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MEMBRANE POTENTIAL, CONTRACTILE ACTIVATION AND RELAXATION RATES IN VOLTAGE CLAMPED SHORT MUSCLE FIBRES OF THE FROG

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SUMMARY

1. Voltage clamped short ($\approx$ 1·5 mm) muscle fibres of the frog can develop maximum tension of 4·3 kg/cm^2.

2. The time course of contractile responses to prolonged depolarization is markedly dependent on the fibre membrane potential. With sufficiently long pulses the responses present a plateau and a spontaneous relaxation phase.

3. At room temperature (20–22 °C), and at membrane potentials of -10 mV the plateau duration is about 2 sec and the spontaneous relaxation rate is 0·50 sec^{-1}. At membrane potentials of -35 mV the plateau duration is 4·6 sec and the spontaneous relaxation rate is 0·28 sec^{-1}.

4. When the fibres are depolarized at room temperature with relatively short pulses ($<$ 2 sec), the contractile responses are cut short at the end of the pulse, and the fibres relax at a rate (11 sec^{-1}) which is independent on the pulse amplitude and duration.

5. The relaxation rate after a short pulse can be affected by membrane potential only in the region near the contractile threshold where further release of contractile activator is expected to occur.

6. The time course of contractile responses to prolonged depolarization may be shortened by conditioning depolarization.

7. The system responsible for the release of contractile activator may be tentatively described by three states, resting, activated, and inactivated, in analogy with the model proposed by Chandler, Rakowski & Schneider (1976) to describe the possible configurations of the potential dependent charge movement in muscle.

INTRODUCTION

Relaxation following a twitch or a tetanus occurs after a muscle fibre membrane has recovered its normal resting potential following the transient depolarization associated with the propagated action potential. When, on the other hand, a muscle fibre is submitted to a long lasting depolarization, as in the case of a K contracture, the contractile response shows a plateau phase, followed by a spontaneous relaxation which takes place while the fibre membrane is still in a depolarized state (Hodgkin

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& Horowicz, 1960). Relaxation in the two cases proceeds at different rates and most probably is the expression of two different situations. It is conceivable that in the first case, once the membrane is fully repolarized, activator release has ceased, and relaxation is determined by processes such as calcium unbinding from contractile proteins and/or Ca uptake by the sarcoplasmic reticulum. In the second case, the fibre membrane is depolarized, and relaxation proceeds much more slowly and might represent in an approximate way the time course of release of contractile activator which is thought to proceed throughout the contractile response but in a continuously diminishing way (Caputo, 1972). While there is no information on whether the fast relaxation following twitches or tetani is affected by the membrane potential level, it is known that the rate of relaxation during potassium contractures does depend on the fibre membrane potential (Hodgkin & Horowicz, 1960). To explain the transient nature of these responses, Hodgkin & Horowicz (1960) proposed a simple model based on the depletion of an activator store. Other possibilities including a mechanism for activator release controlled by a membrane process which is potential and time-dependent have also been considered (Hodgkin & Horowicz, 1960; Caputo, 1972, 1976).

Knowledge of the mechanism underlying the spontaneous relaxation of contractile responses to sustained depolarization could be valuable for understanding the nature of the process which controls Ca release during contractile activation. The present work was carried out to study the membrane potential dependency of relaxation, and to gain knowledge on the extent by which Ca release may be modified by membrane potential changes. In order to do so, we have employed a two micro-electrode voltage clamp technique and short muscle fibres (1 to 1·5 mm) to improve longitudinal homogeneity of potential control (Heistracher & Hunt, 1969; Bezanilla, Caputo & Horowicz, 1971). A preliminary account of some of the experiments reported here has been presented elsewhere (Caputo, 1977).

METHODS

Bundles of five to twenty fibres were dissected from the M. lumbricalis IV digiti of the hind limb of *Rana pipiens*. The fibres in this muscle are disposed in a double pennate arrangement so that the length of the fibres is only a fraction of the muscle length. The fibres used in this work were around 1·5 mm long, and had approximately a 70 μm diameter. Due to their short length special care had to be taken not to stretch and damage them during the dissection and mounting procedure. Once dissected the fibres were mounted in a Lucite chamber, by hooking one tendon to the chamber and the other to the shaft of an RCA 5734 transducer for force measurement. In later stages of the work the transducer used was made with two Pixie elements. The short length of these fibres allows the use of a two micro-electrode clamp system, one for measuring potential, and the other for passing current. The micro-electrodes were made flexible following the procedure described by Heistracher & Hunt, 1969). The voltage micro-electrode was filled with 3 M-KCl and connected via a Ag–AgCl connexion to an amplifier with variable input capacitance compensation (NF1, Bioelectric Instruments). The current electrode was filled with 2 M-K citrate, and was connected to the output of the control amplifier (Philbrick, 1022). The chamber was connected through a saline-filled agar bridge to a pool containing 3 M-KC1 connected by a Ag-AgCl electrode to the virtual ground input of an operational amplifier (Philbrick P-25) wired as current to voltage transducer to measure total membrane current. To achieve the desired control of the membrane, a steady potential (holding potential) and up to three pulses could be applied, through respective resistors to the summing junction of the control amplifier. The output of the voltage measuring amplifier (NFI) was also connected to this summing junction through an adequate resistor. The output of the control

amplifier had a dynamic range of ± 100 V, thus allowing one to pass sufficient current through the citrate micro-electrode to control adequately the membrane potential.

F. Bezanilla, C. Caputo & P. Horowicz (unpublished results) have obtained evidence that although this technique is grossly inadequate for studying phenomena such as the sodium conductance changes underlying the generation of action potentials, it is satisfactory for the study of slower processes such as contractile activation. In fact the advantage of using short fibres resides in the possibility to bring most of the fibre length under effective control of the clamp system. When the current micro-electrode is placed in the centre of the fibre, the following relationship holds (Weidmann, 1952):

$$V = V_0 \cosh \frac{[(L-X)/\lambda]}{\cosh (L/\lambda)}$$

where V_0 is the potential at $X = 0$, X is the distance between current and potential recording electrodes, V is the potential at X, L is the fibre half length, and $\lambda = \sqrt{r_m/r_i}$, where r_m is the membrane resistance × unit length, and r_i is the internal resistance/unit length. Since the value of λ in these fibres is near 2 mm, the potential at a fibre end is about 95% of the value of V_0. Therefore since most of the fibre length can be voltage clamped, it is possible to measure the tension output of the fibre in response to depolarizing pulses. In fact, taking the tension values presented in Table 1 and considering a mean diameter of 70 μm for these fibres a value of 4·3 kg/cm^2 is obtained which is similar to that reported for tetanic tension in frog muscle fibres (Hodgkin & Horowicz, 1960). It is important to stress that the ability to measure the full tension output should be considered a strict requirement for the studies of contractile activation in which tension development is measured under voltage clamp conditions.

The fibres in this work were bathed in a Ringer solution with the following composition: KCl, 2 mM; NaCl, 115 mM; $CaCl_2$, 1·8 mM; Tris buffer pH 7. Tetrodotoxin 10^{-6} g/ml. was added to suppress Na-dependent conductance changes and to improve clamping conditions.

All the experiments were carried out at room temperature (20–22 °C). A few experiments were carried out in the Physiological Laboratory in Cambridge. In these experiments single muscle fibres, dissected from the semitendinosus muscle of *Rana temporaria*, were used to study repriming after K contractures. These experiments were carried out following the procedure described by Hodgkin & Horowicz (1960).

RESULTS

Tension-voltage relationship

Tension development in muscle fibres is related to membrane potential by a steep S-shaped curve (Hodgkin & Horowicz, 1960). For the case of voltage clamped muscle fibres the steepness of the curve depends on the duration of the voltage clamp depolarizing pulse when relatively short pulses (< 100 msec) are applied (Bezanilla *et al.* 1971). Fig. 1 illustrates the tension voltage relationship of the short fibres used in this work, depolarized with test pulses of 1 or 2 sec duration. It may be noticed that the contractile threshold is between −55 and −50 mV, and that beyond this contractile threshold further depolarization of 15 mV causes the fibres to develop about 90% of the total tension. It is important to notice that maximum tension is obtained at membrane potential values less negative than −30 mV.

Relaxation rates during long pulses.

Since the work of Hodgkin & Horowicz (1960), it is known that during potassium contractures, muscle fibres relax spontaneously at a rate that is dependent on the test potassium concentration and hence on the fibre membrane potential. Fig. 2 shows that a similar behaviour is followed by short muscle fibres depolarized with long lasting (> 5 sec) voltage clamp pulses. The figure shows the results obtained with two different fibres, each of them tested with two pulses which depolarized

the membrane to different values (−8 and −35 mV, for one and −9 and −32 mV for the other). It appears that bearing with the results shown in Fig. 1, the tension generated by the fibres in response to the two test pulses differed by a small but significant amount. The time course of the contractile response varies to a much

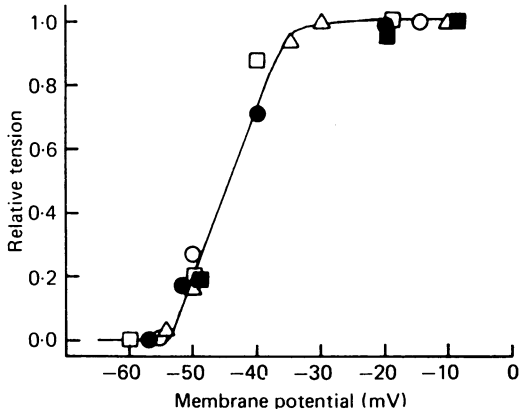


Fig. 1. The relationship between peak tension developed expressed as a fraction of maximum tension and the membrane potential during voltage clamp/depolarizing pulses. The different symbols refer to results obtained with five different fibres. The holding potential of these fibres was −100 mV and the pulses had a duration of 1 sec or longer.

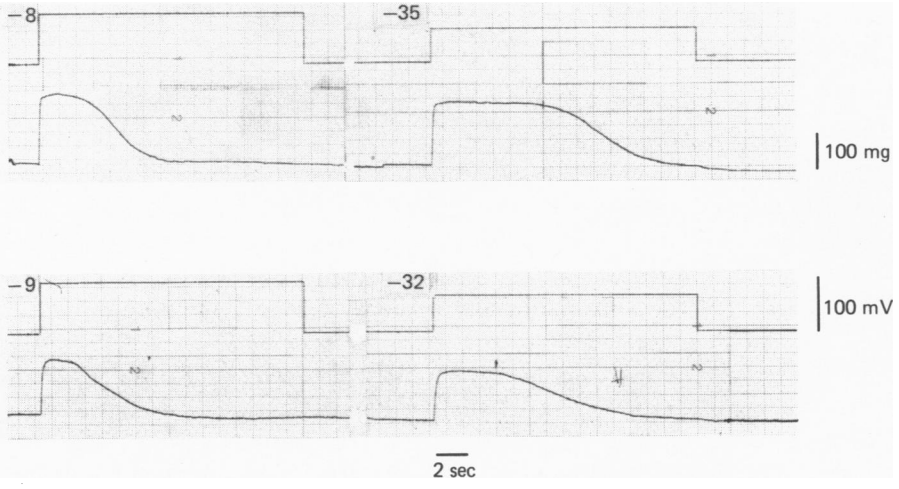


Fig. 2. Contractile responses to prolonged depolarizing pulses obtained with two fibres. Two responses for each fibre are shown. Notice the marked difference in plateau duration and spontaneous relaxation phase obtained at the two different membrane potentials.

greater extent, confirming the findings of Hodgkin & Horowicz (1960). The difference in the time course is due both to a marked prolongation of the plateau phase, and to a slowing of the spontaneous relaxation phase with the smaller test pulse. With respect to the spontaneous relaxation phase, it is worth noticing that it follows an

S-shaped decay, rather than a single exponential one. However, at a first approximation, and only for descriptive purposes, single exponentials may be considered to describe the relaxation phase. Fig. 3*A* shows the semilogarithmic plots of tension decay for one fibre. In this case it may be observed that about 60 % of the relaxation phase can be described by a single exponential, whose rate constant appears to be dependent on the membrane potential. Table 1 summarizes the results obtained with

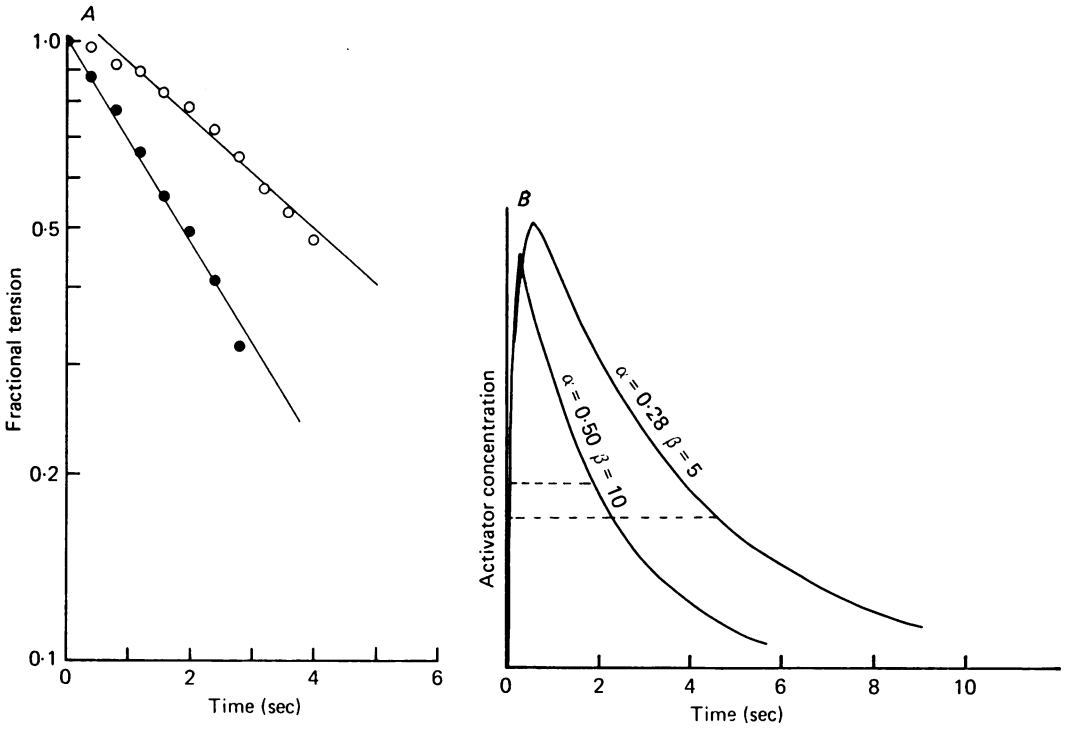


Fig. 3. *A*, semilogarithmic plots of fractional tension versus time, of the spontaneous relaxation phase of two responses to different pulses for the same fibre. Only a fraction of the relaxation phase follows an exponential decay. ●, 92 mV (0·38 sec^{-1}); 0, 65 mV (0·21 sec^{-1}). *B*, plots of the activator concentration in arbitrary scale versus time for two different situations. The activator concentration A from the scheme $P \xrightarrow{\alpha} A \xrightarrow{\beta} E$ is given by

$$A = \frac{\alpha}{\beta - \alpha}\,(e^{-\alpha t} - e^{-\beta t})$$

(Hodgkin & Horowicz, 1960). The two curves were obtained with the values of α and β given in the Figure. The dotted lines represent the plateau corresponding to the mean responses obtained in Table 2.

five different fibres. Changing the mean membrane potential value during the pulse from − 9 to − 36 mV causes the plateau duration to be prolonged from 2·2 to 4·6 sec, the tension to drop by 10 % and the exponential relaxation rate constant to decrease by 45 %. To explain the time course of K contractures, Hodgkin & Horowicz (1960) proposed a simple model according to which spontaneous relaxation is due to the exhaustion of a store of a precursor of the contractile activator which undergoes the transformation

$$P \xrightarrow{\alpha} A \xrightarrow{\beta} E$$

TABLE 1. Fibre response to depolarization and relation to membrane potential

Fibre	Membrane potential during pulse 1 (mV)	Tension (T_1) (mg)	Plateau duration (PD_1) (sec)	Relaxation rate (R_1) (sec^{-1})	Membrane potential during pulse 2 (mV)	Tension (T_2) (mg)	Plateau duration (PD_2) (sec)	Relaxation rate (R_2) (sec^{-1})	$\frac{T_2}{T_1}$	$\frac{PD_2}{PD_1}$	$\frac{R_2}{R_1}$
1	−10	184	2·6	0·68	−35	169	6·4	0·39	0·92	2·46	0·57
2	−9	138	1·5	0·49	−32	121	3·1	0·30	0·88	2·07	0·61
3	−10	124	2·2	0·38	−40	118	3·5	0·15	0·95	1·59	0·39
4	−8	211	2·1	0·38	−35	191	5·0	0·21	0·91	2·38	0·55
5	−8	165	2·4	0·55	−40	145	4·8	0·36	0·88	2·0	0·65
Mean			2·2	0·50			4·6	0·28	0·91	2·1	0·55
± S.E. of mean			± 0·4	± 0·13			± 1·3	± 0·10	± 0·03	± 0·4	± 0·10

where P is the precursor, A is the activator and E an end product, and α and β are the rate constant for the activator formation and destruction. According to this model the duration of the plateau is the time for the concentration of activator to fall from a supramaximal to a maximal value, and the duration of the relaxation phase is the time for the concentration to fall from a maximal to a threshold value. The decay from supramaximal to threshold activator concentration would then follow a simple exponential time course.

TABLE 2. Effect of short pulses on membrane potential and relaxation rate in eleven experimental fibres

Fibre	Pulse duration (msec)	Membrane potential during pulse (mV)	Rate of relaxation (sec^{-1})
10	100	+20	14·5
10	100	−20	13·7
12	500	−30	8·5
12	500	+30	8·4
13	200	−40	11·9
13	200	−30	14·9
14	200	−40	12·7
14	400	−40	11·8
15	1000	−50	7
15	1000	−53	6·5
16	2000	0	13
			11·2 ± 3

Fig. 3*B* shows two curves describing the time course of formation and decay of the activator A, obtained using α and β values of 0·5 and 10 and 0·28 and 5 respectively. It is evident from this example that the model is reasonably satisfactory for explaining the prolongation of the plateau, observed with the smaller depolarization. The sizable difference in peak tension obtained with the two pulse amplitude (see Table 1) would not be expected according to the model.

Relaxation rates after short pulses

An argument against the idea that the duration of the plateau represents the time for the concentration of the activator to fall from a supramaximal to a maximal value is derived from experiments in which the fibres are depolarized with relatively short pulses, see Figs. 4, 5 and 6. In this case, the contractile responses are cut short, and the relaxation that follows the trailing edge of the pulse is much faster than the spontaneous relaxation obtained with the longer pulses. It is important to point out that the model predicts the same relaxation rate for both types of relaxation.

The relaxation observed with short pulses can be described reasonably well by a single exponential (see Fig. 5) whose time constant of about 10 sec^{-1} does not depend on the pulse duration or amplitude. This point is illustrated in Table 2 which presents the results obtained with several fibres depolarized with pulses from 100 to 2000 msec duration to values from −53 to +30 mV. The mean decay constant of the fast relaxation obtained in such experiments is 11·2 sec^{-1} (± 3 S.E. of mean). This

value is clearly different from the value of the decay constant obtained when spontaneous relaxation during long pulses is studied. This difference indicates that the relaxation time course with long or short pulses is the expression of entirely different situations. As already mentioned, when long pulses are used, relaxation can be considered to follow the time course of activator release which proceeds throughout the

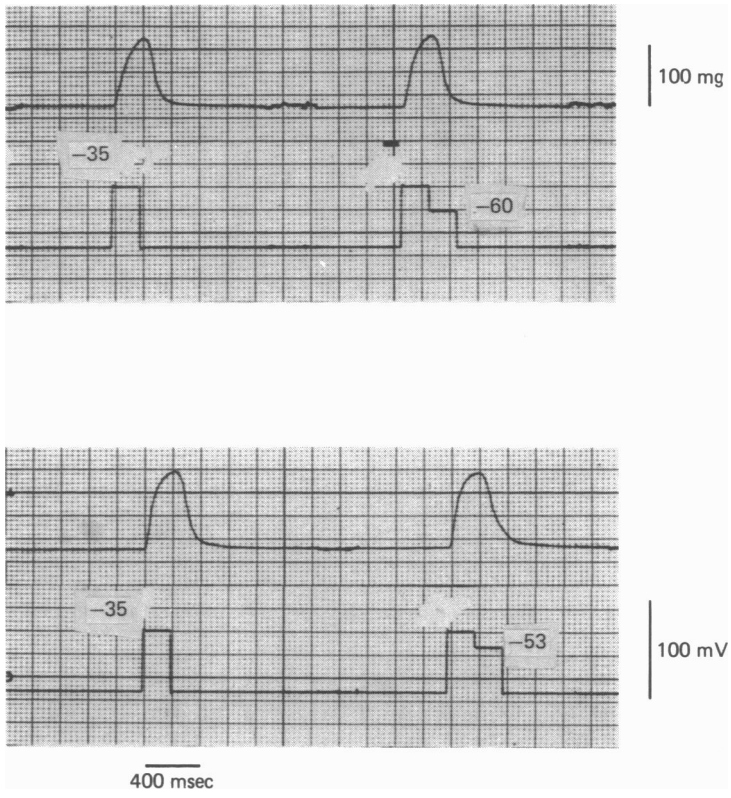


Fig. 4. Effect of changes in the membrane potential level during the fast relaxation phase of contractile responses to relatively short pulses (second and fourth records). The first and third records show the contractile responses to a pulse of 65 mV and 200 msec duration. Notice that fast relaxation starts immediately after the end of the pulse. The numbers give the membrane potential level during the pulses.

response duration (Caputo, 1972) while in the case of short pulses, membrane repolarization puts an abrupt end to Ca release, and the relaxation rates may be indicative of other processes such as Ca unbinding from the contractile proteins or Ca uptake by the sarcoplasmic reticulum.

In the short pulse experiments just described, the fibres relaxed at a time when the membrane had already repolarized to its normal holding potential value. Some experiments were carried out to test the effect of membrane potential variation during the fast relaxation phase following short pulses. Figs. 4, 5 and 6 illustrate the experimental procedures and some of the results obtained. The top two records of Fig. 4 show that the relaxation rate following a pulse of 200 msec, which depolarized the membrane to our absolute value of -35 mV, is the same independently of whether relaxation occurs while the fibre membrane is held at -100 or -60 mV. The

two lower records show that the relaxation rate is markedly decreased when the membrane potential during relaxation has a value of -53 mV. Fig. 5 shows the result of a similar experiment, in this case the tension during the relaxation has been plotted semilogarithmically versus time. It may be observed that in this case, most of the relaxation phase follows a simple exponential decay. The decay constant had a value of 11·9 sec^{-1} when the membrane potential during relaxation was -100 or -60 mV and decreased to 5·9 sec^{-1} when the potential was -55 mV. In a third

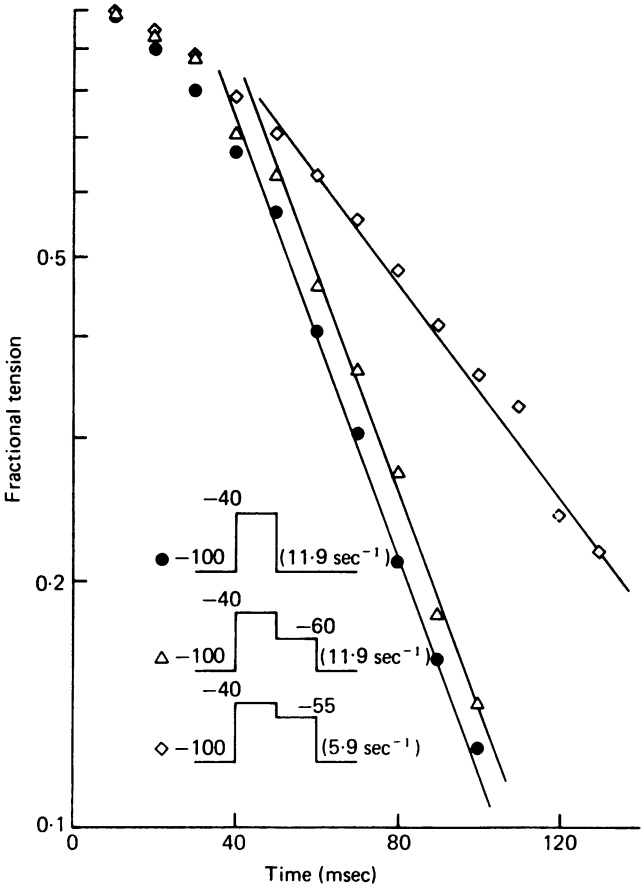


Fig. 5. Effect of changes of the membrane potential level during the fast relaxation phase of contractile responses to short pulses (200 msec). The figures show semilogarithmic plot of fractional tension versus time of the relaxation phase of responses under different conditions. The insert shows the pulse configuration for each case and the corresponding time constants. Notice that for the case of the fast relaxation phase, a single exponential describes most of the relaxation phase.

similar experiment the decay constant was 14·9 sec^{-1} at -100, 15·2 sec^{-1} at -60 and 9·3 sec^{-1} at -50 mV. In other experiments in which the membrane was hyperpolarized during relaxation no effect was observed.

The conclusion that can be derived from these experiments is that at membrane potentials more negative than the contractile threshold, the relaxation rate is not affected by the value of the membrane potential. When the membrane potential is

similar or less negative than the contractile threshold, extra amount of contractile activator is released and this has a marked effect on the relaxation rate. Fig. 6 shows an experiment giving support to this interpretation. In fact in this case, when the membrane potential during the relaxation phase is made increasingly less negative, besides causing a decrease in the relaxation rate one can observe that a second steady tension level is obtained during the second pulse roughly corresponding to the one expected from the tension–voltage relationship.

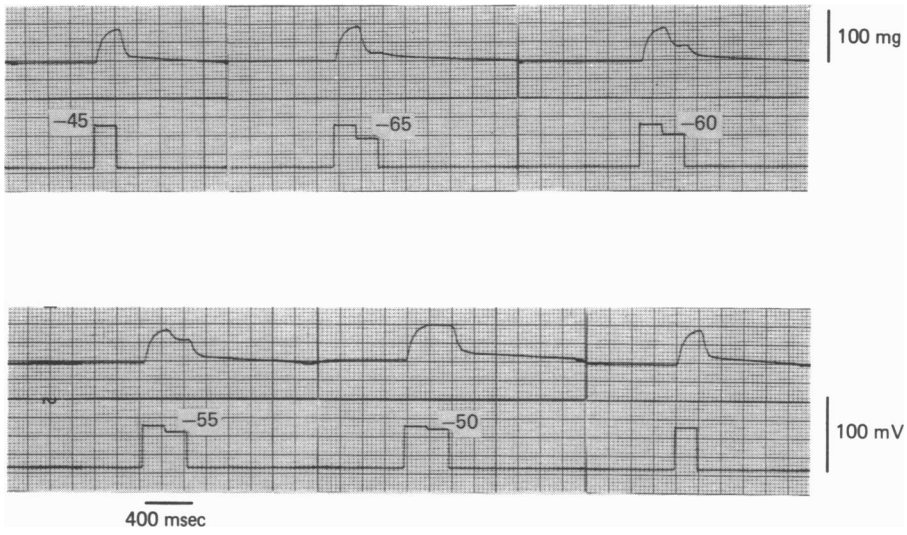


Fig. 6. Effect of changes of membrane potential on the relaxation phase after relatively short pulses (200 msec). In this case it is clear that when the membrane potential is made less negative than a given threshold value, beside changes in the rate of relaxation a second tension level is attained corresponding to the new membrane potential.

Relaxation rates and contractile inactivation

Frankenhaeuser & Lannergren (1967) have shown that contractile activation, studied with K contractures, may be affected by conditioning depolarization, giving rise to the notion of an inactivation process analagous to the one occurring in the case of sodium conductance system in excitable membranes (Caputo, 1972). The term inactivation has also been used to describe the spontaneous relaxation of prolonged contractile responses. Inactivation of Ca release could be the mechanism underlying both phenomena. In fact, as an alternate way to describe the time course of K contractures, a model was proposed according to which, the mechanism responsible for calcium release is inactivated with time (Caputo, 1972). According to this model depolarization of the membrane causes activation of the release mechanism, which may be then rapidly reversed by membrane repolarization. After release activation is on, a second process, inactivation, starts after a certain delay, at a rate that is membrane potential dependent. The delay in the inactivation onset determines the duration of the contracture plateau. In terms of this model an earlier onset of inactivation should cause a reduction in the contractile response duration, and eventually a drop in the tension output. The next experiments show

that as point of fact, the first major effect of conditioning depolarization is a marked—shortening of contractile responses followed by a decrease in the peak tension output.

Fig. 7 shows that the contractile response to a 65 mV test pulse can be effectively shortened by preceding the test pulse with a 25 mV 10 sec conditioning prepulse, while its peak tension is not greatly affected. In this case the prepulse is sufficiently small so as not to elicit tension by itself. It may be seen that the response plateau duration is sharply reduced and the rate of relaxation is increased.

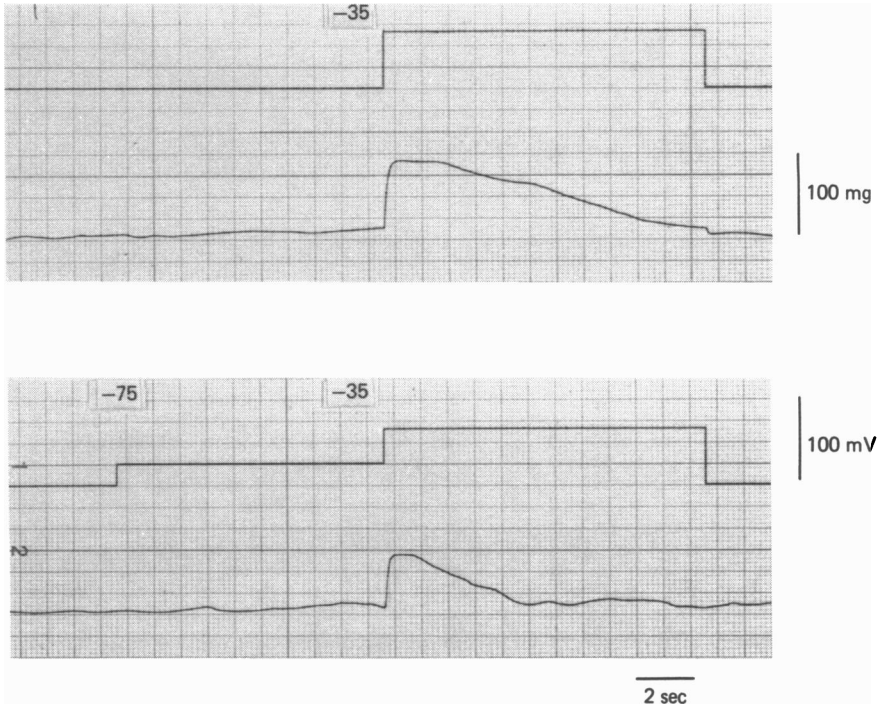


Fig. 7. Effect of subthreshold conditioning depolarization on the time course of a contractile response to a prolonged depolarization. The membrane potential level appears near the pulses.

Fig. 8 shows a further example of this behaviour. It demonstrates also that the effect of the conditioning pulse can be enhanced by prolonging its duration.

In other similar experiments it was found that the rate of relaxation could be further increased with larger conditioning pulses. In these cases, however, the conditioning pulses elicited sizable responses, and the tension developed in response to the test pulse was considerably reduced, in agreement with the findings of Frankenhaeuser & Lannergren (1967).

These experiments give support to the notion of an inactivation process, whose onset and rate may affect both contraction time course and tension output. In the original work of Hodgkin & Horowicz (1960) it was shown that after a K contracture, muscle fibres were unresponsive to subsequent further depolarization unless they were allowed to reprime. This contractile refractiveness was then considered to be the result of the complete inactivation process referred to above. During repriming

after a potassium contracture the fibre can be considered to be in intermediate states of inactivation. From the experiments described above one might expect that during repriming, full tension output is restored before the response recovers its normal time course. An example of such behaviour is shown in Fig. 9. In this experiment repriming after K contractures was studied. In each row the first contracture is the control, followed by a second challenge at different times. It may be observed that the fibre recovers its capacity for full tension output at a time when the normal response time course has not yet been restored.

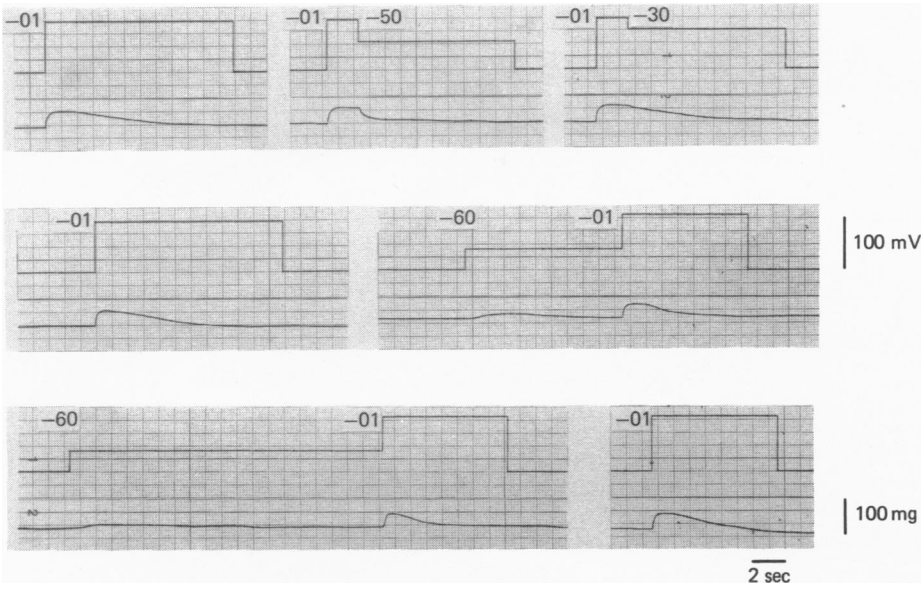


Fig. 8. Effect of duration of conditioning depolarization on the time course of contractile responses to prolonged depolarization. In this case conditioning depolarization was above contractile threshold.

DISCUSSION

In agreement with the work of Hodgkin & Horowicz (1960), the experiments reported here show that the time course of contractile responses elicited by prolonged depolarizations is greatly dependent on the fibre membrane potential. They also show that this time course can be shortened by conditioning depolarizations. These results may be taken to indicate that an 'inactivation' process of the type first described by Frankenhauser & Lannergren (1967) may determine the time course and the maximum tension of these responses. From experiments carried out by interrupting and then resuming again K contractures, it appears that the contractile activator is released continuously throughout these responses (Caputo, 1972). Repolarization of the fibre membrane, however, causes the release to stop immediately. Under voltage clamp conditions the relaxation that follows membrane repolarization occurs more rapidly than that observed in the case of interrupted potassium contractures, most possibly because in the latter case repolarization, produced by removal of the high K contracture medium, is achieved more slowly.

The fast relaxation that follows membrane repolarization is not affected by the membrane potential level, when this is more negative than the contractile threshold. This indicates that at these potentials no further release of contractile activator takes place. The continuous release of contractile activator, the fast relaxation that follows membrane repolarization, which is independent of the pulse magnitude and duration, and the sizable difference in peak tension developed at membrane potentials of -10 or -40 mV, argue against the idea that the plateau duration of these

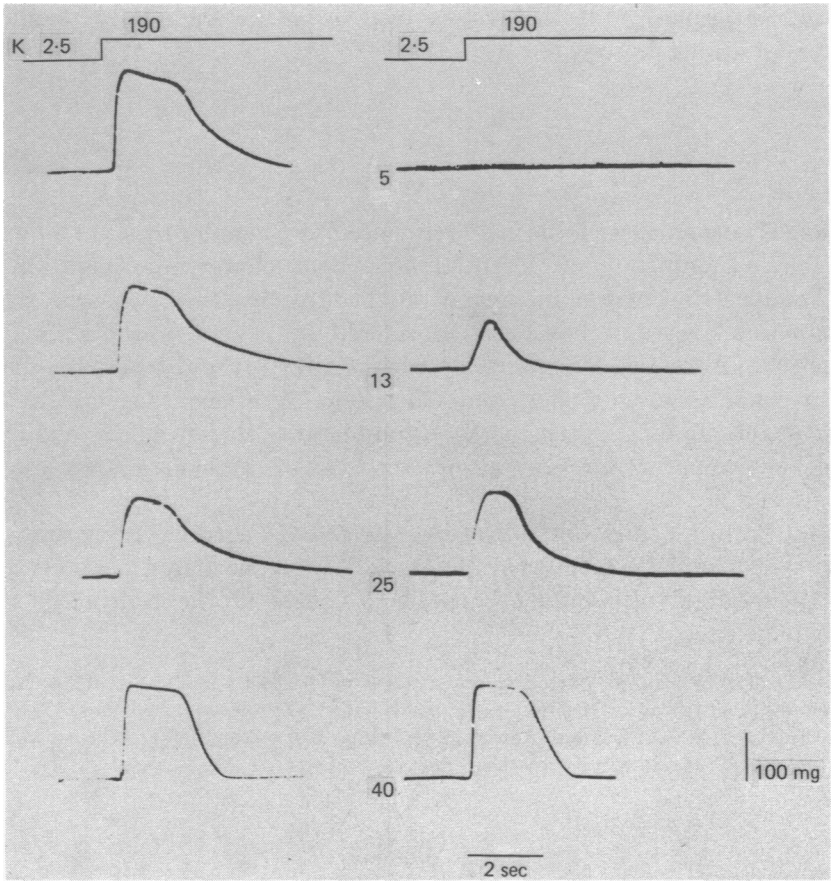
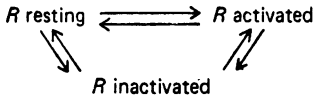


Fig. 9. Repriming after potassium contractures. The second record in each row shows the contractile response after different repriming times (in seconds) after the first contracture. It is clear that maximum tension is restored before the fibre recovers the normal response time course. The bottom two responses were obtained with a different fibre. These experiments were carried out following the procedure of Hodgkin & Horowicz (1960).

prolonged contractile responses represent the time necessary for the activator concentration in the myoplasm to be reduced from a supramaximal to a maximal concentration. Suggestive but not conclusive evidence against the idea that the spontaneous relaxation of potassium contractures was due to exhaustion of a store of activator available for release was obtained by changing differently the time course

of these responses by several experimental procedures (Caputo & Gimenez, 1967; Caputo, 1976).

The results presented in this work are consistent with the idea that calcium release is activated when the fibres are depolarized and then inactivated with time (Caputo, 1972). The time course of this inactivation would determine the time course of prolonged contractile responses. The extent of inactivation present at a given time would determine the amount of tension developed by the fibres. Although more experimental information is needed for a quantitative formulation of this model, it is tempting to propose that the system responsible for Ca release might exist in three different states represented by



This system is analogous with the model proposed by Chandler *et al.* (1976) to describe the possible configuration of potential dependent charge movement in skeletal muscle. The idea that charge movement might directly control calcium release is a most attractive one, and has been considered by several authors (Schneider & Chandler, 1973; Adrian & Almers, 1976; Adrian, 1978). It is also possible that charge movement could serve only as a connecting signal between transverse tubules and sarcoplasmic reticulum, triggering either a conductance change which might facilitate Ca release, or promoting a regenerative Ca release of the type observed in skinned fibres.

It should be mentioned that inwardly directed Ca currents occurring upon depolarization of the fibre membrane under certain particular conditions (Beaty & Stefani, 1976) might also influence the time course of the contractile responses studied here.

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