Calcium Current Activated upon Hyperpolarization of Paramecium tetraurelia

ROBIN R. PRESTON, YOSHIRO SAIMI, and CHING KUNG

From the Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin-Madison, Madison, Wisconsin 53706

ABSTRACT Hyperpolarization of Paramecium tetraurelia under conditions where K+ currents are suppressed elicits an inward current that activates rapidly toward a peak at 25–80 ms and decays thereafter. This peak current (Ihyp) is not affected by removing Cl ions from the microelectrodes used to clamp membrane potential, or by changing extracellular Cl− concentration, but is lost upon removing extracellular Ca2+. Ihyp is also lost upon replacing extracellular Ca2+ with equimolar concentrations of Ba2+, Co2+, Mg2+, Mn2+, or Sr2+, suggesting that the permeability mechanism that mediates Ihyp is highly selective for Ca2+. Divalent cations also inhibit Ihyp when introduced extracellularly, in a concentration- and voltage-dependent manner. Ba2+ inhibits Ihyp with an apparent dissociation constant of 81 μM at −110 mV, and with an effective valence of 0.42. Ihyp is also inhibited reversibly by amiloride, with a dissociation constant of 0.4 mM. Ihyp is not affected significantly by changes in extracellular Na+, K+, or H+ concentration, or by EGTA injection. Also, it is unaffected by manipulations or mutations that suppress the depolarization-activated Ca2+ current or the various Ca2+-dependent currents of Paramecium. We suggest that Ihyp is mediated by a novel, hyperpolarization-activated calcium conductance that is distinct from the one activated by depolarization.

INTRODUCTION

In most excitable cells, membrane hyperpolarization apparently serves simply to suppress spiking, a task that is readily accomplished with a single K+ or Cl− current. In Paramecium, however, membrane hyperpolarization is actively involved in locomotory control, made possible by a coupling of membrane potential change to ciliary beat frequency and direction (Machemer, 1974). Thus, it is perhaps not surprising to discover that several ion currents are elicited during hyperpolarization of Paramecium tetraurelia, including a voltage-dependent K+ current (Oertel, Schein, and Kung, 1978; Preston, Saimi, and Kung, 1990a), a Ca2+-dependent K+ current (Richard, Saimi, and Kung, 1986; Preston et al., 1990a), a Ca2+-dependent Na+ current (Saimi, 1986), and a Ca2+-dependent Mg2+ current (Preston, 1990). The source of Ca2+ for...
activating the three Ca\(^{2+}\)-dependent currents is suggested to be a novel, hyperpolarization-activated Ca\(^{2+}\) current (Saimi, 1986; Hennessey, 1987; Preston et al., 1990a; Preston and Saimi, 1990; Preston, Wallen-Friedman, Saimi, and Kung, 1990b). This current has not been studied in detail previously. Since it clearly has a central role in controlling excitability and behavior in *Paramecium*, and since a Ca\(^{2+}\) conductance that is activated by membrane hyperpolarization is highly unusual, it seemed appropriate to investigate this current in some depth. As reported here, the putative Ca\(^{2+}\) current exhibits some unique properties, including its complete inhibition by Ba\(^{2+}\) and other divalent cations.

**Materials and Methods**

**Cell Stocks and Culture Conditions**

The following strains of *Paramecium tetraurelia*, derived from stock 51s, were used in the present studies: d4-91, fast-2 (cam\(^{11}\)/cam\(^{11}\)) (Kink, Maley, Preston, Ling, Wallen-Friedman, Saimi, and Kung, 1990); d4-95 pawn B (pseB/pseB) (Kung, 1971); d4-623, dancer (Dn/Dn) (Hinrichsen, Saimi, and Kung, 1984); d4-650, pantophobiac (cam\(^2\)/cam\(^2\)) (Kink et al., 1990), and d4-700, eccentric (Preston, R.R., and C. Kung, unpublished observations). All stocks also contained the trichocyst nondischarge mutation nd6 (Lefort-Tran, Aufderheide, Pouphile, Rossignol, and Beisson, 1981) to aid microelectrode insertion. Cells were maintained at room temperature (22–24°C) on a chemically defined, monoxenic growth medium, similar to that described (Preston et al., 1990a). Experimental cells were starved for 2 d and then maintained in logarithmic growth phase for 2 d. This feeding schedule ensured cells of consistent size and quality, and minimized variations in membrane current size within and between daily cell cultures.

**Electrophysiological Recording Techniques**

10 min before experimentation, cells were transferred individually to an adaptation solution containing 4 mM KCl, 1 mM CaCl\(_2\), 0.01 mM EDTA, and 1 mM HEPES, pH 7.2. When necessary, cells were deciliated by 2 min agitation in 5% ethanol (vol/vol) in adaptation solution as described (Ogura, 1981; Preston and Usherwood, 1988). The standard solution used to bathe the cells during electrical recording ("Ca\(^{2+}\)/TEA\(^{+}\) solution") comprised 10 mM tetraethylammonium (TEA) chloride, 0.25 mM Ca(OH)\(_2\), 0.75 mM CaCl\(_2\) (total free Ca\(^{2+}\) = 1 mM), 0.01 mM EDTA, and 1 mM HEPES, pH 7.2. Membrane currents were recorded under voltage clamp using intracellular glass capillary microelectrodes of 12–30 MΩ tip resistance filled with 4 M CsCl (99.9995%; Aldrich Chemical Co., Milwaukee, WI). When recording under Cl\(^{-}\)-free conditions, the electrodes were filled with 3 M Cs-glutamate, Cs-sulphate, or Cs-citrate (99.9% CsOH [Aldrich Chemical Co.] neutralized with the respective acids). The purity of the electrode contents is critical for studies of the putative Ca\(^{2+}\) current: Cs salts of lower grade apparently contain contaminants that suppress this current completely. When necessary, the contents of the experimental chamber (capacity ~1 ml) were changed using a gravity-fed perfusion system with a flow rate of 12.5 ml/min. Generally, data were not collected in new solutions until 8–10 ml had flowed through the chamber. Membrane potential was usually clamped at ~40 mV using hardware and PC-based software from Axon Instruments, Inc., Foster City, CA (Axoclamp 2A, TL-1 interface, and pCLAMP). Command pulses were generally applied at 7-s intervals and resultant currents filtered at 1–2 kHz.

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

Data were leak-corrected and analyzed using pCLAMP. Leak-current amplitudes were estimated from averaged responses to repeated 20-ms steps to between -43 and -52 mV. Tail current amplitudes and time courses were derived from fits performed on currents recorded later than 4.5 ms after returning to holding potential. This excluded contributions from capacitive artefacts, which settle within 0.5 ms of stepping to a new level, and from a small ionic tail component ($\tau = 0.6$ ms). Unless stated otherwise, data are presented as means ± SD, with levels of statistical significance between means determined using a Student's $t$ test. $P$ values of $<0.05$ were considered significant.

FIGURE 1. Isolation of the transient inward current and the time course of its activation and inactivation. (A) Currents elicited from a single specimen by 300-ms steps to -120 mV in Ca$^{2+}$/TEA$^+$ solution before (upper trace) and after (middle trace) inhibiting the peak with 1 mM amiloride. The current that remains after suppressing the peak plateaus at ~40 ms and is sustained thereafter. Subtracting this sustained current from the total membrane response (upper trace) yields the amiloride-sensitive component, $I_{\text{hyp}}$ (lower trace). Broken lines in this and subsequent figures represent holding current level. The traces have been corrected for linear leak current, as described in Materials and Methods. (B) Hyperpolarizing a cell to -120 mV for periods ranging from 10 to 300 ms shows that steps terminating within the peak elicit a prominent inward tail. Note that the envelope of these tails parallels the inward peak current. (C) The protocol in B above was repeated using steps to between -90 and -130 mV. The amplitude of the resultant tail currents at the instant of returning to -40 mV ($I_{\text{tail}}$) were estimated, and have been plotted as a function of step duration. Note that regardless of membrane potential, steps of 300-ms duration fail to elicit an inward tail. Filled circles, tail currents elicited by steps to -90 mV; open circles, steps to -100 mV; filled squares, steps to -110 mV; open squares, steps to -120 mV; triangles, steps to -130 mV. Points are means from five to eight cells.
Isolating the Transient Component of the Current Activated upon Hyperpolarization

As noted in the Introduction, step hyperpolarization of *P. tetraurelia* under voltage clamp elicits two K⁺ currents, a Ca²⁺-dependent Na⁺ current, and a Ca²⁺-dependent Mg²⁺ current. Na⁺ and Mg²⁺ currents are observed only when Na⁺ and Mg²⁺ are present extracellularly (Saimi, 1986; Preston, 1990). The K⁺ currents were suppressed fully by the use of Cs⁺-filled microelectrodes for clamping membrane potential, and by including 10 mM TEA⁺ in the bath solution (Hinrichsen and Saimi, 1984).

After suppressing the K⁺ currents, hyperpolarizing *Paramecium* in the Na⁺-free, Mg²⁺-free Ca²⁺/TEA⁺ solution yields a current that comprises three elements (Fig. 1 A, upper trace). The first, a leak current (approximately −1.1 nA in the example shown), is readily subtracted from the total membrane response using methods described above. The second, a sustained inward current (Fig. 1 A, middle trace), is revealed after inactivating or inhibiting (using amiloride or Ba²⁺; see below) a third, transient component. The sustained current has yet to be characterized, whereas the transient is the focus of the present report. For convenience, we refer to this transient current as "Ihyp" from here on. The sustained component requires ~20 ms longer to stabilize than Ihyp requires to reach a peak, but even so it causes us to underestimate the contribution of Ihyp to Ipeak by only 4% (3.2 ± 1.1%, or 0.11 ± 0.03 nA, n = 5; values were determined for a step to −120 mV, but are representative of error levels at any potential between −70 and −130 mV).

Fig. 1 B confirms that the inward peak is an inactivating current. Here, a cell has been hyperpolarized to −120 mV for periods ranging from 10 to 300 ms. Steps terminating during the inward peak elicit a prominent inward tail current that represents the deactivation of Ihyp. The envelope of tail currents elicited by stepwise increases in stimulus duration parallels the time course of the inward current flowing during the hyperpolarization (Fig. 1 C), suggesting that this current is indeed inactivating. The command protocol was then repeated using steps to −90, −100, −110, or −130 mV; in each case, the resultant tail current envelope mirrored inward peak trajectory. Note that regardless of amplitude, 300-ms steps fail to elicit an inward tail (Fig. 1 C), suggesting that Ihyp inactivates fully by this time.

RESULTS

Hyperpolarization Elicits an Inward Current

Hyperpolarization of *P. tetraurelia* in Ca²⁺/TEA⁺ solution elicits an inward current that activates rapidly toward a peak and then decays to a new level that is sustained at >250–300 ms (Fig. 2 A). The current–voltage relations of the peak current (Ipeak) and the current at 300 ms (I300) are presented in Fig. 2 B. As is evident from Fig. 2 A, the time required for the current to peak depends on membrane potential. Currents evoked by steps to −70 mV require 80−90 ms to peak, whereas currents at −130 mV peak within 25 ms or less.

Voltage steps terminating within the inward peak elicit an inward tail current (Fig. 1 B). This tail contains a fast outward component, the ionic basis for which is unknown, and a slow inward component that decays with a time course that is well described by a single exponential. A 30-ms step to −120 mV, for example, yields a
tail that decays with a time constant ($\tau_{\text{tail}}$) of $12.7 \pm 2.3$ ms, $n = 8$. $\tau_{\text{tail}}$ is not significantly dependent on membrane potential (at $-35$ to $-105$ mV) or on step duration (5–250 ms; not shown), but increases significantly as tail current amplitude decreases (from $11.5 \pm 1.9$ ms for a $-2.1 \pm 0.5$ nA current to $18.7 \pm 2.2$ ms for a $-0.6 \pm 0.1$ nA current, $n = 6$ and 8, respectively, recorded at $-40$ mV). This trend is consistent with the notion that $I_{\text{hyp}}$ inactivates in a $Ca^{2+}$-dependent manner (Standen and Stanfield, 1982), an issue that is addressed elsewhere (Preston, Saimi, and Kung, 1992).

The degree to which $I_{\text{hyp}}$ is inactivated at rest ($-40$ mV) was investigated. Cells were held at membrane potentials ranging from $-10$ to $-120$ mV and then stepped to $-120$ mV for 300 ms to elicit $I_{\text{hyp}}$ (Fig. 3A). At holding potentials positive to $-40$ mV, a variety of depolarization-activated currents are elicited. These currents deactivate slowly ($\tau \approx 60$ ms) upon hyperpolarization, thereby invalidating our usual method of calculating the contribution of $I_{\text{hyp}}$ to the total current. Thus, cells were subjected to the above stimulation protocol both before and during exposure to 1 mM amiloride. Amiloride inhibits $I_{\text{hyp}}$ fully at this concentration (see below) without affecting other currents elicited upon hyperpolarization. Amiloride reduces the depolarization-activated $Ca^{2+}$ transient by $\sim 30\%$ (see below) but has no significant effect on the currents evoked later than 5 ms upon depolarization (not shown). Thus, subtracting currents activated in the presence of 1 mM amiloride from those obtained

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Currents evoked upon hyperpolarization. (A) Hyperpolarization of $P$. tetraurelia in $Ca^{2+}$/TEA$^+$ solution elicits an inward current that activates rapidly toward a peak and inactivates thereafter. $I_{\text{hyp}}$ represents the inactivating portion of these currents. (B) The amplitude of the peak current ($I_{\text{peak}}$) and current at 300 ms ($I_{300}$) are plotted as a function of membrane potential ($V_m$). Data have been leak corrected. Points are means $\pm$ SD from eight cells.}
\end{figure}
in this drug’s absence yields a satisfactory estimate of \( I_{\text{hyp}} \). The resultant steady-state inactivation curve (Fig. 3 B) suggests that \( I_{\text{hyp}} \) is partly inactivated at \(-40\) mV, the holding potential used throughout this report. Holding at more positive levels removes this inactivation, but this maneuver also elicits and then inactivates currents that are normally observed only upon depolarization from \(-40\) mV. The 300-ms step hyperpolarizations used to elicit \( I_{\text{hyp}} \) permit these currents to recover from inactivation, and these interfere severely with our analyses of \( I_{\text{hyp}} \). Thus, we chose to routinely clamp membrane potential at \(-40\) mV and accept that there is a small (\(~8\%\)) consequential decrease in \( I_{\text{hyp}} \) magnitude.

**FIGURE 3.** Steady-state inactivation of \( I_{\text{hyp}} \). (A) Cells were held for 500 ms at membrane potentials ranging from \(-10\) to \(-120\) mV and then stepped for 300 ms to \(-120\) mV to elicit \( I_{\text{hyp}} \). Steps to potentials positive to \(-40\) mV elicit depolarization-activated currents that prevent accurate determinations of \( I_{\text{hyp}} \). Thus, the voltage protocol was repeated in the presence of 1 mM amiloride to elicit all currents other than \( I_{\text{hyp}} \) (see text). These currents were then subtracted from responses in the absence of amiloride to yield \( I_{\text{hyp}} \). Numerals to the left of the current traces indicate holding potentials (in millivolts). (B) The amplitude of \( I_{\text{hyp}} \) shown in A above, relative to its maximum amplitude \((I/I_{\text{max}})\), plotted as a function of holding potential. The curve was fitted using the Boltzmann relation: 

\[
I/I_{\text{max}} = 1/[1 + \exp ((V - V_{1/2})/s)]
\]

where \( V_{1/2} = -75 \) mV and \( s = 0.07 \). This experiment was repeated using five other cells, with similar results. Similar results were obtained after setting holding potentials manually. The broken vertical line indicates \(-40\) mV, the holding potential at which most of the studies described in this report were carried out.

**\( I_{\text{hyp}} \) Is a \( \text{Ca}^{2+} \) Current**

The currents shown in Fig. 2 were elicited in a solution containing 1 mM \( \text{Ca}^{2+} \), 10 mM \( \text{TEA}^+ \), 1 mM HEPES\(^-\), and 12–13 mM \( \text{Cl}^- \). Neither \( I_{\text{peak}} \) nor \( I_{500} \) is affected by removing \( \text{TEA}^+ \) or the HEPES buffer from the bath (not shown). The possibility that \( I_{\text{hyp}} \) may represent \( \text{Cl}^- \) efflux was investigated by replacing the usual CsCl-filled electrodes with electrodes containing 3 M Cs-glutamate. We have assumed, based on
PRESTON ET AL.  Hyperpolarization-activated Ca\(^{2+}\) Current

previous characterizations of Cl\(^{-}\) conductances in other systems and on the selectivity of single channels in *Paramecium* (Martinac, Saimi, Gustin, and Kung, 1988), that glutamate diffusing into the cytoplasm from the microelectrodes (Hinrichsen and Saimi, 1984) would not permeate such a conductance. The bath was filled initially with a Cl\(^{-}\)-free solution containing 30 mM TEA-glutamate in place of TEA-Cl and Ca(OH)\(_{2}\) in place of CaCl\(_{2}\). Hyperpolarizing *Paramecium* under these conditions again yields an inward transient (Fig. 4A, upper), the magnitude and kinetics of

![Figure 4](image)

**FIGURE 4.** Effects of removing individual components of the bath solution on the inward current. (A) Family of currents elicited by 300-ms steps to -107, -115, -122, and -130 mV under Cl\(^{-}\)-free conditions. Upper traces show currents elicited in a solution containing 30 mM TEA-glutamate; lower traces show effects of replacing TEA-glutamate with 30 mM TEA-Cl. (B) Inward transients \(I_{\text{hyp}}\) recorded in the absence (filled circles) or presence of 30 mM [Cl\(^{-}\)]\(_{o}\) (open circles) plotted as a function of membrane potential \(V_{\text{m}}\). Data are means ± SD from nine cells.

(C) Currents elicited using 300-ms steps to -100, -107, or -115 mV in control solution (1 mM Ca\(^{2+}\), 10 mM TEA\(^{+}\), and 1 mM HEPES buffer; upper trace), after removing Ca\(^{2+}\) (replaced with 1 mM Mg\(^{2+}\); middle trace), and upon replacing Ca\(^{2+}\) (lower trace). (D) The amplitude of \(I_{\text{hyp}}\) before (filled circles) and after (open circles) removing [Ca\(^{2+}\)]\(_{o}\) is plotted against membrane potential \(V_{\text{m}}\). Data points are means ± SD from three cells.

which are indistinguishable from those elicited under normal recording conditions (Fig. 2). Similar currents have been observed using microelectrodes filled with Cs-citrate or Cs-sulphate (not shown). Replacing TEA-glutamate in the bath solution with an equivalent concentration of TEA-Cl had no effect on \(I_{\text{hyp}}\) magnitude (Fig. 4A, lower, 4 B), leading us to conclude that this current is not carried by Cl\(^{-}\) (or
glutamate). $I_{\text{hyp}}$ was lost upon removing extracellular Ca$^{2+}$, however (Fig. 4 C, middle, 4 D), suggesting that it represents a Ca$^{2+}$ flux. Membrane potential steps to more than $+40$ mV elicited uncontrolled, depolarization-activated current that prevented us from determining how $I_{\text{hyp}}$ behaves at the predicted equilibrium potential for Ca$^{2+}$ (approximately $+120$ mV, assuming that $[\text{Ca}^{2+}]_i = 0.1 \mu\text{M}$). $I_{\text{hyp}}$ showed no signs of reversal at potentials below $+40$ mV (not shown), however, lending support to our hypothesis that this current is carried by Ca$^{2+}$.

$I_{\text{hyp}}$ Is Distinct from the Ca$^{2+}$ Current Activated upon Depolarization

Depolarization of Paramecium also elicits a Ca$^{2+}$ transient, $I_{\text{Ca(d)}}$. The possibility that $I_{\text{hyp}}$ may be mediated by the same conductance pathway as $I_{\text{Ca(d)}}$ was investigated in

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Effects of pH and Mutation on $I_{\text{hyp}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{\text{hyp}}$ (nA)</td>
</tr>
<tr>
<td>(A)</td>
<td>Control $-1.61 \pm 0.29$ (4)</td>
</tr>
<tr>
<td></td>
<td>Deciliated $-1.68 \pm 0.76$ (5)</td>
</tr>
<tr>
<td>(B)</td>
<td>Pawn B $-1.46 \pm 0.75$ (6)</td>
</tr>
<tr>
<td></td>
<td>Dancer $-1.84 \pm 0.79$ (3)</td>
</tr>
<tr>
<td>(C)</td>
<td>Pantophobiac $-1.22 \pm 0.42$ (9)</td>
</tr>
<tr>
<td></td>
<td>Eccentric $-1.62 \pm 0.27$ (3)</td>
</tr>
<tr>
<td></td>
<td>Fast-2 $-3.10 \pm 0.36$ (5)</td>
</tr>
<tr>
<td>(D)</td>
<td>pH 6.0 $-2.00 \pm 0.62$ (8)</td>
</tr>
<tr>
<td></td>
<td>pH 7.0 $-1.99 \pm 0.28$ (8)</td>
</tr>
<tr>
<td></td>
<td>pH 8.0 $-1.89 \pm 0.29$ (8)</td>
</tr>
</tbody>
</table>

$I_{\text{hyp}}$ was elicited using 300-ms steps to $-115$ mV; data are given as means $\pm$ SD from $n$ cells. (A) Amplitude of $I_{\text{hyp}}$ in control and deciliated cells. The loss of $I_{\text{Ca(d)}}$ in deciliates was confirmed upon depolarization. (B) Amplitudes of $I_{\text{hyp}}$ evoked from pawn B and dancer, mutants with defective depolarization-activated Ca$^{2+}$ currents. (C) Amplitudes of $I_{\text{hyp}}$ in "pantophobiac," a mutant that lacks Ca$^{2+}$-dependent K$^+$ currents, in "eccentric," a mutant that lacks Ca$^{2+}$-dependent Mg$^{2+}$ currents, and in "fast-2," a mutant lacking Ca$^{2+}$-dependent Na$^+$ currents. (D) Effects of pH on $I_{\text{hyp}}$. $I_{\text{hyp}}$ was elicited in TEA$^+$/Ca$^{2+}$ solution at pH 6.0, 7.0, or 8.0.

Several ways. $I_{\text{Ca(d)}}$ is confined to the ciliary membrane of Paramecium, so it is lost completely upon deciliation (Dunlap, 1977). Deciliation had no effect on $I_{\text{hyp}}$, however (Table I A). Many mutations affect $I_{\text{Ca(d)}}$, including pawn B which eliminates this current (Oertel, Schein, and Kung, 1977), and Dancer, which enhances $I_{\text{Ca(d)}}$ by interfering with its inactivation (Hinrichsen and Saimi, 1984). Neither mutation affects $I_{\text{hyp}}$ significantly (Table I B). Finally, the effects of amiloride on $I_{\text{Ca(d)}}$ were investigated. Whereas 1 mM amiloride suppresses $I_{\text{hyp}}$ fully (see below), it inhibits $I_{\text{Ca(d)}}$ by only 27% ($\pm 7\%$, $n = 3$).
Ihog Is Unaffected by Mutations That Inhibit Ca²⁺-dependent Currents, or by Extracellular Na⁺, K⁺, or H⁺ Concentration

The possibility that Ihog represents a Ca²⁺-dependent conductance was examined by injecting specimens with EGTA, a procedure that has been used previously to demonstrate Ca²⁺ dependence to K⁺, Na⁺, and Mg²⁺ currents in Paramecium (Saimi, 1986; Preston, 1990; Preston et al., 1990a). The results of these studies are detailed elsewhere (Fig. 6 of Preston et al., 1992); in short, EGTA slightly increases Ihog amplitude. We also measured Ihog in several mutants that specifically lack the various Ca²⁺-dependent currents of Paramecium (Table I C). Currents exhibiting properties similar to the wild-type Ihog were recorded from mutants lacking both depolarization- and hyperpolarization-evoked, Ca²⁺-dependent K⁺ currents (Preston et al., 1990b), and from “eccentric,” a mutant that lacks Ca²⁺-dependent Mg²⁺ currents (Preston, R.R., and C. Kung, unpublished data) (Table I C). Hyperpolarization of “fast-2,” a mutant that lacks Ca²⁺-dependent Na⁺ currents (Saimi, 1986) elicits an inward transient that, although significantly larger than the wild-type current at comparable membrane potentials (Table I C), appears qualitatively similar. The fast-2 current is suppressed by amiloride and by removing extracellular Ca²⁺, suggesting that it indeed represents the mutant equivalent of Ihog. These data suggest that Ihog is not mediated by any of the previously described Ca²⁺-dependent conductances, nor by a novel [Ca²⁺]i-dependent conductance pathway in Paramecium.

The effects of monovalent cations on Ihog were investigated. As noted above, hyperpolarizing wild-type paramecia in the presence of extracellular Na⁺ elicits a Ca²⁺-dependent Na⁺ current (INa(Ca)) that masks Ihog (Saimi, 1986). Thus, we took advantage of the fact that fast-2 lacks INa(Ca) to examine the effects of [Na⁺]o on Ihog. 10 mM [Na⁺]o had no effect on the size of Ihog in fast-2 (Fig. 5, A and B), suggesting that Na⁺ does not normally permeate the conductance pathway responsible for this current. In a similar vein, the lack of Ca²⁺-dependent K⁺ currents (IK(Ca)) in “pantophobiac” mutants was exploited to test the effects of K⁺ on Ihog. Hyperpolarization of wild-type cells in the presence of extracellular K⁺ evokes a significant inward current via IK(Ca), despite the inclusion of 10 mM TEA⁺ in the bath solution (Preston et al., 1990a). After inhibiting this current by mutation, it is clear that K⁺ has no effect on Ihog (Fig. 5, C and D). Ihog was also unaffected by changing extracellular H⁺ concentration (Table I D).

Effects of Divalent Cations on Ihog

The ability of other divalent cations to substitute for Ca²⁺ in their ability to carry inward current was tested. Cells were hyperpolarized first in the presence of 1 mM Ca²⁺ extracellularly and then Ca²⁺ was replaced with equivalent concentrations of either Ba²⁺, Co²⁺, Mg²⁺, Mn²⁺, or Sr²⁺. In each case, these substitutions caused the loss of Ihog (Fig. 6), as did substituting Cd²⁺, Cu²⁺, Ni²⁺, and Zn²⁺ for Ca²⁺. However, whereas the effects of the first group of cations were fully reversible, those of the latter were not. This reflects the acute sensitivity of Paramecium to heavy metal poisoning (death follows within 20–30 s of exposure to these divalents). Cells in Ba²⁺ have been hyperpolarized to −120 mV for up to 5 s to examine the possibility that such substitutions cause gross shifts in the kinetics of Ihog activation, but such steps...
fail to elicit further inward current (not shown). The slowly developing current observed upon replacing Ca\(^{2+}\) with Sr\(^{2+}\) (Fig. 6, lower traces) reached a plateau at 500–750 ms and was sustained for at least 3 s. Although the conductance responsible for this current has not been identified, it is not inhibited by amiloride or Ba\(^{2+}\), suggesting that it is not a manifestation of \(I_{\text{hyp}}\). A similarly amiloride-insensitive current is observed after replacing Ca\(^{2+}\) with Mg\(^{2+}\), but is too small to be resolved in

**Figure 5.** Effects of monovalent cations on \(I_{\text{hyp}}\) in mutant cells. (A) Currents elicited from fast-2 mutant cam\(^{11}\) by 300-ms steps to -85, -100, -115, and -130 mV. Upper traces were evoked in Ca\(^{2+}\)/TEA\(^{+}\) solution supplemented with 10 mM choline\(^{+}\); lower traces were elicited after replacing choline\(^{+}\) with Na\(^{+}\). (B) Amplitude of \(I_{\text{hyp}}\) in cam\(^{11}\) plotted as a function of membrane potential in Ca\(^{2+}\)/TEA\(^{+}\) solution supplemented with 10 mM choline\(^{+}\) (filled circles) or 10 mM Na\(^{+}\) (open circles). Data are means ± SD from nine cells. (C) Currents elicited from pantophobiac mutant cam\(^{2}\) by 300-ms steps to -100, -107, -115, and -122 mV. Upper traces were evoked in Ca\(^{2+}\)/TEA\(^{+}\) solution supplemented with choline\(^{+}\), whereas lower traces were elicited in Ca\(^{2+}\)/TEA\(^{+}\) solution containing 10 mM K\(^{+}\). (D) Amplitude of \(I_{\text{hyp}}\) in pantophobiac mutant cam\(^{2}\) as a function of membrane potential in Ca\(^{2+}\)/TEA\(^{+}\) solution supplemented with 10 mM choline\(^{+}\) (filled circles) or 10 mM K\(^{+}\) (open circles). Data are means ± SD from 12 cells.

Fig. 6. These observations suggest that the conductance that mediates \(I_{\text{hyp}}\) has an uncommonly high selectivity for Ca\(^{2+}\).

**Inhibition of \(I_{\text{hyp}}\) by Barium and Other Divalent Cations**

The inability of Ba\(^{2+}\) to permeate a Ca\(^{2+}\) conductance is highly unusual, so we examined its effects on \(I_{\text{hyp}}\) in greater detail. Kostyuk, Doroshenko, and Martynyuk
(1985) witnessed a reversible loss of Ca channel current in snail neurons after substituting Ba\(^{2+}\) for Ca\(^{2+}\), but this effect was slow in onset (half-times of several minutes) and was use dependent. In contrast, barium inhibits \(I_{\text{hyp}}\) immediately upon its addition to the bath solution, and there is no evidence to suggest that its effects are use dependent.

Ba\(^{2+}\) inhibits the Ca\(^{2+}\) current in a concentration-dependent manner (Fig. 7, A and B). We assume that this reflects Ba\(^{2+}\) (M) interacting with divalent cation binding sites (B) in the following manner:

\[
nM + B \rightleftharpoons B \cdot M_n
\]

where \(n\) is the number of barium ions required to inhibit \(I_{\text{hyp}}\), and \(k_1\) and \(k_{-1}\) are the forward and reverse rate constants. This equation can be transformed as follows:

\[
\frac{I}{I_{\text{max}}} = \left[1 + \frac{[M]^n}{K_D}\right]^{-1}
\]

where \(I\) and \(I_{\text{max}}\) are values for \(I_{\text{hyp}}\) in the presence and absence, respectively, of Ba\(^{2+}\).

Fitting the data in Fig. 7 B to Eq. 2 yields a dissociation constant \((K_D)\) of 81 \(\mu\)M at -110 mV. Hill analyses furnish a constant \((n)\) of 1.26, sufficiently close to unity to suggest that Ba\(^{2+}\) interacts with the conductance in a 1:1 manner. The inhibition of \(I_{\text{hyp}}\) by Ba\(^{2+}\) is voltage dependent, quantified by determining \(K_D\) values at each of
several different membrane potentials ($V_m$). Fig. 7C shows that $K_D$ is an exponential function of membrane potential, changing $e$-fold for a 30-mV shift in $V_m$. This voltage dependence provides information about the location of barium’s site of action within the transmembrane electrical field. The concentration of Ba$^{2+}$ at its binding site ([Ba$^{2+}$]$_b$) is related to extracellular concentrations ([Ba$^{2+}$]$_o$) by:

$$[\text{Ba}^{2+}]_b = [\text{Ba}^{2+}]_o \exp \left( -\frac{z\delta VF}{RT} \right)$$  \hspace{1cm} (3)

where $z$ is the valency of Ba$^{2+}$, $\delta$ is the fraction of the potential difference across the membrane experienced by Ba$^{2+}$ at the binding site, and $F$, $R$, and $T$ have their usual meanings. Since it takes a 30-mV change in $V_m$ to produce an $e$-fold change in $K_D$ (Fig. 7C), the Ba$^{2+}$ binding site must be located some distance into the membrane’s electrical field ($\delta = 0.42$).

$I_{hyp}$ is also inhibited by Cd$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ (at $-115$ mV, $K_D = 0.76$, 0.67, and 0.93 mM, respectively) with Hill coefficients that are close to unity. These inhibitions are also weakly voltage dependent, but were not investigated in detail. Mg$^{2+}$ and Sr$^{2+}$ inhibit $I_{hyp}$ by $<50\%$ at the highest concentration tested (2 mM).
Inhibition of $I_{hp}$ by Amiloride

$I_{hp}$ is inhibited reversibly by amiloride. This diuretic's effects are concentration dependent (Fig. 8), with a dissociation constant of 0.39 mM at $-110$ mV. $K_D$ values were also obtained at membrane potentials ranging from $-70$ to $-130$ mV, but we could not detect any voltage dependence to the block (not shown). Hill coefficients increased from 2.9 at $-85$ mV to 5.0 at $-130$ mV, suggesting that the inhibition of $I_{hp}$ by amiloride may involve multiple sites of interaction.

![Figure 8. Inhibition of $I_{hp}$ by amiloride.](image)

Discussion

The present report describes a transient inward current activated upon hyperpolarization of *Paramecium tetraurelia*. We believe this current to represent a novel, hyperpolarization-activated $Ca^{2+}$ conductance, a notion that is examined further below.

Evidence That $I_{hp}$ Represents a $Ca^{2+}$ Conductance

$I_{hp}$ is insensitive to changes in intracellular or extracellular $Cl^-$ concentration (Fig. 4, A and B) or to removal of all extracellular cations other than $Ca^{2+}$. The loss of $I_{hp}$...
upon removing extracellular Ca\(^{2+}\) (Fig. 4, C and D) might suggest that this current is normally activated by increases in [Ca\(^{2+}\)]. This seems unlikely, however, for whereas injecting *Paramecium* with EGTA suppresses all of its known [Ca\(^{2+}\)]-dependent currents (Saimi, 1986; Preston, 1990; Preston et al., 1990a), such injections may actually potentiate \(I_{\text{hyp}}\) (Preston et al., 1992). Thus, despite the lack of precedence for such a conductance, we believe that \(I_{\text{hyp}}\) represents a novel, hyperpolarization-activated, and Ca\(^{2+}\)-specific current.

**What Is the Molecular Nature of the Ca\(^{2+}\) Conductance Pathway?**

If we assume that \(I_{\text{hyp}}\) represents Ca\(^{2+}\) influx, then *a priori* this current might be mediated by a transport protein or by a Ca\(^{2+}\)-selective ion channel.

Na\(^{+}\)/Ca\(^{2+}\) exchangers are plasma membrane components of many cells. Thus, the suppression of \(I_{\text{hyp}}\) by amiloride, a renowned Na\(^{+}\) transport inhibitor (Benos, 1982; Kleyman and Cragoe, 1988), might suggest that this current represents Na\(^{+}\)/Ca\(^{2+}\) exchange. This seems unlikely, however. Known Na\(^{+}\)/Ca\(^{2+}\) antiports exchange three sodium ions for each Ca\(^{2+}\), so that under the Na\(^{-}\)-free conditions used here Na\(^{+}\)/Ca\(^{2+}\) exchange would generate outward current. Further, \(I_{\text{hyp}}\) was unaffected by adding 10 mM Na\(^{+}\) extracellularly (Fig. 5, A and B), a maneuver designed to reverse the driving force for Na\(^{+}\) ([Na\(^{+}\)] = 3–4 mM [Hansma, 1979]). The plasma membrane Ca\(^{2+}\)-ATPase that maintains low intracellular Ca\(^{2+}\) concentrations is ubiquitous among eukaryotes and its properties are well understood (reviewed by Carafoli, 1991). This pump transports Sr\(^{2+}\) as efficiently as Ca\(^{2+}\) (Pfleger and Wolf, 1975), an ion selectivity that is in marked contrast to the Ca\(^{2+}\) exclusivity demonstrated by \(I_{\text{hyp}}\) (Fig. 6). In addition, the plasma membrane Ca\(^{2+}\) pump is suggested to be a Ca\(^{2+}\)/H\(^{+}\) exchanger (discussed by Carafoli, 1991), so the inability of a 100-fold change in [H\(^{+}\)]\(_{o}\) to affect \(I_{\text{hyp}}\) (Table I D) argues against its being a Ca\(^{2+}\)/H\(^{+}\) pump current.

**How Does \(I_{\text{hyp}}\) Compare with Ca Channel Currents Described Previously?**

We are unaware of any other examples of a channel conductance that is both highly Ca\(^{2+}\) selective and hyperpolarization activated. The literature contains descriptions of many Ca\(^{2+}\)-permeable "leak" or "background" conductances (e.g., Coyne, Dagan, and Levitan, 1987; Coulombe, Lefèvre, Baro, and Coraboeuf, 1989; Brezden and Gardner, 1990; Franco and Lansman, 1990) and of several second messenger-gated channels (e.g., Benham and Tsien, 1987; Kuno and Gardner, 1987). Although their weak voltage dependence suggests that these permeabilities might support Ca\(^{2+}\) influx during hyperpolarization, none are hyperpolarization activated as such. Further, most distinguish poorly between Ca\(^{2+}\), Ba\(^{2+}\), or even Mg\(^{2+}\), an ion selectivity that clearly sets them apart from \(I_{\text{hyp}}\).

The only example of a Ca\(^{2+}\) current whose selectivity approaches that of \(I_{\text{hyp}}\) was described recently by Hoth and Penner (1992). These authors showed that rat mast cells respond to depletion of [Ca\(^{2+}\)], with an inward Ca\(^{2+}\) flux that, although voltage independent, was lost when either Ba\(^{2+}\), Sr\(^{2+}\), or Mn\(^{2+}\) was substituted for Ca\(^{2+}\).

**How Might Such a High Selectivity of a Channel for Ca\(^{2+}\) Be Achieved?**

All Ca\(^{2+}\) conductances described to date, whether pump or channel mediated, share the common ability of being able to support Sr\(^{2+}\) and/or Ba\(^{2+}\) currents when these
ions are substituted for Ca$^{2+}$ (Pfleger and Wolf, 1975; Hagiwara and Byerly, 1981; Tsien, Hess, McCleskey, and Rosenberg, 1987; Bean, 1989). The loss of $I_{\text{hyp}}$ in Ba$^{2+}$ or Sr$^{2+}$ (Fig. 6) is thus particularly intriguing. Conceivably, these observations might be explained in terms of (a) a conductance pathway that is permeable to Ca$^{2+}$ alone, or (b) a conductance that is regulated by Ca$^{2+}$ binding to a site that is distinct from the permeability mechanism.

**Ca$^{2+}$-specific Permeation**

Present theories of ion channel permeation are based on studies of neuronal Na$^+$, K$^+$, and L-type Ca$^{2+}$ channels (see Hille, 1984; Begenisich, 1987; Tsien et al., 1987; Yellen, 1987), but it seems likely that other channel classes are designed similarly. Permeant ions are envisioned as having to traverse a series of energy maxima and minima, conferred by Van der Waal's repulsive forces between the ion and its surrounds, and by charged groups lining the channel pore. Although the nature of a permeant ion's interaction with these charges may be complex (reviewed by Eisenman and Alvarez, 1991), the energy wells may be considered as ion-binding sites. If $I_{\text{hyp}}$ is indeed a channel-mediated current, then one may envision that its specificity is engendered by two or more binding sites whose affinity for Ca$^{2+}$ permits passage of this cation alone. If channel pores are considered rigid structures, it might be difficult to explain how divalents with similar ionic radii and hydration energies to Ca$^{2+}$ (such as Ba$^{2+}$, Sr$^{2+}$, and Cd$^{2+}$; Rosseinsky, 1965) could be excluded. Channels are not structurally unyielding, however (Pietrobon, Prod'hom, and Hess, 1988). Eisenman and Alvarez (1991) suggested that in addition to causing gross conformational changes, passage of an ion through a channel induces a series of local molecular rearrangements, the nature and extent of which reflect the species of permeant ion. In light of such emerging “deformable selectivity filter” theories (Eisenman and Alvarez, 1991), the lack of precedence for a highly Ca$^{2+}$-selective Ca channel becomes less of a constraint when considering possible permeability mechanisms.

**Ca$^{2+}$-dependent Ca Channel Activation**

The selectivity of $I_{\text{hyp}}$ is more akin to that of a Ca$^{2+}$-binding protein than to a known Ca channel. For example, Ca$^{2+}$ is the only divalent cation capable of activating protein kinase C by > 5% (Takai, Kishimoto, Iwasa, Kawahara, Mori, and Nishizuka, 1979). Also, whereas Cd$^{2+}$ blocks L-type Ca channels at micromolar concentrations (e.g., Lansman, Hess, and Tsien, 1986), $I_{\text{hyp}}$ is relatively insensitive to Cd$^{2+}$, as are Ca$^{2+}$-binding proteins such as calmodulin (Chao, Suzuki, Zysk, and Cheung, 1984). These observations raise the possibility that Ca$^{2+}$ binding to the channel might be a prerequisite for its activation, and that divalents such as Ba$^{2+}$ inhibit $I_{\text{hyp}}$ by displacing Ca$^{2+}$ from this site. Ba$^{2+}$ inhibition of $I_{\text{hyp}}$ is voltage dependent (Fig. 7C), so the site must be located within the membrane's electric field, perhaps even within the channel pore. The existence of a second Ca$^{2+}$-binding site that imparts selectivity for divalent cations over monovalents once the channel has opened must be inferred from the lack of effect of extracellular Na$^+$ and K$^+$ on $I_{\text{hyp}}$ (Fig. 5), but there are no indications as to its location. This scenario is similar to a model developed by Kostyuk and colleagues (Kostyuk, Mironov, and Doroshenko, 1982; Kostyuk, Mironov, and Shuba, 1983; Kostyuk and Mironov, 1986) to account for the selectivity of L-type Ca.
channels. Although current opinion favors models in which L-type channel selectivity is a function of static pore structure (see Tsien et al., 1987), an allosteric model such as that described above provides a plausible explanation for the observed Ca$^{2+}$ exclusivity of \( I_{\text{hyp}} \).

**Pharmacology of \( I_{\text{hyp}} \) and Its Inhibition by Amiloride**

\( I_{\text{hyp}} \) is unaffected by variety of agents that inhibit Ca$^{2+}$ currents in multicellular organisms (Preston, R. R., Y. Saimi, and C. Kung, unpublished results): the only drug shown to inhibit \( I_{\text{hyp}} \) reversibly is amiloride (Fig. 8). While better known as a Na$^+$ transport inhibitor (Benos, 1982), amiloride also inhibits many channel currents, including the T-type Ca$^{2+}$ current of guinea pig atrial myocytes and chick neurons (Tang, Presser, and Morad, 1988) and the L-type Ca$^{2+}$ current of GH3 cells (Garcia, King, Shevell, Slaughter, Suarez-Kurtz, Winquist, and Kaczorowski, 1990). The lack of voltage dependence of the diuretic's effects on \( I_{\text{hyp}} \) is interesting, suggesting that Ba$^{2+}$ and amiloride may inhibit this current via separate mechanisms. We were also interested to note that the Hill coefficient for amiloride's effects on \( I_{\text{hyp}} \) increases with hyperpolarization, perhaps indicating that additional amiloride binding sites become available as membrane potential decreases.

**Possible Functions of \( I_{\text{hyp}} \)**

The Ca$^{2+}$ exclusivity of \( I_{\text{hyp}} \) is intriguing, since it provides the cell with a highly precise ion filter. Why should *Paramecium* need such a filter? In considering this question, it is useful to review briefly the possible function(s) of \( I_{\text{hyp}} \).

*Paramecium* physically contracts when hyperpolarized, a response that is Ca$^{2+}$ dependent (Nakaoka, Tanaka, and Oosawa, 1984) and presumably is effected by the extensive cytoskeletal networks underlying the plasma membrane of this organism. The functions of contraction are uncertain, but may relate to cell movement through silt and debris. Amiloride inhibits contraction (1 mM; Preston, R. R., unpublished observations), suggesting that \( I_{\text{hyp}} \) provides the stimulus for this phenomenon.

A primary function of \( I_{\text{hyp}} \) is to permit activation of the Ca$^{2+}$-dependent currents upon hyperpolarization. *Paramecium*, unlike the specialized excitable cells of higher organisms, has to contend with an environment whose ion composition changes continually. Since *Paramecium* is dependent upon membrane potential for locomotory control, it is critical that the organism maintain stable excitation thresholds regardless of extracellular ion concentration. How *Paramecium* accomplishes this is uncertain, but there are indications that the hyperpolarization-activated currents may be involved (Richard, Hinrichsen, and Kung, 1985). The resting potential of an unstimulated paramecium fluctuates by several millivolts (Moollenar, de Goede, and Verveen, 1976). We suggest that these fluctuations cause \( I_{\text{hyp}} \) and its dependent currents to activate repeatedly, enabling continual readjustment of membrane excitation parameters. Perhaps the unique selectivity of \( I_{\text{hyp}} \) enables this current to be activated frequently without the danger of permitting toxic cations access to the cell interior. The fact that \( I_{\text{hyp}} \) is partly inactivated at -40 mV (Fig. 3) supports the idea that this current may be functionally significant at potentials close to rest and, indeed, small (~0.2 nA) inward transients are readily discerned at 150–200 ms after step hyperpolarization from -40 mV to only -45 mV.
This work was supported by NIH grants GM-22714 and GM-38646, and by a grant from the Lucille P. Markey Charitable Trust.

Original version received 7 November 1991 and accepted version received 7 May 1992.

REFERENCES


