EFFECT OF DANTROLENE SODIUM ON EXCITATION-CONTRACTION COUPLING IN FROG SKELETAL MUSCLE

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Abstract The effect of dantrolene sodium, 1-[5-(p-nitrophenyl)furfurylideneamino]hydantoin sodium hydrate, on electrical and mechanical response in frog skeletal muscles (whole muscles or single fibers) and on the biochemical properties of contractile proteins and fragmented sarcoplasmic reticulum isolated from frog or rabbit skeletal muscle was investigated. The peak tensions of twitch, tetanus and potassium contracture were significantly inhibited by dantrolene, without affecting the magnitude of resting potential, the amplitude and duration of action potential and the negative afterpotential. On the other hand, ATP-induced shortening of glycerol-extracted rabbit psoas muscle fibers, ATPase activity of frog myofibrils and Ca release induced by caffeine, Ca uptake and ATPase activity of fragmented sarcoplasmic reticulum of frog or rabbit muscle were not affected by dantrolene. Caffeine contracture was partially inhibited by dantrolene and was almost unchanged by it in potassium-depolarized muscle fiber. Nitrate ions and low concentration of caffeine rapidly recovered the twitch inhibition induced by dantrolene. These results suggested that dantrolene acts on the membrane of transverse tubules and possibly the triadic junction and that it inhibits the inward movement of Ca and subsequently decreases the release of activator Ca from sarcoplasmic reticulum.

It has been recently reported that dantrolene is a useful skeletal muscle relaxant (Snyder et al., 1967; Chyatte et al., 1971; Chyatte and Birdsong, 1971) and suggested that it has a direct effect on the skeletal muscle and at some step subsequent to membrane depolarization (Ellis and Bryant, 1972; Ellis and Carpenter, 1972). More recently, Putney and Bianchi (1974) suggested that it acts, at least in part, by inhibiting the "triggering" step in excitation-contraction coupling.

Received for publication July 10, 1975
The following abbreviations were used: ATP, adenosine triphosphate; EGTA, ethyleneglycol-bis(β-aminoethyl)-N,N'-tetraacetic acid; Tris buffer, tris(hydroxymethyl)aminomethane-HCl buffer.
In this paper, we also studied the site of action of dantrolene in detail using single muscle fibers as well as whole muscle. Evidence is presented to show that dantrolene acts on the membrane of transverse tubules including the junctions between these tubules and terminal cisternae in triads, which are essential for the excitation-contraction coupling in skeletal muscle, and the mechanism of the action of dantrolene is discussed.

Partial reports on this work were presented at the 54th Meeting of the Physiological Society of Hokkaido (Takauji et al., 1974), and at the 52nd General Meeting of the Physiological Society of Japan (Takauji et al., 1975).

MATERIALS AND METHODS

**Electrical and mechanical experiments.** Whole muscles and single fibers were dissected respectively from the sartorius muscles and the semitendinosus or iliofibularis muscles of *Rana japonica*. In the case of single fibers, after the dissection each fiber was left for about 1 hr in Ringer solution and tested for excitability before being transferred from the dissection dish to the experimental cell.

All experiments were carried out with a cell made of acryl glass. When whole sartorius muscles were used, the pelvic bone was fixed to a hook connected to the cell and the distal tendon was attached by a silk thread to a resistance wire strain gauge (Nihon Kohden, SB-1T). One tendon of the single fiber was fixed with chemical paste (Aron-arufa A, Tōa Gōsei Chemical Co.) on a lever of the strain gauge (Nihon Kohden, SB-1TH) and the other was connected to a glass hook. The fiber was stretched to 1.3 times slack length. Tensions of these muscles and single fibers were recorded on an ink-writing oscillograph (Nihon Kohden, WI-180). If necessary, the flow rate of solution was adjusted, so that the solution in the cell could be replaced at least within a fraction of a second.

Electrical stimulation was applied through two platinum plates (massive electrode) which were set parallel with whole muscle fiber at distance of 4 mm, while it was applied through two platinum wires for single fiber. The duration of rectangular pulse was 1 msec.

Membrane potentials were measured with the intracellular microelectrodes having a resistance of 10–30 MΩ.

Normal Ringer solution contained 105 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂ and 5 mM Tris-HCl buffer (pH 7.1). Potassium Ringer solution was prepared by replacing all NaCl in normal Ringer solution with isosmotic KCl. Both Ringer solution contained 10⁻⁶ g/ml of *d*-tubocurarine. Dantrolene sodium was dissolved in Ringer solution.

**Biochemical experiments.** The preparation of glycerol-extracted muscle fibers from rabbit psoas was performed according to the method of Szent-Györgyi (1949). In the present experiment, fibers preserved for more than 60 days were used. Before use, bundles of the muscle fibers were taken from 50% glycerol
and washed in a Petri dish containing 20% glycerol at room temperature for more than 30 min. Then, thin bundle was isolated from the muscle bundles. The thickness of the thin bundle was about 300 μm.

Myofibrils were prepared from frog leg muscles by a modification of the procedure of Nagai et al. (1960).

Fragmented sarcoplasmic reticulum was prepared from the leg muscles of frog (Rana japonica) and from the leg and back muscles of rabbit, according to the procedures of Taniguchi and Nagai (1970) and Takauji et al. (1967), respectively. We collected the fractions sedimented between 2,000 × g and 12,400 × g from frog and the 12,400–67,500 × g fractions from rabbit.

The ATPase activity of myofibrils and fragmented sarcoplasmic reticulum was measured according to Nagai et al. (1960), and calcium release and calcium uptake according to Weber and Herz (1968) using the Millipore filter method (Martonosi and Feretos, 1964). Radioactivity of the aliquots of reaction mixture and the filtrate obtained through a Millipore filter (type PH, with 0.3 μm average diameter) was counted in a liquid scintillation spectrometer (Horiba, LS-700).

Shortening in glycerol-extracted muscle fibers was determined by measuring the change of length induced by ATP. The initial length of the fibers was 30 mm. The concentration of protein was determined by the biuret reaction as calibrated by nitrogen determination. Concentrations of free calcium were estimated by use of Ca-EGTA buffers (Yamamoto et al., 1970). The water used was glass-distilled and had been passed through an ion exchange column. Experiments were carried out at room temperature (20–25°C). Dantrolene sodium, 1-[5-(p-nitrophenyl)furfurylideneamino]hydantoin sodium hydrate, was supplied from Yamanouchi Pharmaceutical Co., Ltd. The crystalline disodium salt of ATP was purchased from Sigma Chemical Co. 45CaCl2 was purchased from Daiichi Pure Chemicals Co., Ltd. Other chemicals were commercial products of the best reagent grade available.

RESULTS

Twitch and tetanus tensions

Ellis and Carpenter (1972) and Putney and Bianchi (1974) reported that dantrolene inhibits twitch and tetanus tensions in skeletal muscle. We confirmed their results using frog whole sartorius muscle. The inhibition of twitch tension was about 70–80% at 20 min after the addition of 25 μM dantrolene and the extent of inhibition was dependent on the concentration of the drug; the maximal inhibition and the half maximal inhibition were observed with 6.25 μM and 1 μM dantrolene, respectively. The rate of tension development and the rate of relaxation were somewhat reduced by the drug.

Twenty-five μM dantrolene inhibited 17–40% tetanus tension. The effects of
dantrolene on the ratio of twitch to tetanus and on the minimal fusion frequency for complete tetanus were also almost similar to the results of Ellis and Carpenter (1972) and Putney and Bianchi (1974).

**Resting and action potentials**

Figure 1 is a representative result on the effect of dantrolene on resting and action potentials. There was no difference in the resting membrane potential between dantrolene-treated and non-treated sartorius muscles. The mean value of the resting potential was $89.4 \pm 4.1 \text{ mV}$ for five dantrolene treated fibers and $87.7 \pm 4.7 \text{ mV}$ for seven non-treated fibers. We also observed no significant effect of dantrolene on the amplitude and shape of action potential and the negative afterpotential. These results confirm that reported by Ellis and Bryant (1972) and support the findings that there is no effect of the drug on the total membrane resistance or capacitance (Ellis and Bryant, 1972) and on the Na, K, Mg, and H$_2$O contents of sartorius muscle (Putney and Bianchi, 1974).

**Potassium contracture**

In the experiment carried out on a single fiber (Fig. 2), time course of potassium contracture was markedly shortened with treatment of the fiber with $2.5 \mu\text{M}$ dantrolene for 1 min, with which twitch response in a single fiber was completely inhibited (see Fig. 5); particularly the rate of spontaneous relaxation was accelerated, while the peak contracture tension was less strongly inhibited (about 20%).

Moreover, we observed that dantrolene did not have any marked effect on the amplitude of depolarization, which occurred as a result of the solution change from $2.5 \text{ mM} \text{ K}^+$ to $107 \text{ mM} \text{ K}^+$; the amplitude of depolarization in the dantrolene-treated fibers was on the average $69 \text{ mV}$, the same value to that obtained in the normal fibers.

**Caffeine contracture**

Effect of dantrolene on the contracture induced by $7.5 \text{ mM} \text{ caffeine}$ was shown
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Fig. 2. Effect of dantrolene on potassium contracture in a single fiber. A: in K+-Ringer. B: in K+-Ringer containing 2.5 μM dantrolene.

Fig. 3. Effect of dantrolene on caffeine contracture in a single fiber. A: Contracture was induced by 7.5 mM caffeine in normal Ringer. B: Contracture was recorded 1 min after immersion of a single fiber in Ringer containing 2.5 μM dantrolene, at which twitch tension was markedly inhibited. The horizontal black line indicates that the single fiber was in dantrolene-Ringer. Arrow indicates replacing the experimental solution with normal Ringer.

in Fig. 3. Dantrolene treatment of a single fiber for 1 min inhibited the rate and extent of the tension development by caffeine, but the inhibition of tension was incomplete (30–50%). On the other hand, caffeine contracture in a potassium depolarized single fiber was not significantly inhibited by 2.5 μM dantrolene (Fig. 4). Thus these results suggest that the permeability of membrane, especially transverse tubular membrane for caffeine is reduced by dantrolene and the decreased permeability is restored by K+ ions.

Rate of dantrolene action on twitches in a single fiber

Figure 5A shows the rate of twitch inhibition induced by dantrolene. When Ringer solution was rapidly changed with a 2.5 μM dantrolene-containing solution, within 5 sec the twitch tension of single fiber decreased to 50% of maximal tension, and within 10 sec to 70%. Thereafter, the tension gradually decreased. As
Fig. 4. Effect of dantrolene on caffeine contracture in a potassium depolarized fiber. In A and B, the first curve and the second curve were potassium contracture and caffeine contracture, respectively. Caffeine, 7.5 mM. A: in K+-Ringer. B: in K+-Ringer containing 2.5 μM dantrolene.

Fig. 5. Rate of inhibition of twitches by dantrolene. A: a series of twitches of a single fiber in Ringer containing 2.5 μM dantrolene. B: after twitch inhibition dantrolene-Ringer was replaced with normal Ringer. C: replaced with NO$_3^-$-Ringer. D: replaced with 1.5 mM caffeine-Ringer. Horizontal black lines indicate that the single fiber was in dantrolene-Ringer.

shown in Fig. 5B, the depressed twitch tension was very slowly restored upon returning the fiber to the normal Ringer solution. In contrast, recovery from the twitch inhibition by dantrolene was extremely rapid (within 5 sec) in NO$_3^-$-Ringer solution and in 1.5 mM caffeine-Ringer solution (Fig. 5C and D). These results resemble those of PUTNEY and BIANCHI (1974), who used Ringer solution containing 10 mM NaSCN.
Fig. 6. ATP-induced shortening in glycerol-extracted muscle fibers from rabbit psoas in the absence and presence of dantrolene. Shortening was represented by
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\text{shortening} = \frac{\text{initial length} - \text{measured length}}{\text{initial length}} \times 100.
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Composition of incubation medium: 20 mM Tris-HCl buffer (pH 7.2), 50 mM K\(^+\), 1 mM MgCl\(_2\), 1 mM ATP, about 10 \(\mu\)M Ca\(^{++}\) contaminating reaction mixture; total volume, 3 ml; diameter of fiber bundle, 300 \(\mu\)m; length, 30 mm; temp., 22°C. Concentrations of dantrolene: ○, nil; ●, 25 \(\mu\)M; ●, 50 \(\mu\)M.

Fig. 7. ATPase activity of frog myofibrils at various concentrations of dantrolene. Reaction conditions; myofibrils, 1 mg protein/ml; total volume, 2 ml; reaction time, 2 min; temp., 20°C. Other conditions as for Fig. 6.
Activity of contractile proteins

Since dantrolene seems to have a partial inhibitory effect on the tension developed by 7.5 mM caffeine (Fig. 3), we tested whether or not dantrolene has a direct effect on the activity of contractile element (actomyosin-ATP system). Dantrolene (25 and 50 μM) did not affect the shortening induced by ATP of glycerol-extracted muscle fibers isolated from rabbit psoas (Fig. 6). Corresponding to this result, dantrolene in concentrations of 1–60 μM was also without effect on the Mg²⁺-activated ATPase activity of frog myofibrils (Fig. 7). Moreover, although the figure is not shown here the curve showing pCa-dependence of the myofibrillar ATPase activity was not shifted to either side by dantrolene. From these results, it is concluded that dantrolene does not directly act on contractile proteins; actomyosin and troponin-tropomyosin complex.

⁴⁵Ca release, ⁴⁵Ca uptake, and ATPase activity of fragmented sarcoplasmic reticulum

We measured the effect of dantrolene on the activities of fragmented sarcoplasmic reticulum (SR) isolated from frog or rabbit skeletal muscle. Dantrolene had no effect on the ⁴⁵Ca release induced by 10 mM caffeine (Fig. 8). Similar results were obtained in the case of ⁴⁵Ca release induced by 5 mM caffeine. Furthermore, ⁴⁵Ca uptake ability and the basic and extra ATPase activities, which are
Fig. 9. ATPase activity of fragmented sarcoplasmic reticulum from frog skeletal muscle in the absence and presence of dantrolene. Reaction conditions: Ca\(^{2+}\) contaminating reaction mixture, about 10 \(\mu\)M; total volume of reaction mixture, 2 ml; temp., 21°C. Other conditions as stated for Fig. 8. ○, without dantrolene; ●, with dantrolene (10 \(\mu\)M).

Fig. 10. Basic and extra ATPase activities of rabbit fragmented sarcoplasmic reticulum in the absence and presence of dantrolene. Oxalate, 5 mM. Other conditions as for Fig. 9. Arrow indicates addition of CaCl\(_2\) (50 \(\mu\)M). ○, ●: in the absence of dantrolene; △, ▲: in the presence of dantrolene (50 \(\mu\)M). Open symbols: without CaCl\(_2\); filled symbols: with CaCl\(_2\).
another well-known activity of SR, of frog and rabbit fragmented SR were not also affected by dantrolene (Figs. 8, 9, and 10).

Each of experimental results described above is a representative result of several such experiments.

DISCUSSION

Confirming the data of Ellis and Carpenter (1972) and Putney and Banchi (1974), our results demonstrated that dantrolene depresses twitch, tetanus and potassium contracture with respective degree of inhibition (see RESULTS, Fig. 2). Since resting and action potentials (also negative afterpotential) (Fig. 1) and the amplitude of depolarization caused by increasing the external concentration of K+ ions are not significantly affected by dantrolene, it appears that the inhibition of these mechanical responses by dantrolene is not ascribed to a mere change of these membrane potentials. Caffeine was able to cause contracture (Figs. 3 and 4), at the same concentration of dantrolene that strongly inhibited twitch tension (Fig. 5). Furthermore, it appears that dantrolene does not directly act on the sarcoplasmic reticulum and the contractile proteins, because dantrolene had no significant effect on the Ca release induced by caffeine, Ca uptake and basic and extra ATPase activities of fragmented sarcoplasmic reticulum (Figs. 8–10), and ATP-induced shortening of glycerol-extracted muscle fibers (Fig. 6) and ATPase activity of myofibrils were not influenced by dantrolene (Fig. 7). From these results, it is concluded that dantrolene acts on the excitation-contraction coupling process in frog skeletal muscle.

As shown in Fig. 1 and mentioned above, dantrolene did not influence the resting and action potentials, the negative afterpotential and the amplitude of depolarization caused by a high concentration of K+ ions. On the other hand, however, we found that a series of twitches of single fibers was extremely rapidly (within 5 sec) depressed by addition of dantrolene (Fig. 5A) and the dantrolene-depressed twitch tension was restored to almost control level more rapidly (within 5 sec) when dantrolene-Ringer solution was replaced by Ringer solution containing NO3- ions or caffeine in subthreshold concentration than when it was replaced by normal Ringer (Fig. 5B, C, and D). Since the action of NO3- ions and subthreshold concentration of caffeine upon twitches of frog single fibers occurred rapidly (within 1–3 sec) (Hodgkin and Horowicz, 1960; Lütgau and Oetliker, 1968), our findings suggest that dantrolene acts on more superficial sites in excitation-contraction coupling process, probably on the tubular membranes and triadic junction.

It has been reported that 45Ca influx increases relating to twitch and potassium depolarization (Bianchi, 1968). More recently, Oota and Nagai (1974) proposed that 45Ca influx in response to potassium depolarization occurs through the transverse tubular membrane in frog skeletal muscle, on the basis of their
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finding that the increase in $^{45}$Ca influx in response to potassium depolarization was not observed in T-disrupted muscle. Putney and Bianchi (1974) found that dantrolene decreases $^{45}$Ca influx associated with potassium depolarization. From these findings, it may be considered that the inhibitory action of dantrolene is attributed to the depression of the inward movement of Ca which occurs through the membrane of T-system, and to a subsequent decrease in the release of activator Ca from SR.

In a potassium depolarized fiber, contrary to normal muscle fibers, caffeine contracture was not significantly inhibited by dantrolene (Fig. 4). The question remains as to whether (1) K$^+$ ions potentiate caffeine contracture (Matsushima et al., 1962; Caputo, 1966) despite the presence of dantrolene or (2) K$^+$ ions antagonize the action of dantrolene on transverse tubular membrane or triadic junction. However, the latter possibility may be excluded, since potassium contracture itself was depressed by dantrolene (Fig. 2). A simple explanation for the former is that K$^+$ ions may restore the permeability of tubular membrane for caffeine which has been inhibited by dantrolene. Experiments in progress are designed to test this possibility.

In addition to the inhibitory effect of dantrolene on the peak tension of potassium contracture, we also demonstrated that this drug increases the rate of spontaneous relaxation of the contracture (Figs. 2 and 4). A similar phenomenon has been observed in the case of lowering external calcium (Curtis, 1964; Frankenhaeuser and Lannergren, 1967) or addition of local anaesthetics, for example, procaine (Oota, unpublished data). In the former case the restoration or inactivation curve is markedly shifted towards more negative potential values, in other words, the mechanical inactivation is potentiated (Frankenhaeuser and Lannergren, 1967). Therefore, it is considered to be interesting and worthwhile to determine whether or not dantrolene affects the inactivation curve. Further experiments are in progress on this problem.

As demonstrated in the present results, the Ca movement associated with the transverse tubular membrane was affected by dantrolene, whereas that associated with the sarcoplasmic reticulum was unaffected. These findings suggest that there is a difference in the chemical components or molecular structure in these two membranes.

It was further observed that in thin bundles of semitendinosus muscle (about 200 μm in diameter), which contain fast and slow fibers, dantrolene predominantly depressed an initial fast phasic component of potassium contracture but only slightly depressed a second slow tonic component (unpublished data). This result was almost in agreement with that of Ellis and Carpenter (1972); they reported a relatively small (about 25%) depression by dantrolene of potassium contracture in frog rectus abdominis muscles. Since transverse tubular system is very sparse in slow fiber (Page, 1965), this finding may also support our foregoing conclusion that the sites of action of dantrolene is at transverse tubular membranes.
and triadic junctions in frog skeletal muscle. Further experiments on the effect of dantrolene on single slow fibers are also in progress.

The authors are greatly indebted to Yamanouchi Pharmaceutical Co., Ltd. for supplying dantrolene sodium. We thank Miss. E. Ooe, Miss Y. Yokota an Mrs. K. Ogyu for their technical assistance.

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