Ryanodine modifies conductance and gating behavior of single Ca\(^{2+}\) release channel

ERIC ROUSSEAU, JEFFREY S. SMITH, AND GERHARD MEISSNER
Departments of Biochemistry and Nutrition and Physiology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514

Ryanodine modifies conductance and gating behavior of single Ca\(^{2+}\) release channel. Am. J. Physiol. 253 (Cell Physiol. 22): C364-C368, 1987.—Ryanodine affects excitation-contraction coupling in skeletal and cardiac muscle by specifically interacting with the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel. The effect of the drug at the single channel level was studied by incorporating skeletal and cardiac SR vesicles into planar lipid bilayers. The two channels were activated by micromolar free Ca\(^{2+}\) and by millimolar ATP and inhibited by Mg\(^{2+}\) and ruthenium red. Addition of micromolar concentrations of ryanodine decreased about twofold the unit conductance of the Ca\(^{2+}\)- and ATP-activated skeletal and cardiac channels. A second effect of ryanodine was to increase the open probability \((P_o)\) of the channels in such a way that \(P_o\) was close to unity under a variety of activating and inactivating conditions. The effects of ryanodine were long lasting in that removal of ryanodine by perfusion did not return the channels into their fully conducting state.

For the past two decades the effects of the natural plant alkaloid, ryanodine, on excitation-contraction coupling (E-C) have been studied in various types of striated muscles. Pharmacological and electrophysiological studies with intact and skinned fibers produced conflicting data (3, 6, 11, 29, 30). Some results indicated that ryanodine enhances E-C coupling, whereas others implied that this drug acts as an inhibitor. This duality of ryanodine effects has been also observed in studies with cardiac and skeletal muscle sarcoplasmic reticulum (SR) vesicle membrane fractions (7, 12). More recent ryanodine-binding studies and vesicle ion flux measurements have suggested that at submicromolar concentrations ryanodine exerts its activating effect by “opening” the SR Ca\(^{2+}\) release channel (9, 13, 14, 17). The second effect of ryanodine, observed during prolonged incubation at high concentrations (>100 \(\mu\)M), was the loss of the Ca\(^{2+}\) releasing activity of heavy SR vesicles, which implies that the Ca\(^{2+}\) release channel was present in a “closed state” (8, 14, 21).

The technique of vesicle incorporation into planar lipid bilayers is a powerful tool for studying ion channel behavior at the molecular level. This approach is especially useful for the study of ion channels from SR (16) and T-tubule membranes (4) due to the inaccessibility of these membranes by the patch-clamp technique. Lately this approach has allowed us to study the behavior of single Ca\(^{2+}\) release channels from skeletal (22-24) and cardiac SR muscle preparations (20). In each case a large conductance, divalent cation-selective channel was identified that was activated by micromolar free Ca\(^{2+}\) and by millimolar adenine nucleotides and inhibited by Mg\(^{2+}\) and ruthenium red. In this paper, we report the first direct observations of the effects of ryanodine on skeletal and cardiac SR release channels, using the planar lipid bilayer technique. We found that ryanodine has a profound effect on the unit conductance and the gating behavior of the skeletal and cardiac Ca\(^{2+}\) release channels.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

Reagents. ATP and the ATP analogue AMP-PCP were obtained from Sigma (St. Louis, MO) and ruthenium red from Fluka Chemical (Ronkonkama, NY). Phospholipids were purchased from Avanti Biochemicals (Birmingham, AL). HEPES buffer solutions were prepared using double glass distilled water as described by Smith et al. (25). Ryanodine was a generous gift from Dr. Hoffman, Merck Sharp & Dohme (Rahway, NJ). All other reagents were of reagent grade.

Isolation of sarcoplasmic reticulum vesicles. Rabbit skeletal muscle SR vesicles were prepared as described previously (15). Briefly, SR vesicles containing the Ca\(^{2+}\) release channels were recovered from the 37-45% region of a sucrose gradient that contained membranes sedimenting at 2,600-35,000 g.

Canine cardiac muscle SR vesicles were recovered from the 31-40% region of sucrose gradients that contained membranes sedimenting at 10,000-100,000 g (14).

Planar bilayer measurements. The planar lipid bilayer technique for observing calcium channels from SR membranes is described in detail by Smith et al. (25). The procedure is as follows. SR vesicles were fused into Mueller-Rudin planar lipid bilayers containing phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) in the ratio 5:3:2 (50 mg/ml phospholipid in decane), using an asymmetric choline-chloride buffer system. Vesicle fusions were visualized as step-like increases in Cl\(^{-}\) conductance due to the incorporation of Cl\(^{-}\) channels. After the first fusion, the cis-
chamber, which corresponds to the cytoplasmic side of the SR, was perfused with a Ca²⁺-EGTA, 125 mM Tris/250 mM HEPES (pH 7.4), buffer solution. Cis-Ca⁺⁺ and EGTA concentrations were adjusted to obtain different free Ca²⁺ concentrations, which were calculated by a computer program using binding constants published by Fabiato (5). The trans chamber, which corresponds to the intra-SR compartment, was perfused with a 50 mM Ca(OH)₂ or Ba(OH)₂-250 mM HEPES (pH 7.4) buffer. All experiments were performed at room temperature. Unit currents were measured with a home-made double-stage amplifier, as described by Smith et al. (25). The electrical signals were filtered at 4 kHz for storage on videotape using a modified audio processor and videotape recorder, as described by Bezannilla (2). Data recordings were filtered at 300 Hz and digitized at 1 kHz for storage on hard disk and further analysis using an IBM PC-XT microcomputer. The open probability values (Pₒ) were determined from data stored in 50-s files.

RESULTS

Figure 1 shows the current fluctuations of a single skeletal Ca²⁺ release channel before and after the addition of ryanodine. In the upper trace (Fig. 1A), the channel was partially activated by 1.2 μM cis-Ca⁺⁺. Both short and long open events, shown as upward deflections, were seen. Many of the open events were so brief in duration that they were not fully resolvable at the cutoff frequency (300 Hz) used in our studies. Figure 1B shows that at the addition of 4 mM ATP the channel was greatly activated. A few fully resolved closed events were detectable, although most of the time the channel appeared to open so rapidly that individual transitions were difficult to resolve. The current trace in Fig. 1C was taken after the addition of 15 μM ryanodine to the 1.2 μM Ca⁺⁺, 4 mM ATP cis-medium. One minute after the addition of the drug, a sudden step-like change in unit current amplitude was observed, suggesting the formation of a subconducting state. In Fig. 1C, the decrease in unit current amplitude was preceded by a brief period of increased closed transitions. In other experiments, formation of the subconductance state appeared to occur directly from the fully conducting state. The Pₒ of the subconducting state was near unity. Brief events reaching the closed state level were only infrequently observed. In two recordings where we tested the lifetime of the subconductance state we found that the channel remained in the ryanodine induced substate until the bilayers disrupted (up to 30 min).

In agreement with studies of passively loaded SR vesicles (14), the action of ryanodine seemed to be correlated with the degree of channel activity (Pₒ) in the reference condition. In general, ryanodine reacted with the activated channel within several minutes when added to the cis chamber at micromolar concentrations. Ryanodine modification of the channel in the presence of ruthenium red, a potent inhibitor of the channel, or by trans ryanodine, was not observed. Ryanodine did not change the passive electrical properties of the bilayer at concentrations of up to 200 μM in both chambers. Rather, ryanodine appeared to interact specifically with the Ca²⁺ release channel in that the drug was also without an effect on SR single Cl⁻ channel currents observed in the choline-chloride containing fusion buffer.

Figure 2, A–C, shows recordings at two different voltages before and after the channel was modified by ryanodine. In the upper two traces, the channel was activated by 1.2 μM free Ca²⁺ plus 4 mM cis ATP in the absence of ryanodine. The channel displayed a high Pₒ at both voltages, with a Pₒ (0 mV) = 0.70 and Pₒ (–30 mV) = 0.65. The two traces in Fig. 2B show the behavior of the channel after ryanodine had acted on the channel. Ryanodine decreased the mean current amplitude value at 0 and –30 mV, and changed the kinetic behavior of the channel in such a way that long-lived open events predominated. Closed events were short in duration and were separated by open intervals that could last for as long as 10–20 s.

The skeletal and cardiac Ca²⁺ release channels are sensitive to inhibition by millimolar concentrations of Mg⁺⁺ and micromolar concentrations of the polycationic dye ruthenium red. In contrast, in the presence of micromolar concentrations of ryanodine, Mg⁺⁺, and ruthenium red were ineffective in inhibiting ⁴⁰Ca²⁺ release from vesicles (14). A similar resistance to inhibition by Mg⁺⁺ and ruthenium red was observed for the ryanodine modified channel in the bilayers. Addition of 10 μM ruthenium red to the cis-chamber did not appreciably affect the behavior of the modified channel (not shown). The addition of 15 μM cis-ruthenium red and 4 mM free Mg⁺⁺ partially attenuated single channel current (Fig. 2C). In the presence of the two inhibitors, channel fluctuations were poorly resolved.

Figure 2D compares the current voltage relations of the ryanodine unmodified and modified channels. The curves were obtained from single channel fluctuations recorded under the conditions of Fig. 2, A and B. The slope conductance with 50 mM Ca⁺⁺ as the trans current carrier was found to be 83 pS for the unmodified channel and 42 pS for the ryanodine modified channel. The reference conductance value of 83 pS is lower than the value.
RYANODINE AFFECTS Ca\(^{2+}\) RELEASE CHANNEL

The effect of ryanodine on several skeletal Ca\(^{2+}\) release channels is shown in the multichannel recording experiment of Fig. 3. At least four Ca\(^{2+}\) release channels from skeletal muscle SR vesicles were incorporated into the bilayer. In the presence of 2.5 μM cis-Ca\(^{2+}\), the channels displayed a multitude of transitions that were difficult to resolve (not shown). By lowering the free Ca\(^{2+}\) concentration to 1 μM by the addition of EGTA, the reference channel activity could be reduced to essentially one current level. (Fig. 3A). Numerous short open events were seen; superpositions of the current fluctuations of two or three channels were infrequent due to the low open probability of the channels. In the next three traces (B, C, and D), ryanodine successively modified three channels. In each case, the channel passed abruptly into a subconductance state with a high open probability. In Fig. 3D, a few short-lived open events remained, suggesting the presence of at least one additional channel that was not affected by ryanodine. An identical behavior has been observed three times during different multichannel recordings under the same experimental conditions. In each case, ryanodine induced a “staircase” phe-

of 100 pS reported in the previous papers for the skeletal 
Ca\(^{2+}\) release channel (23, 24). This could be due to a 
lower signal-to-noise ratio in the present experiments, 
as well as difficulties in resolving the open events of the 
partially activated channel.

The reversal potential for the unmodified channel was 
close to +30 mV, as previously reported (23). In the 
presence of ryanodine, the extrapolated value of the 
reversal potential was shifted by 5 mV toward the nega-
tive potential. This shift in reversal potential could be 
related to a slight change of the selectivity of the ryan-
odine channel protein complex to Ca\(^{2+}\) and Tris\(^{+}\).

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FIG. 2. Current voltage behavior of a single skeletal Ca\(^{2+}\) release channel in absence and presence of ryanodine. A: single channel currents were recorded before addition of ryanodine with 1.2 μM free Ca\(^{2+}\) (1 mM EGTA, 0.95 mM CaCl\(_2\), 4 mM ATP, 125 mM Tris-250 mM HEPES, pH 7.4 cis, and 50 mM Ca(OH)\(_2\)-250 mM HEPES, pH 7.4 trans. Open probability (P\(_o\), 0 mV) = 0.70 and P\(_o\), (-30 mV) = 0.65. B: channel activity recorded 1 min after addition of 15 μM cis-
ryanodine. P\(_o\), (0 mV) = 0.99 and P\(_o\), (-30 mV) = 0.88. C: channel activity recorded as in B except with 15 μM ruthenium red and 4 mM cis-Mg\(^{2+}\). D: unitary currents of a skeletal Ca\(^{2+}\)-release channel were recorded as a function of holding potential as in A (open circles) and in presence of 15 μM cis-ryanodine (open squares). Values of unit conductance were γ = 83 pS (open circles), and γ = 42 pS (open squares). Data points are average of 3 different experiments.

FIG. 3. Effect of ryanodine on single channel currents of several skeletal Ca\(^{2+}\) release channels. A: opening events (upward deflections) were recorded in absence of ryanodine in 1 μM free Ca\(^{2+}\) (110 μM EGTA, 100 μM CaCl\(_2\), 125 mM Tris-250 mM HEPES, pH 7.4 cis and 50 mM Ca(OH)\(_2\)-250 mM HEPES, pH 7.4 trans. Holding potential was 0 mV Solid line indicates zero current level. B-D: channel recordings were taken 90 s (B), 120 s (C), and 240 s (D) after addition of 30 μM cis-ryanodine. Subconducting states of 3 channels modified by ryanodine are indicated by dashed lines. Note that in D, infrequent open events are evident, indicating presence of 4th channel that has not been affected by ryanodine.
The unmodified channel was found to be 105 pS. This means that micromolar Ca\(^{2+}\) activates the cardiac channel to a greater extent than the skeletal channel (20). With 50 mM Ba\(^{2+}\) as the current carrier, the unit conductance of the unmodified channel was found to be 105 pS. This value was 33% greater than the one previously reported, using 50 mM Ca\(^{2+}\) in the trans-chamber as the current carrier (20). Within 1 min after addition of 10 \(\mu\)M cis-ryanodine, the channel passed into a subconducting state. The unit conductance was lowered from 105 to 41 pS, which corresponded to a 60% decrease. The \(P_o\) of the subconducting state was close to unity, which corresponded to a doubling of the \(P_o\) of the channel. Accordingly, ryanodine had a most profound effect on the \(P_o\) when the Ca\(^{2+}\) release channel was only partially activated, as illustrated in Figs. 3 and 4. Single channel measurements are in good agreement with recent vesicle 45Ca\(^{2+}\) flux measurements that also indicated a decreased sensitivity of an open channel toward its usual activators and inhibitors after exposure to micromolar concentrations of ryanodine (13, 14).

The effect of ryanodine on a small conductance, 5–10 pS, calcium channel from SR (24, 27) has not been studied. The role of this channel in Ca\(^{2+}\) release from SR is not known. In the artificial membranes the small conductance channel does not fit the characteristics determined for Ca\(^{2+}\) release in isolated vesicles (24).

It is of interest to relate the change in single channel behavior seen in the present study to the positive and negative inotropic effects of ryanodine at the cellular level. In skeletal muscle fibers, the positive inotropic effect that is thought to result from an increase of intracellular free Ca\(^{2+}\) concentration, can be related to a progressive time-dependent enhancement of SR Ca\(^{2+}\) release through channels stabilized by ryanodine in an open state (26). Bers et al. (1), using the rapid cooling contracture technique in the presence of ryanodine, found that during pacing, the SR appears to be able to accumulate Ca\(^{2+}\) and that during rest this Ca\(^{2+}\) is rapidly lost. Our bilayer studies and previous vesicle ion flux and binding studies have shown that ryanodine preferably interacts with the channel in its open state (9, 13, 14).

The negative inotropic effect of ryanodine in cardiac muscle has been ascribed to an inactivation of the SR Ca\(^{2+}\) release mechanism (28, 30). However, another possibility, which has not been ruled out, is that ryanodine activates the cardiac Ca\(^{2+}\) release channel (10) and that the different effects seen in skeletal and heart muscle are the consequence of differences in the Ca\(^{2+}\) handling ability of the two tissues. It is well known that cardiac cells are capable of effectively lowering their free cytoplasmic Ca\(^{2+}\) via a Na\(^{+}-\)Ca\(^{2+}\) exchanger located in their surface membrane (18, 19). Accordingly, we propose that submicromolar ryanodine in cardiac muscle, like in skeletal muscle, reduces the Ca\(^{2+}\) handling ability of SR by forming a long-lasting, subconductance state of the Ca\(^{2+}\) release channel. Ryanodine induces SH Ca\(^{2+}\) depletion in a time-dependent and use-dependent manner. In heart cells, released Ca\(^{2+}\) is effectively extruded by the Na\(^{+}-\)Ca\(^{2+}\) exchanger, whereas in skeletal muscle, the myofibrillar Ca\(^{2+}\) concentration may be sufficiently elevated to induce long-lasting contractures.

In conclusion, our present results demonstrate the value of the planar bilayer technique to gain insight into
the molecular mechanism of a drug affecting a specific intracellular membrane channel. Moreover, this information should lead to a better understanding of SR function in more complex systems such as isolated vesicle or intact muscle fibers.

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