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VOLTAGE CLAMP STUDIES ON INSECT SKELETAL MUSCLE

I. THE INWARD CURRENT

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SUMMARY

Membrane currents in the muscle fibre of larval mealworm, *Tenebrio molitor* were investigated under voltage-clamp conditions. An initial inward current, a transient outward current and a delayed outward current were distinguished. Increasing external calcium concentration increased the size of the inward current and shifted the reversal potential in a positive direction. The inward current persisted in Na-free media and TTX-saline. However, the current was suppressed by 15 mM-cobalt ions. Therefore, it was concluded that the inward current was carried by calcium ions. Although the inward current was markedly reduced when external sodium was replaced with glucose, it was concluded that the contribution of Na ions to the current was small. Calcium conductance showed voltage and time dependence. The inactivation variable fell below unity with increasing strength of hyperpolarizing prepulses.

INTRODUCTION

The ionic mechanisms underlying the electrogenesis in muscle fibres of phytophagous endopterygote insects have attracted the attention of many physiologists because of the peculiar ionic levels in their haemolymph. The potassium and magnesium concentrations are usually very high, and the sodium level is low (Florkin & Jeuniaux, 1974). Vertebrate muscle could not function in an extracellular environment characterized by any one of these factors (Katz, 1966).

Studies in the last decade have led to the consensus that action potentials of insect skeletal muscle fibres are generally Ca-dependent (Washio, 1972*a*, *b*; Patlak, 1976; Deitmer & Rathmayer, 1976; Fukuda, Furuyama & Kawa, 1977; Fukuda & Kawa, 1977*b*; Yamamoto & Fukami, 1977; Ashcroft, Standen & Stanfield, 1979). Recently it has been shown that Ca channels alone are responsible for the action potential in muscle fibres of mealworm larvae and that Na and Li ions pass through these channels (Yamamoto & Washio, 1979*a*, 1980).

In the present work an attempt has been made to dissect the individual ionic current

components from the total ionic current in a *Tenebrio* muscle by means of the voltage clamp technique. A preliminary account of this work has appeared (Yamamoto, Fukami & Washio, 1978).

MATERIALS AND METHODS

The experiments were made on the ventral longitudinal muscle fibres of the larval mealworm *Tenebrio molitor*. The dissection was as described previously (Yamamoto & Washio, 1979*b*). The dissected preparation was continually perfused with *Tenebrio* saline containing 70 mM-NaCl, 30 mM-KCl, 5 mM-$CaCl_2$, 10 mM-$MgCl_2$, 445 mM-glucose and 5 mM-HEPES (modified from Kusano & Janiszewski, 1976). The pH of the solution was adjusted to 7·2 with KOH. Ionic concentrations in test solutions were raised by replacing glucose with an osmotically equivalent amount of test ions. Na-free and Ca-free salines were prepared by substitution of an osmotically equivalent amount of glucose or choline chloride for the ions. The short muscle fibres of the immature larvae (10 mm or less in total body length) was used. Fibres were less than 800 μm in length and about 50 μ m in diameter. A mean value for the length constant (λ) using a short cable model (Weidmann, 1952) was 1·4 mm (Yamamoto *et al.* 1978), so it was expected that these small fibres could be clamped satisfactorily by a two microelectrode method with an error of less than 4% at the resting state. However, the error is likely to be considerably more than 4% in the presence of an inward current as the length constant, λ, falls with an increase in membrane conductance. If we assume that the membrane conductance during the peak inward current (at about + 10 mV) is ten times higher than that in the resting state (Werman, McCann & Grundfest, 1961), then λ = 0·44 mm in the active state. Under these conditions, the efficiency of the clamp is about 70%. Glass micro-electrodes were filled with 3 M-KCl or 2 M-K citrate, and had a resistance of 5–10 MΩ. Two microelectrodes were inserted at the centre of a muscle fibre with an inter-electrode distance of less than 50 μm, one being used to record the membrane potential and the other to deliver current. The resting potential of the muscle fibres in the standard solution ranged between 35 and 50 mV. The membrane potential was fed into the control amplifier, to which a commanding and a holding potential were applied. The output of the control amplifier was connected to the current electrode (Fig. 1). Thus, a current flowed through the current electrode until the membrane potential became equal to the holding and commanding potentials, and the membrane potential was clamped at that level. Feedback and preamplifiers were the 8500 Preamp-Clamp (Dagan, Minneapolis, Minn.).

Leakage current correction for analysis of net active membrane current was based on the observation that the steady-state leakage currents are linear functions of hyperpolarizing pulses. Furthermore, membrane currents produced by small displacements of the membrane potential to either side of the holding potential were symmetrical. Thus, it was assumed that leakage current was linear for all values of membrane potential. On this basis, the current-voltage relation was plotted by subtracting currents from opposite polarity pulses (Anderson, 1969).

Experiments were all done at 20–24 °C.

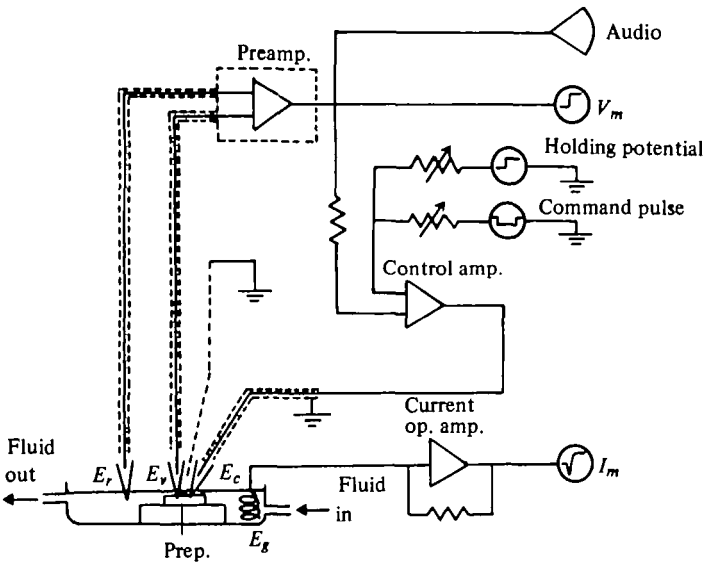

Fig. 1. Voltage-clamp circuit. The membrane potential was recorded differentially, between two glass microelectrodes, one in the cell as a recording electrode (E_v) and another in the chamber as a reference electrode (E_r), using a high input impedance electrometer. A large Ag-AgCl electrode (E_g) used as bath ground electrode was connected directly to the current operational amplifier. With the cell membrane of the preparation (Prep.) in the feedback loop the control amplifier injects or withdraws current to make the membrane potential (V_m) match the command signals through a current electrode (E_c). The current output of the control amplifier thus matches that flowing across the membrane. Current was measured with an operational amplifier connected directly to E_g. Membrane voltage (V_m) and current (I_m) were displayed on a dual beam oscilloscope. A grounded shield placed between the two electrodes helped reduce coupling of the control amplifier output at the current electrode to the voltage electrode.

RESULTS

Separation of membrane current components

Fig. 2 shows a family of membrane currents measured under voltage clamp conditions in the normal saline. The current trace during hyperpolarization was superimposed with inverted polarity on that during depolarization. The membrane current was found to consist of one inward and two outward components. In the currents shown in Fig. 2, the inward current appeared at -20 mV followed by a transient outward current. The inward current increased in size with positive shifts of membrane potential up to about $+5$ mV. With further depolarization, the amplitude of the inward current decreased and the current reversed its sign from inward to outward at about $+25$ mV. The third component is the delayed outward current which was activated with depolarization larger than $+5$ mV.

This paper describes properties of the inward current. The outward currents are analysed in the following paper (Yamamoto & Washio, 1981).

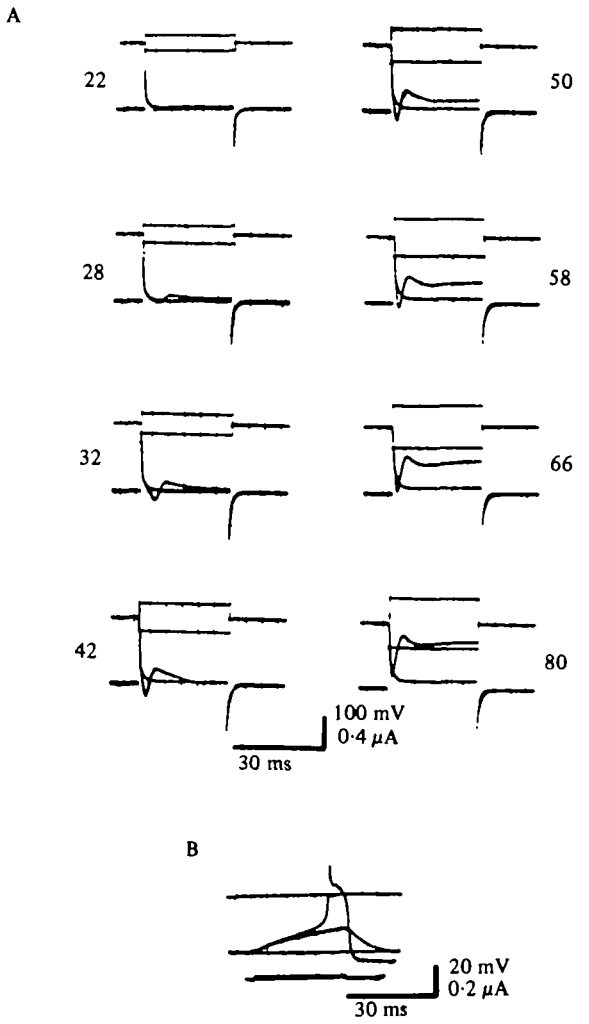

Fig. 2. (A) Membrane currents (lower traces) during voltage clamp of a single muscle fibre. Voltage steps shown in upper traces. The current trace during hyperpolarization was superimposed with inverted polarity on that during depolarization. Displacement from the holding potential of −40 mV is indicated in each record in mV. (B) An action potential and a subthreshold response (middle trace) elicited by current pulses (lower trace) from the same fibre in normal saline. The upper trace represents the reference potential level.

Ionic nature of the initial inward current

Replacement of external Na^+ by glucose reduced the size of the inward current by about 45% (mean of eight experiments). When Na^+ was replaced with choline ions, however, the inward current decreased to a lesser extent (20% reduction, mean of nine experiments). In the example shown in Fig. 3, the inward current suppressed by glucose saline was completely restored after the introduction of choline saline. The reversal potential was not significantly changed after replacement with either glucose or choline ions.

An increase in external calcium concentration raised the inward current, and made the reversal potential more positive (Fig. 4). For a ten-fold increase in Ca concentra-

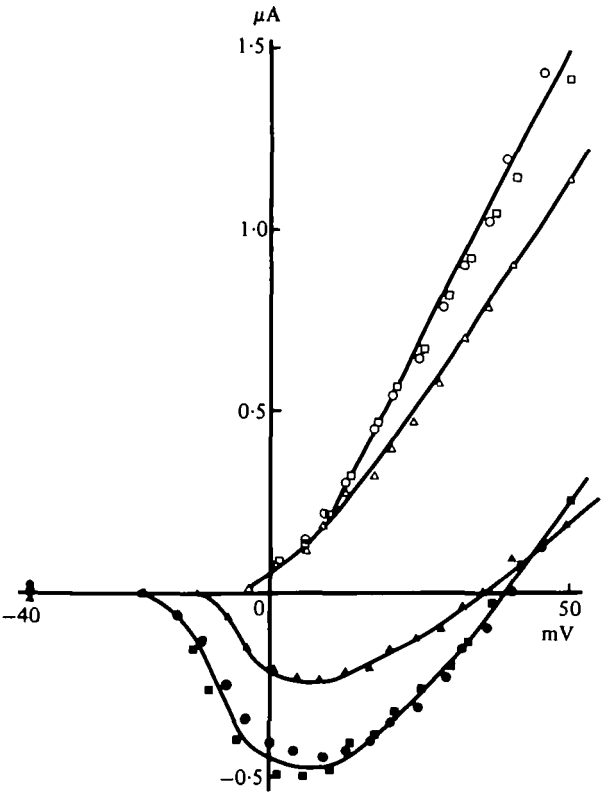

Fig. 3. Current-voltage relations at the peak of the early transient current (filled symbols) and in the steady state (open symbols, obtained at 30 ms after the onset of test pulse). Recordings were made in normal saline (circles), glucose-substituted Na-free saline (triangles), and choline-substituted Na-free saline (squares) from a single muscle fibre. The holding potential was −40 mV.

tion, the reversal potential shifted by between 19 to 28 mV (mean, 22; S.D., 3·4; n = 5). This was not statistically different ($P < 0{\cdot}05$ in t test) from the shift observed in Na-free (choline) saline (range 11–28 mV; mean, 20; S.D., 6·3; $n = 4$).

The small inward current remaining in the Ca-free medium might be carried by Na or Ca in the extracellular spaces. Sodium contribution to the inward current was suggested by the fact that the amplitude of the current was reduced by eliminating sodium from the medium.

Pharmacological properties of the membrane currents

TTX ($3{\cdot}3 \times 10^{-6}$ M) had no effect on either the early inward current or the delayed outward current. In contrast, 15 mM-Co ions added to the medium almost completely eliminated the inward current within a few minutes, and lowered the reversal potential, without affecting the outward current (Fig. 5). This effect of Co ions was fully reversible. 50 mM-tetraethylammonium (TEA) potentiated the inward current, shifted the reversal potential in the positive direction, and abolished the outward current (Fig. 6).

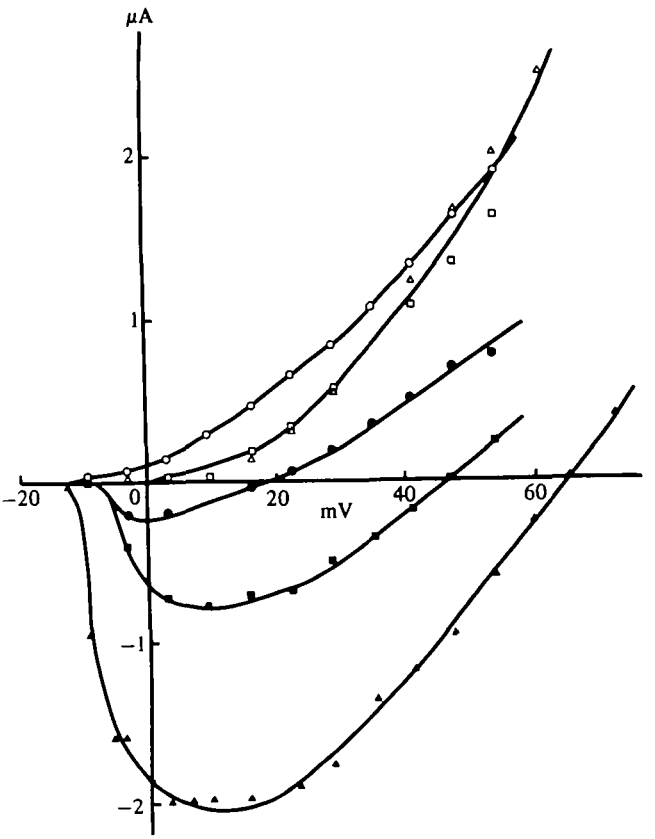

Fig. 4. Current-voltage relations at the peak of the early transient current (filled symbols) and in the steady state (open symbols, obtained at 30 ms after the onset of test pulse) of a single muscle fibre. External solution contained 0 mM-Ca^{2+} (circles), 5 mM-Ca^{2+} (squares) and 50 mM-Ca^{2+} (triangles). The concentrations of other cations were normal. The holding potential was −35 mV. The I-V relation in Ca-free saline was obtained 12 min after replacement of the medium.

Ionic conductances of the inward current

The dependence of the conductance for the transient inward current upon the absolute membrane potential was determined in TEA saline to eliminate contamination by the transient outward current (Fig. 7). The conductance was calculated using the equation

$$G_t = \frac{I_t}{V - V_{rev}}, \qquad (1)$$

where I_t is the maximum inward current measured at an absolute membrane potential V, and V_{rev} is the measured reversal potential. It can be seen that a displacement of the membrane potential of about 5 mV produces an e-fold change in the membrane conductance as in the squid giant axon (Hodgkin & Huxley, 1952*b*).

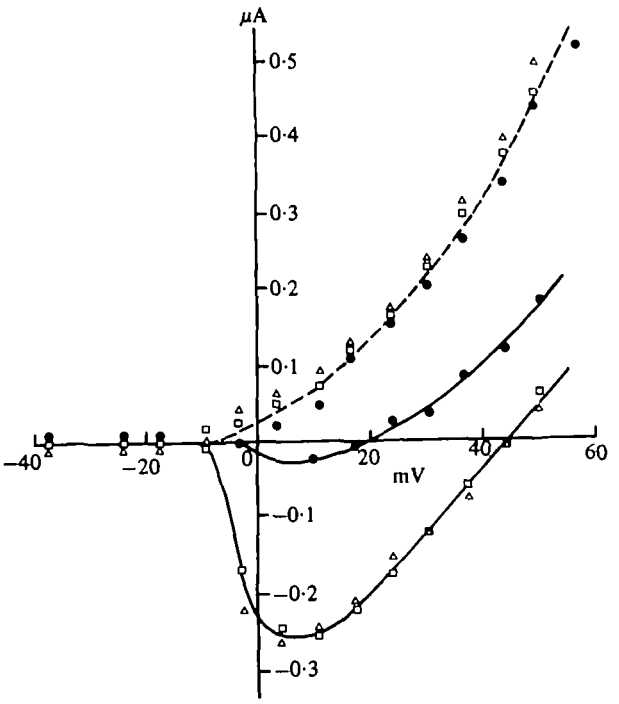

Fig. 5. The peak amplitude of the inward current (continuous lines) and the maximum amplitude of the outward current (interrupted line) before (□) and during (●) treatment with 15 mM-Co^{2+}, and after washing (△) with normal saline are plotted against the membrane potential. The holding potential was −40 mV.

The inactivation process of the inward current

The time courses of inactivation were determined by a double pulse technique in normal saline (Fig. 8). The data are followed closely by the equation (Hodgkin & Huxley, 1952*a*),

$$y = y_\infty - (y_\infty - 1)\exp(-t/\tau_h), \tag{2}$$

where y is the amplitude of current in response to the test pulses, y_∞ is the steady-state value, t is the duration of the prepulse and τ_h is the time constant of inactivation. An increase in the duration of a hyperpolarizing conditioning pulse reduced the size of the inward current whereas an increase in depolarizing pulse duration removed some of the inactivation. A similar observation has been made in molluscan neurones (Geduldig & Gruener, 1970). This makes a marked contrast to the situation of Na channel inactivation where the reverse takes place. The difference may be explained by the existence of the transient outward current (see the next paper: Yamamoto & Washio, 1981).

Steady-state inactivation curves are shown in Fig. 8. In normal saline (Fig. 8A), the data obtained with depolarizing prepulses are fitted well by the equation

$$h_\infty = 1/[1+\exp(V-V_h)/K], \tag{3}$$

where V_h is the conditioning potential at which $h_\infty = 0{\cdot}5$ and K is a shape parameter.

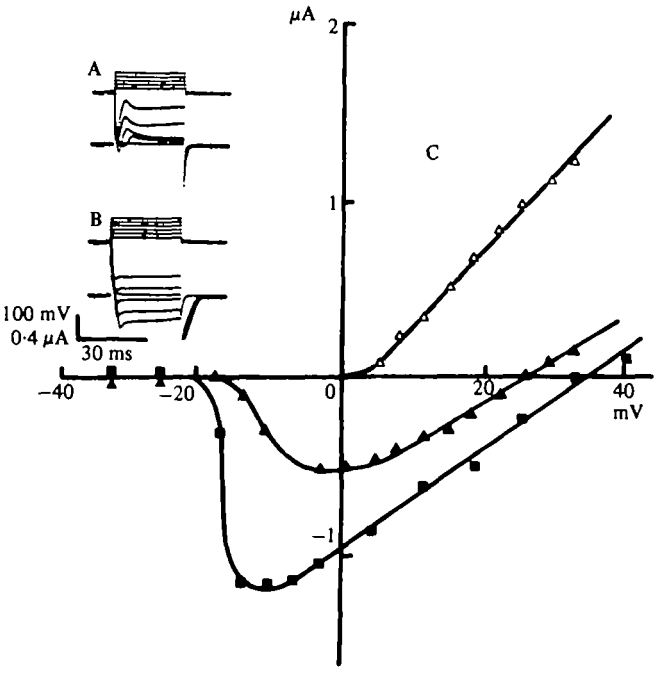

Fig. 6. (A, B) Membrane currents obtained before and after TEA treatment, respectively. Upper traces represent the membrane potential and lower traces represent the membrane current. The hump on the current traces has disappeared and the inward current appears larger after the treatment. (C) Inward current-voltage relations established in normal saline (▲) and in 50 mM-TEA saline (■). The peak inward current was potentiated up to 220% of the control. The outward current observed in normal saline (open triangles) was completely abolished by TEA. The reversal potential for the inward current is very low in this particular fibre due to the strong activation of the transient outward current. It was shifted positive by about 10 mV. A–C were obtained from the same fibre clamped at −40 mV.

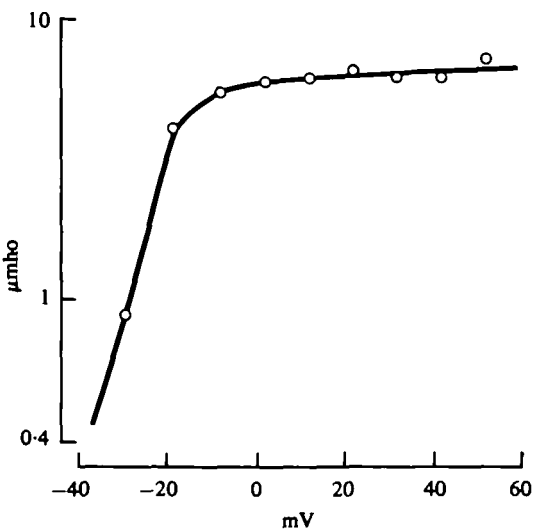

Fig. 7. Conductance for the transient inward current as a function of absolute membrane potential in TEA saline (50 mM).

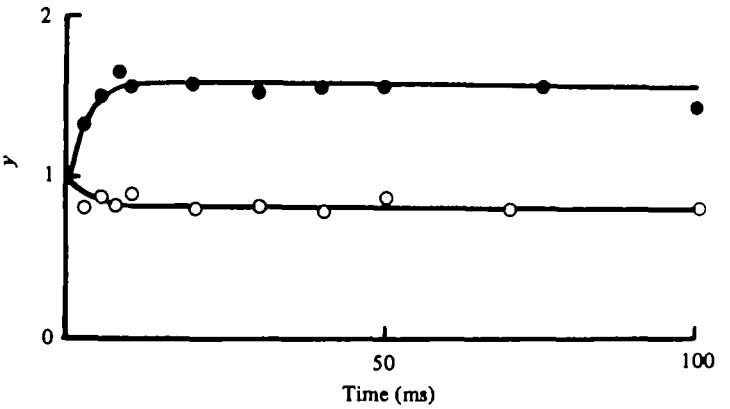

Fig. 8. Time course of inactivation in normal saline. Ordinate: inward current relative to inward current with no prepulse (y). Abscissa: duration of conditioning prepulse. Open circles show the effect of hyperpolarizing prepulse (to -105 mV) while filled circles represent the depolarizing pulse (to 0 mV). Test pulse potentials were: $+20$ mV (●), and -5 mV (○). The continuous lines are drawn to equation (2). The time constant is 17·5 ms. Data were obtained from two different fibres.

This equation is similar to that used by Hodgkin & Huxley (1952*b*) for squid axon. However, with hyperpolarizing pulses, the observed value of h_∞ falls below the predicted value (Fig. 9A). In contrast, in TEA saline hyperpolarization had no effect on inactivation and the data were fitted well by equation 3 (Fig. 9B). In the presence of TEA the inward current was markedly prolonged as shown in Fig. 6B and during a 100 ms prepulse current inactivation did not reach a steady state. However, similar measurements with longer pulses were difficult, because long and strong depolarizing pulses often damaged the cell. For this reason we were unable to obtain reliable results at higher membrane voltages (Fig. 9B). A similar result have been obtained in molluscan neurones (Kostyuk & Krishtal, 1977). The 'hyperpolarizing inactivation' shown in Fig. 9A could again be explained by the early outward current. A detailed discussion is given in the following paper (Yamamoto & Washio, 1981).

DISCUSSION

The results reported here show that in response to a suprathreshold depolarization of mealworm larval muscle there are two initial transient currents, followed by a delayed outward current. The following evidence suggests that the initial inward current is associated with an inward movement of Ca ions down on electrochemical gradient. Firstly, removal of external Ca ions suppressed the transient current, while a ten-fold increase in the external Ca concentration increased the size of the inward current and shifted its reversal potential in a positive direction (Fig. 4). Secondly, the transient current persisted when choline was substituted for Na (Fig. 3) for at least 3 h, and finally, the current was not affected by $3{\cdot}3 \times 10^{-6}$ M-TTX, but was suppressed by 15 mM-Co ions (Fig. 5). In many excitable membranes, the Na channel is readily blocked by 10^{-7} to 10^{-8} M-TTX (Narahashi, Moore & Scott, 1964), and the Ca channel is selectively suppressed by cobalt ions (Hagiwara & Takahashi, 1967; Fukuda & Kawa, 1977*a*).

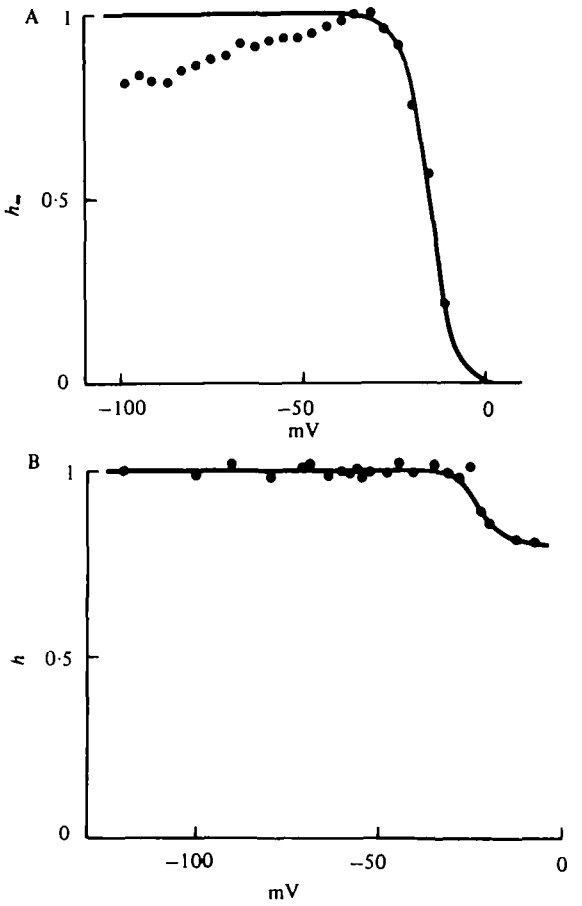

Fig. 9. Inactivation curve in normal saline (A), and TEA-saline (B) of two different fibres. Ordinate, peak inward current relative to inward current with no conditioning pulse. Abscissa, potential of conditioning pulse. Conditioning pulse duration 100 ms. In TEA-saline, the inward current significantly prolonged in duration so the current in response to the test pulse was usually superimposed on the decay phase of the current to the conditioning prepulse (see the test). Holding potential: −55 mV (A), and −40 mV (B). Test pulse potential: −10 mV (A), and −12 mV (B). Continuous line in A drawn to equation (3) with $V_h = -15$ mV, $K = 3$ mV.

The reduction in inward and outward currents observed when glucose was substituted for Na (Fig. 3) probably resulted from the reduced ionic strength of the medium (cf. Murayama & Lakshminarayanaiah, 1977).

The lack of effect of TTX on the transient current suggests that there is no Na channel in this muscle fibre. Therefore, Na ions must enter via the Ca channels, as they do to some extent in frog atrial muscle fibres (Rougier *et al.* 1969) and in molluscan neurones (Kostyuk & Krishtal, 1977). We observed also that the reversal potential slightly shifted when Na^+ ions were removed from the external medium (Fig. 3). According to constant field theory (Goldman, 1943; Hodgkin & Katz, 1949) one would expect only a small shift in the reversal potential after withdrawal of Na^+ ions. We calculated the permeability ratio $P_{Ca}:P_{Na}$ of this channel from two sets

V_{rev} measurements obtained in different external Ca concentrations, 0 and 5 mM, because of uncertaintities of the intracellular ionic activities. According to the constant field theory, we obtained the following expression for V_{rev}, assuming that Na^+, K^+ and Ca^{2+} are the only charge carriers of the current

$$V_{rev} = \frac{RT}{F} \ln \frac{4P'_{Ca}\alpha[Ca]_0 + P_{Na}\alpha[Na]_0 + P_K\alpha[K]_0}{4P'_{Ca}\alpha[Ca]_i + P_{Na}\alpha[Na]_0 + P_K\alpha[K]_i}, \quad (4)$$

$$P'_{Ca} = P_{Ca}\left[\frac{1}{\exp((E_m F/RT) - (V'F/RT)) + 1}\right] \quad (5)$$

where α denotes the activity coefficient of ions, E_m is the membrane potential, and V' is the fixed charge potential resulting from the relative distribution of fixed surface charges in the vicinity of the calcium and potassium channels. R, T and F have their usual thermodynamic meanings. The αs in the external solution were taken as 0·745 for Na^+ and K^+ and 0·33 for Ca^{2+}, according to Reuter & Scholz (1977). We have no experimental data concerning K^+ contribution to the inward current. We first assumed a P_{Na}/P_K permeability ratio to be 1, as did Reuter & Scholz (1977), but later we found that the error introduced by neglecting $\alpha[K]_0$ is negligible. The P_{Ca}/P_{Na} thus obtained was 1:0·04. This ratio is very close to that reported in the slow channel of the mammalian cardiac muscle (Reuter & Scholz, 1977). This permeability ratio predicts a 6 mV shift in V_{rev} when $[Na]_0$ is reduced from 70 mM to 0 mM. We observed a 20 mV shift of the reversal potential for a ten-fold change in $[Ca]_0$, and this again coincides with the prediction by the constant field theory. Furthermore in Ca-free high Na-saline, V_{rev} may be very positive, which is in agreement with the Na-dependent action potentials reported earlier by Yamamoto & Washio (1979*a*).

External application of TEA, which blocks potassium channels (Hille, 1967), resulted in a marked potentiation of the transient current, accompanied by a positive shift in reversal potential (Fig. 6). This suggests that in the absence of TEA, the transient 'inward' current is contaminated with an early outward current. This may explain why the reversal potential is shifted in Co^{2+} saline (Fig. 5) since the shunting effect of the outward current will be greater when the inward current is reduced. A similar shift in the reversal potential by Co^{2+} has been reported in the barnacle muscle fibre (Hagiwara, Hayashi & Takahashi, 1969).

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