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Evidence for Two K^+ Currents Activated Upon Hyperpolarization of *Paramecium tetraurelia*

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Summary. Hyperpolarization of voltage-clamped *Paramecium tetraurelia* in K^+ solutions elicits a complex of Ca^{2+} and K^+ currents. The tail current that accompanies a return to holding potential (−40 mV) contains two K^+ components. The tail current elicited by a step to −110 mV of ≥50-msec duration contains fast-decaying ($\tau \approx 3.5$ msec) and slow-decaying ($\tau \approx 20$ msec) components. The reversal potential of both components shifts by 55–57 mV/10-fold change in external $[K^+]$, suggesting that they represent pure K^+ currents. The dependence of the relative amplitudes of the two tail currents on duration of hyperpolarization suggests that the slow K^+ current activates slowly and is sustained, whereas the fast current activates rapidly during hyperpolarization and then rapidly inactivates. Iontophoretic injection of a Ca^{2+} chelator, EGTA, specifically reduces slow tail-current amplitude without affecting the fast tail component. Both K^+ currents are inhibited by extracellular TEA^+ in a concentration-dependent, noncooperative manner, whereas the fast K^+ current alone is inhibited by 0.7 mM quinidine.

Key Words inward rectification · voltage-dependent K^+ current · Ca^{2+}-dependent K^+ current · *Paramecium*

Introduction

Although considered to be a primitive animal, *Paramecium* performs within a single cell many of the biological functions more commonly associated with higher organisms. The complexity of membrane excitation in *Paramecium* epitomizes the degree of specialization achieved by this protozoan: at least eight ion conductances have been described to date (Machemer, 1988*a*). Since ciliary activity is coupled to changes in the membrane potential of *Paramecium*, this complexity may reflect the organism's need for a fine control over its locomotory behavior.

A regenerative depolarization of *Paramecium* and consequential behavioral events have been well characterized and are described in detail elsewhere (Eckert & Brehm, 1979; Kung & Saimi, 1982; Saimi & Kung, 1987; Machemer, 1988*b*), but this organism also demonstrates a regenerative hyperpolarization when extracellular K^+ concentrations are low (Satow & Kung, 1977). This transient may reflect activation of an inwardly rectifying K^+ current (Oertel, Schein & Kung, 1978). Although originally considered to be an anomaly (Katz, 1949), inward rectification is a property of many cell types (*see* review by Rudy, 1988). The phenomenon is usually attributed to K^+ channels, characterized by their tendency to open during hyperpolarization and close when the cell is depolarized. As a class, inward rectifiers are poorly understood, as is their function. Hille (1984) speculates that their closure upon depolarization may help minimize antagonistic ion current flow during the prolonged depolarizations that are characteristic of oocytes upon fertilization and of heart cells. Since inward rectifiers generally drive membrane potentials toward E_K when activated, they may also serve to modulate cell excitability.

The ionic basis of inward rectification in *Paramecium* has not previously been characterized. Saimi (1986) suggested that hyperpolarization of *P. tetraurelia* activates a Ca^{2+} current, and there have been reports of K^+ currents activated upon hyperpolarization (Oertel et al., 1978; Richard, Saimi & Kung, 1986), but the nature and relative contributions of these currents to the total membrane response has not been determined. The present study re-examines the currents activated upon hyperpolarization of voltage-clamped *Paramecium tetraurelia*, and details two K^+ currents that are kinetically and pharmacologically distinct.

Materials and Methods

Cell Stocks and Culture Conditions

In order to facilitate cell penetration by microelectrodes and to minimize damage resulting from trichocyst release, a trichocyst nondischarge mutant line derived from *Paramecium tetraurelia* wild-type stock 51s, nd-6 (*nd6*/*nd6*: Sonneborn, 1975), was used throughout this study. The electrophysiological and behavioral

characteristics of nd-6 are indistinguishable from those of wild-type *P. tetraurelia* (R.R. Preston & Y. Saimi, *unpublished*). Cells were grown and maintained at room temperature (22–24°C) on a chemically defined, monoxenic medium containing 0.1 g/liter (0.5 mM) glucose, 0.1 g/liter (1.3 mM) NH_4acetate, 0.5 mM K_2HPO_4, 0.2 mM$MgSO_4$, 0.5 mM $CaCl_2$, 5 mM HEPES buffer, 3 mM NaOH, 1 mg/liter stigmasterol, and 7.5 mg/liter phenol red. The culture medium was inoculated with *Enterobacter aerogenes* 24 hr prior to use.

Solutions

All solutions contained 1 mM Ca^{2+}, 0.01 mM EDTA, 1 mM HEPES buffer, pH 7.2. The following ion concentrations (in mM) were added as required: *K^+-solution*: 4 KCl; *K^+-choline solution*: 4 KCl, 10 choline Cl; *K^+-TEA^+ solution*: 4 KCl, 10 tetraethylammonium (TEA^+) chloride; and *TEA^+-solution*: 10 TEA^+Cl.

Electrophysiological Recording

The techniques used to monitor the membrane currents of *Paramecium* under two electrode voltage clamp were similar to those described previously (Naitoh & Eckert, 1972; Hinrichsen & Saimi, 1984). Depending on the nature of the experiment, voltage-recording and current-injection glass capillary microelectrodes were filled with either 3 M KCl or 4 M CsCl, tip resistance 15–25 MΩ. The cell membrane was voltage clamped near resting potential in K^+-solution (−40 mV) using an Axoclamp 2A current- and voltage-clamp module (Axon Instruments). Commands for the Axoclamp were provided by a WPI 1830 series stimulator (World Precision Instruments), or an S-95 tri-level stimulator (Medical Systems). Membrane potential and current signals were digitized and displayed on a Nicolet 201 digital oscilloscope (Nicolet Instrument) and stored on magnetic disks for later analysis. Membrane currents were filtered at 2 kHz (slow membrane events at 1 kHz) using an 8-pole Bessel filter (Frequency Devices, Model 902 LPF). EGTA was injected iontophoretically into cells using a third intracellular glass capillary microelectrode filled with 100 mM K_2EGTA, 1 mM HEPES, pH 7.2. Iontophoretic electrodes filled with the above solution had tip resistances of 100–200 MΩ. When necessary, the contents of the experimental chamber (capacity ≈ 1 ml) were changed using a gravity-fed perfusion system with a flow rate of 4 ml/min.

All experiments were performed at room temperature (22–24°C).

Data Analysis

Tail current time courses were computer-fitted to single or double exponents using a least-squares method ('Asystant+' data analysis program; Asyst Software Technologies). Observations of responses to small voltage steps (<15 mV), or to larger voltage steps (30–90 mV) in the presence of K^+-current inhibitors, suggest that capacitive transients settle within 0.5–1 msec of terminating the voltage command. These transients were excluded from estimations of tail-current amplitudes and time constants by collecting and fitting data at ≥2–3 msec after terminating the voltage step. Tail-current amplitudes at the moment of return to holding level were thus determined by back-extrapolation and are presented relative to holding currents. All data are expressed as means ± SD; levels of statistical significance between means were assessed using a Student's *t* test. Recent evidence suggests that even small (<12 mV) negative steps from holding potential elicit active membrane responses from *Paramecium* (R.R. Preston, *unpublished*; *see also* Machemer, 1988*a*), so currents are presented without leakage correction. The membrane resistance of each cell was tested with 0.1–0.2 nA current pulses at the beginning of the recording period. Cells with resistances of <50 MΩ were discarded.

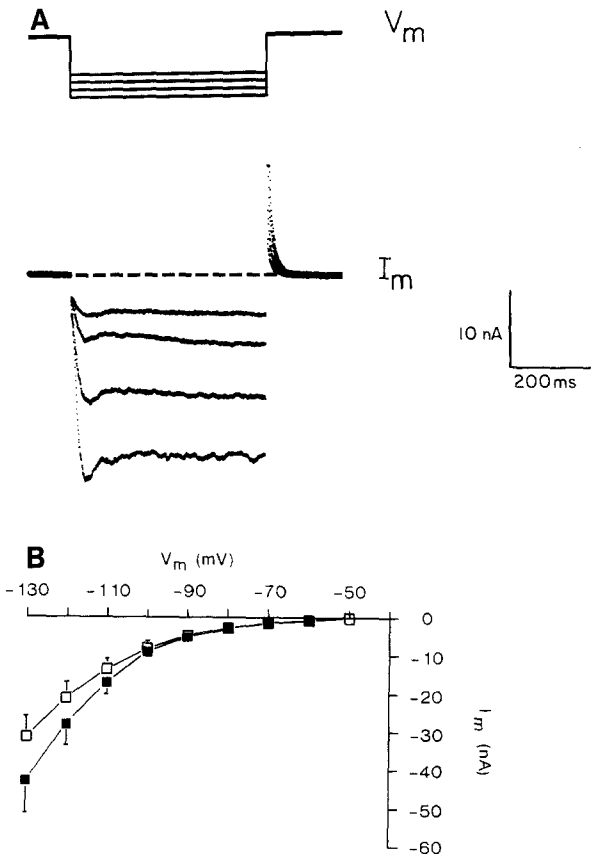

Fig. 1. Currents activated by hyperpolarization of *Paramecium tetraurelia* in K^+-solution. (*A*) 500-msec hyperpolarizations in K^+-solution (−90 to −120 mV in 10-mV steps) activate inward current complexes. The broken lines in these and subsequent figures represent zero current levels. (*B*) The amplitude of these currents (I_m) at their peak (filled symbols) and at 500 msec (open symbols) are plotted as a function of membrane potential (V_m). Data are means ± SD of 16 cells

Results

Hyperpolarization-Activated Currents

In K^+-solution, 500-msec step hyperpolarizations of *P. tetraurelia* under voltage clamp elicit inward currents (Fig. 1*A*). These currents typically peak at 35–95 msec (the current activated by a step to −110

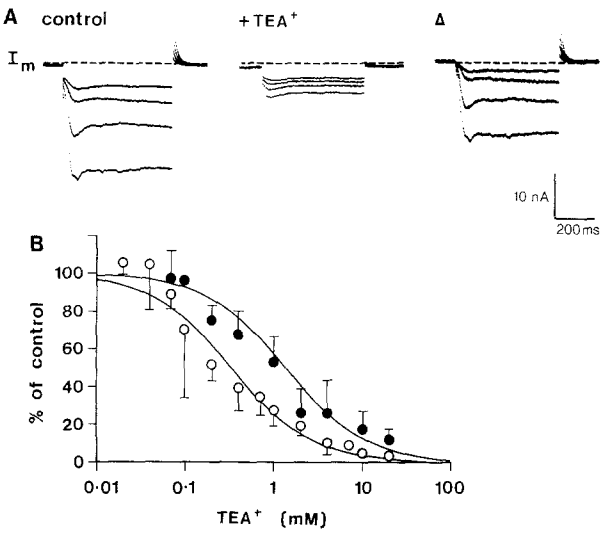

Fig. 2. Inhibition of the K^+ currents by TEA^+. (*A*) Families of currents elicited by 500-msec hyperpolarizations (−90 to −120 mV in 10-mV steps) in K^+-choline solution (left), and responses to identical voltage steps in K^+-TEA^+ solution (middle). Traces to the right show the amount of current that is suppressed by TEA^+, produced by electronic subtraction of the first two sets of traces shown. (*B*) Concentration dependence of inhibition of the fast-decaying (open symbols) and slow-decaying (filled symbols) tail currents by TEA^+. 75- or 750-msec steps to −110 mV were used to elicit tail currents that comprised predominantly fast- and slow-decaying components, respectively. Amplitudes of the individual components were first determined in K^+-solution, and then in the presence of varying concentrations of TEA^+. Choline$^+$ was used as a counterion, maintaining the combined TEA^+/choline$^+$ concentration at 20 mM. Data are the means ± SD of 3–9 specimens. Inhibition curves were fitted assuming a single TEA^+ binding site

mV peaks at 55.5 ± 6.8 msec, $n = 38$), decay in the subsequent 60–100 msec, and then again rise toward a second peak or plateau. The amplitude of the first, peak current and currents at 500 msec are shown as a function of membrane potential in Fig. 1*B*.

These hyperpolarization-activated inward currents in K^+-solution actually represent a complex of currents. These composites include 'leak' current, an inward Ca^{2+} current (Saimi, 1986; Hennessey, 1987; *see below*), and one or more K^+ currents. Adding 10 mM TEA^+ (a commonly used K^+ channel inhibitor) to the extracellular solution reduces the amplitude of the peak current and the current at 500 msec by ≥70% (Fig. 2*A* and Table 1,*A*), and almost completely suppresses the outward tail current observed in a control solution. This suggests that the outward tail current and a major portion of the inward current are carried by K^+. Previous studies have suggested that the K^+ current(s) activated by hyperpolarization of *Paramecium* may have voltage and/or Ca^{2+} dependence (Oertel et al., 1978; Richard et al., 1986). The precise nature and number of K^+ currents, or their relative contributions to the total inward current observed following hyperpolarization, was unknown, however. The composite nature of the inward current precludes a study of the properties of the individual K^+ currents in isolation. However, it may be possible to draw conclusions about the properties of these currents from a study of their tail currents upon returning to holding potential. First, it was necessary to determine the relative purity of the tail current by examining possible contributions from other hyperpolarization-activated currents.

Hyperpolarization-Activated Ca^{2+} Current

K^+ currents were suppressed by bathing cells in K^+-free, 'TEA^+-solution', and by the use of Cs^+-filled voltage-recording and current-injection microelectrodes. Cs^+ is presumed to diffuse from the electrodes and inhibit the K^+ currents internally. The Ca^{2+}-dependent Na^+ current is not observed in the absence of external Na^+, so the currents that remain under these conditions are suggested to comprise 'leak' current and a hyperpolarization-activated Ca^{2+} current.

In TEA^+-solution, a 500-msec step hyperpolarization to −110 mV activates an inward current that peaks at 30–40 msec and then decays rapidly to a new, sustained level (Table 1,*D*). The peak represents a novel Ca^{2+} current (Saimi, 1986; Hennessey, 1987). Termination of the voltage step during the inward peak elicits a small inward tail current (a 40-msec step to −110 mV elicits a tail of −1.3 ± 0.4 nA, mean ± SD, $n = 15$). This tail current rapidly decreases in amplitude with increasing step duration; steps of >70–100 msec elicit tail currents too small to be measured with accuracy.

The inward tail currents in TEA^+-solution are small and inactivating, suggesting that the tails observed following hyperpolarization in K^+-solution are relatively pure K^+ currents. Thus, we can use these tails to characterize the associated inward K^+ current(s), bearing in mind that outward tail currents elicited by short (<100 msec) steps may be contaminated by a small inward component.

Two Kinetically Distinct K^+ Currents Activated Upon Hyperpolarization

The tail current that follows a 500-msec step to −110 mV in K^+-solution decays with a time course that is approximated by the sum of two exponents

Table 1. Values for peak and sustained components of the inward currents activated by 500-msec hyperpolarizations in various solutions

	I_{peak}, nA	I_{500}, nA	n
A K^+-choline	−13.7 ± 4.2	−12.9 ± 3.5	7
K^+-TEA^+	−4.3 ± 1.0	−4.1 ± 1.0	7
B K^+-solution	−17.2 ± 3.1	−13.6 ± 2.5	16
after EGTA	−7.7 ± 1.9	−8.4 ± 1.8	16
C +Quinidine	inhibited	−12.2	2
after EGTA	—	−8.3	2
K^+ currents suppressed[a]			
D Cs^+/TEA^+	−3.9 ± 0.8	−2.5 ± 0.5	15
E Cs^+/TEA^+	−4.2 ± 1.0	−2.8 ± 0.5	7
after EGTA	−3.9 ± 0.9	−3.2 ± 0.3	7
F Cs^+/TEA^+	−3.7 ± 0.7	−2.4 ± 0.6	4
after quinidine	−3.6 ± 0.5	−2.5 ± 0.4	4

A 500-msec step to −110 mV from −40 mV was used to elicit peak currents (I_{peak}) and currents at 500 msec (I_{500}) in various solutions.

[a] CsCl electrodes were used in combination with 10 mM external TEA^+ to suppress K^+ currents. Values are mean ± SD responses of n cells.

A: Amplitudes of currents in K^+-solution supplemented with 10 mM choline Cl are compared with those in K^+-solution supplemented with 10 mM TEA^+. B: Membrane current responses of cells in K^+-solution before and after a 20-sec, −7.5-nA iontophoretic injection of EGTA. C: The current that remains following exposure to 0.7 mM quinidine in K^+-solution is reduced in amplitude by a 20-sec, −7.5-nA iontophoretic injection of EGTA. Hyperpolarization no longer yields a peak after quinidine exposure; this value is therefore omitted from the Table. D: Values for peak and total currents elicited following suppression of K^+ currents. E: Responses of cells bathed in TEA^+-solution before and after a 20-sec, −7.5-nA injection of EGTA. EGTA has no significant effect on the amplitudes of either the peak or the sustained current. F: The peak and sustained components of the currents activated in TEA^+-solution are unaffected by exposure to 0.7 mM quinidine.

(Fig. 3*A*, right). The faster-decaying component has a time constant of 3.7 msec (± 1.3 msec, n = 20), whereas the slower-decaying component has a time constant of 19.6 msec (±6.2 msec, n = 20). This suggests that *Paramecium's* membrane response to hyperpolarization includes two K^+ currents with distinct deactivation kinetics. The two tail-current components are also distinct in their kinetics of activation. Specimens in K^+-solution were hyperpolarized to −110 mV for periods of 10 msec to 2 sec. In each case, the time course of decay of the tail current elicited upon returning to −40 mV was fitted with one or two exponents (Fig. 3*A*), and the amplitude of each component extracted. These amplitudes are plotted against step duration in Fig. 3*B*. The tail current elicited following short (<50 msec) hyperpolarizations decays with a time course that is best fitted with a single exponent ($\tau \approx 3.5$ msec; Fig. 3*A*, left). This current activates rapidly to a peak of ca. 10 nA at 40–60 msec, and then inactivates. A slow-decaying tail component ($\tau \approx 20$ msec) is not observed until a hyperpolarization to −110 mV exceeds 40 msec (Fig. 3*A*, middle and right), and then this current increases slowly in amplitude with increasing step duration to a plateau after 400–800 msec. The time constants of the fast and slow components of the tail current are not affected by increasing step duration (*not shown*). Thus, the K^+ tail current observed following a 500-msec step hyperpolarization comprises two components: a fast-activating, fast-decaying component, and a slow-activating, slow-decaying component. The dependence of the amplitudes of both the fast and slow tail-current components on membrane potential is presented in Fig. 3*C*. Tail-current decay rates are not dependent on the magnitude of the membrane potential change (*not shown*).

Reversal Potentials of the K^+ Currents

K^+ was confirmed as the carrier of the major portion of the inward current upon hyperpolarization of *Paramecium*, and also of the two components of the tail current, by determining the reversal potentials of the tail components. The two K^+ tail components have distinct activation and inactivation kinetics, so that a 750-msec hyperpolarization can be used to elicit a tail current that comprises largely slow component, whereas a 75-msec step elicits a tail current that comprises largely fast component. Cells in K^+-solution were hyperpolarized to −120 mV for 750 msec to activate the inward currents, and then the voltage step terminated by stepping to various membrane potentials (Fig. 4*A*). The amplitude of each resultant membrane current relative to zero current level was determined 12 msec after stepping to the new holding level, so that this amplitude largely reflected the slow component of the tail current. These amplitudes are plotted against membrane potential in Fig. 4*B*. The second line of shallower slope in Fig. 4*B* was generated by measuring membrane current amplitudes 12 msec after terminating a 750-msec voltage step to −100 mV. The two lines intersect at the tail current's reversal potential (E_r). The reversal potential varies by ca. 57 mV/decade change in external K^+ concentration (Fig. 4*C*), which is close to the theoretical 58 mV/decade change expected of a pure K^+ current.

Similar measurements of tail-current amplitudes 2 msec after a 75-msec voltage step yielded reversal potentials that varied by 55 mV/decade change in $[K^+]_o$, suggesting that the peak inward current in K^+-solution and its associated fast tail are also carried by K^+ (Fig. 4*C*).

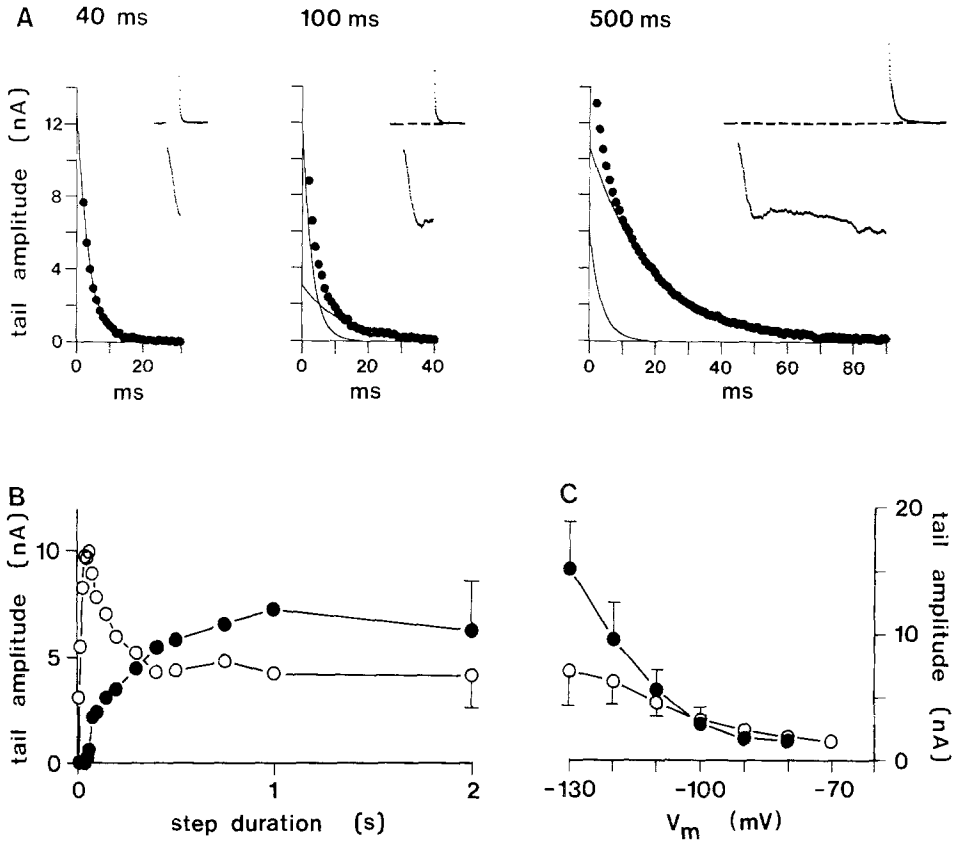

Fig. 3. Tail currents in K^+-solution comprise fast-decaying and slow-decaying exponential components. (*A*) Tail currents elicited by 40-, 100- and 500-msec steps to −110 mV in K^+-solution are displayed graphically. The original current traces are shown in the three corresponding insets. The tail current elicited by a 40-msec hyperpolarization (left) can be fitted with a single exponent (solid line), $\tau \simeq$ 3.6 msec. Hyperpolarizations of increasing duration elicit tail currents that contain a second exponentially decaying component, $\tau \approx 20$ msec (middle and right). (*B*) Specimens were stepped to −110 mV for periods of 10 msec to 2 sec and resultant tail currents fitted to single or double exponents. The open symbols show that the fast-decaying component of the tail current activates rapidly upon hyperpolarization and inactivates thereafter. The slow-decaying component (filled symbols) activates slowly during the voltage step. Points are means from 11–22 specimens. For clarity, error bars are provided for one time point only; other values were similarly variable. (*C*) The amplitudes of the fast (open symbols) and slow (filled symbols) tail-current components are plotted as a function of membrane potential (V_m). Data points are means ± SD of 13 specimens

Ca^{2+} Dependence of the Slow-Activating K^+ Current

Richard et al. (1986) suggested that there may be Ca^{2+} dependence to the inward current elicited by hyperpolarization of *P. tetraurelia*. To determine the relative Ca^{2+} sensitivities of the two currents described here, specimens in K^+-solution were injected iontophoretically with the Ca^{2+} chelator, EGTA. Cells were first hyperpolarized for 500 msec to elicit control responses and then injected with EGTA for 20 sec at −7.5 nA. This injection reduces the amplitude of both the peak inward current and the current at 500 msec compared with control responses (Fig. 5*A* and *B*). EGTA injection also reduces the amplitude of the slow tail current by 60–65% (Fig. 5*C*, lower). Similar EGTA injections had no effect on Ca^{2+}-current amplitude, or on other currents activated upon hyperpolarization in TEA^+-solution (Table 1,*E*), suggesting that the effects on K^+-current amplitude do not reflect a decrease in the amount of Ca^{2+} entering the cell during hyperpolarization.

A 20 sec, −7.5 nA EGTA injection had no effect on the amplitude of the fast tail current (Fig. 5*C*, upper), or on the time constant of either component of the K^+ tail current (*not shown*).

Inhibition by TEA^+ and Quinidine

The relative sensitivities of the two K^+ currents to external TEA^+ was investigated, using 75- and 750-

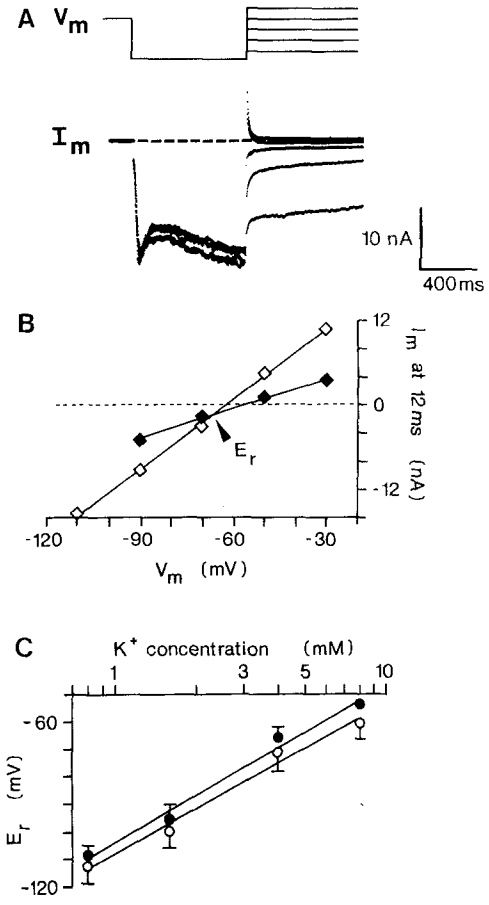

Fig. 4. Reversal potentials of the two K^+ tail currents. (*A*) Cells were hyperpolarized to −120 mV for 750 msec to activate K^+ currents, and then stepped to various potentials to elicit the tail currents shown. (*B*) Tail current amplitudes (I_m) relative to zero current level as a function of membrane potential (V_m). Open symbols indicate currents elicited by a 750-msec step to −120 mV, whereas the filled symbols indicate the amplitudes of tail currents elicited by a 750-msec step to −100 mV. Arrowhead indicates the tail current's reversal potential (E_r). (*C*) K^+ concentration dependence of reversal potentials (E_r). Open symbols show reversal potentials of tail currents elicited by 75-msec hyperpolarizations, filled symbols indicate reversal potentials of tails elicited by 750-msec steps. Data points are means ± SD of 6–9 cells

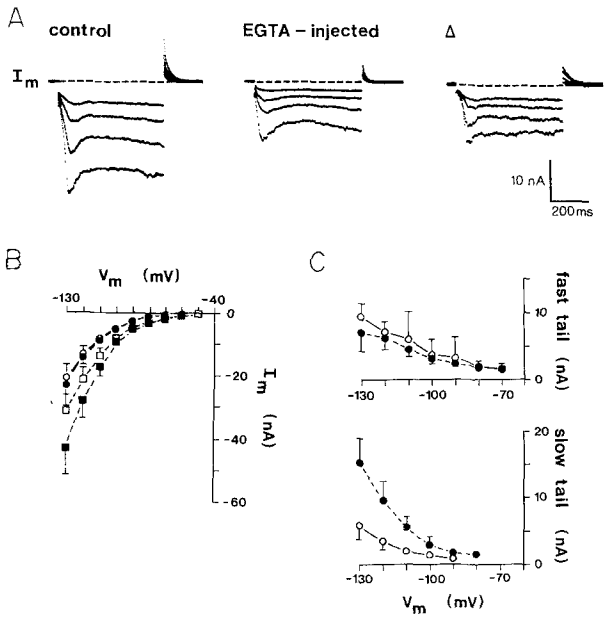

Fig. 5. Effects of EGTA injection. (*A*) Families of inward currents elicited by 500-msec hyperpolarizations in K^+-solution (−90 to −120 mV in 10-mV steps) before (left) and after (middle) a 20-sec, −7.5-nA EGTA injection. Electronic subtraction of these two sets of traces yields the amount of current that is EGTA inhibited (right). (*B*) Effects of EGTA injection on the amplitudes of peak currents (filled symbols) and currents at 500 msec (open symbols) elicited by hyperpolarization in K^+-solution. The squares indicate the amplitudes of these currents (I_m) before EGTA injection as a function of membrane potential (V_m), whereas the circles show the responses of the same cells after a 20-sec, −7.5-nA EGTA injection. (*C*) Effects of EGTA on the amplitudes of the fast (upper) and slow (lower) tail-current components. In both plots, filled circles denote tail-current amplitudes before EGTA injection, the open circles denote amplitudes after injection. Data points are the means ± SD from 13 specimens

msec hyperpolarizations to −110 mV to elicit tail currents that comprise largely fast and slow components, respectively. Control responses were elicited by hyperpolarizations in K^+-solution supplemented with 20 mM choline$^+$, and the amplitudes of the two components of the tail currents extracted. 0.02–20 mM TEA^+ was then added to the solution (maintaining the combined TEA^+/choline$^+$ concentration at 20 mM) and the cells again hyperpolarized to elicit tail currents. TEA^+ inhibits both the fast and slow components of the tail current in a concentration-dependent manner (Fig. 2*B*), although the fast component shows slightly enhanced sensitivity to TEA^+ compared with the slow current (K_i = 0.33 and 1.36 mM, respectively). Hill analyses of the inhibition of the two components yielded coefficients of 0.88 and 0.96 for the fast and slow components, respectively, suggesting that TEA^+ interacts with the channels in a noncooperative manner.

The K^+ currents show differential sensitivity to quinidine. Specimens in K^+-solution were hyperpolarized to −110 mV for 70 or 700 msec to elicit control responses, and then exposed to 0.7 mM quinidine for 1 min. The cells were then washed in K^+-solution, and their membrane responses to hyperpolarization re-examined. Exposure to quinidine causes a reversible loss of the inward peak, and a complete but reversible inhibition of the associated fast tail current (Fig. 6*A*).

The slow-activating K^+ current is comparatively insensitive to quinidine (Fig. 6*B*). The ampli-

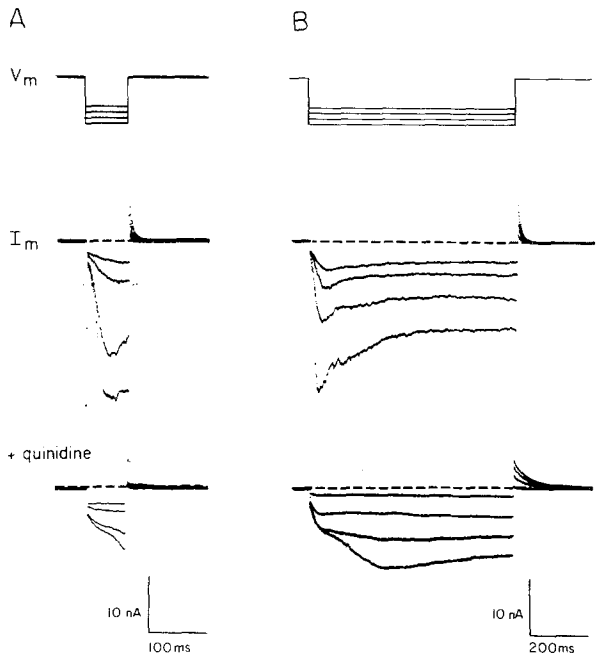

Fig. 6. Inhibition of the voltage-dependent K^+ current by quinidine. (*A*) Families of currents elicited by 70-msec or (*B*) 700-msec hyperpolarizations (−90 to −120 mV in 10-mV steps) in K^+-solution. Lower traces show responses of the same cells to identical voltage steps following exposure to 0.7 mM quinidine. Quinidine completely inhibits the fast-decaying, but not the slow-decaying component of the K^+ tail current, although the time constant of the latter component is increased by quinidine treatment

tude of the inward current elicited at 700 msec by a step to −110 mV decreased from −11.3 ± 2.2 nA to −8.4 ± 1.5 nA (n = 8) after exposure to 0.7 mM quinidine, whereas the slow tail amplitude decreased from 4.5 ± 1.6 nA to 3.1 ± 1.2 nA (n = 8). The decrease in tail-current amplitude is not statistically significant. Quinidine (0.7 mM) also caused a reversible increase in the time constant of the slow tail current (Fig. 6*B*), from 17.2 to 60.0 msec (± 5.8 and 15 msec, respectively, n = 8). EGTA injection reduces the amplitude of both the inward current and the very slow tail current observed following quinidine treatment (Table 1,*C*). Quinidine had no effect on the hyperpolarization-activated Ca^{2+} current (Table 1,*F*).

Discussion

Hyperpolarization of voltage-clamped *Paramecium tetraurelia* in K^+-solution elicits an inward current complex. This complex comprises 'leak' current, a Ca^{2+} current, and two K^+ currents. These K^+ currents are distinct in their kinetics of activation, deactivation, and inactivation, in their Ca^{2+} sensitivities, and in their sensitivities to K^+-current inhibitors.

After the K^+ currents are suppressed with internal Cs^+ and extracellular TEA^+, a step hyperpolarization activates a current that comprises peak and sustained components. The small inward tail observed upon returning to holding potential activates and subsequently inactivates with a time course that mirrors the peak of the inward current. These currents represent a novel, hyperpolarization-activated Ca^{2+} current and its associated tail (Saimi, 1986; Hennessey, 1987; R.R. Preston & Y. Saimi, *in preparation*). The sustained current in TEA^+-solution presumably represents 'leak' current. The 'leak' current may contain a small active element: this possibility is being investigated further (*see also* Machemer, 1988*a*).

The majority of the inward current elicited by hyperpolarization of *Paramecium* in K^+-solution is carried by K^+ (ca. 80% at 500 msec; Table 1). The hyperpolarization-activated tail in K^+-solutions is almost pure K^+ current, although we recognize that hyperpolarizations of ca. 40 msec elicit an outward K^+ tail that contains a small inward Ca^{2+} component. Thus, Fig. 3*B* underestimates the rate at which the fast tail amplitude rises. The Ca^{2+} current contributes less than 3% to the amplitude of tail currents elicited by hyperpolarizations of >70–100 msec in K^+-solution, so we feel justified in using these tails to characterize the associated inward K^+ currents.

Using E_K values calculated from the reversal potentials of the tail currents, the Nernst equation yields internal K^+ concentrations of 61–82 mM. The values are high compared with the 17–18 mM estimated by Oertel et al. (1978) for *P. tetraurelia*, and the 34 mM calculated by Ogura and Machemer (1980) from reversal potentials of K^+ responses in *P. caudatum*. Similar values for $[K^+]_i$ (ca. 18 mM) were provided by Hansma (1974) for *P. tetraurelia* using flame photometry. Since low resistance, high molarity K^+ electrodes were used in the present study, it is possible that diffusion and iontophoresis of the electrode contents during the voltage clamp raised $[K^+]_i$ to artificially high levels.

Evidence for Two K^+ Currents Activated Upon Hyperpolarization

The Two K^+ Currents Are Kinetically Distinct

Tail-current analyses suggest that hyperpolarization of *Paramecium* activates two K^+ currents that may be readily distinguished on the basis of their kinetic properties. The first K^+ current activates rapidly

upon hyperpolarization, peaks, and subsequently inactivates (Fig. 3*B*). The time course of inactivation can be described by the sum of two exponents with time constants of ca. 62 msec and 2.0 sec. These values remain approximations, however. Long hyperpolarizations elicit tail currents that contain both fast and slow components; since the relative contributions of the fast component is small after long voltage steps, it is difficult to determine its amplitude, and hence the time course of reduction in amplitude, with accuracy. The second K^+ current activates gradually during hyperpolarizations of $\geq$50 msec and is sustained (for at least 2 sec, Fig. 3*B*). The difference in deactivation kinetics of the two K^+ currents is readily apparent in the time constants of the two K^+ tails ($\tau \approx 3.5$ and 20 msec).

The Two K^+ Currents Can Be Distinguished by Their Ca^{2+} Sensitivity

EGTA injection reduces the amplitude of the slow K^+ tail current without affecting the fast K^+ tail (Fig. 5*C*). This suggests that the slow tail is Ca^{2+}-dependent, whereas the fast tail is not. EGTA suppression of the slow tail is incomplete, however (Fig. 5*C*, lower). A possible explanation is that the slow current can be activated by Ca^{2+} or voltage, so that a voltage stimulus accounts for activation of the current after EGTA injection. An alternative explanation is that EGTA incompletely suppresses a hyperpolarization-induced rise in $[Ca^{2+}]_i$. However, prolonged injections of EGTA (60 sec) or of BAPTA, a more efficient Ca^{2+} chelator, also failed to inhibit the slow K^+ current by >80% (R.R. Preston, Y. Saimi & C. Kung, *unpublished*). Determining the exact extent of Ca^{2+} dependence of the slow K^+ current would necessitate removing all external Ca^{2+}. However, the membrane of *Paramecium*, like that of many systems, is unstable in the absence of external divalent cations. Exchanging $[Ca^{2+}]_o$ for other divalent cations is inappropriate, since Mg^{2+} and Ba^{2+} commonly inhibit K^+ currents, including those of *Paramecium*.

EGTA significantly reduces the amplitude of the peak inward current (Fig. 5*B*). This reduction is not due to EGTA effects on Ca^{2+} or 'leak' currents (Table 1), and tail-current analyses suggest that the peak occurs before there is a significant activation of the slow K^+ current. We might thus conclude that the reduced peak reflects inhibition of the fast K^+ current by EGTA. EGTA has no effect on the amplitude of the fast K^+ tail current, however (Fig. 5*C*). Possible explanations for this apparent discrepancy include an EGTA-induced shift in the voltage dependence of the peak. Alternatively, the tail currents may not accurately reflect the behavior of the associated inward currents. Since EGTA injection has no effect on the responses to hyperpolarization of pantophobiac mutants that lack the slow K^+ current (Preston et al., 1990*b*), we believe the fast K^+ current to be genuinely Ca^{2+} insensitive. Thus, in the wild type, the peak inward current must contain a Ca^{2+}-sensitive element whose deactivation is not detected in tail analyses. Whether this element represents the slow-activating current is uncertain; we are continuing to investigate this possibility.

Since EGTA reduces the amplitude of both the slow inward current and its associated slow K^+ tail current, we consider this current to be primarily Ca^{2+} dependent. A >70% reduction in the amplitude of the slow tail following inhibition of the hyperpolarization-activated Ca^{2+} current (using 1 mM amiloride; R.R. Preston & Y. Saimi, *in preparation*) is consistent with this hypothesis. The fast K^+ current is presently considered to be voltage-dependent.

The Two K^+ Currents Are Pharmacologically Distinct

The two K^+ currents are distinct in their relative sensitivities to TEA^+ and quinidine. The fast K^+ current is approximately four times more sensitive to TEA^+ than the slow current (Fig. 2*B*). The difference in the sensitivities of the two K^+ currents to quinidine is more significant (Fig. 6). Whereas 0.7 mM quinidine inhibits the fast K^+ current completely, it has little effect on the amplitude of the slow K^+ current (Table 1,*C*). The increase in the time constant of the slow K^+ tail current in the presence of quinidine may simply reflect a nonspecific perturbation of membrane function by this membrane-permeant drug (Yeh & Narahashi, 1976). The very slow current is EGTA sensitive (Table 1,*C*), suggesting that it indeed represents the Ca^{2+}-dependent K^+ current rather than a new, quinidine-induced current. This notion is supported by the inability of quinidine to induce a slow tail current in pantophobiac, a mutant that lacks a Ca^{2+}-dependent K^+ current upon hyperpolarization (R.R. Preston & Y. Saimi, *unpublished*).

Both K^+ currents are inhibited by Ba^{2+} (10 mM), and quinine (1 mM), and both are insensitive to gallamine (10 mM), 4-aminopyridine (10 mM), tolbutamide (1 mM), and the bee venom apamin (10 μM; R.R. Preston, *unpublished*).

Separation of The Two Currents by Mutation

In separate studies (Preston et al., 1990*a*,*b*), the ion currents of two pantophobiac mutants of *P. tetraurelia* are described. These mutants lack the slow K^+ current activated upon hyperpolarization of the wild type, but the fast K^+ current is to all intents normal. Thus, the slow current can be suppressed by mutation independently of the fast current, providing additional evidence for two distinct hyperpolarization-activated K^+ currents in *Paramecium*.

Possible Roles of Inward Rectification in *Paramecium*

Activation of K^+ currents drives the membrane potential of a cell toward E_K, thereby reducing the probability of regenerative depolarization. Thus, inward K^+ rectifiers often function to stabilize the membrane potentials of excitable cells, particularly of cells with endogenous spiking activity, such as pacemakers. Although there are many examples of voltage-dependent inward rectifiers, and even of an inward rectifier that is activated by Ca^{2+} release from intracellular stores (Sauvé et al., 1986, 1987), *Paramecium* is unique in its possession of two K^+ currents that are activated upon hyperpolarization. Saimi (1986) additionally showed a novel Ca^{2+} current and Ca^{2+}-dependent Na^+ current to be activated by negative voltage steps from rest. The complexity of *Paramecium's* responses to hyperpolarization make it less easy to assign specific roles to the individual currents, but it is possible that they also serve to stabilize membrane potential. When the slow K^+ current is enhanced by mutation (restless), the cell's membrane potential behaves like a pure K^+ electrode in response to changes in $[K^+]_o$ (Richard, Hinrichsen & Kung, 1985). Since this current has no obvious role in excitation, and since its activity must be controlled in the wild type (the wild-type membrane does not behave like a K^+ electrode), Richard et al. (1986) suggest that its main function may be in membrane potential regulation. Pantophobiac shows little difference in 'resting' potential compared with the wild type (Saimi et al., 1983), despite its lack of the slow K^+ current, suggesting that the fast K^+ current may also be a determinant of membrane potential. This current may also be involved in the escape response of *Paramecium*. The escape response comprises a sudden increase in forward swimming speed in response to posterior mechano-stimulation, resulting from a K^+-mediated, hyperpolarizing transient. If $[K^+]_o$ is lowered sufficiently, the hyperpolarization becomes regenerative (Satow & Kung, 1977), perhaps reflecting fast K^+-current activation.

Although their functions are not understood fully, the existence of at least four currents activated by hyperpolarization of *Paramecium* clearly indicates that they are important for the day-to-day survival of the unicell. Further analysis of pantophobiacs and other mutants defective in their hyperpolarization-activated currents should provide insight into the functions and possible regulatory pathways of the inward rectifiers in *Paramecium*.

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