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EFFECT OF CALCIUM WITHDRAWAL ON MECHANICAL THRESHOLD IN SKELETAL MUSCLE FIBRES OF THE FROG

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SUMMARY

1. Voltage-clamp experiments were performed on frog skeletal muscle fibres using two intracellular micro-electrodes. The threshold for the Na current and the strength-duration curve for mechanical threshold were determined.

2. The change in threshold for the Na current was studied as a function of the external Ca and Mg concentrations which ranged from 0·1 to 50 mM.

3. The resting potential, effective resistance and threshold for the Na current were unchanged when 1·8 mM-Ca was replaced by 3 mM-Mg, indicating that the surface potential and the electrical properties of the fibres were not modified. The addition of 5 mM-EGTA did not affect these parameters.

4. In *Ca-free* saline (3 mM-Mg and 5 mM-EGTA) the mechanical threshold was significantly increased for short pulses ($\leqslant$ 20 msec). In isolated single muscle fibres this effect was observed shortly after applying the *Ca-free* saline, and was rapidly reversed upon the return to control saline.

5. In *isotonic EGTA* (85 mM-ETGA) the muscle fibres were depolarized and were unable to contract even if they were hyperpolarized to -90 mV for 12 min prior to stimulation. If 3 mM-Mg was added, most fibres contracted locally.

6. In single muscle fibres caffeine contractures were unmodified after a 30 min exposure to *Ca-free* saline.

7. It can be concluded that external Ca withdrawal impairs Ca release from the sarcoplasmic reticulum and that external Ca is not essential for triggering contraction.

INTRODUCTION

Evidence accumulated over the last decade indicates that in frog twitch muscle fibres the main source of Ca involved in contraction is the sarcoplasmic reticulum, while extracellular Ca is not involved directly in contractility (Lüttgau & Glitsch, 1976; Ebashi, 1976; Endo, 1977). This concept has received strong support from the finding that twitches can be elicited *in vitro* when muscles are exposed to salines with

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a very low Ca concentration (less than 10^{-8} M) obtained by adding 1–3 mM of the Ca chelator ethyleneglycol-bis (β-aminoethylether)-N,N'-tetraacetic acid (EGTA) (Armstrong, Bezanilla & Horowicz, 1972; Sandow, Pagala & Sphicas, 1975; Blinks, Rüdel & Taylor, 1978). However, the conclusion that extracellular Ca is not involved directly in contractility has been challenged recently by Barrett & Barrett (1978), who reported that contraction is blocked when the concentration of chelator in the saline is increased to about 85 mM.

Potassium contractures, on the other hand, have been shown to be more sensitive than twitches to changes in external Ca. In salines with Ca omitted, their time course is shortened (Lüttgau, 1963; Caputo & Gimenez, 1967) and when 1 mM-EGTA is added their amplitude is also reduced (Stefani & Chiarandini, 1973; Lüttgau, Melzer & Spiecker, 1977).

Exposures to low Ca concentrations, such as those used in the aforementioned experiments, may affect muscle contractility directly by reducing inward Ca driving force and indirectly by changing membrane electrical properties, such as the surface potential of the fibres. Ca and other divalent cations screen or bind to negatively charged groups in the membrane, and changes in their extracellular concentration can modify the surface potential and the electric field within the membrane. This, in turn, may modify the relationship between membrane potential and excitability threshold (A. F. Huxley in Frankenhaeuser & Hodgkin, 1957; Hille, Woodhull & Shapiro, 1975) and membrane potential and mechanical threshold (Lüttgau, 1963; Frankenhaeuser & Lännergren, 1967; Costantin, 1968; Dörrscheidt-Käfer, 1976).

To discriminate a direct from and indirect influence of extracellular Ca on contractility, the present experiments were performed under ionic conditions in which the electrical properties of the fibres, particularly the surface potential, remained constant. It was found that a drastic reduction of extracellular Ca increased the mechanical threshold for short pulses but did not block contraction. A partial report of these findings has been presented (Chiarandini, Stefani & Sanchez, 1979).

METHODS

Experiments were performed on muscles of cold-adapted *Rana temporaria*. Cutaneous pectoris and tensor fascia lata were used as whole muscle preparations. Single muscle fibres were dissected from the tibialis anterior.

Voltage clamp

A two micro-electrode voltage-clamp technique similar to that described by Costantin (1968) was used to control the membrane potential of the fibres to measure the thresholds of contraction and Na current. The feed-back amplifier consisted of the vertical amplifier of a Tektronix 502 A oscilloscope set at a gain of 50–500 followed by a high output voltage (± 100 V) transistor amplifier which had a gain of 20. The bath was virtually grounded via a current–voltage transducer which was used to measure the injected current. The whole system had a time constant of about 100 μsec. Command pulses were rounded off to a time constant of about 200 μsec.

The membrane potential was measured differentially between the intracellular micro-electrode and a reference external micro-electrode which was positioned near the surface of the cell. Intracellular micro-electrodes had resistances from 8 to 20 MΩ and were filled with 4 M-Na acetate. They were shielded to within 100 μm of their tip with conductive silver paint and insulated with a fast curing resin. The shields of the current and the voltage micro-electrodes were connected to earth and to the drive shield of the voltage follower, respectively. The micro-

electrodes were positioned one in front of the other, perpendicular to the length of the fibre. Since the current electrode behaves as a point source of current, it was inserted deep into the muscle fibre to assure a more uniform membrane potential (Adrian, Costantin & Peachey, 1969). Muscle fibres were voltage clamped to a holding potential of −90 mV. The effective resistance (R_{eff}) was calculated from the ratio between a 10 mV negative voltage step and the corresponding injected current.

Sodium current threshold

Thirty msec command pulses with amplitudes increasing by 1 mV steps were used. The increase of the command pulses produced a proportional increase in the leakage current up to a certain level of depolarization at which it did not increase further. A further depolarization brought about a small but clearly evident ingoing Na current. Threshold was taken as the membrane potential at which the leakage current stopped increasing, less 1 mV.

Mechanical threshold

Mechanical threshold was optically determined in whole muscles and in isolated single muscle fibres. Fibres were visualized under a compound microscope with 10 × and 32 × objectives which gave a final magnification of 200 × or 640 ×, providing good resolution of striations. Na current was abolished with 5×10^{-7} M-tetrodotoxin. Subthreshold pulses were applied and their strength was increased stepwise with one or two stimuli at each step until a localized shortening of sarcomeres became detectable near the voltage micro-electrode, which was in the optical plane. The pulse amplitude was then decreased until the contraction disappeared and this membrane potential was taken as the threshold. The threshold was first determined for a 200 msec pulse and thereafter for pulses of progressively decreasing duration. Pulses were applied at a frequency of 1 every 2 or 3 sec. Measurements were accurate to within 2 mV.

Mechanical recording

Single fibres from tibialis anterior were isolated with stubs of tendon at the ends and transferred to a small flush-through chamber of 0·25 ml. capacity. The fibre was suspended between two fine stainless steel hooks inserted into the tendons, one attached to the chamber and the other to the lever of an isometric transducer RCA 5734 connected to a pen recorder.

Solutions

Control saline contained (mM): NaCl 115, KCl 2·5, $CaCl_2$ 1·8. To study changes of Na current threshold, salines with different concentrations of Ca or Mg were made by adding the required amount of solid $CaCl_2$ or $MgCl_2$ to a solution with 115 mM-NaCl and 2·5 mM-KCl. The calculated Mg contamination was *ca.* 10 μM. A *low-Ca* saline was prepared by omitting Ca from the control saline and adding 3 mM-Mg. It had an estimated Ca concentration of 10–50 μM which was due to Ca contaminant in the chemicals (Stefani & Chiarandini, 1973). A *Ca-free* saline was prepared by adding to the *low-Ca* saline 5 mM-Na_2EGTA. *Isotonic EGTA* saline contained 85 mM-Na_2EGTA, 2·5 mM-KCl and, in some cases, 3 mM-$MgCl_2$ (*isotonic EGTA-Mg* saline). All solutions were buffered to pH 7·4 with 2 mM-imidazole Cl. Tetrodotoxin and caffeine (Sigma Chemical Co., St. Louis, Mo.) were added from concentrated aqueous solutions. Whole muscles were equilibrated in the test solution with constant stirring for about 30 min, unless otherwise stated. Single muscle fibres were exposed to a continuous flow of the solution at a rate of 2·5 ml./min. Experiments on mechanical threshold were carried out at 3·0 °C. The temperature of the solution was controlled in the case of whole muscles with a Peltier cooling device wired to a temperature controller, and in the case of single fibres by precooling the solution. Special care was taken to maintain the temperature of the bathing solution constant since mechanical threshold for short pulses is highly temperature dependent. Caffeine contractures and some experiments on Na current threshold were performed at room temperature (22–24 °C). Values are given as mean ± standard error (S.E.) of the mean, with the number of observations in parentheses.

RESULTS

Influence of divalent cations on electrical parameters

As mentioned in the Introduction, changes in external divalent cations may have a marked influence on the surface potential of muscle fibres, which in turn may modify the relation between membrane potential and tension. An initial set of experiments was performed to determine what concentration of Mg should be present in the saline to maintain the surface potential constant in the absence of Ca. Since the surface

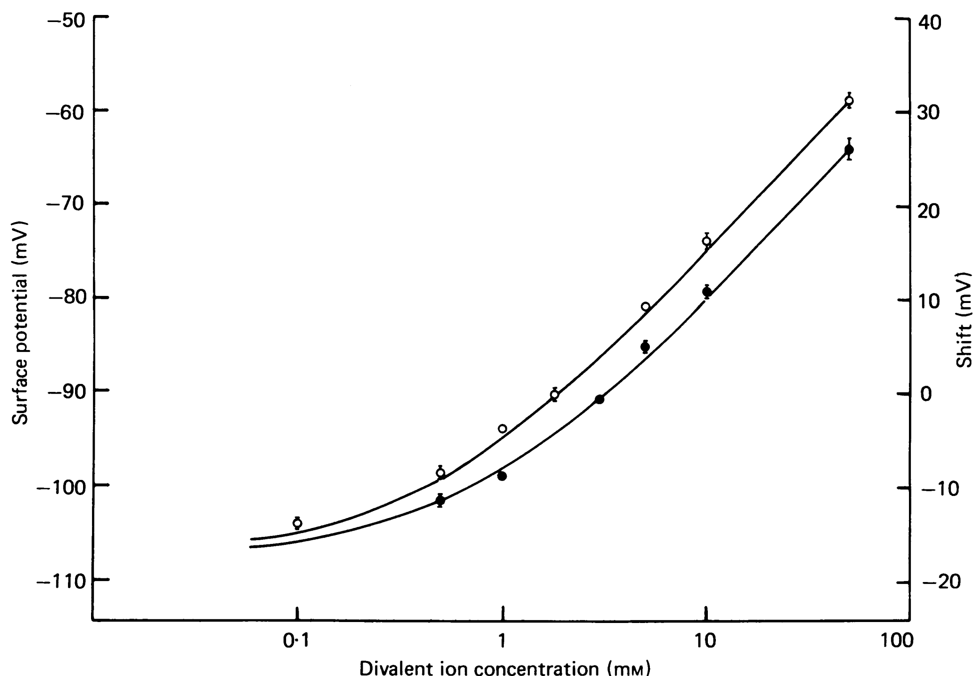

Fig. 1. Relationship between Na current threshold and extracellular Ca (○) or Mg (●) concentrations. Threshold changes (right scale) are referred to the threshold measured with 1·8 mM-Ca. The smooth curves and the magnitude of the surface potential (left scale) were obtained from the model I of Hille *et al.* (1975). Symbols are the mean ± S.E. of five to ten fibres. Temperature: 22 °C.

potential cannot be measured directly, the threshold of Na current was determined on the assumption that any changes of the surface potential brought about by a modification in the external divalent cation concentration will be reflected as a corresponding change in the threshold for the Na current (Hille *et al.* 1975).

Fig. 1 shows the observed shifts (right scale) of Na current threshold as a function of the concentration of Ca (○) (0·1–50 mM) or Mg (●) (0·5–50 mM) in several muscle fibres. These shifts are expressed as deviations from the threshold value in 1·8 mM-Ca. It is clear from Fig. 1 that when 1·8 mM-Ca is replaced by 3 mM-Mg no change in the threshold for Na current is observed. The experimental points are fitted closely by the theoretical curves which are reproduced from Fig. 1 of Hille *et al.* (1975).

These curves correspond to the model I of these authors which was proposed to account for the shifts in the voltage dependence of Na permeability in nerve fibres as a function of the extracellular ionic concentrations. This model has been applied successfully to skeletal muscle to explain related shifts in Na permeability and contraction threshold for long pulses (Campbell & Hille, 1976; Dörrscheidt-Käfer, 1976). The good agreement between our results and the theoretical values indicates that the threshold for Na current is an adequate measure of the surface potential.

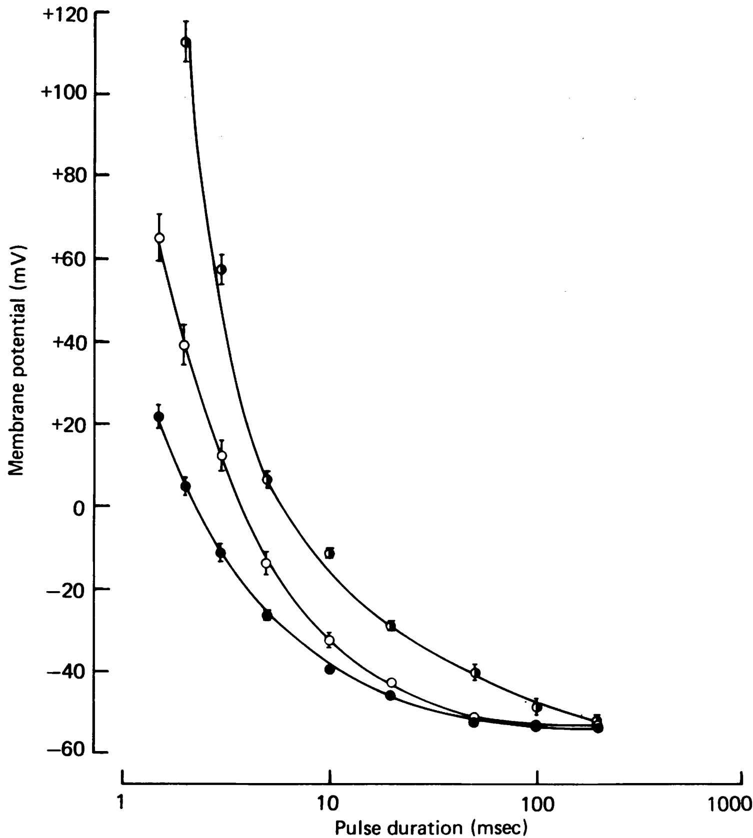

Fig. 2. Strength–duration curve of mechanical threshold in normal saline (●), *Ca-free* saline (○) and *isotonic EGTA-Mg* saline (◑). Symbols are the mean ± S.E. of forty-one, sixteen and five fibres respectively. Temperature: 3·0 °C.

Since most of the experiments on mechanical threshold to be detailed below involved testing the effects of a saline with Ca omitted and containing 3 mM-Mg and 5 mM-EGTA (*Ca-free* saline) a second set of experiments was performed to ascertain if this saline affected the threshold for Na current, the resting potential (E_{rp}) and the R_{eff} of the fibres. It was found that none of these parameters was changed by *Ca-free* saline. They were respectively at 3·0 °C: $-58·6 \pm 0·9$ mV (seven fibres), $-88·5 \pm 1·1$ mV (fifteen) and $0·74 \pm 0·07$ MΩ (fifteen) in control saline, and $-57·5 \pm 1·1$ mV (six), $-86·7 \pm 1·2$ mV (nine) and $0·68 \pm 0·09$ MΩ (nine) in *Ca-free* saline.

Effect of Ca-free and low-Ca salines on mechanical threshold

To investigate the possible role of external Ca in excitation–contraction coupling we studied the effects of *Ca-free* and *low-Ca* salines (see Methods) on the strength–duration curve of mechanical threshold. Fig. 2 shows the effect of *Ca-free* saline in

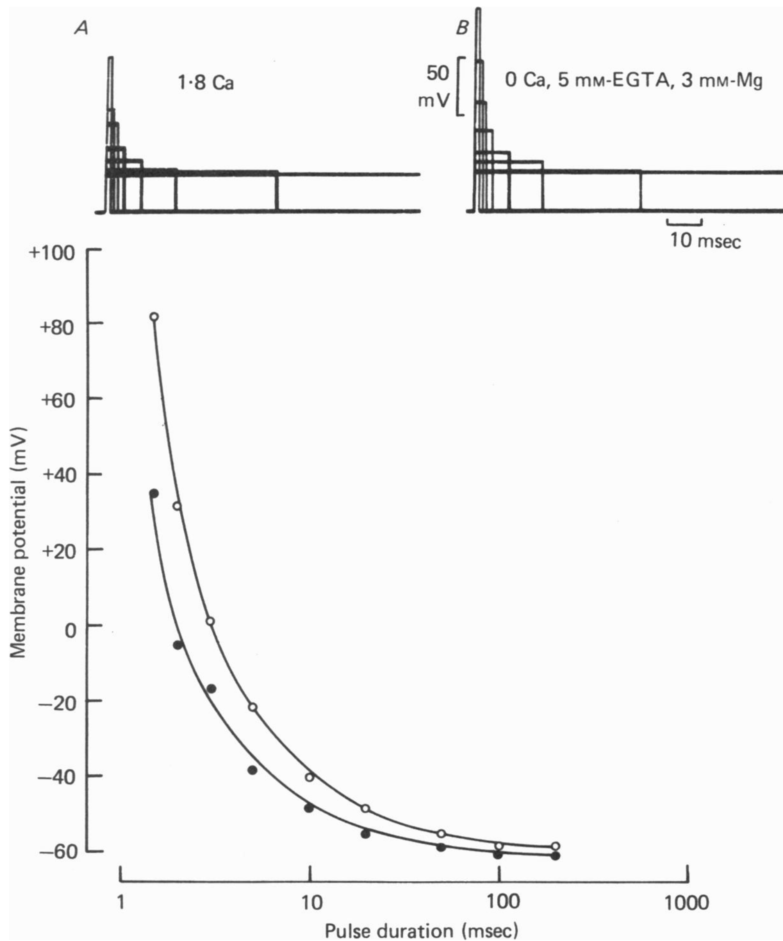

Fig. 3. Strength–duration curves of mechanical threshold in an isolated single fibre in normal (●) and in *Ca-free* saline (○). *A* and *B* show records of threshold pulses for these curves. E_{rp} − 87 mV; R_{eff} 0·40 MΩ (●) and 0·39 MΩ (○). Temperature 3·0 °C. The records were reinforced.

several fibres in whole muscle preparations. The curve obtained after exposing the muscles to *Ca-free* saline constantly stirred for 30 min deviates from the control one for short duration pulses. The difference between the two curves is significant at $P < 0{\cdot}05$ for 20 msec pulses and $P < 0{\cdot}001$ for 10 msec or shorter pulses. The increase of mechanical threshold was observed only if the muscle was exposed to the *Ca-free* saline with stirring. In comparable experiments, performed without agitation, the two strength–duration curves were the same.

In addition to stirring it was found necessary to reduce drastically the Ca concentration of the saline by adding EGTA to detect a change in the mechanical threshold. The strength–duration curves obtained in control and *low-Ca* salines were the same for five fibres tested in each solution.

The fact that stirring the *Ca-free* saline is essential to demonstrate a threshold increase suggests that the exchange of the extracellular Ca with the bulk solution is slow. To minimize this diffusional delay we carried out experiments in isolated single muscle fibres. Fig. 3 shows strength–duration curves obtained in one fibre in control and *Ca-free* salines. As in the case of whole muscles, there is an increase in the mechanical threshold for short pulses. This effect was observed about 2 min after starting the change of solution, which is the time necessary to replace completely the bath solution. It was reversible upon returning the fibre to control saline. Similar results were obtained in two other single fibres.

Effect of isotonic EGTA on electrical properties and mechanical activation

Barrett & Barrett (1978) have reported that muscle fibres immersed in salines containing an isotonic concentration of EGTA or citrate (*i.e.* about 85 mM) do not contract locally when action potentials are evoked by intracellular stimulation. We confirmed this observation in five isolated muscle fibres which were exposed for 1 h to *isotonic EGTA* saline. Under these conditions the fibres were depolarized and had an E_{rp} of $-13{\cdot}8 \pm 1{\cdot}1$ mV and R_{eff} of $0{\cdot}80 \pm 0{\cdot}20$ MΩ. The fibres maintained the membrane resistance throughout the experiment and they were easily hyperpolarized with a steady inward current. No contraction could be detected when depolarizing pulses of 200 mV and 200 msec were applied, even if the fibres were hyperpolarized to a holding potential of -90 mV for periods of 5–12 min immediately prior to stimulation.

A different result was obtained with *isotonic EGTA-Mg* saline. In nine isolated fibres which were exposed to a continuous flow of the saline to wash out any possible Ca accumulation adjacent to them, the E_{rp} averaged $-11{\cdot}0 \pm 1{\cdot}3$ mV and the R_{eff}, $0{\cdot}90 \pm 0{\cdot}20$ MΩ. The addition of 3 mM-Mg did not prevent cell depolarization, most likely because it was partially chelated by EGTA. The fibres were voltage-clamped to -90 mV and contractility was tested with 200 mV depolarizing pulses with a duration of 5 msec. Immediately after applying the holding potential the fibres were refractory to stimulation, but about 2 min afterwards six fibres responded. The other three remained unresponsive even after 5, 8 and 10 min of hyperpolarization. In the six fibres that contracted, mechanical repriming was complete after about 5 min of hyperpolarization. During this period the mechanical threshold changed to less depolarized membrane potentials. For instance, in one fibre the mechanical threshold was 68 mV after 178 sec of hyperpolarization, 48 mV after 206 sec, 20 mV after 244 sec and 22 mV after 306 sec. Similar results were obtained in whole muscles exposed to *isotonic EGTA-Mg* for 1 h with constant stirring. The fibres had an E_{rp} of $-21{\cdot}2 \pm 2{\cdot}2$ mV (nine) and R_{eff} of $1{\cdot}50 \pm 0{\cdot}17$ MΩ (nine). All fibres showed localized tension when stimulated with pulses after restoring contractility by hyperpolarizing the fibres to -90 mV for 4–6 min. The mechanical threshold, however, was much higher than in *Ca-free* saline (Fig. 2).

Caffeine contractures

The increase of mechanical threshold observed in *Ca-free* saline could be due to a partial depletion of Ca in the sarcoplasmic reticulum. To analyse this possibility we studied the effects of Ca withdrawal on caffeine contractures. Fig. 4 shows two

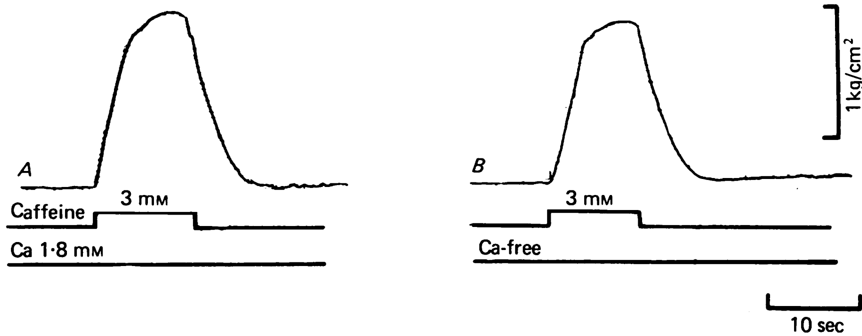

Fig. 4. Caffeine (3 mM) contractures in an isolated single fibre from tibialis anterior muscle. The fibre was exposed to *Ca-free* solution for 30 min before inducing tension (*B*) and then exposed to control saline for 20 min before being tested (*A*). Temperature: 22 °C.

caffeine contractures, one evoked in control saline (*left*) and the other after a 30 min exposure to *Ca-free* saline (*right*). The amplitude and time course of the two contractures are quite similar, suggesting that the sarcoplasmic reticulum is not depleted of Ca. Similar results were obtained in four other fibres.

DISCUSSION

The main finding of this paper is that Ca withdrawal increases significantly the mechanical threshold for pulses of 20 msec or shorter. The mechanism underlying this effect is unknown but some insights into it may be gained by applying the first-order kinetics model used by Adrian, Chandler & Hodgkin (1969) to analyse changes in myoplasmic Ca during a depolarizing pulse. When myoplasmic Ca concentration is neglected on account of its extremely low value (Ebashi & Endo, 1968; Hellam & Podolsky, 1969), this model predicts that for very short pulses, the process of Ca release from the sarcoplasmic reticulum prevails over the Ca uptake. Therefore, it can be postulated that the increase in mechanical threshold reported here might be due to a reduction of the amount of Ca released from the sarcoplasmic reticulum. Such an effect on Ca release could be the result of (*a*) a depletion of Ca in the sarcoplasmic reticulum; (*b*) a decrease of the radial spread of potential along the transverse tubules due to a shortening of the tubular space constant (λ_T); or (*c*) a reduction in the efficiency of the mechanism whereby tubular depolarization triggers release of Ca.

A depletion of Ca in the sarcoplasmic reticulum can be ruled out for the following reasons. Calcium withdrawal did not affect the mechanical threshold for pulses longer than 20 msec or the tensions evoked by a submaximal concentration of caffeine. This alkaloid evokes tension by acting directly on the sarcoplasmic reticulum, without involving membrane depolarization (Weber & Herz, 1968; Lüttgau &

Oetliker, 1968). Furthermore, in single fibres, the increase of mechanical threshold was observed shortly after the change to *Ca-free* saline, and it would be extremely unlikely that a partial depletion of the sarcoplasmic reticulum could have occurred so rapidly (Curtis, 1970).

A decrease of the radial spread of potential could increase the mechanical threshold. This may occur if *Ca-free* saline brought about an increase of the tubular membrane conductance (G_T), which would cause a reduction of λ_T. This possibility is unlikely for two reasons. Firstly, since G_T is about 50 % of the total membrane conductance (Hodgkin & Nakajima, 1972; Adrian & Peachey, 1973), an increase in G_T due to the *Ca-free* saline should have been reflected as a detectable reduction of R_{eff}, which was not observed. Secondly, the radial spread of depolarization down the tubules in a normal fibre is not steeply dependent upon λ_T. For example, according to the model of Adrian, Chandler & Hodgkin (1969), the tubular potential at the centre of a 40 μm fibre at the end of a 1·5 msec voltage-clamp step is 86 % of the potential at the surface. Even if λ_T were reduced to 50 % of its control value by the *Ca-free* solution (requiring G_T to increase fourfold), the tubular potential would be reduced only to 78 % of the potential at the surface at the end of a 1·5 msec pulse. In conclusion, the observed changes in mechanical threshold shown in Figs. 2 and 3 cannot be explained by changes of the radial spread of depolarization down the tubules.

Since neither a depletion of the Ca store in the sarcoplasmic reticulum nor a decrease of λ_T appears to explain the observed increase of mechanical threshold, it seems reasonable to postulate that Ca withdrawal affects the mechanism whereby tubular depolarization induces Ca release from the sarcoplasmic reticulum. However, the present results indicate that external Ca does not have an essential role in this process, since localized tensions could be evoked by depolarizing pulses after prolonged exposures to *Ca-free* and *isotonic EGTA-Mg* salines. Furthermore, contractions could still be elicited after the inside of the fibre had been made sufficiently positive to prevent external Ca entry (Miledi, Parker & Schalow, 1977). The failure of Barrett & Barrett (1978) to evoke tension in isotonic EGTA seems to be related to the lack of any divalent cation in their saline and not specifically to Ca lack, as our positive findings with *isotonic EGTA-Mg* indicate. Similarly, it has been recently reported that muscle fibres can contract in isotonic EGTA with 5 mM-free-Mg (Lüttgau & Spiecker, 1979). In the light of these observations it seems reasonable to abandon the hypothesis that an influx of extracellular Ca is an essential step to trigger Ca release from the sarcoplasmic reticulum (Bianchi & Bolton, 1967; Ford & Podolsky, 1970; Chiarandini & Stefani, 1973; Stefani & Chiarandini, 1973).

There is increasing evidence that a voltage-dependent movement of intramembraneous charges relates membrane depolarization to Ca release (Schneider & Chandler, 1973; for review see Almers, 1978). In a speculative manner, it can be postulated that to some extent charge movement may depend on extracellular Ca, either because Ca enters into the fibre (Sanchez & Stefani, 1978) and binds to negative charges at the inner side of the cell membrane modifying the local electric field (Beaty & Stefani, 1976) or because charge movement depends on a membrane bound Ca which is displaced by EGTA.

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