Effects of External Calcium Deprivation on Single Muscle Fibers

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ABSTRACT Deprivation of external calcium causes sudden potentiation of the twitch response of single muscle fibers. The potentiation was 64 ± 8%. Potentiation is simultaneous with membrane depolarization occurring after Ca^{++} removal. This depolarization amounted to 9 ± 2 mv. Ca^{++} removal also alters the action potential. 3 min after calcium withdrawal, action potential amplitude fell by 36 ± 3 mv; maximum rates of rise and fall of the spike decreased by 55 ± 5 and 63 ± 5% respectively. Changes in shape of the A. P. differ from those seen with other potentiators of the twitch response, such as Zn^{++}. After short exposure to calcium-free media, potassium-induced contractures show potentiation of peak tension. The S-shaped curve relating potassium contracture tension to log $[K]_o$ shifts to the left after such treatment. Calcium deprivation also increased the rate of relaxation of the contractures. This effect depends on the duration of calcium deprivation, and is probably related to the effect of calcium lack on the membrane. The change in relaxation occurred immediately after calcium deprivation, and was reversed by sudden readmission of calcium. Relaxation of twitch and tetanus responses also were affected by Ca lack, but not as rapidly as potassium contractures. The results suggest that external calcium is not directly involved in the process responsible for tension development, supporting the view that this process is mediated by translocation of intracellular calcium. The relaxation process, however, appears to be rapidly affected by deprivation of external calcium.

Normally the contractile response of muscle fibers is triggered by the lowering of the fiber membrane potential to a threshold value at which the excitation-contraction coupling (ECC) process begins to be effective (1–4). The hypothesis which postulated that calcium ions entering the fibers from the extracellular space upon depolarization of the membrane activated the contractile material (5, 6) appears to be inadequate on the basis of theoretically calculated diffusion delays (4, 7, 8). However, the presence in the interior of the fiber of the system of transverse tubules which open out directly into the external space could reduce such delays (9, 10).

There is general agreement that the depolarization of the fiber membrane releases calcium from elements of the sarcoplasmic reticulum and this calcium activates the contractile material (3, 4, 10–14). This latter explanation is supported by studies involving local activation experiments (15), and the localization of calcium deposits at the level of the terminal cisternae of the sarcoplasmic reticulum (11, 12, 16, 17). According to this hypothesis, extracellular calcium should not play the most important role in the process of activation of the contractile material. However, it is known that deprivation of external calcium results in the loss of the mechanical responses of muscle fiber to different stimuli (6, 18–22). The disappearance of the contractile response has been attributed to the depolarization of the fiber membrane which occurs in calcium-free media (18, 20), and also to a change in the ECC system (19, 23).

Using single muscle fibers, Lüttgau (21) has shown that removal of external calcium causes a fast depolarization of about 10 mv, followed by a slower one which progresses at a rate of 1 mv per min. In this case, the loss of the contractile response should occur after a relatively long delay if it is caused by depolarization of the membrane. In agreement with this, it has been found that after a short incubation of whole muscles (6) and single fibers (21, 22) in calcium-free solutions, it is still possible to obtain potassium contractures, which in certain cases may even show potentiation of the peak tension. A potentiation of the twitch response of single fibers in a medium with only one-fourth the normal calcium content has also been reported (24).

The experiments described in this paper were carried out to extend this information further by studying the short-term effects of calcium deprivation on the electrical and mechanical responses of muscle fibers.

Single muscle fibers were used throughout this study to reduce diffusional delays and incubation periods in the experimental solutions. A partial report of this work was presented at the Second International Biophysics Congress in Vienna in September, 1966.

MATERIALS AND METHODS

Single fibers were isolated from the semitendinosus muscles of *Rana pipiens* and *Leptodactylus ocellatus*. The frogs of the genus *Rana* were flown from Oshkosh, Wisconsin, those of the genus *Leptodactylus* were obtained locally. On arrival at the laboratory the animals were kept in a large tank with running water, and generally used within a month.

The dissection procedure, the experimental chamber, and the setup for registering tension have been previously described (25).

A small portion of the fiber, less than 2 mm long, rested on a small pedestal smeared with vaseline. The groove in which the fibers lay, was covered by a glass cover slide for most of the fiber length; a small opening was left above the portion of the fiber sup-

ported by the pedestal for microelectrode penetration. Glass microelectrodes filled with 3 M KCl were used.

Resting membrane potentials were measured using a Keithley 603 amplifier, connected either to a chart recorder or to an oscilloscope. For the measurement of action potentials a Bioelectric NFl amplifier (Bioelectric Instruments Inc., Hastings-on-Hudson, New York) with neutralized input capacity, connected to an oscilloscope, was used. The maximum rates of rise and fall of action potentials were measured by electrical differentiation. A low internal resistance calibrator was connected between the indifferent electrode in the chamber and earth. Stimulation of the fibers was carried out through two platinum electrodes cemented in the bottom of the chamber groove.

The normal Ringer solution had the following composition in mM NaCl 115; KCl 2.5; $CaCl_2$ 1.8; Na_2HPO_4 2.15, and NaH_2PO_4 0.85. Calcium-free solutions were prepared by omitting the $CaCl_2$. Contracture-inducing solutions, with raised potassium content, were prepared by substituting NaCl for KCl. Since the exposure periods of the fibers to high potassium solutions were short, it was not thought necessary to maintain the $(K)_o$ $(Cl)_o$ product constant (26).

All solutions were prepared using tridistilled water. The presence of calcium in this water could not be detected employing normal titration procedures; however, after evaporating the water in order to increase the concentration of any calcium present, and making allowance for any calcium lost during such procedure, it could be estimated that the concentration of calcium in the water used, was less than 10 μM. All the experiments were carried out between 19 and 21°C.

RESULTS

Effect of Calcium Free Media on the Twitch Tension

A marked seasonal difference in the responses of single fibers from *R. pipiens* exposed to calcium-free media has been found. Unless otherwise stated, the experiments reported in this section were carried out either with fibers from winter *R. pipiens* or with fibers from *L. ocellatus*. The term winter frogs is restricted to those specimens of *R. pipiens* obtained between October and April. Details of the seasonal variations observed will be given later on.

Fig. 1 shows the result of an experiment in which a single fiber was suddenly deprived of external calcium. The fiber was stimulated electrically at a frequency of 1 stimulus per sec. It may be seen that the twitch is potentiated immediately after the solution change, and that the fiber is able to maintain the contractile response for a long period before the twitch tension falls below the value obtained in the solution with normal calcium content. It appears that the potentiation of the twitch is a two phase process, each phase covering approximately half the value of the total potentiation, and that one phase develops immediately after the solution change and the other one takes about 30 sec to reach the maximum value.

For eight fibers from *R. pipiens*, tested during the month of February, the

mean potentiation of the twitch response due to such experimental procedure, expressed as per cent of the response under normal conditions was 64 ± 8 (mean ± SEM). The number of potentiated twitches obtained from a given fiber was always greater than 150. After an adequate recovery period, a second exposure resulted in a lesser potentiation or no potentiation at all of the twitch response, and in fewer responses.

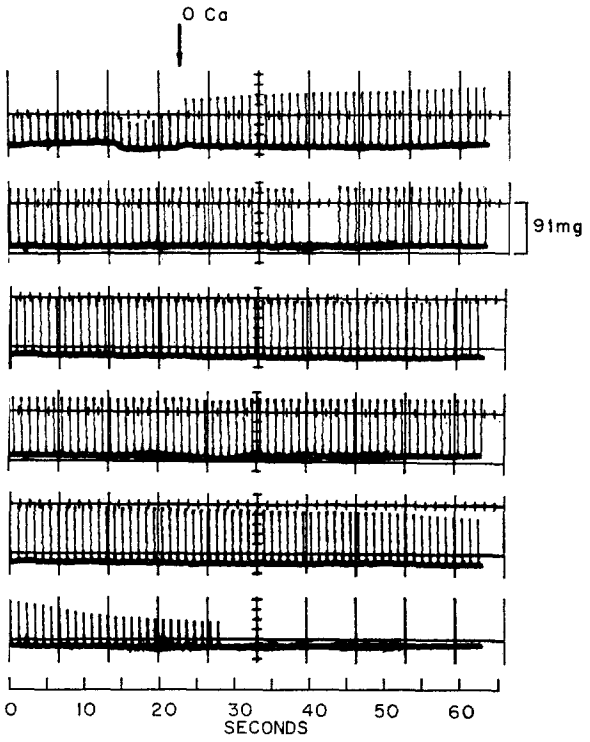

FIGURE 1. Oscilloscope records showing the effect of external calcium removal on the twitch tension of a single muscle fiber from *R. pipiens*. Fiber diameter 70 μ. The fiber was stimulated at a frequency of 1 stimulus per sec. In the second record from the top, stimulation was stopped during approximately 5 sec. Experiment of 16 February 1966.

In the preceding experiments the fibers were stimulated during the exposure to the calcium-free media and the exposure period was never longer than 5 min. In one experiment a single fiber was incubated for 15 min in a calcium-free solution before starting to stimulate it at 1 shock per sec. The twitches obtained after this longer period also showed a potentiation of 74%, but in this case only 30 twitches could be obtained before tension started to decline. The experiments carried out with fibers from *L. ocellatus* gave essentially the same results.

Effect of Calcium-Free Media on Membrane Potentials

Fig. 2 shows the time course of the depolarization of a single fiber membrane caused by sudden deprivation of the external calcium, and the repolarization due to restoration of the normal calcium concentration. The depolarization of 9 mv is complete within 2 sec of the change. The mean depolarization for eight fibers from *R. pipiens* so tested was 9 ± 2 mv. When the exposure to the calcium-free medium is a short one, as in this case, the depolarization is reversible. The depolarization caused by calcium-free solution seems to be associated with the potentiation of the twitch response, although it does not develop in two phases as does the twitch potentiation.

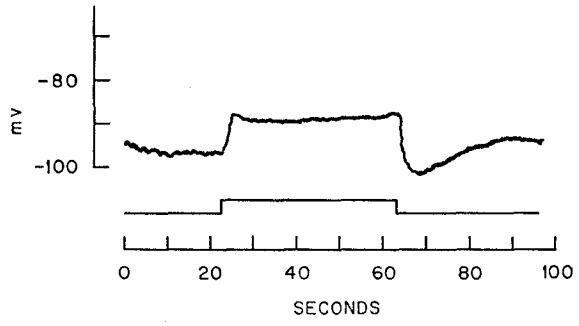

FIGURE 2. Tracing showing the membrane potential changes due to external calcium deprivation and restoration. Solution changes are indicated by the steps in the lower trace. Single fiber from *R. pipiens*. Fiber diameter 70 μ. Experiment of 21 April 1966.

Sandow and Kahn (27) observed a potentiation of the twitch when the external potassium was raised. This observation has been confirmed by the experiments shown in Fig. 3. Single fibers were suddenly exposed to solutions with 5 and 10 mM per liter of potassium ions, and the change in twitch tension and membrane potential measured. In Fig. 3 the changes in twitch tension, expressed in per cent of the normal value, and the changes of membrane potential, expressed in millivolts of depolarization, are plotted against the external potassium concentration. It appears that in this case the depolarizations obtained are associated with a lesser twitch potentiation since doubling the normal potassium concentration causes a twitch potentiation of 14 ± 4% and a depolarization of 11 ± 2 mv, while increasing the external potassium concentration to 10 mM per liter induces a twitch potentiation of 56 ± 9% and a depolarization of 28 ± 2 mv. Furthermore when the external potassium concentration is raised, the twitch potentiation develops immediately, and lacks the slow phase observed when the potentiation is produced by calcium

lack. From these results, it appears, that although a twitch potentiation is associated with the depolarization of the membrane, the potentiation observed when the fibers are deprived of external calcium cannot be explained entirely in these terms.

The possible contribution of changes in the shape of the action potentials to the twitch potentiation was also investigated. To eliminate the possibility of tearing the membrane with the microelectrode during the twitch movement, hypertonic solutions, prepared by adding 115 mM of NaCl to the normal solution, were used (28). The resulting solution did not completely eliminate the mechanical response. Test experiments were carried out to investigate whether the hypertonic solution per se caused appreciable changes

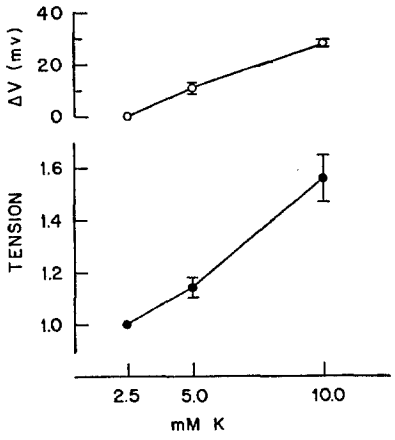

FIGURE 3. Twitch potentiation and membrane depolarization of single muscle fibers from *R. pipiens* obtained by raising the external potassium concentration. The twitch tension (filled circles) is expressed relative to the tension obtained in medium with 2.5 mM of potassium. The filled circles represent the mean (± 1 SEM), for six fibers. The open circles represent the mean depolarization (± 1 SEM) for four fibers. Experiments performed in March 1967.

in the action potentials. The results obtained in such test experiments confirmed the previous finding that hypertonicity does not appreciably affect the shape of the action potentials (28). However, it was observed that hypertonic solutions increased the resting membrane potential by approximately −8 mv.

Fig. 4 shows an experiment in which the action potential and the twitch tension of a single fiber were registered simultaneously. The fiber was incubated in hypertonic medium to reduce the contractile response and then exposed to a calcium-free solution, also hypertonic. It appears that the twitch response, which was almost abolished by hypertonicity (top left record), is partially restored by the calcium-free solutions (top right record). The electrical response of this particular fiber was not drastically affected by the calcium lack, even after a relatively long exposure of 10 min (bottom left record). The record at bottom right shows the fiber responses 5 min after calcium restoration. However, in the case of other fibers calcium deprivation causes more rapid changes in the electrical response, although the effect on the twitch is

qualitatively the same. Fig. 5 shows a case in which after 5 min of exposure to calcium-free solution the action potential of a single fiber looked considerably altered although the twitch appeared to be partially restored.

Table I shows the changes in some parameters of the action potentials of five fibers 3 min after exposure to a calcium-free medium. From these results it appears that the changes in the shape of the action potentials, obtained in calcium-free solutions, do not seem to be of the type obtained with some substances known to be twitch potentiators, i.e. zinc ions (29), although the

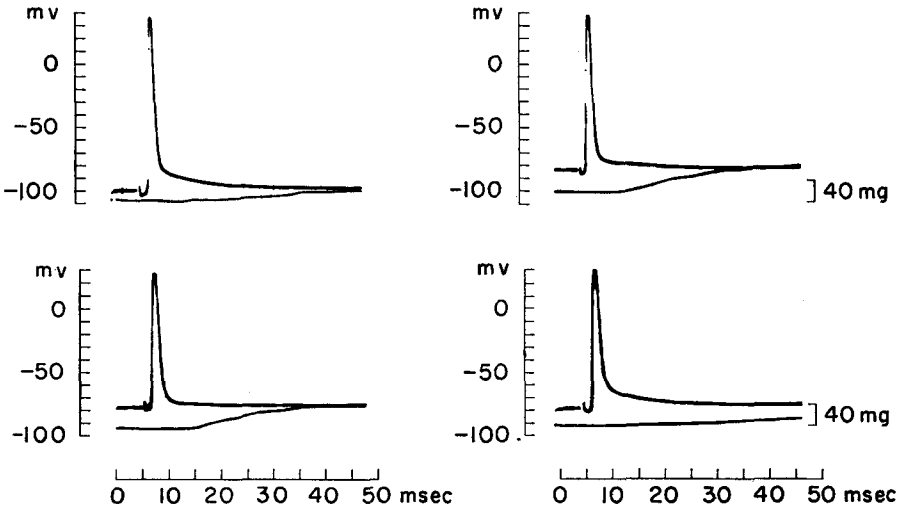

FIGURE 4. Oscilloscope records showing the action potential and twitch tension of a single fiber from *L. ocellatus* in hypertonic medium. Fiber diameter 110 μ. The upper left record was obtained in a solution with normal calcium content. The records at upper right and lower left were obtained respectively 2 and 10 min after withdrawing external calcium. The record at lower right was obtained 5 min after calcium restoration. Experiment of 13 October 1966.

small change observed, due especially to the reduction of the rate of the fall of the spikes, might contribute to the potentiation of the twitch (30).

It is interesting to note that the fibers isolated from *R. pipiens* showed seasonal variations in behavior as already mentioned. From May to September, exposure of such fibers to calcium-free media resulted in short bursts of spontaneous activity followed by mechanical refractoriness to electrical stimulation. Measurements of changes in the membrane potentials were rendered difficult since the fibers contracted upon exposure to calcium-free solutions and dislodged the microelectrode; however, it was determined that in such cases the depolarization exceeded 30 mv. In one experiment it was possible, by adding procaine at a concentration of 2 mM to restore the twitch response of a fiber that had lost it as a consequence of withdrawing the exter-

nal calcium. Experiments conducted in the same period of the year with fibers from *L. ocellatus* gave basically the same results as those obtained with winter *R. pipiens* fibers.

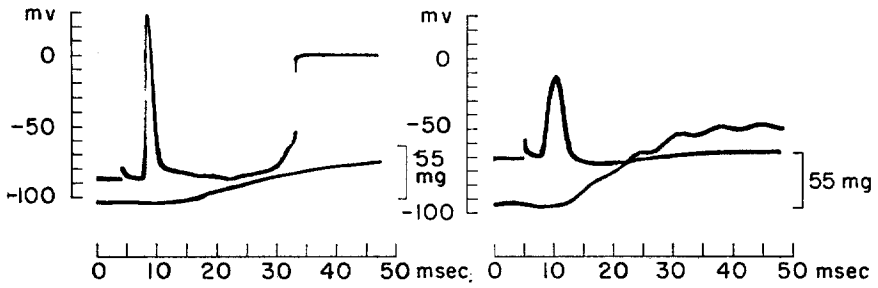

FIGURE 5. Oscilloscope records showing the action potential and twitch tension of a single fiber from *L. ocellatus* in hypertonic medium. Fiber diameter 120 μ. The record at left was obtained in an hypertonic solution with normal calcium content. The record at right was obtained 5 min after calcium deprivation. Experiment of 17 October 1966.

TABLE I

THE EFFECT OF CALCIUM DEPRIVATION ON THE ACTION POTENTIAL OF SINGLE FIBERS

Fiber	A.P. amplitude		Rising phase *dV/dt*		Falling phase *dV/dt*	
	1.8 mM Ca	0 Ca*	1.8 mM Ca	0 Ca*	1.8 mM Ca	0 Ca*
	mv	*mv*	*v/sec*	*v/sec*	*v/sec*	*v/sec*
1.	140	101	458	243	129	80
2.	131.5	96.6	414	241	145	67
3.	127	97	427	228	190	133
4.	128	85	384	152	150	99
5.	128	95	425	253	169	110
Mean±SEM	131±3	94±3	422±12	223±19	157±11	98±12

* The values in 0 Ca were obtained 3 min after the change of solutions.

Effects of Calcium-Free Solutions on the Potassium Contractures

Frank, using whole muscles (6), established that the presence of calcium in the bathing medium was required for the production of potassium contractures. However, Lüttgau (21) and Pauschinger et al. (22), working with single fibers found that after short incubation of the fibers in calcium-free media, potassium contractures, although modified, could still be elicited.

Hodgkin and Horowicz (3) found that the development of tension in single muscle fibers is related to the logarithm of external potassium concentration by a steep S-shaped curve, with tension starting at a determined potassium concentration. Lüttgau (21) showed that raising the concentration of external

calcium resulted in a shift toward the right of the S-shaped curve, *i.e.* tension started at a higher potassium concentration; he also showed that the rate of rise of tension and of relaxation increased, and the plateau of the potassium contracture was remarkably shortened in low calcium solution.

The following experiments were performed to obtain additional information on the effects of calcium lack on the potassium contractures.

Fig. 6 shows the results obtained with two fibers of one frog, when potassium contractures were induced in calcium-free medium. It appears that in this case the S-shaped curve is shifted toward the left; *i.e.*, tension starts at a lower

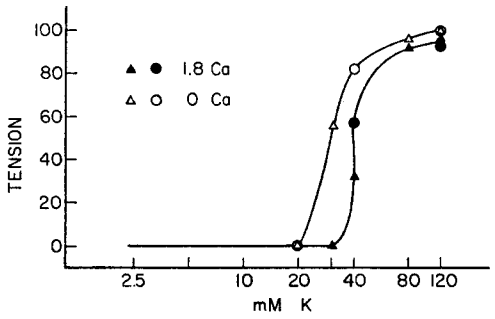

FIGURE 6. Effect of external calcium lack on the relation between contracture peak tension and potassium concentration of single fibers isolated from *R. pipiens*. The contracture peak tension is expressed in per cent of the maximum tension. The two different symbols represent the results obtained with two different fibers from the same frog. Contracture tensions represented by filled symbols were obtained in media with normal calcium content. Contracture tensions represented by open symbols were obtained 10 sec after calcium deprivation. Fibers diameter 70 μ. Experiment of 8 November 1966.

potassium concentration. It is interesting to observe that only a few contractures can be obtained from an individual fiber during calcium deprivation. Results similar to those shown in Fig. 6 were obtained in other fibers. In the particular case shown, however, calcium lack does not appear to induce any appreciable potentiation of the contractures obtained with potassium concentrations higher than 40 mM; in six other fibers such potentiation was observed, as will be shown below.

Figs. 7 and 8 show the modifications of the time course of the contracture after different periods of exposure to calcium-free solutions. The results of Fig. 7 were obtained with a fiber from *L. ocellatus*. Record A shows a normal contracture induced by 80 mM of potassium, records B and C show contractures obtained after 2 and 10 min respectively of exposure to calcium-free media. The results of Fig. 8 were obtained with a fiber from *R. pipiens* in summer. Record A shows a normal contracture, records B, C, and D show contractures obtained after 8, 15, and 25 sec of calcium deprivation, respectively. In both figures, besides the potentiation of the peak contracture tension,

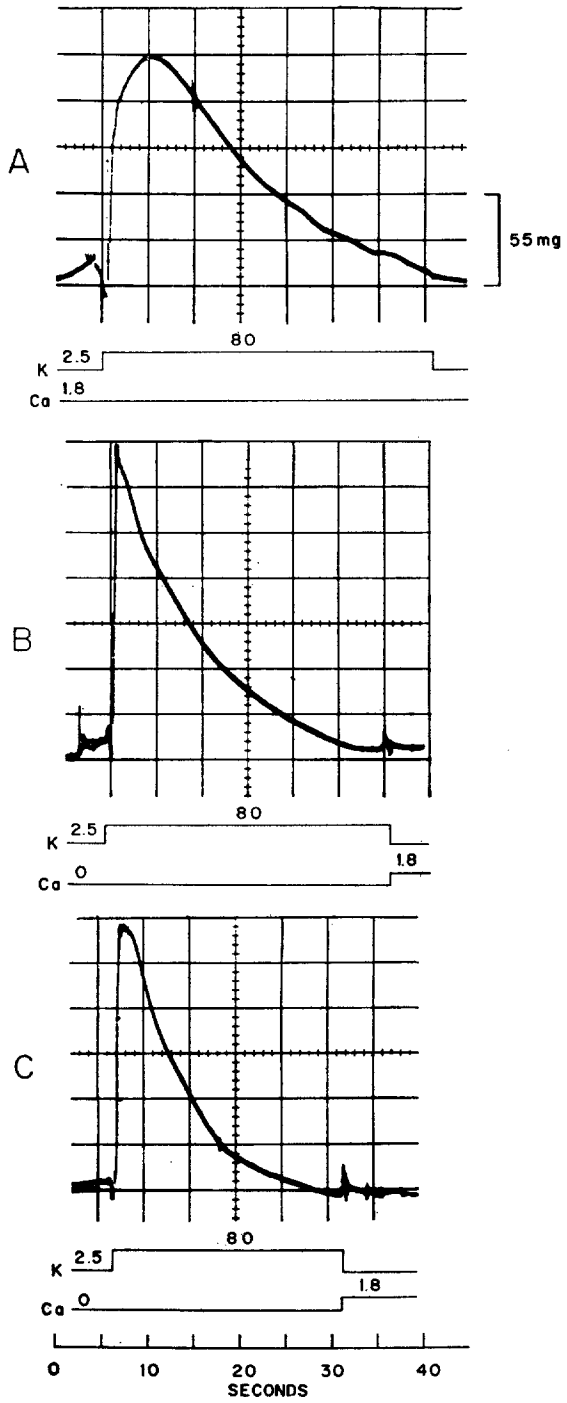

FIGURE 7. Oscilloscope records showing potassium contractures of a single fiber from *L. qcellatus* after different exposure periods to calcium-free solutions. Record A shows a contracture obtained in a solution with normal calcium content. Record B starts after 2 min of exposure of the fiber to a calcium-free solution. Record C starts 10 min after exposure to the calcium-free solution. In the last two cases the contracture medium also lacked calcium. After each contracture the fiber was allowed to rest during 15 min in the normal solution. Fiber diameter 85 μ. Experiment of 28 August 1966.

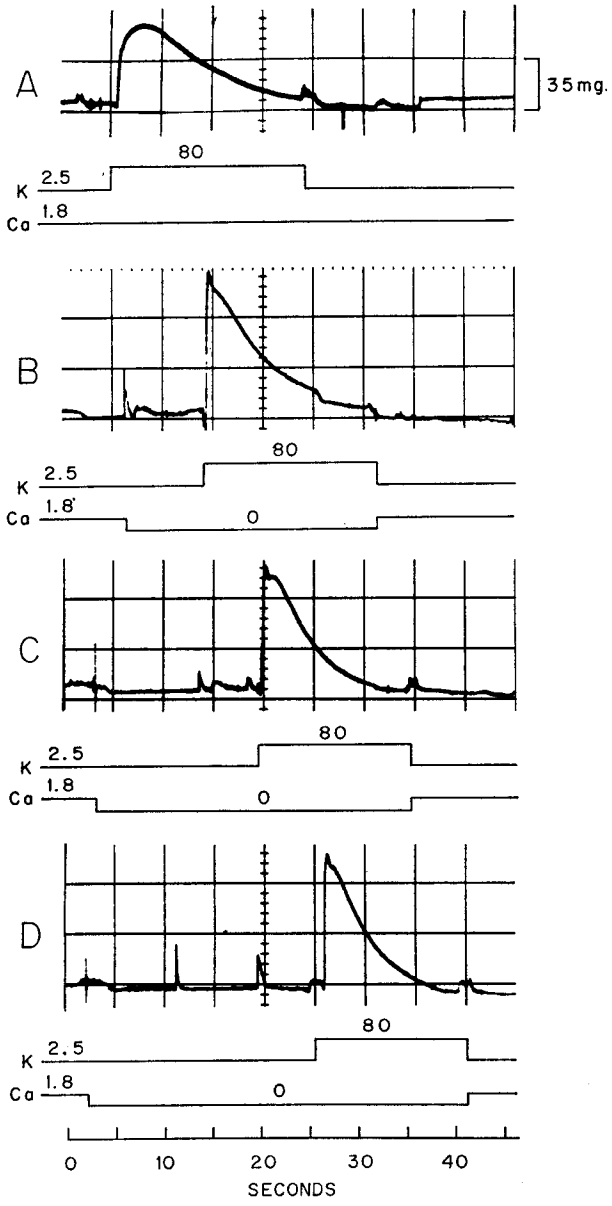

FIGURE 8. Oscilloscope records showing potassium contractures of a single fiber from *R. pipiens* after different exposure periods to calcium-free solutions. The traces below each contracture show the solution changes. After each contracture the fiber was allowed to rest during 15 min in normal Ringer's solution. Fiber diameter 60 μ. Experiment of 21 July 1966.

the effect of calcium lack on the relaxation phase is also shown. The relaxation rate appears to be increased in zero Ca, confirming the results obtained by Lüttgau and Pauschinger et al. (21, 22). Although this effect is basically the same for the fibers of both frogs, there is a clear difference in the time necessary for the effect to appear. This difference is better observed in Fig. 9, in which

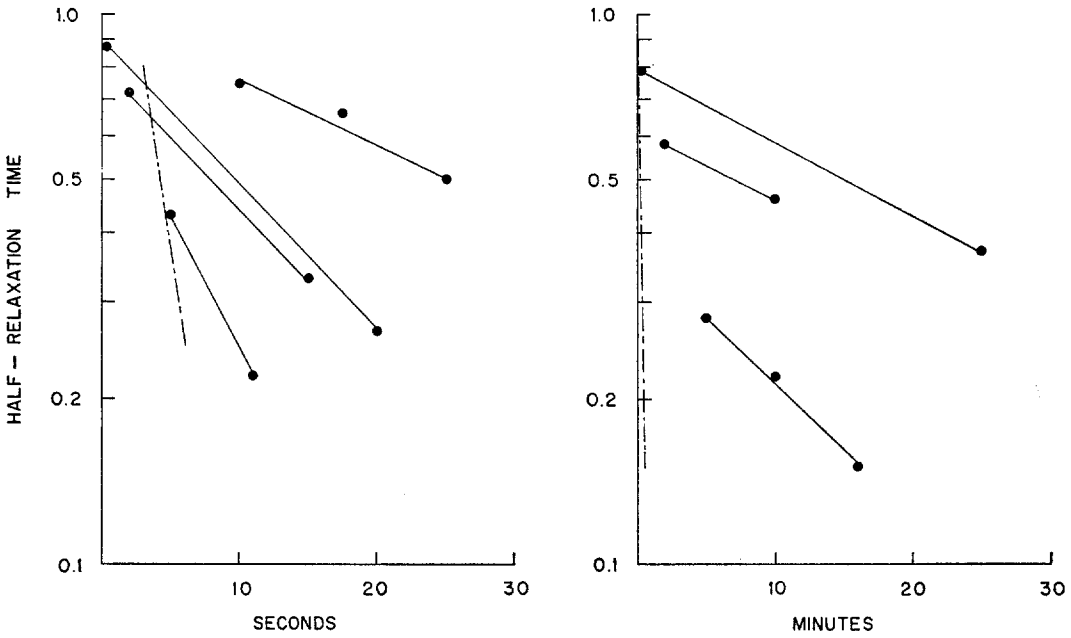

FIGURE 9. The relation between the half-relaxation time of potassium contractures and the exposure period to calcium-free solutions for single muscle fibers from *R. pipiens* (left) and *L. ocellatus* (right). The diameters of the fibers from *R. pipiens* were approximately 60 μ; those of the fibers from *L. ocellatus* were approximately 100 μ. The continuous line unites the experimental points obtained for each fiber; the discontinuous line shows the time course of the loss of a substance by diffusion from cylinders of 60 μ (left) and 100 μ (right). Experiments with *R. pipiens* fibers were performed during the month of August, 1966.

the results obtained with four fibers from summer *R. pipiens* and three from *L. ocellatus* are shown. The relaxation rate is shown in terms of the half-relaxation time; that is, the time necessary for the peak tension to fall to half its initial value. The ordinate has been expressed as a fraction of the half-relaxation time obtained in solutions with full calcium content. The great difference observed cannot be explained considering the difference in fiber diameter. Experiments carried out with fibers from winter *R. pipiens* gave the same results as those obtained with *L. ocellatus* fibers. This point needs more experimental work; however, it is probable that such difference in behavior is a

consequence of the different membrane potential changes caused by calcium lack in the fibers from *R. pipiens* in different periods of the year.

The experiment shown in Fig. 10 was carried out to test whether the relaxation phase of the contractures could be immediately affected by external

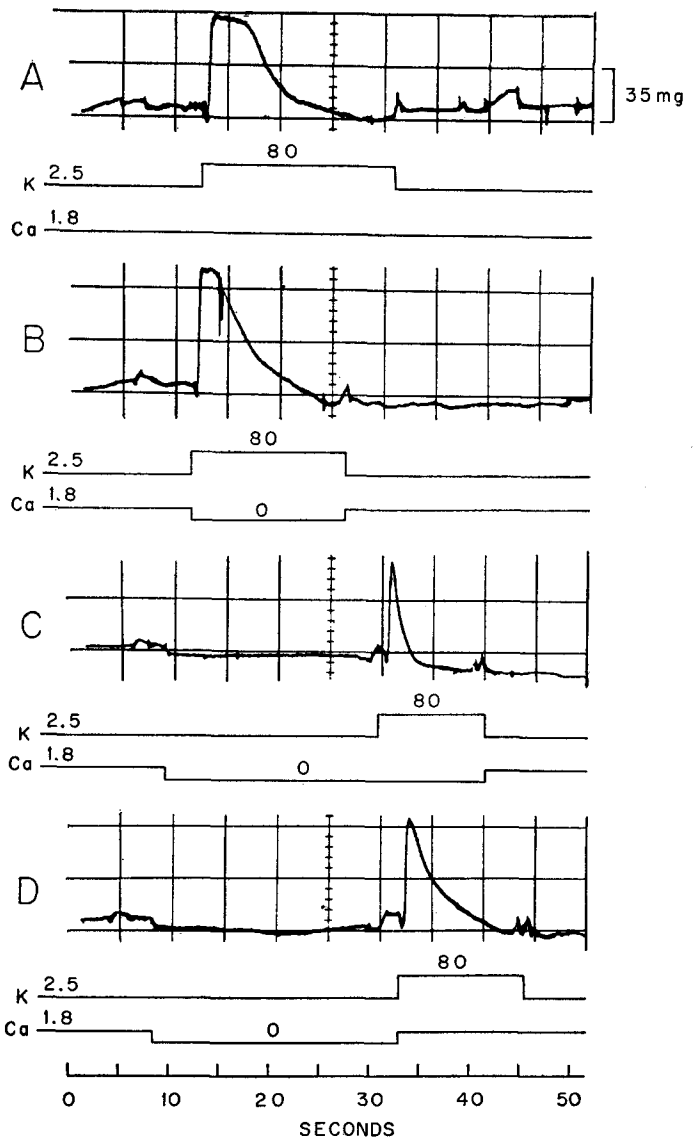

FIGURE 10. Oscilloscope records showing the sudden effects of external calcium deprivation and restoration on the relaxation phase of potassium-induced contractures. Single fiber from *R. pipiens*. Fiber diameter 65 μ. Experiment of 26 July 1966.

calcium change. A fiber from a summer *R. pipiens* was chosen because such fibers show the effect of calcium lack more rapidly. Record A shows a normal contracture with a half-relaxation time of 5.2 sec, in record B, the high potassium solution lacked calcium, and in this case the contracture half-relaxation time was 4.5 sec. In C a potassium contracture was induced after a 25 sec exposure to a calcium-free medium, the contracture-inducing solution also lacked calcium, and the half-relaxation time was 0.8 sec. Finally, in D, the contracture was also induced after 25 sec of exposure to a calcium-free solution, but in this case the contracture solution had a normal calcium content; here the half-relaxation time was 2.1 sec. From this experiment it appears that the relaxation phase is affected immediately by changes in the external calcium, contrary to what happens in the case of the tension development phase.

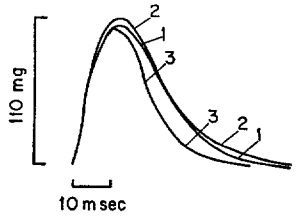

FIGURE 11. Traces of isometric twitches of a single fiber from *L. ocellatus*. Fiber diameter 100 μ. Twitch 1 was obtained in normal Ringer's solution. Twitches 2 and 3 were obtained respectively 2 and 20 min after calcium deprivation. Experiment of 9 August 1966.

Calcium Deprivation Effect on the Relaxation Phase of Twitches and Tetani

Fig. 11 shows an experiment performed with a fiber from *L. ocellatus*, in which twitches were elicited before and after calcium deprivation. Twitch 1 was obtained in a medium with 1.8 mM of calcium, twitches 2 and 3 were obtained 2 and 20 min after calcium deprivation. Twitch 3 was obtained after

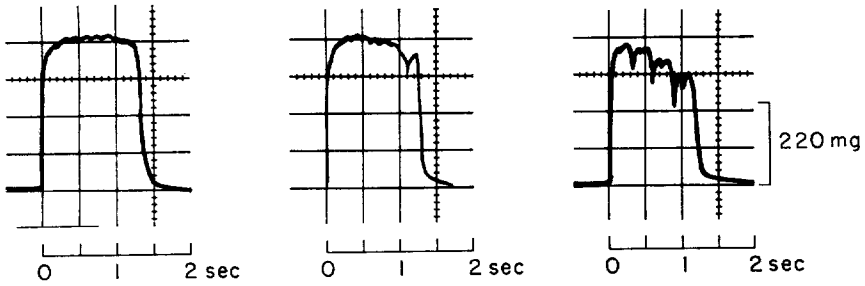

FIGURE 12. Oscilloscope records showing isometric tetanic contractions of a single fiber from *L. ocellatus*, stimulated at a frequency of 125 shocks per sec during approximately 1.5 sec. Fiber diameter 100 μ. Left record shows a tetanus obtained in normal Ringer's solution. Center and right records show tetani obtained respectively 10 and 22 min after calcium deprivation. The fiber was left in the calcium-free solution after the tetanus shown in the center. Experiment of 24 August 1966.

the potentiation caused by calcium deprivation had disappeared. The twitch tension is similar to that of twitch 1, but the relaxation phase is faster.

Fig. 12 shows the effect of calcium lack on electrically elicited tetani. The fiber was stimulated at a frequency of 125 stimuli per sec. No potentiation of the maximal tension is obtained after calcium deprivation. It appears also that the fiber is not able to sustain the tetanic tension for the duration of stimulation. The tension falls, as shown with tetani obtained 10 and 22 min after the solution change.

DISCUSSION

In the case of the extensor longus muscles, it has been reported that the disappearance of the mechanical response caused by calcium lack is determined by the rate at which calcium ions leave the extracellular space (6, 31). According to this, in the case of single muscle fibers, the loss of the mechanical response should be determined by the rate at which solutions are changed. The results obtained in the experiments reported here show clearly that this is not the case. Single fibers from *L. ocellatus* or from winter specimens of *R. pipiens* maintain the mechanical response to electrical stimulation and to raised potassium concentrations for relatively long periods after calcium deprivation. These results are in agreement with the view that activation of the contractile material is mediated by translocation of intracellular calcium and not by the entry of external calcium (4, 9, 11).

When the dimensions of the transverse tubules are considered, it seems reasonable to ignore the possibility that the long delay in the loss of the contractile response might depend on the time required for calcium ions to leave the tubular space. In fact the volume of the transverse tubules has been found to be 0.3% of that of the fibers; unless the calcium concentration inside the tubules is much greater than that of the extracellular space, the calcium present in the tubules should not be sufficient to maintain the contractile activity of the fibers.

Deprivation of external calcium induces a potentiation of both twitch and contracture responses. In the case of the twitch, the potentiation probably is caused by a combination of several factors, such as depolarization of the membrane, lowering of the contractile threshold, and small changes in the action potential shape. In the case of the potassium contractures, the depolarization caused by calcium lack probably affects the region of low potassium concentrations of the S-shaped curve relating tension to the logarithm of external potassium concentrations (3). However, the fact that a potentiation of the peak contracture tension is observed also at higher potassium concentrations suggests that the potentiation is mediated by a shift in the contractile threshold caused by calcium lack; it is opposite in direction to the shift observed when external calcium is raised (21). It is important to recall that

Curtis (32) using the e. longus muscle did not observe any shift in the S-shaped curve, when the muscles were exposed to a solution containing only 100 μM of calcium, although this could be due to his use of methyl sulfate in partial replacement of chloride; moreover, he also reported that this calcium concentration did not induce any depolarization of the fiber membrane. On the other hand, Krolenko and Tsifrinovich (24) have observed a twitch potentiation when the calcium concentration was lowered to one-fourth of the normal value. It seems probable, then, that calcium lack, by altering the fiber membrane properties, also changes the contractile threshold of the fiber.

The depolarization caused by calcium lack has been explained in terms of an increase in permeability to all ions (32). This interpretation is in agreement with the role of stabilizer generally attributed to calcium (33). In order to stabilize the membrane, calcium is probably adsorbed at the outer surface of the membrane (34). Therefore the seasonal variations observed in the case of fibers from *R. pipiens* may be caused by a variation in the adsorption constant of calcium to the membrane.

The changes observed in the action potential after calcium deprivation are similar to those reported for toad muscle fibers (35).

It is clear from these experiments that extracellular calcium is not the most important factor involved in the mechanism responsible for tension development. It is possible that external calcium replenishes the cellular exchangeable calcium when this has been exhausted. This view is supported by the fact that caffeine contractures, which take place without important changes in the fiber membrane potential (36), are not affected as rapidly as potassium contractures by external calcium deprivation (37, 25) although after a certain number of contractures, the tension starts to decline.

It is also clear that the mechanism which regulates the relaxation process after contraction is affected rapidly by external calcium concentration changes Recently, Curtis (38) has studied the calcium fluxes in single fibers and has found that the fiber calcium is distributed in two fractions, one of which is rapidly exchangeable and amounts to 47% of the total, and the other which appears to be nonexchangeable. The exchangeable fraction can be reduced to zero when external calcium is removed. It is probable that this fraction is in a loose bound form and is in equilibrium with the cellular free calcium whose concentration has been estimated to be approximately 10^{-7} M (16, 39). It is probable, then, that a decrease of the Curtis exchangeable fraction (38) results also in a decrease of the free calcium, which should bring the fiber toward a more relaxed state (13). This view seems to be supported by our experimental finding that external calcium lack increases the relaxation rates after different types of contractile activity.

Recently, Foulks and Perry (40) have found that increased calcium concentration prolongs the relaxation phases of potassium contractures. Their

results suggest also that the relaxation process is dependent on the membrane potential level. It is then possible that the effects of calcium deprivation on the relaxation rates of muscle fibers described in this paper are mediated by changes that occur at the level of the cellular membranes.

The authors wish to thank Drs. F. Herrera and L. Villegas for careful reading of the manuscript. This work was aided in part by grant 5R05-TW00122 from the Public Health Service of the United States to one of the authors (C.C.).

Received for publication 3 February 1967.

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