J. Physiol. (1983), **345**, *pp.* 549–565
With 9 text-figures
Printed in Great Britain

INACTIVATION OF CALCIUM CONDUCTANCE CHARACTERIZED BY TAIL CURRENT MEASUREMENTS IN NEURONES OF *APLYSIA CALIFORNICA*

BY ROGER ECKERT* AND DOUGLAS EWALD

From the Department of Biology and Ahmanson Laboratory of Neurobiology, University of California, Los Angeles, CA 90024, and Friday Harbor Laboratories, Friday Harbor, WA, U.S.A.*

(*Received 22 February 1983*)

SUMMARY

1. Calcium tail currents, recorded at −40 mV after repolarization from 7 or 10 ms voltage-clamp depolarizations in axotomized *Aplysia* neurones in the presence of tetrodotoxin and tetraethylammonium, were used to investigate the inactivation of the calcium conductance without interference from contaminating potassium currents.

2. Prior depolarization with a prepulse (V_1) resulted in a reduction in size of the tail currents recorded following the test pulse (V_2). The reduction occurred in both the fast ($\tau_1 < 0{\cdot}4$ ms) and slow ($\tau_2 \approx 2{\cdot}0$ ms) components of the tail current.

3. The degree of inactivation remained constant when tail currents were measured at potentials ranging up to 30 mV on either side of the potassium equilibrium potential. Thus, any changes in potassium current must have contributed virtually nothing to the changes in tail current amplitude seen following presentation of the prepulse.

4. Inactivation was greatest following prepulses to potentials (+10 to +40 mV) that produce maximal entry of calcium ions, and declined to about zero as the prepulse approached the calcium equilibrium potential.

5. For V_1 potentials above +50 mV, the prepulse caused an apparent short-term facilitation of V_2 tail currents in EGTA-injected neurones. This effect, detected up to 50 ms following the pulse, is of uncertain origin.

6. Pressure injection of calcium ions caused reduction in the size of the tail current, which was restored by subsequent injection of EGTA.

7. Tail current amplitude was reduced by presentation of the prepulse for all test pulse voltages, but the measured inactivation declined exponentially towards a minimum with test pulses of increasingly positive potential.

8. Removal of inactivation following a 200 ms prepulse was greatly accelerated by injection of EGTA. The EGTA-resistant inactivation remaining at short times decayed with a time constant of about 0·12 s. The relation of tail current reduction to prepulse voltage is consistent with the interpretation that the EGTA-resistant inactivation remaining at short times depends on entry of calcium ions during the prepulse, as does the EGTA-sensitive inactivation remaining at later times.

* Address for reprint requests.

9. It is proposed that the 'EGTA-resistant' phase of inactivation results from loading of EGTA with calcium ions near the inner surface of the membrane during sustained calcium entry, allowing the intracellular calcium concentration to rise.

10. The results provide further evidence for a calcium-mediated inactivation of the calcium conductance. No evidence was found in these cells for voltage-dependent inactivation of calcium channels. However, a voltage-dependent facilitation of the inward current was detected at short times in EGTA-injected neurones following prepulses to positive membrane potentials.

INTRODUCTION

Voltage-clamp studies on the ciliate *Paramecium caudatum* (Brehm & Eckert, 1978; Brehm, Eckert & Tillotson, 1980), and on caesium-loaded molluscan neurones (Tillotson, 1979; Eckert & Tillotson, 1981) first indicated that the calcium conductance undergoes inactivation dependent on the accumulation of free calcium ions at the cytoplasmic side of the surface membrane during calcium current flow. The calcium current recorded during a test depolarization (V_2) is reduced in amplitude if calcium ions enter the cell during a prior depolarization (V_1). Procedures that reduce the entry of calcium during V_1 or that interfere with the intracellular rise in ionized free calcium were found to diminish the drop in amplitude of the peak calcium current recorded during V_2. Similar findings suggesting a calcium-mediated inactivation of the calcium conductance have been reported for insect muscle (Ashcroft & Stanfield, 1981), myocardium (Marban & Tsien, 1981) and *Helix* neurones (Brown, Morimoto, Tsuda & Wilson, 1981; Plant & Standen, 1981; Standen, 1981). The dependence of calcium channel inactivation on intracellular calcium ions in these tissues differs from the well-established voltage-dependent inactivation of the sodium channel (Hodgkin & Huxley, 1952). In addition, calcium currents found in some tissues, such as frog muscle and polychaete eggs, appear not to exhibit calcium-dependent inactivation (Almers & Palade, 1981; Fox & Krasne, 1981; Cota & Stefani, 1982). Thus, it is important to rule out possible alternative mechanisms that might account for the calcium-dependent reduction in the measured inward current seen in some tissues.

One alternative explanation for a calcium-dependent reduction in the measured inward current is the activation of a calcium-dependent outward current that could sum with the calcium current to diminish the recorded net inward current. One class of potassium channels is activated under depolarization by an elevation of internal free calcium ions due to current flow (Meech & Standen, 1975; Eckert & Tillotson, 1978; Gorman & Hermann, 1979; Lux, Neher & Marty, 1981; Marty, 1981). Even in caesium-loaded cells and in cells bathed in tetraethylammonium (TEA) ions, outward current can be recorded at high positive membrane potentials, and at lower potentials some partial outward current may be hidden in the net inward current. If present, such a partial outward current could produce effects mistaken for changes in the calcium current. Previous work (Brehm *et al.* 1980; Eckert & Tillotson, 1981; Eckert, Tillotson & Brehm, 1981; Plant & Standen, 1981) has addressed the possibility that the reduction of inward current results simply from a calcium-dependent enhancement of an outward potassium current, and several lines of evidence suggest that the reduction in current is due to a real inactivation in calcium current, although the

presence of a simultaneous outward current was not fully ruled out. In the experiments described below we have avoided the problem of contaminating potassium currents by measuring calcium tail currents with the membrane potential (V_m) sufficiently close to the potassium equilibrium (E_K) to assure virtual isolation of the calcium current (Eckert & Ewald, 1981, 1983). High concentrations of extracellular TEA further diminished the likelihood of potassium current at those potentials. The findings provide further evidence for a true inactivation of the calcium conductance by an action of intracellular calcium ions. Preliminary reports have appeared elsewhere (Eckert, 1981; Eckert & Ewald, 1981, 1982*a*).

METHODS

Cells L2–L6 of *Aplysia californica* were used. The methods of preparation, solutions, temperature, measurement techniques and computer analyses were identical to those described in the preceding paper (Eckert & Ewald, 1983). In most of the experiments a double-pulse technique was used in which a long (100 or 200 ms) conditioning pulse, V_1, preceded a short (7 or 10 ms) test pulse, V_2, by typically 1000 ms. The V_1–V_2 pair was repeated at precise 20 s intervals; this was extended to longer intervals in some experiments in order to minimize further any use-dependent accumulation of inactivation.

Inactivation was determined as equivalent to the ratio $(I_0-I)/I_0$, termed inactivation index, in which I is the amplitude of a test pulse tail current when the test pulse was preceded by V_1, and I_0 is the amplitude of the tail current without presentation of V_1. Tail current amplitudes were taken 400 μs following the end of the V_2 command. At this point the true membrane potential, following all but the largest test pulses, was estimated to be within 2 mV of the measured value, and the fast component of current decay, τ_1, had an amplitude about ten times that of the slower component, τ_2 (Eckert & Ewald, 1983).

RESULTS

Effects of V_1 on V_2 tail currents

Presentation of V_1 1000 ms before V_2 caused a decrease in over-all amplitude of the tail current recorded at a V_m of -40 mV following the termination of the 7 ms V_2 (Fig. 1*A*). The inward current during V_2 was also depressed as reported earlier (Tillotson & Horn, 1978; Tillotson, 1979; Eckert & Tillotson, 1981). The time course of the inward current tail recorded following 7–10 ms test pulses can be fitted by the sum of a fast and a slow exponential having time constants τ_1 and τ_2, respectively (Eckert & Ewald, 1983). The τ_1 component is an order of magnitude larger in amplitude than the slower component, and a reduction in its amplitude accounts for most of the loss in over-all amplitude seen with presentation of V_1. Presentation of V_1 produced no significant changes in the time constants of the two components (Fig. 1*B*).

In the experiments reported here, V_m following the test pulse was normally returned to a holding potential of -40 mV, which was within 15 mV or less of E_K (determined from potassium tail currents before addition of TEA to the bath). It was necessary to determine whether a hidden, use-dependent potassium current could produce any changes in net amplitude of the inward current tails if there was a discrepancy between E_K and V_m. Measurements of the tail currents following V_2 were made with V_1 'on' and 'off' at values of V_m both positive and negative to E_K (Fig. 2*A*). The time constant τ_1 showed some voltage dependence, as described earlier

(Eckert & Ewald, 1983), but was independent of V_1. Likewise, the ratio $(I_0-I)/I_0$ remained constant (*ca.* 0·35 in this experiment) for potentials of −70 to −10 mV ($E_K = -47$ mV) (Fig. 2*B*). It appears, then, that under the conditions of these experiments precise agreement between E_K and V_m was not a significant factor in the reduction of the tail current amplitude brought about by a prepulse.

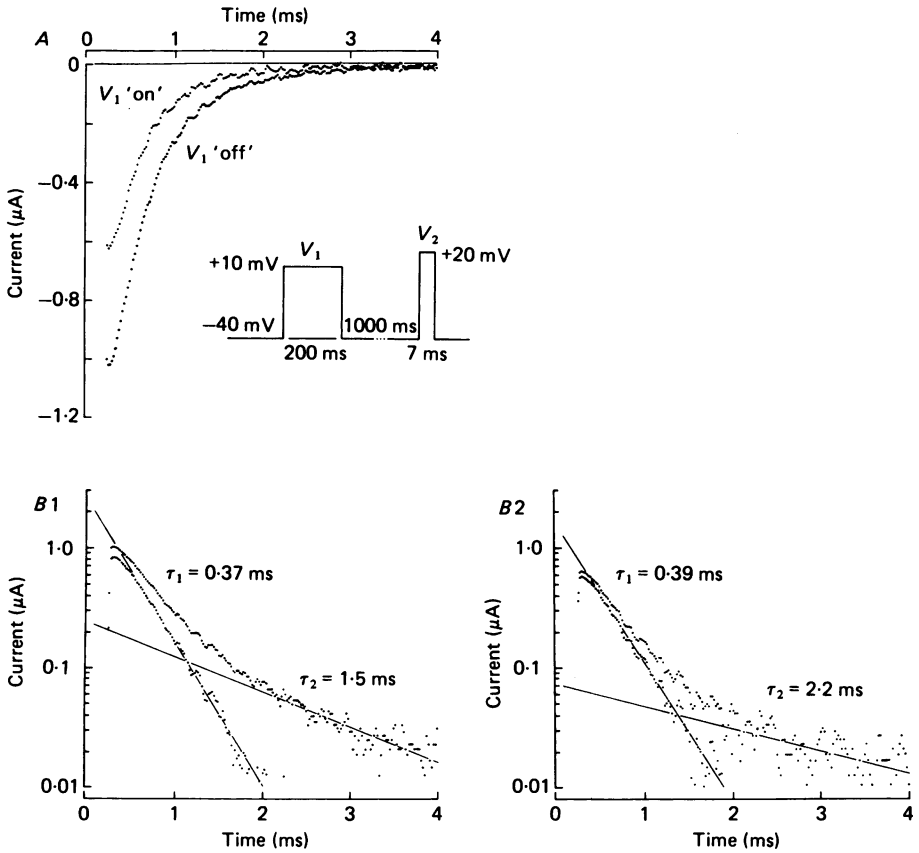

Fig. 1. Reduction in amplitude of V_2 tail current associated with presentation of V_1. Inset shows protocol. The 7-ms-long V_2 is presented either alone (V_1 'off') or preceded by a conditioning pulse (V_1 'on'). *A*, tail currents recorded following termination of V_2. With V_1 'on' the tail current is reduced in amplitude. *B*1, computer-generated fits of the first (τ_1) and second (τ_2) exponential components of the tail current described earlier (Eckert & Ewald, 1983), with V_1 'off'. *B*2, as for *B*1 but with V_1 'on'.

Calcium-dependent inactivation

Since calcium entry during V_1 depends on the driving force, $V_m - E_{Ca}$ (where E_{Ca} is the calcium equilibrium potential), as well as on elevated calcium conductance, it is possible to vary calcium entry by varying V_1. By making V_1 equal to or close to E_{Ca}, entry of calcium ions is prevented, and the calcium-dependent process suppressed even though the calcium conductance is fully activated (Katz & Miledi, 1966; Eckert & Lux, 1977). This effect can be seen in Fig. 3, in which the amplitudes of τ_1 and

τ_2 tail current components are plotted against V_1 voltage before and after injection of EGTA, which is reported to lower the intracellular calcium (Alvarez-Leefmans, Rink & Tsien, 1981). Before EGTA injection the amplitudes of both tail current components pass through a minimum at values of V_1 between +10 mV and +40 mV, the range of voltages at which maximum calcium entry is detected with calcium

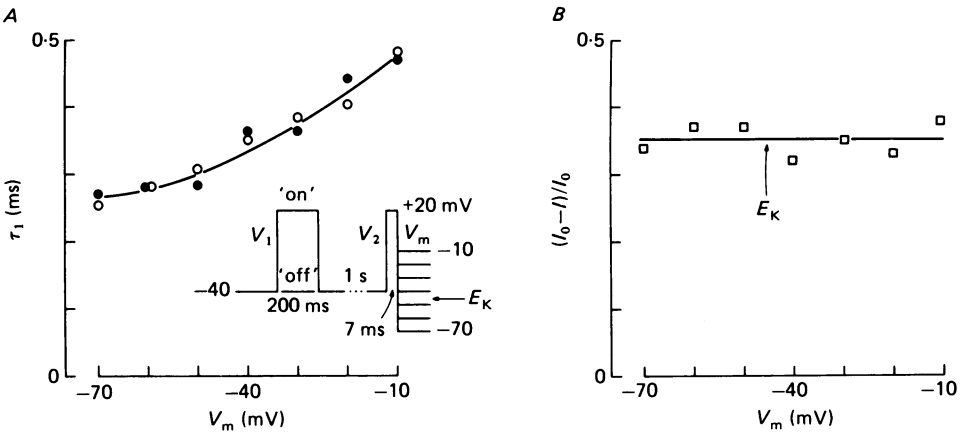

Fig. 2. Tail currents measured at different membrane potentials with V_1 'off' and 'on'. *A*, time constant of the τ_1 component of tail current plotted against the potential, V_m, to which membrane was clamped following V_2. The open circles indicate V_1 'on'; the filled circles, V_1 'off'. *B*, inactivation of the τ_1 component plotted against V_m. The amplitude of τ_1 was measured 400 μs following the end of V_2. Inactivation was determined as the fractional loss of amplitude seen with V_1 'on'. Loss of τ_1 amplitude was independent of V_m. The value of E_K was determined before TEA application from the reversal potential of potassium tail current.

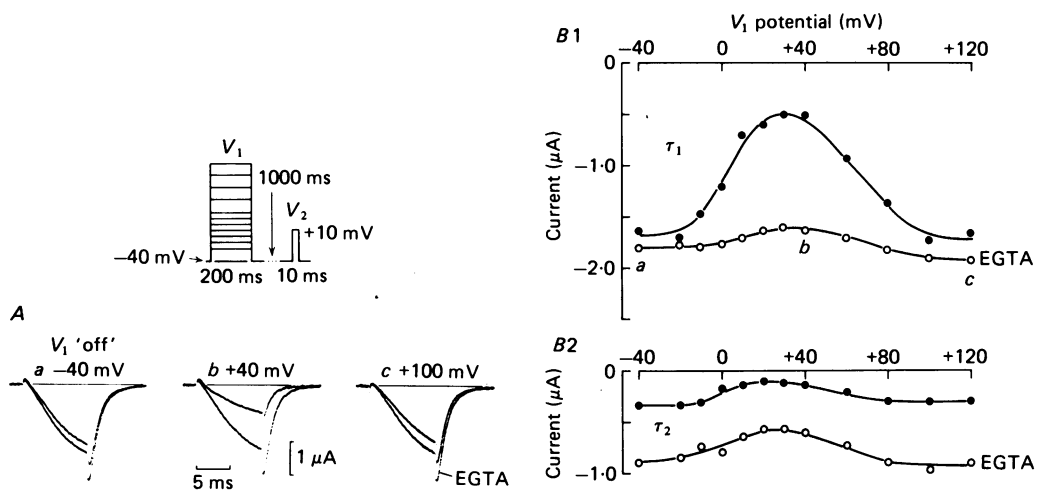

Fig. 3. Inactivation as a function of V_1 potential. *A*, representative V_2 current traces made with V_1 at −40 (i.e. 'off'), +40 and +100 mV before (smaller currents) and after (larger currents) injection of EGTA. *B*1 and *B*2, computer-assisted measurements of the τ_1 and τ_2 phases, respectively, made 500 μs following the end of V_2, plotted against V_1 potential. Filled circles are before, and open circles after injection of EGTA.

indicators (Eckert & Tillotson, 1978; Gorman & Thomas, 1980). The current recorded *during* V_2 exhibits a similar relation to V_1 potentials (Brehm & Eckert, 1978; Tillotson, 1979; Eckert & Tillotson, 1981; Plant & Standen, 1981). Inactivation of both components of the tail current diminished and disappeared as V_1 approached +120 mV. Following injection of EGTA, the amplitude of the τ_1 component became

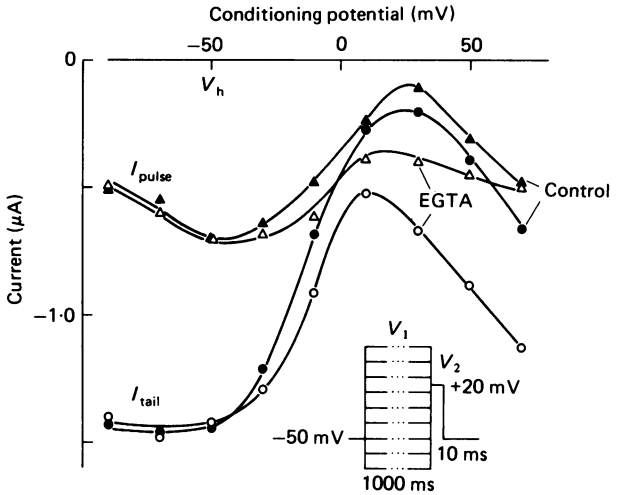

Fig. 4. Effect of prolonged V_1 conditioning step on V_2 calcium currents. The 10 ms V_2 was preceded without interval by a 1000 ms V_1. Triangles indicate current recorded during the test pulse just before its termination (I_{pulse}); circles indicate tail current amplitude measured 500 μs after termination of the test pulse (the τ_1 and τ_2 components were not separately determined). Open symbols indicate currents measured after injection of EGTA. The decrease in I_{pulse} amplitude at potentials more negative than −50 mV is attributed to removal of inactivation, by increased negative potentials, of the early transient potassium current, $I_{K(A)}$.

nearly constant for all values of V_1, with only a small inactivation remaining for the mid-range voltages of V_1 (Fig. 3*B*1). Reduction in amplitude of the τ_2 component by calcium entry during V_1 was more resistant to injected EGTA than was the reduction exhibited by the τ_1 component (Fig. 3*B*2).

In a related experiment, analogous to the Hodgkin & Huxley (1952) h_∞ experiment, the V_1–V_2 interval was reduced to zero, and V_1 was prolonged to 1000 ms (Fig. 4). The amplitude of the V_2 tail current (τ_1 and τ_2 components measured together) went through the characteristic minimum for mid-range V_1 voltages. However, the effect of the EGTA injection in suppressing inactivation was smaller under these conditions than it was when a 1 s interval separated V_1 and V_2. The lower effectiveness of EGTA in suppressing inactivation in the absence of a V_1–V_2 interval may indicate either: (i) a calcium-independent, voltage-dependent component of inactivation that is revealed at short times and is removed during long intervals, or (ii) a failure, under the load of calcium entry during a prolonged V_1, of the injected EGTA to buffer adequately the calcium in the micro-environment close to the inner surface of the membrane.

As an alternative method of increasing the intracellular calcium ion activity,

calcium chloride was pressure-injected (Standen, 1981) into the cell during a sequence of 10 ms test depolarizations. The injection caused the tail currents to drop to approximately half their control amplitudes (Fig. 5). Subsequent injection of EGTA from a second, pre-inserted pipette restored the tail current to its original amplitude. There was no measurable change in time constants τ_1 and τ_2 following injection of calcium or EGTA.

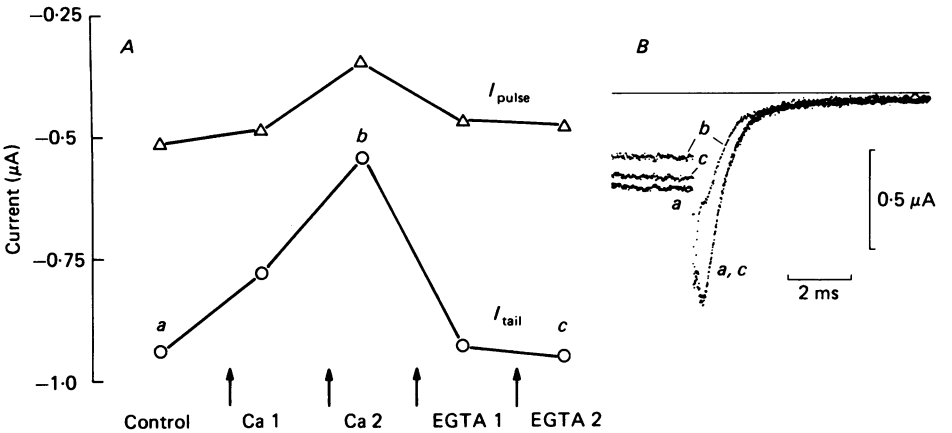

Fig. 5. Inactivation of calcium conductance by calcium injection, and removal of the inactivation by injection of EGTA. *A*, amplitudes of I_{pulse} (triangles) and I_{tail} (circles) elicited by a 10 ms pulse to +20 mV. Two pressure injections of calcium chloride from a pipette containing 100 mM-calcium chloride produced partial inactivation of the currents. Subsequent ionophoretic injection of EGTA reversed the inactivation. *B*, superimposed records of the currents before (*a*) and after the two calcium injections (*b*), and after the second EGTA injection (*c*). Semilogarithmic plots of the tail currents (not shown) yielded values of 0·52 ms for τ_1, which remained unchanged by calcium and EGTA injection. The value of τ_2 changed from 2·2 ms before calcium injection to 4·7 ms following calcium injection. After injection of EGTA τ_2 was 2·6 ms.

Effect of prior calcium entry on current–voltage relations

Amplitudes of the current measured just before termination of the test depolarization (I_{pulse}) were determined along with amplitudes of the tail current (I_{tail}) for depolarizations ranging from −10 to +80 mV (Fig. 6*A*). For each value of V_2 the test pulse was presented alternately with V_1 (to +20 mV) 'on' and 'off'. Presentation of V_1 caused a reduction in I_{tail} at all values of V_2, and also caused a reduction in I_{pulse} at potentials where I_{pulse} was net inward.

The net outward I_{pulse} seen at potentials above about +50 mV, ascribed to an outward current exceeding I_{Ca}, was also diminished by the presentation of V_1. The behaviour of this outward current suggests that it consists primarily of the voltage-dependent delayed rectifier current, $I_{K(V)}$. Whereas the calcium-dependent current $I_{K(Ca)}$ decreases in these cells in response to a prepulse that causes inactivation of the calcium current (Eckert & Ewald, 1982*b*), it is also sensitive to injected EGTA, which this current is not; the non-specific current is reported not to undergo inactivation (Byerly & Hagiwara, 1982); and the transient outward current $I_{K(A)}$ is

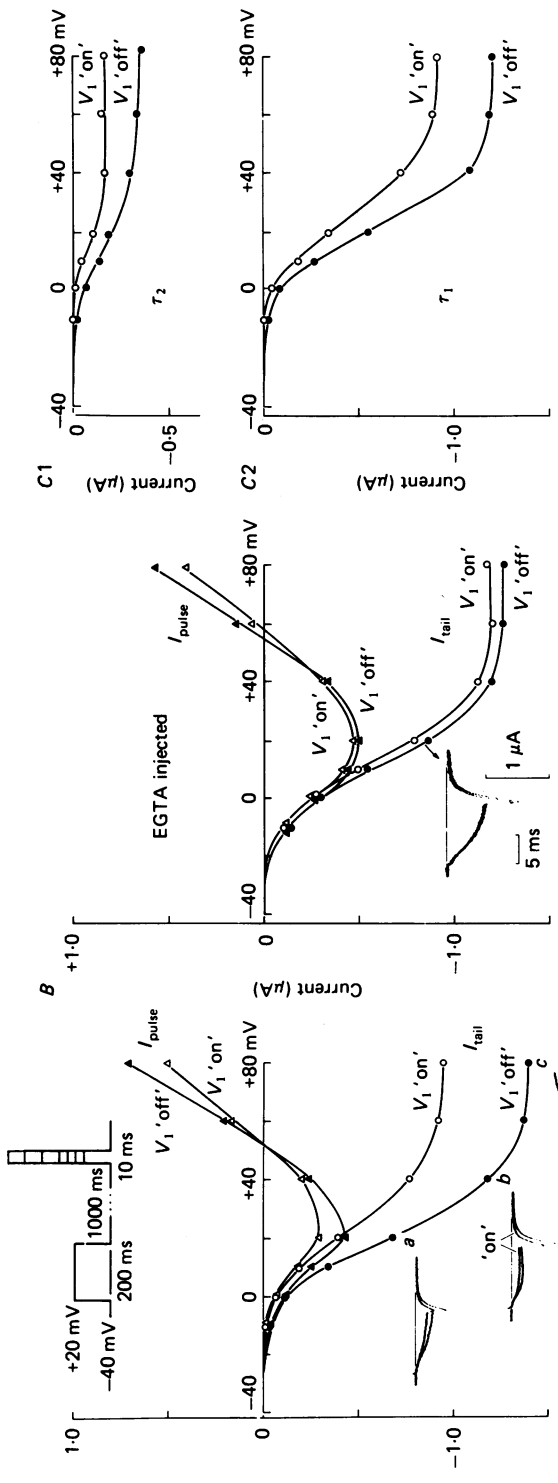

Fig. 6. Effect of prepulse on current–voltage relations of test pulse currents. *A*, amplitude of I_{pulse} (▲) measured at the end of a 10 ms V_2, and amplitude of I_{tail} (●) as a function of V_2 potential. V_2 currents recorded following presentation of a 200 ms V_1 to +20 mV ending 1000 ms before the test pulse are indicated by the corresponding open symbols (△, ○). Inserts show superimposed traces with and without V_1 'on' for V_2 values to +20 mV (*a*), +40 mV (*b*) and +80 mV (*c*). Note that at high V_2 voltages presentation of V_1 causes a *drop* in the outward current contaminating I_{pulse}, which is opposite in direction to the concomitant reduction in I_{tail}. *B*, the corresponding current–voltage relations following EGTA injection. *C1* and *C2*, I_{tail} separated into τ_2 and τ_1 components, respectively, plotted against V_2 potential. The computer-assisted measurements were made at 500 μs following the end of V_2.

essentially absent at a holding potential of −40 mV (Hermann & Gorman, 1981*a*). Thus, $I_{K(V)}$ remains the most likely candidate for outward current contamination.

Following injection of EGTA, inactivation manifested as reduced amplitude of I_{tail} was greatly diminished (Fig. 6*B*), whereas the inactivation of the outward I_{pulse} seen at high potentials remained essentially unaltered. Failure of injected EGTA to suppress the outward current suggests that any residual outward current contamination of I_{pulse} that may remain under the conditions of these experiments is independent of intracellular calcium accumulation, and thus cannot be responsible for calcium-dependent changes in amplitude of the tail current. Since the characteristics of the outward current seen during test pulses to high potentials cannot account for the reduction in tail current produced by the prepulse, the most likely cause of the reduction in the current is a partial inactivation of the calcium conductance.

The τ_1 and τ_2 components plotted separately (Fig. 6*C*) both exhibit sigmoidal current–voltage relations. However, the τ_2 component exhibited a greater percentage inactivation than τ_1.

Voltage dependence of the inactivation index

It was noted earlier that the decrease in the V_2 calcium current resulting from calcium entry during V_1 appears most pronounced for small values of V_2, becoming less pronounced with increasing depolarization (Eckert & Tillotson, 1981; Eckert *et al.* 1981). These earlier experiments were limited by outward-current contamination at high potentials, so we have used tail current measurements to extend those observations. Averaging techniques were used for currents recorded for the lower values of V_2 (i.e. up to −10 mV) to enhance the signal to noise ratio of the small tail currents, and special precautions were taken to avoid any systematic errors of measurement (Fig. 7*A*). Thus, V_2 was presented in randomized order with V_1 alternately 'on' and 'off' so as to avoid systematic accumulation of use-dependent inactivation. The inactivation index became smaller as V_2 became more positive (Fig. 7*B*), with the relation approaching a minimum inactivation of about 0·2 at very positive values of V_2.

A source of possible artifact in such an experiment is the inactivation due to calcium entry during the test pulse but before the point of measurement. Thus, the greater the entry of calcium during the test pulse itself, the less percentage inactivation will be contributed by calcium remaining from the prepulse. Except at low to mid-range potentials, this argument does not seem to apply here, since the amount of calcium entering the cell during V_2 decreases as V_2 increases beyond about +50 mV (Eckert & Tillotson, 1978; Gorman & Thomas, 1980) where there is a progressive loss of driving force acting on calcium ions (see also Fig. 3).

Another possible source of artifact depends on the limited frequency response of our tail current recordings. If it is assumed that there is an increasing percentage loss of recorded amplitude with increasing tail current amplitude, a preferential loss of apparent inactivation might result with the increasing activation that occurs with increasing V_2. This possibility seems unlikely since the relation between the inactivation index and V_2 amplitude approximates a declining exponential over its range (Fig. 7*B*), while the activation curve is sigmoidal (Fig. 7*A*).

The '$d+h$' protocol used to eliminate linear symmetrical currents should also be considered as a possible source of artifact in Fig. 7. For pulse amplitudes above 60 mV a $d/2$ modification of the protocol was used to avoid excessively strong hyperpolarizations from the −40 mV holding potential (Armstrong & Bezanilla, 1974). The curve in Fig. 7*B* shows no break at this voltage (i.e. +20 mV). Finally,

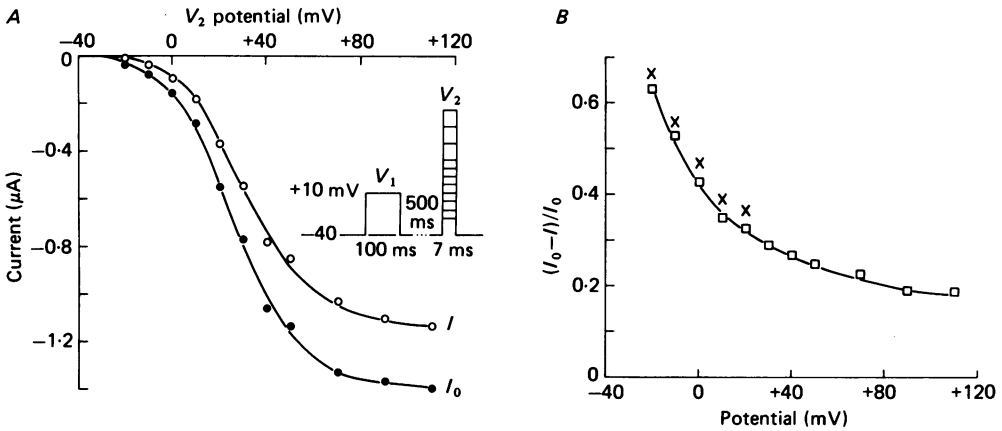

Fig. 7. Effect of test pulse membrane voltage on calcium-mediated inactivation. *A*, V_2 was presented at each potential once with V_1 'off' (●) and once with V_1 'on' (○). The order of presentation of potentials was randomized to avoid systematic use-dependent effects. The tail current was measured 400 μs following the end of V_2 without separation into τ_1 and τ_2 components. Measurements for potentials below 0 mV were made by averaging several recordings to improve the signal to noise ratio. *B*, inactivation index determined from *A* plotted against V_2 potential. The index declines e-fold in 36 mV. The unconnected points (×) indicate the values determined from measurements of the pulse currents for potentials where outward-current contamination was minimal.

it can be seen in Fig. 7*B* that there is correspondence between the plots based on tail current measurements (□) and those based on measurements of pulse current (×) made just before repolarization. For relatively low voltages these are believed to be essentially free from contaminating outward current. Although the useful range of pulse current measurements is limited, the correspondence with tail current measurements is instructive, since the pulse current measurements are not subject to high-frequency attenuation. In addition, this is the range of potentials within which most of the decline in the inactivation index takes place.

Removal of inactivation with time

Tillotson & Horn (1978) reported that the calcium current recorded in caesium-loaded *Aplysia* neurones recovered from inactivation over a period of seconds along a time course approximated by the sum of two time constants. A similar time course was reported for non-loaded *Helix* neurones (Plant & Standen, 1981). We investigated the ionic dependence of recovery from inactivation using tail current measurements (Fig. 8). Each V_1–V_2 interval was repeated twice, first with V_1 'on,' and then with V_1 'off,' and the 200-ms-long V_1 being presented every 60 s. The V_2 tail current amplitude recorded with V_1 'on' (I) was related to the amplitude with V_1 'off' (I_0)

obtained on the previous cycle. This method corrected for the accumulation of inactivation left over from previous cycles, which was minimized but not fully eliminated by the long cycle times. The removal of inactivation typically occurred along a time course that approximated a single or double exponential for intervals up to 3 s. Later phases were not determined nor peeled off to determine the time constant of the initial phase.

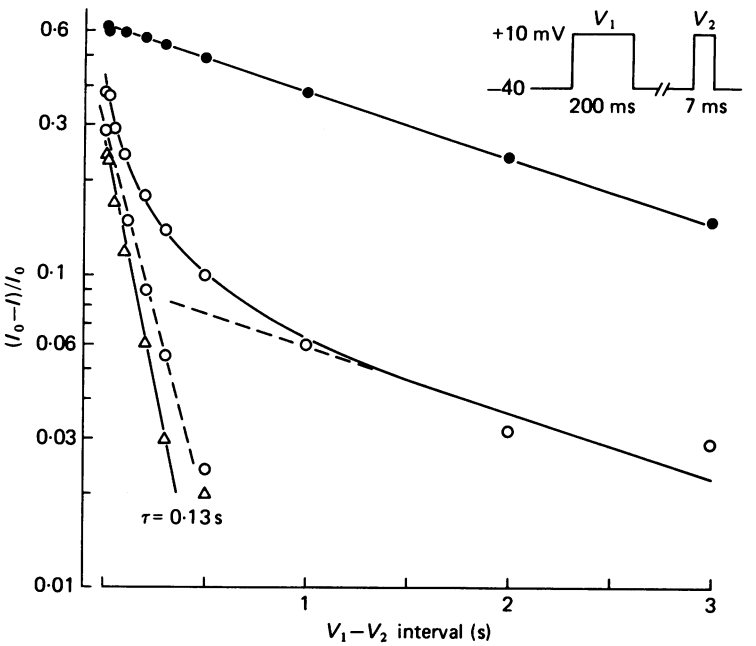

Fig. 8. Time course of removal of inactivation. A 7 ms V_2 to +10 mV was given with a 200 ms V_1 to +10 mV alternately 'on' and 'off'. Tail currents were measured 400 μs following the end of V_2. I_0, tail current with V_1 'off'; I, tail current with V_1 'on'. The inactivation index, $(I_0-I)/I_0$, was plotted against the V_1-V_2 interval obtained in 20 mM calcium artificial sea water before (●) and after (○) injection of EGTA. The dashed plot shows the fast, EGTA-resistant phase after subtraction of the slower phase. Subsequently, extracellular calcium was replaced by 20 mM-barium (△).

As seen earlier (Fig. 3), injection of EGTA reduced up to 10-fold the inactivation remaining 1 s following the end of V_1. In contrast, a much smaller reduction in inactivation was measured at short times following V_1 (Fig. 8). The initial phase of removal of inactivation was accentuated following injection of EGTA so that little inactivation remained for later phases of removal. The time constant of this EGTA-resistant phase of removal of activation was 0·12 s ($\pm$0·03, $n = 4$), several times faster than the rate of removal seen before EGTA injection.

Substitution of barium (not shown) for calcium in the bath without EGTA injection also decreased the time constant of the first phase of recovery to a value similar to that seen following EGTA injection. Replacement of calcium with barium following EGTA injection (Fig. 8) further decreased slightly the inactivation seen at both early and late times, but left the time constant of the EGTA-resistant phase of recovery

essentially unchanged. The preferential loss of the late phase of recovery in barium may indicate that the slow phase in particular, depends on the properties of the cytoplasmic calcium buffer, which appears to have a lower affinity for barium than for calcium (Connor, Ahmed & Ebert, 1981).

Calcium dependence remains at short times in EGTA-injected cells

To determine whether the EGTA-resistant phase of recovery might represent the removal of a voltage-dependent component of inactivation, we compared the dependence on V_1 potential of the EGTA-resistant early phase of recovery with the

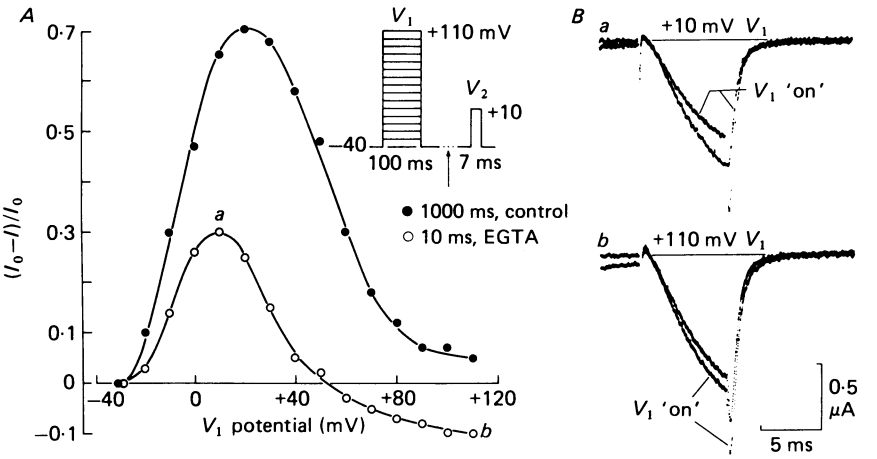

Fig. 9. Dependence of V_2 current on V_1 potential at a 1000 ms V_1–V_2 interval before injection of EGTA (●) and at a 10 ms interval following EGTA injection (○). These conditions were selected to compare behaviour of the currents predominating in the EGTA-resistant and EGTA-sensitive phases of removal of inactivation seen in Fig. 8. *A*, tail current amplitudes were measured 400 μs following the end of V_2 against V_1 potential. *B*, V_2 current traces obtained after EGTA injection at two V_1 potentials (*a*, +10 mV; *b*, +110 mV) corresponding to the labelled points in *A*. The prolonged tail current (third component: Eckert & Ewald, 1983*a*) from V_1 is still apparent in panel *b* as a declining inward current before onset of V_2. The enhanced amplitude of the V_2 current in *b* due to the prepulse cannot be ascribed to simple addition of the slow V_1 tail since the V_2 current increases with time during the depolarization whereas the slow V_1 tail was decreasing with time.

EGTA-sensitive recovery seen at later times (Fig. 9*A*). Thus, the effect of V_1 voltage on V_2 tail current was determined in two voltage series in the same cells: one series, using a 1000 ms V_1–V_2 interval, was carried out before injection of EGTA, while the other, using a 10, 20 or 50 ms interval, was made after the cell was injected with enough EGTA to suppress the inactivation remaining 1000 ms after V_1 to 0·05 or less. The inactivation measured before EGTA injection at 1 s (see Fig. 8) consisted primarily of the slow EGTA-sensitive component, whereas that measured after EGTA injection using short intervals consisted primarily of the rapid EGTA-resistant component.

The plots generated from these two sets of measurements are dissimilar in shape (Fig. 9*A*). The EGTA-sensitive component (●) produced the usual plot consistent

with the model of inactivation dependent on the entry and accumulation of calcium ions during V_1; this component was largely blocked by injected EGTA (Figs. 3 and 8). The EGTA-resistant component (○) produced a more complex plot in which the peak of inactivation occurred in response to somewhat lower V_1 voltages, and the falling limb descended more steeply. For V_1 potentials above +50 mV, the plot went below zero due to an increase in the amplitude of the V_2 tail current following strong V_1 depolarizations (see below). These differences in the plots generated before and after EGTA injection were seen in all nine cells in which this experiment was carried out.

Short-term, voltage-dependent facilitation of the inward current

An important factor contributing to the form of the short-interval EGTA-injected plot is shown in Fig. 9*B*. A prepulse to +10 mV produced substantial inactivation, seen as a decrease in both I_{pulse} and I_{tail}, whereas a prepulse to +110 mV produced enhancement of both I_{tail} and I_{pulse}. The facilitation seen at short intervals in the EGTA-injected cell may also be present at V_1 potentials as low as 0 mV, distorting the plot of inactivation against V_1 potential (Fig. 9, open circles), shifting the peak relative to the peak of simple calcium-dependent inactivation (closed circles). At higher potentials, where calcium entry diminishes, facilitation exceeds inactivation and thus appears to be voltage dependent. The increased rate of activation of the inward current (Fig. 9*Bb*) indicates that the enhanced amplitude does not result from a simple summation upon the inward tail current remaining from V_1. Fenwick, Marty & Neher (1982) ascribe an apparent calcium-dependent facilitation in chromaffin cells to a small positive shift in V_m. In the present case calcium entry appears not to be responsible, for the facilitation increases as V_1 approaches E_{Ca}. However, there remains the possibility of a small (1–2 mV), transient, uncontrolled positive displacement of V_m following V_1, although no such tail was apparent in the voltage recordings.

The voltage-dependent facilitation present in these cells is normally obscured by the more prominent calcium-dependent inactivation of the calcium current. However, in cells that exhibit little or no inactivation of the calcium conductance, voltage-dependent facilitation of the inward current may have physiological significance.

DISCUSSION

Calcium-mediated reduction of inward current is not an artifact of contaminating potassium currents

Using calcium tail currents measured under conditions that virtually eliminate possible contamination by potassium current, we have confirmed earlier observations, based on simple current measurements (Tillotson, 1979; Eckert & Tillotson, 1981), that prior calcium entry causes a reduction of inward current. In the earlier measurements there remained some question as to possible contamination by a calcium-dependent outward current; however, in the experiments reported here the loss of net inward current cannot be attributed to partial cancellation of the inward current by enhanced activation of a calcium-dependent potassium current. The results likewise cannot be attributed to augmentation of the 'non-specific' conductance (Kostyuk, Krishtal & Shakhovalov, 1977; Byerly & Hagiwara, 1982) since the

non-specific current is reported to have a reversal potential near 0 mV, and any augmentation of that conductance would produce an increased rather than a decreased net inward current at −40 mV; moreover, this current, which would be inward at −40 mV, is reported not to inactivate. The findings thus provide further evidence for a reduction of the calcium current that occurs as a consequence of entry of calcium ions during current flow.

Direct evidence for virtual freedom of the calcium tail current measurements from contamination by other currents is seen in Fig. 6, where both the tail currents and current during the pulse are plotted with V_1 'on' and 'off'. Presentation of V_1, besides diminishing inward currents, caused a reduction in net outward current recorded during V_2 at potentials above about +50 mV. The outward current behaved like the voltage-dependent potassium current, and remained virtually unaffected by injection of EGTA. The reduction by a prepulse of a contaminating potassium current summing with the calcium tail current should produce an *increase*, rather than a decrease, in measured tail current, since E_K was never smaller than −40 mV in these experiments.

The apparent weakening of calcium-mediated inactivation with depolarization (Fig. 7; see also Eckert & Tillotson, 1981; Eckert *et al.* 1981) also argues against activation of $I_{K(Ca)}$ as a source of artifact causing an apparent loss of inward current, since activation of $I_{K(Ca)}$ undergoes an increase rather than a decrease with increasingly positive potential (Gorman & Hermann, 1979). Finally, it has been demonstrated (Eckert & Lux, 1977; Eckert & Ewald, 1982*b*) that prior entry of calcium ions decreases rather than increases $I_{K(Ca)}$ during a subsequent depolarization, which would contribute to an increase rather than a decrease in net inward current.

Hyperpolarization does not inactivate the calcium conductance

The usefulness of the tail current technique in eliminating interference from potassium currents is particularly well illustrated in Fig. 4. Comparison of the amplitude of I_{pulse} with I_{tail} reveals a difference in the behaviour of these two currents with regard to hyperpolarization during V_1. Hyperpolarizing conditioning steps cause a reduction in I_{pulse}, which has been taken as evidence for inactivation of the calcium conductance by hyperpolarization (Adams & Gage, 1979). Since a similar decrease in amplitude is not seen in the tail current measurements, the hyperpolarization apparently does not alter the calcium current itself, but instead removes inactivation of an incompletely blocked outward current which sums with the calcium current so as to reduce the net inward current. Activation by hyperpolarization is characteristic of the transient potassium current, $I_{K(A)}$ (Hagiwara & Saito, 1959; Connor & Stevens, 1971; Neher, 1971). Neither Gorman & Thomas (1980), using arsenazo III in *Aplysia* neurones to measure calcium ion entry, nor Plant & Standen (1981), using current measurements on *Helix* neurones, were able to demonstrate any inactivation of the calcium conductance by prior hyperpolarization.

Inactivation index diminishes with increasing positivity of the test pulse

Unlike the behaviour of classical voltage-dependent inactivation, calcium-mediated inactivation is insensitive to the membrane voltage (except as it determines entry and accumulation of calcium ions) before the test pulse (Eckert & Tillotson, 1981). The

results shown in Fig. 7 suggest, however, that calcium-mediated inactivation may be sensitive to the voltage of the test depolarization. We have not been able to determine an artifactual origin of the drop in inactivation index that occurs with increasing V_2 amplitude. Moreover, loss of efficacy of calcium dependence with increased positive potential is also seen during prolonged calcium currents (Eckert *et al.* 1982, 1983; Chad *et al.* 1984). On the other hand, others have concluded that the calcium-mediated inactivation shows no voltage dependence (Plant & Standen, 1981; Ashcroft & Stanfield, 1982).

Increased internal positive potential should drive calcium ions towards an anionic receptor site if the latter is located within the field of the membrane. Thus, it seems unlikely at first consideration that the apparent decrease in inactivation associated with increased positive membrane potential results from a direct effect of membrane voltage on the movement of calcium ions within the field of the membrane. However, consideration of simple binding site models of calcium-mediated inactivation of the calcium channels (Eckert *et al.* 1982, 1983; Standen & Stanfield, 1982; Chad *et al.* 1984) suggests possible membrane-field-related mechanisms that might explain diminished inactivation at positive test pulse potentials (J. Chad & R. Eckert, unpublished observations).

Origin of the EGTA-resistant phase of recovery

The 'EGTA-resistant' phase of recovery apparent at short V_1–V_2 intervals (Fig. 8) might be taken as evidence for a component of inactivation that develops independently of calcium entry and accumulation. The existence of significant calcium-independent inactivation in these cells seems unlikely, however. Experimental changes in the size of the calcium current, produced independently of membrane voltage, indicate that the inactivation in these cells is primarily current-dependent, exhibiting little or no voltage-dependence other than some loss of inactivation with test pulses of increased positive potential (Eckert & Ewald, 1982*a*; Chad *et al.* 1984; Fig. 7 in this paper).

The biphasic shape of the short-interval plot made after injection of EGTA (Fig. 9*A*) appears to have its origin in two effects: (i) calcium-mediated inactivation resulting from EGTA-resistant accumulation of free calcium ions near the inner surface of the membrane (see below), during V_1, and (ii) facilitation of the inward current in response to high positive values of V_1. At high V_1 potentials this effect is uncovered by diminished calcium-dependent inactivation. At intermediate potentials both facilitation and inactivation occur producing a shift in the peak of the plot (Fig. 9*A*) and an accelerated descent of the falling limb.

According to this interpretation, some accumulation of calcium ions takes place near the membrane of the EGTA-injected cell, and the inactivation that remains at short V_1–V_2 intervals in the EGTA-injected cell (i.e. EGTA-resistant phase) is identical in origin to the EGTA-sensitive inactivation seen at long intervals in the uninjected cell. We propose that the apparent EGTA insensitivity of the inactivation seen at short times results from diffusion-limited mixing of uncomplexed EGTA with free calcium ions freshly replenished by the steady current. It is reasonable to suppose that during current flow much of the free EGTA close to the membrane becomes saturated, allowing some current-dependent accumulation of free calcium ions to

occur. Thus, the accelerated early phase of recovery from inactivation, which exhibits a time constant of about 0·12 s, may result from diffusion-limited processes in the crucial region near the inner surface of the membrane. This interpretation receives support from the behaviour seen when barium replaces calcium, when the accelerated early phase of recovery exhibits a similar rate.

We thank Drs J. Chad, G. Augustine and J. Deitmer for helpful comments and discussions, and J. Chad for invaluable guidance and help in the computer software applications. The work was supported by USPHS NS 8364 and NSF BNS 80-12346, and the Epilepsy Foundation of America.

REFERENCES

ADAMS, D. J. & GAGE, P. W. (1979). Characteristics of sodium and calcium conductance changes produced by membrane depolarization in an *Aplysia* neurone. *J. Physiol.* **289**, 143–161.

AKAIKE, N., LEE, K. S. & BROWN, A. M. (1978). The calcium current of *Helix* neuron. *J. gen. Physiol.* **71**, 509–531.

ALMERS, W. & PALADE, P. T. (1981). Slow calcium and potassium currents across frog muscle membrane: measurements with a Vaseline-gap technique. *J. Physiol.* **312**, 159–176.

ALVAREZ-LEEFMANS, F. J., RINK, T. J. & TSIEN, R. Y. (1981). Free calcium ions in neurones of *Helix aspersa* measured with ion-selective micro-electrodes. *J. Physiol.* **315**, 531–548.

ARMSTRONG, C. M. & BEZANILLA, F. (1974). Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. gen. Physiol.* **63**, 533–552.

ASHCROFT, F. M. & STANFIELD, P. R. (1981). Calcium dependence of the inactivation of calcium currents in skeletal muscle fibers of an insect. *Science, N.Y.* **213**, 224–226.

BREHM, P. & ECKERT, R. (1978). Calcium entry leads to inactivation of calcium channel in *Paramecium*. *Science, N.Y.* **202**, 1203–1206.

BREHM, P., ECKERT, R. & TILLOTSON, D. (1980). Calcium-mediated inactivation of calcium current in *Paramecium*. *J. Physiol.* **306**, 193–203.

BROWN, A. M., MORIMOTO, K., TSUDA, Y. & WILSON, D. L. (1981). Calcium current-dependent and voltage-dependent inactivation of calcium channels in *Helix aspersa*. *J. Physiol.* **320**, 193–218.

BYERLY, L. & HAGIWARA, S. (1982). Calcium currents in internally perfused nerve cell bodies of *Limnea stagnalis*. *J. Physiol.* **332**, 503–528.

CHAD, J., ECKERT, R. & EWALD, D. (1984). Kinetics of calcium-dependent inactivation of calcium current in voltage-clamped neurones of *Aplysia californica*. *J. Physiol.* (in the Press).

CONNOR, J., AHMED, Z. & EBERT, G. (1981). Diffusion of Ca^{2+}, Ba^{2+}, H^+ and arsenazo III in neural cytoplasm. *Neurosci. Abstr.* **7**, 15.

CONNOR, J. A. & STEVENS, C. F. (1971). Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol.* **213**, 21–30.

COTA, G. & STEFANI, E. (1982). Calcium channel inactivation in the presence of dantrolene sodium in frog twitch muscle fibers. *Neurosci. Abstr.* **8**, 943.

DOROSHENKO, P. A. & TSYNDRENKO, A. Y. (1978). Action of intracellular calcium on the inward calcium current. *Neirofiziologiya* **10**, 203–205. (Translated in *Neurophysiology* (1978) **10**, 143–145.)

ECKERT, R. (1981). Calcium-mediated inactivation of voltage-gated Ca channels. In *The Mechanism of Gated Calcium Transport Across Biological Membranes*, ed. OHNISHI, S. T. & ENDO, M. New York: Academic Press.

ECKERT, R. & EWALD, D. (1981). Ca-mediated Ca channel inactivation determined from tail current measurements. *Biophys. J.* **33**, 145a.

ECKERT, R. & EWALD, D. (1982*a*). Ca-dependent inactivation of Ca conductance in *Aplysia* neurons exhibits voltage dependence. *Biophys. J.* **37**, 182a.

ECKERT, R. & EWALD, D. (1982*b*). Residual calcium ions depress activation of calcium-dependent current. *Science, N.Y.* **216**, 730–733.

ECKERT, R. & EWALD, D. (1983). Calcium tail currents in voltage-clamped intact nerve cell bodies of *Aplysia californica*. *J. Physiol.* **345**, 533–548.

ECKERT, R., EWALD, D. & CHAD, J. (1982). A single Ca-mediated process can account for both rapid and slow phases of inactivation exhibited by a single calcium conductance. *Biol. Bull.* **163**, 398.

ECKERT, R., EWALD, D. & CHAD, J. (1983). Calcium-mediated inactivation of calcium current in neurons of *Aplysia californica*. In *The Physiology of Excitable Cells*, ed. GRINNELL, A. D. & MOODY, W. J., JR. New York: Alan Liss.

ECKERT, R. & LUX, H. D. (1977). Calcium-dependent depression of a late outward current in snail neurons. *Science, N.Y.* **197**, 472–475.

ECKERT, R. & TILLOTSON, D. (1978). Potassium activation associated with intraneuronal free calcium. *Science, N.Y.* **200**, 437–439.

ECKERT, R. & TILLOTSON, D. (1981). Calcium-mediated inactivation of the calcium conductance in caesium-loaded giant neurones of *Aplysia californica*. *J. Physiol.* **314**, 265–280.

ECKERT, R., TILLOTSON, D. & BREHM, P. (1981). Calcium mediated control of Ca and K currents. *Fedn Proc.* **40**, 2226–2232.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982). Sodium and calcium channels in bovine chromaffin cells. *J. Physiol.* **331**, 599–635.

FOX, A. P. & KRASNE, S. (1981). Two calcium currents in egg cells. *Biophys. J.* **33**, 145a.

GORMAN, A. L. F. & HERMANN, A. (1979). Internal effects of divalent cations on potassium permeability in molluscan neurones. *J. Physiol.* **196**, 393–410.

GORMAN, A. L. F. & THOMAS, M. V. (1980). Intracellular calcium accumulation during depolarization in a molluscan neurone. *J. Physiol.* **208**, 259–285.

HAGIWARA, S. & SAITO, N. (1959). Voltage–current relations in nerve cell membrane of *Onchidium verruculatum*. *J. Physiol.* **148**, 161–179.

HERMANN, A. & GORMAN, A. L. F. (1981*a*). Effects of 4-aminopyridine on potassium currents in a molluscan neuron. *J. gen. Physiol.* **78**, 63–86.

HERMANN, A. & GORMAN, A. L. F. (1981*b*). Effects of tetraethylammonium on potassium currents in a molluscan neuron. *J. gen. Physiol.* **78**, 87–110.

HODGKIN, A. L. & HUXLEY, A. F. (1952). The dual effect of membrane potential on sodium conductance of the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

KATZ, B. & MILEDI, R. (1966). Input–output relation of a single synapse. *Nature, Lond.* **212**, 1242–1245.

KOSTYUK, P. G., KRISHTAL, D. A. & SHAKHOVALOV, Y. A. (1977). Separation of sodium and calcium currents in the somatic membrane of mollusc neurones. *J. Physiol.* **270**, 545–568.

LUX, H. D., NEHER, E. & MARTY, A. (1981). Single channel activity associated with the calcium dependent outward current in *Helix pomatia*. *Pflügers Arch.* **389**, 293–295.

MARBAN, E. & TSIEN, R. W. (1981). Is the slow inward current of heart muscle inactivated by calcium? *Biophys. J.* **33**, 143a.

MARTY, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature, Lond.* **291**, 497–500.

MEECH, R. & STANDEN, N. (1975). Potassium activation in *Helix* neurones under voltage clamp: a component mediated by calcium influx. *J. Physiol.* **249**, 211–239.

NEHER, E. (1971). Two fast transient current components during voltage clamp on snail neurons. *J. gen. Physiol.* **58**, 36–53.

PLANT, T. D. & STANDEN, N. B. (1981). Calcium current inactivation in identified neurones of *Helix aspersa*. *J. Physiol.* **321**, 273–285.

STANDEN, N. B. (1981). Ca channel inactivation by intracellular Ca injection into *Helix* neurones. *Nature, Lond.* **293**, 158–159.

STANDEN, N. B. & STANFIELD, P. R. (1982). A binding-site model for calcium channel inactivation that depends on calcium entry. *Proc. R. Soc.* B **217**, 101–110.

TILLOTSON, D. (1979). Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1497–1500.

TILLOTSON, D. & HORN, R. (1978). Inactivation without facilitation of calcium conductance in caesium-loaded neurones of *Aplysia*. *Nature, Lond.* **273**, 512–514.

ZUCKER, R. (1981). Tetraethylammonium contains an impurity which alkalizes cytoplasm and reduces calcium buffering in neurones. *Brain Res.* **208**, 473–478.