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THE EFFECTS OF CALCIUM DEPRIVATION UPON MECHANICAL AND ELECTROPHYSIOLOGICAL PARAMETERS IN SKELETAL MUSCLE FIBRES OF THE FROG*

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SUMMARY

1. The effects of Ca^{2+} deprivation upon mechanical and electrophysiological parameters of single muscle fibres from the m. semitendinosus and the m. iliofibularis of the frog were investigated.

2. When the external free Ca concentration was reduced in steps of one order of magnitude from 10^{-3} to 10^{-9} M, using up to 10 mM-EGTA and in the presence of 3 mM-Mg^{2+}, the maximum force of K contractures declined by 5–15%, the plateau of maximum force shortened, and in most cases the phase of spontaneous relaxation lengthened.

3. In Ringer solution containing 10^{-9} M-Ca^{2+} and 1 mM-Mg^{2+} 85% of maximum tetanic force could be maintained for at least 15 sec (5 Hz; 3 °C).

4. The reduction of external Ca^{2+} from 10^{-3} to 10^{-9} M and its replacement by Mg^{2+} induced a 20–30 mV shift towards more negative potentials of the 'steady state' inactivation curve (which relates maximum force upon full depolarization to the logarithm of the K concentration or the corresponding membrane potential during the conditioning period).

5. The same alteration in concentrations of divalent cations caused little or no change in the shape and potential dependence of the activation curve (which relates maximum force to the logarithm of the external K concentration of the corresponding membrane potential).

6. The threshold potential for the onset of delayed rectification (point voltage clamp) and that for the initiation of an action potential did not change when external Ca^{2+} was reduced to 10^{-9} M and replaced by Mg^{2+}.

7. When the concentration of $EGTA^{2-}$ was increased to 80 mM (in the presence of 5 mM-Mg^{2+}) twitch height dropped to very small values within a few minutes. However, tetanic force (50 Hz) reaching 20–85% of the original value could still be induced after 1 hr in high $EGTA^{2-}$.

8. The experiments show that external Ca^{2+} acts upon excitation–contraction coupling mainly by impeding 'inactivation'. A hypothesis is proposed in which the plateau of maximum force during a contracture is the consequence of a regenerative Ca_i^{2+}-dependent shift of the inactivation curve towards more positive potentials.

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INTRODUCTION

In his well-known review on excitation–contraction coupling in skeletal muscle fibres, Sandow (1952) proposed that an influx of Ca ions into the fibre during an action potential initiates contraction. Since then this influx has been demonstrated with flux measurements (Bianchi & Shanes, 1959) and electrophysiological methods (Beaty & Stefani, 1976*a*, *b*). In addition, a regenerative Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum has been observed in skinned fibres (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1970), suggesting that even a relatively small influx of Ca ions may ultimately result in a large increase in myoplasmic free Ca. The 'stabilizing' action of Ca^{2+} and other divalent cations due to a decrease in the external surface potential (cf. Lüttgau, 1963) is generally accepted, but the physiological role of external Ca^{2+} in activation is still controversial.

In EGTA-buffered solutions with 10^{-9} M-Ca^{2+}, i.e. a concentration which is lower than the internal free Ca concentration, normal twitches are still possible (Armstrong, Bezanilla & Horowicz, 1972), which suggests that an influx of external Ca^{2+} ions is not a prerequisite for a twitch. However, contractures become smaller and shorter (Stefani & Chiarandini, 1973), which does suggest a role for external Ca^{2+} in activation. The authors cited used $EGTA^{2-}$ at low concentrations (1 mM) to reduce the ionic concentration of Ca to the desired level. In the T-system with a small volume and a restricted access this buffer capacity might be insufficient, since Ca ions presumably are continuously entering the tubular space from internal stores. To circumvent this problem, Barrett & Barrett (1978) applied $EGTA^{2-}$ at concentrations as high as 90 mM to single muscle fibres. This treatment depolarized the fibres. After repolarization by an internally applied current the authors were able to induce action potentials which were no longer followed by a mechanical response. This again suggests that external Ca ions are involved in excitation–contraction coupling.

We have re-investigated the action of external Ca^{2+} upon excitation–contraction coupling with solutions in which Ca^{2+} was replaced by Mg^{2+}. In addition we extended this analysis by evaluating further parameters. The results show (*a*) that force can be induced by depolarization under conditions where a passive influx of Ca^{2+} can no longer be expected, (*b*) that Ca^{2+} deprivation mainly accelerates the inactivation process and (*c*) that external Ca^{2+} promotes (via a Ca^{2+} influx?) a maintenance of tension during a K contracture or tetanic stimulation.

Preliminary accounts of some of the experiments described here were presented at meetings of the Physiological Society (Lüttgau, Melzer & Spiecker, 1977) and the Deutsche Physiologische Gesellschaft (Spiecker, Grabowski & Lüttgau, 1978).

METHODS

Semitendinosus and iliofibularis muscles of the frog (*Rana temporaria*) were used in these experiments. Single fibres were isolated as described previously (Lüttgau, 1965). The experiments were carried out in a Perspex chamber of the Hodgkin–Horowicz type, which allowed a quick change of external solutions. Isometric tension was recorded with a force transducer (Endevco, type 8107/20 or RCA 5734).

The fibres were usually left in the experimental chamber for 20–30 min before experiments started with a control tetanus at 50 Hz. Contractures were induced by solutions with an elevated K concentration. After spontaneous relaxation these solutions were replaced by Ringer solution in which fibres recovered for 15–20 min before a new contracture cycle was induced. If not otherwise stated the experiments were carried out at room temperature (19–24 °C).

Electrophysiological methods. Resting and action potentials were measured in fibres of whole semitendinosus muscles with conventional micro-electrodes. Voltage-clamp experiments were performed with two internal micro-electrodes as, for example, described by Costantin (1968; point voltage clamp). A detailed description of the electronic device, which was assembled by W. Grabowski and E. Wettwer in our laboratory, will appear elsewhere (R. Fink & E. Wettwer, in preparation).

Solutions. In Table 1 the composition of the main solutions used in our experiments is given, together with the calculated free concentrations of Mg and Ca. The solutions with the suffixes 1 and 2 differed from each other only in so far as they contained either Ca^{2+} or Mg^{2+} and $EGTA^{2-}$ (see Table 1, columns 12–14). We aimed at replacing ionic Ca in 1 by the same amount of ionic Mg in 2. Small deviations, for example in solutions D, O, P and Q, resulted from a correction of earlier calculations in which we neglected the binding of Mg^{2+} to $EGTA^{2-}$ and that of both divalent cations to other anions in solution. The electrophysiological solutions (D and E) and the depolarizing solutions with constant $K \times Cl$ product (I–Q) were made up with Na gluconate or Na propionate because Na methylsulphate was found to have an appreciable Ca^{2+} contamination (see below). The pH in all solutions was adjusted with NaOH, HCl or propionic acid to pH $7{\cdot}00 \pm 0{\cdot}02$.

The procedure of calculating the ionic concentration of divalent cations in the solution needs a more detailed description.

Low stabilized concentrations of free Ca can be adjusted by employing Ca buffers such as EGTA. According to the mass action law, the free Ca is given by

$$[Ca^{2+}] = \frac{1}{K'_{Ca}} \frac{[CaEGTA^{2-}]}{[H_2EGTA^{2-}]}. \quad (1)$$

The exact value of $[Ca^{2+}]$ can be computed only if the apparent binding constant K'_{Ca} and the ratio $[CaEGTA^{2-}]/[H_2EGTA^{2-}]$ is known. The apparent binding constant depends on pH and the ionic composition of the employed solutions. It can be estimated by combining the absolute binding constants of the corresponding ions with EGTA, as described by Portzehl, Caldwell & Rüegg (1964). The value of K'_{Ca} used in the calculations was $4{\cdot}35 \times 10^6$ M^{-1} for the conditions pH 7·00, 20 °C and $[Mg^{2+}] = 3$ mM. To determine the ratio $[CaEGTA^{2-}]/[H_2EGTA^{2-}]$ the concentration of Ca^{2+} and $EGTA^{2-}$ in the applied stock solutions has to be known exactly. However, it is rather difficult to prepare $CaCl_2$ or $EGTA^{2-}$ solutions of precise concentrations, as both substances contain an unknown fraction of water and traces of ions which may combine with $EGTA^{2-}$. Other stock solutions needed for Ringer, etc., also contain impurities of Ca^{2+} and additional ions which might be bound by $EGTA^{2-}$. An elegant method of determining the relative concentration of free cations which bind EGTA with high affinity constants is the titration procedure described by Moisescu & Pusch (1975). It takes advantage of the pH change during the binding of Ca^{2+} to $EGTA^{2-}$ according to the reaction

$$H_2EGTA^{2-} + Ca^{2+} \rightleftharpoons CaEGTA^{2-} + 2H^+ \quad (2)$$

and of the uptake of H^+ by a pH buffer. In principle, the reaction is applicable for any pH lower than 9. The equation presented with H_2EGTA^{2-}, however, would be appropriate only for a pH between 7·5 and 4. As an example, the determination of Ca^{2+} traces in a solution with 60 mM Na methylsulphate is shown in Fig. 1. NaOH was used to adjust the pH in this solution to 7·5–7·7. The titration solution contained 10^{-2} M-Na_2H_2EGTA. It was adjusted to pH 8·00 and buffered with $MOPS^-$, a buffer which like TES^-, $HEPES^-$ or $Tris^+$ does not form complexes with Ca^{2+} (unpublished measurements).

The titration curve was V-shaped. The decrease in pH turned into an increase when all traces of Ca^{2+} were bound to $EGTA^{2-}$, i.e. when protons were no longer released and the pH rose under the influence of the $MOPS^-$ buffer. In Fig. 1 the end point of Ca^{2+} complexation or

TABLE 1. Composition of solutions (mM). Columns 1–12 present the total concentrations and columns 13 and 14 give the calculated concentrations of free Ca and Mg. The contamination with Ca^{2+} and other $EGTA^{2-}$-binding cations usually ranged from 5×10^{-3} to 10^{-2} mM. pH 7·00 ± 0·02. T = 20–23 °C (electrophysiol. expt. T = 5–7 °C; soln $D_{1,2}$ and $E_{1,2}$)

	K^+	Na^+	$Tris^+$	Ca^{2+}	Mg^{2+}	Cl^-	Sucrose	MS^-	$Prop^-$	$Gluc^-$	$MOPS^-$	$EGTA^{2-}$	Ca^{2+}	Mg^{2+}
Soln	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ringer														
A	2·5	117·5	—	1·8	—	121	—	—	—	—	2·5	—	1·8	—
Tris Ringer for Ca–Mg exchange														
B_1	2·5	—	117	3·2	—	119·5	—	—	—	—	—	—	3·2	—
B_2	2·5	10	117	—	3·6	119·5	—	—	—	—	—	5	10^{-6}	3·05
C_1	2·5	—	117	1·2	3	123·5	—	—	—	—	—	—	1·2	3
C_2	2·5	0–6·6	117	—	3	119·5	—	—	—	—	—	0–3·3	0·2–7·10^{-6}	2·7–3
Electrophysiological Solutions														
D_1	2·5	117·5	—	4	—	25·5	350	—	—	90	2·5	—	1·6	—
D_2	2·5	117·5	—	—	4	25·5	350	—	—	90	2·5	1·22	$3 \cdot 10^{-6}$	2·7
E_1	2·5	117·5	—	4	—	25·5	350	—	90	—	2·5	—	3·1	—
E_2	2·5	117·5	—	—	4	25·5	350	—	90	—	2·5	1·22	$3 \cdot 10^{-6}$	3
High EGTA														
F	2·5	215	—	—	21·2	44·9	—	—	—	—	5	96·2	$5 \cdot 10^{-8}$	5
Maximum contracture solution														
G_1	190	5	—	3·2	—	—	—	190	6·4	—	5	—	3·2	—
G_2	190	10	—	—	3·5	—	—	190	7	—	5	2·5	10^{-6}	3·2
H_1	190	—	4	1·2	3	6·4	—	190	6	—	—	—	1·2	3
H_2	190	—	4	0·3–5	3	4	—	190	6	—	—	0–10	0·1–7·10^{-6}	2·5–3

TABLE 1—*continued.*

Soln	K^+ 1	Na^+ 2	$Tris^+$ 3	Ca^{2+} 4	Mg^{2+} 5	Cl^- 6	Sucrose 7	MS^- 8	$Prop^-$ 9	$Gluc^-$ 10	$MOPS^-$ 11	$EGTA^{2-}$ 12	Ca^{2+} 13	Mg^{2+} 14
Constant K × Cl product solutions														
I_1	93·6	33·4	—	3·5	—	3·2	—	93·6	35·2	—	5	—	3·2	—
I_2	93·6	33·4	—	—	3·8	3·2	—	93·6	30·8	—	5	2·5	10^{-6}	3·2
K_1	62·8	64·2	—	3·7	—	4·8	—	62·8	64·6	—	5	—	3·2	—
K_2	62·8	64·2	—	—	4·2	4·8	—	62·8	60·8	—	5	2·5	10^{-6}	3·2
L_1	42·2	84·8	—	3·9	—	7·2	—	42·2	83·4	—	5	—	3·1	—
L_2	42·2	84·8	—	—	4·4	7·2	—	42·2	79·4	—	5	2·5	10^{-6}	3·2
M_1	28·4	98·6	—	3·9	—	10·6	—	28·4	93·8	—	5	—	3·1	—
M_2	28·4	98·6	—	—	4·5	10·6	—	28·4	90	—	5	2·5	10^{-6}	3·2
N_1	19	108	—	4·0	—	15·8	—	19	98·2	—	5	—	3·0	—
N_2	19	108	—	—	4·5	15·8	—	19	94·2	—	5	2·5	10^{-6}	3·2
O_1	19	108	—	4·0	—	15·8	—	19	98·2	—	5	—	3·0	—
O_2	19	113	—	—	4·0	15·8	—	19	94	—	5	2·5	10^{-6}	2·8
P_1	13	114	—	4·0	—	23	—	13	97	—	5	—	3·0	—
P_2	13	119	—	—	4·0	23	—	13	93	—	5	2·5	10^{-6}	2·8
Q_1	8·7	118·2	—	4·0	—	34·6	—	8·7	89	—	5	—	3·05	—
Q_2	8·7	123·2	—	—	4·0	34·6	—	8·7	85·8	—	5	2·5	10^{-6}	2·86

Abbreviations: MS^-, methylsulphate; $Prop^-$, propionic acid; $Gluc^-$, gluconic acid; $MOPS^-$, 3-(N-morpholino)propane sulphonic acid; $EGTA^{2-}$, ethylene glycol bis (β-aminoethyl ether)-N,N′-tetraacetic acid. Apparent binding constants used were: $K'_{CaEGTA} = 4{\cdot}35 \times 10^6\ M^{-1}$, $K'_{MgEGTA} = 40{\cdot}4\ M^{-1}$, $K_{CaProp^-} = 10^{0{\cdot}50}\ M^{-1}$, $K_{MgProp^-} = 10^{0{\cdot}54}\ M^{-1}$, $K_{CaGluc^-} = 10^{1{\cdot}22}\ M^{-1}$, $K_{MgGluc^-} = 10^{0{\cdot}70}\ M^{-1}$. The data were calculated from the absolute binding constants (Sillén & Martell, 1964, 1971), as described by Portzehl, Caldwell & Rüegg (1964). For further details concerning these solutions see Spiecker (1979).

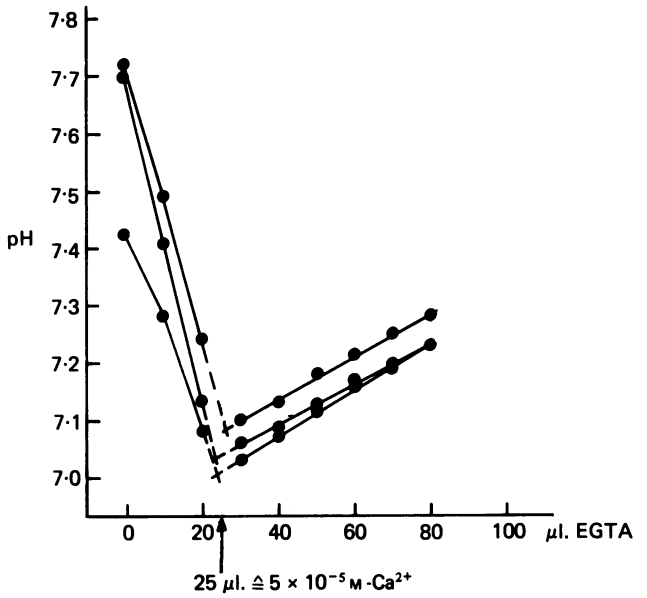

Fig. 1. Titration of Ca^{2+} and other ions which form complexes with $EGTA^{3-}$ in 5 ml. 60 mM-Na methylsulphate solution. The titration solution contained 10^{-2} M-H_3EGTA^{2-} and was buffered with 10^{-7} M-MOPS$^-$ (pH 8·0). The titration was repeated three times. T = 20 °C.

proton release, estimated as the intersection of the two straight lines, was reached after the addition of 25 µl. of the titration solution. This corresponded to 5×10^{-5} M-Ca^{2+} (with the inclusion of other cations which were bound by $EGTA^{3-}$). In different stock solutions impurities between 5×10^{-6} and 2×10^{-4} M were found and considered in our calculations.

RESULTS

The time course of potassium contractures and tetani at different external Ca^{2+} concentrations

In a first series of experiments we investigated the action of external Ca^{2+} upon the time course of force development after a complete depolarization with solutions containing 190 mM-K. The external Ca^{2+} concentration was reduced in steps of one order of magnitude from values above to those below the supposed internal concentration of ionic calcium (e.g. 10^{-7} M; see Endo, 1977). The total Mg concentration was kept constant at 3 mM, and its ionic concentration fell from 3 mM at 10^{-3} M-Ca^{2+} to 2·5 at 10^{-9} M-Ca^{2+}. The fibres were equilibrated for 5 min in solution C_2 with varying concentrations of $EGTA^{3-}$ to obtain the concentration of free Ca required. After this period they were exposed to contracture solution H_2 with the corresponding concentration of free Ca. Fig. 2 shows an experiment with Ca^{2+} concentrations extending from 10^{-3} to 7×10^{-9} M in which the fibre survived twelve contractures without showing signs of fatigue or deterioration. The maximum force decreased by no more than 15% when external [Ca^{2+}] was lowered. In this fibre the tension rose again at 7×10^{-9} M to the control level, but in other preparations the tension fell by 10–15%.

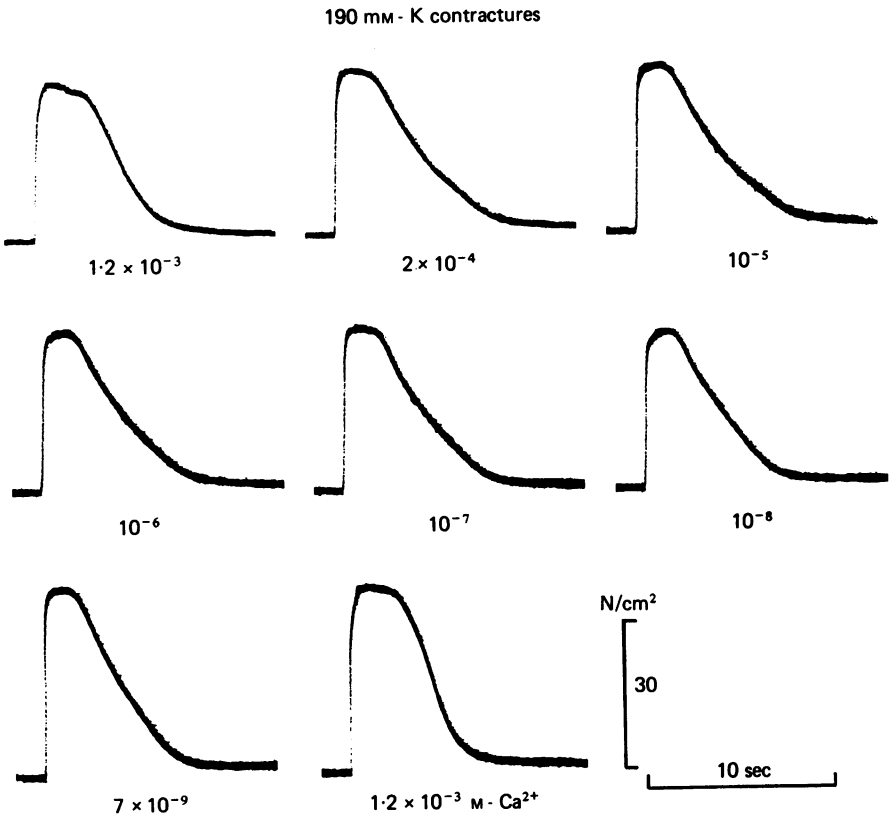

Fig. 2. Contractures induced with 190 mM-K^+ (soln H_2) at different Ca^{2+} concentrations. Control contractures with 1·2 mM-Ca^{2+} and 3 mM-Mg^{2+} (soln H_1), of which only the first and last one are displayed, alternated with those in low Ca^{2+} solutions. The fibre recovered in Ringer (soln A) for 15–20 min after each contracture. The Mg^{2+} concentration in low Ca^{2+} solutions ranged from 3·0 (at 10^{-4} M-Ca^{2+}) to 2·5 mM (at 7×10^{-9} M-Ca^{2+}). T = 21 °C.

Additional effects of the low Ca^{2+} solutions were a reduction in the duration of the plateau of maximum force (by 30–50 %) and an increase in the half time of relaxation from the plateau region to the resting level (rather irregularly by 0–200 %). These alterations occurred at relatively high Ca^{2+} concentrations (10^{-4}–10^{-5} M) and only minor effects were observed when the external Ca^{2+} concentration was lowered from values above to those below the normal internal free-Ca concentration.

Barrett & Barrett (1978) recently suggested that much higher concentrations of $EGTA^{2-}$ are needed to obtain the desired low Ca^{2+} concentrations in the restricted area of the transverse tubular system. For this reason they applied Ringer solution in which NaCl was replaced by Na-$EGTA^{2-}$. Under these conditions twitches could no longer be induced in single muscle fibres from the cutaneous pectoris muscle of the frog. We were able to confirm their results in whole semitendinosus muscles. As indicated by the authors the high $EGTA^{2-}$ solution caused a strong depolarization so that a repolarization was needed before action potentials could be induced. For this reason we modified their solution by adding $MgCl_2$ (5 mM-Mg^{2+}) without reducing

the free EGTA concentration (80 mM; solution F). When this solution was applied resting potentials and input resistances, measured in whole muscles with the microelectrode technique, became only slightly smaller or remained constant for at least 60–120 min. The solution was then employed in five single fibre experiments.

After the application of high $EGTA^{2-}$, twitch force declined in two phases (Fig. 3*B*). Within 1–3 min it dropped to 10–50 % of the original value. Thereafter force declined more slowly so that even after 20–80 min twitch force was 2–20 %.

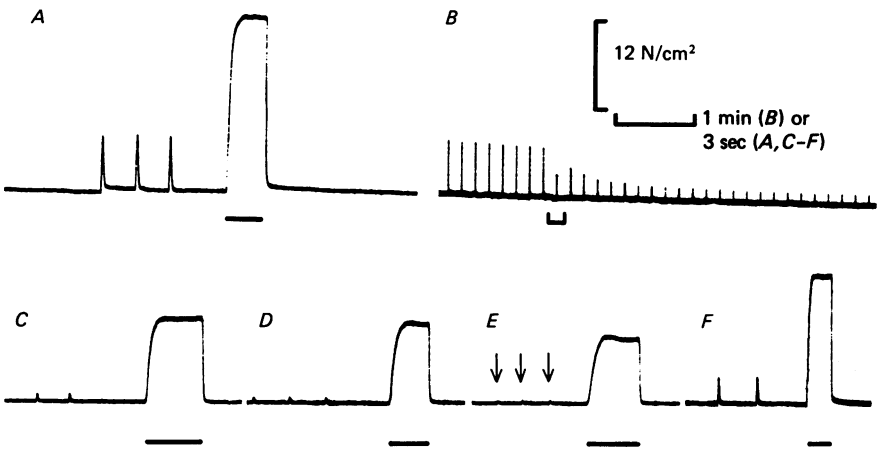

Fig. 3. Twitches and tetani (50 Hz; indicated by horizontal bars) in Ringer (*A*) and 7, 14 and 24 min (*C*, *D*, *E*) after the application of high $EGTA^{2-}$ (soln F). *B*, twitches at 0·1 sec^{-1} before and after the exchange of solutions (the bracket shows the time needed for the exchange). *F*, recovery 20 min after the reapplication of Ringer. T = 22 °C.

Tetanic force (50 Hz) declined less markedly than twitch force. After 1 hr about 20–85 % of the original value was observed. The twitch:tetanus relation of 1:2–5 in Ringer changed to 1:10–50 after 45 min in high $EGTA^{2-}$. In a few cases we observed a short phase of spontaneous twitching or a slight contracture when high EGTA was applied. This effect was probably due to a phasic depolarization which is to be expected after a sudden reduction in $[Cl^-]_o$ (Hodgkin & Horowicz, 1959).

Fibres survived well in high $EGTA^{2-}$. Propagated action potentials could still be induced after 120 min. Twitches and tetani recovered after the reapplication of Ringer (Fig. 3), but in most cases recovery remained incomplete.

The results suggest that $EGTA^{2-}$ readily diffuses into the T-system, reaching its main parts within 1–3 min and causing the fast decrease in force. The further decline in twitch height might be explained by the diffusion of $EGTA^{2-}$ into remote branches of the T-system (Franzini-Armstrong, 1973), a substantial loss of Ca^{2+} from internal stores, and additional effects of Ca^{2+} removal upon the membranes of the transverse tubular system.

The role of external Mg^{2+} was investigated in more detail at low $EGTA^{2-}$ concentrations. Fig. 4 shows contracture experiments with three different fibres. The first (*A*) developed a contracture with maximum force upon depolarization in 1·8 mM-

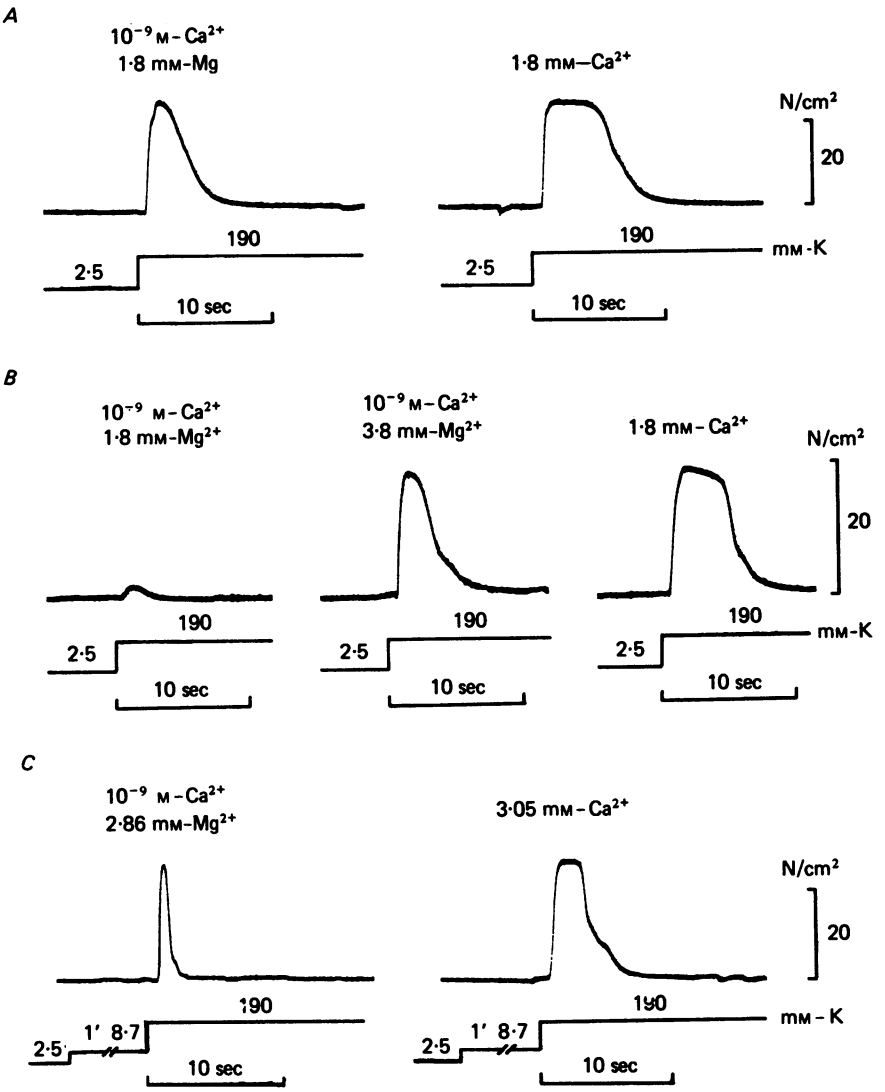

Fig. 4. The role of Mg ions in the preservation of contracture force in Ca^{2+}-poor solutions. Three different fibres. Before each contracture, the fibres were equilibrated in a $Tris^+$ Ringer solution with the Ca^{2+} and Mg^{2+} concentrations which were afterwards applied with the following contracture solution. *A* and *B*: soln $B_{1,2}$ and $G_{1,2}$; free Ca or Mg slightly modified. *C*: soln. $Q_{1,2}$ and $G_{1,2}$. T = 22 °C.

Mg^{2+}, while the second fibre (*B*) showed only a small contracture under the same condition but developed maximum force when the concentration of Mg^{2+} was doubled. All contractures in Mg^{2+}-containing solutions were shorter than those in the control solution with the corresponding Ca^{2+} concentration. This is particularly striking in the third experiment (*C*). Here the fibre was left for one minute in a conditioning solution with 8·7 mM-K (−70 mV; solution Q_2) before the contraction was induced by a complete depolarization. In 2·86 mM-Mg^{2+} a very short contracture

was observed while the corresponding Ca^{2+} contracture revealed the normal plateau. The role of Mg^{2+} in maintaining contractility rests upon its ability to preserve a high resting potential and to retard inactivation (Lüttgau, 1963). The latter can sufficiently be explained by a screening effect upon external surface charges (Dörrscheidt-Käfer, 1976). Ca ions, in this respect, act in a similar fashion to Mg ions. The more powerful action of Ca^{2+}, in particular upon plateau duration, might then be related to the inflow of Ca ions upon depolarization (Beaty & Stefani, 1976*a*).

The influence of Ca^{2+} inflow was also tested by the application of D600, a drug which especially in its L-form blocks the Ca-channel (skeletal muscle fibres: Beaty & Stefani, 1976*a*). The drug (kindly provided by Dr A. Oberdorf, Knoll AG, Ludwigshafen) was usually applied for 10 min in an equilibration solution and subsequently in the contracture solution. The first effect, a reduction in plateau duration, was observed at a concentration of $\sim 10^{-5}$ M L-D600. At concentrations between $2–8 \times 10^{-5}$ M the effect upon the time course of contractures was similar to that of low Ca^{2+} solutions, although less distinct. The height of contractures declined, and the duration of the plateau decreased by no more than 20%. We suggest that these effects were due to an incomplete blockage of the Ca^{2+} influx upon depolarization, which is consistent with electrophysiological measurements (Palade & Almers, 1978).

Finally, we observed that in a low Ca^{2+} Ringer solution with 10^{-9} M-Ca^{2+} and 1 mM-Mg^{2+} tetani at 5 Hz (3 °C) were only slightly less than in normal Ringer solution. They could be maintained for at least 15 sec with only a 15% decrease in maximum height. The results show that in a low Ca^{2+} solution force can be maintained if the process of inactivation is held up by keeping the membrane potential at the resting level between two successive action potentials. The shortening of the plateau of contractures in low Ca^{2+} solutions is, thus, a sign of an accelerated inactivation rather than of a Ca^{2+} depletion in the sarcoplasmic reticulum.

The effect of substituting Mg^{2+} for external Ca^{2+} upon the activation curve

The development of force is related to the membrane potential by a steep S-shaped curve with force starting at -50 mV (Hodgkin & Horowicz, 1960). In this section we describe experiments which show how far the replacement of external Ca^{2+} by Mg^{2+} influences this relation.

The fibres were always equilibrated for 5 min in $Tris^+$-Ringer solution (solution $B_{1,2}$) which contained the same ionic concentration of Ca or Mg as the subsequently applied K-rich solution (3·0–3·2 mM throughout). Na^+ was replaced by $Tris^+$ in order to prevent twitching upon depolarization. Contractures in the potential range between -50 and -10 mV were induced by elevating the external K concentration at a constant $K \times Cl$ product (solutions $I_{1,2}$–$N_{1,2}$). The experiment was usually started with a contracture induced by a complete depolarization (solution G_1; 'control contracture') at 3·2 mM-Ca^{2+}. It was followed by two contractures at a test K concentration with either Ca^{2+} or Mg^{2+}. After each contracture the fibre recovered in normal Ringer solution (solution A) for 15 min, followed by 5 min in a new equilibration solution. This cycle of contractures was always terminated by a second control contracture at 190 mM-K (solution G_1) and the mean maximum force of the two controls was taken as 100%. In Fig. 5, the evaluation of experiments

with four different fibres can be seen. It shows that neither the threshold potential nor the shape of the activation curve is significantly altered when external Ca^{2+} is replaced by Mg^{2+}. Near the threshold, i.e. at -50 mV, a large scatter in force development between 0 and 35% was observed. This effect, which was confirmed in additional experiments, might characterize an unstable region in which small alterations in the kinetics of activation and inactivation have drastic effects upon maximum force.

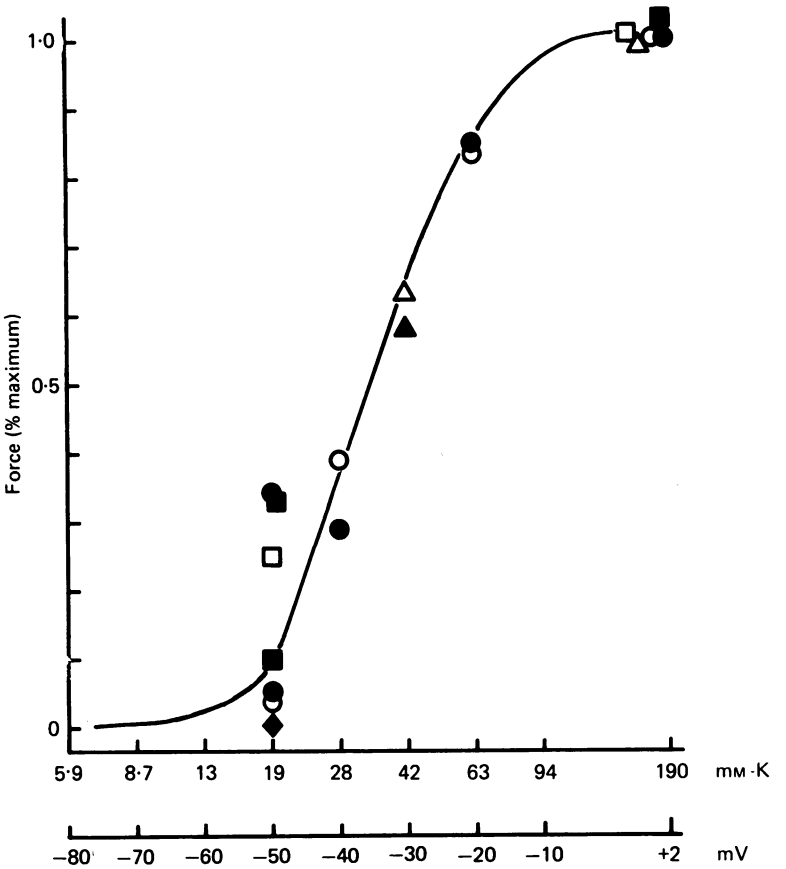

Fig. 5. The dependence of force development in percentage of maximum force (ordinate) on the external K concentration (logarithmic scale) or the corresponding membrane potential (abscissa). Filled symbols: 3·0–3·2 mM-Ca^{2+}; open symbols: 3·05–3·2 mM-Mg^{2+}, 10^{-9} M-Ca^{2+}. Methylsulphate and propionate as foreign anions. Different symbols indicate four different fibres. T = 21–22 °C.

In earlier experiments (Lüttgau *et al.* 1977) we used sulphate as the only foreign anion for preparing solutions with a constant K × Cl product and of equal osmolarity (cf. Hodgkin & Horowicz, 1959). $MgSO_4$ (4 mM) was added to both the Ca^{2+}-rich and the Ca^{2+}-poor (10^{-9} M) solution. $Tris^+$-Ringer as equilibration solution contained either 1·2 mM-Ca^{2+} and 3 mM-Mg^{2+} or 10^{-6} mM-Ca^{2+} and 3 mM-Mg^{2+}. Under these conditions the 'Ca^{2+}-free' activation curve was shifted to more positive values, an effect which was probably due to the rather strong binding of Mg^{2+} to sulphate. At high concentrations of sulphate free Mg was reduced to values below 1 mM. Therefore, we assume that the smaller contractures in Ca^{2+}-poor solutions, in particular

at zero membrane potential, may partly be explained by the 'low Mg^{2+}-effect' described in the previous section.

The effect of substituting Mg^{2+} for external Ca^{2+} upon the 'steady state' inactivation curve

In the 'steady state' the degree of contractile inactivation is related to the logarithm of the external K concentration or membrane potential by an S-shaped curve with inactivation starting at -50 mV (Hodgkin & Horowicz, 1960). In this section we investigated the effect of replacing external Ca^{2+} by Mg^{2+} upon this

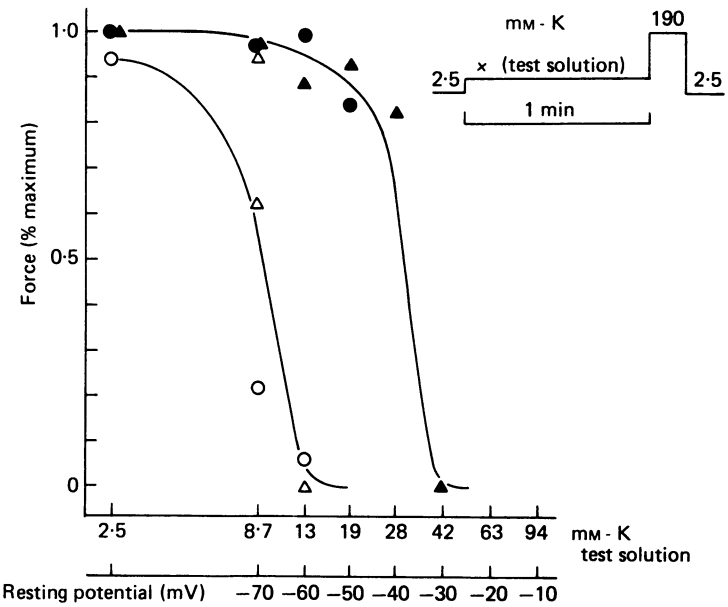

Fig. 6. 'Steady state' inactivation curve. The experimental procedure is shown in the inset. Force development upon full depolarization with 190 mM-K in percentage of maximum force is plotted on the ordinate against the potassium concentration applied in the test solution (logarithmic scale) or the corresponding membrane potential. Two different fibres. Filled symbols: 3·0–3·1 mM-Ca^{2+}; open symbols: 2·8–3·2 mM-Mg^{2+}, 10^{-9} M-Ca^{2+}. T = 20–22 °C.

relationship. The following procedure was adopted: The fibre was left for 5 min in a modified Ringer solution with $Tris^+$ instead of Na^+ (solution $B_{1,2}$). The concentrations of Ca^{2+} or Mg^{2+} were roughly the same as those used in the subsequent solutions. After this equilibration period the fibre was bathed in the test solution with an elevated K concentration at a constant K × Cl product (solution $L_{1,2}$–$Q_{1,2}$), which after 1 min was replaced by the contracture solution with 190 mM-K methylsulphate (solution $G_{1,2}$). Before and after two contractures at a test conditioning K concentration with either Ca^{2+} or Mg^{2+}, maximum force was checked by 'control' contractures (190 mM-K; solution G_1) without a preceding application of an elevated K^+ test solution. Maximum force was taken as the average maximum force of the control contractures.

In Fig. 6, force development upon full depolarization with 190 mM-K in percentage

of maximum force is plotted against the K concentration (logarithmic scale) in the test solution. It can be seen that the replacement of Ca^{2+} by about the same concentration of Mg^{2+} shifted the inactivation curve by 20–30 mV towards more negative potentials. A similar shift was observed in further experiments after replacing Ca^{2+} by Mg^{2+}, although the anion composition of the solutions differed from those shown in Table 1 (compare Lüttgau *et al.* 1977).

The present finding that replacing Ca^{2+} by Mg^{2+} leads to a large shift in the potential relation of steady-state inactivation while the activation curve remains unaltered could be expected from earlier measurements of Frankenhaeuser & Lännergren (1967). These authors reduced external Ca^{2+} from 2 to 0·2 mM, without replacing it by another divalent cation, and measured a shift of -27 and -5 mV for the inactivation and activation curve, respectively. A comparison with our results suggests that the main shift of the inactivation curve occurs at moderate Ca^{2+} concentrations.

The effect of Ca^{2+} removal upon the threshold for the increase in K conductance and the initiation of the action potential

Since Ca^{2+} deprivation caused a large shift in the potential dependence of contraction-inactivation towards more negative potentials we were interested to find out whether or not electrophysiological parameters, that is the threshold for the increase in K conductance or that for the initiation of an action potential, might be altered in a similar way.

The measurements of the K conductance were performed with two micro-electrodes in a point voltage clamp technique (Costantin, 1968; Kao & Stanfield, 1968) using whole semitendinosus muscles. Fig. 7 illustrates the experimental procedure. From the holding potential of -90 mV, depolarizing voltage steps of about 200 msec duration were applied and recorded on a storage oscilloscope together with the corresponding outward current. The total current–voltage relation, (I_t) evaluated after 200 msec (Fig. 7), reveals two mainly linear segments with a low and a high conductance, respectively. Membrane current density (I_m, dashed line, arbitrary units) was obtained from I_t by Cole's theorem (see Costantin, 1968). The threshold for the onset of delayed rectification was determined from the I_m plot. It was defined as the most negative potential at which the slope increased by at least 10%.

From the results collected in Table 2 it can be deduced that the threshold potentials in solutions with either Ca^{2+} or Mg^{2+} were nearly equal, whereby the value with Mg^{2+} in propionate Ringer indicates a trend to more positive potentials. In this context it should be noted, that the concentrations of divalent cations varied by up to 1 mM. According to voltage-clamp measurements by Costantin (1968; Fig. 2), a reduction in Ca^{2+} or Mg^{2+} by 1 mM in the concentration range of 2–4 mM of divalent cations causes a shift in threshold by only a few mV towards more negative potentials. Such a small difference would be lost in the dispersion of our data.

As an additional electrophysiological parameter, we measured the threshold for the initiation of a regenerative action potential. For this purpose we applied rectangular depolarizing current pulses of 100 msec duration intracellularly. The current was increased until an action potential occurred within a latency period between 10 and 30 msec. The threshold potential was defined as the intersection of the tangent along the electrotonic potential during the current pulse and that along

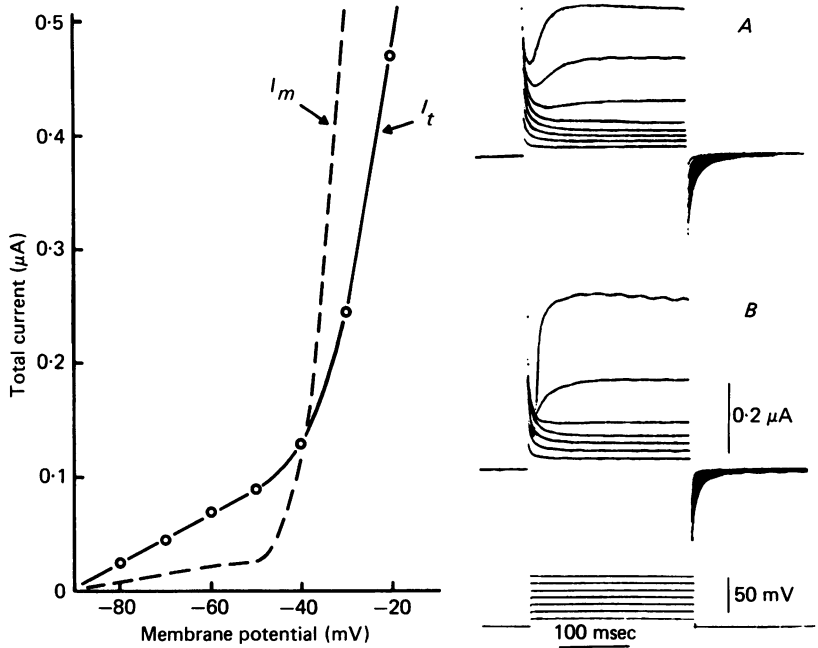

Fig. 7. Voltage-clamp experiments. *Right side*: membrane currents during step depolarizations from a holding potential of −90 mV. Hypertonic chloride Ringer solution with TTX, 1 mg/l. *A*: 4 mM-Ca^{2+}; *B*: 3 mM-Mg^{2+}, 10^{-9} M-Ca^{2+}. The potential steps shown below induced the current in *B*. T = 7 °C. The difference in conductance between fibre *A* and *B* was probably due to different fibre diameters (not measured). Even in high EGTA the input resistances were, if at all, only slightly smaller than those measured in Ringer.

Left side: current–voltage relationship of the experiment in *B*. The total clamp current (I_t), read at the end of a 200 msec pulse, was plotted on the ordinate against the corresponding membrane potential on the abscissa. From this curve membrane current density (I_m; arbitrary units) was derived using Cole's theorem (dashed line).

the upstroke of the action potential. The results are included in Table 2. Again, little change was observed in the threshold when Ca^{2+} was replaced by Mg^{2+}. Alterations in the threshold of K^+ and Na^+ currents, which could be observed when Cl^- was partly replaced by foreign anions, indicate specific anionic effects. They are probably related to similar effects of foreign anions which were found and discussed by Kao & Stanfield (1968).

Membrane potentials after Ca^{2+} deprivation

The effect of solutions with a low Ca^{2+} content upon the membrane potential was investigated in a series of control measurements with whole semitendinosus muscles.

In the equilibration solution B_2 (1–4 mM-Mg^{2+}, $Tris^+$ instead of Na^+, Ca^{2+}-free), we found no obvious decrease in resting potential as compared with normal Ringer. In high $EGTA^{2-}$ (solution F) the reduction in $[Cl]_o$ caused a transient depolarization. Thereafter we observed little or no depolarization for periods as long as 1–2 hr.

In 'Ca^{2+}-free' and $EGTA^{2-}$- containing solutions in which Ca^{2+} was not replaced by Mg^{2+} the resting potential declined steadily. After the application of 1–2 mM-$EGTA^{2-}$ it reached -80 to -50 mV within 20–60 min. Depolarization appeared to be less when Na^{+} was replaced by $Tris^{+}$.

Further tests showed that the membrane potential at elevated K concentrations (constant $K \times Cl$ product; solutions I to Q) remained (within 1–3 mV) the same when either Ca^{2+} or Mg^{2+} was present and when Cl^{-} was partially replaced by sulphate or propionate as impermeable anions.

TABLE 2. The threshold potential for the onset of delayed rectification (voltage clamp technique) and the regenerative action potential in solutions with different anions and either a normal or a low concentration of ionic Ca (Ca^{2+} replaced by Mg^{2+}). Means ± S. D. of means followed by number of fibres used in parentheses.

The bathing solution contained in addition 350 mM-sucrose to suppress contractile activity and, in the voltage clamp experiments, TTX, 1 mg/l., to block Na conductance. T = 5–7 °C

Solution	Threshold potential: Delayed rectification (mV)	Threshold potential: Action potential (mV)
Cl Ringer (modified soln A)		
4×10^{-3} M-Ca^{2+}	$-51{\cdot}9 \pm 3{\cdot}2$ (7)	$-50 \pm 3{\cdot}9$ (10)
3×10^{-3} M-Mg^{2+}, 10^{-9} M-Ca^{2+}	$-52{\cdot}9 \pm 2{\cdot}7$ (7)	$-46 \pm 3{\cdot}17$ (10)
Propionate Ringer (soln $E_{1,2}$)		
$3{\cdot}1 \times 10^{-3}$ M-Ca^{2+}	$-63{\cdot}1 \pm 3{\cdot}6$ (7)	$-42{\cdot}4 \pm 2{\cdot}5$ (8)
3×10^{-3} M-Mg^{2+}, 3×10^{-9} M-Ca^{2+}	$-60{\cdot}3 \pm 4{\cdot}8$ (7)	$-43{\cdot}7 \pm 2{\cdot}8$ (8)
Gluconate Ringer (soln $D_{1,2}$)		
$1{\cdot}6 \times 10^{-3}$ M-Ca^{2+}	$-66{\cdot}9 \pm 3{\cdot}6$ (7)	$-51{\cdot}1 \pm 3{\cdot}7$ (9)
$2{\cdot}7 \times 10^{-3}$ M-Mg^{2+}, 3×10^{-9} M-Ca^{2+}	$-64{\cdot}9 \pm 4{\cdot}4$ (7)	$-47{\cdot}4 \pm 3{\cdot}6$ (9)

DISCUSSION

The present results agree with those of Armstrong, Bezanilla & Horowicz (1972) in showing that contractile activity in skeletal muscle fibres could still be maintained in 'Ca^{2+}-free' solutions which contained 1–5 mM-$EGTA^{2-}$. When, however, $EGTA^{2-}$ was further increased to 80 mM, twitch force declined within a few minutes to a small fraction of the original value. The main effect of Ca^{2+} deprivation, measured at low $EGTA^{2-}$, was a shift of the steady-state inactivation curve towards more negative potentials. It can be assumed that an increase in $EGTA^{2-}$ caused a further reduction in tubular [Ca^{2+}] and an additional shift of the inactivation curve. The fall in twitch force in Mg^{2+}- containing high $EGTA^{2-}$ solutions might be explained by the assumption that inactivation of contraction largely outstripped the activation process when the muscle was depolarized.

The immediate block of contraction activation in Mg^{2+}-free solutions with a high $EGTA^{2-}$ concentration, as observed by Barrett & Barrett (1978) and confirmed in our experiments, must have been due to the omission of Mg^{2+}. Besides other deleterious effects, which were indicated by a loss of ion selectivity of the membrane

(depolarization, reduction in input resistance), the omission of divalent cations in the external solution should cause an increase in external surface charge density. This effect could produce a further shift of the inactivation curve towards more negative potentials and lead to a complete inactivation of EC-coupling.

The fast action of $EGTA^{2-}$ suggests that it readily diffuses into the T-system and that, at least in high $EGTA^{2-}$, tubular $[Ca^{2+}]$ falls below the normal myoplasmic value. Nevertheless, after 1 hr tetanic force still reached 20–85% of the original value as long as Mg^{2+} was present. A passive influx of Ca^{2+} across the transverse tubular membrane can, therefore, not be regarded as a prerequisite for the release of Ca^{2+} from the sarcoplasmic reticulum. Further arguments against a direct role of the Ca^{2+} influx in activation have recently been put forward by other authors. Palade & Almers (1978) found that the influx of Ca^{2+}, first measured with electrophysiological methods by Beaty & Stefani (1976*a*), is turned on rather slowly upon depolarization, so that an effective Ca^{2+} influx cannot be expected during the early phase of contraction activation. In addition, Miledi, Parker & Schalow (1977) described experiments in which they showed, with the help of the Ca indicator dye arsenazo III, that at short depolarizing pulses the internal release of Ca^{2+} is unaffected by conditions designed to reduce or abolish the entry of external Ca^{2+} (e.g. at voltage clamp potentials more positive than the Ca^{2+}-equilibrium potential).

The large effect of Ca^{2+} deprivation upon the potential dependence of inactivation, while further parameters are not affected, deserves a more detailed discussion. Two earlier findings suggest that the potential relation of inactivation depends on internal $[Ca^{2+}]$: (1) dantrolene Na^+ reduced $[Ca^{2+}]_i$ (Hainaut & Desmedt, 1974) and caused a shift of the inactivation curve towards more negative potentials (Takauji & Nagai, 1977); (2) caffeine induced an increase in $[Ca^{2+}]_i$ and a shift of the inactivation curve towards more positive potentials (Lüttgau & Oetliker, 1968). Consequently, the shift of the inactivation curve towards more negative potentials under Ca^{2+} deprivation could be the result of a decrease in $[Ca^{2+}]$ near the inner rather than the outer side of the tubular membrane. This process could occur between the transverse tubules and the sarcoplasmic reticulum.

Inactivation is not only influenced by $[Ca^{2+}]$ but also by temperature. Caputo (1972*a*) observed a strong shift of the inactivation curve towards negative potentials when the temperature was lowered. This effect suggests that metabolic processes are involved in inactivation. Considering the results discussed above the proposed potential shift of inactivation could be explained by a local increase in negative surface charges (perhaps produced by a Ca^{2+}-dependent phosphorylation of internal membrane sites).

The suggested $[Ca^{2+}]_i$ dependence of inactivation allows a new interpretation of the time course of contractures which we wish to present for further discussion. Depolarization of the tubular membrane causes the release of Ca^{2+} from the adjoining sarcoplasmic reticulum. This coupling process remains under the control of the membrane potential of the tubular wall as has been shown experimentally by Caputo (1972*b*) and worked out theoretically in the model described by Chandler, Rakowski & Schneider (1976). The early increase in $[Ca^{2+}]_i$ causes a shift of the inactivation curve towards more positive potentials and thus a retardation of inactivation (plateau of the contracture). This might be a regenerative process,

since a retardation of inactivation allows the Ca^{2+} release to continue, thus resulting in an increase in $[Ca^{2+}]_i$ and a further shift of the inactivation curve. In pursuing this idea it could be assumed that relaxation occurs if Ca^{2+} uptake by the sarcoplasmic reticulum becomes larger than Ca^{2+} influx plus Ca^{2+} release ('exhaustion' of Ca^{2+} release, delayed onset of active Ca^{2+} uptake; cf. Lüttgau & Moisescu, 1978). This process might likewise be regenerative, since a decrease in $[Ca^{2+}]_i$ would shift the inactivation curve towards more negative potentials, resulting in a further reduction of Ca^{2+} release.

In addition we assume that the regenerative Ca^{2+} release is identical with the Ca^{2+}-induced Ca^{2+} release phenomena observed in skinned fibres by Endo, Tanaka & Ogawa (1970) and Ford & Podolsky (1970). Endo (1977) recently suggested that the latter effect is unlikely to occur under physiological conditions. He argues that the concentration necessary to induce the effect, namely 3×10^{-4} M-Ca^{2+}, will not be reached by a Ca^{2+} influx from the T-system. In our proposal, however, it is assumed that the regenerative process starts after an initial activation has caused the release of Ca^{2+} from the sarcoplasmic reticulum. Under these conditions the necessary Ca^{2+} concentration may well be attained.

As demonstrated by the high $EGTA^{2-}$ experiments the influx of Ca^{2+} is not necessary for the *initiation* of contraction. Our experiments, however, suggest that the delayed Ca^{2+} influx supports the coupling process during longer-lasting activity. Contractures became shorter when Ca^{2+} was replaced by Mg^{2+} in the external solution or when the influx of Ca^{2+} was reduced by L-D600. In addition Blinks, Rüdel & Taylor (1978) showed that in Ca^{2+}-free-solutions the aequorin response remained unaltered during twitches but became smaller during a tetanus. In summary we suggest that the coupling between depolarization of the T tubular membrane and the release of Ca^{2+} from the sarcoplasmic reticulum depends on the concentration of internal free Ca and on metabolic processes. Several phenomena like phasic responses (Costantin, 1971), regenerative processes (Endo, 1977), staircase effects as observed in aequorin experiments (Blinks *et al.* 1978) and force development during two pulse voltage clamp experiments (Bezanilla, Caputo & Horowicz, 1972; see Caputo, 1978) could be interpreted reasonably well by the proposed model. Our experiments, however, do not exclude alternative explanations (see Hodgkin & Horowicz, 1960). At present, it is still not possible to decide whether a spontaneous relaxation or a fall in the aequorin luminescent response is due to an exhaustion of Ca stores (cf. Winegrad, 1968, 1970) or a potential dependent inactivation process.

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