Ryanodine Stabilizes Multiple Conformational States of the Skeletal Muscle Calcium Release Channel

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Nanomolar to micromolar ryanodine alters the gating kinetics of the Ca\(^{2+}\) release channel from skeletal sarcoplasmic reticulum (SR) fused with bilayer lipid membranes (BLM). In the presence of asymmetric CsCl and 100 mM CaCl\(_2\) cis, ryanodine (RY) (5–40 nM) activates the channel, increasing the open probability (p_0; maximum 300% of control) without changing unitary conductance (468 picosiemens (pS)). Statistical analyses of gating kinetics reveal that open and closed dwell times exhibit biexponential distributions and are significantly modified by nanomolar RY. Altered channel gating kinetics with low nanomolar RY is fully reversible and correlates well with binding kinetics of nanomolar [\(^3\)H]RY with its high affinity site (K_0 = 0.7 nM) under identical experimental conditions. RY (20–50 nM) induces occasional 1/2 conductance fluctuations which correlate with [\(^3\)H]RY binding to a second site having lower affinity (K_{20} = 23 nM). RY (5–50 nM) in the presence of 500 mM CsCl significantly enhances Ca\(^{2+}\)-induced Ca\(^{2+}\) release from actively loaded SR vesicles. Ryanodine ≥50 nM stabilizes the channel in a 234-pS subconduction which is not readily reversible. RY (≥70 µM) produces a unidirectional transition from the 1/2 to a 1/4 conductance fluctuation, whereas RY ≥200 µM causes complete closure of the channel. The RY required for stabilizing 1/4 conductance transitions and channel closure do not quantitatively correlate with [\(^3\)H]RY equilibrium binding constants and is attributed to significant reduction in association kinetics with ≥200 nM [\(^3\)H]RY in the presence of 500 mM CsCl. These results demonstrate that RY stabilizes four discrete states of the SR release channel and supports the existence of multiple interacting RY effector sites on the channel protein.

Calcium (Ca\(^{2+}\)) release channels of junctional sarcoplasmic reticulum (SR)† play an essential role in excitation-contraction coupling of striated muscle. The plant alkaloid ryanodine is a conformationally sensitive probe specific to the Ca\(^{2+}\) release channels of skeletal and cardiac SR (1–4). Modulation of the high affinity (K_0 = 1–3 nM) [\(^3\)H]ryanodine-binding site in skeletal and cardiac SR by ligands parallels many of the pharmacological properties exhibited by the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism studied with skinned and intact muscle fibers and junctional SR vesicles including activation by micromolar Ca\(^{2+}\) and inactivation by Ca\(^{2+}\) >300 µM, modulation by adenine nucleotides, caffeine, and anthraquinones, and inhibition by Mg\(^{2+}\) and ruthenium red (2–6). Purification and reconstitution of the ryanodine receptor-Ca\(^{2+}\) channel complex of skeletal and cardiac SR into planar lipid bilayers has demonstrated chemically gated cation-permeable pores having similar large conductances which are modulated by the same relevant ligands that influence the high affinity binding of [\(^3\)H]ryanodine (see Refs. 7 and 8 for reviews). It is generally agreed that channel activation unmaskas the high affinity sites for ryanodine and hence binding of nanomolar ryanodine reflects a ligand-induced open state of the Ca\(^{2+}\) channel.

However, equilibrium and kinetic analyses of the binding of [\(^3\)H]ryanodine suggest a more complex interaction between the alkaloid and the Ca\(^{2+}\) channel oligomer. The existence of multiple interacting binding sites for [\(^3\)H]ryanodine on the channel oligomer has remained controversial (9–13), and the exact mechanism by which ryanodine produces its heterogeneous effects on SR Ca\(^{2+}\) permeability remains unresolved (10, 12). Recent evidence suggests the existence of allosterically modulated binding of ryanodine to as many as four sites, which can be best described by negative cooperativity (11–13). Receptor occupation results in a sequential activation/inactivation of the Ca\(^{2+}\) release channel; even 1 mM ryanodine, which is considered to be only inhibitory to the channel based on single channel measurements (11), is initially, if only briefly, able to fully activate Ca\(^{2+}\) release from SR vesicles (12). There appears to be a lack of correlation between binding constants of [\(^3\)H]ryanodine in SR vesicle preparations compared to the activity of the alkaloid in reconstituted SR channels in planar bilayers. In the absence of preincubation of SR with the alkaloid, concentrations ≥10 µM are required to lock the channel in a persistent subconductance state (the apparent consequence of ryanodine binding to "low affinity" sites), while concentrations in excess of 1 mM are needed to completely block single channel activity (the apparent consequence of ryanodine binding to "low affinity" sites) (14–17). The quantitative discrepancy between ryanodine receptor binding and the ability of the alkaloid to influence the gating of the purified channel in an artificial bilayer has been attributed to its slow association kinetics (17). An important outstanding question which may clarify the multiplicity of ryanodine effector sites on the SR channel complex is whether...
or not ryanodine stabilizes distinct gating states at the level of single channels in a BLM that quantitatively correlate with the equilibrium constants and kinetic behavior under identical assay conditions.

In this paper three distinct approaches, [3H]ryanodine receptor binding analyses, Ca2+ loading and release measurements from actively loaded SR vesicles, and single channel measurements from rabbit skeletal SR fused with planar lipid bilayers, are utilized under identical assay conditions to demonstrate that discriminating concentrations of ryanodine stabilize distinct-gating behaviors of single Ca2+ channels in a BLM which can be predicted by a sequential mechanism.

**EXPERIMENTAL PROCEDURES**

**Preparation of SR Vesicles—**Purified SR vesicles from rabbit skeletal muscle were prepared by the method of Saft et al. (18) for receptor analysis and Ca2+ transport measurements or by the method of Mace Lennan (19) for BLM experiments. Protein concentrations were determined by the method of Lowry et al. (20) after removal of Hesper buffer by precipitating the protein with 2% perchloric acid, centrifuging at 30,000 × g, and dissolving the pellet in 1 N NaOH.

**Reconstitution of SR into Lipid Bilayer—**Reconstitution experiments were carried out following fusion of SR vesicles to a planar BLM as described previously (15). Vesicles were made with a 53:46 mixture of phosphatidylethanolamine (PE) and phosphatidylserine (PS) at 50 mg/ml in decane. The bilayer was formed across a 150-μm hole drilled in a polystyrene cap, separating two chambers each of 0.7 ml. 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, 0.3-0.7 mM CaCl2, 5 mM HEPES, pH 7.2, whereas the trans chamber contained 100 mM CsCl, 5 mM HEPES, pH 7.2. Following a single step-like fusion event, a 2-fold excess of EGTA, pH 7.2, was added to the cis chamber which was subsequently perfused with an identical buffer with no added CaCl2.

**Ca2+ Release—**All current recordings were measured with respect to the trans (ground) side. Either a List Medical patch clamp amplifier (Instratech), stored on VCR tape, and subsequently analyzed for single channel fluctuations in length (approximately 3200 pm) or a Tektronix 2440 digital storage oscilloscope. The single channel data were digitized with a Scientific Solutions analog to digital converter (Labmaster) and subsequently analyzed for single channel activity. In a typical experiment, CaCl2 (100 μM) was added to the cis chamber to activate the Ca2+ channel and gating properties were acquired. Ryanodine (5 nM to 200 μM) was added from a 100 × stock to the cis chamber, and after 3-5 min single channel fluctuations were measured for at least 2 min.

**Analysis of Single Channel Kinetics—**Data was digitized with a Scientific Solutions analog to digital converter (Labmaster TM-40) without additional filtering and analyzed using a 386 PC computer with pCLAMP (version 5.5; Axon Instruments, Burlingame, CA). Each of the data points shown is the average ± S.E. of six to 10 data blocks randomized, each consisting of approximately 2000 events per block, taken 5-10 min after the addition of ryanodine. The experiments were replicated at least three times on each of three different SR preparations. The data were fit to a biexponential:

\[ p = W_1 e^{-\tau_1 t} + W_2 e^{-\tau_2 t} \quad \text{(Eq. 1)} \]

where \( p \) is the probability of the channel being open or closed after \( t \) seconds, \( \tau_1 \) and \( \tau_2 \) are time constants, and \( W_1 + W_2 = 100\% \).

**Measurement of [3H]Ryanodine Binding—**Specific equilibrium binding of [3H]ryanodine (95 or 60 Ci/mmol, with 99 or 97.2% purity, respectively; Du Pont-New England Nuclear) was performed under conditions identical to those used for obtaining single channel measurements from actively loaded SR vesicles, and single channel fluctuations from rabbit skeletal SR fused with planar lipid bilayers, are utilized under identical assay conditions to demonstrate that discriminating concentrations of ryanodine stabilize distinct-gating behaviors of single Ca2+ channels in a BLM which can be predicted by a sequential mechanism.

**Analysis of Binding Data—**Saturation analyses from equilibrium binding data were fitted to one- or two-site models, and the dissociation constant (Kd) and the maximal binding capacity (Bmax) were determined with the LIGAND computer program. Bimolecular association rate constants (koff) were calculated using the nonlinear regression analysis program ENZFITTER (Elsevier BioSoft). All experiments were repeated at least three times on separate days.

**Spectrophotometric Determination of Ca2+ Release—**Ryanodine release from SR vesicles was determined by modification of the method of Palade (21). Briefly, 100 μg of SR protein was stirred at 25 °C in buffer solution, consisting of 95 mM KCl, 500 mM CsCl, 20 mM K-MOPS, 7.5 mM sodium pyrophosphate, 250 μM antipyrilaza III, 1 mM MgATP, 20 μg/ml creatine phosphokinase, and 5 mM phosphocreatine, pH 7.0, in a 1-ml final volume. Changes in free Ca2+ were monitored by measuring the absorbance at 710 nm and subtracting the absorbance at 790 nm, at 1-s 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 loaded with six consecutive additions of 20 nM of CaCl2 using a Hamilton syringe, allowing the absorbance to return to the base line between additions. Ca2+ release was induced by a bolus addition of 40 nM of CaCl2 in the presence or absence of ryanodine.

**Results**

**Receptor Binding Studies in the Presence of CsCl—**The multiplicity of [3H]ryanodine binding sites in skeletal SR membranes is evident when equilibrium (4.5-h incubation) binding is performed within two discriminating concentration ranges of radioligand at 25 °C in the presence of 500 mM CsCl and 100 μM CaCl2 (the same conditions used in subsequent BLM studies). Saturation curves with [3H]ryanodine in the range of 0.5-500 nM reveal a best fit by two sites having Kd values of approximately 0.7 and 22 nM (nH = 0.76), whereas binding in the range of 50-5050 nM reveal an additional site which exhibits significantly lower affinity (Kd = 1.3 μM, nH = 0.4) (Table I). Fig. 1A shows the total, nonspecific, and specific equilibrium binding of 0.5-5050 nM [3H]ryanodine to 40 μg of SR protein.

<table>
<thead>
<tr>
<th>Binding</th>
<th>Site</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
<th>nH</th>
<th>Commission</th>
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</thead>
<tbody>
<tr>
<td>0.5-50</td>
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<td>3.7</td>
<td>7.2</td>
<td>0.76</td>
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</tr>
<tr>
<td>50-5050</td>
<td>50</td>
<td>3.7</td>
<td>7.2</td>
<td>0.76</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Table I**

Effect of 500 mM CsCl and temperature on binding constants of [3H]ryanodine

Data at 250 mM KCl and 37 °C are taken from Ref. 12. Equilibrium binding of 0.5-50 and 50-5050 nM [3H]ryanodine is measured as described above. All experiments are performed in duplicate and repeated twice. Data are averages ± S.E. of three independent determinations.
Ryanodine and Sarcolplasmic Reticulum Channel States

**Experimental Procedures.** Nonspecific binding was determined in the presence of 100-fold excess of unlabeled ryanodine. Data are shown from one representative experiment performed in duplicate which were repeated twice with similar results.

Equilibrium binding of [3H]ryanodine shown on graph B. Scatchard analysis of specific binding of 0.5-150 nM [3H]ryanodine, data points taken from graph A, C, specific binding of 550-5050 nM [3H]ryanodine, data points taken from graph A. D, Scatchard analysis of specific binding of 0.5-151 nm [3H]ryanodine shown on graph C. E, Scatchard analysis of specific binding of 550-5050 nM [3H]ryanodine shown on graph C. See K_d and B_max values in text. Data are shown from one representative experiment performed in duplicate which were repeated twice with similar results.

skeletal SR in the presence of 500 mM CsCl, 100 μM CaCl_2 at 25 °C. Eq. 1, B and C, show the specific binding of 0.5-150 and 550-5050 nM [3H]ryanodine, respectively, which are derived from Fig. 1A. Fig. 1D is a Scatchard analysis of the data in Fig. 1B, and Fig. 1E is a Scatchard analysis of the data in Fig. 1C. The specific binding of 0.5-150 nM [3H]ryanodine is best fit by a two-site model with K_d values of 0.5 and 19.5 nM and B_max values of 4.6 and 9.7 pmol/mg of protein. The binding of 550-5050 nM [3H]ryanodine can be best analyzed by a one-site model (Fig. 1E) with a K_d of 1.3 μM and a B_max of 26.2 pmol/mg of protein. The specific binding has plateaued from 100 to 500 nM [3H]ryanodine (Fig. 1, A and B) and rises again above 500 nm up to and beyond 5050 nm. This indicates the possibility of a fourth site which is not saturated at 5050 nm concentration (Table I). Table I summarizes the K_d and B_max values of the different binding sites in 250 mM KCl buffer at 37 °C (taken from Ref. 12) and in 500 mM CsCl buffer at 25 °C. The presence of CsCl markedly increases the affinity of [3H]ryanodine for the higher affinity states (K_d and K_a), whereas it increases the K_d of the lower affinity state. Comparing the two conditions, the maximal binding capacities of the first three sites are in very good agreement. The existence of a fourth, lowest affinity state, which is difficult to quantify with equilibrium experiments in 250 mM KCl at 37 °C, is not detectable with the range of [3H]ryanodine utilized in the present experiments (50-5050 nM) with 500 mM CsCl at 25 °C.

The rate of association of nanomolar [3H]ryanodine with its binding sites is significantly slower at 25 °C than 37 °C in the absence of CsCl (not shown). In the presence of 500 mM CsCl, the rates of association of [3H]ryanodine (1 nM - 3 μM) at 25 °C is biphasic (Fig. 2). Although k_{on} is a linear function of [3H]ryanodine (r^2 = 0.90) in the range of 1-50 nM (Fig. 2, inset), there is a dramatic drop in the apparent rate of association at [3H]ryanodine > 250 nM, which is also linearly dependent on the concentration of ryanodine (r^2 = 0.99). The t_{1/2}, to equilibrium under these conditions are 112, 22, and 4.9 min at 1, 10, and 50 nM [3H]ryanodine, and 6, 8, 50, and 41 min at 250, 500, 1000, and 3000 nM [3H]ryanodine (Fig. 2). The decrease in k_{on} at high ryanodine is in agreement with decreased affinity measured in equilibrium experiments. Under these conditions (500 mM CsCl and 25 °C) equilibrium is stable at all [3H]ryanodine concentrations tested for >420 min.

Nano-molar Ryanodine and Single Channel Kinetics—Fusion of SR vesicles to a BLM in the presence of a CsCl gradient and Ca^2+ in the cis chamber results in discrete fluctuations in current corresponding to single gating events of the Ca^2+ release channel. High Cs+ has previously been shown to inhibit SR K+ channels (23). CaCl_2 (100 μM; Fig. 3A, first trace) or mM ATP added to the cis side results in an increased probability of finding the channel in an open state (p_o), whereas micromolar ruthenium red or millimolar Mg^2+ inhibits channel activity (data not shown). In the presence of

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**Fig. 1.** Multiple binding affinities for [3H]ryanodine in the presence of CsCl. Equilibrium binding of 0.5-5050 nM [3H]ryanodine to 40 μg of skeletal SR in the presence of 500 nM CsCl and 100 μM CaCl_2 at 25 °C. Equilibrium binding was measured as described under "Experimental Procedures." Nonspecific binding was determined in the presence of 100-fold excess of unlabelled ryanodine. A, specific binding (○) was determined after subtracting nonspecific binding (Δ) from total binding (□). B, specific binding of 0.5-151 nM [3H]ryanodine, data points taken from graph A. C, specific binding of 550-5050 nM [3H]ryanodine, data points taken from graph A. D, Scatchard analysis of specific binding of 0.5-151 nM [3H]ryanodine shown on graph C. E, Scatchard analysis of specific binding of 550-5050 nM [3H]ryanodine shown on graph C. See K_d and B_max values in text. Data are shown from one representative experiment performed in duplicate which were repeated twice with similar results.

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**Fig. 2.** Biphasic association rate for [3H]ryanodine in the presence of 500 mM CsCl. The experiments are performed as described under "Experimental Procedures" in the presence of 1 nM, 10 nM (specific activity 20 Ci/mm), 50 nM (specific activity 15.5 Ci/mm), 250 nM (specific activity 3.6 Ci/mm), 500 nM (specific activity 2.7 Ci/mm), 1000 nM (specific activity 3 Ci/mm), and 3000 nM (specific activity 2.8 Ci/mm) [3H]ryanodine. Inset shows that the observed association rate constant (k_{on}) is linearly dependent on the concentration of ryanodine in the 1-50 nM range. The decline in k_{on} between 250 nM and 1 μM is also linear. Data are the average ± S.D. of three determinations.
Ryanodine and Sarcoplasmic Reticulum Channel States

FIG. 3. Addition of nM ryanodine increases channel open time in a reversible manner. Fusion of SR vesicles to a PEPS (5:3) BLM is performed as described under “Experimental Procedures.” Following fusion, the cis chamber is perfused with 500 mM CaCl2, 5 mM HEPES, pH 7.2, and the trans chamber contains 100 mM CaCl2 (4 mM HEPES, pH 7.2. The following sequential additions are made to the cis chamber: A: trace 1, 100 μM CaCl2; trace 2, 10 nM ryanodine; trace 3, the solution on the cis side was perfused with Ca2+-free buffer and 100 μM CaCl2 subsequently added. All traces are recorded at +20 mV. The solid bar to the left of each trace represents the closed state, whereas the dashed line represents the full conductance (468 ± 205 pS) open state of the channel. The time mark in trace 2 is 40 ms, whereas in all other traces it is 20 ms. The data is representative of four experiments. B, p0, for open states (circles) and for closed states (triangles) of the SR channel is modified by nM ryanodine. Each data point is derived from 6-11 randomly selected data blocks (each 5 s long) which were acquired 5 min after the addition of ryanodine. Dwell times (mean ± S.D.) for open state (C) and for the closed state (D) are modified by nanomolar ryanodine. Data obtained from (B) are best fit with a biexponential equation with two time constants (τ2, squares; τ1, circles) as described under “Experimental Procedures.”

100 μM CaCl2 cis, the p0 for the open state of the channel ranged between 0.12 and 0.36 with four different SR preparations. Addition of ryanodine (5-40 nM) to the cis chamber in the presence of 100 μM Ca2+ increases the p0 of the open state of the channel with a concomitant decrease in p0 of the closed state in a dose-dependent manner. Fig. 3B summarizes a typical result from one of three SR preparations where ryanodine (40 nM) increases the p0 (open state) of the full conductance 2.7-fold (p0 from 0.12 to 0.32) and decreases p0 (closed state) 1.5-fold. These actions of nanomolar ryanodine are typically acquired 5-10 min after the addition of the alkaloid. The dwell times of the open and closed states are best fit by the sum of two exponentials having time constant τ1 and τ2 (where τ1 < τ2). Kinetic analysis shows that as p0 (open state) increases with increasing ryanodine, so does τ2 and, to a lesser extent, τ1 (Fig. 3C). Conversely, as p0 (closed state) decreases with increasing ryanodine, τ2 and, to a lesser extent, τ1 also decrease (Fig. 3D). In one SR preparation, addition of ryanodine (10 nM) to the cis chamber in the presence of 100 μM Ca2+ dramatically slowed the fluctuation of the channel (Fig. 3A, second trace), increasing the p0 (open state) from 0.36 to 0.70 (n = nine independent determinations). To test the reversibility of the ryanodine-modified channel, the cis chamber is perfused with a Ca2+-free medium. Subsequent addition of Ca2+ (100 μM) to the cis side results in the near complete disappearance of prolonged open states (n = 4; p0 = 0.30; Fig. 3A, third trace). Ryanodine induces slower transitions without altering the voltage-current relationship (Fig. 4, Δ versus □). With a 5:1 CsCl gradient, the voltage intercept at zero current (−36 ± 17 mV) and the unitary conductance (468 ± 205 pS) remains unchanged in
the presence or absence of 5–40 nM ryanodine.

Ryanodine Stabilizes 1/2 and 1/4 Single Channel Transitions—Although 5–40 nM of the alkaloid significantly increases \( p_o \) of the full conductance state of the channel (Fig. 3A, trace 2, and Fig. 3B), ryanodine (10–40 nM) results in occasional subconductance states of approximately 50% of the full conductance state (Fig. 5, trace 2). Raising the ryanodine concentration to 50 nM (Fig. 5, trace 3) results in a transition from the more slowly fluctuating full conductance state to long-lived 1/2 conductance fluctuations after a delay of nearly 10 min. The delay in appearance of a 1/2 conductance state from the full conductance state is very dependent on the ryanodine concentration. For example, addition of 10 \( \mu \)M ryanodine (Fig. 5, trace 4) results in rapid (within a few seconds) elimination of the full conductance state, and only discrete 1/2 subconductance (225 ± 68 pS) fluctuations are observed. At micromolar concentrations removal of free ryanodine by perfusion of the cis side with Ca\(^{2+}\)-free buffer, followed by addition of 100 \( \mu \)M Ca\(^{2+}\) (Fig. 5, traces 5 and 6) are ineffective in restoring the rapid full conductance gating behavior of the channel. A final addition of ruthenium red (20 \( \mu \)M) blocked the subconductance state in bilayers modified by \( \mu \)M ryanodine (not shown).

Ryanodine (≥70 \( \mu \)M) cis causes the channel to fluctuate in 1/2 and 1/4 subconducting states which are most clearly demonstrated when several channels are incorporated in the BLM (Fig. 6, traces 1–4, and Table II). The ryanodine-

50 ms \[ 30 \text{ pA} \]

FIG. 5. Ryanodine induces occasional and persistent 1/2 conductance fluctuations with increasing concentration. Additions to the cis chamber are as follows: trace 1, 100 \( \mu \)M CaCl\(_2\); trace 2, 10 nM ryanodine; trace 3, an additional 40 nM ryanodine; trace 4, 10 \( \mu \)M ryanodine; traces 5 and 6, the cis side is perfused with a Ca\(^{2+}\)-free buffer, and 100 \( \mu \)M CaCl\(_2\) is subsequently added. The lower solid line to the left of each trace represents the closed state of the channel, whereas the upper solid line represents the full open state. In all traces the center dashed line represents the 1/2 conductance state of the channel. Current trace 2 clearly demonstrates both full and occasional 1/2 conductance fluctuations in the presence of 10 nM ryanodine. This experiment was repeated four times.

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stabilized 1/4 states have been observed in each of 11 independent bilayer experiments on at least four different SR preparations. Interestingly, in all observations of the 1/4 state (several hundred), they always appear as closures from the 1/2 conductance state. Openings from a closed state to a 1/4 conductance state have not been observed and return of the 1/4 state to the full conductance state have not been observed. In comparison to the 1/2 conductance state, the 1/4 states are short-lived (Table II). Both 1/2 and 1/4 subconductances are emanating from the Ca\(^{2+}\) release channel since both show a similar, though not identical, voltage intercept at zero current (Fig. 4) and both are inhibited by ruthenium red (Fig. 6, trace 5). The slight shift to more positive reversal potentials seen with the 1/2 and 1/4 states of the channel is suggestive of a change in selectivity of the channel's permeation pore for Cs\(^+\):Cl\(^-\), from 20:1 (at −35 mV) to 9:1 (at −28 mV). However, the degree of variability associated with the calculated reversal potentials precludes definitive conclusions regarding a change

TABLE II

<table>
<thead>
<tr>
<th>Type of transition</th>
<th>( p_o )</th>
<th>Open time</th>
<th>Conductance</th>
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<tr>
<td>1/2 state</td>
<td>0.35</td>
<td>12.2 s</td>
<td>225 ± 68 pS</td>
</tr>
<tr>
<td>1/4 state</td>
<td>0.008</td>
<td>8.5 ms</td>
<td>129 ± 25 pS</td>
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The \( p_o \) for the 1/2 state transition represents the probability of finding the channel in the 1/2 open state, whereas \( p_o \) for the 1/4 state transition is the probability of the channel closing from the 1/2 state to the 1/4 state. Before the addition of ryanodine, the single channel conductance is 468 ± 25 pS. These results are calculated from two of six independent bilayer experiments.

FIG. 6. Addition of 100 \( \mu \)M ryanodine causes SR channels to fluctuate in 1/2 and 1/4 subconducting states. Trace A shows the effect of 100 \( \mu \)M ryanodine added to the cis chamber in the presence of several channels. Trace B is an expanded view of a section of trace A showing a 1/4 conducting state transition. Trace C shows further transitions to the 1/4 state. In trace D, 20 \( \mu \)M ruthenium red is added to the cis chamber. The solid bar to the left of each trace represents the closed state of the channel(s). The center dashed line in traces B through D represents 1/4 state transitions, whereas all other dashed lines represent 1/2 state transitions.
in pore selectivity. Ryanodine at concentrations ≥ 200 μM causes complete closure of the channel, and no subsequent gating is observed.

**Ca**<sup>2+</sup> Transport Measurements—SR (100 μg of protein) was actively loaded by six additions of 20 nmol of CaCl<sub>2</sub> followed by a bolus addition of 40 nmol of CaCl<sub>2</sub> in the absence and in the presence of 5 nM, 50 nM, 2 μM, and 200 μM ryanodine. Fig. 7 shows that even in the presence of 500 mM CaCl<sub>2</sub>, SR vesicles can be loaded with Ca<sup>2+</sup> to near capacity and that ryanodine enhances the initial rate of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release in a concentration-dependent manner. Subsequent to an initial activation phase, ryanodine (2 and 200 μM; traces 4 and 5, respectively) induces a dose- and time-dependent inactivation of efflux (Fig. 7). The biphasic effects of μM ryanodine on Ca<sup>2+</sup> efflux from vesicles is supported by the behavior of Ca<sup>2+</sup> channels in BLM, which reveal that μM ryanodine initially prolongs opening of a number of channels in the bilayer followed by a unidirectional drop in conductance (Figs. 6 and 7).

**DISCUSSION**

During excitation-contraction coupling in striated muscle, the release of Ca<sup>2+</sup> from SR appears to be mediated by a tetrameric protein complex possessing extensive hydrophilic portions in its structure which has been identified as the ryanodine receptor/Ca<sup>2+</sup> release channel complex (24-28). There is apparent consensus based on the properties of high affinity [3H]ryanodine binding (2-4, 11, 12), sedimentation profile (27, 29), and morphology (30, 31) that the Ca<sup>2+</sup> release channel complex, the ryanodine receptor, and the tetrameric junctional foot protein are synonymous. However, an alternative model has been recently proposed suggesting a role for a lower molecular weight Ca<sup>2+</sup> channel protein (32). Equilibrium and kinetic constants for the binding of nanomolar [3H]ryanodine to its high affinity binding sites greatly depend on the presence of channel activators or inhibitors (2, 3, 10, 33) and has contributed significantly to our understanding of the structure and function of the Ca<sup>2+</sup> release channel. However, the published data concerning the molecular interactions of ryanodine with its binding sites on the Ca<sup>2+</sup> channel protein and the exact consequence binding has on channel function has remained unclear. First, there is no consensus as to the heterogeneity of binding sites for [3H]ryanodine on the skeletal and cardiac SR channel protein. Recently, conclusions concerning the positive (3, 9, 10) and negative (11-13) cooperativity have been drawn. Second, there is a significant lack of correlation between binding constants for [3H]ryanodine and the concentrations of the alkaloid required to alter single Ca<sup>2+</sup> channel activity in BLM experiments. The present work addresses the complexities of the coupling between receptor occupancy and single channel behavior. To minimize the confounding influence of temperature and channel activators or inhibitors, radioligand binding, BLM, and vesicle transport studies were all performed under identical assay conditions in the presence of optimal (100 μM) Ca<sup>2+</sup> for channel activation and high affinity binding of [3H]ryanodine.

The association of [3H]ryanodine with its high affinity binding site is extremely dependent on temperature having a Q<sub>10</sub> of >3.5 (13, 34), suggesting transitions in conformation governed by significant entropic and enthalpic constraints. In this paper we demonstrate that binding of nanomolar [3H] ryanodine to its high affinity binding sites proceeds rapidly at 25 °C in the presence of 500 mM CsCl (Fig. 2) when compared to association kinetics seen in the absence of high CsCl (3, 13, 34). The significance of this finding relates to the rapidity with which nanomolar ryanodine could be expected to alter single channel-gating behavior and the quantitative dose-response relationship in BLM experiments. The calculated K<sub>obs</sub> values reported in Fig. 2 and the typical BLM record which is acquired under identical experimental conditions and analyzed between 5 and 10 min after the addition of ryanodine allow quantitative predictions to be made. Ryanodine at 1, 5, 10, 20, and 40 nM is expected to reach at least 2.4, 7.5, 12.4, 22.1, and 41.5% of equilibrium occupancy, respectively, 5 min after addition of ryanodine to the cis chamber of the BLM. Induction of prolonged open dwell times (and reduced closed dwell times) of the full conductance fluctuation becomes clearly evident at concentrations >5 nM ryanodine, and both parameters increase in a dose-dependent manner with ryanodine (Fig. 3). It is not surprising that ryanodine at concentrations near K<sub>ci</sub> (~1 nM) calculated from equilibrium binding experiments has little influence on single channel-gating behavior, since only a small number of productive collisions would be predicted in the time frame of bilayer experiments. Increasing ryanodine concentration in the range of 5-40 nM is expected to enhance the frequency of productive collisions between the alkaloid and its high affinity effector site, assuming the formation of a readily reversible complex. The binding of nM [3H]ryanodine to its highest affinity sites has been shown to be readily reversible (12) as

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**Fig. 7.** Time- and concentration-dependent activation and inactivation of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release by ryanodine in the presence of 500 mM CaCl<sub>2</sub>. 100-μg SR vesicle preparation was actively loaded with six additions of 20 nmol of CaCl<sub>2</sub> as described above. Ca<sup>2+</sup> release was induced by a bolus addition of 40 nmol of CaCl<sub>2</sub> in the absence (trace 1) and presence of 5 nM, 50 nM, 2 μM, or 200 μM ryanodine (traces 2-5, respectively). The data are from representative experiments which were repeated at least three times with similar results. The black arrow indicates the addition of 1 μg of A23187. The relative initial Ca<sup>2+</sup> release rates are 32, 45, 54, 76, and 87 nmol/mg/min at 0, 5 nM, 50 nM, 2 μM, and 200 μM ryanodine, respectively.
are the transitions induced by nM ryanodine in the BLM reported here (Fig. 3). Our results with rabbit SR suggest that the binding constant having the highest affinity for \(^{[3}H\)ryanodine \((K_{\text{on}})\) corresponds to longer open times of the full conductance state of the channel (Fig. 4). A similar conclusion regarding the open and closed dwell times of the full conductance state has been recently reached by Bull and coworkers (35) working with frog SR in BLM, suggesting that the present findings with nanomolar ryanodine are not merely limited to a single species or to methodology but reflect a common mechanism of ryanodine action on the SR channel.

The appearance of occasional 1/2 conductance fluctuations increase in frequency with increasing ryanodine concentration in the range of 20–50 nM as predicted by the increase in \(k_{\text{obs}}\) in this range of concentrations. These results taken together suggest that the second high affinity binding conformation resolved by radioligand equilibrium binding analysis \((i.e. K_{\text{on}})\) is correlated with the onset of occasional \((\text{readily reversible})\) 1/2 conductance states which appear directly from the modified \((\text{slower fluctuating})\) full conductance state. A sequential change in the conformation of single channels induced by ryanodine could account for this gating behavior. In support of negative cooperativity in the range of 0.5–50 nM \(^{[3}H\)ryanodine, we have recently reported that the dissociation of 50 nM \(^{[3}H\)ryanodine proceeds in a biphasic manner with fast and slow components which are in excellent agreement with \(K_{\text{on}}\) and \(K_{\text{off}}\) (12). Micromolar concentrations of ryanodine, known to cause a transition to the long-lived 1/2 conductance state, has most frequently been attributed to high affinity ryanodine binding. However, we demonstrate here that the rapidity with which \(500 \text{nM} \) ryanodine induces a unidirectional transition from the mixed channel behavior \((\text{slowly fluctuating full conductances and occasional 1/2 conductance fluctuations})\) to the long-lived 1/2 conductance state is also highly dependent on the concentration of the alkaloid. Ryanodine \((50 \text{nM})\) requires \(>5 \text{ min}\) to induce the unidirectional transition to the 1/2 conductance state, whereas \(0.2–10 \mu \text{M} \) ryanodine affects a very rapid transition. The \(k_{\text{obs}}\) values for the binding of \(^{[3}H\)ryanodine in CsCl drop linearly between 0.2 and 1 \(\mu \text{M}\) \(^{[3}H\)ryanodine \((\text{and possibly beyond})\), suggesting a dramatic change in channel conformation coincident with the transition to the long-lived 1/2 conductance state. The inability to reverse the 1/2 conductance state induced by 10 \(\mu \text{M} \) ryanodine (Fig. 5) after perfusion of the cis chamber strongly suggests that the transition may have a significant energy barrier. In support of this hypothesis, we recently reported a highly persistent action of \(\mu \text{M} \) ryanodine on channel function (36).

Two important points of disagreement regarding the action of ryanodine on the SR \(\text{Ca}^{2+}\) release channel can be resolved by the present data: 1) the apparent discrepancy between negative and positive cooperativity and 2) the lack of correlation between the apparent equilibrium constants for ryanodine and the concentrations required to alter channel gating behavior in BLM studies. First, CsCl reduces the apparent affinity of the third site \((K_{\text{on}})\) compared to 250 mM KCl at 37 °C (Table I). Second, and of utmost importance to understanding the mechanism of ryanodine action, is the finding that the rate of \(^{[3}H\)ryanodine association dramatically decreases at high nanomolar to micromolar concentrations of the alkaloid (Fig. 2). A disproportionate decrease in the value of \(K_{\text{on}}\) relative to \(K_{\text{on}}\) can fully account for the low affinity binding \((K_{\text{on}})\). Paradoxically, increasing ryanodine \((0.4–50 \mu \text{M})\) in the dissociation medium is known to slow the off-rate of \(nM \) \(^{[3}H\)ryanodine from its binding sites, suggestive of positive cooperativity (2–4, 9, 12). However, positive cooperativity would not be expected to slow the association kinetics with increasing \(^{[3}H\)ryanodine (Fig. 2). The apparent paradox can be explained by a sequential mechanism in which the kinetic constants obtained depend on the order in which \(^{[3}H\)ryanodine and unlabeled ryanodine concentrations are raised during kinetic experiments. If the high affinity site for ryanodine is near the channel pore, sequential occupation of additional sites on the channel oligomer upon elevating the ryanodine concentration, could cause partial occlusion of the former and account for the slowing of dissociation.

The dramatic drop in \(k_{\text{obs}}\) at concentrations of the alkaloid greater than 250 nM can also account for the lack of correlation between the putative low affinity binding constant \((K_{\text{on}})\) and the concentrations required to induce additional transitions in the gating behavior of the channel. In the time frame of BLM experiments \((5–10 \text{ min})\) only 6.7 and 8.1% of equilibrium receptor occupancy is expected at 1 and 3 \(\mu \text{M} \) ryanodine, respectively (Fig. 2). Based on these results, a low affinity binding constant \((K_{\text{on}})\) measured under equilibrium \((4.5 \text{ h})\) conditions would be expected to lack quantitative correlation with the concentrations necessary to induce additional changes in single channel behavior. In fact, the presence of CsCl appears to significantly increase the calculated value of \(k_{\text{obs}}\) and \(K_{\text{on}}\) is beyond detectability with radioligand binding analysis (Table I). These results are consistent with the high concentrations of ryanodine needed to observe the unidirectional 1/4 conductance transitions \((\geq 70 \mu \text{M})\) and those needed to effect channel closure \((\geq 200 \mu \text{M})\). Multiple substates of the unitary \(\text{Na}^{+}\) conductance have been described with purified \(\text{Ca}^{2+}\) release channels in BLM (37). A unitary conductance of 400 pS in symmetrical \(\text{Na}^{+}\) and subconductances 75, 50, and 25% have been determined with these purified channel preparations (37). These states are difficult to resolve in native heavy SR fused with a BLM, perhaps due to the faster gating kinetics of the native channel which preclude resolution of subconductance states with current BLM techniques (37). In conclusion, the present results suggest that discriminating concentrations of ryanodine can stabilize four discrete gating behaviors of the native channel in SR which strongly supports the existence of multiple, interacting effector sites for ryanodine on the channel complex. A sequential mechanism by which ryanodine alters channel function can reconcile the literature and account for the complex behavior of \(^{[3}H\)ryanodine at its binding sites.

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REFERENCES