Ryanodine sensitizes the cardiac Ca^{2+} release channel (ryanodine receptor isoform 2) to Ca^{2+} activation and dissociates as the channel is closed by Ca^{2+} depletion

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In single-channel recordings, the rabbit cardiac Ca^{2+} release channel (RyR2) is converted to a fully open subconductance state with about 50% of full conductance by micromolar concentrations of ryanodine. At +30 mV, corresponding to a luminal to cytoplasmic cation current, the probability of opening (P_o) of ryanodine-modified channels was only marginally altered at pCa 10 (pCa = $-\log_{10}$ Ca concentration). However, at −30 mV, the P_o was highly sensitive to Ca^{2+} added to the cis (cytoplasmic) side and, at pCa 10, was reduced to less than 0.27. The EC_{50} value for channel opening was about pCa 8. No significant Ca^{2+} inactivation was observed for ryanodine-modified channels at either −30 mV or +30 mV. The opening of unmodified Ca^{2+} channels is Ca^{2+} sensitive, with an EC_{50} value of about pCa 6 (two orders of magnitude less sensitive than ryanodine-modified channels) and IC_{50} values of pCa 2.2 at −30 mV and 2.5 at +30 mV. Mg^{2+} decreased the P_o of ryanodine-modified channels at low Ca^{2+} concentrations at both −30 and +30 mV. Caffeine, ATP, and ruthenium red were modulators of the P_o of ryanodine-modified channels. In a [³H]ryanodine binding assay, [³H]ryanodine dissociation from the high-affinity binding site was found to be Ca^{2+} sensitive, with an IC_{50} of pCa 7.1. High concentrations of unlabeled ryanodine prevented [³H]ryanodine dissociation, but ruthenium red accelerated dissociation. These results suggest that ryanodine sensitizes Ca^{2+} activation of the Ca^{2+} release channel and desensitizes Ca^{2+} inactivation through an allosteric interaction. [³H]Ryanodine dissociates from the high-affinity site when the channel is closed by removal of Ca^{2+}, implying that high-affinity ryanodine and Ca^{2+} binding sites are linked through either short- or long-range interactions, probably involving conformational changes.

Ryanodine, a neutral plant alkaloid, was of early interest to physiologists because of its inhibitory effects on cardiac and skeletal muscle function (1). The specific, high-affinity binding of [³H]ryanodine to a class of Ca^{2+} release channels made possible their purification from skeletal and cardiac muscle sarcoplasmic reticulum and accounts for the name, ryanodine receptor (RyR) (2–5). More recently, the property of binding to RyR Ca^{2+} release channels only in the open state (6–11) has made [³H]ryanodine binding a useful functional probe of the opening of Ca^{2+} release channels by other ligands.

High-affinity ryanodine binding *in vitro* is influenced by temperature, pH, ion concentration, and ligands (see review in ref. 12). Among known ligands, Ca^{2+} is considered to be a prerequisite for channel opening and thus for ryanodine binding. Ryanodine bound to the high-affinity site in RyRs dissociates very slowly. At higher concentrations, ryanodine slows the rate of dissociation of ryanodine bound to the high-affinity site of RyR1 by occupation of the low-affinity sites (6, 13–15). Ruthenium red, a channel blocker (6), and Ca^{2+}, a channel activator, also delay dissociation of bound ryanodine (14). It is not known why these compounds, which have such different effects on RyR channel function, affect the dissociation of ryanodine from its high-affinity sites in a similar way.

Ryanodine activates Ca^{2+} release from the sarcoplasmic reticulum, but prolonged exposure of microsomes to micromolar concentrations of ryanodine inhibits Ca^{2+} release (2, 16–19). Single-channel recordings have revealed that ryanodine activates Ca^{2+} release channels at nanomolar concentrations and stabilizes four discrete channel states at higher concentrations (20). A common finding is that micromolar concentrations of ryanodine convert Ca^{2+} release channels to a subconductance state at about 50% of full conductance and a probability of opening (P_o) very close to unity (21). Alterations in conductance by ryanodine have been attributed mainly to modified ion handling, a consequence of allosterically determined conformational changes (22). The mechanism of action of ryanodine on Ca^{2+} release channels, particularly its ability to alter channel gating, is still poorly understood.

In this study, we used single-channel recordings across planar lipid bilayers to examine possible mechanisms for the ryanodine modification of rabbit cardiac muscle RyR (RyR2 isoform) Ca^{2+} release channels. We found that ryanodine-modified channels were much more sensitive to Ca^{2+} than normal channels and were modulated by Mg^{2+}, caffeine, and ATP in the presence of 1 mM EGTA. In addition, the dissociation of bound [³H]ryanodine from the high-affinity site of RyR2 was observed when the channel was closed by decreased Ca^{2+}, but not by the addition of ruthenium red.

Materials and Methods

Preparation of Sarcoplasmic Reticulum Microsomes. Heavy sarcoplasmic reticulum microsomes were isolated from rabbit heart muscle as described (23). Microsomes were suspended in 0.25 M sucrose, 125 mM KCl, and 10 mM Tris·HCl (pH 8.0) and stored at −70°C.

Partial Purification of RyR2 Protein and Single-Channel Recording. Cardiac microsomes were solubilized and partially purified by density sucrose gradient centrifugation, and single-channel properties were recorded in a planar lipid bilayer system, as described (24, 25). Free Ca^{2+} was calculated with the use of the apparent binding constants described by Fabiato (26).

[³H]Ryanodine Dissociation Assay. A previous protocol (27) was modified to determine dissociation of bound [³H]ryanodine to cardiac microsomes. In brief, cardiac microsomes were incubated with 10 nM [³H]ryanodine in a buffer containing 0.5 mM KCl, 10 or 100 μM free Ca^{2+}, 0.2 mM EGTA, and 25 mM Hepes (pH 7.2) for 90 min at 37°C to reach equilibrium. At that point, additional agents, taken from highly concentrated stock to minimize changes in volume, were added, and the incubation
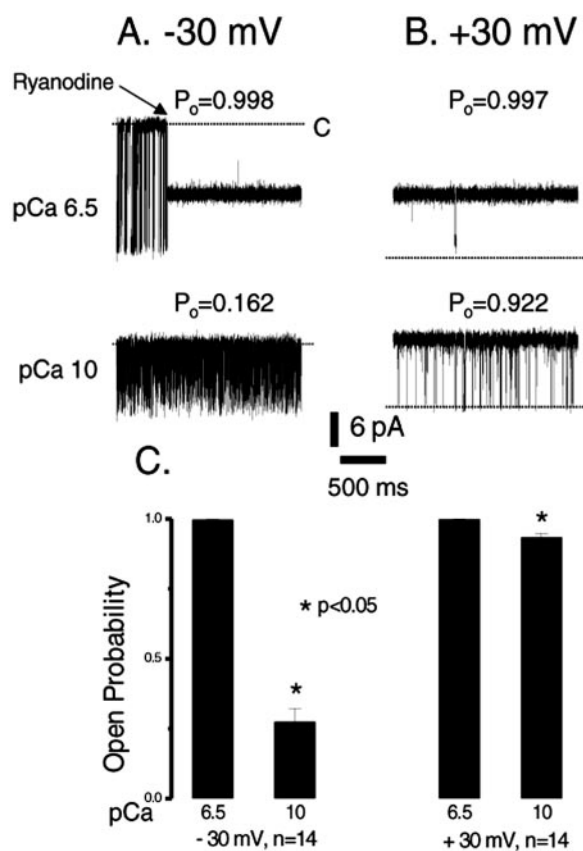
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Fig. 1. Effect of EGTA on ryanodine-modified Ca^{2+} release channels. Single-channel currents are shown as downward (*A*) or upward (*B*) inflections from the closed level at -30 mV or $+30$ mV, indicated by a dotted line in each trace. Tracings were recorded in symmetrical 250 mM KCl solution. The upper traces in both *A* and *B* were recorded in the absence of EGTA to give a pCa of 6.5, and the lower traces were recorded in the presence of 1 mM EGTA, which lowered the Ca^{2+} concentration to a pCa of 10. Modulation of the channel by ryanodine is shown in the upper trace at -30 mV (*A*). The ryanodine-modified channel had about half the conductance of the normal channel. The P_o shown above each trace is for the ryanodine-modified channel. Traces were filtered at 1 kHz and digitized at 10 kHz. The averaged P_o for the ryanodine-modified channels measured in *A* and *B* before and after the addition of 1 mM EGTA is shown in *C*.

proceeded. At different time points, duplicate 0.2-ml samples, containing about 50 μg protein, were filtered on Whatman GF/B membrane filters, presoaked with a washing buffer composed of 25 mM Hepes (pH 7.4) and 0.25 M KCl, and the filters were washed with 12 ml of washing buffer. Background binding was measured in the presence of 10 μM unlabeled ryanodine (1,000-fold excess). [^{3}H]Ryanodine bound to the filter was quantified by liquid scintillation counting.

Results

Ryanodine Sensitizes Ca^{2+} Activation of RyR2. In the absence of EGTA in the 1-ml cis chamber, the addition of 3 μl of the purified Ca^{2+} release channel, which was suspended in 100 μM Ca^{2+}, would raise the Ca^{2+} concentration of the cis chamber to about pCa 6.5 (pCa $= -\log_{10}$ Ca concentration). Under these conditions, a K^+ current of about 21 pA with an open probability (P_o) of about 0.10 could be observed with a single RyR2 channel incorporated into a planar lipid bilayer at a holding potential of either $+30$ mV or -30 mV in symmetrical 250 mM KCl (not shown). The addition of 1–10 μM ryanodine to the cis chamber locked the channel in a subconductance state with $\approx$50% conductance of about 12 pA and full P_o (Fig. 1*A*). These are the

archetypal properties for modulation of Ca^{2+} release channels by ryanodine (21).

The question of how ryanodine increases the probability of channel opening has not yet been answered. In our experiment, only ryanodine and Ca^{2+} were present as possible activators of Ca^{2+} channel opening. Thus, changes in channel gating could result from either a direct or an indirect action of ryanodine. To test the hypothesis that the high P_o for ryanodine-modified Ca^{2+} release channels is mediated through an alteration in Ca^{2+} sensitivity, we lowered the Ca^{2+} concentration to pCa 10 by the addition of 1 mM EGTA to the cis chamber in the same single-channel recording as that shown at pCa 6.5 in Fig. 1*A*. At -30 mV, where the current flows from the cytoplasmic side to the luminal side, the P_o of ryanodine-modified Ca^{2+} release channels was reduced from nearly 1.0 at pCa 6.5 to less than 0.27 at pCa 10 (Fig. 1 *A* and *C*). At $+30$ mV and pCa 10, however, the P_o was reduced from 0.997, observed at pCa 6.5, by a small, but significant extent, to about 0.922 (Fig. 1 *B* and *C*). These values did not change when 1 mM EGTA was also added to the trans chamber. Under these conditions, the channel remained in a subconductance state, the 350-pS conductance for K^+ being about 50% of the control. These results demonstrate that ryanodine alone is not capable of opening the channel fully: ryanodine sensitizes the channel to Ca^{2+}-induced opening. This sensitization is more readily evident at -30 mV.

The Ca^{2+} dependence of channel opening in ryanodine-modified Ca^{2+} release channels was measured. At -30 mV, incremental additions of Ca^{2+} to the cis chamber, beginning at pCa 10, induced incremental increases in P_o (Fig. 2 *A* and *C*). Plots of P_o against pCa at -30 mV show a smooth curve for Ca^{2+} dependence of P_o, rising from 0.197 to 0.992 between pCa 10 and pCa 6.2, with a plateau above pCa 6 (Fig. 2 *A* and *C*). The EC_{50} value, calculated from the dose–response curve for Ca^{2+} activation at -30 mV, was pCa 7.9 $\pm$ 0.3 ($n = 3$). In contrast, there was limited inhibition of channel opening in the presence of 1 mM EGTA at $+30$ mV (Figs. 1 and 2 *B* and *C*). The P_o increased after incremental additions of Ca^{2+} to the cis chamber, dipped slightly as Ca^{2+} concentrations were raised to pCa 7.4 in all three recordings, and then increased again to the fully open state at Ca^{2+} concentrations above pCa 6 (Fig. 2 *B* and *D*). The lifetime of the open state (γ_o) was increased, and the lifetime of the closed state (γ_c) was decreased in parallel with the elevation of Ca^{2+} (Fig. 2 *B* and *D*). There was no evidence of inactivation of channel opening by concentrations of Ca^{2+} up to 10 mM for ryanodine-modified Ca^{2+} release channels at either negative or positive potentials (Fig. 2).

The Ca^{2+} dependence of channel opening and closing was also measured in normal channels (Fig. 3). The results were not nearly as pronounced as those seen with ryanodine-modified Ca^{2+} release channels, inasmuch as a strong Ca^{2+} dependence of channel opening and closing was observed at both -30 mV and $+30$ mV. As the Ca^{2+} concentration was elevated in the cis chamber, P_o was increased at both $+30$ and -30 mV (Fig. 3). Dose–response curves (Fig. 3 *C* and *D*) yielded EC_{50} values of 6.1 $\pm$ 0.3 and 6.0 $\pm$ 0.1 ($n = 5$) for -30 and $+30$ mV, respectively. These curves illustrate that the Ca^{2+} sensitivity at -30 mV (pCa 7.9 vs. pCa 6.1) is two orders of magnitude higher in ryanodine-modified Ca^{2+} release channels and, at $+30$ mV (pCa >10 vs. 6.0), is more than four orders of magnitude higher. The average of five independent experiments showed a decrease in P_o at pCa values between 3 and 1.5, so that the IC_{50} value was 2.2 $\pm$ 0.4 at $+30$ mV and 2.5 $\pm$ 0.3 at -30 mV ($n = 5$) (Fig. 3 *A* and *B*). At -30 mV, the increase in P_o, which reached a maximum at approximately pCa 5, was associated with an increase in γ_o and a decrease in γ_c. The pattern was qualitatively similar at $+30$ mV.

Ryanodine-modified Ca^{2+} release channels were tested for modulation by other compounds. In the sequential traces shown in Fig. 4, the presence of 1 mM EGTA (pCa 10) in the cis
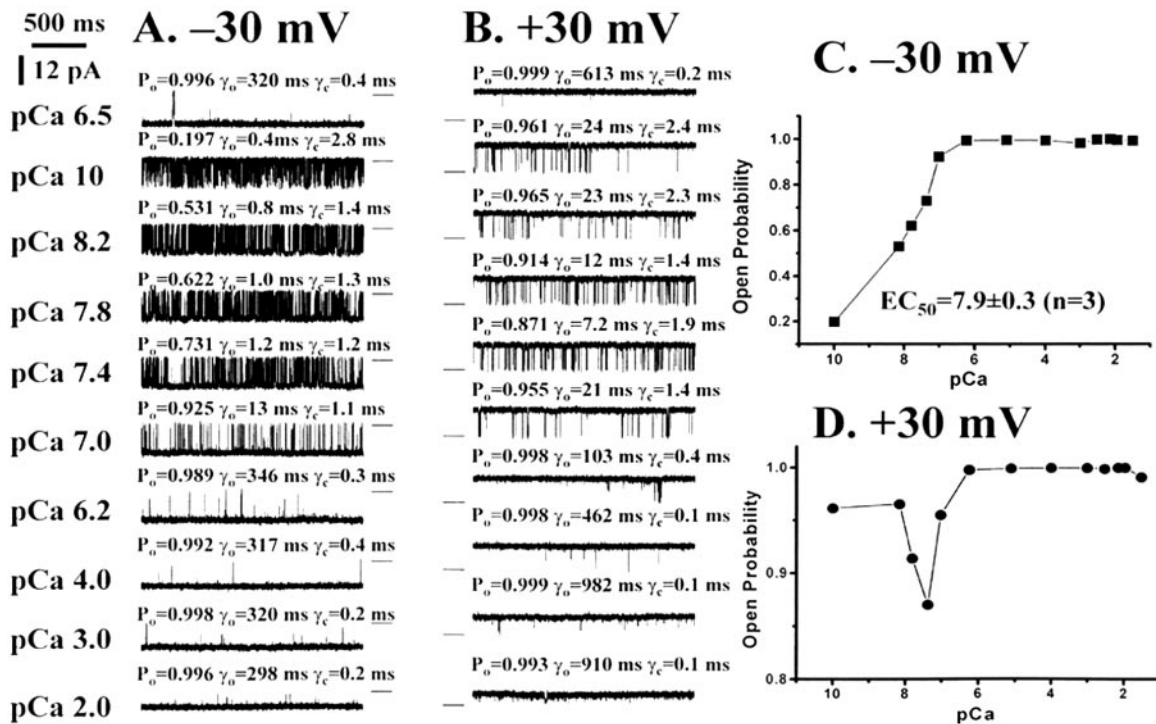
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Fig. 2. Ca^{2+} activation of ryanodine-modified Ca^{2+} release channels. Single-channel currents, at −30 mV or +30 mV, are shown as downward (*A*) or upward (*B*) inflections from the closed level, which is indicated by a short line to the right (*A*) or left (*B*) of each trace. Currents were recorded in symmetrical 250 mM KCl and 1 mM EGTA at various free Ca^{2+} concentrations, indicated on the left of *A*. P_o, open-state lifetime (γ_o), and closed-state lifetime (γ_c) are shown above each trace. Traces were filtered at 1 kHz and digitized at 10 kHz. Resulting Ca^{2+} activation curves are shown in *C* for −30 mV and *D* for +30 mV. (Please note the change in scale between *C* and *D*.)
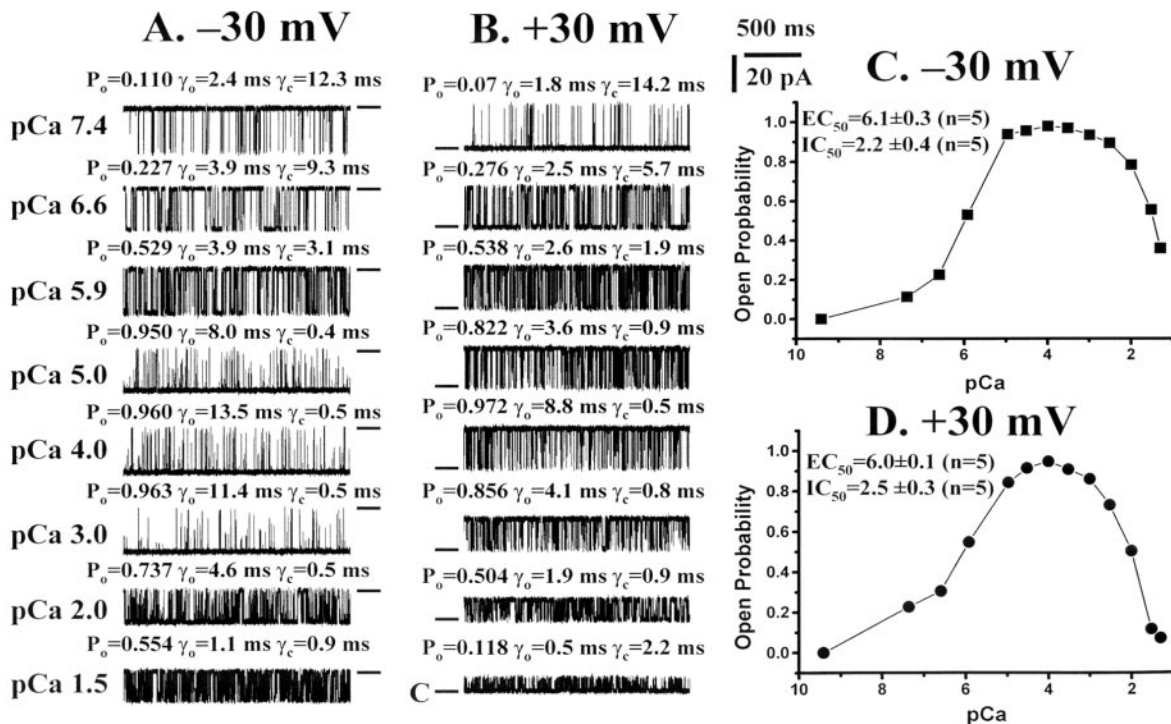

Fig. 3. Ca^{2+} activation and inactivation of ryanodine-modified Ca^{2+} release channels. Single-channel currents, at −30 mV or +30 mV, are shown as downward (*A*) or upward (*B*) inflections from the closed level, which is indicated by a short line to the right (*A*) or left (*B*) of each trace. Currents were recorded in symmetrical 250 mM KCl and 50 μM EGTA at various free Ca^{2+} concentrations, indicated on the left of *A*. P_o, open-state lifetime (γ_o), and closed state lifetime (γ_c) are shown above each trace. Traces were filtered at 1 kHz and digitized at 10 kHz. Resulting Ca^{2+} dose–response curves are shown in *C* for −30 mV and *D* for +30 mV.
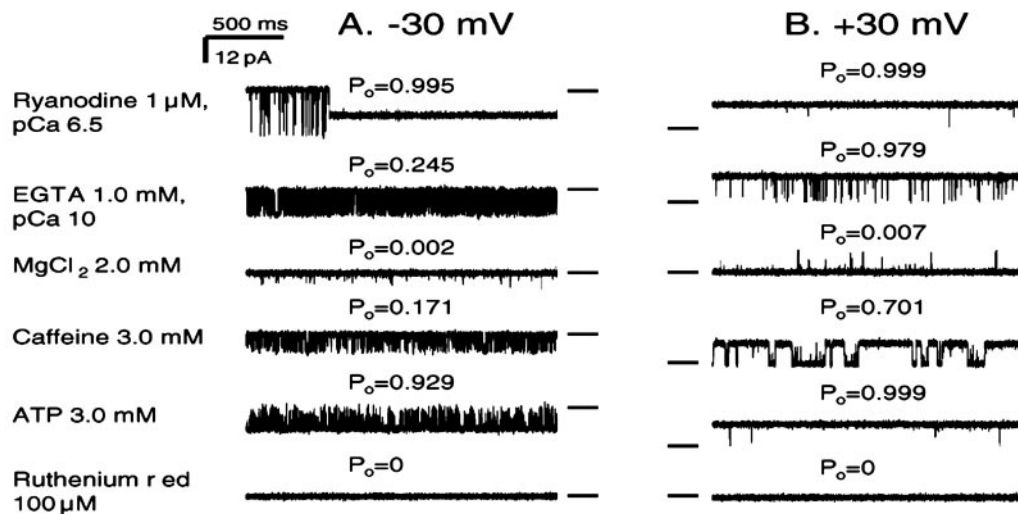
Fig. 4. Modulation of ryanodine-modified Ca^{2+} release channels by Mg^{2+}, ATP, caffeine, and ruthenium red. Single-channel currents, shown as downward (*A*) or upward (*B*) inflections from the closed level at −30 mV or +30 mV, indicated by a short line to the right (*A*) or left (*B*) of each trace, were recorded in symmetrical 250 mM KCl solution. The top traces in both *A* and *B* were recorded at pCa 6.5, and the lower traces are selected from continued recordings in the presence of agents, labeled at the left of *A*. Modulation of the channel by ryanodine is shown in the top trace at −30 mV (*A*). The ryanodine-modified channel exhibited about half of the conductance of the normal channel. P_o, shown above each trace, is for the ryanodine-modified channel. Traces were filtered at 1 kHz and digitized at 10 kHz.

chamber reduced P_o from 0.995 to 0.245 at −30 mV, but to only 0.979 at +30 mV. After the addition of 2 mM $MgCl_2$ to the cis chamber, the P_o was decreased at both holding potentials, from 0.245 to 0.002 at −30 mV and from 0.979 to 0.007 at +30 mV. At pCa 5, however, the P_o for the ryanodine-modified Ca^{2+} release channels was close to 1 at both −30 and +30 mV, and this P_o was unchanged, even when up to 10 mM $MgCl_2$ was added to the cis side (data not shown).

In the presence of 2 mM $MgCl_2$ in the cis chamber, Ca^{2+} activation curves showed that the EC_{50} was shifted from pCa ≈7.9 to pCa ≈6.0 for ryanodine-modified Ca^{2+} release channels and from pCa ≈6.0 to pCa ≈4.0 for normal channels, a shift in Ca^{2+} sensitivity of about two orders of magnitude for both normal and modified channels at both −30 and +30 mV. These observations illustrate that Mg^{2+} is a powerful inhibitor of both normal and ryanodine-modified Ca^{2+} release channels at both positive and negative voltages. The fact that miniscule Ca^{2+} concentrations can overcome Mg^{2+} inhibition, however, shows that the Ca^{2+} affinity for the site of Ca^{2+} activation of the channel is much higher than Mg^{2+} affinity in ryanodine-modified Ca^{2+} release channels.

In ryanodine-modified Ca^{2+} release channels, both caffeine and ATP increased P_o that had been depressed by 1 mM EGTA and $MgCl_2$. These different ligands are both believed to act through Ca^{2+}, by increasing the sensitivity of the channel to Ca^{2+} activation. P_o was reduced to 0 by the addition of ruthenium red at both +30 and −30 mV (Fig. 4), in a manner similar to that observed with unmodified channels.

Dissociation of [³H]Ryanodine Associated with Closure of Channel. If ryanodine binds only to open channels, and if these channels close, then it is reasonable to assume that ryanodine might dissociate from the channel to allow it to close. To test this hypothesis, we attempted to detect the dissociation of [³H]ryanodine bound to the high-affinity site of RyR2 by depletion of Ca^{2+}.

Fig. 5 shows time courses for the dissociation of bound [³H]ryanodine under different experimental conditions. Cardiac muscle microsomes were incubated for 90 min at 37°C in the binding buffer (pCa 4) before different agents were added and

incubation was continued. The addition of 1 mM EGTA (pCa 7.7) initiated dissociation of bound [³H]ryanodine in a time-dependent manner, whereas the control was unchanged. The time course could be fitted to a single-exponential equation with a dissociation rate constant of 74.9 ± 6.3 min^{-1} ($n = 4$). The addition of 1 mM Ca^{2+} at 120 min led to full recovery of [³H]ryanodine binding after 60 min. The addition of 2 mM EGTA, reducing the pCa to 8.0, produced a deeper curve than that for pCa 7.7, with a dissociation rate constant of 19.1 ± 1.4
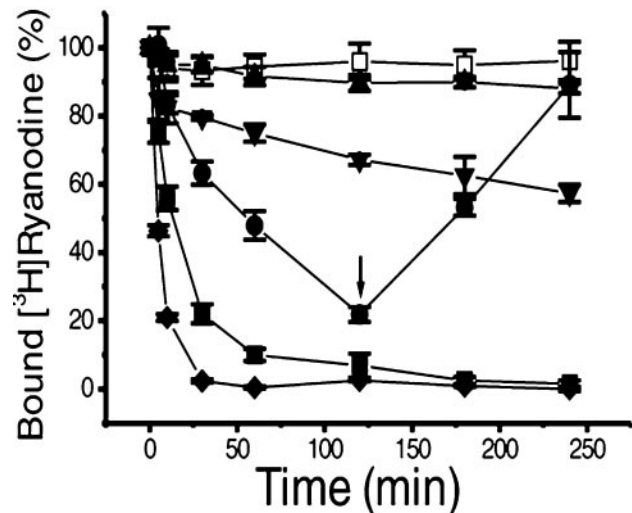

Fig. 5. Dissociation of [³H]ryanodine from the high-affinity binding site of cardiac muscle RyR2. Microsomes were incubated with 10 nM [³H]ryanodine in a binding buffer containing 100 μM free Ca^{2+} for 90 min at 37°C to reach equilibrium. Incubation was then continued after the addition of different agents: □, no addition (control); ●, 1 mM EGTA; ■, 2 mM EGTA; ◆, 2 mM EGTA plus 100 μM ruthenium red; ▲, 2 mM EGTA plus 100 μM ryanodine; ▼, 100 μM ruthenium red. At different time points, duplicate 0.2-ml aliquots were filtered. The arrow indicates the addition of 1 mM $CaCl_2$ at a time point of 120 min for the 1 mM EGTA curve. The data are normalized to the values obtained after the first 90-min incubation, which is shown as time 0.
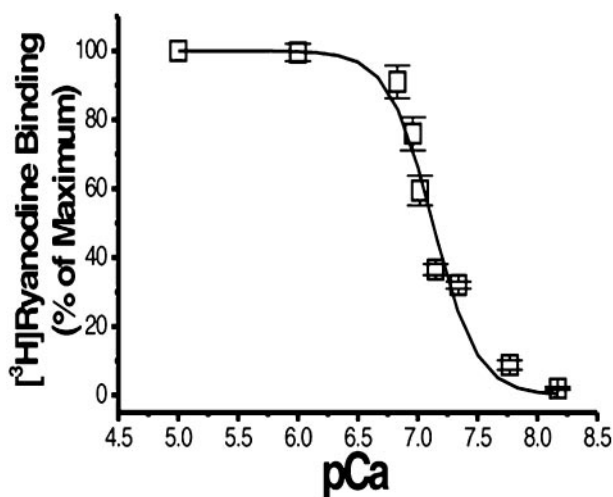
Fig. 6. Ca^{2+}-dependent dissociation of [^{3}H]ryanodine from the high-affinity binding site of RyR2. Microsomes were incubated with 10 nM [^{3}H]ryanodine in a binding buffer containing 10 μM free Ca^{2+} for 90 min at 37°C to reach equilibrium. The reaction mixes were then incubated for 2 h with different concentrations of EGTA to set the desired pCa values. Duplicate 0.2-ml aliquots were filtered at the time points indicated. The data are normalized to the values obtained after the first 90-min incubation.

min^{-1} ($n = 4$, $P < 0.001$). Because the equilibrium state for [^{3}H]ryanodine binding was not altered under these conditions, the dissociation behavior can be attributed mainly to the lowering of Ca^{2+} by EGTA, which closes the channels.

Because occupation of low-affinity binding sites by ryanodine delays dissociation of [^{3}H]ryanodine bound to high-affinity sites (6, 13–15), the effect of 100 μM ryanodine on the dissociation initiated by lowered Ca^{2+} was tested. In the presence of 2 mM EGTA and 100 μM ryanodine, no significant dissociation of [^{3}H]ryanodine was observed up to 240 min of incubation at 37°C when compared with the control (Fig. 5). There was also no significant dissociation when only 100 μM ryanodine was present (data not shown). This finding suggests that occupation of low-affinity sites by higher concentrations of ryanodine maintained the binding of [^{3}H]ryanodine to high-affinity sites, even in the presence of Ca^{2+} concentrations so low that channels should have been closed.

Ruthenium red is a powerful blocker of Ca^{2+} release channels that acts by binding to sites located in the conduction pore of the channel (28). Its mode of action is obviously distinct from that of channel closure induced by Ca^{2+} depletion. Ruthenium red also delays dissociation of ryanodine bound to the high-affinity site in a nonequilibrium state (6). Accordingly, we attempted to determine whether ruthenium red affects the dissociation of [^{3}H]ryanodine bound to the high-affinity site of the channel. In the presence of 100 μM ruthenium red, a very slow dissociation of [^{3}H]ryanodine with a rate constant of 363.1 $\pm$ 36.5 min^{-1} ($n = 3$) was observed when compared with the control (Fig. 5). These results suggest that ruthenium red has little effect on high-affinity [^{3}H]ryanodine binding, even though it blocks the Ca^{2+} release channels. When added together with 2 mM EGTA, however, 100 μM ruthenium red speeded dissociation of [^{3}H]ryanodine bound to the high-affinity site, with a rate constant of 6.7 $\pm$ 0.3 min^{-1} ($n = 4$, $P < 0.001$ when compared with the rate in the presence of 2 mM EGTA).

In the presence of different concentrations of Ca^{2+}, dissociation of [^{3}H]ryanodine from the high-affinity site of RyR2 was increased as Ca^{2+} concentrations decreased (Fig. 6), showing a typical curve of Ca^{2+} dependence. The IC_{50} value obtained by

fitting the curve with a sigmoid equation was 7.12 $\pm$ 0.02 pCa units ($n = 4$), and the slope was 2.3 $\pm$ 0.3.

Discussion

Increased Asymmetric Ca^{2+} Sensitivity of Ryanodine-Modified Ca^{2+} Release Channels. In this study, we explored the effect of ryanodine binding to RyR2 on Ca^{2+} sensitivity at both positive and negative voltages. We found that ryanodine binding induces a higher sensitivity to Ca^{2+} when channel function is measured at positive voltages. At -30 mV, the current flows from the cytoplasmic side to the luminal side, whereas, at $+30$ mV, the current flows from the luminal side to the cytoplasmic side, the direction of Ca^{2+} flow that is physiologically relevant. Single-channel recordings at $+30$ mV show that ryanodine-modified Ca^{2+} release channels retain a P_o close to unity in the presence of 1 mM EGTA (pCa 10), and at -30 mV the P_o is about 0.2. Under these conditions, unmodified channels have a P_o of 0.

In earlier studies (1), cardiac and skeletal muscle function was shown to be modulated by ryanodine. Since then ryanodine has been shown to increase Ca^{2+} permeability from sarcoplasmic reticulum and, at higher concentrations, to inhibit Ca^{2+} release (refs. 2, 16, 29, 30, and see reviews in refs. 10 and 11). Ryanodine blocks the channel in a subconductance state at about 50% of full conductance and a P_o near unity (21). This action has been regarded as a direct effect of ryanodine. Our results help to clarify the action of ryanodine on Ca^{2+} release by showing that increased Ca^{2+} permeability is due to an indirect effect of ryanodine on Ca^{2+} sensitivity.

The binding of ryanodine, through allosteric interactions, could convert Ca^{2+} release channels to a specific conformation, associated with a 50% reduction of the conductance (31). Altered ion handling resulting from conformation changes induced by ryanodine has been considered to be a major reason for alterations in conductance (22). Evidence presented in this study shows that the high P_o induced by ryanodine is likely to be caused by increased Ca^{2+} sensitivity. In earlier studies of the effects of site-directed mutagenesis of residues lining the pore, ryanodine restored or enhanced the response of some mutant channels to caffeine activation. This effect of ryanodine may have occurred through a ryanodine-induced increase in Ca^{2+} sensitivity, because caffeine can sensitize Ca^{2+} activation (25, 32). Thus, the high-affinity binding of ryanodine to Ca^{2+} release channels is likely to induce a conformational change that alters not only conductance but also the affinity of Ca^{2+} for Ca^{2+} activation.

Inhibition by Mg^{2+} of Ryanodine-Modified Ca^{2+} Release Channels. The increased Ca^{2+} sensitivity of ryanodine-modified Ca^{2+} release channels was asymmetric, inasmuch as higher Ca^{2+} sensitivity was observed when the current flowed from the lumen to the cytoplasm. When Mg^{2+} was added at pCa 10, however, the asymmetry was lost and channel opening was inhibited to an equal extent when current flowed in either direction. The inhibited channel was then activated in either direction by subsequent addition of caffeine and ATP (Fig. 4) or Ca^{2+} (data not shown).

Mg^{2+} competes with Ca^{2+} at both the activation and inactivation sites (33, 34). In this study, Ca^{2+} bound to low-affinity sites did not inhibit channel opening of ryanodine-modified Ca^{2+} release channels. This lack of inhibition of channel opening by bound Ca^{2+} suggests that the inhibition by Mg^{2+} of channel opening is due to the competitive binding of Mg^{2+} to the high-affinity Ca^{2+} binding site. Mg^{2+} is considered to be an important regulator in controlling Ca^{2+} release from the sarcoplasmic reticulum of skeletal muscle, even though it has low binding affinity (35). Our results, together with those of others (33, 34), suggest that Mg^{2+} competition with the high-affinity Ca^{2+} sites could be equally important in controlling Ca^{2+} release

under both physiological and pathological conditions and in both cardiac and skeletal muscles.

Dissociation of [³H]Ryanodine Bound to RyR2 Ca²⁺ Release Channels Is Associated with Closure of Channels. In this study, we have modified a protocol (27) to detect dissociation of [³H]ryanodine bound to the high-affinity site of RyR2 by lowering Ca^{2+} under equilibrium binding conditions. It is known that [³H]ryanodine binds to the high-affinity site only when the channel is in the open state, with a Ca^{2+} EC_{50} value of about pCa 6 for Ca^{2+} association. Results from this study suggest that closing of the channel by removal of Ca^{2+} resulted in dissociation of bound [³H]ryanodine with a Ca^{2+} IC_{50} value for dissociation of about pCa 7.1—one order of magnitude higher than the pCa value for Ca^{2+} association. Thus both [³H]ryanodine association and [³H]ryanodine dissociation are primarily Ca^{2+} dependent, reflecting a close relationship between Ca^{2+} and ryanodine binding sites. This relationship is particularly shown to be true when we consider that ryanodine binding to the high-affinity site increases Ca^{2+} sensitivity of Ca^{2+} release channels and that Ca^{2+} is a basic activator of RyR channels. Although a direct interaction between these two sites is possible, most observations are consistent with the view that the high-affinity site for ryanodine binding is exposed or formed by conformational changes resulting from the opening of the channel by Ca^{2+}. When the channel is closed by removal of Ca^{2+}, the ryanodine-binding site is either cryptic or disrupted (6, 13–15).

In an earlier report, Chu *et al.* (6) showed that ruthenium red, at high concentrations, slowed the rate of dissociation of [³H]ryanodine from the high-affinity site. In contrast, we found that ruthenium red accelerated the dissociation of [³H]ryanodine that was induced by removal of Ca^{2+}. Ruthenium red, at 100 μM, closed RyR channels, but it induced only a very slow dissociation of [³H]ryanodine bound to the high-affinity site under equilibrium conditions. Removal of Ca^{2+} also closed channels but led to the complete dissociation of [³H]ryanodine. It is believed that ruthenium red blocks Ca^{2+} release channels by binding to sites located in the conduction pore (28) or inhibits the channels by binding to cytosolic and luminal sites (36). Our results demonstrate that ruthenium red and removal of Ca^{2+} have very different effects on the dissociation of [³H]ryanodine from the high-affinity binding site, even though their actions are cooperative.

The protocol for measuring ryanodine dissociation may become a useful tool for distinguishing among the actions of different compounds. It may also become a useful tool for measurement of channel function, because Ca^{2+}-dependent closure of the channel, detected by [³H]ryanodine dissociation from its high-affinity binding site, provides the converse measure to Ca^{2+}-dependent opening of the channel, which is measured by [³H]ryanodine association.

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