EFFECTS OF SOME DIVALENT CATIONS ON THE DENERVATED SKELETAL MUSCLE OF THE RAT

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There have been few studies on actions of divalent cations on mammalian skeletal muscles (1) as compared to amphibian muscles, which have been used in the study of the role of calcium in the excitation-contraction coupling process (2, 3). Related studies on mammalian denervated skeletal muscle are also few.

Denervated skeletal muscles were generally reported to be more sensitive to acetylcholine (ACh) (4, 5), but Kiku-iri (6) indicated that twitch and tetanus evoked by direct electrical stimulation were reduced gradually with the lapse of time after denervation in the sartorius muscle of the frog, while the pattern of caffeine-contracture in the denervated muscle was not different from that in the innervated muscle. Gutmann and Sandow (7) reported that a decrease in twitch and tetanus tensions became progressively greater with increased duration of denervation in the extensor digitorum longus muscle of rats, whereas the denervated muscle became more sensitive to caffeine.

The aim of the present study was to investigate effects of some divalent cations, electrical stimulation and substituting strontium ions for calcium ions in the bath solution on the rat denervated skeletal muscle.

METHODS

Male rats of the Wistar strain were denervated, under ether anesthesia, by removing approx. 1.5 cm of obturator nerve in the abdomen supplying the gracilis anticus muscle. These were maintained postoperatively for 15–26 days. The average size of the muscle preparations was 0.5–1.0 mm × 2 mm × 15 mm (thickness × width × length). The isolated muscle was suspended in a 12 ml organ bath filled with Locke-Ringer solution gassed with a mixture of 98% O₂ and 2% CO₂ and kept at 32°C. In order to stimulate the muscle electrically, massive electrodes were used and the rectangular current pulses of 1 msec duration and of sufficient strength to produce the supramaximal contraction were applied. In this experiment Locke-Ringer solution containing d-tubocurarine (10⁻³ mM) was used as bath fluid to eliminate indirect action. The Locke-Ringer solution had the following composition (mM): NaCl, 146.86; KCl, 5.63; CaCl₂, 2.14; MgCl₂, 2.09; NaHCO₃, 5.93;

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glucose, 2.78. The muscles were arranged under 0.2 g initial tension for the isometric and 0.3 g initial load for the isotonic recordings of responses. All divalent cations were used as chloride.

RESULTS

Non-specific potentiation

Fig. 1 illustrates the mechanical responses of denervated and innervated muscles to electrical stimulation applied through massive electrodes. The size of twitch and tetanus tension evoked by direct electrical stimulation of the muscle was increased after denervation in isotonic and isometric conditions. In addition, the denervated muscle became more sensitive to drugs and ions, such as ACh, hexanoylcholine (HexCh), nicotine (Nc), barium ions, caffeine or potassium ions (Fig. 2), which was the same in isometric and isotonic conditions. Therefore it can be said that denervation nonspecifically increases responses of the muscle. ACh in concentration of $6 \times 10^{-2}$ mM was sufficient to produce the maximal ACh-contracture, which was inhibited by d-tubocurarine chloride ($10^{-4}$ mM). Caffeine-contracture in denervated muscles was characterized by enhancement of the peak tension as well as increase of the time required to reach this tension.

Actions of divalent cations in Locke-Ringer solution

With respect to twitch by direct electrical stimulation, barium ions in a low concentration (0.5 mM) caused transient increase immediately followed by decrease in size and in moderate concentration (3 mM) produced only decrease in the denervated muscle, whereas a higher concentration (10 mM) of the same ions caused transient decrease immediately followed by increase in the innervated muscle. Strontium ions in a concentration of 10 mM produced only decrease in both muscles.

![Fig. 1. Isotonic twitch and tetanus in innervated and denervated muscles. A, the innervated muscle. B, the muscle 24 days after denervation. Vertical lines indicate twitch responses. Duration was 1 msec. Frequency in the case of tetanus was 50 cycles per sec. Muscles were pretreated with d-tubocurarine ($10^{-4}$ g/ml) 40 min prior to testing. Load on the muscle was 0.3 g.](image-url)
Fig. 2. Comparison between isometric responses to drugs of denervated muscle and those of innervated muscle.

Fig. 3. Potentiating effects of barium and strontium ions on ACh- and caffeine-contracture in Locke-Ringer solution. Time of divalent cation pretreatment was 3 min.

a. in (A), a, d in (B) : control response.
b. in (A), b, e in (B) : response after pretreatment with 8 mM Sr\(^{2+}\).
c. in (A), c, f in (B) : response after pretreatment with 8 mM Ba\(^{2+}\) and 1 mM Ba\(^{2+}\), respectively.

KCl-contractures in Locke-Ringer solution tended to be enhanced in innervated muscles, whereas they were considerably reduced in denervated muscles when pretreated with strontium (6 mM) or barium ions (6 mM) for 3 min. Strontium and barium ions in Locke-Ringer solution potentiated ACh- and caffeine-contractures (Fig. 3). Barium ions were much more effective than strontium ions. Since caffeine-contracture was increased by smaller concentrations of barium ions, the action of barium was found to be
considerably specific to caffeine-contracture. The time required to reach the peak tension of caffeine-contracture in denervated muscles decreased strikingly with enhancement of the peak tension by 1 mM barium ions. Cobalt, nickel and manganese ions (1 mM) reduced the drug-induced contractures in both muscles.

Effects of substituting divalent cations for calcium ions

The following study determines the effects of substituting 2 mM of some divalent cations for calcium ions. In order to deplete calcium in the muscle as much as possible, the following procedure was carried out. Caffeine was applied to muscles immersed in calcium-free solution containing 1 mM EGTA every 30 min during an exposure period of 2.5 hr after exposing the muscle to calcium-free solution, 6-9 mM and 18 mM caffeine were applied to the denervated and innervated muscles, respectively as a first step. Next, 27 mM caffeine was used on both muscles (Fig. 4). This course is termed Ca-depletion in this paper. After this treatment, contractures were not evoked in either muscle by test drugs, namely $6 \times 10^{-5}$ mM ACh, 27 mM caffeine or 153 mM KCl. The muscles were next exposed to a calcium-free solution containing 2 mM of each divalent cation and 1 mM EGTA for 30 min prior to testing. The calcium-free solution substituted with 2 mM

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**Fig. 4.** Schematic experimental process for Ca-depletion and treatment with 2 mM strontium solution containing 1 mM EGTA.

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**Fig. 5.** Effects of 2 mM strontium solution on ACh- and caffeine-contractures after Ca-depletion. The underlined show drug-induced responses in 2 mM strontium solution after Ca-depletion.

Upper trace: denervated muscle.
Lower trace: innervated muscle.
divalent cations and 1 mM EGTA for calcium is termed divalent cation-solution hereafter, for example, when strontium ions are used, the solution is termed strontium solution.

The amplitude of contractions of the denervated muscle produced by the test drugs, ACh, caffeine and excess KCl, was increased in strontium solution; the time necessary to reach the peak tension of caffeine-contracture was markedly shortened, whereas in innervated muscles there was almost no change (Fig. 5). Each drug-induced contracture in strontium solution was repeated every 40 min for a total of seven repetitions. The first response by ACh, KCl or caffeine was 220%, 280% or 250% against the control response by each drug. The seventh response by each drug was 170%, 250% or 190% respectively in denervated muscle. On the other hand, values for KCl- and caffeine-contractures were 140% and 108% in first responses, and 80% and 60% in the seventh, respectively in innervated muscles (Fig. 6).

Cobalt, nickel, or manganese solution did not restore the production of drug-induced contractures in either muscle. In the case of barium solution, KCl- or caffeine-contracture in innervated muscles and ACh-contracture in denervated muscles were sometimes restored, but responses were much smaller in amplitude than control responses in Locke-Ringer solution and the next responses declined more intensively than they did during the initial exposure to barium solution. For this reason, strontium appears to be more effectively utilized in the excitation-contraction coupling process than calcium in the denervated muscle preparation. The following experiments were carried out to investigate this effect of strontium in the denervated muscle. When the denervated muscle was tested with 42 mM KCl during a 10 min exposure to strontium solution in place of Locke-Ringer
solution, the response was increased, as shown in Fig. 7b. Subsequently re-exposing the muscle to Locke-Ringer solution for 2 min led to a greater contracture (Fig. 7c). A similar phenomenon was observed in cases of exposure to strontium solution for 25 min (Fig. 7e, f) or 10 sec (not shown in the figure) instead of 10 min exposure. These phenomena suggest that responses were influenced by divalent cations taken up into the muscle cells rather than by those in the outer solution. After eliminating calcium (Ca-depletion) and placing in 2 mM strontium solution, the response was potentiated even more (Fig. 7g). A similar phenomenon was observed on ACh-contracture. Similarly, caffeine-contracture was also enhanced much more by the above procedure (Fig. 7k). To adequately demonstrate the action of cations, the experiment in Fig. 8 was carried out. Being placed in 2 mM strontium solution after Ca-depletion, ACh-contracture was potentiated as mentioned above (Fig. 8b).

When the muscle was exposed to Locke-Ringer solution in place of 2 mM strontium solution for 1 min, ACh-contracture returned slightly to the control response in Fig. 8a (Fig. 8c), however even on re-exposure to 2 mM strontium solution for 20 min, the response decreased much more (Fig. 8d). In addition, this response to ACh was repeated for three times in 2 mM strontium solution with no change observed (Fig. 8e). When placed again in 2 mM strontium solution after Ca-depletion, ACh-contracture was increased to the same

![Fig. 7. Effects of 2 mM strontium solution on KCl- and caffeine-contracture before and after Ca-depletion.](image)

- a, h, control response in Locke-Ringer solution.
- b, i, response after treated with 2 mM strontium solution for 10 min in place of Locke-Ringer solution.
- c, f, response after re-exposure to Locke-Ringer solution for 2 min following the response (b) and (e), respectively.
- d, response after immersion in Locke-Ringer solution for 20 min following the response (c).
- e, j, response after treatment with 2 mM strontium solution for 25 min in place of Locke-Ringer solution.
- g, k, response after calcium depletion and placed in 2 mM strontium solution.
FIG. 8. Comparison between response of the denervated muscle to ACh and caffeine after re-exposure to Locke-Ringer solution and those after re-exposure to 2 mM strontium solution.

a, response in Locke-Ringer solution.
b, f, g, response in 2 mM strontium solution after Ca-depletion.
c, response after treatment with Locke-Ringer solution for 1 min in place of 2 mM strontium solution.
d, i, response after washing with 2 mM strontium solution for 20 min.
e, response repeated three times in 2 mM strontium solution after d.
h, response after treatment with Locke-Ringer solution for 3 min in place of 2 mM strontium solution.
j, response after washing with 2 mM strontium solution for further 25 min.

extent as in Fig. 8b (Fig. 8f). Caffeine-contracture was suppressed considerably to the control response in Locke-Ringer solution (Fig. 8h), but was gradually restored after washing with 2 mM strontium solution (Fig. 8i, j).

DISCUSSION

Denervated muscles became nonspecifically sensitized to stimulants, namely ACh and HexCh which reacted with the receptor, KCl and electrical stimulation which acted on the whole muscle membrane, and caffeine which produced the contracture by causing release of calcium ions from the sarcoplasmic reticulum. Furthermore, papaverine and dodecyltrimethylammonium (8), which relaxed the intestinal smooth muscle of the guinea pig, contracted the denervated muscle.

When mammalian skeletal muscles were denervated, the resting membrane potential became low with the result of a reduction in the potassium conductance of the membrane (9). It was also demonstrated that barium ions caused depolarization of the membrane due to a reduction of the potassium conductance in the innervated muscle of the frog (10), therefore, the membrane of denervated muscles may be more depolarized by treatment with barium ions. Consequently, the amplitude of the contraction evoked by KCl or electrical stimulation was suppressed by barium ions. On the other hand, the contracture
produced by ACh or caffeine was enhanced.

The action of barium ions was more specific to enhancement of the caffeine-contracture in both the innervated and the denervated muscles than that of contractures induced by ACh as demonstrated in Fig. 3. Barium ions however showed a more specific action on the denervated muscle as caffeine-contracture was enhanced more intensively in the denervated than the innervated muscle (Fig. 3) and simultaneous treatment with 2 mM barium ions and caffeine, potentiated only the caffeine-contracture in the denervated muscle. Strontium ions showed similar effects to barium ions on the above responses, but effects were less as compared with those of barium ions.

An increase in the amplitude of drug-induced contractures by 2 mM strontium solution in denervated muscles (Fig. 6) appeared to be due to uptake of strontium ions into cells from which calcium ions seemed to have been depleted in part by the process shown in Fig. 4. This is concluded as contractures were not produced completely by drugs of much higher concentration (such as 27 mM caffeine, 153 mM KCl) on the condition of the Ca-depletion (Fig. 4), and the seventh response by each drug was not greatly reduced when each drug-induced contracture in 2 mM strontium solution (containing 1 mM EGTA) was repeated every 40 min for a total of seven times (Fig. 6).

As shown in Fig. 7, re-exposing the muscle to Locke-Ringer solution for 2 min (Fig. 7c) led to a greater KCl-contracture than a 10 min exposure to 2 mM strontium solution which replaced Locke-Ringer solution (Fig. 7b). This is similar to cases of a 25 min (Fig. 7e, f) or a 10 sec exposure (not shown in the figure) instead of 10 min exposure to strontium solution. This increase could be due to uptake of strontium ions into the muscle cell, although direct proof has not been obtained experimentally.

On the other hand the ACh-response (Fig. 8c) after exposure to Locke-Ringer solution for 1 min in place of 2 mM strontium solution was decreased much more even in re-exposing 2 mM strontium solution four times for 80 min (Fig. 8d, e). This decrease appeared to be due to binding of calcium ions with the surface membrane or uptake of same ions into muscle cells, although this has not been experimentally justified. In addition, ACh- and caffeine-contractures in Locke-Ringer solution were slightly increased even in pretreating 8 mM strontium for 3 min (Fig. 3), but KCl-contracture was rather reduced in the denervated muscle. For this reason, calcium appeared to antagonize the potentiating action of strontium by adhering strongly to the surface membrane. These phenomena suggest the possibility that utilization of strontium ions may be enhanced by denervation. Caffeine-contracture was restored gradually after washing with 2 mM strontium solution in place of Locke-Ringer solution (Fig. 8i, j). In comparison with the effect of 2 mM strontium solution on the ACh-contracture (Fig. 8d, e), caffeine appears to mobilize the divalent cations in cells, namely in this case, release of calcium and uptake of strontium ions. Furthermore, the action of caffeine on denervated muscle appears to be also regulated by the movement of the divalent cations at the cell membrane since the caffeine-contracture in the denervated muscle was potentiated by simultaneous treatment with 2 mM barium ions and caffeine. This phenomenon was observed in spite of the fact that muscle contraction by caffeine is
reportedly due to release of calcium ions from the sarcoplasmic reticulum (11, 12).

Loomis and Konker (13) reported that d-tubocurarine, ACh or histamine applied intravenously induced contractures of the denervated anterior tibial muscle of the rat in situ, thereby indicating no effective antagonism between curare and ACh. The present experiments indicated that d-tubocurarine induced no contracture of isolated denervated muscles and antagonized the action of ACh as reported by Elmqvist and Thesleff (14), while histamine itself induced no contracture and relaxed ACh- or barium-contracture. These discrepancies remain to be clarified.

SUMMARY

Responses of the gracilis anticus muscle of the rat to ACh, KCl, caffeine or electrical stimulation were non-specifically potentiated after denervation. Strontium and barium ions potentiated ACh- and caffeine-contractures in the Locke-Ringer solution. Barium ions were more effective than strontium ions. Caffeine-contracture in denervated muscles was characterized by prolongation of the time required to reach the peak tension as well as enhancement of peak tension. Barium ions reduced markedly the time required to reach the peak tension, with increase of the peak tension. In substituting strontium for calcium, drug-induced contractures did not change in the innervated muscle, but were enhanced in the denervated muscle. On the other hand, cobalt, nickel and manganese ions did not restore drug-induced contractions in either muscle but barium ions restored it partially. From this increasing action of strontium for the contraction of denervated muscle it was postulated that strontium may be utilized more substantially in this denervated muscle, perhaps in the cell or on the surface membrane.

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