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CALCIUM INACTIVATION IN SKELETAL MUSCLE FIBRES OF THE STICK INSECT, *CARAUSIUS MOROSUS*

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SUMMARY

1. Inactivation of Ca currents in skeletal muscle fibres of the stick insect, *Carausius morosus*, was studied using a three-electrode voltage-clamp method.

2. The extent of inactivation showed a voltage-dependence similar to that of the Ca current, inactivation being absent in the absence of a Ca current, maximal at potentials where Ca currents are largest, and reduced at potentials close to E_{Ca}.

3. Ca currents inactivated along a double exponential time course, both when measured from the decline of Ca current during a single pulse and when measured using a two pulse protocol. In 20 mM-Ca-Ringer the fast time constant of inactivation had a mean value of 27 msec and that of the slow time constant was 134 msec, at 0 mV and 5 °C.

4. The rate of inactivation was slowed, and its extent reduced, in low $[Ca]_o$, where Ca currents are smaller. Similarly, inactivation was faster and more complete in high-Ca-Ringer.

5. The rate of recovery from inactivation also followed a double exponential time course, with time constants of 638 msec and 4 sec following a 500 msec inactivating pulse in 20 mM-Ca-Ringer at 5 °C. Recovery appeared to be related to the amount of Ca entry during the inactivating pulse, being slower in high $[Ca]_o$ and following longer inactivating pulses.

6. Inactivation was slowed and reduced in extent when Ba^{2+} or Sr^{2+} carried current. Inactivation in Ba solutions may be due to depletion of Ba^{2+} from the lumen of the transverse tubules.

7. Ba^{2+} does not compete with Ca^{2+} for the inactivation mechanism.

8. It is concluded that inactivation of Ca currents in stick insect muscle fibres is primarily mediated by Ca^{2+} entry.

INTRODUCTION

Calcium currents have been studied in both invertebrate and vertebrate skeletal muscle fibres, and in a number of other excitable cell membranes (for a recent review see Hagiwara & Byerly, 1981). The activation of these Ca currents, although it occurs more slowly, does not appear to differ fundamentally from that of Na currents, and may be described by expressions similar to those used by Hodgkin & Huxley (1952*b*) to describe activation of Na currents in squid axons (Kostyuk & Krishtal, 1977*a*;

Kostyuk, Krishtal & Pidoplichko, 1981; Henček & Zachar, 1977; Llinás, Steinberg & Walton, 1976, 1981).

The kinetics of Ca inactivation, however, differ considerably between preparations, and may even vary with the experimental conditions. For example, inactivation of Ca currents in intact crustacean muscle is thought to be voltage-dependent (Henček & Zachar, 1977), whereas in fibres perfused with the Ca chelator EGTA the Ca currents do not appear to inactivate at all (Keynes, Rojas, Taylor & Vergara, 1973). Similarly, in EGTA-perfused frog muscle Ca inactivation can be completely accounted for by depletion of Ca^{2+} from the lumen of the transverse tubular (T-) system (Almers, Fink & Palade, 1981), although in intact frog muscle fibres inactivation was found to be potential-dependent (Stanfield, 1977; Sanchez & Stefani, 1978; Cota, Nicola Siri & Stefani, 1981). These differences may be related to an earlier finding of Hagiwara & Nakajima (1966), who found that even in the presence of high external [Ca], intracellular calcium must be reduced to below 5×10^{-7} M before it is possible to elicit Ca-dependent regenerative activity in barnacle muscle fibres. Internal Ca^{2+} also blocks Ca channels in molluscan neurones (Kostyuk & Krishtal, 1977*b*; Akaike, Lee & Brown, 1978) and tunicate eggs (Takahashi & Yoshii, 1978), Ca currents being half blocked at concentrations between 10^{-8} M (neurones) and 10^{-5} M (eggs). In invertebrate muscle at least, one possible explanation for the marked differences in Ca inactivation with the experimental conditions may therefore be that intracellular Ca inactivates the Ca channel, and further, that the Ca^{2+} which flows across the membrane normally inactivates the Ca channel, as has been suggested for *Paramecium* (Brehm & Eckert, 1978) and for molluscan neurones (Tillotson, 1979).

In this paper, we examine the activation and inactivation properties of the Ca permeability of muscle fibres of an insect (*Carausius morosus*). Our results show that the activation is voltage-dependent and may be described by Hodgkin–Huxley kinetics (Hodgkin & Huxley, 1952*b*) but add arguments in support of our previous finding that inactivation depends on Ca entry (Ashcroft & Stanfield, 1980, 1981).

METHODS

Experiments were carried out on the ventral longitudinal muscle fibres of the stick insect, *Carausius morosus* (order *Phasmida*), using a three-electrode voltage clamp method to control membrane potential (Adrian, Chandler & Hodgkin, 1970*a*; Ashcroft & Stanfield, 1982). In this method, membrane current density is related to the voltage difference between two impaling micro-electrodes, recording membrane potentials V_1 and V_2, by the expression

$$I_m = \frac{a(V_2 - V_1)}{3l^2 R_i} \text{ A . cm}^{-2}. \quad (1)$$

Membrane current per unit volume of fibre is given by

$$j_m = \frac{2(V_2 - V_1)}{3l^2 R_i} \text{ A . cm}^{-3}. \quad (2)$$

In all our experiments, l, the distance between the two recording electrodes and between one of these electrodes (V_1) and the apodemal end of the fibre, was set at 250 μm. The fibre radius, a, was calculated electrically from the response to a 10 mV hyperpolarization; short fibre cable theory was used since the fibres are only 1·5 mm long (Ashcroft & Stanfield, 1982). R_i, the sarcoplasmic resistivity, was assumed to be 322 Ω cm in hypertonic solution at the temperature of our experiments (Ashcroft, 1980). The holding potential was set at -60 mV in all our experiments.

We allowed an interval of 1 sec between pulses that elicited Ca current for recovery, and in two pulse experiments each double pulse was alternated with a test pulse by itself to control for rundown of the Ca current.

The leakage currents in this muscle show outward rectification. As described previously (Ashcroft & Stanfield, 1982) this rectification was fitted by constant field theory (Goldman, 1943; Hodgkin & Katz, 1949) making the assumption that leakage currents were carried entirely by Cl^- when the external solution contained tetraethylammonium ions (TEA^+).

Solutions. The standard Ringer solution contained (mM): $CaCl_2$, 20; $MgCl_2$, 50; KCl, 20; HEPES, 5 (pH 7·4 with KOH); and TEACl, 120 (to reduce K currents). The solution was made hypertonic (×2·5) with 400 mM-sucrose to block contraction (Hodgkin & Horowicz, 1957). In a few experiments, 4-aminopyridine (4-AP) was added to the Ringer solution at 4 mM in an attempt to reduce K currents further, but as it produced little additional block we did not use it routinely.

Strontium and barium Ringer solutions were made by substituting Sr^{2+} or Ba^{2+} for Ca^{2+}. Ringer solutions containing different Ca concentrations were made by replacing Ca^{2+} with Mg^{2+} or *vice versa*.

All our experiments were carried out at 2–6 °C.

RESULTS

In *Carausius* muscle fibres, depolarization elicits three ionic currents: an inward current, carried by calcium ions, and two potassium currents, a transient outward and a delayed outward current (Ashcroft & Stanfield, 1982). These K currents are substantially blocked by 120 mM-TEA^+, which was present in all the solutions used in the experiments described in this paper. However, outward currents are measurable at potentials positive to +30 mV in Ringer containing TEA and 1 mM-La^{3+} (to block Ca currents). Since outward currents are shifted to potentials approximately 10 mV more positive in the presence of La^{3+} (Ashcroft & Stanfield, 1982), this suggests that voltage-dependent outward currents will not influence the time course of the Ca current at potentials negative to +20 mV in our solutions.

Activation of calcium currents

We have fitted the activation of the Ca current with an expression similar to that first proposed by Hodgkin & Huxley (1952*b*) to describe activation of Na currents in the squid giant axon. Thus we suppose that

$$P_{Ca} = \bar{P}_{Ca} m^3 h, \tag{3}$$

where P_{Ca} is the potential and time dependent Ca permeability coefficient obtained from

$$I_{Ca} = P_{Ca} \frac{4VF^2}{RT} \frac{[Ca]_i \exp(2VF/RT) - [Ca]_o}{\exp(2VF/RT) - 1}, \tag{4}$$

and where $\bar{P}_{Ca}$ is the maximum Ca permeability co-efficient at a given $[Ca]_o$. m denotes the fraction of proposed activation gates in an open position, and $(1-h)$ gives the fraction of Ca permeability inactivated at a given membrane potential and time. As we describe in this paper (see below), h is not simply voltage-dependent, as it is in squid axon, but depends on Ca entry.

We have chosen to describe the Ca current in terms of a permeability, rather than a conductance, because the instantaneous current–voltage relation is not linear but shows a pronounced inward rectification (Ashcroft & Stanfield, 1982). Such rectification is expected in view of the large concentration gradient for Ca^{2+} across the fibre membrane and, in insect muscle, it may be described

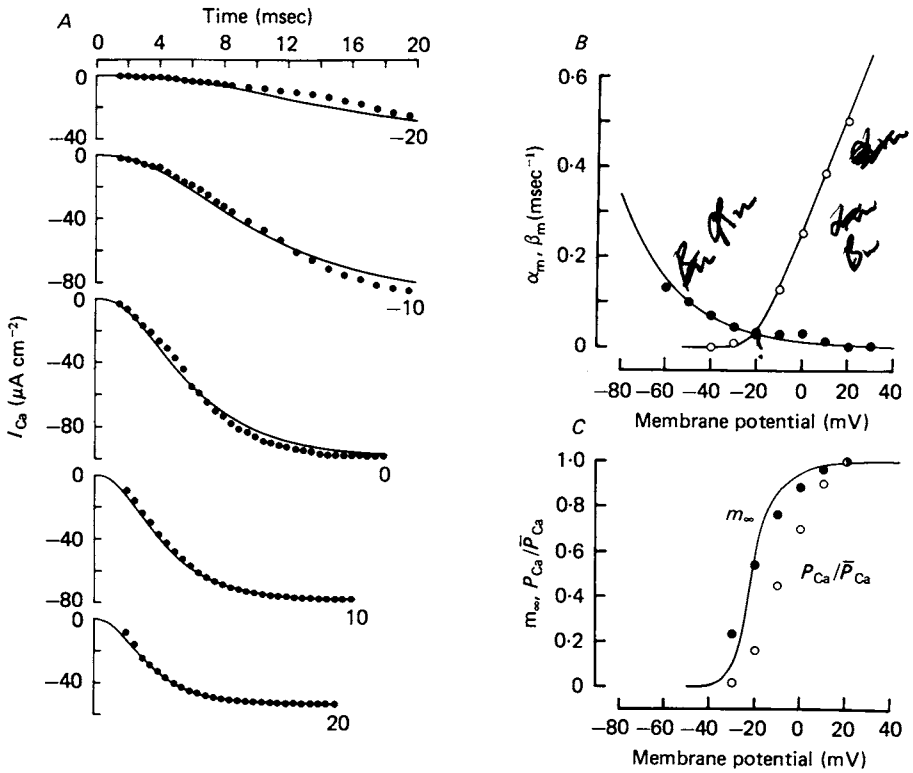

Fig. 1. *A*, calcium currents corrected for inactivation, leakage and capacity currents as described in the text (●). The numbers adjacent to each trace indicate the membrane potential. The curve through the experimental points is drawn to eqn. (5) of the text, with τ_m = 15 msec (−20 mV), 6 msec (−10 mV), 3·5 msec (0 mV), 2·5 msec (+10 mV), 2·0 msec (+20 mV). RP, −55 mV. HP, −60 mV. Fibre diameter, 92·6 μm. Temp. 4·6 °C. *B*, potential dependence of the rate constants for opening (α_m, ○) and closing (β_m, ●) of the Ca channel. The experimental points were obtained from $\alpha_m = m_\infty/\tau_m$, and $\beta_m = 1/\tau_m - \alpha_m$. Same fibre as in *A*. The line through the filled circles is drawn to

$$\alpha_m = \frac{0{\cdot}013\,(V - V_m)}{1 - \exp(-(V - V_m)/3)},$$

and that through the open circles to

$$\beta_m = 0{\cdot}0306 \exp(-(V - V_m)/25),$$

where V_m is −20 mV. *C*, voltage-dependence of the Ca permeability, normalized to give $P_{Ca}/\bar{P}_{Ca}$ (○) = 1·0 at +20 mV, and of the steady-state activation of the Ca permeability, m_∞ (●) in 20 mM-Ca-Ringer. Same fibre as in *A*. P_{Ca} was calculated as described in the text. m_∞ was obtained experimentally from $\sqrt[3]{(P_{Ca}/\bar{P}_{Ca})}$. The continuous line is drawn to eqn (6) of the text using values of α_m and β_m obtained from the curves fitted in *B*.

by constant field theory (but see also Llinás *et al.* 1981). We recognize, however, that because of the saturation of Ca currents at high $[Ca]_o$, Ca currents are more correctly described in terms of rate theory (Hille, 1975).

Fig. 1 *A* shows Ca currents recorded in 20 mM-Ca-Ringer corrected for inactivation, leakage and capacity currents. Ca currents were corrected for inactivation after fitting

the decline of the current to a double exponential (or in some cases to a single exponential, see below, p. 357); leakage currents were subtracted as described in Methods, and capacity currents were subtracted by scaling the capacity recorded for small voltage steps. Calcium permeabilities were obtained from the maximum Ca current after correction for leakage and inactivation, using eqn. (4), and the relation between $P_{Ca}/\bar{P}_{Ca}$ and membrane potential is plotted as the open circles in Fig. 1*C*. $[Ca]_i$ was assumed to be 10^{-7} M. The maximum Ca permeability in 20 mM-Ca-Ringer was $6{\cdot}14 \times 10^{-5} \pm 0{\cdot}18 \times 10^{-5}$ cm/sec ($n = 7$).

Fig. 1*A* also shows that the activation of the Ca current follows a sigmoidal time course, the rate of rise and the final amplitude of the current increasing as the membrane potential is made more positive. The fit through the experimental points is drawn to

$$I = I_{max}\,[1-\exp(-t/\tau_m)]^3. \tag{5}$$

We have used an m^3 relation to fit the rising phase of the Ca current, although an m^4 relation also gives a reasonable fit. Estimation of the power of m from the turn on of the Ca current is affected by the time taken for the membrane potential to settle at a new level. If the membrane is not clamped sufficiently fast this may lead to an apparent delay in the onset of the Ca current, and thus to an error in the estimation of the power of m. Such an effect is likely to become more pronounced at positive potentials. It is therefore possible that the value of m we find in insect muscle would be smaller were we able to clamp the membrane potential more rapidly. In molluscan neurones, activation of Ca currents is best fitted by an m (Akaike *et al.* 1978) or an m^2 relation (Kostyuk *et al.* 1981) whereas an m^5 relation is required for presynaptic Ca currents (Llinás *et al.* 1981) and an m^6 for the Ca currents of crustacean muscle (Henček & Zachar, 1977).

Fig. 1*B* gives the potential dependent rate constants for the opening (α_m) of and closing (β_m) of the activation gate (for details of how these were obtained, see Figure legend). These were used to calculate the continuous curve illustrating the steady-state activation of the Ca permeability, m_∞, in Fig. 1*C* from

$$m_\infty = \alpha_m/(\alpha_m+\beta_m). \tag{6}$$

The experimental points (filled circles) are simply $\sqrt[3]{(P_{Ca}/\bar{P}_{Ca})}$.

These results show that the activation of the Ca permeability in *Carausius* muscle may be described by Hodgkin–Huxley kinetics.

Voltage-dependence of inactivation

We initially attempted to fit the steady-state inactivation of the Ca current as if it were simply potential-dependent (Ashcroft, Standen & Stanfield, 1979). We used a two pulse protocol to examine this, with an inactivating pulse of 1 sec duration preceding a test pulse to 0 mV. Each double pulse was alternated with a test pulse by itself to control for any run-down of the Ca current. The result of four such experiments is shown in Fig. 2*B*, where steady-state inactivation, called h_∞, is measured as the peak Ca current elicited by a test pulse which followed an inactivating prepulse, expressed as a fraction of that flowing in the absence of a pre-pulse.

The experimental points have been fitted by

$$h_\infty = [1+\exp((V-V_h)/k_h)]^{-1}, \tag{7}$$

where V is the membrane potential during the inactivating pre-pulse; V_h, the potential at which $h_\infty = 0{\cdot}5$, is -20 mV, and $k_h = 6$ mV. This expression is similar to that used to describe steady-state inactivation of Na currents in the squid axon (Hodgkin & Huxley, 1952*a*, *b*) and has also been frequently used to describe inactivation of Ca currents (Standen, 1975; Reuter & Scholz, 1977; Henček & Zachar, 1977). However, at potentials positive to -10 mV it is apparent that the experimental points deviate from the predicted curve.

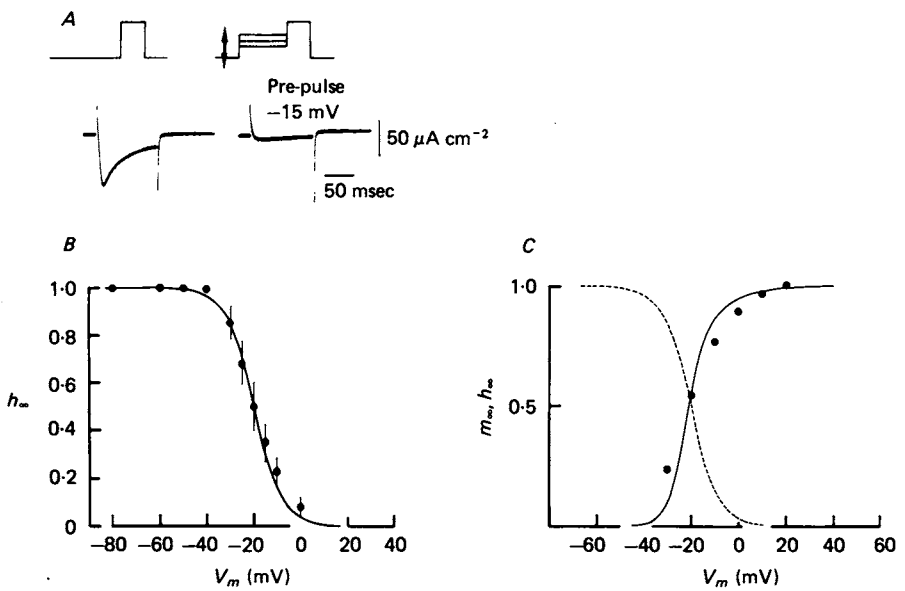

Fig. 2. *A*, pulse protocol (above) used to determine the voltage-dependence of inactivation. The test pulse depolarized the membrane to 0 mV and the amplitude of the 1 sec pre-pulse was varied. Membrane currents (below) recorded at 0 mV in the absence (left) and presence (right) of a pre-pulse to -15 mV. *B*, steady-state inactivation (h_∞) measured for four fibres using a two pulse protocol, shown in *A*, in 20 mM-Ca-Ringer. Peak Ca current during the second pulse, expressed as a fraction of that in the absence of an inactivating pre-pulse (h_∞), is plotted against the membrane potential during the pre-pulse. The smooth curve is drawn to eqn. (7) of the text with $V_h = -20$ mV and $k_h = 6$ mV. *C*, voltage-dependence of steady-state inactivation (dashed line) compared with that of steady-state activation (continuous line) of the Ca permeability. The dashed line is identical with that in *B*, and the continuous line is the same as that shown in Fig. 1*C*. The holding potential was -60 mV.

Fig. 2*C* compares the voltage-dependence of steady-state inactivation (h_∞) with that of steady-state activation (m_∞) of the Ca permeability. It shows that the value of h_∞ only begins to fall from unity as that of m_∞ begins to rise from zero, as if the onset of inactivation is linked to the opening of Ca channels. In contrast, in the squid axon h_∞ is 0·5 at the resting potential, whereas m_∞ has a value of zero (Hodgkin & Huxley, 1952*b*; Armstrong, 1981).

To investigate these points further, we adopted a slightly different pulse protocol, illustrated in Fig. 3*A*. In this case, we used an inactivating pre-pulse of 300 msec followed by a period of 100 msec at the holding potential (-60 mV). A test pulse to

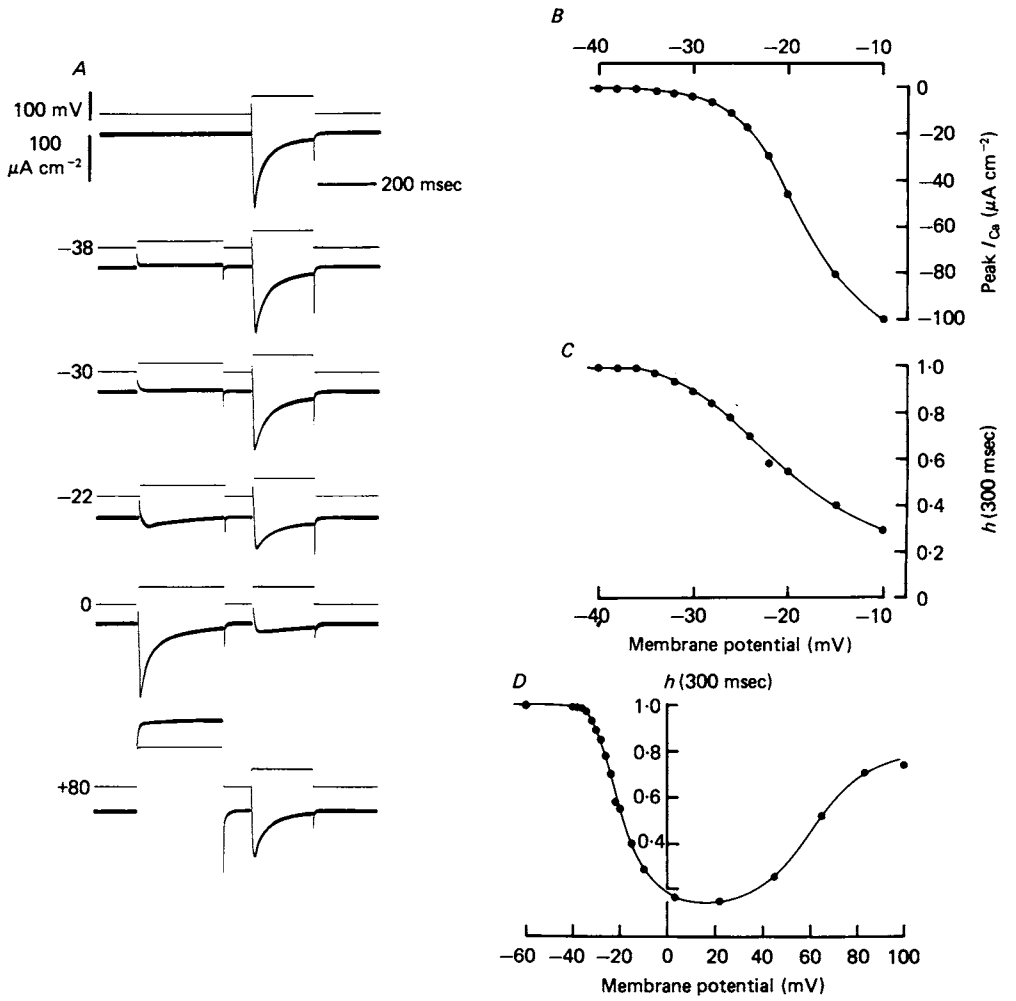

Fig. 3. *A*, records of voltage (above) and current (below) for a series of paired pulses in 20 mM-Ca-Ringer. The numbers adjacent to each trace indicate the membrane potential during the first pulse: the second pulse depolarized the membrane to 0 mV. The duration of the first pulse was 300 msec. RP, −44 mV. HP, −60 mV. Fibre diameter, 77 μm. Temp. 3·3 °C. *B*, voltage-dependence of the Ca current. Peak Ca current during the first pulse (leak corrected) plotted against membrane potential, for the same fibre as in *A*. *C*, voltage-dependence of inactivation. Peak Ca current during the second pulse, as a fraction of that in the absence of an inactivating 300 msec pulse, plotted against the membrane potential during the first pulse. Same fibre as in *A*. *D*, voltage-dependence of inactivation over a larger potential range than in *C*.

0 mV then measured the amount of Ca current remaining. The interval at the holding potential served two purposes: first, it allowed the activation kinetics of the Ca permeability to return to their resting state (Meves, 1978); secondly, it allowed a considerable part of any K permeability activated during the first pulse to return to rest before the test pulse was applied. As we have already stated, K currents were

substantially blocked by TEA^+ in our experiments. But any residual K current (see, for example, Fig. 3*A*, depolarization to +80 mV) may interfere with the measurement of Ca current during the subsequent test pulse. In *Carausius* muscle, delayed K current declines with a time constant of approximately 75 msec at −60 mV (Ashcroft & Stanfield, 1982), so that any residual K permeability activated should fall by about 74% during the 100 msec interval at −60 mV.

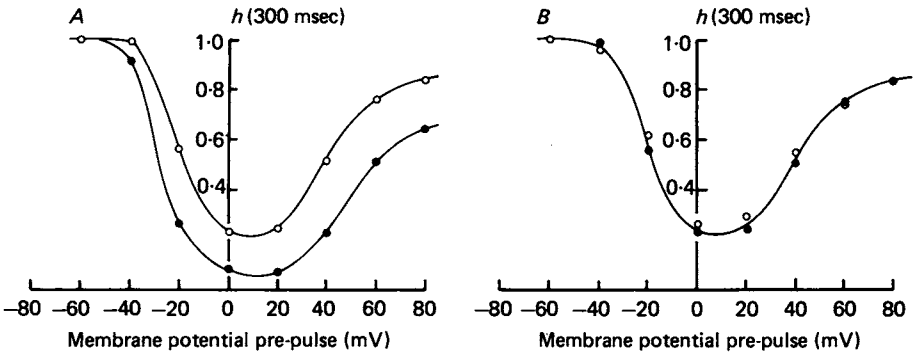

Fig. 4. *A*, effect of the duration of the pulse interval on inactivation. Peak Ca current during the test pulse, as a fraction of that in the absence of a first pulse, is plotted against the membrane potential during the first pulse. Pulse interval, 25 msec (●), 200 msec (○). First pulse duration 300 msec. 20 mM-Ca-Ringer. *B*, effect of test pulse amplitude on inactivation. Test pulse potential 0 mV (●), +20 mV (○). First pulse duration 300 msec, pulse interval 100 msec. 20 mM-Ca-Ringer.

Fig. 3*D* shows that the voltage-dependence of inactivation is similar to that of the Ca current, inactivation being maximal at around 0 mV and falling again at more positive potentials as E_{Ca}, the equilibrium potential for Ca^{2+}, is approached. We have suggested elsewhere that this voltage-dependence arises because inactivation is dependent on Ca entry (Ashcroft & Stanfield, 1981). If Ca entry is indeed a prerequisite for inactivation, no inactivation should occur unless Ca current is elicited by the first pulse. We therefore examined inactivation at potentials close to the threshold for the Ca current, and found that both the Ca current and inactivation appeared to be initiated at about the same potential (−34 mV, compare Fig. 3*B* and *C*). This contrasts with the Ca current of the Mexican frog (*Rana moctezuma*) where inactivation is voltage-dependent and initiated at potentials 20 mV negative to the threshold of the Ca current (Cota *et al.* 1981).

An alternative explanation of these results might be that the development of an outward K current masks the failure of the Ca current to inactivate, as has been suggested for neurones of dorid molluscs (Connor, 1979). In insect muscle, the delayed and early outward currents which are activated by depolarization are unlikely to produce such an effect: these K currents simply become larger with increasing depolarization and are anyway unlikely to contribute when the inactivating pulse is negative to about +20 mV. A K current activated by Ca entry (Meech, 1978; Meech & Standen, 1975) might be responsible, however, since it would have a dependence on potential and $[Ca]_o$ similar to that we describe for inactivation. While we have

been unable to remove K^+ from the internal solution to prevent K^+ currents from flowing, we have attempted to test this issue in the following ways.

First, we designed the pulse protocol to allow substantial relaxation of residual K permeability during the interval at −60 mV. Increasing this interval to allow for complete recovery of the delayed current (Fig. 4*A*) does not alter the form of the inactivation curve, though less inactivation is seen at all potentials because the Ca current also recovers more during longer intervals. Secondly, since Ca-activated K conductance is strongly voltage-dependent (Gorman & Thomas, 1980) the inactivation curve should be affected by the test pulse potential if it is merely an artifact of Ca-dependent K current activation. However, we saw no effect on the inactivation curve when we changed the test pulse potential from 0 mV to +20 mV (Fig. 4*B*).

Rate of inactivation

We have examined the rate of inactivation in two ways, either by measuring the rate of decline of Ca currents during a single depolarizing pulse, or by using a pulse protocol similar to that of Fig. 3 and changing the duration of the first pulse.

When measured from a single depolarizing pulse, the decay of the Ca current followed a double exponential time course (Fig. 5*A*). At 0 mV, the fast time constant, τ_1, had a mean value of $27{\cdot}4 \pm 2{\cdot}2$ msec ($n = 22$) and the slow time constant, τ_2, varied between 100 and 250 msec (mean, $134{\cdot}2 \pm 12{\cdot}3$ msec; $n = 22$), when measured from a 600 msec pulse. A similar double exponential time course has been described for the inactivation of Ca currents of dialysed snail neurones (Kostyuk & Krishtal, 1977*a*), where the time constants were somewhat slower, even at a higher temperature ($\tau_1 = 50$ msec; $\tau_2 = 320$ msec at 0 mV, 20 °C and in 10 mM-Ca-Ringer). On the other hand, Henček & Zachar (1977) described the inactivation of Ca currents in crayfish muscle as following a single exponential (during a 200 msec depolarization), with a time constant of 16 msec at 0 mV (16 °C; 13·5 mM-Ca solution).

Fig. 5 shows the potential-dependence of the fast time constant (*B*) and of the slow time constant (*C*) in 5 mM, 20 mM and 50 mM-Ca-Ringer for fibres where these were measured over a voltage range between −10 and +20 mV. Neither of the time constants appears to be voltage-dependent. At potentials negative to about −20 mV the decay of Ca currents usually followed a single exponential (during a 600 msec pulse), and fibres which had significantly smaller Ca currents tended to show a single exponential time course of inactivation at all potentials. We have plotted results only from those currents in which a clear double exponential time course was observed.

We have considered the possibility that the two time constants result from the presence of a large series resistance, as it is difficult to distinguish two exponentials where Ca currents are small as in 5 mM-Ca solution (see below) or close to threshold. However, tails of Ca current which flow when the membrane is returned to more negative membrane potentials after a depolarization appear to decay with a single exponential in spite of their large size (see Ashcroft & Stanfield, 1981, 1982). Further, in mixtures of Ca^{2+} and Ba^{2+}, currents are similar in size to those in 20 mM-Ca^{2+} but decay with a single exponential (Fig. 10). If a substantial fraction of the Ca channels is located in the T-system, which is unlikely to be well controlled by the voltage clamp, regenerative activity in the T-tubules might be reflected at the surface membrane as a second, slower component of inactivation. Again, however, this explanation cannot account for the single time constant observed in mixtures of Ca^{2+} and Ba^{2+}. It seems more probable that the Ca currents do indeed inactivate along a double exponential time course.

When we used a two pulse protocol to investigate inactivation, using a pre-pulse to 0 mV, whose duration was varied, followed by a 100 msec interval at the holding potential, and then a test pulse to 0 mV, Ca currents also appeared to inactivate along a double exponential time course (Fig. 6). However, the best fit values of τ_1 and τ_2 were somewhat slower ($\tau_1 = 64{\cdot}7 \pm 1{\cdot}5$ msec; $\tau_2 = 892 \pm 36$ msec; $n = 3$) than those measured from the decay of the Ca current during a single 1 sec pulse in the same fibre ($\tau_1 = 40{\cdot}9 \pm 4{\cdot}8$ msec; $\tau_2 = 250 \pm 10$ msec; $n = 3$). Fig. 6*B* and *C* shows the effect of potential on the rate of inactivation, measured using a two pulse protocol.

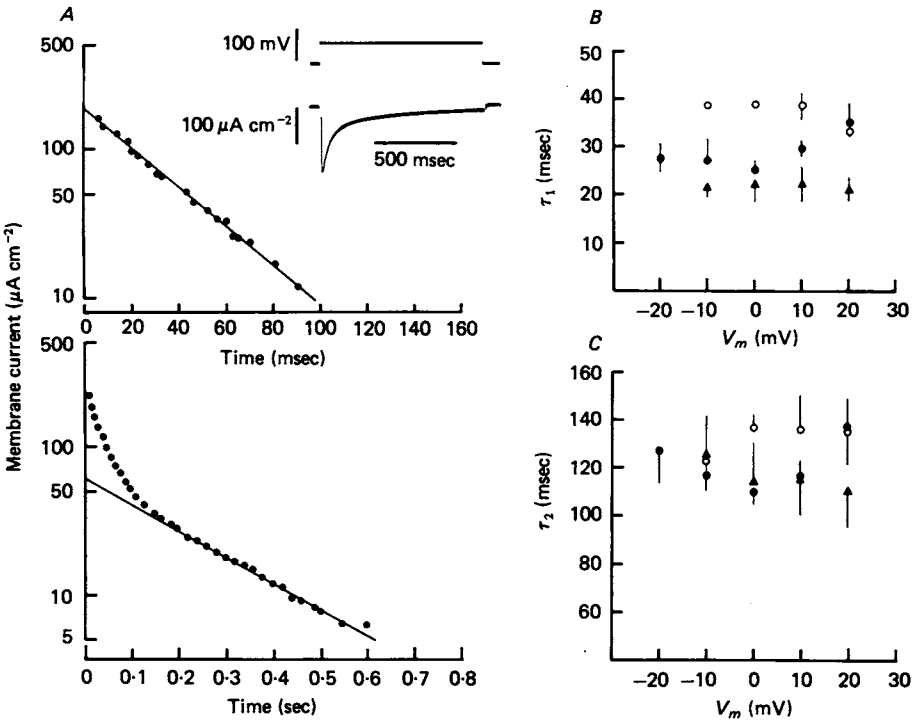

Fig. 5. *A*, time course of inactivation measured during a 1 sec pulse to 0 mV. *Inset*, membrane potential and membrane current records for a 1 sec pulse to 0 mV. *Below*, membrane current minus current at 1·0 sec, plotted against time elapsed since the peak value of the inward current. The line is drawn to an exponential with a time constant of 200 msec. *Above*, fast exponential obtained by subtracting the slow exponential from the experimental points. The line is drawn to a time constant of 32 msec. *B*, voltage-dependence of the fast time constant of inactivation (τ_1). 5 mM-Ca ($n = 4$), ○; 20 mM-Ca ($n = 13$, $n = 3$ at -20 mV), ●; 50 mM-Ca ($n = 6$), ▲. Only those fibres in which inactivation followed a double exponential time course are shown. *C*, voltage-dependence of the slow time constant of inactivation (τ_2). 5 mM-Ca ($n = 4$), ○; 20 mM-Ca ($n = 13$, $n = 3$ at -20 mV), ●; 50 mM-Ca ($n = 6$), ▲.

Inactivation was faster at 0 mV, where Ca currents are larger, than at -20 mV. At $+100$ mV we were unable to measure any time dependence of inactivation, the Ca current being about 20 % inactivated at all times (see also Fig. 3*D*). Since Ca currents are likely to be very small at this potential, this reduction in the extent of inactivation at $+100$ mV supports our hypothesis that Ca entry is required for inactivation. That

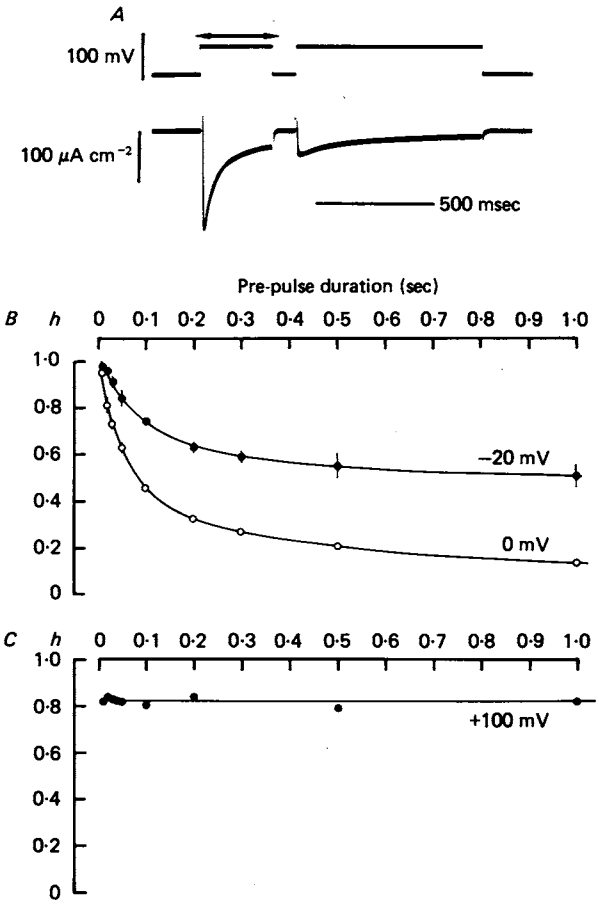

Fig. 6. *A*, voltage (above) and current (below) records for a pair of pulses to 0 mV in 20 mM-Ca-Ringer. The rate of inactivation was examined by changing the duration of the first pulse. RP, −51 mV. HP, −60 mV. Fibre diameter, 111·4 μm. Temp. 5·6 °C. *B*, rate of inactivation measured for three fibres at −20 mV (●) and for three fibres at 0 mV (○). Peak inward current during the test pulse which follows a pre-pulse is plotted as a fraction of that in the absence of an inactivating pre-pulse (*h*). The test pulse depolarized the membrane to 0 mV. The line is drawn to a double exponential with a fast time constant of 90 msec and a slow time constant of 324 msec at −20 mV, taking steady-state inactivation as 0·5. At 0 mV the line is fitted with time constants of 59 msec and 490 msec (steady-state inactivation = 0·1). *C*, rate of inactivation at +100 mV (one fibre). Line drawn by eye.

inactivation which is present may be due to a rapidly inactivating voltage-dependent component (too fast to be measured in our experiment) or to Ca entry during the tail current that follows the first pulse.

As in the case of a single pulse, an increase in the rate of inactivation was found when external [Ca] was increased to 50 mM.

We are not certain why the two pulse protocol produced somewhat slower rates of inactivation. It is possible that the interval may slightly distort the time course

of inactivation, since the rate of recovery from inactivation depends on the duration of the pre-pulse (see below, Fig. 8*C*). In *Carausius* muscle, recovery from inactivation occurs more slowly following a long inactivating pulse than after a pre-pulse of shorter duration. This effect would tend to enhance the depression of Ca currents produced by long inactivating pulses in our two pulse experiments, and so might lead to an apparent slowing of inactivation when measured using the two pulse protocol. The activation of a small amount of K current on depolarization, or series resistance effects, might also produce an apparent speeding of inactivation during a single pulse.

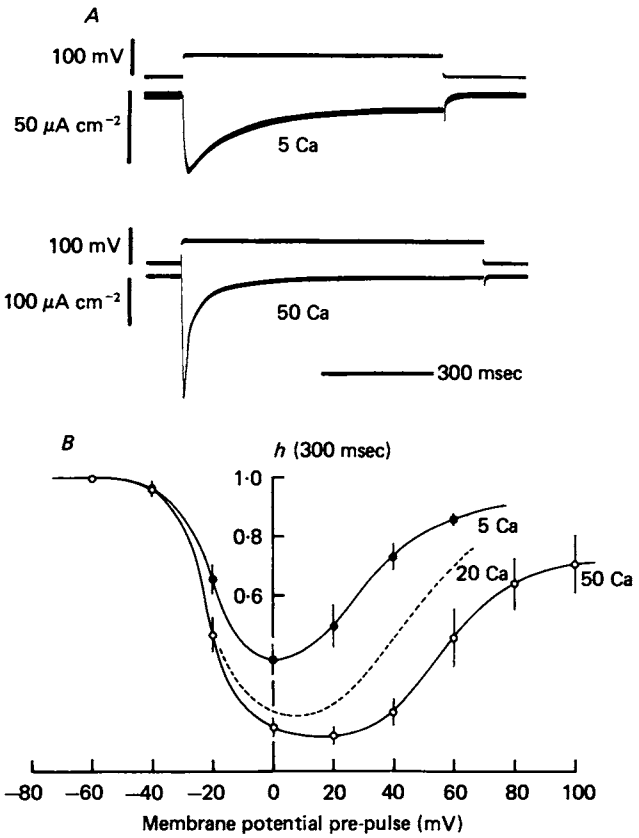

Fig. 7. *A*, membrane potential and membrane current recorded for a depolarization to 0 mV in 5 mM-Ca-Ringer (above) and in 50 mM-Ca-Ringer (below). Records taken from different fibres. *B*, voltage-dependence of inactivation following a 300 msec pre-pulse (two pulse protocol) for five fibres in 5 mM-Ca-Ringer (●) and for four fibres in 50 mM-Ca-Ringer (○). The dashed line indicates the extent of inactivation in 20 mM-Ca-Ringer (ten fibres).

Effect of $[Ca]_o$ *on inactivation*

If the inactivation of Ca currents occurs as a consequence of the entry of Ca^{2+}, a change in the rate of Ca entry may lead to a similar change in the rate of inactivation. Fig. 7*A* shows that increasing $[Ca]_o$ to 50 mM did result in an increase in the rate of inactivation and that reducing $[Ca]_o$ to 5 mM slowed inactivation. Ca currents were about 50 % smaller in 5 mM-Ca-Ringer and about 1·5 times larger in 50 mM-Ca-Ringer.

Measuring the decay of the Ca current during a 600 msec depolarization in 50 mM-Ca^{2+} gave a mean value for τ_1 of $22{\cdot}0 \pm 2{\cdot}9$ msec and for τ_2 of $112{\cdot}4 \pm 11{\cdot}5$ msec ($n = 10$). When $[Ca]_o$ was reduced to 5 mM, the Ca current generally decayed with a single exponential having a time constant of $98{\cdot}9 \pm 6{\cdot}7$ msec ($n = 9$), perhaps because the slower time constant was too slow (and the current slowly inactivating too small) to measure in this solution. In those fibres where inactivation followed a double exponential time course (four out of thirteen) the mean time constants were $\tau_1 = 38{\cdot}7 \pm 0{\cdot}6$ msec; $\tau_2 = 136{\cdot}5 \pm 5{\cdot}4$ msec ($n = 4$). These fibres had Ca currents which were about twice as large as those in which inactivation followed a single exponential.

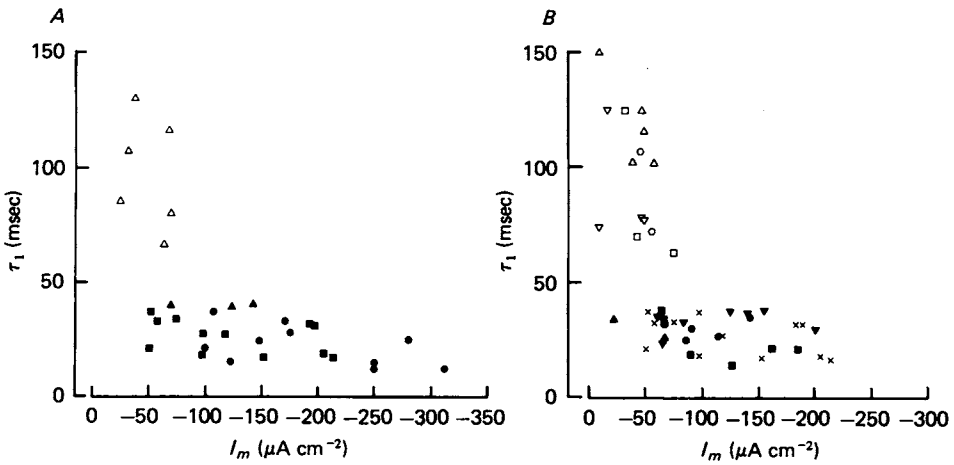

Fig. 8. *A*, relationship between τ_1 and peak Ca current, at 0 mV. △ ▲, 5 mM-Ca; □ ■, 20 mM-Ca; ○ ●, 50 mM-Ca-Ringer. Open symbols were taken from fibres in which inactivation followed a single exponential: we have assumed that this represents the faster of two exponentials. *B*, relation between τ_1 and peak inward current for fibres in 20 mM-Ca-Ringer. Membrane potential: △ ▲, −20 mV; □ ■, −10 mV; ×, 0 mV; ○ ●, +10 mV; ▽ ▼, +20 mV. Open symbols taken from currents in which inactivation followed a single exponential.

Time constants of inactivation were increased in 50 mM-Ca and reduced in 5 mM-Ca at all potentials (Fig. 5*B* and *C*). Since Ca currents saturate at high $[Ca]_o$ in *Carausius* muscle (Ashcroft & Stanfield, 1982), reducing $[Ca]_o$ from our standard concentration of 20 mM has a greater effect on current amplitude then increasing it by an equal amount, which may account for the more pronounced effect of reducing $[Ca]_o$ on inactivation.

The effect of [Ca] on inactivation suggests that the rate of inactivation may be more closely related to the amount of Ca entry than to membrane potential. In Fig. 8 we have plotted the value of τ_1 against the size of the peak inward current at 0 mV for fibres in 5, 20, and 50 mM-Ca^{2+} (*A*); and for fibres in 20 mM-Ca^{2+} at different membrane potentials (*B*). We have also plotted results from those fibres in which inactivation followed a single exponential, assuming that it represents the faster of two time constants, to enable us to include fibres that had small Ca currents. τ_1 appears to become briefer as the rate of Ca entry increases.

The extent of inactivation was reduced in 5 mM-Ca and increased in 50 mM-Ca-Ringer (Fig. 7*B*). The shift in the inactivation curve with Ca concentration may be related to similar shift in the current–voltage relation of the Ca current (Ashcroft & Stanfield, 1982).

The rate of Ca inactivation also became slower when the Ca current was reduced by inhibitors of the Ca channel. In 20 mM-Ca-Ringer containing 5 mM-Ni^{2+}, Ca currents were reduced by 20% and, at 0 mV, inactivated with time constants of $32{\cdot}3 \pm 3{\cdot}3$ msec and $130{\cdot}2 \pm 12{\cdot}3$ msec ($n = 3$): compare Ni-free, $27{\cdot}4 \pm 2{\cdot}2$ msec and $134{\cdot}2 \pm 12{\cdot}3$ msec ($n = 22$). The rate of inactivation was further decreased in the presence of 10 mM-Ni^{2+} ($\tau_1 = 35{\cdot}7 \pm 3{\cdot}5$ msec, $\tau_2 = 94{\cdot}0 \pm 9{\cdot}3$ msec, 0 mV, $n = 3$; Ca currents were about the same size as in 5 mM-Ca-Ringer).

Thus both the rate and extent of inactivation appear to be related to the amount of Ca entry.

Steady-state inactivation

The Ca current of insect muscle does not completely inactivate but declines to some steady level (see, for example, Fig. 9*A*). If Ca inactivation is dependent on Ca^{2+} entering and binding to a site at or near the inner side of the membrane, then in the steady state, at a given membrane potential, Ca^{2+} should enter at approximately the same rate as that at which it is removed by metabolic processes. We have therefore estimated the rate at which Ca^{2+} enters in the steady state per unit volume of fibre. The amount of Ca^{2+} that enters per unit volume of fibre (mole/l.) in a time t is given by Q/zF where F is the Faraday and

$$Q = \int_0^t j_m \, dt \tag{7}$$

j_m, the current per unit volume of fibre is defined in eqn. (2). Ca currents were corrected for leakage, and Ca entry was calculated for a period of 100 msec after the current had reached a steady state. At 0 mV the rate of Ca entry measured in one fibre was around 70 μmole/l. sec in 20 mM-Ca-Ringer. Similar rates were obtained in 5 mM and 50 mM-Ca-Ringer (25–150 μmole/l. sec); the size of the non-inactivating current did not appear to change with Ca concentration. Removal of Ca^{2+} from the sarcoplasm will be influenced by many processes but probably, in muscle fibres, will be primarily effected by Ca uptake into sarcoplasmic reticulum (s.r.). The rate of uptake of Ca^{2+} into s.r. by skinned frog skeletal muscle was estimated by Endo (1977), on the basis of certain assumptions to be 70 μmole/l. of fibre per second at 0 °C. This suggests that our assumption that Ca entry may be in equilibrium with Ca removal at the steady state may be reasonable.

Recovery from inactivation

We have investigated the rate of recovery from inactivation with a three pulse protocol, illustrated in Fig. 9*A*. An inactivating pulse of 500 msec to 0 mV was followed, after a variable interval at the potential at which recovery was being measured, by a test pulse to 0 mV. In 20 mM-Ca-Ringer, recovery from inactivation followed a double exponential time course (Fig. 9*B*), with a fast time constant, τ_1, of 638 ± 12 msec and a slow time constant, τ_2, of $4{\cdot}1 \pm 0{\cdot}8$ sec ($n = 4$) at -60 mV

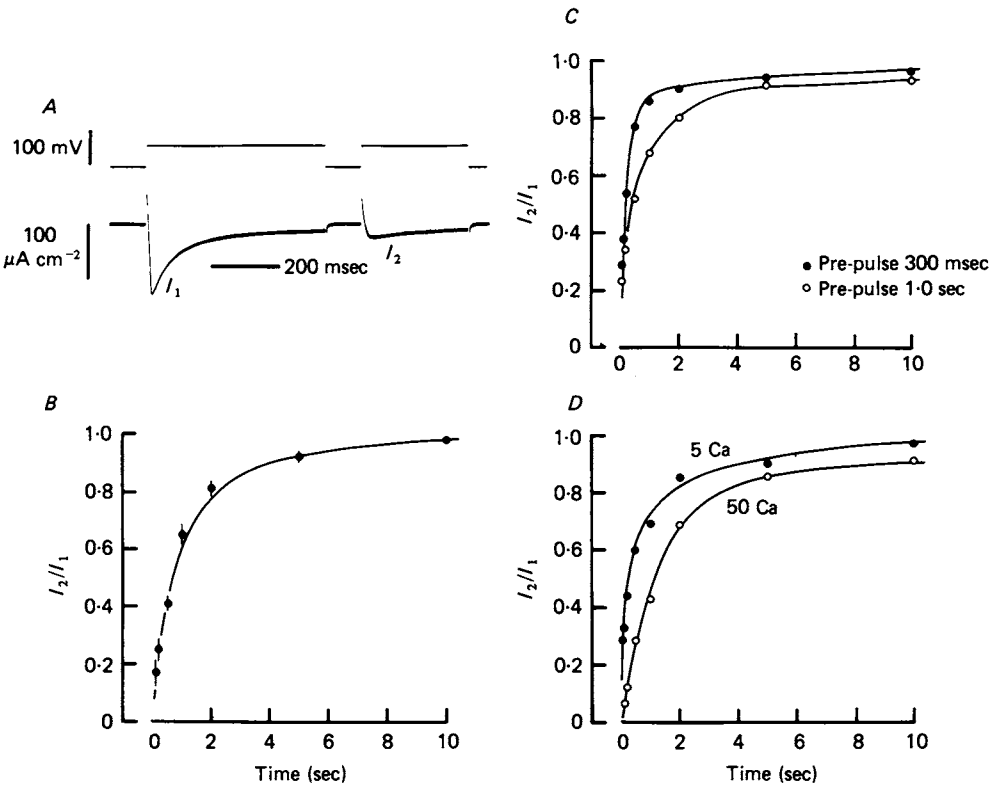

Fig. 9. *A*, voltage (above) and current (below) records for a pair of pulses to 0 mV in 20 mM-Ca-Ringer. The rate of recovery from inactivation was studied by changing the duration of the interval. RP, −46 mV. HP, −60 mV. Fibre diameter, 88 μm. Temp. 3·0 °C. *B*, mean rate of recovery from inactivation measured at −60 mV and 5 °C in 20 mM-Ca-Ringer, following a 500 msec inactivating pre-pulse to 0 mV ($n = 4$). The peak current during the second pulse (I_2) as a fraction of that during the inactivating pulse (I_1), is plotted against the duration of the interval between the pulses. The curve is drawn to a double exponential with a fast time constant of 750 msec and a slow time constant of 3·6 sec. *C*, effect of the duration of the inactivating pulse on the rate of recovery at −60 mV. Records were taken from the same fibre. Pre-pulse duration, (●) 300 msec, (○) 1 sec. Curves fitted by eye. *D*, effect of Ca concentration on the rate of recovery at −60 mV following a 500 msec pre-pulse. (●) 5 mM-Ca-Ringer. (○) 50 mM-Ca-Ringer. Records are taken from different fibres. The curve through the filled circles is drawn to a double exponential with time constants of 375 msec and 3·7 sec; that through the open circles is drawn to time constants of 1·2 sec and 13·4 sec.

and 5 °C. A similar double exponential time course of recovery from inactivation has been described for the Ca current of snail neurones (Tillotson & Horn, 1978; Adams & Gage, 1979) and the rates are comparable with those we find.

We consistently observed a 'dip' or apparent delay in the recovery curve at around 20 msec (in 20 mM-Ca-Ringer), similar to that described for the recovery of Na currents from inactivation in the squid giant axon (Chandler & Meves, 1970*a*; Gillespie & Meves, 1981).

When recovery was allowed to occur at −80 mV, its rate was little changed from that measured at −60 mV in the same fibre. However, both time constants were

approximately doubled at −40 mV (where Ca entry just begins) the faster time constant increasing to around 1·5 sec and the slower to about 10 sec. At this potential the Ca current never fully recovered, reaching a steady-state where currents remained about 20% inactivated. Recovery from inactivation was also slower when the duration of the inactivating pulse was increased (Fig. 9*C*) or when the Ca concentration was raised (Fig. 9*D*), suggesting that the rate of recovery from inactivation may be related to the amount of Ca^{2+} that has entered during the inactivating pulse.

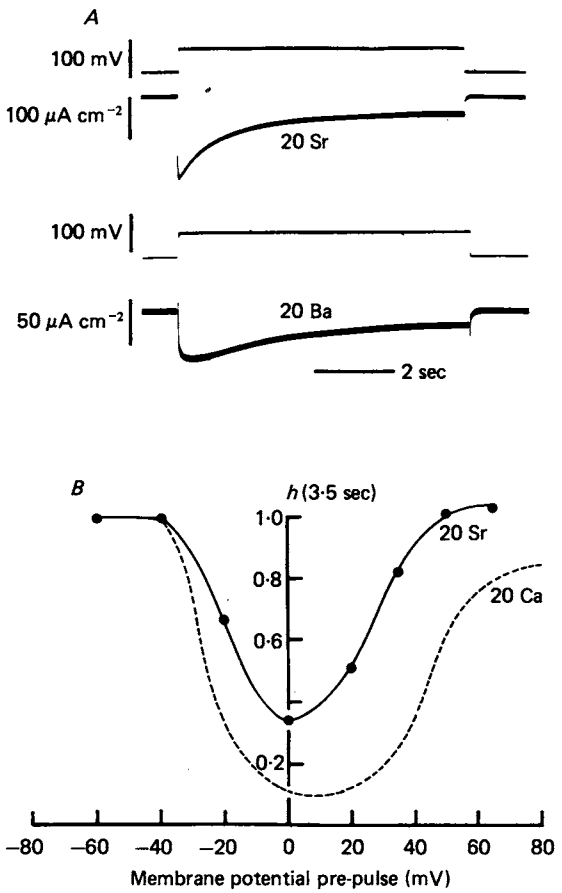

Fig. 10. *A*, membrane potential and membrane current recorded for a depolarization to 0 mV in 20 mM-Sr-Ringer (above) and in 20 mM-Ba-Ringer (below). Sr: RP, −53 mV; HP, −60 mV; temp. 5·4 °C. Ba: RP, −43 mV; HP, −60 mV; temp. 2·1 °C. *B*, potential-dependence of steady-state inactivation in 20 mM-Sr-Ringer measured with a first pulse of 3·5 sec (●) followed by a 100 msec interval at the holding potential. The dotted line indicates steady-state inactivation in 20 mM-Ca-Ringer (600 msec first pulse).

If recovery from inactivation primarily reflects the rate of removal of Ca^{2+} from the vicinity of the Ca channel, and if our assumption that in the steady-state Ca^{2+} enters at a rate similar to that at which it is removed by metabolism is correct, then recovery should occur at a rate similar to or slower than the rate at which Ca^{2+} enters in the steady-state. To some extent this is supported by the finding that recovery

at negative membrane potentials (-60 and -80 mV) is independent of potential. For a 500 msec depolarization to 0 mV, Ca entry averaged 60–70 μmole/l. of fibre volume in 20 mM-Ca-Ringer. If the rate of removal is about 70 μmole/l. of fibre per second (see above, p. 362), this suggests that recovery will take somewhat longer than 1 sec to occur, since it is probable that as sarcoplasmic [Ca] falls during recovery, so will the rate of Ca-disposal. Experimentally the time constants for the rate of recovery were about 650 msec and 4 sec, so that the agreement is not unreasonable.

Inactivation in Sr and Ba solutions

As we have previously described (Ashcroft & Stanfield, 1981) Sr^{2+} and Ba^{2+} also permeate the Ca channel of insect muscle, but when these ions carry current inactivation is very much slowed and reduced in extent. The decay of Sr current during an 8 sec pulse (Fig. 10*A*) followed a double exponential time course, as does that of Ca current, with time constants of 540 ± 16 msec and $1{\cdot}9 \pm 0{\cdot}1$ sec ($n = 4$) in 20 mM-Sr-Ringer at 0 mV: compare mean values of 27·4 msec and 134·2 msec in 20 mM-Ca^{2+}. Inactivation of Ba currents, however, appeared to follow a single exponential with a time constant of about 2 sec at 0 mV. (It is possible that an 8 sec pulse was not sufficient to allow inactivation to reach its steady-state in Ba-Ringer and that a second, much slower, element of inactivation was missed. Longer pulses, especially in Ba-Ringers, tended to damage the fibre, making it leaky, or caused the clamp to deteriorate.)

Fig. 10*B* compares the potential-dependence of inactivation in 20 mM-Sr-Ringer with that in 20 mM-Ca-Ringer; the first pulse was 3·5 sec in Sr and 600 msec in Ca solution, so that in both cases inactivation approached the steady-state. There was less steady-state inactivation in Sr than in Ca-Ringer suggesting that Sr^{2+} does not bind as readily as Ca^{2+} to the inactivation site. This difference is even more pronounced than it appears since recovery from inactivation in Sr^{2+} occurs so slowly that little will have occurred during the 100 msec interval at the holding potential, whereas the Ca current will have recovered by about 20% (Fig. 9*B*).

Using an inactivating pre-pulse of 1 sec duration, recovery from partial inactivation (50%) followed a double exponential time course with time constants of 542 ± 278 msec and $3{\cdot}1 \pm 0{\cdot}5$ sec ($n = 3$) in 20 mM-Sr-Ringer at -60 mV.

Depletion may contribute to inactivation in Ba solutions

We have argued elsewhere (Ashcroft & Stanfield, 1981) that inactivation of Ca currents is not the result of Ca depletion from a small extracellular space, or of Ca accumulation at the inner membrane surface *per se*, since currents which are of similar size, as Ca and Sr currents are, do not inactivate at the same rate (see also Almers *et al.* 1981). However, since Ba currents inactivate so slowly, the possibility exists that depletion of Ba^{2+} from a small extracellular space, such as the T-system, underlies inactivation in Ba solutions. Although we do not have direct evidence that Ca channels occur in the T-system in insect muscle, Henček & Zachar (1978) have elegantly shown that Ca channels occur in the T-system of crayfish muscle, even if they are not necessarily limited to that site. And in frog muscle, the Ca channels appear to be confined to the T-system (Nicola Siri, Sanchez & Stefani, 1980). Almers *et al.* (1981) have shown that in frog muscle fibres perfused with EGTA, inactivation

of currents carried by alkali earth metal ions (Ca^{2+}, Ba^{2+}, Sr^{2+}) is due to depletion alone. These currents are inactivated with time constants of 0·2–2 sec in 10 mM-Ca solution at room temperature, a rate comparable with that found in our experiments in Ba-Ringer.

A rough test of this possibility may be made by computing the size of the space supposed to be completely depleted of Ba^{2+} during the voltage pulse. Following Adrian, Chandler & Hodgkin (1970*b*), we assume that the decay of current is due to depletion of the permeating ion from a space where we may ignore replenishing, and write

$$Q = \int_{t=0}^{t=t'} [j_m(t) - j_m(\infty)]\, dt, \tag{9}$$

where t' is the duration of the pulse and j_m is the current per unit volume at time t. Q is the charge which flows across the membrane and may also be defined as

$$Q = \rho\, zF[Ba]_o, \tag{10}$$

where ρ is the fractional fibre volume assumed to be depleted of Ba^{2+}, $z = 2$, and F is Faraday's constant. Using such a method, we found ρ to be 0·008 of the fibre volume. Almers *et al.* (1981), using a more complete modelling, found that for frog muscle the volume of the fibre depleted of Ca was about 0·004 of the fibre volume, similar to the volume enclosed by the T-system (Peachey, 1965). In insect muscle fibres, the volume of the T-system may be somewhat greater since membrane capacity is higher than in frog muscle fibres of comparable diameter (Ashcroft, 1980), and in the hypertonic solutions we use, the T-system is probably swollen (see Franzini-Armstrong, Heuser, Reese, Somlyo & Somlyo, 1978).

Thus inactivation of Ba currents in *Carausius* muscle may, at least partly, be the result of depletion.

Inactivation in mixtures of Ca^{2+} and Ba^{2+}

Although Ba currents inactivate very slowly, it is still possible that Ba^{2+} binds to the inactivation site without producing an inactivating effect, and thus acts as a competitive inhibitor to the inactivating action of Ca^{2+}. To test for such possible competition, we investigated the rate and extent of inactivation in solutions containing 5 mM-Ca^{2+} and 15 mM-Ba^{2+} (Fig. 11). In this solution, the magnitude of the inward current was approximately that expected from inward currents recorded in Ringer containing only Ca^{2+} or Ba^{2+} as permeant ions: we saw no evidence for anomalous mole fraction effects of the kind described for other permeability mechanisms (Hagiwara & Takahashi, 1974; Hagiwara, Miyazaki, Krasne & Ciani, 1977). Inward currents measured at 0 mV were therefore larger than in 5 mM-Ca^{2+} alone ($-94·6 \pm 21·3$ μA cm^{-2} ($n = 4$) in 5 mM-Ca + 15 mM-Ba; $-50·5 \pm 10·6$ μA cm^{-2} ($n = 11$) in 5 mM-Ca). The time course of inactivation followed a single exponential with a time constant of $63·2 \pm 8·0$ msec ($n = 5$) at 0 mV, slightly faster than that obtained in 5 mM-Ca-Ringer ($98·9 \pm 6·7$ msec; $n = 9$). The extent of inactivation at 300 msec (Fig. 11*B*) was similar to that found in 5 mM-Ca-Ringer. There did, however, appear to be a 5 mV positive shift in the curve relating inactivation (h) and membrane potential perhaps because Ba^{2+} may be more effective than Mg^{2+} at binding to

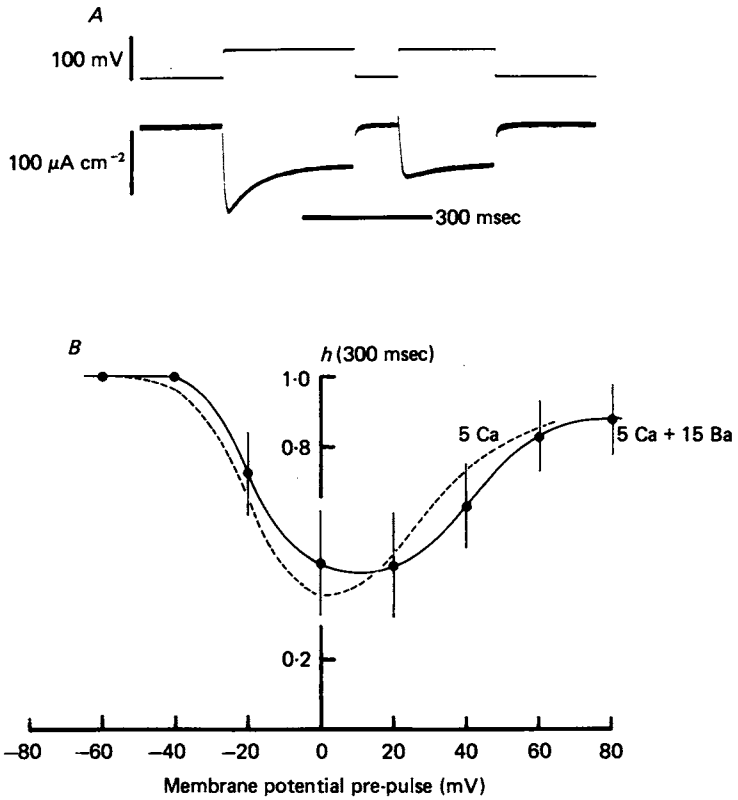

Fig. 11. *A*, membrane potential and membrane current recorded for a pair of pulses to 0 mV in 5 mM-Ca, 15 mM-Ba-Ringer. RP, −48 mV. HP, −60 mV. Temp. 3·7 °C. *B*, potential-dependence of inactivation in four fibres in 5 mM-Ca, 15 mM-Ba solution (●). The dashed line indicates the extent of inactivation in 5 mM-Ca-Ringer.

membrane surface charges, with the result that the activation kinetics are shifted (see also Ashcroft & Stanfield, 1982). The results suggest that there is little competition between Ba^{2+} and Ca^{2+} for the site supposed to control inactivation.

DISCUSSION

The results presented in this paper provide evidence in support of the hypothesis that inactivation of Ca currents in *Carausius* muscle fibres depends on Ca entry. Inactivation is greatest at potentials where Ca currents are largest, and is reduced at potentials where there is little Ca influx such as close to the threshold for the Ca current and close to the Ca equilibrium potential. When Ca entry is reduced, either by decreasing $[Ca]_o$ or by the presence of a blocking ion in the external solution, both the rate and extent of inactivation are also reduced: similarly, inactivation is faster and more complete in high Ca-Ringer.

These results, however, may also be interpreted in terms of a potassium current which is activated by Ca entry. In this case the apparent inactivation would show

a potential-dependence similar to that of the Ca current, and would be affected by $[Ca]_o$ and Ca inhibitors in a way comparable to that we describe. Since Ca inactivation follows a double exponential time course it is also necessary to consider whether one of the two time constants of inactivation (possibly the faster) is an artifact of K current activation. Kostyuk (1980) has suggested that the fast component of inactivation in perfused *Helix* neurones is the result of activation of a Ca-dependent non-selective outward current, the true time course of Ca inactivation being considerably slower.

As we have already stated K currents were substantially blocked in our experiments, and we do not believe that residual Ca-activated K currents account for our results because:

(*a*) The amplitudes of Ca current tails decay with a rate that is independent of the membrane potential at which they are measured, and that is similar to the rate of inactivation of the Ca current used to elicit them (Ashcroft & Stanfield, 1981). Were inactivation due to the development of an outward current, tail currents measured at E_K should decline more slowly, if at all, than the rate at which the Ca current inactivates.

(*b*) In spite of the voltage-dependence of Ca-activated K currents, the extent of inactivation was unaffected by test pulse potential (Fig. 4*B*). Ca-activated K current in *Aplysia* neurones increased *e*-fold every 25 mV (Gorman & Thomas, 1980), so that a similar voltage-dependence in *Carausius* muscle would produce a large effect on inactivation when the test pulse potential was increased 20 mV, Ca currents appearing more completely inactivated at the more positive test pulse potential. In *Aplysia* neurones, Eckert & Tillotson (1981) found that in two pulse experiments, the extent of inactivation produced by a given pre-pulse was actually reduced at more positive values of test pulse potential, an effect that is in the opposite direction from that expected from Ca-dependent K current activation.

Plant & Standen (1981) have recently shown that the two pulse protocol may be used to separate Ca-dependent inactivation from the effects of Ca-activated K currents even where Ca-activated K currents are present.

(*c*) In two pulse experiments, contamination by residual K currents is substantially reduced because K current tails decline very considerably during the interval. Under these conditions inactivation still followed a double exponential time course, suggesting that this is not an artifact of K current activation.

We consider that the development of a Ca-activated K current does not contribute to inactivation of Ca currents in *Carausius* muscle at potentials negative to about +10 to +20 mV, for the reasons stated above. At more positive potentials the decay of Ca currents is undoubtedly influenced by K currents, delayed current becoming measurable at potentials positive to +20 mV even in the presence of 120 mM-TEA (Ashcroft & Stanfield, 1981).

The shape of the inactivation curve (Fig. 3*D*) is superficially similar to that reported for Na inactivation in squid axon, where inactivation also recovers at very positive potentials (Chandler & Meves, 1970*a*, *b*). Chandler & Meves described Na inactivation as the sum of two components, h_1 and h_2, representing two types of Na conductance with different inactivation properties. The h_1 variable, describing the transient Na conductance, declines exponentially with depolarization, whereas h_2,

which describes the maintained Na conductance, is increased at positive potentials. Strong depolarization favours the h_2 state so that there is less inactivation at these potentials. It is unlikely that such a mechanism is responsible for our results, however, as it does not account for the effects of $[Ca]_o$ or of Sr^{2+} and Ba^{2+} on inactivation.

In Ca-Ringer, inactivation did not recover completely at positive potentials (Figs. 3*D* and 7*B*), even though little Ca^{2+} is likely to enter at potentials positive to about +80 mV because of the strong inward rectification of the instantaneous current–voltage relation (Ashcroft & Stanfield, 1982). That inactivation which was present showed very little time-dependence (Fig. 6*C*). It therefore seems possible that Ca entry during the current tail that follows the first pulse is sufficient to account for the inactivation that occurs at positive membrane potentials. A similar argument has been made by Katz & Miledi (1967) to account for the delay in transmitter output that accompanies very large depolarizations at the squid giant synapse and frog neuromuscular junctions treated with tetrodotoxin. The fact that when Sr^{2+} or Ba^{2+}, ions which produce little inactivation, carried current, inactivation recovered completely at positive potentials (Fig. 10*B*; Ashcroft & Stanfield, 1981) supports this idea. However, we cannot exclude the possibility that a small, rapidly inactivating, voltage-dependent component of inactivation is present at very positive membrane potentials.

In *Carausius*, Ca currents do not inactivate completely but decline to some steady level. A maintained current is predicted if Ca entry mediates inactivation, as an equilibrium will finally be reached between Ca entry and Ca inactivation. Non-inactivating Ca currents similar to those we describe have also been reported for *Paramecium* (Eckert & Brehm, 1979), and for perfused molluscan neurones (Akaike *et al.* 1978). It seems possible that this steady current may reflect an underlying equilibrium between Ca entry and the rate of its removal from the vicinity of the Ca channel. Close to threshold, the rate of Ca entry may be small enough that it is balanced by the rate of Ca removal by the fibre, thus accounting for the fact that Ca currents appear to inactivate very little at these potentials.

If inactivation is mediated by Ca^{2+}, then the extent of inactivation is likely to be influenced by the rate of removal of Ca^{2+} from the vicinity of the Ca channel, and hence by the Ca-buffering properties of the fibre. The fact that recovery from inactivation is slower following a large Ca influx provides some support for this idea. It could also account for the difference in inactivation reported for intact and EGTA-perfused muscle fibres (see, for example, Keynes *et al.* 1973; Henček & Zachar, 1977), since Ca buffering would be significantly increased in the presence of EGTA. Eckert & Tillotson (1981) have recently shown that Ca inactivation in *Aplysia* neurones is both slowed and reduced in extent after injection of EGTA.

Calcium ions might mediate inactivation either during their passage through the Ca channel, or by binding to a site located at, or near, the inner mouth of the channel. We consider it less likely that Ca inactivation results from a simple self block of the Ca channel (as would occur if Ca^{2+} entered the channel at a rate faster than it left), since we do not expect such a mechanism to predict time constants as slow as we observe. If, however, the probability of Ca^{2+} binding to an inactivation site within the channel were extremely low (so that few ions which passed through the channel were bound), such slow time constants might be obtained. It seems more probable

that Ca^{2+} mediates inactivation by interaction with some site associated with the Ca channel and accessible to intracellular Ca^{2+}. Intracellular Ca^{2+} has been reported to block Ca-dependent regenerative activity in barnacle muscle at about 5×10^{-7} M (Hagiwara & Nakajima, 1966). In molluscan neurones Kostyuk & Krishtal (1977*b*) found Ca currents were half blocked at a concentration of $2{\cdot}7 \times 10^{-8}$ M, although Akaike *et al.* (1978) report a somewhat lower affinity of the Ca-binding site (around 10^{-6} M). It seems plausible that Ca^{2+} flowing through membrane channels might block its own entry by binding to such a site.

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