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EFFECT OF RYANODINE ON SKELETAL MUSCLE RETICULUM CALCIUM ADENOSINE TRIPHOSPHATASE (CaATPase)

ALAN S. FAIRHURST

Department of Medical Pharmacology and Therapeutics,
California College of Medicine, University of California, Irvine, Calif. 92664, U.S.A.

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Abstract—Ryanodine activates the CaATPase of a heavy (2000–8000 *g*) sub-unit of skeletal muscle sarcoplasmic reticulum, with K_A (for half-maximal activation) of $1{\cdot}49 \times 10^{-5}$ M in the presence of 5×10^{-3} M MgATP and 10^{-6} M free Ca. K_A is only $3{\cdot}7 \times 10^{-6}$ M when the muscle fraction is exposed to ryanodine in the absence of Mg, and K_A in the presence of 5 mM free Mg is $3{\cdot}3 \times 10^{-5}$ M, with Mg competitively inhibiting the effect of the alkaloid. Ryanodine does not affect ADP–ATP exchange rate or the steady state level of phosphoprotein, but activates CaATPase by altering Ca permeability and decreasing intravesicular free Ca. The effect of ryanodine increases with temperature, is not affected by extensive dialysis and appears irreversible, with 1 mole of drug bound per 10^7 g of membrane protein; the drug has little effect on standard fragmented sarcoplasmic reticulum (FSR) preparations.

THE ALKALOID ryanodine produces an irreversible contracture of vertebrate skeletal muscle and negative inotropism in cardiac muscle[1–3] and appears to be a highly specific agent with which to investigate muscle function. Procita[4], Elison and Jenden[5] have suggested that one site of action of the alkaloid lies within the contractile protein complex of skeletal muscle, although in cardiac muscle the troponin–Ca interaction is not affected by the drug.[6] In a study attempting to correlate the pharmacological action on skeletal muscle with effects on subcellular membranal preparations, it was shown[7] that ryanodine inhibits net Ca uptake in oxalate-containing media by a heavy fraction of muscle, sedimenting between 2000 and 8000 *g*, but has little effect on Ca uptake by the more widely studied fragmented sarcoplasmic reticulum (FSR) fraction isolated between 12,000 and 35,000 *g*; such interference *in vivo* with an intracellular Ca transport mechanism in skeletal muscle could lead to a continued high level of free Ca in the vicinity of the contractile proteins, and resultant prolonged activation of the contractile complex and contracture. Although this heavy muscle fraction is not homogeneous and contains some contaminating mitochondrial fragments, these do not contribute to the Ca uptake activity of the fraction,[7,8] and the drug-sensitive component appears to be derived from the terminal sac sub-units of the sarcoplasmic reticulum,* so that these elements would thus appear to comprise one site of action of ryanodine. Weber and Herz[8] and Fuchs[9] have presented data which suggest that the site of action also of caffeine in skeletal muscle is within a membranous component of a heavy fraction of muscle, rather than the light FSR fraction.

The effect of ryanodine has been studied also on calcium efflux from a heavy muscle fraction which had been pre-loaded with calcium oxalate,[10] and the alkaloid was found

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to alter membrane Ca permeability and cause a marked increase in Ca efflux, which could partly account for the inhibition of net Ca uptake observed in earlier studies. However, the rather unphysiological conditions of these efflux experiments, particularly the absence of ATP necessary to suppress concurrent Ca uptake during the efflux measurements, somewhat limited the significance of the findings. Therefore, another experimental approach was sought for evidence that ryanodine acts on a part of the sarcoplasmic reticulum by modifying Ca permeability rather than by interfering directly with the Ca transport system. One parameter for such study is the Ca-activated ATPase of the heavy muscle fraction, since Weber[11] has shown that, in the absence of oxalate, Ca transport and the associated CaATPase are controlled in part by the level of free Ca accumulating internally in the SR vesicles as a result of Ca transport, and that the stimulatory effect of surface active agents on CaATPase of fragmented SR preparations is due to an increase in Ca permeability leading to a decrease in internal free Ca concentration. In early studies with ryanodine,[12] an effect had been demonstrated on CaATPase in experiments in which small non-saturating amounts of Ca were rapidly accumulated in the presence of oxalate, and the alkaloid was found to prolong Ca uptake time and increase the amount of ATP hydrolysis, to produce an apparent uncoupling of Ca transport. However, the rapidly changing levels of the free Ca and the presence of oxalate in the medium limited the extent of such experiments. In the present study, free Ca levels in the medium were, therefore, maintained constant within the physiological range by the use of CaEGTA* buffers, and the effect of the alkaloid was studied in the presence of ATP. The data presented here indicate that ryanodine at pharmacologically significant concentrations causes a pronounced stimulation of CaATPase and a decreased rate of net Ca transport by increasing the Ca permeability of the membranes of the drug-sensitive component of the heavy muscle fraction, and suggest that this effect is one of the bases of the pharmacological action of this alkaloid on skeletal muscle.

MATERIALS AND METHODS

Fraction 2–8 was isolated between 2000 and 8000 *g* from a homogenate of rabbit skeletal muscle as described previously,[10] extracted for 5 min at 0° with 0·6 M KCl to remove any contaminating actomyosin, pelleted and stored in 100 mM KCl–30 mM imidazole at pH 7·0 and used within 24 hr. Further fractionation by differential centrifugation or on sucrose density gradients causes significant damage as indicated by increased CaATPase activity, so that the fraction was used without additional purification. A reference fragmented sarcoplasmic reticulum (FSR) preparation was obtained from the same homogenate by collecting material sedimenting between 12,000 and 35,000 *g* which was extracted with 0·6 M KCl, then fractionated on a linear 30–50 per cent sucrose gradient for 90 min at 100,000 *g* and the top white band of material harvested. MgATPase activity was assayed for 10 min at 30° in 5 ml of medium containing approximately 0·5 mg of protein; 1 mM EGTA; 5 mM ATP; 5 mM $MgCl_2$; 100 mM KCl and 30 mM imidazole at pH 7·0. After removal of protein with 5% trichloroacetic acid, inorganic phosphate was assayed by the method of Fiske and Subbarow.[13] CaMgATPase was determined in a similar medium but with the EGTA replaced by 5 mM CaEGTA buffer which maintained the free Ca concentration of

* EGTA: ethylene glycol-bis-(aminoethyl ether)-*N*,*N*′-tetra-acetic acid.

10^{-6} M or other levels as noted in the text; CaATPase activity was calculated by subtracting MgATPase rates from those of CaMgATPase. NaKMgATPase assays were performed with the MgATPase medium but containing additionally 100 mM NaCl and 20 mM KCl. Tritium-labeled ryanodine was prepared in this laboratory[14] and used at a specific activity of 483 mCi/m-mole. Equilibrium binding studies were performed by incubating 15 mg of fraction in 5 ml of 10 mM KCl–30 mM imidazole at pH 7·0 containing labeled drug for 5 min at 30°, then cooling and centrifuging at 100,000 *g* for 30 min. Aliquots of supernatants and pellets were counted in Bray's solution containing soluene and corrected for quenching with internal standards. The volume of supernatant trapped in the pellets was estimated by measurement of ^3H_2O similarly trapped in parallel experiments and corrections were applied for this. In dialysis experiments, the tubing was boiled in 5% $NaHCO_3$ and washed thoroughly to prevent inactivation of the muscle fraction. The fraction was incubated with labeled drug for 5 min at 30° and then dialyzed against 30 vol. of KCl buffer at approximately 5° with six changes in dialyzing medium; the experiments were completed within 18 hr, since otherwise significant activation of CaATPase was observed in the control (no drug) experiment. Phosphoprotein formation was assayed by the method of Makinose[15] but after incubation at 30° for 5 sec, using ATP^{32} prepared as described by Glynn and Chapell;[16] this phosphoprotein could be decomposed by 0·2 M hydroxylamine at pH 5·3 and thus resembles the phosphoprotein of FSR. ADP^{32}–ATP exchange was assayed at 30° as described by Makinose.[15] Calcium uptake was measured in the presence of oxalate,[7] and protein was assayed by the method of Lowry *et al.*[17]

RESULTS

A comparison of the ATPase activities of fraction 2–8 and FSR was made under various conditions to establish whether the CaATPase activity of fraction 2–8 is regulated by the internal free Ca concentration, as has been described for FSR preparations by Weber;[11] these data are shown in Fig. 1. In expt. A, EGTA was

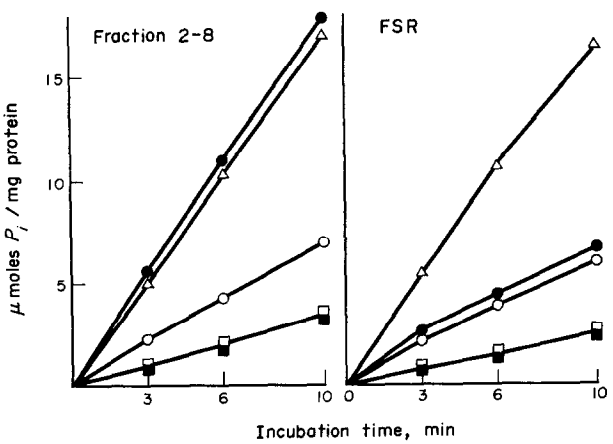

FIG. 1. ATPase activities of fraction 2–8 and FSR under various conditions. Activities represent: (A, ■) MgATPase; (B, □) MgATPase with 10^{-4} M ryanodine; (C, O) CaMgATPase; (D, △) CaMgATPase with 5 mM oxalate; and (E, ●) CaMgATPase with 10^{-4} M ryanodine. Protein concentration was 0·14 mg/ml.

present to suppress ionization of any endogenous Ca, and the low activity, therefore, represents MgATPase, which is not affected by ryanodine (expt. B). In the presence of CaEGTA, which maintains a free Ca concentration of approximately 10^{-6} M in the medium, the ATPase rate is doubled and is essentially linear over the 10-min assay period in expt. C. Addition of oxalate (expt. D) substantially increases the CaATPase of both fraction 2–8 and FSR; since the action of oxalate is merely to suppress the concentration of free Ca within the vesicles as a result of internal precipitation of Ca oxalate,[18] this result indicates that CaATPase of fraction 2–8 as well as that of FSR is limited by the internal Ca^{2+} concentration. Experiment E shows the effect of 10^{-4} M ryanodine, fraction 2–8 being markedly stimulated whereas FSR is little affected by the alkaloid. The effects of lower concentrations of ryanodine on fraction 2–8 are shown in Fig. 2 where it can be seen that detectable stimulation of

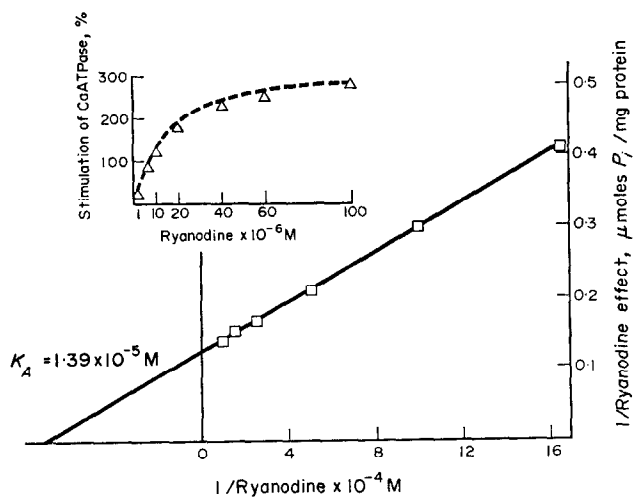

FIG. 2. Effect of different concentrations of ryanodine on CaATPase of fraction 2–8. Ryanodine effect is defined as the absolute stimulation of CaATPase produced by the drug. Protein was 0·13 mg/ml.

CaATPase is obtained at 10^{-6} M ryanodine, at which concentration the characteristic pharmacological effects of this alkaloid are obtained with isolated intact muscle preparations. Further analysis of this experiment is shown in the double reciprocal plot of ryanodine concentration v. ryanodine effect, indicating that the concentration of alkaloid needed for half-maximal activation (K_A) of the membrane CaATPase is $1{\cdot}49 \times 10^{-5}$ M. The control CaATPase activity of fraction 2–8 preparations increases with aging, with a parallel decrease in net Ca^{45} uptake (Table 1) and, since the ryanodine effect is defined here as that increase in CaATPase due to the alkaloid, the apparent K_A determined under these conditions varies somewhat from preparation to preparation depending on the age of the fraction and integrity of the membranes, with a range of $1{\cdot}3$ to $2{\cdot}5 \times 10^{-5}$ M.

TABLE 1. ALTERATION OF CaATPASE AND Ca UPTAKE ACTIVITIES WITH AGING OF FRACTION 2–8*

Preparation	CaATPase (μmole/mg/ 10 min)	Ca^{45} uptake (μmole/mg/ min)
Fresh	3·8	1·5
One-day-old	4·6	1·2
Two-days-old	6·2	0·9

* CaATPase data corrected for MgATPase. Ca^{45} uptake was measured in the presence of 5 mM oxalate. Protein concentration was 0·14 mg/ml in CaATPase experiments and 0·025 mg/ml in Ca uptake experiments.

Although it has not been possible to remove traces of mitochondrial contaminants from fraction 2–8, and these have been shown to make no contribution of Ca uptake by this fraction, the effects of azide and oligomycin were examined on MgATPase and CaATPase of fraction 2–8 since these agents inhibit CaATPase of cardiac mitochondria[19] by some 85 per cent. Table 2 shows that azide stimulates CaATPase slightly

TABLE 2. EFFECT OF MITOCHONDRIAL INHIBITORS ON MgATPASE AND CaMgATPASE OF FRACTION 2–8 AND EFFECTS OF Na^+, K^+ AND OUABAIN*

Additions	MgATPase	MgCaATPase	CaATPase
None	4·07	8·60	4·53
Azide, 5 mM	2·40	7·93	5·53
Oligomycin, 3 μg/mg protein	2·90	7·87	4·97
Ethanol	3·68	8·65	4·97
Ryanodine, 10^{-4} M	4·00	16·72	12·72
Azide + ryanodine	2·40	14·90	12·50
Oligomycin + ryanodine	2·40	16·44	14·04
NaCl, 100 mM + KCl, 20 mM	4·15	—	—
NaCl + KCl + ouabain, 0·2 mM	4·20	—	—

* Assays as in Methods; values indicate micromoles of phosphate liberated per milligram per 10 min. CaATPase was calculated from (CaMgATPase − MgATPase). Protein concentration was 0·13 mg/ml.

while the effect of oligomycin is the same as that of the ethanol solvent employed with this antibiotic, and that the increment in CaATPase due to ryanodine is unaffected by azide or oligomycin; also, NaKATPase activity of this preparation is quite small. The influence of temperature was studied on the effect of ryanodine since the action of the alkaloid in intact muscle is markedly dependent on temperature.[20] Also Fuchs[9] has shown that Ca uptake by a somewhat similar rabbit muscle sedimenting between 1,500 and 10,000 *g* exhibits sensitivity to caffeine only at higher temperatures. Table 3 shows that the effect of ryanodine increases with temperature so that the effects of ryanodine *in vivo* could be expected to be greater than that shown at 30° in Fig. 2.

TABLE 3. INFLUENCE OF TEMPERATURE ON RYANODINE EFFECTS ON CaATPASE OF FRACTION 2–8*

Activity assayed	Temperature		
	25°	30°	37°
MgATPase	2·29	2·75	4·35
CaATPase	2·52	3·89	5·9
CaATPase + ryanodine, 10^{-4} M	9·39	13·97	23·8

* Activities were assayed as in Methods, but at temperatures indicated. Data indicate μmole phosphate per milligram per 10 min. CaATPase values are corrected for MgATPase. Protein was 0·12 mg/ml.

Since both oxalate and ryanodine stimulate CaATPase of fraction 2–8, and since oxalate acts by suppressing the internal free Ca level, fraction 2–8 was exposed concurrently to both of these agents to determine whether their effects were additive. Figure 3 shows that CaATPase activity increases with increasing oxalate concentration

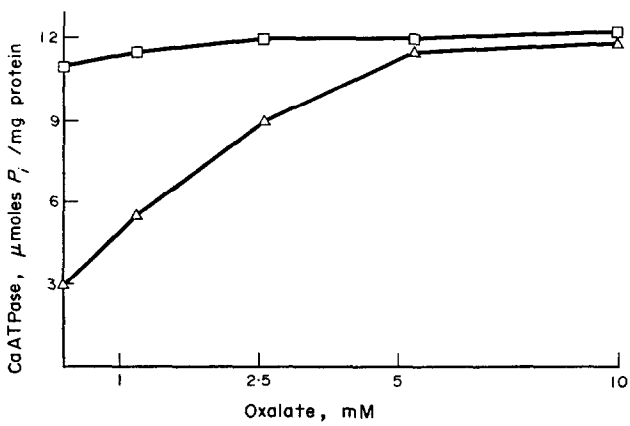

FIG. 3. Effect of ryanodine and potassium oxalate on CaATPase of fraction 2–8. △—△, ryanodine absent; □—□, 10^{-4} M ryanodine added. MgATPase activity, which is unchanged by oxalate or ryanodine, has been subtracted from the data. Protein was 0·1 mg/ml.

and that ryanodine further stimulates CaATPase; however, little additional effect of ryanodine is seen in the presence of an optimal concentration of 10 mM oxalate. Since no evidence has been found for formation of a metal–ryanodine complex, these results suggest that ryanodine acts by increasing the Ca permeability of the membranes and thus causing a decrease in the internal Ca concentration, or that the alkaloid acts by modifying the interaction between the internal free Ca and the Ca transport system, to reduce the extent of product inhibition of Ca transport. Fraction 2–8, therefore, was treated with 0·5% desoxycholate (DOC) to increase membrane Ca permeability and allow access of Ca to the inner surface of the membranes; the effect of ryanodine was examined after such treatment. Figure 4 shows that the DOC-treated material exhibits high CaATPase activity which is inhibited by Ca levels greater than 10^{-5} M, and that the addition of ryanodine to the incubation medium produces no significant additional

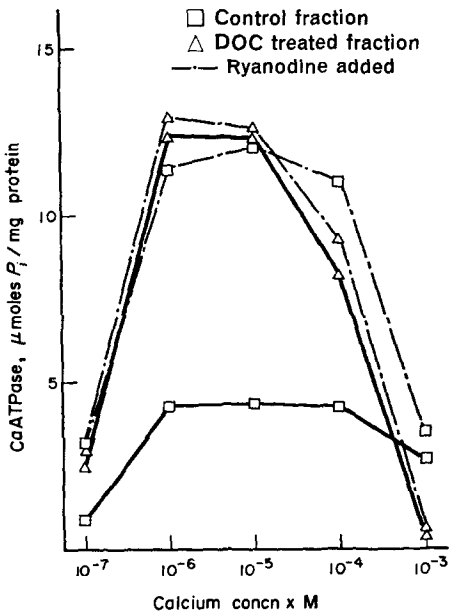

FIG. 4. Effect of level of ionized Ca and 10^{-4} M ryanodine on CaATPase activity of fraction 2–8 before and after treatment with 0·5% desoxycholate (DOC). Protein was 0·14 mg/ml. Five mM total EGTA was present in the 10^{-7} M to 10^{-5} M Ca experiments, with $CaCl_2$ added and free Ca calculated from the binding constants of Chaberek and Martell[21] for EGTA and of Burton[22] for ATP.

effect at any Ca level, suggesting that the effect of ryanodine on intact membranes of fraction 2–8 is not due to an effect on the last step in the Ca transport and the associated CaATPase sequence, namely the dissociation of the calcium–carrier complex at the inner surface of the membranes. Figure 3 also shows that the effect of 10^{-4} M ryanodine on the CaATPase of the standard 2–8 fraction is quantitatively similar to that produced by DOC, although the mechanisms by which these agents affect membrane structure are not known. Other experiments in this laboratory have shown that treatment of fraction 2–8 with Triton X-100 or by sonication also induces high CaATPase activity which is then unaffected by ryanodine, so that intact membranes are apparently necessary for demonstration of an effect of the alkaloid.

Although few details are known of the postulated steps in the over-all CaATPase reaction sequence in sarcoplasmic reticulum preparations, two such partial reactions are experimentally accessible and were examined as possible sites of action of ryanodine. The initial step in the CaATPase sequence, i.e. the interaction of ATP with the membranes, can be measured by assaying ATP–ADP^{32} exchange rates. Table 4 shows that ATP–ADP exchange is too rapid to be considered rate-limiting in the over-all CaATPase reaction, being some 7-fold greater than the CaATPase rate, and is not affected by ryanodine. During CaATPase activity, fraction 2–8 forms a phosphoprotein which is sensitive to hydroxylamine degradation similar to that of FSR preparations[15] and thus may comprise an intermediate of the Ca transport system of fraction 2–8. Although the rate of formation of this phosphoprotein is too great to permit kinetic studies, the steady state level was examined and is not significantly affected by ryanodine or by oligomycin.

TABLE 4. EFFECT OF RYANODINE ON ADP–ATP EXCHANGE RATE AND ON PHOSPHOPROTEIN FORMATION BY FRACTION 2–8*

Present in assay	Exchange rate (μmole/mg/10 min)	P-protein labeled (μmole/10^6 g protein)
Mg, EGTA	5·0	0·26
Mg, CaEGTA, pCa = 6	46·5	1·40
Mg, CaEGTA + ryanodine, 10^{-4} M	44·5	1·30
Mg, CaEGTA + oligomycin, 3 μg/mg	—	1·32

* Assays as described in Methods. Protein concentration in exchange experiments was 0·015 mg/ml and 0·5 mg/ml in P-protein experiments.

From studies with isolated intact skeletal muscles,[2,3] it is known that the pharmacological effects of ryanodine are not altered by prolonged washing of the muscle and are apparently irreversible, so that it was of interest to determine whether the effect of the alkaloid on fraction 2–8 was similarly irreversible. Repeated washing involving high speed sedimentation and resuspension of the muscle fraction causes damage, so the fraction was incubated with tritium-labeled ryanodine and then dialyzed extensively to remove the drug. Table 5 shows that the activation of CaATPase produced initially by exposure to 10^{-4} M ryanodine remains essentially constant during the experiment, while the concentration of drug in the dialysis sac and the amount of ryanodine bound per milligram of membrane protein are markedly reduced, suggesting either that ryanodine produces a damaged, stable modification of the membranes which persists after removal of the drug or that a ryanodine–membrane complex is formed which is stable but which involves very small amounts of drug, corresponding in this experiment to approximately 1 mole of ryanodine per 10^7 g of membrane protein.

TABLE 5. BINDING OF LABELED RYANODINE TO FRACTION 2–8 AND CONCURRENT CaATPASE ACTIVITY*

	Assay time (hr)			
	0	9	15	18
Ryanodine in sac (nmole/ml)	117·5	2·95	0·44	0·285
Ryanodine bound (nmole/mg protein)	33·69	0·86	0·13	0·0955†
CaATPase activity (μmole/mg/10 min)	13·8	13·75	13·7	14·0
CaATPase of control dialyzed fraction	3·5	3·65	3·8	3·9

* Fraction 2–8 was incubated with labeled ryanodine in KCl buffer for 5 min at 30°, then dialyzed against 30 vol. of KCl buffer at 5° with six changes in medium over 18 hr. Samples were removed at times indicated and analyzed for total ryanodine and for bound ryanodine after ultrafiltration through XM-50 Amicon filters. The control experiment was identical but ryanodine was omitted. CaATPase was determined as in Methods, data corrected for Mg ATPase activity. Initial protein concentration in sac was 2·6 mg/ml and in CaATPase assays was 0·14 mg/ml.

† After 18 hr of dialysis, 1 mole of ryanodine remains bound per $1·047 \times 10^7$ g of protein.

Equilibrium binding of ryanodine was then studied by incubating fraction 2–8 at 30° with various concentrations of labeled alkaloid and then separating the components by ultracentrifugation. Assays of CaATPase activity were run on aliquots from the same experiments to allow correlation of activation of CaATPase with amounts of drug bound to the fraction. Since Mg is known to block effects of ryanodine on Ca efflux[10] from fraction 2–8 and to modify the effects of the alkaloid on isolated intact muscle,[23] ryanodine binding and effects on CaATPase were studied also in the presence of 5 mM Mg^{2+}. Figure 5 shows that ryanodine binding increases linearly with

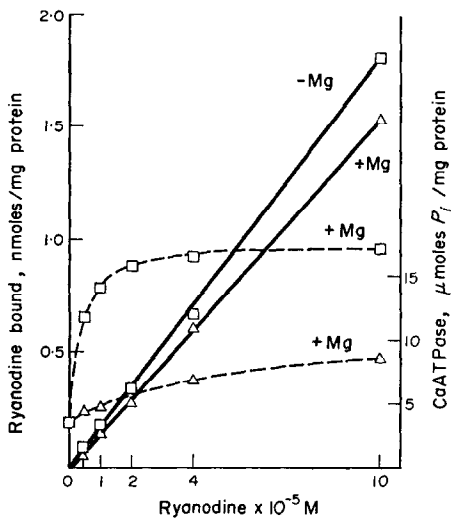

FIG. 5. Effect of 5 mM $MgCl_2$ on the equilibrium binding of radioactive ryanodine to fraction 2–8 (——) and on the effect of ryanodine on CaATPase activity of fraction 2–8 (- - - -). The fraction was preincubated at 30° for 5 min with labeled ryanodine ± $MgCl_2$, then assayed for binding and CaATPase activity as in Methods. Protein concentration during binding was 3 mg/ml and 0·13 mg/ml in CaATPase assays.

drug concentration, with no evidence of saturation of binding sites at 10^{-4} M ryanodine although saturation of CaATPase activation occurs. Mg has relatively little effect on ryanodine binding, but markedly reduces the activation of CaATPase produced by the drug, suggesting that Mg reduces the pharmacologically significant interaction between the membranes and ryanodine, or prevents activation of CaATPase and membrane damage subsequent to ryanodine binding. The relationship between ryanodine and Mg was studied further by preincubating fraction 2–8 with different concentrations of drug in the presence or absence of Mg and then assaying CaATPase activity in media containing the standard total Mg concentration (5 mM). The double reciprocal plot of ryanodine concentration vs. ryanodine effect (Fig. 6) shows that when fraction 2–8 is exposed to the alkaloid in the presence only of buffer and KCl, the K_A for ryanodine is $3{\cdot}7 \times 10^{-6}$ M and that when the fraction is exposed to ryanodine in the presence of 5 mM Mg^{2+}, the K_A is increased to $3{\cdot}3 \times 10^{-5}$ M. Since in both these series of experiments the concentration of total Mg in the CaATPase assay medium is the same, these results show that the effect of the alkaloid is not reversed by subsequent exposure to Mg, and that when the muscle fraction is exposed concurrently to Mg and ryanodine, the metal competitively inhibits the effect of the alkaloid.

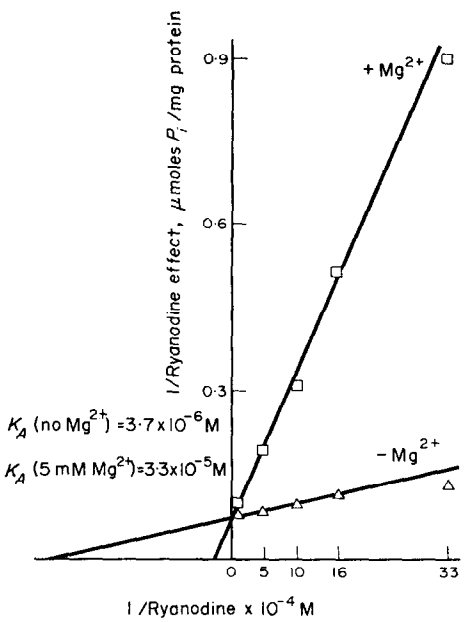

FIG. 6. Effect of 5 mM $MgCl_2$ in preincubation medium on K_A for ryanodine activation of CaATPase of fraction 2–8. The fraction was preincubated at 30° for 5 min with ryanodine ± $MgCl_2$, then assayed for CaATPase activity in systems containing the standard 5 mM $MgCl_2$. Ryanodine effect is defined as the absolute increase in CaATPase due to the drug. Protein in assay was 0·14 mg/ml.

DISCUSSION

A number of agents have been shown to affect CaATPase activity of skeletal muscle FSR preparations, but only ryanodine, caffeine and methadone[24] have so far been found to affect preferentially CaATPase and the associated net Ca transport of the heavy muscle fraction 2–8. To the extent that effects of ryanodine at this subcellular level are irreversible, occur at pharmacologically significant micromolar concentrations in systems containing physiological levels of Ca ions and ATP and are similarly influenced by magnesium, it is reasonable to believe that the cellular structures from which fraction 2–8 are derived comprise a site of action of ryanodine *in vivo* and may be involved in the physiological control of skeletal muscle activity. The low NaKATPase of fraction 2–8 indicates little contamination by sarcolemmal fragments; the studies with azide and oligomycin confirm earlier findings that mitochondria are not involved in the actions of caffeine and ryanodine on this fraction, while electron microscope examination of the fraction suggests that many of the vesicular structures may be derived from the terminal sacs of the SR, which have been implicated as Ca storage and release sites.[25]

The stimulation of CaATPase by ryanodine presumably is due to an effect on some rate-limiting step in the over-all CaATPase sequence. Panet *et al.*[26] have suggested that phosphoprotein hydrolysis is such a step, although their hydrolysis rates would appear to be lower than those occurring *in vivo*, and the present study shows that ryanodine has no effect on steady state phosphoprotein levels. However, under the experimental conditions of this report, it would seem more likely that the rate-limiting

step is associated with the intravesicular free Ca levels, since Hasselbach and Makinose[18] and also Weber[11] have shown that such Ca has a regulating function on the Ca transport activity of the FSR, and it has been shown above that CaATPase of fraction 2–8 is similarly regulated by intravesicular Ca^+ concentration. The mechanism whereby this Ca regulates CaATPase is not known, but apparently is not directly affected by ryanodine, since the experiments with DOC (Fig. 4) show that the inhibitory effects of high Ca are essentially unchanged by the alkaloid. The data are consistent with the hypothesis that ryanodine alters the regulated state of CaATPase by decreasing intravesicular Ca via an effect on membrane Ca permeability, since the stimulatory effect of ryanodine on CaATPase is not seen when an optimal concentration of oxalate is present to suppress intravesicular free Ca to levels sufficiently low to permit maximal CaATPase activity, but that the effect of the alkaloid is apparent when added to a system where the intravesicular Ca is at a high inhibitory level due to the Ca transport in the absence of oxalate. This suggests either that the effect of ryanodine is antagonized by a low intravesicular free Ca level, or that the alkaloid acts by suppressing the intravesicular Ca concentration. However, ryanodine is known to be an effective inhibitor of net Ca uptake in the presence of oxalate[7] so that obviously the effect of the drug is not antagonized by oxalate or by low intravesicular free Ca. Thus it would appear that the activating effect of ryanodine on CaATPase may depend on some action which suppresses intravesicular Ca, and since no evidence has been found for a ryanodine–Ca complex, it is likely that this effect is due to a ryanodine–membrane interaction resulting in an increased permeability of the membranes, as was also indicated by effects on Ca efflux.[10]

The present heterogeneity of fraction 2–8 precludes meaningful analysis of membrane composition, but the different sensitivities of this fraction and FSR to ryanodine, caffeine and methadone may provide an approach to the correlation of structure and function in the membranes of the heavy fraction. The irreversible activation of CaATPase by low concentrations of ryanodine indicates an interaction with a component or configuration perhaps specific to fraction 2–8, and this component may be physiologically significant in the transient modification of SR Ca permeability during muscle activation. The pyrrole–carboxylate moiety of ryanodine also appears important in this interaction, since the alcohol ryanodol has little pharmacological activity and does not affect CaATPase; it may also be significant that another pyrrole–carboxylate ester, batrachotoxin, has a different but very specific effect on ion transport in electrogenic membranes.[27]

Since fraction 2–8 is not homogenous, the linearity of ryanodine binding with ryanodine concentration (Fig. 5) probably reflects the summation of binding to numerous sites on extraneous protein and to non-specific as well as specific sites on the CaATPase complex under investigation, while the small effect of Mg on ryanodine binding supports this supposition that specific sites comprise only a small fraction of total binding sites. Although these data do not exclude the possibility that Mg does not interfere with specific binding *per se* but instead prevents membrane damage subsequent to ryanodine binding, the competitive nature of the inhibition of the ryanodine effect by Mg may indicate either direct competition or some allosteric effect of the metal, and since skeletal muscle contains some 8 mM total Mg it is likely that the effect of ryanodine *in vivo* is affected by the local concentration of ionized metal. This stabilizing-protective effect of Mg is not limited either to the interaction

with ryanodine or to the SR membranes, since Weber[28] has shown that the uncoupling effect of caffeine on a heavy SR fraction is greatly reduced in the presence of high Mg, while Peters and Fouts[29] have shown that Mg modifies the activity and kinetic and binding constants of another intracellular membrane system, the hepatic drug-metabolizing complex.

Only a very small amount of ryanodine, about 1 mole/10^7 g of membrane protein, remains bound to fraction 2–8 after dialysis, although CaATPase remains fully activated. It may be that this represents residual drug incidentally present in the membranes rather than drug directly related to the activated configuration of the CaATPase complex, since if the size of the Ca tranport unit is 10^5 g (i.e. the same size as the transport unit of FSR),[30] it seems unlikely that ryanodine interacts with only one site per 100 transport units, although this cannot be excluded with the present data. The other possibility, that ryanodine induces a stable modification in the membranes which is associated with an activated CaATPase function and which persists after removal of the drug, must be considered although there seems little pharmacological precedent for such a "hit and run" effect as once postulated[31] but later rejected[32] in studies on the mechanism of action of reserpine.

If such an interaction occurs *in vivo* in skeletal muscle, Ca flux across the reticulum membranes would be increased and the ryanodine-induced net shift of Ca ions into the sarcoplasmic compartment would then lead to continued activation of the actomyosin complex and contracture; this effect of the alkaloid might then be supplemented by a modification of the contractile element response by ryanodine as suggested by Procita[4] and by Elison and Jenden.[5] Although these studies have not yet been extended to cardiac muscle, a similar effect of ryanodine on a sub-unit of the cardiac SR might produce a shift of Ca ions into sarcoplasm followed by loss across the sarcolemma, since a large Ca flux occurs across this barrier during heart activity, leading to the pharmacological effect of loss of contractility in this muscle.

Although the present data suggest a relationship between the action of ryanodine on a subcellular muscle fraction and the induction of irreversible contracture in intact skeletal muscle, they do not account for the alkaloid-induced contractile failure of skeletal muscle observed *in vitro* in calcium-depleted muscle[33] and *in vivo* with low frequency stimulation.[34] It would seem that a further site of action of ryanodine must be postulated which assumes a critical role under the conditions used to elicit contractile failure. Although it remains difficult to explain the low frequency failure of Katz *et al.*,[34] this additional effect of ryanodine may involve a change in sarcolemmal Ca permeability as suggested by Hajdu,[33] since if this occurred in addition to the inhibition of net Ca uptake by a component of SR, loss of Ca across the sarcolemma might lead to contractile failure and thus to an effect of the drug on skeletal muscle which grossly resembles the effect on cardiac muscle.

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