and while it was carried out one of us (E.H.) held a U.S. Public Health Service Fellowship (SF-172).

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EFFECT OF SOME CONTRACTURE-PRODUCING AGENTS ON GLYCEROL-EXTRACTED MUSCLE FIBER RELAXED WITH RELAXING FACTOR

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SUMMARY

1. Caffeine, Ca ion, carnosine, DNP, nicotine and cyanide produced contraction of the fiber relaxed with the crude extract. Acetylcholine, choline, histamine and fluoride had no influence on the relaxed fiber.

2. In the absence of the relaxing factor, caffeine and carnosine had no marked influence on the tension development of the fiber in concentrations ranging from 2 m*M* to 10 m*M*. DNP lowered the tension development to about 50 % of the control value, but cyanide increased it to about 150 % at 10 m*M*. Nicotine increased it to 130 % at 5 m*M* and lowered it slightly at 10 m*M*.

3. These agents had no influence on the fiber relaxed with EDTA except Ca ion and nicotine.

4. Caffeine, DNP and carnosine did not contract the fibers relaxed with crude extract which were preincubated with ATP and Mg. However, Ca ion, nicotine and cyanide constantly produced contraction of the relaxed fibers.

5. On the basis of these findings, the production of relaxing substance by the granules in the presence of ATP and Mg and the significance of the granules in the excitation–contraction coupling were suggested.

INTRODUCTION

Glycerol-extracted muscle fibers contract on addition of ATP, but this contraction is not followed by relaxation. In the past few years, since MARSH[1] discovered a physiological relaxing factor in an extract of skeletal muscle, many investigations on the nature of the relaxing factor have been carried out[2–12]. It is now generally recognized that the granules, microsomes, isolated from the extract of muscle are essential for the relaxation of the fiber[6–12].

Recently, on the basis of detailed experiments on the inhibiting action of granules on myofibrillar ATPase, NAGAI *et al.*[13] have suggested that a very effective and labile relaxing substance might be produced by granules in the presence of ATP, Mg and oxalate. GERGELY[12] also has reported that the relaxation of fibers may be brought about by some relaxing substance produced by the granules in the presence of ATP and a "co-factor".

It has been known for a long time that many agents, acetylcholine, caffeine, nicotine, calcium, DNP and cyanide, produce contracture of skeletal muscle[14–16]. According to HAYASHI[17], carnosine causes contraction upon intracellular injection. It is interesting to recall at this point the investigation of HASSELBACH[18] in which he observed that the relaxation of glycerol-extracted muscle fiber induced by MARSH–BENDALL factor was inhibited by addition of caffeine, resulting in recontraction of the fiber. It is also well known that the relaxing factor becomes inactive when Ca ions are added. GERGELY[12] reported that carnosine is an inhibitor of the relaxing factor when assayed on both single fiber and myofibrills. Therefore, it can be expected that certain agents which produce contracture of living muscle may show significant effects on the relaxing action of granules in the presence of ATP, Mg and others.

In view of these facts, the present study was carried out for the purpose of throwing some light on the understanding of the nature of the relaxing substance and providing some clue as to the relationship between the excitation and the contraction of muscle.

MATERIALS AND METHODS

The glycerol extraction of muscle fibers from rabbit psoas was carried out according to the method of SZENT-GYÖRGYI[19]. In the present experiment, fibers which had been preserved for 60 days were mainly used. Before the isometric experiment, the above-mentioned muscle bundles were cut and dissected into shorter and fine bundles, about 2 cm long and 0.5 mm in diameter, in a Petri dish containing about 50 ml of 20 % glycerol–aqueous solution, and then stored at 0°. After about 12 h, a single fiber was isolated from the above fine muscle bundles under the binocular microscope. The fiber was then mounted on the tensionmeter* as described by WEBER[20]. The diameter of the single fiber was 60–70 μ.

The granules fraction was generally prepared from rabbit muscle according to the procedure of PORTZEHL[9] and BENDALL[21]. The extract was centrifuged at 13,000 $\times$ g for 10 min. This separated the large particles of the mitochondria fraction, which were discarded. The supernatant was termed the crude extract. Then this crude

Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

extract was spun at 80,000 × *g* for 50 min to precipitate the microsomal particulates, *i.e.*, the relaxing granules. The final residue was resuspended in a solution containing 80 m*M* KCl, 20 m*M* histidine and 5 m*M* potassium oxalate and stored at 0°, and termed the granules fraction.

ATP was obtained from the Sigma Chemical Co. in the form of a crystalline disodium salt. It was dissolved in 20 m*M* aqueous solution which was adjusted to pH 7.0 with KOH before use. Carnosine** used in this experiment was a product of the Nutritional Biochemical Corp. and was only for chemical and investigational use. Other chemicals were obtained from the Kanto Chemical Co. All solutions were prepared with deionized water.

The fibers were tested in solutions containing 0.04 *M* Tris–acetate buffer of pH 7.0, 0.12 *M* KCl, 2 m*M* $MgCl_2$, 2 m*M* ATP and, if desired, the relaxing factor and the contracture-producing agents under investigation. The concentration of the crude extract was expressed in volume per cent of total reaction mixture.

RESULTS

Relaxing activities of the three fractions

The relaxing activities of each of the fractions, *i.e.*, the crude extract, the granules and the supernatant, which had been prepared according to the described method were studied. The fibers developed a maximum tension of about 2400 g/cm² of fiber cross section with 2 m*M* ATP and 2 m*M* Mg at pH 7.0 and room temperature. The granules and supernatant which were obtained from the same volume of the crude extract were far less relaxing than the crude extract (final concentration 10 %). The granules and supernatant recombined in their original proportions were as active as the crude extract, and lowered the tension of the fiber to about 10 % of the maximum tension. These results were in agreement with those of GERGELY[12].

Large amounts of isolated fresh granules corresponding to 60 % crude extract produced relaxation without the addition of supernatant, but became rapidly inactive in a few days. The activity of the crude extract remained for about 7–10 days.

The effect of the contracture-producing agents on the relaxed fibers

At the outset, a single fiber relaxed with crude extract was immersed in the test solution containing caffeine, 2 m*M* ATP, 2 m*M* Mg and 10 % crude extract. As shown in Fig. 1, the relaxed fibers again contracted with increasing concentrations of caffeine. The concentration of caffeine was increased from 0.5 m*M* to 10 m*M*. When relaxation of the fiber was produced with 10 % of crude extract, the second contraction of the fiber appeared on the addition of 1 m*M*, and reached the maximum degree of contraction with 10 m*M* caffeine. The effective concentration of the caffeine changed with variation in the concentration of crude extract. This effect of caffeine was not affected by the addition of 5 m*M* procaine.

Similar results were also obtained in isotonic experiments using fiber bundles, 0.3 mm in diameter.

As can be seen in Fig. 2, Ca ion, nicotine, cyanide, carnosine and DNP showed

* The authors wish to thank Prof. H. H. WEBER, Dr. W. HASSELBACH and Mr. W. HEINEMANN of the Max Planck Institute in Heidelberg for making the tensionmeter.

** The authors wish to thank Prof. S. OKAMOTO of the Kobe Medical College for the gift of carnosine.

effects similar to those shown by caffeine. It was also shown that the effects of these agents depended on their concentrations. Ca ion which produced a satisfactory effect in concentrations of about 10^{-4} *M* was most effective as compared with other agents which exhibited their ultimate effects on the fiber relaxed with crude extract in concentrations of 10^{-3} *M*. As illustrated in the figure, the rates of contraction of the fibers in solutions containing the same concentration (5 m*M*) of these agents were in the following order: Ca $\gg$ nicotine $\geq$ caffeine > cyanide > carnosine > DNP. The magnitudes of the maximum tension developed after applying these agents were in the following order: Ca $\gg$ carnosine > cyanide > nicotine $\geq$ caffeine > DNP. In the experiments with caffeine and nicotine, the decreases in tension of the fibers after reaching their maximum tension were exceedingly great compared with any other agents.

In contrast to these agents, acetylcholine, histamine, fluoride and choline had no influence on the relaxation of fibers.

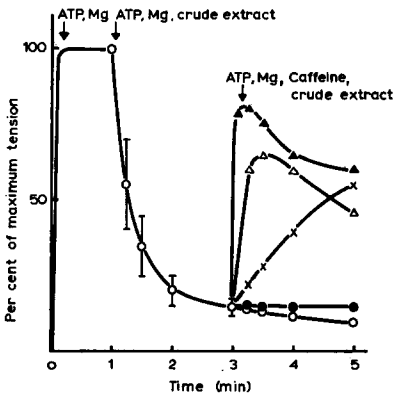

Fig. 1. Effect of caffeine on the fiber relaxed with crude extract. First arrow indicates when fibers were immersed in the test solution containing 2 m*M* ATP, 2 m*M* Mg, 40 m*M* Tris–acetate buffer (pH 7.0) and 0.12 *M* KCl; second arrow, when the ATP solution was replaced with another ATP solution containing 10 vol. % of crude extract and third arrow, replacement with another ATP solution containing 10 % of crude extract and caffeine. Caffeine concentration in m*M* is given by ○, nil; ●, 0.5; ×, 2; △, 5; ▲, 10. Room temperature (17–20°).

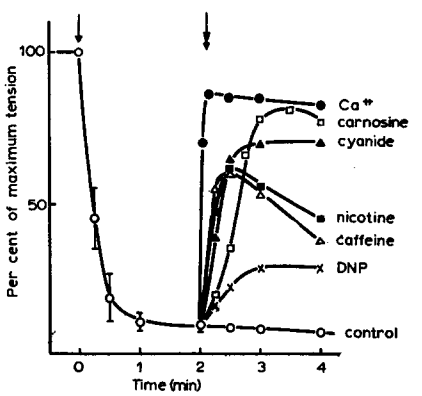

Fig. 2. Effect of several agents on the fiber relaxed with crude extract. First arrow indicates when fibers were immersed in the ATP solution containing 10 % of crude extract; second arrow, in the ATP solution containing crude extract and agent. Other conditions were the same as stated for Fig. 1; symbols: ○, nil; ●, 0.2 m*M* Ca; △, 5 m*M* caffeine; ▲, 5 m*M* cyanide; □, 5 m*M* carnosine; ■, 5 m*M* nicotine; ×, 5 m*M* DNP.

The effect of these agents on the tension development

To determine whether the effects of these agents are produced by inhibiting the relaxing factor or by activating the tension development, the experiments shown in Fig. 3 were carried out.

Caffeine and carnosine had no significant influence on the tension development of fibers in concentrations ranging from 2 m*M* to 10 m*M*. The tension development was inhibited with increasing concentrations of DNP and at 10 m*M* the maximum tension was lowered to about 50 % of that of the control. Cyanide increased the tension development with increasing concentrations and at 10 m*M* it was increased

to about 150% of the control value. Nicotine increased the tension development about 30% at 5 m*M* and lowered it slightly at 10 m*M*.

The effect on the EDTA relaxation

The experiment shown in Fig. 4 demonstrated that no agents except Ca ion and nicotine influenced the fiber relaxed with 2 m*M* EDTA in the presence of 2 m*M* ATP and 2 m*M* Mg.

Influence of preincubation of the crude extract with ATP on the caffeine and DNP effects

Earlier, NAGAI *et al.*[13] presented results showing that the inhibiting activity of granules on the myofibrillar ATPase was increased by preincubation of the granules with ATP, Mg and oxalate. They suggested that the relaxing substance may be produced by the incubation of the granules with ATP.

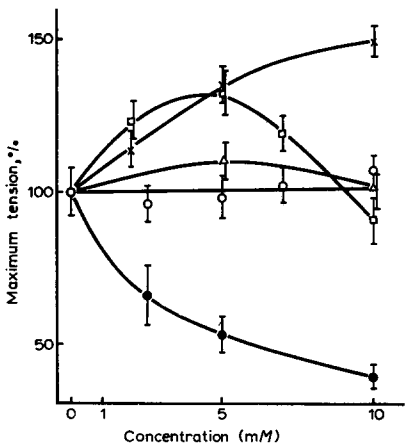

Fig. 3. Effect of several agents on the maximum tension of a single fiber produced with 2 m*M* ATP and 2 m*M* Mg. Ordinate: the maximum tension of fiber developed without agent was taken as 100. Abscissa: concentration of agents in m*M*/l; symbols: ○, caffeine; ●, DNP; ×, cyanide; △, carnosine; □, nicotine.

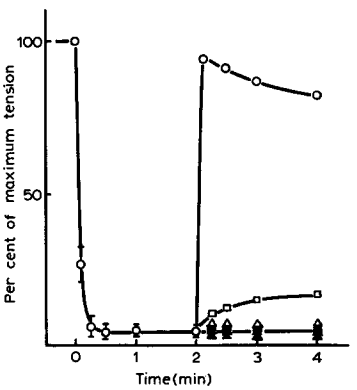

Fig. 4. Effect of agents on the fiber relaxed with EDTA. The relaxation was produced with 2 m*M* EDTA in the presence of 2 m*M* ATP and 2 m*M* Mg. After 2 min the EDTA solution was exchanged with another EDTA solution containing the test agent. Other conditions were the same as stated for Fig. 1; symbols: ○, 2 m*M* Ca; ● 7 m*M* DNP; △, 5 m*M* cyanide; ▲, 5 m*M* carnosine; ×, 5 m*M* caffeine; □, 5 m*M* nicotine.

Accordingly, it would be of interest to know whether the effects of caffeine and the other agents are due to the destruction of the granules or to the inhibition of the production of the relaxing substance.

As shown in Fig. 5, 5 m*M* caffeine and 7 m*M* DNP added to the test solution before the addition of the crude extract produced contraction. However, when 5 m*M* caffeine and 7 m*M* DNP were added to the solution after preincubation of the crude extract with ATP and Mg for 10–30 min, it did not produce contraction of the relaxed fibers. Similar results were obtained with carnosine.

On the other hand, preincubation of the crude extract had no effect on the action of Ca ion, cyanide and nicotine at concentrations of 0.2, 5 and 5 m*M*, respectively. The solutions containing these agents produced contraction of the relaxed fibers, even

when each agent was added to the solution after preincubation of the crude extract with ATP and Mg for 30 min*.

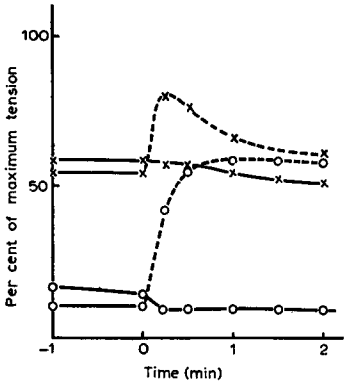

Fig. 5. Influence of preincubation of the crude extract with ATP and Mg on the caffeine and DNP effects. At 0 min, the solution used to decrease the tension of fiber was replaced with another solution to which 5 mM caffeine or 7 mM DNP was added after preincubation of the crude extract with 2 mM ATP and 2 mM Mg for 30 min (solid lines), and with one to which caffeine or DNP was initially added without preincubation (broken lines). The crude extract used in the caffeine experiment was used one week after preparation. Other conditions were the same as stated for Fig. 1; symbols: O, DNP; ×, caffeine.

DISCUSSION

The agents, caffeine and others, evidently inhibited the relaxing activity of the crude extract containing the granules. As shown in Fig. 1, there may be a stoichiometric relation between the amount of the agents and that of the granules. At the same time, these agents which have different effects on the tension development of the fibers, uniformly contracted the relaxed fiber (Figs. 2 and 3). Furthermore, these agents, except Ca ion and nicotine, have no influence on the fiber relaxed with EDTA (Fig. 4). These findings may indicate a specific interaction between these agents and granules. This is further supported by the evidence that the inhibiting activity of granules on the myofibrillar ATPase was removed by the addition of these agents[22].

The results obtained in Fig. 5 indicate that caffeine and DNP do not inhibit the relaxing activity of the crude extract preincubated with ATP and Mg for 10–30 min. Similar results were obtained with carnosine. These findings obtained under the present experimental conditions not only give further weight to the suggestion that a relaxing substance is produced by the granules in the presence of ATP and Mg but also indicate that caffeine and DNP, as well as carnosine, inhibit the chemical reaction which produces the relaxing substance instead of inactivating the substance produced by the granules. On the other hand, Ca ion, nicotine and cyanide inhibited the relaxing activity even when they were added after preincubation of the crude extract for 30 min. Although direct evidence has not yet been obtained, these findings may suggest that these agents might be able to combine with the relaxing substance produced by the preincubation of the crude extract.

* The authors and Dr. K. KONISHI in our laboratory were able to show that 5 mM caffeine and 5 mM DNP prevent the inhibiting action of granules on the myofibrillar ATPase and that these agents also become less effective on the action of the granules preincubated with ATP, Mg and oxalate for 7 min. Furthermore, they observed that Ca ion, nicotine and cyanide at concentrations of 5 mM constantly prevent the inhibiting action of the granules[22].

Furthermore, it is very interesting to note that some of these agents, caffeine[23] and DNP[15], can produce contracture in muscles which are depolarized by application of K_2SO_4 or by removal of Na ion. Accordingly, as previously described by WEBER[24] on the action of Ca ion, one may consider as follows: a living muscle keeps its resting state by maintaining the concentration of a very labile relaxing substance produced by the granules at constant equilibrium. If the process producing the substance is stopped by contracture-producing agents or if the substance combines with these agents, contracture of the muscle would be caused by the decrease in the concentration of the relaxing substance.

It is known that intracellular injection of Ca ion[25] or carnosine[17] causes contraction. On the other hand, AXELSSON *et al.*[23] observed that the intracellular injection of caffeine did not cause contraction. Procaine inhibited the caffeine contracture of frog sartorius muscle[26], nevertheless it has no influence on the contraction of the relaxed glycerol fiber produced by caffeine. These findings appear to contradict the above speculation. Though detailed study on this point is required, the findings obtained in the present work may give a clue to the understanding of the relationship between the excitation and the contraction of muscle and to further investigations for the identification of the relaxing substance.

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