J. Physiol. (1981), **317**, *pp.* 303–316
With 6 text-figures
Printed in Great Britain

EFFECTS OF EXTERNAL CALCIUM REDUCTION ON THE KINETICS OF POTASSIUM CONTRACTURES IN FROG TWITCH MUSCLE FIBRES

BY G. COTA AND E. STEFANI*

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

(*Received 3 October 1980*)

SUMMARY

1. The amplitude and time course of K contractures (Cl^- constant) of single twitch muscle fibres of the frog have been analysed in three external Ca^{2+} concentrations.

2. The resting potential, effective resistance, threshold for the Na current and K-induced depolarizations were not modified by replacing 1·8 mM-Ca^{2+} by 3 mM-Mg^{2+} in absence (*low-Ca* saline: 3–6 μM-Ca^{2+}) or in presence of 5 mM-EGTA (*Ca-free* saline: $\leqslant 10^{-9}$ M-Ca^{2+}).

3. The tension development during the initial phase of K contractures was independent of external Ca^{2+} while the amplitude, the duration and the time constant of spontaneous relaxation decreased progressively as Ca^{2+} concentration was diminished.

4. When the concentration of Mg^{2+} was increased to 5 mM in *Ca-free* saline K contractures were slower and smaller than those in 3 mM-Mg^{2+}.

5. In *Ca-free* saline the activation curve (peak tension *vs.* logarithm of external K^+ concentration) shifted by 3–5 mV towards more positive potentials while the inactivation curve (peak tension of the test contracture *vs.* logarithm of external K^+ concentration during the conditioning period) shifted by 16–18 mV towards more negative potentials. Both curves became steeper in *Ca-free* saline.

6. The effects of external Ca^{2+} reduction were not modified by replacing all chloride for methanesulphonate.

7. Direct effects of external Ca^{2+} on excitation–contraction coupling during K contractures could involve the inward Ca current and/or specific interactions between external Ca^{2+} ions and the coupling mechanism.

INTRODUCTION

It is well established that twitch muscle fibres of the frog contract upon electrical stimulation when exposed to very low Ca^{2+} concentrations ($\sim 10^{-9}$ M), obtained by the addition of 1–5 mM-ethyleneglycol-bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) (Armstrong, Bezanilla & Horowicz, 1972; Sandow, Pagala & Sphicas, 1975; Blinks, Rüdel & Taylor, 1978). However, in similar conditions K contractures become shorter and smaller (Stefani & Chiarandini, 1973; Lüttgau & Spiecker, 1979) and the

* To whom reprint requests should be addressed.

'steady-state' inactivation curve for K contractures shifts towards more negative potentials (Lüttgau & Spiecker, 1979). Moreover, in Mg^{2+} containing high EGTA (80 mM) solutions, the twitch amplitude falls within minutes to a small fraction of the original value (Lüttgau & Spiecker, 1979). Furthermore, reduction of external Ca^{2+} increases the mechanical threshold for short pulses ($\leqslant$ 20 msec) (Chiarandini, Sánchez & Stefani, 1980). These findings suggest that external Ca^{2+} may have some role in excitation–contraction coupling.

In the present experiments, we have further analysed the amplitude and time course of K contractures in three external Ca^{2+} concentrations: 1·8 mM; 3–6 μM and $\leqslant 10^{-9}$ M. For this purpose 1·8 mM-Ca^{2+} was replaced by 3 mM-Mg^{2+} to prevent changes of the surface potential and other electrical properties (Cota & Stefani, 1979).

METHODS

Muscles of *English* frogs *Rana temporaria* and of *West Mexican* frogs *Rana pipiens* were used throughout these experiments. Single fibres were dissected from tibialis anterior muscle (Chiarandini & Stefani, 1973). Sartorius and cutaneous pectoris muscles were used as whole muscle preparations. All experiments were made at room temperature (22–24 °C). *R. temporaria* were cold-adapted (4–6 °C) and fed once a fortnight. *R. pipiens* were kept at room temperature or at 4–6 °C and fed once a week.

Electrical recording. Membrane potential, thresholds of Na current and of contraction, and effective resistance (R_{eff}) were measured as described by Chiarandini *et al.* (1980). Briefly, a two-micro-electrode voltage-clamp system with a 100 μsec time constant was used. Fibres were clamped at a holding potential of −90 mV. Mechanical threshold was optically determined. R_{eff} was calculated from the ratio between a 10 mV step and the total current injected, measured at the end of a 30–50 msec pulse.

Mechanical recording. Muscle fibres, once isolated, were allowed to rest for 45–90 min and then mounted in the experimental chamber and stretched to 1·1 of their slack length. The experimental chamber consisted of a channel 2 mm wide and 3 mm high (0·25 ml. capacity) into which solutions could be introduced. The fibres were freely suspended in the channel as described by Costantin (1971). One tendon was attached to the anode pin of an isometric RCA 5734 force transducer and the other one was fixed to the bottom of the channel by a stainless-steel hook. This end of the fibre was located 5–7 mm from the site of saline inflow. The mechanical output was registered with a rectilinear (Gould Brush model 220) and/or a curvilinear (Grass model 79 D) pen recorder. At the end of the experiment the fibre diameter was measured with a microscope by using an eye-piece micrometer at several places along the fibre, which was considered as circular in cross section. The mean of these values was taken (Frankenhaeuser & Lännergren, 1967).

Solutions. Three basic solutions were used: a *control* saline with (mM) NaCl 117·5, KCl 2·5, $CaCl_2$ 1·8; a *low-Ca* saline prepared by omitting $CaCl_2$ from the *control* saline with 3 mM-$MgCl_2$ added, and a *Ca-free* saline prepared by adding 5 mM-Na_2EGTA to the low-Ca saline. In the *low-Ca* saline a Ca^{2+} contamination of 3–6 μM was measured by using the pH-metric method developed by Moisescu & Pusch (1975). The calculated concentration of ionized Ca^{2+} in the *Ca-free* saline was $\leqslant 10^{-9}$ M (Stefani & Chiarandini, 1973). These three basic solutions were made *Cl-free* by replacing all Cl^- by methanesulphonate ($CH_3SO_3^-$) (methanesulphonic acid, Eastman Organic Chemicals, Rochester, N.Y.). Determinations with a Ca^{2+}-selective electrode (Orion 93–20) demonstrated that Ca^{2+} was practically fully ionized in the *control Cl-free* saline (Kenyon & Gibbons, 1977). The ionized Ca^{2+} in sulphate salines with 8 mM-$CaSO_4$ (solution E from Hodgkin & Horowicz, 1959) was 4 mM. The K^+ concentration in the *Cl* and *Cl-free* solutions was increased isotonically by replacing K^+ by Na^+. The solutions were buffered to pH 7·40 ± 0·01 with 2 mM-imidazole Cl. Solutions in contracture experiments contained 10^{-7} M-tetrodotoxin (Sigma Chemical Co., St. Louis, MO.). Some experiments were performed with depolarizing solutions with constant $K^+ \times Cl^-$ product made up as described by Lüttgau & Spiecker (1979). Deionized-bidistilled water was used throughout the experiments.

Solution replacement. During K contractures, special care was taken to assure a thorough

exchange of the solution in the chamber by applying the different salines with continuous flow. Muscle fibres were pre-exposed to the tested solutions during 60–90 sec with a continuous perfusion of 1–1·5 ml./sec. Thereafter, K-contracture solutions were introduced at 2–3 ml./sec. Tests with Methylene blue indicated that about 99% of the solution in the chamber was exchanged in less than one second. K-contracture solutions were initially washed out with *control* saline at 1–1·5 ml./sec (2–5 min). Subsequently, between successive contractures, the fibres were allowed to recover in *control* saline flowing at 3–5 ml./min. In most cases, muscle fibres were briefly exposed to the contracture solution (4–6 sec) at intervals of 15–25 min. When the decay phase of the K contracture was studied, the exposure time was increased until spontaneous relaxation was nearly complete (8–12 sec). In these cases, contractures were induced at intervals of 40 min or longer. In this way fibres were kept in good condition for 8–12 hr (Hodgkin & Horowicz, 1960; González Serratos, 1975).

Data analysis. The experimental data were fitted to the proposed function according to the Patternsearch routine (Colquhoun, 1971), which minimizes the squared differences between the data and the function. The Patternsearch routine was run in Fortran IV on a microcomputer (SOL 20, Processor Technology). The fitting error is the sum of the squared differences divided by the number of observations. Values are given as mean ± standard error of the mean with the number of observations between parentheses. Statistical significance was determined following the Student's *t* test.

RESULTS

Effect of Ca-free saline on electrical parameters

In frog sartorius (*R. temporaria*) Chiarandini *et al.* (1980) found that the replacement at 3 °C of *control* saline by *Ca-free* saline identical to ours did not change the resting potential ($E_{r.p.}$), R_{eff} and threshold for Na current. Shifts of the latter parameter were assumed to reflect corresponding changes of the surface potential (Hille, Woodhull & Shapiro, 1975). We confirmed these observations at room temperature. The values of threshold for the Na current, $E_{r.p.}$ and R_{eff} were respectively: $-55{\cdot}4 \pm 0{\cdot}3$ mV (10), $-86{\cdot}6 \pm 1{\cdot}7$ mV (10) and $0{\cdot}41 \pm 0{\cdot}06$ MΩ (10) in *control* saline, and $-55{\cdot}8 \pm 0{\cdot}3$ mV (6), $-88{\cdot}2 \pm 0{\cdot}9$ mV (6) and $0{\cdot}39 \pm 0{\cdot}05$ MΩ (6) in *Ca-free* saline. A similar conclusion was reached when using fibres from *R. pipiens*. The lack of effect of *Ca-free* saline on $E_{r.p.}$ was also confirmed in three isolated muscle fibres which were perfused with *Ca-free* saline at 2–3 ml./sec.

Since K contractures are produced by the depolarization which results from the increase of external $[K^+]$, it was necessary to ascertain that the K-induced depolarizations in *control* and *Ca-free* salines were similar. We measured the $E_{r.p.}$ with different K^+ concentrations (2·5–120 mM), keeping Cl^- constant. The experiments were performed in small bundles of muscles from *Rana temporaria* and the measurements were carried out 2–10 min after solution replacement. In six fibres the $E_{r.p.}$ in *control* saline and in *Ca-free* saline was respectively: $-92{\cdot}2 \pm 1{\cdot}2$ mV and $-93{\cdot}0 \pm 1{\cdot}4$ mV (2·5 mM-K^+); $-55{\cdot}5 \pm 0{\cdot}9$ mV and $-57{\cdot}2 \pm 1{\cdot}1$ mV (20 mM-K^+); $-38{\cdot}8 \pm 1{\cdot}1$ mV and $-40{\cdot}0 \pm 1{\cdot}3$ mV (40 mM-K^+); $-27{\cdot}5 \pm 1{\cdot}5$ mV and $-28{\cdot}7 \pm 1{\cdot}6$ mV (70 mM-K^+); $-13{\cdot}7 \pm 1{\cdot}0$ mV and $-15{\cdot}0 \pm 1{\cdot}2$ mV (120 mM-K^+). Equivalent results were obtained in sartorius muscle from *R. pipiens*. In *Cl-free* saline the electrical properties were not modified when 1·8 mM-Ca^{2+} was replaced by 3 mM-Mg^{2+} with 5 mM-EGTA added.

Effect of external Ca^{2+} reduction on the time course of K contractures

The time course of contractures induced by 80 mM-K^+ in *control*, *low-Ca* and *Ca-free* salines was examined. Isolated fibres were exposed for 90 sec to a continuous flow (1–1·5 ml./sec) of a solution with the tested Ca^{2+} concentration and with 2·5 mM-K^+, before the perfusion with the corresponding contracture solution. Previous experiments had shown that a 60 sec exposure was sufficient to produce maximum effect. Fig. 1 shows K contractures in two isolated fibres (*A* and *B*) from *R. pipiens* in *control*

saline (*a*), *low-Ca* saline (*b*) and *Ca-free* saline (*c*). It can be seen that when external Ca^{2+} is reduced the peak tension drops, the duration is shortened and the spontaneous relaxation rate increases.

The initial 90 % of the rising phase of the tension can be empirically described by the function:

$$T(t) = T_\infty (1-\exp(-t/\tau_a))^n, \quad (1)$$

where $T(t)$ is tension, T_∞ the tension when $t \to \infty$ if spontaneous relaxation was not present and τ_a the time constant of the tension rise. After finding that the best fit

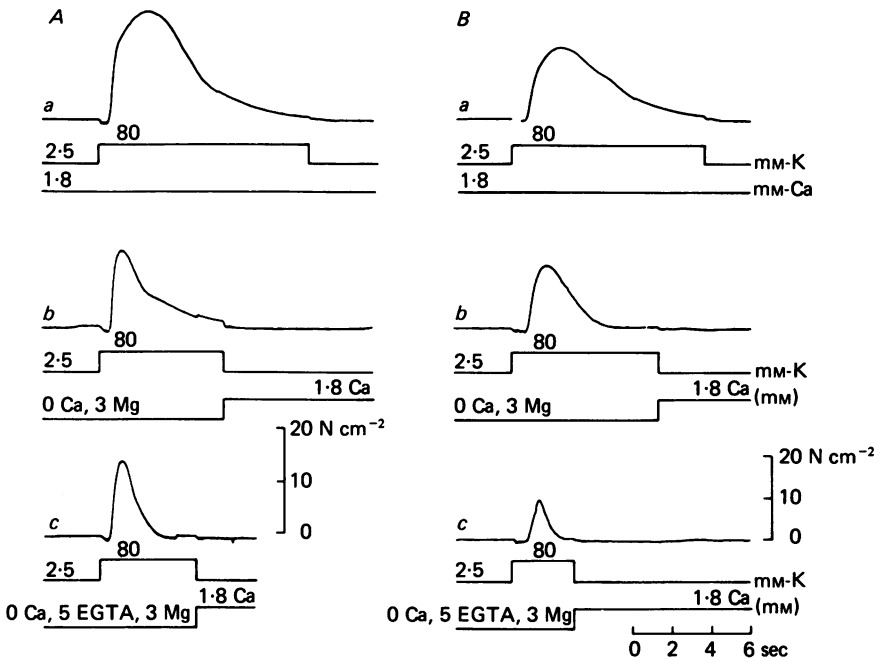

Fig. 1. Effects of external Ca^{2+} reduction on the amplitude and time course of contractures with 80 mM-K^+. Two different fibres from *R. pipiens*. *A*, diameter = 77 μm; *B*, diameter = 96 μm; *a* is in *control* saline, *b* in *low-Ca* saline and *c* in *Ca-free* saline. Contractures were obtained in the following sequence: *A*, *c–b–a*; *B*, *c–b–a*.

for n was 2, T_∞ and τ_a were fitted. In five fibres from *R. pipiens* τ_a was: $0{\cdot}36 \pm 0{\cdot}04$ sec in *control* saline, $0{\cdot}34 \pm 0{\cdot}03$ sec in *low-Ca* saline and $0{\cdot}36 \pm 0{\cdot}04$ sec in *Ca-free* saline. T_∞ and the measured peak tension had similar values in *control* saline. When external Ca^{2+} was reduced, T_∞ remained unmodified, while the peak tension amplitude diminished to 80 ± 3 % in *low-Ca* saline and to 53 ± 7 % in *Ca-free* saline ($P < 0{\cdot}01$ for paired data). The lack of effect of external Ca^{2+} reduction on τ_a and T_∞ indicates that the initial phase of K contracture is independent of external Ca^{2+}. As mentioned before, K contractures were shortened when external Ca^{2+} was reduced. The duration at half peak tension was: $3{\cdot}8 \pm 0{\cdot}3$ sec in *control* saline, $2{\cdot}1 \pm 0{\cdot}2$ sec in *low-Ca* saline and $0{\cdot}9 \pm 0{\cdot}1$ sec in *Ca-free* saline ($P < 0{\cdot}01$ for paired data).

Fig. 2 shows the semilogarithmic plot of the decay of the K contracture *vs.* time for the fibres shown in Fig. 1. Zero time corresponds to the time when the decay phase

started. Although the spontaneous relaxation phase follows a complex time course (Caputo & Fernández de Bolaños, 1979), it can be fitted relatively well with a single exponential if the initial and last 10 % of the decay phase are not included. The time constant of relaxation (τ_r) for the five fibres studied was: $2{\cdot}1 \pm 0{\cdot}3$ sec in *control* saline, $1{\cdot}5 \pm 0{\cdot}2$ sec in *low-Ca* saline ($P < 0{\cdot}10$ for paired data), and $0{\cdot}5 \pm 0{\cdot}1$ sec in *Ca-free* saline ($P < 0{\cdot}01$ for paired data). Similar results on the parameters studied were obtained by using different K^+ concentrations.

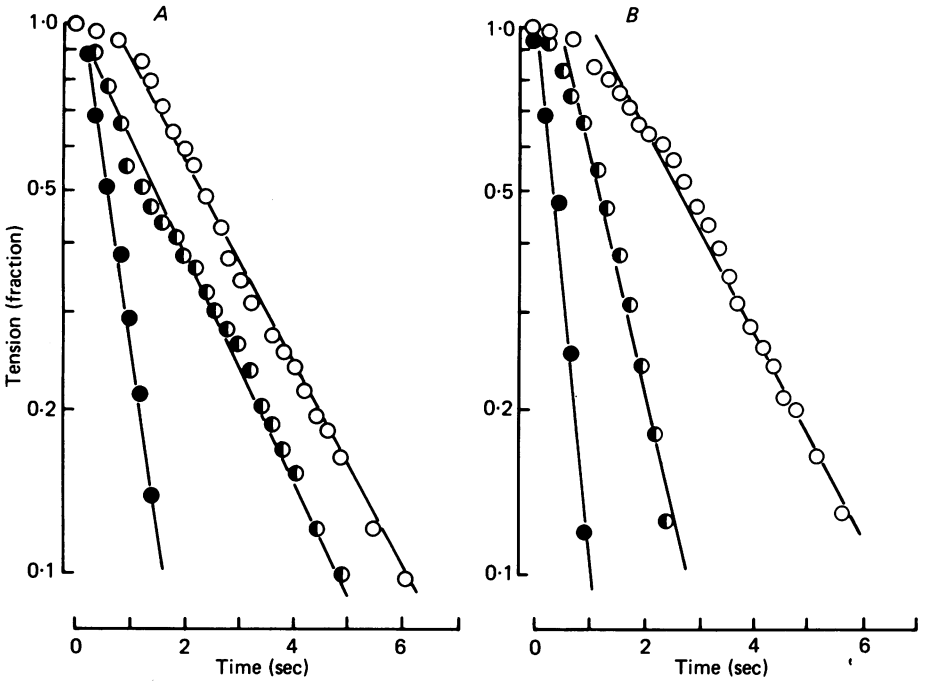

Fig. 2. Time course of the decay of K contractures (spontaneous relaxation phase) in *control* saline (○), *low-Ca* saline (◐) and *Ca-free* saline (●). Semilogarithmic plots. Two different fibres from *R. pipiens*.

Effect of Ca-free saline on the activation curve

We have studied the effect of the *Ca-free* saline on the *activation curve* (peak tension *vs.* logarithm of external K^+ concentration) in five other fibres of *R. pipiens* and in four of *R. temporaria*. We selected single fibres with similar diameters, since the degree of depolarization needed to produce maximal tension is dependent on the fibre diameter (González Serratos, 1975). Out of thirteen fibres of *R. temporaria* tested, the maximal tension (120 mM-K^+) decreased in eight of them to 83 ± 5 % in *Ca-free* saline. In *R. pipiens* the maximal tension decreased to 81 ± 4 % in seven fibres out of eight tested ($P < 0{\cdot}01$ for paired data).

Fig. 3 shows K contractures in *control* (*A*) and in *Ca-free* salines (*B*) from a fibre of *R. pipiens*. For a given K^+ concentration, the contractures in *Ca-free* saline were smaller and shorter. Fig. 4 shows the relationship between the peak tension and the logarithm of the K^+ concentration ([K]) in *control* saline (○) and *Ca-free* saline (●);

A is for a fibre of *R. temporaria* and *B* is for the fibre shown in Fig. 3 (*R. pipiens*). We have fitted the experimental points to

$$T([K]) = \frac{T_m}{1+\exp(-(\ln[K]-\ln[\overline{K}])/k)}, \quad (2)$$

where $T([K])$ is the peak tension measured in the different K^+ concentrations used, T_m is the maximal tension obtained which corresponds to the peak tension in 120 mM-K^+ and was taken as 1·00 in control saline, $[\overline{K}]$ is the K^+ concentration

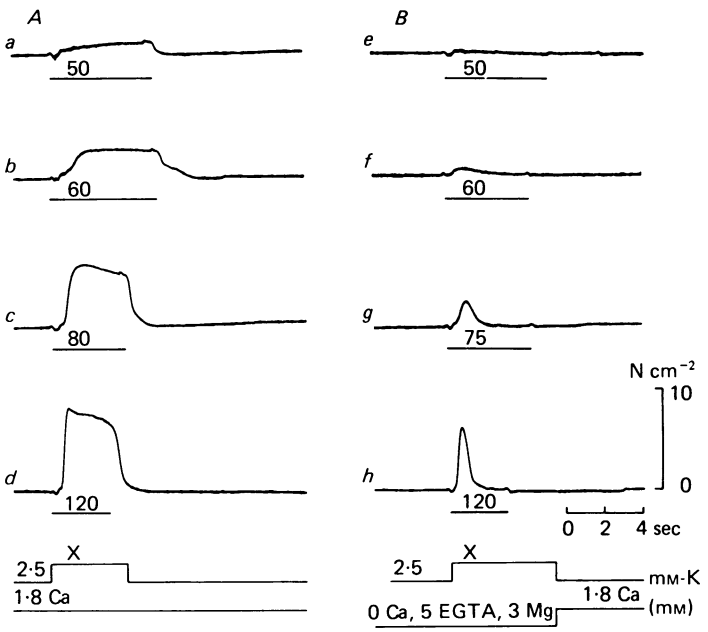

Fig. 3. Contractures obtained with brief exposures to different K^+ concentrations (indicated under each record) in a single fibre, diameter = 81 μm of *R. pipiens* in *control* (*A*) and *Ca-free* salines (*B*).

corresponding to the half amplitude of the *activation curve* and k is related to the steepness of the curve. This is a general equation used to describe the voltage dependence of activation processes (Hodgkin & Huxley, 1952; Schneider & Chandler, 1973). The continuous lines in Fig. 4*A* and *B*, represent the best fit of the data to eqn. (2). The activation parameters for nine fibres are summarized in Table 1. The results demonstrate that when muscle fibres are exposed to *Ca-free* saline, $[\overline{K}]$ increases and k decreases. In conclusion, the activation curve in *Ca-free* saline shifted towards more positive potentials and was steeper. Similar effects of external Ca^{2+} reduction were obtained in *Cl-free* solutions (Table 1).

The activation curves in *Cl-free* salines shifted to the left in relation to the curves in *Cl* solutions (see Table 1, *B*). This can be related to the smaller depolarizations obtained with different external K^+ concentration in *Cl* salines due to the muscle membrane Cl^- permeability (Hodgkin & Horowicz, 1959). Accordingly, the mechanical threshold, optically determined through the micro-electrode voltage-clamp technique (100 msec pulses, cutaneous pectoris muscle of *R. temporaria*) was not modified when Cl^- was replaced for methanesulphonate: $-52{\cdot}4 \pm 0{\cdot}7$ mV (5) in *Cl* saline and $-53{\cdot}8 \pm 0{\cdot}8$ mV (6) in *Cl-free* saline.

TABLE 1. Effects of *Ca-free* saline on the activation curve. Best fit parameters for fibres from *R. temporaria* (*A*) and for *R. pipiens* (*B*). The differences of the parameters in *control* and in *Ca-free* salines (*Cl* solutions) are significant for paired data at the indicated level. *R. temporaria*: T_m $0.05 < P < 0.10$; $[\overline{K}]$ $P < 0.1$; k $0.01 < P < 0.2$. *R. pipiens*: T_m $P < 0.02$; $[\overline{K}]$ $P < 0.05$; $kP < 0.01$. T_m and k for all fibres $P < 0.005$.

			1·8 mMCa^{2+}				Ca-free				
	Cell	Diameter (μm)	$[\overline{K}]$ (mM)	k	Fitting error (10^{-3})	n	T_m	$[\overline{K}]$ (mM)	k	Fitting error (10^{-3})	n
Cl solutions											
(*A*)	E·01	80	39·1	0·151	1·10	6	1·00	50·8	0·122	0·66	6
	E·03	85	48·1	0·140	0·18	7	0·71	67·2	0·145	0·24	7
	E·04	91	51·7	0·184	0·22	6	0·89	64·3	0·131	0·26	5
	E·05	105	43·9	0·211	1·38	6	0·80	56·0	0·110	0·42	6
	Mean ± S.E. of mean	91	45·7	0·17	—		0·85	59·6	0·13	—	
		±5	±2·4	±0·01	—		±0·05	±3·3	±0·01	—	
(*B*)	E·06	97	75·1	0·164	0·82	6	0·83	87·4	0·113	1·43	7
	E·07	83	81·3	0·115	0·90	4	0·92	83·7	0·050	0·01	4
	E·08	81	67·1	0·162	0·24	6	0·77	80·1	0·121	0·48	5
	E·09	102	71·3	0·160	1·23	8	0·60	77·0	0·107	0·04	8
	E·10	104	76·0	0·140	0·45	8	0·71	79·8	0·088	0·01	7
	Mean ± S.E. of mean	94	74·1	0·15	—		0·77	81·6	0·10	—	
		±5	±2·1	±0·01	—		±0·05	±1·6	±0·01	—	
Cl-free solutions											
(*B*)	I·01	105	34·0	0·223	1·42	7	0·90	48·0	0·200	0·42	5
	I·03	81	25·0	0·197	0·09	5	0·90	35·7	0·141	0·14	5

Lüttgau & Spiecker (1979) have analysed the effect of external Ca^{2+} reduction upon contractures induced by solutions with constant $K^+ \times Cl^-$ product made up by replacing chloride for propionate and methylsulphate. They found little or no change in the shape and potential dependence of the activation curve when 3 mM-Ca^{2+} was replaced by 3 mM-Mg^{2+} and 2·5 mM-EGTA. We confirmed their results in three fibres from *R. temporaria* using solutions with the same ionic composition.

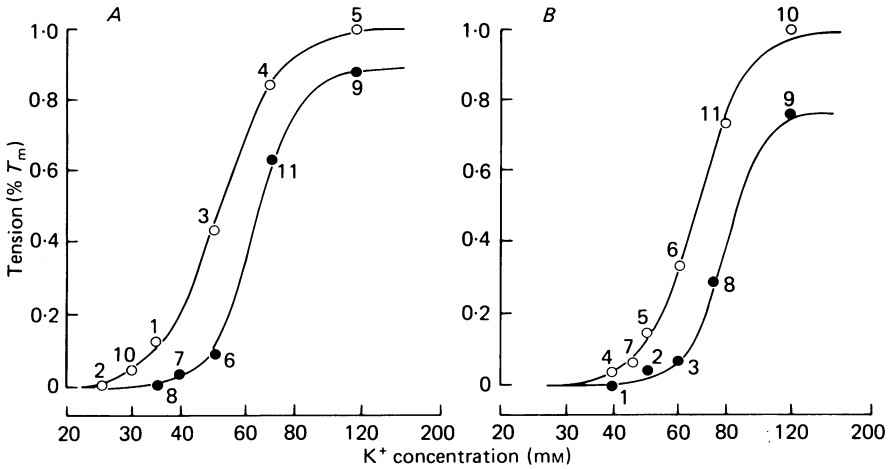

Fig. 4. Activation curves. Relationship between peak tension and external K^+ concentration in *control* (○) and *Ca-free* salines (●). *A* in a fibre from *R. temporaria*. *B* in the fibre shown in Fig. 3 (*R. pipiens*). Numbers show the sequence in which contractures were elicited. The smooth curves were drawn according to eqn. (2). The best fit parameters are shown in Table 1.

Since the activation curves of Fig. 4 suggest that the mechanical threshold is increased in *Ca-free* saline, we carried out experiments to study, in detail, the tension development close to mechanical threshold. In two fibres of *R. pipiens* the K^+ concentration that elicits minimum detectable tension (~0·3 mg) increased reversibly in *Ca-free* saline from 40 to 45 mM-K^+ and from 45 to 50 mM-K^+. This corresponds to a change of about 2 mV in membrane potential.

The parameter $[\overline{K}]$ of the activation curves was found to be lower in *R. temporaria* than in *R. pipiens* (Table 1). Since *R. temporaria* were cold-adapted while *R. pipiens* were kept at room temperature, it was thought that the difference in $[\overline{K}]$ was possibly a temperature adaptation effect (Kovács & Schneider, 1978; Gilly & Hui, 1980). We therefore performed experiments in fibres from cold-adapted *R. pipiens* (2 weeks at 4–6 °C). Cold adaptation significantly reduced $[\overline{K}]$ from 74·1 ± 2·1 mM to 59·7 ± 2·1 mM ($P < 0·02$), without affecting k (0·15 ± 0·01 and 0·17 ± 0·01).

Effect of external Ca^{2+} reduction on the inactivation curve

Lüttgau & Spiecker (1979) have demonstrated a 20–30 mV shift towards more negative potentials of the inactivation curve when external 3 mM-Ca^{2+} is replaced by 3 mM-Mg^{2+} and 2·5 mM-EGTA is added. We have extended these observations in our ionic conditions.

The inactivation of K contractures was studied with the following procedure (Frankenhaeuser & Lännergren, 1967): the fibres were initially perfused at 1–1·5 ml./sec with *control*, *low-Ca* or *Ca-free* salines with 2·5 mM-K^+ for 1 min. Subse-

quently, the corresponding conditioning solution (2·5–45 mM-K^+) was applied for 1 min and finally the test contracture (70 or 120 mM-K^+) was elicited (Fig. 5 inset). To analyse these curves it has to be considered the fact that 1 min of conditioning period does not reach steady state (Frankenhaeuser & Lännergren, 1967). The points in the inactivation curves are the peak tensions of test K contractures obtained with different conditioning solutions, expressed as a fraction of the peak tension of the test

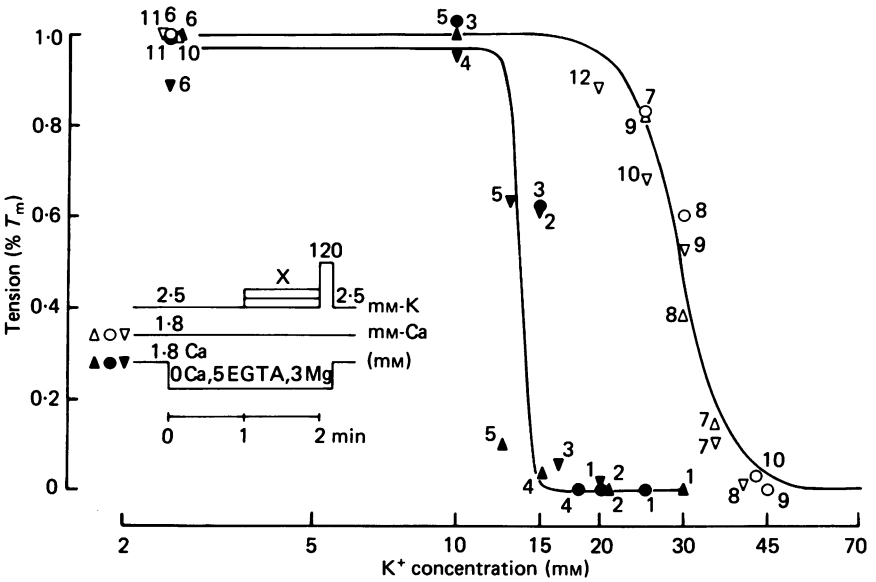

Fig. 5. Inactivation curves. Relationship between maximal tension (120 mM-K^+) and external K^+ concentration during the conditioning period in two fibres from *R. temporaria* (○–●, △–▲) and one fibre from *R. pipiens* (▽–▼) (130, 90 and 100 μm in diameter, respectively) in *control* (open symbols) and *Ca-free* salines (filled symbols). The inset shows the experimental procedure. Measurements were made in the sequence indicated by numbers. The smooth curves were drawn according to eqn. (3) which was fitted to data at K^+ concentration higher than 8 mM. The best fit parameters are given in the text.

contracture in *control* saline with a 2·5 mM-K^+ prepulse. The experimental points were fitted to the relation:

$$T([K]) = \frac{T_m}{1+\exp((\ln[K]-\ln[\bar{K}])/k)}. \quad (3)$$

The parameters of this equation are the same as those of eqn. (2).

The inactivation curve (120 mM-K^+) studied in three fibres of *R. temporaria* and two fibres of *R. pipiens* became steeper and shifted towards more negative potentials by about 17 mV in *Ca-free* saline and 7 mV in *low-Ca* saline, suggesting that the curve shifts progressively as external Ca^{2+} is reduced. Fig. 5 shows the inactivation curves for two fibres of *R. temporaria* (○–●, △–▲) and one fibre of *R. pipiens* (▽–▼), in *control* saline (open symbols) and in *Ca-free* saline (filled symbols). In the last solution the peak tension decays abruptly between 10 and 20 mM-K^+ conditioning pulses. The average values for the parameters in *control* saline and *Ca-free* saline were respectively: $[\bar{K}] = 29{\cdot}7 \pm 1{\cdot}1$ mM and $13{\cdot}7 \pm 1{\cdot}0$ mM ($P < 0{\cdot}01$); $k = 0{\cdot}12 \pm 0{\cdot}01$ and

0·025 ± 0·007 ($P < 0·02$). A similar shift (about −14 mV, two fibres of *R. temporaria*) of the inactivation curve was obtained in *Ca-free* saline with 70 mM-K^+ test contractures. The peak tension between 2·5 and 10 mM-K^+ was about 70 % of the value in *control* saline; thereafter between 10 and 20 mM-K^+ the contracture inactivated abruptly.

Effect of increasing Mg^{2+} on K contractures in Ca-free saline

Although no change in the surface potential near Na channels is observed when 1·8 mM-Ca^{2+} is replaced by 3 mM-Mg^{2+}, one cannot neglect the possibility that such replacement might not be appropriate for the regions of the membrane controlling

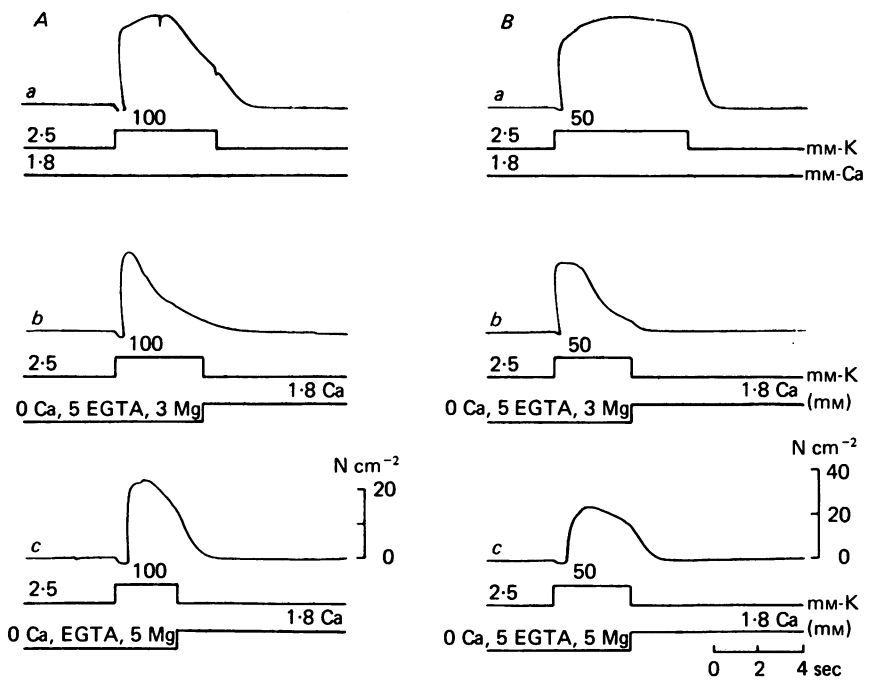

Fig. 6. Effect of increasing the external Mg^{2+} concentration on K contractures. Two different fibres from *R. temporaria*. *A* is with 100 mM-K^+ (diameter = 87 μm) and *B* is with 50 mM-K^+ (diameter = 105 μm); *a* is in *control* saline, *b* in *Ca-free* saline with 3 mM-Mg^{2+} and *c* in *Ca-free* saline with 5 mM-Mg^{2+}. Records were obtained in the following sequence: *A*, *c–b–a*; *B*, *c–b–a*.

the voltage dependency of contraction. It is conceivable that different regions of the membrane may have different types and/or density of fixed charge (Hille *et al.* 1975; Di Francesco & McNaughton, 1979; Dörrscheidt-Käfer, 1979). For example, the reduction of K contractures observed in *Ca-free* saline (3 mM-Mg^{2+}) could be attributed to inappropriate Ca^{2+} replacement leading to a more negative outer surface potential and to a partial mechanical inactivation. If this were the case, one could expect to remove inactivation and the tension to increase if the external Mg^{2+} concentration was increased. Fig. 6 shows K contractures (100 mM-K^+ in *A* and 50 mM-K^+ in *B*) in *control* saline (*a*), in *Ca-free* saline with 3 mM-Mg^{2+} (*b*) and in *Ca-free*

saline with 5 mM-Mg^{2+} (*c*). As we have previously shown, K contractures were smaller and shorter in 3 mM-Mg^{2+} *Ca-free* saline. In 5 mM-Mg^{2+} *Ca-free* saline, the time course of contractures was slower and the amplitude for 100 mM-K^+ changed slightly; but the contractures clearly decreased when using lower K^+ concentrations (50 mM-K^+). Similar findings were obtained in five fibres tested (30–50 mM-K^+ contractures). These results indicate that in 3 mM-Mg^{2+} *Ca-free* saline the contractile mechanism was not inactivated.

The slower time course and the reduction of tension produced by increasing Mg^{2+} from 3 to 5 mM could be explained by a less negative outer surface potential leading to a shift to the right of the *activation curve*. For example, the tension produced by 50 mM-K^+ in 5 mM-Mg^{2+} *Ca-free* saline (Fig. 6*B*, *c*) would correspond to the tension produced by 42 mM-K^+ according to the activation curve for K contractures in 3 mM-Mg^{2+} *Ca-free* saline, and the corresponding calculated shift was 3–4 mV. Accordingly, a similar reduction of 4–5 mV of the outer surface potential can be obtained when external Mg^{2+} is increased from 3 to 5 mM (Chiarandini *et al.* 1980).

DISCUSSION

Replacement of Ca^{2+} by Mg^{2+}. The thresholds for mechanical activation (pulses $\geqslant$ 100 msec) and for Na current (Chiarandini *et al.* 1980; this paper) were not modified when 1·8 mM-Ca^{2+} was replaced by 3·0 mM-Mg^{2+}, indicating that this replacement did not change the surface potential near the Na channels or at the membrane regions where excitation–contraction coupling occurs. Correspondingly, K contractures in 3 mM-Mg^{2+} *Ca-free* saline (2·5 mM-K^+ prepulse) were not inactivated, and they became slower and decreased when Mg^{2+} was increased to 5 mM in a predictable manner due to the shift in the threshold for Na current.

In view of the foregoing, the reported effects of external Ca^{2+} reduction on K contractures can be attributed to a direct effect on the excitation–contraction coupling process.

When external Ca^{2+} is reduced without replacement by any other divalent cation, initially the peak tension increases, the contracture shortens and has a faster time of relaxation (Lüttgau, 1963; Caputo & Giménez, 1967; Frankenhaeuser & Lännergren, 1967; Caputo, 1972) and the inactivation curve is displaced to lower K concentrations (Frankenhaeuser & Lännergren, 1967). Finally, the tension decreases and eventually the fibres do not contract (Lüttgau, 1963; Caputo & Giménez, 1967). These observations can be related to combined effects on electrical properties (surface potential and $E_{r.p.}$) and to direct effects on excitation–contraction coupling. For example, muscle fibres are depolarized when external Ca^{2+} is reduced (Lüttgau, 1963; Caputo & Giménez, 1967) and the threshold for the Na current shifts (from 2 mM-Ca to 0·2 mM-Ca) by 13–15 mV toward more negative potentials (Campbell & Hille, 1976; Chiarandini *et al.* 1980).

Lüttgau & Spiecker (1979) did not find modifications in the *activation curve* when replacing 3 mM-Ca^{2+} with 3 mM-Mg^{2+} (2·5 mM-EGTA, contractures at constant $K^+ \times Cl^-$ product). By using the same solutions, we have confirmed their results. This lack of effect of *Ca-free* saline on the activation curve could be related to an inappropriate substitution of Ca^{2+} by Mg^{2+} which could lead to an increase in the external surface potential. Along this line, Lüttgau & Spiecker (1979) found a shift of the inactivation curve of -20 to -30 mV, while we found a smaller one of -17 mV.

Direct effects of external Ca^{2+} on K contractures. The present research demonstrates that external Ca^{2+} plays a supporting role for maintaining the tension during the

contracture: the peak tension, the duration and the time constant of spontaneous relaxation decrease progressively as external Ca^{2+} is reduced. Notwithstanding, external Ca^{2+} is not essential: the tension development during the initial phase of the contracture was not modified by the *Ca-free* saline and furthermore, in agreement with previous authors, we confirmed the observation that tension can still be elicited in the virtual absence of Ca inflow from external sources (Armstrong *et al.* 1972; Stefani & Chiarandini, 1973; Miledi, Parker & Schalow, 1977; Lüttgau & Spiecker, 1979; Chiarandini *et al.* 1980). The lack of effect of *Ca-free* saline on the initial phase of K contractures supports the view that initially this process is mediated by translocation of intracellular Ca^{2+} (Caputo & Giménez, 1967).

With the actual evidence at hand, it is not possible to elucidate the mechanism(s) of the direct effects of external Ca^{2+} on K contractures. Several possibilities, or a combination of them, can explain equally well these results. They will be briefly summarized: (1) the inward Ca current (I_{Ca}) during the depolarization (Beaty & Stefani, 1976*b*; Stanfield, 1977; Sánchez & Stefani, 1978) which is mainly located in the transverse tubular system (Nicola Siri, Sánchez & Stefani, 1980) could be of sufficient magnitude to constitute an additional factor which contributes directly to the final myoplasmic Ca^{2+} concentration (Potreau & Raymond, 1980); (2) the I_{Ca} activated during the prolonged depolarization may load the sarcoplasmic reticulum (s.r.) thus making more Ca^{2+} available for release (Nicola Siri *et al.* 1980); (3) the I_{Ca} could account for an activation of the contractile proteins involving a Ca-induced Ca^{2+} release mechanism (Weiss & Bianchi, 1965; Stefani & Chiarandini, 1973; Chiarandini & Stefani, 1976; Potreau & Raymond, 1980) of the type observed in skinned fibres (for review, see Endo, 1977); (4) following the model proposed by Caputo & Fernández de Bolaños (1979), external Ca^{2+} reduction would produce an earlier onset of the inactivation process at an increased rate, leading to a reduction of contracture duration, an increase in the spontaneous relaxation rate and an eventual fall in the peak tension (see Ebashi, 1976); (5) the coupling between depolarization of the transverse tubular membrane and the release of Ca^{2+} from the s.r. could depend on metabolic processes and on internal free Ca^{2+} concentration which could modify the density and/or type of negative surface charges at the inner side of the tubular membrane, altering the K contracture inactivation curve (Lüttgau & Spiecker, 1979); and finally (6), specific interactions of external Ca^{2+} (Shlevin, 1979) and/or internal Ca^{2+} (Beaty & Stefani, 1976*a*) with the asymmetrical capacity currents may occur; these are thought to be associated with excitation–contraction coupling (Schneider & Chandler, 1973; Adrian & Almers, 1976; Chandler, Rakowski & Schneider, 1976; Mathias, Levis & Eisenberg, 1980; see Almers, 1978).

The authors are most grateful to Dr D. J. Chiarandini for revising the manuscript, and to Emma Bensimón for secretarial work, This work was supported by the CONACyT of Mexico, grants PCCBNAL 790022 and PCAIEUA 790057.

REFERENCES

ADRIAN, R. H. & ALMERS, W. (1976). Charge movement in the membrane of striated muscle. *J. Physiol.* **254**, 339–360.

ALMERS, W. (1978). Gating currents and charge movements in excitable membranes. *Rev. Physiol. Biochem. & Pharmacol.* **82**, 96–190.

ARMSTRONG, C. M., BEZANILLA, F. M. & HOROWICZ, P. (1972). Twitches in presence of ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid. *Biochim. biophys. Acta* **267**, 605–608.

BEATY, G. N. & STEFANI, E. (1976*a*). Calcium dependent electrical activity in twitch muscle fibres of the frog. *Proc. R. Soc.* B **194**, 141–150.

BEATY, G. N. & STEFANI, E. (1976*b*). Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **260**, 27*P*.

BLINKS, J. R., RÜDEL, R. & TAYLOR, S. R. (1978). Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J. Physiol.* **277**, 291–323.

CAMPBELL, D. T. & HILLE, B. (1976). Kinetics and pharmacological properties of the sodium channel of the frog skeletal muscle. *J. gen. Physiol.* **67**, 309–323.

CAPUTO, C. (1972). The time course of potassium contractures of single muscle fibres. *J. Physiol.* **223**, 483–505.

CAPUTO, C. & FERNÁNDEZ DE BOLAÑOS, P. (1979). Membrane potential, contractile activation and relaxation rates in voltage clamped short muscle fibres of the frog. *J. Physiol.* **289**, 175–189.

CAPUTO, C. & GIMÉNEZ, M. (1967). Effects of external calcium deprivation on single muscle fibers. *J. gen. Physiol.* **50**, 2177–2195.

CHANDLER, W. K., RAKOWSKI, R. F. & SCHNEIDER, M. F. (1976). Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol.* **254**, 285–316.

CHIARANDINI, D. J., SÁNCHEZ, J. A. & STEFANI, E. (1980). Effect of calcium withdrawal on mechanical threshold in skeletal muscle fibres of the frog. *J. Physiol.* **303**, 153–163.

CHIARANDINI, D. J. & STEFANI, E. (1973). Effects of manganese on the electrical and mechanical properties of frog skeletal muscle fibres. *J. Physiol.* **232**, 129–147.

CHIARANDINI, D. J. & STEFANI, E. (1976). Ca and excitation–contraction coupling in frog skeletal muscle fibres. In *Electrobiology of Nerve, Synapse & Muscle*, ed. REUBEN, J. P., PURPURA, D. P., BENNETT, V. L. & KANDEL, E. R. New York: Raven Press.

COLQUHOUN, D. (1971). *Lectures on Biostatistics*. Oxford: Clarendon Press.

COSTANTIN, L. L. (1971). Biphasic potassium contractures in frog muscle fibers. *J. gen. Physiol.* **58**, 117–130.

COTA, G. & STEFANI, E. (1979). Efecto de cationes divalentes sobre el potencial de superficie externo en fibras musculares esqueléticas de rana. *Proc. XXII Congreso Nacional de Ciencias Fisiológicas*, 1978, México: Soc. Méx. Ciencias Fisiológicas.

DIFRANCESCO, D. & MCNAUGHTON, P. A. (1979). The effects of calcium on outward membrane currents in the cardiac Purkinje fibre. *J. Physiol.* **289**, 347–373.

DÖRRSCHEIDT-KÄFER, M. (1979). Excitation–contraction coupling in frog sartorius and the role of the surface charge due to carboxyl group of sialic acid. *Pflügers Arch.* **380**, 171–179.

EBASHI, S. (1976). Excitation–contraction coupling. *A. Rev. Physiol.* **38**, 293–313.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**, 71–108.

FRANKENHAEUSER, B. & LÄNNERGREN, J. (1967). The effect of calcium on the mechanical response of single twitch muscle fibres of *Xenopus laevis*. *Acta physiol. scand.* **69**, 242–254.

GILLY, W. F. & HUI, C. S. (1980). Mechanical activation in slow and twitch skeletal muscle fibres of the frog. *J. Physiol.* **301**, 137–156.

GONZÁLEZ SERRATOS, H. (1975). Graded activation of myofibrils and the effect of diameter on tension development during contractures in isolated skeletal muscle fibres. *J. Physiol.* **253**, 321–339.

HILLE, B., WOODHULL, A. M. & SHAPIRO, B. I. (1975). Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. *Phil. Trans. R. Soc.* B **270**, 301–318.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & HOROWICZ, P. (1960). Potassium contractures in single muscle fibres. *J. Physiol.* **153**, 386–403.

HODGKIN, A. L. & HUXLEY, A. F. (1952). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

KENYON, J. L. & GIBBONS, W. R. (1977). Effect of low-chloride solutions on action potentials of sheep cardiac Purkinje fibers. *J. gen. Physiol.* **70**, 635–660.

KOVÁCS, L. & SCHNEIDER, M. F. (1978). Contractile activation by voltage clamp depolarization of cut skeletal muscle fibres. *J. Physiol.* **277**, 483–506.

LÜTTGAU, H. C. (1963). The action of calcium ions on potassium contractures of single muscle fibres. *J. Physiol.* **168**, 679–697.

LÜTTGAU, H. C. & SPIECKER, W. (1979). The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *J. Physiol.* **296**, 411–429.

MATHIAS, R. T., LEVIS, R. A. & EISENBERG, R. S. (1980). Electrical models of excitation–contraction coupling and charge movement in skeletal muscle. *J. gen. Physiol.* **76**, 1–31.

MILEDI, R., PARKER, I. & SCHALOW, G. (1977). Measurements of calcium transients in frog muscle by the use of arsenaso III. *Proc. R. Soc.* B. **198**, 201–210.

MOISESCU, D. G. & PUSCH, H. (1975). A pH metric method for the determination of the relative concentration of calcium to EGTA. *Pflügers Arch.* **355**, Suppl. R122.

NICOLA SIRI, L., SÁNCHEZ, J. A. & STEFANI, E. (1980). Effect of glycerol treatment on the calcium current of frog skeletal muscle. *J. Physiol.* **305**, 87–96.

POTREAU, D. & RAYMOND, G. (1980). Calcium-dependent electrical activity and contraction of voltage-clamped frog single muscle fibres. *J. Physiol.* **307**, 9–22.

SÁNCHEZ, J. A. & STEFANI, E. (1978). Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **283**, 197–209.

SANDOW, A., PAGALA, M. K. D. & SPHICAS, E. C. (1975). Excitation–contraction coupling: effects of 'zero' Ca^{2+} medium. *Biochim. biophys. Acta* **404**, 157–163.

SCHNEIDER, M. F. & CHANDLER, W. K. (1973). Voltage dependent charge movement in skeletal muscle: a possible step in excitation–contraction coupling. *Nature, Lond.* **242**, 244–246.

SHLEVIN, H. H. (1979). Effects of external calcium concentration and pH on charge movement in frog skeletal muscle. *J. Physiol.* **288**, 129–158.

STANFIELD, P. R. (1977). A calcium dependent inward current in frog skeletal muscle fibres. *Pflügers Arch.* **368**, 267–270.

STEFANI, E. & CHIARANDINI, D. J. (1973). Skeletal muscle: dependence of potassium contractures on extracellular calcium. *Pflügers Arch.* **343**, 143–150.

WEISS, G. B. & BIANCHI, C. P. (1965). The effect of potassium concentration on ^{45}Ca uptake in frog sartorius muscle. *J. cell. comp. Physiol.* **65**, 385–392.