

# Ultraslow Contractile Inactivation in Frog Skeletal Muscle Fibers

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**ABSTRACT** After a contracture response, skeletal muscle fibers enter into a state of contractile refractoriness or inactivation. Contractile inactivation starts soon after membrane depolarization, and causes spontaneous relaxation from the contracture response. Here we demonstrate that contractile inactivation continues to develop for tens of seconds if the membrane remains in a depolarized state. We have studied this phenomenon using short (1.5 mm) frog muscle fibers dissected from the *Lumbricalis brevis* muscles of the frog, with a two-microelectrode voltage-clamp technique. After a contracture caused by membrane depolarization to 0 mV, from a holding potential of  $-100$  mV, a second contracture can be developed only if the membrane is repolarized beyond a determined potential value for a certain period of time. We have used a repriming protocol of 1 or 2 s at  $-100$  mV. After this repriming period a fiber, if depolarized again to 0 mV, may develop a second contracture, whose magnitude and time course will depend on the duration of the period during which the fiber was maintained at 0 mV before the repriming process. With this procedure it is possible to demonstrate that the inactivation process builds up with a very slow time course, with a half time of  $\sim 35$  s and completion in  $>100$  s. After prolonged depolarizations ( $>100$  s), the repriming time course is slower and the inactivation curve (obtained by plotting the extent of repriming against the repriming membrane potential) is shifted toward more negative potentials by  $>30$  mV when compared with similar curves obtained after shorter depolarizing periods (10–30 s). These results indicate that important changes occur in the physical state of the molecular moiety that is responsible for the inactivation phenomenon. The shift of the inactivation curve can be partially reversed by a low concentration ( $50 \mu\text{M}$ ) of lanthanum ions. In the presence of 0.5 mM caffeine, larger responses can be obtained even after prolonged depolarization periods, indicating that the fibers maintain their capacity to liberate calcium.

## INTRODUCTION

The spontaneous relaxation from a potassium contracture, which occurs while the fiber membrane remains in a depolarized state, was originally attributed to exhaustion of a contractile activator (Hodgkin and Horowicz, 1960) and later to the onset of an inactivation process that ends calcium release from the sarcoplasmic reticulum

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(Frankenhaeuser and Lannergren, 1967; Caputo, 1972). After the contracture response of a muscle fiber, it enters a state of contractile refractoriness, from which it can be released by membrane repolarization (Hodgkin and Horowicz, 1960). The steady-state relationship between extent of repriming and the membrane potential value during the repolarization period provides information about the voltage dependency of the inactivation process. Since the spontaneous relaxation occurs with a relatively fast time course, with a rate constant of  $\sim 0.5 \text{ s}^{-1}$  at  $20^\circ\text{C}$  (Caputo and Fernandez de Bolaños, 1979), one might think that inactivation proceeded with the same time course (Caputo, 1972). However, there are indications that the inactivation process develops much more slowly. The initial evidence for the slowness of the contractile inactivation was provided by Hodgkin and Horowicz (1960) who reported that contractile repriming was decreased after prolonged exposure to high potassium solutions. Contractile inactivation can also be caused by conditioning depolarizations, induced by high potassium solutions (Frankenhaeuser and Lannergren, 1967; Nagai et al., 1979), or by voltage-clamp steps (Caputo et al., 1984; Caputo and Bolaños, 1987) and it has been reported that in these cases it could take several seconds or even minutes to develop, depending on the depolarization level (Nagai et al., 1979). For this article we studied the time course of development and the voltage dependence of contractile inactivation, characterizing the repriming process after prolonged depolarization periods to 0 mV. We show that inactivation proceeds with an ultraslow time course, with a half time of  $\sim 35 \text{ s}$ , and that when it is completed in  $\sim 100 \text{ s}$ , important changes are found in the repriming time course and in the voltage dependence of the repriming process. These results suggest that changes in the physical state of the molecular entities are responsible for the phenomenon, and most probably they are also responsible for the intramembrane charge movement signals. A short communication of this work has been presented at the Biophysical Society (Caputo and Bolaños, 1989b).

#### MATERIALS AND METHODS

The experiments were performed with bundles of muscle fibers dissected from the lumbricalis muscles of *Rana pipiens*. The dissecting and handling as well as all the other experimental procedures were similar to those already described (Caputo et al., 1984; Caputo and Bolaños, 1987). In brief, the membrane potentials of individual fibers were controlled and displaced to the desired values using a two-microelectrode voltage-clamp technique. Isometric tension developed by voltage-clamped single fibers was measured with a tension transducer (model 400; Cambridge Instruments, Inc., Cambridge, MA). The experiments were carried out at  $21^\circ\text{C}$  using a normal Ringer's solution whose composition in millimoles per liter was as follows: 115 NaCl; 2.5 KCl, 1.8  $\text{CaCl}_2$ , 10 mM Tris buffer, pH 7.4; osmolarity 260 mosmol; tetrodotoxin was added at a concentration of  $2 \times 10^{-7} \text{ M}$ .

#### RESULTS

##### *Ultraslow Buildup of Inactivation*

Fig. 1 shows the results of an experiment testing the effect of prolonged depolarization on the repriming capacity of a muscle fiber. The upper records show the results

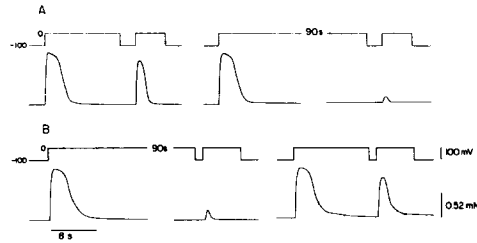


FIGURE 1. Effects of the duration of a first depolarizing pulse on the contractile response to a second pulse in two muscle fibers, *A* and *B*. Both fibers were held at a membrane potential of  $-100$  mV and depolarized to  $0$  mV. In the two runs made with fiber *A*, the first depolarization durations were 10 and 90 s, respectively. In the runs of fiber *B*, they were 90 and 10 s. See the text for further details.

obtained with one fiber. In the first panel, the fiber was depolarized from  $-100$  to  $0$  mV and developed a contracture, which was ended by the spontaneous relaxation. The depolarization lasted 10 s, after which the fiber was repolarized to  $-100$  mV for 2 s, and then depolarized again to  $0$  mV. The repriming period of 2 s was sufficient for the fiber to develop a second response whose peak tension amounted to  $\sim 84\%$  of the first one. After a rest period of at least 5 min the fiber was depolarized again to  $0$  mV; this time the depolarization was maintained for 90 s. After this period the same repriming procedure only enabled the fiber to develop a small response that amounted to 10% of the first one. The lower row of Fig. 1 shows a similar experiment carried out with another fiber following the inverse procedure.

Fig. 2 summarizes the results of several experiments in which the same repriming protocol as that shown in Fig. 1 was followed. In the graph, the reprimed tension, that is the tension in response to the second depolarization expressed as a fraction of the tension of the first response is plotted against the duration of the first

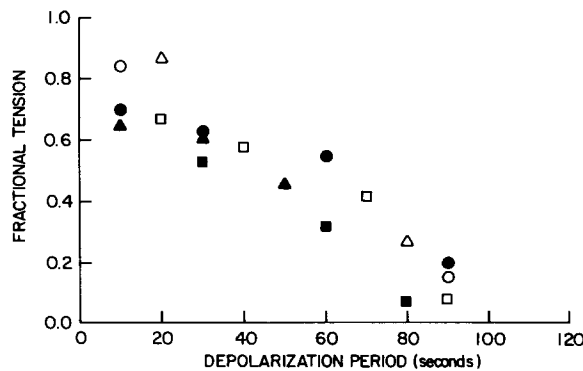


FIGURE 2. Ultraslow time course of inactivation buildup, determined in experiments similar to those shown in Fig. 1. The graph shows the extent of contractile repriming, i.e., the ratio between the peak tensions of the second and first responses plotted against the duration of the first depolarization. In all cases the fibers were depolarized to  $0$  mV from a holding potential of  $-100$  mV. Repriming at  $-100$  mV lasted 2 s. In the graph each type of symbol corresponds to the results obtained with the same fiber.

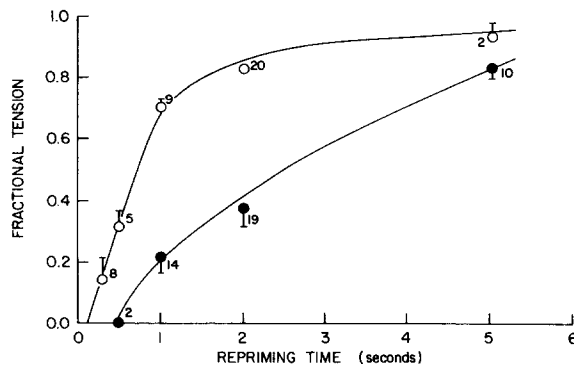


FIGURE 3. Time course of contractile repriming after relatively short (10 s; *open symbols*) or prolonged (>80 s; *filled symbols*) depolarizations. The graph represents the extent of repriming, plotted against the repriming duration. Each point represents the mean  $\pm$  the SEM of different determinations, whose number appears beside each point.

depolarizing pulse. The figure demonstrates that the buildup of the inactivation process occurs with a half time of  $\sim 35$  s, and that completion takes  $>100$  s.

Fig. 3 shows that the time course of contractile repriming after a first depolarization depends on the duration of the depolarization period. In the graph the fractional reprimed tension is plotted against the repriming duration, that is, the interval between depolarizations. In these experiments the repriming membrane potential was  $-100$  mV. The open and filled circles represent the results obtained when the first depolarization was relatively short (10 s) or rather prolonged ( $>80$  s), respectively.

Fig. 4 shows the effect of the membrane potential at which the fiber was held during the interval between depolarizations, on the fiber repriming capacity. The upper record of Fig. 4 *A* of the figure shows a run in which, after a 10-s depolarization, repriming for 1 s at  $-100$  mV restored  $\sim 36\%$  of the initial tension. The lower run demonstrates that with a repriming potential of  $-150$  mV, tension restoration was 86%. Fig. 4 *B* shows similar runs, using another fiber, in which the first depolarization lasted 80 s. In this case repriming at  $-100$  and  $-150$  mV caused tension restoration of 10 and 81%, respectively. Similar experiments were carried

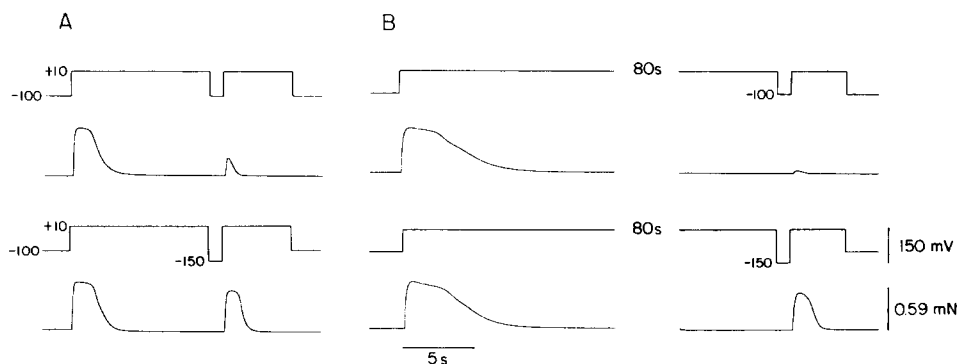


FIGURE 4. Effect of the level of the fiber membrane potential during repriming on the extent of repriming in two different fibers, *A* and *B*. In these experiments the repriming duration was 1 s, and the repriming potential after short or long depolarization was varied.

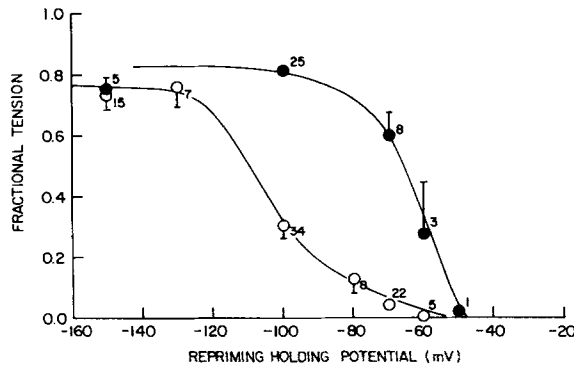


FIGURE 5. Relationship between the extent of repriming and the repriming membrane potential, after short (10 s; *filled symbols*) or prolonged (>80 s; *open symbols*) depolarizations. The experiments used for this figure were similar to those shown in Fig. 4. Each point represents the mean  $\pm$  the SEM of different determinations, whose number appears beside the symbols.

out using different repriming potentials and fixed repriming durations (1 or 2 s) after short or prolonged depolarizations. The pooled results are illustrated in Fig. 5. The extent of repriming, plotted vs. the repriming potential, is shown. In the graph, a comparison is made between the results obtained for repriming after short (*filled symbols*) and prolonged (*open symbols*) depolarizations. The curves shown in Fig. 5 are similar to those normally used to represent the fiber steady-state inactivation property, and to those usually obtained using repriming intervals sufficient to produce 100% contractile restoration at  $-100$  mV, except that they were obtained with repriming periods of only 1 or 2 s. Thus the present curves do not represent steady-state inactivation properties but rather transient ones.

The results presented above suggest that the state of the mechanism responsible for contractile repriming differs in the two cases, as is evidenced by the differences in the repriming time course and in the repriming voltage sensitivity.

#### *The Effect of Caffeine*

The possibility that the slow inactivation process could be a consequence of calcium depletion from the sarcoplasmic reticulum was tested in a few experiments in which caffeine was used. Fig. 6 summarizes the results of several experiments similar to those shown in Fig. 2. The figure demonstrates that in the presence of 0.5 mM caffeine the onset and completion of the ultraslow inactivation is considerably

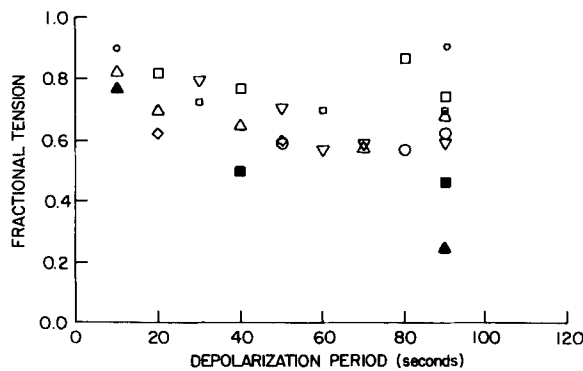


FIGURE 6. Effect of caffeine (0.5 mM) on the development of inactivation. The graph is similar to that shown in Fig. 2, with which it should be compared for a clearer demonstration of the caffeine effects. The open symbols were obtained in the presence of caffeine, the filled ones in its absence. Each symbol corresponds to the results obtained with the same fiber.

delayed, showing that in the presence of the drug, large contractile responses can be obtained after sustained depolarizations. This effect is possibly due to the capacity of caffeine to sensitize the calcium release mechanism to membrane depolarization, or in other words to improve depolarization-contraction coupling (Lüttgau and Oetliker, 1968; Caputo et al., 1981; Delay et al., 1986).

#### *The Effect of Lanthanum*

Fig. 7 shows that lanthanum, at a low concentration ( $50 \mu\text{M}$ ) has an effect apparently similar to that of caffeine. In the upper row the records show contractile repriming in the absence of this ion after depolarizations of 40 or 80 s. The records in the lower row were obtained in the presence of lanthanum, using the same experimental protocol. It is clear that in its presence the duration of the first responses was

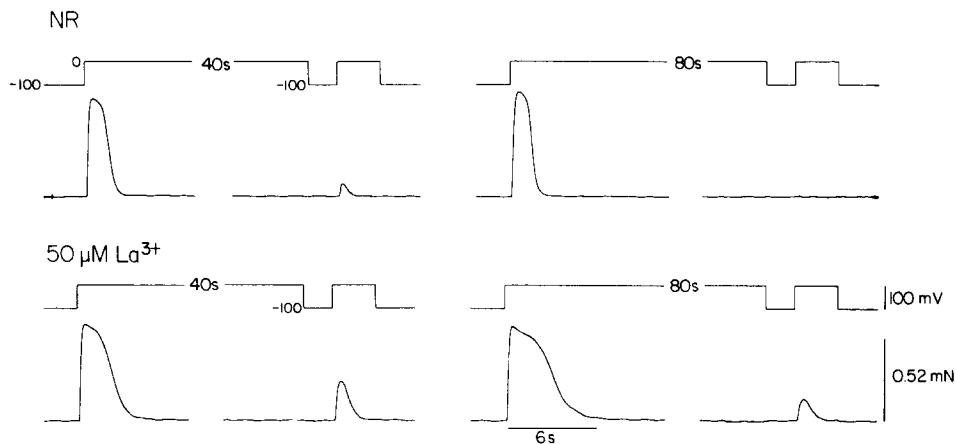


FIGURE 7. Effects of lanthanum ions ( $50 \mu\text{M}$ ) on the repriming capacity of a single fiber after depolarizations of 40 or 80 s in duration. In these experiments the fibers were reprimed at  $-100 \text{ mV}$  for 2 s. It appears that lanthanum improves repriming, possibly by reducing the extent of inactivation.

appreciably prolonged, confirming earlier findings (Andersson and Edman, 1974; Bolaños et al., 1986), and that repriming was improved in both cases. Fig. 8 shows the effect of  $50 \mu\text{M}$  lanthanum on the relationship between the extent of repriming and the repriming potential. A repriming time of 2 s at different membrane potential values was allowed after prolonged depolarizing pulses. The open symbols refer to the experiments carried out in the absence of lanthanum ions, and the filled ones in its presence. It appears that lanthanum shifts the inactivation curve toward less negative potentials, the region where it falls when shorter depolarizations are applied (see Fig. 5).

#### DISCUSSION

In their classical work of 1960, Hodgkin and Horowicz characterized the inactivation process by studying repriming from the inactivation associated with a full-sized

potassium contracture. In their experiments exposure to the high potassium solution elicited the first contracture which lasted <20 s. With this protocol they obtained the steady-state inactivation curve, relating the extent of recovery to the potassium concentration and hence to the membrane potential during the recovery period that lasted 60 s. In this work we demonstrate that contractile inactivation, induced by membrane depolarization to 0 mV, is a very slow process, building up with a half time of ~30 s and reaching completion in >100 s. Furthermore we show that when inactivation is fully developed, after a prolonged depolarization, contractile repriming is delayed and its voltage sensitivity, expressed by the relationship between extent of repriming and repriming membrane potential, is shifted by >30 mV toward more negative potentials. These results indicate that after sustained depolarization, the inactivation process, which initially causes the spontaneous relaxation from the contracture, and later brings contractile refractoriness, continues to progress, possibly because of conformational changes of the molecular moiety serving as the

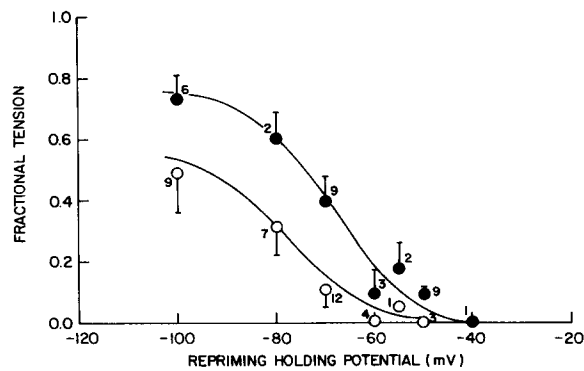


FIGURE 8. Effect of 50  $\mu$ M lanthanum ions on the relationship between repriming capacity and the fiber membrane potential during the repriming period (which was 2 s long in these experiments) after prolonged depolarizations. The points represent the results (mean  $\pm$  SEM) in the absence (*open symbols*) and presence (*filled symbols*) of the trivalent cation. The results obtained in the absence of lanthanum are similar to those shown in Fig. 5, but were obtained in a different group of fibers.

voltage sensor for depolarization-contraction coupling. These results confirm and extend the initial observation of Hodgkin and Horowitz (1960) and those of Nagai et al. (1979), who studied the time course of inactivation of potassium contractures induced by conditioning depolarizations with subthreshold potassium concentrations. They found that conditioning the fibers with 20 and 30 mM potassium, complete inactivation was achieved within 6 and 20 min, respectively, indicating that this process is clearly voltage dependent. However, when potassium contractures are used, measurements of the repriming time course can be affected by the delay of membrane repolarization after the change of solutions (Caputo, 1972).

Starting with the work of Lüttgau (1963) numerous authors have studied the effect of extracellular calcium on contractile activation and inactivation, and they have shown that contractile inactivation is enhanced in the absence of extracellular calcium (Caputo and Gimenez, 1967; Frankenhaeuser and Lannergren, 1967; Caputo, 1972; Lüttgau and Spiecker, 1979; Brum et al. 1988b). High calcium

concentration and lanthanum, on the other hand, have the opposite effect, prolonging the contracture time course (Andersson and Edman, 1974; Bolaños et al., 1986). In this work we show that lanthanum, at low concentration is effective in shifting the inactivation curve obtained after prolonged depolarizations toward less negative membrane potentials, suggesting that it prevents the attainment of the final state of inactivation. These results are supported by the recent communication of Feldmeyer (1989) showing that lanthanum ions are also effective in suppressing or reducing contractile inactivation or paralysis caused by Ca-free solutions, or by D-600 (Eisenberg et al., 1984).

The ultraslow inactivation dealt with here is a totally different process from the fast decline of calcium release observed by different authors (Palade and Vergara, 1982; Melzer et al., 1984; Schneider et al., 1987) and Schneider and Simon (1988), who have used optical techniques to monitor intracellular calcium. This last phenomenon appears to be mostly due to inactivation of the sarcoplasmic reticulum calcium channels, which are responsible for calcium release during contractile activation. It is interesting to note that while this phenomenon is clearly detected as a reduction of the optical signal and of the rate of calcium release it does not have an evident contractile counterpart, since the mechanical responses to voltage-clamp steps, or raised external potassium, do not show a decaying phase with the same time-course characteristics.

One could consider the possibility that the phenomenon studied in this work could be the result of the depletion of some substance involved in excitation-contraction coupling. Although calcium depletion in the sarcoplasmic reticulum can be discarded in view of the results obtained with caffeine, other substances could be considered. For instance it has been proposed that inositol-triphosphate could participate in contractile activation (Vergara et al., 1985; Volpe et al., 1985). While serious questions have been raised against this possibility (Walker et al., 1987), there are indications (Donaldson, 1989) that in depolarized, inactivated fibers the sensitivity to this compound is increased. Information regarding the fate of this or related compounds during ultraslow inactivation could be important for clarifying the role of inositol-phosphates in excitation-contraction coupling.

Independent of the role of chemical messengers in depolarization-contraction coupling, there is little doubt that the operation of the voltage sensor for depolarization-contraction coupling is associated with intramembrane charge movement (Schneider and Chandler, 1973). It is known that prolonged membrane depolarization causes charge immobilization or transformation of charge 1 into charge 2 (Chandler et al., 1976; Brum and Rios, 1987). In a fully polarized fiber, the charge that moves in the potential range between  $-100$  and  $+40$  mV, termed charge 1, is considered to represent the electrical manifestation of the process that provides voltage sensitivity for contractile activation. After prolonged depolarization, this charge is reduced and a second type, termed charge 2, appears to move in the potential range between  $-100$  and  $-180$  mV. There is recent evidence (Brum and Rios, 1987; Caputo and Bolaños, 1989a) in favor of the idea that upon depolarization, the molecular moiety responsible for charge 1 undergoes a conformational change, acquiring the properties corresponding to charge 2. Recently Brum et al. (1988a) have shown that the interconversion of charge 1 into charge 2 is facilitated



under calcium-free conditions. Agents like D-600 and nifedipine also appear to act in a similar way. On the other hand, there is some indication that in the case of charge movement as well, lanthanum acts in the opposite way, partially reversing the shift of the charge-potential relationship induced by membrane depolarization (Caputo and Bolaños, unpublished results). The conversion of charge 1 into charge 2 could constitute the molecular basis of contractile inactivation. The development of ultraslow inactivation could be explained assuming that after membrane depolarization, the voltage sensor for depolarization-contraction coupling may successively occupy different physical states, with slow transitions from one state to another and a long time interval (~100 s) between the first and the last one. The progression between these states could be favored by certain experimental conditions, like calcium-free solutions or D-600 and impeded by others, like lanthanum.

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