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Ryanodine Induces Persistent Inactivation of the Ca^{2+} Release Channel from Skeletal Muscle Sarcoplasmic Reticulum

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SUMMARY

Junctional sarcoplasmic reticulum (SR) membranes isolated from rabbit skeletal muscle were pretreated with 0.1–500 μM ryanodine under equilibrium conditions optimal for receptor binding, followed by the removal of bound alkaloid by several washes in Ca^{2+}- and ryanodine-free buffer. Pretreatment with >100 nM ryanodine results in a concentration-dependent decrease in the B_{max} of the high affinity sites and a complete loss of measurable low affinity binding sites that persist for >48 hr. Quantitative analysis of residual ryanodine using three different methods demonstrates that the inhibition is not the result of residual ryanodine bound to its receptor. Ca^{2+} transport measurements made with antipyrylazo III show that actively loaded ryanodine-pretreated SR exhibits a persistent insensitivity to ryanodine- and daunomycin-induced Ca^{2+} release that is not seen with washed control vesicles. Lipid bilayer membranes fused with SR vesicles exhibit rapidly fluctuating single-channel events with a conductance of 468 pS in asymmetric CsCl solutions. Ryanodine (10 μM) produces a unidirectional transition to a slowly fluctuating half-conductance state that is not reversed by perfusion of the bilayer with Ca^{2+}-free buffer and subsequent addition of dithiothreitol. However, dithiothreitol added in the ryanodine pretreatment medium offers marked protection against ryanodine-induced loss of binding sites and allows complete restoration of native gating behavior of single channels in bilayer lipid membrane. Using three different experimental approaches, the data demonstrate that the alkaloid at micromolar concentration persistently alters SR Ca^{2+} release channel function, perhaps by uncoupling four negatively cooperative binding sites. The oxidation of critical receptor thiols is implicated in the process.

[^{3}H]Ryanodine is a specific, conformationally sensitive probe for the Ca^{2+} release channels of muscle SR (1, 2). The functional state of the channel dramatically governs the association and dissociation kinetics and hence the apparent affinity of [^{3}H] ryanodine for its binding site (2–4). Conditions that enhance the open probability (P_o) of the Ca^{2+} channel also enhance the rate of binding of nanomolar [^{3}H]ryanodine with its high affinity site (K_{d_1} = 1–4 nM). Low (nanomolar) Ca^{2+}, which promotes channel closure, decreases the rate of association of the alkaloid and enhances its dissociation, thereby increasing the apparent K_d of [^{3}H]ryanodine for its binding sites. However, the interaction of ryanodine with the SR channel is complicated by the ability of the alkaloid itself to alter channel function. Under conditions optimal for binding of [^{3}H]ryanodine to its high affinity site (e.g., 50–100 μM Ca^{2+}), the alkaloid is known both to activate and to inhibit Ca^{2+} release from SR vesicles in a concentration-dependent manner (5, 6). Furthermore, nanomolar to micromolar ryanodine has been demonstrated to stabilize distinct gating behaviors of single channels fused with bilayer lipid membranes (see Discussion and Refs. 7–10). To account for the heterogeneous effects of the alkaloid, ryanodine has been postulated to bind in a positively (11) or negatively (5, 9, 12) cooperative manner to as many as four sites on the channel oligomer. An important and perplexing question that still plagues mechanistic interpretations of the exact action of ryanodine on the skeletal and cardiac SR Ca^{2+} release channels concerns the very slow off-rate of nanomolar [^{3}H]ryanodine when dissociation is affected by a large excess (micromolar) of unlabeled ryanodine (2, 11). Although this behavior suggests classic positive cooperativity, it has recently been demonstrated that [^{3}H]ryanodine at >200 nM can also dramatically diminish the predicted association rate (7). The apparent paradox could be explained by a sequential mechanism if low nanomolar ryanodine alters channel function in a fully reversible manner (i.e., obeys the law of mass action), whereas ryanodine at >100 nM induces a unidirectional (irreversible) change in channel

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ABBREVIATIONS: SR, sarcoplasmic reticulum; BLM, bilayer lipid membrane; DTT, dithiothreitol; EC_{50}, concentration of compound resulting in 50% of maximal activation; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; IC_{50}, concentration of compound resulting in 50% of maximal inhibition; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N'*,N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

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function. A sequential model of this sort would predict that higher concentrations of ryanodine would induce a transition in conformation constrained by a significant energy barrier. Once overcome, the effects of ryanodine would be predicted to persist regardless of receptor occupancy.

The present work utilizes three distinct approaches, [^{3}H] ryanodine receptor binding analyses, Ca^{2+} loading and release measurements from actively loaded SR vesicles, and single-Ca^{2+} channel measurements from planar lipid bilayers fused with SR vesicles, to provide the first direct evidence for 1) an apparently irreversible action of ryanodine on the SR Ca^{2+} release channel complex of rabbit skeletal muscle SR that persists after dissociation of the alkaloid from its binding sites and 2) the involvement of oxidation of critical receptor sulfhydryls in the process. These findings may account for the complex molecular actions of ryanodine and can reconcile the apparent contradictory results concerning the positive or negative cooperativity among [^{3}H]ryanodine binding sites.

Materials and Methods

Preparation of SR vesicles. Purified SR vesicles from rabbit skeletal muscle were prepared by the method of Saito *et al.* (13) for receptor analysis and Ca^{2+} transport measurements or by the method of MacLennan (14) for BLM experiments.

Pretreatment with ryanodine. Junctional SR vesicles were incubated in 20 mM HEPES buffer (pH 7.1) containing 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride, at a final concentration of 100 μg/ml, in the absence or presence of several concentrations of ryanodine in a 20-ml volume. After a 2-hr incubation at 37° the samples were centrifuged at 109,000 × *g* at 4° for 1 hr. The pellets were lightly rinsed and resuspended in 20 ml of Ca^{2+}-free HEPES buffer, shaken at 37° for 2 hr in order to dissociate the reversibly bound ryanodine, and subsequently centrifuged for 1 hr at 109,000 × *g* at 4°. The final pellet was resuspended in Ca^{2+}-free HEPES buffer at a final protein concentration of approximately 5 mg/ml and was assayed as described below.

Experiments aiming to "protect" SR receptors from inactivation by micromolar ryanodine with 0.5–1 mM DTT were performed by treating four identical aliquots of SR membranes in the following ways. One aliquot served as untreated control, one received 1 μM ryanodine, one received the protective agent (DTT), and one aliquot received both ryanodine and DTT. All aliquots were processed as described above. Experiments examining the effect of DTT (0.5 and 1 mM) on the binding properties of [^{3}H]ryanodine were performed by incubating 25 μg of SR preparation in a 850-μl volume in the absence or presence of 0.5 or 1 mM DTT for 2 hr at 37°. [^{3}H]Ryanodine was subsequently added in 150-μl aliquots to give final concentrations of 0.5–100 nM, and this reaction mixture was incubated for 3 hr at 37° before quenching by filtration as described below. Measurement of dissociation rate constants in the absence or presence of 1 mM DTT was measured as described by Pessah *et al.* (15).

Protein concentrations were determined by the method of Lowry *et al.* (16) after removing the HEPES buffer by precipitating the protein with 2% perchloric acid, centrifuging at 30,000 × *g*, and dissolving the pellet in 1 N NaOH.

Measurement of [^{3}H]ryanodine binding. Specific high or low affinity binding of [^{3}H]ryanodine was measured as described before (5). Briefly, 30 μg of SR vesicles were incubated in duplicate for 3 hr with 1 nM (for high affinity sites) or 50 nM (for low affinity sites) [^{3}H] ryanodine, in a final volume of 1 ml of 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$, 20 mM HEPES, pH 7.1 at 37°. To determine the binding constants (K_d and B_{max}) of the high and low affinity binding sites, equilibrium experiments were performed in the presence of 0.5–500 or 50–5000 nM [^{3}H]ryanodine, respectively. The assays were terminated by addition of 2.5 ml of ice-cold wash medium (consisting of 20 mM Tris·HCl, 250 mM KCl, 15 mM NaCl, and 50 μM $CaCl_2$, pH 7.1), followed by rapid filtration through GF/B glass fiber filters in a cell harvester (Brandel, Gaithesburg, MD); one additional 2.5-ml wash was used to rinse the filters. Radioactivity was measured by scintillation counting with an efficiency of approximately 43%. In all cases nonspecific binding of [^{3}H]ryanodine was defined by the addition of a 100-fold excess of unlabeled ryanodine. Filter binding of [^{3}H]ryanodine in the absence of vesicular preparation was negligible. The effect of DTT on the association kinetics of the binding of 1 nM [^{3}H]ryanodine was measured by quenching the reaction by rapid filtration at times ranging from 10 to 240 min after the addition of the SR vesicles, which were preincubated in the absence or presence of 1 mM DTT at 37° for 2 hr. Dissociation of [^{3}H]ryanodine from the receptor equilibrium complex of SR vesicles preincubated in the absence or presence of 1 mM DTT for 2 hr at 37° was determined by 100-fold dilution of the assay buffer at times ranging from 15 min to 5 hr.

Analysis of binding data. At least eight different concentrations of the test compounds were used to assess their potency on the binding of [^{3}H]ryanodine. The EC_{50} or IC_{50} values were computed by the ENZFITTER program (Elsevier-Biosoft). Equilibrium binding data from saturation analysis were fitted to a one- or two-site model and the dissociation constant (K_d) and the maximal binding capacity (B_{max}) were determined by nonlinear regression analysis with the LIGAND computer program.

The percent protection by DTT was calculated according to the following equation, which takes into account the residual effect of DTT:

$$\% \text{ Protection} = 100 \times [P - (R \times DTT/C)]/[DTT - (R \times DTT/C)]$$

where C and R are [^{3}H]ryanodine binding after treatment without and with ryanodine, respectively; DTT is [^{3}H]ryanodine binding after treatment with DTT; and P is [^{3}H]ryanodine binding after treatment with DTT plus ryanodine together. There is 100% protection when [^{3}H] ryanodine binding after treatment with the protector (DTT) and inhibitor (ryanodine) together equals the [^{3}H]ryanodine binding after treatment with the protector alone (i.e., DTT). There is 0% protection when the [^{3}H]ryanodine binding after treatment with the protector and inhibitor together equals the binding observed with the inhibitor alone, corrected for the enhancing effect of the protector (i.e., $R \times DTT/C$).

Spectrophotometric determination of Ca^{2+} release. Rapid Ca^{2+} release from SR vesicles was determined by the method of Palade (17), which utilizes pyrophosphate as a precipitating anion. Briefly, 100 μg of SR protein were stirred at 35° in buffer solution, consisting of 95 mM KCl, 20 mM K-MOPS, 7.5 mM sodium pyrophosphate, 250 μM antipyrylazo III, 1 mM MgATP, 20 μg/ml creatine phosphokinase, and 5 mM phosphocreatine, pH 7.0, in a 1-ml final volume. Free Ca^{2+} concentration changes were monitored by measuring the absorbance at 710 nm and subtracting the absorbance at 790 nm, at 1-sec intervals, using a diode-array spectrophotometer (model 8452A; Hewlett Packard, Palo Alto, CA). The mixture was allowed to equilibrate for 1 min with constant stirring. The vesicles were actively filled with consecutive additions of 12 nmol of $CaCl_2$ using a Hamilton syringe, allowing the absorbance to return to the baseline between additions. Ca^{2+} release was induced by daunorubicin or ryanodine at basal or elevated extravesicular Ca^{2+} concentration. For additional details, see figure legends.

Analysis of transport data. The absorbance signals were calibrated by adding known amounts of $CaCl_2$ from a National Bureau of Standards stock to the complete transport mixture in the presence of 2 μg/ml A23187 to prevent Ca^{2+} accumulation. The functional absorbance of the dye ($A_{710} - A_{790}$) was a linear function of the added $CaCl_2$ in the range of 10 to 100 μM, yielding least squares correlation coefficients of >0.99, and was stable during the course of the experiments. The initial (fast) rates of release of accumulated Ca^{2+} were analyzed by measuring the change in mAU/sec, using the least squares best linear fit to the steepest slope, with the Kinetics Software Program (Hewlett Packard). Rates of Ca^{2+} release were normalized and reported as nmol

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of Ca^{2+}/mg/sec or as relative release rates. The functional absorbance of antipyrylazo III is linearly proportional to free Ca^{2+} concentration. The component of Ca^{2+} release directly attributable to the presence of daunorubicin or ryanodine was determined by subtracting the rate of Ca^{2+} release attributable to the presence of activator Ca^{2+} alone.

Reconstitution of SR into a lipid bilayer. Reconstitution experiments were carried out after fusion of SR vesicles to a planar BLM. Bilayers were made with a 5:3 mixture of phosphatidylethanolamine and phosphatidylserine at 50 mg/ml in decane. The bilayer was formed across a 150-μm hole drilled in a polystyrene cup, separating two chambers of 0.7 ml each. SR vesicles suspended in 0.3 M sucrose were added to the *cis* chamber (final SR protein concentration was between 5 and 10 μg/ml) of the BLM setup. The *cis* chamber contained 500 mM CsCl, 0.7 mM $CaCl_2$, 5 mM HEPES, pH 7.2, whereas the *trans* chamber contained 100 mM CsCl, 5 mM HEPES, pH 7.2. After step-like fusion events, 1.5 mM EGTA, pH 7.2, was added to the *cis* chamber, which was then perfused with an identical buffer with no added Ca^{2+} or EGTA. All current recordings were measured with respect to the *trans* (ground) side.

A List patch-clamp amplifier (model L/M-EPC7) was used to amplify picoampere currents. Data was filtered at 3 kHz, processed with a VR-10 digital data recorder (Instratech), stored on videocassette tape, and analyzed for channel activity. Data were digitized with a Scientific Solutions analog to digital converter (Labmaster TM-40) and analyzed using pCLAMP (Axon Instruments, Burlingame, CA).

Measurement of Ca^{2+} (Mg^{2+})-ATPase activity. The Ca^{2+}-dependent component of ATPase was determined by subtracting Ca^{2+}-independent ATP hydrolysis in the presence of 1 mM EGTA from total activity in the presence of 100 μM Ca^{2+}. Measurements were made spectrophotometrically in the presence of the ionophore A23187 by enzymatically coupling ADP production to the oxidation of NADH with phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (18).

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in 3–17% linear gradient polyacrylamide gels with a 3% stacking gel, in a Bio-Rad Mini-Protean dual slab cell (Bio-Rad, Richmond, CA) with the buffer system of Laemmli (19). Gels were stained with Coomassie blue.

Chemicals. [^{3}H]Ryanodine (95 Ci/mmol or 60 Ci/mmol, with 99% or 97.2% purity, respectively) was obtained from New England Nuclear (Wilmington, DE). Unlabeled ryanodine was purified according to the method of Waterhouse *et al.* (20), yielding a purity of ≥99%. Antipyrylazo III and neomycin were purchased from Sigma (St. Louis, MO), and ruthenium red was obtained from Aldrich (Milwaukee, WI). All other chemicals were of the highest quality commercially available.

Results

Persistent Alteration of Channel Function by Micromolar Ryanodine

Receptor binding studies. Rabbit SR vesicles were pretreated in the absence or presence of several concentrations of ryanodine under conditions that maximize receptor binding (in the presence of 50 μM Ca^{2+}). In these experiments, the alkaloid is subsequently removed from the vesicles by several washes in Ca^{2+}-free medium and the pretreated SR (referred to subsequently as ryanodine-treated SR) is assayed for the binding of 1 nM [^{3}H]ryanodine. Pretreatment with increasing ryanodine (up to 10 μM) decreases the binding of [^{3}H]ryanodine in a concentration-dependent manner (~82% maximum inhibition). Pretreatment with higher (100 μM) ryanodine concentrations does not further decrease the binding (Table 1).

In order to assess the amount of residual dissociable ryanodine remaining in the treated SR membranes, three experimental approaches are taken. First, the washed vesicles are pelleted and resuspended to their original volume in 100% methanol.

TABLE 1

Effect of ryanodine pretreatment on the binding of 1 nM [^{3}H] ryanodine

Binding of [^{3}H]ryanodine after pretreatment was measured as described in Materials and Methods. All experiments were performed in duplicate and repeated *n* times, as indicated. Data are mean ± standard error.

[Ryanodine][a]	Bound	*n*	Inhibition[b]
μM	pmol/mg of protein		%
0	0.748 ± 0.133	9	
0.1	0.700 ± 0.253	3	14.05 ± 3.82
1.0	0.365 ± 0.114[c]	6	52.16 ± 8.52[c]
10.0	0.142 ± 0.049[c]	5	82.54 ± 2.23[c]
100.0	0.175 ± 0.027	4	81.47 ± 5.59

[a] Ryanodine concentration for pretreatment.
[b] Calculated as the ratio of [^{3}H]ryanodine binding and respective washed control for each individual experiment and shown as the average of *n* experiments.
[c] Significantly different from previous concentration, $p < 0.05$, unpaired Student *t* test.

TABLE 2

Binding of 1 nM [^{3}H]ryanodine in the presence of methanol extracts of ryanodine-pretreated SR vesicles

Binding to 30 μg of untreated SR vesicles was performed in the presence of methanol extracts of the treated vesicles as described in the text. Each experiment was performed in triplicate. Data are mean ± standard error. Specific binding in the presence of 10 μl of methanol is 0.85 ± 0.15 pmol/mg of protein.

[Ryanodine][a]	Bound	Inhibition of the binding of 1 nM [^{3}H]ryanodine	*n*[b]
μM	pmol/mg of protein	%	
0	0.85 ± 0.16		4
0.1	0.84	0	1
1.0	0.72 ± 0.18	15.0 ± 5.7	4
10.0	0.70 ± 0.20	17.0 ± 10.7	3
100.0	0.63 ± 0.10	25.8 ± 17.0	3

[a] Ryanodine concentration for pretreatment.
[b] *n*, number of experiments.

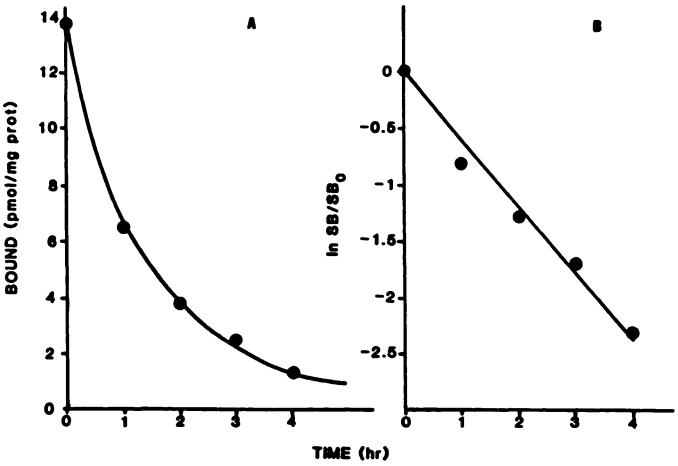

Fig. 1. Dissociation of 1 μM [^{3}H]ryanodine by 100-fold excess of Ca^{2+}-free medium. Association of 1 μM [^{3}H]ryanodine is measured in the presence of 50 μM Ca^{2+} as described in Materials and Methods and then dissociation is induced by a 100-fold excess of Ca^{2+}-free assay medium. Data shown are from one experiment assayed in duplicate and are representative of three independent determinations performed on different SR preparations and on different days. A, Specific residual binding at different times after dilution. *B*, linear regression analysis, where *SB* is specific binding at time *t* and SB_0 is specific binding at time 0.

Ten microliters (a volume equivalent to that used in experiments assessing the integrity of [^{3}H]ryanodine binding sites in pretreated SR) of this denatured methanol extract are assayed for the ability to inhibit the binding of 1 nM [^{3}H]ryanodine to

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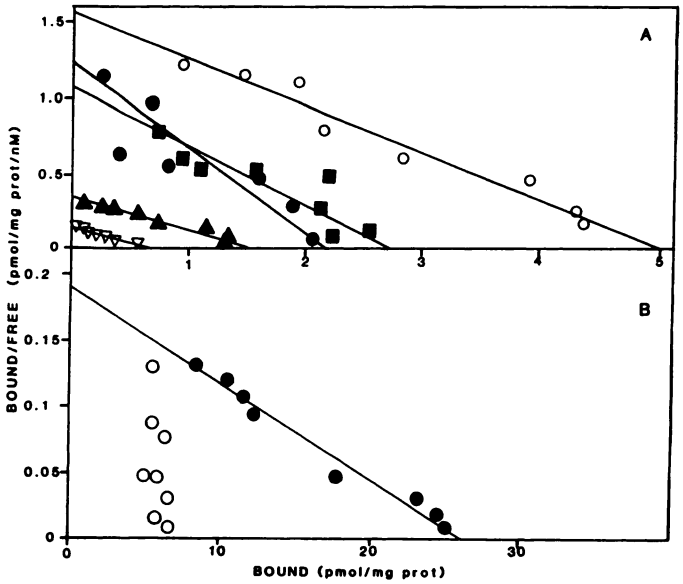

Fig. 2. A, Scatchard analysis of the saturating binding of 1–64 nM [^{3}H]ryanodine to control (○), washed control (●), and 0.1 (■), 1 (▲), and 10 (▽) μM ryanodine-treated SR vesicles. K_d values are 3.2, 2.5, 1.7, 4.4, and 4.8 nM, respectively, and B_{max} values are 4.9, 2.7, 2.1, 1.5, and 0.63 pmol/mg of protein, respectively. Data shown are from one set of representative experiments, which were repeated three, five, and four times at 0.1, 1, and 10 μM ryanodine, respectively. Although the B_{max} of washed control varied from preparation to preparation, the decline in B_{max} was consistently observed in samples pretreated with ryanodine. B, Scatchard analysis of the binding of 50–5000 nM [^{3}H]ryanodine to washed control (●) and 1 μM ryanodine-treated (○) SR vesicles. Binding of ryanodine is performed as described in Materials and Methods. Loss of the low affinity ryanodine binding sites is representative of three independent determinations. See other details in the text.

TABLE 3

Scatchard analysis of the binding of [^{3}H]ryanodine after treatment with 1 μM ryanodine

Binding of 1 nM [^{3}H]ryanodine in the presence of 1–64 nM unlabeled ryanodine was performed in duplicate as described in Materials and Methods. Values shown were calculated by the LIGAND computer program and are mean ± standard error.

	Unlabeled ryanodine binding		n^a
	K_d	B_{max}	
	nM	pmol/mg of protein	
Unwashed control	3.4 ± 0.3	7.3 ± 0.9	7
Washed control	3.7 ± 0.7	6.1 ± 0.9	6
Ryanodine (1.0 μM)-treated	4.9 ± 1.1	3.9 ± 1.0[b]	5

[a] n, number of independent experiments, each performed in duplicate.
[b] Significantly different from washed control, $p < 0.05$, unpaired Student t test.

30 μg of native SR (i.e., SR not pre-exposed to ryanodine). Control samples receive 10 μl of 100% methanol to exclude the effect of methanol on the binding of [^{3}H]ryanodine. Methanol (1% final concentration) has no significant effect on the binding of [^{3}H]ryanodine. The data summarized in Table 2 show that the reduction in the binding of [^{3}H]ryanodine seen in Table 1 after treatment with and removal of unlabeled ryanodine is not simply due to the presence of residual unlabeled ryanodine in the membrane preparation. Second, to determine the amount of ryanodine nonspecifically associated with pretreated SR membranes, 100 μg of protein/ml in a 20-ml volume is pretreated with 1 μM [^{3}H]ryanodine (specific activity, 2111 dpm/pmol) in the absence and in the presence of a 100-fold excess of unlabeled ryanodine. After washing and centrifugation, 20-μl aliquots (75 μg of protein) are measured for membrane-bound radioactivity. The residual [^{3}H]ryanodine bound in these experiments is compared with that obtained when 1 μM [^{3}H]ryanodine (specific activity, 2111 dpm/pmol) is bound to 75 μg of washed control SR. Nearly all of the specifically bound [^{3}H]ryanodine is removed by the wash protocol. Quantitatively, 0.14 ± 0.06% (two independent determinations ± range) of the binding remains associated with the washed SR. This level is equivalent to 1.4 nM ryanodine in the final SR sample. Because 10-μl aliquots of these pretreated/washed membranes are routinely assayed for the binding of 1 nM [^{3}H]ryanodine in a final volume of 1 ml, only 14 pM "contaminating" ryanodine is introduced into the assay, a concentration that could not inhibit the binding of labeled ryanodine. Furthermore, the nonspecific binding remains the same after the wash procedure and constitutes 25.3 ± 4.3% of the total binding, which accounts for the 25% reduction of 1 nM [^{3}H]ryanodine binding by the methanol extracts of treated SR (Table 2).

TABLE 4

Binding of 1 nM [^{3}H]ryanodine to control washed (CON) and 1 μM ryanodine-treated (RYA) SR samples 24 and 48 hr after pretreatment

Treatment with 1 μM ryanodine (2 hr at 37°) and binding of 1 nM [^{3}H]ryanodine were performed in triplicate as described in Materials and Methods. Samples were kept on ice between the measurements.

Time after pretreatment	Condition	Bound	Inhibition
hr		pmol/mg of protein	%
0	CON	0.499 ± 0.03	
0	RYA	0.271 ± 0.01	45.7
24	CON	0.543 ± 0.04	
24	RYA	0.314 ± 0.01	42.2
48	CON	0.429 ± 0.01	
48	RYA	0.268 ± 0.01	37.5

A third line of evidence demonstrates that the reversibly bound ryanodine must be largely dissociated from the receptors after a 2-hr incubation in low Ca^{2+} medium. The dissociation of 1 μM [^{3}H]ryanodine by dilution with medium containing 50 μM Ca^{2+} proceeds slowly ($t_{1/2}$ = 4.1 hr) (5). After 2 hr of association with 1 μM [^{3}H]ryanodine, 100-fold dilution of the assay medium with a solution nominally free of Ca^{2+} results in more rapid dissociation ($t_{1/2}$ = 57 ± 7 min; three independent determinations) (Fig. 1). These results predict 80% dissociation of the specifically bound ryanodine (at 1 μM) within 2 hr, and 94% of the specifically bound ryanodine dissociates within 4 hr (Fig. 1). Given the initial pelleting step, which effectively removes unbound ryanodine, and the ~2000-fold dilution of the pellet volume in the wash step, it is not surprising that the present protocol effectively minimizes "carryover" of unlabeled ryanodine.

Scatchard analysis of the binding of 1–64 nM [^{3}H]ryanodine to SR pretreated with 0.1, 1, and 10 μM ryanodine shows a significant decrease in the maximal binding capacity of the high affinity site with only a slight change in the K_d of the high affinity site, compared with SR that was handled identically but not exposed to ryanodine (Fig. 2A). Variation in K_d and B_{max} values observed among different SR preparations and the fact that each pretreatment experiment requires a large amount of SR necessitated that different sets of experiments be performed on different membrane preparations. Therefore, the respective K_d and B_{max} values from the control, washed control, and ryanodine-treated samples within a given experiment are always generated simultaneously from the same SR prepara-

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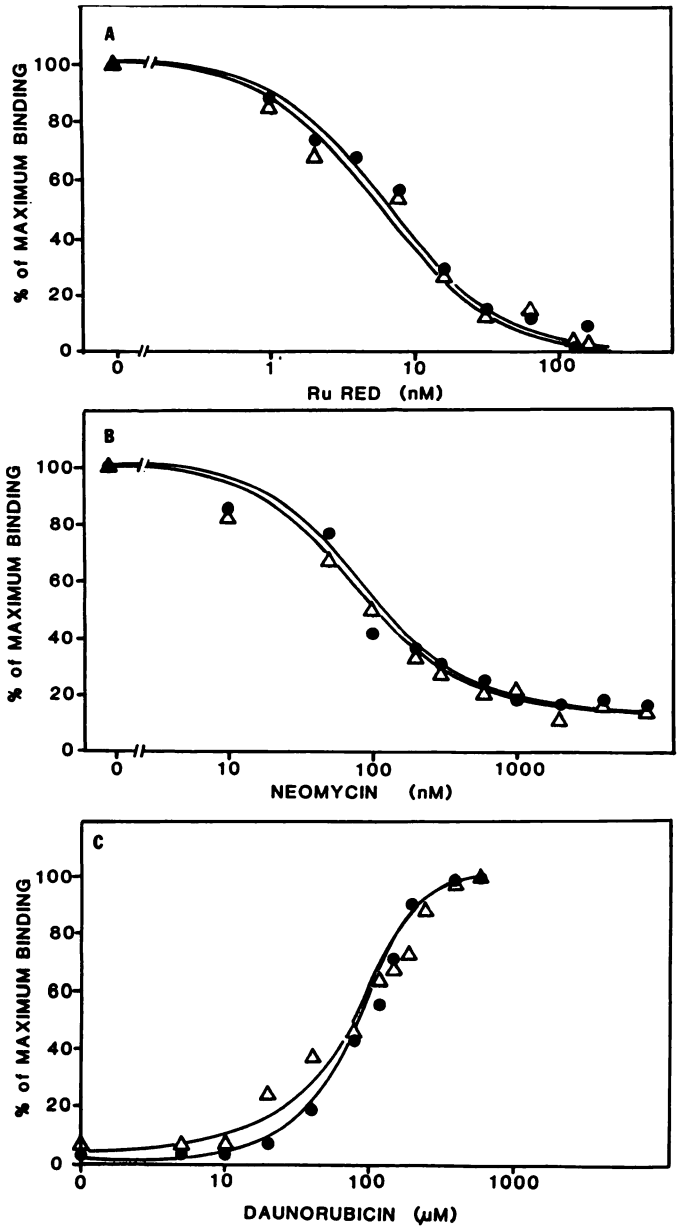

Fig. 3. Effect of ruthenium red (A), neomycin (B), and daunorubicin (C) on the binding of 1 nM [^{3}H]ryanodine to washed control (●) and 1 μM ryanodine-treated (△) SR. The treatment in the absence and presence of ryanodine and the binding of 1 nM [^{3}H]ryanodine are performed as described in Materials and Methods, in the presence of 50 μM Ca^{2+} (A and B) or 2 μM Ca^{2+} and 1 mM Mg^{2+} (C). Data shown are from four determinations performed on two different preparations. The respective maximum specific binding (and the respective IC_{50} or ED_{50} concentrations) for control and 1 μM ryanodine-treated samples are 0.83 and 0.55 pmol/mg of protein (IC_{50} = 7.0 ± 0.7 and 6.1 ± 0.7) (A), 1.02 and 0.68 pmol/mg of protein (IC_{50} = 96.6 ± 7.4 nM and 95.0 ± 5.4 nM) (B), and 0.82 and 0.40 pmol/mg of protein (in the presence of 250 μM daunorubicin) (EC_{50} = 90.6 ± 3.4 and 93.5 ± 6.6 μM) (C).

tion, to allow direct comparisons to be made. Although washing and centrifugation in a sucrose-free and high-salt (250 mM KCl, 15 mM NaCl) medium reduce the binding capacity of washed versus unwashed controls by 24.4 ± 7.0% (six experiments), each of the samples exposed to ryanodine is paired with a control sample treated identically but not exposed to ryanodine, and the effect of ryanodine treatment is thereby compared with its paired control. The average binding constants of unwashed

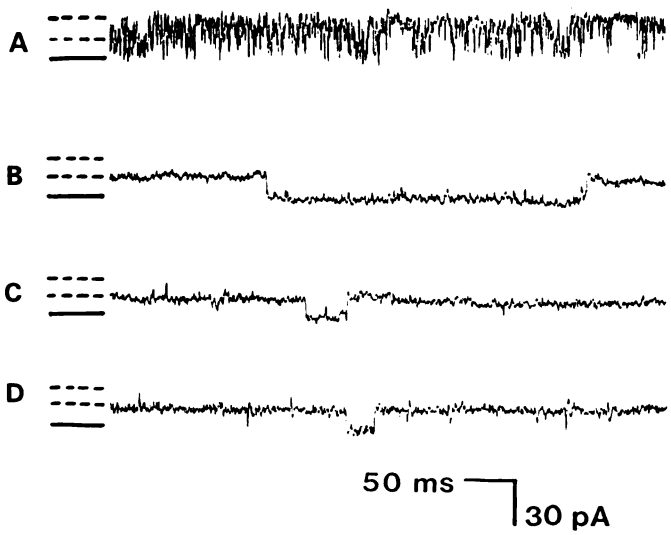

Fig. 4. Addition of micromolar ryanodine leads to a long-lived subconductance state that is not reversible. Additions to the *cis* chamber are as follows: A, 100 μM $CaCl_2$; B, 10 μM ryanodine; C, the *cis* side perfused with Ca^{2+}-free buffer and 100 μM $CaCl_2$ subsequently added; D, 1 mM DTT. All traces are recorded at +20 mV. *Solid bar to the left of each trace*, closed state; *dashed lines*, full conductance (*upper dashed line*) and (*lower dashed line*) half-conductance states of the channel. This experiment was repeated four times.

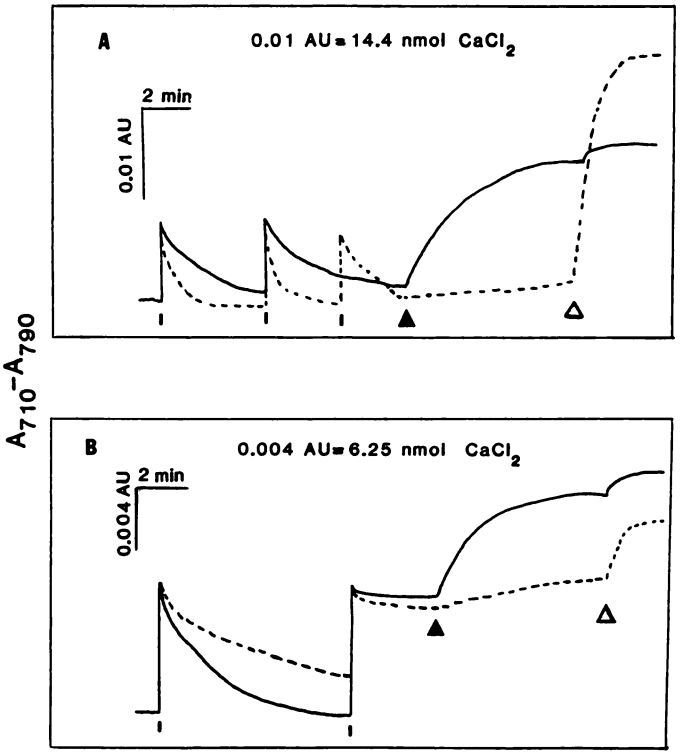

Fig. 5. Pretreatment of skeletal SR with ryanodine results in irreversible inhibition of ryanodine-induced Ca^{2+} release. Skeletal SR membranes are pretreated with 100 μM (A) (– – –) or 100 nM (B) (– – –) ryanodine and washed as described in Materials and Methods. Paired controls are treated identically without ryanodine (A and B) (——). Washed membranes (80 and 40 μg of protein in A and B, respectively) are actively loaded with sequential additions of 12 nmol of $CaCl_2$ as shown (*vertical bars*). ▲, Addition of 30 μM ryanodine; △, addition of 2 μg of A23187.

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TABLE 5

Effect of tryanodine pretreatment on the activity of Ca^{2+} (Mg^{2+})-ATPase

Ca^{2+}-dependent ATPase activity of washed control (CON) and 10 μM (10 RYA) and 100 μM (100 RYA) ryanodine-treated SR (5 μg of protein) was determined in the presence of A23187 as described in Materials and Methods. Values are average ± standard error of three independent determinations. Experiments 1 and 2 were performed on two different SR preparations.

Condition	Activity
	μmol of P_i/mg of protein/min
Experiment 1	
CON	2.49 ± 0.32
10 RYA	2.14 ± 0.06
Experiment 2	
CON	4.37 ± 0.28
100 RYA	4.21 ± 0.06

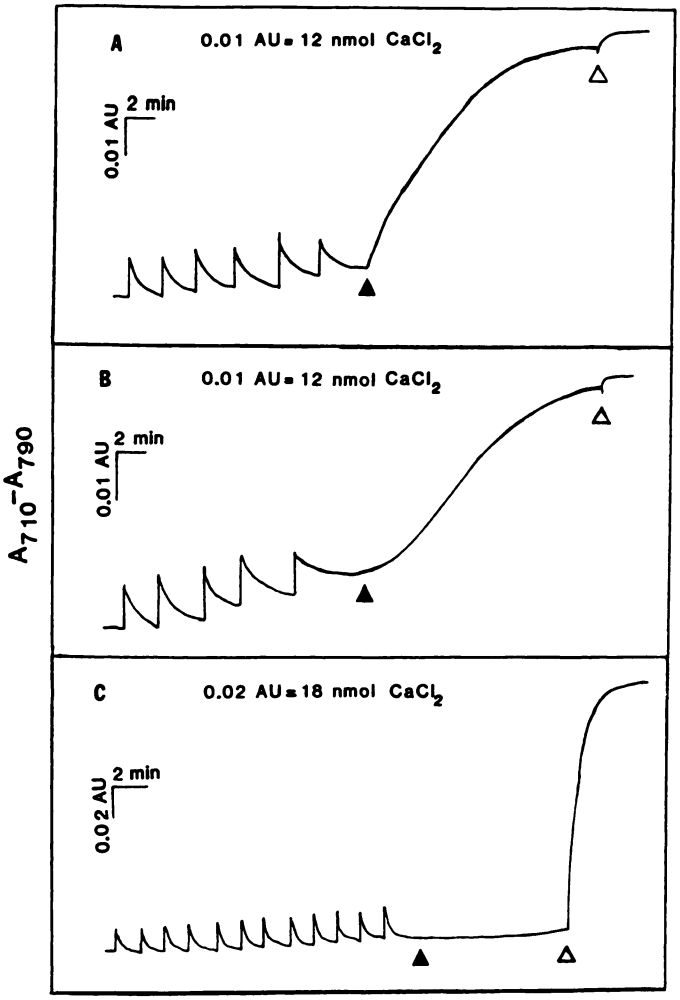

Fig. 6. Pretreatment of skeletal SR with ryanodine results in irreversible inhibition of anthraquinone-induced Ca^{2+} release. Skeletal SR membranes are pretreated in the absence (A) or in the presence of 100 nM (B) or 100 μM (C) ryanodine and subsequently washed and centrifuged as described above. Washed membranes (132 μg) are actively loaded with sequential additions of 12 nmol of $CaCl_2$ to near their respective capacities, as shown. ▲, Addition of 50 μM daunorubicin. A23187 is added at the end of each experiment (△) to release accumulated Ca^{2+}.

"native" SR, washed control SR, and 1 μM ryanodine-treated SR are summarized in Table 3. The binding of 50 nM [^{3}H]ryanodine in the presence of 50–5000 nM unlabeled ryanodine to washed control SR is shown in Fig. 2B. This experiment is performed on a preparation different from that shown in Fig.

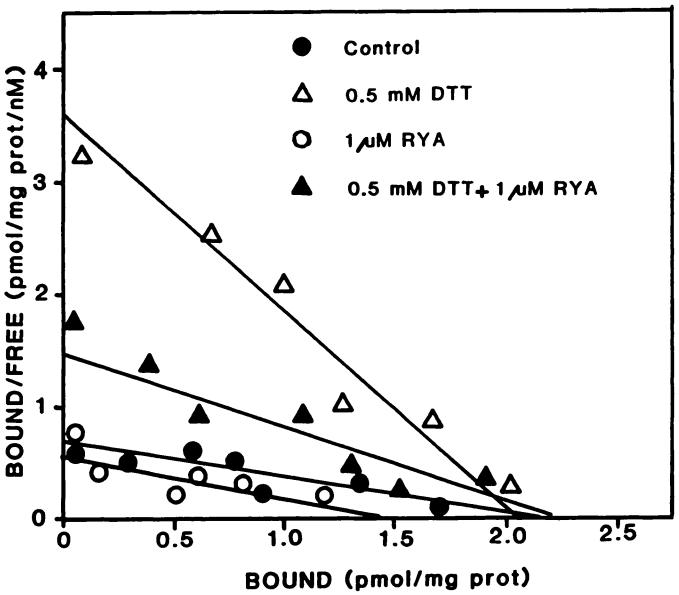

Fig. 7. Scatchard analysis of the binding of 0.5–100 nM [^{3}H]ryanodine to washed control (●) and 1 μM ryanodine- (○), 0.5 mM DTT- (△), and 1 μM ryanodine plus 0.5 mM DTT (▲)-treated membranes. Treatment of the membranes and assay of the binding of [^{3}H]ryanodine are performed as described in Materials and Methods. K_d and B_{max} of washed control membranes are 3.1 ± 0.7 nM and 2.2 ± 0.3 pmol/mg of protein, respectively. After pretreatment with 0.5 mM DTT, the K_d is 0.6 ± 0.1 nM and B_{max} is 2.0 ± 0.1 pmol/mg of protein. After pretreatment with 1 μM ryanodine, K_d is 2.66 ± 0.5 nM and B_{max} is 1.4 ± 0.2 pmol/mg of protein; after pretreatment with DTT and ryanodine together, K_d is 1.5 ± 0.03 nM and B_{max} is 2.2 ± 0.2 pmol/mg of protein. Data shown are from one experiment assayed in duplicate. This experiment was repeated twice more with different SR preparations and DTT gave essentially the same level of protection (i.e., offered near-complete protection against the ryanodine-induced decrease in B_{max}).

2A, which has a lower binding capacity. The K_d and B_{max} of the high affinity site of the preparation shown in Fig. 2B after washing and centrifugation are 7.6 nM and 9.3 pmol/mg of protein, respectively. For the low affinity site the K_d and the B_{max} are 138 nM and 26.5 pmol/mg of protein, respectively (Fig. 2B). After treatment with 1 μM ryanodine, the binding of [^{3}H]ryanodine at 50 nM is found to be fully saturated and does not change with increasing concentrations of [^{3}H]ryanodine, clearly showing the loss of the low affinity sites after pretreatment with ryanodine (Fig. 2B). The average value for the specific binding of 50–850 nM [^{3}H]ryanodine is 6.1 ± 0.16 pmol/mg of protein, which is 34.5% less than the washed control value in this preparation. Loss of low affinity binding has been consistently observed in ryanodine-treated SR (three independent experiments) and appears to be a significant step in the molecular action of the alkaloid. The effect of transient exposure of SR to 1 μM ryanodine is very persistent, inasmuch as the binding of 1 nM [^{3}H]ryanodine measured 24 and 48 hr after the treatment (samples were kept on ice between the assays) remains depressed (Table 4).

The pharmacological sensitivity of the binding of [^{3}H]ryanodine to the remaining high affinity sites after treatment with ryanodine has been compared with the corresponding washed control. Ruthenium red and neomycin, competitive inhibitors of the high and low affinity binding of ryanodine (21, 22), respectively, yield similar IC_{50} values for washed control and ryanodine-treated SR. The potency of daunorubicin, a potent

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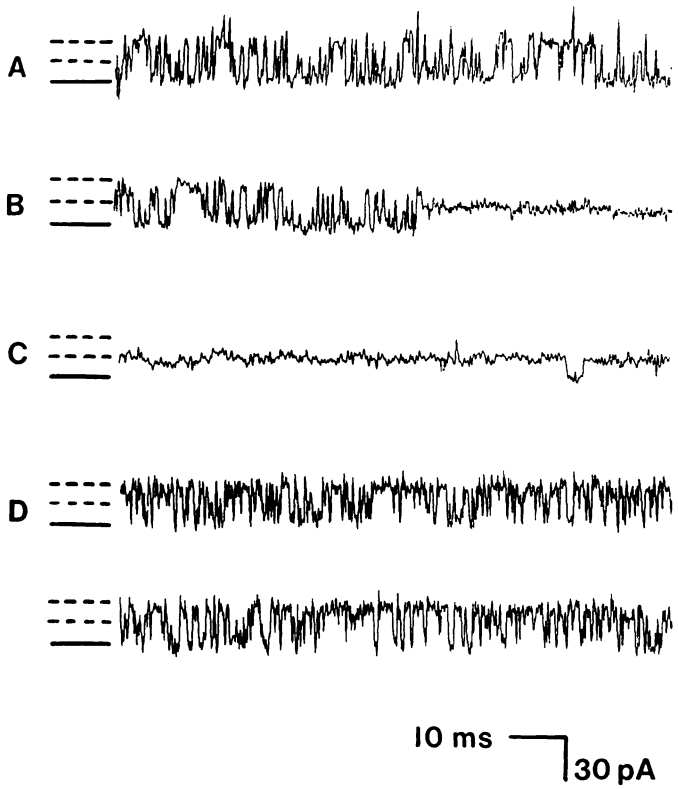

Fig. 8. DTT protects against the irreversible action of micromolar ryanodine. The following additions were made to the *cis* chamber: A, 0.5 mM DTT plus 50 μM $CaCl_2$; B, 10 μM ryanodine; C, 50 μM $CaCl_2$ added after perfusion of the *cis* chamber with Ca^{2+}-free buffer; D, 0.5 mM DTT. *Solid bar to the left of each trace*, closed state of the channel; *lower dashed line*, half-conductance state; *upper dashed line*, full conductance state of the channel. This experiment was repeated three times.

activator of the binding of ryanodine (23, 24), is also unchanged by ryanodine pretreatment (Fig. 3).

SDS-polyacrylamide gel electrophoresis of 20 μg of washed control and 0.1, 100, and 500 μM ryanodine-treated SR microsomes shows no detectable difference among the washed control and the ryanodine-treated samples with respect to the intensity and location of biochemical markers of junctional SR, including the high molecular weight junctional foot protein (M_r ~400,000), the Ca^{2+} pump protein (M_r 110,000), and calsequestrin (M_r 55,000) (data not shown).

Single-channel measurements. Lipid bilayer membranes fused with SR membranes show very rapid (mean open time, 0.5 msec) discrete fluctuations in current in the presence of 100 μM $CaCl_2$, having a conductance of 468 pS (Fig. 4A). Ryanodine (10 μM) in the presence of 100 μM Ca^{2+} rapidly induces slowly fluctuating gating events having approximately one half the conductance (225 pS; Fig. 4B). Perfusion of the BLM chamber with a ryanodine- and Ca^{2+}-free buffer within 5 min of addition of the alkaloid, followed by the addition of 100 μM Ca^{2+} (Fig. 4C), is ineffective in restoring the native gating behavior of the channel, as is the addition of 1 mM DTT (Fig. 4D). A final addition of ruthenium red (5 μM) blocks the ryanodine-modified subconductance state (data not shown).

Ca^{2+} transport measurements. Pretreatment with 0.1 or 100 μM ryanodine alters the Ca^{2+} transport properties of SR membrane vesicles. In the washed control vesicles, 30 μM ryanodine causes rapid efflux of Ca^{2+} (0.95 nmol/mg/sec; ± 12%), whereas the initial rate of Ca^{2+} release from vesicles pretreated with 100 μM ryanodine is completely inhibited, even though they load significantly more Ca^{2+} (Fig. 5A). Membranes pretreated with 100 nM ryanodine load significantly less Ca^{2+} than the corresponding control vesicles (Fig. 5B) and exhibit a much smaller release of Ca^{2+} in response to 30 μM ryanodine, even at elevated (15 μM) extravesicular Ca^{2+}. The altered Ca^{2+} uptake characteristics of SR pretreated with ryanodine are not due to modification of the Ca^{2+} pump protein, because the rate of Ca^{2+}-dependent P_i production shows no significant differences between washed control and 10 or 100 μM ryanodine-treated samples (Table 5).

Another important consequence of pretreatment of SR with ryanodine is the inhibition of anthraquinone-induced release of Ca^{2+}. Washed control vesicles exposed to 50 μM daunorubicin respond with rapid efflux of Ca^{2+} (initial rate, 1.3 ± 0.01 nmol/mg/sec; mean ± standard error) (Fig. 6A), whereas the daunorubicin-induced initial rate of Ca^{2+} release is significantly lower after treatment with 100 nM ryanodine (0.48 ± <0.1 nmol/mg/sec) (Fig. 6B) and totally inhibited in membranes pretreated with 100 μM ryanodine (Fig. 6C).

Role of Critical Thiols in the Persistent Action of Ryanodine

Receptor binding studies. The oxidation of critical sulfhydryl groups plays an important role in the gating of the Ca^{2+} release channel of SR and dramatically influences the binding of [^{3}H]ryanodine (2, 25). DTT, an effective thiol-reducing agent, is used to examine the possible role of sulfhydryl modification in the irreversible action of ryanodine. Four aliquots of SR protein are treated as described in Materials and Methods. DTT (1 mM) protects the ryanodine receptors from the action of 1 μM ryanodine by 50.8 ± 10.9% (mean ± standard error of three independent determinations, each performed in triplicate; calculated according to the equation described in Materials and Methods). The ability of DTT to alter the binding of [^{3}H]ryanodine to untreated control SR is assessed by preincubating the membranes with DTT (0.5 and 1.0 mM) at 37° for 2 hr before assay for the binding of 0.5–100 nM [^{3}H]ryanodine, as described in Materials and Methods. Scatchard analysis reveals that DTT enhances the affinity of ryanodine for the high affinity binding sites; the K_d decreases from 3.4 nM to 1.2 nM at 0.5 mM DTT and to 1.1 nM at 1 mM DTT. There is no significant change in the B_{max} with 0.5 mM DTT (3.82 pmol/mg of protein in the absence of DTT and 3.76 pmol/mg of protein in the presence of 0.5 mM DTT). DTT at 1 mM slightly decreases the B_{max} to 3.2 pmol/mg of protein. The enhanced affinity of ryanodine for the high affinity binding site produced by DTT pretreatment is attributed to an increase of the association rate from 0.0020 min^{-1} nM^{-1} to 0.0046 min^{-1} nM^{-1}, as well as a decrease in the dissociation rate from 0.01169 min^{-1} to 0.00898 min^{-1}. These changes in rates of association and dissociation can account for the 3-fold increase in binding affinity seen with equilibrium binding experiments.

Scatchard analyses of the binding of 0.5–100 nM [^{3}H]ryanodine to washed control SR or to SR pretreated with either 1 μM ryanodine, 0.5 mM DTT, or 0.5 mM DTT plus 1 μM ryanodine demonstrate the ability of DTT to protect the maximal binding capacity of SR (Fig. 7). The ability of DTT to protect against ryanodine-induced reduction in B_{max} has been consistently observed in three separate SR preparations.

Single-channel measurements. The protective effect of DTT has also been assessed at the level of the single channel in BLM preparations. Addition of DTT (0.5 mM) to the *cis*

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chamber does not alter channel gating (Fig. 8A) and the introduction of ryanodine (10 μM) results in the prolonged subconductance state seen with ryanodine alone (Fig. 8B). Removal of ryanodine by perfusion (with Ca^{2+}-free medium) of the *cis* chamber, followed by the additions of Ca^{2+} (50 μM) and DTT (0.5 mM), restores the full conductance, rapidly fluctuating state (Fig. 8, C and D).

Discussion

The present results demonstrate for the first time a persistent, seemingly irreversible, uncoupling of SR Ca^{2+} release channel activation after treatment with micromolar ryanodine. Transient exposure of skeletal SR to 0.1–500 μM ryanodine exhibits persistent alterations in 1) the binding of [^{3}H]ryanodine to high and low affinity sites, 2) the Ca^{2+} transport properties of actively loaded vesicles, and 3) the gating behavior of single channels in BLM. Four lines of evidence suggest that these effects are not the result of residual ryanodine occupying sites on the channel complex. First, free ryanodine is effectively removed in the first centrifugation step. The rinsed pellet comprises no more than 10-μl volume and is diluted ~2000-fold in nominally Ca^{2+}-free medium. Even 1 μM [^{3}H]ryanodine dissociates markedly faster in a Ca^{2+}-free dilution medium (Fig. 1 and Ref. 9). After 2 hr of dissociation, the SR is repelleted and again suspended in a Ca^{2+}-free medium, which promotes further dissociation of any remaining, specifically bound, ryanodine. Second, pretreatment with and removal of 1 μM [^{3}H] ryanodine indicates the presence of approximately 14 pM free ryanodine per assay, which could not and does not reduce the binding of 1 nM [^{3}H]ryanodine. Third, methanol extraction of the treated SR vesicles shows that the vesicles contain mainly the nonspecifically bound ryanodine after the washing procedure. This ryanodine is most likely partitioned with the lipid phase. Fourth, small amounts of "contaminating" ryanodine reversibly bound to receptor would be expected to significantly decrease the apparent affinity of the remaining receptors for [^{3}H]ryanodine in subsequent binding assays without altering B_{max} (i.e., via classical competition). Indeed, the present results clearly indicate that ryanodine treatment causes significant reduction in B_{max} with no change in K_d. Additional evidence is presented in Fig. 3, which demonstrates that the potency of neomycin and ruthenium red, competitive inhibitors of [^{3}H] ryanodine binding, remain unchanged after pretreatment.

Despite the near-complete removal of ryanodine from pretreated SR, the subsequent binding of 1 nM [^{3}H]ryanodine is persistently depressed, as is the maximal binding capacity measured at 0.5–100 nM [^{3}H]ryanodine, compared with the corresponding controls. The reduction of B_{max} is persistent even 48 hr after the pretreatment, indicative of an irreversible action. However, the remaining high affinity sites retain similar sensitivity to the inhibitors ruthenium red and neomycin and to the activator daunorubicin, suggesting that the remaining binding sites are pharmacologically intact. Further analysis of the binding with an extended range of [^{3}H]ryanodine concentrations indicates that the ryanodine pretreatment precludes detection of the low affinity binding states seen with control membranes. Ryanodine and daunomycin, which enhance the release rate of Ca^{2+} from both cardiac (24) and skeletal (23) SR, are completely ineffectual in inducing the release of Ca^{2+} from actively loaded vesicles pretreated with 100 μM ryanodine, even though significantly more Ca^{2+} is actively accumulated by the vesicles. This finding is consistent with those obtained from receptor-binding studies, because SR pretreated with the alkaloid exhibits reduced numbers of functional channels as measured by [^{3}H]ryanodine binding capacity. Although the binding data show no significant change in the EC_{50} concentration of daunorubicin in washed control or 1 μM ryanodine-treated SR, the efficacy of daunorubicin decreases with ryanodine pretreatment. For instance, 100 μM daunorubicin enhances the binding of 1 nM [^{3}H]ryanodine in washed control vesicles 26 times, in 100 nM ryanodine-treated vesicles 27 times, in 1 μM ryanodine-treated vesicles 13 times, and in 100 μM ryanodine-treated vesicles only 7 times (data not shown), which is consistent with the transport data. Additional evidence comes from the persistent alteration of single channels in BLM, where brief exposure (typically ≤5 min) to micromolar ryanodine stabilizes a half-conductance state that is not reversed by perfusion of the BLM chamber with a Ca^{2+}-free medium unless DTT is present before addition of ryanodine. The persistent inhibition appears to be a general characteristic of ligands that interact with the ryanodine site. Irreversibility is also seen with nanomolar ruthenium red or neomycin, inhibitors that recognize a site that overlaps with the ryanoid recognition site (22). For example, SR membranes pretreated with ruthenium red at its IC_{50} (25 nM), in the same manner as reported here with ryanodine, have been found to have persistently diminished binding capacity of [^{3}H]ryanodine, by 50% (22).

A sequential mechanism has been recently proposed (5, 12) to account for the ability of ryanodine to stabilize different gating behaviors of single channels in BLM, including 1) a reversible slowly fluctuating full conductance (7, 8), 2) a reversible half-conductance (7), 3) a persistent half-conductance (7–10), 4) unidirectional quarter-conductance transitions (7), and 5) full closure (7, 8). In BLM studies, ryanodine-induced gating behaviors 1 and 2 (described above) show excellent quantitative correlation with apparent K_{d_1} (1–4 nM) and K_{d_2} (20–50 nM) obtained from equilibrium measurements under identical experimental conditions. [^{3}H]Ryanodine in the range of 1 to 50 nM exhibits simple bimolecular association rates, and dissociation is best described by negative cooperativity (5, 7). However, channel states 3, 4, and 5 described above are unidirectional transitions and lack quantitative correlation with putative low affinity [^{3}H]ryanodine equilibrium binding constants (7). Because single-channel measurements in a BLM are obtained within 10 min of addition of ryanodine, whereas equilibrium binding experiments are performed over several hours, the quantitative discrepancies between the two methods can only be explained if association rates drop with increasing ryanodine. Recently, Buck *et al.* (7) have demonstrated that association rates indeed decrease linearly above 200 nM ryanodine. The perplexing very slow off-rate of nanomolar [^{3}H] ryanodine seen when dissociation is affected by a large excess (micromolar) of unlabeled ryanodine (2, 11) can be accounted for by a sequential mechanism. Although the slowing of dissociation suggests classical positive cooperativity, the finding that micromolar [^{3}H]ryanodine also significantly diminishes the predicted association rate indicates that the kinetic constants obtained experimentally depend on the order in which [^{3}H] ryanodine and unlabeled ryanodine concentrations are added during kinetic experiments. These results, taken together with the inability to reverse the half-conductance state induced by 10 μM ryanodine after perfusion of the bilayer chamber,

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strongly suggest a dramatic change in channel conformation coincident with the ryanodine-induced transition to the long-lived half-conductance state (channel transition 3; described above), a transition that appears to be associated with a significant energy barrier.

SDS-gel electrophoresis of washed control and SR treated with ryanodine (100 nM to 500 μM) shows no overt differences in the location or staining density of the M_r 360,000 ryanodine receptor subunit, demonstrating that the persistent action of micromolar ryanodine is not the result of accelerated proteolysis of the junctional foot protein subunit but more likely the result of altered subunit-subunit interactions within the oligomeric complex. The observation that DTT (0.5 mM) not only affords significant protection against the loss of receptor capacity but also permits reversal of the ryanodine-induced half-conductance state is most interesting. Critical thiols on the ryanodine receptor complex are thought to play an important role in gating the Ca^{2+} channel (25). The present results suggest that a shift in redox equilibrium of critical thiols of the channel complex may account, wholly or in part, for the hypothesized energy hump associated with conformational transition 3 (described above) induced by μM ryanodine.

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