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THE EFFECTS OF RYANODINE ON MODEL SYSTEMS DERIVED FROM MUSCLE—III

RECONSTITUTED ACTOMYOSIN*

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Abstract—Actomyosin was reconstituted from highly purified myosin A and actin of varying purity. Its ATPase activity was enhanced by ryanodine as much as three-fold if the actin was extracted at room temperature; further purification of actin yielded actomyosin which was insensitive to ryanodine. The enhancement showed a characteristic time course, increasing progressively with time for 10–30 min after the addition of ryanodine. After preincubation of actomyosin with ryanodine in the absence of the ATP, the ATPase rate was greatly increased over a control system without ryanodine as soon as MgATP was added. Preincubation of either actin or myosin A with ryanodine had no effect on the ATPase rate measured after the other compounds were added. It is concluded that a factor extractable with actin at room temperature is required for the activating effect of ryanodine on actomysin ATPase, which probably depends upon its stabilizing effect upon an active contractile protein complex.

SEVERAL factors have recently been described which modify the physicochemical and enzymatic properties of actomyosin. A protein extractable with tropomyosin has been shown to confer calcium dependence on reconstituted actomyosin insofar as superprecipitation and ATPase are concerned; this factor was at first thought to be tropomyosin itself.[1–5] Two protein factors have been identified that modify the polymerization of actin and consequently its interaction with myosin A. These are opposite in their effects and are now known as α- and β-actinin.[6,7] While several other protein factors have been reported with similar kinds of property, their identity and correspondence with metin, tropomyosin, or α or β-actinin have not been established.

The experiments reported in this paper were designed to investigate further the possibility that sensitivity of muscle models to ryanodine may be conditioned by a factor extractable from muscle which influences the properties of actomyosin.

MATERIALS AND METHODS

Preparation of myosin A. This was done according to the procedures of Szent-Györgyi,[8] and was carried out entirely in the cold (~2°). Instead of using ATP for the final purification, the impure yield was redissolved in 0·2 M KCl, centrifuged at 16,000 *g* for 1 hr, and then reprecipitated in 0·033 M KCl. The cycle was repeated three times. The final yield was taken up in 0·2 M KCl. An equal volume of ice-cold glycerol was added and the preparation stored at −18°. For an experiment, an

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aliquot was withdrawn and diluted with 3 vol. of cold glass-distilled water. After centrifugation, the resulting precipitate was washed twice more in 0·05 M KCl and finally taken up in 0·2 M KCl.

Preparation of actin. The residue obtained from the preparation of myosin A was used for the preparation of acetone powders according to the method of Szent-Györgi.[8] For the extraction of actin the procedure described by Mommaerts[9] was employed, with a solution 2×10^{-4} M with respect to both ATP and ascorbic acid at pH 7·5 (100 ml for each 5 g acetone powder). The crude extract was centrifuged at 16,000 *g* for 1 hr; KCl and $MgCl_2$ were added to the supernatent to bring it to 0·1 M and 0·01 M respectively. Polymerization was allowed to proceed for 1 hr at 18°, after which the solution was centrifuged at 78,000 *g* in Spinco rotor No. 30 for 3 hr. The precipitate was dissolved in 0·05 M KCl (30 ml for each 5 g acetone powder) and centrifuged at 16,000 *g* for 1 hr. The supernatant is referred to subsequently as crude actin. For further purification, the precipitate obtained with the 78,000-*g* spin was redissolved in the ATP solution by gentle homogenization in a Potter–Elvehjem homogenizer and then centrifuged for 1 hr at 16,000 *g*. The polymerization and high-speed spin were repeated. The final yield was again dissolved in ATP solution (15 ml for each 5 g acetone powder) and dialyzed overnight against 1 l. of magnetically stirred ATP solution. The resulting G-actin was polymerized in 0·1 M KCl as required and is referred to as 'purified' actin.

For the preparation of actin extracted at room temperature, one third of the appropriate volume of ATP solution was used for the initial extraction at 2° for 30 min. The preparation was filtered with suction. The residue was extracted at room temperature twice, with half the remaining ATP solution, and the extracts pooled. Both the cold and the room-temperature extracts were then processed separately in exactly the same manner as that described for the preparation of crude actin. These preparations will be referred to as cold-extracted and room temperature-extracted actin respectively. The preparation of reconstituted actomyosin will be described in the legend of each figure.

Measurement of ATPase. The procedure has been described previously.[10]

Viscometry. Relative viscosity of actin was determined in an Ostwald viscometer of 10-ml capacity and flow time of 79 sec for distilled water. Temperature was maintained at 23°.

Protein determination. The method of Westley and Lambeth[11] or Lowry *et al.*[12] was used, with bovine serum albumin as standard.

Materials. ATP, disodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. Salt reagents were of analytical grade. Ascorbic acid and bovine serum albumin were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif.

RESULTS

Four types of reconstituted actomyosin were used in experiments of the type illustrated in Fig. 1. In addition to those shown, reconstituted actomyosin prepared from myosin A and crude, cold-extracted actin (4:1) was stored in 50% glycerol overnight. After twice precipitating at ionic strength 0·2 and redissolving in 0·6 M KCl, this preparation yielded curves intermediate between the lower two of Fig. 1. It is evident from these curves that ryanodine stimulates the ATPase of reconstituted

actomyosin. The stimulation became progressively less as the purity of actin was increased. The preparation which involved highly purified actin and myosin A was the least sensitive. The glycerol-extracted reconstituted actomyosin was somewhat more sensitive. As the actin preparation became less pure, the stimulation became more marked. These results strongly suggest the participation of a factor extractable with actin and removable by the purification used.

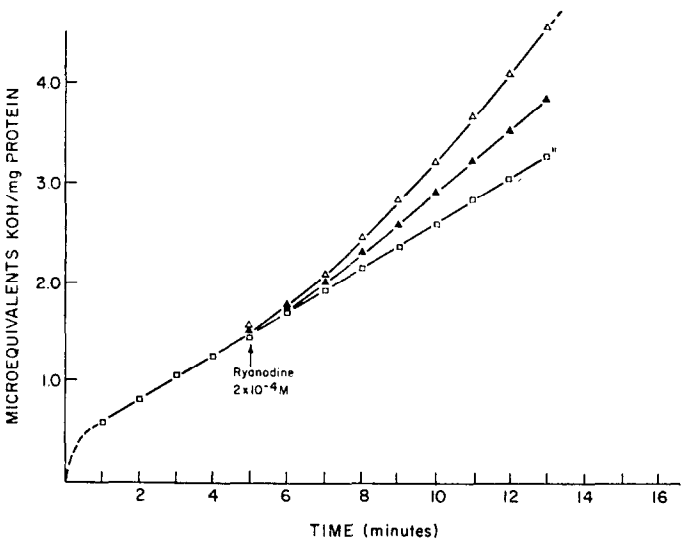

FIG. 1. The effect of ryanodine on ATPase activity of actomyosin reconstituted from highly purified myosin A and actin prepared in different degrees of purity. Conditions: 94 mM KCl, 2 mM MgATP, 0·2 mM $CaCl_2$, 1·25 mg protein, pH 6·7, 30°; □, purified actin; ▲, crude actin; △, room-temperature actin. All the data superimposed until ryanodine was added.

The response to ryanodine in these experiments showed a characteristic time course which was repeatedly observed. The ATPase rate progressively increased with time, so that the titration curve was concave upward (Fig. 1). The reasons for this escalating response are considered further below.

The ryanodine concentration employed in the experiments of Fig. 1 and most subsequent work was 2×10^{-4} M. Similar results were obtained when this was reduced to 5×10^{-5} M, but greater dilutions produced inconsistent effects.

Although added calcium was not necessary for the ATPase activity of reconstituted actomyosin, the escalating response to ryanodine was seen only if additional calcium was made available (Fig. 2). For this reason, 0·2 mM was used in all experiments, except as noted. When 1·0 mM calcium was included in the reaction mixture, there was a very marked stimulation of ATPase even in the absence of ryanodine. Addition of the drug caused no further stimulation. The ryanodine effect and its Ca^{2+} dependence were even more marked when the ATP concentration was increased to 5·0 mM and Mg^{2+} reduced to 1·0 mM (Fig. 3). These data recall previously reported experiments on myosin B and myofibrils,[10] in which maximal ryanodine effects were observed under similar conditions.

The escalating response to ryanodine suggests that a relatively slow interaction

occurs between two or more components of the system, the net effect of which is to elevate the ATPase activity. In order to ascertain whether ATP is required for such an interaction, reconstituted actomyosin was incubated with ryanodine for varying lengths of time before ATP was added. Initial ATPase rates were estimated by the least-squares method. For zero-time preincubation, ATP and ryanodine were added simultaneously. Controls were similarly treated, but no ryanodine was included

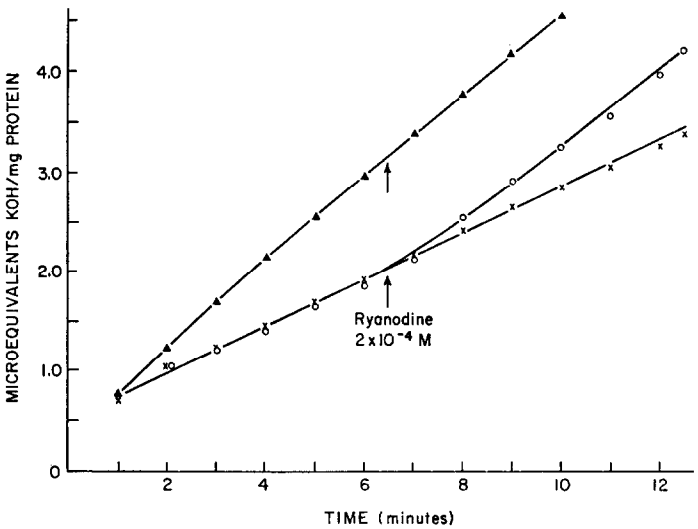

FIG. 2. The influence of calcium concentration on the ryanodine effect on actomyosin reconstituted from purified myosin A (1 mg) and room-temperature actin (0·3 mg). Conditions: 94 mM KCl, 2 mM MgATP, pH 6·7, 30°; ▲, with 1·0 mM $CaCl_2$; ○, with 0·2 mM $CaCl_2$; ×, with no added $CaCl_2$. Ryanodine added as indicated at arrows.

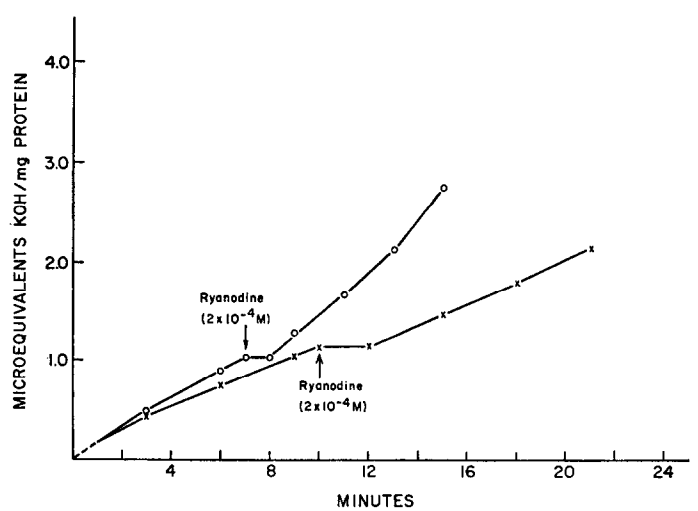

FIG. 3. Calcium dependence of ryanodine effect on reconstituted actomyosin in presence of excess ATP. Conditions as in Fig. 2, except 1 mM $MgCl_2$, 5 mM ATP; ○, with 0·2 mM $CaCl_2$; ×, with no added $CaCl_2$.

(Fig. 4). It will be seen that while the ATPase rates of the controls remained constant throughout, that of the ryanodine-treated system increased with the duration of preincubation. A similar result was obtained when the total time elapsing between the start of preincubation and the addition of ATP was identical in all cases. Variation in duration of exposure to ryanodine was achieved by adding ryanodine at the appropriate times.

In these experiments a maximal effect of ryanodine was seen immediately after the ATPase reaction was started by MgATP addition, provided the other components

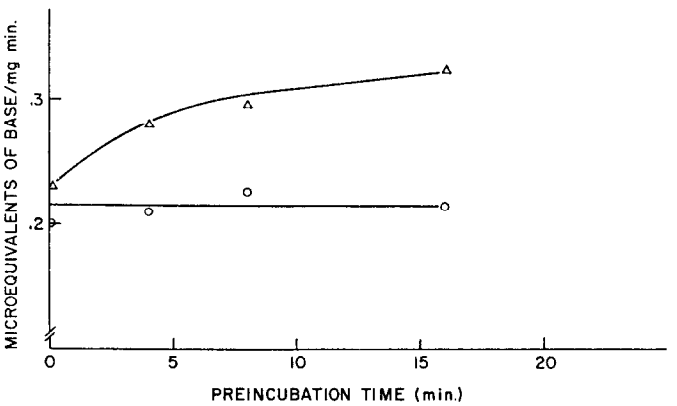

FIG. 4. The effect of preincubation with ryanodine on ATPase activity of actomyosin reconstituted from purified myosin A (1 mg) and room-temperature actin (0·3 mg). Conditions: 94 mM KCl, 0·2 mM $CaCl_2$, pH 6·7 ,30°. The system was preincubated for the times indicated with (△) and without (○) ryanodine (2×10^{-4} M), and the reaction was then started with 2 mM MgATP. Ordinate is initial ATPase rate after addition of substrate.

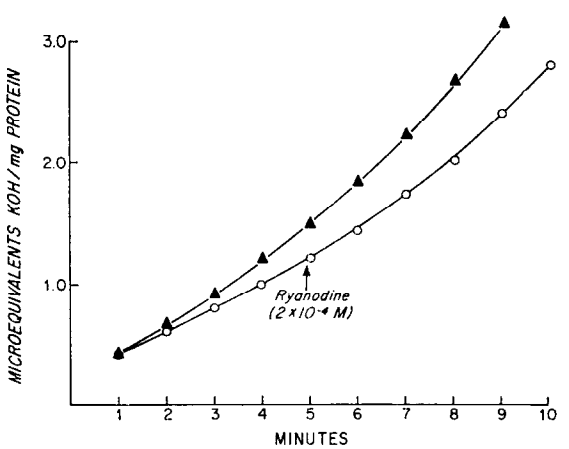

FIG. 5. The effect of preincubation of ryanodine with room-temperature actin (0·3 mg) on ATPase activity of actomyosin reconstituted from it. Conditions: 94 mM KCl, 0·2 mM $CaCl_2$, pH 6·7, 30°. The system was preincubated with (▲) and without (○) 2×10^{-4} M ryanodine for 5 min. The ATPase reaction was then started at zero time by the simultaneous addition of myosin A (1 mg) and 2 mM MgATP. An additional aliquot of ryanodine sufficient to increase its concentration by 2×10^{-4} M was added to the control system only (○) at the time indicated.

were preincubated for a sufficient time. This is consistent with the view that the escalating response normally observed results from a slow interaction, which can go to completion before the ATPase reaction is started. Since ATP and Mg^{2+} were not included in the preincubation mixture, these components are evidently not required for the activating interaction.

The weight ratio of myosin A to F-actin usually employed in the formation of synthetic actomyosin varies from 2·5 to 4. Since both the correct ratio and the completeness of the interaction are uncertain, the possibility exists that a proportion of either or of both of these proteins may remain in uncombined form. It is also generally

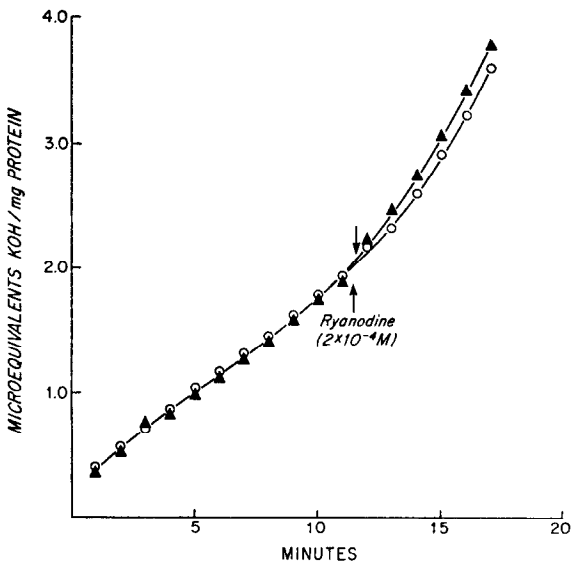

FIG. 6. The effect of preincubation of ryanodine with purified myosin A (2 mg) on ATPase activity of actomyosin reconstituted from it with room-temperature actin. Conditions: 94 mM KCl, 0·2 mM $CaCl_2$, pH 6·7, 30°. The system was preincubated with (▲) and without (○) 2×10^{-4} M ryanodine for 4 min. The ATPase reaction was then started at zero time by simultaneous addition of room-temperature actin (0·6 mg) and 2 mM MgATP. An additional aliquot of ryanodine sufficient to increase its concentration by 2×10^{-4} M was added to both systems at the time indicated.

believed that in presence of ATP, actomyosin dissociates into its constituent proteins.[13–15] A specific interaction of ryanodine with either myosin A or actin was therefore sought. In the experiments of Fig. 5 ryanodine was preincubated with F-actin. Myosin A and ATP were then added to start the reaction. A similar system without ryanodine served as control. An escalating ATPase response was observed immediately upon starting the reaction, whereas a similar effect was seen in the control system only after subsequent addition of ryanodine. It may be concluded that while the postulated activation reaction cannot proceed under these preincubation conditions (i.e. without myosin A), ryanodine retains its ability to induce the reaction when myosin A and ATP are added.

In the experiments of Fig. 6, myosin A was preincubated with and without ryanodine for 4 min, at which time F-actin and ATP were added to start the reaction. It is seen

that the ATPase rates of both control and test system were identical in spite of the ryanodine in the test system. Addition of the drug to both systems, which constituted a second dose in the test system, was followed by a typical escalating response of equal magnitude in each. When preincubated with myosin A, ryanodine therefore not only fails to activate the system but loses its ability to induce a response when F-actin and ATP are subsequently added.

The basis of this result was further investigated by preparing actomyosin containing an excess of myosin A (weight ratio of 8:1, myosin A:F-actin). This was not affected by the usual concentration of ryanodine (2×10^{-4} M) added during ATP hydrolysis, but showed the typical escalating response when a second equal increment of ryanodine was added. Sensitivity to ryanodine was therefore reduced by the excess myosin A present in the system.

In another approach to this question, ryanodine (2×10^{-4} M) was preincubated with myosin A alone and subsequently added to a standard actomyosin preparation while ATPase activity was being measured. A duplicate experiment was run in which preincubated myosin A was added instead of the ryanodine/myosin A mixture. The rates observed after the additions showed no escalation, and were 0·386 and 0·380 μmole/mg protein/min for ryanodine and control respectively. These data confirm the conclusion that the effect of ryanodine on reconstituted actomyosin is reduced or prevented by prior exposure to myosin A or concurrent exposure to an excess of myosin A.

The proposal by Demos and Sogaert[16] and Maruyama[17] of the presence of a factor in so-called natural actin which inhibits the formation of the network structure of F-actin suggested an examination of the effect of ryanodine on the viscosity of crude actin preparations. For this purpose the pellet of F-actin obtained after the first ultracentrifugation (see Materials and Methods) was depolymerized in ATP–ascorbic acid solution by gentle homogenization in a Potter–Elvehjem homogenizer. No evidence was found that ryanodine influenced the polymerizability of actin at 1, 4, and 8 mM ATP. The relative viscosity of F-actin as a function of ATP concentration was also compared after 16 min of polymerization in the presence and absence of ryanodine. No effect of ryanodine on viscosity was found. There was a slight fall of viscosity in both test and control systems at the higher concentrations of ATP.

DISCUSSION

The previous papers in this series led to a tentative conclusion that an unidentified factor in myofibrils and glycerol-extracted fibers may be necessary for the demonstration of an effect of ryanodine on muscle models. Support for the existence of such a factor in actomyosin preparations is now presented. It appears to be associated with impure actin preparations, from which it is removed by the usual purification procedures; its extraction from acetone powder was accomplished more readily at room temperature than at 0°.

Reconstituted actomyosin prepared from crude actin of this type showed a characteristic response to ryanodine when ATPase activity was measured. Then enzymatic rate increased progressively for 10–30 min after addition of ryanodine, eventually achieving a level two to three times the initial rate. A progressive increase in reaction rate is not uncommonly seen in enzymatic systems and may be attributable to the slow destruction of or removal of an inhibitor, to the slow generation of an activator, or

to an alteration of the form of the enzyme itself. The first two possibilities seem unlikely in the present system, since (a) a corresponding change may be induced by preincubation of the components with ryanodine in the absence of its substrate, MgATP; but (b) no similar activation of either the partial or complete system occurs unless and until ryanodine is added. Although it is possible to construct complex hypothetical reaction sequences that would explain the observed rate escalation in terms of an inhibitor or activator change, the available data are insufficient to support extensive conjecture, and the alternative of a change in the enzyme system itself is certainly more attractive.

In a recent study of actomyosin extracted from ryanodine-treated muscle,[18] Procita reported that it behaved as a uniform single-component system in which actin and myosin are so tightly bound as to resist conditions under which dissociation would normally occur. Fibers prepared from this actomyosin generated consistently more tension than those prepared from conventional actomyosin, and this was interpreted in terms of the uniformity and stability of the protein complex.

If ryanodine is capable of stabilizing an active contractile protein complex, it might be manifested in several ways, including not only increased tension generation by fibers of 'actomyosin' but also perhaps the increased tension produced by glycerol-extracted fibers under the influence of ryanodine,[19] myofibrillar and actomyosin ATPase activation,[10] and corresponding changes in other muscle models. Such a hypothesis therefore provides a reasonable explanation of many of the effects described in the present series of reports.

Nevertheless, it appears unlikely that this contractile protein complex involves only actin and myosin. The characteristic effect of ryanodine on reconstituted actomyosin was seen only when this was prepared from crude actin, and it disappeared when highly purified actin was employed. It seems reasonable to conclude that the participation of some other factor is required, and the evidence indicates that extraction of this factor from acetone powder with actin is promoted at room temperature.

It is now generally recognized that, unlike native actomyosin, the Mg^{2+}-activated ATPase of actomyosin reconstituted from highly purified actin and myosin has no absolute requirement for Ca^{2+} and cannot be inhibited by calcium chelators. Such a requirement may be conferred upon reconstituted actomyosin by a protein extractable with tropomyosin but apparently not identical with it.[1–5] Since the conditions favoring extraction of tropomyosin and this associated protein from acetone powder of muscle also promote the extraction of actin yielding ryanodine-sensitive reconstituted actomyosin, the ryanodine-sensitizing factor may be similar to and possibly identical with one of these proteins. Further work will clearly be required to establish its identity and functional relationship to these and other recently described factors which modify the properties of actin and actomyosin.

Ryanodine has now been shown to influence two distinct mechanisms in muscle: a specific reticular component which concentrates calcium,[20,21] and a contractile protein complex. The effects on each system require calcium in subsaturating amounts and are discernible as an increase in Mg^{2+} activated ATPase, which appears to be sensitized to calcium ions. Each effect can be demonstrated under conditions in which the other sensitive system is absent or has been inactivated. Uncoupling of the calcium-pumping system has been demonstrated in granular fractions of muscle homogenates which have been extensively washed in Weber–Edsall solution or 0·6 KCl to remove

possible actomyosin contamination.[20] Effects on contractile protein systems have been demonstrated in the present series of reports after prolonged glycerol extraction and after treatment with desoxycholate, which eliminates calcium-pumping granules. Evidently, these two effects must be accepted as actions on two discrete systems rather than a single effect which artifactually appears in both systems because of their heterogeneity and overlapping composition. It is interesting that either of these basic actions might explain the gross effects of the alkaloid on skeletal muscle; perhaps its very high potency is in part the result of two mutually supporting biochemical lesions. On the other hand, potent alkaloids generally owe their pharmacological effects to a single fundamental effect and, in view of the similarity of the conditions required and net effect produced, it is tempting to speculate that a common molecular mechanism may underlie the effect of ryanodine on both the calcium-pumping vesicles and the contractile proteins.

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