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EFFECTS OF RYANODINE ON MODEL SYSTEMS DERIVED FROM MUSCLE—V

RECONSTITUTED ACTOMYOSIN AND α-ACTININ*

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Abstract—The ATPase activities of actomyosins reconstituted from G- and F-actins and myosin A, and the superprecipitation of F-actomyosin were investigated in the presence of α-actinin or ryanodine or both. The ATPase activity initiated upon the addition of G-actin to myosin A was consistently depressed by α-actinin, but the ATPase of F-actomyosin was stimulated. This stimulation was greater when ryanodine was also present. The superprecipitation of F-actomyosin was also consistently stimulated by α-actinin. When both the drug and α-actinin were present, the superprecipitation not only was greater than that in the presence of α-actinin alone, but continued to increase for a considerable time (> 5 min) beyond the point when maximum value had been reached in the presence of α-actinin alone.

Ultracentrifugal studies showed that the α-actinin–actomyosin complex in the presence of ryanodine sedimented faster than the same complex in the absence of ryanodine. The double-spiked character of the main peak was present, but the trailing peak, attributed to the presence of α-actinin in the complex, was much greater. These findings are interpreted as indicating enhancement of the incorporation of α-actinin into the α-actinin–actomyosin complex. Taken together with the results of the ATPase and superprecipitation studies, they constitute positive data on the effects of ryanodine on muscle proteins which should be helpful in elucidating the mechanism of action of the alkaloid which leads to the production of spastic paralysis in skeletal muscle.

The muscle protein, α-actinin, has been shown to be capable of accelerating the superprecipitation of reconstituted actomyosin and to enable superprecipitation to take place at higher ionic strength. Its ability to cause an increase in the degree of shrinking of actomyosin was believed to be due to the strengthening of the interaction between actin and myosin. Inspite of these remarkable effects, it was concluded that α-actinin is not an essential contractile protein, but a regulator or modifier of the actomyosin-ATP system [1]. Subsequent to these observations, a series of papers appeared [2–4] in which it was reported that the ATPase activity, as well as the turbidity response of actomyosin to ATP, was enhanced by this muscle protein.

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Earlier, Elison and Jenden [5–7] reported in a series of papers on the enhanced ATPase activities of myofibrils, natural actomyosin and reconstituted actomyosin in the presence of ryanodine. This effect of ryanodine was especially marked on the ATPase activity of reconstituted actomyosin, which was found to be enhanced up to 3-fold if the actin was extracted at room temperature. The enhancement showed a characteristic time course, increasing progressively for 10–30 min after the addition of ryanodine, and was not seen when pure preparations of actin were used to reconstitute the actomyosin. It was concluded that a factor extractable with actin at room temperature was required for the enhancement of actomyosin ATPase by ryanodine. It is pertinent to ask whether the unknown factor might not be α-actinin itself. Further relevancy to this question is afforded by the report that ryanodine itself was capable of strengthening the interaction between actin and myosin in the natural actomyosin of skeletal muscle [8]. This question motivated us to undertake the study of the influence of α-actinin on the ATPase and the superprecipitation of reconstituted actomyosins in the presence or absence of ryanodine.

MATERIALS AND METHODS

Purification of actin. From a rabbit killed by exsanguination, the muscles of back and legs were removed and immediately placed on ice. Acetone powders were prepared by the method of Szent-Gyorgyi [9] and highly purified actin by the method of Maruyama [10, 11]. F-actin was prepared from G-actin by adding 0·1 M KCl.

Purification of myosin A. This was done according to the method previously described [7].

Purification of α-actinin. This was done by the method of Seraydarian *et al.* [2].

Determination of ATPase activity. This was done on a Radiometer pH-stat according to the method previously described [6]. In these experiments, G-actin or F-actin was added last to initiate the ATPase reaction.

Protein determination. The method of Lowry *et al.* [12] was used with bovine serum albumin as a standard.

Sedimentation studies. This was done on a Spinco–Beckman model E analytical ultracentrifuge at 27°, bar angle 60° with observations between 32,000 and 56,100 rev/min.

Superprecipitation studies. These were done using reconstituted F-actomyosin in a Beckman-DUspectrophotometer using 1-cm glass cells of 4-cm height in the Gilford arrangement for reading at 27°. The temperature was maintained by circulating water at 27° through a jacket embracing the entire cuvette holder. After the initiation of the reaction, no stirring was done, since this has been shown to be of no advantage [2].

Materials. ATP, disodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. Salt reagents were of analytical grade. Ryanodine was a gift of S. B. Penick & Co., New York, N.Y.

RESULTS

Actomyosin ATPase

In an earlier publication [13], data were presented which allowed the conclusion that β-actinin not only was capable of retarding the conversion of G-actin to F-actin but also of reversing the reaction. It was also shown that ryanodine could oppose these effects. The results of similar studies done with α-actinin are depicted in Figs. 1 and 2. One of the most consistent observations was the progressive increase in ATPase rate when G-actin was added to myosin A (uppermost plot in Fig. 1). As with β-actinin, α-actinin added to G-actin before addition of the combination to myosin A depressed the initial rate of ATPase, and the progressive increase in ATPase rate also followed. Increasing the proportion of α-actinin to G-actin depressed the initial rate further. Inclusion of 2×10^{-5} M ryanodine enhanced the ATPase rate somewhat, but it still remained considerably below the rate of control (G-actin and myosin A alone). Thus, with regard to ATPase activities of actomyosin reconstituted in the manner described, α-actinin behaved qualitatively similar to β-actinin. Its effect on the ATPase of reconstituted F-actomyosin was considered (Fig. 2). β-Actinin has been shown to inhibit the ATPase of any type of actomyosin. α-Actinin on the other hand inhibited the ATPase of G-actomyosin but stimulated the ATPase of actomyosin reconstituted from F-actin and myosin A. It has never been found to be inhibitory on this type of actomyosin, and stimulation of up to 50 per cent has been observed under the conditions of our experiments (50 mM KCl). Ryanodine has been found to stimulate the ATPase of actomyosins only when these have been depressed by β-actinin beforehand [13]. α-Actinin stimulated the ATPase of F-actomyosin even without prior depression by β-actinin, and ryanodine was capable of intensifying this stimulation considerably. In other words, α-actinin conferred upon the F-actomyosin sensitivity for stimulation by the alkaloid.

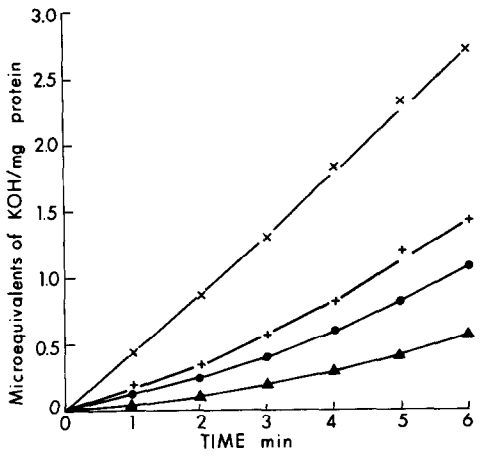

Fig. 1. Influence of α-actinin on the ATPase activity of actomyosin reconstituted from G-actin and myosin A. Conditions: G-actin/myosin A = 1·5/4·0 (w/w), 0·05 M KCl, 2·5 mM ATP, 2·5 mM $MgCl_2$, 0·2 mM $CaCl_2$, pH 6·8, 30°. Key: (× -- ×) control; (●--●) α-actinin included, to 1/6 of G-actin; (▲--▲) α-actinin included, to 1/3 of G-actin; (+ -- +) same as (× -- ×), but 2×10^{-5} M ryanodine included, added ahead of proteins.

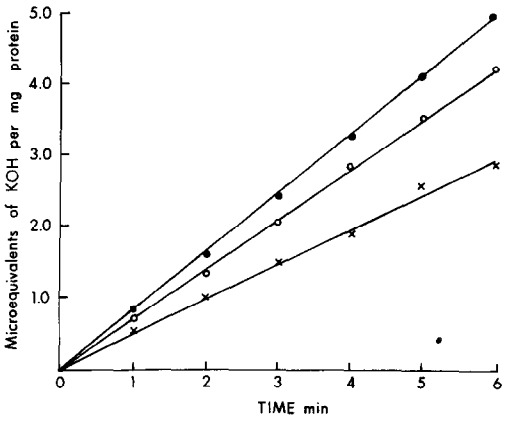

Fig. 2. Influence of α-actinin on the ATPase activity of actomyosin reconstituted from F-actin and myosin A. Conditions: F-actin/myosin A, 1·5/4·0 (w/w), 0·05 M KCl, 2·5 mM ATP, 2·5 mM $MgCl_2$, 0·2 mM $CaCl_2$, pH 6·8, 30°. Key: (× -- ×) control; (○--○) α-actinin included in the amount of 1/3 (w/w) of the actin; (●--●) same as (○--○), but 2×10^{-5} M ryanodine added ahead of proteins.

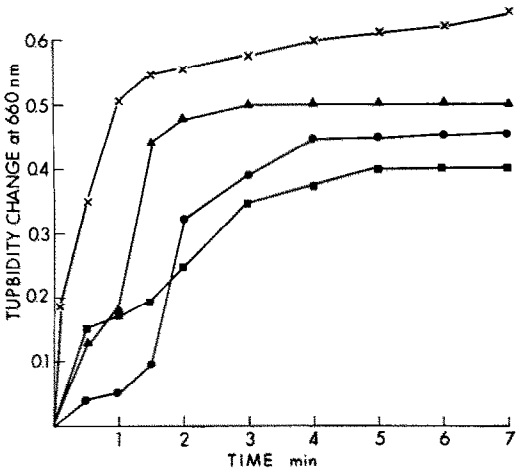

Fig. 3. Influence of ryanodine on the superprecipitation of reconstituted F-actomyosin in the presence of α-actinin observed at 660 mm. Conditions: 0·8 mg/ml of reconstituted actomyosin, 50 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, 0·2 mM CaCl, 60 mM Tris-maleate, pH 6·8, 27°. Key: (●--●) control; (■--■) control + 0·2 × 10^{-4} M ryanodine; (▲--▲) control + 0·2 mg/ml of α-actinin; (× -- ×) control + 0·2 mg/ml of α-actinin + 2 × 10^{-5} M ryanodine.

Influence of ryanodine on the superprecipitation of actomyosin in the presence and absence of α-actinin

Figure 3 shows the results of superprecipitation studies on F-actomyosin. The slowest initial rate was seen with F-actomyosin alone. In the presence of either α-actinin or ryanodine, the initial rate was significantly greater. Under all these three conditions, the superprecipitation reached a maximum after about 4 min. With the system including both α-actinin and ryanodine, it was noted that the initial rate was considerably faster, the intensity of the superprecipitation was greater, and the process continued to increase for a considerable time (at least 5 min) beyond the time when all the other three systems had reached a maximum. This behavior was similar to that observed with the ATPase rate of actomyosin reconstituted from impure F-actin and myosin A [7].

In the experiments of Fig. 4, ryanodine, wherever used, was kept constant at 2 × 10^{-5} M, and α-actinin was used at two different concentrations. Again it was noted that the initial rates of superprecipitation were faster than control when either α-actinin or ryanodine or both were present. The two different concentrations of α-actinin affected only the initial rates differently and in a manner reflecting the difference in concentrations, but the maximum superprecipitation reached in the presence of both concentrations was about equal, and was reached, as in the preceding experiments, after about 4 min. In the presence of ryanodine, the superprecipitations at both concentrations of α-actinin were enhanced, the enhancement by the drug being greater in the presence of the higher α-actinin concentration. In confirmation of the previous findings already described, the superprecipitation continued to increase beyond the time when F-actomyosin either alone or in the presence of α-actinin, but without ryanodine, had reached a maximum.

When the concentration of ryanodine was varied from 2 × 10^{-5} M to 2 × 10^{-4} M in the presence of α-actinin present to 40 per cent of the actomyosin, the enhancement of superprecipitation was greater with the higher concentration of ryanodine. This was not interpreted as being due to the addition of separate stimulant effects. This conclusion followed from the plots of Fig. 4.

The lower concentration of α-actinin appeared to be saturating, since raising the concentration from 20 to 40 per cent of the actomyosin did not produce more superprecipitation, although the initial rates did reflect the differences in concentration, being faster for the higher of the two. If the effect of ryanodine merely added to that of α-actinin, then the upper two plots of Fig. 4 should coincide since, as already pointed out, the two concentrations of α-actinin were saturating. This was clearly not the case. Based on these observations, the results are taken to mean that the alkaloid increased the capacity of F-actomyosin–α-actinin interaction, such interaction being manifested by superprecipitation as reported by Briskey *et al.* [4].

Interaction between α-actinin and F-actomyosin in the presence and absence of ryanodine

In the experiments of Fig. 5, α-actinin was added to an already formed F-actomyosin in ultracentrifugal sedimentation studies of F-actomyosin (upper traces).

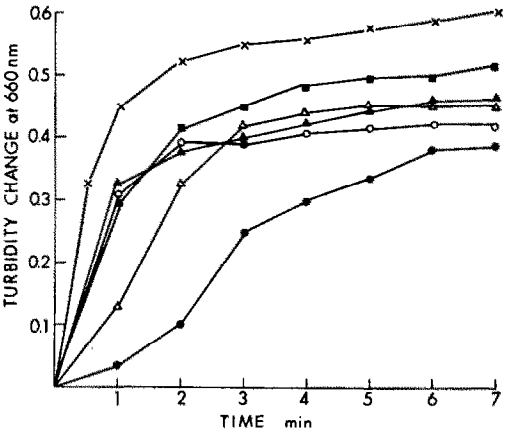

Fig. 4. Influence of ryanodine on the superprecipitation of actomyosin reconstituted from F-actin and myosin A in the presence of varying amounts of α-actinin, observed at 660 mm. Conditions: 0·8 mg/ml of 50 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, 60 mM Tris-maleate, pH 6·8, 27°. Key: (●--●) control; (△--△) control + 0·2 mg/ml of α-actinin; (▲--▲) control + 0·4 mg/ml of α-actinin; (○--○) control + 0·2 × 10^{-4} M ryanodine; (■--■) control + 0·2 mg/ml of α-actinin and 2 × 10^{-5} M ryanodine; (× -- ×) control + 0·4 mg/ml of α-actinin and 2 × 10^{-5} M ryanodine.

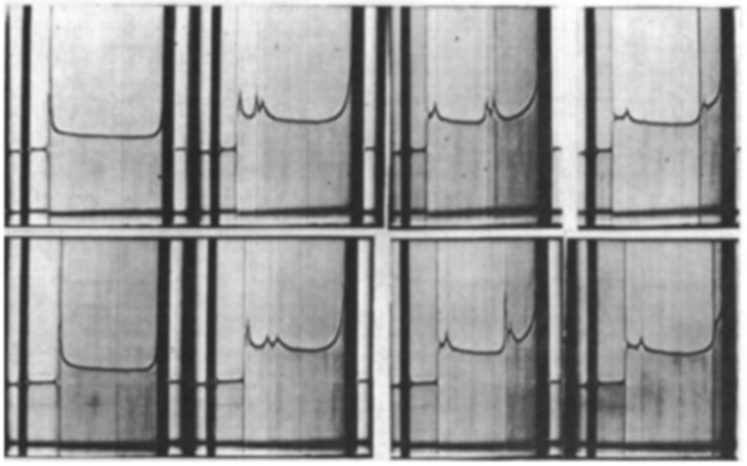

Fig. 5. Ultracentrifugal sedimentation, pure reconstituted F-actomyosin, 4·8 mg/ml, in the presence of α-actinin present to 20% of the F-actomyosin (upper diagram), and pure reconstituted F-actomyosin, 4·8 mg/ml, in the presence of both α-actinin (20% of the actomyosin) and 2×10^{-5} M ryanodine.

The double-spiked character of the main peak, correlated with the presence of α-actinin in combination with actomyosin as already shown by Briskey *et al.* [4], is clearly evident. The inclusion of ryanodine produced a pattern which showed that the α-actinin–F-actomyosin complex sedimented faster, and that the trailing peak in the double-spiked main peak is much higher than the corresponding peak in the absence of ryanodine. These findings strongly suggested that ryanodine enhanced the complexation between F-actomyosin and α-actinin.

DISCUSSION

An essential step in the isolation of α-actinin is to have maximum skeletal muscle relaxation in the animal source. This is usually achieved by paralyzing the preanesthetized animal with *d*-tubocurarine before exsanguination. Maximum yields are obtained by this procedure. Presumably in the relaxed muscle, the protein exists in an environment that affords greater ease of isolation. The poorer yields obtained from non-paralyzed animals suggest that the α-actinin is incorporated into or bound by the contractile machinery in states of non-relaxation. That α-actinin is indeed incorporated into the actin component of actomyosin during superprecipitation is shown by the work of Briskey *et al.* [4].

The results described in this paper corroborate the findings of Briskey *et al.* [4] with respect to both enhancement of ATPase activity and superprecipitation of actomyosin by α-actinin. But the study of the influence of ryanodine yielded some interesting information. The ATPase of myosin A added to G-actin follows a progressively increasing rate. α-Actinin, like β-actinin, depresses this rate [13]. Ryanodine stimulates this depressed rate, but never to equal or exceed the basic rate of the control. On the other hand, the ATPase activity of reconstituted F-actomyosin is always enhanced by α-actinin under the conditions of these experiments. Ryanodine is capable of enhancing this ATPase further. This latter observation is more in harmony with the known actions of the alkaloid on skeletal muscle although, as pointed out by Briskey *et al.* [4], changes in ATPase activity in the presence of α-actinin may not be indicative of a functional rate for the protein. Facilitation of the conversion of G-actin to F-actin by α-actinin is not indicated by the results presented.

As regards the influence of ryanodine on the superprecipitation of F-actomyosin, two points need to be considered. Ryanodine itself is capable of enhancing superprecipitation in the absence of α-actinin. The effect of these two substances together on superprecipitation of F-actomyosin is greater than their individual effects. If this is due to additive effects, then it suggests that ryanodine acts like α-actinin in this respect. This is open to further investigation. On the basis of the present evidence, however, it appears more likely that the alkaloid enhances the capacity of F-actomyosin to bind α-actinin, and such an effect is manifested by enhancement of superprecipitation.

While clear-cut evidence has not been presented for this effect of ryanodine as a basis for its action in producing irreversible contracture in skeletal muscle, and doubt still exists whether superprecipitation represents the contraction process, the results presented constitute good evidence to show direct interaction between the drug and the contractile machinery of skeletal muscle. As already pointed out in conjunction with the discussions of methods of purification, the α-actinin

becomes less capable of being isolated in conditions of non-relaxation of skeletal muscle, implying binding or incorporation into contractile actomyosin. If α-actinin is indeed essential for states of contraction to come about, and to be maintained, then it follows that any agents facilitating the incorporation or binding of α-actinin into the contractile machinery should be able to enhance contraction, and any agent that interferes with the reversibility of this interaction should cause persistent contraction. Both of these effects are consistent with the known actions of ryanodine [14].

In should be pointed out that doses much in excess of effective doses *in vivo* were used in the experiments of this report. Doses of this magnitude were used by us in previous work [5–7, 13]. This discrepancy is not easy to explain. Some hypothetical explanations could be offered. Drugs which are effective at the very low concentrations of 10^{-7} to 10^{-8} M would be expected to require greater receptor specificity and stringent cytoarchitectural arrangement. Conceivably, these conditions are no longer met after cellular fractionation. Also, we were able to observe young heart cells cultured from newborn rats survive for over 45 min in the presence of 10^{-5} M ryanodine. This observation suggested that during maturation some factor develops in the cardiac cell which confers upon it the 1000-fold increase in sensitivity which the mature cell exhibits. It is not beyond reason to assume that such modifying factors became lost during the purification. The same considerations could apply to skeletal muscle as well.

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