Voltage-dependent inactivation of a calcium channel

(calcium currents/inactivation/egg cell membrane)

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ABSTRACT The inactivation of Ca currents in unfertilized eggs of the marine polychaete *Neanthes arenaceodentata* was investigated by using a voltage clamp technique. These Ca currents do not appear to be masked by other currents in the voltage range studied. Inactivation increased monotonically with increasing depolarization and occurred at potentials more negative than the inward Ca current. Currents elicited by depolarizations at different Ca concentrations had approximately the same time course of inactivation, even though the size of the currents varied by almost an order of magnitude. There was complete inactivation in solutions containing Ca, Sr, or Ba (all permeant) for depolarizations beyond -30 mV absolute; the time course of the inactivation process was similar for all three permeant ions. Increasing depolarizations toward the expected equilibrium potential for Ca, Ba, or Sr did not produce any lessening of the inactivation. Thus, it appears that the inactivation seen in *Neanthes* eggs is a purely voltage-dependent phenomenon.

Ca has been found to be important in a wide variety of cell functions (1-4), raising questions as to how it crosses cell membranes and is itself regulated.

Inward-going Ca currents are found in many cell membranes (5-14). Some of these inward currents relax with time (7-14). This paper deals with an inward-going Ca current, found in the egg cell membrane of the marine polychaete, *Neanthes*, that has been found to relax with time under maintained depolarization. There are several possibilities for Ca relaxation that will be considered. First, there could be no inactivation of the Ca conductance at all; rather, the relaxation process could be a turn on of a conductance that produces a net outward current and thereby masks the inward current and makes it appear as if there is inactivation. Second, there could be a Ca-conductance decrease induced by the Ca current itself. Third, an external depletion or an internal accumulation of Ca would decrease the gradient for Ca across the cell membrane and thus reduce the inward current. Fourth, there could be a voltage-dependent inactivation of the Ca conductance.

The Hodgkin and Huxley model of voltage-dependent sodium inactivation (15) has been used to explain Ca-channel inactivation also (7-11). Ca itself has been shown to have an inhibitory effect on Ca currents (16-18). Recently, a Ca-induced Ca inactivation has been discovered in *Aplysia* and in *Paramecium*, whereby the Ca entering the cell causes a decrease in the Ca current by a mechanism that is still unknown (13, 14). The authors of these reports have suggested that perhaps all Ca-channel inactivation is induced by Ca entry into the cell. This paper presents voltage-clamp data that are inconsistent with the universality of a Ca-induced Ca inactivation; in *Neanthes* eggs, there is a Ca-conductance decrease that is clearly voltage dependent and remarkably similar to the inactivation seen in Na channels such as those of the squid giant axon (15).

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Table 1. Solutions used

Name	NaCl, mM	KCl, mM	Permeant divalent,* mM	MgCl ₂ , mM	Tris, mM	pH
ASW	470	10	10 Ca	50	0	7
60 Ca	470	10	60 Ca	0	0	7
10 Ca	460	10	10 Ca	0	10	7.6
10 Ba	460	10	10 Ba	0	10	7.6
10 Sr	460	10	10 Sr	0	10	7.6
40 Ca	460	10	40 Ca	20	0	7

* As chloride.

MATERIALS AND METHODS

Adult *Neanthes arenaceodentata* were purchased from Donald J. Reish (California State University, Long Beach, CA). Eggs found in the coelom of the mature female adult of *Neanthes* account for about 75% of the total body weight. The mature eggs are large, up to $650\text{ }\mu\text{m}$ in diameter. The eggs were ejected from the mature female immediately after severing the anterior and posterior portions of the worm, by using a slow jet of seawater from a syringe through a needle inserted into the coelom.

The solutions used are given in Table 1. Experiments done in unbuffered solutions at pH 7.0 were repeated (except for those in which Co was present) at pH 7.6 (with added Tris) with no change in the results.

The membrane potential was controlled by a two-microelectrode voltage clamp. The holding potential was usually set equal to the zero-current potential of the egg—i.e., between -70 and -85 mV. When Sr or Ba was used in place of Ca in the bathing medium, the holding potential was set more negative than -80 mV to remove any inactivation. Step pulses had rise times of approximately 2 msec for a 100-mV jump. The microelectrodes used for current passing and potential monitoring had resistances of 5-10 M Ω and were filled with 3 M KCl. A reference electrode was positioned near the egg to minimize any bath series resistance. The bath was held at virtual ground by an I to V converter (joined to the bath by a 3 M KCl/agar bridge), which also measured total bath current.

The experimental data were filtered by a six-pole Bessel analog filter having a corner frequency at 860 Hz, after which they were digitized at a sampling rate of 2900 points per sec by an eight-bit A/D converter (Cromemco D+7AI/0 board) and then stored in a Northstar Horizon II microcomputer. The data were subsequently plotted on a Houston Instruments Omnigraphic X-Y plotter driven by a D/A converter (Cromemco D+7AI/0 board).

In some cases, the computer was also used to subtract the leak and noninactivating currents from the current records (see Fig. 1).

Abbreviation: ASW, artificial seawater.

FIG. 1. Computer-aided leak and noninactivating-current subtraction procedure. (A) Current record obtained during depolarization to -35 mV. (B) Current record seen by using a two-pulse procedure (*Inset*): The conditioning pulse is to a potential that totally inactivates the inward current (-15 mV); the test pulse then returns the potential to -35 mV (the conditioning and test pulses are separated by a 10-msec interval). The computer subtracts the current record following the test pulse point by point from the current trace in A; this generates C, a record of all the inactivating currents at -35 mV. In B, the final outward current in the prepulse to -15 mV is smaller than the final outward-current level in the -35 mV pulse. This is thought to be due to K current flowing through an anomalous rectifier. The voltage dependence of the anomalous rectifier is such that there is less conductance at -15 mV than at -35 mV, thus, a smaller outward current is observed. The kinetics of this K conductance are such that they do not interfere with the Ca records and can be subtracted out. Experiment done in 10 Ca solution. Dashed line represents the zero-current level.

RESULTS AND DISCUSSION

Ca Is the Charge Carrier. Three pieces of evidence suggest that the inward currents are carried by a net inward Ca flux (Fig. 1A). First, increasing the concentration of Ca in the bath increased the size of the inward current; conversely, decreasing bath Ca decreased the size of the inward current. Second, 10 mM Co decreased this inward current significantly. Third, replacement of Na by tetramethylammonium ion did not change the current.

Current Relaxation Is Due to a Conductance Decrease. Two lines of evidence show that the time-dependent current relaxation observed is due to a decreased conductance for a net inward current rather than a conductance increase for a net outward current. First, the instantaneous tail currents mediated by these Ca channels and observed on repolarizing the membrane to near the resting potential decreased with time. An example of this phenomenon is shown in Fig. 2. An egg was depolarized from a holding potential of -80 mV to -22 mV for 10, 20, 30, or 150 msec before stepping to -50 mV. The inward "tail currents" observed on repolarizing the cell to -50 mV (-50 mV was chosen rather than the holding potential because the tail currents were greatly slowed when the potentials were returned to more positive than the holding potential and thus could be viewed with much better resolution) were largest when the depolarization at -22 mV was maintained for 10 msec and

became progressively smaller the longer the depolarization was maintained, until, at 150 msec, no more tail current was observable. Because the early tail-current size is directly related to the instantaneous conductance at the time the depolarization to -22 mV was interrupted, the decrease in tail-current magnitude with time is a clear indication of a conductance decrease with time. Qualitatively, the same results were obtained if, after the depolarization to -22 mV, the potential was stepped to -55 mV or -60 mV instead of -50 mV (if an inward tail current and an outward current were just compensating each other, the lack of dependence on the repolarization potential would be very unlikely).

Second, two pieces of data can be used to show that the two most common types of conductances known to produce outward currents—those due to a voltage-gated K channel (delayed rectifier) and those due to a Ca-induced K channel—do not activate in the voltage range studied (more negative than 0 mV): The currents seen during depolarizations to -29 mV in artificial seawater (ASW) and 60 Ca solution are shown in Fig. 3A. A voltage-gated K conductance should have been activated to the same extent in both solutions and thus have produced the same amount of net outward current (as reflected by the difference between the peak inward and the final outward current levels). However, the difference between the steady-state and the peak inward currents in 60 Ca was much larger than that in ASW. Furthermore, similar differences were observed for the two solutions at any given potential between -40 mV and 0 mV, arguing that these current relaxations are not due to current through a volt-

FIG. 2. Time dependence of tail-current size. The inward-current conductance was activated with a depolarization and then monitored by stepping to a new, more negative potential and measuring the instantaneous or tail-current components. (A) Current record obtained after a depolarization to -22 mV. Arrows on the trace represent different times at which the membrane was repolarized to -50 mV: a, 10 msec; b, 20 msec; c, 30 msec; d, 150 msec. (B, C, D, and E) Tail currents seen after 10-, 20-, 30-, and 150-msec depolarization, respectively. Note that the early part of the tail current decreases with time such that, at 150 msec, there is no longer any early inward tail current. Experiment done in 40 Ca solution. Upper scale is for trace A and lower scale is for traces B-E.

FIG. 3. Comparison of inward currents in ASW and 60 Ca solution. (A) Current traces generated by depolarizations to -29 mV in ASW and 60 Ca solution. Note that the final outward current levels are the same in both solutions. Lower dashed line represents the zero-current level. (B) Plot of peak inward current, subtracted for leak, versus membrane potential in ASW ($\circ$) and 60 Ca solution ($\square$). (C) Plot of time constants of the relaxation process versus membrane potential in ASW and 60 Ca solution.

age-gated K channel. A Ca-induced K conductance is unlikely because the conductances in 10 Ca, 10 Ba, or 10 Sr solutions all relaxed such that there was no longer any net inward current (Fig. 4A). In studies where Ca-activated K channels have been seen, there have been large differences in the efficacy of Ca or Sr versus Ba in activating the conductance (19).

Neither internal accumulation nor external depletion is a likely cause for the relaxation of the inward currents for two reasons. First, the egg, which is spherical, has a large volume

FIG. 4. Comparison of inward currents in 10 Sr, 10 Ca, and 10 Ba. (A) Current traces after depolarization to -40 mV in 10 Sr, 10 Ca, and 10 Ba. Dashed line represents the zero-current level. Note that there is no outward current component in the 10 Ba solution at the end of the 150-msec depolarization, as there is in the 10 Sr and 10 Ca solutions. This is thought to be a block by Ba on an anomalous rectifier current. (B) Time constants of the relaxation process versus membrane potential in 10 Sr, 10 Ca, and 10 Ba solutions.

($\approx 6.5 \times 10^7 \mu\text{m}^3$), and the maximum inward current is very small (< 20 nA). Thus, the amount of Ca entering the cell is usually less than 1.5 fM per depolarization. The very small amounts of Ca involved make accumulations or depletions unlikely. Although no electron microscopy has yet been done on the eggs, capacitance measurements suggest that there is no excess membrane available for infoldings (assuming $1.2 \mu\text{F}/\text{cm}^2$ of membrane). A second reason for discarding accumulation or depletion comes from two-pulse experiments following the protocol shown in Fig. 1 *Inset*, in which the conditioning pulse approaches the expected Ca equilibrium potential and assuming internal free Ca to be 1–0.1 μM (20, 21). As depletion or accumulation effects should have been minimal during the positive conditioning pulses used, they cannot account for the absence of the time-dependent current during the test pulse shown in Fig. 5A (+138-mV pulse).

The Inactivation Process Is Voltage-Dependent. The data of Fig. 3 can also be used to show that the inactivation process seen in *Neanthes* eggs is a voltage-dependent process and is not Ca induced. If the conductance decrease were induced by Ca entering the cell, the greater net influx of Ca per unit time (i.e., the larger inward current) should induce in 60 Ca solution a more rapid inactivation of Ca conductance than that in ASW; however, it does not. A plot of the time constant of inactivation

FIG. 5. Steady-state inactivation in Sr, Ca, and Ba. Experiments were performed by using a two-pulse procedure (see Fig. 1 *Inset*). In this series of experiments, the conditioning pulses were depolarizations to different potentials and lasted for 150 msec, and the test pulses were depolarizations to -25 mV for 150 msec; the voltage was returned to the holding potential for 10 msec between the conditioning and the test pulses. The amount of inactivation seen during the test pulse depended on the amplitude of the conditioning pulse. (A) Current traces in 10 Ca solution. Number to the right of the current trace indicates potential of the conditioning pulse. (Note that, in the $+138$ -mV trace, the A/D converter of the computer was saturated during the conditioning pulse.) Dashed line represents the zero-current level. (B) Plot of peak inward current elicited by a conditioning pulse divided by peak inward current seen with no conditioning pulse (I/I_0) versus membrane potential. (C) Same inactivation plot as for 10 Ca in B but with an expanded potential axis. Also included is a leak-subtracted current-voltage (I-V) plot for the peak inward Ca current. Arrows denote the most-negative potential at which inward current was seen in the I-V plot and the most-negative potential at which inactivation was seen in the inactivation plot. Note that inactivation was present before there was any detectable inward current.

versus membrane potential in both ASW and 60 Ca solution (see Fig. 3C) shows that, at any given potential, the time constants are virtually the same in the two solutions; yet, a plot of the peak

inward current versus potential (see Fig. 3B) shows that the size of the two currents are very different at each given potential. In addition, even though, at potentials more positive than -10 mV, the peak inward currents get smaller, the inactivation process proceeds more rapidly (see Fig. 3B and C). These data are inconsistent with Ca-induced inactivation.

Fig. 4A and B also shows that the inactivation process is voltage dependent in that the inactivation of the inward current in Ba is complete and the time constants of the inactivation process at a given potential are similar in Sr, Ba, and Ca solutions. Fig. 5A and B shows that inactivation increases monotonically with depolarization (there is an e -fold change in I/I_0 for a 5-mV change in potential) and that the currents stay inactivated as the expected reversal potentials for Sr, Ba, or Ca are approached. These observations are again in contrast to reports on Ca-induced inactivation for which inactivation in Ba is incomplete, the kinetics of the inactivation process change greatly in both Sr and Ba, and inactivation decreases as the potential approaches the equilibrium potential for Ca (or similarly for Ba or Sr) (13, 14).

Finally, Fig. 5C shows the steady-state inactivation curve for Ca of Fig. 5B on an expanded scale, including the leak-subtracted I-V curve at the peak of the inward Ca current. It can be seen that there is a region, denoted by the arrows in Fig. 5C, where inactivation of the conductance is present although there is no observable inward Ca current. Again, this observation indicates a voltage-dependent inactivation.

Thus, there appear to be real differences in Ca-channel inactivations; i.e., Ca channels inactivate in a potential-dependent or Ca-induced manner. It will be interesting to see whether preparations are found where both Ca-induced and voltage-dependent inactivation exist simultaneously.

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