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SOME EFFECTS OF REMOVAL OF EXTERNAL CALCIUM ON PIG STRIATED MUSCLE

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SUMMARY

1. Bundles of about 800 cells from the m. thyreopharyngicus of pigs were used to measure activation and inactivation during contracture by K^+ depolarization.

2. When $[Ca^{2+}]$ in the medium was lowered to less than 5×10^{-10} M for 3 min (replacing Ca^{2+} by Mg^{2+}) the activation occurred at the same $[K^+]$ in the medium as in normal solution (3 mM-Ca^{2+}) but inactivation was shifted to lower external $[K^+]$. The absolute value of this shift in terms of membrane potential is uncertain, because $[K^+]$ at the cell surface is unknown.

3. Exposure for 4 min to Ca^{2+}-free medium (Ca^{2+} being replaced by Mg^{2+}) had no effect on contractility tested after a subsequent rest of 22–25 min in normal solution ($[Ca^{2+}] = 2$ mM). However, if the muscle underwent one maximal K^+ contracture in Ca^{2+}-free medium the response (tetanus or K^+ contracture) after the same interval in normal solution was strongly reduced, although the membrane potential recovered fully. K^+ contractures in normal solution could be repeated without loss of contractile force.

4. A K^+ contracture in Ca^{2+}-free medium had very little effect on the response to caffeine, tested after 25 min in normal solution.

5. It seems that Ca^{2+} is lost into Ca^{2+}-free medium only during depolarization, from a site which is not accessible to Ca^{2+} from outside at the resting membrane potential, or from inside at any membrane potential. This site might be located inside the transverse-tubular membrane and, when loaded with Ca^{2+}, might represent the positive group of the model of Chandler, Rakowski & Schneider (1976*b*), the movement of which during depolarization activates and inactivates the Ca^{2+} release from the sarcoplasmic reticulum.

INTRODUCTION

There is agreement that the transient contractile response to maintained depolarization in skeletal muscle is due to the superimposition of a rapid activation process and an inactivation of slower time course (Hodgkin & Horowicz, 1960), and that activation reflects the opening and inactivation reflects the closure of Ca^{2+} channels in the membrane of the terminal cysternae of the sarcoplasmic reticulum (s.r.). Lüttgau & Spiecker (1979) have made the important observation that in Ca^{2+}-free solution less depolarization is required for inactivation than in normal Ringer

solution but that activation is not affected by removal of Ca^{2+} (provided that Ca^{2+} is replaced by Mg^{2+}).

If altering the surface potential is avoided (by keeping the concentration of divalent cations constant (McLaughlin, Szabo & Eisenmann, 1971; Lüttgau & Spiecker, 1979)) the only way in which a potential change across a membrane can be imagined to change the membrane's performance is by movement of charged groups inside the membrane. 'Gating' of this sort at the transverse-tubular membrane (Almers, 1975; Adrian & Almers, 1976; Adrian, Chandler & Rakowski, 1976; Chandler, Rakowski & Schneider, 1976*a*, *b*) seems to be implied by either of the current theories of excitation–contraction coupling. Regardless of whether depolarization of the transverse-tubular membrane operates the Ca^{2+} gate of the s.r. by mechanical means (Chandler *et al.* 1976*b*) or by causing current flow across the terminal cysternae membrane (Miller, 1978; Mathias, Levis & Eisenberg, 1981), charge movement in the transverse-tubular membrane must be involved. In the first case it moves a 'plug' in the adjacent terminal cysternae membrane and in the second case it increases the ionic permeability at the junction between the transverse-tubular membrane and the terminal cysternae membrane. If Ca^{2+} deprivation affects inactivation but not activation it must interfere with some but not all of the charge movements that occur.

The different effect of Ca^{2+} deprivation on activation and inactivation observed by Lüttgau & Spiecker (1979) seems to allow two conclusions: (1) activation and inactivation do not depend on a single elementary process and (2) inactivation is not a simple consequence of activation. The experiments also show that external Ca^{2+} is not necessary for the initiation of contraction. The authors further propose that the rise in internal Ca^{2+} brought about by the slow inward Ca^{2+} current (Sanchez & Stefani, 1978; Almers & Palade, 1981; Almers, Fink & Palade, 1981) is required to keep the inactivation process from occurring at more negative potentials than those needed for activation. The claim that it is *internal* Ca^{2+} that matters is based on the fact that caffeine and dantrolene also shift the inactivation curve (in opposite direction) along the potential axis, and that the Ca^{2+}-entry blocker D-600 has an effect similar to that of Ca^{2+} deprivation.

The present experiments are concerned with the question as to whether external Ca^{2+} has to enter the intracellular space to cause the effect seen by Lüttgau & Spiecker (1979). They first confirm the finding in a mammalian striated muscle. They further show that excitation–contraction coupling fails in solutions of normal Ca^{2+} content for at least 20 min after a K^+-contracture in Ca^{2+}-free medium, notwithstanding an unimpaired effect of caffeine under the same conditions. This seems to indicate that during stimulation in Ca^{2+}-free solution Ca^{2+} is lost from a site which cannot be replenished by the slow inward Ca^{2+} current after readmission of Ca^{2+}. The idea is advanced, therefore, that this site is membrane-bound and accessible to Ca^{2+} from outside, but only during depolarization. An attempt is made to bring these observations into context with the postulated role of movable charges in excitation–contraction coupling.

TABLE 1. Composition of solutions used (mM)

	KCl	KCH_3SO_3	NaCl	$NaCH_3SO_3$	Na_2 EGTA	Ca^{2+}	Mg^{2+}	$Na^+_{tot.}$	$Cl^-_{tot.}$
D_{-40}	—	23·8	24·5	91·7	—	3	—	126·2	30·5
d_{-40}	—	23·8	24·5	86·7	2·5	—	3	126·2	30·5
D_{-32}	—	34·7	14·9	90·4	—	3	—	115·3	20·9
d_{-32}	—	34·7	14·9	85·4	2·5	—	3	115·3	20·9
D_{-29}	—	41·8	11·3	86·9	—	3	—	108·2	17·3
D_{-25}	—	50·4	8·6	81	—	3	—	99·6	14·6
d_{-25}	—	50·4	8·6	76	2·5	—	3	99·6	14·6
D_{-20}	—	60·8	5·9	73·3	—	3	—	89·2	11·9
D_{-16}	—	73·3	3·9	62·8	—	3	—	76·7	9·9
d_{-16}	—	73·3	3·9	57·8	2·5	—	3	76·7	9·9
D_{-13}	—	80·5	3·0	56·5	—	3	—	69·5	9
D_{-11}	—	88·4	2·2	49·4	—	3	—	61·6	8·2
d_{-11}	—	88·4	2·2	44·4	2·5	—	3	61·6	8·2
D_{-9}	—	97·1	1·5	41·5	—	3	—	53	7·5
D_{-6}	—	106·6	0·8	32·6	—	3	—	43·4	6·8
d_{-6}	—	106·6	0·8	27·6	2·5	—	3	43·4	6·8
D_{-4}	—	117·5	0·2	22·8	—	3	—	33	6·2
D_{-1}	—	128·5	5·6	5·9	—	3	—	21·5	5·6
D_{+1}	—	141·2	—	—	—	3	—	10	5·1
M_{+9}	—	190	—	—	—	3	—	10	—
m_{+9}	—	190	—	—	2·5	—	3	15	—
N_{-67}	4·8	—	135	—	—	2	—	145	144
n_{-67}	4·8	—	135	—	2·5	—	2	150	144

Solutions are marked by capital letters if containing Ca^{2+}, with lower case letters if Ca^{2+}-free. Subscripts indicate the membrane potential (mV) measured in the m. thyreopharyngicus in Ca^{2+}-containing solutions (see insert in Fig. 2). N and n are the 'normal' solutions; D and d, and M and m, are the depolarizing solutions. The latter are composed such that $[Na^+]+[K^+] = 150$ mM and $[K^+]\times[Cl^-] \sim 725$ mM^2. They contain 3 mM-Ca^{2+} instead of 2 mM because Na methanesulphonate weakly complexes Ca^{2+} (see text). In Ca^{2+}-free, EGTA-containing solutions Mg^{2+} replaces Ca^{2+} to keep the surface potential constant. The $[Ca^{2+}]$ in Ca^{2+}-free solutions is $< 5\times10^{-10}$ M. All solutions contained Na MOPS (10 mM) and glucose (10 mM). Ca^{2+} and Mg^{2+} were added as chlorides except in solutions D_{-1}, D_{+1}, M_{+9} and m_{+9} which contained Ca^{2+} and Mg^{2+} as methanesulphonate salt. Solution D_{+1} also contained 5·1 mM-Tris Cl in addition to the other ingredients.

METHODS

Material

Muscle bundles of $1{\cdot}4\pm0{\cdot}06$ mm diameter and 25 ± 4 mm length (mean $\pm$ S.E. of mean, $n = 15$) were prepared from the m. thyreopharyngicus of 6 month old pigs, slaughtered under CO_2 anaesthesia. The mm. thyreopharyngici insert at the linea obliqua on either side of the thyroid cartilage and meet at the back of the pharynx in a tendineous raphe. The muscles were separated at the raphe, dissected from the underlying connective tissue but not detached from the cartilage. Then a 5 mm wide strip from the edge or underside was cut in the direction of the fibres and from this the final bundle was teased with blunt instruments. The bundle was tied with cotton thread at both ends within the muscular part before the preparation was detached from the cartilage.

Since the fibre diameter was found to be approximately 50 μm, the bundles contained about 800 cells per cross-section. They were mounted vertically in a muscle bath of 20 ml kept at 37 °C, between a Perspex holder and the spring of a Grass force–displacement transducer FT 03 C. After equilibration in the bath the resting length of the bundle was adjusted until maximal tension obtained upon supramaximal stimulation. The resting load was about 1 g. Stimulation with rectangular pulses of 0·2 ms singly or at 50 Hz was from a Grass S 4 stimulator via two silver

electrodes arranged in the longitudinal direction near the middle of the bundle. The transducer signal from twitches was amplified and recorded with a Tektronix oscilloscope 502 A and that from tetani and contractures with a Grass polygraph ink-writing recorder. The bundles developed $1{\cdot}59 \pm 0{\cdot}37$ kg/cm^2 maximal (tetanic) tension ($n = 15$). The duration of a single twitch measured at half-maximal tension was $58{\cdot}6 \pm 8{\cdot}9$ ms ($n = 4$).

Bathing solutions

These are described in Table 1. All solutions were buffered to pH 7·35 (at 37 °C) with 10 mM-Na morpholinopropane sulphonate (MOPS), contained 10 mM-glucose and were equilibrated with O_2 before and during contact with the muscle. 'Normal solution' (N) contained (mM): NaCl, 135; KCl, 4·8; Na MOPS, 10; $CaCl_2$, 2. In depolarizing solutions (D and d; M and m) K^+ replaced Na^+. The sum $[Na^+]+[K^+]$ was constant = 150 mM and the product $[K^+] \times [Cl^-]$ was constant = ~ 725 mM^2; Cl^- was replaced by methanesulphonate ($CH_3SO_2O^-$). Solutions contained either 2 mM-$CaCl_2$ (N) or 3 mM-$CaCl_2$ (D and M) viz. 2 mM-$MgCl_2$ (n) or 3 mM-$MgCl_2$ (d and m) + 2·5 mM-Na EGTA ('Ca^{2+}-free'). Measurements with a Ca^{2+}-selective electrode showed that the dissociation constant of the binary complex $CaCH_3SO_2O^+$ is of the order of 0·1 M. To avoid depolarizing solutions of different total Ca^{2+} concentrations a compromise was made by using 3 mM-Ca^{2+} instead of 2 mM in $CH_3SO_2O^-$-containing solutions. $[Ca^{2+}]$ varied in these from 2·51 to 1·21 mM when $CH_3SO_2O^-$ was varied between 20 and 150 mM. Maximal contractures were elicited with 190 mM-KCH_3SO_2O, 10 mM-Na MOPS, 3 mM-$Ca(CH_3SO_2O)_2$ (solution M) (or 2·5 mM-Na EGTA + 3 mM-$Mg(CH_3SO_2O)_2$ (solution m)).

Concentrations. The concentration of potassium in the solutions was verified by emission flame photometry (EEL instrument) and of total calcium and magnesium by atomic absorption flame photometry (IL 151 instrument). Contaminating Ca^{2+} in the Ca^{2+}-free solutions was found to be $3{\cdot}2$–$13{\cdot}6 \times 10^{-6}$ M. After addition of 2·5 mM-EGTA this amounted to a free Ca^{2+} concentration of $1{\cdot}08$–$4{\cdot}7 \times 10^{-10}$ M. It was calculated by an iteration procedure taking into account the H^+, Ca^{2+}, Mg^{2+} and EGTA concentrations. The free Mg^{2+} concentration in the 'Ca^{2+}-free', 2·5 mM-EGTA, 3 mM-Mg^{2+} solutions was 2·6 mM.

The following dissociation constants for EGTA complexes were used: K_{H_1} $3{\cdot}47 \times 10^{-10}$, K_{H_2} $1{\cdot}4 \times 10^{-9}$, K_{H_3} $2{\cdot}8 \times 10^{-3}$, K_{Ca} $2{\cdot}24 \times 10^{-11}$, K_{Mg} 4×10^{-6} (Sillen & Martell, 1964).

Measurements

Bundle cross-section area was calculated from the wet weight and the length measured at 37 °C and 1 g tension, assuming cylindrical shape. The *fibre diameter* was obtained by counting the cells along the mean diameter of histological cross-sections at four levels of a typical bundle and dividing the bundle diameter, determined *in vivo*, by the obtained number. The result was approximately 50 μm.

The time course of the *mean extracellular concentration of K^+ or Ca^{2+} inside the bundle* after a change of the external solution was calculated according to eqn. (1) for diffusion into a cylinder given by Almers *et al.* (1981).

$$\frac{C}{C_o} = 1 - 4 \cdot \sum_{n=1}^{n=\infty} \frac{\exp(-\kappa \alpha_n^2 / a^2)}{\alpha_n^2} \qquad (1)$$

with C = mean concentration in the cylinder, C_o = external concentration, κ = diffusion coefficient (the values for the diffusion coefficients in free solution $D_K = 19 \times 10^{-6}$ cm^2/s, $D_{Ca} = 7{\cdot}7 \times 10^{-6}$ cm^2/s (Almers *et al.* 1981) and $D_{EGTA} \sim 4 \times 10^{-6}$ cm^2/s were divided by 2 to account for tortuosity of the diffusion path), a = radius; α_n are the roots of $J_0(x) = 0$, $J_0(x)$ being the Bessel function of zero order and the first kind. D_{EGTA} is not available. The value 4×10^{-6} cm^2/s was chosen by analogy to molecules of similar size.

Membrane potential was measured with conventional micro-electrodes in surface cells of the bundle. For this the bundle was mounted in a horizontal Perspex trough of 5 ml volume, thermostatically controlled at 37 °C, and equilibrated for 1 h isotonically at a load of 1·2 g in oxygenated solution N. Changing the bathing fluid was done by suction and addition of pre-warmed oxygenated solution from a pipette. Before the measurement the muscle was clamped at the length reached. The trough was covered by two Perspex lids tightened with Vaseline. A stream of oxygen was passed between the bath surface and the lid, along the full length of the trough during waiting periods and along half the length during measurements. The intracellular potential was displayed via a cathode-follower-stage on a Tektronix 502 A oscilloscope.

Reagents

Chemicals used were analytical grade from Fluka or Merck. Glucose was Analar B.D.H. Methanesulphonic acid was from Fluka and the Na, K and Ca salts were prepared by neutralizing the acid with the respective hydroxides. 3-*N*(morpholino)propanesulphonic acid (MOPS) was from Sigma and ethyleneglycol-bis-(*β*-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA) was from Fluka. Both were converted to the Na salt with NaOH. Caffeine base was of pharmacopoea helvetica VI grade; at a concentration of 20 mM in solution N it caused the pH to drop from 7·35 to 7·2 (37 °C). Water was distilled in a Scorah apparatus after passage over an ion-exchange column. The degree of scatter is given as standard error of the mean throughout.

RESULTS

Effect of Ca^{2+} removal on potential dependence of activation and inactivation

K^+ contractures were elicited by replacing Na^+ by K^+. The sum of cations and the product $[K^+] \times [Cl^-]$ were kept constant. In Ca^{2+}-free solutions with 2·5 mM-EGTA Ca^{2+} was replaced by Mg^{2+} in order to keep the external surface potential constant (McLaughlin *et al.* 1971). The Ca^{2+} concentrations in the 'Ca^{2+}-free' solutions were calculated to be $< 5 \times 10^{-10}$ M. The bundles were tested with high $[K^+]$ solutions after 3 min exposure to Ca^{2+}-free solutions. As a measure for activation peak tension was taken. For inactivation experiments the muscle was exposed to the test K^+ concentration for 1 min and subsequently a solution of 190 mM-K^+ was applied. The difference between the 190 mM-K^+ contracture after the test solution and that obtained starting from 4·8 mM-K^+ was used to assess the degree of inactivation during the 1 min exposure to the test solution. Contracture tension was referred to maximal tetanic tension at the given time of the experiment in normal solution and the latter was normalized to the initial tetanic tension.

No precautions, such as Na^+-free solutions or tetrodotoxin poisoning, were taken to prevent action potentials. The possibility that the contractures were due to propagated action potentials can be refuted on the following grounds. In a bundle of the dimensions used about 10 % of the cells are situated in the surface layer and will be exposed to the depolarizing solution within seconds after the bath change. Assuming a threshold potential between −50 and −40 mV (Lüttgau & Spiecker, 1979) these cells should produce at least 10 % of the single twitch tension in 20 mM-K^+ solution when firing several action potentials. This was clearly not the case (Fig. 2*A*), possibly owing to accommodation.

Fig. 1 shows the protocol of the experiment and Fig. 2 summarizes the results from several experiments. The abscissa in mM-K^+ is converted to mV membrane potential according to the measured concentration dependence of the membrane potential (E_m) shown in the insert of Fig. 2. The continuous line in the insert corresponds to E_m (mV) $= 61{\cdot}5 \log [([K^+]_o + 0{\cdot}05 [Na^+]_o)/([K^+]_i + 0{\cdot}05 [Na^+]_i)]$ with $[K^+]_i = 134{\cdot}6$ mM and $[Na^+]_i = 15$ mM. This indicates that the Na^+ permeability relative to the K^+ permeability is somewhat higher and the $[K^+]_i$ slightly lower than generally accepted for mammalian muscle (Sreter & Woo, 1963; Dockry, Kernan & Tangney, 1966; Kernan & MacDermott, 1976; Eisner, Kernan & MacDermott, 1978). It may be seen from Fig. 2 that the position of the activation curve in Ca^{2+}-free medium is the same as in the normal Ca^{2+} concentration but that removal of Ca^{2+} shifts the inactivation curve to potentials 16 mV more negative at 50 % tension. The inactivation curve is less steep in Ca^{2+}-free solutions. At low $[K^+]$ the shift is somewhat larger. This is

qualitatively similar to what Lüttgau & Spiecker (1979) found for single frog muscle fibres (shift by 30 mV).

The tension curve recorded from bundles is the result of many fibres going through a contraction–relaxation cycle whose onset is staggered in time according to the diffusion of K^+ into the fibre from the surface to the centre of the bundle. Application of the equation for diffusion into a cylinder (Almers *et al.* 1981) with a diffusion coefficient half that in free solution (to allow for tortuosity of the diffusional path) shows that it takes about 3 min for the average $[K^+]$ in the bundle

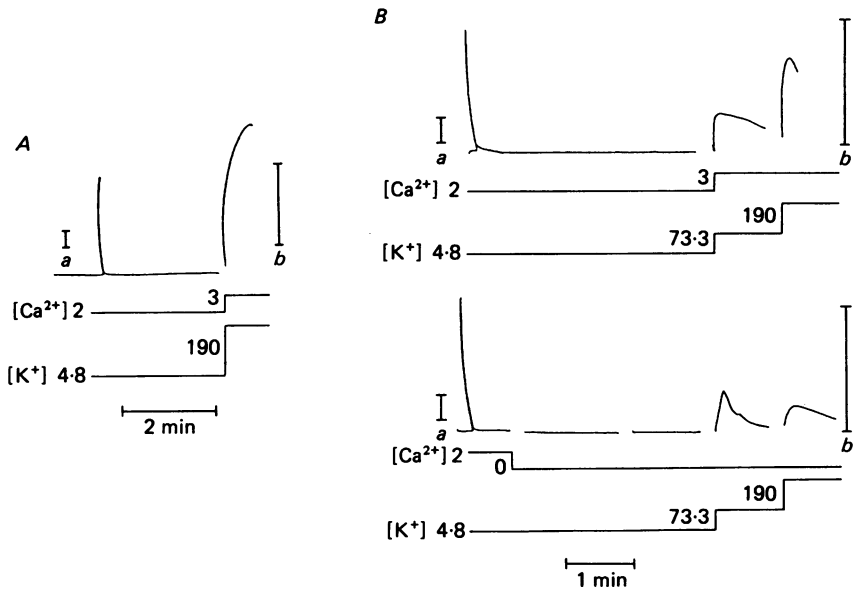

Fig. 1. Type of experiment to test activation and inactivation. *A*, first upstroke: tetanic response to 0·2 ms stimuli at 50 Hz. Second deflexion: maximal K^+ contracture in the presence of Ca^{2+}. *B*, two sets of records from the same muscle. Above: tetanic response and a submaximal and a maximal contracture in the presence of Ca^{2+}. Below: the same except that 3 min before the first contracture Ca^{2+} was removed from the bath and replaced by 3 mM-Mg^{2+} + 2·5 mM-EGTA (solution n). Note that the amplitude of the first contracture is similar with and without Ca^{2+} but that without Ca^{2+} its decay is faster and that the second contracture is strongly reduced. Total $[Ca^{2+}]$ and $[K^+]$ are given in mM under the curves. Tension calibration 5 g: *a* for tetani, *b*, for contractures. Gaps in the record mark changes of the bath. Peak tension of the first contracture is taken to indicate activation, reduction of peak tension of the second contracture to indicate inactivation.

to reach 90% of that in the external solution. This is approximately 3 times the duration of the whole K^+ contracture. Relating the contractile response to the $[K^+]$ inside the bundle, which varies as a function of time and distance from the surface, is an intractable problem. Clearly, the effective $[K^+]$ at the fibre surface was less than the nominal concentration in the test solutions. No significance should be attached, therefore, to the absolute values of the abscissae in Fig. 2. However, since the diffusion characteristics for K^+ were presumably the same with and without Ca^{2+}, the shift of the inactivation curve, if not correct in mV, is certainly real in its direction.

A similar reservation as for K^+ applies for the extracellular Ca^{2+} concentration inside the bundle in the Ca^{2+}-free solutions, particularly because it is a function of outward Ca^{2+} and inward EGTA diffusion. The mean free Ca^{2+} concentration at 3 min can be estimated to be roughly 10^{-7} M from the diffusion of Ca^{2+} and EGTA in a cylinder (eqn. (1)). Further, the Ca^{2+} concentration in the transverse tubules and in the extracellular space might exceed the calculated value for the following reason. Even at rest in normal media there is a steady efflux of Ca^{2+} from frog muscle (Curtis, 1966)

that might also occur in mammalian muscle and could amount to a net outflow into Ca^{2+}-free medium. Thus the transverse-tubular Ca^{2+} concentration is certainly higher than the nominal concentration in the 'Ca^{2+}-free' media, yet apparently low enough to have an effect on inactivation.

It could be argued that the different action of Ca^{2+} deprivation on activation and inactivation is due to the fact that activation was tested after 3 min in Ca^{2+}-free medium and inactivation after 4 min. However, inspection of the time course of the

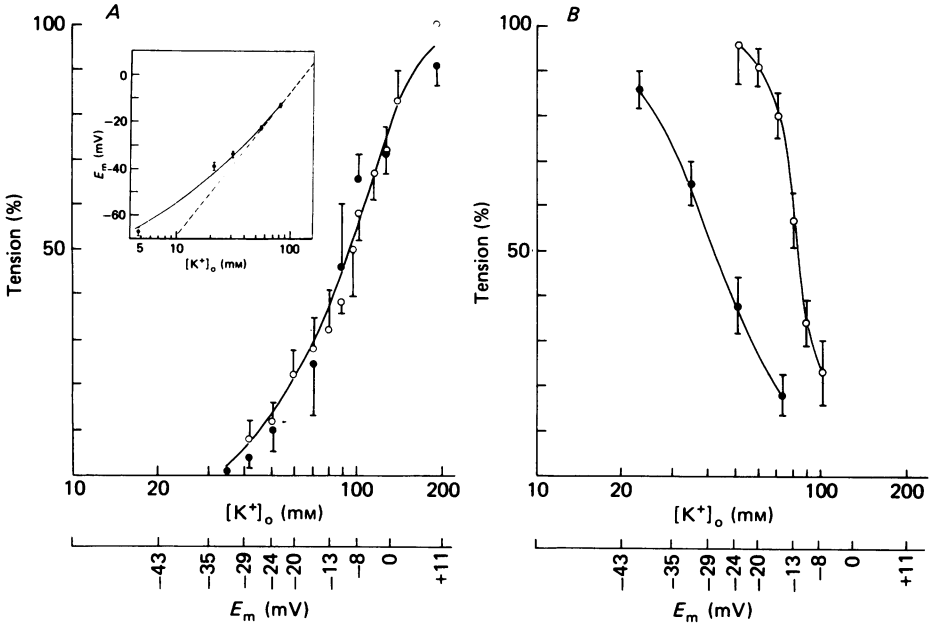

Fig. 2. Activation (*A*) and inactivation (*B*) curves obtained as described in Fig. 1. Insert: dependence of membrane potential on $[K^+]_o$ as measured in surface fibres of the bundle. Points: mean of four experiments ± S.E. of mean. Continuous line: $E_m = 61{\cdot}5 \log [([K^+]_o + 0{\cdot}05[Na^+]_o)/([K^+]_i + 0{\cdot}05[Na^+]_i)]$ with $[K^+]_i = 134{\cdot}6$ mM and $[Na^+]_i = 15$ mM. Dashed line: slope of 61·5 mV per tenfold change of $[K^+]_o$. Main Figure: ordinate: peak tension of K^+ contractures as a percentage of that caused by 190 mM-K^+ (corrected for decline of tetanus during time of experiment). Abscissa: $[K^+]_o$ in mM (log scale) or mV corresponding to continuous curve of insert. Points: mean of three to thirteen experiments ± S.E. of mean. ○, $[Ca^{2+}] = 3$ mM, $[Mg^{2+}] = 0$; ●, $[Ca^{2+}] < 5 \times 10^{-10}$ M, $[Mg^{2+}] = 2{\cdot}6$ mM. Notice that the activation curve is not affected by removal of Ca^{2+} but that the inactivation curve is shifted by 16 mV to more negative membrane potentials at 50% tension.

mean $[Ca^{2+}]_i$ calculated according to eqn. (1) shows that after 3 min the change per minute is of the order of 10% of the concentration reached. It seems probable, therefore, that the different effect of lowering the $[Ca^{2+}]_o$ on activation and inactivation is real.

Lasting effect of K contracture in Ca^{2+}-free solution

In the foregoing experiments it was observed that muscles stimulated once in Ca^{2+}-free solution remained less responsive even after prolonged exposure (22–25 min) to a solution of normal Ca^{2+} content. This behaviour was studied systematically in the way illustrated in Fig. 3. Bundles were either stimulated for 1 min by 190 mM-K^+

solutions with normal Ca^{2+} content or in Ca^{2+}-free solutions, or left resting in the Ca^{2+}-free solutions. After 22–25 min in solutions of normal Ca^{2+} content they were tested by tetanic stimulation and a second K^+ contracture (190 mM). The outcome is shown in Table 2. The Ca^{2+}-free period *per se* had no demonstrable effect. However, a preceding contracture in Ca^{2+}-free solution strongly reduced the tetanic force and even more so the contracture tension in normal solution. This is remarkable because

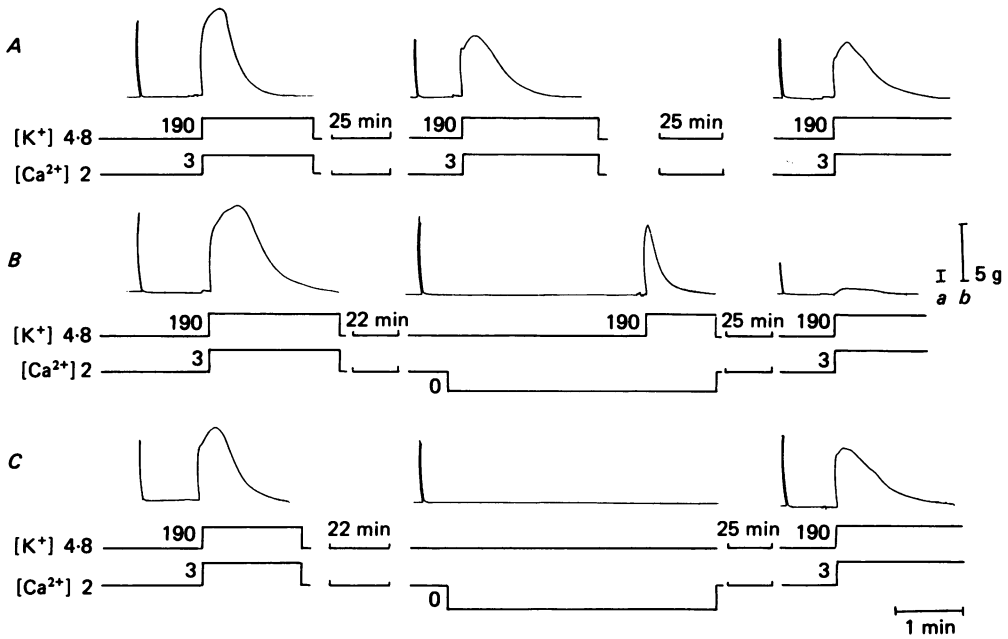

Fig. 3. Experiment with three muscle bundles from the same muscle, showing the consequence of a contracture in Ca^{2+}-free medium. Concentrations given below the curves are in mM. First upstroke: short tetanus at 50 Hz, 0·2 ms stimuli. Normal medium (solution N) except when $[K^+] = 190$ and/or $[Ca^{2+}] = 0$ mM (solution M, m or n). In Ca^{2+}-free solutions 2·5 mM-EGTA was present and Ca^{2+} was replaced by Mg^{2+} as in Figs. 1 and 2. *A*, three tetani and contractures in the presence of Ca^{2+} at 25 min intervals. *B*, two contractures as in *A*, separated by one contracture in Ca^{2+}-free medium ($[Ca^{2+}]_o < 5 \times 10^{-10}$ M, replaced by Mg^{2+}). *C*, same as *B* but omitting contracture in Ca^{2+}-free solution. Tension calibration 5 g: *a* for tetani, *b* for contractures. Notice irreversible impairment of contractility in *B*. For summary of five experiments of this type see Table 2.

recovery for 25 min in a Ca^{2+}-containing medium is long enough to bring the average Ca^{2+} concentration in the bundle to more than 90 % of that in the external solution. If depolarization is interrupted at the height of contracture ($\sim$ 15 s) the recovery is nearly complete. In order to attribute some physiological significance to this irreversible impairment it must be shown that the combination of low-Ca^{2+} treatment with depolarization does not grossly damage the permeability barrier represented by the plasma membrane, since mammalian muscle seems to be more sensitive to the 'skinning' action of Ca^{2+}-free EGTA solutions than frog muscle (Wood, Zollman & Reuben, 1975). Therefore, the membrane potential (E_m) was measured in surface fibres before and after 3 min in Ca^{2+}-free normal medium (solution n) and a further

TABLE 2. Effect of contractures in Ca^{2+}-free solutions

	A	B	C	*P* between A and B	*P* between B and C	*P* between C and A
Tetanus (*t*) (2 mM-Ca^{2+})	0·846 ± 0·048	0·431 ± 0·040	0·938 ± 0·057	< 0·0002	< 0·0002	0·25
Contracture (*c*) (3 mM-Ca^{2+})	0·753 ± 0·067	0·191 ± 0·048	0·741 ± 0·068	< 0·0002	< 0·0002	> 0·5
c/*t*	0·902 ± 0·090	0·418 ± 0·074	0·796 ± 0·070	0·00025	0·006	> 0·5

Contraction parameters (tetanic tension, *t* and maximal contracture tension, *c*) obtained 25 min after treatment A, B and C, expressed as fraction of value before the treatment. Treatment A: [Ca^{2+}] 3 mM, [K^+] 190 mM for 2 min. Treatment B: [Ca^{2+}] < 5×10^{-7} mM for 4 min, [K^+] 190 mM during the fourth minute. Treatment C: [Ca^{2+}] < 5×10^{-7} mM, [K^+] 4·8 mM for 4 min. Mean ± S.E. of mean of five experiments. *c*/*t* was calculated for individual experiments and averaged.

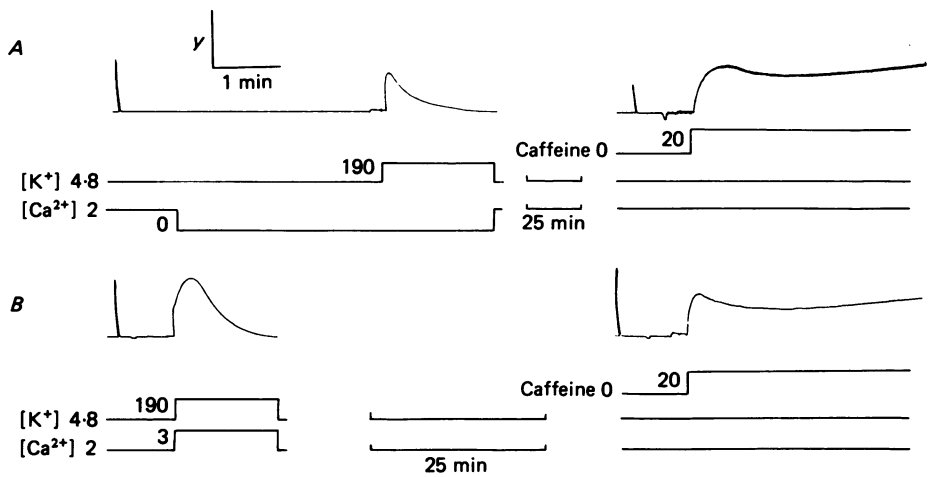

Fig. 4. Tetanus and caffeine contracture after maximal contracture ([K^+] 190 mM) in Ca^{2+}-free medium ([Ca^{2+}]$_o$ < 5×10^{-10} M) (*A*) or in Ca^{2+} (3 mM)-containing solution (*B*). Concentration underneath curves in mM. Tension calibration: $y = 25$ g for tetanus, $y = 5$ g for K^+ contracture, $y = 2{\cdot}5$ g for caffeine contracture. Notice that unlike the tetanus the caffeine response is not depressed by the preceding K^+ contracture in Ca^{2+}-free medium.

2 min in Ca^{2+}-free 190 mM-K^+ medium (solution m) under isometric conditions. In seven muscles from seven animals E_m was 66·8 ± 1·7 mV (S.E. of mean, fifty-nine impalements) before the treatment. Between 10 min and 30 min after returning to Ca^{2+}-containing normal medium (solution N) E_m was 63·5 ± 1·7 mV (sixty-two impalements). This shows that the impairment of the mechanical performance is not due to the muscle's failure to repolarize. Furthermore, the membrane potential responded normally to K^+ depolarization after the treatment.

For an explanation of this irreversible impairment of contractility the first possibility coming to mind is that Ca^{2+} from the s.r. is lost during the contracture in Ca^{2+}-free medium. It has clearly been shown that under depolarization a Ca^{2+} channel opens up in the plasma membrane and particularly in the transverse-tubular membrane.

Sanchez & Stefani (1978) have shown that for the largest Ca^{2+} currents they observed about 100–150 nmol/g could enter the sarcoplasma in the first second and Almers *et al.* (1981) have provided good evidence that this channel does not truly inactivate, allowing for a Ca^{2+} flux which is governed by the diffusional resistance in the transverse-tubular lumen for any length of time of depolarization.

In the present experiments the channel might have carried Ca^{2+} in the outward direction provided that the $[Ca^{2+}]$ in the extracellular space had fallen below the $[Ca^{2+}]$ prevailing in the myoplasma water after Ca^{2+} release from the s.r. Fig. 4 shows that this cannot be the proper explanation of the observation. Whereas in similar experiments the tetanic tension was reduced to 36 % after a contracture in Ca^{2+}-free medium, the contractile force elicited by a high concentration of caffeine was hardly reduced at all (to 89 %, $n = 3$). If caffeine liberates Ca^{2+} from the s.r., but in a way which is different from that by depolarization (Lüttgau & Oetliker, 1968), this experiment seems to show that the manoeuvre in Ca^{2+}-free solution does not measurably deplete the s.r. Ca^{2+} stores. Therefore, the failure to respond normally to depolarization (and action potentials) must be ascribed to a defect which lies closer to the potential-dependent step. Fig. 4 compares the tetanus to the caffeine action. However, the defect cannot be due to failure of action potentials, because the K^+ contracture suffered even more than the tetanic response. Besides, membrane potential (present experiments) and action potentials (Stefani & Chiarandini, 1973; Lüttgau & Spiecker, 1979) are quite resistant to short exposure to Ca^{2+}-free solutions.

DISCUSSION

The findings thus are as follows. (1) As in frog skeletal muscle, the potential dependence of the activating process of m. thyreopharyngicus of the pig is not affected by removal of external Ca^{2+}. In contrast, in Ca^{2+}-free solution the inactivation process is more sensitive to depolarization, which is again similar to the behaviour seen in frog muscle. (2) Stimulation (exposure to 190 mM-K^+ for 1 min) in the absence of external Ca^{2+} brings about an irreversible impairment of the contractile response to electrical stimulation or upon depolarization by K^+. The fact that a nearly normal response to caffeine is still possible under these conditions shows that the refractory state cannot be explained by Ca^{2+} depletion of the s.r.

Even an exposure to Ca^{2+}-free 5 mM-Na EGTA solution for 30 min does not alter the amplitude and time course of the caffeine contracture in frog skeletal muscle (Chiarandini, Sanchez & Stefani, 1980).

Furthermore, it has been shown that the effect is not due to a decay of the resting potential.

The most likely mechanism by which a change in membrane potential can bring about activation and inactivation is movement of charged groups within the transverse-tubular membrane. With this in mind, the present results might be interpreted as follows. (1) When the muscle is depolarized in Ca^{2+}-free medium Ca^{2+} is lost from a site which is not the s.r. and which cannot be replenished by the slow Ca^{2+} inward current. This Ca^{2+} loss must reduce the membrane charge that moves during activation, because it slows the rate of tension rise in a second contracture (Fig. 3). (2) The effects seen during the first depolarization after removal of Ca^{2+} are

the shift of inactivation to more negative potentials and the shortening of the contracture, suggesting an increase in the membrane charge moving during inactivation.

The observed outward current indicating a charge movement in the membrane corresponds to activation. A second phase of outward current related to an inactivating charge movement as suggested by the model of Chandler *et al.* (1976*b*) has not been detected experimentally and is, therefore, hypothetical.

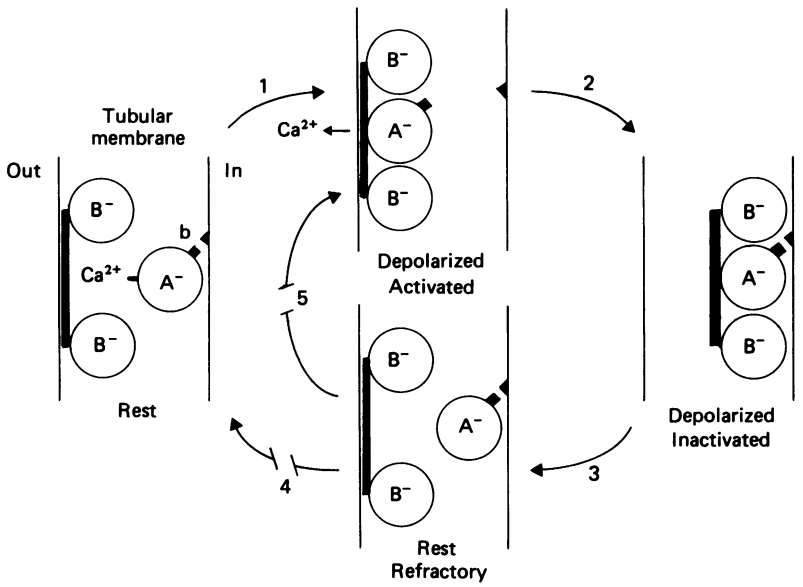

Fig. 5. Possible role of external Ca^{2+} in the model for the transverse-tubular membrane (Chandler, Rakowski & Schneider, 1976*b*). A^- is a group to which bound Ca^{2+} imparts positive charge. It equilibrates with external Ca^{2+} in the out-position only. The outward movement of $(ACa)^+$ occurring when the membrane is depolarized in some way activates the Ca^{2+} release from the adjacent terminal cysterna. BB is a negatively charged complex which brings $(ACa)^+$ back to the in-position, thus inactivating the Ca^{2+} channel in the terminal cysterna. If depolarization is elicited in Ca^{2+}-free medium Ca^{2+} is lost from A^- in step 1. Hence, step 2 requires less depolarization and is faster than in normal Ca^{2+} medium. After step 2 A^- is inaccessible to external Ca^{2+}; step 4 or 5 are impossible, the system is refractory even if external Ca^{2+} is readmitted. A hypothetical bond (b) keeps A^- in place at the normal membrane potential. Its formation is assumed to be a slow process (see Discussion).

Introducing a single type Ca^{2+}-site of high affinity in the model of Chandler *et al.* (1976*b*) leads to a decrease of the 'activator charge' and an increase of the 'inactivator charge' when Ca^{2+} is lost from this site. In the model (Fig. 5) A is a positive group that moves rapidly (against low viscous resistance) outwardly and BB is a negative charge (exceeding that of A) moving slowly (against high viscous resistance) inwardly upon depolarization. Outward movement of A somehow causes Ca^{2+} release from the terminal cysternae and movement of BB brings A back again, thus terminating Ca^{2+} release, i.e. causing inactivation. The observed effects are accounted for by attributing the positive charge of A to bound Ca^{2+} which can equilibrate with external Ca^{2+} when A is in the out- but not when it is in the in-position.

In Ca^{2+}-free medium depolarization moves A outwardly, A loses its Ca^{2+}, the complex ABB, therefore, is more negative than before and a lesser potential is required to bring ABB into the in-position. Upon repolarization ABB dissociates into A and BB but, since A is now negative, a second depolarization will not move it any more, even if Ca^{2+} is readmitted to the medium. A negative charge on A seems necessary to make it apt to complex Ca^{2+}. To explain why A stays in the in-position at the normal membrane potential it may be assumed that there is some chemical bonding which, together with the repulsion from BB, keeps it in place.

The observation that single twitches are possible in Ca^{2+}-free media (Armstrong, Bezanilla & Horowicz, 1972; Lüttgau & Spiecker, 1979) for a long time shows that the proposed loss of Ca^{2+} into the medium does not occur in twitch activity. Similarly, K^+ depolarization of some seconds' duration (Lüttgau & Spiecker, 1979; Stefani & Chiarandini, 1973; present experiments) does not induce the refractory state. It may be necessary to hold A (Fig. 5) for a while in the in-position to permit it to re-attach. Immediate repolarization might allow it to oscillate between the two positions without macroscopic tension generation, thus recapturing some of the Ca^{2+} that has not had time to diffuse away.

The fact that contracture tension is more vulnerable to stimulation in Ca^{2+}-free media than tetanic tension (Table 2) needs some explanation. As it takes about 3 min to bring the mean extracellular K^+ concentration in the bundle to 90% of that in the applied solution, the more central fibres will not be exposed to depolarizing concentration in 1 min (Fig. 3) and thus will not undergo the irreversible Ca^{2+} loss postulated. The second immersion into K^+ solution thus will reach only incapacitated fibres, while electrical stimulation will activate the unimpaired fibres in the core. An alternative explanation is offered by the fact that central fibres depolarize slowly (Dulhunty, 1980). In Ca^{2+}-free solutions such fibres might become inactivated before activating membrane potentials are reached. For this reason they might be protected against the loss of Ca^{2+} from site A.

While Lüttgau & Spiecker (1979) found that the Ca^{2+}-entry blocker D-600 had similar effects as Ca^{2+}-free media, Gonzales-Serratos, Valle-Aguilera, Lathrop & del Carmen (1982) recently reported that diltiazem does not shorten K^+ contractures, nor reduce twitch or tetanus force but on the contrary enhances it at concentrations and exposure times that abolish the slow inward Ca^{2+} current. Caputo (1972) has shown that in frog muscle K^+ contractures shortened by removal of Ca^{2+} can be prolonged by 1·8 mM-Ni^{2+} ions which are known to bind to Ca^{2+} sites. Both observations support the idea that external Ca^{2+} does not need entering the myoplasma to exert its effects.

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