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The Effects of Ryanodine on Passive Calcium Fluxes across Sarcoplasmic Reticulum Membranes*

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Frank A. Lattanzio, Jr.‡, Robert G. Schlatterer‡, Michael Nicar§, Kevin P. Campbell¶, and John L. Sutko‡§‖

From the Departments of ‡Physiology and §Internal Medicine, The University of Texas Health Science Center, Dallas, Texas 75235 and the ¶Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242

Ryanodine at concentrations of 0.01–10 μM increased, while greater concentrations of 10–300 μM decreased the calcium permeability of both rabbit fast twitch skeletal muscle junctional and canine cardiac sarcoplasmic reticulum membranes. Ryanodine did not alter calcium binding by either sarcoplasmic reticulum membranes or the calcium binding protein, calsequestrin. Therefore, the effects by this agent appear to involve only changes in membrane permeability, and the characteristics of the calcium permeability pathway affected by ryanodine were those of the calcium release channel. Consistent with this, the actions by ryanodine were localized to junctional sarcoplasmic reticulum membranes and were not observed with either longitudinal sarcoplasmic reticulum or transverse tubular membranes. In addition, passage of the junctional sarcoplasmic reticulum membranes through a French press did not diminish the effects of ryanodine indicating that intact triads were not required. Under the conditions used for the permeability studies, the binding of [^{3}H]ryanodine to skeletal junctional sarcoplasmic reticulum membranes was specific and saturable, and Scatchard analyses indicated the presence of a single binding site with a K_d of 150–200 nM and a maximum capacity of 10.1–18.9 pmol/mg protein. [^{3}H] ryanodine binding to this site and the increase in membrane calcium permeability caused by low concentrations of ryanodine had similar characteristics suggesting that actions at this site produce this effect. Depending on the assay conditions used, ryanodine (100–300 μM) could either increase or decrease ATP-dependent calcium accumulation by skeletal muscle junctional sarcoplasmic reticulum membranes indicating that the alterations of sarcoplasmic reticulum membrane calcium permeability caused by this agent can be determined in part by the experimental environment.

In muscle and other tissues, physiologically important fluxes of calcium occur through channels present in sarcoplasmic and endoplasmic reticulum membranes (*cf.* Refs. 1–3). The identification of specific pharmacological effectors of these ion channels is an important step in their ultimate characterization. Findings by Fairhurst and his colleagues (4–6) and the data presented in this report and recently by Meissner (7) suggest that the plant alkaloid ryanodine is a specific modulator of the calcium channel present in skeletal and cardiac muscle SR[1] membranes.

Ryanodine is a potent effector of the mechanical activity of intact skeletal and intact and skinned cardiac muscle cells (8–10), and it has been found by a number of investigators to affect ATP-dependent accumulation of calcium by isolated skeletal and cardiac muscle SR membranes (4, 11–16). Although the actions by ryanodine on both intact muscle cells and isolated SR membranes can be explained by alterations in SR membrane calcium permeability, whether this is actually the case is uncertain for the following reasons. First, the molecular basis of the effects by ryanodine on ATP-dependent calcium accumulation by isolated SR vesicles is unclear, since this agent has been reported to both increase (12–15) and decrease (4, 11, 14, 16) this variable. Moreover, in one case the latter effect was only observed in the presence of ruthenium red (16), an agent that affects both the calcium release channel (17, 18) and the calcium pump (19) present in HSR membranes. Second, alternative explanations exist for these *in vitro* effects of ryanodine. For example, changes in the efficiency of calcium transport by the calcium pump could be involved (12). Third, interpretation of the *in vitro* measurements of ATP-dependent calcium accumulation by SR vesicles is confounded by the use of calcium precipitating agents, such as oxalate (11–15) and phosphate (16), that can complicate the observed calcium binding/permeability characteristics (20). Moreover, effects by ryanodine on passive calcium fluxes across SR membranes and hence on the calcium permeability of the membrane that are consistent with the observed alterations of active calcium accumulation have not been observed under the assay conditions used in these studies. (*i.e.* Refs. 12 and 14). Finally, it is not clear whether the observed changes in active calcium accumulation by isolated SR vesicles share a cause and effect relationship with the changes in the mechanical activity of intact muscle preparations caused by this agent, because much greater ryanodine concentrations are required to affect the former than the latter (9).

Therefore, in order to determine whether ryanodine directly affects the calcium permeability of SR membranes in a man-

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‖ To whom correspondence should be addressed: Dept. of Pharmacology, University of Nevada School of Medicine, Howard Bldg., Reno, NV 89557.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; HSR, junctional sarcoplasmic reticulum; LSR, longitudinal sarcoplasmic reticulum; T-T, transverse tubule; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

ner consistent with alterations of the conductance characteristics of the SR calcium release channel, we have conducted the following experiments. First, we investigated the effects of ryanodine on passive calcium fluxes across SR membranes in the absence of either calcium pump activity or calcium precipitating agents. Second, we evaluated whether ryanodine might mimic changes in membrane permeability. Third, we determined whether, consistent with actions on the calcium release channel, the actions of ryanodine were localized to junctional SR membranes, the putative site of this channel. Fourth, we compared the characteristics of [^{3}H]ryanodine binding to those observed for the effects of this agent on HSR membrane calcium permeability to assess whether a specific binding site associated with the latter changes could be identified. And fifth, we investigated whether differences in experimental conditions might underlie the observations of opposite effects by ryanodine on ATP-dependent calcium accumulation by isolated SR vesicles. Some of the results of these studies have been presented in preliminary form (21).

EXPERIMENTAL PROCEDURES

Materials—Ryanodine (lot no. 704-RWP-1) was purchased from the S. B. Penick and Co. (Lyndhurst, NJ), ruthenium red from Sigma, A23187 and ionomycin from Behring Diagnostics, Percoll from Pharmacia P-L Biochemicals, and ^{45}Ca from ICN Pharmaceuticals (Irvine, CA). All other materials were of reagent grade or better. The ryanodine used for this work was a mixture of primarily ryanodine and dehydroryanodine. Ryanodine was purified using high pressure liquid chromatography (22) and found to give the same results as the mixture in the experiments presently reported (data not shown).

Membrane Preparation Procedures—Adult male rabbits were heparinized, anesthetized with sodium pentobarbital (30 mg/kg, intravenously), and bled via the jugular veins. White skeletal muscle was removed from the back and hind limbs and placed immediately in ice-cold normal saline. The muscles were trimmed of nonmuscle tissue, minced, and used for the preparation of SR and T-T membranes by the following methods. For the majority of experiments HSR and LSR membranes were prepared using the method of Saito *et al.* (23). In some preparations the overnight discontinuous sucrose density gradient centrifugation step was changed by using higher capacity gradients centrifuged for 3 h at 41,000 rpm in a Beckman 45Ti rotor. For other preparations the sucrose gradient was replaced with a self-forming Percoll gradient (29.2% in 0.25 M sucrose) that was centrifuged for 1 h at 20,000 rpm in a Sorvall SS-34 rotor. Comparable membrane fractions were obtained with each of these modifications, and no differences in their characteristics were observed in the present studies. T-T membranes were prepared by the method of Rosemblatt *et al.* (24). HSR and LSR membrane fractions were also obtained using this procedure and were found to be functionally similar to the R4 and R2 fractions, respectively, of Saito *et al.* (23). For the experiments shown in Fig. 10, crude HSR membranes were prepared as described by Fairhurst (4). Crude cardiac SR membranes were prepared from canine myocardium using the procedure of Jones *et al.* (12). All membrane preparations were stored at a concentration of 20–40 mg/ml in liquid N_2 in 0.3 M sucrose, 10 mM imidazole (pH 7.4), or kept at 0–4 °C, if used within 48 h of preparation.

Calcium Flux Assays—Passive calcium fluxes were determined as both calcium influx and efflux using protocols similar to those described by Meissner (25). Briefly, if stored in liquid N_2, the membranes were rapidly thawed in a 37 °C water bath and then placed on ice. The membranes were diluted to a concentration of 0.1–0.2 mg/ml in solution 1 that contained 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8), and 20 μM $CaCl_2$ and allowed to equilibrate for 30 min at 0–4 °C. The membranes were then collected by centrifugation at 35,000 rpm in a Beckman 42.1 rotor for 90 min and resuspended in solution 1 at a concentration of approximately 5 mg/ml.

Measurements of calcium influx were initiated by the rapid mixing of 3.6 μl of ^{45}Ca/^{40}Ca admixtures with 46.4 μl of the reaction mixture composed of solution 1 and the membrane protein. At the designated times the uptake of calcium was terminated by the rapid addition of 2.5 ml of an ice-cold terminating solution that contained 0.3 M sucrose, 10 μM ruthenium red, 0.5 mM $LaCl_3$, 0.5 mM $HgCl_2$, and 10 mM Pipes/Tris (pH 6.8). This terminating solution has been found to effectively quench the increase in SR membrane calcium permeability caused by ryanodine.[2] The reaction solution was then filtered through Whatman GF/A filters that were washed with two additional 5-ml aliquots of ice-cold terminating solution. The vesicular content of calcium was measured using liquid scintillation counting techniques as the radioactivity retained on the filters. Unless otherwise indicated the vesicles were preincubated with either control or ryanodine-containing solutions for 30 min prior to the addition of calcium. Uptake intervals shorter than 1 s were achieved using an electronically timed rapid mixing system.[2]

For measurements of calcium efflux, the vesicles (5 mg of protein/ml in solution 1) were passively loaded with calcium (0.1–5 mM) for variable periods ranging from 2 to 120 min at 37 °C, and then 5-μl aliquots were diluted into 500 μl of either 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8), 1 mM EGTA (passive calcium efflux) or into the same solution containing 10 μM free calcium (calcium-stimulated calcium efflux). Efflux periods ranging from 1 to 20 s were terminated either by the dilution of this mixture with 5 ml of ice-cold terminating solution and filtering as described above or by washing the vesicles on GF/A filters with a solution containing 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8), and 5 mM $LaCl_3$. With the latter method which was used for efflux intervals longer than 10 s, the 15 s required for the filtration/wash step was included as part of the efflux period. Efflux intervals of 1–10 s were timed using a metronome, and zero time points were obtained by diluting samples directly into the ice-cold terminating solution prior to filtration or by extrapolation back to the zero time point.

ATP-dependent calcium accumulation was assayed using either the conditions of Seiler *et al.* (15) or those of Fairhurst (4). For the former, calcium uptake was measured in a medium containing 50 mM histidine (pH 7.0), 3 mM $MgCl_2$, 3 mM potassium oxalate, 100 mM KCl, 50 μM $^{45}CaCl_2$, and 3 mM Na_2ATP. The assay conditions of Fairhurst (4) involved an uptake medium containing 20 mM imidazole (pH 7.0), 30 mM KCl, 5 mM potassium oxalate, 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$, and 5 mM Na_2ATP. A crude HSR membrane fraction (4) was used for these studies, and membrane protein was preincubated for 10 min at the temperature used for the assay in the absence and presence of ryanodine prior to the initiation of the reaction by the addition of ATP. Calcium accumulation was permitted to proceed for 10 min and was then terminated as described above.

In some experiments the vesicular calcium content was also determined using atomic absorption spectroscopy. For these studies, Whatman GF/A filters were placed in a solution containing 3 mM $LaCl_3$ and 162 mM HCl 30 min prior to use to lower background calcium levels. The filters were rinsed four times with 5-ml aliquots of terminating solution just prior to filtration of the vesicles. Filters treated in this manner were also used for the parallel isotopic flux measurements conducted to ensure that the ability of the filters to retain intact vesicles was not altered. After collection of the membranes, the filters were soaked in 1 ml of the $LaCl_3$/HCl solution overnight, and the calcium present in the medium was then determined using atomic absorption spectroscopy.

Calcium Binding Assays—Calcium binding to SR membranes and isolated calsequestrin was measured using both thin layer equilibrium dialysis (26) and fluorescence measurements (27). Calsequestrin was prepared as described previously (28).

[^{3}H]Ryanodine Binding Assay—[^{3}H]Ryanodine was prepared by catalytic reduction of dehydroryanodine as described previously (22). Membranes (0.1 mg of protein/ml) were incubated with [^{3}H]ryanodine (specific activity, 60.8 Ci/mmol) in solution 1 for 60 min at 37 °C. The membranes were collected by filtration on Whatman GF/A filters and washed with three 5-ml aliquots of solution 1. The filters were dried overnight, and the counts retained on the filters were determined using liquid scintillation counting techniques. Nonspecific binding was measured in the presence of a 100-fold excess of nonlabeled ryanodine. [^{3}H]Ryanodine was added to a concentration of 10 nM; greater concentrations of this agent were prepared as admixtures of labeled and nonlabeled drug. The maximal effects by ryanodine on the passive calcium permeability of SR membranes were observed after 30 min of exposure of the membranes to this agent at 37 °C, whereas the binding of [^{3}H]ryanodine to these membranes under similar conditions required at least 60 min to attain maximal levels. The basis for the difference in the times required for

[2] F. A. Lattanzio, Jr., G. Meissner, and J. L. Sutko, manuscript in preparation.

these two events is not clear, but its existence suggests that the binding of [^{3}H]ryanodine may not always be directly related to measurable changes in membrane permeability in this experimental system.

Protein concentrations were determined using the method of Lowry *et al.* (29) with bovine serum albumin as a standard. Unless otherwise indicated all assays were conducted at 37 °C, and the results reported are representative of those obtained in triplicate using at least four different membrane preparations. Variability between triplicate determinations was less than 10%.

RESULTS

Effects by Ryanodine on Passive Calcium Fluxes across Skeletal and Cardiac SR Membranes—Ryanodine exerted two distinct effects on passive calcium fluxes across skeletal muscle HSR membranes. First, as shown in Fig. 1, at a concentration of 1 μM, it produced comparable increases in both calcium influx (*Panel A*) and efflux (*Panel B*). Second, at greater concentrations of 100–300 μM, ryanodine decreased both passive calcium fluxes. Data from calcium efflux measurements are shown in Fig. 2. In these latter experiments, the calcium channel was activated by 10 μM free calcium present in the efflux medium (*cf.* Ref. 25) in order to enhance detection of a decrease in this variable. Under these conditions, low concentrations of ryanodine (less than 1 μM) did not further increase calcium efflux, whereas high concentrations of this agent (greater than 100 μM) decreased this variable reversing the stimulation caused by either calcium or low (1 μM or less) concentrations of ryanodine. These results suggest that ryanodine can alter the calcium permeability of HSR membranes by effecting the movement of calcium through the calcium release channel. As can also be seen in Fig. 2, under the assay conditions used for these studies, ryanodine was less potent as an inhibitor of calcium efflux across HSR membranes than ruthenium red, another agent that is thought to block the calcium release channel (17, 18).

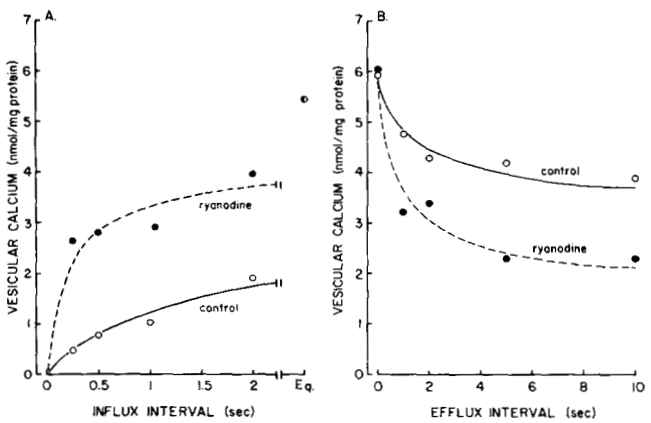

FIG. 1. **The time course of passive calcium movements into (*Panel A*) and out of (*Panel B*) skeletal muscle HSR vesicles in the absence (○) and presence (●) of 1 μM ryanodine.** In *Panel A*, the value (◐) denoted as *Eq.* on the abscissa represents the equilibrium vesicular calcium content measured 20 min after the addition of calcium in the presence of the calcium ionophore ionomycin (1 μM) which was added to ensure that an equilibrium had been reached. The calcium content at equilibrium was the same in both nontreated and treated vesicles.

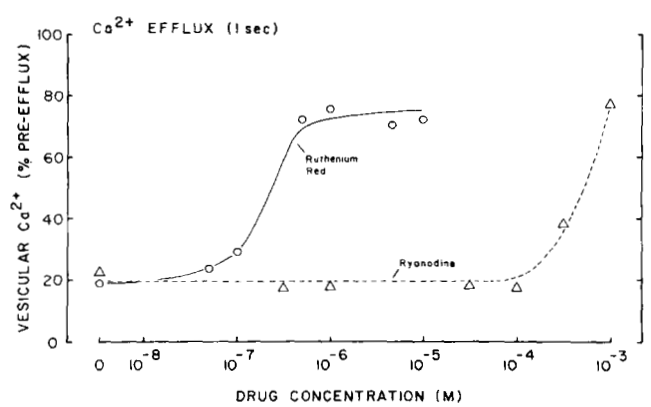

FIG. 2. **The inhibitory effects of ryanodine (△) and ruthenium red (○) on calcium-stimulated calcium efflux from skeletal muscle HSR vesicles.** Membranes were equilibrated with 0.1 mM calcium in the absence and presence of the concentrations of ryanodine or ruthenium red shown along the abscissa. The efflux medium contained 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8), and 10 μM $CaCl_2$ ($CaCl_2$/EGTA mixture). Calcium efflux was terminated after 1 s. The calcium remaining in the vesicles after the efflux period is expressed as a percentage of that present prior to its initiation.

In two experiments with different crude preparations of cardiac SR membranes, ryanodine caused both the increase and decrease in calcium efflux at concentrations of 1 and 300 μM, respectively (Fig. 4). The smaller magnitude of the changes observed with the cardiac membranes may be due to the inhomogeneity of this preparation, since it contains membranes derived from both longitudinal and terminal regions of the SR (13). The cardiac preparation was not further purified for these studies as described by Jones and Cala (13), since this involves loading the vesicles with calcium oxalate which would complicate measurement of passive calcium fluxes. In addition, under the assay conditions used in the present experiments, the calcium channel present in the cardiac membranes may be predominantly open in the absence of ryanodine. This would diminish the apparent effectiveness

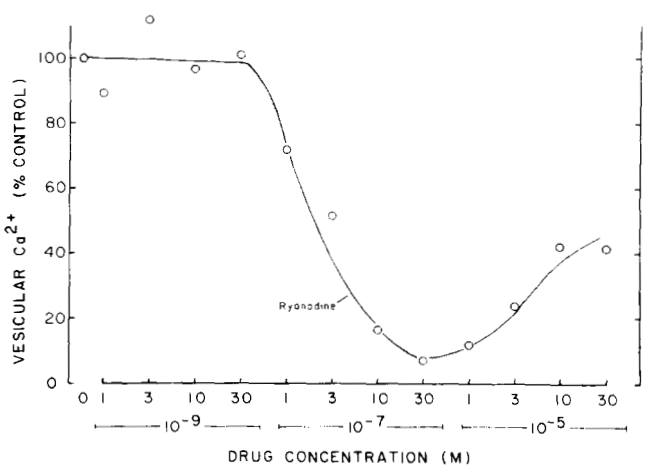

FIG. 3. **The concentration dependence of the effects exerted by ryanodine on calcium efflux from skeletal muscle HSR vesicles during a 20-s interval expressed as a percentage of that occurring from nontreated membranes.** Vesicles were incubated for 30 min at 37 °C prior to the addition of calcium (5 mM) 2 min prior to the start of the efflux period. Similar calcium contents were present in both nontreated and treated vesicles at the start of the efflux period.

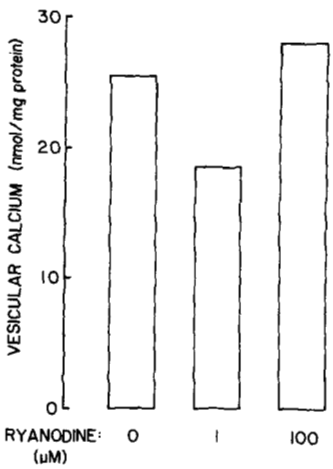

FIG. 4. **The effects of ryanodine at concentrations of 1 and 100 μM on calcium efflux from cardiac SR vesicles measured during a 20-s interval.** The vesicles were preincubated with either 0, 1, or 100 μM ryanodine for 30 min at 37 °C, and calcium (5 mM) was added 10 min prior to the initiation of the efflux period. Non-treated and treated vesicles contained between 41.6 and 44.7 nmol of calcium/mg of protein prior to the initiation of the efflux period. The results are expressed as the quantity of calcium present in the vesicles at the end of the efflux interval.

TABLE I

Thin-layer dialysis (26) determinations of the effects of ryanodine on the calcium binding by purified calsequestrin isolated from rabbit HSR membranes or by intact HSR vesicles

Calsequestrin (1 mg of protein/ml) or HSR membranes (5 mg of protein/ml) were incubated with either 0 or 1 μM ryanodine for 30 min at 37 °C. The protein or membranes were then placed in dialysis tubing and equilibrated with ^{45}Ca for 4 h at room temperature in the continued presence of ryanodine (for treated samples). The incubation and dialysis solutions also contained 0.1 M KCl and 10 mM Pipes/Tris (pH 6.8). Similar results were obtained when the dialysis was conducted for 3 h at 37 °C.

Ca^{2+} concentration	Calcium bound			
	Calsequestrin		HSR membrane protein	
	No ryanodine	1.0 μM ryanodine	No ryanodine	1.0 μM ryanodine
	nmol/mg		*nmol/mg*	
100 μM	42.3	41.7	20.6	22.6
500 μM	273.6	283.1	67.4	66.3
1.0 mM	432.0	428.6	97.4	102.3

of this agent to increase cardiac SR membrane calcium permeability.

Ryanodine Does Not Affect Calcium Binding by SR Membranes—The changes in calcium fluxes caused by ryanodine could result from a change in the calcium binding, as well as the calcium permeability characteristics of the SR membranes. As shown in Table I, neither low nor high concentrations of ryanodine affected calcium binding by either native skeletal HSR membranes or the primary calcium binding protein present, calsequestrin. Identical results were obtained for purified calsequestrin using either equilibrium dialysis or measurements of calcium-induced changes in native protein fluorescence. Calcium binding by intact cardiac SR membranes was similarly unaffected by ryanodine (data not shown). Therefore, the most likely explanation for both of the effects by ryanodine is that they result from an alteration of the calcium permeability of SR membranes involving changes in the conductance characteristics of the calcium release channel.

Localization of the Effects of Ryanodine to Skeletal HSR Membranes—If ryanodine does act on the SR calcium release channel, then its actions should be restricted to those subfractions of SR membranes that contain the channel. The availability of relatively homogeneous preparations of skeletal muscle T-T, HSR and LSR membranes permitted us to test whether this was the case. As shown in Fig. 5, 1 μM ryanodine did not affect calcium influx into either LSR (*Panel A*) or T-T (*Panel B*) membranes. High concentrations of ryanodine (100–300 μM) were similarly ineffective in altering either the influx or efflux of calcium from these membranes (data not shown). These results are consistent with a similar localization of [^{3}H]ryanodine binding to HSR membranes described below. In addition, the actions of ryanodine on HSR membrane permeability were not dependent on the presence of

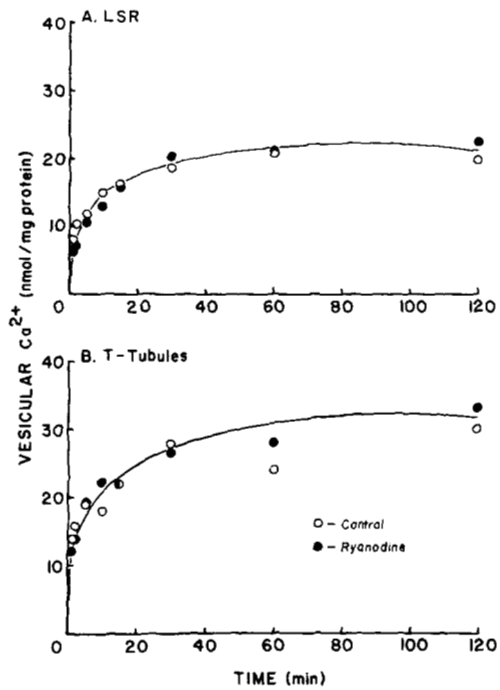

FIG. 5. **The time course of calcium uptake into skeletal muscle LSR (*Panel A*) and T-T (*Panel B*) membranes measured in the absence (O) and presence (●) of 1 μM ryanodine.** Calcium (5 mM) was added to the membrane suspension at zero time, and calcium uptake was terminated at the times indicated on the abscissa. The vesicles were preincubated in the absence and presence of ryanodine for 30 min at 37 °C prior to the start of the uptake period.

TABLE II

Effect of French press treatment on the actions of low and high concentrations of ryanodine on the calcium permeability of skeletal muscle HSR membranes

HSR membranes were passed through a French press at 6,000 p.s.i. twice, and then control and treated preparations were placed onto a discontinuous sucrose gradient consisting of 20, 28, 36, and 45 % (w/v) sucrose. Three bands were obtained after treatment with the French press at the 20/28, 28/36, and 36/45% sucrose interfaces. Control membranes yielded only one band at the 36/45% sucrose interface. The membrane bands were diluted with 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8), 20 μM $CaCl_2$, and centrifuged for 90 min at 110,000 × g_{max}. The pellets were resuspended in 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8) containing 0, 1, or 300 μM ryanodine and incubated for 30 min at 37°C. Calcium (5 mM) was added, and the calcium efflux occurring over a 20-s interval was determined 2 min later.

	HSR vesicular calcium	
	Control	French press
	nmol/mg protein	
Control	58.2	59.3
Ryanodine (1 μM)	14.1	6.5
Ryanodine (300 μM)	43.1	40.6

intact triads, since as shown in Table II, two passages of HSR membranes through a French press, a treatment that disrupts intact triads (30), did not diminish the increase in calcium efflux caused by 1 μM ryanodine.

Comparison of [^{3}H]Ryanodine Binding and the Effects by Ryanodine on SR Membrane Calcium Permeability—Two laboratories have recently demonstrated that skeletal muscle HSR membranes specifically bind [^{3}H]ryanodine (16, 31). Therefore, we compared the characteristics of [^{3}H]ryanodine with those of the alterations in calcium permeability produced by this agent to determine whether either the increase or decrease in the latter variable could be attributed to the site(s) that bind the radioligand. The ability of ryanodine to increase, as well as decrease, the calcium permeability of HSR membranes suggested that it either had two distinct sites of action or a single site with two different ryanodine binding conformations was involved.

In agreement with the previous findings (16, 31), [^{3}H] ryanodine was found to bind to skeletal muscle HSR (Fig. 6), but not to LSR or T-T (data not shown) membranes in a specific and saturable manner. A K_d of 150–200 nM and a maximum binding capacity of 10.1–18.9 pmol/mg membrane protein ($n = 4$) were observed. The K_d values are comparable to those obtained by Fleischer *et al.* (16) using similar membranes and binding conditions but are greater than those reported by Pessah *et al.* (31) who used a different membrane preparation and a low ionic strength binding medium. The maximum number of sites present is similar to those found previously by both laboratories. In the present experiments, the binding of [^{3}H]ryanodine to four of six preparations tested yielded a linear Scatchard plot indicating the existence of a single homogeneous population of binding sites. In two experiments a curvilinear Scatchard plot was obtained, indicating the possible presence of a second lower affinity binding site. The reasons for the inconsistency of this observation are not clear. Two [^{3}H]ryanodine binding sites have been identified in cardiac SR membranes (31).

We have found a number of parallels between the specific binding of [^{3}H]ryanodine to skeletal muscle HSR membranes and the ability of this compound to increase the calcium permeability of these membranes. First, under comparable incubation conditions the K_d of [^{3}H]ryanodine binding was similar to the concentration that caused a 50% increase in the calcium permeability of HSR membranes (150–200 nM *versus* 200–300 nM).

Second, as shown in Fig. 7*A*, when added simultaneously or prior to ryanodine, ruthenium red at concentrations similar to those at which it has been reported to inhibit [^{3}H]ryanodine binding (31) prevented the increase in HSR membrane calcium permeability caused by low concentrations of ryanodine. Third, both [^{3}H]ryanodine binding and the increase in calcium permeability caused by low concentrations of ryanodine were inhibited by the presence of multivalent cations, such as magnesium and calcium (Ref. 31 and Figs. 7*B* and 8), as well as lanthanum, mercury, and copper during the exposure of the membranes to ryanodine. The evaluation of the abilities of cationic species such as ruthenium red and magnesium to prevent the increase in HSR membrane calcium permeability caused by ryanodine by interfering with the binding of this latter agent is complicated by the direct decreases in calcium permeability caused by these agents. This problem was circumvented in the present studies by taking advantage of the fact that in the presence of 0.1 M KCl, lanthanum ions block

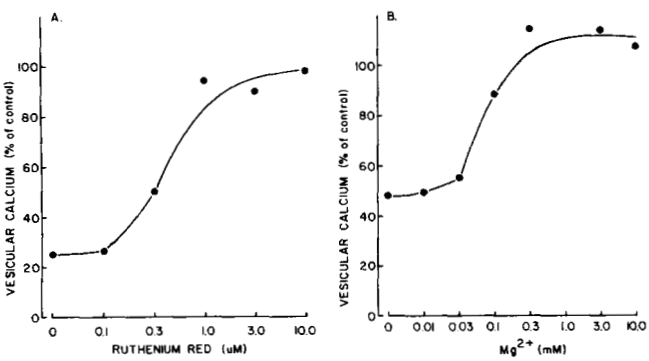

FIG. 7. **Inhibition by ruthenium red (*Panel A*) and magnesium (*Panel B*) of the increased calcium efflux from skeletal muscle HSR membranes caused by 1 μM ryanodine.** HSR vesicles were exposed to 0.1 mM calcium, 1 μM A23187, and to either ruthenium red or magnesium at the concentrations indicated along the abscissa in the absence and presence of 1 μM ryanodine. The rationale for the inclusion of the calcium ionophore A23187 is discussed in the text. The calcium efflux occurring during a 20-s interval was measured in the presence of 5 mM $LaCl_3$ in 0.1 M KCl, 10 mM Pipes/Tris (pH 6.8). The quantity of calcium present in ryanodine-treated vesicles at the end of the efflux period is expressed as a percentage of that remaining in nontreated vesicles.

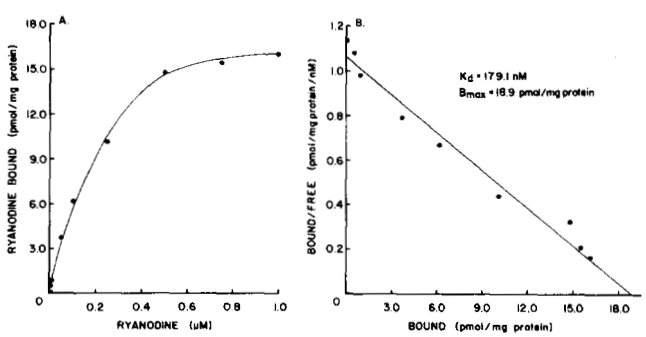

FIG. 6. **Saturation (*Panel A*) and Scatchard (*Panel B*) plots of the binding of [^{3}H]ryanodine to skeletal muscle HSR membranes.** The specific binding, which ranged from 10 to 40% of the total binding has been plotted. HSR membranes were incubated with the various concentrations of ryanodine shown along the abscissa of *Panel A* (10 nM [^{3}H]ryanodine; specific activity, 60.8 Ci/mmol) for 60 min at 37 °C as described under "Experimental Procedures." Each point represents the mean of triplicate samples, and the data are representative of the results obtained with four different membrane preparations (K_d range, 150–200 nM; B_{max} range, 10.1–18.9 pmol/mg protein).

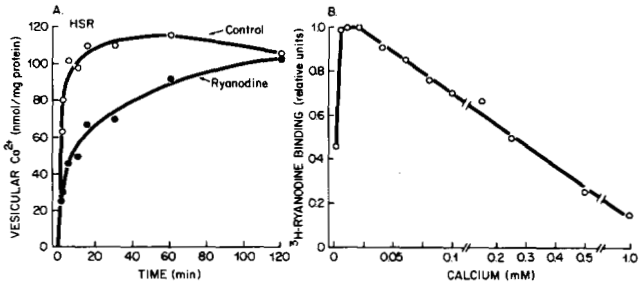

FIG. 8. **Inhibition by calcium of the increased calcium efflux from skeletal muscle HSR membranes caused by 1 μM ryanodine (*Panel A*) and of the binding of [^{3}H]ryanodine to these membranes (*Panel B*).** In *Panel A*, HSR membranes were incubated for 30 min at 37 °C in the absence (○) and presence (●) of 1 μM ryanodine. Calcium (5 mM) was added to the vesicle suspension, and at the times indicated on the abscissa aliquots were removed and the quantity of calcium remaining in the vesicles after a 20-s efflux period was determined. In *Panel B*, the calcium concentrations shown on the abscissa were present during the 60-min interval permitted for [^{3}H]ryanodine (10 nM) binding. Calcium concentrations less than 50 μM were achieved using a calcium/EGTA buffer. In this experiment the maximal [^{3}H]ryanodine bound was 2.5 pmol/mg HSR membrane protein.

calcium fluxes mediated by the calcium ionophore, A23187, but not those occurring through the ryanodine-activated calcium channel.[2] This permitted us to use the ionophore to ensure that the vesicles completely equilibrated with calcium in the presence of either magnesium or ruthenium red during the incubation period when the effects by these agents on ryanodine binding would be exerted. The presence of lanthanum ions (5 mM) during the efflux period prevented contributions by the ionophore to calcium efflux and left the ryanodine-activated calcium channel as the only differentiating factor between the effluxes measured in the absence and presence of ryanodine. Neither magnesium nor ruthenium red block the ryanodine-activated channel in the presence of 0.1 M KCl[2]; therefore, direct effects by these agents on the channel during the efflux period were not a factor. In Fig. 8*A*, the increase in HSR membrane calcium permeability induced by ryanodine was diminished with increasing time of exposure of the membranes to calcium (5 mM). In parallel experiments, calcium levels greater than 25 μM were found to decrease the binding of [^{3}H]ryanodine to HSR membranes (Fig. 8*B*). Thus, the inhibition by calcium of the increase in HSR membrane calcium permeability caused by ryanodine is, at least in part, due to an inhibition of ryanodine binding at the site responsible for this effect.

Fourth, [^{3}H]ryanodine binding and the increased calcium permeability caused by low concentrations of ryanodine exhibited a marked dependence on temperature. As shown in Table III, both variables decreased as the temperature maintained during exposure of the membranes to ryanodine was lowered from 37 to 25 °C. This dependence on temperature was a function of ryanodine binding and not of the expression of its effects on membrane permeability, since similar results were obtained at assay temperatures of either 25 or 37 °C, if the membranes had been first exposed to ryanodine at 37 °C (data not shown). A similar temperature dependence has been reported for the actions of high concentrations of ryanodine to increase ATP-dependent calcium accumulation by cardiac SR membranes (14). And fifth, the expression of both [^{3}H] ryanodine binding and its functional consequences exhibit similar dependencies on incubation time becoming maximal after 2 h at 37 °C. These results suggest that the binding site identified using [^{3}H]ryanodine is that responsible for the increased HSR membrane calcium permeability caused by this agent.

The requirement for high concentrations of ryanodine to decrease calcium permeability (*cf.* Fig. 3) could reflect actions by ryanodine at a low affinity binding site, or that the conversion of one ryanodine binding conformation to another was very slow if a single binding site is involved. To investigate the nature of the site or site conformation involved further, we assessed whether washing membranes previously exposed to either low or high concentrations of ryanodine with ryanodine-free solutions reversed the effects of this agent. As shown in Fig. 9, the increase the in HSR membrane calcium permeability caused by low concentrations of ryanodine was partially reversed by this procedure. In contrast, the ability of high concentrations of this agent to preclude the increase in HSR membrane calcium permeability caused by low concentrations of ryanodine was unaffected, since the wash step did not result in an increase in the calcium permeability of membranes previously exposed to high concentrations of ryanodine (*i.e.* there was not a shift to the left along the ryanodine concentration response curve shown in Fig. 3). Therefore, the site or site conformation at which ryanodine acts to decrease HSR membrane calcium permeability may either have a relatively high affinity or a slow rate of dissociation for this ligand. Additional studies are required to clarify further the nature of this site.

Assay Condition-dependent Expression of the Actions by Ryanodine—Different workers have reported that high concentrations (100–300 μM) of ryanodine can both increase and decrease ATP-dependent calcium accumulation by both cardiac and skeletal SR membranes (11–16). We investigated whether these conflicting results might be due to the use of different assay conditions by the different laboratories. In particular we compared those employed by Fairhurst (4) and Seiler *et al.* (15), who found a decrease and increase, respectively, in this variable using skeletal HSR membranes. As shown in Fig. 10, ryanodine both increased and decreased ATP-dependent calcium accumulation by the crude HSR membranes originally used by Fairhurst dependent on the assay conditions employed. Although the specific condition(s) responsible for determining the effects of ryanodine that are

TABLE III

The effects of incubation temperature on the actions of ryanodine on the calcium permeability of skeletal muscle HSR membranes and on the binding of [^{3}H]ryanodine to these membranes

Skeletal muscle HSR vesicles were preincubated in either the absence or presence of either [^{3}H]ryanodine or unlabeled ryanodine for 30 min at either 25 or 37 °C. The calcium efflux occurring during a 20-s interval was then assayed 2 min later. [^{3}H]Ryanodine binding was determined as described under "Experimental Procedures." The same temperature used during the preincubation period was maintained during the duration of the experiment. At 37 °C, [^{3}H]ryanodine binding was 13.7 pmol/mg of HSR membrane protein.

Temperature	Vesicular calcium		[^{3}H]Ryanodine binding
	Control	1 μM ryanodine	
	nmol/mg protein		*% of 37 °C value*
25	39.6	30.2	9.3
37	64.0	11.0	100

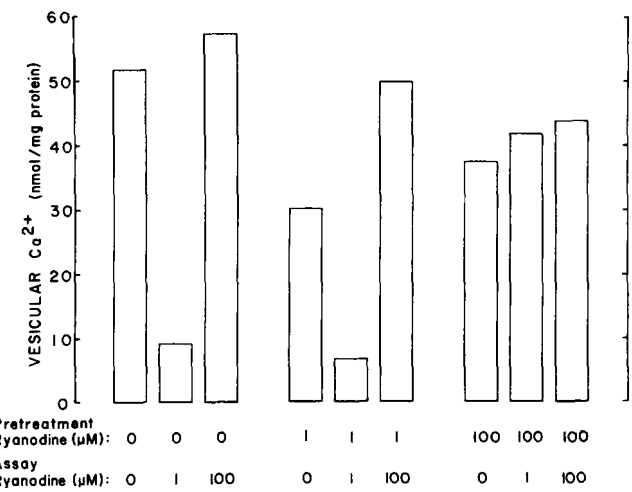

FIG. 9. **Assessment of the reversibility of the effects of low and high concentrations of ryanodine.** Skeletal muscle HSR membranes were exposed to either 0, 1, or 100 μM ryanodine (pretreatment) for 30 min at 37 °C. They were then diluted 520-fold and washed with ryanodine-free solution and collected by centrifugation. The membranes were resuspended in ryanodine-free solution and incubated for a second 30-min period in the absence and presence of either 1 or 100 μM ryanodine. Calcium was added during the final 5 min of the incubation period, and calcium efflux occurring during a 20-s interval was then determined (assay). The results are expressed as the quantity of calcium present in the vesicles at the end of the efflux period.

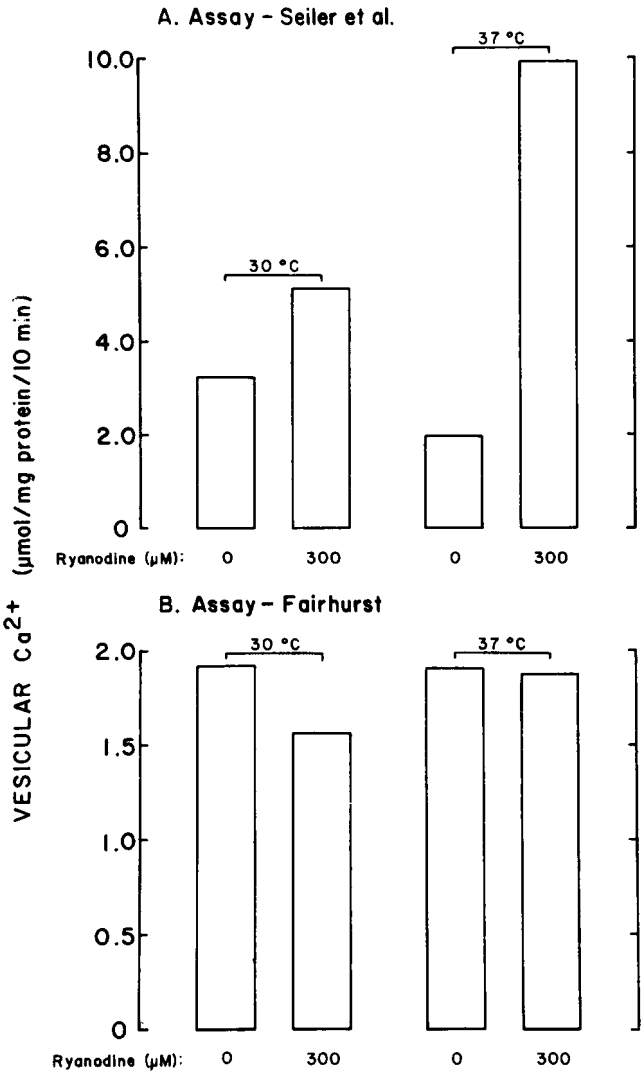

FIG. 10. **Assay condition-dependent alteration of ATP-dependent calcium accumulation by a crude preparation of skeletal muscle HSR membranes (4).** Calcium accumulation was assayed in the presence or absence of 300 μM ryanodine at both 30 and 37 °C using the assay conditions of either Seiler *et al.* ((15) *Panel A*) or Fairhurst ((4) *Panel B*) described under "Experimental Procedures."

expressed is not clear, these results do indicate that the basis of the disagreement between the results obtained in different laboratories may lie in the effects exerted by this condition on the susceptibility of the SR calcium channel to the actions of ryanodine. The existence of a condition-dependent channel conformation that binds ryanodine would be consistent with the use dependence observed for the effects of this agent on intact tissues (*cf.* Ref. 9).

DISCUSSION

The present results demonstrate that ryanodine can both increase and decrease the calcium permeability of skeletal and cardiac SR membranes. These data confirm and extend those reported earlier by Fairhurst and Jenden and their colleagues (4–6, 8), and they are in agreement with those recently reported by Meissner (7). Moreover, the following observations suggest that ryanodine produces these effects by acting directly on the calcium release channel. First, the actions of ryanodine are localized to membranes derived from junctional or terminal regions of the SR. The calcium release channel is similarly thought to be localized in these membranes (32). Second, our results and those of Meissner (7) indicate that the calcium permeability affected by ryanodine has the characteristics expected for the calcium release channel (25). Third, ryanodine does not appear to affect directly a component of the SR membrane ATP-dependent calcium transport system, since LSR membranes which also contain this system were unaffected by ryanodine. Fourth, the rapid time course of the ryanodine-sensitive fluxes across HSR membranes (*cf.* Fig. 1) is consistent with the flux rates thought to occur through a channel (33). Two additional observations have been made. First, the specific [^{3}H]ryanodine binding site present in skeletal HSR membranes appears to be that affected by ryanodine to cause an increase in calcium permeability of these membranes. Second, the ability of nanomolar concentrations of ryanodine to change SR membrane calcium permeability is similar to concentration dependence of its effects on intact muscles (9) and is consistent with a cause and effect relationship existing between these two events.

In its ability to both increase and decrease the flux of calcium through the SR membrane calcium channel, ryanodine resembles the dihydropyridine calcium channel effectors, such as Bay K 8644, that similarly exert different effects on calcium fluxes through plasma membrane calcium channels (34, 35). The existence of the two effects by ryanodine on HSR membrane calcium permeability suggests that there are two binding sites or binding site conformations for this compound that affect the calcium release channel. The exact nature of these sites and their relationship to the calcium release channel remain to be elucidated, but the present data suggest the possibility that the SR and plasma membrane calcium channels share similar general regulatory features and that endogenous effectors may exist for both channels. The ability of ryanodine to affect the calcium release channel when present in nanomolar concentrations makes it a potentially useful experimental probe of the biochemical and biophysical characteristics of the protein(s) involved.

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