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INWARD CALCIUM CURRENT IN TWITCH MUSCLE FIBRES OF THE FROG

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SUMMARY

1. Voltage clamp experiments using the three micro-electrode voltage clamp technique were performed on sartorius muscles of the frog.

2. By blocking potassium currents with tetraethylammonium and replacing chloride ions with sulphate a slow inward current was detected.

3. The slow inward current is mainly carried by calcium, since it is abolished by cobalt and D-600, it depends on external calcium, and is not affected by removing external sodium or by tetrodotoxin (TTX).

4. The slow inward current has a mean threshold of -40 mV, reaches a mean maximum value at *ca.* 0 mV of 81 μAcm^{-2} and has a mean reversal potential of $+38$ mV.

5. The calcium current is inactivated by the application of 2 sec conditioning prepulses according to a sigmoid curve with $V_h = -42$ mV and $k = 6{\cdot}2$ mV.

6. The slow time course of this calcium current makes it rather unlikely that it participates in contraction during a twitch, but it might be activated during long depolarizations as potassium contractures.

INTRODUCTION

In frog skeletal muscle a significant increase in calcium uptake has been observed during muscle activity (Bianchi & Shanes, 1959). However, only recently has a calcium dependent action potential in frog twitch muscle fibres been described (Beaty & Stefani, 1976*a*). This calcium spike can be detected by blocking the delayed potassium current with tetraethylammonium (TEA) and removing the chloride shunt by using an impermeant anion as sulphate. The present experiments were performed to provide additional information on this calcium spike by studying the underlying membrane currents. We used the three intracellular micro-electrodes voltage clamp technique which was developed for muscle fibres by Adrian, Chandler & Hodgkin, (1970*a*). Preliminary results on the calcium current using the voltage clamp technique have been reported (Beaty & Stefani, 1976*b*; Stanfield, 1977).

METHODS

Experiments were performed at room temperature (22–26 °C) on sartorius muscles of *Rana pipiens*. The voltage clamp technique for muscle fibres developed by Adrian *et al.* (1970*a*) was used: three micro-electrodes were inserted into a fibre near its end. Two electrodes, filled with 3 M-potassium chloride, were inserted at a distance $x = l$ and $x = 2l$ from the end of the fibre, in order to record membrane potentials V_1 and V_2 respectively. A third electrode filled with 2 M-potassium citrate was inserted at $x = 2l + l'$, and was used to deliver current intracellularly. The current electrode was screened to earth to within 2–3 mm from its tip. Electrode resistances ranged from 5 to 20 MΩ. The potential differences V_1 and V_2 were recorded via a high impedance unity gain voltage follower (W.P. Instruments, Inc., model 750). The membrane potential was generally controlled at $x = l$; however, to measure membrane capacity, stability and frequency response were improved by controlling the potential closer to the current electrode at $x = 2l$. The feedback amplifier consisted of a Tektronix 502 oscilloscope set at a gain of 500–2000 followed by a high voltage operational amplifier (171 J Analog Devices) in the inverting configuration set at a gain of 10. The output of the 502 oscilloscope amplifier was taken from the cathode follower at the final stage of the vertical amplifier.

The potential difference between the inside of the fibre (V_1 or V_2) and the bathing fluid was recorded on one beam of a storage oscilloscope and was also led to the inverting input of the feed-back amplifier. The non-inverting input was connected to the outputs of a calibrator in series with a square wave generator to produce the holding potential (E_h) and the command pulses respectively. The command pulses were rounded with time constants of 0·1–0·4 msec to avoid initial damped oscillations. The output of the feedback amplifier was connected to the current micro-electrode via a chlorided silver wire. The bath was virtually grounded via a non-polarizable chlorided silver plate of 5 mm × 30 mm placed at one side of the chamber. The bath electrode was connected to the inverting input of high input impedance operational amplifier (Model 1026, Phillbrick) in the ammeter configuration with a 50 KΩ resistance in the feed-back loop. The voltage across this feed-back resistor was amplified × 20 and inverted, thus 1 mV ≡ 1 nA. To improve stability the recording system of the total current (I_o) was rounded off to 0·3 msec by a shunting capacity across the 50 kΩ feed-back resistor. The total current I_o was displayed in the third beam of the oscilloscope.

To calculate membrane current density (I_m, A.cm^{-2}) Adrian *et al.* (1970*a*) have shown that the I_m is approximately equal to:

$$I_m = \frac{a\,(V_2 - V_1)}{3l^2 R_i}, \tag{1}$$

where a is the fibre radius, V_1 and V_2 are the membrane potentials recorded at $x = l$ and $x = 2l$ respectively and R_i is the specific internal resistance of the fibre. The membrane current was then recorded as $V_2 - V_1$ on the second beam of the oscilloscope. The differentiality of the electronics was within 0·1 %. Adrian *et al.* (1970*a*) showed that the approximation for the membrane current is correct to within 5 % if l is less than 2 space constants (λ): this occurs when $(V_2 - V_1)/V_1$ is less than 6. In these experiments l varied from 125 to 300 μm, l' from 20 to 50 μm, and $(V_2 - V_1)/V_1$ was always less than 6. To calculate I_m and the total capacity per unit surface (C_m, μFcm^{-2}), l was directly measured with the microscope, R_i was taken at 22 °C as 160 Ωcm and 250 Ωcm for isotonic and hypertonic saline respectively (see Adrian *et al.* 1970*a*) and the fibre radius was calculated according to Adrian *et al.* (1970*a*).

Since we were not familiar with this technique we have initially reproduced most of the experiments of Adrian *et al.* (1970*a*, *b*) obtaining similar results. For example, the specific membrane resistance (R_m) and the total capacity per unit surface (C_m) were respectively in normal saline 3100 ± 340 Ωcm^2 (12) and 5·9 ± 0·5 μFcm^{-2} (16) (mean ± S.E., (number of observations)).

Solutions. The normal saline contained 115 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$; the chloride-free saline, 40 mM-Na_2SO_4, 1·25 mM-K_2SO_4, 9 mM-$CaSO_4$ and 113 mM-sucrose (Hodgkin & Horowicz, 1959*b*). Tetraethylammonium sulphate ($(TEA)_2SO_4$) was added to the chloride-free saline by isotonic replacement of Na_2SO_4. Sodium was varied by isotonic replacement of Na_2SO_4 with Tris (hydroxymethyl) aminomethane sulphate ($(Tris)_2SO_4$) or $(TEA)_2SO)_4$. Magnesium and cobalt were added as solid $MgSO_4$ and $CoSO_4$. D-600 (Knoll Pharmaceuticals) (methoxy

derivative of iproveratril) was dissolved directly in the saline, tetrodotoxin (TTX) (Calbiochem) was added from a 10^{-4} M-aqueous solution, and dantrolene sodium, 1-(5-(*p*-nitrophenyl) furfuryl-ideneamino) hydantoin sodium hydrate) (Norwich Pharmacol. Co.) from a 10^{-3} M solution. To reduce contractions 350 mM-sucrose was added to the saline (Hodgkin & Horowicz, 1957; Howarth, 1958). All solutions were buffered to pH 7·2 with 4 mM-Tris maleate. Muscles were first equilibrated in normal saline, then in chloride-free saline; TEA and sucrose were added after the resting potentials of the fibres had recovered.

Mechanical artifacts. Since in these experiments it is necessary to deliver large and long depolarizing pulses, mechanical artifacts become a major difficulty. Initial experiments were made by adding 350 mM-sucrose to the saline; however, in most cases contraction was not completely abolished and records showed mechanical artifacts. We then used the procedure described by Stefani & Schmidt (1972), mounting the muscle around a polyethylene rod and stretching the muscle until no movement to electrical stimulation was detected under the microscope. This procedure is successful for impalling fibres in the middle of the muscle, but becomes impractical for penetrating muscle fibres close to their tendon. We used in the present experiments, hypertonic saline with the addition of 10^{-5} M-dantrolene. This compound reduces twitch tension and potassium contractures (Ellis & Carpenter, 1972; Putney & Bianchi, 1974). Besides, the calcium spike in frog muscle (Beaty & Stefani, 1976*a*) was practically unaffected. By combining hypertonic sucrose and dantrolene, movements were almost abolished and we were able to deliver large depolarizing pulses in a repetitive manner without mechanical artifacts and/or membrane damage. Since the experiments were performed at room temperature, resting potentials of muscle fibres deteriorate rapidly when adding hypertonic sucrose; therefore measurements were made within 15–60 min after adding the sucrose. During voltage clamp, membrane potentials were maintained at -90 mV (E_h). Values are expressed as mean ± S.E., number of observations are in brackets.

RESULTS

Effect of hypertonicity on resting potential at room temperature (22–24 °C). The mean resting potential in chloride-free saline was $-93{\cdot}1 \pm 0{\cdot}7$ mV (20), as was reported by Hodgkin & Horowicz (1959*b*). A similar value of $-94{\cdot}2 \pm 0{\cdot}2$ mV (11) was obtained with the addition of 40 mM-TEA. When 350 mM-sucrose was added to this saline, the resting potentials initially increased during the first 5 min to $-104{\cdot}1 \pm 0{\cdot}9$ mV (10). Thereafter the potentials slowly declined to $-90{\cdot}3 \pm 1{\cdot}9$ mV (10) at *ca.* 15 min, to $-82{\cdot}7 \pm 1{\cdot}4$ mV (6) at 30 min and to $-65{\cdot}6 \pm 1{\cdot}2$ mV (10) at 60 min after the addition of sucrose. In these experiments we used muscle fibres exposed for 15–60 min to the hypertonic sucrose having resting potentials ranging from -65 to -85 mV. It was therefore necessary to deliver a steady inward current during voltage clamp to maintain the membrane potential at -90 mV (E_h).

Slow inward current. In order to record small calcium inward currents during depolarizing steps it becomes necessary to block opposing outward going potassium and chloride currents. This can be achieved almost completely by replacing external chloride by a non-permeant anion such as sulphate (Hutter & Noble, 1960) and by adding external TEA, a blocker of potassium outward currents (Armstrong & Binstock, 1965; Stanfield 1970). In addition, sodium currents were eliminated by complete replacement of Na by TEA, and by blocking sodium channels with TTX (Narahashi, Moore & Scott, 1964; Hille, 1968; Adrian *et al.* 1970*a*).

The muscle fibre in Fig. 1 was bathed in chloride and sodium-free saline containing 80 mM-TEA and 5×10^{-7} M-TTX. The resting potential of the cell was -78 mV, and it was maintained at -90 mV. Each pair of traces shows records of the imposed membrane potential (square pulses) and of membrane currents measured as $V_2 - V_1$. In this and subsequent experiments command pulses were delivered every 5–10 sec.

In *a*, the leakage current associated with a subthreshold pulse which drove the membrane potential to −43 mV can be seen. In *b*, the command pulse was increased to −38 mV, and evoked a small and slowly increasing inward current which appeared after a long delay. This current increased in amplitude and became faster and steeper

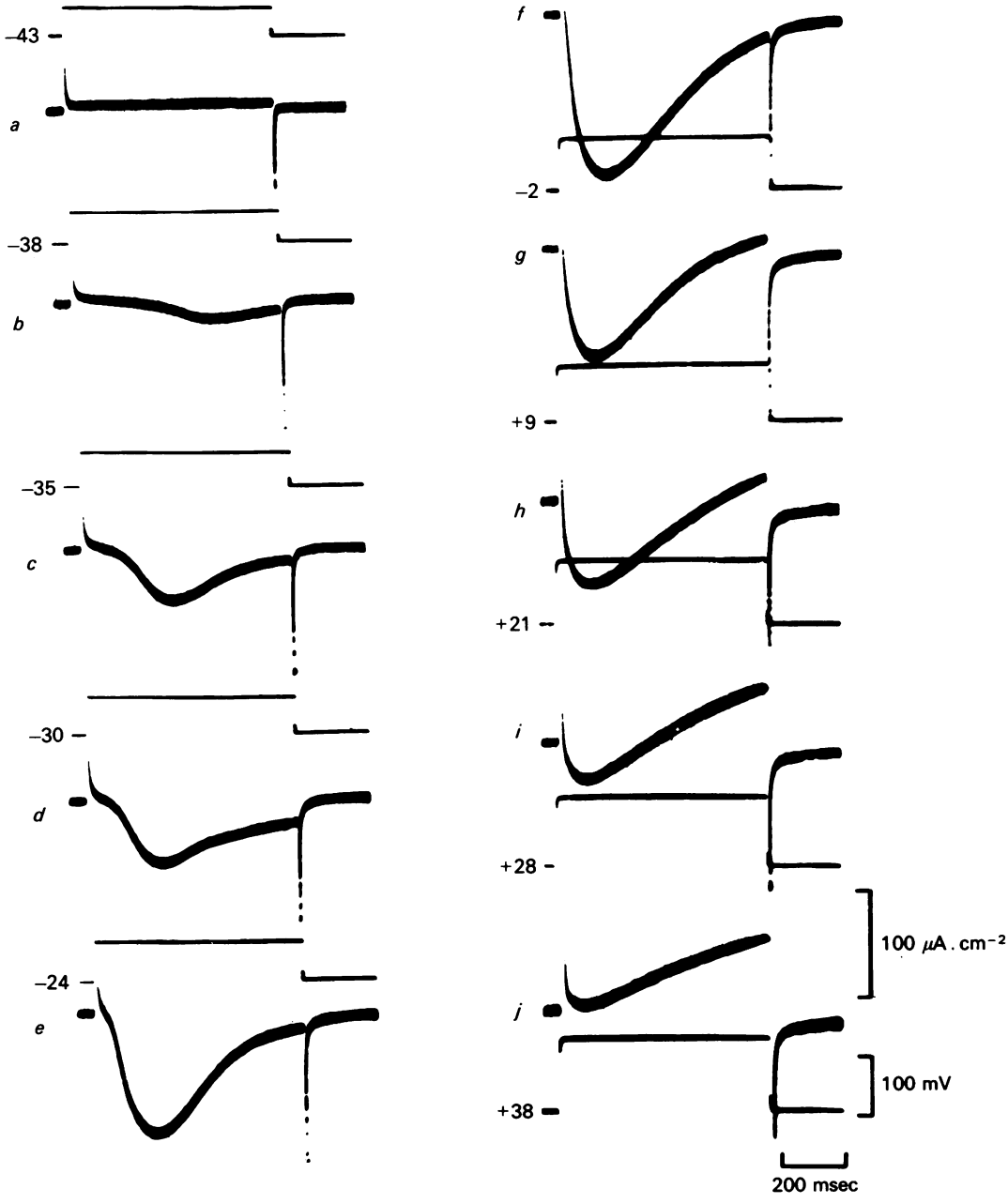

Fig. 1. Records of membrane currents during command pulses to different potentials (numbers at left). Chloride and sodium-free saline with 80 mM-TEA and 5×10^{-7} M-TTX. E_h − 90 mV; electrode spacing 265 μm; radius 29·6 μm; 1 mV ≡ 5·6 μAcm^{-2}.

as the command pulse was increased (records *b* to *f*), being maximal at −2 mV (*f*). With command pulses driving the membrane potential to positive values, the inward current decreased in amplitude approaching a reversal potential (records *g* to *j*). These inward currents were not maintained during the pulse but slowly declined. For example at −24 mV (*e*), the current reached a peak at 220 msec and decayed exponentially with a half time of 200 msec. With large steps the decline of the current was followed by a large slowly developing outward current (records *g* to *j*). When pulses were terminated, fast inward tail currents were difficult to differentiate from the capacitative surges; then followed a second inward tail current which decayed slowly, and resembled the second slow potassium channel described by Adrian *et al.* (1970*b*).

These inward currents are much slower than sodium and initial potassium currents. Furthermore, they are highly temperature dependent, their time to peak being several seconds at 2–6 °C. Thus we decided to perform experiments at room temperature. Slow inward currents were recorded in almost every fibre studied in 40 or 80 mM-TEA. In a few fibres, however, the current did not clearly turn inward. This was due either to high values of leakage currents due to muscle damage during micropipette insertion or, in some cases, to incomplete block of the potassium outward current. In conclusion, by removing potassium and chloride outward currents, a slow inward current is recorded that is not carried by sodium, since it is elicited in the presence of TTX and in a sodium-free solution.

Ionic mechanism of the slow inward currents. Beaty & Stefani (1976*a*, *b*) have shown that the slow spike that is elicited in a chloride-free saline with TEA depends on external calcium. Along these lines, the slow inward current we have recorded in these experiments was obtained in a saline without sodium and with the addition of 5×10^{-7} M-TTX indicating that calcium must carry a major part of this current. This is further supported by the calcium dependency of this inward current. Fig. 2 shows records of membrane currents and command pulses when calcium was replaced by magnesium in the chloride free TEA saline. In *a*, the leakage current associated with a depclarizing pulse to −20 mV is shown. When the cell was further depolarized (to −10 mV in *b* and to +8 mV in *c*), there was an initial outward current which spontaneously decayed during the pulse. This outward current corresponds to remaining potassium currents which were not blocked by TEA (Stanfield, 1970). In all fibres tested no inward current could be recorded in the absence of calcium and the slowly developing outward current was not clearly seen. In these experiments membrane currents were recorded as soon as possible (5–10 min) after the calcium replacement by magnesium, since the membrane resistance progressively decays. Similar results were obtained when 10 mM-cobalt sulphate or 10^{-4} M-D-600 were added to the chloride-free TEA saline.

Fig. 3 shows current–voltage relations obtained in chloride-free TEA saline with calcium replaced by magnesium. In this figure the leak current (curve *a*, filled circles) and the TEA resistant potassium current measured at the peak can be seen (curve *b* open circles).

Current–voltage relation of the inward current. Fig. 4 shows the current–voltage relation of the fibre shown in Fig. 1. Curve *a* (filled circles) is the leak current. Open circles (curve *b*) represent the value of the inward current from the base line to the

peak for different voltage steps. The inward current has a threshold of −40 mV, it follows a continuous curve reaching a maximum value near 0 mV, and then it declines rather abruptly to the reversal potential. In this fibre the reversal potential after substraction of the leak current is +40 mV (assuming that the leak conductance is

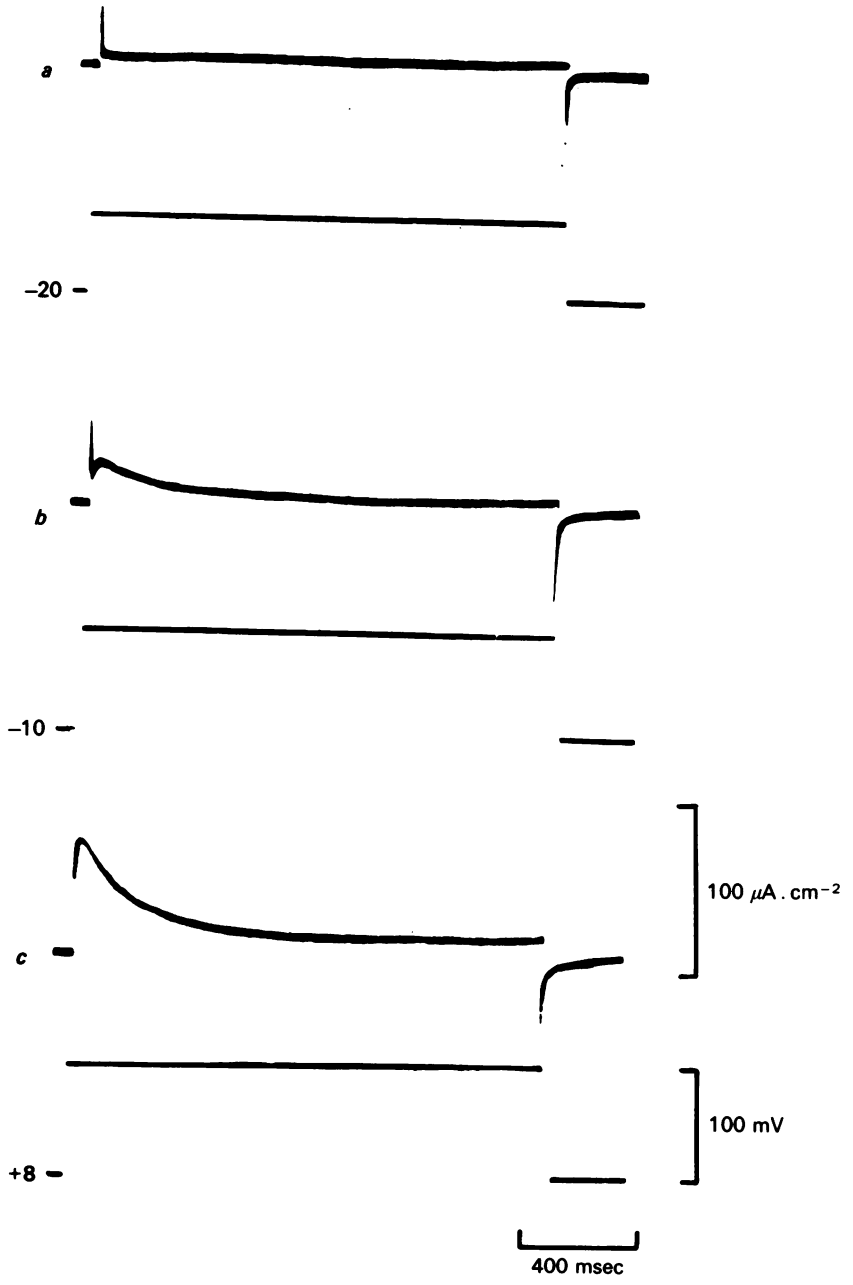

Fig. 2. Records of membrane currents during command pulses to different potentials (numbers at left). Chloride-free saline with 40 mM-TEA, 40 mM-Na, 10 mM-Mg and 0-Ca. Note the absence of inward current and the TEA resistant potassium outward currents. E_h −90 mV; electrode spacing 240 μm; radius 30 μm; 1 mV ≡ 7 μAcm^{-2}.

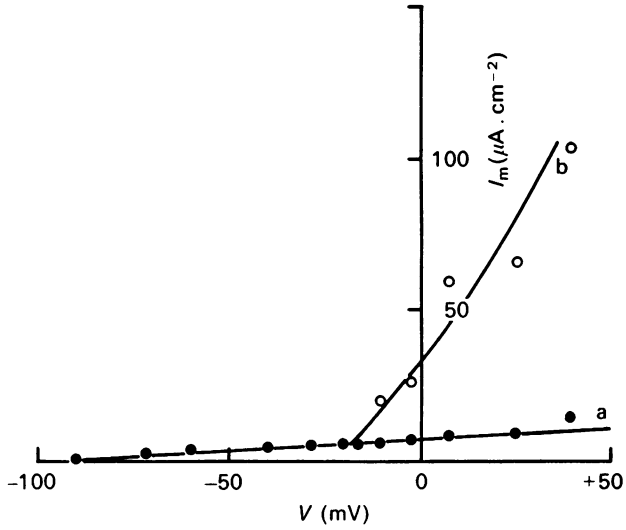

Fig. 3. Relation between membrane current and voltage during depolarizing steps of the fibre shown in Fig. 2. Filled circles (curve *a*) are the steady-state currents and open circles (curve *b*) are peak outward current. Leak conductance 0·08 mmho cm^{-2}.

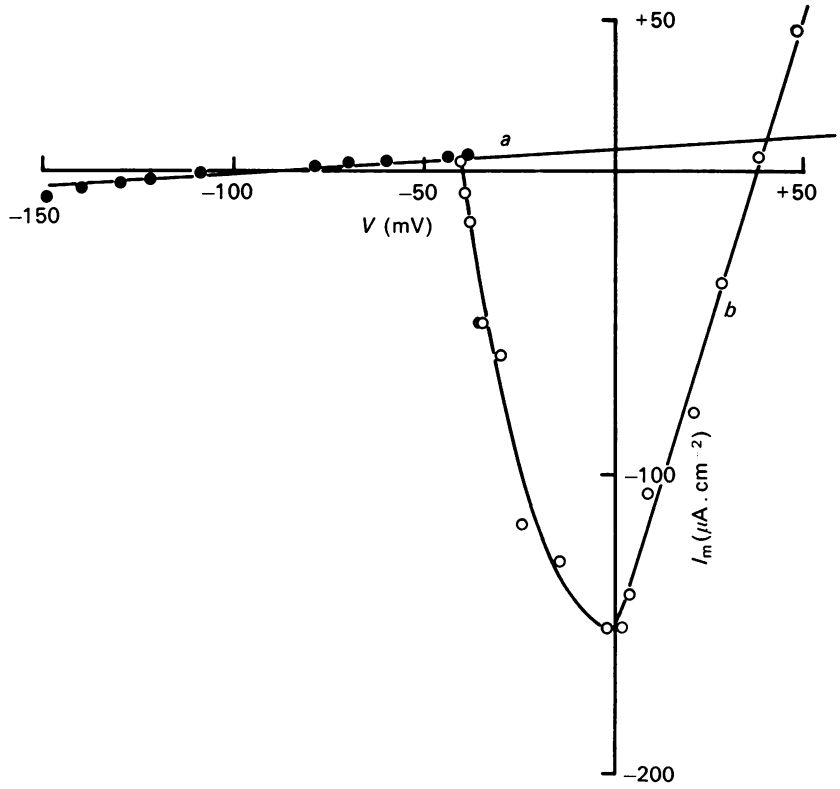

Fig. 4. Relation between membrane current and voltage during depolarizing steps of fibre in Fig. 1. Filled circles (curve *a*), subthreshold steady-state currents and open circles (curve *b*), peak inward currents. Reversal potential is +40 mV measured at the intercept of curve *a* and *b*. Leak conductance 0·07 mmho cm^{-2} and conductance change during maximum inward current 4·0 mmho cm^{-2}.

linear). The maximum current after leak substraction is 158 μA cm^{-2}, and the associated conductance 4·0 mmho cm^{-2}. In several fibres the mean extrapolated reversal potential after substracting leak currents was +38 ± 2 mV (14). The inward current measured at *ca.* 0 mV was 81 ± 10 (14) μA cm^{-2} and the associated conductance change 2·3 ± 0·3 mmho cm^{-2} (14). The mean leak conductance in these fibres was 0·11 ± 0·03 mmho cm^{-2}.

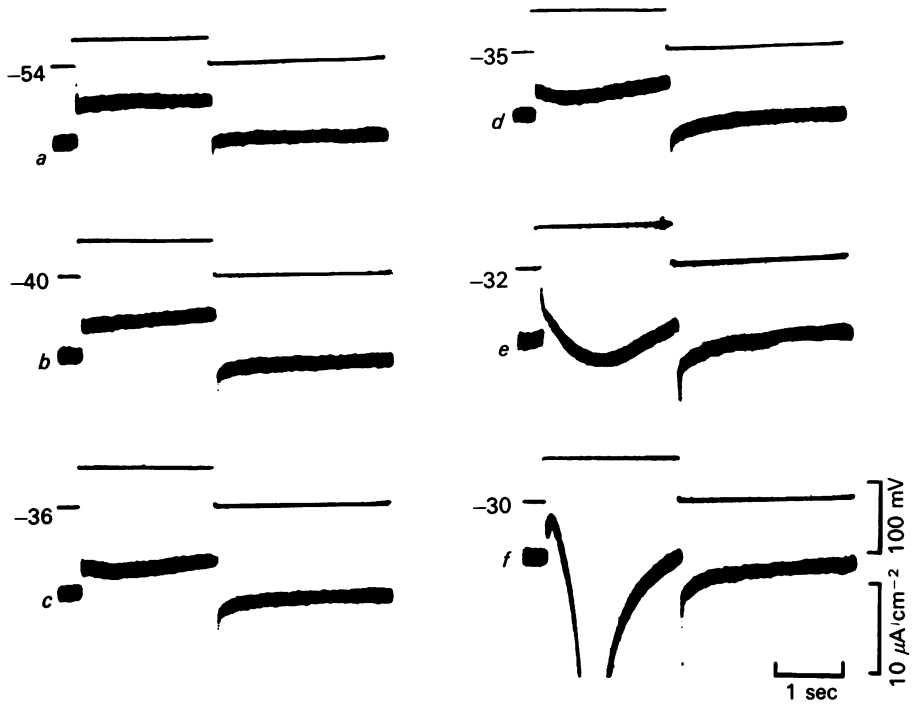

Fig. 5. Records of membrane currents during command pulses to different potentials (numbers at left). Chloride-free saline, 40 mM-sodium, 40 mM-TEA and 5×10^{-7} M-TTX. E_h −90 mV; electrode spacing 265 μ; a = 20·2 μ; 1 mV ≡ 3·8 μAcm^{-2}. Note inward current close to threshold.

The steep decline of the inward current at positive potentials shown in Fig. 4 (curve *b*) can be explained by a simultaneous activation of potassium outward currents, which as already shown (Figs. 2 and 3) are not completely blocked by TEA (Stanfield, 1970) and can be activated during large depolarizations. These results clearly indicate that the slow inward current is at least produced by two simultaneously oppossing currents, a calcium inward and the remaining potassium current. Because of these potassium currents, the actual reversal potential of the calcium current cannot be measured directly since it depends upon the ratio between calcium and potassium conductances. It was uncertain to subtract the activated outward potassium currents since they are non-linear with voltage and are time dependent, thus it becomes necessary to completely block the delayed current in order to measure with some accuracy the reversal potential of the calcium current.

Threshold. Records of voltage and membrane currents obtained at membrane potentials close to threshold values are shown in Fig. 5. In *a*, a pulse to −54 mV is

delivered, and the linear leak current can be seen. In *b* the pulse brings the potential to −40 mV. The current slowly increased outwardly being smaller at the beginning than in *a*. At the end of the pulse there is an inward tail current that declines slowly. The reduction of the outward current with a larger step can be explained by a small superimposed inward current which becomes more evident during the tail current at the end of the pulse. Thus, at this potential (−40 mV) two current points are plotted, the current at the beginning and at the end of the voltage step (Fig. 6). Records *c* and *d* show more clearly the inward going current which becomes evident in records *e* and *f*. In *f* the inward current is preceded by a small potassium outward current.

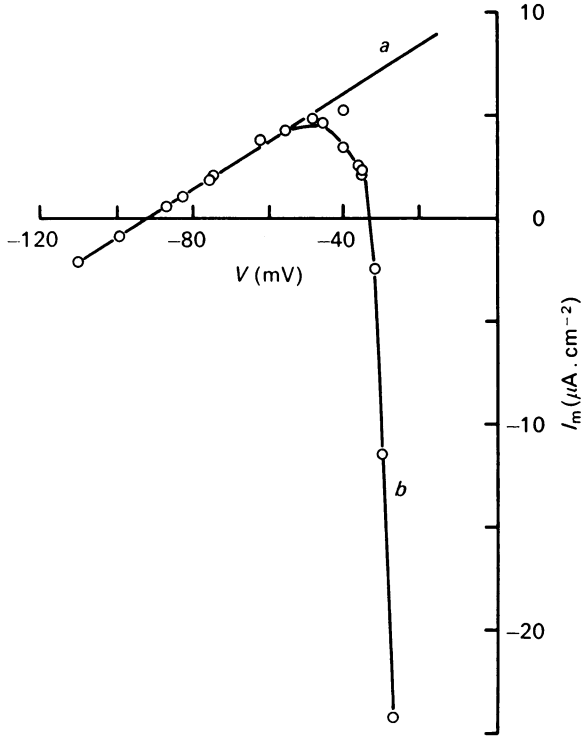

Fig. 6. Relation between membrane current and potential during depolarizing steps of fibre in Fig. 5. Curve *a* is the leak current. Note the threshold for the current at −46 mV. Leak conductance 0·12 mmho cm^{-2}.

Fig. 6 shows the current voltage relation in the same fibre. Curve *a* is the leak current and *b* the activated currents. In this fibre, the current threshold was −46 mV and the leak conductance 0·12 mmho cm^{-2}. The mean threshold in different fibres was −40·9 ± 1·3 (7). This value is somewhat over-estimated because of opposing potassium currents.

Inactivation. The inward calcium current is not maintained during a long depolarizing pulse thus suggesting that the current is inactivated. However, the decay of the current can also be explained by a simultaneous slow activation of potassium currents. Fig. 7 shows one experiment to analyse these possibilities. The fibre was held at −90 mV. In *a* the test pulse to −24 mV and the associated inward current are

shown. Note the initial potassium outward current followed by the slow inward calcium current. At the end of the pulse the current ends abruptly and a slow tail current is seen. In the following records, the test pulse was maintained constant at −24 mV and was preceded by a conditioning prepulse of 2 sec whose amplitude was varied. The amplitude of the inward current during the test pulse decreases as the amplitude of the prepulse increases. For example, the inward current is greatly reduced in *e* by a prepulse to −36 mV, which did not elicit by itself any slow inward

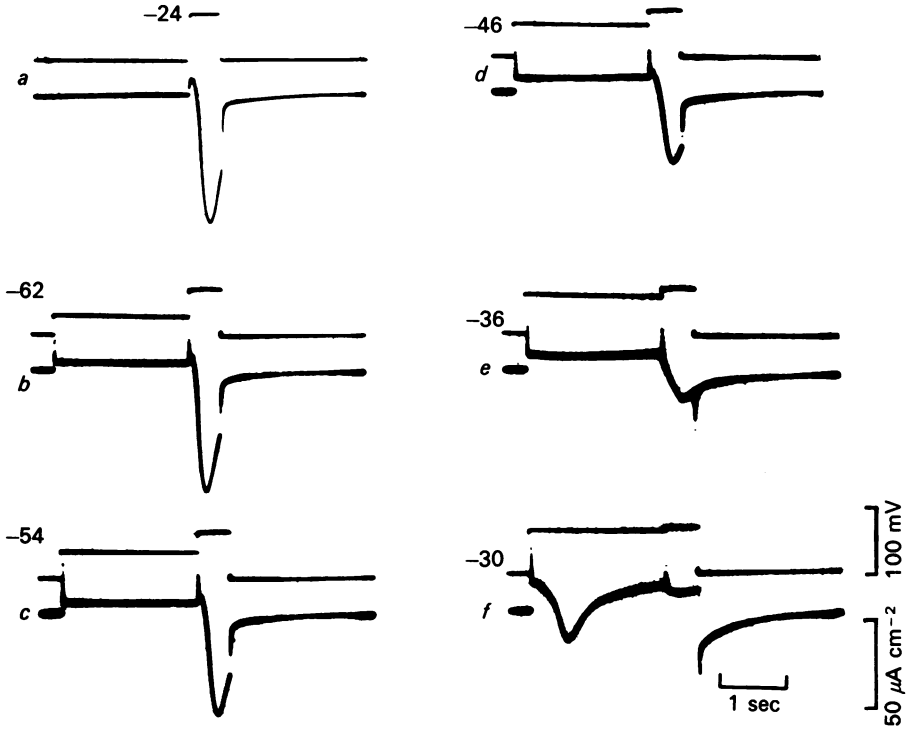

Fig. 7. Records of membrane currents (lower traces) during conditioning prepulses (upper traces) to different potentials (numbers at left), and test pulses always to −24 mV. The prepulse lasted 2 sec. Chloride and sodium-free saline with 80 mM-TEA and 5×10^{-7} M-TTX. E_h −90 mV; electrode spacing 265 μ; radius 20·2: 1 mV ≡ 3·8 μA cm^{-2}.

current. In *f* the prepulse elicited a slow inward current and there is a small current left during the test pulse. The inactivation curve depends on the duration of the prepulses and reaches a steady state with prepulses of 2 sec. The curve can be fitted by the relation proposed for sodium inactivation (Hodgkin & Huxley, 1952; Adrian *et al.* 1970*a*):

$$h_{Ca} = [1 + \exp(V - V_h)/k]^{-1}.$$

In our experiments V_h was −42 mV and k was 6·2 mV.

DISCUSSION

During muscle activity there is an increase in calcium uptake (Bianchi & Shanes, 1959; Curtis, 1966). This calcium uptake is quite small and in the normal frog saline it was not detected as an electrical signal because of large opposing potassium (Hodgkin & Horowicz, 1959*a*) and chloride currents (Hutter & Noble, 1960). Along these lines, by greatly reducing potassium and chloride currents, a calcium dependent action potential was found by Beaty & Stefani (1976*a*). Using the voltage clamp technique a slow inward membrane current underlying the calcium spike is described in the present paper. The slow inward current we found is mainly carried by calcium ions since it is abolished by removing external calcium or by applying cobalt or D-600 which are known to block calcium conductance in other tissues (Hagiwara & Nakajima, 1966; Hagiwara & Takahashi, 1967; Kohlhardt *et al.* 1972, 1973; Baker *et al.* 1973). Accordingly, this current is not sodium dependent and it is not affected by TTX. These findings are consistent with the observations of Beaty & Stefani (1976*a*, *b*), and Stanfield (1977).

Since TEA did not completely block potassium currents, which in turn were large with strong depolarizations, the recorded inward current is composed of calcium inward current and the remaining potassium outward current. Both currents have a somewhat similar voltage and time dependency and it is difficult to separate them; therefore, the calcium reversal potential and inactivation could not be measured accurately. Remaining potassium current prevents any detailed quantitation of calcium current. Besides, after the calcium current, there is a slowly developing outward current, probably carried by potassium, which is not clearly seen when the calcium current is absent. This may suggest that this slow outward current depends on a preceding calcium entry (Meech & Standen, 1975; Fink & Lüttgau, 1976).

Another interesting point is whether the calcium current goes through a specific channel or through one of the ionic channels already described for muscle fibres (Adrian *et al.* 1970*a*, *b*). A specific channel for calcium has been found in heart muscle (Reuter, 1968; Vassort *et al.* 1969; Reuter & Scholz, 1977), in barnacle muscle cells (Hagiwara, Hayashi & Takahashi, 1969; Keynes, Rojas, Taylor & Vergara, 1973), in undifferentiated cells such as the tunicate egg (Okamoto, Takahashi & Yoshii, 1976), and in slow muscle fibres of the frog (Stefani & Uchitel, 1976). Our experiments show that this calcium current in muscle does not go through the early sodium channel since it is much slower and it is TTX resistant. We cannot exclude, however, another faster calcium current through the sodium channel. It is also very unlikely that the calcium current goes through the early potassium channel since both currents have different time course. We may tentatively conclude that calcium enters via a specific channel. However, with the present data we cannot completely rule out some participation of the slow potassium channel (Adrian *et al.* 1970*b*).

Even though we do not have at present a precise quantitation of the calcium current, its slow time course makes it most likely that it does not play any role during twitch contraction since it would not be activated during a single action potential. This is agreement with Armstrong, Bezanilla & Horowicz (1972), who recorded twitches in calcium free saline ($< 10^{-8}$ M). Albeit, during a prolonged depolarization as a potassium contracture (Hodgkin & Horowicz, 1960), it is possible that this

calcium conductance would be activated producing a calcium influx. Accordingly it was reported that potassium contractures are reduced in calcium-free saline (10^{-9} M) containing 4 mM-magnesium or with the addition of manganese (Stefani & Chiarandini, 1973; Chiarandini & Stefani, 1973, 1976). It becomes relevant therefore to calculate from our records the calcium entry and the final calcium concentration in myoplasm. For example, by integrating the current from Fig. 1*e*, the total calcium entry per unit surface during the step was found to be unexpectedly large, $2{\cdot}3 \times 10^{-10}$ M cm^{-2}, leading, assuming free diffusion, to a final concentration of $1{\cdot}5 \times 10^{-4}$ M. Values of the same order were obtained in several other fibres and are in agreement with the reduction of the plateau of potassium contractures reported in calcium-free salines.

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