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EFFECT OF GLYCEROL TREATMENT ON THE CALCIUM CURRENT OF FROG SKELETAL MUSCLE

BY L. NICOLA SIRI, J. A. SÁNCHEZ AND E. STEFANI

From the Department of Physiology and Biophysics, Centro de Investigación del IPN, Apartado Postal 14-740, México 14, D.F.

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SUMMARY

1. Current and voltage clamp experiments were carried out on frog skeletal muscle fibres. For voltage clamp, the three micro-electrode technique near the fibre end was used.

2. Calcium spikes and currents were recorded in TEA sulphate saline. The addition of 400 mM-glycerol did not appreciably modify them.

3. Muscle fibres were detubulated with the glycerol method. They showed sodium propagating action potentials, with sodium and potassium currents of expected amplitudes.

4. Calcium spikes and currents were reduced or abolished in detubulated muscle fibres.

5. An analysis of fibre capacitance showed a linear correlation between the remaining I_{Ca} and the degree of electric discontinuity between the transverse tubular system and the surface membrane.

6. These results indicate that I_{Ca} is mainly located in the transverse tubular system. This localization is compatible with some role during mechanical activation.

INTRODUCTION

A calcium dependent action potential was recently found in frog skeletal muscle fibres by blocking potassium currents with tetraethylammonium (TEA) and replacing chloride with sulphate (Beaty & Stefani, 1976). In the same saline under voltage clamp conditions, a slow inward calcium current was described (Sánchez & Stefani, 1978). An important point is to know whether the calcium current is located in the transverse tubular system or in the surface membrane. A tubular localization would be compatible with some role during excitation–contraction coupling. The localization was investigated by correlating calcium current amplitude with the degree of disruption of the transverse tubular system after glycerol treatment, a procedure which uncouples the system electrically from the surface membrane of the muscle fibre (Fujino, Yamaguchi & Suzuki, 1961; Howell & Jenden, 1967; Eisenberg & Eisenberg, 1968; Gage & Eisenberg, 1969*a*, *b*; Eisenberg & Gage, 1969; Dulhunty & Gage, 1973; Chandler, Rakowski & Schneider, 1976). Preliminary results have been reported (Nicola Siri, Sánchez & Stefani, 1977).

Reprints request should be sent to Dr E. Stefani, Department of Physiology and Biophysics, Centro de Investigación del IPN, Ap. 14-740, México 14, D.F.

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METHODS

Experiments were performed at room temperature (22–26 °C) on sartorius and tensor fascia lata muscles of *Rana pipiens* and *R. temporaria*. Conventional techniques for injecting current intracellularly and recording transmembrane potential were used. For voltage clamping, the three micro-electrode technique developed by Adrian, Chandler & Hodgkin (1970) was used and it is described in detail elsewhere (Sánchez & Stefani, 1978). Three micro-electrodes impaled a muscle fibre near its end. Two electrodes positioned at distances l and $2l$ from the fibre end were used to record the membrane potential V_1 and V_2 respectively. V_1 or V_2 was controlled

TABLE 1. Solutions (mM)

References	Na^+	K^+	Ca^{2+}	Mg^{2+}	TEA^+	Cl^-	SO_4^{2-}	Sucrose
A (Normal saline)	115	2·5	1·8	—	—	121·1	—	—
B	115	2·5	5	5	—	137·5	—	—
C (Na-sulphate saline)	80	2·5	9	—	—	—	50·25	113
D (TEA-sulphate saline)	—	2·5	9	—	80	—	50·25	113

by a feed-back amplifier that injected current intracellularly through the third micro-electrode positioned at the distance $2l+l'$. The potential differences V_1 and V_2 were generally recorded differentially referred to a third micro-electrode positioned close to the muscle fibre. The micro-electrodes were shielded to within 100 μm of their tip with conductive silver paint and insulated with fast curing resin. The shield of the current micro-electrode was connected to earth. To minimize capacitance to ground, the voltage micro-electrodes were screened to the drive shield of the voltage follower which gave a time constant of 10–30 μsec for 10–30 MΩ micro-electrodes with the optimum capacitance compensation. This compensation was carefully adjusted by passing square pulses through the ground electrode. The membrane potential was generally controlled at $x = l$; however, to measure membrane capacitance, stability and frequency response were improved by controlling the potential closer to the current electrode at $x = 2l$. The bath was virtually grounded via a non-polarizable chlorided silver plate connected to the inverting input of a high input impedance operational amplifier in the ammeter configuration with a 1 MΩ resistance in the feed-back loop.

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

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

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 resistivity of the myoplasm. The membrane current was then recorded as $V_2 - V_1$. Adrian *et al.* (1970) showed that the approximation for the membrane current is correct to within 5% if l is less than two space constants (λ): this occurs when $(V_2 - V_1)/V_1$ is less than 6. In these experiments l varied from 125 to 400 μm, l' from 20 to 50 μm, $(V_2 - V_1)/V_1$ was always less than 6. l was directly measured with the microscope, and R_i was taken at 22 °C as 160 and 250 Ωcm for isotonic and hypertonic saline respectively (see Adrian *et al.* 1970). The fibre radius, I_m, and the total capacitance per unit surface (C_m, μF cm^{-2}) were calculated following Adrian *et al.* (1970). The holding potential was −90 mV.

Solutions also contained 1 mM-Tris-maleate buffer (pH 7·4), 10^{-5} M-dantrolene sodium (1-(5(*p*-nitrophenyl)furfuryl ideneamino) hydantoin sodium hydrate) (Norwich Pharmacol. Co.), and occasionally 5×10^{-7} M-tetrodotoxin (Calbiochem). Averaged values are expressed as mean ± standard error; the number of observations is in brackets.

RESULTS

Effects of 400 mM-glycerol on calcium spike and calcium current

In these experiments we attempted to determine whether the calcium current I_{Ca} is located primarily in the surface membrane or in the transverse tubular system by using the glycerol treatment which has been reported to disrupt the continuity of the system with the surface membrane. Previous to the glycerol treatment, it was important to know whether the addition of glycerol by itself to the saline would modify the calcium response.

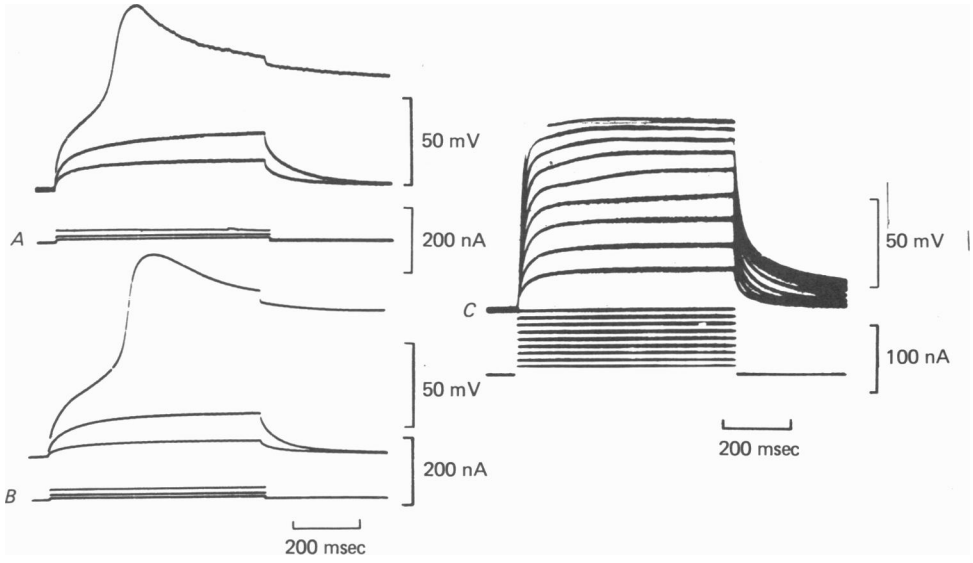

Fig. 1. Effect of glycerol treatment on the calcium response. Intracellular recording of membrane potential (upper traces) to injection of square pulses of current (lower traces). In *A*: TEA-sulphate. In *B*: TEA-sulphate + 400 mM-glycerol. In *C*: approximately 1 hr after glycerol withdrawal. Note the regenerative response in *A* and *B*, that disappears after detubulation in *C*. E_{rp} − 84 mV in *A*, − 85 in *B* and − 78 in *C*. The fibres were polarized to − 85 mV.

Fig. 1*A* shows a calcium response in isotonic TEA sulphate (soln. *D*). Fig. 1*B* shows a similar response after bathing the muscle for 1 hr in TEA sulphate (soln. *D*) + 400 mM-glycerol. The responses were practically identical indicating the lack of effect of glycerol. Similar results were obtained when, to this saline, sodium was added and the calcium spike was preceded by a sodium action potential as reported by Beaty & Stefani (1976). This conclusion was strengthened by the recordings of I_{Ca} under voltage clamp. To abolish contraction, 200 mM-sucrose was added to soln. *D* + 400 mM-glycerol just before electrical recording. Muscle fibres were clamped to − 90 mV and 800 msec steps of increasing amplitudes were delivered (Fig. 2). A slowly developing inward current can be seen with a threshold close to − 42 mV (Fig. 2*b*, *c*). The current becomes larger and faster with stronger depolarizations reaching a maximum peak amplitude at − 10 mV (Fig. 2*c–f*). It spontaneously decays and with stronger depolarization leads to a late outward potassium current

towards the end of the pulse (Fig. 2*f–h*). After substracting the leak current, the calcium current had a maximum amplitude of 65 μA cm^{-2} and inverted at + 35 mV. The maximum inward current ranged from 40 to 100 μA cm^{-2} in different fibres tested. These observations are essentially similar to those reported by Stanfield (1977) and Sánchez & Stefani (1978), indicating that the addition of glycerol to the saline does not impair calcium currents.

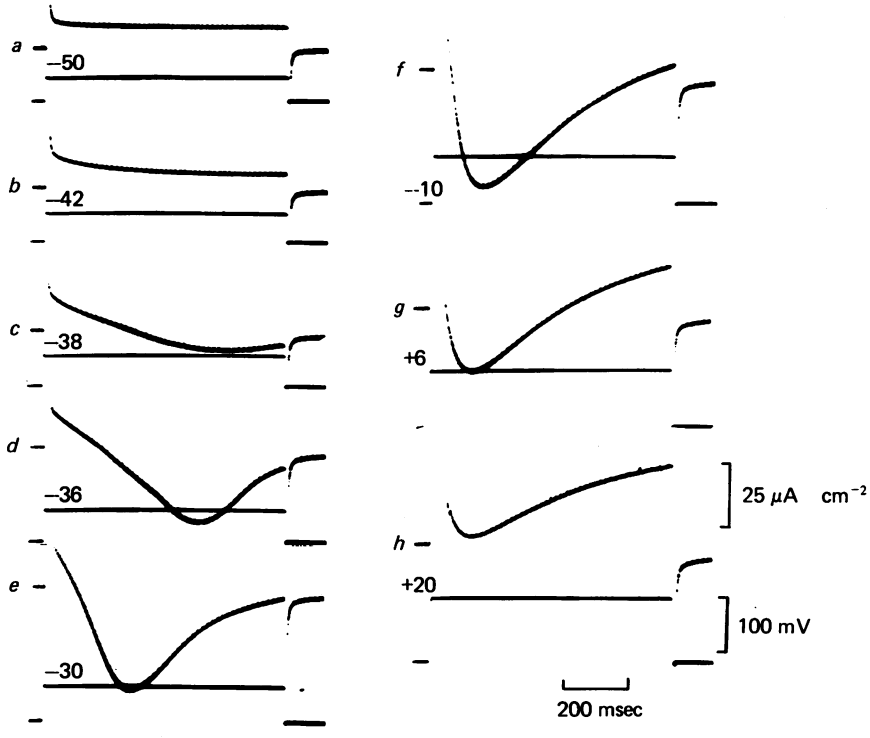

Fig. 2. Records of membrane currents during voltage clamp to different potentials (mV, numbers at left). TEA sulphate (soln. *D*) + 400 mM-glycerol + 200 mM-sucrose. E_h − 90 mV, G_m 0·20 mmho cm^{-2}, radius 18 μm, 1 mV ≡ 12 μA cm^{-2}, E_{rp} − 91 mV.

Effects of glycerol treatment on calcium spike and calcium current

Muscles were initially soaked in TEA sulphate (soln. *D*). They were then equilibrated for 1 hr with 400 mM-glycerol added, and finally transferred again to soln. *D*. Measurements were started in this solution after 1 hr of equilibration. Muscle fibres tended to be depolarized and only fibres with resting potentials (E_{rp}) more negative than − 70 mV were used.

Fig. 1*C* shows the electrical response of a treated fibre. The E_{rp} was − 78 mV and the fibre was polarized to − 85 mV with a steady current. The effective resistance was 2·1 MΩ. The transmembrane voltage increases with the current steps without any indication of regenerative activity. In the fifth step a small hump in the voltage recording can be seen which can be attributed to some remaining calcium activity. This point was further investigated measuring membrane currents.

Muscles were equilibrated for 1 hr in normal saline (soln. *A*) + 400 mM-glycerol,

then transferred to a saline with 5 mM-calcium and 5 mM-magnesium for 1 hr (soln. *B*) (Eisenberg, Howell & Vaughan, 1971). The muscles were then placed in sodium sulphate (soln. *C*) for about 10 min. At this stage, detubulated fibres showed propagating action potentials, sodium and early potassium currents of expected amplitudes with a normal membrane resistance, indicating that glycerol treatment left the surface membrane intact, as has been previously shown (Gage & Eisenberg,

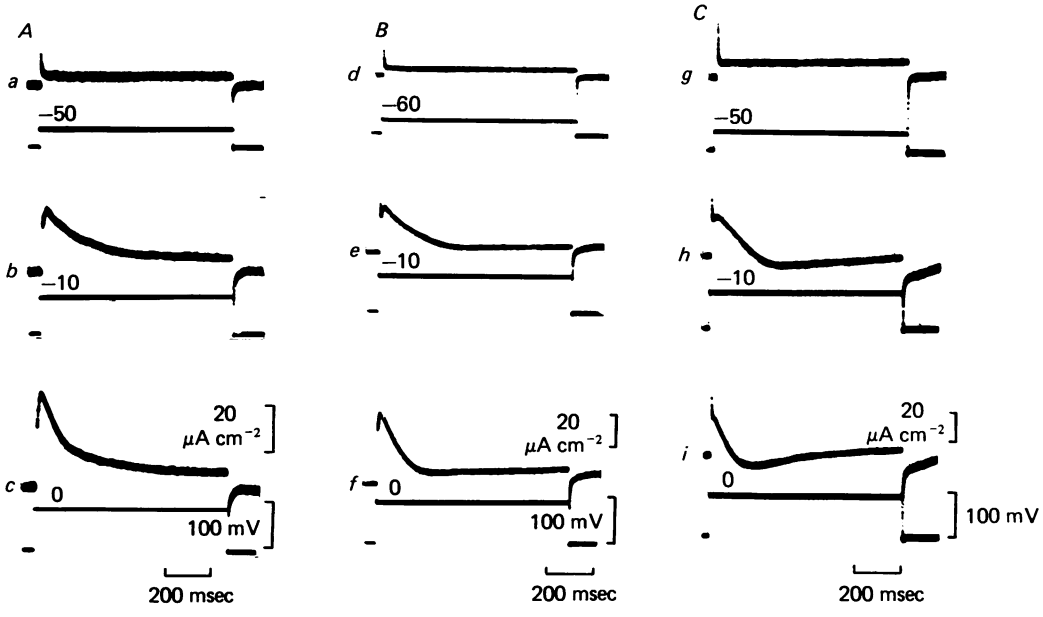

Fig. 3. Records of membrane currents during voltage pulses to different potentials (mV, numbers at left). Three muscle fibres (*A*, *B*, *C*) after glycerol treatment, showing different degrees of detubulation. Fraction of expected tubular capacitance 0·16 in *A*, 0·26 in *B* and 0·42 in *C*. Note the correlation between degree of detubulation and I_{Ca}. In *A*: $E_{rp} = -82$ mV, $G_m = 0{\cdot}09$ mmho cm^{-2}, radius 18 μm, 1 mV $\equiv$ 4·7 μA cm^{-2}; In *B*: $E_{rp} = -79$ mV, $G_m = 0{\cdot}15$ mmho cm^{-2}, radius 18 μm, 1 mV $\equiv$ 2·3 μA cm^{-2}; In *C*: $E_{rp} = -76$ mV, $G_m = 0{\cdot}22$ mmho cm^{-2}, radius 19 μm, 1 mV $\equiv$ 3·1 μA cm^{-2}.

1969*b*). The E_{rp} ranged from -65 to -92 mV with a mean value of -81 mV. To measure I_{Ca} the muscles were finally transferred to TEA sulphate (soln. *D*). The glycerol method was successful in most cases in abolishing contraction when short pulses (5 msec) were delivered. However, to detect calcium current, long pulses ($\simeq$ 1 sec) were necessary. These occasionally produced local contractions during strong depolarizations, dislodging micro-electrodes and producing gross mechanical artifacts. Successful recordings were obtained in sixteen fibres from eight muscles.

Fig. 3 shows currents in three detubulated fibres (*A*, *B* and *C*) from different muscles. In fibre *A* there is no indication of I_{Ca}. There is an initial outward potassium current which reaches a peak (*b*, *c*) and inactivates almost exponentially with a time constant of 130 msec (*c*). In fibres *B* and *C*, the initial potassium current decays steeply due to a remaining ingoing calcium current which clearly turned inward in fibre *C* (*h*–*i*). In these fibres, the late potassium current, which normally follows the calcium current (Fig. 2*f*–*h*) was not detected. In conclusion, calcium spikes and

currents are greatly reduced or abolished in detubulated muscle fibres. A similar finding was reported by Potreau & Raymond (1978).

Calcium current and electrical properties in detubulated fibres

Fibres in normal saline had a total capacitance per unit surface (C_m) of $7{\cdot}0 \pm 0{\cdot}4$ μF cm^{-2} (11). C_m is the sum of the surface capacitance (C_s) (0·9 μF cm^{-2}) in parallel with the tubular capacitance (C_t) (Hodgkin & Najakima, 1972). The tubular capacitance (C_t) can be directly obtained from $C_m - C_s$, and it can be calculated as if the fibre were intact (expected tubular capacitance, C'_t), from the fibre electrical radius and the normal volume capacitance ($2{\cdot}7 \times 10^3$ μF cm^{-3}; Schneider, 1970). Thus, the ratio C_t/C'_t is the fraction of expected tubular capacitance (f.e.t.c.) which is an indication of the remaining electrically connected tubules with the surface membrane (Chandler *et al.* 1976). As expected, in normal fibres this fraction was close to 1 ($1{\cdot}01 \pm 0{\cdot}07$ (11)). In detubulated fibres C_m was $4{\cdot}9 \pm 0{\cdot}3$ μF cm^{-2} (39) and the fraction of expected tubular capacitance was $0{\cdot}43 \pm 0{\cdot}03$ (39). Similar values of capacitance and capacitance fraction in detubulated fibres were obtained by Chandler *et al.* (1976). The membrane conductance per unit surface in TEA sulphate (soln. *D*) was $0{\cdot}10 \pm 0{\cdot}01$ mmho cm^{-2} (9) for normal fibres and $0{\cdot}15 \pm 0{\cdot}02$ mmho cm^{-2} (9) for detubulated fibres.

Further information about the localization of I_{Ca} was then obtained by comparing in each fibre the residual maximal inward I_{Ca} and the f.e.t.c. (Chandler *et al.* 1976). For example, in Fig. 3, fibres *A*, *B* and *C* had a f.e.t.c. of 0·16, 0·26 and 0·42 respectively. We can see that the fibres with larger f.e.t.c. had larger I_{Ca}. Maximum calcium currents were measured from *I–V* curves after leak substraction, and compared with the fraction of expected capacitance. Fig. 4 shows the voltage–current relation in fibre *B* of Fig. 3. Curve *a* is the leak current, curve *b* represents the value of the peak outward current, and the points on curve *c* are the values of the current from the base line to the peak ingoing current. In this fibre the membrane current did not turn inward, but after substracting the leak current, a remaining calcium current of about 10 μA cm^{-2} was measured. This value is somewhat underestimated because of the unblocked potassium currents (Sánchez & Stefani, 1978). Fig. 5 shows the relation between the residual I_{Ca} and the degree of electric discontinuity between the transverse tubular system and the surface as a fraction of the expected capacitance. As reported by Chandler *et al.* (1976), there is a great variability of the glycerol treatment on the degree of detubulation on different fibres. Since the fibres plotted in Fig. 5 had comparable radii (25 ± 1 μm (16)), we would expect a linear relation between I_{Ca} and the capacitance fraction (f.e.t.c.) if the density of calcium channels were constant fibre to fibre, and unaffected by glycerol treatment. The points can be fitted by the least-squares method with a linear function:

$$I_{Ca} = (-5 \pm 10)\ \mu\text{A cm}^{-2} + (70 \pm 20)\ \mu\text{A cm}^{-2} \times \text{f.e.t.c.}$$

The correlation coefficient was 0·84 with a confidence interval for the parameters of 95%. The good linear correlation between I_{Ca} and f.e.t.c. indicates that most of this current is located in the transverse tubular system. In these experiments we could not discriminate any I_{Ca} arising from the surface membrane since, for f.e.t.c. = 0, the *y* intercept is not significantly different from 0. A small contribution of a surface I_{Ca} would have remained undetected because of the experimental

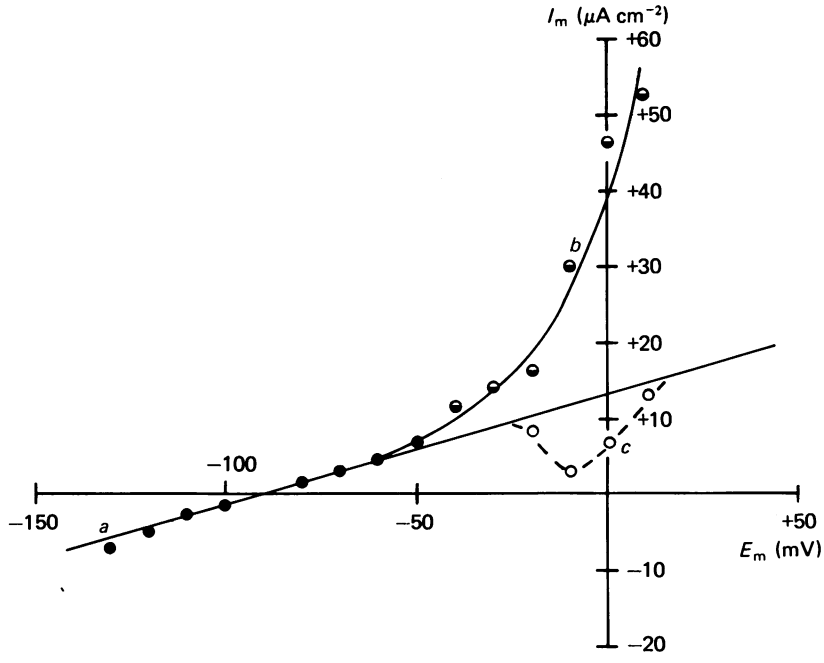

Fig. 4. Relation between membrane current and membrane potential of fibre *B* in Fig. 3. Curve *a* (●), subthreshold steady-state currents; curve *b* (◒) peak outward currents and curve *c* (○), peak ingoing currents.

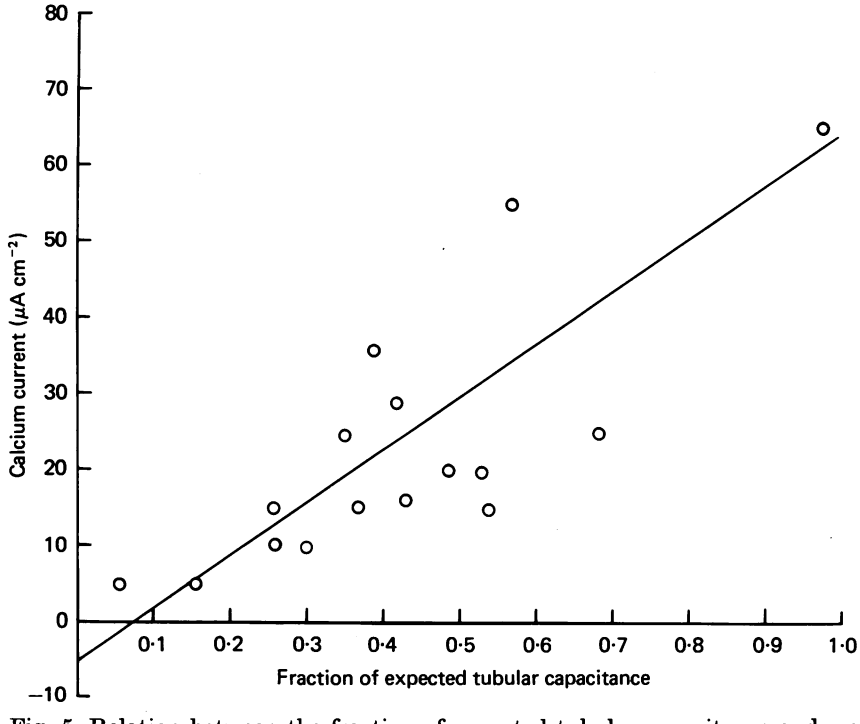

Fig. 5. Relation between the fraction of expected tubular capacitance and maximum calcium current measurement during membrane potentials from −10 to +10 mV. The points were fitted with a straight line by the least-squares method with correlation coefficient of 0·84.

errors and the uncertainty of the I_{Ca} measurement due to opposing non linear potassium currents. The I_{Ca} value extrapolated for a f.e.t.c. of 1, is about 65 μA cm^{-2}, which is close to the average calcium current recorded in untreated fibres (81 ± 10 μA cm^{-2} (14)) (Sánchez & Stefani, 1978).

DISCUSSION

The main conclusion of the present results is that I_{Ca} in muscle is located in the transverse tubular system. This view is supported by the diminution of calcium spike and current in detubulated muscle fibres and by the linear correlation between I_{Ca} and f.e.t.c., which is a measure of electrical discontinuity between the transverse tubular system and the surface membrane. These results albeit cannot rule out a small I_{Ca} located in the surface membrane, due to the errors involved in measuring residual I_{Ca}, mainly because of outward potassium currents. The tubular localization of I_{Ca} is supported by the lack of a direct deleterious effect of glycerol, since calcium spikes and I_{Ca} of normal amplitudes were recorded in TEA sulphate + 400 mM-glycerol. In keeping with this idea, the extrapolated value of I_{Ca} for fibres with f.e.t.c. = 1 was close to the value expected for normal fibres.

Apparently, this is not the case for charge movement, where a direct effect of glycerol treatment was proposed on the basis that the treatment disrupted 0·7–0·8 of the charge movement but only 0·4 of the tubules (Chandler *et al.* 1976).

Inward calcium currents are followed by slowly developing potassium currents (Fig. 2*g*, *h*). These depend on the preceding I_{Ca}, since they disappear when I_{Ca} is blocked; thus it was thought that this potassium conductance is activated by a calcium influx (Meech & Standen, 1975; Fink & Lüttgau, 1976; Sánchez & Stefani, 1978). Although the late potassium current was absent after detubulation, we cannot distinguish between a tubular or surface localization since the effect may be due to the reduction of I_{Ca}.

Another interesting point is whether intratubular calcium content is sufficient to account for the measured calcium entry. The calculation has been done as follows. We have assumed that all I_{Ca} was located in the transverse tubular system, that up to the time of peak I_{Ca} (*ca.* 200 msec) the tubular space was not refilled with calcium from the external solution, and that the finite pool of 10 mM-calcium sulphate is rapidly ionized to 1 mM-calcium. The second assumption seems reasonable for a fibre with a 30 μm radius, since the half time for potassium outflow from the transverse tubular system is 700 msec (Kirsch, Nichols & Nakajima, 1977). By integrating I_{Ca} up to the time of peak amplitude (Fig. 1*f* of Sánchez & Stefani, 1978), the total calcium entry per unit external surface was 1×10^{-10} mole cm^{-2}. The amount of calcium in the transverse tubular system per unit external surface, calculated using 30 μm for the fibre radius and 0·023 for the tubular system's fraction volume in hypertonic sucrose solutions (Freygang, Goldstein, Hellam & Peachey, 1964; Peachey & Schild, 1968), is 7×10^{-10} mole cm^{-2}. In view of these calculations it seems reasonable to suggest that during I_{Ca} a reduction of the intratubular calcium concentration occurs, and that this reduction should be considered when analysing the time course of the current.

The tubular localization of the I_{Ca} is compatible with some role during muscle

contraction. In view of the slow time course of I_{Ca}, it seems unlikely that it would be activated during a twitch, albeit it may play a role in tension evoked by maintained electrical activity such as potassium contractures or tetanus. In fact, the amplitude and time course of potassium contractures are reduced after external calcium removal (Stefani & Chiarandini, 1973; Lüttgau, Melzer & Spiecker, 1977), and recently a correlation was reported between inward calcium current and tension development (Potreau & Raymond, 1978). Along this line, an increase in calcium content of the sarcoplasmic reticulum has been found in fatigued muscle after tetanic stimulation (González Serratos, Somlyo, McClellan, Shuman, Borrero & Somlyo, 1978). Also, twitches are potentiated after either a potassium contracture or a tetanic stimulation (Ramsay & Street, 1941; Chiarandini & Stefani, 1974). It may be possible that the I_{Ca} activated during maintained electrical activity may load the sarcoplasmic reticulum with calcium, making more calcium available for release during depolarization. Alternatively, the mobilization of the recently described charge movement associated with excitation–contraction coupling (Schneider & Chandler, 1973; Adrian & Almers, 1976; see Almers, 1978) may be related in some way to calcium entry.

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