Activation of calcium channels in sarcoplasmic reticulum from frog muscle by nanomolar concentrations of ryanodine

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ABSTRACT Sarcoplasmic reticulum vesicles isolated from fast-twitch frog skeletal muscle presented two classes of binding sites for ryanodine, one of high affinity (Kd_1 = 1.7 nM, $Bmax_1$ = 3.3 pmol per mg) and a second class with lower affinity (Kd_2 = 90 nM, $Bmax_2$ = 7.0 pmol per milligram).

The calcium channels present in the sarcoplasmic reticulum membranes were studied in vesicles fused into lipid bilayers. Low concentrations of ryanodine (5 to 10 nM) activated a large conductance calcium channel after a short delay (5 to 10 min). The activation, which could be elicited from conditions of high or low fractional open time, was characterized by an increase in channel fractional open time without a change in conductance. The open and closed dwell time distributions were fitted with the sum of two exponentials in the range of 4 to 800 ms. The activating effect of ryanodine was due to an increase of both open time constants and a concomitant decrease in the closed time constants. Under conditions of low fractional open time (<0.1), the time spent in long closed periods (>800 ms) between bursts was not affected by ryanodine. Higher concentrations of ryanodine (250 nM) locked the channel in a lower conductance level (≈40%) with a fractional open time near unity.

These results suggest that the activating effects of nanomolar concentrations of ryanodine may arise from drug binding to high affinity sites. The expression of the lower conductance state obtained with higher concentrations of ryanodine may be associated with the low affinity binding sites observed in frog sarcoplasmic reticulum.

INTRODUCTION

Ryanodine, one of the active principles found in the bark of the shrub *Ryania speciosa Vahl*, is a plant alkaloid that interferes with the physiological response of cardiac and skeletal muscle (Sutko et al., 1985). The toxic effects of ryanodine depend on its concentration, muscle stimulation paradigms, and muscle type (Jenden and Fairhurst, 1969). In the case of skeletal muscle, ryanodine produces irreversible contractures (Katz et al., 1970).

Biochemical experiments have shown that ryanodine acts specifically at the level of the sarcoplasmic reticulum (SR); depending on its concentration it either stimulates or blocks calcium release from SR vesicles isolated from rabbit skeletal muscle (Fleischer et al., 1985, Meissner, 1986; Lattanzio et al., 1987).

The ryanodine receptor protein has been isolated and purified from the terminal cisternae of SR (Campbell et al., 1987; Inui et al., 1987*a*; Lai et al., 1988). The purified receptor and the "feet" structures, that link the SR and the transverse tubule membranes at the level of the triad (Franzini-Armstrong, 1970), share similar morphological features (Lai et al., 1988), a finding that has led to the proposal that the ryanodine receptor forms the feet structures.

The ryanodine receptor from rabbit skeletal muscle reconstituted into planar lipid bilayers (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988; Smith et al., 1988), displays properties similar to those of the high conductance calcium channels present in heavy SR vesicles (Smith et al., 1985, 1986; Lai et al., 1988). These results have been interpreted as evidence that the ryanodine receptor protein is identical to a calcium channel protein of SR terminal cisternae membranes (Smith et al., 1988); a direct role of this protein in excitation-contraction coupling has been proposed (Ma et al., 1988).

Addition of micromolar amounts of ryanodine to either the native SR vesicles or the purified ryanodine receptor fused into bilayers decreases channel conductance and increases dramatically the fractional open time (P_o) (Rousseau et al., 1987; Imagawa et al., 1987; Lai et al., 1988; Smith et al., 1988). These effects of the drug may explain why addition of ryanodine increases calcium release in isolated SR vesicles (Fairhurst and Hasselbach, 1970; Meissner, 1986; Lattanzio et al., 1987) and induces contractures in whole muscles (Katz et al., 1970). However, binding studies have shown that isolated SR vesicles bind ryanodine with high affinity (Kd: 5–50 nM) (Pessah et al., 1986; Inui et al., 1987*a*; Lattanzio et al., 1987; Lai et al., 1988; Hasselbach and Migala, 1988) whereas the effects on the channel in native SR vesicles have been

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obtained with much higher (micromolar) concentrations of ryanodine.

Heavy SR vesicles isolated from frog skeletal muscle fuse into lipid bilayers and display a large conductance calcium channel that shares several properties with the calcium channel studied in SR isolated from rabbit muscle (Suárez-Isla et al., 1988). In addition, the calcium channel of SR from frog is activated by micromolar concentrations of inositol (1,4,5)-trisphosphate (Suárez-Isla et al., 1988).

Using SR vesicles fused into planar lipid bilayers, we show here that ryanodine, at concentrations as low as 5 nM, activated the large conductance calcium channel of SR from frog by increasing P_o without changing single channel conductance. We have confirmed that higher concentrations of ryanodine lock the channel in a low conductance state with a P_o near unity, the same effects reported for the calcium channel present in SR from rabbit muscle (Rousseau et al., 1987).

EXPERIMENTAL PROCEDURES

SR preparation

Heavy SR membranes were isolated from frog skeletal muscle as follows. Three to four adult frogs (*Caudiverbera caudiverbera*), weighing from 400 to 700 g each, were pithed and 100 to 150 g of muscle were removed from the hind legs. The muscle was cleaned of blood vessels, connective tissue, and large nerves, and was finely minced before homogenization in a blendor (Waring Products, New Hartford, CT) for 30 and 20 s at low speed with 4 vol of 0.1 M KCl, 20 mM Tris-maleate, pH 7.0. All these steps were carried out at 4°C in a cold room. The microsomes were isolated by differential sedimentation, removing mitochondria by sedimentation at 10,000 *g* for 30 min. To remove contractile proteins, the resulting suspension was made 0.6 M in KCl by addition of solid salt, and the microsomes were collected by sedimentation at 100,000 *g*. The microsomal pellet was washed twice: the first time with 0.1 M KCl, 20 mM Tris-maleate, pH 7.0 and the second time with 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0. The washed microsomal pellet was resuspended in 15 to 20 ml of 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0 and was loaded on top of three discontinuous sucrose gradients made of layers of equal volumes of 25, 27.5, 37, 45, and 50% sucrose solutions (wt/vol, adjusted by refractometry). After overnight centrifugation at 100,000 *g* at 4°C in a SW 25.1 rotor, bands were collected by aspiration, diluted 20-40-fold with 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0, and sedimented at 100,000 *g* for 60 min at 4°C. The resulting pellets were resuspended in a small volume of 0.3 M sucrose, 20 mM Tris-maleate pH 7.0, frozen rapidly by immersion in solid CO_2/acetone, and stored frozen at −20°C.

The band collected as a large pellet from the 50% sucrose layer (Heavy SR) was used in the experiments described here.

Heavy SR vesicles were prepared from rabbit skeletal muscle as described in detail elsewhere (Fernández et al., 1980.)

Ryanodine binding

[^{3}H]-Ryanodine binding assays were routinely carried out by incubating heavy SR vesicles for 90 min at 37°C in a solution containing 0.1 mg/ml of protein, 0.5 M KCl, 0.1 mM $CaCl_2$, 0.5 mM AMP-PNP, 20 mM MOPS-Tris, pH 7.1 and 100 nM [^{3}H]-ryanodine. Modifications from these conditions are specified in the text. [^{3}H]-Ryanodine was synthesized from purified ryanodine as described in detail elsewhere (Sutko et al., 1986).

To stop the reaction, samples were filtered through Whatman GF/A filters (2.4 cm discs); the filters were washed with 3 × 5 ml of ice-cold 0.5 M KCl, 0.1 mM $CaCl_2$, 20 mM MOPS-Tris, pH 7.1. Specific [^{3}H]-ryanodine binding was determined by subtracting the nonspecific binding obtained in the presence of 10 μM unlabeled ryanodine.

Single channel recording and analysis

Planar phospholipid bilayers were formed from an equal mixture of palmitoyloleoyl phosphatidylethanolamine (POPE) and phosphatidylserine (PS), unless specified otherwise. Lipids, obtained from Avanti Polar Lipids, Inc., (Birmingham, AL) were dissolved in decane to a final concentration of 25 mg/ml.

Fusion of sarcoplasmic reticulum membrane vesicles to negatively charged Mueller-Rudin bilayers has been previously described (Suárez-Isla et al., 1988). Membrane vesicles (50 to 100 μg protein) were added to one compartment, called *cis*, where the voltage was applied; the composition of the *cis* solution (4 ml) was 100 mM choline-Cl, 5 mM $CaCl_2$, 25 mM Hepes/Tris, pH 7.4. The other compartment, called *trans*, contained 25 mM Hepes/Tris, pH 7.4.

The membrane was held at 0 mV and fusion was monitored as appearance of a chloride conductance. After fusion, the *cis* compartment was perfused with 20–40 ml of a solution containing 225 mM Hepes/Tris, pH 7.4. The calcium concentration in the *cis* compartment after washing was calculated to be 10–40 μM in the majority of the experiments, except in the case where Ca^{2+} chelating agents were used. The *trans* compartment solution was replaced with Hepes/Ca or Hepes/Ba, pH 7.4 to a final Ca or Ba concentration of 37 mM. The experiments were

carried out at room temperature (22–24°C). The *trans* compartment was held at virtual ground through an operational amplifier (type LF157-AH; National Semiconductor Corp., Santa Clara, CA) in a current-to-voltage configuration.

Current signals were modulated in frequency and recorded on tape. For analysis, data were filtered at 200 Hz using an eight-pole low pass Bessel filter (902 LPF, Frequency Devices Inc., Haverhill, MA) and digitized at 1 KHz with a version of the Axolab/Axess acquisition and analysis system (Axon Instruments, Inc., Burlingame, CA). For event detection, a threshold level halfway between the open and closed current levels was used and all events shorter than 1 ms were excluded from the analysis. No further corrections for missed events was attempted. Analysis of the distribution of open and closed times was carried out with the pClamp software, version 5.01 or 5.03 (Axon Instruments, Inc., Burlingame, CA). Open and closed dwell time distributions containing 2,000–3,000 events were fitted with the sum of two exponentials in the range of 4 to 800 ms. P_o was computed from records of 100 s or longer.

RESULTS

Binding experiments

Ryanodine binding to heavy SR isolated from frog muscle was routinely measured after 90 min incubation of the vesicles with the drug at 37°C, although apparent equilibrium was reached after 15 min incubation under the standard conditions described in Experimental Procedures. At 25°C, 50% of maximal binding was obtained after 10 min incubation and apparent equilibrium was reached after 30 min.

Omitting AMP-PNP from the incubation solution reduced ryanodine binding at low ionic strength (0.1 M KCl) by 14-fold, from 4.2 ± 1.5 pmol per milligram of protein (two experiments) to 0.3 pmol per mg of protein. Higher ionic strength enhanced ryanodine binding and decreased the sensitivity to AMP-PNP. Thus, increasing [KCl] from 0.1 M to 0.5 M increased ryanodine binding from 4.2 to 6.9 ± 0.6 pmol per mg (three experiments). Omitting AMP-PNP in the presence of 0.5 M KCl decreased ryanodine binding from 6.9 to 5.4 pmol per milligram of protein. Lowering temperature from 37° to 25°C decreased to about half the amount of ryanodine bound, from 6.9 to 3.7 pmol per milligram. Binding required a calcium concentration higher than 0.1 μM, and maximal binding was obtained at 0.1 mM [$CaCl_2$].

A study of ryanodine binding as a function of ryanodine concentration revealed the presence of at least two types of binding sites (Fig. 1), one with high affinity (Kd_1 = 1.7 mM, $Bmax_1$ = 3.3 pmol per milligram). A second site with much lower affinity was approximately fitted using values of Kd_2 = 90 mM and $Bmax_2$ = 7.0 pmol per milligram. Nonspecific binding did not reach more than 15% of total binding in the range of concentrations studied. Ryanodine binding to heavy SR from rabbit skeletal muscle showed only one class of binding sites, with very similar Kd (1.6 nM) and Bmax (4 pmol per milligram) as the high affinity sites present in SR from frog; higher nonspecific binding was obtained in this case (~60% of total binding at 100 nM ryanodine, not shown).

Single channel experiments

a. Channels with high P_o

Heavy SR vesicles from frog muscle display a calcium channel (Suárez-Isla et al., 1988), which in this case (pH 7.4, 0 mV) had a P_o of 0.12 (Fig. 2 *A*), and a single channel conductance of 85 pS. Addition of 1 mM ATP to the *cis* side increased P_o to 0.88. Subsequent addition of 5 nM ryanodine produced, after a lag period of 5 min, a further increase in P_o to 0.99 (Fig. 2 *A*), without changing the current amplitude, or modifying the single-channel conductance (not shown). The activating effect (increase in P_o without conductance change) lasted for 10 min in this experiment, the time recorded at low ryanodine concentrations.

Further addition of 50 nM ryanodine to the *cis* side, had a complex effect on channel kinetics (Fig. 2 *A*). Channel gating was characterized by the appearance of many unresolved rapid fluctuations between the closed and the maximally open current level of 2.8 pA. The P_o measured in this case was 0.75. Increasing ryanodine concentration to 250 nM produced, after a lag period of 15 s, a drastic decrease in the current flowing through the channel (from 2.8 to 1.3 pA) (Fig. 2 *A*). At this concentration the channel remained open for several minutes, with very infrequent closures. P_o increased to a value near 1.0.

In a channel that displayed initially a high P_o in the absence of ATP, low concentrations of ryanodine (10 nM) also increased P_o from 0.75 to 0.94 (Fig. 2 *B*), without modifying the current amplitude. No changes in current amplitude were observed 19 min after ryanodine addition.

The open and closed dwell time distributions of the channel could be adequately fitted by the sum of two exponentials in the range of 4 to 800 ms (Fig. 3). Addition of 10 nM ryanodine, in the absence of ATP, produced significant changes in both time constants, increasing the open time constants and decreasing the closed time constants (Fig. 3). The fitted values for the open time constants in the control were 4.9 and 34.4 ms; addition of

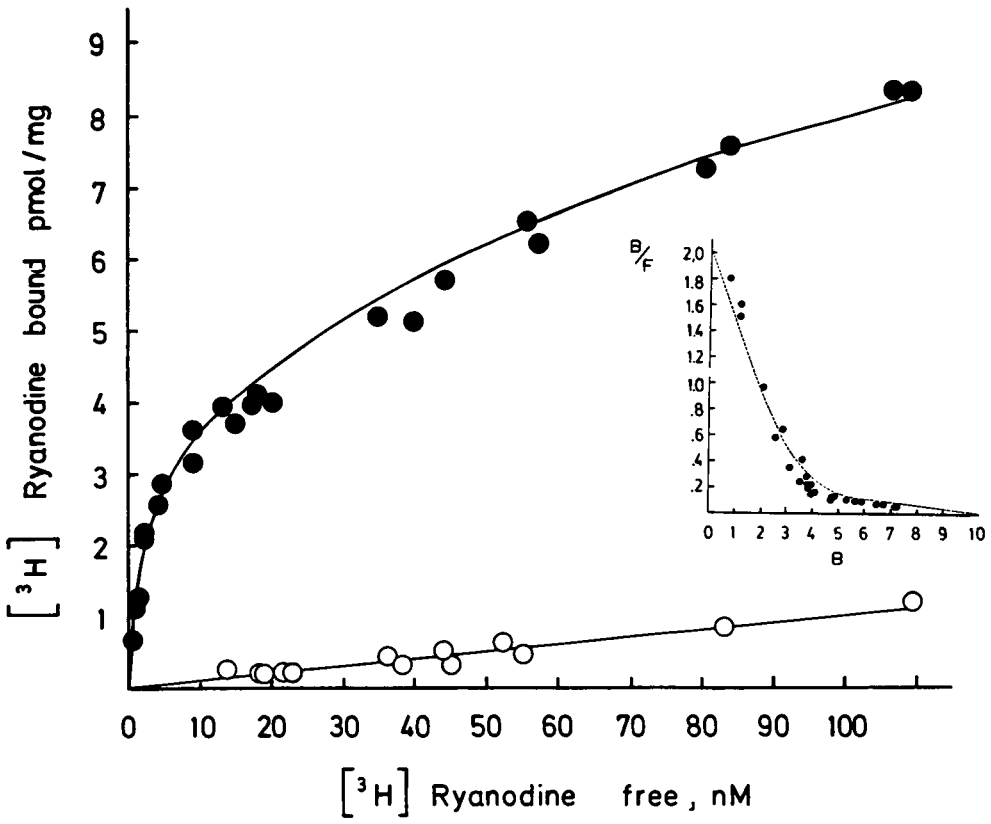

FIGURE 1 [^{3}H]-Ryanodine binding to SR vesicles isolated from frog skeletal muscle. The curve for total binding (*solid circles*) was fitted as the sum of two hyperbolas (Kd_1 = 1.7 nM; Kd_2 = 90 nM; $Bmax_1$ = 3.3 pmol/mg; $Bmax_2$ = 7 pmol/mg) plus a straight line (*open circles*) for nonspecific binding (slope = 0.01 pmol/mg × nM). The insert shows a Scatchard plot for specific binding; data were fitted with a curve using the same Kd and Bmax values as above.

10 nM ryanodine increased them to 10.8 and 86.0 ms. respectively (Fig. 3). The slow closed time constant for the control was 21.4 ms and decreased to 12.0 ms after addition of 10 nM ryanodine. The values of the fast closed time constants were at the limit of the time resolution of our recording system (~3 ms) and thus the fitted values are subject to some uncertainty in this case (control ≈4 ms; ryanodine ≈3 ms) and may not represent reliably the fast kinetic component. In any case even if we disregard these values the changes in the other three time constants are consistent with the observed increase in P_o elicited by the drug (Fig. 2 *B*).

b. Channels with low P_o

Ryanodine has been postulated to bind to the open channel (Pessah et al., 1987). Hence, we investigated its effects on channel activity under initial conditions of low P_o. To reduce P_o before the addition of ryanodine, we lowered the [Ca^{2+}] in the *cis* side by perfusing this compartment with 40 ml of a solution of 225 mM Hepes/Tris, pH 7.4 containing 1 μM-free Ca (100 μM HEDTA,[1] 38 μM Ca/Hepes). In this condition the channel displayed a bursting pattern of openings and closings separated by long-lasting silent periods (Fig. 4). We defined as a long-lasting silent period any closed event longer than 800 ms.

Addition of 5 nM ryanodine to the *cis* compartment activated the channel after a lag period of 8 min. Unconditional P_o increased from 0.06 to 0.19, with no change in the current flowing through the channel (Fig. 4), even 16 min after the addition of the drug. P_o was also calculated during the burst periods, where it increased from 0.15 to 0.42. The fractional time spent in the long-lasting closures did not vary and was 0.58 before and 0.55 after ryanodine addition, respectively. Thus, the major change produced by ryanodine took place within the bursting periods.

The open and closed dwell time distributions were fitted by the sum of two exponentials in the interval of 4 to 800 ms. 5 nM ryanodine increased the slow open time constant from the control value of 9.3 to 29.3 ms and

[1]HEDTA: *N*-hydroxyethylethylene-diaminotriacetic acid.

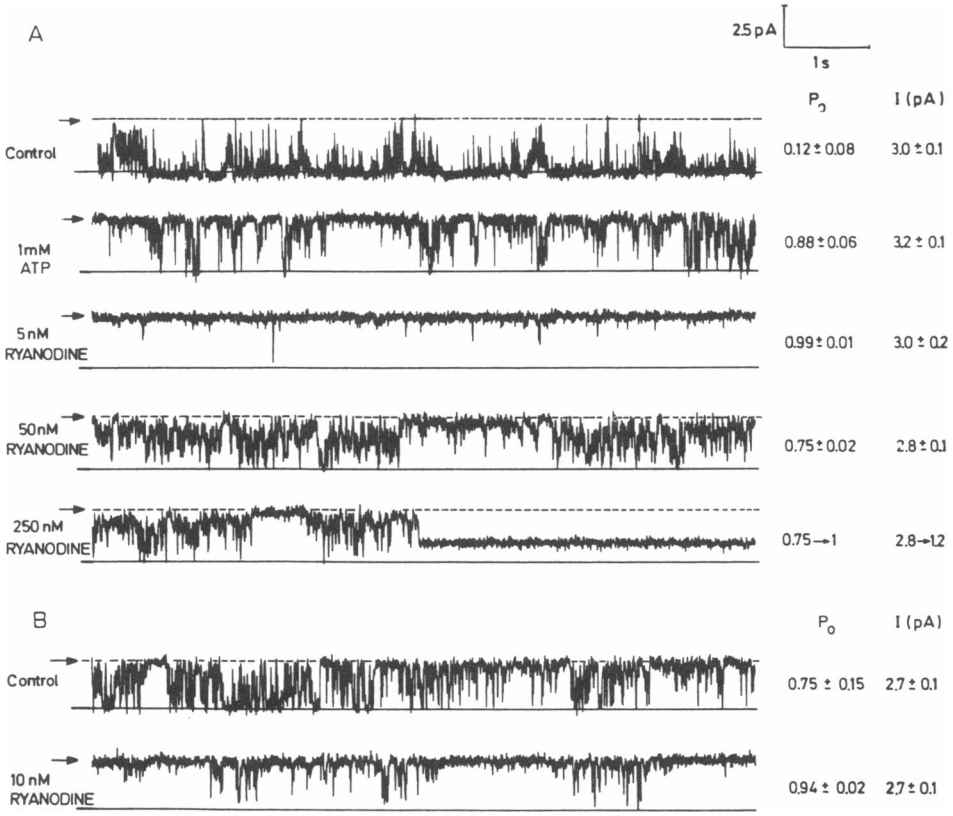

FIGURE 2 Single-channel fluctuations of 0 mV. For display, records were filtered at 100 Hz and digitized at 500 Hz. The solid line indicates the zero current level and the broken line the open current level. *Cis* solution: 225 mM Hepes/Tris, pH 7.4, $[Ca^{2+}] \geq 10$ but lower than 40 μM. *Trans* solution: 37 mM Ca/Hepes, 15 mM Hepes/Tris, pH 7.4. The single-channel conductance was 85 pS in *A* and 94 pS in *B*. Depicted P_o were calculated over a period of at least 100 s.

decreased the closed time constants from 7.8 and 59.6 ms to 6.6 and 34.1 ms respectively. The fast open time constants were near the limit of resolution of our system (control $\approx$ 2 ms, ryanodine $\approx$ 4 ms) and did not represent reliably the fast kinetic component.

These results indicate that the activating effects of ryanodine at low concentrations are also expressed when the initial P_o is low (<0.1) and that the kinetic effects are similar to those observed at higher P_o. Furthermore, the changes observed can be ascribed to kinetic modification of intraburst time constants.

DISCUSSION

Binding experiments

We show here that SR vesicles isolated from frog muscle have two classes of ryanodine binding sites, with Kds of 2 nM and 90 nM, respectively. More than one class of sites have been described for other SR preparations. Thus, in SR vesicles from cardiac muscle two populations of sites were observed (Inui et al., 1987*b*), and two sites seem to be present in SR isolated from chicken skeletal muscle (K. Murakami and J. L. Sutko, unpublished observations).

In the case of SR vesicles isolated from rabbit skeletal muscle, a single class of high affinity ryanodine binding sites has been found at high ionic strength (Pessah et al., 1986; Inui et al., 1987*a*; Hasselbach and Migala, 1988; Lai et al., 1988). Using SR vesicles isolated from rabbit, we detected the presence of high affinity sites (Kd = 2 nM) but not of the low affinity sites (with Kd $\approx$ 100 nM) observed in SR from frog. It is conceivable that lower affinity sites might be present, but under our experimental conditions detection of sites with affinities lower than 200 nM was precluded by the presence of a large nonspecific binding component. However, nonsaturable binding components have been observed at lower ionic strength using μM concentrations of ryanodine (Chu et al., 1988), indicating the presence of low affinity sites in SR from rabbit. Recent experiments have confirmed these findings, since Meissner et al. (1989) under conditions of high ionic strength have detected high (Kd = 4 nM) and low affinity [^{3}H]-ryanodine binding sites in heavy SR frac-

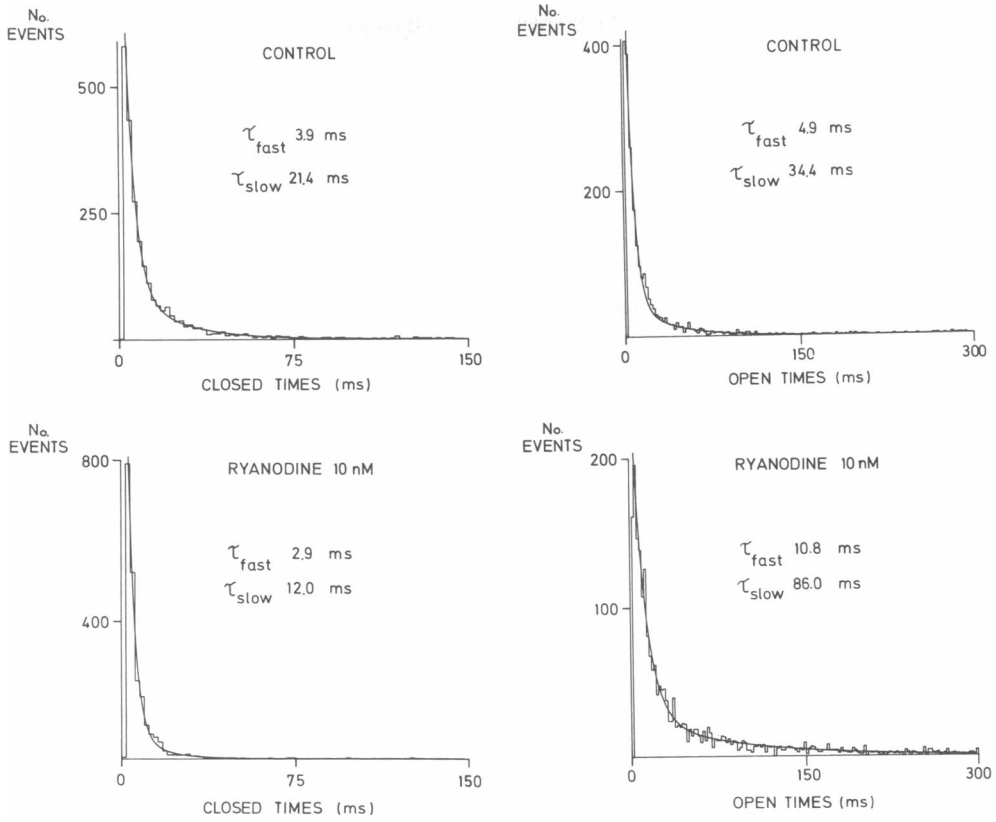

FIGURE 3 Effect of ryanodine on the open and closed dwell time distributions. Unconditional interval duration histograms were constructed from records of the channel shown in Fig. 2 *B*. Records were filtered at 200 Hz and digitized at 1 KHz. Smooth lines correspond to the bi-exponential fit with the time constants indicated in the figure.

tions isolated from rabbit skeletal muscle; moderate tryptic digestion leads to loss of high affinity sites but does not modify low affinity binding. Ca^{2+} release from trypsin-digested vesicles remains sensitive to activation by Ca^{2+} and adenine nucleotides and is inhibited by Mg^{2+} and ruthenium red (Meissner et al., 1989).

Single channel experiments

Studies carried out with native SR vesicles from rabbit fused into lipid bilayers have shown that μM concentrations of ryanodine modify channel properties, causing both a drastic increase in probability of channel opening and a decrease in channel conductance (Rousseau et al., 1987; Imagawa et al., 1987, Smith et al., 1988). The purified channel exhibits a similar behavior in the presence of μM concentrations of ryanodine (Lai et al., 1988), or after two hours of incubation with 20 nM ryanodine (Smith et al., 1988).

We show in the present work for the first time that there is a stimulating effect of nanomolar ryanodine on channel opening probability with no effect on channel current. This effect is due to a modification of the kinetic constants of channel openings and closings. We have also confirmed in frog membranes previous observations made in SR from rabbit, namely, that higher concentrations of ryanodine leave the channel in an open state characterized by a P_o near unity and by a significant reduction of the current flowing through the channel (Rousseau et al., 1987; Imagawa et al., 1987; Lai et al., 1988; Smith et al., 1988).

The stimulating effect of 5–10 nM ryanodine, which was observed with or without ATP present in several single channel experiments, took place only a few minutes (5 to 10) after addition of the drug to the *cis* compartment, regardless whether the channel had a high or a low P_o before addition of ryanodine. These effects are much faster than those previously described for low concentrations of ryanodine (Smith et al. 1988).

Furthermore, our results show that the stimulatory effects of nanomolar ryanodine are expressed mainly during the bursting periods, in which the channel is rapidly oscillating between open and closed states. This observation is consistent with the assertion that ryanodine binds to the open channel.

The purified calcium channel seems to have several

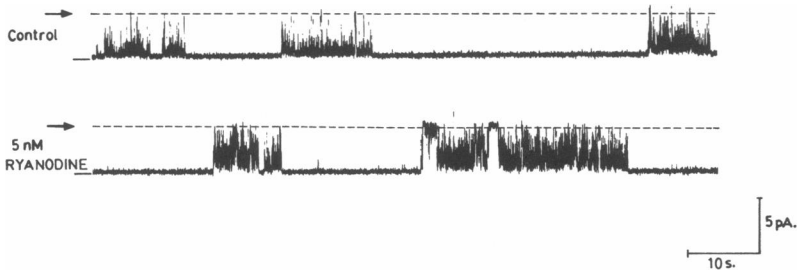

FIGURE 4 Effect of ryanodine on a channel with low P_o. The membrane was formed with a mixture of POPE:PS:PC = 25:15:10 (milligram/milliliter in decane). *Cis* solution: 225 mM Hepes/Tris, pH 7.4, 1-μM free Ca (100 μM HEDTA, 38 μM Ca/Hepes). *Trans* solution: 37 mM Ba/Hepes, 15 mM Hepes/Tris, pH 7.4.

sub-conductance states (Smith et al., 1988). It is tempting to speculate that low concentrations of ryanodine stabilize the state with the highest conductance. Higher concentrations of ryanodine would favor states with lower conductances (see Fig. 2 *A* at 50 nM ryanodine), and 250 nM ryanodine would stabilize the channel in the lower sub-conductance state. Thus, ryanodine could be used to express these conductance states.

Is there a correlation between binding and single-channel experiments?

The inhibitory effect of ryanodine on calcium uptake in SR vesicles takes place at nanomolar concentrations and the apparent affinity of the drug derived from dose response curves for calcium uptake ability coincides with the affinity determined for [^{3}H]-ryanodine binding (Hasselbach and Migala, 1988). These authors concluded that the calcium channels of SR should be activated by nanomolar concentrations of the drug. The present results support such assertion, since we show that ryanodine binds to high affinity sites of SR and opens the calcium channels at nanomolar concentrations (5–10 nM). Thus, although the channel experiments were not performed in the same conditions as the binding experiments, our results suggest that the activating effects of low concentrations of ryanodine arise from drug binding to high affinity sites.

It is conceivable that the low affinity ryanodine binding sites that we found in the SR from frog are also associated with the calcium channel, so that the expression of the low conductance open state observed at high (micromolar) concentrations of ryanodine reflects the occupancy of such sites by the drug. It is interesting to note in this regard that moderate trypsin digestion abolishes high affinity binding sites while preserving channel activation and inhibition characteristics (Meissner et al., 1989), suggesting that low affinity sites may be somehow related to channel function in SR from rabbit. Nevertheless, to establish the functional association of the two types of binding sites with channel behavior requires further experiments at the macromolecular level.

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