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

GLYCEROL-EXTRACTED MUSCLE FIBERS*

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Abstract—Glycerol-extracted rabbit psoas muscle fibers were placed in a calcium-buffered electrolyte solution, and tension responses to ATP and ryanodine were measured. When [Ca^{2+}] was suboptimal, ryanodine enhanced tension development in fibers extracted for 1–5 days or for 120 days. The enhancement was seen also after treatment with desoxycholate. Although ryanodine is known to affect the calcium pump, the present results cannot be attributed to this, because the calcium pump is inactivated by prolonged glycerol extraction or by desoxycholate. A direct effect of ryanodine on contraction seems probable, and the results are consistent with a sensitization to Ca^{2+}.

THE INSECTICIDAL properties of the alkaloid ryanodine are related primarily to its ability to produce flaccid paralysis.[1] In other species, death due to the drug is associated with irreversible rigor in skeletal musculature.[2,3] However, the myocardium responds to ryanodine with a progressive contractile failure,[4–6] and the smooth muscle of mammalian bladder and uterus is not notably affected by the drug.[7] Although there is no obvious correlation between the response of a muscle to ryanodine and its functional or morphological characteristics, the drug may nevertheless serve as a useful tool in analyzing the mechanisms controlling the contraction–relaxation cycle and the dependence of other physiological properties on them.

The study of the contraction–relaxation cycle of muscle has been facilitated by techniques and methodology developed in recent years. The glycerol-extracted fiber[8] is one preparation on which tension generation can be studied. The relaxing factor system originally described by Marsh,[9,10] and later found to consist of a particulate fraction and a soluble component,[11] affords a means by which the relaxation phase can be investigated. The discovery of the calcium-pumping activity of the particulate fraction by Hasselbach and Makinose[12] led to the conclusion that the relaxing action of this component is due to its capacity to lower Ca^{2+} levels below the level required for contraction. Strength was given to this view by the findings of Weber and co-workers and Ebashi[13–15] that calcium is a mandatory requirement for the ATPase and superprecipitation of actomyosin as well as by the older observation that a nonphysiological agent such as the calcium chelator EDTA is capable of inducing relaxation after ATP-induced contraction of glycerinated fibers and of preventing superprecipitation of actomyosin.[16] These observations opened up yet another area

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in which the action of contracture-producing agents such as ryanodine might be fruitfully studied.

Although Edwards *et al.*[1] concluded that ryanodine acts specifically on the contractile process, Blum *et al.*[17] reported that ryanodine did not affect the tension developed by glycerinated rabbit skeletal muscle in the presence of ATP. Pick and Tullius[2] found no effect of the alkaloid on the ATPase activity of crude actomyosin, and Blum *et al.*[17] made a similar observation with actomyosin of greater purity. On the other hand, Jenden and Haslett[18] showed reversal by ryanodine of the spontaneous relaxation of freshly glycerinated rabbit psoas fibers after tension development in the presence of ATP or ATP-regenerating systems. Seraydarian *et al.*[19] further noted that, with prolonged stimulation in the absence of oxygen, the rate of relaxation decreased considerably more in the ryanodine-treated frog sartorius muscle than it did in the stimulated untreated muscle, although the height of the twitch and the rate of contraction changed to the same extent in both treated and untreated muscle. These findings were interpreted to mean that the effect of ryanodine is primarily on relaxation.

Fairhurst and Jenden[20] reported an uncoupling effect of ryanodine on the calcium pump of skeletal muscle granules. This might lead to the persistence of Ca^{2+} levels in the sarcoplasm high enough to maintain a contracted state in muscle, and hence delay relaxation. It is also conceivable that the rigor-producing effect of ryanodine is exerted through interaction with a soluble relaxing factor, either by destroying it, once formed, or by preventing its synthesis. Both of these possibilities were rejected by Elison *et al.*[21] on the basis of direct experimental test. During these experiments, however, several observations were made which suggested a direct interaction of ryanodine with the contractile machinery itself. Fibers which had developed maximal tension in presence of ATP were found to develop about 20 per cent more tension upon addition of ryanodine. The present report will describe experiments in which the direct effect of ryanodine on the contractile machinery is reinvestigated.

METHODS AND MATERIALS

Preparation of glycerinated fibers. Standard procedures were employed for the preparation of glycerol-extracted rabbit psoas fibers, which were stored at −18° before use. Any modification of this procedure will be described in the pertinent section.

Preparation of relaxing factor. Skeletal muscle was homogenized in a Waring Blendor in 3 vol. of a cold solution containing 30 mM KCl, 5 mM $MgCl_2$, and 30 mM potassium phosphate at pH 6·5. Debris was separated by centrifugation at 1000 *g* for 5 min in a Sorvall refrigerated centrifuge. The supernatant was filtered through several layers of cheesecloth and then centrifuged at 10,000 *g* for 15 min. The supernatant was further centrifuged for 90 min in the Spinco preparative centrifuge, with the No. 40 rotor at maximal speed (140,000 *g* at the bottom of the tube). The clear pinkish supernatant is referred to as the soluble muscle extract.

Measurement of tension. For the measurement of tension, a group of fibers less than 100μ in diameter was mounted horizontally in a 2-ml Plexiglas bath with one end fixed and the other attached to an RCA 5734 transducer tube by a 6-cm extension. The preparation was immersed in a solution containing 30 mM KCl, 5 mM $MgCl_2$, 30 mM potassium phosphate at pH 7, and 1 mM mercaptoethanol, at a temperature

of 22°. Tension development was induced with ATP. Modifications of this medium will be given in the appropriate result sections. Wherever possible, comparisons between different conditions were made on matched lengths of the same fiber.

Materials. ATP, disodium salt, and creatine phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were of analytical grade. EGTA [ethyleneglycol *bis*-(β-aminoethylether)-*N*,*N*′-tetraacetic acid] was a gift of Geigy Chemical Co.

RESULTS

Spontaneous relaxation in briefly glycerinated fibers

Muscle fibers extracted for 1–5 days in glycerol retain the ability to relax spontaneously after ATP-induced tension development (Fig. 1) especially in the presence

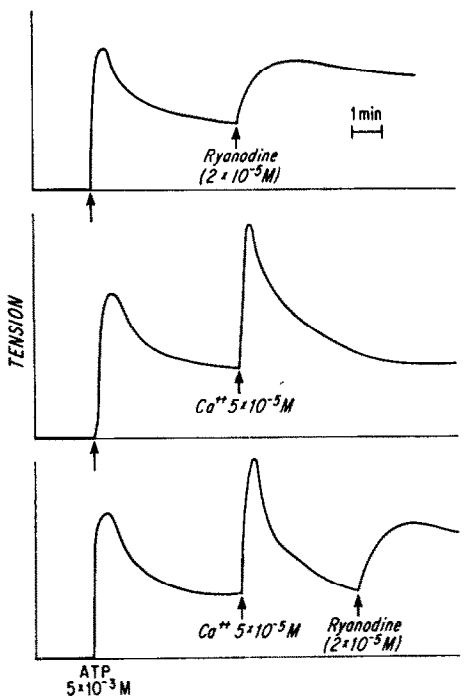

FIG. 1. Effect of ryanodine and calcium on spontaneous relaxation of three 24-hr glycerinated rabbit psoas fibers. Conditions: 30 mM KCl, 5 mM $MgCl_2$, 30 mM potassium phosphate at pH 6·5, 10 mM creatine phosphate, 22°. Additions as indicated.

of an ATP-regenerating system. The relaxed fiber responds to small amounts of added calcium by a brief contraction followed by further relaxation. Ryanodine (2×10^{-5} M) produced a more persistent contraction, whether or not it is preceded by the addition of calcium.

Spontaneous relaxation in these fibers has been attributed to persistence of the relaxing factor system,[18] and active calcium pickup has been demonstrated in fibers prepared in this way.[22, 23] Reversal of the relaxation by ryanodine might reasonably

be interpreted in terms of its uncoupling effect on the calcium pump,[20] but a direct effect on the contractile system cannot be excluded.

Influence of desoxycholate treatment of fibers

Desoxycholate treatment destroys the calcium-concentrating ability of both isolated granules[24] and briefly glycerinated fibers,[22] and may therefore be employed to differentiate these two alternative mechanisms of action of ryanodine. Fig. 2 illustrates

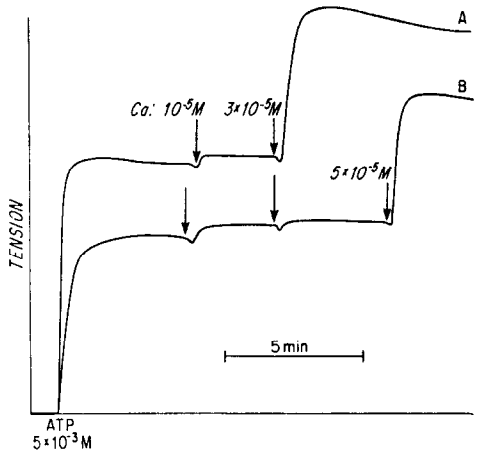

FIG. 2. Influence of desoxycholate pretreatment on the response of 24-hr glycerinated rabbit psoas fibers to stepwise addition of calcium, in the presence (A) and absence (B) of ryanodine (2×10^{-5} M, added initially). Conditions: 30 mM KCl, 5 mM $MgCl_2$, 30 mM potassium phosphate at pH 6·5, 10 mM creatine phosphate, 1 mM EGTA, 22°. Additions as indicated.

some experiments of this type, in which 1 mM EGTA was also included in the reaction medium. The tension response to ATP was well maintained in these experiments, and no significant spontaneous relaxation was observed. Addition of stepwise increments of calcium produced little effect until a total calcium concentration of 9×10^{-5} M was achieved, corresponding to $2{\cdot}0 \times 10^{-7}$ M Ca^{2+}, which almost doubled the tension.* A paired fiber which had been exposed to 2×10^{-4} M ryanodine yielded a somewhat greater initial tension after ATP, which was almost doubled when a total calcium concentration of 4×10^{-5} M (8×10^{-8} M Ca^{2+}) was reached. This experiment was confirmed repeatedly, and strongly suggests that the effects of ryanodine do not depend exclusively on the calcium pump. It is also significant that considerable tension development occurred in the presence of 1 mM EGTA with no added calcium other than that present as reagent impurities.

Experiments on chronically glycerinated fibers

Further confirmation was obtained in experiments on desoxycholate-treated chronically glycerinated fibers (Fig. 3), in which it is very unlikely that any functioning residue

* The concentration of Ca^{2+} in EGTA calcium buffer solutions used in this and the two following papers was calculated from the equation

$$p[Ca^{2+}] = 2pH - 7{\cdot}28 + \log_{10}[EGTA]/[CaEGTA].$$

This is an accurate approximation to the complete formal equation[25] in the pH range 4·0–7·5, where $pK_{Ca} = 11{\cdot}0$, $pK_1 = 9{\cdot}43$ and $pK_2 = 8{\cdot}85$,[26] provided that $[Mg^{2+}]/[Ca^{2+}]$ does not approach K_{Mg}/K_{Ca} ($6{\cdot}3 \times 10^5$). These conditions are satisfied throughout the present experiments.

of calcium-pumping structures remains. As before, tension after ATP was well maintained, and somewhat greater in paired fibers exposed to ryanodine. Once again, a further increase in tension could be elicited by the addition of calcium; the threshold Ca^{2+} concentration was lower in ryanodine-treated fibers ($1{\cdot}0 \times 10^{-7}$ M) than in paired controls ($8{\cdot}5 \times 10^{-7}$ M).

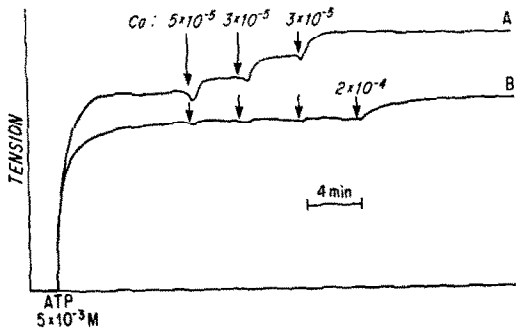

FIG. 3. Influence of desoxycholate pretreatment on the response of 120-day glycerinated rabbit psoas fibers to stepwise addition of calcium in the presence (A) and absence (B) of ryanodine (2×10^{-5} M, added initially). Conditions as in Fig. 2. All additions after ATP refer to total calcium.

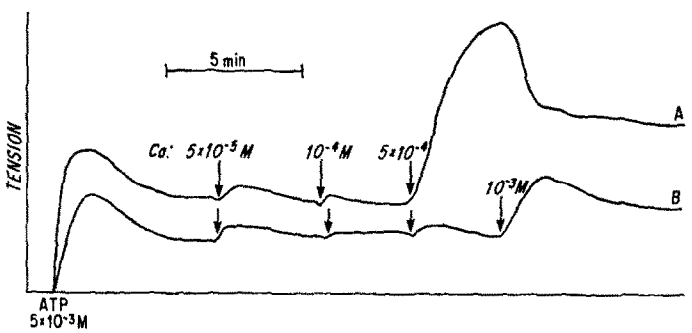

FIG. 4. Effect of stepwise increments of calcium concentration on the spontaneous relaxation of ATP-induced contraction of 120-day glycerinated rabbit psoas fibers, with (A) and without (B) ryanodine. Conditions as in Fig. 2. All additions after ATP refer to total calcium.

Chronically extracted fibers not treated with desoxycholate yielded similar results, although the tension generated by ATP was not well maintained if EGTA but no added calcium was present at the time of addition (Fig. 4). Ryanodine-treated fibers yielded greater tensions than paired controls, and stepwise addition of calcium revealed a greater sensitivity to calcium in ryanodine-treated fibers.

If calcium was included in the system initially, either buffered with EGTA or in excess (5×10^{-5} M), chronically extracted fibers showed no spontaneous relaxation after ATP but could be induced to relax by about 50 per cent by the addition of a soluble muscle extract (Fig. 5). In distinction to most soluble extracts of skeletal muscle previously referred to in the literature as soluble relaxing factor, this effect is independent of the Ca^{2+} concentration and has previously been reported in calcium-buffered systems.[27] Fibers relaxed in this way retain sensitivity to ryanodine; the

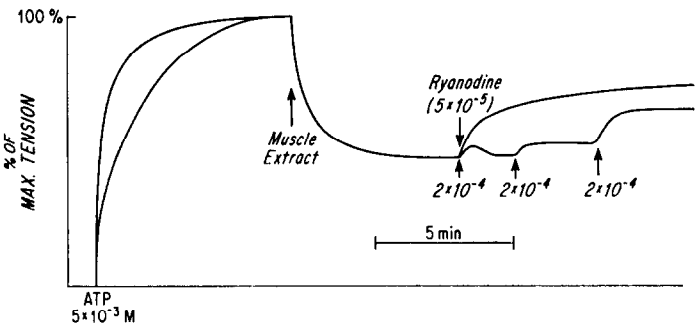

FIG. 5. Influence of ryanodine on 120-day glycerol-extracted fibers after ATP and a soluble muscle extract (0·3 ml) at times indicated. Conditions as in Fig. 1, except that 5×10^{-5} M $CaCl_2$ was included in both fibers, and 1 mM EGTA was also included in the fiber yielding the lower record.

concentration required is lower (5×10^{-5} M) when Ca^{2+} is present in excess than when a lower buffered Ca^{2+} level is maintained (6×10^{-4} M).

DISCUSSION

The results described indicate that an effect of ryanodine on glycerol-extracted muscle fibers can be demonstrated under conditions in which residual activity of calcium-pumping elements is very unlikely. While clear-cut evidence of interference with the calcium pump by ryanodine has previously been published,[20] it now appears probable that this alkaloid exerts an additional action on the contractile elements themselves. Although Edwards *et al.* suggested in 1948[1] that ryanodine might affect the contractile mechanism directly, no explicit evidence was adduced, and subsequent work failed to demonstrate an action on actomyosin ATPase or light scattering, or on chronically glycerinated muscle fibers.[2, 17] This failure is probably related to an inappropriate choice of conditions; in particular, the critical role played by calcium in regulating contractile events was not appreciated at that time.

An effect of ryanodine on briefly (1–5 days) glycerinated fibers was demonstrated by Jenden and Haslett,[18] and interpreted in terms of interference with a relaxing system retained in these fibers. The subsequent finding that ryanodine impaired the efficiency of the calcium pump[20] which remains active in briefly extracted fibers[22, 23] appeared to provide a satisfactory basis for the gross effects of ryanodine on both glycerol-extracted fibers and intact muscle. It is hard to reconcile the present data with such a unitary view of its mechanism of action. Desoxycholate in the concentrations employed here has previously been shown to inactivate the intrinsic relaxing activity and calcium pump of skeletal muscle,[24] and the failure of treated fibers to relax spontaneously after ATP-induced tension development is in agreement with this. The calcium-concentrating ability of rabbit psoas fibers disappears within about one week of glycerol extraction.[22] Yet ryanodine induced an increase in tension in fibers extracted in glycerol for 3–4 months, after a subsequent exposure to desoxycholate. Clearly, the actions of ryanodine cannot be entirely mediated by effects on the calcium pump.

The chelator EGTA was employed as a metal buffer to control Ca^{2+} concentration in the relevant range, and allowed an assessment to be made of the interaction of this

ion and ryanodine in the presence of a relatively high concentration of Mg^{2+}. A positive interaction was consistently observed. When an excess of Ca^{2+} was present (EGTA omitted: Fig. 5) the concentration of ryanodine required to induce further contraction was much less (5×10^{-5} M) than when a lower buffered Ca^{2+} level ($1{\cdot}0 \times 10^{-7}$ M) was maintained. On the other hand, experiments in which the Ca^{2+} concentration was elevated in a stepwise manner consistently showed that ryanodine lowers the Ca^{2+} concentration required for contraction.

In summary, the experiments described lead to the conclusion that in addition to the known effect of ryanodine on the calcium pump, this alkaloid exerts a direct action on contractile elements which may involve a sensitization to calcium ions.

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