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# Ryanodine as a Probe for the Functional State of the Skeletal Muscle Sarcoplasmic Reticulum Calcium Release Channel

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## SUMMARY

In this paper, we study the modulation of the rabbit fast twitch skeletal muscle calcium release channel by assaying the kinetics of [$^3$H]ryanodine binding, $^{45}Ca^{2+}$ flux, and single-channel activity. The effects of modulators of the $Ca^{2+}$ release channel (confirmed here with both flux and single-channel data) were examined for effects on [$^3$H]ryanodine binding to terminal cisternae vesicles. We find that activators of the release channel, such as adenine nucleotides (1 mM) and caffeine (1 mM), enhance the rate of association of [$^3$H]ryanodine, whereas inhibitors, such as $Mg^{2+}$ (1 mM) and ruthenium red (100 nM), decrease the rate of association. High concentrations of either ryanodine or ruthenium red, which close the channel, slow the dissociation of [$^3$H]ryanodine, suggesting that at these concentrations the inhibitory effects of both ryanodine and ruthenium red occur as the result of binding at a site distinct from but interacting cooperatively with the high affinity site. Our data are consistent with a model in which the high affinity ryanodine binding site is within a conformationally sensitive area of the channel, such that conditions that open the channel (ATP, caffeine, etc.) enhance the rate at which [$^3$H]ryanodine reaches its binding site and other conditions that close the channel (the binding of ryanodine and ruthenium red to a low affinity site) slow the dissociation of [$^3$H]ryanodine from the high affinity site. Some conditions that inhibit channel activity (high concentrations of $Mg^{2+}$ and $Ca^{2+}$) slow association but do not affect dissociation of bound [$^3$H]ryanodine, suggesting a completely different state of the channel from that which is inactive in the presence of high concentrations of ryanodine or ruthenium red. In summary, the functional state of the fast twitch skeletal muscle calcium release channel can be characterized by the changes in the kinetics of [$^3$H]ryanodine binding. Different modulators (activators/inhibitors) affect different aspects of ryanodine binding (association/dissociation).

Ryanodine, a plant alkaloid, exhibits toxic effects on muscle tissue (1, 2). The site of action of ryanodine is apparently a "heavy" fraction of SR (3) on a $Ca^{2+}$ release channel in isolated skeletal muscle terminal cisternae (4–6). The channel protein has been isolated, purified as a homotetramer with a subunit of $M_r$ = 400,000–450,000 in both skeletal and cardiac muscle (7–11), and reconstituted into a phospholipid planar bilayer, where it functions as a ligand-gated $Ca^{2+}$ channel (7, 10, 12) that is activated by ryanodine in a manner comparable to that in isolated vesicles (13). The $Ca^{2+}$ release channel corresponds morphologically to the foot structure (10, 14, 15) at the transverse tubule-SR junction, where it is likely to be involved in excitation-contraction coupling (16).

The binding of [$^3$H]ryanodine to terminal cisternae is reported to be influenced by several factors, such as effectors of $Ca^{2+}$ release [e.g., $Ca^{2+}$, $Mg^{2+}$, caffeine, and adenine nucleotides (6, 17–19)], ionic strength (6, 8, 19), pH (19, 20), and temperature (18). Cardiac SR has two classes of binding sites for [$^3$H]ryanodine (8, 9, 19). Recent evidence (21) suggests that this is also true for fast twitch skeletal muscle. At present, the available evidence indicates that there is one high affinity ryanodine binding site per tetramer of both the cardiac and skeletal muscle SR $Ca^{2+}$ release channels (10, 11).

The effects of ryanodine on $Ca^{2+}$ flux are influenced by similar factors, with high ryanodine concentrations blocking a $Ca^{2+}$ efflux pathway (18, 22, 23) rather than affecting the $Ca^{2+}$, $Mg^{2+}$-dependent ATPase (24, 25). In this study, we have used ryanodine to probe the functional state of the $Ca^{2+}$ release channel. We correlate the biphasic effects of ryanodine on $Ca^{2+}$ flux of isolated terminal cisternae and single calcium channels reconstituted into bilayers with [$^3$H]ryanodine binding in the presence of $Ca^{2+}$ channel effectors. We present an in-depth analysis of the effects of the modulators on the association and dissociation kinetics of [$^3$H]ryanodine, on its equilibrium binding and on the activity of the release channel.

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**ABBREVIATIONS:** SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; AMP-PNP, 5′-adenylylimidodiphosphate, tetralithium salt; AMP-PCP, β,γ-methyleneadenosine-5′-triphosphate, sodium salt; BSA, bovine serum albumin.

## Experimental Procedures

**Materials.** ATP, AMP-PNP, and AMP-PCP were purchased from Sigma Chemical Co. (St. Louis, MO). Ryanodine was from CalBiochem (La Jolla, CA). [$^3$H]Ryanodine (60 Ci/mmol) and $^{45}CaCl_3$ were from Dupont-New England Nuclear (Wilmington, DE). Phospholipids were obtained from Avanti (Pelham, AL).

**SR preparation.** Junctional terminal cisternae (26) and heavy SR vesicles, obtained in a 40% sucrose gradient after density centrifugation (27), were isolated from the back and hindlegs of rabbit fast twitch skeletal muscle.

**Passive $^{45}Ca^{2+}$ loading.** SR vesicles (1 mg of protein/ml) were passively loaded with 1 mM $^{45}CaCl_2$ (150,000 cpm/nmol) for 2 hr at 25°, in a medium containing 20 mM MOPS (pH 7.0), 300 mM KCl, and varying $MgCl_2$ concentrations, before the addition of ryanodine. At appropriate time intervals after the addition of ryanodine and/or adenine nucleotide (time zero), about 30 μg (30 μl) of SR were diluted 33-fold into 1 ml of $Ca^{2+}$ release-inhibiting medium, containing 10 mM $MgCl_2$, 1 mM EGTA, 300 mM KCl, and 20 mM MOPS (pH 7.0), at 25°. The entire volume was immediately filtered through Millipore filters (0.45 μm, type HA; Millipore Corp., Bedford, MA). The filters were washed with 2 ml of release-inhibiting medium and counted for radioactivity in 10 ml of Bray's scintillation fluid (Research Products International, Mount Prospect, IL).

**Equilibrium [$^3$H]ryanodine binding.** [$^3$H]Ryanodine was incubated for 16 hr at room temperature (22–25°) with 5–20 μg of SR membranes in 250 μl of binding buffer containing 300 mM KCl, 100 μM $CaCl_2$, and 20 mM MOPS (pH 7.0), with or without adenine nucleotides and $MgCl_2$. The [$^3$H]ryanodine was diluted 1:10 with unlabeled ryanodine for concentrations ranging from 0.4 to 100 nM. Nonspecific binding was defined in the presence of 10 μM unlabeled ryanodine. The entire sample volume was filtered through Whatman GF/F glass fiber filters. The filters were washed with five 5-ml aliquots cold 0.3 M NaCl and counted by liquid scintillation, after the addition of 10 ml of Ready Protein (Beckman, Stanford, CA) and 1 hr of shaking. Scatchard plots were analyzed by linear regression.

**Equilibrium binding data analysis.** The binding data were fit to a single-site model, based on a nonlinear least squares Marquardt method, to estimate the free parameters $B_{max}$, $K_d$, and $K_i$. Parameter uncertainties were provided by $\chi^2$ values. Scatchard analysis was performed using a least squares fit linear regression program.

**[$^3$H]Ryanodine association kinetics.** Heavy SR vesicles (0.03–0.1 mg of protein with 7–30 pmol/mg of [$^3$H]ryanodine binding sites) were added to [$^3$H]ryanodine (5–35 nM), binding buffer, and 100 μg/ml BSA, at room temperature, in 300 mM KCl, 20 mM MOPS (pH 7.0), in the presence of the indicated modulators (see Table 3). At 15-min intervals, 200–500-μl aliquots were filtered, washed, and processed for radioactivity, as described above. Equilibrium values were obtained at 8 hr. An association plot of ln $B_{eq}/(B_{eq} - B)$ over time was analyzed, where $B_{eq}$ and $B$ are the amounts of [$^3$H]ryanodine bound at equilibrium (8 hr) and time $t$, respectively. $k_{obs}$ was derived from the slope. Association curves where the amount of [$^3$H]ryanodine bound at equilibrium exceeded 5% of the [$^3$H]ryanodine added were discarded.

**[$^3$H]Ryanodine dissociation.** Heavy SR vesicles (0.5–2.5 mg of protein/ml, with 7–30 pmol/mg of binding sites) were equilibrated for 15 hr at 4° with [$^3$H]ryanodine (5–30 nM), in 300 mM KCl, 20 mM MOPS (pH 7.0), with the indicated modulators (see Table 2). In order to initiate dissociation of bound [$^3$H]ryanodine at time zero (room temperature), the membranes were diluted to less than 0.1 nM [$^3$H] ryanodine in binding buffer containing 100 μg/ml BSA and the indicated concentrations of unlabeled ryanodine and/or other effectors (see Table 2). Aliquots (200 μl) were filtered at the indicated intervals for a total period of 48 hr. The filters were processed for radioactivity as described above.

Protein was estimated by the method of Lowry *et al.* (28), using BSA as standard.

**Bilayer techniques.** Bilayers were formed over a 100-μm diameter aperture in the wall (thickness, 50 μm) of a Derlin cup. For SR vesicles, a 5:3:2 mixture of phosphatidylethanolamine from bovine heart, phosphatidylcholine from egg, and phosphatidylserine from bovine brain (50 mg/ml in decane) was used, according to the method of Mueller *et al.* (29). The side of the bilayer to which the membrane preparations were added is defined as *cis* (0.5-ml chamber). The other side of the bilayer is defined as *trans* (4.0 ml).

Salt agar bridges, equilibrated in the solutions present in each chamber, were used to connect the chambers to Ag/AgCl electrodes immersed in 2 M KCl. The Ag/AgCl electrodes were connected to the appropriate inputs of the amplifier (27).

The following bilayer-forming procedure was used. A drop (1–2 μl) of the bilayer-forming solution was applied to the edges of the aperture and allowed to air dry (10 min). Both chambers were filled with buffer solution [25 mM $Cs_2SO_4$, 10 mM MOPS (pH 7.4), 8 μM $CaCl_2$.] The aperture was occluded by application of a small amount of the bilayer-forming solution, using a glass rod. While the chambers were stirred, the ionic gradient was established (125 mM $Cs_2SO_4$) and the vesicle preparation was added. Excess lipid was removed gently until the capacitance across the aperture reached a value of 150–300 pF.

In general, the *cis* chamber solution was made hypertonic to the solution in which the membranes were resuspended. After addition of membranes, the final protein concentration in the *cis* chamber was in the range of 1–20 μg/ml. An osmotic gradient was established between the *cis* and *trans* chambers by addition of a concentrated $Cs_2SO_4$ (the current carrier) solution to the *cis* chamber. These conditions increased the frequency of vesicle fusion with the bilayer.

## Results

**[$^3$H]Ryanodine binding.** The affinity of SR membranes for [$^3$H]ryanodine is markedly dependent on the presence of compounds known to alter calcium release from the SR. The $K_d$ values obtained by Scatchard analysis of [$^3$H]ryanodine binding under a variety of conditions are summarized in Table 1. Purified junctional terminal cisternae have between 20 and 35 pmol of binding sites/mg of protein. Heavy SR membranes exhibit similar high affinity binding for ryanodine but have a lower site density (Fig. 1A). As shown in Fig. 1B, under the conditions of our assay, nonspecific binding is low.

Compounds that are known to affect the release of $Ca^{2+}$ from the SR are adenine nucleotides, $Ca^{2+}$, $Mg^{2+}$, ruthenium red, and caffeine. We have characterized the effects of these compounds on [$^3$H]ryanodine binding. The apparent $K_d$ (Table 1) for the binding of [$^3$H]ryanodine is affected by adenine nucleotides (Fig. 1, A and B), $Mg^{2+}$ (Fig. 1, A and B), ruthenium red (Fig. 1C), and ionic strength (Fig. 2). ATP, AMP-PNP, and AMP-PCP increase the apparent affinity of the site for [$^3$H]ryanodine. Caffeine, an activator of calcium release, causes only small increases in the apparent affinity of the binding site for ryanodine. Differences in the $B_{max}$ values under these condi-

TABLE 1

**Dissociation constants**

Numbers in parentheses are number of experiments.

| Buffer | $K_d$ (equilibrium) | $K_d$ (kinetic)* |
|---|---|---|
| | *nM* | |
| Control | 7.94, 8.35 | 5.8 |
| Control, 1 mM $Mg^{2+}$ | 36.6, 33.1 | |
| 1 mM AMP-PNP | 2.15 ± 0.77 (5) | 2.8 |
| 1 mM AMP-PCP | 1.95 | 2.5 |
| 1 mM AMP-PNP, 1 mM $Mg^{2+}$ | 3.70 ± 1.67 (4) | 7.6 |
| 1 mM AMP-PNP, 1 mM caffeine | 1.42 | 1.1 |
| 1 mM AMP-PCP, 1 mM caffeine | 1.23 | |
| 1 mM AMP-PNP, 100 nM ruthenium red | 6.16 | |

* $K_d = k_{-1}/k$.

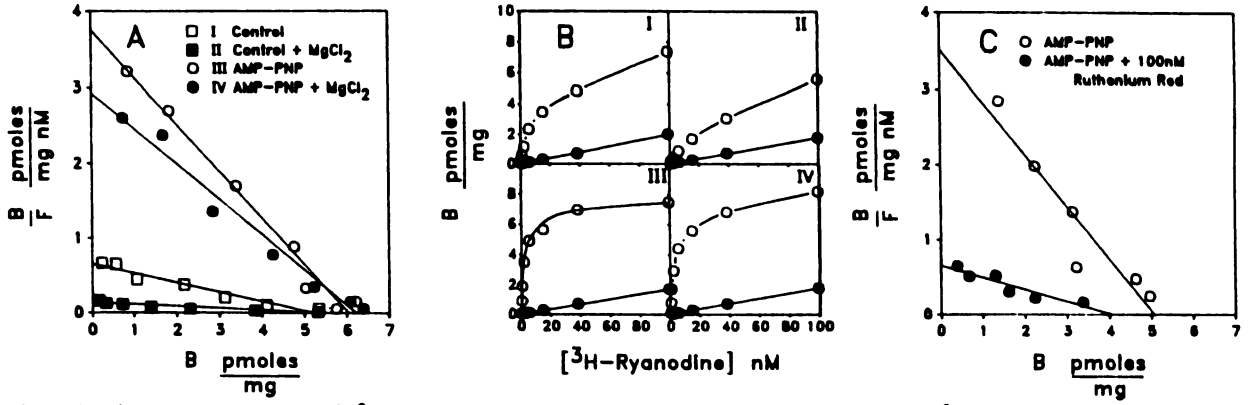


**Fig. 1.** Analysis of equilibrium binding of [$^3$H]ryanodine to SR membranes. A, Scatchard analysis of [$^3$H]ryanodine binding (as described in Experimental Procedures) was performed in a control binding buffer containing 300 mM KCl, 100 $\mu$M $CaCl_2$, 20 mM MOPS (pH 7.0) (I) (□) ($K_d$ = 7.94 nM, $B_{max}$ = 5.23 pmol/mg, $r$ = 0.972), with 1 mM $MgCl_2$ (II) (■) ($K_d$ = 36.6 nM, $B_{max}$ = 5.35 pmol/mg, $r$ = 0.938), with 1 mM AMP-PNP, (III) (○) ($K_d$ = 1.61 nM, $B_{max}$ = 6.05 pmol/mg, $r$ = 0.990), and with 1 mM AMP-PNP plus 1 mM $MgCl_2$ (IV) (●) ($K_d$ = 2.11 nM, $B_{max}$ = 6.22 pmol/mg, $r$ = 0.987). B, Equilibrium binding curves for [$^3$H]ryanodine binding for the Scatchard plots described in A. ○, Total binding; ●, non-specific binding. Nonspecific binding is defined as that obtained in the presence of 10 $\mu$M unlabeled ryanodine. I–IV correspond to A. C, Scatchard analysis of [$^3$H]ryanodine binding in control binding buffer containing 1 mM AMP-PNP (○) ($K_d$ = 1.44 nM, $B_{max}$ = 5.07 pmol/mg, $r$ = −0.942) and with 100 nM ruthenium red (●) ($K_d$ = 6.16 nM, $B_{max}$ = 4.06 pmol/mg, $r$ = −0.925).

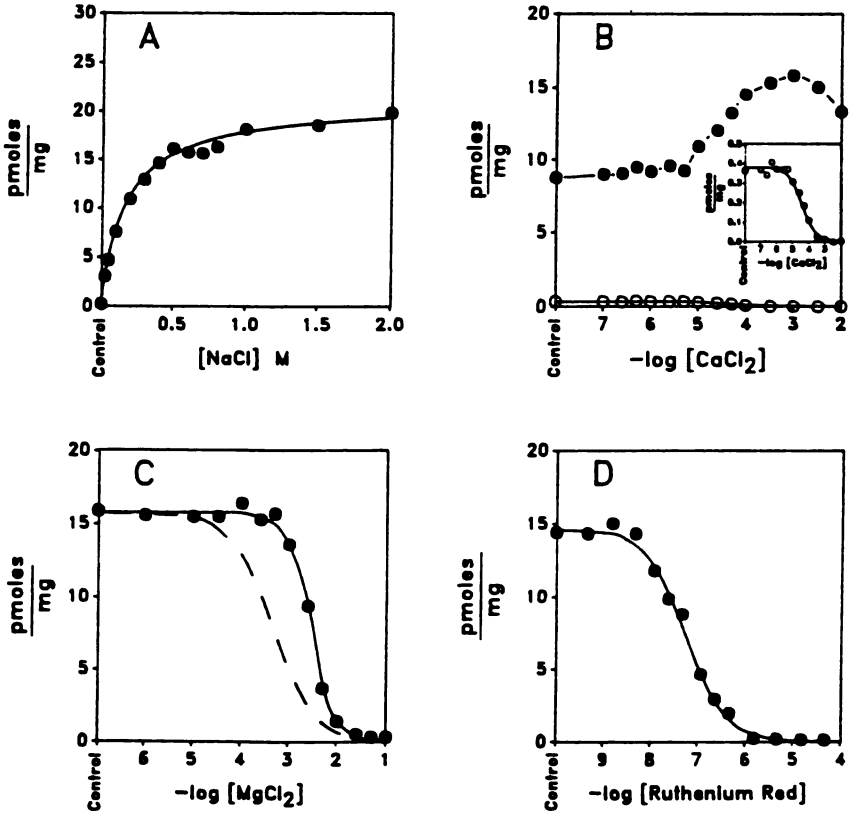


**Fig. 2.** Effect of ionic strength, $Ca^{2+}$, $Mg^{2+}$, and ruthenium red on [$^3$H]ryanodine binding to heavy SR membranes. Membranes (10 $\mu$g in a 250-$\mu$l assay volume) were incubated 16 hr with 5.0 nM [$^3$H]ryanodine, in a binding buffer containing 1 mM AMP-PCP, 20 mM MOPS (pH 7.0), 100 $\mu$g/ml BSA, and 100 $\mu$M $CaCl_2$, in the presence of 25 mM to 2.0 M NaCl (A), 10 $\mu$M to 10 mM $CaCl_2$ (○, control; ●, control plus 1 M NaCl; *inset*, control on expanded scale (B), 1 $\mu$M to 100 mM $MgCl_2$ with 0.3 M KCl (C), and 1 mM to 100 $\mu$M ruthenium red with 0.3 M KCl (D). Filtration and scintillation counting were carried out as described in Experimental Procedures.

tions were found not to be statistically significant, except for ruthenium red, which consistently gave a lower $B_{max}$ than in controls. This is presumably due to some binding of ruthenium red to a low affinity site that noncompetitively alters high affinity binding. This will be discussed in a later section.

High concentrations of NaCl also increase binding by increasing affinity. KCl will substitute for NaCl and the effect, therefore, appears to be one of ionic strength. No specific binding of [$^3$H]ryanodine can be detected at salt concentrations below 10 mM. In addition, dilution of bound [$^3$H]ryanodine into buffer without salt results in extremely rapid dissociation ($t_{1/2} \approx 1$ min). $Ca^{2+}$ is also required for [$^3$H]ryanodine binding; the addition of 1 mM EGTA decreases binding by 99 and 96% in the absence and presence of 1 M NaCl, respectively, for the experiment shown in Fig. 2B. In the presence of NaCl, $Ca^{2+}$ appears to stimulate binding even further (Fig. 2B). Fig. 2A show the effects of increasing concentrations of NaCl on [$^3$H] ryanodine binding at 100 $\mu$M $Ca^{2+}$ and Fig. 2B show the effects of increasing concentrations of $Ca^{2+}$ at two different ionic strengths. Higher concentrations of $Ca^{2+}$ (>2 mM) inhibit binding (Fig. 2B, *inset*), decreasing the affinity and $B_{max}$ for binding.

$Mg^{2+}$ and ruthenium red increase the apparent $K_d$ for binding of [$^3$H]ryanodine. The inhibition of [$^3$H]ryanodine binding by increasing concentrations of $Mg^{2+}$ and ruthenium red is shown

in Fig. 2, C and D, respectively. The inhibition of [$^3$H]ryanodine binding by low concentrations of ruthenium red is suggestive of competitive inhibition, with a $K_i$ of 17 and 28 nM in two independent determinations.

To characterize further the interactions of these compounds with the ryanodine binding site, we examined the kinetics of ryanodine binding in the presence of activators (caffeine, AMP-PNP, and AMP-PCP) and inhibitors (ruthenium red, 10 mM $Mg^{2+}$, and 10 mM $Ca^{2+}$) of calcium release. Dissociation was initiated by a greater than 100-fold dilution of the radiolabeled ryanodine and was monitored for a total of 48 hr. Full dissociation occurred in all buffers and the dissociation could, in each case, be fitted to a single exponential. Dissociation constants calculated from these experiments are shown in Table 2. As can be seen in Table 2, neither 10 mM $Mg^{2+}$, caffeine, AMP-PNP, nor AMP-PCP significantly altered the rate of dissociation; neither do 10 mM $CaCl_2$, 1 mM EGTA, or 1 M NaCl (data not shown). However, as summarized in Table 2 and shown in Fig. 3, high concentrations of both ruthenium red and unlabeled ryanodine greatly slow the rate of dissociation of bound [$^3$H] ryanodine. The ruthenium red effect is seen only at concentrations above 1 $\mu$M and the ryanodine effect is only seen at concentrations greater than 5 $\mu$M.

Association rate constants were determined under pseudo-first-order conditions, either from the slope of a plot of $k_{obs}$ versus [$^3$H]ryanodine concentration or using the independently determined $k_{-1}$ values (Table 1) and the equation (for review, see Ref. 30):

$$k_1 = \frac{k_{obs} - k_{-1}}{[[^3\text{H}]\text{ryanodine}]}$$

where $k_1$ is the association rate constant, $k_{-1}$ is the dissociation rate constant, and $k_{obs}$ is the slope of a ln $[B_{eq}/(B_{eq} - B)]$ versus time plot, where $B_{eq}$ is the amount of [$^3$H]ryanodine bound at equilibrium and $B$ is that bound at time $t$. Association rate constants were calculated using only those experiments in which less than 5% of the added [$^3$H]ryanodine was bound at equilibrium and are summarized in Table 3. As can be seen, AMP-PNP and AMP-PCP increase the rate of association of [$^3$H]ryanodine. Both 1 M NaCl (or KCl) and caffeine can also increase the rate and this appears to be additive to the effect of AMP-PCP. Both ruthenium red (100 nM) and $Mg^{2+}$ (1 mM) decrease the rate of association.

**$^{45}Ca^{2+}$ flux studies.** We observed ryanodine effects on the ability of preloaded junctional terminal cisternae to retain $Ca^{2+}$ over a period of 24 hr. We examined the influence of various effectors, such as $Mg^{2+}$ and adenine nucleotide, in the same reaction conditions as for the [$^3$H]ryanodine binding studies. In the absence of these effectors, the vesicles are able to retain $Ca^{2+}$ over a long period of time. Ryanodine induces the opening of the $Ca^{2+}$ release channel, resulting in a loss of SR $Ca^{2+}$ in as little as 30 min (Fig. 4A). A biphasic effect is observed with higher ryanodine concentrations (100 $\mu$M), consistent with an earlier report (23). Under our conditions, there is an initial activation, followed by a gradual inactivation of the channel upon prolonged incubation, thereby allowing the SR to retain vesicular $Ca^{2+}$ and sometimes to reaccumulate external $Ca^{2+}$. At the onset of $Ca^{2+}$ loading, the presence of $Mg^{2+}$, which closes the channel, reduces the loading as well as $Ca^{2+}$ release. The presence of $Mg^{2+}$ also slows the time course of the ryanodine activation, thus delaying ryanodine-induced $Ca^{2+}$ release. This is consistent with the ability of $Mg^{2+}$ to slow the rate of association of [$^3$H]ryanodine. We further show that the addition of AMP-PNP opens the release channel and there is a rapid loss of $Ca^{2+}$ from SR, despite the presence of $Mg^{2+}$ (Fig. 4B), consistent with the observations of Pessah *et al.* (6) that $Mg^{2+}$ inhibition of the channel is overcome by adenine nucleotides. The biphasic behavior of high concentrations of ryanodine is qualitatively the same as in Fig. 4A.

**Single-channel analysis.** The effects of high and low concentrations of ryanodine on the activity of the release channel were also investigated using SR membranes reconstituted into bilayers (Fig. 5). These experiments were performed both with (Fig. 5B) and without (Fig. 5A) ATP in the buffers. In these experiments, $Cs^+$ was used as a current carrier (31). With heavy SR membranes, a rapidly flickering channel with a slope conductance of 505 pS in 125 mM $Cs_2SO_4$ *cis*, 25 mM $Cs_2SO_4$ *trans* was recorded. Both the *cis* and *trans* chambers also contained 10 mM MOPS (pH 7.4) and 8 $\mu$M $CaCl_2$. The addition of ryanodine at 1 $\mu$M produced long openings with a slope conductance of 250 pS. The onset of this effect was faster in the presence of ATP (Fig. 5B). Higher concentrations of ryanodine first activated and then inhibited channel activity.

## Discussion

In this paper, we present an in-depth kinetic and equilibrium analysis of the modulation of [$^3$H]ryanodine binding by factors that regulate the activity of the $Ca^{2+}$ release channel. One of the factors, ruthenium red, is studied in detail for the first time. We have demonstrated the following points. 1) Adenine nu-

TABLE 2
**Dissociation rate constants**
Numbers in parentheses are numbers of experiments.

| Added to incubation buffer | Added to dilution buffer | $k_{-1}$ |
|---|---|---|
| | | $min^{-1}$ |
| 0 | 0 | 0.0020, 0.0026 |
| 100 $\mu$M $Ca^{2+}$ | 100 $\mu$M $Ca^{2+}$ | |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 0.0025 ± 0.0002 (12) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 0.0028 ± 0.0006 (8) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 10 mM $Mg^{2+}$, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 0.0025 ± 0.0007 (3) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 1 mM Caffeine, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 0.0021 & 0.0020 |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 100 $\mu$M Ryanodine, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 0.00075 ± 0.00008 (3) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 10 $\mu$M Ruthenium red, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PNP | 0.00063 ± 0.00017 (3) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 100 $\mu$M Ryanodine, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 0.00094 ± 0.00011 (4) |
| 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 10 $\mu$M Ruthenium red, 100 $\mu$M $Ca^{2+}$, 1 mM AMP-PCP | 0.00090 ± 0.00002 (4) |

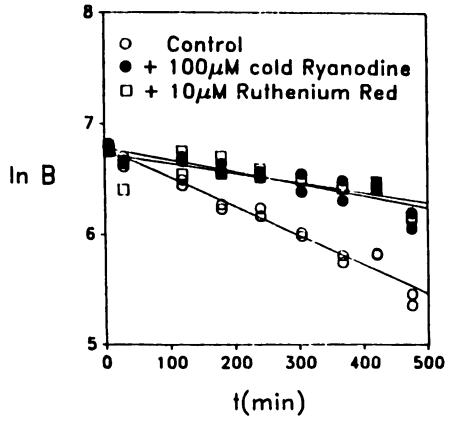


**Fig. 3.** Dissociation of bound [$^3$H]ryanodine. SR vesicles (1.8 mg of protein/ml, 28 nM in sites) were equilibrated with [$^3$H]ryanodine (25 nM) in a binding buffer containing BSA and 1 mM AMP-PCP. To initiate dissociation of bound [$^3$H]ryanodine, the vesicles were diluted to 0.05 nM [$^3$H]ryanodine in binding buffer containing BSA. ○, Control; □, with 10 μM ruthenium red; ●, with 100 μM unlabeled ryanodine. Aliquots (1.2 μg) were filtered at the indicated times.

TABLE 3

**Association rate constants**

Numbers in parentheses are number of experiments.

| Added to incubation buffer | $k_1$ |
|---|---|
| | $nM^{-1}\ min^{-1}$ |
| 0 | 0.0005 |
| 100 μM $Ca^{2+}$ | 0.0004 ± 0.0002 (6) |
| 100 μM $Ca^{2+}$, 1 mM caffeine | 0.00108 |
| 100 μM $Ca^{2+}$, 1 mM AMP-PNP | 0.0010 ± 0.0004 (7) |
| 100 μM $Ca^{2+}$, 1 mM AMP-PCP | 0.0010 ± 0.0005 (9) |
| 100 μM $Ca^{2+}$, 1 mM AMP-PCP, 1 mM caffeine | 0.0019 ± 0.0005 (11) |
| 100 μM $Ca^{2+}$, 1 mM AMP-PCP, 1 mM $Mg^{2+}$ | 0.0003 ± 0.0002 (6) |
| 100 μM $Ca^{2+}$, 1 mM AMP-PCP, 1 M NaCl | 0.0020 ± 0.0004 (8) |
| 100 μM $Ca^{2+}$, 1 mM AMP-PCP, 100 nM ruthenium red | 0.00011, 0.00012 |

cleotides, high ionic strength, caffeine, and micromolar $Ca^{2+}$ increase the affinity of the receptor for [$^3$H]ryanodine by increasing the rate of association. 2) Millimolar $Mg^{2+}$, millimolar $Ca^{2+}$, and <1 μM ruthenium red decrease the apparent affinity by slowing the rate of association. 3) The interaction of low concentrations of ruthenium red ($K_i \cong 20$ nM) with the high affinity ryanodine binding site appears to be competitive. 4) High ionic strength also enhances the rate of association and this effect is additive to that of adenine nucleotides. 5) The presence of a high salt concentration shifts the $Ca^{2+}$ inhibition curve to the right, indicating that $Na^+$ can bind to this $Ca^{2+}$ binding site. An additional increase in binding is seen at concentrations of $Ca^{2+}$ below the inhibitory concentration, suggesting multiple interacting $Ca^{2+}$ binding sites on the ryanodine receptor. 6) The association rate constant for [$^3$H]ryanodine indicates that the binding is slower than is expected for diffusion-controlled binding. 7) Both ryanodine and ruthenium red appear to have low affinity binding and this slows the rate of dissociation of [$^3$H]ryanodine from the high affinity site; whether this binding is either saturable or specific is not known. 8) Adenine nucleotides activate the channel and apparently affect the rate of both activation and inhibition of the channel by ryanodine. 9) $Mg^{2+}$ inhibits channel activity, most probably by a mechanism distinct from that of ryanodine. 10) It is unclear whether the inhibitory effect of ruthenium red on the release channel is due to its binding at the high or low affinity

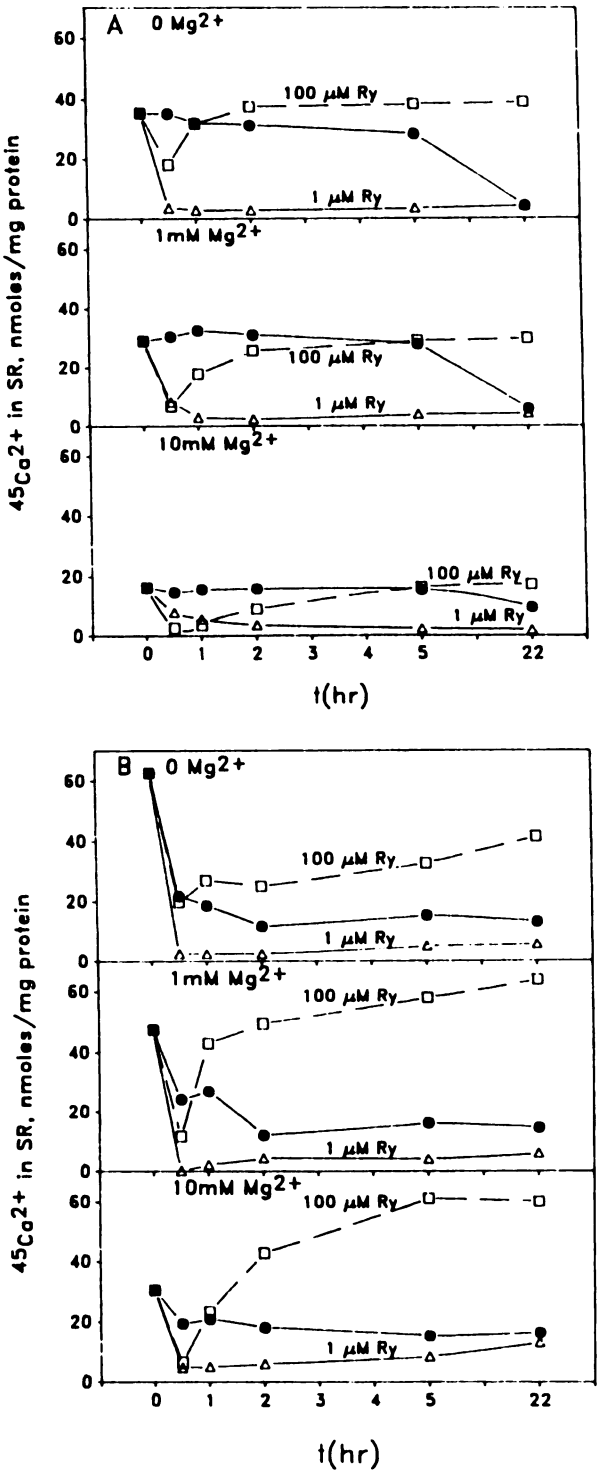


**Fig. 4.** The effect of ryanodine on $Ca^{2+}$ efflux of preloaded junctional terminal cisternae in the presence of $Mg^{2+}$. A, Junctional terminal cisternae vesicles were passively loaded, prior to time zero, with 1 mM $^{45}CaCl_2$ and varying $MgCl_2$ concentrations, as described in Experimental Procedures. At time zero, the vesicles were exposed to 0 (●), 1 μM (△), or 100 μM (□) ryanodine (*Ry*). At the indicated time intervals, 30 μl of SR were diluted into 1 ml of $Ca^{2+}$ release-inhibiting medium. The entire volume was immediately filtered through Millipore filters. The filters were washed with 2 ml of release-inhibiting medium and processed for radioactivity measurements. B, The vesicles were passively loaded with 1 mM $^{45}CaCl_2$ and varying mg. $Cl_2$ concentrations, as in A. After 2 hr of loading (time zero), 1 mM AMP-PNP and ryanodine were added simultaneously to the loading mixture. The different ryanodine concentrations were the same as in A. When 1 M NaCl was present in the loading medium, there was no measurable passive $^{45}Ca^{2+}$ loading (not shown).

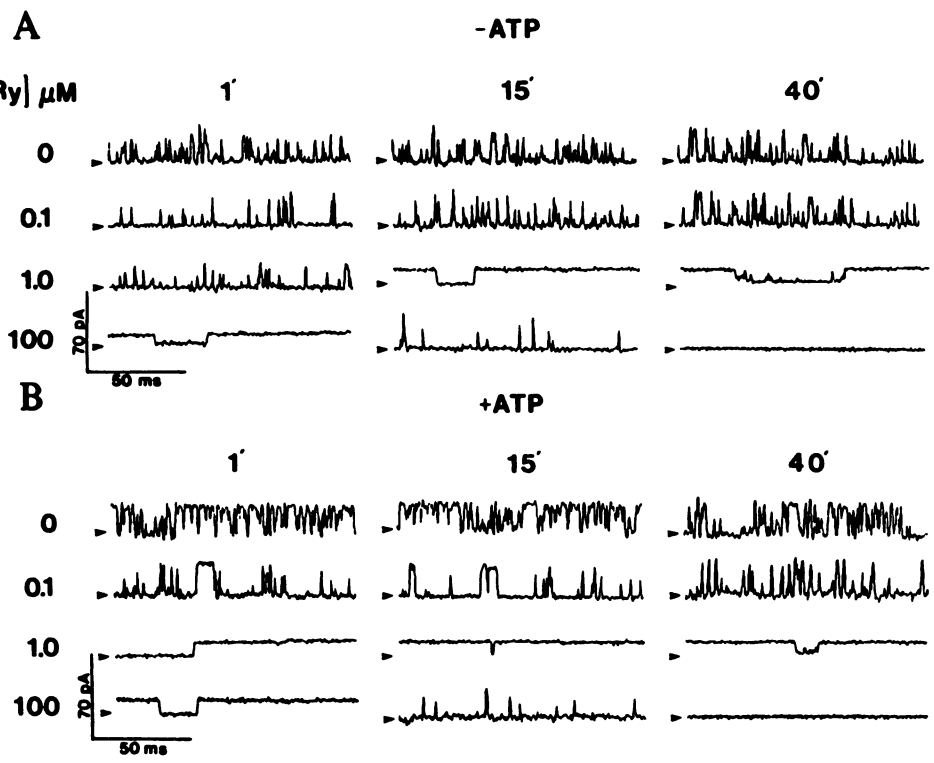


**Fig. 5.** Temporal effects of different ryanodine (*Ry*) concentrations on calcium release channel activity incorporated into bilayers, in the presence or absence of ATP. Heavy SR membranes were incorporated into bilayers phosphatidylethanolamine/phosphatidylcholine/phosphatidylserine, 5:3,2, 50 mg/ml decane). The channel incorporation was obtained with a $Cs_2SO_4$ gradient (125 mM cis, 25 mM *trans*); after the channel incorporation, the gradient was eliminated, in order to avoid bilayer weakness, and the records were obtained with a holding potential of −35 mV but with voltage pulses of +50 mV for up to 45 min thereafter. Cesium current is observed as upward deflections, with the *arrow* representing the closed level. The channel records were obtained without filter cut-off. After the addition of membranes in the *cis* chamber, the final protein concentration was in the range of 5–12 μg/ml. Ryanodine and ATP were added to both chambers to a final concentration of 100 nM, 1 μM, or 100 μM (ryanodine) and 1 mM (ATP). The records shown are representative of the channel activity in the conditions indicated. They were performed in independent observations in at least two different experiments. The recording data were stored and processed as previously reported (27).

site; however, the $K_i$ for the high affinity site is considerably lower than the reported (32) concentration of ruthenium red required for half-maximal inhibition of $Ca^{2+}$ release (~0.8 μM), as reflected by enhancement of $Ca^{2+}$ uptake. This requires further investigation. 11) Ryanodine bound at the high affinity site apparently activates the calcium release channel, but ryanodine at the low affinity site inhibits channel activity. The addition of high concentrations of ryanodine produces first activation followed by inhibition of channel activity.

The slowing of the rate of dissociation of [$^3$H]ryanodine from the high affinity site by ruthenium red or high concentrations of ryanodine suggests an allosteric modulation of the site by these ligands bound at a second site. The currently available data from other laboratories suggest that the ryanodine receptor exists as a tetramer of four identical $M_r$ 450,000 subunits (10) and that the oligomeric complex has only one high affinity [$^3$H]ryanodine binding site per tetramer. The question arises as to whether 1) the binding of ryanodine to one of four initially identical sites induces a conformational change, resulting in lower affinity for ryanodine at the remaining three sites (i.e., negative cooperativity), as suggested by McGrew *et al.* (21); 2) whether three of the potential sites are low affinity before binding ryanodine; or 3) whether there is only one binding site per tetramer. The finding that ryanodine and ruthenium red at high concentrations can slow the dissociation of ryanodine from the high affinity site suggests that low affinity sites do exist and that they can interact allosterically with the high affinity site. We cannot, however, discriminate between negative cooperativity and preexisting differences in the sites in explaining the existence of high and low affinity sites. We also cannot at this time quantitate the number of low affinity sites nor do we have any information about their specificity. We have recently shown that the presence of micromolar concentrations of ryanodine during the purification of the detergent-solubilized complex increases the recovery of the tetrameric structure, suggesting that ryanodine bound at the low affinity site stabilizes this oligomer (33). This finding is consistent with allosteric interaction between subunits of the tetramer.

It has been observed that tryptic digestion of the skeletal muscle ryanodine receptor results in low affinity binding (34), suggesting the possibility that the low affinity site may arise from proteolysis. The reproducibility of the effects of 100 μM ryanodine and 10 μM ruthenium red on the rate of [$^3$H]ryanodine dissociation argues against this interpretation of our data.

The finding that compounds that are known to activate the release channel enhance the rate at which ryanodine reaches its high affinity binding site and compounds that lower release channel activity decrease the rate of association leads to the conclusion that the high affinity binding site is in a very conformationally sensitive part of the protein, such as in the channel itself or associated with the gating mechanism. The ability of ryanodine to lock the channel in an open state of reduced conductance supports this conclusion. In addition, the effect of $Ca^{2+}$ and $Mg^{2+}$ on ryanodine binding suggests that high concentrations of these ligands are inhibiting channel activity by a very different mechanism than ruthenium red and high concentrations of ryanodine, in that the high concentrations of $Mg^{2+}$ and $Ca^{2+}$ slow association of [$^3$H]ryanodine, whereas ruthenium red and ryanodine slow its dissociation.

A question that needs to be addressed is the effect of 1 M NaCl on the binding of [$^3$H]ryanodine. The apparent affinity is increased upon increasing the NaCl concentration, via an increase in the rate of association of ryanodine, an effect that is additive to the effect of adenine nucleotides and low concentrations of $Ca^{2+}$. In addition, dilution of bound [$^3$H]ryanodine into low ionic strength (<10 mM salt) buffer results in an extremely rapid dissociation ($t_{1/2}$ at room temperature of approximately 1 min). An association rate of ryanodine that is

slower than a diffusion-controlled rate can have several explanations. It may mean that the binding site is in a relatively inaccessible location or that the channel must open in order for ryanodine to bind. It has been previously suggested that NaCl affects the conformation of the ryanodine receptor (35). Alternatively, some other molecule may be occupying the binding site and ryanodine must wait for dissociation of this molecule before it can bind to the site. High salt is known to remove loosely associated proteins and molecules and may, therefore, be enhancing the apparent association of ryanodine by removing an endogenous occupant of the site. Because the effects of ionic strength (both high and low) on the properties of the protein appear to be totally reversible (data not shown), we conclude that it is most likely that conformation of the binding site is dependent on ionic strength.

During the preparation of this manuscript, a paper by McGrew *et al.* (21) was published. These workers also found two binding sites for [$^3$H]ryanodine is skeletal muscle. Also in agreement with our results, when they examined the rate of dissociation of bound [$^3$H]ryanodine, they found that binding of ryanodine at the low affinity site correlated both with slower dissociation of bound ryanodine and with closing of the release channel.

In summary, our data suggest that the binding of ryanodine is dependent on the conformation of the protein. Compounds that enhance the release of $Ca^{2+}$ from the SR, such as adenine nucleotides and caffeine, enhance the association rate for [$^3$H] ryanodine but have no effect on its dissociation rate. High concentrations of $Mg^{2+}$ and $Ca^{2+}$, which inhibit SR $Ca^{2+}$ release, slow the rate of association of [$^3$H]ryanodine but like the activators do not alter dissociation. In contrast, high concentrations of ryanodine and ruthenium red slow the dissociation of bound [$^3$H]ryanodine. Ruthenium red at lower concentrations appears to be competitive with [$^3$H]ryanodine for binding to the high affinity site, suggesting a biphasic action similar to that of ryanodine.

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