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# THE BIOSYNTHESIS OF A MUSCLE-RELAXING SUBSTANCE

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## SUMMARY

Under appropriate conditions it is possible to show that, during the incubation of muscle microsomes with ATP, a soluble dialyzable substance is found which is capable of inhibiting tension generation in glycerinated muscle fibers. A hypothesis has thus been made that the muscle-relaxing activity of MARSH extract is achieved through the formation of this substance. A number of conditions have been found, however, where the microsomes were active, but no soluble substance could be demonstrated. The conditions most favorable for forming the soluble muscle-relaxing substance have been investigated and some preliminary characterization of it has been achieved. Evidence is presented which suggests that ATP is a substrate for the formation of the substance.

## INTRODUCTION

Although the muscle-relaxing activity of extracts of muscle[1] is associated with the microsomal fraction of the extract[2–5], some doubt exists as to whether the microsomes per se produce relaxation. Both LORAND[6] and WEBER[7] have noted that the size of the microsomes would preclude their penetration into a glycerinated muscle fiber and consequently an essential area of the fiber would be beyond the action of the microsome. It thus becomes necessary to postulate that the microsomes produce relaxation by forming or releasing some readily diffusible substance. The observations that inhibition of tension[8] and inhibition of myofibrillar ATP-ase[9,10] is preceded by a latency which is abolished by preincubation suggests that such a substance is indeed formed, or released, or both. Attempts by NAGAI *et al.*[10] to demonstrate the presence of such a substance, however, met with failure.

Abbreviations used: ATP, adenosinetriphosphate; CP, creatine phosphate; CPT, creatine phosphoryltransferase; CTP, cytidine triphosphate; SMRS, soluble musele-relaxing system.

The present report shows that a dialyzable substance is formed or released by the interaction of ATP and either skeletal or cardiac muscle microsomes. This substance, like the Marsh extract, inhibits the tension induced by ATP in single glycerinated rabbit psoas muscle. PARKER AND GERGELY[11] have briefly reported a similar substance capable of inhibiting myofibrillar ATP-ase. Evidence is also presented which indicates that this substance is synthesized during the microsome-ATP incubation and not merely released.

## METHODS

Muscle microsomes were prepared from rabbit skeletal muscle and dog cardiac muscle by differential centrifugation. The extract to be centrifuged was obtained by homogenizing chilled muscle in a Waring blendor for 40 sec with 3 volumes of a salt–sucrose solution (70 m*M* KCl, 2 m*M* $MgCl_2$, 20 m*M* Tris, 2.5 m*M* K-oxalate, 20 % sucrose). Brief periods of homogenization are essential when cardiac muscle is being used[12]. The homogenate was centrifuged for 25 min at 11,000 × *g* and the sediment discarded. The muscle microsomes were then obtained by centrifuging the supernatant for 1 h at 40,000 × *g*. The microsomes were resuspended in cold salt–sucrose solution so that 1 ml contained microsomal material from 2.5 g muscle. Resuspension using a Potter-Elvehjem homogenizer was necessary in order to obtain consistently active preparations.

Muscle-relaxing activity was assayed by its capacity to inhibit contraction in single glycerinated rabbit psoas fibers extracted for more than 90 days in 50 % glycerol at —18° (see ref. 13). Contraction was induced with a solution of 5 m*M* ATP, 2 m*M* $MgCl_2$, 70 m*M* KCl, 20 m*M* Tris, 2.5 m*M* K-oxalate, called the contraction solution. Experiments with glycerinated fibers were performed at pH 7 and room temperature, at a fiber length of 100 % rest length.

Protein was determined by the method of LOWRY *et al.*[14].

## RESULTS

Fig. 1 shows the influence of incubation on the rate at which microsomes cause relaxation. Microsomes were added to contraction solution (see METHODS) either immediately or 48 min before relaxing activity of the incubation mixture (*i.e.* contraction

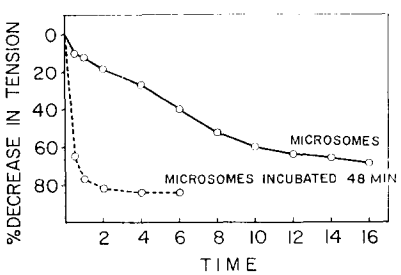


Fig. 1. Influence of preincubation of muscle microsomes on their relaxing activity. The curves depict the time course of fall in tension of a contracted fiber following the addition of microsomes or preincubated microsomes. The muscle fibers had been extracted for more than 6 months. ATP, 5 m*M*; Mg, 2 m*M*; $\mu$ = 0.16; room temperature; microsomes, 35 $\mu$g protein/ml contraction solution. For other details see text.

solution and microsomes) was to be tested. The intervals of time (0 and 48 min) and the concentration of granules used (35 $\mu$g protein/ml contraction solution) in this experiment were chosen arbitrarily to demonstrate most clearly the difference

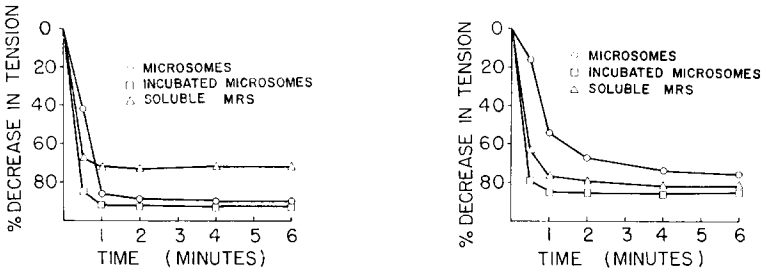


Fig. 2. Comparison of the tension-inhibiting activity of microsomes, incubated microsomes and SMRS. 2a shows such a comparison for microsomes prepared from rabbit skeletal muscle. The SMRS was prepared by centrifuging the incubation mixture for 1 h at 104,000 $\times$ *g*. 2b shows a similar experiment using microsomes prepared from dog myocardium. Incubation period was 30 min. Microsomes; 2a 380 $\mu$g protein/ml contraction solution. 2b, 1.1 mg protein/ml contraction solution. Conditions otherwise as in Fig. 1.

resulting from incubation. When a large amount of microsomes was employed the difference between incubated and non-incubated systems was much less striking (see Fig. 2a, 2b). The results shown in Fig. 1 confirm that a noticeable period exists before the microsomes become fully active and that this lag period disappears if the microsomes are preincubated. In most experiments the incubated and non-incubated microsomes eventually produced about the same degree of relaxation, suggesting that the material responsible for relaxation is being formed during the incubation.

*A soluble muscle-relaxing system (SMRS)*

If the incubation mixture described above, *i.e.* microsomes and contraction solution, was subjected to high speed centrifugation (104,000 $\times$ *g* for 1 h) to remove the microsomes, relaxing activity was still found in the supernatant. This supernatant will be referred to as the soluble muscle-relaxing system. Fig. 2a and 2b show the results of experiments conducted with rabbit skeletal muscle microsomes (Fig. 2a) or with dog cardiac microsomes (Fig. 2b), in which the activity of the SMRS is compared to that of incubated and non-incubated microsomal contraction solution mixture. Usually the SMRS did not produce as much relaxation as the non-sedimented incubation mixture. The relaxation produced by the SMRS was fully reversed by addition of calcium chloride.

*Conditions favoring the formation of SMRS*

It has never been possible to predict in advance the period of incubation or the amount of microsomes which would produce a maximally active SMRS. With each preparation of microsomes and even with the same preparation on different days the optimal incubation period and microsomal concentration varied. Table I is a summary of our experience to date. Both the average amount and the range of relaxing activity we observed after various incubation periods with various amounts of microsomes are shown in the table. Two points seem indicated: (a) within the range of concentrations at which microsomes are effective, the degree of inhibition of

TABLE 1

INFLUENCE OF INCUBATION TIME AND MICROSOMAL CONCENTRATION ON THE SMRS

MRF column shows the maximal depression (%) in tension obtained after the microsomes had been incubated for the time indicated. SMRS column shows the maximal depression (%) in tension obtained after the microsomes had been incubated for the time indicated and the microsomes had been spun down by high speed centrifugation. Conditions for determining inhibition of tension were identical to those described in Figs. 1 and 2.

| *Microsome concn. (mg/ml)** | *Incubation period (min)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *6* | | *15* | | *30* | | *60* | | *120* | |
| | *MRF* | *SMRS* | *MRF* | *SMRS* | *MRF* | *MSRS* | *MRF* | *SMRS* | *MRF* | *SMRS* |
| 31 | | | 80 (76–84) | 30 (11–43) | 77 (75–80) | 61 | 75 (70–80) | 42 (32–52) | 76 (62–90) | 38 (35–40) |
| 62 | | | | | 69 (65–80) | 39 (19–69) | 82 (78–84) | 47 (40–62) | 85 (80–91) | 45 (26–74) |
| 124 | 68 (64–73) | 19 (8–25) | 80 (77–82) | 59 (23–69) | 86 (84–89) | 41 (17–73) | 88 (85–91) | 46 (16–77) | | |
| 248 | | | | | 79 (78–81) | 63 (61–64) | 81 (75–85) | 66 (60–72) | | |
| 496 | | | | | 83 (79–85) | 41 (32–50) | 84 (82–86) | 20 (0–33) | | |

* Since many different microsome preparations were used in this study, there was no absolute constancy of protein concentration. The amount of microsomes used, however, was always related to the amount of muscle from which they were prepared; thus a concentration of 31 mg/ml indicates that microsomes prepared from 31 mg of muscle were incubated per each ml of contraction solution.

tension is independent of the amount of microsomal material used; (b) the microsome–ATP mixture becomes fully active (*e.g.* 6 min) before the SMRS becomes fully active.

Fig. 3 shows in one series of experiments how the period of incubation influenced the formation of the SMRS. After 5 min of incubation only slight activity in the SMRS could be demonstrated. After 10 min the activity in this fraction was increased and by 20 min was maximal. Incubation for 60 min produced no further increment in activity. The 5-min incubation demonstrated again an interval of time when the microsome-contraction solution mixture was fully active and yet the activity of the soluble component of the system was low.

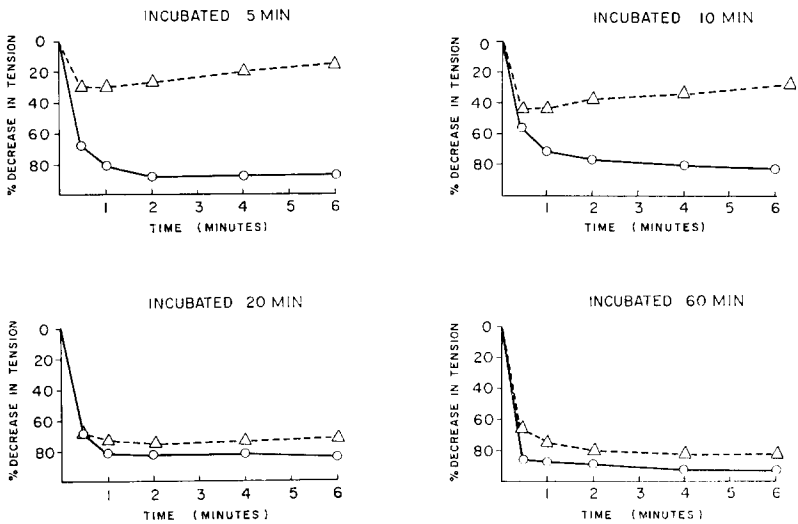


Fig. 3. Comparison of the tension-inhibiting activities of SMRS and microsomes after various periods of incubation. O—O, time course decrease in tension in the incubation mixture; △—△, time course decrease in tension in the soluble MRS prepared from the corresponding incubation mixture. Microsomes 350 $\mu$g protein/ml contraction solution. Conditions otherwise as in Fig. 1.

The influence of ATP concentration on the relaxing activity of the SMRS has been investigated. Direct comparisons of relaxing activities using 0.5, 1 and 5 m$M$ ATP were performed in the presence of 5 m$M$ CP and CPT (400 $\mu$g/ml). The transphosphorylating system was added to overcome ATP diffusion difficulties and the depression of ATP concentration by microsomal ATP-ase. Although the microsome preparations produced relaxation at 0.5 and 1 m$M$ ATP no relaxing activity could be demonstrated in the SMRS. With one group of muscle fibers the cofactor described by BRIGGS *et al.*[15] was required to produce relaxation at both 0.5 and 1 m$M$ ATP, but not at 5 m$M$ ATP. The same microsome preparation when assayed on glycerinated fibers from another rabbit did not require cofactor. Since at 5 m$M$ ATP the cofactor was not essential for the biosynthesis of active SMRS, it was not used in the preceding experiments. Such a use would have unnecessarily complicated the interpretation of the reaction leading to the formation of the active SMRS. The observation that the cofactor is required with some fibers and not with others, suggests that the fiber might be the site of action of the SMRS. Such a site has previously been suggested by WEBER[7].

*SMRS concentration–activity relationships*

A total of 12 experiments were run to determine the effect of diluting SMRS on its activity. Fig. 4 shows that for each 50% dilution there was approx. a 29% decrease in activity. The log of activity appears to be directly related to the concentration. The slope of this curve is very noticeably different from that relating the relaxing activity of muscle microsomes to their concentration[15]. Once an effective concentration of microsomes is reached, maximal relaxation is rapidly achieved.

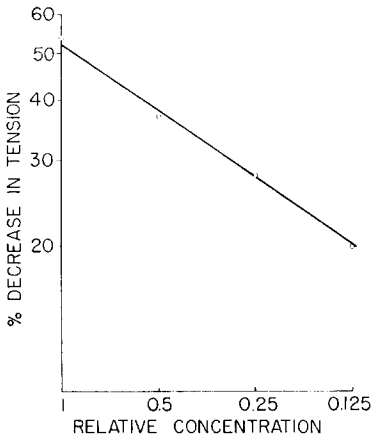


Fig. 4. Concentration–activity relationship. The concentration of the SMRS as prepared by centrifugation of the incubation mixture of microsomes and ATP is taken arbitrarily as 1. After each successive equal dilution with contraction solution the concentration of SMRS is taken to be 0.5, 0.25 and 0.125. Four determinations of the per cent decrease in tension were made at each concentration. Three different preparations of microsomes were used.

*The substrate for the formation of the relaxing agent in the SMRS*

Some preliminary studies have been carried out in an attempt to establish whether the microsomes play a purely catalytic role in the formation of the relaxing agent, and, in addition, furnish substrate for its formation, or whether they merely release a preformed relaxing agent. This question was tested by determining whether the microsomes could be used repeatedly to form an effective SMRS. If they furnish substrate, their ability to form the SMRS should be decreased on a second or third incubation. NAGAI *et al.*[10] have carried out similar experiments in which they tested the ATPase-inhibiting activity of microsomes after they had been repeatedly suspended in ATP. They found that after three such resuspensions the microsomes had

TABLE II

CAPACITY OF MUSCLE MICROSOMES TO FORM SMRS ON SUCCESSIVE REINCUBATIONS

The per cent decrease in tension reported is the maximal value. The microsomes were incubated in contraction solution for 30 min and centrifuged for 60 min at 104,000 × *g*. Conditions for measuring per cent decrease in tension were as described in Fig. 1.

| *Expt.* | *Incubation mixture* | *SMRS 1* | *SMRS 2* | *SMRS 3* |
|---|---|---|---|---|
| | *% decrease in tension* | *% decrease in tension* | *% decrease in tension* | *% decrease in tension* |
| 1 | 65 | 62 | 68 | — |
| 2 | 76 | 47 | 39 | 38 |
| 3 | 63 | 58 | 47 | 41 |

lost none of their capacity to inhibit ATP-ase. Table II shows that the capacity of the microsomes to form SMRS similarly was not significantly depressed when they werereused a second or third time. It was surprising, that considering the lability of the microsomes, there was not a greater loss in activity.

*Preliminary characterization of the soluble muscle relaxing agent*

*Dialysis*: The relaxing activity of the SMRS was found to be dialyzable. One volume of extraction solution was put into cellophane dialysis tubing and dialyzed against 10 volumes of SMRS. Dialysis was carried out for 16 h at 3°. The tension-inhibiting activity of the SMRS before dialysis, after dialysis, and the activity of the dialysate are shown in Table III. With but one exception, a great deal of activity was lost during the dialysis procedure. When the dialysate was compared to the dialyzed solution, however, the dialysate contained, with one exception, nearly comparable activity. Most important, activity did cross the dialyzing membrane.

TABLE III

DIALYSIS OF SMRS

Each column shows the per cent decrease in tension observed with the given SMRS. The control shows the activity before dialysis. The dialysate shows the activity of the SMRS within the dialysis tubing. The dialyzed solution shows the activity of the solution being dialyzed after dialysis.

| *Control* | *Dialyzed solution* | *Dialysate* |
|---|---|---|
| 46 | 15 | 18 |
| 45 | 28 | 11 |
| 40 | 46 | 40 |
| 38 | 27 | 21 |

*Stability to heat and pH*: Table IV shows the results of a series of experiments designed to test the influence of pH on the stability of the SMRS. When the SMRS was brought to pH 1.5 or 11 for 1 h at 0°, most of the tension-inhibiting activity was destroyed, and less deviation from neutrality resulted in less loss of activity. Heating at pH 7.0 was very deleterious; *e.g.* heating at 70° for 5 min resulted in about 50% loss of activity and heating at 70° for 10 min resulted in more than 80% loss of activity. The SMRS proved to be reasonably stable at room temperature. Activity was not measurably decreased in 2 h, the longest interval studied to date.

The relaxing activity in the SMRS is adsorbed by charcoal at pH 7.0. It is not

TABLE IV

STABILITY OF SMRS AT VARIOUS pH

The columns show the per cent decrease in tension produced by the SMRS before and after exposure to the indicated pH for 1 h at 0°.

| *Control* | *pH 1.5* | *Control* | *pH 3.0* | *Control* | *pH 4.5* | *Control* | *pH 9* | *Control* | *pH 10* | *Control* | *pH 11* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 46 | 7 | 42 | 37 | 70 | 29 | 42 | 24 | 78 | 18 | 60 | 8 |
| 49 | 11 | 61 | 30 | 43 | 42 | 61 | 40 | 50 | 13 | 49 | 18 |
| | | | | 50 | 22 | | | 55 | 35 | | |
| | | | | 38 | 25 | | | | | | |

as readily adsorbed, however, as ATP is. This characteristic has on occasion been successfully applied to the separation of ATP from the mixture.

## DISCUSSION

The isolation by centrifugation of a dialyzable agent capable of inhibiting tension generation suggests the hypothesis that the function of the microsome in the muscle-relaxing system is not to cause relaxation directly, but to synthesize some agent which then produces relaxation. The time required to biosynthesize such an agent is thus very likely the cause of the delay observed before obtaining full inhibitory effects upon the addition of microsomes. The fact that the compound is dialyzable means that it could diffuse readily into the glycerinated muscle preparation, a point against the view that the microsome is the actual relaxing agent.

The inability to demonstrate active SMRS under all conditions in which the microsomes are effective, *e.g.* low ATP concentrations (0.5 and 1.0 m$M$) and brief periods of incubation at higher ATP concentrations (5 m$M$), raises some questions about this hypothesis. No certain explanation of the difficulty seems apparent at the moment. The inability to demonstrate the relaxing agent at low ATP concentration may in part be a reflection of the decreased relaxing ability of the microsomes at these concentrations of ATP[13] and the decreased amounts of relaxing factor that this represents. Even under apparently optimal conditions the success of preparing the relaxing agent was unpredictable. Obviously there are factors of still unknown significance playing a role in the system.

WEBER[7], in his discussion of the role of the microsomes in the relaxing mechanism, suggests two possibilities, either a relaxing agent is biosynthesized by the microsomes or the microsomes release a preformed agent to the myofibril. He suggests that perhaps ATP serves as a carrier in the system, since its presence is clearly requisite. This same explanation could be applied to the results obtained by preincubation and centrifugation. Certain observations seem to suggest that a preexisting relaxing agent in the microsome is unlikely. If such an agent did indeed exist, it should be possible by some means to release it from the microsome. Such techniques as freezing and thawing, sonication and treatment with organic solvents[16] have failed to produce any evidence of such a substance. Furthermore, the specificity of the relaxing system for ATP or CTP (see ref. 17), no other trinucleotide being effective, suggests a biochemical reaction rather than merely the release of a preexisting agent. The differing slopes relating tension-inhibiting activity to concentration of microsomes or SMRS suggest that the SMRS is of a different composition and hence must be formed during incubation.

The nature of the biosynthetic reaction leading to the formation of the relaxing agent remains to be elucidated. The experiments carried out so far add some information on this point. The following facts seem pertinent:

1. Only ATP and CTP of many nucleotides investigated are capable of producing relaxation[17].

2. The amount of microsomes used to form SMRS in the experiments reported above did not prove significantly related to the activity of the SMRS obtained. The capacity to form the SMRS was, however, related to ATP concentration. NAGAI[13] has presented contrary evidence, however, suggesting that a quantitative relationship

exists for the amount of microsomes needed to relax a given amount of myofibrils.

3. On successive incubations of microsomes with ATP the microsomes successively form the active agent. If the microsomes are donating a substance to this reaction no significant loss in this capacity was observed when the microsomes had been used for three successive incubations.

These data suggest that the ATP and, by analogy presumably CTP, function as one of the substrates for the formation of the active relaxing agent. Whether or not the microsomes also contribute substrate seems difficult to decide.

It is quite possible that ATP also functions as the energy source for the biosynthetic reaction. EBASHI[18] has attempted to discover, for example, whether the relaxing activity of microsomes could be dissociated from their ATP-ase activity. He found that, in all cases in which the ATP-ase had been destroyed, the relaxing function had been lost. He did find some situations in which the relaxing function was lost, but not the ATP-ase; he never found the converse to be true.

If ATP serves a substrate function in the formation of the relaxing agent, this has some important implications for the observation made by HASSELBACH AND WEBER[19] that relaxing factor shifts the region of over-optimal ATP concentrations to lower concentrations. This statement has had a certain mechanistic challenge. However, if low ATP concentration will not support the synthesis of the relaxing agent, but will support contraction[20], then a shift in overoptimal concentration need not be invoked to explain the results.

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