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EFFECTS OF RYANODINE ON MODEL SYSTEMS DERIVED FROM MUSCLE—IV

RECONSTITUTED ACTOMYOSIN AND β-ACTININ*

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Abstract—The influence of β-actinin on the ATPase activity of skeletal muscle actomyosin reconstituted from either G- or F-actin, and myosin A and the modification of this influence by ryanodine were studied. The ATPase activity of actomyosin reconstituted from G-actin and myosin A showed a progressive increase in rate. This finding suggested a gradual conversion of G- to F-actin, the latter then complexing with myosin A to form the active F-actomyosin. β-Actinin slowed this progressive increase in ATPase rate. Preincubation studies showed that ryanodine, which opposed this slowing effect, did not inactivate β-actinin. The ATPase activity of actomyosin reconstituted from myosin A and F-actin which has been preincubated with β-actinin was also reduced, but in this instance the progressive increase seen with G-actin was no longer observed. The results presented are compatible with the known effects of β-actinin, namely the retarding action on the conversion of G- to F-actin and the dispersing effect on F-actin. F-actin complexed with Myosin A in reconstituted actomyosin was less susceptible to the dispersing effect. Ryanodine opposed both types of action. Since the activity of β-actinin was not reduced by preincubation with ryanodine, it is concluded that the alkaloid stabilizes F-actin against the dispersing effect of β-actinin.

WITHIN the last decade or so, evidence from a variety of sources has directed attention to direct interaction of ryanodine with muscle contractile proteins as its possible site of action. Although earlier investigations failed to reveal any interference with the ATPase activity of myosin B,[1,2] with the light-scattering response of myosin B to ATP, or with shortening of glycerol-treated rabbit fibers induced by ATP,[1] a later study revealed[3,4] that a maximum contracture could be induced in fibers which had been extracted with glycerol for only 24–48 hr. These fibers responded to ATP with a brief contraction followed by relaxation.[5] Subsequently it was reported that ryanodine enhanced ATP-induced contraction of rabbit skeletal muscle fibers which had been extracted for at least 3 months, a period of time which had been shown to be sufficient for inactivation of the relaxing factor system.[6] Although the earlier work of Blum *et al.*[1] and Pick and Tullius[2] failed to reveal any effect of ryanodine on the ATPase activity of myosin B, a later investigation showed definite enhancement of the ATPase activity of myofibrils.[7] This finding led to renewed attention on the ATPase activity of actomyosin as a possible site for the action of ryanodine. Elison and Jenden,[8] having confirmed the lack of effect of ryanodine on the ATPase activity of myosin B, investigated the effect of the alkaloid on actomyosin reconstituted *in vitro* from actin and

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highly purified myosin A. The ATPase activity was found to be enhanced by the drug up to 3-fold if the actin was extracted at room temperature and not subjected to high purification, but pure preparations of actin yielded reconstituted actomyosin, which was unaffected by the alkaloid. The enhancement showed a characteristic time course, increasing progressively for 10–30 min after addition of ryanodine, and was observed only if subsaturating amounts of calcium were included in the incubation medium. They concluded that a factor extractable with actin is required for the enhancement of actomyosin ATPase by ryanodine.

This report is concerned with data from experiments which help to explain the escalating rates of ATPase activity of reconstituted actomyosin observed in earlier work,[8] with some evidence for the identity of the factor involved, and with the influence of ryanodine on the activity of this factor.

METHODS AND MATERIALS

Purification of actin. Skeletal muscle was obtained from a stunned rabbit killed by exsanguination. The muscle of back and legs was removed and immediately placed on ice. Acetone powders from skeletal muscle were prepared by the method described by Szent-Gyorgyi,[9] and highly purified G-actin was prepared by the method of Martonosi.[10] F-actin, whenever needed, was prepared from G-actin by adding 0·1 M KCl.

Preparation of myosin A. This was done according to the method previously described.[8]

Reconstitution of actomyosin. This was done usually by adding indicated amounts of actin and myosin A. In some instances, reconstituted F-actomyosin was reisolated by ionic precipitation before use.

Preparation of β-actinin. This was done according to the method of Maruyama[11,12] except for the last dialysis step. Instead of using 5 mM $KHCO_3$, 0·1 M KCl or glass-distilled H_2O was used, depending on whether the protein was to be used in experiments involving F-actin or experiments involving G-actin.

Measurement of ATPase. The inorganic phosphate generated by ATPase activity was followed by titrating with KOH in a pH-stat as described previously.[7]

Protein determination. This was done according to the method of Lowry *et al.*,[13] using bovine serum albumin as a standard.

Materials. ATP, disodium salt, was purchased from Sigma Chemical Company, St. Louis, Mo. Salt reagents were analytical grade. Ascorbic acid and bovine serum albumin were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif. Ryanodine was obtained as a gift from S. B. Penick & Company, N.Y.

RESULTS

Figure 1 shows the results of experiments designed to compare the activities of reconstituted actomyosins and the influence of β-actinin and calcium on these activities. In all instances where G-actin was used to form actomyosin, a progressive increase in rate was seen which approached the constant ATPase rate of F-actomyosin as a limiting value. This result suggested a gradual conversion of G- to F-actin, the latter then combining with myosin A to yield the most active form of actomyosin, namely F-actomyosin. In the presence of β-actinin, the progressive increase in rate was slowed considerably. It seems likely that this effect of β-actinin is related to its retarding effect

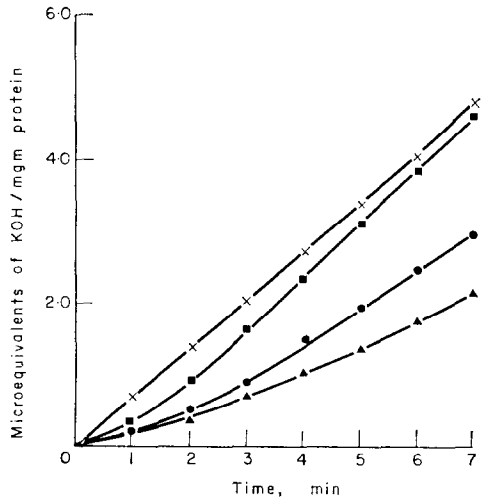

FIG. 1. Effect of β-actinin on the ATPase activity of actomyosin reconstituted from skeletal muscle G-actin and myosin A, β-actinin added to G-actin before addition of myosin A. Conditions: G- or F-actin/myosin A = 1·5/4·0, w/w; 0·1 M KCl; 2·5 mM ATP; 2·5 mM $MgCl_2$; 0·2 mM $CaCl_2$ except where indicated; pH 6·5; 30°. ×—×, F-actin + myosin A; ■—■, G-actin + myosin A; ●—●, G-actin + myosin A + β-actinin (β-actinin/G-actin, 1/2, w/w;), ▲—▲, G-actin + myosin A + β-actinin (β-actinin/G-actin, 1/2, w/w), no $CaCl_2$.

on the conversion of G- to F-actin and its dispersing effect on F-actin previously reported by Maruyama.[11] Although calcium was capable of enhancing the ATPase

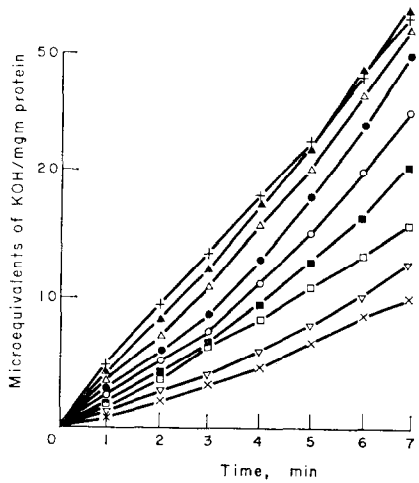

FIG. 2. Influence of ryanodine on the ATPase activity of actomyosin reconstituted from skeletal muscle G-actin and myosin A in the presence of varying amounts of β-actinin which was added to G-actin before addition of myosin A. Control conditions: G-actin/myosin A = 1·5/4·0, w/w; 0·1 M KCl; 2·5 mM $MgCl_2$; 2·5 mM ATP; 0·2 mM $CaCl_2$; pH 6·5; 30°. +—+, control and control + 2×10^{-5} M ryanodine (identical); △—△, control + β-actinin (β-actinin/G-actin, 1/10, w/w); ▲—▲, is △—△ + 2×10^{-5} M ryanodine; ○—○, control + β-actinin (B-actinin/G-actin, 1/5, w/w); ●—●, is ○—○ + 2×10^{-5} M ryanodine; □—□, control + β-actinin (β-actinin/G-actin, 1/2, w/w); ■—■, is □—□ + 2×10^{-5} M ryanodine; ×—×, control + β-actinin (β-actinin/G-actin, 1/1, w/w); ▽—▽ is ×—× + 2×10^{-5} M ryanodine.

activity of G-actomyosin somewhat, it was not an absolute requirement. It was included in all subsequent experiments, except where indicated.

Figure 2 shows the results of experiments designed to study the effect of ryanodine on the ATPase activity of reconstituted G-actomyosin in the presence of varying amounts of β-actinin. The escalating ATPase rate was observed in all instances whether or not β-actinin was present, but in the presence of this protein the rate was depressed. This depression increased with the increasing ratio of β-actinin to G-actin and was opposed significantly by ryanodine. The alkaloid had no influence at all on the ATPase rate of the system where no β-actinin was present.

The experiments of Fig. 3 were designed to study the influence of preincubation on the activity of β-actinin. In these experiments, β-actinin was preincubated in the media for ATPase measurements together with ryanodine or with ryanodine and G-actin. G-actin preincubated by itself served as a control. The ATPase activity of the preincubation system was started by adding myosin A or G-actin and myosin A. It will be seen that the ATPase rate of the control was constant. Since this rate was of the same magnitude as that of F-actomyosin, it served as evidence for the conversion of all the G- to F-actin during the preincubation period. On the other hand, the addition of myosin A to a system in which β-actinin, G-actin and ryanodine were preincubated resulted in a reduced but escalating ATPase rate. The fact that preincubation of G-actin alone resulted in actomyosin with maximum activity upon addition of myosin A, whereas preincubation of G-actin with β-actinin and ryanodine for the same length of time resulted in actomyosin with depressed but escalating ATPase rate, must mean that β-actinin remained capable of delaying the conversion of G-to F-actin in the presence of ryanodine. That β-actinin indeed did not lose activity in the presence of ryanodine is corroborated by the finding that the addition of G-actin and myosin A to the preincubation system containing β-actinin and ryanodine resulted in the typical strongly depressed and escalating ATPase rate.

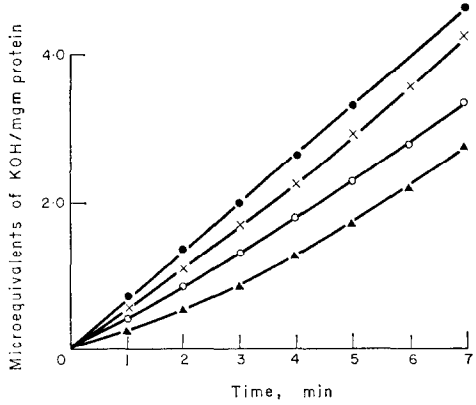

FIG. 3. Influence of preincubated β-actinin on the ATPase activity of actomyosin reconstituted from skeletal muscle G-actin and myosin A. Conditions: G-actin/myosin A = 1·5/4·0, w/w; 0·1 M KCl; 2·5 mM ATP; 2·5 mM $MgCl_2$; 0·2 mM $CaCl_2$; myosin A or G-actin followed by myosin A added to preincubation mixture to start reaction; ×—×, β-actinin/G-actin (1/5, w/w) + ryanodine, preincubated for 5 min; ○—○, β-actinin + G-actin (1/3, w/w) + ryanodine, preincubated for 5 min; ▲—▲, β-actinin + ryanodine, preincubated for 5 min, then G-actin added (β-actinin/G-actin, 1/5, w/w) followed by myosin A; ●—●, no β-actinin, but myosin A added to G-actin which had been preincubated for 5 min with 2×10^{-5} M ryanodine.

The addition of myosin A to F-actin, which was preincubated for 5 min with β-actinin, resulted in a smaller ATPase rate as compared to the control in which F-actin preincubated by itself was involved (Fig. 4). This depression in rate increased with an increase in the ratio of β-actinin to F-actin. The inclusion of ryanodine in such preincubation mixtures resulted in all instances in diminution of the rate-depressing effect of β-actinin. This opposing effect of ryanodine was not seen with the control in which no β-actinin was involved. It should be pointed out that rather high ratios of β-actinin to F-actin were necessary in these experiments. It should also be noted that in no instance was the escalating ATPase rate seen in contrast to the observations with G-actin and myosin A systems.

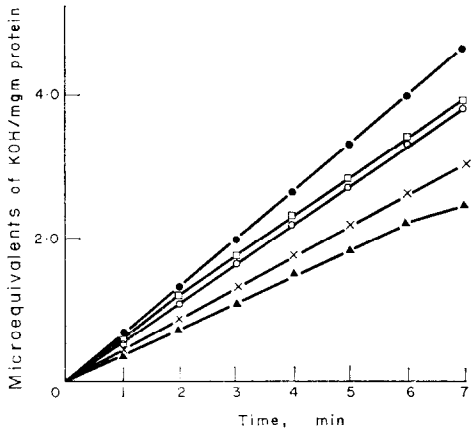

FIG. 4. ATPase activity of F-actomyosin made up of myosin A and F-actin, the latter having been preincubated with β-actinin in the presence and absence of ryanodine. Control conditions: F-actin/myosin A = 1·5/4·0, w/w; 0·1 M KCl; 2·5 mM ATP; 2·5 mM $MgCl_2$; 0·2 mM $CaCl_2$; 30°; pH 6·5. (Myosin A was added to the preincubation system to start ATPase activity.) ●—●, control using F-actin preincubated by itself (F-actin preincubated with ryanodine yielded same plot); ○—○, F-actin preincubated with β-actinin for 5 min (β-actinin/F-actin, 1/2, w/w); □—□, same as ○—○, but 2×10^{-5} M ryanodine present in preincubation system; ▲—▲, F-actin preincubated with β-actinin for 5 min (β-actinin/F-actin, 1/1, w/w); ×—×, same as ▲—▲, but 2×10^{-5} M present in preincubation system.

In the experiments of Fig. 5, F-actomyosin was preincubated with β-actinin and with or without ryanodine. After appropriate periods of incubation ATP was added. The amount of base needed to titrate the resultant phosphoric acid was plotted against incubation time. Several observations can be made. There was a definite diminution in ATPase rate which became more pronounced with increasing incubation time. This diminution in rate was significantly less when ryanodine was also present in the preincubation system. The upper two curves of Fig. 5 demonstrate that this influence of ryanodine was more pronounced with increasing incubation time. Two conclusions could be drawn. It appears that β-actinin in relatively high doses was capable of exerting a disrupting influence on the ATPase activity of preformed F-actomyosin, presumably by disrupting the F-actin component, and that ryanodine had a stabilizing effect against this action of β-actinin.

The ATPase activity of reconstituted actomyosin highly purified by re-precipitation was studied in the presence of β-actinin and varying concentrations of ATP. By

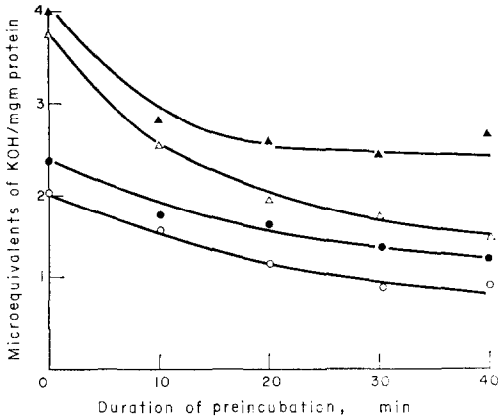

FIG. 5. Influence of β-actinin on the ATPase activity of reconstituted skeletal muscle F-actomyosin as a function of duration of preincubation of β-actinin and F-actomyosin. Conditions: F-actin/myosin A = 1·5/4·0; β-actinin/F-actin, 1/1, w/w; 0·1 M KCl; 2·5 mM ATP; 2·5 mM $MgCl_2$; 0·2 mM $CaCl_2$; 30°; pH 6·5; (ATP added to preincubation system to start reaction). ○—○, ATPase rates, 3 min after addition of ATP to preincubation system of F-actomyosin and β-actinin and preincubated for the times indicated; ●—●, same as ○—○, but 2×10^{-5} M ryanodine present in the preincubation system; △—△, ATPase rates 6 min after addition of ATP to preincubation system of F-actomyosin and β-actinin and preincubated for the times indicated; ▲—▲, same as △—△, but 2×10^{-5} M ryanodine present in preincubation system.

plotting the ATPase activities at 3 and 6 min in terms of base used to titrate the generated phosphoric acid, the curves of Fig. 6 were obtained. It will be seen that the ATPase activities peaked at 2·5 mM ATP concentration when it was equimolar with Mg^{2+}. At higher concentrations, ATP became inhibitory. These observations were in agreement with earlier reports on the activity of natural actomyosin[7] in which it was

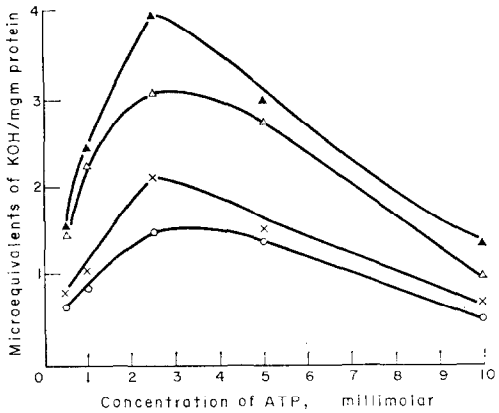

FIG. 6. Influence of both ryanodine and β-actinin on the ATPase activity of reconstituted skeletal muscle F-actomyosin in the presence of varying concentrations of ATP. All systems contain F-actin/myosin A, 1·5/4·0, w/w; 0·1 M KCl_2; 2·5 mM $MgCl_2$; 0·2 mM $CaCl_2$; 30°; pH 6·5; β-actinin/F-actin, 1/2, w/w; ○—○, ATPase rates 3 min after addition of ATP to start reaction, no ryanodine; ×—×, same as ○—○, but 2×10^{-5} M ryanodine included; △—△, ATPase rates 6 min after addition of ATP to start reaction, no ryanodine; ▲—▲, same as △—△, but 2×10^{-5} M ryanodine included.

also pointed out that ryanodine was capable of exerting an opposing effect to the inhibitory action of ATP only at the higher concentrations. The results of the present experiments showed in addition that the ATPase activity of reconstituted actomyosin at all concentrations of ATP used was higher in the presence of ryanodine. In other words, the presence of β-actinin extended the stimulant action of the drug to concentrations of ATP where it is not normally inhibitory.

DISCUSSION

In an earlier publication,[8] it was reported that reconstituted actomyosin prepared from crude actin and myosin A showed a characteristic response to ryanodine when ATPase activity was measured. The enzymatic rate increased progressively after addition of ryanodine, eventually achieving a level two to three times the initial value. Since actomyosin prepared from highly purified actin and myosin A did not exhibit this behavior, it was concluded that the participation of an unknown factor, extractable with actin at room temperature, was required. The results presented in this report serve to explain the escalating pattern of the ATPase rate and to confirm the prediction of the involvement of a protein factor. The progressively increasing ATPase rate, seen only when highly purified G-actin was added to myosin A, approached the constant ATPase rate of F-actomyosin as a limiting value. It is concluded that this rate pattern was due to gradual conversion of G- to F-actin, the latter then combining with myosin A to form the highly active F-actomyosin. The lowering of initial rates and the slower increase in rates in the presence of increasing concentrations of β-actinin are interpreted as being due to the retarding action of this protein on the G–F-actin transformation as well as its ability to disperse formed F-actin.[11,12] The results of various experimental designs showed that ryanodine was capable of opposing this action of β-actinin.

Since β-actinin remains active after preincubation with ryanodine, the opposing effect of ryanodine on its action must occur through a mechanism other than its inactivation by the drug. Stabilization of the formed F-actin against dispersion by β-actinin is the most likely explanation. Several factors speak for this conclusion. The addition of myosin A to F-actin which has been preincubated with β-actinin resulted in actomyosin with much reduced activity as compared with the control. If ryanodine was also present in the preincubation mixture, the resulting actomyosin was much more active though not quite as active as the control. It is concluded that during the preincubation β-actinin exerted its dispersive action on F-actin and that ryanodine was capable of opposing this action. This effect of ryanodine resulted in preservation of F-actin which in combination with myosin A yielded the most active form of actomyosin. The depressed ATPase activity of reconstituted actomyosin in the presence of β-actinin is also taken as an indication for the ability of β-actinin to disperse the actin component of actomyosin. Rather high ratios of β-actinin to F-actin were necessary, showing the greater stability of F-actin. When ryanodine was also included, the ATPase rate was much higher and the difference in rates between control and experimental became more pronounced with an increase in time of incubation. This result speaks for a stabilization of the actin component of actomyosin against the dispersing action of β-actinin.

The ATPase activity of reconstituted actomyosin in the presence of β-actinin is elevated by ryanodine at all concentrations of ATP used. Since a stimulation of

ATPase activity never occurred in the absence of β-actinin, it can be concluded that ryanodine did not stimulate the inherent ATPase activity *per se*, but ATPase activity depressed by β-actinin. It is known that in the presence of high concentrations of ATP actomyosin dissociates into F-actin and myosin A.[14–16] It is not beyond imagination that this dissociation could be facilitated even at lower concentrations of ATP by β-actinin which by dispersing F-actin caused the equilibrium F-actin + myosin A ⇌ F-actomyosin to proceed in favor of dissociation. By opposing the dispersion of F-actin, ryanodine shifted the equilibrium in favor of F-actomyosin and a higher ATPase rate. Procita[17] reported that he was able to extract greater quantities of actomyosin from the skeletal muscle of rabbits treated with ryanodine than from untreated animals. The results obtained and the conclusions drawn in this report are in harmony with his findings.

Results have been presented which are taken as evidence for the ability of ryanodine to stabilize F-actin and F-actomyosin against β-actinin, a protein factor whose actions on these contractile proteins of muscle are related to relaxation. The effect of ryanodine is in keeping with the known paralytic action on skeletal muscle. It must be pointed out that doses much in excess of effective doses *in vivo* were used in the experiments of this report. Such high doses are, however, not unknown in our experience. We have used them in our earlier work[7,8] and recent observations (unpublished) showed that cultured heart cells survived for at least 45 min in the presence of 2×10^{-5} M ryanodine. We have no explanation at this time for the discrepancy between effective doses *in vivo* and *in vitro*.

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