Calcium-dependent Inactivation of the 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 The Ca$^{2+}$ current activated upon hyperpolarization of Paramecium tetraurelia decays over a period of 150–200 ms during sustained steps under voltage clamp. At membrane potentials between −70 and approximately −100 mV, the time course of this inactivation is described by a single exponential function. Steps negative to approximately −100 mV elicit currents that decay biexponentially, however. Three lines of evidence suggest that this current’s inactivation is a function of intracellular Ca$^{2+}$ concentration rather than membrane potential: (a) Comparing currents with similar amplitudes but elicited at widely differing membrane potentials suggests that their time course of decay is a sole function of inward current magnitude. (b) The extent of current inactivation is correlated with the amount of Ca$^{2+}$ entering the cell during hyperpolarization. (c) The onset and time course of recovery from inactivation can be hastened significantly by injecting cells with EGTA. We suggest that the decay of this current during hyperpolarization involves a Ca$^{2+}$-dependent pathway.

INTRODUCTION

Ca channels are common to most, if not all cells (Hagiwara and Byerly, 1981; Tsien, Hess, McCleskey, and Rosenberg, 1987; Bean, 1989; Hess, 1990). The rise in intracellular Ca$^{2+}$ concentration that results from their activation can have profound effects on cell function (Rasmussen and Barrett, 1984), so the activity of many such channels is regulated (see Dolphin, 1990; Schultz, Rosenthal, Hescheler, and Trautwein, 1990; Trautwein and Hescheler, 1990). This regulation may occur at several levels, depending on cell and Ca channel type, but it most commonly takes the form of a temporary inactivation during sustained excitation.

Two common Ca channel inactivations have been documented (reviewed by Eckert and Chad, 1984). The first is triggered by the passage of Ca$^{2+}$ through the channel. Although the mechanisms involved have yet to be defined fully, it has been suggested...
that Ca\(^{2+}\)-dependent inactivation of L-type currents involves changes in the phosphorylation state of the channel, or of an associated regulatory element (see Chad, Kalman, and Armstrong, 1987; Trautwein and Hescheler, 1990). The second type of inactivation occurs independently of the Ca\(^{2+}\) flux, apparently being invoked by the membrane potential change itself. The mechanism of such voltage-dependent Ca channel inactivation is unknown, but could be a property inherent to the channel's molecular structure.

Hyperpolarization of *Paramecium tetraurelia* elicits a novel inward Ca\(^{2+}\) transient (*I_{Ca(h)}*; Preston, Saimi, and Kung, 1992). Mutational and other analyses have shown *I_{Ca(h)}* to be distinct from the depolarization-activated current described previously (see Preston et al., 1992). Since *I_{Ca(h)}* provides the rise in intracellular Ca\(^{2+}\) concentration necessary for activating three Ca\(^{2+}\)-dependent currents during membrane hyperpolarization (Saimi, 1986; Preston, 1990; Preston, Saimi, and Kung, 1990a), we were interested in determining how this current is regulated. As described below, *I_{Ca(h)}* inactivates and recovers from inactivation in a Ca\(^{2+}\)-dependent manner.

MATERIALS AND METHODS

The membrane currents of *Paramecium tetraurelia* were examined under two-electrode voltage clamp using methods that have been described (Preston et al., 1992). Unless noted otherwise, all data are expressed as means ± SD. The significance of differences between means was determined using a Student's *t* test.

RESULTS

Step hyperpolarization of *P. tetraurelia* from −40 mV elicits an inward Ca\(^{2+}\) transient, *I_{Ca(h)}* (Fig. 1 A). We showed previously that returning to holding potential before this current has decayed fully elicits an inward tail that reflects the deactivation of *I_{Ca(h)}* (Fig. 1 of Preston et al., 1992). We showed further that hyperpolarizing cells for periods ranging from 10 to 300 ms elicits a series of such tail currents, the envelope of which parallels the time course of the inward current flowing during the voltage step. Thus, it is likely that the observed decay of *I_{Ca(h)}* represents a true inactivation of this current, rather than activation of an overlapping, outward current.

*I_{Ca(h)}* varies considerably in magnitude and in its kinetics of inactivation from cell to cell. This is not unusual for an ion current in *Paramecium*, or for Ca\(^{2+}\) currents in general (see Mentrard, Vassort, and Fischmeister, 1984; Jones and Marks, 1989; Slesinger and Lansman, 1991). We noted, however, that hyperpolarization to approximately −100 mV usually elicits currents whose decay phase is well described by a single exponential, \(\tau = 30–40\,\text{ms}\) (Fig. 1 A). As the amplitude of the step hyperpolarization increases, however, a second, faster component appears within the decay of *I_{Ca(h)}*, so that two exponential functions are now required to describe this current's inactivation (Fig. 1 A). Fig. 1 B compares the relative membrane potential dependence of the two inactivation time constants. Although absolute values may vary from cell to cell, the slow component's time constant increases significantly with decreasing membrane potential, whereas \(\tau_{\text{fast}}\) is not membrane potential dependent (Fig. 1 B). Note that the relative contribution of the fast-decaying component of inactivation to *I_{Ca(h)}* increases with membrane hyperpolarization (Fig. 1 C).
Figure 1. Hyperpolarization-activated Ca\(^{2+}\) current and its inactivation. (A) Family of currents elicited by 300-ms steps from \(-40\) mV. The inactivating, peak current represents \(I_{\text{Ca}(hi)}\). Currents elicited by steps up to approximately \(-100\) mV decay with a time course that can be fitted with a single exponential function, \(\tau = 30\)–40 ms. At more negative potentials, a second component to inactivation appears, so that two exponential functions (\(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\)) plus a constant are now required to describe the decay of this current adequately. Traces have been corrected for linear leak current. The two extracted traces (right) were elicited by steps to \(-85\) mV (upper right) and \(-122\) mV (lower right). Their decay phases were computer fitted to single or double exponential functions, and then the fitted curves (solid lines) were superimposed over the current records. The lower trace also shows the contribution of \(\tau_{\text{slow}}\) to the biphasic decay of this current. The current at \(-85\) mV decays with a time constant of 33 ms, whereas \(\tau_{\text{fast}} = 8\) ms and \(\tau_{\text{slow}} = 58\) ms at \(-122\) mV. The broken lines in this and subsequent figures represent holding current levels (approximately \(-0.1\) nA throughout). (B) Time constants (\(\tau\)) of the fast-decaying (\(\tau_{\text{fast}}\); filled circles) and slow-decaying (\(\tau_{\text{slow}}\); open circles) components of inactivation plotted as a function of membrane potential. The plot includes data collected from cells yielding currents that decayed biphasically at membrane potentials positive to \(-100\) mV. Data are means \pm SD from eight cells. (C) Amplitudes of the fast-decaying (filled circles) and slow-decaying (open circles) components of inactivation (\(I\)) as a function of membrane potential (\(V_m\)). The relative amplitudes of the two components were determined by fitting the decay phase of \(I_{\text{Ca}(hi)}\) to two exponentials and then back-extrapolating to the center of the peak. Values are presented relative to current amplitude at 300 ms. Data are means \pm SD from eight cells.
Onset of Inactivation

We next determined the time course of inactivation's onset, using classical paired-pulse protocols (Fig. 2A). Inactivation was invoked using conditioning hyperpolarizations (\(V_{\text{cond}}\)) of variable magnitude (to between -50 and -140 mV) and duration (10–600 ms). The effects of these conditioning steps on \(I_{\text{Ca(h)}}\) were tested using a 300-ms test step to -120 mV (\(V_{\text{test}}\)), applied 400 ms after \(V_{\text{cond}}\). The 400-ms respite between \(V_{\text{cond}}\) and \(V_{\text{test}}\) in these and subsequent experiments was needed to allow deactivation of an uncharacterized hyperpolarization- and time-dependent “leak”

\[V_{\text{cond}}: -100 \text{ mV}
\]

\[V_{\text{test}}: -120 \text{ mV}
\]

\[2 \text{ nA} \quad 100 \text{ ms}
\]

**FIGURE 2.** Onset of \(I_{\text{Ca(h)}}\) inactivation. (A) Effects of increasing the duration of a conditioning hyperpolarization (\(V_{\text{cond}}\): -100 mV) on a subsequent test current (\(V_{\text{test}}\): 300 ms, -120 mV). The upper trace shows a control current (\(I_{\text{max}}\)) elicited by \(V_{\text{test}}\) in the absence of \(V_{\text{cond}}\). The effects of conditioning hyperpolarizations on test currents (\(I\)) are shown in the subsequent four traces: \(V_{\text{cond}}\) increases progressively from 20 to 50, 80, and 200 ms. \(V_{\text{test}}\) follows \(V_{\text{cond}}\) by 400 ms in each case. (B) The amplitude of \(I_{\text{Ca(h)}}\) relative to control values (\(I/I_{\text{max}}\)) plotted as a function of the duration of a prior conditioning hyperpolarization to -50 mV (filled circles), -70 mV (open circles), -90 mV (filled squares), or -150 mV (open squares). In these and subsequent experiments, \(I_{\text{Ca(h)}}\) control values were determined both before and after every two paired-pulse protocols. Data are means from 4–10 cells. (C) Relative test current amplitudes (\(I/I_{\text{max}}\)) plotted against the magnitude of \(V_{\text{cond}}\) for conditioning steps of 50 ms (filled circles), 150 ms (open circles), 300 ms (filled squares), 450 ms (open squares), and 600 ms duration (triangles). Inhibition curves were fitted by eye. Data are means from 5–11 cells.
current. Increasing the duration of V_{cond} from 10 ms to ~200 ms at any given membrane potential causes the amplitude of a subsequent test current to decline steeply, and with a time course that is approximated by a single exponential function plus a constant (Fig. 2 B). Time constants for the onset of inactivation were variable, especially at membrane potentials positive to -100 mV, but we could detect no significant voltage dependence to \( \tau_{\text{onset}} \) (\( \tau_{\text{onset}} = 35 \pm 6 \) ms when \( V_{\text{cond}} = -100 \) mV, and \( 28 \pm 12 \) ms when \( V_{\text{cond}} = -130 \) mV, \( n = 4 \) and 8, respectively). Increasing the duration of \( V_{\text{cond}} \) beyond 300 ms failed to invoke a second, temporally distinct phase of inactivation, regardless of its amplitude (Fig. 2 B). \( V_{\text{cond}} \) was usually limited to 600 ms to avoid damaging cells, but we have applied conditioning hyperpolarizations of up to 5 s without observing further decreases in the response to \( V_{\text{test}} \). Note that although steps to -50 mV elicit currents of ~0.3 nA or less, they inactivate \( I_{\text{Ca(h)}} \) significantly (filled circles).

In Fig. 2 C, relative test current amplitude \( (V_{\text{test}} = -120 \) mV) has been plotted against the amplitude of \( V_{\text{cond}} \) for steps of 50-, 150-, 300-, 450-, and 600-ms duration. Note that regardless of duration, the extent of inactivation induced by the \( V_{\text{cond}} \) increases steeply between -40 and -80 mV, levels off at between -80 and -110 mV, and then increases further with hyperpolarization toward -140 mV.

**Recovery from Inactivation**

Although the previous experiment (Fig. 2) failed to resolve two components to the onset of \( I_{\text{Ca(h)}} \) inactivation, these data do not rule out the possibility that the biexponential decay of this current during hyperpolarization (Fig. 1) reflects two kinetically distinct inactivation mechanisms. If so, it might be possible to detect two phases within the time course of recovery from inactivation. \( I_{\text{Ca(h)}} \) recovery was also monitored using paired-pulse stimulation protocols (Fig. 3 A). The first protocol used a conditioning hyperpolarization of only 60-ms duration (-110 mV), tailored to elicit the fast component of inactivation alone (Fig. 3 A, upper trace). The time course of recovery from this inactivation was monitored using a 300-ms test hyperpolarization \( (V_{\text{test}}) \) to -110 mV, applied at various times after \( V_{\text{cond}} \). Recovery was slow compared with inactivation's onset, proceeding with a time constant, \( \tau_{\text{rec}} \), of 405 ms (\( \pm 128 \) ms, \( n = 10 \); Fig. 3 B, open circles). \( I_{\text{Ca(h)}} \) was next inactivated fully using a 300-ms \( V_{\text{cond}} \) (-110 mV; Fig. 3 A, lower trace); again, recovery was monitored using a 300-ms step to -110 mV. Recovery again proceeded with a time course that was well fitted by a single exponent (\( \tau_{\text{rec}} = 950 \pm 307 \) ms, \( n = 10 \); Fig. 3 B, filled squares), even though the inactivating current had decayed biexponentially.

Note that \( I_{\text{Ca(h)}} \) takes significantly longer to recover after a 300-ms inactivating step than it does after a 60-ms step. The factors governing the time course of recovery from inactivation are considered in a later section.

**Time Course of Inactivation Reflects the Magnitude of the Inactivating Current**

As noted above, \( I_{\text{Ca(h)}} \) magnitude varies significantly from cell to cell. During the early stages of these studies, it became obvious that in cells expressing small currents, the time course of \( I_{\text{Ca(h)}} \) inactivation could invariably be fitted with a single exponent regardless of membrane potential (over a -70- to -130-mV range). In other cells, however, even hyperpolarizations to -85 mV were sufficient to activate large currents that decayed biexponentially. Thus, the time course of \( I_{\text{Ca(h)}} \) inactivation appears to
be an expression primarily of current magnitude rather than absolute membrane potential. This possibility was investigated more systematically using methods similar to those described by Chad, Eckert, and Ewald (1984).

Cells were first hyperpolarized to membrane potentials ranging from $-70$ to $-120$ mV for 300 ms to elicit a family of control currents (Fig. 4 A; note that currents at $-100$ and $-110$ mV inactivate biphasically). The same cells were then stepped repeatedly to $-120$ mV (300 ms) while slowly adding 1 mM amiloride to the bath. Amiloride suppressed $I_{Ca(h)}$ (Preston et al., 1992) so that this repeated hyperpolarization produced currents of decreasing amplitude as inhibitor concentration slowly increased (Fig. 4 B). From these two sets of records (Fig. 4, A and B), it is possible to select and compare directly the time course of inactivation of two currents that have similar amplitudes but that were activated by disparate steps, and vice versa. Fig. 4 C

**FIGURE 3.** Time course of recovery from inactivation. (A) Upper trace, a 60-ms conditioning step to $-110$ mV elicits the fast component of inactivation alone. $V_{\text{test}}$ (300 ms, $-110$ mV) was applied at various times after $V_{\text{cond}}$ ($V_{\text{cond}}$ and $V_{\text{test}}$ were separated by 200 ms in the example shown) to monitor $I_{Ca(h)}$ recovery. Lower trace, conditioning and test currents were both elicited by 300-ms steps to $-110$ mV, with a 200-ms interval between the two. (B) The time courses of recovery from inactivation induced by the above conditioning steps are compared. Open circles, $V_{\text{cond}} = 60$ ms; filled squares, $V_{\text{cond}} = 300$ ms. Recovery was monitored by determining the amplitude of the current induced by $V_{\text{test}}$ ($I$) relative to control currents induced by a similar step in the absence of $V_{\text{cond}}$ ($I_{\text{max}}$). Recovery from inactivation proceeds with a time course that is well described by a single exponential function, irrespective of conditioning current decay rates ($t_{\text{rec}} = 452$ ms when $V_{\text{cond}} = 60$ ms, and 933 ms when $V_{\text{cond}} = 300$ ms).
FIGURE 4. Time course of inactivation is related to current size. (A) Family of currents elicited by 300-ms steps from -40 mV in the absence of amiloride. Numerals to the left of the current traces indicate the membrane potential at which the currents were elicited (in millivolts). Traces have been leak corrected. (B) Currents elicited from the same cell by repeated 300-ms steps to -120 mV during gradual (over a period of 60–90 s) replacement of the bath solution with one containing 1 mM amiloride. The amplitude of $I_{Ca}$ decreased as the concentration of amiloride in the bath increased toward 1 mM (denoted by the cross-hatched wedge). (C) Comparison of the time course of inactivation of two currents elicited from a single cell by 300-ms step hyperpolarizations to -120 mV. The upper trace shows a control current elicited before amiloride application. This current's decay phase has been computer-fitted to two exponential functions (solid lines). The lower trace shows a current evoked by an identical step during amiloride perfusion. The current's decay can now be adequately described by a single exponential. (D) Comparison of the time course of decay of two currents with similar amplitudes that were activated by disparate steps. The currents were elicited from a single cell before and during amiloride application. The upper trace was evoked using a 300-ms step to -80 mV in the absence of amiloride. The lower trace was elicited using a step to -120 mV during amiloride perfusion. The currents inactivate with similar time courses: solid lines superimposed over the current traces were computer-fitted using the time constants indicated.
compari the time course of inactivation of two currents that were elicited by identical steps, but that had very different amplitudes. A 300-ms step to −120 mV first elicited a control $I_{\text{Ca(h)}}$ of −3.3 nA that inactivated with a time course described by the sum of two exponents ($\tau_{\text{fast}} = 17 \text{ ms}, \tau_{\text{slow}} = 112 \text{ ms}$). An identical step to −120 mV during amiloride perfusion elicited a current of greatly reduced amplitude (−1.6 nA; Fig. 4 C, lower trace) that decayed monoexponentially ($\tau = 33 \text{ ms}$). Two currents that had similar amplitudes but were activated by different steps are compared in Fig. 4 D. The upper trace is a −0.6-nA control current evoked by a 300-ms step to −80 mV in the absence of amiloride. The current inactivates with a time constant of 40 ms. The lower trace was elicited by a step to −120 mV during amiloride perfusion. Although this current activates faster than the current at −80 mV in the absence of amiloride, its amplitude and the time course of its inactivation are almost identical (−0.6 nA, $\tau = 39 \text{ ms}$). Similar results were obtained using Ba$^{2+}$ as an inhibitor (not shown). Thus, the occurrence of two phases to the decay of $I_{\text{Ca(h)}}$ depends on current amplitude (and hence the Ca$^{2+}$ influx; see Discussion), not on membrane voltage per se.

### Inactivation of $I_{\text{Ca(h)}}$ Is Dependent upon Prior Ca$^{2+}$ Entry

If $I_{\text{Ca(h)}}$ inactivation is dependent on [Ca$^{2+}$], the extent to which this current inactivates during hyperpolarization should be correlated with the amount of Ca$^{2+}$ entering the cell during inactivation. This assumption was tested as follows. $I_{\text{Ca(h)}}$ was inactivated using conditioning hyperpolarizations to between −50 and −140 mV and of variable duration (50–400 ms). These steps elicited Ca$^{2+}$ fluxes of variable size (Fig. 5 A). After a 400-ms return to holding potential, a second test hyperpolarization ($V_{\text{test}} = −120 \text{ mV}, 300 \text{ ms}$) was applied to determine the extent to which $I_{\text{Ca(h)}}$ had been inactivated. The amount of Ca$^{2+}$ entering during inactivation was determined by integrating the area under the peak of the current evoked by $V_{\text{cond}}$. (Current levels at 300 ms were used as a baseline for these calculations; when $V_{\text{cond}}$ duration was <300 ms, baselines were determined from a series of 300-ms control currents at equivalent membrane potentials.) The results, which are plotted in Fig. 5 B, show a clear correlation between the extent to which $I_{\text{Ca(h)}}$ is inactivated by $V_{\text{cond}}$ and the amount of Ca$^{2+}$ entering the cell during the inactivating step.

### Effects of EGTA on $I_{\text{Ca(h)}}$ Inactivation

If $I_{\text{Ca(h)}}$ is inactivated by rising [Ca$^{2+}$], it should be possible to modify this process by enhancing the Ca$^{2+}$ buffering capacity of the cytoplasm. Fig. 6 shows the consequences of injecting Paramecium iontophoretically with the Ca$^{2+}$ chelator, EGTA. A −7-nA, 15-s injection ([EGTA]$_i$ = 1.4 mM) consistently increased the amplitude of both $I_{\text{peak}}$ and $I_{300}$ (Fig. 6 B), but only slightly. In some cells EGTA injection affected the time course of decay of $I_{\text{Ca(h)}}$. For example, a 300-ms step to −130 mV before EGTA injection elicited a current that inactivated biphasically, with time constants of 8 and 151 ms. After EGTA injection, an identical step elicited a current that decayed monoexponentially ($\tau = 51 \text{ ms}$). Increasing [EGTA]$_i$ to ~4.2 mM (−7 nA, 45 s) failed to affect $I_{\text{Ca(h)}}$ amplitude or decay rates further; at levels in excess of 5 mM, cell condition deteriorated rapidly (not shown). We also injected cells with BAPTA, which
chelates Ca\(^{2+}\) with greater efficiency than EGTA (Tsien, 1980). Moderate injections (−7 nA, 5 s) increment \(I_{\text{peak}}\) and \(I_{300}\) to an extent similar to EGTA, but BAPTA injections are considerably more lethal (not shown), perhaps indicating that cell survival is critically dependent on a basal level of [Ca\(^{2+}\)]\(_i\).

![Figure 5](image)

**Figure 5.** Relationship between inactivation of \(I_{\text{Ca(h)}}\) and Ca\(^{2+}\) entry. (A) Conditioning hyperpolarizations of variable magnitude (−50 to −140 mV) and duration (50–400 ms) were used to elicit similarly variable inward Ca\(^{2+}\) fluxes. A 300-ms test hyperpolarization to −120 mV was used to ascertain the effects of these fluxes on \(I_{\text{Ca(h)}}\) 400 ms after \(V_{\text{cond}}\). Sample traces from three different cells are shown. Each set of traces comprises five currents: a control current elicited in the absence of \(V_{\text{cond}}\), and four test currents preceded by a \(V_{\text{cond}}\) to −70, −90, −110, or −130 mV. \(V_{\text{cond}}\) duration is 50 ms in the first set of traces (left), 100 ms in the second, and 200 ms in the third (right). (B) Relative test current amplitude (\(I/I_{\text{max}}\)) is plotted as a function of the amount of Ca\(^{2+}\) entering the cell during \(V_{\text{cond}}\). The total Ca\(^{2+}\) flux (in nanocoulombs) was quantified by integrating the area under the peak of currents elicited by a \(V_{\text{cond}}\) of 50–400 ms duration to various membrane potentials (−50 to −140 mV). The graph represents data pooled from nine cells. (C) Cells in B were injected with EGTA and the relationship between the extent of \(I_{\text{Ca(h)}}\) inactivation and Ca\(^{2+}\) influx was redetermined. EGTA was iontophoresed into the cell (−10 nA, 20 s) from a third intracellular glass capillary microelectrode filled with 100 mM K\(_2\)EGTA and 1 mM HEPES, pH 7.0.

We also examined the effects of EGTA on the relationship between Ca\(^{2+}\) influx and consequential \(I_{\text{Ca(h)}}\) inactivation. A −10-nA, 20-s EGTA injection significantly reduces the extent to which \(I_{\text{Ca(h)}}\) is inactivated by Ca\(^{2+}\) influx (Fig. 5 C), as compared with preinjection values (Fig. 5 B).
FIGURE 6. Effects of injecting EGTA. (A) Currents activated by 300-ms steps to $-130$ mV before and after iontophoretic injection of EGTA ($-7$ nA, 15 s). Traces have been leak corrected. (B) Peak currents ($I_{\text{peak}}$; circles) and currents at $300$ ms ($I_{300}$; squares) are plotted as a function of membrane potential ($V_m$). Open symbols represent control currents, whereas filled symbols denote currents elicited after iontophoretic injection of EGTA. Data are means ± SD from five cells, and have been leak corrected.

**Effects of EGTA on Recovery from Inactivation**

These results suggest that $I_{\text{Ca}(h)}$ may be regulated by $[\text{Ca}^{2+}]_i$. This Ca$^{2+}$ sensitivity is also manifest in the time course of recovery from inactivation. $I_{\text{Ca}(h)}$ was inactivated using conditioning hyperpolarizations of 300-ms duration to between $-80$ and $-130$ mV (Fig. 7). The progress of recovery from inactivation was monitored with a test step to $-120$ mV (300 ms). As reported above, $I_{\text{Ca}(h)}$ recovers from inactivation with an exponential time course ($\tau_{\text{rec}}$; Fig. 3). Little difference was observed between the rates of $I_{\text{Ca}(h)}$ recovery from conditioning steps to $-80$, $-90$, $-100$, or $-110$ mV. $\tau_{\text{rec}}$ increases significantly with further increases in $V_{\text{cond}}$, however, from $\sim 700$ ms to $>1,600$ ms using a $V_{\text{cond}}$ of $-130$ mV (Fig. 7, filled circles). EGTA was then injected.

FIGURE 7. Effects of EGTA on the time course of recovery from inactivation. Inset shows stimulation protocol used to determine time constants for recovery of $I_{\text{Ca}(h)}$ from inactivation ($\tau_{\text{rec}}$). $V_{\text{cond}}$ (300 ms) was varied from $-80$ to $-130$ mV. $V_{\text{test}}$ (300 ms, $-120$ mV) was applied at various times after $V_{\text{cond}}$. $\tau_{\text{rec}}$ is plotted as a function of $V_{\text{cond}}$ amplitude before (filled circles) and after (open circles) a 15-s, $-7$-nA EGTA injection. Data are means ± SE from three to eight cells.
iontophoretically and \( \tau_{\text{rec}} \) redetermined. EGTA decreased \( \tau_{\text{rec}} \) in all instances, but its effects were greatest in speeding recovery from the inactivation induced by conditioning steps to \(-120\) and \(-130\) mV (Fig. 7, open circles).

Finally, we were interested to note that the use of conditioning steps to \(-110\) mV and below often causes the onset of \( I_{\text{Ca(h)}} \) recovery to be delayed by up to \(800\) ms (Fig. 8, filled circles). This delay is suppressed fully by injecting cells with EGTA (Fig. 8, open circles).

**DISCUSSION**

Hyperpolarization of *Paramecium* under voltage clamp elicits an inactivating \( \text{Ca}^{2+} \) current, \( I_{\text{Ca(h)}} \). At membrane potentials positive to approximately \(-100\) mV, this current inactivates with an exponential time course, whereas two exponential functions are required to describe \( I_{\text{Ca(h)}} \) decay at \(-100\) mV and below. The time course of decay is a function of inward current magnitude, not membrane potential. Both inactivation and recovery from inactivation are affected by procedures that modify intracellular \( \text{Ca}^{2+} \) concentration, pointing toward the involvement of one or more \( \text{Ca}^{2+} \)-sensitive steps in these events.

**Evidence for a \( \text{Ca}^{2+} \)-dependent Inactivation Mechanism**

Previous studies on *Paramecium* (Brehm and Eckert, 1978; Brehm, Eckert, and Tillotson, 1980; Hennessey and Kung, 1985), mollusk neurons (Tillotson, 1979; Eckert and Ewald, 1983; Plant, Standen, and Ward, 1983; Chad et al., 1984), frog atria (Mentrard et al., 1984), mammalian pancreatic B cells (Plant, 1988), and cardiac Purkinje fibers (Kass and Sanguinetti, 1984; Lee, Marban, and Tsien, 1985) have taken affirmation of one or more of the following criteria as evidence that \( \text{Ca}^{2+} \) currents inactivate in a \( \text{Ca}^{2+} \)-dependent manner. (a) Inactivation slows or is removed...
when Sr$^{2+}$, Ba$^{2+}$, Na$^+$, or Cs$^+$ replace Ca$^{2+}$ as the permeant ion. (b) Ca$^{2+}$ entry is required for inactivation, so that depolarization to the equilibrium potential for Ca$^{2+}$ opens these channels but fails to inactivate them. (c) Agents that decrease [Ca$^{2+}$]$_i$ reduce the extent of inactivation and hasten subsequent recovery.

The fact that $I_{Ca(h)}$ is inhibited by Ba$^{2+}$ and Sr$^{2+}$ (Preston et al., 1992), and that this current is activated by hyper- rather than depolarization, denied us the luxury of using the two most compelling demonstrations of Ca$^{2+}$-dependent Ca channel inactivation. Nonetheless, there are strong indications that $I_{Ca(h)}$ inactivates in a current-dependent and, indeed, [Ca$^{2+}$]$_i$-dependent manner.

**The Rate and Extent of Inactivation Are Functions of Inward Current Magnitude**

Fig. 4 D compares the decay rates of two currents, one evoked by a step to $-80$ mV and the other by a step to $-120$ mV. The amplitude of the latter was reduced to that of the first by adding amiloride to the bath solution. The fact that the two currents inactivate with near-identical time courses demonstrates clearly that the rate of $I_{Ca(h)}$ decay is determined by inward current magnitude rather than membrane potential per se. Chad et al. (1984) conducted a similar study on the Ca$^{2+}$ dependence of $I_{Ca}$ inactivation in *Aplysia* neurons, using Co$^{2+}$ to partly suppress this current. The authors also took similarities in the decay rates of currents that had been evoked at very different voltages to be strong evidence for the participation of a Ca$^{2+}$-dependent step in inactivation.

The relationship between $I_{Ca(h)}$ magnitude (in terms of net Ca$^{2+}$ influx) and the extent of induced inactivation is shown in Fig. 5. Here, conditioning hyperpolarizations of widely different duration and to a wide range of membrane potentials were used to inactivate $I_{Ca(h)}$. Plotting the extent of induced inactivation against the amount of Ca$^{2+}$ entering the cell during these diverse conditioning steps shows that the two factors are strongly correlated (Fig. 5 B). Moreover, this correlation is weakened by EGTA injection (Fig. 5 C), again supporting the idea that Ca$^{2+}$ influx and inactivation are causally related.

**Inactivation and Recovery from Inactivation Are Modified by Increased Intracellular Ca$^{2+}$ Buffering**

Injecting *Paramecium* with EGTA affects both $I_{Ca(h)}$ inactivation and recovery from this inactivation, although to different extents. The decay of $I_{Ca(h)}$ is largely unaffected by EGTA; rather, its effects are restricted to small increases in peak and sustained ($I_{500}$) inward current amplitudes (Fig. 6 B). Amplitude increases after EGTA injection are predicted by models of Ca$^{2+}$-dependent Ca$^{2+}$ current inactivation (Standen and Stanfield, 1982) and have been demonstrated experimentally (reviewed by Eckert and Chad, 1984). Ca$^{2+}$ chelators are generally unable to suppress such inactivations fully (Brehm et al., 1980; Eckert and Tillotson, 1981; Eckert and Ewald, 1983; Kalman, O'Lague, Erxleben, and Armstrong, 1988; Plant, 1988). Computer modeling (Chad and Eckert, 1984; Simon and Llinás, 1985; Sherman, Keizer, and Rinzel, 1990) suggests that when a Ca channel opens, a region of high Ca$^{2+}$ concentration forms immediately below its inner mouth. This domain is submicroscopic (tens of nanometers) and dissipates almost instantly upon channel closure, but the concentration of Ca$^{2+}$ within the domain approaches that of the extracellular solution. The
Ca²⁺ Current Inactivation

channel inactivation site is assumed to be located within this domain. Thus, the steepness of the Ca²⁺ concentration gradient, combined with the limitations of EGTA as a Ca²⁺ buffer, renders inactivation relatively insensitive to injected EGTA. This is particularly true for \( I_{\text{Ca(h)}} \), where the Ca²⁺ concentration gradient between domain and cytoplasm is exacerbated by the massively increased driving force for Ca²⁺ influx during hyperpolarization. We attempted to circumvent EGTA's limitations by injecting cells with BAPTA, but its effects were similar to those of EGTA (unpublished results). The failure of BAPTA to remove inactivation completely may again reflect the steepness of the Ca²⁺ concentration gradient during hyperpolarization, or perhaps that the Ca²⁺ inactivation site is located deep within the putative channel pore. We should also note that the currents that depend on Ca²⁺ influx via \( I_{\text{Ca(h)}} \) to activate during hyperpolarization are also relatively EGTA resistant (Saimi, 1986; Preston, 1990; Preston et al., 1990a), perhaps indicating that \( I_{\text{Ca(h)}} \) and its dependent currents are shielded from agents introduced into the bulk cytoplasm.

In contrast to the lackluster effects of EGTA on \( I_{\text{Ca(h)}} \) amplitude and decay rates, this chelator significantly accelerates rates of recovery from inactivation (Figs. 7 and 8). Several authors (Brehm et al., 1980; Shimoni, 1981; Ashcroft and Stanfield, 1982; Mentrand et al., 1984) have suggested that the time course of recovery reflects the rate at which free Ca²⁺ is removed from the vicinity of the Ca channel and, indeed, Ca²⁺ current restitution is adequately described in mathematical models by a simple Ca²⁺ sequestration mechanism (Standen and Stanfield, 1982). There are indications that the return of \( I_{\text{Ca(h)}} \) from an inactivated state may similarly be governed by Ca²⁺ removal rates. Recovery of \( I_{\text{Ca(h)}} \) from conditioning steps to between -80 and -110 mV proceeds with a time constant of \( \sim 700 \) ms (Fig. 7, filled circles). Increasing \( V_{\text{cond}} \) beyond -110 mV is accompanied by a dramatic slowing of recovery, a trend that is reversed fully by injecting EGTA (Fig. 7, open circles). Further, the use of conditioning currents to -110 mV and below often delays the onset of recovery from inactivation by several hundred milliseconds (Fig. 8, filled circles), a delay that is overcome fully by injecting EGTA (Fig. 8, open circles). These findings are consistent with the idea that \( I_{\text{Ca(h)}} \) inactivates in a Ca²⁺-dependent manner, and that large hyperpolarizations cause a Ca²⁺ concentration build-up at the inner membrane surface that temporarily prevents removal of inactivation. By reducing intracellular free Ca²⁺ concentration, EGTA allows recovery to proceed normally.

**Biexponential Decay of \( I_{\text{Ca(h)}} \) and Ca²⁺-dependent Inactivation**

Although the biexponential decay of \( I_{\text{Ca(h)}} \) at membrane potentials of -100 mV and below (Fig. 1) might suggest intuitively that a minimum of two distinct steps are involved, this phenomenon can readily be explained in terms of a single, Ca²⁺-dependent process. Two comprehensive models for Ca²⁺-dependent Ca²⁺ current inactivation have been developed, the first by Standen and Stanfield (1982), the second by Chad et al. (1984). The modeled currents exhibit many features in common with each other and with \( I_{\text{Ca(h)}} \). Both groups noted that a minimum of two exponents were required to describe the currents' decay during the voltage step: to quote Chad et al. (1984), "the occurrence of one or two phases is implicit in the hyperbolic relation between the proportion of channels remaining not inactivated and the build-up of free Ca²⁺ during current flow." Chad et al. (1984) also predicted...
that the relative contribution of the fast-decaying component to the inactivation should increase with current amplitude (cf. Fig. 1 C), while Standen and Stanfield (1982) suggested that this component's time constant should be independent of current magnitude (cf. Fig. 1 B). Although the decay of $I_{Ca(h)}$ can be accounted for by a single inactivation mechanism, we readily acknowledge that more than one such pathway could be involved in regulating this current's expression.

Conclusions

The studies presented above suggest that the decay of $I_{Ca(h)}$ during hyperpolarization is Ca$^{2+}$ dependent. We could find no evidence to suggest that $I_{Ca(h)}$ inactivation or recovery from inactivation is affected by membrane voltage per se. The molecular basis for the Ca$^{2+}$-dependent inactivation has yet to be determined, but it is interesting to note that its kinetics are altered in several calmodulin-defective mutants of *P. tetraurelia* (Preston, Wallen-Friedman, Saimi, and Kung, 1990b; Preston, R.R., unpublished observations). Further analyses of these defects may yield important insights into the mechanisms of Ca$^{2+}$-dependent inactivation of $I_{Ca(h)}$ and perhaps of other depolarization-activated Ca$^{2+}$ currents.

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