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Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons

(Ca channel inactivation/nystatin/cesium loading/*Aplysia* neurons)

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ABSTRACT **Inactivation of the Ca channel of *Aplysia* neurons was studied in the absence of potassium current in cells that were cesium-loaded with the aid of the ionophore nystatin. Inactivation was substantially decreased by methods that limited Ca entry. Depolarizations commensurate with the equilibrium potential of Ca resulted in minimal inactivation. Replacement of extracellular Ca by Ba also decreased inactivation. It is concluded that inactivation of the Ca channel is a function of the extent of Ca entry rather than membrane potential, thus differing fundamentally from the purely voltage-dependent mechanism for sodium inactivation.**

The importance of transient changes in levels of intracellular free Ca in various cell functions has led to increased interest in the properties of the Ca channel that is activated by membrane depolarization. Voltage-dependent Ca entry has been demonstrated in muscle fibers (1), nerve cell bodies (2), axons (3), presynaptic terminals (4), and dendrites (5). Rapid increases in intracellular Ca concentration function in such diverse processes as excitation-contraction coupling (6), synaptic transmission (7), and the activation of a Ca-mediated potassium conductance (8).

Voltage-clamp analyses of Ca current (I_{Ca}) and the behavior of the Ca channel have been hampered by the presence of other simultaneous ionic currents, especially that of potassium (I_K), that sum algebraically with I_{Ca}. A number of experimental procedures have been devised to circumvent this problem in molluscan neurons. Use of the calcium-sensitive molecules arsenazo III (9) and aequorin (10–12) allows monitoring of changes in intracellular Ca levels and indirect measurement of Ca entry. Direct measurement of I_{Ca} has been made possible by minimizing competing currents with internal perfusion (13–15) and nystatin-mediated loading to replace intracellular K (16, 17). In the present study the ionophore nystatin was used to substitute the impermeant ion Cs^+ for intracellular K^+, thereby minimizing I_K during depolarization.

In previous voltage-clamp studies, inactivation of the Ca channel has been observed both as a relaxation of I_{Ca} during a single depolarizing pulse (14, 15, 18) and as a decreased peak amplitude I_{Ca} during the second of two closely spaced depolarizing pulses (17). In two studies on molluscan neurons (15, 19), of which only one used a method for minimizing I_K (15), it was suggested that the mechanism of Ca inactivation is similar to that for Na inactivation. The experiments of the present report offer quite a different conclusion: that Ca inactivation results from the extent of Ca entry by a process not directly dependent upon membrane potential and therefore differs fundamentally from the purely voltage-dependent Na channel inactivation in the Hodgkin–Huxley formulation (20). Some of these experiments have appeared in abstract form (21).

MATERIALS AND METHODS

Experiments were performed on single identified neurons of *Aplysia californica* that were kept in a self-contained seawater system. The visceral ganglion was dissected and treated with protease (Sigma) to facilitate the removal of the connective tissue layer surrounding the cells. The giant somata R-15, R-2, and R-14 (22) were exposed and Cs was substituted for intracellular K with the use of the ionophore nystatin by using a slightly modified version of the nystatin procedure described previously (16). The exposed cells were initially bathed in a Cs-loading solution (Table 1) containing nystatin (Calbiochem; 60 mg/liter). After 15–20 min of loading, which has been shown to lower intracellular K concentration by 90% in these cells (16), the nystatin was washed out of the membrane with nystatin-free loading solution. After 30 min of washing, the cells were bathed in a test solution (generally 100Ca0Na). The cell of choice was then impaled with voltage-monitoring and current-passing electrodes (filled with 3 M CsCl; 3–6 MΩ and 0.3–1.0 MΩ, respectively). The reference electrode (filled with 3 M CsCl in 2% agar; 0.3–0.5 MΩ) was positioned within a few millimeters of the cells to minimize bath series resistance.

Membrane potential was controlled under voltage clamp as described (11). The holding potential was set to −40 mV (the approximate resting potential) and step pulses had rise times of 0.4–1.0 msec. Within any single experiment, pulse pairs were separated by 30 sec to allow for recovery processes. The potential of the recording electrode was checked for drift after each experiment.

The preparation was bathed at 15°C (±1°C) in test solutions containing no K, limiting intracellular accumulation and possible development of I_K, and no Na, eliminating both Na current and possible artifacts due to action potentials in uncontrolled regions of the axon. Signals were stored electronically on tape and were reproduced on a strip chart recorder.

RESULTS

The effectiveness of the Cs-loading procedure for the elimination of outward I_K is illustrated in Fig. 1*A*. Shown are typical records of 100-msec voltage steps to +20 mV in both an untreated (left) and a Cs-loaded (right) cell in solutions containing no Na. Through use of the loading procedure, Cs^+ ions, known for their ability to block K channels (23), were substituted for an estimated 90% of the normal intracellular K^+ ions (16). The net inward current that remained after Cs loading varied with the extracellular Ca concentration (unpublished observations) and was blocked by cobalt (17), indicating that the current is carried by Ca. Blockage of the I_{Ca} by Co left a time-invariant outward current that became prominent only at large (+60 mV)

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Abbreviations: I_{Ca}, Ca current; I_K, K current; PI and PII, first and second depolarizing pulses, respectively; I_{PII}, current during PII.

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Table 1. Composition of test solutions*

Test solutions	Composition, mM						
	NaCl	KCl	$CaCl_2$	$BaCl_2$	$MgCl_2$	Tris†	$CoCl_2$
10Ca0Na	0	0	10	0	140	410	0
100Ca0Na	0	0	100	0	50	410	0
100Ba0Na	0	0	0	100	50	410	0
0Ca20Co	0	0	0	0	140	410	20

* The Cs-loading solution was 300 mM CsCl/394 mM sucrose/100 mM $MgSO_4$/10 mM Tris. Nystatin was dissolved in methanol (2.5 mg/ml) and added to loading solution at a final concentration of 60 mg/liter.
† Solutions were adjusted to pH 7.7 with HCl.

positive membrane potentials. There was an apparent absence of outward current through channels that exhibit the time-variant kinetics characteristic of voltage-activated K channels. In addition, extracellular application of 50 mM tetraethylammonium chloride, known to block the Ca-activated K channel in *Helix* (24) and *Aplysia* (25) neurons, had no significant effect on the peak amplitude and the relaxation kinetics of I_{Ca} recorded during single depolarizing pulses in Cs-loaded cells (unpublished observations). Furthermore, no net outward current was ever observed (after subtraction of leakage current) with step depolarizations to +20 mV for 5 sec. Thus, neither the amplitude nor the time course of the I_{Ca} recorded in Cs-loaded cells should be contaminated by either a voltage-dependent or Ca-activated I_K.

The amplitude of I_{Ca}, recorded from a Cs-loaded R-2 cell, is plotted against the membrane potential of a clamp pulse in Fig. 1*B*. The time-invariant outward leak, which increases nonlinearly with membrane potential, was measured in a Ca-free solution containing the Ca channel blocker Co and subtracted from current measured in 100 Ca0Na. This leakage correction procedure gives a more accurate representation of the current-voltage relationship for Ca and shows that I_{Ca} amplitude reaches a maximum in the voltage range +40 to +60 mV and is decreased with further increases in membrane potential. The current approached zero in the range of estimated Ca equilibrium potential, around +160 mV.

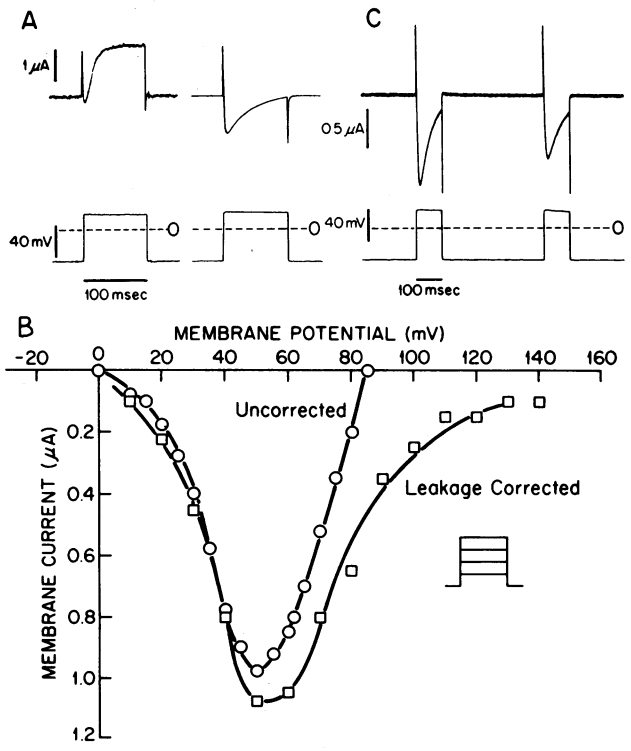

FIG. 1. Net current during voltage-clamp depolarization. (*A*) Comparison of currents during 100-msec pulses to +20 mV from a holding potential of −40 mV in two different R-2 cells: an untreated cell (left record) and a Cs-loaded cell (right). Both cells were bathed in 10Ca0Na test solution (see Table 1). (*B*) Peak amplitude of I_{Ca} plotted against membrane potential of a single 100-msec clamp pulse. o, Directly measured current recorded in 100Ca0Na (Table 1); □, current corrected for leakage by subtraction of the time-invariant outward current recorded in the same cell in 0Ca20Co (Table 1). Holding potential (V_h) = −40 mV, cell R-2. (*C*) I_{Ca} recorded during identical clamp pulses to +22 mV in a Cs-loaded R-2 cell; V_h = −40 mV, 15°C.

Fig. 1*C* shows membrane currents obtained with a Cs-loaded R-2 cell bathed in 100Ca0Na during a pair of identical depolarizing pulses (to +22 mV, and lasting 100 msec) separated by 400 msec. The peak amplitude of the inward I_{Ca} during the second pulse (PII) was clearly smaller than that produced during the first pulse (PI). The effect of varying the potential of PI on the peak amplitude of the inward current recorded during a constant-potential PII is shown in Fig. 2. The pair of 100-msec-duration pulses was separated by an interval of 200 msec, and PII was held constant at +20 mV. Fig. 2*A* shows representative records in 100Ca0Na. The peak amplitude of the current during PII (I_{PII}) decreased and then increased again as the potential of PI increased. I_{PII}, normalized by dividing by the amplitude of I_{PII} obtained without PI, was plotted against the membrane potential of PI (Fig. 2*C*). I_{PII} reached its minimum in the range of PI potential +25 to +70 mV. Subsequently, I_{PII} approached unity as PI approached +150 mV.

These experiments suggest that the decrease of I_{PII} depends on the extent of Ca entry during PI rather than on the potential of PI. This is most easily seen by comparing the Ca curve of Fig. 2*C* to the leakage-corrected I_{Ca} curve of Fig. 1*B*. The peak amplitude of I_{PII} was minimal in the PI voltage range where peak I_{PI} was maximal. Then, as the voltage of PI was further increased toward Ca equilibrium potential E_{Ca} (estimated in snail neurons to be +130 to +200 mV) (14), there was a progressive return toward control values of the peak amplitude of I_{PII} as the amplitude of peak I_{PI} approached zero. The small remaining reduction of I_{PII} after the large depolarizing PIs (>+120 mV), recorded in Ca solution, may be the result of Ca entry during the tail current following the repolarization of PI.

Fig. 2*B* shows the results obtained with the same cell when extracellular Ca was replaced with Ba in 100Ba0Na. There were notable differences between results obtained in the Ba and in the Ca solutions. The inward current in the Ba medium is identified as barium current; its peak amplitude was greater than that of I_{Ca} during identical depolarizing voltage steps. Ba appears to carry a larger current than Ca in the Ca channel (Fig. 2*B*), and yet I_{PII} was decreased significantly less in Ba than in Ca solutions. The Ba influx recorded during PI, which was greater than that of Ca for a given depolarization, should result in a greater decrease in driving force on Ba than occurs on Ca,

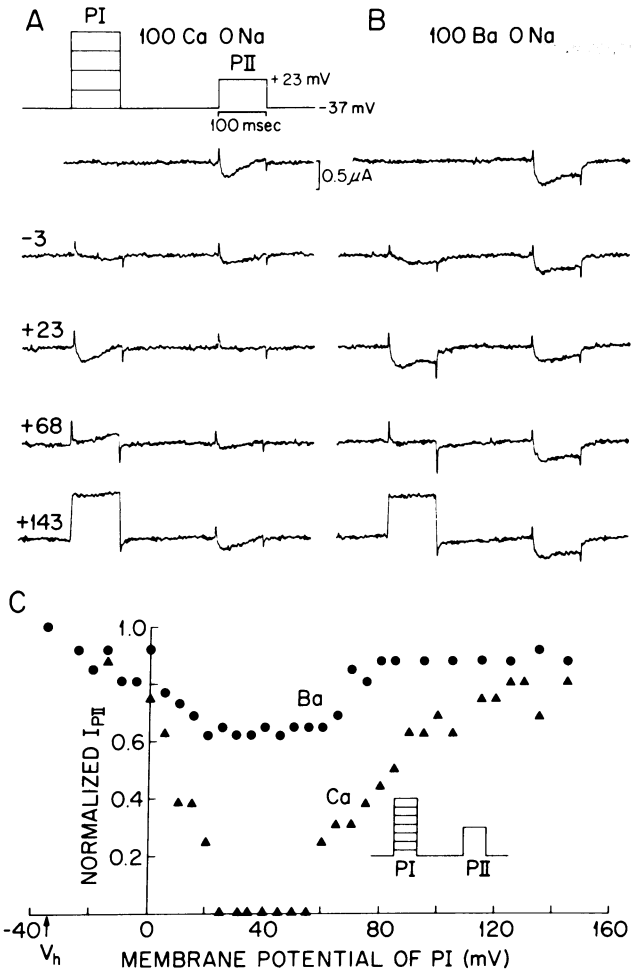

FIG. 2. Effect of varying the voltage of PI on current during PII. Holding potential (V_h) was −37 mV and temperature was 15°C (±1°C). The 100-msec pulses were separated by 200 msec. Voltage during PII was +20 mV. (*A* and *B*) Records of the variable-amplitude PI experiment in 100Ca0Na and 100Ba0Na test solutions. Numbers at left of each record give membrane potential (mV) during PI. (*C*) Plot of normalized current (ratio of peak PII current with PI to that without PI) against voltage of PI in Ca (▲) and Ba (●) solutions.

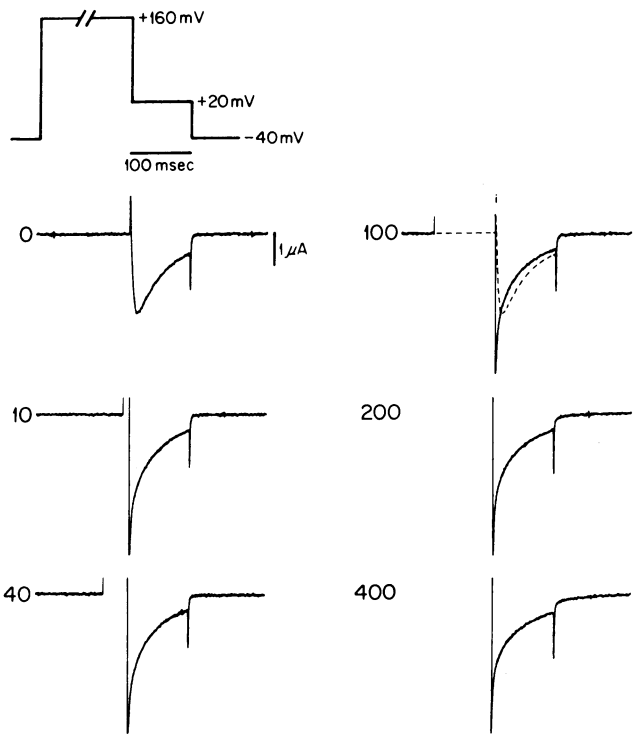

FIG. 3. Test for voltage-dependent effect on peak Ca current reduction. Shown are current records from R-2 during 100-msec test pulses to +20 mV preceded with no interval by a prepulse of variable duration to +160 mV. Prepulse durations (msec) are given at the left of each current record. The test pulse current, recorded without prepulse, is repeated as a tracing over the tail current following the prepulse in the 100-msec record. The outward leakage during the prepulse was off scale.

as a result of its entry. This argues against the possibility that the depression of I_{PII}, when carried by Ca, is due to a reduction in driving force resulting from Ca accumulation near the inner surface of the membrane during PI. In view of the minimization of outward I_K by the Cs-loading procedure and the apparent absence of a significant change in driving force on Ca, it is concluded that the reduction of peak amplitude of I_{PII} is the result of decreased Ca conductance and may therefore be properly termed "Ca inactivation."

The two-pulse experiment does not eliminate the possibility that, in addition to the Ca-dependent inactivation described above, there is voltage-dependent inactivation of I_{Ca} that is removed in the interval between the two pulses and is thus not assayed. The conditions of the experiment of Fig. 3 were chosen to maximize any voltage-dependent effect: the interval separating the pulses was eliminated to avoid time-dependent removal, and a very large prepulse of progressively longer duration was used to limit the extent of Ca entry to maximize any time- and voltage-dependent effect. Except for its immediate strong onset following the capacitive transient, the tail current (recorded during the test pulse following the prepulse) resembled the control inward I_{Ca} (recorded without a prepulse). This can be seen in the tail current record following the 100-msec prepulse over which the control current was traced (broken line). Changing the duration of the prepulse caused little decrease in the tail currents.

Two observations indicate that the Ca channels remained fully activated during the prolonged +160-mV prepulse: (*i*) the tail current was maximal immediately following the prepulse and (*ii*) the tail current resembled the control current except for its more rapid and strong onset and slightly faster relaxation. The slightly greater depression of the tail current compared to the control I_{Ca} can be explained as a consequence of the initial strong entry of Ca through fully activated channels immediately upon termination of the prepulse.

DISCUSSION

The major conclusion of the two-pulse experiments is that Ca inactivation, measured as the depression of the peak amplitude of I_{Ca} during PII, depends on the extent of Ca entry during PI rather than on the potential of PI. Two observations indicate that this conclusion is also true for Ca inactivation measured as the relaxation of I_{Ca} during single depolarizing-voltage pulses: when Ba is substituted for extracellular Ca, the net inward barium current shows significantly less relaxation for a given potential level (Fig. 2*B*); neither the apparent kinetics of relaxation nor the amplitude (at times following peak amplitude of the control current) of I_{Ca} are significantly affected by a variable-duration, large-amplitude prepulse to a potential approaching Ca equilibrium potential (Fig. 3).

The term "inactivation" has been chosen to describe the depression of I_{Ca} measured either with voltage-pulse-pairs or during single pulses. Evidence has been presented that indicates

that this is not the result of activation of a residual outward I_K. The possibility remains that the apparent Ca-dependent inactivation of I_{Ca} is a function of a reduction of driving force due to significant accumulation of Ca near the inner surface of the membrane. The much smaller depression of barium current compared to I_{Ca}, in spite of the larger barium influx at a given potential, argues against this interpretation. Another line of evidence against this explanation comes from experiments with perfused cells [barnacle muscle (26), snail neuron (14), and tunicate egg (18)] in which intracellular free Ca was buffered to an increased level with respect to normal free Ca. Although the free Ca was increased to levels much lower than would significantly affect driving force on Ca, the increase causes substantial or complete depression of I_{Ca}. The evidence, then, favors a change in Ca conductance over a reduction in driving force to account for the present findings made in Cs-loaded cells whose natural buffering capacity is presumably intact.

These findings suggest alternative interpretations of several experimental results. The conclusion (19) that the inactivation mechanism of I_{Ca} in *Helix aspersa* is similar to that of Na inactivation is in clear conflict with the above findings. Na inactivation is strongly potential dependent (20). The lack of direct dependence on membrane potential demonstrated here in a molluscan system and in *Paramecium* (27) indicates that inactivation of the Ca channel occurs by a process qualitatively different from the inactivation of the Na channel. It has also been reported that Ca accumulation is responsible for the reduction in Ca-dependent outward current during the second of a pair of identical clamp pulses (28). Ca inactivation, as demonstrated in the present study with a paired-pulse experiment, results in depression of Ca entry during the second pulse. This reduced Ca entry and accumulation may be sufficient to account for the observed depression of Ca-activated K current.

It can be concluded from these results that, although the Ca channel exhibits voltage-dependent activation, its inactivation is not a direct consequence of membrane depolarization. Instead, Ca entry during depolarization is responsible for inactivation of the Ca channel. Because this mechanism has been found in two phylogenetically disparate groups [i.e., Mollusca and Protozoa (27)], it is likely to be a general feature of Ca channels throughout the animal kingdom.

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