DISSOCIATION OF EXCITATION AND CONTRACTION IN SKELETAL MUSCLE INDUCED BY DEUTERIUM OXIDE AND DANTROLENE-SODIUM

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Abstract The effect of D_2O and dantrolene-Na (DAN) on the electrical and mechanical responses of single muscle fiber (mouse EDL and frog semitendinosus), Ca^{2+} uptake and release of sarcoplasmic reticulum (SR) and contractile proteins was investigated to elucidate the coupling mechanism between depolarization and contraction.

Both agents inhibited tension development induced by depolarization and caffeine, especially the initial phase of contraction (more pronounced in fast muscle) without affecting excitation of membrane and contractile machinery. The effect of DAN can be antagonized by increasing Ca^{2+} concentration and lowering the temperature of the medium. Ca^{2+} uptake and release abilities of isolated SR were not altered significantly in DAN, but were reduced considerably in D_2O .

These results suggest that there is a Ca^{2+} -mediated trigger mechanism for the rapid release of activator Ca^{2+} from internal storage (SR). DAN and D_2O interfere predominantly the action of the site(s) of this mechanism, probably on the T-tubules. In addition, D_2O decelerates Ca^{2+} release from SR *per se*.

It has been shown by KAMINER and KIMURA (1972) that barnacle muscle fiber does not contract with stimulus in deuterium oxide (D_2O) and the transient increase of intracellular Ca^{2+} associated with membrane depolarization is not detected. BEZANILLA and HOROWICZ (1975) showed the depression of fluorescence intensity (indicator of Ca^{2+} movement) from *Nile Blue A* stained frog muscle in D_2O . Recently, EASTWOOD *et al.* (1975), SANDOW *et al.* (1976), and YAGI and ENDO (1976) reported the inhibition of mechanical response in intact and skinned skeletal muscle fibers. Contraction of heart muscle is also inhibited by the presence of D_2O (EICHKORN *et al.*, 1975); this situation might be brought about by depressing Ca^{2+} influx.

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In Ringer solution containing dantrolene-Na (DAN, muscle relaxant; ELLIS and CARPENTER, 1972), contraction of skeletal muscle is reduced by 80%, although the excitation of membrane remains apparently normal. Ca^{2+} movement in muscle cell as monitored by aequorin is depressed in DAN medium (HAINAUT and DESMEDT, 1974). Since D_2O as well as DAN seem to have no direct effect on the contractile system, enzymes of cell membranes and the sarcoplasmic reticulum (SR) (HOTTA and MORALES, 1960; INOUE *et al.*, 1975), the action of these agents may be to dissociate the coupling between excitation of cell membranes and Ca^{2+} release from SR. Though it is certain that the T-system plays a crucial role in the process of signal transmission from the surface to the inside of the cell in skeletal muscle, the mechanism leading to the increase of myoplasmic Ca^{2+} is still unknown (EBASHI, 1976). OOTA *et al.* (1976) suggested that entry of a small amount of Ca^{2+} into the cell due to depolarization of T-tubule membrane might act as a trigger for large release of Ca^{2+} from SR. Recent reports on the action of DAN by PUTNEY and BIANCHI (1974), TAKAUJI *et al.* (1975) and HONMA *et al.* (1976) seem to support the presence of trigger Ca^{2+} on the T-tubule membrane. This idea contradicts the proposal of SANDOW *et al.* (1975) that depolarization of the T-tubule membrane *per se* is responsible for Ca^{2+} release from the SR.

In order to elucidate the mechanism of increase of myoplasmic Ca^{2+} upon stimulation, we investigated the effect of D_2O and DAN on depolarization-induced contraction of skeletal muscle fibers. Although the mechanism of action of D_2O and DAN may be quite different, their primary site of action should be the T-tubule membrane where the signal is transmitted into the SR. The effect of these drugs at the sub-cellular level, *i.e.*, Ca^{2+} uptake, Ca^{2+} release and Ca^{2+} -activated ATPase activity of SR and on the contractile element were also investigated.

The facts observed in these experiments indicate that the trigger mechanism mediated by Ca^{2+} located in the T-system possibly takes a part during the course of excitation-contraction coupling.

MATERIALS AND METHODS

Muscle fibers and bathing solution. The experiments were performed on the fiber bundles isolated from the ext. digitorum longus (EDL) muscle of mouse and the semitendinosus muscle of frog. The bathing medium for mouse EDL was Tyrode Ringer solution composed (in mM) of NaCl, 135; KCl, 2.65; glucose, 5.5; MgCl_2 , 2; and tris-maleate, 10 (pH 7.0), saturated with 95% O_2 and 5% CO_2 , and that for frog semitendinosus was NaCl, 105; KCl, 2.5; glucose, 10; and tris-maleate, 10 (pH 7.0). The concentration of Ca^{2+} in the solutions was adjusted by addition of concentrated CaCl_2 . At low concentrations of Ca^{2+} , below 0.1 mM, free Ca^{2+} in the medium was controlled by addition of EGTA taking its apparent association constant with Ca as $4.4 \times 10^5 \text{ M}^{-1}$ (OGAWA, 1968). No correction was made for that in D_2O . EGTA was not used for Ca^{2+} concentrations above

0.1 mM.

DAN (supplied by Yamanouchi Pharmaceutical Corp.) was saturated in the Ringer solution by adding an excess amount; the solution was then filtered. The concentration was determined spectrophotometrically (608 for 10 mg/l at 390 nm, ELLIS and CARPENTER, 1974). In most cases, 13 mg/l (40 μ M) of DAN in Ringer solution was used.

Deuterium oxide (99.7%) was purchased from Merck Co. and distilled once in an all glass distillation apparatus (B.P., 101°C). To determine the pH of the Ringer solution composed of D₂O corresponding to the pH of the normal Ringer, a factor of 0.44 was added to the reading of conventional pH meter (MIKKELSON and NIELSEN, 1960).

Stimulation and recording of response. Stimulation of the fibers was achieved with an intracellular electrode having the impedance of 2–3 M ohms with 3 M KCl. The maximum current across the membrane obtained through the electrode was about 300 nA. The threshold current for excitation of the membrane was approximately 50 nA on the average. A pair of platinum electrodes was also used for stimulation of the fiber bundles extracellularly. The membrane potential change induced by the depolarizing current was detected through the same electrode used for the intracellular stimulation utilizing the preamplifier and the ATAC-250 addscope. Tension was recorded on a polyrecorder (PMP-3004, Nihon-Kohden) through a semiconductor transducer (SP-5-12, Toyota) connected to the muscle fibers. Details of the experimental setup and method of measurement were described in an earlier report (YAMAMOTO *et al.*, 1976).

Preparation of muscle model. Glycerinated muscle fibers were prepared by treating frog semitendinosus in 50% glycerol at –20°C for about one week. Before use, glycerol was washed out with a solution containing 0.1 M KCl and 20 mM tris-maleate (pH 7.0). The muscle fibers were separated into small bundles (cross section; about 0.1 mm² or diameter; 0.3 mm) and attached to the tension transducer. Tension development was initiated by addition of ATP (3 mM) in the bathing solution (0.1 M KCl, 1 mM Mg²⁺, 20 mM tris-maleate, pH 7.0).

Preparation of SR and measurement of Ca²⁺ uptake, release and ATPase activity. Fragmented sarcoplasmic reticulum (SR) was obtained from rabbit skeletal muscle according to the method of EBASHI and LIPMAN (1962). The purified sample was suspended in a buffer containing 0.1 M KCl, 1 mM MgCl₂ and 20 mM tris-maleate. This solution was used also for Ca²⁺ binding and ATPase activity measurements. Ca²⁺ binding of SR was performed in the presence of 3 mM ATP and 1 mM MgCl₂ by the Millipore filtration method using ⁴⁵Ca. Release of bound Ca²⁺ from SR was performed as follows: Ca²⁺ was loaded to SR in the presence of ATP and Mg²⁺ for 5 min and then the medium was diluted with the solution containing 3 mM EGTA instead of ATP to 20 times followed by filtration at 30 sec, 1, 5, and 10 min after dilution. The remaining bound Ca(⁴⁵Ca) to SR on the filter was measured. ATPase activity of SR was

measured in the presence of 3 mM ATP, 1 mM MgCl_2 and P_i liberated from ATP was analyzed by FISKE and SUBBAROW (1925) method. DAN or D_2O did not interfere the colorimetric measurement of P_i by this method.

RESULTS

Twitch and tonic responses in normal Ringer (Ca^{2+} ; 2 mM)

Both mouse EDL and frog semitendinosus muscle in normal Ringer solution responded mechanically and electrically to the intracellular stimulus, as shown in Fig. 1a, b. The responses shown here are those from single fiber although a bundle was used. When the stimulation was made by a long pulse current (1 sec), the tension was maintained during the application of current (tonic response, Fig. 1c, not inhibited by TTX). In mouse EDL, the duration of action potential and contraction was considerably shorter than those of frog semitendinosus. Sometimes, mouse EDL exhibited repeated excitation upon stimulus: this phenomena caused an apparent increase of twitch tension and duration of contraction of the fibers, as shown in Fig. 1d. This repetitive excitation was abolished by increasing the Ca^{2+} concentration in the medium.

Response in D_2O and DAN Ringer

In D_2O Ringer (99%) solution, the mechanical response of the frog semitendinosus upon stimulus was markedly reduced without noticeable change in resting and action potentials (Fig. 2a). The tonic response was reduced to a lesser degree than that of twitch (Fig. 2b). Upon returning to normal Ringer, the fiber recovered its mechanical response immediately (Fig. 2c). Inhibition of twitch tension varied depending on the content of D_2O in the Ringer, as shown in Fig. 2d. The reduction of tension was also observed in mouse EDL muscle fiber in D_2O although the tension generated was much smaller than that of frog semitendinosus muscle fiber.

In the Ringer solution containing DAN (40 μM), the twitch tension of frog semitendinosus muscle fiber was reduced to 20 to 30% of that in normal Ringer. Resting and action potentials were not affected by DAN although the current required to cause the excitation of membrane increased somewhat (Fig. 3a, b). Tonic response was also inhibited, either in the presence or absence of TTX (Fig. 3c). Recovery from DAN effect could be achieved by returning the fiber to normal Ringer, but it took several minutes and in many cases, the recovery was not complete whereas in D_2O , both inhibition and recovery were instantaneous.

Effect of temperature

The twitch response of the fiber in D_2O and DAN Ringer at temperatures from 15 to 30°C (mouse EDL) and 4 to 20°C (frog semitendinosus) were measured and compared with those in normal Ringer. The duration of action potential and contraction time of both muscles became short at high temperatures. Figure

Fig. 1. Response of single muscle fiber in normal Ringer upon intracellular stimulation. Top trace, current of stimulus; middle trace, mechanical response (tension); bottom trace, electrical response (membrane potential change). (a) Twitch response, frog semitendinosus at 13°C, (b) twitch response, mouse EDL at 28°C, (c) twitch and tonic response, frog semitendinosus at 13°C, (d) summation of twitch tension, mouse EDL at 28°C.

Fig. 2. Response of single muscle fiber in D_2O Ringer. Frog semitendinosus at $13^\circ C$; top, current; middle, tension; bottom, action potential. Dotted line indicates the corresponding response in normal Ringer. (a) Twitch response in 99% D_2O Ringer, (b) twitch and tonic response in 99% D_2O . Note that the calibration for dotted line is 10 mg. (c) Recovery of mechanical response from D_2O . 10 sec after replacement of D_2O Ringer to normal Ringer. (d) Mechanical response in the Ringer containing different amount of D_2O , ---, 50%; --, 75%; —, 90%.

Fig. 3. Response of single muscle fiber in DAN Ringer, frog semitendinosus at $13^\circ C$. Top, current; middle, tension; bottom, action potential, (a) Twitch response in normal Ringer, (b) twitch response in DAN Ringer ($40 \mu M$), (c) twitch and tonic response in DAN Ringer. Dotted line indicates the control level.

Fig. 4. Twitch response of single muscle fiber in D₂O Ringer at different temperatures. Mouse EDL, intracellular stimulation (50 nA, 10 msec). Top, mechanical response in normal Ringer; middle, mechanical response in 50% D₂O Ringer; bottom, action potential. The temperature of the measurement is indicated at the top of each trace. D₂O gave no effect on the action potential.

Fig. 5. Effect of temperature on twitch tension of single muscle fiber in DAN Ringer. Mouse EDL, intracellular stimulation (50 nA, 10 msec). Upper trace, twitch tension; lower trace, action potential. (a) Response in normal Ringer at 31°C. (b) Response in DAN Ringer at 31°C after 15 min incubation. (c) Response in DAN Ringer at 15°C, after the experiment (b), the temperature of the medium was lowered from 31°C to 15°C while the fiber was resting in the same medium. It took several min to lower the temperature of the bath. (d) Response in normal Ringer at 15°C, 15 min after the replacement of DAN Ringer with normal Ringer. (e) Response in normal Ringer at 31°C, after the experiment (d), the temperature of the bathing medium was raised from 15°C to 31°C to see the extent of recovery by comparing with experiment (a).

4 shows the mechanical response of mouse EDL at various temperatures in normal Ringer (top traces) and in 50% D₂O Ringer (middle traces) solutions. Apparently, twitch tension in D₂O was inhibited more strongly at high temperature ranges and contraction time was prolonged at all temperatures. Action potential was not affected in the presence of D₂O.

Inhibition of twitch tension by DAN was also temperature dependent. At 31°C, DAN reduced the twitch tension developed by mouse EDL to 30% of its original value after 15 min incubation (Fig. 5b). When the temperature was lowered to 15°C, the peak tension was not reduced appreciably in DAN Ringer (Fig. 5c,d).

Responses of frog semitendinosus muscle fiber to DAN were qualitatively similar to those of mouse EDL. The upper traces of Fig. 6 represent the twitch tension of fiber bundles (for this experiment, extracellular electrode was used) in normal Ringer at various temperatures. The lower traces indicate the response in DAN Ringer at the corresponding temperatures. Inhibition of twitch tension was clearly pronounced at high temperature ranges. The initial rate of tension development was decreased in the presence of DAN, but the time to peak tension was not altered.

Fig. 6. Effect of DAN on twitch response at low temperature ranges, frog semitendinosus muscle fiber bundles, extracellular stimulation (20 V, 10 msec). Upper trace, twitch tension in normal Ringer; lower trace, twitch tension in DAN Ringer (40 μ M). The temperature of measurement is indicated at the top of each trace.

Effect of external Ca^{2+} concentration

Twitch amplitude of muscle fiber was somewhat reduced in high Ca^{2+} Ringer, probably because of stabilization of membrane (FRANKENHAEUSER and LÄNNER-GREN, 1967). Also, increase of Ca^{2+} concentration in the medium antagonized the inhibitory effect of DAN on twitch tension, as shown in Fig. 7. The same antagonistic effect of Ca^{2+} in twitch response in D_2O were noticed, however, to a lesser degree than those in DAN Ringer. In low Ca^{2+} media (less than 0.1 mM), contraction of EDL was augmented at first, followed by the loss of contractility within several minutes. Ca^{2+} requirement for the function of mouse EDL seems to be more strict than for frog semitendinosus muscle which can respond to the stimulus in Ca^{2+} -free medium for a considerable length of time (SANDOW *et al.*, 1975).

Fig. 7. Effect of external Ca^{2+} concentration on twitch response in DAN Ringer, mouse EDL at 28°C , frog semitendinosus at 15°C . Both fiber bundles were stimulated extracellularly (20 V, 10 msec). The ratio of peak twitch tension in DAN (15 min incubation) and that in normal Ringer are plotted at various Ca^{2+} concentrations. Δ , mouse EDL; $\circ$, frog semitendinosus.

Fig. 8. Potassium and caffeine contracture of muscle in D_2O (99%). Frog semitendinosus. Dotted line indicates the corresponding control in normal Ringer. (a) K^+ contracture in 110 mM K Ringer at 15°C , (b) caffeine contracture (5 mM) at 15°C . Note that the tension developed 5 min after the addition of caffeine reached to the same level of the control.

K⁺ and caffeine contractures

Experiments on K⁺ and caffeine induced contractures were performed on whole semitendinosus muscle of frog by placing specimens in K⁺ Ringer (110 mM) or Ringer containing 5 mM caffeine. As shown in Fig. 8a, D₂O (99%) abolished the tension induced by high K⁺ almost completely. The contracture induced by caffeine was also inhibited in D₂O Ringer, especially in the early phase, however, tension increased gradually and after 5 min, it reached the same level to that of control (Fig. 8b). The presence of DAN (40 μ M) in Ringer solution also reduced the tension development by high K⁺ or caffeine, to a lesser degree than those in D₂O.

ATP-induced contraction of glycerinated muscle fibers

Glycerinated muscle fiber in the measuring medium (0.1 M KCl, 1 mM Mg²⁺, 20 mM tris-maleate) developed tension upon addition of ATP (3 mM), as shown in Fig. 9. In a medium made up of 99% D₂O or that containing 40 μ M of DAN, the fiber bundles generated tension as large as that in the control medium. Actual recording of tension was varied depending on the sizes of fiber bundles, but the maximum tension per unit cross section fell into the same ranges in all media (Fig. 9).

Fig. 9. ATP-induced contraction of glycerinated muscle fiber bundles; frog semitendinosus muscle fiber bundles. Medium; 0.1 M KCl, 1 mM Mg²⁺, 20 mM tris-maleate (pH or pH_D; 7.0) at 25°C. Tension was induced by addition of 3 mM ATP. The diameter of the bundles was checked under the microscope and tension per mm² calculated. —, 99% D₂O; --, 40 μ M DAN; ---, control.

Ca²⁺ uptake, release and ATPase activity of fragmented sarcoplasmic reticulum

Fragmented sarcoplasmic reticulum (SR) from rabbit skeletal muscle exhibited ATP-dependent Ca²⁺ uptake. In the control medium (0.1 M KCl, 1 mM Mg²⁺, 0.1 mM Ca²⁺ (⁴⁵Ca), 20 mM tris-maleate, pH 7.0) containing 1 mM ATP, the sample of this preparation binds about 100 nmol of Ca²⁺ per mg of protein. The rate and the maximum Ca²⁺ uptake of SR in DAN medium were slightly less than those of corresponding controls, but they were not statistically significant (Fig. 10a).

Fig. 10. Ca²⁺ uptake by fragmented sarcoplasmic reticulum (SR) and release, rabbit skeletal muscle. Medium; 0.1 M KCl, 1 mM Mg²⁺, 20 mM tris-maleate (pH or pHD; 7.0) at 25°C. (a) Time course of Ca²⁺ uptake, □, no ATP (no difference between control, D₂O and DAN); ○, 1 mM APT, control; ●, 1 mM APT, DAN; △, 1 mM ATP, D₂O. (b) Ca²⁺ release. Ca²⁺ taken up in H₂O with ATP for 5 min was taken as 100%. The bound Ca²⁺ to SR after dilution with medium containing no ATP (0.1 mM EGTA) are plotted. ○, diluted with H₂O medium; ●, diluted with DAN medium; △, diluted with D₂O (99%) medium.

On the other hand, in 99% D₂O medium, Ca²⁺ uptake of SR was reduced significantly, as shown in Fig. 10a. This reduction of Ca²⁺ uptake was not due to the irreversible change of SR in D₂O because the pretreated SR with 99% D₂O took up Ca²⁺ to the same extent as that of original SR in H₂O.

The Ca²⁺ which had been loaded to the SR in H₂O medium could be released by washout with large volumes of medium containing EGTA. When the dilution medium consisted of D₂O, Ca²⁺ release from SR was markedly decelerated.

Fig. 11. Time course of liberation of P_i from ATP by SR in the presence and absence of Ca²⁺ in D₂O and DAN. Medium; 0.1 M KCl, 1 mM Mg²⁺, 20 mM tris-maleate (pH or pHD; 7.0) at 25°C. ○, ●, control medium; △, ▲, D₂O medium; □, ■, DAN medium. Open and filled symbols indicate measurements in the presence of (0.1 mM Ca²⁺) and absence of (0.1 mM EGTA) Ca²⁺, respectively.

There was no significant difference between Ca^{2+} release in control and DAN medium (Fig. 10b).

Hydrolysis of ATP by SR in the presence of Mg^{2+} was greatly accelerated by the addition of Ca^{2+} (extra splitting, HASSELBACH and MAKINOSE, 1966) in the control medium as well as in that containing DAN, *i.e.*, DAN gave no effect on ATPase activity of SR. On the other hand, in D_2O (99%), ATP hydrolysis of SR was decelerated, especially in early phase of reaction (Fig. 11). The disappearance of extra splitting of ATP by which the energy for Ca^{2+} transport may be supplied, is consistent with the reduction of ATP-dependent Ca^{2+} uptake ability of SR in D_2O (Figs. 10a and 11).

DISCUSSION

D_2O and DAN, as well as the other drugs which modify E-C coupling, have been used in the investigation of physiological function of muscle although their modes of action are not yet fully understood. The experiments performed here confirmed the following characteristic features of the drugs already reported (ELLIS and CARPENTER, 1972; PUTNEY and BIANCHI, 1974; TAKAUJI *et al.*, 1975; BEZANILLA and HOROWICZ, 1975; YAGI and ENDO, 1976; SANDOW *et al.*, 1976): 1) inhibition of twitch tension without affecting the membrane potential; 2) depression of caffeine- and K^+ -induced contracture; and 3) no effect on the machinery for contraction. Also, the effect of DAN was most marked in twitch response of fast mammalian muscle (ELLIS and CARPENTER, 1972). DAN seems to interfere with the mechanism leading to the rapid release of Ca^{2+} (trigger for release of activator Ca). However, most of the experiments on mammalian skeletal muscle were performed on a bundle or whole muscle, but not on single fibers, probably due to technical difficulties. We used intracellular stimulation so that the response recorded was from the single fiber in which the electrode was placed.

As reported previously (YAMAMOTO *et al.*, 1976), muscle fibers exhibited twitch and tonic responses upon stimulus (Fig. 1). Both twitch and tonic responses were markedly depressed in D_2O or DAN Ringer without noticeable change in the action potential although the elevation of threshold current in DAN was noticed. Since the tonic response may be attributed to the increase of myoplasmic Ca^{2+} utilized by depolarization of T-tubule membrane (YAMAMOTO *et al.*, 1976), the reduction of tonic tension by D_2O and DAN suggests that Ca^{2+} entry through the T-system is blocked.

Contraction of muscle fibers at high temperature is much faster than that at low temperature. Correspondingly, the duration of action potential is also temperature dependent, as illustrated in Fig. 4. In mouse EDL, the peak tension of twitch response was reduced considerably even in normal Ringer by simply lowering the temperature. However, in D_2O Ringer (50%) at low temperatures, reduction of twitch tension was not drastic (in 99% D_2O , tension development was

too small to measure), so that the relative inhibition represented as the ratio of the peak tension was apparently small at low temperature ranges. The effect of DAN was also potentiated at high temperature ranges as examined in mouse EDL and frog semitendinosus muscle fibers (Figs. 5 and 6). The time to peak tension was not altered in D₂O or DAN at each temperature but the initial rate of rise was reduced. These facts indicate that some rate-limiting chemical process which is temperature-dependent may be involved in the mechanism of rapid signal transmission into the cell. D₂O and DAN prevent the release of activator Ca²⁺ from the SR by decelerating this chemical process. The involvement of trigger Ca²⁺ in this mechanism was postulated by the depression of DAN effect at high external Ca²⁺ concentration (Fig. 7). These observations are in agreement with those of ELLIS and CARPENTER (1972) and TAKAUJI *et al.* (1975) that DAN inhibited predominantly the initial phasic component of K⁺ contracture, probably mediated by trigger Ca²⁺ located on the T-system.

K⁺ and caffeine contracture were inhibited by D₂O, much more markedly than by DAN. The difference between D₂O and DAN can be explained by assuming that D₂O blocks not only the trigger mechanism but also decelerates the release of activator Ca²⁺. In fact, this was shown in several experiments. In isolated SR, both the rate and amount of uptake and release of Ca²⁺ were reduced to a considerable degree in D₂O medium whereas DAN gave a small effect (Fig. 10 and TAKAUJI *et al.*, 1975), although VAN WINKLE (1976) recently reported that DAN significantly suppressed the Ca²⁺ release from SR in isolated system. In the intact fibers, the intensive contraction with long duration which reflected the large release of Ca²⁺ from internal storage (explosive contraction in low Ca²⁺ medium, YAMAMOTO *et al.*, 1976) was hardly observed in D₂O Ringer (trace is not shown; it occurs in DAN Ringer).

SANDOW *et al.* (1976) characterized the effect of D₂O on mechanical properties of frog muscle fibers as (1) prolongation of latency period and (2) decrease of initial rate of tension development (dp/dt). They suggested that these effects were caused by (1) lowered rate and amount of Ca²⁺ release from SR and (2) a reduced speed of diffusion of Ca²⁺ to the contractile filaments in D₂O. YAGI and ENDO (1976) also demonstrated that the Ca²⁺ release in skinned fibers was reduced in D₂O. EASTWOOD *et al.* (1975), on the other hand, could not detect any effect of D₂O on internal membrane system (SR) although they recognized the depression of K⁺ contracture of crayfish muscle fibers with prolongation of relaxation time. The differences between their results and those shown here as well as those of SANDOW *et al.* (1976) and YAGI and ENDO (1976) probably attributed to the differences of the materials (crayfish and mammalian or frog muscle) although it seems that observation of EASTWOOD *et al.* (1975) can be interpreted as the reflection of retardation of Ca²⁺ uptake ability of SR in D₂O. Lack of enhancement of ATP hydrolysis of SR by Ca²⁺ from which the energy of Ca²⁺ transport may be supplied (HASSELBACH and MAKINOSE, 1966) in D₂O is quite consistent with

the fact that SR exhibits reduced Ca^{2+} uptake in this medium.

It is evident that from these results, there is a trigger mechanism responsible for the rapid release of activator Ca^{2+} . However, role of Ca^{2+} in the trigger mechanism cannot be immediately defined as for the Ca^{2+} -induced Ca^{2+} release; this mechanism may play only minor role in the E-C coupling process (THORENS and ENDO, 1975). An attractive theory presented by SCHNEIDER and CHANDLER (1973) shedded some light on this problem, *i.e.*, the movement of charged particles in the T-system opens the Ca^{2+} channel in the SR membrane (CHANDLER *et al.*, 1976). It seems that a certain chemical process mediated by Ca^{2+} located on T-system, other than electrical events, is involved in the coupling between depolarization of T-tubule membrane and Ca^{2+} release from SR.

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