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Mechanism of action of ryanodine on cardiac sarcoplasmic reticulum *

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Ryanodine was found to initially inhibit calcium uptake by cardiac sarcoplasmic reticulum. This initial depression was followed by a later marked stimulation of calcium uptake. These effects were noted when calcium uptake was measured in the presence or absence of oxalate. The requirement for preincubation with ryanodine was highly dependent on ryanodine concentration and temperature. The mechanism of action of ryanodine clearly was not an effect on oxalate entry or calcium oxalate precipitation because the effects were also observed in the absence of oxalate. Ryanodine also had no effect on passive calcium efflux from actively loaded vesicles. Because ryanodine had no effect on Ca^{2+}-ATPase activity under defined conditions of an ATP-regenerating system and no calcium gradient, we suggest ryanodine does not change the stoichiometry of the pump. Our results are consistent with the hypothesis that ryanodine closes a calcium channel in a subpopulation of the vesicles.

Introduction

Ryanodine is a neutral alkaloid isolated from *Ryania speciosa* that causes irreversible contracture in mammalian skeletal muscle and a decline in contractile force of mammalian cardiac muscle [1]. In these intact tissues there are several possible sites of action of ryanodine. Of these, the sarcoplasmic reticulum has attracted considerable attention. In isolated skeletal sarcoplasmic reticulum vesicles, ryanodine decreases calcium uptake and stimulates Ca^{2+}-ATPase activity presumably by increasing calcium efflux [2,3]. It has been reported that ryanodine decreases calcium uptake in cardiac sarcoplasmic reticulum [4,5] and that this appeared to be an apparent uncoupling of calcium transport from ATP hydrolysis [5]. In these studies calcium uptake was measured in the presence of oxalate which enhances uptake by precipitating transported calcium. When uptake was measured in the absence of oxalate, ryanodine either had no effect [4] or slightly stimulated uptake [5]. More recent studies have shown that ryanodine increases oxalate-supported calcium uptake in isolated cardiac sarcoplasmic reticulum vesicles [6–8] while Ca^{2+}-ATPase activity was slightly inhibited [6,8]. In these later studies, Jones and co-workers [6] initially attributed these effects of ryanodine to the closure of a ryanodine-sensitive calcium efflux

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Abbreviations: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

pathway, but later speculated that the effect can be explained by an increase in the pumping efficiency of the Ca^{2+}-ATPase [7]. We have investigated the effects of ryanodine on an isolated cardiac sarcoplasmic reticulum preparation in order to distinguish between alternative mechanisms of action of the drug. Our results suggest that there is a calcium channel distinct from the pump which is present in a subpopulation of the sarcoplasmic reticulum and which is closed by ryanodine.

Methods

Sarcoplasmic reticulum preparation. Cardiac sarcoplasmic reticulum vesicles were isolated from minced canine ventricular myocardium as described previously [9]. The protein concentration was estimated by the method of Lowry et al. [11] using bovine serum albumin as a standard.

Oxalate-supported calcium uptake + ATPase rates. The rate of oxalate-supported calcium uptake was estimated by assaying at various times the disappearance of ^{45}Ca from filtrates of the reaction bath using 0.45 μm Millipore filters [10]. The reaction bath was maintained at either 37°C or 27°C and contained 100 mM KCl, 20 mM imidazole buffer, 10 mM NaN_3, 10 mM potassium oxalate, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.2 mM added $CaCl_2$ (0.8 μCi ^{45}Ca/ml) and 0.02–0.06 mg/ml cardiac sarcoplasmic reticulum protein. Total calcium in the bath was estimated by atomic absorption spectrophotometry using a Perkin-Elmer Model 380 AAS, following extraction of the bath with 6% trichloroacetic acid, 10% HCl and 0.5% $LaCl_3$. Total calcium varied but was found to be about 250 μM. Most of the contaminating calcium was due to calcium in the Na_2ATP supplied by P and L Biochemicals (7 μmol/mmol). The pH of the uptake bath was adjusted to pH 6.8 or 7.1 as desired with HCl.

ATPase activity during oxalate-supported calcium uptake was measured from the same reaction bath used to measure calcium uptake. 2-ml aliquots of the reaction bath were filtered through 0.45 μm Millipore filters. An aliquot (0.5 ml) was taken for determination of ^{45}Ca and a 1.0 ml aliquot was transferred to a tube containing 1.0 ml chilled (4°C) 10% trichloroacetic acid. The released phosphate in the filtrate was assayed by colorimeteric methods [12]. The Ca^{2+}-ATPase activity was calculated as the difference in ATPase rate in a bath containing 0.2 mM Ca compared to one containing 2.5 mM EGTA. The 'basal' ATPase activity was less than 10% of the total ATPase activity.

Steady-state calcium uptake and passive efflux. Steady-state calcium uptake and passive efflux of calcium were estimated as described earlier [9]. The reaction bath (27°C) contained 100 mM KCl, 20 mM imidazole buffer (pH 6.8), 10 mM sodium azide, 100 μM Na_2ATP, 2.1 mM $MgCl_2$, 4 μM added calcium, 0.074 mg/ml cardiac sarcoplasmic reticulum proteins, 0.8 μCi ^{45}Ca/ml and 300 μM ryanodine, when added. Total calcium in the bath was determined by atomic absorption spectrophotometry as described above and was found to be 9.7 μM. The calcium content of the sarcoplasmic reticulum was determined by counting aliquots of the filtrates following Millipore filtration. Net passive efflux was begun by adding 2.5 mM EGTA to quench the operation of the Ca^{2+}-ATPase. Previous studies have shown that this results in a passive efflux of calcium through a route distinct from the pump [9,13].

ATPase activity. ATPase activity in the absence of a calcium gradient and as a function of calcium concentration was measured by a coupled enzyme reaction. The 3 ml reaction mixture was kept at 37°C and contained 100 mM KCl, 20 mM imidazole buffer (pH 6.8), 0.151 mg cardiac sarcoplasmic reticulum protein per ml, 1 mM phospho*enol*pyruvate, 0.142 mM NADH, 0.02 mg/ml lactate dehydrogenase, 0.013 mg/ml pyruvate kinase, 2 μM A23187, and 300 μM ryanodine, when added. The cardiac sarcoplasmic reticulum was preincubated in 1.8 ml containing 167 mM KCl, 33 mM imidazole, 500 μM ryanodine and 3.3 μM A23187 for 4 min at 37°C prior to initiation of the reaction by addition of ATP, $MgCl_2$, $CaCl_2$, NADH, lactate dehydrogenase, pyruvate kinase and phospho*enol*pyruvate previously equilibrated to 37°C. The calcium ionophore, A23187, was added in 5 μl DMSO immediately after addition of the cardiac sarcoplasmic reticulum. The final concentration of DMSO (0.17%) has previously been shown to have no effect on Ca^{2+}-ATPase activity [14]. The free calcium concentration was

varied by including 0–5000 μM EGTA in the solution containing $CaCl_2$. The free calcium concentrations were calculated by Dr. Alex Fabiato using apparent stability constants of $1.11 \cdot 10^{-6}$ M^{-1} for Ca^{2+}-EGTA, $3.06 \cdot 10^{3}$ M^{-1} for Ca^{2+}-ATP. $1.02 \cdot 10^{-4}$ M^{-1} for Mg^{2+}-ATP and $3.0 \cdot 10^{1}$ M^{-1} for Mg^{2+}-EGTA [15]. Total calcium was estimated by atomic absorption spectrophotometry as described above and was found to be 106 μM. ATPase activity was monitored by the decrease in absorbance of NADH at 340 nm using an Aminco DW-2 sectrophotometer. The ATPase rate was calculated using an extinction coefficient of $6.2 \cdot 10^{3}$ $M^{-1} \cdot cm^{-1}$ for NADH [16].

Materials. Ryanodine was purchased from S.P. Penick Corp. The calcium ionophore, A23187, was from Calbiochem. Na_2ATP was from P&L Laboratories. Reagents for the coupled enzyme assay (NADH, phospho*enol*pyruvate, lactate dehydrogenase and pyruvate kinase) were purchased from Sigma Chemical Co.

Results

An examination of the time-course of the effect of ryanodine on cardiac sarcoplasmic reticulum showed that oxalate-supported calcium uptake is initially depressed by ryanodine, followed by a later stimulation of uptake (Fig. 1). At 300–500 μM ryanodine, less than 0.5 min was required before stimulation of uptake was noticeable. The two curves of uptake in the presence of ryanodine in Fig. 1 are superimposable.

Because of this requirement for preincubation, we undertook a more detailed study of the rate of oxalate-supported calcium uptake as a function of ryanodine concentration and time of preincubation. In this case calcium uptake was calculated from a linear regression on six points taken within the first 0.6 min after beginning the reaction with ATP, Mg and Ca. The uptake rate calculated in this way was assigned to the time of pre-incubation rather than to the time corresponding to the mid-point of the assay. The results, shown in Fig. 2, indicate that the rate of calcium uptake can be greatly enhanced and that the stimulation is faster at higher concentrations of ryanodine. A consequence of this observation is that the dose-response curve for ryanodine depends on the time of

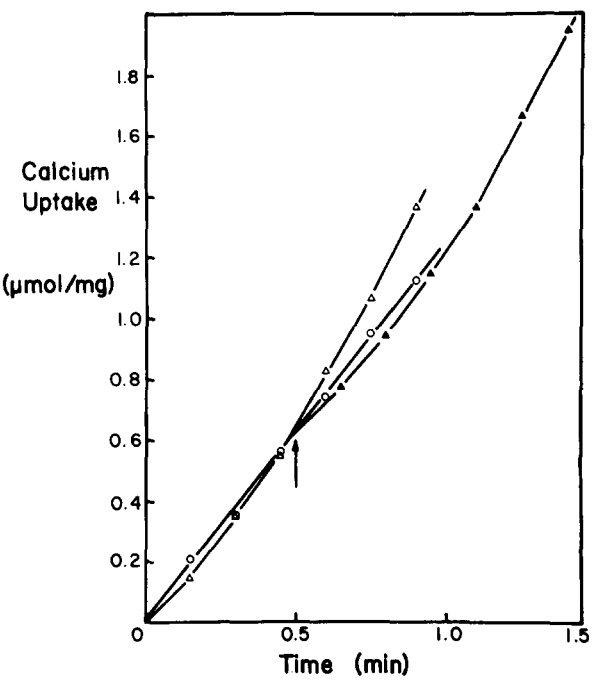

Fig. 1. Early effect of ryanodine on oxalate-supported calcium uptake by cardiac sarcoplasmic reticulum vesicles. Ca^{2+} uptake was determined by Millipore filtration as described in Methods. The bath was kept at 37°C, the pH was 7.1 and the cardiac sarcoplasmic reticulum concentration was 77 μg/ml. The uptake reaction was started by the addition of Mg, ATP and Ca pre-equilibrated to 37°C. Ryanodine concentration was zero (○), or 500 μM (△, ▲). Ryanodine was added at the start of uptake (△) or 0.5 min after beginning uptake (▲) at the arrow.

pre-incubation. The data shown in Fig. 3 indicate that considerable stimulation can occur at concentrations of ryanodine below 20 μM. Although we have not yet extended these studies, it appears likely that the cardiac SR will be sensitive to ryanodine below 1 μM.

The stimulation of oxalate-supported calcium uptake was also dependent on the temperature of preincubation (Fig. 4). In this case maximum stimulation was reached by about 4 min with 300 μM ryanodine at 37°C, but 10 min of pre-incubation were required at 27°C. The first-order plot of ryanodine stimulation gives a rate constant of 1.16 min^{-1} at 37°C and 0.24 min^{-1} at 27°C, suggesting a highly temperature-dependent step in the process (data not shown).

To determine whether or not ryanodine stimulation of calcium uptake was due to alteration of

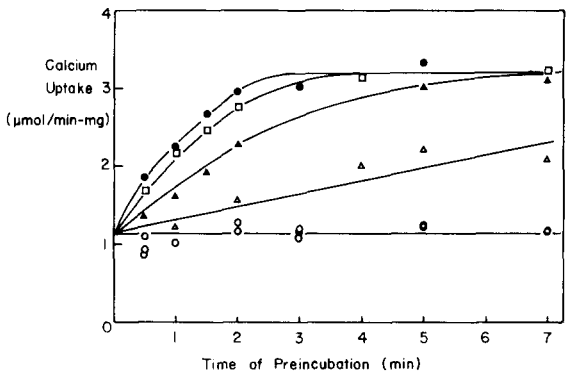

Fig. 2. Effect of time of preincubation and ryanodine concentration on calcium oxalate uptake rate of cardiac sarcoplasmic reticulum. Reaction conditions are identical to those described in Methods except that the pH was 6.8, the temperature was 37°C, and the cardiac sarcoplasmic reticulum was 147 μg/ml. Ryanodine was added for the indicated periods of preincubation at a final concentration of (○) zero; (△) 50 μM; (▲) 150 μM; (□) 300 μM; and (●) 500 μM. Actual preincubation concentrations of ryanodine were 1.25-times the final concentrations indicated here. Data shown are representative of the curves obtained with three different cardiac sarcoplasmic reticulum preparations.

oxalate entry or the kinetics of calcium oxalate crystallization, we investigated calcium uptake in the absence of oxalate. The results, shown in Fig.

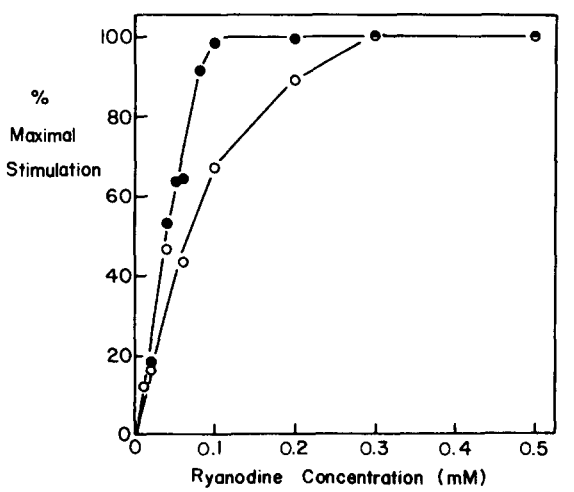

Fig. 3. Dose-response curve for ryanodine stimulation of oxalate-supported calcium uptake rate of cardiac sarcoplasmic reticulum. Reaction conditions were similar to those in Fig. 2 except that the preincubation was for 3 min (○) or 5 min (●) with the indicated final concentrations of ryanodine.

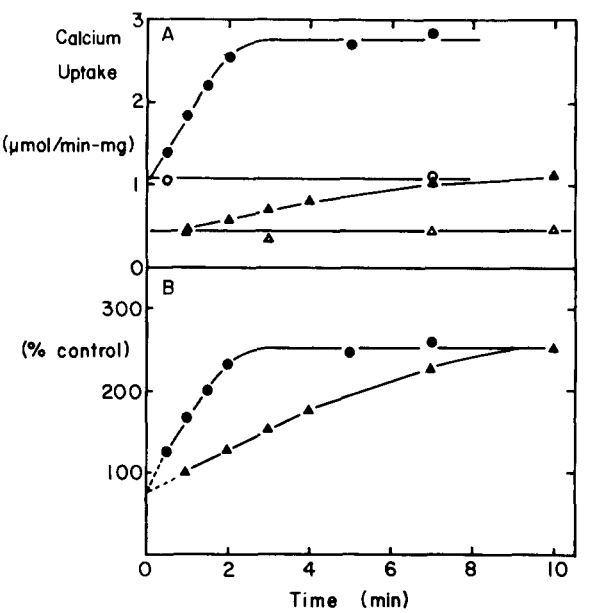

Fig. 4. Effect of temperature on ryanodine stimulation of oxalate-supported calcium uptake rate of cardiac sarcoplasmic reticulum. Reaction conditions were identical to those described in the legend of Fig. 2. Temperature was 37°C (●,○) or 27°C (▲,△). 375 μM ryanodine (●,▲) was preincubated with cardiac sarcoplasmic reticulum for the periods indicated before beginning the uptake reaction. Panel (B) shows that the percent stimulation is the same at both 27°C and 37°C.

5, indicate that short periods (0.5 min) of ryanodine pre-incubation at 27°C actually inhibit steady-state calcium uptake, while longer periods

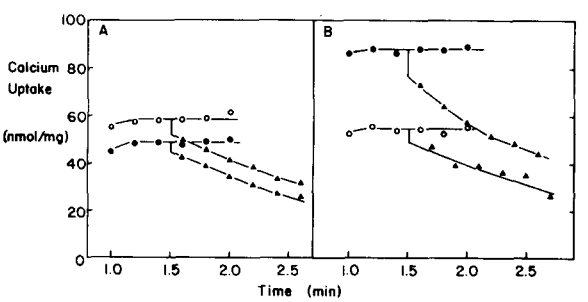

Fig. 5. Effect of ryanodine on steady-state calcium uptake in the absence of oxalate. Reaction conditions were as described in Methods. Preincubation was for 0.5 min (A) or 10 min (B) in the absence (○,△) or presence (●,▲) of 500 μM ryanodine. Final ryanodine concentration during measurement of uptake was 300 μM. Steady-state uptakes (○,●); measurement of passive efflux by addition of 2.5 mM EGTA 1.5 min after beginning the reaction (△,▲). Data shown are the mean of four separate determinations on a single preparation of cardiac sarcoplasmic reticulum.

(10 min) cause an increase in steady-state uptake. After 10 min preincubation with ryanodine, passive efflux at steady-state was 38 nmol · min^{-1} · mg^{-1} while after 10 min preincubation without ryanodine the passive efflux was 25 nmol · min^{-1} · mg^{-1}. The higher passive efflux in the presence of ryanodine is due to the higher load [13]. The first-order rate constant for passive efflux is a measure of the passive permeability to Ca and this was not significantly different for any of the four efflux curves shown in Fig. 5.

The data of Fig. 6 further document the requirement for preincubation of ryanodine for stimulation of uptake in the absence of oxalate at 27°C. The similarity of this curve for ryanodine in Fig. 6 and Fig. 4 suggests that ryanodine exerts the same effect whether oxalate-supported uptake or oxalate-independent uptake is used to assay the cardiac sarcoplasmic reticulum function.

To determine whether or not the stoichiometry of the Ca^{2+} pump was altered by ryanodine, Ca^{2+}-ATPase activity was assayed under defined conditions. These conditions included a regenerating system for ATP and inclusion of A23187 to make the membranes hyperpermeable. This was necessary to avoid differences in internal calcium

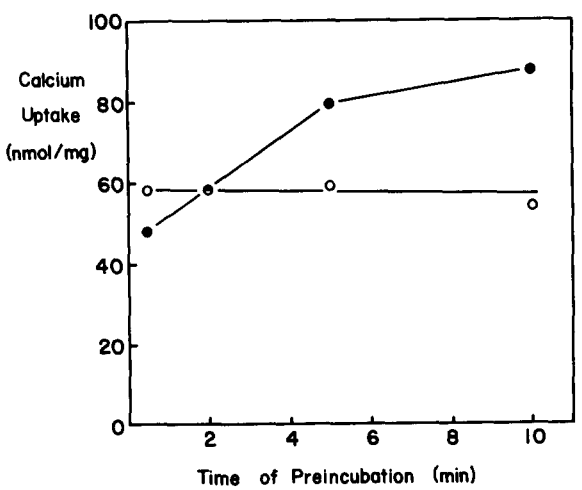

Fig. 6. Effect of pre-incubation on steady-state calcium uptake in the absence of oxalate. Reaction conditions were as described in Methods. Time of preincubation refers to the time of preincubation of the cardiac sarcoplasmic reticulum with (●) or without (○) 500 μM ryanodine prior to beginning the reaction by adding ATP, Ca and Mg.

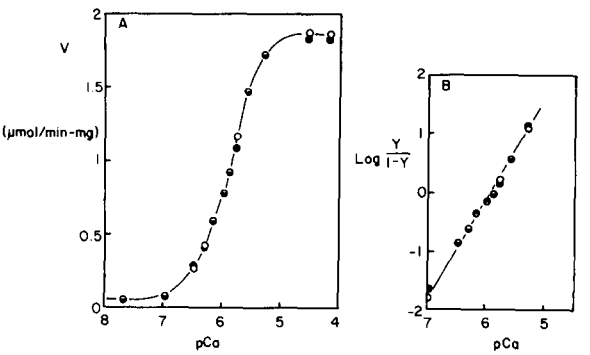

Fig. 7. Effect of ryanodine on Ca^{2+}-ATPase activity as a function of free calcium. Ca^{2+}-ATPase activity was assayed as described in Methods. Panel (A) shows the ATPase activity as a function of p*Ca*, for cardiac sarcoplasmic reticulum preincubated for 4 min in the presence (●) or absence (○) of ryanodine. The Hill plot (B) was calculated after subtraction of ATPase activity at p*Ca* = 7.7. In this case V_{max} was 1.80 and $Y = V/V_{max}$. The slope of the Hill plot was 1.72.

concentration that could arise due to differences in calcium transport. The results (Fig. 7) show that ryanodine was without effect on the Ca^{2+} pump over the range of activator calcium concentration. The Hill coefficient for both conditions (± ryanodine) at 37°C was 1.72. Similar results were obtained with a different sarcoplasmic reticulum preparation at 27°C with 0.5 min preincubation with 200 μM ryanodine, except the Hill coefficients were 1.89 and 1.87 with and without ryanodine, respectively (data not shown). This indicates that ryanodine does not alter the Hill coefficient at early or late stages of its effect on cardiac sarcoplasmic reticulum.

The effect of ryanodine on the apparent coupling ratio is shown in Table I. In agreement with previously reported data, ryanodine stimulated calcium uptake and increased the apparent coupling ratio [6–8]. Although a slight decrease in Ca^{2+}-ATPase activity was noted, in agreement with others [6–8], the depression was not significant.

Discussion

Results reported in this paper confirm the observation that ryanodine stimulates oxalate-supported calcium uptake by cardiac sarcoplasmic reticulum while slightly inhibiting Ca^{2+}-ATPase

activity [6–8]. The effect of ryanodine is thus to increase the apparent coupling ratio. This could occur in several ways as depicted schematically in Fig. 8, Ryanodine could (a) increase oxalate entry or (b) crystallization, (c) decrease efflux of calcium through a passive route or (d) through a gated channel (e) ryanodine could increase the inherent stoichiometry of the pump. We have systematically attempted to distinguish among these possible mechanisms of action.

Although we have not eliminated the possibility that ryanodine alters oxalate entry or crystallization, this mechanism appears unlikely because ryanodine exerts a similar stimulation of uptake when oxalate is not present (Fig. 6). Also, it is postulated that oxalate entry is dependent on hydrogen ion ejection by the calcium pump [17], so lack of an effect on Ca^{2+}-ATPase activity would likely have no effect on oxalate entry unless the effect is an uncoupling of hydrogen ion ejection. In unpublished results using previously published techniques [14] we have shown that ryanodine does not change the crystallization kinetics of calcium oxalate.

We have directly eliminated the possibility that ryanodine increases passive efflux by estimating this efflux from actively loaded vesicles (Fig. 5). Ryanodine had no effect on the permeability for calcium of cardiac sarcoplasmic reticulum membranes. This conclusion differs from that of Chamberlain et al. [29] who reported that ryanodine decreased the passive efflux from passively loaded cardiac sarcoplasmic reticulum vesicles. the discrepancy between these results may be due to the method of loading. Gerdes et al [30] suggest that active loading is less likely to lead to experimental artefacts than extended preincubation of the vesicles in high calcium concentrations. In support of Gerdes et al., incubation of cardiac sarcoplasmic reticulum vesicles in our hands at 4°C for 18 h leads to marked inhibition of Ca^{2+}-ATPase activity (data not shown).

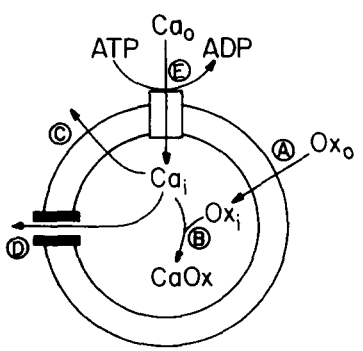

Fig. 8. Model of the possible sites of action of ryanodine on calcium uptake by cardiac sarcoplasmic reticulum. Ryanodine could increase oxalate entry (A); enhance calcium oxalate crystallization (B); decrease passive calcium efflux (c); decrease efflux through a gated channel (D); increase the stoichiometry of the pump (E).

The two remaining modes of action of ryanodine include an alteration of pump stoichiometry and a decrease in calcium efflux from a calcium channel distinct from the pump. Jones et al. [6] first suggested that ryanodine might decrease calcium efflux from a calcium specific channel. This is consistent with experiments conducted with papillary muscles from cat, dog, rabbit and guinea pig [18–20]. However, recent studies point to the possibility of an inherent slippage of the Ca^{2+} pump of skeletal sarcoplasmic reticulum, suggesting that the pump could have an inherently variable stoichiometry [21]. Jones and Cala [7] suggest ryanodine increases the calcium pumping efficiency rather than blocking calcium efflux. Their evidence was that calcium uptake by a subpopulation of sarcoplasmic vesicles is increased 8-fold while Ca^{2+}-ATPase activity is unchanged [7]. This evidence is really no different from that reported previously [6,8] or in Table I of the present report. We attempted to distinguish between these two remaining mechanisms by investigating the dependence of Ca^{2+}-ATPase activity on calcium concentration. The assumption here is that the activator calcium is also the transported calcium. The lack of effect of ryanodine on the calcium dependence of ATPase activity suggests either that the stoichiometry of the pump is unchanged by ryanodine or that the activator calcium is not transported calcium.

The data obtained by us suggests that ryanodine acts by closing a calcium channel. This hypothesis can be reconciled with the observations of others by recognizing that the vesicles may be heterogeneous with respect to the channel. If the channels are present in only a fraction of sarcoplasmic reticulum vesicles and these channels are

TABLE I

EFFECT OF RYANODINE ON CALCIUM UPTAKE AND ATPase ACTIVITY

Oxalate-supported calcium uptake was measured in a 10 ml reaction bath kept at 37°C and containing final concentrations of 100 mM KCl, 20 mM imidazole buffer (pH 6.8), 10 mM potassium oxalate, 10 mM NaN_3, 5 mM Na_2ATP, 5 mM $MgCl_2$, 0.2 mM added $CaCl_2$, 0.3 mM ryanodine. Cardiac sarcoplasmic reticulum protein (0.024–0.048 mg/ml) were pre-incubated for 4.0 min in 8 ml medium containing 125 mM KCl, 25 mM imidazole, 12.5 mM potassium oxalate, 12.5 mM NaN_3 and 0.375 mM ryanodine, when added, prior to addition of 2 ml of solution containing ATP, Mg and Ca. Rates were calculated from the linear regression of four points taken between 0 and 2.0 min after beginning the reaction. The values given are the mean ± S.E. for single determinations of six different cardiac sarcoplasmic reticulum preparations.

	Calcium uptake (μmol $\cdot min^{-1} \cdot mg^{-1}$)	Ca^{2+}-ATPase (μmol $\cdot min^{-1} \cdot mg^{-1}$)	Apparent coupling ratio
Control	2.08 ± 0.29	3.25 ± 0.41	0.65 ± 0.03
+ ryanodine	4.46 ± 0.53 [a]	2.91 ± 0.24 [b]	1.52 ± 0.10 [c]

[a] Significantly different, $P < 0.01$, from control.
[b] Not significantly different from control.
[c] Significantly different, $P < 0.001$, from control.

stuck open, then this fraction of sarcoplasmic reticulum vesicles will not accumulate calcium because of the rapid leak through the channel. If ryanodine closes the channel then oxalate-supported calcium uptake could ensue in these vesicles while Ca^{2+}-ATPase activity is only minimally affected. The minor effect on Ca^{2+}-ATPase activity is due to the low intravesicular calcium maintained by calcium oxalate precipitation.

It has been shown that cardiac sarcoplasmic reticulum vesicles can be separated into two classes: vesicles with high uptake rates that are not sensitive to ryanodine and vesicles with low uptake rates that are stimulated by ryanodine [7]. A third class of vesicles was also not ryanodine-sensitive but, because of the high level of $(Na^+ + K^+)$-ATPase, these were viewed as sarcolemma contaminants. The calcium uptake of the sarcoplasmic reticulum preparation can be described as

$$U_T = f_i U_i + f_s U_s + f_h U_h \quad (1)$$

where U_T is the observed overall uptake; U_i, U_s and U_h are the specific activities of calcium uptake in vesicles that lack an open channel, have an open channel or are hyperpermeable, respectively; f_i, f_s and f_h are the fractions of cardiac sarcoplasmic reticulum protein in the ryanodine-insensitive, ryanodine-sensitive and hyperpermeable vesicles, respectively. The Ca^{2+}-dependent ATPase is given similarly as

$$V_T = f_i V_i + f_s V_s + f_h V_h \quad (2)$$

The values of U_i, V_i, U_h, V_h, f_i and f_h are independent of ryanodine concentration; U_i is large and U_h is zero. For the sake of simplicity we can assume $V_i = V_s = V_h$ because internal calcium does not reach inhibitory levels during oxalate-supported calcium uptake in cardiac sarcoplasmic reticulum [14]. The value of U_s is

$$U_s = nV_s - J_g - J_p \quad (3)$$

where n is the real coupling ratio so that nV_s is the net calcium influx from the pump; J_g is the calcium efflux through the hypothetical channel and J_p is the net efflux through the passive pathway distinct from the pump and the channel. If ryanodine closes the channel, then J_g is decreased and thus U_s is increased. Since J_p is usually quite small compared to V_s [9,13], complete closure of the channel will give an overall uptake of approximately

$$U_T = f_i n V_i + f_s n V_s \quad (4)$$

In the absence of ryanodine, J_g will overwhelm the pump so that $U_s = 0$. Thus, in the absence of ryanodine the uptake rate will be

$$U_T = f_i n V_i \quad (5)$$

The Ca^{2+}-ATPase rate from Eqn. 2 and the assumption $V_i = V_s = V_h$ will be

$$V_T = V_i \quad (6)$$

so the apparent coupling ratio will be

$$R = \frac{U_T}{V_T} = f_i n \quad (7)$$

while at maximal stimulation by ryanodine the

uptake will be

$$U_T = f_i n V_i + f_s n V_s \tag{8}$$

while Ca^{2+}-ATPase will be

$$V_T = V_i$$

Since $V_i = V_s$, the apparent coupling ratio will be

$$R = \frac{f_i n V_i + f_s n V_s}{V_i} = (f_i + f_s) n \tag{9}$$

If the true coupling ratio is 2.0, then the apparent coupling ratio will be less than 2.0 so long as f_h is greater than zero, because $f_i + f_s + f_h = 1.0$ by definition. If we accept n as exactly 2.0, then the data of Table I suggest that $f_i = 0.33$, $f_s = 0.43$ and $f_h = 0.24$ for these preparations of cardiac sarcoplasmic reticulum.

This interpretation of heterogeneity of vesicles with respect to a calcium channel is supported by a variety of studies in which sensitivity to caffeine or ryanodine differs among vesicles [2,3,7,22].

The results reported herein also offer an explanation for the biphasic effects of ryanodine on contractile force developed by cat papillary muscles [18] and the marked temperature dependence of the kinetics of contracture in skeletal muscle [23]. In an effort to understand these effects, a kinetic model of the effects of ryanodine was developed. The model was required to explain (a) the initial inhibition of uptake and later stimulation of uptake with no effect on the calcium pump (b) dependence of the rate of stimulation on ryanodine concentration. In the model which follows, we have assumed that there are two types of vesicles. One has channels stuck in an open configuration and the other has channels stuck in a closed configuration. The open configuration allows net calcium efflux through the channel so that calcium uptake is depressed. The equations describing this model can be written as

$$C + R \underset{k_{r1}}{\overset{k_{f1}}{\rightleftharpoons}} CR1 \underset{k_{r2}}{\overset{k_{f2}}{\rightleftharpoons}} OR1 \underset{k_{r3}}{\overset{k_{f3}}{\rightleftharpoons}} CR2 \tag{11}$$

for the competent vesicles initially having a closed channel; and

$$O + R \underset{k_{r4}}{\overset{k_{f4}}{\rightleftharpoons}} OR2 \underset{k_{r5}}{\overset{k_{f5}}{\rightleftharpoons}} CR3 \tag{12}$$

for vesicles initially having an open channel. In this model, then, ryanodine binds to a closed channel, causes it to open momentarily and then close again in a new, more stable configuration with ryanodine bound to it. Ryanodine may also bind to an open channel, causing it to eventually close. The details of the simulation of this model are available on request from the BBA Data Bank *. In brief, the results of the simulation clearly depend on the values of k_{fi} and k_{ri} in Eqn. 11 and Eqn. 12. In order to obtain the time and concentration dependence but high affinity of the ryanodine effect, it is necessary that the association of ryanodine is slow but the final binding is essentially irreversible. Thus one of the important features of this model is that k_{r3} and k_{r5} are very small compared to k_{f3} and k_{f5}. The simulation was performed using the electronic network simulation program SPICE2 [13,27,28].

The results from the simulation of Eqn. 11 and Eqn. 12 are shown in Fig. 9. This result is clearly analogous to the observation reported in Fig. 2. This analogy, of course, does not prove the validity of the model. At least two alternatives are possible. The first is that ryanodine could bind to the inner surface of the sarcoplasmic reticulum vesicles and the requirement for preincubation could be explained by the time necessary for passive entry of ryanodine into the vesicles. A second alternative is that ryanodine first inhibits uptake by inhibition of the calcium pump, followed by stimulation of uptake by closure of a calcium channel. If ryanodine stimulates uptake on the inside surface, then the rate of stimulation should increase with time. The data of Fig. 2 are insufficiently precise to definitely support or refute this prediction, but simulations suggest this is unlikely. The inhibition of Ca^{2+}-ATPase activity in the presence of oxalate is quite small (Table I) and appears insufficient to explain the early inhibition of uptake observed in the absence of oxalate. The inhibition of Ca^{2+}-ATPase activity is no doubt

* See footnote on p. 77.

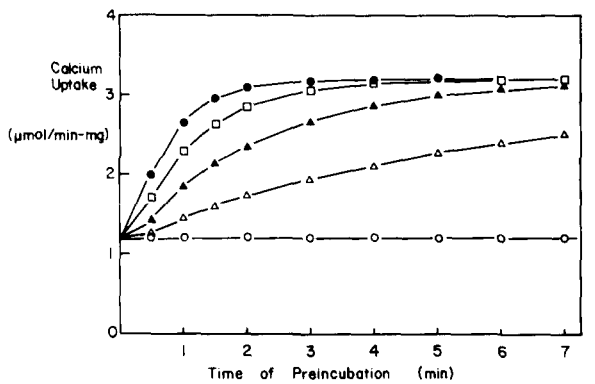

Fig. 9. Simulation of the effect of ryanodine on calcium uptake by cardiac sarcoplasmic reticulum. The stimulations used Eqn. 1 where $f_i = 0.21$, $f_c = 0.34$ and $f_h = 0.45$ were calculated for this preparation, and $U_h = 0$. The value of U_i and U_s depended on the proportion of channels that were open or closed. This proportion was calculated by the simulation program described in the Appendix (see Data Deposition footnote on p. 77). The values of U_i were calculated as $U_i = 5.8 \cdot f_i$ and U_s was calculated as $U_s = 5.8 \cdot f_s$ where f_i was the fraction of channels closed in the ryanodine-sensitive vesicles and f_s was the fraction of channels closed in the ryanodine-sensitive vesicles. The simulations were conducted for preincubation with no ryanodine (○); 50 μM ryanodine (△); 150 μM ryanodine (▲); 300 μM ryanodine (□); and 500 μM ryanodine (●).

due to an increased intravesicular calcium concentration since no inhibition was observed when the calcium ionophore A23181 was used to dissipate the calcium gradient (Fig. 7). All of this information points to a slow association step followed by an essentially irreversible closure of the calcium channel.

The type of binding mechanism suggested here for ryanodine has some precedent. Pratt et al. [24] have suggested that glucocorticoid binding to its receptor occurs via formation of an intermediate glucocorticoid receptor complex. Their evidence was that the initial rate of binding was linear only at low steroid concentrations and the affinity constant measured at equilibrium was much higher than the ratio of the association and dissociation rate constants (k_a/k_d). These binding reactions take hours to reach equilibrium [25] and the dissociation constants are quite slow [25,26]. However, other causes for the kinetics of glucocorticoid binding have been suggested [26].

Regardless of the validity of the model described by Eqns. 11 and 12, the present results support the hypothesis that ryanodine closes a calcium channel in cardiac sarcoplasmic reticulum and that this channel is present in a subpopulation of isolated cardiac sarcoplasmic reticulum vesicles.

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