STUDIES ON RAPID COOLING CONTRACTURE OF FROG TOE MUSCLE IMMERSSED IN HYPERTONIC AND HYPOTONIC SOLUTIONS

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Summary The tension development of rapid cooling contracture (RCC) of toe muscle of *Rana nigromaculata* treated with 1.0 mM caffeine in various hypertonic or hypotonic solutions was measured by mechano-electric transducer. The tension output of RCC decreased in hypertonic NaCl solution in which the concentration of NaCl was increased and in hypertonic solution prepared by addition of KCl to the normal Ringer solution. The RCC tension output was not altered in isotonic solution in which NaCl was isotonicly substituted for KCl in normal Ringer solution, but it decreased in test solution in which KCl was hypertonicly increased. However, the RCC tension output was not altered in a solution in which urea was hypertonicly added to the normal Ringer solution.

The RCC tension output increased in hypotonic solution prepared by reducing the concentration of NaCl in normal Ringer solution; its value was maintained at an osmotic strength of 200–210 m Osm/kg H₂O and was reduced at 160 m Osm/kg H₂O.

In conclusion, the tension development of rapid cooling contracture in hypertonic and hypotonic solutions was similar to the data on crayfish muscle (APRIL *et al.*, 1968), in which the ionic strength of the intracellular space is a major determinant of the mechanical response to a given activation of Ca mechanical coupling.

Since the report by OVERTON (1902) it has been recognized that twitch tension development of skeletal muscle is enhanced in hypotonic solutions with a certain limit of osmotic strength and inhibited in hypertonic solution while ordinary action potential is generated (HODGKIN and HOROWICZ, 1957). SANDOW (1965) has expressed these phenomena in terms of "dissociation of electromechanical coupling." It has been argued, however, that these phenomena might be due mainly to mechanical alteration of intracellular contractile elements rather

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than to an electromechanical relationship, *i.e.*, changes of myoplasmic viscosity and intracellular ionic strength with water transference of muscular cells under differing osmotic strength also influence contractile elements (HOWARTH, 1958; PODOLSKY and SUGI, 1967; HILL, 1968).

In other words, intracellular dehydration caused by immersion in hypertonic solution narrows interfilamental space to produce a close package between A- and I-filaments, thus increasing development of filamentary resting tension (FRT) (DYDÝNSKA and WILKIE, 1968; HILL, 1968).

In crayfish muscle Ca activation was inhibited in hypertonic solution and enhanced in hypotonic solution (APRIL *et al.*, 1968). However, CAPUTO (1966) reported that the caffeine contracture in frog muscle was enhanced in hypertonic solution.

This paper deals with the behavior of rapid cooling contracture (RCC) (FUJII and SAKAI, 1969; SAKAI *et al.*, 1971) of frog toe muscle that was treated with a subcontracture concentration of caffeine and soaked in hypertonic or hypotonic solution.

These studies aim at comparison of RCC probably caused by activation of transiently increased Ca^{++} ions in myoplasm with the above-mentioned experimental data on Ca activation of APRIL *et al.* (1968). A part of this paper was also presented at the seminar of the U.S.-Japan Cooperative Program in Tokyo (1971).

MATERIALS AND METHODS

Toe muscles (extensor longus digiti IV) of the Japanese frog *Rana nigromaculata* were used. After measurement of the resting length of the toe muscle *in situ* and dissection of the muscle specimen, they were immersed in normal Ringer solution for 30–60 min and then fixed to the recording apparatus and twitch tension and tetanus (Po) were recorded at room temperature. The composition of the Ringer solution was as follows: 116.8 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 2.0 mM Tris-HCl (pH 7.0); 6 mg% *d*-tubocurarine was also added to the test solution.

Hypertonic or hypotonic test solutions were prepared by increasing or decreasing the concentration of NaCl in normal Ringer solution, and KCl hypertonic or urea hypertonic solution was made by increasing KCl or adding urea to normal Ringer solution. Before the experiment the osmotic strength of the test solution was measured by a freezing point osmometer of Advanced Instrument Inc., Ltd.

The average osmotic strength of the normal Ringer solution was 230 m Osm/kg H_2O . The glycerol hypertonic solution used in the present experiment was 400 mM glycerol Ringer solution with about 2.7 times the osmotic strength of normal Ringer solution (the same used by HOWELL and JENDEN, 1967).

T-tubule disrupted muscle was prepared by immersion of the muscle in hy-

pertonic glycerol Ringer solution for 1 hr and then replacement in normal Ringer solution. During immersion of the muscle in glycerol hypertonic solution the T-tubule disruption was confirmed by observation of twitch tension development or tetanus. Rapid cooling was carried out by the procedure devised by SAKAI (1965): After treatment at room temperature with a subcontracture concentration of caffeine or thymol for 5–10 min, the muscle preparation in a 50-ml glass cylinder of normal Ringer solution at room temperature, was cooled rapidly by replacing the another cylinder with a previously cooled solution. Muscle tension was recorded on an oscillograph (Nihon-Kohden Co., Ltd.).

The experiments in the present paper were performed between October 1969 and February 1970 and between July and September 1971.

RESULTS

The tension development of RCC in hypertonic solution

Figure 1 indicates the experimental results of RCC in various hypertonic solutions of NaCl. Twitch tension and tetanus (P_o) in normal Ringer solution at room temperature were recorded and then caffeine at 1 mM final concentration was added to the test solution. To confirm potentiation of twitch caused by caffeine, 20 min later two successive RCCs in normal Ringer solution with 1 mM caffeine were observed.

In the present experiment maximal tension development of RCC was used as the standard value for calculation of all tension values, and usually it took about 20–30 sec of cooling time to reach the peak of tension. Immediately after maximal tension was observed, rewarming was carried out.

After RCCs were elicited two times at an interval of 20 min in normal Ringer solution, the muscular preparation was soaked in hypertonic solution. The hypertonicity of the test solution was raised stepwise to 440 m Osm/kg H_2O , and RCCs in the hypertonic solutions were recorded at 20 min intervals.

As shown in Fig. 1, RCC tension development in hypertonic solutions gradually decreased with rising hypertonicity, as did twitch tension development. In Ringer solution containing 1 mM caffeine, RCC tension development depends upon the thermal difference of the extracellular solution and varies by season even under exactly the same thermal conditions. In the present experiment, summer frogs were used, and the maximum tension of caffeine-RCC in normal Ringer solution was $77 \pm 10\%$ ($N=21$) against tetanus (P_o).

Figure 2 shows the relationship between relative tension of RCC in various hypertonic solutions and concentration of NaCl in the test solution. As shown in this figure, even if osmotic strength of the test solution was linearly raised, relative tension development of RCC dropped reciprocally. This result was similar to the data of APRIL *et al.* (1968), who investigated the relationship between tension output of crayfish and extracellular osmolarity of test solution. The

Fig. 1. The inhibitory effect of hypertonic solution on rapid cooling contracture (RCC). At first tetanus tension and twitch were recorded, and then twitch potentiation of 1 mM caffeine was observed. Twenty minutes after addition of caffeine successive RCCs were recorded at intervals of 20 min with rapidly lowering temperature from 23 to 0°C. RCC in various hypertonic solutions prepared by addition of NaCl to the normal Ringer solution were recorded at 20-min intervals. Upper row: signal of rapid cooling. Middle row: osmotic strength of the solution and concentration of NaCl added to the normal Ringer solution. Lower row: tetanus tension (P_o), twitch, and RCCs.

tension development of RCC almost vanished in hypertonic Ringer solution of 2.5 times normal osmolarity but on returning to normal Ringer solution it appeared again.

Figure 3 shows the RCC in hypertonic solution in which KCl was substituted for NaCl, which has been previously discussed with respect to the RCC of depolarized skeletal muscle (SAKAI, 1965). In this experiment, taking into consideration unusual intracellular metabolism and potassium contracture of striated muscle in hypertonic solution containing more than a certain concentration of KCl (SASLOW, 1937; HOWARTH, 1958; MIYAZAKI *et al.*, 1962; NOVOTNÝ *et al.*, 1962; NOVOTNÝ and VYSKOČIL 1966), RCCs were performed at intervals of 30 min.

No remarkable inhibition of RCCs in KCl hypertonic solution was observed,

Fig. 2. Relative tension of RCC produced in various hypertonic solutions. The relative tension of RCC in various hypertonic solutions is expressed as relative value against the maximal tension of RCC in normal Ringer solution. NaCl hypertonic solution was prepared by addition of NaCl to normal Ringer solution, and also KCl and urea hypertonic solution were prepared by addition of KCl and urea as described in the figure. Points of relative tension of RCCs in NaCl and KCl hypertonic solution were obtained from the results shown in Figs. 1 and 2. The value of osmotic strength corresponds to relative tension of RCC observed in various kinds of hypertonic solutions, such as urea hypertonic solution.

but RCC in NaCl hypertonic solution was strongly inhibited, as shown in Fig. 2. The relative tension of RCCs obtained in a hypertonic solution of KCl-added Ringer solution is indicated in Fig. 2, which shows that RCC was reciprocally inhibited, even if osmotic strength in the test solution was linearly raised.

Fig. 3. RCC observed in KCl hypertonic solution. KCl hypertonic solution was prepared by addition of KCl to normal Ringer solution. RCCs were observed at 30-min intervals. Upper row: signal of rapid cooling. Middle row: osmotic strength and concentration of KCl added to the normal Ringer solution. Lower row: maximal tension of twitch and tetanus and RCC. In this experiment RCC was recorded by rapidly lowering temperature from 27 to 0°C.

In an isotonic solution in which KCl was substituted for NaCl, RCC could be produced as in normal Ringer solution, but in a raised concentration of KCl, RCC was partially inhibited. In 450 m Osm/kg H₂O solution it vanished completely, but on returning the muscle to normal Ringer, RCC was again produced, transiently increased muscle weight returning to the normal value.

In addition, in hypertonic solution made by addition of urea as a nonelectrolyte agent, tension development of RCC was examined. As shown in Fig. 2, in urea hypertonic solution RCC was not inhibited. In this experiment the muscular preparation was immersed in urea hypertonic solution for 30 min to avoid the transient effect of osmotic strength on RCC (APRIL *et al.*, 1968).

The experiments discussed so far were all performed on intact muscle fibers. However, the RCC of T-tubule disrupted muscle was also investigated using

1 mM caffeine or 50 μ M thymol, and the results were similar to the results reported by SAKAI *et al.* (1971).

The tension development of RCC in hypotonic solution

Tetanus, twitch tension and then 1 mM caffeine RCC in normal Ringer solution and hypotonic solution, prepared by decreasing NaCl concentration in normal Ringer solution, were recorded. When the muscle preparation was soaked in hypotonic solution, it was slightly swollen, unlike the specimens in hypertonic solution, and RCC was enhanced. This enhancement of RCC showed the

Fig. 4. The relative tension of RCC in hypotonic solution. Hypotonic solution was prepared by reducing NaCl concentration in normal Ringer solution, and the osmotic strength of the test solution is shown in the upper graph. The relative tension of RCC in hypotonic solution was enhanced at 200–220 m Osm/kg H₂O, and in hypotonic solution less than 200 m Osm/kg H₂O showed a decreasing trend. When urea was added to the various hypotonic solutions as shown in the figure, the relative tension of RCC in the hypotonic solution was not affected.

maximum value in 200–210 m Osm/kg H_2O of test solution, and below that osmotic strength RCC tension development decayed gradually (Fig. 4).

In a hypotonic solution of 160 m Osm/kg H_2O the RCC tension output fell more than that in normal Ringer solution. Figures 2 and 4 show these results; in Fig. 4, the relationships between relative tension of RCC, osmotic strength of the test solution, and concentration of NaCl in the test solution are presented.

The phenomenon of RCC enhancement in hypotonic solution resembled the experimental results of Ca^{++} activation induced by microinjection of calcium ions (APRIL *et al.*, 1968), but the ratio of the enhancement of tension development was small, which might be due to the osmotic effect, as discussed below, *i.e.*, swelling of the muscle cell induced by low osmotic strength might disturb mechano-chemical coupling in spite of full activation of the whole muscle cell. Even when urea as a nonelectrolyte agent was added to each hypotonic solution as shown in Fig. 4, the relative tension of RCC was not influenced.

Fig. 5. The effect of hypertonic and hypotonic solutions on RCC. The relative tension of RCCs was calculated from the first peak tension of RCC. (A) ○—○: 1 mM caffeine rapid cooling contracture in normal Ringer solution. ●—●: 1 mM caffeine rapid cooling contracture in 102 mM NaCl hypotonic Ringer solution. (B) 1 mM caffeine RCC in 91.4 mM NaCl hypotonic Ringer was enhanced and inhibited in 2.5 times Ringer solution. Hypertonic Ringer solution was prepared by adding sucrose to normal Ringer solution.

Considering that in a test solution of slightly reduced osmotic strength RCC is known to be enhanced, the following two experiments were performed. First, in normal Ringer and 102 mM NaCl hypotonic solution containing 1 mM caffeine, rapid coolings were carried out continuously at intervals of 10 min, the results of

which are shown in Fig. 5A. Each value in this figure was obtained from the average of 8 cases. Then, in 91.4 mM NaCl hypotonic Ringer, 2.5 times sucrose hypertonic and again normal Ringer solution, rapid coolings were carried out in the presence of 1 mM caffeine (Fig. 5B). In any hypotonic solution RCCs were enhanced more than in normal Ringer solution.

DISCUSSION

It has been recognized by many investigators that Ca microinjection of single muscle fibers can cause Ca-activated contracture (HEILBRUNN and WIERCINSKI, 1947; NIEDERGERKE, 1955; CALDWELL and WALTER, 1963; APRIL *et al.*, 1968). Recently many investigators (PODOLSKY, 1962; EBASHI and ENDO, 1968; ENDO *et al.*, 1970; PODOLSKY and TEICHHOLZ, 1970) reconfirmed the above phenomena by direct addition of Ca ions to skinned muscle fibers (NATORI, 1954).

On the other hand, it is well known that the bound Ca of sarcoplasmic reticulum is released by caffeine (BIANCHI, 1962; HERZ and WEBER, 1965) and produces muscular contracture (AXELSSON and THESLEFF, 1958; MATSUMURA, 1959; FRANK, 1962; LÜTTGAU and OETLIKER, 1968; GEBERT, 1968). A certain concentration of caffeine above subcontracture concentration at room temperature produces muscular contracture, which is caused by accumulation of released Ca ions in myoplasm above the threshold concentration for contracture (PORTZEHL *et al.*, 1964).

In muscle fibers treated with a subcontracture concentration of caffeine, reversible contractures are produced by rapid cooling and rewarming (CONWAY and SAKAI, 1960; LÜTTGAU and OETLIKER, 1968); these have been called rapid cooling contractures (RCC) (FUJII and SAKAI, 1969; SAKAI *et al.*, 1971).

The mechanism of this RCC might be understood as follows:

- 1) In the presence of caffeine the bound Ca of sarcoplasmic reticulum is easily released, and the number of released Ca ions increases in myoplasm.
- 2) Due to rapid lowering of temperature the Ca accumulation ability of sarcoplasmic reticulum is reduced and released Ca^{++} gets close to the threshold concentration necessary for muscular contracture.

The total released Ca^{++} increases transiently and produces reversible muscular contracture. According to OGAWA (1970) release of Ca from the fragmented sarcoplasmic reticulum by caffeine was enhanced at low temperature in comparison with that at room temperature.

The RCC, therefore, might also be explained as one of the patterns of Ca activation (SAKAI, 1965, 1967; FUJII and SAKAI, 1969). On the other hand, the RCC can be observed even in the T-tubule disrupted muscle fibers, as reported previously (SAKAI *et al.*, 1971). There was no difference of tension output of RCC between T-tubule disrupted and non-disrupted muscle fibers, *i.e.*, SR, especially lateral cisternae and longitudinal tubules, might be considered to be a major portion of Ca uptake and release in the RCC.

The concept of the RCC mechanism mentioned above agrees with the experimental data of WINEGRAD (1965, 1968, 1970), in which the terminal cisternal of muscle cells might play a main role in Ca release in muscular contraction.

Troponin of myofilaments has an affinity for Ca ions (EBASHI *et al.*, 1967; PODOLSKY and TEICHHOLZ, 1970). It is still not known if this combining ability of Ca and troponin is influenced by lowering temperature, but the fact of RCC occurrence implies that it is not disturbed by cold, and sarcoplasmic reticulum will be rather influenced by low temperature. As mentioned above, the mechanism of RCC has been considered to exist in Ca mechano-coupling, and it is essentially similar to Ca^{++} activation induced by microinjection of Ca^{++} ions, the concentration of which is more than the threshold for contracture.

With respect to the hypertonic solution, however, the problem of whether Ca pumping action especially Ca releasing action is influenced or not still remains. Of interest here is ISAACSON's report (1969) that in hypertonic solution containing radioactive Ca Ca efflux in the presence of caffeine was not inhibited in spite of the disappearance of contracture.

The effect of osmolarity on sarcoplasmic reticulum is another problem that should not be ignored. It seems, however, that the contractile element, rather than sarcoplasmic reticulum, is strongly influenced by extracellular osmolarity.

The tension development of RCC in hypertonic solution is similar to the inhibitory trend of twitch in hypertonic solution (HODGKIN and HOROWICZ, 1957) and muscular contraction evoked by Ca^{++} injection into a muscle cell (APRIL *et al.*, 1968). In muscle fibers soaked in hypertonic solution prepared with KCl which can be transferred through membranes (BOYLE and CONWAY, 1941), the intracellular ionic strength of potassium is enhanced (HARRIS, 1961). In this muscle preparation, the RCC was partially inhibited (see Fig. 3). Those phenomena are not explained by electromechanical decoupling alone (SANDOW, 1965); rather they suggest that the viscosity of contractile elements would be raised by cellular shrinkage (HOWARTH, 1958), and also narrowing of interfilamental space would be produced by changes in ionic strength (BRANDT *et al.*, 1967; APRIL *et al.*, 1968).

The major determinant of the above inhibition of RCC could be Ca mechanical coupling (SANDOW, 1970). This concept is also supported by the present data (Fig. 2) that tension development of RCC was not inhibited in hypertonic solution prepared with such nonelectrolyte agents as urea. When the muscle preparation was soaked in urea hypertonic solution, transient dehydration occurred, and then intracellular ionic strength was reduced with transference of the nonelectrolyte agent into muscle cell (APRIL *et al.*, 1968).

The RCC of skeletal muscle soaked in 200–210 m Osm/kg H_2O hypotonic solution showed the maximal tension, and so did local contractions evoked by Ca^{++} injection into a muscle in hypotonic solution of similar osmotic strength. Therefore, it seems that within a certain range of osmotic strength the RCC ten-

sion output was raised, and in extraordinarily low or high osmotic strength, RCC tension output was inhibited.

According to HARRIS (1961), in hypotonic solution water is transferred into a cell, interfilamental space gets wider, and contractile elements themselves are also destructive. However, the reason why RCC tension development is enhanced or inhibited in extraordinarily hyper- or hypotonic solution is still unknown, although those phenomena are constantly observed.

Recently it was reported that ATPase activity of myosin B treated with trypsin would depend upon KCl concentration in the medium (MARUYAMA *et al.*, 1964), which may help elucidate our findings that varying ionic strength the physico-chemical properties of troponin can be altered (EBASHI, 1963; EBASHI and EBASHI, 1964).

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