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

MYOFIBRILS AND NATURAL ACTOMYOSIN*

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Abstract—ATPase activity of myosin B and myofibrils was measured in calcium-buffered solutions at various concentrations of Mg^{2+} and ATP in the presence and absence of ryanodine. No effect of ryanodine on myosin B ATPase was found except when ATP was present in excess over Mg^{2+}, when the inhibitory effect of excess ATP was suppressed by ryanodine. A small but consistent increase in myofibrillar ATPase was produced by ryanodine at $[Ca^{2+}] = 10^{-6} - 3 \times 10^{-5}$ M; this increase was more pronounced when ATP was present in excess over Mg^{++}. Ryanodine may act in part by stabilizing the actomyosin complex against the dissociating effect of ATP.

RYANODINE has been shown to increase ATP-induced tension developed by glycerinated fibers.[1] Evidence has also been presented to show that the drug sensitized the contractile elements of skeletal muscle to calcium.[1] Although it has been reported that ryanodine was without effect on the ATPase activity of myosin B,[2,3] it appeared worthwhile to reinvestigate the effect of the drug on the ATPase activities of myofibrils and myosin B under the conditions in which positive effects mentioned above were observed.

The experiments of this paper were undertaken to determine whether the tension-enhancing and calcium-sensitizing effects of ryanodine are reflected in changes in ATPase activities of myofibrils and myosin B.

MATERIALS AND METHODS

Preparation of myofibrils. The procedure for the purification is based on the method described by Gergely.[4] Leg and skeletal muscle of male rabbits was mixed with 3 volumes of ice-cold 65 mM succinate buffer at pH 7·5, and minced in a Waring–Blendor for 60 sec. After adding an equal volume of succinate buffer the preparation was centrifuged at 600 *g* for 15 min, yielding three indistinctly separated layers. The upper fatty layer was removed by suction, and the bottom layer of connective tissue debris was discarded. The middle layer was carefully separated by decantation, rehomogenized for 30 sec, and spun at 600 *g* for 30 min. The fluffy supernatant was filtered through two layers of cheesecloth and centrifuged for another 30 min at 1200 *g*. The precipitate was resuspended with the Waring Blendor in 3 vol. of succinate buffer, centrifuged again at 1200 *g*, and the cycle repeated three more times. The final precipitate was taken up in succinate buffer, mixed with an equal volume of ice-cold glycerol, and stored at −18° for at least 2 weeks before use.

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For an experiment, an aliquot was withdrawn and centrifuged at 10,000 *g* for 15 min. The precipitate was washed once in approximately 50 vol, of succinate buffer and twice with 0·05 M KCl. The final precipitate was suspended in 0·05 M KCl by gentle homogenization in a Potter–Elvehjem homogenizer. Protein was determined by the method of Westley and Lambeth,[5] with bovine serum albumin as standard.

Preparation of actomyosin. Leg and back muscle of rabbit, immediately chilled on ice, was minced in a mincer with holes of 2-mm dia. Three volumes of Weber–Edsall solution, pH 6·8, were added and the tissue extracted with gentle stirring for about 24 hr. An equal volume of Weber–Edsall solution was added and the mixture centrifuged for 30 min at maximum speed in an International model V centrifuge in 250-ml capacity cups. The supernatant was filtered through two layers of cheesecloth. Actomyosin was precipitated out by adding enough doubly distilled water to reduce the KCl concentration to 0·2 M and adjusting the pH to 6·8. After 2 hr, any supernatant was removed by suction and the remainder again centrifuged as before. To the precipitate 1·2 M KCl was added to a final concentration of 0·6 M, and additional 0·6 M KCl was added until a slightly turbid solution was obtained. The molarity of KCl was again reduced to 0·2 M to precipitate the protein, and the process of redissolution and reprecipitation was repeated three more times. The final precipitate was dissolved in 1·2 M KCl. An equal volume of ice-cold glycerol was added and the preparation stored at −18° for at least 2 weeks before use.

For an experiment an aliquot was withdrawn and the molarity of KCl reduced to 0·2 M by adding ice-cold water to precipitate the protein. The precipitate was washed twice in 0·2 M KCl and then suspended in 0·2 M KCl by gentle homogenization.

Ca buffer. All experimental systems contained a calcium buffer consisting of calcium and EGTA [ethyleneglycol *bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid]. The concentration was always 1 mM. The concentrations of calcium will be given in the legends of the figures.

Measurement of ATPase activity. The time course of liberation of H^+ from the actomyosin-ATP system was determined by a constant pH titration at pH 6·7, with a Radiometer model TTT titrator. The total volume of reaction mixture was 10 ml and the temperature was 30°. Before starting the reaction the pH of the reaction mixture was brought to 6·7 with either 1 N KOH or HCl. The steady-state slope of the plot of the time course was determined by the method of least squares.

Materials. All reagents were analytical grade. ATP, as the disodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. EGTA was a gift of the Geigy Chemical Co.

RESULTS

Influence of ryanodine and calcium ions on myosin B ATPase

Fig. 1 illustrates the typical sigmoid curve relating ATPase rate to $[Ca^{2+}]$, which has previously been reported. Duplicate experiments in which 2×10^{-4} M ryanodine was included show no significant difference from controls at any Ca^{2+} concentration.

Influence of ryanodine on myosin B ATPase as a function of $[Mg^{2+}]$ *and* $[ATP]$

There is a lack of agreement on the question of the true substrate of actomyosin ATPase. Many workers feel[6,7] that ATP is the substrate, whereas Perry and Grey[8] are

of the opinion that Mg-ATP is the substrate. It is also well known that ATP influences the state of association of actomyosin in a very complex manner.[9–12] Effects of ryanodine were therefore sought at several ATP and Mg^{2+} concentrations.

When Mg^{2+} and ATP were provided in equimolar concentrations, ATPase rate increased monotonically with substrate concentration (Fig. 2), and no significant

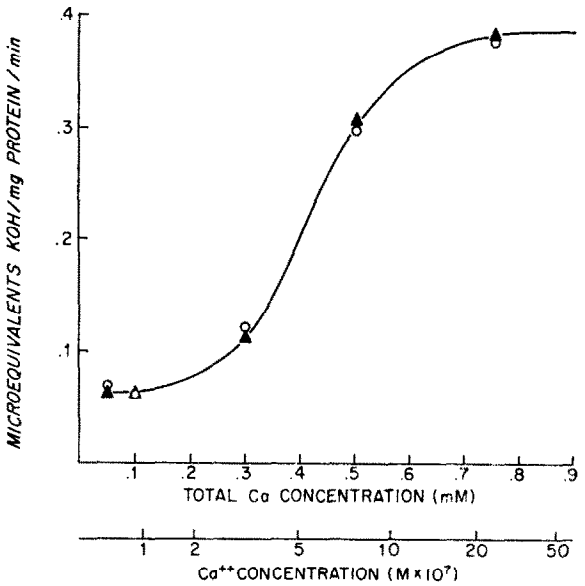

FIG. 1. Effect of ryanodine on myosin B ATPase as a function of Ca^{2+} concentration. Conditions: 85 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 1·25 mg protein, pH 6·7, 30°. Myosin B glycerinated in 50% glycerol for at least 2 weeks before use; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

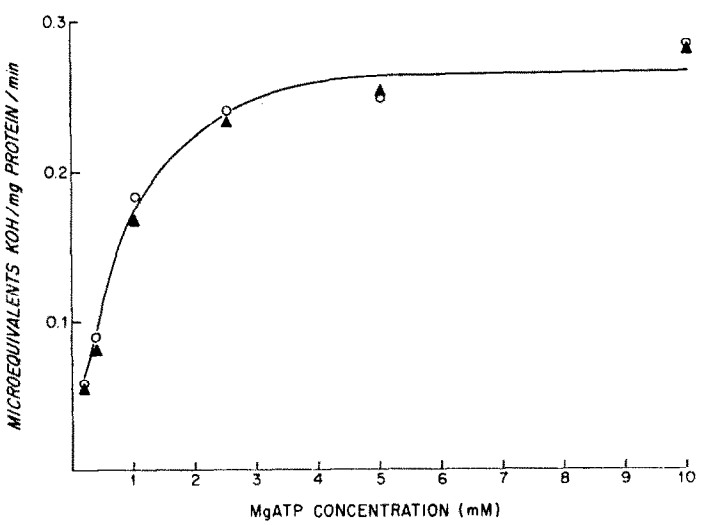

FIG. 2. Effect of ryanodine on myosin B ATPase as a function of Mg-ATP concentration. Conditions: 85 mM KCl, 1 mM EGTA, 0·75 mM $CaCl_2$, 1·25 mg protein, pH 6·7, 30°; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

influence of ryanodine was found at any point. When the amount of ATP was varied while the magnesium concentration was kept constant at 5 mM (Fig. 3), the actomyosin ATPase rate appeared to approach an asymptote as the ATP concentration was increased to 5 mM. Beyond this point a sharp increase occurred, followed by a rapid decline. There was no effect of ryanodine on the ATPase rate until an inhibitory concentration of ATP was reached, when a significant suppression of the inhibition was produced by the alkaloid.

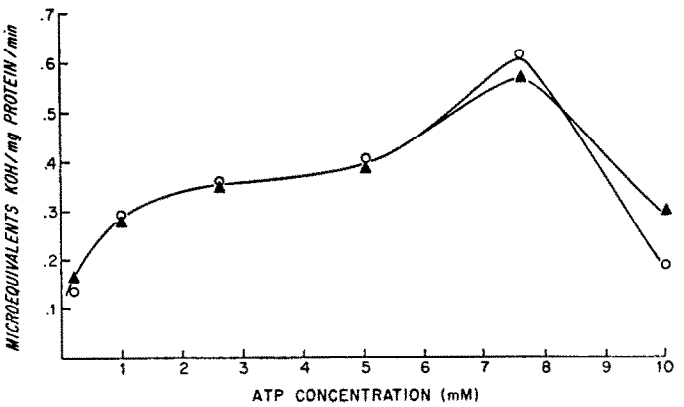

FIG. 3. Effect of ryanodine on myosin B ATPase as a function of ATP concentration. Conditions: 85 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0·75 mM $CaCl_2$, 1·25 mg protein, pH 6·7, 30°; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

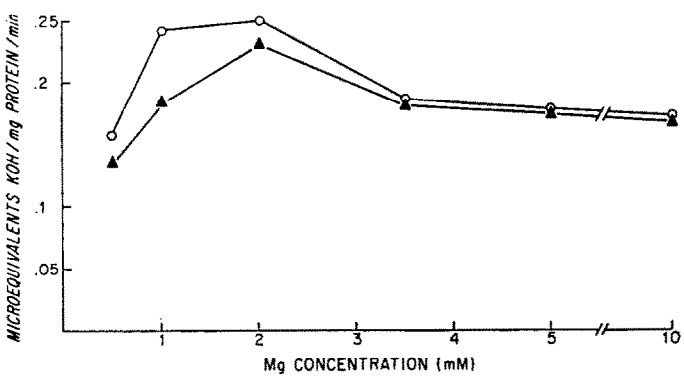

FIG. 4. Effect of ryanodine on myosin B ATPase as a function of Mg concentration. Conditions: 85 mM KCl, 5 mM ATP, 1 mM EGTA, 0·75 mM $CaCl_2$, 1·25 mg protein, pH 6·7, 30°; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

In the experiment of Fig. 4, myosin B was incubated with 5 mM ATP and varying concentrations of magnesium. While excess Mg^{2+} did not influence ATPase rate, lowering the amount of magnesium resulted in increased ATPase activity. Ryanodine had no influence on the system when excess Mg^{2+} was present, but clearly suppressed the effect of reducing [Mg^{2+}] below [ATP].

In both experiments an effect of ryanodine was seen only when ATP was present in molar excess over Mg^{2+}, and in each case its direction was such as to suppress the effect of excess ATP over Mg^{2+}.

Influence of ryanodine on myofibrillar ATPase

In contrast to its lack of effect on myosin B, ryanodine produced a small but consistent increase in myofibrillar ATPase activity at Ca^{2+} concentrations up to 3×10^{-5} M (Fig. 5). This enhancement was more pronounced at lower Ca^{2+} concentrations,

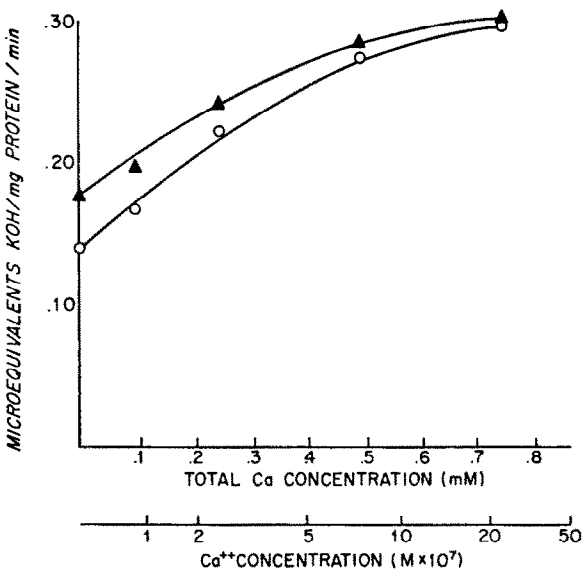

FIG. 5. Effect of ryanodine on the myofibrillar ATPase as a function of Ca^{2+} concentration. Conditions: 85 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 2 mg protein, pH 6·7, 30°. Myofibrils extracted with 50% glycerol for at least 2 weeks before use; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

and was particularly marked in the presence of a large excess of ATP over Mg^{2+} (Fig. 6). A similar enhancement of myosin B ATPase by ryanodine was observed when ATP was present in excess over Mg^{2+}; in both cases ryanodine partially suppressed the normally inhibitory effect of excess ATP, but this inhibition was more readily produced in myosin B (ATP 10 mM, Mg 5 mM) than in myofibrils (ATP 10 mM, Mg 1 mM) (cf. Figs. 3 and 6). Curiously, if the ATP concentration is maintained at 5 mM and $[Mg^{2+}]$ is reduced, ryanodine exerts a net inhibitory effect by suppressing the increased ATPase rate normally seen in both myosin B (Fig. 4) and myofibrils (Fig. 7). Myofibrillar inhibition under these conditions was observed in the range 10^{-7}–10^{-6} M Ca^{2+} (Fig. 8).

DISCUSSION

The results described indicate that when ATP and Mg^{2+} are present at a concentration of 5 mM, a progressive activation of myosin B and myofibrillar ATPase occurs as the Ca^{2+} concentration is raised from 10^{-7} to 10^{-5} M. Similar results have previously

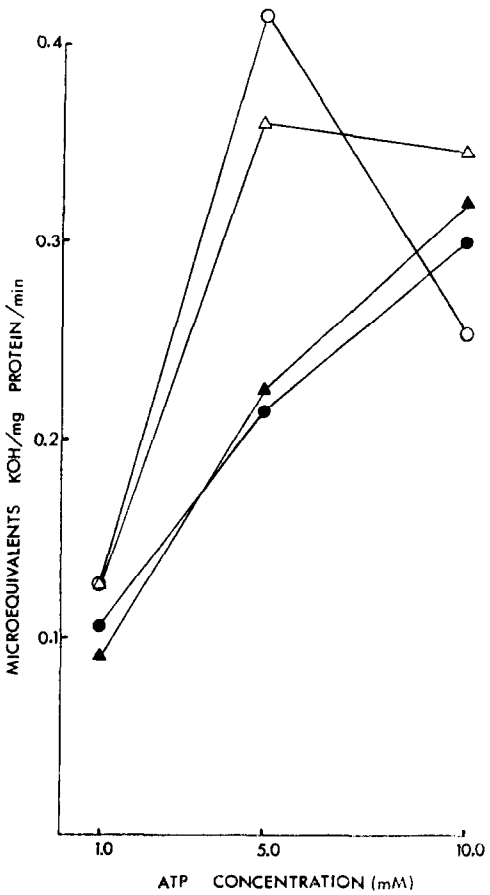

FIG. 6. Effect of ryanodine on myofibrillar ATPase as a function of ATP concentration. Conditions: 85 mM KCl, 1 mM EGTA, 0·25 mM $CaCl_2$, 2 mg protein, pH 6·7, 30°. Triangles: with 2×10^{-4} M ryanodine; circles: no ryanodine. Filled symbols: 5 mM $MgCl_2$; open symbols: 1 mM $MgCl_2$.

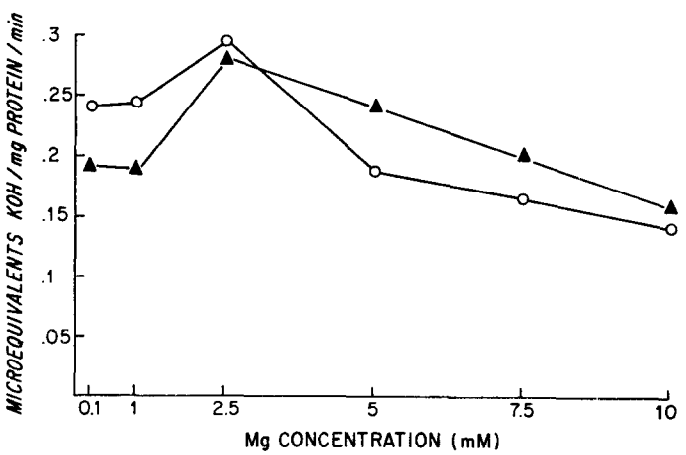

FIG. 7. Effect of ryanodine on myofibrillar ATPase as a function of Mg concentration. Conditions: 85 mM KCl, 5 mM ATP, 1 mM EGTA, 0·25 mM $CaCl_2$, 2 mg protein, pH 6·7, 30°; ▲, with 2×10^{-4} M ryanodine; ○, without ryanodine.

been reported in the literature.[13, 14] While ryanodine produces no change in ATPase activity of myosin B under these conditions, myofibrillar ATPase activity is consistently increased by the alkaloid, the effect being more marked at the lower range of Ca^{2+} concentration. These conditions are similar to those in which glycerinated muscle fibers were shown to produce greater tensions in the presence of ryanodine.

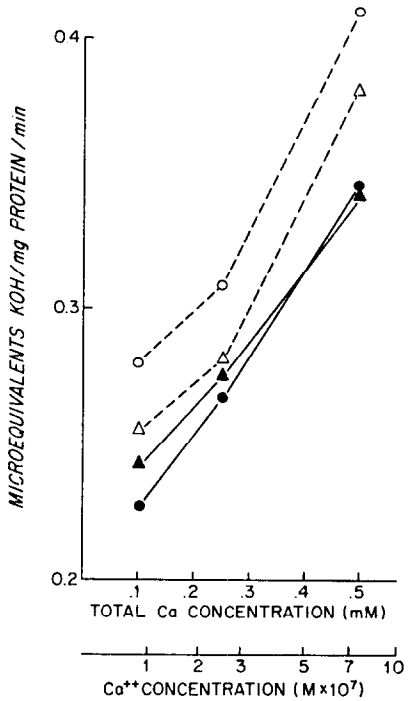

FIG. 8. Effect of ryanodine on myofibrillar ATPase as affected by Ca^{2+} and Mg. concentration. Conditions: 85 mM KCl, 5 mM ATP, 1 mM EGTA, 2 mg protein, pH 6·7, 30°. Triangles: with 2×10^{-4} M ryanodine; circles: no ryanodine. Filled symbols: 5 mM $MgCl_2$; open symbols: 1 mM $MgCl_2$.

Extraction and purification of myosin B not only destroys the organization of the contractile units present in myofibrils and glycerinated muscle fibers but may eliminate other protein components which modify its physicochemical and enzymatic properties. Insensitivity of myosin B to ryanodine under these conditions may therefore be attributable to either of these factors.

When ATP was present in excess over Mg^{2+}, and Ca^{2+} concentration was buffered in the range $1\text{–}5 \times 10^{-7}$ M, ATPase activity of both myosin B and myofibrils was modified by ryanodine. At high ATP levels (ATP 10 mM, Mg^{2+} 1–5 mM) ryanodine suppressed the inhibition usually seen (relative to equimolar ATP and Mg^{2+}), while at lower levels (ATP 5 mM, Mg^{2+} 1 mM) ryanodine suppressed the increase in enzymatic rate seen in its absence. In both cases the effect of the alkaloid was in a direction which tended toward the rate seen when ATP and Mg^{2+} are present in equimolar amounts.

The basis for the effects of excess ATP is in dispute. Inhibition has been ascribed to substrate inhibition[8] and to dissociation of actomyosin into actin and myosin[9–11] at the ionic strength used (0·1). The data summarized in Fig. 2 imply that substrate inhibition of natural actomyosin by Mg-ATP does not occur under the conditions of the present experiments. If actomyosin dissociation is the basis for the effects of excess ATP, then the data presented would be consistent with the possibility that this dissociation is inhibited by ryanodine. Evidence in favor of this has recently been presented by Procita.[15] The relationsip between these phenomena and the differing sensitivity of myosin B and myofibrils to ryanodine at 5 mM Mg^{2+} and ATP is obscure; however, the association of actin with myosin is controlled in part by another protein factor, α-actinin,[16] which greatly extends the range of ATP concentration over which superprecipitation and allied phenomena will occur. It seems possible that this or related factors may be involved in the mechanism of action of ryanodine.

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