J. Physiol. (1981), **314**, *pp.* 265–280
With 7 text-figures
Printed in Great Britain

CALCIUM-MEDIATED INACTIVATION OF THE CALCIUM CONDUCTANCE IN CAESIUM-LOADED GIANT NEURONES OF *APLYSIA CALIFORNICA*

BY ROGER ECKERT AND DOUGLAS L. TILLOTSON*

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

(*Received 23 January 1980*)

SUMMARY

1. The intracellular potassium in giant neurones of *Aplysia californica* was replaced with caesium by a method utilizing the ionophore nystatin. Because caesium ions have low permeability through potassium channels, outward currents during voltage-clamp depolarization were strongly curtailed after the caesium loading procedure and the subsequent wash-out of the ionophore.

2. The calcium current elicited by a test voltage-clamp depolarization (pulse 2) was depressed following the entry of calcium elicited by a prior depolarization (pulse 1).

3. The percentage depression of the test current was a linear function of the pulse 1 current–time integral, and thus appears to be related linearly to the amount of calcium carried into the cell during pulse 1. This linear relation was maintained when calcium entry was varied by changes in external calcium concentration, by altered pulse 1 amplitude and altered pulse 1 duration. Depression was substantially reduced by injection of EGTA, and by substitution of barium for extracellular calcium.

4. The calcium current was unaffected by prior hyperpolarization of the membrane or by prior depolarizations to about E_{Ca}. Depression of the current was not altered by addition of extracellular 50 mM-TEA or by a strong hyperpolarization between the conditioning and test pulses.

5. The rate relaxation of the inward current during a given depolarization depended on the rate of entry and accumulation of free calcium. Relaxation under a given command potential became slower when calcium was partially replaced with magnesium so as to produce a smaller calcium current, or when accumulation of intracellular free calcium was retarded by injected EGTA or by barium substitution for extracellular calcium.

6. Evidence is considered that accumulation of calcium ions at the cytoplasmic surface of the membrane leads to inactivation through an action upon the calcium conductance. Reduced driving force and intracellular surface-charge neutralization do not adequately account for the observed depression of the calcium current resulting from intracellular accumulation of calcium ions.

* Present address: Department of Physiology, Boston University School of Medicine, 80 E. Concord St., Boston, MA 02118, U.S.A.

0022-3751/81/4520-0755 $07.50

INTRODUCTION

Measurements of the membrane calcium current attempted in various nerve cells have been hindered by the presence of simultaneous and imprecisely defined outward currents carried by potassium ions. Various approaches have been tried to circumvent this problem. A method that allows minimization of outward currents by replacement of intracellular free potassium with a less permeant cation, caesium, employs the polyene molecule nystatin, an ionophore that reversibly forms membrane channels selective for monovalent cations (Russell, Eaton & Brodwick, 1977). The formation and maintenance of these channels appears to be a steep function of the extracellular nystatin concentration, which makes it possible after intracellular loading with caesium to remove nearly all of the functional nystatin channels by wash-out of the ionophore. In addition to their low permeability, intracellular caesium ions are reported to block the voltage-dependent potassium current (Bezanilla & Armstrong, 1972). The effectiveness of the caesium-loading procedure for the elimination of the major outward currents was demonstrated in previous reports (Tillotson & Horn, 1978; Tillotson, 1979).

We have applied the caesium-loading method to investigate two manifestations of inactivation of the calcium conductance in neurones of *Aplysia*: (1) the *relaxation* of the calcium current recorded during a depolarization (described variously by Kostyuk & Krishtal, 1977*a*, *b*; Akaike, Lee & Brown, 1978; and Takahashi & Yoshii, 1978), and (2) the *depression* of the inward current elicited by the second depolarization of a pulse pair (Tillotson & Horn, 1978; Akaike *et al.* 1978; Tillotson, 1979). The inward current evoked by the second pulse is minimal for conditioning potentials of intermediate value (i.e. +10 to +40 mV). Conditioning potentials below and above this range produced less depression of the test current, while high conditioning potentials (i.e. > +100 to +120 mV) produce little or no depression. This behaviour has been ascribed to an inactivation of the calcium conductance dependent on the prior entry of calcium (Tillotson, 1979). This report presents further evidence for this interpretation.

METHODS

Experiments were performed on single identified neurones in the visceral ganglion of *Aplysia californica*. The caesium-loading technique and other methods were identical to those described earlier (Tillotson, 1979). The holding potential, V_h, was set to −40 mV to approximate the resting potential of the untreated cell, and to avoid any residue of early outward current (Hagiwara & Saito, 1959; Connor & Stevens, 1971).

Pulse pairs were delivered every 30 sec, allowing adequate time for recovery processes. The recording solution was free of sodium and contained 100 mM-calcium (Table 1), except as noted otherwise. This high calcium concentration was used to maximize calcium currents relative to any residual currents carried by other ions. The results obtained in 10 mM-, 30 mM-, and 100 mM-calcium were qualitatively similar. Current trajectories and inactivation of peak currents were routinely examined for depolarizations of up to +20 mV only, to minimize any contamination from residual outward currents.

RESULTS

Inward currents in the caesium-loaded neurone

Despite drastic temporary alteration of electrical properties (i.e. loss of the membrane potential and of measurable input resistance) during the caesium-loading

TABLE 1. Composition of solutions(mM)

Caesium-loading	CsCl	Sucrose	$MgSO_4$	Tris*	Nystatin†
	300	394	100	10	
Test solutions	NaCl	KCl	$CaCl_2$	$MgCl_2$	Tris*	
10 Ca	0	0	10	140	410	
30 Ca	0	0	30	120	410	
100 Ca	0	0	100	50	410	
100 Ca/50 TEA	0	0	100	50	360	50 TEA
100 Ba	0	0	0	50	410	100 $BaCl_2$
0 Ca/20 Mn	0	0	0	130	410	20 $MnCl_2$

* Tris (hydroxymethyl) aminomethane (Sigma). Solutions were adjusted to pH 7·7 with HCl.
† Nystatin dissolved in methanol (2·5 mg ml^{-1}) was added to loading solution at a final concentration of 60 mg $l.^{-1}$

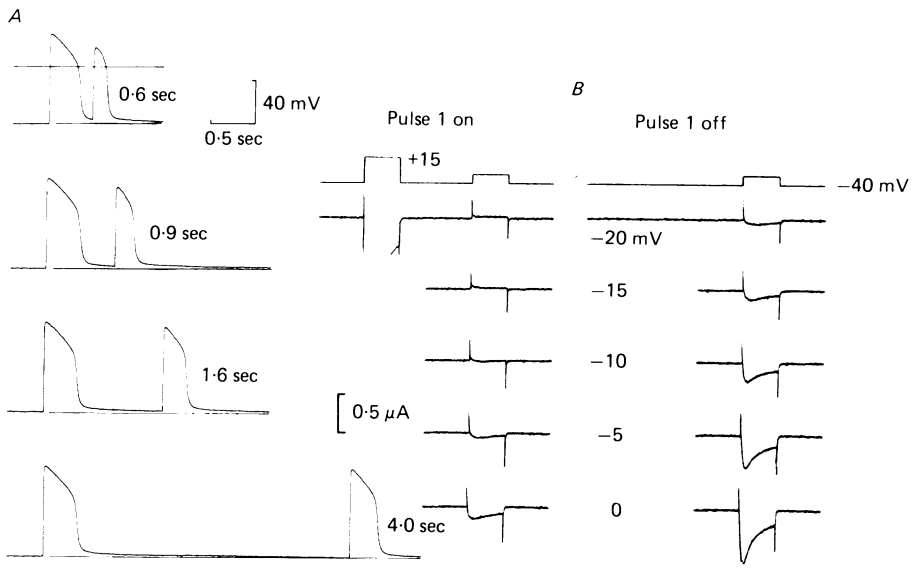

Fig. 1. *A*, action potentials elicited under current clamp in 100 Ca. Polarizing current used to hold at about −52 mV. Superimposed 50 msec outward current stimuli evoked pairs of action potentials, with interpulse times adjusted as indicated at right. R-15 neurone, 15 °C. *B*, inactivation of calcium current. Calcium entry was produced by a 200 msec, +15 mV depolarization (pulse 1) that ended 400 msec before the onset of a variable pulse 2. Left-hand column, pulse 1 on; right-hand column, same pulse 2 potentials without pulse 1. Pulse 2 voltages indicated between left and right columns. R-2 neurone; 15 °C; 100 Ca; $V_h = -40$ mV.

procedure, the caesium-loaded cells retained their ability to produce calcium action potentials (Fig. 1*A*). These underwent a decrease in duration with repetitive firing. In potassium-containing neurones the action potential exhibits, instead, a progressive increase in duration during repetitive firing, which has been ascribed to an accumulating depression or inactivation of potassium currents (Eckert & Lux, 1977; Aldrich,

Getting & Thompson, 1979). The shortening of the calcium action potential seen in the caesium-loaded neurone is consistent with a cumulative reduction in the calcium current described below.

In some double-pulse voltage-clamp experiments the pulse 2 voltage was varied while pulse 1 was of fixed amplitude. Each pulse 2 voltage was presented alternately with and without pulse 1 (Fig. 1*B*). At low potentials ($\leqslant -10$ mV) of pulse 2 the calcium current was virtually eliminated if preceded by pulse 1. There was no

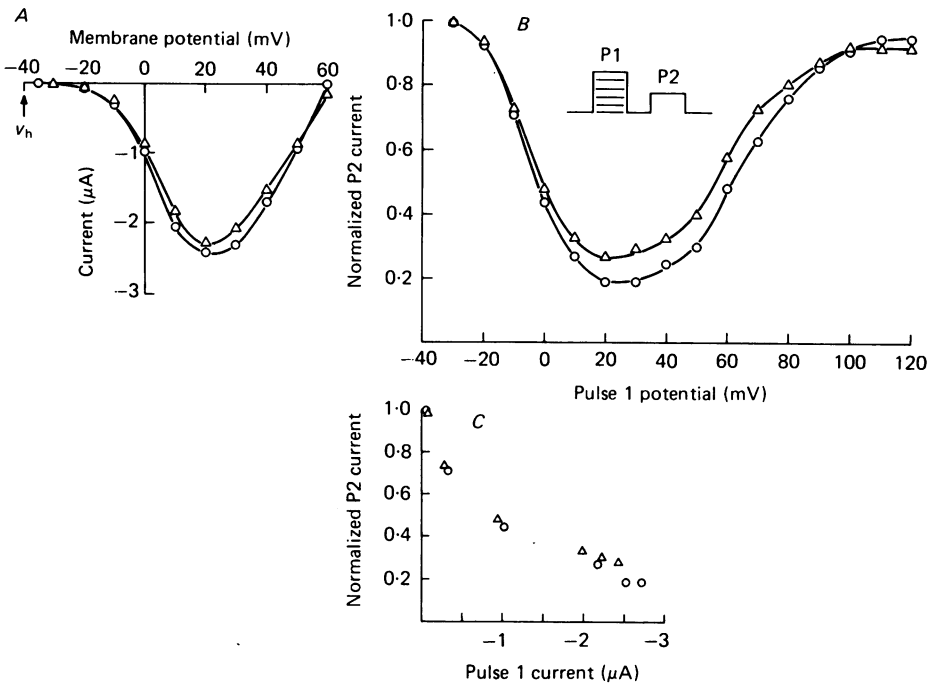

Fig. 2. Inward currents are unaffected by TEA in the caesium-loaded neurone. *A*, current–voltage relations of peak inward currents plotted in 100 Ca and in 100 Ca/50 TEA. *B*, depression of pulse 2 current by prior depolarizing pulse. Relative pulse 2 peak inward current (i.e. with respect to pulse 2 current strength when pulse 1 is absent) is plotted against potential of variable pulse 1. Both pulses of 200 msec duration, separated by 400 msec interval. Pulse 2, +5 mV. *C*, relative pulse 2 currents plotted against pulse 1 leakage-corrected peak currents. Circles, 100 Ca (control); triangles, 100 Ca/50 TEA (Table 1). Same R-2 neurone throughout; 15 °C; $V_h = -40$ mV.

indication of a time-dependent outward current when the inward current was fully suppressed; instead the current trace was essentially time-invariant, showing a small outward leakage current. There was never an indication of a significant outward displacement of the current trajectory that would result from a potassium conductance persisting from pulse 1. When the current was not fully suppressed (associated with lower pulse 1 calcium entry or large pulse 2 voltages) the trajectory of the partially suppressed inward current was attenuated proportionally more at early times than at later times during the pulse (Fig. 1*B*).

The addition of 50 mM-TEA (Table 1) had little effect, if any, on the inward current

recorded in caesium-loaded neurones (Fig. 2*A*). Further, TEA produced little change in the amount of pulse 2 current depression brought about by various pulse 1 voltages (Fig. 2*B*). The small reduction seen following the introduction of TEA may have resulted from the gradual decrease that occurred in the calcium current during the

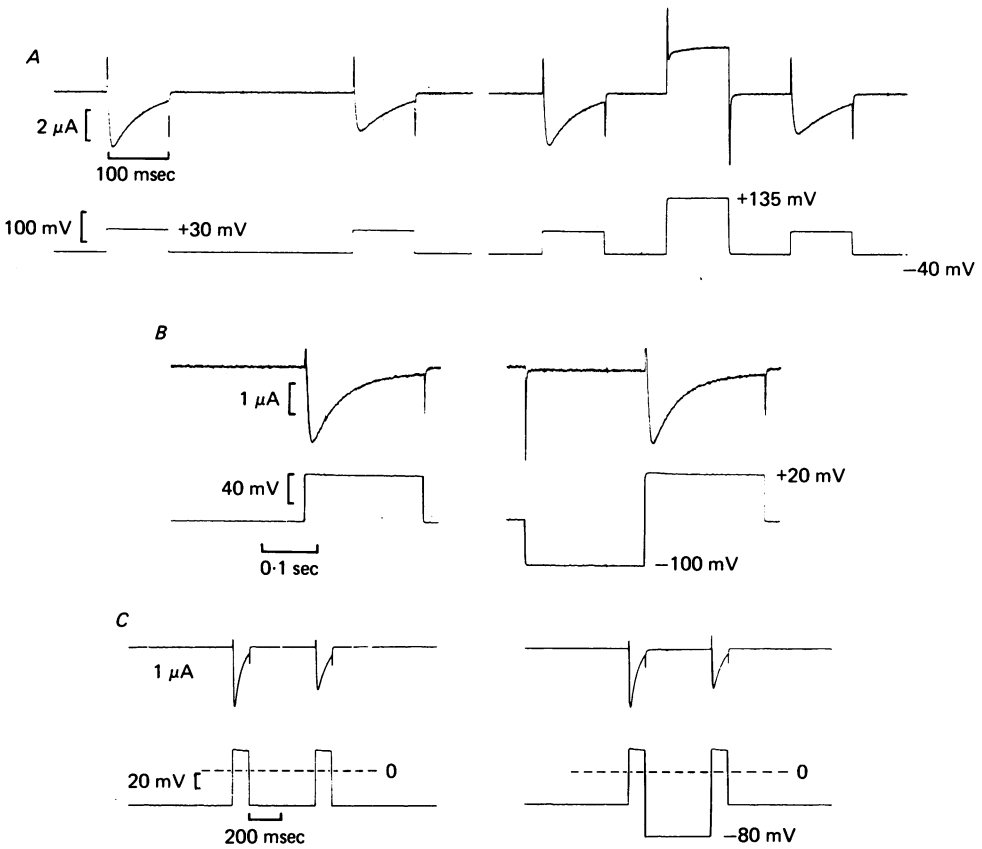

Fig. 3. Strong positive or negative conditioning voltages do not alter the calcium current. *A*, on the left, twin pulses to +30 mV with a 300 msec interval between them. Pulse 2 current reached 70% amplitude of pulse 1 current. On the right, same twin pulses with a 100 msec pulse to +135 mV interposed. No change in pulse 2 current. Caesium-loaded R-2 neurone; 15 °C; 100 Ca; $V = -40$ mV. *B*, a 200 msec conditioning step to −100 mV preceding a depolarization to +20 mV (right) compared with control (left). Caesium-loaded R-2 neurone; 100 Ca; 15 °C; $V_h = -40$ mV. *C*, twin pulses to +28 mV with interpulse hyperpolarization to −80 mV (right) compared with control (left). Caesium-loaded R-2 neurone; 100 Ca; 15 °C; $V_h = -40$ mV.

course of the experiment, so that there was a slightly reduced pulse 1 calcium entry during the TEA part of the experiment. When depression of pulse 2 current is plotted against pulse 1 current amplitude (Fig. 2*C*) the relation between pulse 1 current and pulse 2 depression is seen to be essentially unchanged by addition of TEA. Extracellular TEA in a concentration of 50 mM blocks nearly all of the voltage and calcium-activated outward current in *Aplysia* neurones at voltages of up to and somewhat beyond those of our test pulses (i.e. +20 mV: Hermann & Gorman, 1979).

Failure of TEA to alter the behaviour of the inward current in these experiments is taken as further indication that the depression on the inward current by a prior depolarization reflects a reduction in the calcium current rather than the activation of a hidden outward current.

Calcium inactivation is unaffected by large conditioning voltages

Pulses to approximately the value of E_{Ca} do not produce inactivation of the calcium current (Tillotson, 1979). The possibility exists that inactivation is blocked or reversed by such large potentials. A small removal of inactivation of the sodium conductance has been reported for large conditioning pulses in squid axon (Chandler & Meves, 1970). The experiment shown in Fig. 3*A* tested the possibility that high positive potentials in the vicinity of E_{Ca} might remove inactivation remaining from a prior, smaller depolarization. Two identical 100 msec depolarizations to +30 mV were delivered with a 300 msec interval between them. The peak current registered during pulse 2 was 70% that registered during pulse 1. This was unchanged when a 100 msec pulse to +135 mV was delivered between pulses 1 and 2, indicating that the interposed depolarization to approximately the value of E_{Ca} neither added nor removed any of the inactivation remaining 300 msec after the end of pulse 1. This failure provides some assurance that high voltages do not remove inactivation of the calcium conductance.

Possible activation or inactivation of the calcium conductance by a conditioning hyperpolarization was also examined. A 200 msec hyperpolarization to −100 mV from a holding potential of −40 mV had no effect on the current evoked by an immediate step to +20 mV, the hyperpolarization neither activating nor inactivating the calcium current (Fig. 3*B*). The failure of a hyperpolarization to decrease the inward current indicates that there was no contamination in the caesium-loaded neurone from the early outward current normally carried by potassium through channels that are inactivated at low (i.e. −40 mV) holding potentials (Hagiwara & Saito, 1959; Connor & Stevens, 1971; Neher, 1971; Standen, 1974). The depression of inward current by prior hyperpolarization of R-15 neurone reported by Adams & Gage (1979) may have been caused by incomplete suppression of the early potassium current by TEA and 4-aminopyridine in their experiments. Hyperpolarization during interpulse intervals failed to remove any of the inactivation exhibited by the pulse 2 calcium current (Fig. 3*C*). The failure of hyperpolarization to alter pulse 2 inactivation by pulse 1 is further evidence that moderate as well as strong depolarizations do not produce voltage-dependent inactivation of the calcium conductance. If a voltage-induced inactivation remained following pulse 1 it should have been removed to some extent by the hyperpolarization.

Inactivation is quantitatively related to calcium entry

The findings presented thus far indicate that inactivation of the calcium conductance is not coupled directly to membrane voltage, but may depend upon entry of calcium ions. It has been reported that elevated intracellular calcium concentration depresses or blocks the calcium action potential of perfused barnacle muscle (Hagiwara & Nakajima, 1966) and that the activation of the calcium conductance in molluscan neurones and tunicate eggs is depressed by elevation of intracellular calcium

(Kostyuk & Krishtal, 1977*b*; Akaike *et al.* 1978; Takahashi & Yoshii, 1978). Inactivation of the calcium conductance related to prior calcium entry may thus result from the persistence, near the inner surface of the membrane, of calcium that entered and accumulated during the prior depolarization. If this is so, the inactivation should be a function of the total amount of calcium that entered during the course

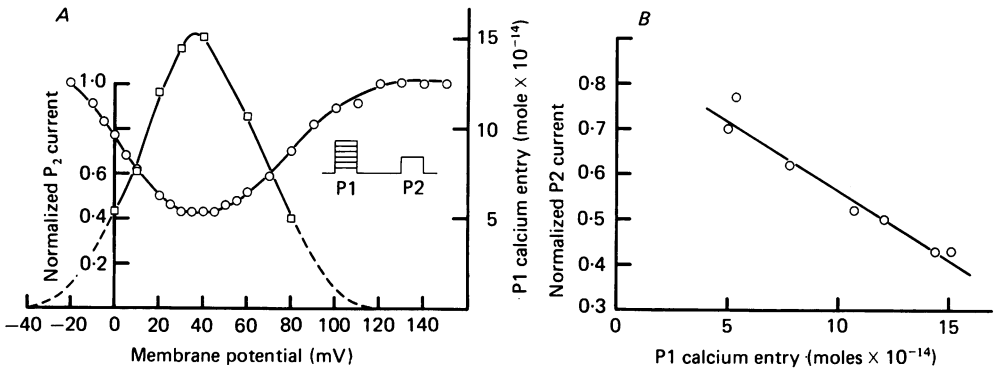

Fig. 4. Inactivation quantitatively related to prior calcium entry. Pulse interval, 400 msec; pulse duration, 200 msec; pulse 2 fixed at +20 mV; pulse 2 potential varied in 100 Ca. *A*, relative pulse 2 current (circles), and calcium entry (squares) during pulse 1, plotted against pulse 1 potential. Calcium entry was determined from the corrected pulse 1 current–time integral as described in the text. *B*, same data plotted as relative pulse 2 current against calcium entry during pulse 1. Caesium-loaded R-2 neurone; 15 °C; $V_h = -40$ mV.

of a conditioning pulse, and hence a function of the time integral of the calcium current during pulse 1. Thus, a brief, strong entry of calcium accompanying a large depolarization should produce the same degree of inactivation as an equal amount of calcium entering during a long, weak pulse, providing pulse 1 is short relative to the time constant of dispersal of the accumulated calcium.

These predictions were tested by varying the amplitude and duration of pulse 1 and by repeating the experiment in 10 Ca and 100 Ca (Figs. 4 and 5). This produced a wide array of current–time integrals for given potentials and durations of pulse 1. The interval from the end of pulse 1 to the beginning of pulse 2 was kept constant and no greater than 250 msec. The areas under current trajectories produced during pulse 1 were cut from chart records and weighed for conversion to current–time integrals. Correction for leakage was made by subtraction of current–time integrals recorded at the same potentials after cobalt or manganese had been substituted for calcium in the bath. The net current–time integrals were then converted arithmetically to moles of calcium ions.

In Fig. 4*A* the relative pulse 2 current is seen to be minimal (i.e. maximally inactivated) following pulse 1 depolarizations to potentials that elicited the strongest calcium entry. At both higher and lower pulse 1 potentials producing weaker calcium entry the pulse 2 current was correspondingly stronger. The relation between pulse 2 current and the pulse 1 current–time integral, plotted in Fig. 4*B*, is linear over the range investigated.

In a variation of this experiment, amplitude and duration were altered. Pulse 1 was delivered for durations ranging from 10 to 200 msec at both 0 and +10 mV (Fig. 5*A*). This was done in 10 Ca with pulse 1 of +20 mV and repeated in 100 Ca with pulse 1 at 0 mV to produce current intensities of a similar range to the series generated in the lower calcium concentration. In another variation the duration of pulse 1 was varied from 5 to 250 msec. In both experiments (Fig. 5*A* and *B*) the pulse 2 peak current intensity was again linearly related to the pulse 1 current–time integral.

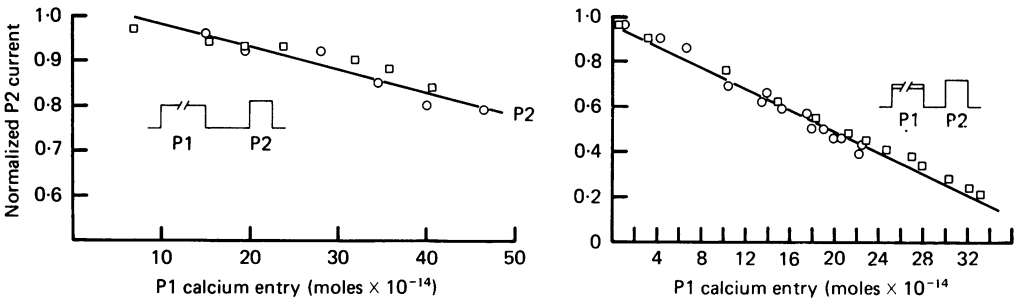

Fig. 5. Inactivation quantitatively related to pulse 1 calcium entry. *A*, pulse 2 inactivation relative to pulse 1 calcium entry in two calcium concentrations, at two pulse 1 amplitudes, and for varied durations of pulse 1. Duration varied between 10 and 200 msec. Extracellular solutions: 100 Ca (circles); 10 Ca (squares). The pulse 1 potential was set at 0 mV in 100 Ca and +20 mV in 10 Ca to produce comparable pulse 1 calcium currents. Pulse 2 was to +24 mV in both solutions. Pulse interval was 200 msec. Caesium-loaded R-2 neurone; 15 °C; $V_h = -40$ mV. *B*, pulse 1 duration was varied between 5 and 250 msec to potentials of 0 (circles) and +20 mV (squares). Pulse 2 potential was +20 mV. Pulse interval was 100 msec. Relative pulse 2 current plotted against pulse 1 calcium entry, determined from corrected current–time integrals as described in the text. Caesium-loaded R-14 neurone; 15 °C; 100 Ca; $V_h = -40$ mV. The slope in *A* is shallower, perhaps because of the greater interval between the pulses.

The time integral of the pulse 1 calcium current is a measure of the amount of calcium that entered during pulse 1. The increment in the intracellular calcium concentration near the inner surface of the membrane at the end of pulse 1 should be proportional to the quantity of calcium that entered during the pulse. If it is assumed that the increment in intracellular calcium remaining after a fixed interval is also proportional to the total calcium entry during pulse 1, then the linearity observed between the time integral of pulse 1 current and the inactivation of pulse 2 current indicates a linear relationship between the increment in intracellular calcium and the decrement in calcium conductance over the range investigated. If inactivation were independently related to membrane potential and/or pulse duration, the data points in Figs. 4*B* and 5 would have been scattered. The close correlation between inactivation and total calcium entry provides further evidence that inactivation results from the accumulation of intracellular calcium ions.

It is implicit in the data in Fig. 5*A* that inactivation should become less pronounced for a given time–voltage paradigm as the extracellular calcium concentration is lowered. This behaviour is seen in Fig. 6*A*, which shows the currents in a cell stimulated at 10 Hz with four 15 msec pulses to +20 mV. In 100 Ca the pulse 4

current was 25% smaller than the pulse 1 current. In 10 Ca pulse 4 exhibited only 10% inactivation. In 10 Ca the pulse 1 current amplitude was 1·5 μA and in 100 Ca it was 4·5 μA, or three times as large. The inactivation in each of the two calcium concentrations was thus approximately proportional to the amplitude of the pulse 1 currents recorded in the two solutions, and hence approximately proportional to the calcium entry and accumulation. This is consistent with the linear relation

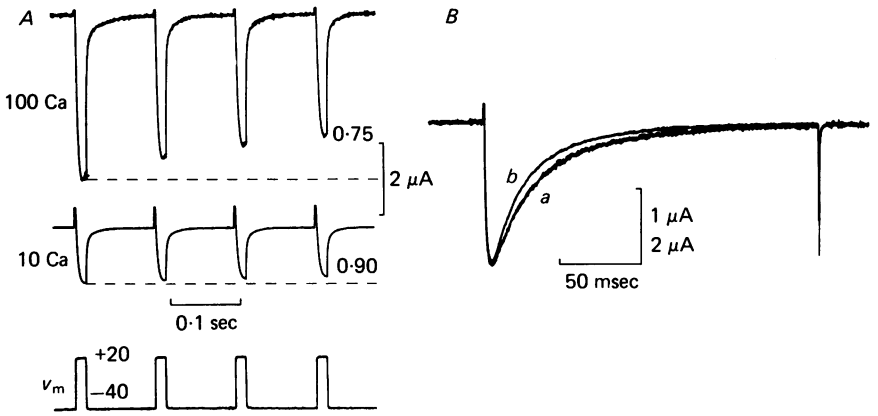

Fig. 6. Inactivation influenced by extracellular calcium concentration. *A*, inactivation of calcium current during train of brief depolarizations (four 15 msec depolarizations to +20 mV with 100 msec intervals between pulses) in 100 Ca and 10 Ca. Numbers at right indicate ratio of pulse 4 current to pulse 1 current. Caesium-loaded R-2 neurone; 15 °C; $V_h = -40$ mV. *B*, relaxation of calcium current in two extracellular concentrations: *a*, 30 Ca; *b*, 100 Ca. Pulse to +20 mV in both solutions. Peak amplitudes normalized for comparison of relaxations. Note different current gains. Caesium-loaded R-2 neurone; 15 °C; $V_h = -40$ mV.

between calcium entry and degree of inactivation seen in Figs. 4*B* and 5. Although the inactivation is weaker in the normal 10 Ca than in the 100 Ca solution used in these experiments, the inactivation seen at normal calcium levels is significant and can be presumed to occur during the normal functioning of the neurone.

Since inactivation remaining at the beginning of pulse 2 is causally related to prior calcium entry during pulse 1, it is reasonable to postulate that the relaxation of the calcium current during pulse 1 occurs also as a consequence of calcium accumulation leading to a calcium-mediated inactivation. If this is true, the relaxation of the calcium current should be slowed if the initial rate of entry of calcium is reduced by lowering its extracellular concentration. This was tested in a cell bathed first in 100 Ca and then in 30 Ca and clamped to +20 mV in both solutions. In 30 Ca the current was smaller, and as predicted exhibited a slower rate of relaxation (Fig. 6*B*). The higher rate of relaxation of the larger current can be ascribed to the more rapid accumulation of intracellular calcium and hence more rapid development of inactivation that must accompany the stronger calcium current.

Interference with accumulation of intracellular free calcium reduces inactivation

The accumulation of intracellular free calcium is limited by the calcium-buffering mechanisms of the cell (Baker, 1972). The accumulation can be further reduced by injection of a calcium-chelating agent. The retardation of elevated intracellular calcium levels should shift the relations between calcium entry and calcium inactiv-

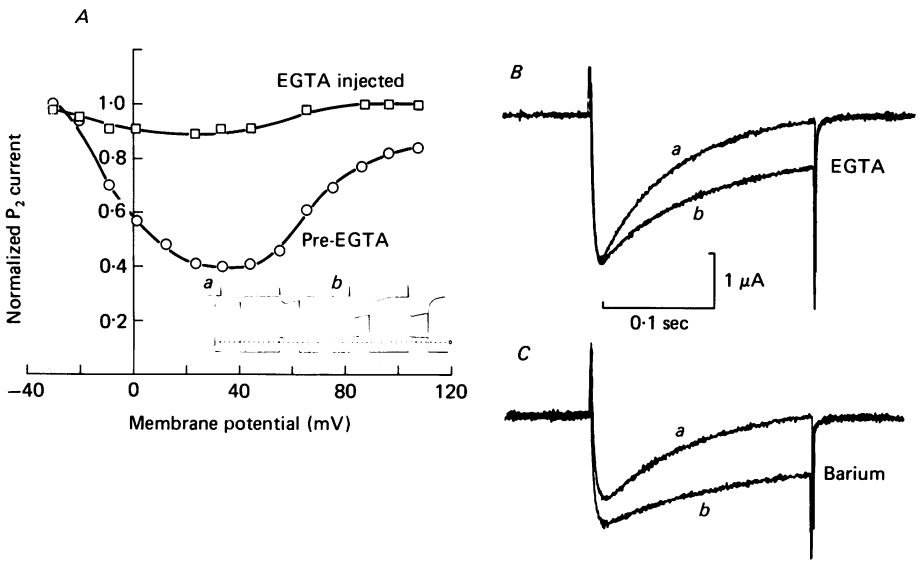

Fig. 7. Interference with intracellular free calcium accumulation. *A*, relative pulse 2 current plotted against pulse 1 potential before (circles) and after (squares) ionophoretic injection of EGTA into caesium-loaded R-14 neurone. Ionophoresis from EGTA-containing micropipette was under voltage clamp, and took 15 min at 0·5 μA. Depolarizing pulses were 100 msec long and separated by 200 msec. Pulse 2 was to +22 mV. 15 °C; 100 Ca; $V_h = -40$ mV. Inset, calibration bars: 0·5 μA and 40 mV. *B*, sample traces of calcium currents during clamp depolarizations to +20 mV before (*a*) and after (*b*) ionophoretic injection of EGTA. Caesium-loaded R-2 neurone. *C*, effect of replacing extracellular calcium with barium. Sweep *a*, current recorded at +20 mV in 100 Ca; sweep *b*, current in 100 Ba (Table 1). R-2 neurone; 15 °C; $V_h = -40$ mV.

ation so that a given calcium current will produce a smaller elevation in free calcium and hence less inactivation. This was tested by measuring calcium inactivation before and after injection of the chelating agent EGTA (Portzehl, Caldwell & Rüegg, 1964). The EGTA was introduced ionophoretically from a pipette filled with 5 mM-EGTA using a current of 0·5 μA applied for 15 min. Inactivation of pulse 2 current was almost eliminated following EGTA injection (Fig. 7*A*, inset). In Fig. 7*B* it is seen that injection of EGTA also results in a markedly decreased rate of relaxation of the inward current. This can be ascribed to chelation of free calcium entering the cytoplasm during current flow so that accumulation of free ion near the inner surface of the membrane is reduced. An increase in the peak calcium current is sometimes also seen following EGTA injection (Fig. 7*A*, inset). This may indicate a steady-state partial inactivation before EGTA injection. Reduced calcium activation has been

reported in response to elevated intracellular calcium (Kostyuk & Krishtal, 1977*b*; Akaike *et al.* 1978).

Another method of interfering with calcium entry and accumulation during depolarization is to substitute it with another ion capable of carrying current through the calcium channel. Barium can replace calcium in carrying the inward current in molluscan neurones (Eckert & Lux, 1975). The consequences of barium substitution, as seen in Fig. 7*C*, resemble those of EGTA injection: the inactivation of the pulse 2 current was strongly reduced (Tillotson, 1979). The peak current carried by barium was greater than that carried by an equal concentration of calcium. The relaxation of the barium current was significantly slowed (Fig. 7*C*). These results suggest that barium ions, while readily entering the cell in place of calcium, are less effective than calcium in mediating the inactivation. Interference with the elevation of free calcium ions in the cytoplasm by barium substitution, EGTA injection, or membrane voltage approaching E_{Ca}, affects the activation of the calcium-dependent potassium current (Meech & Standen, 1975; Eckert & Tillotson, 1978). In addition barium has been reported to block the voltage-dependent potassium current in *Aplysia* neurones (Gorman & Hermann, 1979). The slowed relaxation of inward current after injection of EGTA or barium substitution for calcium in molluscan neurones was until recently attributed entirely to a reduced activation of potassium current (Eckert & Lux, 1976; Connor, 1979), while concurrent effects on the inactivation of the calcium current were overlooked.

DISCUSSION

These experiments demonstrate that the entry and intracellular accumulation of calcium during depolarization lead to a depression of the inward current. This is manifested during depolarization as a slow relaxation of the current to a low level, and, following a period of repolarization, as a depression of peak calcium current elicited by a subsequent test depolarization. Reduction of the inward current by prior calcium entry could arise in at least three ways. (1) The net current might be contaminated with a variable outward current that sums with the calcium current, giving the illusion of diminished inward current. (2) Calcium ions accumulating in the unstirred layer at the inner side of the membrane might alter the current through non-specific effects such as reduced driving force on calcium or altered membrane voltage due to surface-charge screening. (3) There may be a specific reduction in the conductance of the depolarized membrane to calcium. The evidence presented above favours the third possibility. The reduction in calcium conductance is here termed calcium-mediated inactivation, by analogy with the voltage-dependent inactivation exhibited by the sodium system (Hodgkin & Huxley, 1952). Calcium ions may play a role in blocking the sodium channels that carry the dark current in visual receptor cells of vertebrates (Hagins & Yoshikami, 1974), and in the *Limulus* photoreceptor may block the light-activated channels (Lisman & Brown, 1972; Brown & Blinks, 1974). It is well established that intracellular calcium activates a potassium channel in nerve cells (Meech, 1972, 1974*a*, *b*). The calcium-mediated inactivation of the calcium channel appears to be the first example of an electrically activated conductance pathway inactivated by entry of the ion species that normally carries the current through that pathway.

Depression of the inward current is not attributable to enhanced outward current

To produce the present results, an outward current would have to be activated by calcium and exhibit facilitation (i.e. become enhanced during pulse 2). A facilitating calcium-dependent potassium current may be present in an elasmobranch receptor organ (Clusin & Bennett, 1977). However, potassium currents in molluscan neurones have been shown to inactivate only as a consequence of prior depolarization (Eckert & Lux, 1977; Aldrich, Getting & Thompson, 1979). Several lines of evidence argue against the possibility that an outward current summing with the calcium current explains the present results. In double-pulse experiments (i.e. Fig. 1*B*) the pulse 2 current could be fully inactivated by prior calcium entry. In these instances the pulse 2 current plot simply went to zero instead of crossing the pulse 1 voltage axis in a smooth curve to become a time-dependent outward current. Moreover, in cases of full suppression, the pulse 2 current trajectory simply became 'flat' with time. To explain this as the consequence of an enhanced pulse 2 outward current due to residual pulse 1 calcium would require that the hypothetical outward current precisely balance and thereby cancel the calcium current with identical intensity and time course for the duration of the depolarization (i.e. -20 to -10 mV in Fig. 1*B*). This would seem rather fortuitous. Extracellular TEA, which strongly blocks both calcium-activated and voltage-activated outward currents in *Aplysia* neurones (Hermann & Gorman, 1979), had no effect on the inward current (Fig. 3*A*) nor on its inactivation (Fig. 3*B*, *C*)..

Depression of the calcium current is not due to reduced driving force

Accumulation of calcium ions in the unstirred layer at the inner surface of the plasmalemma during calcium entry should reduce the driving force acting on extracellular calcium. However, several considerations make it difficult to explain relaxation and depression of the calcium current in this way. Barium ions carry a somewhat stronger and more slowly relaxing inward current than do calcium ions (Fig. 7*C*). Moreover, photometric measurements of the decline of free intracellular calcium and barium ions following calcium and barium currents in *Aplysia* neurones (D. L. Tillotson & A. L. F. Gorman, unpublished observations) and dorid neurones (Ahmed & Connor, 1979) injected with arsenezo III reveal that the elevated free barium concentration drops more slowly than does free calcium. Thus the entry and accumulation of barium ions must produce a greater increment in the cytoplasmic concentration of ionized barium than the corresponding increment in concentration of free calcium ions. Nonetheless, the barium current undergoes markedly less relaxation (Fig. 7*C*) and less pulse 2 inactivation (Tillotson, 1979).

Calcium entry of a given amount should cause a relatively greater decrease in driving force under conditions of low as compared with high extracellular calcium concentration. Depression in the peak amplitude of pulse 2 current was plotted against the absolute entry of calcium during pulse 1 in solutions containing 10 and 100 mM-calcium (Fig. 5*A*). The peak current values fell close to the same line in both solutions, indicating that it is the absolute intracellular calcium concentration rather than reduced driving force that accounts for depression of the pulse 2 current. Moreover, it is evident from a consideration of the independence principle (Goldman,

1943; Hodgkin & Katz, 1949; Hagiwara, 1975) that large increments in intracellular calcium concentration will produce negligible changes in calcium current (assuming a fixed conductance) so long as the intracellular calcium remains several orders of magnitude below the extracellular concentration. Thus, a rise in intracellular calcium to 10^{-5} mole $l.^{-1}$, for example, would produce less than a 1% reduction in calcium current at +20 mV. However, the current exhibits far greater reduction in response to calcium entry and accumulation (Figs. 1*B*, 2, 4, 5).

In experiments utilizing Ca-EGTA buffers in perfused cells (barnacle muscle: Hagiwara & Nakajima, 1966; Hagiwara, Hayashi & Takahashi, 1969; snail neurone: Kostyuk & Krishtal, 1977*b*; Lee, Akaike & Brown, 1978; tunicate egg: Takahashi & Yoshii, 1978) the calcium spike or calcium current was found to be diminished significantly or eliminated when intracellular calcium was elevated. These effects also could not be accounted for by a reduced driving force on calcium. Kostyuk & Krishtal (1977*b*) anticipated the present findings by suggesting that the calcium current might exhibit a self-blocking action.

Depression of calcium current is not due to intracellular surface-charge screening

It also seems unlikely that depression of the calcium current by intracellular calcium results from a shift in current–voltage relations to more positive potentials due to screening by calcium ions of surface charges on the cytoplasmic side of the membrane. Screening of fixed negative charges at the inner surface of the membrane (Gilbert, 1971) should produce a decrease in potential difference across the membrane. This would be expected to enhance rather than depress the calcium conductance for a given increment of further depolarization from potentials less positive than that of maximal activation (approx. +40 mV). Depolarization itself does not produce inactivation of the calcium conductance (Tillotson, 1979).

By process of elimination, then, we propose that the calcium-mediated depression of the calcium current described in this paper represents a decrease in calcium conductance brought about by an action of calcium ions that enter the cell. The mechanism of calcium-mediated inactivation of the calcium channel requires further study. At this time we can merely propose that there is an interaction between calcium ions and a site associated with the calcium channel that is accessible from the cytoplasmic side of the surface membrane, and that this interaction leads to a diminished calcium channel population or channel conductance or both.

This model of a calcium-mediated inactivation of the calcium conductance predicts that the conductance will not undergo full inactivation under prolonged depolarization. Homoeostatic calcium-buffering mechanisms will prevent unlimited build-up of free intracellular calcium. Negative feedback between the buffer-limited elevation of intracellular calcium during calcium entry and the calcium-mediated inactivation of the calcium conductance should produce a steady-state equilibrium during a depolarization between the voltage-mediated activation and the calcium-mediated inactivation. The calcium current should therefore persist at a reduced level following the initial surge and stabilization of intracellular calcium near the inner surface of the membrane. Such a persistent current has been recorded in snail neurones (Eckert & Lux, 1976), in dorid neurones (Connor, 1979) and in *Paramecium* (Brehm & Eckert, 1978). Enhancement of the cytoplasmic calcium-buffering capability through injection

of EGTA, or barium substitution for calcium, decreases elevation of intracellular calcium levels and thus increases the strength of the steady-state current, as predicted by the model (Fig. 7).

These findings permit a re-interpretation of some earlier reports. For example, the suggestion has been made that calcium inactivation is a function of membrane voltage and time (Standen, 1974; Adams & Gage, 1979). However, the calcium conductance in molluscan neurones (Tillotson, 1979), *Paramecium* (Brehm & Eckert, 1978; Brehm, Eckert & Tillotson, 1980) and insect muscle (Ashcroft & Stanfield, 1980) does not inactivate in response to strong positive potentials; calcium inactivation in each of these systems appears to depend entirely upon the extent of calcium entry. Depression of the calcium-activated potassium outward current during pulse 2 was reported to depend on calcium entry during pulse 1 (Eckert & Lux, 1976). Depressed activation of the potassium current, it now appears, may be a consequence of the reduced calcium entry and accumulation during pulse 2 that results from the calcium-mediated inactivation of calcium conductance.

Renewed caution should also be applied in the interpretation of those experiments in which intracellular free calcium levels are altered, as by injection of EGTA or by barium substitution for calcium (e.g. Connor, 1979). Interference with the activation of a calcium-dependent potassium current and with the inactivation of the calcium current, clearly have similar electrical consequences, shifting the net membrane current toward a less inward or a more outward direction.

We are indebted to Mr J. Meyer and Mr R. Rivera for valuable technical assistance, and to Drs P. Brehm, R. Horn, W. Moody and S. Hagiwara for helpful discussion and criticism. Supported by U.S.P.H.S. grant NS-08364 to R.E. and U.S.P.H.S. S07 RR07009. Major portions of the data presented here were obtained by D.L.T. for a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Biology, University of California, Los Angeles.

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.

Ahmed, Z. & Connor, J. A. (1979). Measurement of calcium influx under voltage clamp in molluscan neurones using the metallochromic dye arsenazo III. *J. Physiol.* **286**, 61–82.

Akaike, N., Lee, K. S. & Brown, A. M. (1978). The calcium current of *Helix* neurons. *J. gen. Physiol.* **71**, 509–531.

Aldrich, R. W., Getting, P. A. & Thompson, S. H. (1979). Mechanism of frequency-dependent broadening of molluscan neurone soma spikes. *J. Physiol.* **291**, 531–544.

Ashcroft, F. M. & Stanfield, P. R. (1980). Inactivation of Ca currents in skeletal muscle fibres of an insect depends on Ca entry. *J. Physiol.* **308**, 36*P*.

Baker, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. molec. Biol.* **24**, 177–223.

Bezanilla, F. & Armstrong, C. M. (1972). Negative conductance caused by entry of sodium and cesium into potassium channels of squid axons. *J. gen. Physiol.* **60**, 588–608.

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, J. E. & Blinks, J. R. (1974). Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. *J. gen. Physiol.* **64**, 643–665.

CHANDLER, W. K. & MEVES, H. (1970). Evidence for two types of sodium conductance in axons perfused with sodium fluoride solution. *J. Physiol.* **211**, 653–678.

CLUSIN, W. T. & BENNETT, M. V. L. (1977). Calcium activated conductance of skate electroreceptors. *J. gen. Physiol.* **69**, 145–182.

CONNOR, J. A. (1979). Calcium current in molluscan neurones: measurement under conditions which maximize its visibility. *J. Physiol.* **286**, 41–60.

CONNOR, J. A. & STEVENS, C. F. (1971). Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. *J. Physiol.* **213**, 1–19.

ECKERT, R. & LUX, H. D. (1975). A non-inactivating inward current recorded during small depolarizing voltage steps in snail pacemaker neurons. *Brain Res.* **83**, 486–489.

ECKERT, R. & LUX, H. D. (1976). A voltage-sensitive persistent calcium conductance in neuronal somata of *Helix*. *J. Physiol.* **254**, 129–151.

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.

GILBERT, D. L. (1971). Fixed surface charges. In *Biophysics and Physiology of Excitable Membranes*, ed. ADELMAN JR, W. J., pp. 359–378. New York: Van Nostrand Reinhold.

GOLDMAN, D. E. (1943). Potential, impedance and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

GORMAN, A. L. F. & HERMANN, A. (1979). Internal effects of divalent cations on potassium permeability in molluscan neurones. *J. Physiol.* **296**, 393–410.

HAGINS, W. A. & YOSHIKAMI, S. (1974). A role for Ca^{2+} in excitation of retinal rods and cones. *Expl Eye Res.* **18**, 299–305.

HAGIWARA, S. (1975). Ca-dependent action potential. In *Membranes*, vol. 3, ed. EISENMAN, G., pp. 359–381. New York: Marcel Dekker.

HAGIWARA, S., HAYASHI, H. & TAKAHASHI, K. (1969). Calcium and potassium currents of the membrane of a barnacle muscle fibre in relation to the calcium spike. *J. Physiol.* **205**, 115–129.

HAGIWARA, S. & NAKAJIMA, S. (1966). Effects of the intracellular Ca ion concentration upon the excitability of muscle fiber membrane of a barnacle. *J. gen. Physiol.* **49**, 807–818.

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. (1979). External and internal effects of tetraethylammonium on voltage-dependent and Ca-dependent K^+ current components in molluscan pacemaker neurons. *Neurosci. Lett.* **12**, 87–92.

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.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

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

KOSTYUK, P. G. & KRISHTAL, O. A. (1977*b*). Effects of calcium and calcium-chelating agents on the inward and outward current in the membrane of mollusc neurones. *J. Physiol.* **270**, 569–580.

LEE, K. S., AKAIKE, N. & BROWN, A. M. (1978). Properties of internally perfused, voltage-clamped, isolated nerve cell bodies. *J. gen. Physiol.* **71**, 489–507.

LISMAN, J. E. & BROWN, J. E. (1972). The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *J. gen. Physiol.* **59**, 701–719.

MEECH, R. W. (1972). Intracellular calcium injection causes increased potassium conductance in *Aplysia* nerve cells. *Comp. Biochem. Physiol.* **42**, 493–499.

MEECH, R. W. (1974*a*). Calcium influx induces a post-tetanic hyperpolarization in *Aplysia* neurons. *Comp. Biochem. Physiol.* **48A**, 387–395.

MEECH, R. W. (1974*b*). The sensitivity of *Helix aspersa* neurons to injected calcium ions. *J. Physiol.* **237**, 259–277.

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

NEHER, E. (1971). Two fast transients during voltage clamp in snail neuron. *J. gen. Physiol.* **58**, 36–53.

PORTZEHL, H., CALDWELL, P. C. & RÜEGG, J. C. (1964). The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. biophys. Acta* **79**, 581–591.

RUSSELL, J. M., EATON, D. C. & BRODWICK, M. S. (1977). Effects of nystatin on membrane conductance and internal activities in *Aplysia* neurons. *J. Membrane Biol.* **37**, 137–156.

STANDEN, N. B. (1974). Properties of a calcium channel in snail neurones. *Nature, Lond.* **250**, 340–343.

TAKAHISHI, K. & YOSHII, M. (1978). Effects of internal free calcium upon the sodium and calcium channels in the tunicate egg analysed by the internal perfusion technique. *J. Physiol.* **279**, 519–549.

TILLOTSON, D. (1979). Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. nat. Acad. Sci. U.S.A.* **76**, 1497–1500.

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