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Sites of Action of D_2O in Intact and Skinned Crayfish Muscle Fibers

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Summary. The effect on tension development of replacing 90% of the H_2O of the bathing saline with D_2O was studied on intact single fibers, and on skinned fibers before and after the latter were treated so as to eliminate Ca-accumulation by the sarcoplasmic reticulum (SR). Excitation-contraction coupling (ECC) of intact fibers is not abolished, but is depressed by D_2O so that higher depolarizations are required to elicit a given tension. The reduction in tension at a given level of depolarization is not due to inhibition of the contractile system. The latter showed an enhanced Ca sensitivity; that is, skinned fibers respond to Ca concentrations that are 1–2 orders of magnitude smaller in D_2O than in H_2O saline. When bathed in D_2O saline, intact fibers or skinned fibers with functional SR can still accumulate and release Ca in sufficient quantities to allow repeated induction of maximum tensions. Relaxation is slowed in all three types of preparation, perhaps because of an increased affinity of troponin to Ca in D_2O salines.

The initiation and development of tension are modified in muscles bathed in D_2O salines. Goodall (1958) has reported a 12-fold reduction in the rate of isotonic tension development and has suggested this effect is due to a change in the contractile system. While Kaminer (1960) and Kaminer and Kimura (1972) emphasize the inhibitory effects of D_2O on excitation-countraction coupling (ECC), Svensmark (1961) could not dissociate possible D_2O effects on the initiating and tension producing processes. The recent inference that "Ca release", a step in the coupling of excitation and contraction, is prevented by D_2O (Kaminer & Kimura, 1972) is of particular interest to those studying ECC. Agents or treatments of fibers that selectively disrupt one of the steps in ECC are valuable as tools for studying this, at present poorly understood, process.

In order to further define the specificity of the inhibitory effects of D_2O we have compared the development of isometric tension by intact and skinned fibers from the same muscle preparation. The comparison

includes a study of the (*i*) membrane properties of intact fibers (*cf.* Suarez-Kurtz, Reuben, Brandt & Grundfest, 1972), (*ii*) interactions of the contractile system and the SR in skinned fibers (*cf.* Reuben, Wood, Zollman & Brandt, 1975), and (*iii*) tension regulation by Ca and substrate in skinned fibers devoid of a functional sarcoplasmic reticulum (SR) (*cf.* Reuben, Brandt, Berman & Grundfest, 1971; Brandt, Reuben & Grundfest, 1972; Orentlicher, Reuben, Grundfest & Brandt, 1974). A preliminary report on this work has appeared (Eastwood & Reuben, 1973).

Materials and Methods

General

Single muscle fibers from the carpopodite flexor of crayfish (*Orconectes* sp.) were used throughout this study. The fibers varied in length from 3 to 6 mm and from 100 to 300 μ in diameter. After dissection the fibers were mounted in a Plexiglas chamber designed for rapid solution exchange, temperature control (20 °C), and stirring (*see* Orentlicher *et al.*, 1974). Isometric tension was recorded with a Bionix F250 transducer (Biological Electronics, Inc., Elcevito, Calif.) mounted on a manipulator that allowed for adjustment of sarcomere length (about 8 μ). In the experimental regimes the behavior of the preparation in saline made up with H_2O was compared to that in salines made with 90% or more of the H_2O replaced by D_2O (obtained from J.T. Baker Chemical Co., Phillipsburg, N.J.). The p.d. was estimated by adding 0.4 to the reading obtained using a pH meter with a standard glass electrode (Glasoe & Long, 1960).

Intact Fibers

Intact fibers were dissected in standard crayfish saline containing (in mM): 200 NaCl, 13.5 $CaCl_2$, 5 KCl, 4 Tris maleate, pH 7.4. The potential difference across the cell membrane was measured and current for altering the transmembrane potential was injected with conventional micropipettes and electronic equipment. Calcium was injected iontophoretically according to a procedure described previously (Reuben, Brandt & Grundfest, 1974). Uniform depolarizations of the fibers were produced by isosmotic substitution of K^+ for Na^+. Na-free saline was prepared by substituting Tris for Na^+.

Skinned Fibers

Following dissection, a single fiber was soaked for about 30 min in a medium containing (in mM): 170 K propionate, 10 EGTA, 4 imidazole, 2 ATP, 1 Mg, pH 7.0 (relaxing solution) before the surface membrane was removed by microdissection (*see* Reuben *et al.*, 1971). After a "priming" period in propionate media (200 K propionate, 4 imidazole, 2 ATP, 1 Mg, pH 7.0) containing small (μM) amounts of added Ca^{++}, fibers generated tension (*P*) in response to an anion change (substitution of Cl^- for propionate) or exposure to 10–20 mM caffeine. These tensions are transient (Reuben, Brandt & Grundfest, 1967; Orentlicher *et al.*, 1974), and as many as 30 responses have been recorded in fibers that were primed between challenges in a saline containing a few μM of ionized Ca (*unpublished observations*).

Brij-Treated Fibers

The SR of skinned fibers treated with 0.5% solution of the nonionic detergent Brij-58 for 20 min can no longer accumulate Ca (Orentlicher *et al.*, 1974). Thus, the Brij-treatment makes available a simplified preparation in which the function of the contractile and regulatory proteins can be tested without perturbation of the myoplasmic Ca-concentration by the Ca regulatory action of the SR. Tension and relaxation records were obtained on Brij-treated skinned fibers exposed to solutions in which EGTA buffered the free Ca^{++} and from fibers exposed to solutions in which EDTA buffered both the substrate (MgATP) and the free Ca^{++} (Brandt *et al.*, 1972). All the binding constants used are the same as those published previously by Reuben *et al.* (1971) except that K_{apparent} for Ca-EGTA is assumed to be $2 \times 10^6\,\text{M}^{-1}$ at pH 7.0.

Results

Skinned Fibers Brij-Treated to Eliminate the SR

The contractile proteins appear to become more sensitive to Ca when D_2O is substituted for H_2O. The records of Fig. 1 show this for tensions induced by adding 0.1 mM (*A*) or 1.0 mM (*B*) Ca to a bathing solution containing 1 mM Mg and 5 mM ATP. The first two (left) and last (lower right) tension records in Fig. 1*A* were obtained in H_2O saline while the

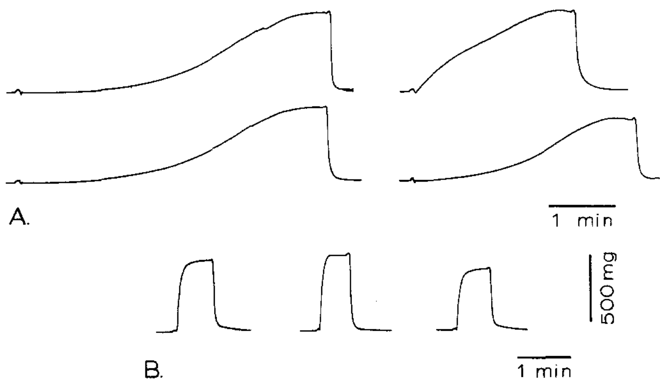

Fig. 1. D_2O enhances sensitivity of the contractile system to Ca. Skinned fiber treated with Brij-58 to disrupt the SR. (*A*) Tensions evoked on introducing 0.1 mM Ca to bathing solutions containing 5 mM ATP and 1 mM Mg. The order of the recordings was upper left, lower left, upper right and lower right. (Tension terminated by introduction of relaxing solution.) *Two records on left:* solvent is H_2O. Artifacts denote addition of Ca. Note slow onset of tension and decline of amplitude due to repetitive Ca exposure. *Right upper* record: solvent is 90% D_2O, change having been made 2 min before start of record. Note more rapid development of tension on adding Ca and the reversal of the downward trend of the amplitude. *Lower record:* Fiber returned to control solution 2 min before applying Ca. Tension developed more slowly and the amplitude was lower. (*B*) Same fiber, response to addition of 1 mM Ca. *First and third records:* responses in control saline. *Middle record:* response 2 min after changing to 90% D_2O

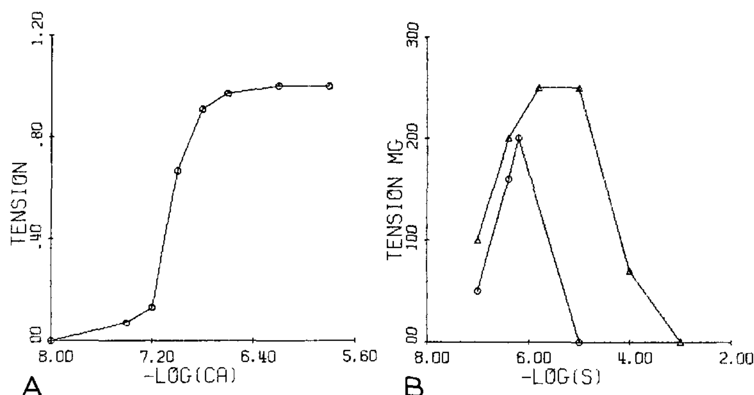

Fig. 2. Skinned fiber as in Fig. 1. (*A*) P/pCa relation obtained in 90% D_2O is shifted to higher pCa as compared with relation in H_2O saline that was previously reported (Brandt *et al.*, 1972). (*B*) P/pS relations in pCa 10 (○) and pCa 8 (△) obtained in 90% D_2O. When H_2O is the solvent the relation for pCa 8 is identical with that for pCa 10 (*cf.* Brandt *et al.*, 1972)

third tension was recorded 2 min after exchanging the control medium for one containing 90% D_2O. In the presence of D_2O the tension saturated more rapidly and was larger than both the previous activation (lower left) and the subsequent control. Fig. 1 *B* records the responses of the same fiber to activation with 1.0 mM Ca. The tension evoked in D_2O (middle record) is larger than in either control and P_0 is more rapidly attained.

A curve relating isometric tension (P) to pCa for fibers bathed in 1 mM substrate is shown in Fig. 2*A*. When the fiber is in D_2O saline half maximum tension ($P/P_0=0.5$) is attained at pCa 7.0 ($pCa= -\log[Ca^{++}]$) which is one order of magnitude greater than that in H_2O saline where the mean pCa value for half maximal tension is 6.0 ± 0.04[1] (Brandt *et al.*, 1972). Thus, the calcium sensitivity in 1 mM substrate is shifted one p unit towards higher pCa values by substituting D_2O for H_2O.

One troublesome point in considering these data is the requirement that the measured pH be 6.6 in D_2O to be comparable to pH 7.0 in H_2O (Glasoe & Long, 1960). If this correction does not compensate for all possible changes in the affinity of the chelators for the divalent cations, the supposition that the fiber's Ca sensitivity is changed could be an artifact of the experimental regime. Alternatively, the shift of the curve may be due to a decrease in the inhibitory effect of substrate and not an increase in Ca sensitivity.

1 This is calculated (from that given in the earlier report) on the assumption that K_{apparent} for CaEGTA at pH 7.0 is 2×10^6 M^{-1}.

There are several ways to test for these possibilities and one is based on the ability of EDTA containing salines to buffer Mg (hence to buffer the substrate concentration) and the Ca concentration simultaneously (*see* Brandt *et al.*, 1972 for experimental details). If we assume that the sensitivity to Ca is increased by an order of magnitude and the sensitivity to substrate is unaffected, then measuring tension as a function of substrate (pS) at pCa 8 and again at pCa 10 will test this assumption. If the premise is correct, the pCa 10 curves will be identical in two solvents, but the pCa 8 curve will broaden and be elevated in D_2O. This is because we have demonstrated (Brandt *et al.*, 1972) that both pCa 8 and pCa 10 in H_2O media are above the range at which Ca influences the tension-substrate curve, while lower pCa progressively broadens and elevates the curve (*cf.* Figs. 1 and 5, Brandt *et al.*, 1972). Fig. 2*B* is a record of such a test. Higher concentrations of substrate are required in D_2O to induce complete relaxation in pCa 8 than in pCa 10. Such a shift in the tension-pS curve in D_2O must be due primarily to an increase in the Ca affinity of the myofibrillar regulatory protein, troponin. It cannot be due to a change in substrate sensitivity since the pCa 10 curve in D_2O and H_2O (Brandt *et al.*, 1972) are identical. An alternate explanation based on an affinity change of EDTA for Ca is less likely since it would require a selective change for Ca relative to that for Mg as well as coordinated change in the binding constants of EGTA and ATP for divalent cations. We interpret the data in Fig. 2, and similar data from two other fibers in which $P/P_0=0.5$ occurred at $p\text{Ca}=8$ as convincing evidence that the affinity of troponin for Ca increases by about 1 to 2 orders of magnitude when 90% of the H_2O is replaced with D_2O.

An increased affinity of troponin for Ca in D_2O media could also be reflected in the rapidity by which Ca can be removed from this protein. To examine this point relaxation was induced (Fig. 3) in fibers that had

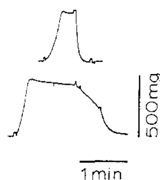

Fig. 3. Retardation of relaxation by D_2O. Tensions evoked by EGTA-buffered Ca (pCa 5) rise more steeply in D_2O (*lower record*) than in H_2O (*upper record*) and attain higher amplitude. Relaxation was rapid in H_2O medium upon washing the fiber with relaxing solution containing 5 mM EGTA. In D_2O relaxing solution (5 mM EGTA) relaxation was slow, but was increased by elevating the EGTA concentration to 10 mM. Artifacts represent 2 washes with 5 mM EGTA followed (30 sec) by 10 mM EGTA

been activated by pCa 5 (EGTA buffer) by washing the fibers with relaxing solutions containing 5 to 10 mM EGTA without added Ca. In the presence of D_2O two washes with 5 mM EGTA induced a slow relaxation in the fiber shown in the lower record of Fig. 3, while a third wash with 10 mM EGTA appreciably accelerated the relaxation. In H_2O media relaxation was abrupt with a single 5 mM EGTA wash (Fig. 3, upper trace).

Skinned Fibers with SR Intact

The four tensions of Fig. 4*A* were all evoked upon replacing the K propionate in the bathing medium with KCl. With H_2O as the solvent, two transient tensions were elicited by this change in anion (1st and 2nd records). The third tension was elicited after 90% of the H_2O was replaced by D_2O. This latter tension was about 25% larger than the controls and relaxation was negligible until D_2O was replaced by H_2O. The fourth tension, which followed the return to an H_2O medium, was similar to the previous control tensions in amplitude, but the relaxation phase was slightly prolonged. The increased amplitude of anion induced tensions and the marked slowing of the relaxation phase were observed in the two other experiments performed in D_2O saline as described above. The amplitude increased by 19% in one fiber and 30% in another.

The enhanced sensitivity to Ca of skinned fibers without a functional SR (Figs. 1 and 2) is also seen in fibers with an intact SR (Fig. 4*B*). The responses elicited by 0.1 mM Ca in H_2O saline (1st and 2nd records)

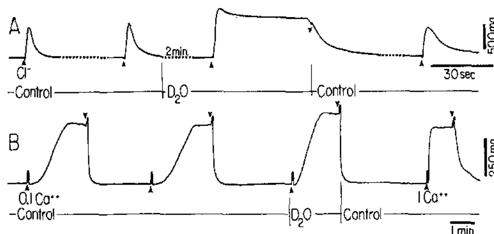

Fig. 4. Skinned fibers with SR intact. (*A*) Tensions were elicited by replacing propionate with Cl. The third record was made 2 min after the solution was changed from H_2O to D_2O. Note the very large and persistent tension in contrast to the brief transient responses of the controls. Relaxation was induced by returning the fiber to the control saline. The last response, with the fiber in the control saline, was again smaller and transient, but relaxation was somewhat slower. (*B*) Another fiber. Tensions were induced by adding Ca. The response to 0.1 mM Ca develops earlier, rises faster and to a higher amplitude in D_2O saline. Note that the amplitude for 1 mM Ca in the control saline was lower than for 0.1 mM Ca in D_2O

developed more slowly than in the D_2O saline (3rd record) and were smaller in amplitude. The fiber was activated a fourth time in H_2O medium by exposure to 1.0 mM Ca. This resulted in a steady-state tension comparable to that attained with 0.1 mM Ca in H_2O, indicating that the lower concentration of Ca was sufficient to saturate troponin and induce a maximal tension.

Intact Fibers

Iontophoretic Ca Injection. Fig. 5 is a high gain recording of tensions (upper trace) evoked by intracellular iontophoretic injections (lower trace) of Ca and compares the responses of a fiber bathed in normal saline (top record) to the responses after replacing 90% of the H_2O with D_2O (lower record).

The increased rate of rise and higher amplitude of the tensions for a fixed quantity of Ca injected and the prolonged relaxation of the responses when the fiber is bathed in D_2O may directly relate to the increased affinity of troponin for Ca. However, other factors cannot be excluded by this experiment. For example, in D_2O the compliance of the inactive sarcomeres may be decreased or Ca regulation by the surface or SR membranes may be functionally depressed. In order to distinguish between these possible actions of D_2O its effect on tension elicited by other modes of stimulation were studied.

Application of Caffeine. Repeated applications of 20 mM caffeine to intact crayfish fibers evoke maximum tension if approximately 20-min intervals are allowed to elapse between exposures (Chiarandini, Reuben, Girardier, Katz & Grundfest, 1970*b*). However, the repriming interval may be reduced to a fraction of a minute, if after removing caffeine, the fiber is activated by an applied current or by elevating K_0. After

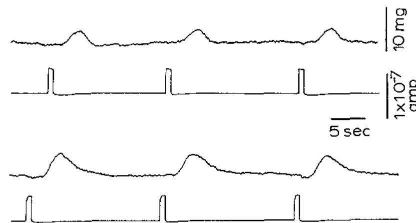

Fig. 5. Intact fiber, responses to Ca injected ionophoretically from an intracellular microelectrode. *Lower traces* in each set denote delivery of Ca by a 2-sec pulse of current (6×10^{-8} A). *Upper traces* denote tensions with the fiber in the control solution (above) and in D_2O (below). Note larger tension and slower relaxation in the latter case

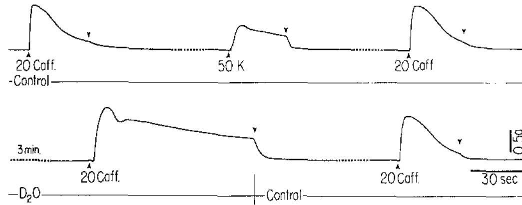

Fig. 6. Intact fiber, responses to 20 mM caffeine. First control response was followed by application of 50 mM K, which evoked a small tension and speeded repriming of responsiveness to a second application of caffeine. The responsiveness was again reprimed with 50 mM K (record not shown) and the solution containing D_2O was introduced 3.5 min before caffeine was applied. The tension was about 25% higher and relaxation was very slow, until caffeine was removed by washing in the control solution (▼). After another repriming with 50 mM K (record not shown) the fiber responded to caffeine as in the two earlier controls. Broken lines indicate deletion of 1 min from the record unless indicated

the first and second maximal caffeine-induced tensions recorded in Fig. 6 the fiber was briefly exposed to 50 mM K (isosmotic), then a third caffeine tension was evoked after about $3^1/_2$ min in the 90% D_2O medium. This tension was about 20% larger than that of the controls and relaxation was greatly retarded. The effects of D_2O on the caffeine-induced tensions were reversible as indicated by the fourth tension that followed the same repriming procedure.

In five other experiments in which caffeine was applied to fibers bathed in D_2O media, P_0 increased by 10 to 12% in two and was unchanged in three. In all such fibers the rate of relaxation was greatly reduced. Although this latter observation, as well as the other results obtained from intact fibers may be directly related to an increased affinity of troponin for Ca,we still cannot exclude an action of D_2O on the membrane systems which remove Ca from the myoplasm. In fact, the following experiments show that D_2O retards repriming of caffeine tensions which depends on extracellular Ca and presumably on a transfer of Ca across the surface membrane (Chiarandini *et al.*, 1970*b*).

Fig. 7 consists of records taken from a fiber which was placed in D_2O saline about 30 sec after an initial caffeine-induced tension. Twenty minutes later it was exposed to caffeine in D_2O, but no tension was evoked. Following a further 60-min soak in the D_2O saline a large caffeine tension with an erratic rate of rise was elicited. Although the peak tension is higher than in either the initial or in the following controls, the irregular rate of rise is typical for that of an incompletely reprimed fiber in H_2O saline. Repriming between caffeine tensions, which in H_2O saline is essentially complete within 20 min (Chiarandini *et al.*, 1970*b*) is clearly slowed for fibers bathed in a D_2O medium.

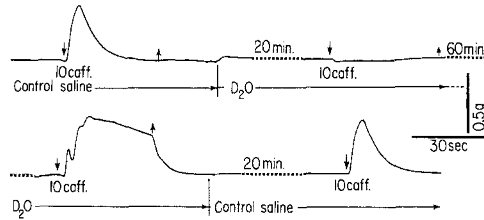

Fig. 7. Effect of D_2O on repriming after caffeine-induced tension. The first tension was induced by 10 mM caffeine in control saline. Arrows indicate application and removal of caffeine. The fiber was then kept for 20 min in the D_2O saline before a second application of caffeine. No response was evoked at this time, but a challenge with cafeine after 60 min in D_2O saline evoked a large, asynchronous, long-lasting response. The last record, obtained 20 min after the fiber had been returned to the control saline, shows that a 20-min repriming period is sufficient in H_2O saline to elicit another tension comparable to the initial control response

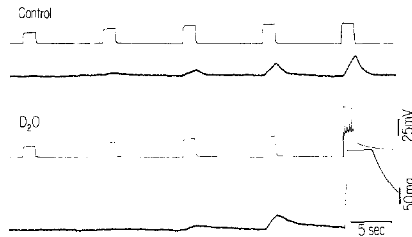

Fig. 8. Responses of intact fiber to depolarization with intracellularly applied currents. *Top trace* in each set shows the changes in membrane potential for increasing current (1.5-sec pulses). *Lower traces,* tensions. In the control saline tension was observed with depolarization of 20 mV (second pulse), but in D_2O tension did not appear until a depolarization of 27 mV was reached (third pulse). The subsequent pulse induced a small activation of the membrane and the last pulse induced a train of spikes. Note that the latter causes a large, sustained tension, indicating that ECC was not eliminated by D_2O. The effective resistance (R_E) of the fiber membrane in H_2O was 2×10^5 ohms and it increased to 3×10^5 ohms in the D_2O

Depolarization with Applied Intracellular Currents. The relation between tension and membrane depolarization induced by applied intracellular currents is modified in fibers exposed to D_2O. In Fig. 8 depolarizing pulses (1.5 sec) produced a detectable tension (recording sensitivity of 50 mg/cm) in a fiber bathed in normal saline when the membrane potential was reduced by about 20 mV (upper line) and the tension increased in amplitude with greater depolarization. After replacing the normal saline with a D_2O saline the first detectable tension required a depolarization of about 27 mV (bottom line). The tension increased with greater depolarization until the membrane potential was reduced by 35 mV when a train of spikes and a large tension were elicited. All-or-none spikes cannot be elicited in these fibers bathed in control saline unless they are treated

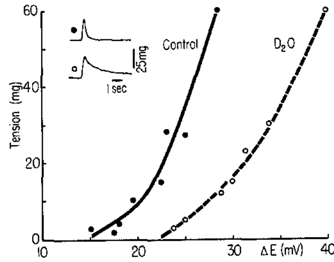

Fig. 9. Tension evoked by different degrees of depolarization (ΔE), in control saline (●) and in D_2O (○). Insets show time course of responses which developed 30 mg tension

with spike-inducing agents (*cf.* Suarez-Kurtz *et al.*, 1972). Two of the D_2O-induced changes are further illustrated in Fig. 9 in which membrane depolarization (1.5 sec pulses) is plotted against tension. The data were obtained from a fiber bathed first in normal saline (filled circles) and then in D_2O medium. Tensions of comparable amplitude were slower to relax in the D_2O medium (inset records).

The effects of D_2O on tension threshold and on relaxation rate shown in Figs. 8 and 9 were consistently observed in the 12 fibers tested. However, only 40% of the fibers produced spikes and in these fibers there was an increase in resting input resistance (R_E) which averaged 25%. No detectable change in R_E was observed in the nonspiking fibers. The spikes were not blocked by replacing all the Na^+ by $Tris^+$, but were abolished by adding 20 mM Mn^{++}. The spikes in D_2O behave similarly to those induced by procaine and caffeine in H_2O (Takeda, 1967; Chiarandini, Reuben, Brandt & Grundfest, 1970*b*) which are associated with rapid increases in the Ca^{++} conductance.

The large tensions which accompany the spikes in D_2O media are particularly noteworthy. Excitation-contraction coupling is obviously functional in fibers bathed in D_2O saline, although it is depressed since there is an increase in the electrical threshold for tension. This depression is noted in spite of an increase in Ca sensitivity of the contractile system (*cf.* Figs. 1, 2, and 3) which would require smaller quantities of Ca to produce a given tension.

Depolarization by Increased Extracellular K Concentration. Tension responses in D_2O salines were compared to those in H_2O salines for seven fibers depolarized by increasing extracellular K to 100 or 200 mM. Fig. 10 is a record from one such experiment. The tension response to 200 mM K is 33% smaller and relaxation about 4 times slower in D_2O saline (middle record) than the preceeding one in H_2O saline (left record)

Fig. 10. Tensions evoked by depolarization of an intact fiber with 200 mM K. *Middle record,* H_2O replaced by D_2O. In the two controls tension was transient, subsiding while K was still elevated. In D_2O relaxation was slow until control saline replaced the D_2O solution

and relaxation accelerates when H_2O is introduced in place of D_2O. A third tension in H_2O saline is larger than the preceding tension and relaxation rate is as fast as the control rate. The mean reduction in tension amplitude for the seven fibers depolarized with high K was 46.6% and relaxation times varied from no change to nearly a 12-fold increase. It must be noted that relaxation in these experiments, as in the caffeine experiments, takes place in the presence of the stimulus, i.e., in high K or caffeine and must be a composite of the inactivation of excitation and the rate of Ca removal from the myoplasm.

Discussion

The crayfish muscle fiber has been dissected by our experimental procedures into three functional systems. (1) The surface membrane, including the invaginations and T-system, (2) the sarcoplasmic reticulum, defined here as an internal membrane system involved in the uptake, storage, and release of Ca^{++}, and (3) the myofilament proteins, both regulatory and contractile. We detected effects of D_2O on both the first and third systems, but obtained no evidence that clearly implicated a modification of the second.

Myofilament System

We interpret the data of Fig. 2*A* to indicate that the Ca sensitivity of the myofilaments is shifted about 1 to 2 *p*Ca units higher in the presence of D_2O. That this shift is an increased sensitivity of the myofilament regulatory proteins to Ca and not a decrease in the effectiveness of substrate to inhibit tension can be deduced from the data of Fig. 2*B*. In the absence of Ca^{++} (*p*Ca 10), the substrate curve (*P*/*pS*) in D_2O saline registers full relaxation by *pS* 5, but at *p*Ca 8 full relaxation is registered at *pS* 3. In H_2O saline the *P*/*pS* relation is constant for the range *p*Ca 8 to *p*Ca 10

(*see* Fig. 6 of Reuben *et al.,* 1971, and Figs. 1 and 5 of Brandt *et al.,* 1972), and identical to the *p*Ca 10 curve in D_2O.

We have ascribed the slow relaxation of activated skinned fibers in the presence of D_2O to the D_2O-induced increase in Ca sensitivity (Fig. 2*A*). This conclusion is consistent with the observation that larger concentrations of EGTA are required in D_2O in contrast to H_2O to produce comparable relaxation rates of skinned fibers exposed to the same Ca concentrations (Fig. 3). A greater fraction of the Ca must be chelated by EGTA in the presence of D_2O in order to reduce the ionized Ca concentration below activation levels. Due to the apparent increased affinity of troponin for Ca an effect of D_2O on the rate of Ca sequestration by the SR could be obscured.

The conclusion that Ca-sensitivity of the regulatory proteins is enhanced by D_2O may explain the altered rise-time of tension in skinned fibers and the slower relaxation rate in both skinned and intact fibers. Shorter rise-times of tension induced by the application of Ca to skinned fibers in D_2O are apparent in Fig. 1*A*, Fig. 3 and Fig. 3*B*. This can be anticipated if lower concentrations of Ca are effective in activating tension. No effect of D_2O on rise-time of a transient Cl-induced tension in skinned fibers is resolved in our records, but relaxation is slowed and should be if the myofilaments are more sensitive to low concentrations of Ca^{++}.

Some degree of prolongation in relaxation time, such as that observed in intact fibers bathed in D_2O saline, after a Ca-injection (Fig. 5), or depolarized with current pulses (Figs. 8 and 9) or high $[K^+]_0$ (Fig. 10) is reasonable if the SR must reduce the myofibrillar Ca^{++} concentration to a lower than normal value. Slowed relaxation of caffeine tensions (Figs. 6 and 7) is also expected, since Ca^{++} must be reduced to levels 10-fold lower than in H_2O. In this case, however, the surface membrane (Chiarandini *et al.,* 1970*b*) is presumably the sequestering organelle because the SR does not accumulate Ca in the presence of caffeine (Weber & Herz, 1968; Orentlicher *et al.*, 1974). Since higher sensitivity of troponin to ionized Ca will slow relaxation, we have not been able to unequivocally assign any effect of D_2O to the SR, although it has been reported recently that commercial D_2O preparations contain a powerful inhibitor of this Ca sequestering system (Huxtable & Bressler, 1974). Our data exclude a major action of D_2O or its contaminants on SR function, since Ca can be accumulated and subsequently released by anion substitution in skinned fibers and by caffeine in intact fibers.

The increased tension observed on the application of Ca^{++} (Fig. 1*A* and *B* and Fig. 4*B*) or Cl^- (Fig. 4*A*) to skinned fibers in D_2O, and the

increased tension induced by caffeine in intact fibers (Figs. 6 and 7) bathed in D_2O is less readily explained than the altered time course of tension. Alteration of the binding constant of the regulatory proteins *per se* cannot account for it, but there are several possible explanations: (1) Enhanced Ca^{++} affinity of the regulatory proteins results in a more rapid activation of the myofibrils within the depths of the fiber which might improve the synchrony of activation. Asynchronous activation of adjacent sarcomeres during the inward diffusion of Ca is believed to account in part for the fact that skinned fibers develop smaller maximum tension than do intact fibers (Reuben *et al.*, 1971). (2) It has been reported that D_2O slows ATP hydrolysis in glycerinated rabbit muscle (Svensmark, 1961). A conceivable mechanism for the reduction of ATP hydrolysis could involve cross bridges remaining "made" for a longer time. At any instant this would result in a greater proportion of the cross bridges being available to support tension. (3) D_2O may affect the actomyosin bonds in some fundamental manner which increases the force each bond develops. Our data do not allow us to distinguish which, if any, of these mechanisms is involved in the production of the higher tension in fibers bathed in D_2O saline.

Excitation at the Surface Membrane

We found four effects of D_2O which are related to the surface membrane and its invaginations: (1) increased repriming time for caffeine tensions, (2) decreased conductance, (3) induction of Ca-spike electrogenesis, and (4) increased threshold for tensions induced by depolarization.

Decreased membrane conductance has been demonstrated in nerve fibers bathed in D_2O salines (Conti & Palmieri, 1968). The large (25%) decrease in resting conductance we observed in 40% of the crayfish muscle fibers implies that the mobility of K^+, Cl^-, or both, in the membrane may be reduced in the presence of D_2O since the K^+ and Cl^- conductances make up the major portion of the resting membrane conductance (Orentlicher & Reuben, 1971). Since the source of the Ca^{++} to reprime the caffeine tension is believed to be the extracellular fluid (Chiarandini *et al.*, 1970*b*), we infer from the increased repriming time for caffeine-induced tension (Fig. 7) that the resting membrane conductance for Ca^{++} is also decreased in D_2O. However, depolarization of fiber exposed to D_2O induces Ca-spikes and under this condition the relative Ca conductance must be increased. This may occur due to a TEA-like action of D_2O on K-activation and/or an increase in depolarizing Ca-activation.

Excitation-contraction coupling is not blocked in the presence of D_2O in crayfish fibers as has been suggested for barnacle (Kaminer & Kimura, 1972) and frog fibers (Sandow, Sphica & Pagala, 1974); instead larger depolarizations are required to produce a tension whose amplitude is equivalent to that of H_2O (Figs. 8, 9). The link between membrane depolarization and Ca mobilization in intact fibers exposed to D_2O must be more depressed than is apparent from Figs. 8 and 9 because the Ca-sensitivity of the contractile apparatus is enhanced in D_2O. The caffeine-induced tensions in intact fibers (Figs. 6 and 7) and the Cl-induced tensions in skinned fibers (Fig. 4*A*) bathed in D_2O media indicate that neither Ca uptake or release from the SR is blocked by D_2O or a contaminant in the D_2O (*cf.* Huxtable & Bressler, 1974). We can conclude that the shift of the depolarization-tension relation is due to an effect of D_2O on some membrane event critical to ECC, but preceding Ca mobilization by the SR. This critical step could be a membrane Ca-flux (Suarez-Kurz *et al.*, 1972; Stefani & Chiarandini, 1973; Atwater, Rojas & Vergara, 1974; Suarez-Kurtz & Reuben, 1975).

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