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CALCIUM CURRENT-DEPENDENT AND VOLTAGE-DEPENDENT INACTIVATION OF CALCIUM CHANNELS IN *HELIX ASPERSA*

BY A. M. BROWN, K. MORIMOTO, Y. TSUDA AND D. L. WILSON

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550, U.S.A.

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SUMMARY

1. Inactivation of the Ca channels has been examined in isolated nerve cell bodies of *Helix aspersa* using the suction pipette method for voltage clamp and internal perfusion.

2. Satisfactory suppression of outward currents was essential. This was achieved over most of the voltage range by substitution of Cs ion for K ion and by the use of TEA intra- and extracellularly and 4-AP extracellularly. A small time- and voltage-dependent non-specific current remained at potentials above +60 mV.

3. In these solutions, Ca current approaches E_{Ca} but cannot be detected in the outward direction. The Ca channel appears to be impermeable to Cs and Tris ions.

4. Inactivation of Ca currents occurs as a bi-exponential process. The faster rate is 10–20 times the slower rate and is about one twentieth the rate of activation. The development of inactivation during a single voltage-clamp step and the onset of inactivation produced by prepulses followed after brief intervals by a test pulse, have roughly similar time courses.

5. The rates of inactivation increase monotonically at potentials more positive than about −25 mV. The amount of steady-state inactivation increases with membrane depolarizations to potentials of about +50 mV. At more positive potentials, steady-state inactivation is reduced.

6. Intracellular EGTA slows the faster rate of inactivation of I_{Ca} and reduces the amount of steady-state inactivation measured with a standard two pulse protocol. The effect is specifically related to Ca chelation and hydrogen ions are not involved. This component of inactivation is referred to as Ca current-dependent inactivation and is consistent with observations that increased Ca_i inactivates the Ca channel. The process does not depend upon current flow alone since Ba currents of comparable or greater magnitude have smaller initial rates of inactivation. Furthermore, application of Ba ion intracellularly in large concentrations has no effect on steady-state inactivation.

7. The bi-exponential inactivation process that persists in the presence of $EGTA_i$ is similar to that occurring when extracellular Ba ion carries current through the Ca channel. Steady-state inactivation also persists and is similar in the two cases. Therefore it is concluded that inactivation is voltage-dependent as well as Ca current-dependent.

8. Diffusion models that included reasonable values for the effect of binding on diffusion, even when combined with declining influxes, did not account for this 'mixed' form of calcium- and voltage-dependent inactivation. A compartmental model in which the particular kinetic model of voltage-dependent inactivation was not critical described the Ca current-dependent inactivation.

INTRODUCTION

There is general agreement that during maintained depolarization of the cell membrane, Ca current reaches a peak and then subsides. The result was confirmed by non-electrophysiological measurements of intracellular Ca activities using Ca indicators such as Arsenazo III which showed that Ca_i activities rose to a maximum asymptote during maintained depolarizations (Ahmed & Connor, 1979; Gorman & Thomas, 1980; Smith & Zucker, 1980). The fall of inward current is referred to as inactivation and may have several causes. The most frequent is an accompanying outward flow of current resulting in a reversal potential that is much less positive than the expected electrochemical equilibrium potential for Ca ion (Standen, 1975; Reuter & Scholz, 1977; Connor, 1977; Kostyuk, Krishtal & Shakhovalov, 1977; Adams & Gage, 1979). The outward current may flow through the Ca channel (Reuter & Scholz, 1977) or through K channels. It should be possible to reduce contamination from the latter source either by large doses of pharmacological blockers or by examining I_{Ca} at the null potential for ionic flow through K channels. When K channels are suppressed by TEA and 4-AP and Cs substitution for K intra- and extracellularly, I_{Ca} become vanishingly small at potentials that approach E_{Ca} (Akaike, Lee & Brown, 1978; Brown, Akaike, Tsuda & Morimoto, 1980), and inactivation still occurs (Brown *et al.* 1980). Tillotson (1979) used the null potential method and both he and Eckert & Tillotson (1978) proposed that the inactivation was Ca-dependent because the amplitudes of tail currents were not reduced during very positive steps to membrane potentials of +160 mV where I_{Ca} was small but were reduced at less positive potentials where I_{Ca} was substantial. The results were obtained in the R_{15} neurone of *Aplysia californica* and further support for this interpretation has been presented by Brehm & Eckert (1978) for *Paramecium* and by Ashcroft & Stanfield (1980) for muscle fibres of the stick insect.

A different conclusion has been reached by a number of other investigators however, namely that inactivation of I_{Ca} is voltage-dependent and similar in nature to the inactivation process of I_{Na} in axons (Standen, 1975; Kostyuk *et al.* 1977; Akaike *et al.* 1978; Adams & Gage, 1979). In these experiments inactivation was not examined at potentials greater than +50 mV. The purpose of the present experiments was to analyse these two proposed mechanisms of I_{Ca} inactivation. The experiments were done on voltage-clamped, internally perfused, isolated, large identifiable nerve cell bodies of *Helix aspersa*. It was found that inactivation of I_{Ca} was bi-exponential in agreement with the results of Kostyuk *et al.* (1977) and Tillotson & Horn (1978) and depended upon both voltage and Ca current. Ba current, I_{Ba}, also inactivates but the inactivation in this case appears to be entirely voltage-dependent. A simple model in which Ca binds to sites at the nerve membrane surface that produce

inactivation accounted for the difference in initial inactivation between I_{Ca} and I_{Ba}. The observation that potential-dependent inactivation could be partially overcome at positive membrane potentials suggests either that inactivated channels can be reopened or that two populations of Ca channels are present. The occurrence of fast and slow rates of inactivation may also indicate two types of channel.

METHODS

Large single, identifiable neurones were isolated from the suboesophageal ganglia of *Helix aspersa* by dissection and ligature of the axon as close to the cell body as possible. Cells in the left visceral hemiganglion that are labelled 2 or 4 by Kerkut, Walker, Lambert, Gayton & Loker (1975) were used in most experiments. The cell body was partially aspirated into a suction pipette and then pulled free of surrounding cells. The axon was either ligated or cut with fine scissors near the cell body. We used the suction pipette method (Lee, Akaike & Brown, 1977, 1978; Akaike *et al.* 1978) for voltage clamp and internal perfusion and complete details are provided in a recent publication (Lee, Akaike & Brown, 1980). The most important modification is the use of a separate glass micro-electrode to record membrane potential, E_m, which provides the reference for the command potential applied to the cell. This modification was necessary because the I–R drop across the tip of a single pipette could not be compensated for and serious errors resulted when large Ca or Ba currents were flowing in the big neurones selected presently. The micro-electrodes had tip diameters of about 1 μm, resistances less than 2 MΩ and were filled with 3 M-KCl. The possibility that 3 M-KCl solution in the micro-electrode leaked significantly into the neurone under negative pressure was examined by comparing the results using micro-electrodes filled with 0·5 M-Cs asparate. The latter gave measured junction potentials of $-4{\cdot}2 \pm 1{\cdot}2$ mV ($n = 7$) but after correction there were no differences between the two methods. The micro-electrode was inserted near the axonal pole of the soma and the suction pipette was applied to the opposite pole. In some experiments micro-electrodes were coated with a conductive silver paint, insulated and connected by a driven shield to a unity gain operational amplifier. There were no significant differences however when the driven shield was not used. The voltage clamp amplifier was a Tecktronix 502A differential amplifier which was operated at peak gains of 100–1000 and a band width of DC to 1 or 10 kHz. Current was measured with a current-to-voltage converter one side of which was held at virtual ground. Series resistance compensation was provided by adding a portion of the membrane current to the command voltage to nullify the voltage drop across the series resistance. In current clamp the R_s compensation is set to nullify the instantaneous change in E_m that occurs when the current steps are turned on and off. The gain of the feed-back circuit was increased often to the point of ringing in the current trace. This was essential for tail current experiments because the tail currents subside in 1·0 msec or less. However, ringing caused current points at the onset and cessation of voltage clamp steps that sometimes could not be corrected by linear subtraction. In these cases tail currents were separated from capacitative current transients by semilogarithmic plots of current against time and extrapolated backwards to the zero time of the recovery potential.

The leakage current is defined as the current that remains after blockage of I_{Ca} and has linear and non-linear components. The linear residual currents along with the linear portion of the capacitative current transient could be subtracted by adding the current responses to equal and opposite voltage steps. Non-linear leakage current was called non-specific current, I_{NS}, and was evaluated separately. Current and voltage data were digitized at rates of 1–500 μsec with 10-bit resolution using a signal averager which compensated for data overflow (Nicolet-1170) and the data files were stored on hard or floppy disks for subsequent analyses.

The Ca current was separated by suppression of K, Na and non-specific currents. K and non-specific currents were suppressed as follows: Cs was substituted for K in external and internal solutions, tetraethylammonium, TEA, was added to external and internal solutions and 4-aminopyridine (4-AP) was added to the external solution. Na current was suppressed by substitution of Tris (hydroxymethyl) aminomethane, for Na. Compositions of these solutions are given in Table 1. Regulation of intracellular Na, K and Cl activities using the suction pipette method has already been demonstrated (Lee *et al.* 1978, 1980).

TABLE 1. Solutions used for extra- and intracellular perfusion of *Helix* neurones.

	mM	NaCl	TrisCl	KCl	CsCl	TEACl	Kasp	Cs aspartate	TEAOH	4-AP	$CaCl_2$	$MgCl_2$	Glucose
External solution pH 7·55	Normal solution	85	5	5	—	—	—	—	—	—	10	15	5·5
	Ca current solution	—	35	—	5	50	—	—	—	5	10*	15	5·5
Internal solution pH 7·35	Normal solution	—	2·0‡	—	—	—	135	—	—	—	—	—	—
	Cs aspartate solution†	—	—	—	—	—	—	135	10	—	—	—	—

* $CaCl_2$ was replaced by $BaCl_2$ in Ba current experiments.
† EGTA was added as required and pH was adjusted by Tris; EGTA-Ca buffered solutions were also used as required (see text).
‡ In some experiments HEPES buffer was used.

Ca buffering was accomplished using EGTA in concentrations of 0·1, 1, 3, 10 and 25 mM and Ca levels set between 10^{-6} and 10^{-9} M. The association constant used for calculating Ca activities in the presence of EGTA at pH 7·2 was $10^{7.13}$. We calculated a value of $10^{4.11}$ for the association constant for Ba EGTA (Martell & Sillén, 1972). Values of Ca activity were calculated using a single ion Ca activity coefficient of 0·3 in solutions of 0·1 M ionic strength. The effects of EGTA itself were assessed by holding Ca activity constant at different EGTA concentrations. The effectiveness of Ca buffering was estimated from the suppression of Ca-activated K current, $I_{K,Ca}$. In these experiments K replaced Cs, and TEA and 4-AP were omitted. Ba currents were produced by equimolar substitution of Ba for Ca.

Steady-state inactivation, h_∞, was measured as the ratio of test pulse current in the presence of a prepulse to test pulse current in the absence of a prepulse. Complete inactivation required prepulses of several seconds in duration. At very positive membrane pulses, prepulses of such a long duration often had deleterious effects. Therefore in many experiments prepulses of about 50 msec duration that rarely produce complete inactivation were used. In such cases the ratio of test pulse currents with and without conditioning prepulses is termed h'_∞. This parameter is similar to the P11/P1 parameter used by Eckert & Tillotson (1978). The time course of inactivation was examined in the following ways. First, the rate of fall of I_{Ca} during a voltage clamp step was analysed. Secondly, the time course of the onset of inactivation was determined using prepulses of varying durations up to several seconds at each command potential, the latter ranging from −50 to +100 mV and examining their effects on a test pulse to about +10 mV which produced a large I_{Ca}. The development of inactivation was also examined by measuring the amplitude of tail currents during step depolarizations. The command potentials were returned to the holding potential, V_H, of −50 mV or to potentials above and below this level. Experiments were done at temperatures of 18–21 °C.

RESULTS

Characteristics of the voltage clamp

Command potentials rose with a time constant of 10–30 μsec. Potentials recorded at the suction pipette were identical to potentials recorded by the micropipette after correction for the *I-R* drop across the suction pipette tip (Fig. 1*A*). The capacitative current transient relaxed with a single time constant which ranged from 100 to 300 μsec and was normally about 250 μsec (Fig. 1*B*). The membrane capacitance, C_M, was estimated from the time constant of the monoexponential hyperpolarizing or depolarizing potential change produced by an inward or outward step of current. C_m was $2–4 \times 10^{-9}$ F which gave values of $1–3 \times 10^{-6}$ F cm^{-2} for spherical neurones 200 μm diameter. The shunt resistance, R_{sh}, was estimated according to Lee *et al.* (1978, 1980) by comparing input resistance R_{in} measured with either one micropipette and a bridge circuit or two micropipettes before and after application of the suction pipette and rupture of the aspirated membrane. The usual R_{in} was $10^7–2 \times 10^7$ Ω with 10^{-9} A current steps giving voltage deflexions of 10–20 mV. When the suction pipette was applied, voltage deflexions measured by the pipette were 50–100 mV. After rupture of the aspirated membrane, cells had voltage deflexions measured by the micropipette of never less than 5 mV and often of 19 mV, so that R_{sh} ranged from 1 to 20 times R_{in}. In some cases, there were no differences between voltage deflexions before and after partial aspiration. R_{sh} was not evaluated in each cell and as a practical matter cells were judged to be satisfactory if they had resting potentials of −45 mV or more, input resistances of $5 \times 10^6–2 \times 10^7$ Ω and action potentials 80–110 mV in amplitude. After suppression of K and Na currents these neurones had inward I_{Ca}s of about 50 nA or more before leakage correction.

The series resistance R_s, was calculated in three ways: from the zero time intercept of the logarithmic plot of capacitative current, from the time constant of the capacitative current transient, or from the step change in voltage produced by a small hyperpolarizing current step. It was 40kΩ or 20 Ω cm². Peak I_{Ca} in our experiments approaches 10^{-7} A so that the peak voltage drop across R_s could be smaller than 5 mV

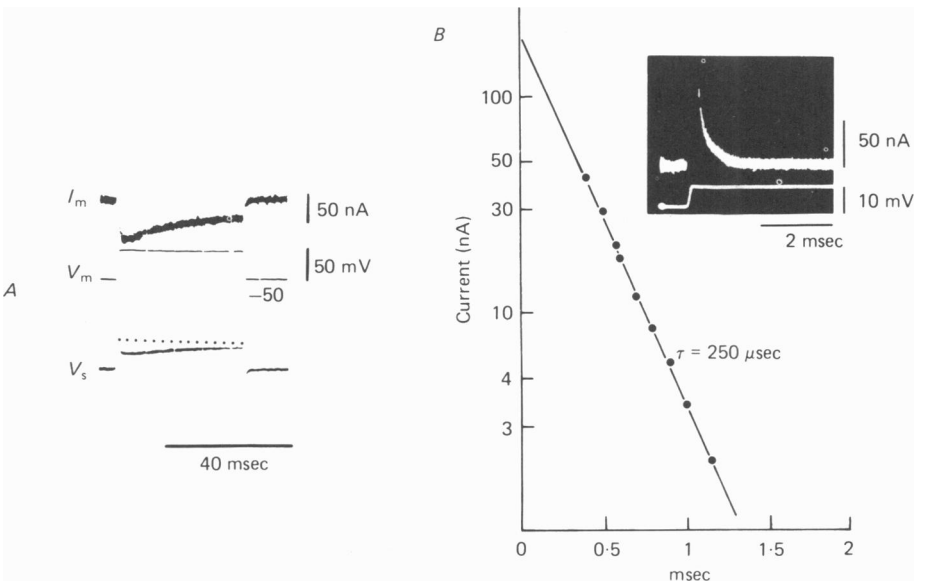

Fig. 1. Voltage-clamp characteristics. *A*, comparison of membrane potentials measured by micro-electrode and suction pipette under voltage clamp. The membrane potential measured by the micro-electrode (V_m) rose quickly to the command potential and remained steady during current. However, the inward current due to I_{Ca} caused a potential drop across the suction pipette tip, proportional to I_{Ca} × suction pipette tip resistance, as shown in the measurement of membrane potential with the suction pipette (V_s). V_s was measured outside the voltage-clamp loop. Interrupted line in V_s shows the membrane potential corrected for the potential drop across the pipette tip. Note that V_m and corrected V_s are isopotential showing that the space clamp was satisfactory. Holding potential, V_H, was −50 mV. I_{Ca} in *A* was the net current after addition of equal but opposite voltage-clamp pulses. *B*, capacitative current transient of a neurone under the voltage clamp. Figure inset shows the membrane current produced by a small depolarizing voltage step of 10 mV from V_H of −50 mV. Membrane current was measured from the steady-state current level, and plotted on a semilogarithmic scale. The capacitative transient current was fitted with a single exponential function having a time constant of 250 μsec. The time constants varied depending on the size of each neurone, and ranged from 100 to 500 μsec. The diameter of this neurone was 100 μm.

which was less than ten percent of the applied membrane potential in uncompensated R_s experiments.

Characteristics of I_{NS}

The presence of a voltage- and time-dependent non-specific current complicates analysis of inactivation. The effect cannot be excluded by studying tail currents at recovery or repolarizing potentials equivalent to E_K because such experiments must often be done over a range of command potentials extending from −150 to +150 mV

for long periods of time precisely the conditions in which E_K is changing (Eaton, 1972). Moreover, I_{Ca} tail currents relax in less than 1·0 msec making their measurement very difficult (Smith & Zucker, 1980). An alternative method is to examine I_{NS} after suppression of I_{Na}, I_K and I_{Ca} (Kostyuk *et al.* 1977; Akaike *et al.* 1978; Byerly, Hagiwara, Masuda & Yoshii, 1979). In the present experiments I_{Na} was suppressed

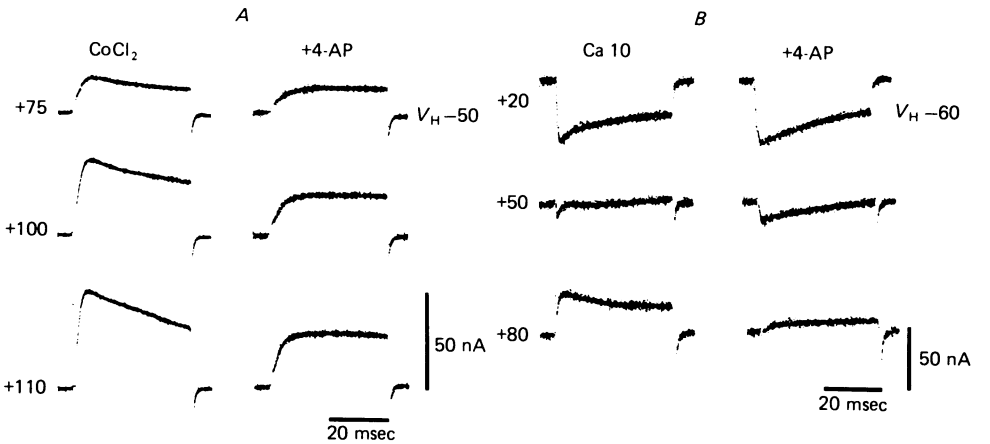

Fig. 2. Effects of 4-aminopyridine (4-AP) on calcium and non-specific currents. *A*, non-specific leakage current was elicited by depolarizing pulses using the Ca extracellular solution shown in Table 1 and the Cs internal solution without TEA. Extracellular $CaCl_2$ was replaced by $CoCl_2$. After 2–3 min exposure to 5 mM-4-AP, the transient outward current was markedly depressed. Holding potential was −50 mV. *B*, effects of 5 mM-4-AP on I_{Ca} produced by depolarizing pulses. After 4-AP the inward current is increased and the rate of inactivation is slowed. Holding potential was −60 mV in this Figure, but there was no difference when the holding potential was −50 mV. When 4-AP was present extracellularly and TEA intracellularly, the transient outward current shown in *A* was never seen even at higher membrane potentials. Note that I_{NS} shown in the right-hand panel of *A* is larger than the outward current that occurs when I_{Ca} is flowing, i.e. as shown in the bottom trace of the far right column in *B*.

by substituting Tris ion for Na extracellularly, I_K was suppressed by substituting Cs for K intra- and extracellularly and I_{Ca} was suppressed by substituting Co for Ca extracellularly. TEA 50 mM was added to the extracellular solution to suppress the flow of Cs through the voltage-dependent late K channel. TEA 10 mM was also added to the intracellular solutions. Under these conditions I_{NS} has a component probably due to the passage of Cs ions through the transient K channel. Thus addition of 4-AP (Thompson, 1977) blocks the transient overshoot of I_{NS} carried by Cs ions while having a smaller effect on the steady I_{NS} (Fig. 2*A*). This transient can alter the apparent kinetics of I_{Ca} so that early inactivation appears faster (Fig. 2*B*). 4-AP is the preferred method for blocking the transient outward current since the other method, namely the use of holding potentials or prepulses to −40 mV, produces only partial blockage and also partially inactivates I_{Ca}.

The I_{NS} that occurs in the presence of TEA and 4-AP may take one of two forms differing mainly in amplitude. For the larger I_{NS}, voltage-dependence appears at zero mV and the currents are considerable at positive potentials (60 nA at +100 mV). The larger I_{NS} rises slowly and may have a second even slower component, is associated

with smaller inward currents and occurs in neurons which often deteriorate within 1–2 hr. For small I_{NS} (Fig. 3), voltage dependence begins at potentials of +60 mV or greater and the current is about 5 nA at +100 mV. The small I_{NS} is also time-dependent (Fig. 2*A*; Kostyuk *et al.* 1977) and the activation process can be fit with a single exponential function having a time constant of 2–4 msec at +100 mV. Neither type of I_{NS} is affected by substitution of isethionate$^-$ or methane sulphonate$^-$ for Cl^- extracellularly. The larger I_{NS}s appeared to be suppressed by as much as 20–30 % by the addition of 10 mM-TEA intracellularly but they were never reduced

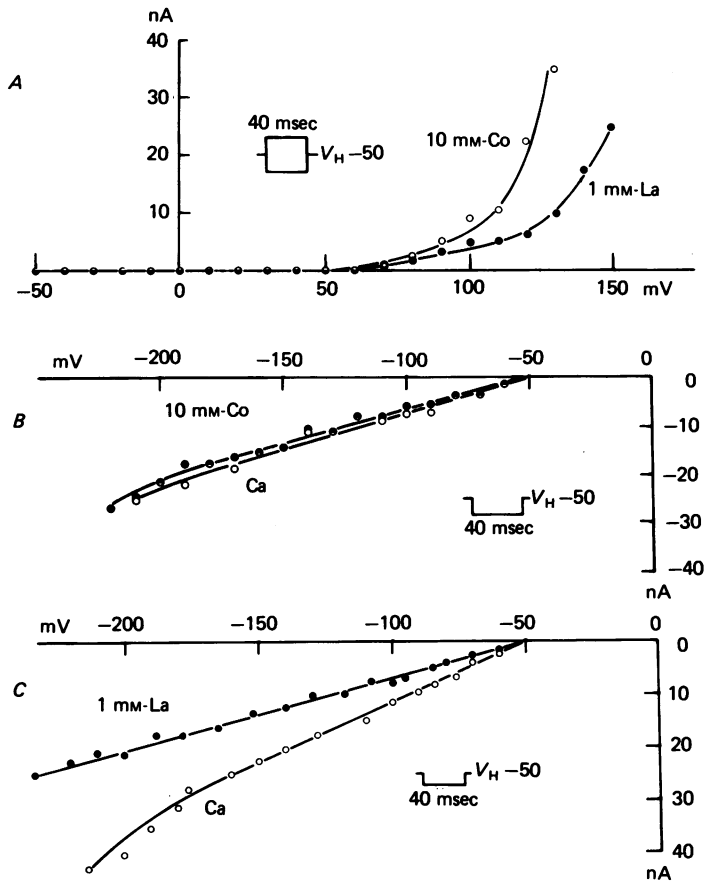

Fig. 3. *I–V* relationships of leakage and non-specific currents. *A*, the voltage-dependent I_{NS} was obtained after replacing 10 mM-$CaCl_2$ with 10 mM-$CoCl_2$. Depolarizing and hyperpolarizing pulses of equal amplitude from V_H were added. The currents were linear between −50 and +60 mV and the voltage-dependent I_{NS} only appeared at potentials above +60 mV. I_{NS} showed little time dependence during the pulse except for an initial rising phase with a time constant of 3–4 msec (Fig. 2*A*). Note that La ion produced a large reduction in I_{NS} and a shift to more positive potentials. *B* and *C* compare effects of Co and La on hyperpolarizing currents. Co had almost no effect (*B*) but La suppressed the leakage currents (*C*). Leakage current shows voltage dependence at potentials more negative than −170 mV in some neurones. Cd produced effects similar to those of La and these effects occurred in cells which did not show voltage dependence at severely hyperpolarized potentials such as the one shown in *B*.

to the amplitude of the smaller I_{NS}. The small I_{NS}s also appeared to be reduced although to a lesser extent by the addition of intracellular TEA. The leakage current also shows voltage dependence at potentials more negative than −170 mV (Fig. 3*C*) and when potentials of this magnitude were used the non-linearity was corrected for. Over their linear range (−150 to +60 mV) the leakage currents have no time dependence and the subtraction procedure we have used cancels the currents at potentials below those that activate outward I_{NS} (Fig. 3*A*).

Initial experiments usually had larger I_{NS}s. As our methods for cell isolation improved, I_{NS}s were smaller. Nevertheless we continued to use 4-AP and TEA extracellularly and TEA intracellularly to guarantee small I_{NS}s since the latter were determined only after blockage of I_{Ca}, the current in which we were most interested.

It is possible that I_{NS} may be increased by I_{Ca}, as is the case for K currents (Meech & Standen, 1975). In fact Byerly *et al.* (1979) found that I_{NS} associated with Cs-substituted K solutions was reduced by intracellular EGTA or extracellular La^{3+} and suggested that it was linked to I_{Ca}, although the N-shaped *I–V* curve characteristic of $I_{K,Ca}$ was absent. In our experiments, addition of EGTA in doses of 1–10 mM intracellularly had no effect on smaller I_{NS} which was the form of I_{NS} in all experiments referred to hereafter. This is presumably due to the fact that Cs flow through Ca-activated K channels is negligible. In addition, outward tail currents due to I_{NS} were never observed in voltage clamp experiments to steps of +10 mV even when the potential was restored to values as high as zero mV. If a Ca-dependent I_{NS} caused by Cs ions were present, it should be revealed by these procedures. The effects of Co to be described in the next section are further support for this statement. Addition of EGTA intracellularly had no effects on leakage currents measured at potentials between −50 and −150 mV and substitution of Ba for Ca extracellularly was likewise without effect at these potentials.

Cd and La ions are frequently used to block I_{Ca} although Cd has been reported to depress non-specific outward currents in *Helix* neurones (Kostyuk *et al.* 1977). La applied extracellularly clearly reduced I_{NS} and leakage conductance (Fig. 3) and similar effects were observed with Cd applied extracellularly. These effects complicate analysis of I_{Ca} and therefore we examined the effects of another I_{Ca} blocker, Co ion. Blockage of I_{Ca} by substituting Co for Ca extracellularly is reversible and does not affect the *I–V* relationship at potentials below −50 mV (Fig. 3*B*). As opposed to La and Cd, it appears that Co has little effect on leakage conductance although it completely suppresses I_{Ca} (Fig. 4*A*). It is also unlikely that Co suppresses I_{NS} since the outward current after I_{Ca} blockage with Co is larger than the outward current in the presence of I_{Ca} (Fig. 4*A*; also compare the currents in the presence of 4-AP before and after Co in Fig. 2*A*, *B*); this may not be the case when La or Cd are used. The increase in outward current after Co is accounted for by blockage of the inward Ca currents flowing at these potentials. The results indicate that Co substitution for Ca extracellularly may be the most specific method for ionic blockage of I_{Ca}. The 10 mM dose seems most suitable since it is equimolar with the normal Ca level and increasing it to 20 mM had no additional effects. Co had similar effects when Ba was substituted for Ca extracellularly.

To summarize, the conditions for optimum study of I_{Ca} are suppression of I_{Na} with Tris, J_K with Cs and I_{NS} with TEA and 4-Ap. I_{NS} is not activated by I_{Ca} under

these experimental conditions, is negligible at potentials up to +60 mV and may be corrected for at higher potentials. We may now determine the time course and amplitude of I_{Ca} without interference from other currents.

Ca currents

Ca currents appeared at potentials 10–20 mV more positive than the usual resting or holding potential, V_H, of −50 mV, and at 10 mM-$[Ca]_o$ the maximum current on

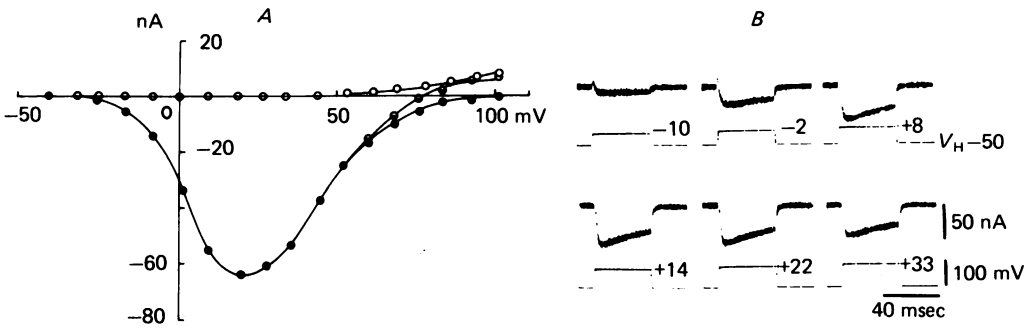

Fig. 4. *A*, current–voltage relationship for I_{Ca} before and after correction for I_{NS}. Ca_i was buffered to 10^{-8} M with EGTA. Currents were measured after addition of hyperpolarizing and depolarizing pulses of equal amplitude from the holding potential of −50 mV. Peak currents are shown by half-filled circles, I_{NS} that remains after Co substitution for Ca is shown by open circles and corrected *I–V* relationship is shown by filled circles. Note that outward current is increased after blockage of I_{Ca}. *B*, Ca currents (top traces) in response to depolarizing pulses and corresponding membrane potentials (bottom traces with values in mV) are shown in pairs. *A* and *B* were recorded from the same cell.

the *I–V* plot occurred at +10 to +20 mV (Fig. 4*A*). The currents become very small at positive potentials and after correction for leakage and non-specific currents, a reversal potential is not observed at potentials of 100–160 mV, despite approaching E_{Ca} when calculated using bulk Ca_o activity of 3 mM and bulk Ca_i activities of 10^{-7}–10^{-8} M had values of +130 to +160 mV. The onset of the current appeared without delay (Fig. 4*B*) and the rising phase had no inflexion; it could be fit by a single exponential function having a time constant referred to as τ_m using Hodgkin–Huxley notation (1952) (Akaike *et al.* 1978; Brown, Lee & Akaike, 1979; but see Kostyuk *et al.* 1977 and Kostyuk, Krishtal, Pidoplichko & Shakhovalov, 1979 for a different interpretation). Tail currents may fall as single exponential functions and τ_m values were computed from the tails as well. The two sets were equivalent at potentials above zero mV. At lower potentials τ_ms calculated from the rising phase were larger but measurement was also less certain since I_{Ca}s were small at these potentials. Half-time measurements showed a similar correspondence between the turn-on and turn-off of the I_{Ca} activation process.

After rising to a peak the Ca currents fell. For short pulses of 50 msec or less the falling phase could be fit by a single exponential function (Fig. 4*B*) but for long pulses two exponential functions were clearly required (Figs. 5 and 6*A*). We assume a model for inactivation of the form $[a\exp^{-t}/\tau_{h_1}(V)+b\exp^{-t}/\tau_{h_2}(V)+h_\infty(V)]$ where we refer to the time constant of the faster falling phase as τ_{h_1} and the time constant of the

slower phase as τ_{h_2}. τ_{h_1} is 0·05–0·1 times τ_{h_2} and is 20–30 times τ_m. The voltage dependences of τ_m, τ_{h_1} and τ_{h_2} have similar shapes as shown in Fig. 5*B* with largest values appearing to occur between −20 and −30 mV. The time constants show greatly reduced voltage dependence at potentials above 10 mV. τ_{h_1} values showed some variability at potentials below +20 mV probably because steady state inacti-

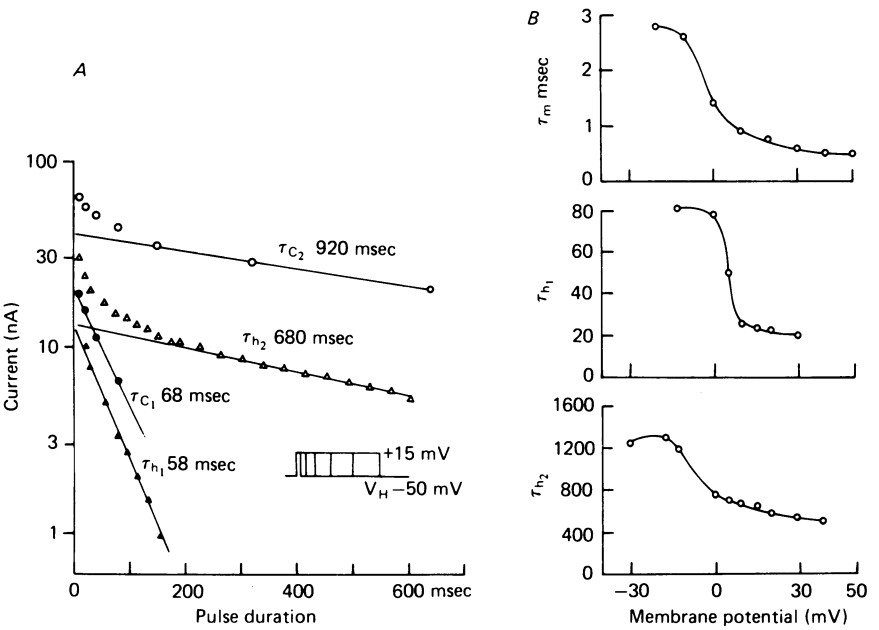

Fig. 5. I_{Ca} kinetics. *A*, plots of inactivation rates using single pulses (τ_hs) and prepulses (τ_cs) with τ defined in the text. Command potentials were +15 mV. Current records were similar to those shown in Fig. 6*A*, 7 and 10. The biexponential nature of inactivation is clear and the differences in taus and amplitudes among the two sets of values are probably not significant. *B*, activation and inactivation time constants of I_{Ca} and their relationship to membrane potential. The voltage dependence of the time constants of activation (τ_m), fast inactivation (τ_{h_1}) and slow inactivation (τ_{h_2}) are average values from four cells. τ_{h_2} was more difficult to measure as discussed in the text. The intracellular solutions did not contain EGTA.

vation did not reach zero at less positive potentials (Fig. 9), and it was sometimes difficult to estimate the exact level of the steady current. At potentials where steady state currents reached zero after 1·0 or more seconds, there was however little difference between τ_{h_1} values estimated from zero current or from the current level after 40–100 msec. τ_{h_2} values have more uncertainty because it was often difficult to assign a minimum asymptotic value. Pulse duration of 1–3 sec for I_{Ca} and 5–10 sec for I_{Ba} were required to produce complete inactivation (Fig. 9) and because such durations were harmful they were not usually applied. Under most circumstances the current that persisted at the end of the pulse was used as the asymptotic value. Despite these uncertainties it was obvious that τ_{h_2} was much greater than τ_{h_1} and this was also true for Ba currents and Ca currents in the presence of intracellular EGTA. The taus obtained with prepulses τ_{c_1} and τ_{c_2} are discussed in a subsequent section.

The Ca currents we have described could be obtained for experimental times as great as 6 hr without large changes in their amplitudes. We have never found Ca currents to be short-lived in healthy, perfused cells.

The results to this point show that I_{Ca} inactivates but do not indicate whether the process is current- or voltage-dependent. These possibilities must be examined in

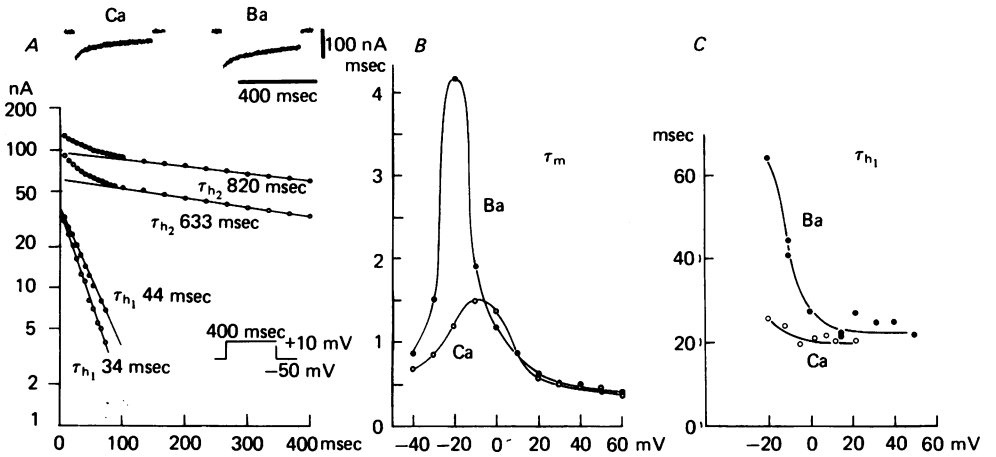

Fig. 6. Comparison of Ca and Ba currents. *A*, *B* and *C* are from different cells. *A*, effects of equimolar substitution of Ba (10 mM) for Ca in the extracellular solution. Intracellular solutions did not contain EGTA. Depolarizing and hyperpolarizing pulses were applied and added. The response reaches a new steady value within 5 min of substitution. The currents decay with two time constants, open circles for I_{Ca}, filled circles for I_{Ba}. Taus are larger for I_{Ba} although as noted already τ_{h_2} values are more difficult to measure. *B*, activation time constants (τ_m) were measured as described in the text and their relationships to membrane potential were similar for I_{Ca} and I_{Ba}. τ_m values for I_{Ba} at potentials more negative than −10 mV are larger but at more positive potentials they are similar. *C*, inactivation time constants (τ_{h_1}) were measured using 40 msec pulses. The current at 40 msec was assumed to be the asymptotic value approached by fast inactivation. A small effect from the slow inactivation process is negligible at such short times. This was confirmed in other experiments using pulses sufficiently prolonged to reach the steady state of complete inactivation. In these cases correction of τ_{h_1} after subtraction of τ_{h_2} had little effect on the τ_{h_1} values.

several ways since no single test is conclusive. The methods used were substitution of Ba for Ca extracellularly, intracellular perfusion with Ca buffers, and prepulses that increased membrane potential but reduced I_{Ca} amplitude. The results are presented next and provide evidence that inactivation is both Ca current-and voltage-dependent.

Ba currents

Substitution of Ba for Ca resulted in larger currents which inactivated more slowly (Fig. 6*A*) similar to results reported by Magura (1977), Connor (1979) and Adams & Gage (1980). The difference is not due to the inability of Ba to activate K currents (Hermann & Gorman, 1979), since in our experiments these currents and I_{NS} were already negligible. The mechanism for the increase in I_{Ba} will be described in a subsequent paper. The effects of substitution reached new steady values within about 5 min. I_{Ba} had a lower threshold voltage and larger peak amplitudes at potentials

up to about +20 mV. At potentials more positive than +20 mV I_{Ca} peaks were greater than I_{Ba} peaks, i.e. the peak *I–V* relationships crossed each other (Fig. 7).

The onset of I_{Ba} was a monoexponential function similar to the onset of I_{Ca} and τ_m values for I_{Ba} and I_{Ca} were similar at most potentials (Fig. 6*B*). The peak of the τ_m (*V*) relationship for I_{Ba} was more negative by a similar amount to the shift in

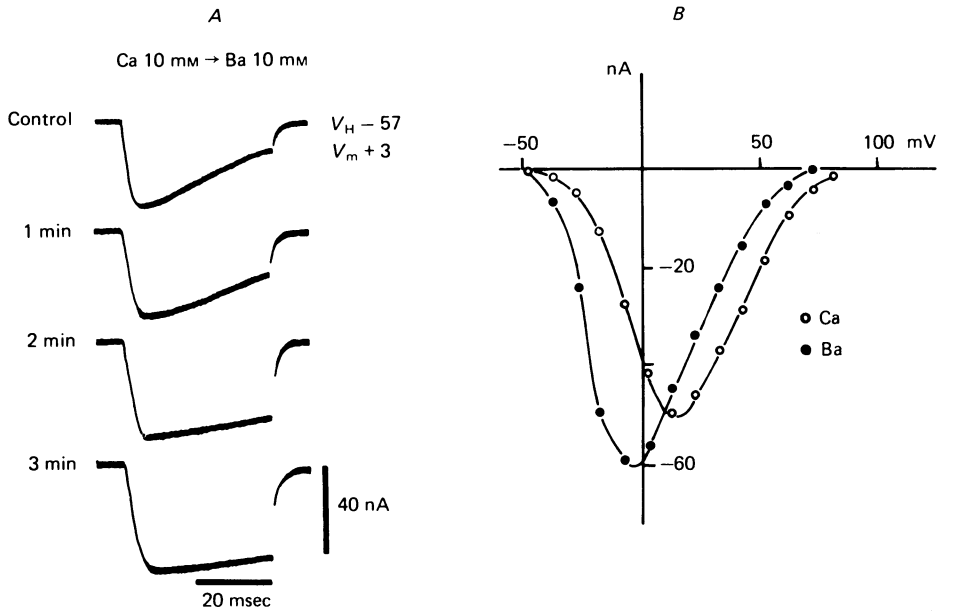

Fig. 7. Comparison of initial inactivation rates and *I–V* relationship for I_{Ca} and I_{Ba}. *A*, effects of equimolar substitution of Ba for Ca in the extracellular solution. Intracellular solutions did not contain EGTA. Depolarizing and hyperpolarizing pulses were applied and added. The response reached a new steady value within 5 min. *B*, *I–V* relationships for I_{Ca} and I_{Ba}.

the nadirs of the *I–V* relationships. The initial decay of I_{Ba} was always much more prolonged than the decay of I_{Ca} (Figs. 6 and 7) even when scaled to equivalent amplitudes. The differences in τ_{h_1} values were about 3-fold at −20 mV but were smaller at more positive potentials (Fig. 6*C*). τ_{h_2} values were compared at only a few voltages because of the difficulties referred to in the previous section. In Fig. 6, τ_{h_2} was larger for I_{Ba} but in Fig. 13 *A*, τ_{h_2} was slightly larger for I_{Ca} at potentials of zero and −10 mV and slightly smaller at potentials of 10 and 20 mV. As we shall see in the next section, intracellular EGTA had little or no effect on τ_{h_2} (Fig. 8*B*).

Effects of intracellular EGTA (EGTA$_i$) on I_{Ca} kinetics

The effects of intracellular EGTA, $EGTA_i$, in concentrations of 0·1, 1, 3, 10 and occasionally 25 mM at Ca activities, Ca_is, ranging from 10^{-6} to 10^{-9} M were examined at potentials below those that activate I_{NS} (+60 mV). Initially a small increase in peak inward currents occurred and the maximum peak current of the *I–V* curves was shifted about 10 mV in the positive direction. After 15–30 min peak currents decreased slightly. The largest effect observed was a reduction in the rate of

inactivation of I_{Ca} especially the fast process (Fig. 8); τ_{h_2} appeared to increase slightly but this could be due to a shift in the τ_{h_2} (V) relationship to more positive potentials. The temporary increase of peak current occurred more quickly at $EGTA_i$s of 25 mM at constant Ca_i but these concentrations of EGTA tended to reduce I_{Ca}s at longer times. Therefore concentrations of 0·1, 1·0 or 10 mM-$EGTA_i$-buffered solutions at Ca_i

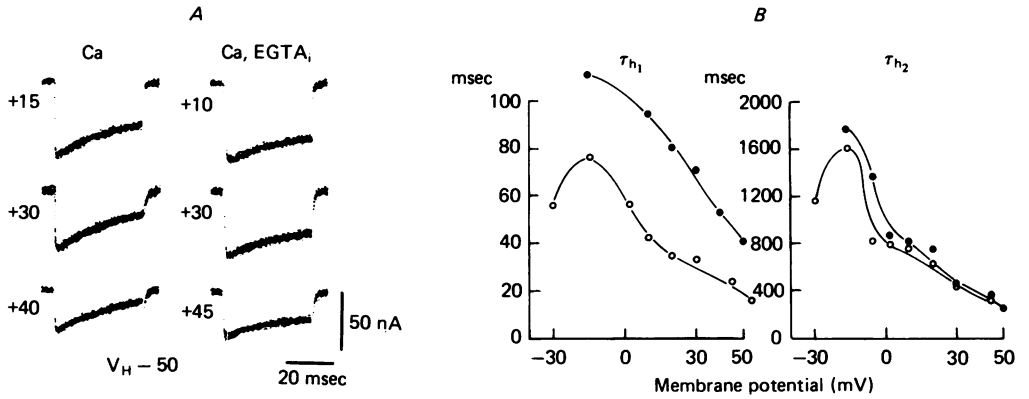

Fig. 8. *A*, effects of EGTA (25 M) applied intracellularly on Ca current. Calcium current (10 mM-Ca_o) at membrane potentials indicated in millivolts are shown before (left-hand side) and after (right-hand side) application of EGTA intracellularly. Depolarizing and hyperpolarizing pulses of equal amplitude were applied and added. 25 mM-EGTA lowered pH and this was adjusted to 7·3 by extra CsOH or Tris-base. This data was taken from the Tris experiment and there was no difference in the Cs experiment. The osmolarity of the Cs solution was about 30 % greater than that of the control and the Tris-buffered EGTA solutions have a 10 % increase in osmolarity. Osmolarity changes of 10–30 % produced by changes in Cs aspartate only had no effect. *B*, effects of intracellular EGTA on the inactivation time constants of the Ca current. Ca current (10 mM-Ca_o) was produced by 600 msec pulses to allow measurement of the fast and slow inactivation time constants τ_{h_1} and τ_{h_2}. The time constants before (open circles) and after (filled circles) addition of 25 mM-EGTA to the intracellular solution are shown.

of 10^{-8} M were used. Decreases in Ca_i from 10^{-8} to 10^{-9} M at constant levels of $EGTA_i$ produced no clear increases in Ca current. However, increases in Ca_i to 5×10^{-7} and 10^{-6} M produced reductions of Ca current particularly in the case of 10^{-6} M. At this level of Ca_i, large increases in leakage current occurred after intervals of 10–15 min and Ca currents were abolished (Akaike *et al.* 1978; Kostyuk *et al.* 1977).

The results show that addition of EGTA to the internal perfusate appeared to change I_{Ca} into a current resembling I_{Ba}. However, the effects are not identical even at $[EGTA]_i$s of 25 mM. Thus, substitution of Ba_o for Ca_o in the presence of $EGTA_i$-buffered internal perfusate led to a further small reduction in the rate of decay of current. The situation is complicated by the fact that substitution of Ba_o for Ca_o produces hyperpolarizing shifts in the I–V relationship and a shift of voltage-dependent inactivation parameters in this direction would account, at least qualitatively, for the observation.

The effectiveness of EGTA-buffered internal perfusate was tested further by examining the effects of $EGTA_i$ on the Ca-activated K current, $I_{K,Ca}$ (Meech, 1974). In these experiments K replaced Cs and TEA and 4-AP were omitted. $EGTA_i$s of

10 and 25 mM at Ca_is of 10^{-7}–10^{-9} M reduced steady-state K currents and tail currents indicating block of $I_{K,Ca}$. Substitution of Co for Ca extracellularly had no further effect on K currents.

Effect of prepulses on I_{Ca} and I_{Ba}

Steady-state inactivation of h_∞, measurements using the two pulse paradigm of Hodgkin & Huxley (1952) require prepulses of sufficient duration to reach a new

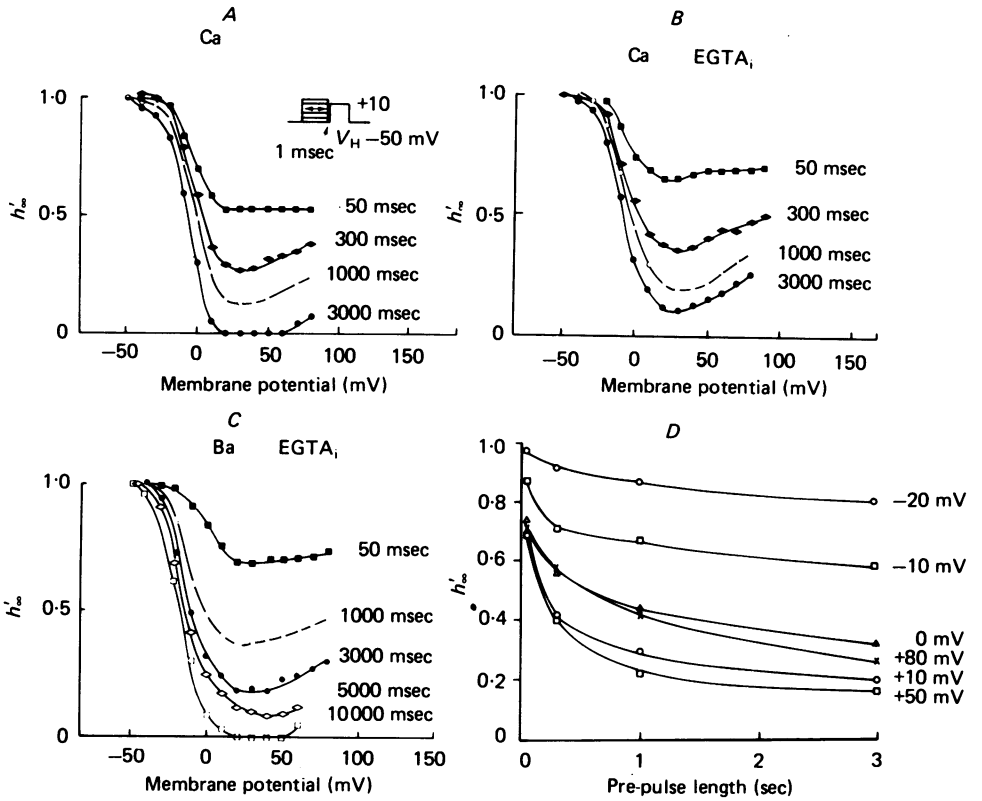

Fig. 9. Steady-state inactivation indicated as h'_∞ for I_{Ca} and I_{Ba} in one neurone. See text for discussion of h'_∞. Stimulation paradigm shown in the inset of *A* which was obtained in 10 mM-Ca_0 and zero intracellular EGTA. *B* was obtained with intracellular EGTA; *C* was obtained with 10 mM-Ba substituted for 10 mM-Ca extracellular in the presence of intracellular EGTA. There was an interval of one msec between the prepulse and the test pulse, but this pause did not affect the results (cf. Fig. 10). Holding potential was −50 mV. The change in h' for I_{Ca} shown in *A* as a function of duration is equivalent to the rate of development of inactivation and is shown in *D*. The plot also shows the potential dependence of rate of inactivation. The half-times at the potentials shown are similar to the τ_{h_2} values at these potentials.

steady state. This necessitated prepulse durations of 3–5 sec in the case of Ca and 6–10 sec in the case of Ba. Such durations limited the number of trials that could be done without deterioration of the neurone and, as noted in the Methods section, we often used prepulse durations of 50–100 msec as a measure of inactivation although the process was incomplete and we denote this process by the parameter h'_∞ (Figs. 9 and

10). The relationships of h_∞ and h'_∞ to membrane potential are very similar (Fig. 9). h_∞ and h'_∞ have values of 1·0 at −50 mV and these are unchanged at −60 to −100 mV in the solutions used presently. As prepulse potentials become more positive, test pulse current peak amplitudes are reduced. They reach their lowest values at about +15 mV and then increase at more positive potentials (Tillotson, 1979) resembling in the case of I_{Ba} or I_{Ca} with $EGTA_i$ where Ca current-dependent inactivation is avoided, the situation found for the Na channel in internally perfused squid axon (Chandler & Meves, 1970). The values of h'_∞ depend upon duration of the prepulse as well as amplitude decreasing to values of zero with sufficiently prolonged prepulses (Fig. 9). For such curves h'_∞ becomes h. The descent of h'_∞ (V) curves was less steep and was shifted slightly to higher potentials. Compared to I_{Ca} longer prepulse durations were required to produce equivalent values of h_∞ and h'_∞ for I_{Ba}.

The addition of 0·1, 1·0 and 10 mM-EGTA to the internal perfusate increased h_∞ and h'_∞ for I_{Ca} at each voltage and shifted the h_∞ (V) and h'_∞ (V) relationships to slightly more positive potentials (Fig. 9). The over-all shape of these relationships was not greatly changed and the extent of inactivation was reduced at very positive potentials, as is the case for I_{Ca} without $EGTA_i$ and I_{Ba}. These doses of $EGTA_i$ did not affect the time course of the Ba current produced by substitution of extracellular Ba for Ca although as noted earlier the rate of inactivation of I_{Ca} was reduced. Unlike the case for I_{Ca} $EGTA_i$ did not shift the h_∞ (V) or h'_∞ (V) relationships of I_{Ba} to more positive potentials. It should be emphasized that both Ca current with $EGTA_i$ and Ba current, while inactivating more slowly than Ca current, still show almost complete inactivation if voltage step durations between +20 and +60 mV are prolonged sufficiently.

The extent of inactivation produced by a prepulse may vary from cell to cell. For example, in two cells prepulse durations of 50 msec were used and one cell showed complete inactivation of test I_{Ca} between +10 and +100 mV similar to the results of Akaike *et al.* (1978), whereas the other cell was only partially inactivated. The effects for I_{Ba} were not different between the two cells; however, if anything the prepulse effect was smaller in the cell showing the largest fall in test I_{Ca}.

The results show that considerable inactivation persists at prepulse potentials greater than +75 mV when there is very little influx of Ca ion and Ca-dependent inactivation should be small. The h_∞ (V) curves give no sign that if extrapolated they would reach values of 1·0 at potentials of +130 to +160 mV which equal the calculated values for E_{Ca} in the bulk solutions normally used in the present experiments. Furthermore, the relationship of τ_{h_1} and τ_{h_2} to membrane potential does not appear to be increasing at potentials between +10 and +50 mV where prepulse I_{Ca} is reduced.

One test of the correspondence of h_∞ or h'_∞ to prepulse influx of Ca ions is to integrate the prepulse current and plot either h_∞ or h'_∞ against this value (Tillotson, 1979). Integrated current was determined by cutting out the current segments, weighing them and calibrating the weighed segment against a weighed segment of known integrated current. The results from two experiments are shown in Fig. 11 and demonstrate that a minimum amount of Ca influx is necessary before any inactivation can be demonstrated. Other evidence for this is the fact that intervals ranging from 1 to 5 msec between pre- and test-pulses had no effect on h'_∞ (Fig. 10).

Hence it is unlikely that the flow of I_{Ca} during current tails having τ_ms of 1 msec or less, or during the settling of the voltage clamp for which the integrated current is 10^{-3} times the minimum amount of Ca_i required in Fig. 11, can produce inactivation. Fig. 11 shows that h'_∞ is inversely related to integrated I_{Ca} to +10 mV but also shows that h'_∞ is mainly independent of Ca influx at more positive potentials. At these

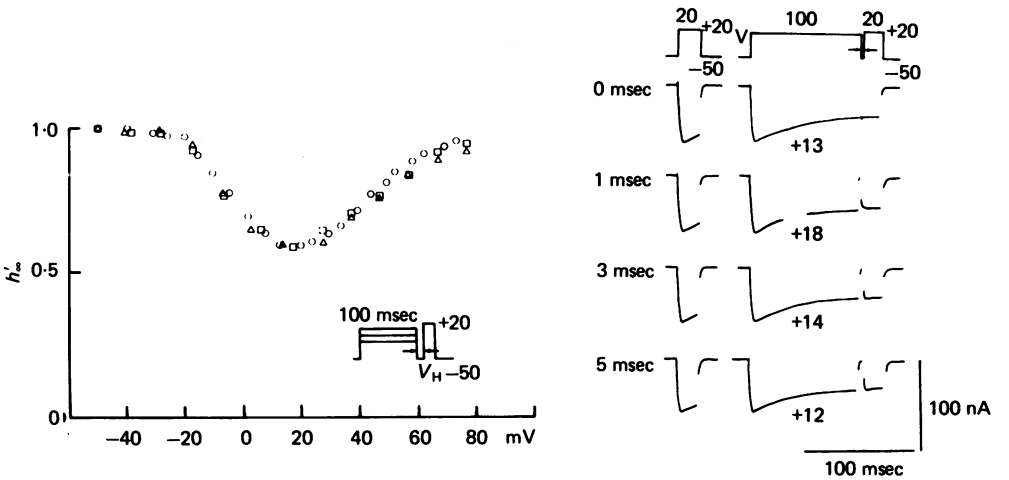

Fig. 10. Effects of short intervals between prepulse and test pulse on h'_∞ (V) relationship. The pause between the prepulse (100 msec duration) and the test pulse (20 msec duration, +20 mV) was changed from 0 to 5 msec in a solution with $CaCl_2$ at 10 mM extracellularly. Intervals over this range produced no differences in the voltage dependency of h'_∞ (left-hand Figure). Traces of the actual currents produced by test pulse with or without prepulse are shown in the right-hand Figure. Holding potential was −50 mV. ○, 0 msec; □, 1 msec; △, 3 msec.

potentials, possible differences in the rate of Ca accumulation do not seem important because τ_m for I_{Ca} is halved between +10 and +50 mV, whereas h'_∞ is virtually unchanged. Fig. 11 shows in addition that h'_∞ may increase as integrated I_{Ca} increases or decreases, indicating the absence of any simple relationship between integrated I_{Ca} and h'_∞ over a substantial range of potentials. The complexity could be due to associated effects of membrane potential either on inactivated channels or on a different population of Ca channels and as noted already is similar to the effects observed at positive membrane potential on the Na channels in squid giant nerve fibers (Chandler & Meves, 1970).

The amount of inactivation and the rate at which it develops can be estimated from experiments which generated the curves shown in Fig. 9*A*, *B*, *C*. The interval between conditioning and test pulse was 1·0 msec and the potential during the interval was set at −50 mV. This procedure provides an alternative to the measurement of tail currents which as noted is very difficult. Under these conditions m_0 for the start of the test pulse should be less than 0·1 and h_∞ during the interval should change very little if at all since τ_{h_1} is likely to be in the range of 20 msec. The test pulse duration is sufficiently long for m to reach m_∞ and we measure peak currents. The results are shown in Fig. 9*D* and indicate that the amount of inactivation is greatest at +50 mV

despite the fact that maximum Ca influx occurs at about +15 mV. Furthermore, the amount of inactivation is substantial at +80 mV when Ca influx is very small. The rate of development of inactivation increases as the potential is made more positive although the differences are small at potentials above zero mV. The half-times correspond to values obtained for τ_{h_2}. Although a faster process corresponding to τ_{h_1}

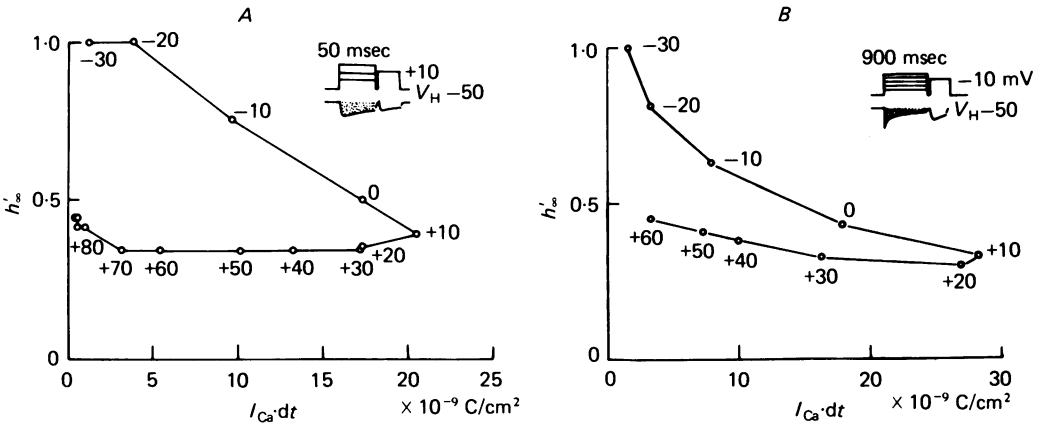

Fig. 11. Relationships between the steady-state inactivation parameter (h'_∞) and Ca influx during the prepulse. *A* and *B* were taken from two different cells. h'_∞ values were obtained using short (50 msec, *A*) and long (900 msec, *B*) prepulse durations as shown in the Figure insets. Extracellular Ca concentration was 10 mM. Ca influx during the prepulse was measured by integration of the Ca current (see text for description of method). Membrane potentials of each prepulse are shown in millivolts with each data point.

is probably present at early times, the data in these experiments were insufficient to establish this point.

In two experiments, the rate at which inactivation developed was examined in more detail at shorter prepulse duration to obtain an estimate of the faster process. As shown in Fig. 5*A* the rate of onset could be fitted with two exponential functions termed τ_{c_1} and τ_{c_2}. At +35 mV τ_{c_1} was larger than τ_{h_1} but the difference may not be significant; τ_{c_2} was also larger than τ_{h_2}. Tillotson & Horn (1978) made similar observations in *Aplysia* neurones.

Results like those shown in Fig. 5*A* were obtained for I_{Ca} with $EGTA_i$ and I_{Ba} but the amount of inactivation was reduced in both cases and the rate at which it developed was slowed particularly in the case of I_{Ba}.

Effects of changes in Ba_i on I_{Ba} and I_{Ca}

Increases in Ca_i activity to levels of $1{\cdot}6 \times 10^{-6}$ M abolish I_{Ca} after 20–30 min (Akaike *et al.* 1978); similar findings were reported by Kostyuk *et al.* (1977). I_{Ba} is also abolished by these levels of Ca_i (N. Akaike & A. M. Brown, unpublished results). These effects are attributed to inactivation of Ca channels by increased levels of Ca_i (Kostyuk, 1980). Therefore, it seemed reasonable to determine whether increases in Ba_i activity would have any effect on kinetics of I_{Ba} or I_{Ca}. Moreover, it is possible that Ca-dependent inactivation is partly due to current flow alone through the Ca channel inverting the argument used for the relief of TEA blockage of K channels

by K current flow (Armstrong, 1975). We examined these questions by perfusing the cell's interior with Ba_i concentrations ranging from 10^{-6} to 10^{-3} M. In some cases (i.e. 10^{-4}–10^{-5} M) Ba_i was buffered with $EGTA_i$ (0·1–1·0 mM). Changes in Ba_i from 10^{-6} to 10^{-3} M had no effect on the time course of I_{Ba} (Fig. 12) although the amplitude was reduced. However, the inactivation rate of I_{Ca} was clearly slowed and the effects

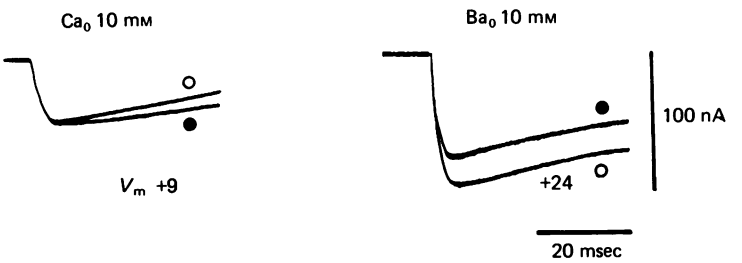

Fig. 12. Ca and Ba currents in the presence of intracellular Ba. Neurone was perfused for 30 min with intracellular solution containing 1 mM-$Ba(OH)_2$. In the presence of Ba_i the rate of inactivation of I_{Ca} was slowed whereas only the amplitude of I_{Ba} was changed.

were greater after longer periods of exposure. In cells with low concentrations of Ba_i peak inward I_{Ca} was unchanged or in some cells, increased slightly. With 10 mM concentrations of Ba_i, peak I_{Ca} was reduced.

We have already demonstrated that substitution of Ba_o for Ca_o prolongs current flow by reducing the rate of inactivation. By contrast, addition of 10 mM-Ca to an extracellular solution containing Ba shortens the time course of the inward current. However, if Ba_i is present at 10^{-3} or 10^{-4} M, substitution of Ca for Ba in the extracellular fluid has a smaller effect on the time course of current flowing through the Ca channel. To summarize, the results show that accumulation of Ba_i in amounts as great as 10^{-3} M has no effect on steady-state inactivation or the rate of inactivation of I_{Ba}. Furthermore, increases in Ba intracellularly seem to reduce the rate of inactivation of I_{Ca}. It is unlikely, therefore, that Ba accumulation during I_{Ba} enhances inactivation. Moreover, Ca-dependent inactivation has no component due to current flow alone.

DISCUSSION

Considerable effort was expended in these experiments to isolate I_{Ca} from contaminating outward currents. The success could be evaluated in part by comparing the reversal potential for I_{Ca} with the Nernst potentials of +130 or +160 mV calculated from bulk activities used presently. The observed reversal potential was about +80 mV but after correction for the currents that remain following I_{Ca} blockage a reversal potential could not be measured; rather the inward current became vanishingly small at potentials above +100 mV. This result differs from results of Standen (1975), Reuter & Scholz (1977), Kostyuk *et al.* (1977), Connor (1979) and Adams & Gage (1979), all of whom showed reversal currents at potentials of less than +80 mV. The experiments of Standen (1975) and Kostyuk *et al.* (1977) were done on *Helix* as were the present experiments so that species differences are unlikely.

The simplest explanation is that isolation of I_{Ca} was more complete in these experiments because either the currents flowing through K channels were suppressed more fully or Cs flow through the Ca channel is negligible. The latter seems likely and would account for the important observation that blockage of Ca currents with Co is followed by an increase in non-specific outward current. In our earlier experiments (Akaike *et al.* 1978) null potentials approaching E_{Ca} were observed although 4-AP and TEA were not used. However, average values for leakage correction were used rather than individual corrections as used presently which might explain the earlier results.

Satisfactory suppression of outward currents in Cs-substituted extra-and intracellular solutions required the addition of 4-AP and large doses of TEA. Addition of EGTA intracellularly had no further effect under these conditions which makes it unlikely that a Ca-activated outward Cs current was present in our experiments. In addition, slow outward tail currents have never been observed even at recovery potentials of zero mV and Co blockage of I_{Ca} was followed by an increase rather than a decrease in outward current. Hence, the slowed inactivation rate of I_{Ba} cannot be attributed to a blocking action of Ba on Cs flow through K channels, since this flow is negligible under the present solution conditions. The choice of I_{Ca} blocker may also be important when ionic blockers are being used. Thus, Cd and La unlike Co, produce significant block of leakage and non-specific outward currents which would reduce the current amplitudes used for correction of I_{Ca} measurements.

Despite the precautions we have taken, uncertainty remains regarding I_{Ca}s at potentials above +80 mV since in this region the non-specific current approaches I_{Ca} in amplitude. It is difficult to lower E_{Ca} much below +100 mV because increasing Ca_i to values above 10^{-6} M has deleterious effects on the cell (Akaike *et al.* 1978; Kostyuk *et al.* 1977; Hagiwara & Naka, 1964) as does reductions in Ca_o. However, at potentials below +60 mV the current records probably reflect I_{Ca} and I_{Ba} faithfully. They indicate that I_{Ca} inactivates in a bi-exponential manner and this is supported by the results of the prepulse experiments. Both rates of inactivation are smaller than those reported in our earlier paper (Akaike *et al.* 1978). In that study small cells with maximum inward Ca currents of 20 nA or less were especially selected to minimize series resistance effects from the single suction pipette used in the experiments. TEA and 4-AP were not used either. The shortened time course may therefore have been due to outward currents although differences in cell sizes could also have been involved.

From the present results two mechanisms of inactivation can be excluded as major factors. The first is a significant change in electrochemical potential resulting from accumulation of Ca or Ba ions intracellularly during current flow. If this were the case steady state inactivation should be greater and rate of inactivation faster for I_{Ba} than for I_{Ca} since I_{Ba} is larger than I_{Ca} and activates at a similar rate. Yet the opposite is the case even when the currents are scaled to the same peak amplitudes. The second is depletion of Ca or Ba at the extracellular face of the membrane and it may be excluded for similar reasons, although such a mechanism may be a factor in I_{Ca}s flowing across skeletal muscle transverse tubules (Almers, Fink & Palade, 1981). Nevertheless, it is possible that either or both of these two mechanisms are operating presently to a small extent, since they might be involved in the cross-over

of the *I–V* curves for I_{Ba} and I_{Ca} that occurs at very positive potentials (Fig. 7*B*).

On the other hand two mechanisms appear likely to play large roles, namely Ca current-dependent inactivation due to influx of Ca ion and voltage-dependent inactivation. The fact that $EGTA_i$ reduces the initial rate of inactivation and the amount of steady-state inactivation of I_{Ca} is strong support for the former mechanism. The effect of $EGTA_i$ on the initial rate of inactivation does not appear to be due primarily to changes in the membrane field since the large reduction in the faster rate of inactivation, $\tau_{h_1}^{-1}$, can not be accounted for by the 10 mV shift in the *I–V* relationship produced by $EGTA_i$ (Fig. 8*B*). The decrease in the amount of steady-state inactivation is associated with a small shift to more positive potentials which only accounts for part of the decrease (Fig. 9*B*). Similar effects of $EGTA_i$ on steady-state inactivation have been reported by Tillotson (1979) and Brehm, Eckert & Tillotson (1980). Attributing these results to Ca current-dependent inactivation may be extended to account for the observations that at high Ca_is of about 10^{-6} M the Ca channel is inactivated (Kostyuk *et al.* 1977; Akaike *et al.* 1978) and that outward current flow of Ca cannot be observed when the electrochemical gradient is in the outward direction (Akaike *et al.* 1978; Kostyuk, 1980).

The slower inactivation process with time constant τ_{h_2} was more difficult to evaluate as noted earlier and this complicated comparisons among I_{Ca}, I_{Ca} with $EGTA_i$ and I_{Ba}. It appears that there are no significant differences in τ_{h_2} among these three cases and that the effects of Ca-dependent inactivation are manifested in the first 40–100 msec of current influx.

The Ca-dependent inactivation could involve H^+ ions since the influx of Ca ions and their subsequent buffering would release protons. This appears unlikely, however, because the internal perfusion fluid was buffered to pH 7·35 using Tris or HEPES buffers so that pH changes should not have occurred. Since the effects of EGTA were obvious, it appears that Ca ion is the direct cause of the current-dependent inactivation.

However, inactivation persists in the presence of $EGTA_i$ in amounts which prevent totally the absorbance changes due to increases in Ca_i produced by I_{Ca} (Ahmed & Connor, 1979). It could be argued that $EGTA_i$ does not totally buffer the Ca_i accumulating near the inner mouth of the channel but the Ca_i activity would have to be less than 10^{-8} M because higher Ca_i would have been detected by Arsenazo III (Gorman & Thomas, 1980). Our earlier data (Akaike *et al.* 1978) as well as that of Kostyuk (1980) suggested that inactivation of I_{Ca} by Ca_i requires activities of 10^{-7} M or greater. Nevertheless, the observation that Ba substitution for Ca extracellularly in the presence of $EGTA_i$ further slowed inactivation may support the argument for incomplete buffering and persistent Ca-dependent inactivation. This explanation is not sufficient though because I_{Ba} also inactivates and the inactivation is unaffected by changes in intracellular concentrations of Ba_i from 10^{-6} to 10^{-2} M the highest value being 10^6 times greater than the Ca_i activities normally present. As noted, Ba_i slows inactivation of I_{Ca} which is strong evidence that Ba ions cannot produce inactivation. Instead, they seem to reduce or protect against the Ca-dependent inactivation produced by the influx of Ca ions. The results show that the effect of current flow is Ca specific and also show that voltage-dependent inactivation occurs. They argue

against the interpretation of Tillotson (1979) that inactivation of I_{Ca} is Ca-dependent only. Further evidence against this view is the independence of h_∞ (V) from Ca_i accumulation at potentials more positive than +10 mV (Fig. 11), and the fact that the h_∞ (V) relationship does not extrapolate to values close to 1·0 at prepulse potentials beyond E_{Ca} (Fig. 9). The extent of inactivation and its rate of development were also similar at prepulse potentials that ranged from +15 mV where Ca influx is maximal to +80 mV where it is very small indicating again that for short intervals between prepulse and test pulse, voltage-dependent inactivation is important.

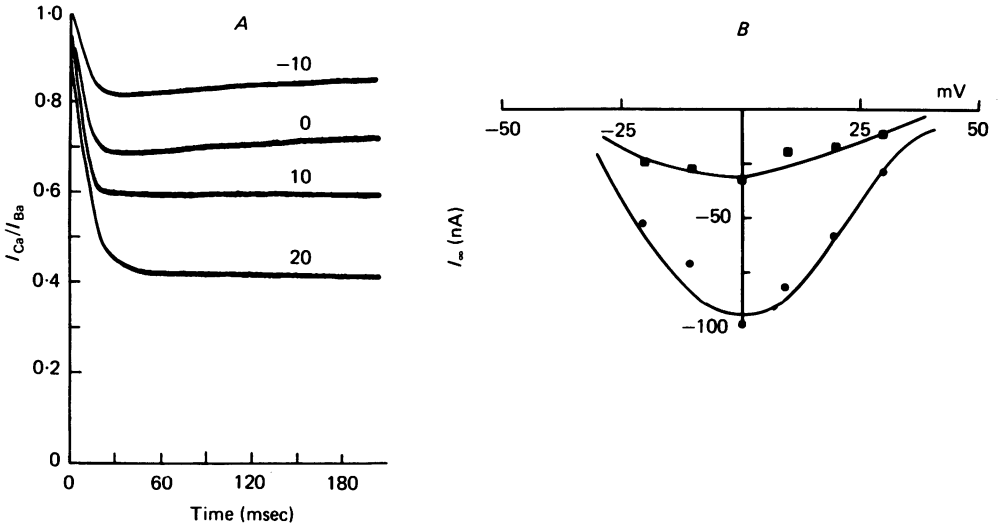

Fig. 13. *A*, time course of inactivation due to accumulation of intracellular Ca. Current records for Ca were divided by current records for Ba at the indicated voltage levels. The result is a scaled version of inactivation due to Ca binding to an intracellular site with the greatest reduction in I_{Ca} occurring at early times similar to τ_{h_1}. Note that the shape of the curves is relatively voltage-independent although the largest effect occurs at potentials where peak I_{Ca} is greatest (see Fig. 7). At zero and −10 mV the ratio of the currents increases slightly at longer times suggesting that I_{Ba} was inactivating more quickly at these times. As noted in the text it is uncertain whether τ_{h_2} values for I_{Ca} and I_{Ba} differ significantly. *B*, plot of I_{Ba} in nA at 200 msec during a potential step (●) is compared to the probability that the inactivating site is occupied by Ca_i kCa(∞), (■), calculated using eqn. (4) which gives the amount of inactivation. The similarity of the two plots suggests that Ca accumulation at the binding site has levelled off at these long times and that the amount of Ca accumulation is proportional to the amplitude of the inward current.

A key argument for the absence of any voltage-dependent inactivation was the observation that tail current amplitude was unaffected by maintained prepulses when prepulse potential was +160 mV (Tillotson, 1979). However, tail current measurements for I_{Ca} are difficult because the tails relax with taus of less than one millisecond (Fig. 6*C* of Smith & Zucker, 1980). Accurate measurement requires $R_s C_M$ values of less than 200 μsec, values which are far lower than those that would be expected when the rise time of the voltage command step is 0·4–1·0 msec (Tillotson, 1979).

It should be noted that the case for Ca-dependent inactivation has been made using relatively long intervals between prepulse and test pulse. We have used mainly short

intervals comparable to those used to study inactivation of the Na system in axon and it is possible that at the longer intervals processes other than the ones we have been examining may have occurred. At these short times it appears that inactivation can be explained partly by a specific effect of Ca and partly by a potential-dependent effect. The latter may account for half or more of the inactivation that occurs. The potential-dependent inactivation parameter decreases and then increases as potential is increased and similar results were observed for the sodium system in squid giant axons (Chandler & Meves, 1970). The subject has been reviewed recently by Meves (1978). The model proposed by Chandler & Meves (1970) accounts for two rates of inactivation as well as reopening of the Na channels at positive potentials and may apply to the situation for Ca channels. A detailed examination of this model was not undertaken presently; rather the combined effects of Ca- and voltage-dependent inactivation upon the kinetics and amplitude of I_{Ca} were assessed without a critical reliance upon any particular model of voltage-dependent kinetics. We start by attributing the difference in the time course of inactivation between I_{Ca}, I_{Ca} with $EGTA_i$ and I_{Ba} to the accumulation of Ca ions at the inner surface of the membrane which bind with a site to produce inactivation. The reaction is assumed to be at equilibrium and nonsaturating. Part of I_{Ca} inactivation and all of the inactivation of I_{Ba} and I_{Ca} with $EGTA_i$ are attributed to a voltage-dependent process. We assume the Chandler-Meves (1970) version of voltage-dependent subunits where the Ca channel may have two inactivated states h_1 and h_2, although the $h_1 \times h_2$ model of Kostyuk & Krishtal (1977) also applies. Thus, for Ba or for Ca in the presence of EGTA we have

$$I_{Ba} = \bar{G}_{Ba} f(v)\, m\, (h_1 + h_2), \tag{1}$$

and for Ca we have

$$I_{Ca} = \bar{G}_{Ca} f(v)\, m\, (h_1 + h_2)\, (1 - k[Ca(t)]_s), \tag{2}$$

where $f(v)$ is the voltage dependence of the normalized current through the open channel and $[Ca(t)]_s$ is the Ca concentration at the inner surface of the membrane where the dependence on time is shown explicitly. The product $k[Ca(t)]_s$ is the probability that the subunit is in the inactive state and includes the binding constant between Ca ion and site. These two equations provide a convenient way to evaluate the time course of $[Ca(t)]_s$:

$$\frac{I_{Ca(t)}}{I_{Ba(t)}} = \frac{\bar{G}_{Ca}}{\bar{G}_{Ba}} (1 - k[Ca(t)]_s). \tag{3}$$

Also, we have evaluated the amount of Ca dependent inactivation as a function of voltage with the following equation

$$k[Ca(t = \infty)] = 1 - \frac{I_{Ca}(t = \infty)/I_{Ba}(t = \infty)}{I_{Ca}(t = o)/I_{Ba}(t = o)}, \tag{4}$$

where the ratio in the denominator is evaluated at a time early in the voltage pulse, before the Ca has a chance to accumulate; thus, the ratio of these currents is simply the ratio of the $\bar{G}$s.

In Fig. 13*A* we have evaluated $I_{Ca}(t)/I_{Ba}(t)$. We see that all the curves thus obtained decrease rapidly, with approximately the same time course, and by about

40 msec they have reached an almost constant value. From eqn. (3), we take this to imply that the Ca-dependent inactivation term, $(1-k([\mathrm{Ca}(t)]_s)$, decreases rapidly to a steady-state value with a time course which is not a strong function of voltage; thus, the Ca at the inner surface accumulates rapidly to a constant value during a voltage clamp pulse.

This result can also be seen in Fig. 8. The Ca dependent inactivation has a relatively fast time course and affects τ_{h_1} rather than τ_{h_2}. Hence the actions of $EGTA_i$ are to prolong τ_{h_1} with little or no effect on τ_{h_2}.

In Fig. 13*B*, we have evaluated the steady-state values for the Ca accumulation, $k\mathrm{Ca}(\infty)$, using eqn. (4). The same plot shows the Ca current after 200 msec of a voltage clamp step. This *I–V* relationship is similar to that for peak Ba currents (Fig. 7). It is encouraging that $k\mathrm{Ca}(\infty)$ peaks at the same potentials where the steady and peak Ba currents are greatest. Hence, maximal Ca current-dependent inactivation occurs at those potentials where the voltage-dependent portion of the conductance is maximal. The time course at $[\mathrm{Ca}(t)]_s$ may be compared to those expected from the solution of diffusion in a sphere with a constant flux at the surface (Gorman & Thomas, 1980; Carslaw & Jaeger, 1959; Crank, 1975). Since one can arrive at an effective diffusion constant which includes a homogeneous, equilibrium chemical reaction inside the cell it would seem that this solution should approximate our physical situation (Anderson, Brown & Yasui, 1979; Crank, 1975; Carslaw & Jaeger, 1959). The time course of the Ca concentration at this inner surface when realistic values are applied (diffusion constant = 10^{-7} cm^2/sec and cell radius = 100 μm) shows no sign of reaching a steady value at 50 msec (Gorman & Thomas, 1980) where we observed an almost steady value of Ca-dependent inactivation.

One possibility was that reduction in Ca influx due to voltage-dependent inactivation might cause a steady value of $[\mathrm{Ca}]_s$. To test this, we derived a solution for $[\mathrm{Ca}]_s$ in which the influx had a time course similar to that of I_{Ba} rather than the steady flux assumed for the initial diffusion model. This model also gave increasing values of $[\mathrm{Ca}]_s$ with time, although the rates of increase were reduced compared to those obtained with a constant influx. The diffusion model might still apply if the reaction at the inner surface were to saturate. However, Ca-dependent inactivation becomes larger with increases in membrane potential that produce greater Ca influx (Fig. 13) so that saturation does not appear to have occurred in the present experiments.

Thus, the above model fails even when it incorporates binding and a steadily declining influx of Ca. This may not be surprising since the process of Ca uptake may not occur homogeneously and may instead occur at submembrane structures like those described by Henkart, Reese & Brinley (1978). Na–Ca exchange at the plasma membrane can be excluded since Tris was substituted for Na extra- and intracellularly. Additionally, the uptake processes may not occur instantaneously (Gorman & Thomas, 1980). In the absence of information on Ca uptake, an alternative approach was to use a compartmental model to analyse our results. We imagined a compartment near the surface of the membrane from which Ca removal is proportional to concentration. The rate of change of Ca concentration in the compartment would be

$$\frac{\mathrm{d}[\mathrm{Ca}]_s}{\mathrm{d}t} = k_1 I_{Ca} - d[\mathrm{Ca}]_s, \tag{5}$$

where k_1 is a constant relating the influx of current to a concentration in the compartment and d is a constant related to the removal processes. The solution for a step input of I_{Ca} is

$$[Ca]_s = \frac{k_1}{d}(1 - e^{-t/d}). \quad (6)$$

With the compartmental model, $[Ca]_s$ should reach an asymptotic value and the approach to this value should be exponential, a result which reasonably describes the early results at +10 and +20 mV shown in Fig. 13 and the steady state results shown in Fig. 13*B*. However, the situation is probably more complicated since at zero and −10 mV for times greater than 30–40 msec I_{Ba} appears to be inactivating more quickly than I_{Ca} (Fig. 13*A*).

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