A*). Similarly, the currents elicited by depolarization of "p*ntA*^{1}, *rst*" include a strong tail current characteristic of $I_{K(Ca,d)}$ (Table 1; Fig. 5*B*). Depolarization of the "*rst, teaA*" double mutant elicits

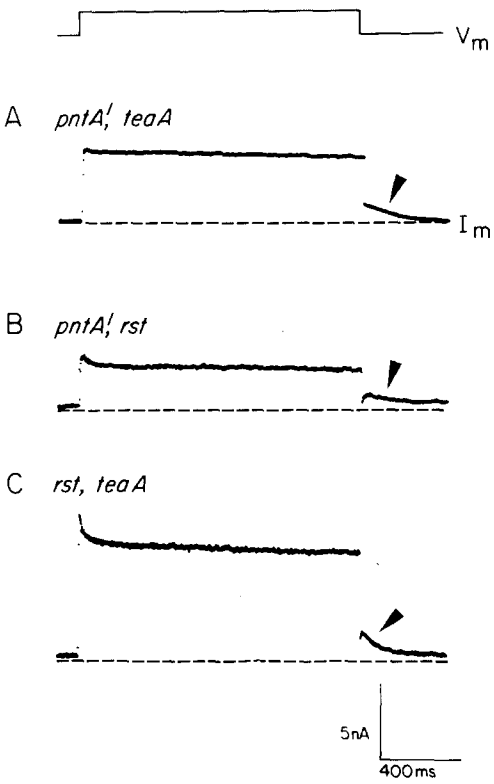

Fig. 5. Ca^{2+}-dependent K^+ currents activated upon depolarization of double mutants. Depolarizations (1500-msec to −10 mV elicit outward currents that include a Ca^{2+}-dependent component in all three double mutants: (*A*) "p*ntA*^{1}, *teaA*," (*B*) "p*ntA*^{1}, *rst*," and (*C*) "*rst, teaA*." Arrowheads indicate the tail currents of $I_{K(Ca,d)}$

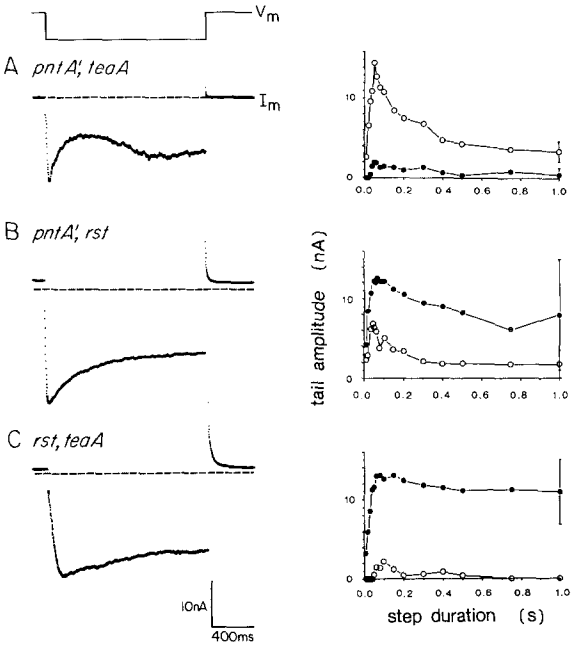

Fig. 6. Ca^{2+}-dependent K^+ currents activated by hyperpolarization of double mutants. Traces to the left show the responses of (*A*) "p*ntA*^{1}, *teaA*," (*B*) "p*ntA*^{1}, *rst*" and (*C*) "*rst, teaA*" double mutants to 1500-msec hyperpolarizations to −110 mV. Graphs to the right plot amplitudes of the fast and slow components of the tail current elicited following steps to −110 mV against the duration of hyperpolarization, as in Fig. 1. $I_{K(Ca,h)}$ is weak in "p*ntA*^{1}, *teaA*" compared with the wild type, whereas $I_{K(Ca,h)}$ activates with a time course that is characteristic of *rst* in both *rst* double mutants. Note that $I_{K(h)}$ is weak in both of the *rst* double mutants. Data are the means of *A* 6, *B* 9, and *C* 10 specimens

currents that are not significantly larger than those of TEA-insensitive A alone (Table 1, Fig. 5*C*), suggesting that the effects of the two single mutations on the amplitude of $I_{K(Ca,d)}$ are not additive.

b) $I_{K(Ca,h)}$: A return to holding potential following hyperpolarization of "p*ntA*^{1}, *teaA*" is accompanied by a tail current that comprises both fast and slow components (Fig. 6*A*, left). The fast component clearly corresponds to $I_{K(h)}$ (Fig. 6*A*, right), and is generally increased in amplitude compared with that of the wild type. The slow component is greatly reduced in amplitude compared with this current in either the wild type or TEA-insensitive A (Table 2), and in many cells it was possible only to resolve a single, fast component. In examples where two tail-current components could be resolved, the slow component decays with a time constant of ca. 25 msec and is TEA^+-sensitive (*not shown*), suggesting that this current indeed represents $I_{K(Ca,h)}$ rather than a third K^+ current or a hyperpolarization-dependent manifestation of $I_{K(Ca,d)}$.

Hyperpolarization of "p*ntA*^{1}, *rst*" elicits both $I_{K(h)}$ and $I_{K(Ca,h)}$ (Fig. 6*B*, left). The time course of activation of $I_{K(Ca,h)}$, as judged by tail-current amplitude, mirrors the time course of $I_{K(Ca,h)}$ activation in restless (Fig. 6*B*, right). The time course of activation of $I_{K(h)}$ in "p*ntA*^{1}, *rst*" is not significantly different from that of the wild type.

Similarly, $I_{K(Ca,h)}$ activates in "*rst, teaA*" with a time course that is characteristic of restless (Fig. 6*C*, right), whereas $I_{K(h)}$ is generally reduced in amplitude compared with that of the wild type (Table 2).

Calcium Currents of Single and Double Mutants

Since the K^+ currents described here are dependent upon Ca^{2+} for their activation, it is possible that the mutation-induced differences in their amplitudes and activation kinetics reflect aberrant rates or amounts of Ca^{2+} entry into the cell during voltage steps.

The source of Ca^{2+} for activation of $I_{K(Ca,d)}$ in *Paramecium* is a well-characterized inward Ca^{2+} transient ($I_{Ca(d)}$) that activates rapidly upon depolar-

ization, peaks at ca. 2 msec, and then inactivates. Suppression of K^+-currents by internal Cs^+ (diffusing from the microelectrodes) and 10 mM external TEA^+ allows this current to be measured in isolation; in the wild type, the peak amplitude of $I_{Ca(d)}$ upon depolarization to −10 mV is −8.6 ± 2.2 nA (n = 11). The mutants showed no significant differences in the time to peak or peak amplitude of this current compared with that of the wild type. Hyperpolarization of the wild type to −110 mV under these same conditions activates an inward current that peaks at −3.9 ± 0.8 nA (n = 11) after 40–70 msec, and then inactivates to a sustained level of −2.5 ± 0.5 nA (n = 11). The peak represents a hyperpolarization-activated Ca^{2+} current (Saimi, 1986; Hennessey, 1987; R.R. Preston & Y. Saimi, *in preparation*). There were no significant differences between the mutants and the wild type in terms of the amplitude of the peak, or of the sustained portion of the current, or in the time to peak.

Discussion

Two Ca^{2+}-dependent K^+ currents of *P. tetraurelia*, $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$, have been described. One of these is activated upon depolarization, the other upon hyperpolarization. Both currents are missing in pantophobiac A^1, whereas in the TEA-insensitive A mutant, $I_{K(Ca,d)}$ activates earlier and more strongly than in the wild type. A third mutation, *rst*, causes both an increase in the amplitude of $I_{K(Ca,d)}$ and the early activation of $I_{K(Ca,h)}$. Construction of three double mutants, "p*ntA*^{1}, *teaA*," "p*ntA*^{1}, *rst*," and "*rst*, *teaA*," shows that at least some degree of Ca^{2+}-dependent K^+ current function can be restored to pantophobiac A^1 by the *rst* and *teaA* mutations, but the early activation of $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ in TEA-insensitive A and restless, respectively, cannot be corrected by introducing the second mutation. These observations suggest that at least two gene products are involved in the functioning of both $I_{K(Ca,d)}$ (p*ntA* and *teaA*) and $I_{K(Ca,h)}$ (p*ntA* and *rst*) in the wild type.

The present studies also hint at a possible compensatory regulation of the amplitudes of the voltage-dependent and Ca^{2+}-dependent K^+ currents in *Paramecium*. This notion is best appreciated when the effects of mutation on the hyperpolarization-activated current are examined (Figs. 2 and 6); when $I_{K(Ca,h)}$ is weakened or removed by mutation (p*ntA*^{1}), $I_{K(h)}$ is generally increased in amplitude compared with the wild type. Conversely, when $I_{K(Ca,h)}$ is enhanced by mutation (*rst*), $I_{K(h)}$ is generally reduced in amplitude. By implication, the cell optimally responds to a given voltage step with a set amount of current, so elimination of one K^+ current causes a compensatory increase in the amplitude of the other. Similar mechanisms may regulate the relative amplitudes of the K^+ currents activated upon depolarization of *P. tetraurelia* ($I_{K(d)}$ is apparently enhanced in pantophobiac A^1; Fig. 1), but this possibility has not been investigated systematically.

Evidence for the Existence of Two Separate Ca^{2+}-Dependent K^+ Conductances in *Paramecium*

Although two independent mutations of *P. tetraurelia* (p*ntA*^{1} and *rst*) simultaneously affect the function of both $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$, this report provides further evidence that the depolarization- and hyperpolarization-activated currents are mediated by two separate Ca^{2+}-dependent K^+ channel populations. First, the *teaA* mutation affects $I_{K(Ca,d)}$ independently of $I_{K(Ca,h)}$. Second, the K^+-channel inhibitor, TEA^+, specifically blocks $I_{K(Ca,h)}$ and not $I_{K(Ca,d)}$. This differential action of TEA^+ on the two Ca^{2+}-dependent K^+ currents is not due to the membrane potential dependence of TEA^+ inhibition; the simultaneous activation of the two Ca^{2+}-dependent conductances in restless shows that inhibition of $I_{K(Ca,h)}$ by TEA^+ occurs without concomitant loss of $I_{K(Ca,d)}$ (Fig. 4). Thus, it is likely that *Paramecium* indeed possesses two distinct Ca^{2+}-dependent K^+ conductances. It is of interest that recordings of single-channel currents from isolated *Paramecium* membrane patches have revealed a 70-pS, Ca^{2+}-dependent K^+ channel that is preferentially activated by hyperpolarization, and a second, 150-pS, Ca^{2+}-dependent K^+ channel that activates upon depolarization (Martinac et al., 1988; Saimi & Martinac, 1989). Although there is no proof that these single-channel activities correspond to whole-cell currents, and there have been no systematic investigations of ion channel activities in the mutants, it is tempting to speculate that the two Ca^{2+}-dependent K^+ channel species correspond to the two Ca^{2+}-dependent K^+ currents reported here.

Interactions Between Gene Products that Regulate the Two Ca^{2+}-Dependent K^+ Conductances

How do the p*ntA*^{1}, *teaA*, and *rst* mutations affect Ca^{2+}-dependent K^+ current function in *Paramecium*, and what is the extent of interaction between the gene products? From a knowledge of the principles of ion channel function in other systems, one can envision two independent but parallel pathways that regulate the activities of $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ in *Paramecium*.

Ca^{2+}-dependent K^+ channels generally retain their activity and Ca^{2+} sensitivity when their sup-

porting membrane is excised from the cell, suggesting that the factors necessary for channel function are all membrane associated, if not an integral part of the channels themselves. Several previous studies have suggested a role for calmodulin (CaM) in channel function (Okada et al., 1987; Onozuka et al., 1987), perhaps as a Ca^{2+}-sensing channel subunit. Thus, it is of interest that *pntA*[1] is known to cause an amino acid substitution in the third Ca^{2+}-binding domain of CaM (Schaefer et al., 1987). A dependence on the normal Ca^{2+}-sensing properties of this protein for Ca^{2+}-dependent K^+ current function in *Paramecium* might explain the loss of both currents in pantophobiac A[1]. Recent evidence suggests that the activity of many K^+ channels may be modulated by covalent modification (Levitan, 1985, 1988). Thus, in addition to affecting the structure of the channels themselves, it is possible that the *Paramecium* mutations affect a channel regulatory or modulatory pathway.

It is possible to envision a hyperpolarization-activated, Ca^{2+}-dependent K^+ channel in *Paramecium* whose Ca^{2+} sensitivity is dependent upon an associated Ca^{2+}-binding subunit, perhaps CaM. If CaM may be considered as a switch for current activation, phosphorylation (or a similar modification pathway) might be considered as a gain control. In the wild type, a continual phosphorylation/dephosphorylation cycle maintains the current at a level determined by the cell's current needs. The *rst* mutation may affect a component of this cycle, so that when activated, the amplitude of the current is always maximal. In this state, the channels may be able to respond to even a weak Ca^{2+}/CaM or voltage signal, a notion supported by preliminary studies on the effects of injecting Ca^{2+} chelators into restless (R.R. Preston & Y. Saimi, *unpublished*). A similar pathway may regulate the activity of $I_{K(Ca,d)}$. As above, CaM acts as a switch, but in this example, the channels' gain control may be regulated by a *teaA* mutation-sensitive pathway.

Presumably the two Ca^{2+}-dependent K^+ channels have a common ancestor, so a degree of cross-talk between the two channel regulatory pathways is not unexpected. Thus, the *teaA*-sensitive pathway allows limited expression $I_{K(Ca,h)}$ in "*pntA*[1], *teaA*," and the *rst* pathway allows $I_{K(Ca,d)}$ to function in "*pntA*[1], *rst*," and also enhances $I_{K(Ca,d)}$ in restless.

While the evidence for a role of the *teaA* and *rst* gene products in regulation of channel activity is limited, such speculation provides a starting point for biochemical characterizations of these channels, and of mutant cells with abnormalities in the activity of these channels. One of the strengths of *Paramecium* as model organism for the study of membrane excitation is the ease with which ion channel components can be manipulated genetically (Saimi & Kung, 1987). In future, it may be interesting to generate mutants that suppress the phenotypes of *pntA*[1], *teaA*, and *rst*. These mutations may involve hitherto unknown ion channel regulatory pathways, the elucidation of which may be of relevance to membrane excitation in many organisms.

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References

Ganetzky, B., Wu, C.-F. 1986. Neurogenetics of membrane excitability in *Drosophila*. *Annu. Rev. Genet.* **20:**13–44

Hennessey, T.M. 1987. A novel calcium current is activated by hyperpolarization of *Paramecium tetraurelia*. *Soc. Neurosci.* **13:**108 (*abstr.*)

Hennessey, T. M., Kung, C. 1987. A calcium-dependent potassium current is increased by a single-gene mutation in *Paramecium*. *J. Membrane Biol.* **98:**145–155

Hinrichsen, R. D., Amberger, E., Saimi, Y., Burgess-Cassler, A., Kung, C. 1985. Genetic analysis of mutants with a reduced Ca^{2+}-dependent K^+ current in *Paramecium tetraurelia*. *Genetics* **111:**433–445

Levitan, I.B. 1985. Phosphorylation of ion channels. *J. Membrane Biol.* **87:**177–190

Levitan, I.B. 1988. Modulation of ion channels in neurons and other cells. *Annu. Rev. Neurosci.* **11:**119–136

Martinac, B., Saimi, Y., Gustin, M.C., Kung, C. 1988. Ion channels of three microbes: *Paramecium*, yeast and *Escherichia coli*. *In:* Calcium and Ion Channel Modulation. A.D. Grinnell, D. Armstrong, and M.B. Jackson, editors. pp. 415–430. Plenum, New York

Meech, R.W. 1978. Calcium-dependent potassium activation in nervous tissues. *Annu. Rev. Biophys. Bioeng.* **7:**1–18

Okada, Y., Yada, T., Ohno-Shosaku, T., Oiki, S. 1987. Evidence for the involvement of calmodulin in the operation of Ca-activated K channels in mouse fibroblasts. *J. Membrane Biol.* **96:**121–128

Onozuka, M., Furuichi, H., Kishii, K., Imai, S. 1987. Calmodulin in the activation process of calcium-dependent potassium channel in *Euhadra* neurones. *Comp. Biochem. Physiol.* **86A:**589–593

Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature (London)* **307:**693–696

Preston, R.R., Saimi, Y., Kung, C. 1990*a*. Evidence for two K^+ currents activated upon hyperpolarization of *Paramecium tetraurelia*. *J. Membrane Biol.* **115:**41–50

Preston, R.R., Wallen-Friedman, M.A., Saimi, Y., Kung, C. 1990*b*. Calmodulin defects cause the loss of Ca^{2+}-dependent K^+ currents in two pantophobiac mutants of *Paramecium tetraurelia*. *J. Membrane Biol.* **115:**51–60

Richard, E.A., Hinrichsen, R.D., Kung, C. 1985. A single gene mutation that affects a potassium conductance and resting membrane potential in *Paramecium*. *J. Neurogenet.* **2:**239–252

Richard, E.A., Saimi, Y., Kung, C. 1986. A mutation that increases a novel calcium-activated potassium conductance of *Paramecium tetraurelia*. *J. Membrane Biol.* **91:**173–181

Saimi, Y. 1986. Calcium-dependent sodium currents in *Paramecium:* Mutational manipulations and effects of hyper- and depolarization. *J. Membrane Biol.* **92:**227–236

Saimi, Y., Hinrichsen, R.D., Forte, M., Kung, C. 1983. Mutant analysis shows that the Ca^{2+}-induced K^+ current shuts off one type of excitation in *Paramecium*. *Proc. Natl. Acad. Sci. USA* **80:**5112–5116

Saimi, Y., Kung, C. 1987. Behavioral genetics of *Paramecium*. *Annu. Rev. Genet.* **21:**47–65

Saimi, Y., Martinac, B. 1989. A calcium-dependent potassium channel in *Paramecium* studied under patch clamp. *J. Membrane Biol.* **112:**79–89

Satow, Y., Kung, C. 1976. A 'TEA^+-insensitive' mutant with increased potassium conductance in *Paramecium aurelia*. *J. Exp. Biol.* **65:**51–63

Satow, Y., Kung, C. 1980. Ca-induced K^+-outward current in *Paramecium tetraurelia*. *J. Exp. Biol.* **88:**293–303

Schaefer, W.H., Hinrichsen, R.D., Burgess-Cassler, A., Kung, C., Blair, I.A., Watterson, D.M. 1987. A mutant *Paramecium* with a defective calcium-dependent potassium conductance has an altered calmodulin: A nonlethal selective alteration in calmodulin regulation. *Proc. Natl. Acad. Sci. USA* **84:**3931–3935

Sonneborn, T.M. 1970. Methods in *Paramecium* research. *Methods Cell. Physiol.* **4:**243–339

Stanfield, P.R. 1983. Tetraethylammonium ions and the potassium permeability of excitable cells. *Rev. Physiol. Biochem. Pharmacol.* **97:**1–67

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