Ryanodine Sensitizes the \( \text{Ca}^{2+} \) Release Channel (Ryanodine Receptor) to \( \text{Ca}^{2+} \) Activation *

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Running Title: Molecular mechanism of ryanodine action

* This work was supported by research grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Alberta to S.R.W.C.

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ABSTRACT

Ryanodine, a plant alkaloid, is one of the most widely used pharmacological probes for intracellular Ca\(^{2+}\) signaling in a variety of muscle and non-muscle cells. Upon binding to the Ca\(^{2+}\) release channel (ryanodine receptor), ryanodine causes two major changes in the channel: a reduction in single channel conductance and a marked increase in open probability. The molecular mechanisms underlying these alterations are not well understood. In the present study, we investigated the gating behavior and Ca\(^{2+}\) dependence of the wild type (wt) and a mutant cardiac ryanodine receptor (RyR2) after being modified by ryanodine. Single channel studies revealed that the ryanodine-modified wt RyR2 channel was sensitive to inhibition by Mg\(^{2+}\) and to activation by caffeine and ATP. In the presence of Mg\(^{2+}\), the ryanodine-modified single wt RyR2 channel displayed a sigmoidal Ca\(^{2+}\) dependence with an EC\(_{50}\) value of 110 nM, while the ryanodine-unmodified single wt channel exhibited an EC\(_{50}\) of 120 µM for Ca\(^{2+}\) activation, indicating that ryanodine is able to increase the sensitivity of the wt RyR2 channel to Ca\(^{2+}\) activation by ~1,000 fold. Furthermore, ryanodine is able to restore Ca\(^{2+}\) activation and ligand response of the E3987A mutant RyR2 channel that has been shown to exhibit ~1,000 fold reduction in Ca\(^{2+}\) sensitivity to activation. The E3987A mutation, however, affects neither \(^{3}\text{H}\)ryanodine binding to nor the stimulatory and inhibitory effects of ryanodine on the RyR2 channel. These results demonstrate that ryanodine does not ‘lock’ the RyR channel into an open state as generally believed, rather it sensitizes dramatically the channel to activation by Ca\(^{2+}\).
INTRODUCTION

Ryanodine, a plant alkaloid, binds specifically with high affinity to and alters the function of intracellular Ca\textsuperscript{2+} release channels (ryanodine receptors, RyRs). Because of its high affinity and specificity, ryanodine has been widely used as a specific ligand for the identification, purification, cloning, and functional characterization of RyRs (1-7). The unique and specific action of ryanodine on RyR function also has made it an invaluable pharmacological probe for intracellular Ca\textsuperscript{2+} signaling in a variety of cells (8,9), and for understanding the mechanisms of ion conduction and channel gating of RyRs (10,11).

Ryanodine has dual actions on the Ca\textsuperscript{2+} release channel depending on its concentration. Ca\textsuperscript{2+} flux studies using sarcoplasmic reticulum (SR) vesicles revealed that at nanomolar to micromolar concentrations, ryanodine stimulates Ca\textsuperscript{2+} release from SR vesicles, while at higher concentrations (micromolar to millimolar range), ryanodine inhibits Ca\textsuperscript{2+} release (12-15). Consistent with these Ca\textsuperscript{2+} release studies, [\textsuperscript{3}H]ryanodine binding analysis established the existence of low and high affinity ryanodine binding sties (16-20). Binding of ryanodine to the high affinity site was correlated with channel activation, whereas occupation of the low affinity ryanodine binding site was related to channel inhibition (19-21). These opposite effects of ryanodine have been clearly demonstrated at the single channel level. Addition of micromolar concentrations of ryanodine caused the RyR channel to enter into a long-lived open state with a reduced single channel conductance (22,23). Exposure to high concentrations (submillimolar to millimolar) of ryanodine led to a persistent blockade of single RyR channels (11,21).
The molecular mechanisms underlying these complex changes in channel conductance and gating behavior of RyR upon ryanodine modification are not well understood. 

[^3H]ryanodine binding studies have suggested that complete blockade of the RyR channel by high concentrations of ryanodine may result from the occlusion of the channel conduction pore by ryanodine (19). Detailed characterization of the effects of ryanodine or ryanodine derivatives on ion handling by RyR has been reported. These studies have led to the proposal that reduction in channel conductance upon ryanodine modification is likely to result from allosteric alterations in ion permeation and ion binding, and/or from partial block by ryanodine (10,24). Little is known about the molecular mechanism by which ryanodine increases the open probability (Po) of the RyR channels. Ryanodine is thought to ‘lock’ the RyR channel into a subconductance open state upon binding, and the ryanodine-modified RyR channel is thought to be insensitive to modulation by other ligands such as Ca^{2+}, Mg^{2+}, and ruthenium red (5,11,12,22).

The view that ryanodine ‘locks’ the RyR channel in an open substate has recently been contested by Tanna et al. (25) who observed that the Po of the channel before ryanodine modification influenced the Po of the ryanodine-modified state. Low Po channels displayed more closing events than high Po channels after modified by ryanodine. This observation suggests that ryanodine does not simply ‘lock’ the channel into an open state. This observation also implies that activation of the RyR channel by ryanodine would be finite and regulatable. To test these possibilities, we have examined the gating properties and the Ca^{2+} dependence of the ryanodine-modified wild type cardiac ryanodine receptor (RyR2) and a
mutant RyR2 that exhibits a markedly reduced sensitivity to activation by Ca\textsuperscript{2+}. Our results demonstrate that ryanodine-modified channels are sensitive to modulation, and that ryanodine increases dramatically the sensitivity of the RyR2 channel to activation by Ca\textsuperscript{2+}. 
EXPERIMENTAL PROCEDURES

Materials  Ryanodine was obtained from Calbiochem. [3H]ryanodine was from NEN Life Science Products. Brain phosphatidylserine was from Avanti Polar Lipid. Synthetic 1,2-Dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were from Northern Lipids.

Preparation of cell lysates from transfected HEK293 cells
HEK293 cells grown for 24-26 hr after transfection using Ca2+ phosphate precipitation were washed three times with PBS (137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl) plus 2.5 mM EDTA and were harvested in the same solution by centrifugation. Cells were solubilized in lysis buffer containing 25 mM Tris, 50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.6 % egg phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mix (1 mM benzamidine, 2 mg/ml leupeptin, 2 µg/ml pepstatin A, 2 µg/ml aprotinin, and 0.5 mM PMSF) on ice for 1 hr. Cell lysate was obtained after removing the unsolubilized materials by centrifugation in microcentrifuge at 4 °C for 30 min.

Single channel recordings in planar lipid bilayers
Recombinant wt and E3987A mutant RyR2 proteins used for single channel recordings were purified from cell lysates by sucrose density gradient centrifugation as described previously (26). Brain phosphatidylserine and synthetic 1,2-Dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), dissolved in chloroform, were combined, in a 1:5 ratio (w/w), dried under nitrogen gas and suspended in 30 µl of n-decane at a concentration of 15 mg lipid /ml. Bilayers were formed across a 250-µm hole in a Delrin partition separating two chambers. The trans chamber (600 µl) was
connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments Inc.). The *cis* chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM Hepes, pH 7.4, was used for all recordings. A 2-4 µl aliquot of the sucrose density gradient-purified recombinant wt or mutant RyR2 proteins was added to the *cis* chamber. Unless indicated otherwise, spontaneous channel activity was always tested for sensitivity to EGTA and Ca²⁺, thereby providing information about the Ca²⁺ sensitivity, orientation in the bilayer and stability of the incorporated channel. All subsequent additions were made to that chamber in which the addition of EGTA inhibited the activity of the incorporated channel. This chamber presumably corresponds to the cytoplasmic side of the Ca²⁺ release channel. Recordings were filtered at 2,500 Hz. The data were analyzed using pClamp 6.0.3 software (Axon Instruments Inc.). Free Ca²⁺ concentrations were calculated using the computer program of Fabiato and Fabiato (27).

**Ca²⁺ release measurements in HEK293 cells**

Free cytosolic Ca²⁺ concentration in transfected HEK293 cells was measured using the fluorescence Ca²⁺ indicator dye fluo-3, AM as described previously (26) with some modifications. Transfected cells were collected and loaded with 10 µM fluo-3, AM in KRH buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 6 mM glucose, 1.2 mM MgCl₂, 2 mM CaCl₂, and 25 mM Hepes, pH 7.4) plus 0.1 mg/ml BSA and 250 µM sulfinpyrazone (an organic-anion transport inhibitor used for inhibiting the leakage of the fluo-3 dye) (28) with or without 500 µM ryanodine at room temperature for 2 hr.. After 2 hr loading, cells were washed with KRH buffer three times and resuspended in 150 µl KRH buffer plus 0.1 mg/ml BSA and 250 µM...
sulfinpyrazone. The fluo-3 loaded cells were added to 2 ml KRH buffer in a cuvette. Fluorescence intensity of fluo-3 at 530 nm was measured in an SLM-Aminco series 2 luminescence spectrometer with 480 nm excitation at 25 °C.

**[^3H]Ryanodine Binding** Equilibrium[^3H]ryanodine binding to cell lysates was carried out as described previously (26). Briefly, binding mixture (300 µl) containing 30 µl of cell lysate, 500 mM KCl, 25 mM Tris, 50 mM Hepes, pH 7.4, 1 mM CaCl₂, 2.5 mM caffeine, 15 nM[^3H]ryanodine, and the protease inhibitor mix was incubated at 37 °C for 2 hr. To optimize[^3H]ryanodine binding to the Ca²⁺ sensing mutant E3987A, 1 mM CaCl₂ and 2.5 mM caffeine were included in the binding mix (26). The binding mix after 2 hr incubation was diluted into 5 ml dissociation buffer containing 25 mM Tris, pH 7.5, and 250 mM KCl, with or without 200 µM unlabelled ryanodine, and was further incubated at 37 °C for 0-240 min. The amount of[^3H]ryanodine that remained bound was determined by membrane filtration assay using Whatman GF/B filters presoaked with 1% polyethylenimine. The filters were washed and the radioactivities associated with the filters were determined by liquid scintillation counting. Nonspecific binding was determined by measuring[^3H]ryanodine binding in the presence of 30 µM unlabelled ryanodine. All binding assays were done in duplicate.
RESULTS

Ryanodine-modified single RyR2 channels were sensitive to modulation. Early single channel studies showed that ryanodine increased the open probability (Po) of the RyR channel to near unity, and that at micromolar activating Ca\(^{2+}\) concentrations, the ryanodine-modified channels were inhibited only partially by Mg\(^{2+}\) and ruthenium red (22). These observations have led to the notion that ryanodine-modified RyR channels are insensitive to modulation (11). An alternative explanation for the partial inhibition is that at micromolar Ca\(^{2+}\) concentrations, ryanodine activation may be too strong to be completely suppressed by Mg\(^{2+}\) and ruthenium red. To test this possibility, we examined the effect of Mg\(^{2+}\) on ryanodine-modified single RyR2 channels at nanomolar Ca\(^{2+}\) concentrations. Fig. 1 shows that a single mouse wt RyR2 channel displayed brief openings and minimal Po at submicromolar Ca\(^{2+}\) concentrations. Addition of ryanodine abruptly shifted the channel into a state with long-lived openings and a marked increase in Po, and a reduced single channel conductance (Figs. 1a,b). The free Ca\(^{2+}\) concentration was then reduced to ~12 nM by addition of 0.43 mM EGTA, which resulted in only a small reduction in Po (Fig. 1c). Subsequent addition of 2 mM MgCl\(_2\), however, decreased the Po of the ryanodine-modified channel markedly (Fig. 1d). The Mg\(^{2+}\) inhibited, ryanodine-modified RyR2 channel could be reactivated by addition of caffeine and by addition of ATP (n=4) (Figs. 1e, f). Thus, in the presence of low concentrations of activating Ca\(^{2+}\), the ryanodine-modified RyR2 channels, like the ryanodine-unmodified
channels, are sensitive to modulation by various channel regulators.

**Ryanodine increased the sensitivity of single RyR2 channels to activation by Ca\(^{2+}\)**

To investigate the mechanism of ryanodine activation, we examined the Ca\(^{2+}\) dependence of ryanodine-modified single wt RyR2 channels. A single RyR2 channel was modified by ryanodine in the presence of ~300 nM Ca\(^{2+}\) and 2 mM Mg\(^{2+}\) (Fig. 2Aa). The free Ca\(^{2+}\) concentration was subsequently reduced to various levels by addition of EGTA. As seen in Fig. 2A, the ryanodine-modified single RyR2 channel was fully activated at ~300 nM Ca\(^{2+}\) and was inhibited at ~50 nM Ca\(^{2+}\), displaying a sigmoidal Ca\(^{2+}\) response (Fig. 2C). Analysis of the Ca\(^{2+}\) dependence using the Hill equation yielded an EC\(_{50}\) of 100 nM and a Hill coefficient of 3 in the presence of 2 mM Mg\(^{2+}\) (Fig. 2C). On the other hand, the ryanodine-unmodified channel in the presence of 2 mM Mg\(^{2+}\) was activated by Ca\(^{2+}\) at micromolar concentrations and was inactivated at ~10 mM Ca\(^{2+}\) (Fig. 2B). The Ca\(^{2+}\) response of the ryanodine-unmodified channel in the presence of Mg\(^{2+}\) could be described by an EC\(_{50}\) of 100 µM and a Hill coefficient of 2 for Ca\(^{2+}\) activation, and an IC\(_{50}\) of 6 mM and a Hill coefficient of 2 for Ca\(^{2+}\) inactivation (Fig. 2C). These data demonstrate that ryanodine is able to enhance the sensitivity of single RyR2 channels to Ca\(^{2+}\) activation by ~1,000 fold (Fig. 2C).
In the absence of Mg\(^{2+}\), the EC\(_{50}\) for Ca\(^{2+}\) activation of single wt RyR2 channels is \(\sim 0.3\) µM (26). Based on the above estimation of the extent of ryanodine activation, single wt RyR2 channels upon modification by ryanodine would be expected to be active at subnanomolar Ca\(^{2+}\) concentrations in the absence of Mg\(^{2+}\). As shown in Fig. 3, the ryanodine-modified single wt RyR2 channels in the absence of Mg\(^{2+}\) were extremely sensitive to Ca\(^{2+}\) activation. Significant channel activity could still be detected even when the Ca\(^{2+}\) concentration was reduced to as low as \(\sim 0.3\) nM (Figs. 3A,C). Some channels remained fully active at a Ca\(^{2+}\) concentration less than 1 nM (Figs. 3B,C). These residual activities, however, could be abolished by a subsequent addition of 2 mM Mg\(^{2+}\) (Fig. 2). Thus, together, these observations indicate that ryanodine dramatically increases the Po of the RyR channel by sensitizing the channel to Ca\(^{2+}\) activation.

**Ryanodine restored the activity of the E3987A Ca\(^{2+}\) sensing mutant** To demonstrate further the sensitizing effect of ryanodine on Ca\(^{2+}\) activation, we utilized a Ca\(^{2+}\) sensing deficient mutant of RyR2. We have shown previously that a single point mutation E3987A reduced the sensitivity to Ca\(^{2+}\) activation of single RyR2 channels by \(\sim 1,000\) fold, as measured by single channel recordings in planar lipid bilayers (26). We reasoned that if ryanodine is able to enhance the sensitivity of the wt RyR2 channel to Ca\(^{2+}\) activation by \(\sim 1,000\) fold, ryanodine would be able to restore Ca\(^{2+}\) activation of the E3987A mutant RyR2 channel to a level
similar to that of the wt. To this end, we examined the Ca\textsuperscript{2+} dependence of single E3987A mutant RyR2 channels after being modified by ryanodine. As shown in Fig. 4, upon modification by ryanodine, a single E3987A mutant RyR2 channel was converted to a state with a reduced single channel conductance and a markedly increased Po, as were observed with the wt channel (Fig. 1) (26). It is important to note that the ryanodine-modified single E3987A mutant channels never open to the ryanodine-unmodified full conductance level. In addition, different from the ryanodine-unmodified mutant channels, the ryanodine-modified single E3987A mutant channel was highly sensitive to Ca\textsuperscript{2+} activation. The Ca\textsuperscript{2+} dependence of some ryanodine-modified single E3987A mutant channels could be characterized by an EC\textsubscript{50} of 0.42 \(\mu\)M and a Hill coefficient of 3.1. These values are very similar to those of single wt RyR2 channels in the absence of ryanodine (EC\textsubscript{50} = 0.26 \(\mu\)M, Hill coefficient = 3.1) (26).

Hence, ryanodine is able to restore Ca\textsuperscript{2+} activation of the E3987A mutant RyR2 channels. Furthermore, like that observed with the ryanodine-modified wt RyR2 channels, some ryanodine-modified E3987A mutant channels remained highly active even at ~100 nM Ca\textsuperscript{2+} (Fig. 4e). These residual activities, however, could be abolished by subsequent addition of Mg\textsuperscript{2+} (see below). These observations indicate that upon ryanodine modification the sensitivities to Ca\textsuperscript{2+} activation of both the wt and E3987A mutant RyR2 channels were enhanced dramatically.

**Ryanodine-restored E3987A Ca\textsuperscript{2+} sensing mutant channels were sensitive to modulation by**
Mg$^{2+}$ and caffeine

It is apparent from Fig. 4 that the ryanodine-restored E3987A mutant channels behaved like the wt channel with respect to Ca$^{2+}$ dependent activation. To investigate whether ryanodine-restored single E3987A mutant channels also respond to channel modulators in a similar fashion as does the wt, we determined the effect of Mg$^{2+}$ on the Ca$^{2+}$ dependence of the restored mutant channels. As shown in Fig. 5, in the presence of 2 mM Mg$^{2+}$, ryanodine-restored single E3987A mutant channels were fully activated at ~400 µM Ca$^{2+}$ and were inhibited at micromolar levels (Figs. 5a-d). It should be noted that the ryanodine-unmodified single E3987A mutant channels exhibited little activity in the presence of 2 mM Mg$^{2+}$ (data not shown). Analysis of the Ca$^{2+}$ dependence reveals that the EC$_{50}$ and the Hill coefficient values (115 µM and 2.1) of the ryanodine-restored single E3987A mutant channels are very similar to those of the ryanodine-unmodified wt channels (100 µM and 2.0) in the presence of 2 mM Mg$^{2+}$ (Figs. 2 and 5). Thus, the ryanodine-restored single E3987A mutant channels exhibit Ca$^{2+}$ and Mg$^{2+}$ responses similar to those of the ryanodine-unmodified wt channels.

In other words, ryanodine rescues the Ca$^{2+}$ and Mg$^{2+}$ responses of the E3987A mutant RyR2 channel. Comparison of the EC$_{50}$ values of the ryanodine-modified wt and ryanodine-restored E3987A mutant channels in the presence of 2 mM Mg$^{2+}$ (Figs. 2 and 5) also reveals that the relative sensitivities of single E3987A mutant and wt RyR2 channels differ by ~1,000 fold (Fig. 5e). This estimated difference is in agreement with that estimated previously by
single channel recordings in the absence of ryanodine but in the presence of ATP and caffeine.

The ryanodine-restored E3987A mutant channels were also sensitive to caffeine. The Ca\(^{2+}\) dependence of the Mg\(^{2+}\) inhibited, ryanodine-restored E3987A mutant channels was shifted by 2 mM caffeine to the left by \(\sim 100\) fold, from 115 \(\mu\)M to \(\sim 1\) \(\mu\)M (Fig. 6A). Note that the ryanodine unmodified E3987A mutant channels in the presence of 2 mM caffeine and 2 mM Mg\(^{2+}\) showed minimal Ca\(^{2+}\) activated activities (Fig. 6B). Hence, ryanodine is able to restore the ligand response of single E3987A mutant channels.

**Ryanodine restored caffeine response of HEK293 cells expressing the E3987A Ca\(^{2+}\) sensing mutant**

It is clear that ryanodine is able to restore the function and regulation of single E3987A mutant channels in lipid bilayers. To examine whether ryanodine is able to restore function of the E3987A mutant in the cell context, we pretreated HEK293 cells expressing the wt and E3987A mutant RyR2 channels with or without 500 \(\mu\)M ryanodine and determined caffeine-induced Ca\(^{2+}\) release in these cells. Pretreatment of wt RyR2 expressing HEK293 cells with ryanodine abolished caffeine-induced Ca\(^{2+}\) release (Figs. 7a,b), whereas, pretreatment with ryanodine of E3987A mutant expressing HEK293 cells converted the mutant channel from caffeine-insensitive to caffeine sensitive (Figs. 7c,d). We have examined also the effect of ryanodine pretreatment on the caffeine response of HEK293 cells expressing the wt RyR3 and the corresponding Ca\(^{2+}\) sensing mutant E3885A in RyR3. Similarly, ryanodine pretreatment restored the caffeine-response of the E3885A mutant
expressing cells (Figs. 7g,h), but abolished that of the wt RyR3 expressing cells (Figs. e,f).

Therefore, ryanodine is able to restore the activities of the Ca\(^{2+}\) sensing mutants of RyR2 and of RyR3 in the context of HEK293 cells.

**E3987A mutant RyR2 channels were sensitive to blockade by high concentrations of ryanodine**

High concentrations of ryanodine are known to block the RyR channel completely by binding to the low affinity binding site. However, 500 \(\mu\)M ryanodine did not appear to block completely the Ca\(^{2+}\) sensing mutants of RyR2 or RyR3 in HEK293 cells (Fig. 7). These observations raise a question of whether the E3987A mutant RyR2 channel is sensitive to blockade by high concentrations of ryanodine. To address this question, we investigated the effect of high concentrations of ryanodine on the activity of single E3987A mutant channels incorporated into lipid bilayers. Fig. 8 shows that a single E3987A mutant channel activated by 400 \(\mu\)M Ca\(^{2+}\) and 2 mM caffeine was modified by 500 \(\mu\)M ryanodine into a reduced subconductance state with increased Po. Shortly after modification (within 10-120 seconds), the ryanodine-modified E3987A mutant channel was blocked completely and irreversibly (n=6). These data indicate that single E3987A mutant channels are sensitive to block or occlusion by ryanodine at high concentrations.

It has been shown also that binding of ryanodine to the low affinity binding site can slow down the dissociation of bound \(^{3}\text{H}\)ryanodine from the high affinity binding site (19-21). We have demonstrated previously that the E3987A mutant RyR2 protein is capable of binding \(^{3}\text{H}\)ryanodine with binding affinity identical to that of the wt RyR2 (26). To
demonstrate further that the E3987A mutant channels also contain the low affinity ryanodine binding site, we examined the effect of excess unlabelled ryanodine on the dissociation of bound $[^3H]ryanodine. Fig 9 shows that in the absence of unlabeled ryanodine, $[^3H]ryanodine bound to wt and E3987A mutant RyR2 proteins dissociated with an initial dissociation rate constant ($K_{off}$) of $0.011 \pm 0.0011 \text{ min}^{-1}$ (n=4) and $0.016 \pm 0.0012 \text{ min}^{-1}$ (n=3), respectively. Addition of 200 $\mu$M unlabelled ryanodine slowed down the dissociation of bound $[^3H]ryanodine from both the wt and E3987A mutant RyR2 channels substantially. The $K_{off}$ values for the wt and E3987A mutant RyR2 channels in the presence of 200 $\mu$M ryanodine were decreased to $0.0012 \pm 0.0001 \text{ min}^{-1}$ (n=3) and $0.0024 \pm 0.0003 \text{ min}^{-1}$ (n=3), respectively. Taken together, these data indicate that the E3987A mutant RyR2 channel, like the wt, possesses both the high and low affinity ryanodine binding sites.
DISCUSSION

It is generally believed that upon binding ryanodine ‘locks’ the RyR channel into an open state, and that the ryanodine-modified RyR is insensitive to modulation (5,11). These views, however, have not been examined in detail and have recently been questioned (25). In the present study, we demonstrate that ryanodine is able to increase the sensitivity of single wt RyR2 channels to Ca^{2+} activation by ~1,000 fold. In addition, we show that ryanodine is able to restore Ca^{2+} response of the Ca^{2+} sensing deficient mutant E3987A, which has been shown to exhibit ~1,000 fold reduction in Ca^{2+} sensitivity. Furthermore, both the ryanodine-modified wt and ryanodine-modified E3987A mutant RyR2 channels are sensitive to modulation by channel modulators such as Mg^{2+} and caffeine. These results provide direct evidence that ryanodine does not ‘lock’ the RyR channel into an open state, rather it enhances dramatically the channel activity by sensitizing the channel to Ca^{2+} activation.

Our results also reveal that Ca^{2+} activation and ryanodine binding are functionally coupled. While Ca^{2+} activation has been shown to influence ryanodine binding to the RyR channel (29,30), our present study shows that ryanodine binding to the channel can in turn influence Ca^{2+} activation. The molecular basis underlying this functional coupling, however, has yet to be understood. Since ryanodine binds to the open state of the channel, it is possible that ryanodine could enhance Ca^{2+} activation by stabilizing the open state or destabilizing the closed state of the channel. Alternatively, ryanodine binding may cause conformational changes that result in an increase in the affinity of the channel for activating Ca^{2+}, thereby
enhancing the sensitivity of the channel to Ca\textsuperscript{2+} activation. In this regard, it is of interest to note that the ryanodine-modified wt and E3987A mutant channels were relatively insensitive to addition of EGTA, but sensitive to subsequent addition of Mg\textsuperscript{2+}. It appeared that the activating Ca\textsuperscript{2+} ions dissociated slowly from the activation site in the ryanodine-modified channels and were unable to be chelated completely by EGTA, but could be readily replaced by Mg\textsuperscript{2+}. One possible explanation for these observations is that the activating Ca\textsuperscript{2+} ions may have been partially occluded in the Ca\textsuperscript{2+} activation site in the ryanodine-modified channels.

Information on the locations of the ryanodine binding and Ca\textsuperscript{2+} activation sites may provide some insights into the coupling mechanism between ryanodine binding and Ca\textsuperscript{2+} activation. Both the high and low affinity ryanodine binding sites have been localized to a 75 kDa COOH terminal fragment, starting at arginine 4475, of RyR (31,32). Recently, we have shown that mutations in the proposed pore-forming segment (4820-4829) in RyR2 impaired or abolished [\textsuperscript{3}H]ryanodine binding, but retained caffeine sensitive channel activity (33), suggesting that the pore-forming segment is the major determinant of ryanodine binding. We have shown also that mutation E3987A dramatically reduces the Ca\textsuperscript{2+} sensitivity to activation of RyR2, but does not affect ryanodine binding to either the high or low affinity site (26) (Fig. 8). These observations indicate that glutamate 3987 is not involved in ryanodine binding, and that the sites for ryanodine binding and Ca\textsuperscript{2+} activation are distinct. Hence, ryanodine most likely affects Ca\textsuperscript{2+} activation through an allosteric mechanism. However, such a mechanism
does not exclude the possibility that the ryanodine binding and Ca$^{2+}$ activation sites, although separate, are located in a close proximity in the three dimensional structure of RyR. It would be of great interest to map the ryanodine binding and Ca$^{2+}$ activation domains onto the three dimensional (3D) structure of RyR. Recent localization of the amino terminus of recombinant RyR3 onto its 3D structure may make such domain mapping possible (34).

Our observation that ryanodine is able to restore the Ca$^{2+}$ and other ligand responses of the RyR2 Ca$^{2+}$ sensing mutant, E3987A, is in line with the results of a recent study of the corresponding mutant, E4032A, in RyR1 (35). It was shown that the corresponding mutation E4032A severely diminished the responsiveness of RyR1 to stimulation by depolarization, caffeine and Ca$^{2+}$. Treatment with ryanodine restored the responsiveness of the E4032A mutant RyR1 to these stimuli. Although the observations as a whole are consistent, some potential inconsistencies exist between our study and that by Fessenden et al. One inconsistency is concerned with the single channel conductance of the ryanodine-modified mutant channels. It was stated that some ryanodine-pretreated single E4032A mutant RyR1 channels exhibited frequent gating transitions to the fully open state having single channel conductance similar to that of the ryanodine-untreated wt RyR1 channels (35). In contrast, in our study we have never observed gating transitions to the fully open state of the wt in the ryanodine-modified E3987A mutant RyR2 channels. In all cases, upon modification by ryanodine, single channel conductances of both the wt and E3987A mutant RyR2 channels were reduced and the resulting modified channels gate between the closed and subconductance states. The reason for this potential discrepancy is not clear. It is possible that
the corresponding mutation may have differential effects on RyR1 and RyR2 isoforms.

Another potential discrepancy lies in the sensitivities of the E4032A mutant RyR1 and the E3987A mutant RyR2 to blockade by high concentrations of ryanodine. The observation that treatment of E4032A-expressing cells with 500 µM ryanodine did not affect their response to stimuli led Fessenden et al. to conclude that the E4032A mutant RyR1 channel is insensitive to blockade by high concentrations of ryanodine. We also have observed that treatment of the E3987A mutant RyR2-expressing cells with 500 µM ryanodine restored the caffeine response (Fig. 7). However, single channel and [$^3$H]ryanodine binding studies clearly indicate that the E3987A mutant RyR2 channel is sensitive to irreversible block by high concentrations of ryanodine, and that the E3987A mutant channel possesses both the high and low affinity ryanodine binding sites and responds to ryanodine in a manner identical to that of the wt (Figs. 8, 9). Hence lack of complete blockade of RyR channel activities by high concentrations of ryanodine in intact cells may not necessarily indicate that the channel is insensitive to ryanodine block. It is not clear whether single E4032A mutant RyR1 channels in lipid bilayers could be modified by ryanodine and be blocked by high concentrations of ryanodine.

It is of interest to know that treatment of wt RyR1 expressing cells with 40 mM caffeine plus 500 µM ryanodine resulted in a long-lasting Ca$^{2+}$ transient (35). If ryanodine at 500 µM were able to block completely the wt RyR1 channel, one would expect a short-lived Ca$^{2+}$ transient. This observation thus suggests that under these conditions, 500 µM ryanodine was unable to block completely the wt RyR1 channel in intact cells, although it has been
shown from single channel studies in lipid bilayers that the wt RyR1 channel can be blocked completely by high concentrations of ryanodine (11). The reasons for the apparent lack of complete inhibition of the E4032A mutant RyR1 or E3987A mutant RyR2 channels in intact cells by high concentrations of ryanodine are not clear. The efficacy of blockade by high concentrations of ryanodine in intact cells might be different from that in lipid bilayers.

Although ryanodine sensitizes both the wt and E3987A mutant RyR2 channels to Ca\textsuperscript{2+} activation, the outcomes of ryanodine treatment on wt and E3987A mutant RyR2-expressing cells could be dramatically different. In the case of E3987A mutant RyR2, ryanodine is able to shift the Ca\textsuperscript{2+} sensitivity of the mutant channel to a level comparable to that of the wt, and thus rescues the ligand response of the mutant-expressing cells. On the other hand, the ryanodine-modified wt RyR2 channel has extremely high sensitivity to Ca\textsuperscript{2+} activation. At the resting Ca\textsuperscript{2+} concentrations, the ryanodine-modified wt RyR2 channel would be fully activated and thus become unresponsive to further stimulation by caffeine. Hence, the dramatically different outcomes observed with the wt and E3987A mutant RyR2-expressing cells with or without ryanodine treatment are most likely to be the consequence of their marked differences in Ca\textsuperscript{2+} sensitivity to activation, rather than the result of differential actions of ryanodine on the wt and E3987A mutant RyR2 channels.

In summary, we have demonstrated that ryanodine sensitizes the RyR channel to Ca\textsuperscript{2+} activation and that ryanodine-modified channel is regulatable. Further localization of the Ca\textsuperscript{2+} activation and ryanodine binding sties in the linear sequence and on the three dimensional
structure of RyR should lead to a better understanding of the functional coupling between Ca$^{2+}$ activation and ryanodine binding. Since ryanodine does not irreversibly activate the RyR, as previously thought, ryanodine or ryanodine analogues could be used as specific activators of the RyR channel. In particular, ryanodine derivatives that have fast unbinding rate and are less potent in Ca$^{2+}$ sensitization would be useful therapeutic agents for manipulating Ca$^{2+}$ release.

**Acknowledgments** We would like to thank Dr. Wayne R. Giles and the CIHR Group on Ion Channels and Transporters for continuous support, and Dr. Paul M. Schnetkamp for the use of his luminescence spectrometer. This work was supported by research grants from the Canadian Institutes of Health Research and from the Heart and Stroke Foundation of Alberta to S.R.W.C.
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LEGENDS

Fig.1. Ligand gating behavior of the ryanodine-modified single wt RyR2 channels

Single channel activities of the wt RyR2 were recorded in a symmetrical recording solution containing 250 mM KCl, 25 mM Hapes, pH 7.4. The trans chamber was connected to the input of the headstage amplifier and the cis chamber was held at virtual ground. Single channel activities shown in panel a were inhibited by addition of 0.1 mM EGTA to the trans chamber and were reactivated by addition of 67 μM CaCl2 to the same chamber, indicating that the cytoplasmic side of the incorporated channel was facing the trans chamber. Panel b shows modification of the same channel by 17 μM ryanodine in the presence of 128 nM cytoplasmic free Ca2+. Subsequent additions of EGTA (c), Mg2+ (d), caffeine (e) and ATP (f) were made to the cytoplasmic side of the same channel (the trans chamber). The open probability (Po), arithmetic mean open time (To) and the arithmetic mean closed time (Tc) are indicated on the top of each panel. A short line to the right of each current trace indicates the base line. The holding potential was -20 mV. Openings are downward.

Fig.2. Ca2+ responses of ryanodine-modified and ryanodine-unmodified single wt RyR2 channels in the presence of Mg2+

Single channel activities were recorded as described in the legend to Fig. 1. The Ca2+ responses of ryanodine-modified (A) and ryanodine-unmodified (B) single wt RyR2 channel in the presence of 2 mM Mg2+ are shown. The single channel shown in A was inhibited by addition of EGTA to the trans chamber, indicating that the cytoplasmic side of the
incorporated channel was facing the \textit{trans} chamber. After reactivating the channel by raising
the Ca$^{2+}$ concentration to 282 nM, 17 $\mu$M ryanodine was added to the \textit{trans} chamber
(cytoplasmic side of the channel) to obtain a ryanodine-modified channel (Aa). Panels Aa-
Ad show current traces of the ryanodine-modified single wt RyR2 channel at various
cytoplasmic free Ca$^{2+}$ concentrations. Openings are downward. The single channel shown in
B was inhibited by addition of EGTA to the \textit{cis} chamber, indicating that the cytoplasmic side
of the incorporated channel was facing the \textit{cis} chamber. The cytoplasmic free Ca$^{2+}$
concentration was then increased to the indicated level by addition of concentrated CaCl$_2$
solution (panels Ba-Bf). Openings are upward. The holding potential was $-20$ mV in A and
$+20$ mV in B. In order to record single channel currents in the same direction, a positive or
negative holding potential was applied depending on the orientation of the incorporated
channel in the bilayer. The direction of all single channel currents shown is from the luminal
to the cytoplasmic side of the channel. The relationships between open probability (Po) and
Ca$^{2+}$ concentrations (pCa) of the ryanodine-modified (solid circles) and the ryanodine-
unmodified (open circles) single wt RyR2 channels in the presence of 2 mM Mg$^{2+}$ are shown
in Panel C. Data points shown were individual measurements obtained from 4 ryanodine-
modified and 8 ryanodine-unmodified single wt RyR2 channels. Curves shown represent fits
using the Hill equation. The open probability (Po), arithmetic mean open time (To) and the
arithmetic mean closed time (Tc) are indicated on the top of each panel. A short line to the
right of each current trace indicates the base line.
Fig. 3. Ca\textsuperscript{2+} responses of ryanodine-modified single wt RyR2 channels in the absence of Mg\textsuperscript{2+} Single channel activities were recorded as described in the legend to Figs. 1 and 2. Single channels shown in A and B were incorporated into the lipid bilayers with their cytoplasmic side facing the cis chamber, based on their sensitivity to EGTA and Ca\textsuperscript{2+}. All subsequent additions were made to the cis chamber. Single wt RyR2 channels were first converted to the modified substate by addition of 17 \( \mu \)M ryanodine. The cytoplasmic free Ca\textsuperscript{2+} concentrations were then reduced to the indicated level by addition of concentrated EGTA solution. In the absence of Mg\textsuperscript{2+}, the Ca\textsuperscript{2+} responses of the ryanodine-modified wt RyR2 channels varied. Panel A shows a ryanodine-modified wt RyR2 channel that was inhibited by lowering the Ca\textsuperscript{2+} concentration. Panel B shows a ryanodine-modified wt RyR2 channel that was only partially inhibited by reducing the Ca\textsuperscript{2+} concentration. Panel C shows the Po-pCa relationships of the ryanodine-modified single wt RyR2 channels that were inhibited by addition of EGTA (open circles, \( n=3 \)) and that were partially inhibited by addition of EGTA (open squares, \( n=2 \)). Solid lines represent fits using the Hill equation. The dash line indicates the Ca\textsuperscript{2+} response of the ryanodine-unmodified single wt RyR2 channels (26). The holding potential was +20 mV. Openings are upward.

Fig. 4. Ca\textsuperscript{2+} dependence of ryanodine-modified single E3987A mutant RyR2 channels A single E3987A mutant RyR2 channel was activated by 200 \( \mu \)M Ca\textsuperscript{2+} (a) and was subsequently modified by addition of 60 \( \mu \)M ryanodine (b). The Po of the E3987A mutant
channel at 200 μM Ca^{2+} is very low. To reduce the time required for ryanodine modification of the mutant channels, a relatively high concentration (60 μM) of ryanodine was employed. After ryanodine modification, the cytoplasmic free Ca^{2+} concentrations were reduced to various levels as indicated in each panel by addition of concentrated EGTA solution (b-e). The Po-pCa relationships of the ryanodine-modified single E3987A mutant RyR2 channels are shown in Panel f. In the absence of Mg^{2+}, the Ca^{2+} responses of the ryanodine-modified single E3987A mutant RyR2 channels also varied. Solid circles indicate ryanodine-modified E3987A mutant channels (n=4) that were inhibited by addition of EGTA, while solid squares indicate ryanodine-modified single mutant channels (n=4) that were less sensitive to EGTA. The dash line indicates the Ca^{2+} response of the ryanodine-unmodified single E3987A mutant channels (26). The single channel shown was incorporated into the bilayer with its cytoplasmic side facing the cis chamber, based on its sensitivity to EGTA and Ca^{2+}. The holding potential was +20 mV. Openings are upward.

**Fig.5. Effect of Mg^{2+} on the Ca^{2+} dependence of ryanodine-modified single E3987A mutant RyR2 channels**

A single E3987A mutant RyR2 channel was first activated by 400 μM Ca^{2+} and was subsequently modified by addition of 33 μM ryanodine, followed by the addition of 2 mM Mg^{2+} (a). The cytoplasmic free Ca^{2+} concentrations were then reduced to various levels as indicated in each panel by addition of concentrated EGTA solution (b-d). The Po-pCa relationships of the ryanodine-modified single E3987A mutant RyR2 channels...
in the presence of 2 mM Mg\(^{2+}\) is shown in Panel e. Data points indicated by solid circles were obtained from five single ryanodine-modified E3987A mutant channels and were fitted with the Hill equation. The dash line indicates the Ca\(^{2+}\) response of the ryanodine-modified E3987A mutant channels in the absence of Mg\(^{2+}\), taken from Fig. 4. The holding potential was +20 mV. Openings are upward.

**Fig. 6. Effect of caffeine on the Ca\(^{2+}\) dependence of ryanodine-modified single E3987A mutant RyR2 channels**

A single E3987A mutant RyR2 channel was first activated by 100 µM Ca\(^{2+}\) plus 2 mM caffeine and was subsequently modified by addition of 25 µM ryanodine, followed by the addition of 2 mM Mg\(^{2+}\) (Aa). The cytoplasmic free Ca\(^{2+}\) concentrations were then reduced to various levels as indicated by addition of concentrated EGTA solution (Ab-Ad). In panel B, a single E3987A mutant channel was first activated by 200 µM Ca\(^{2+}\) plus 2 mM caffeine and was then inhibited by 2 mM Mg\(^{2+}\). The cytoplasmic free Ca\(^{2+}\) concentrations were subsequently increased to various levels as indicated by addition of concentrated CaCl\(_2\) solution (Bb, Bc). The Po-pCa relationships of the ryanodine-modified (solid circles, n= 4) and ryanodine-unmodified (open circles, n= 6) single E3987A mutant RyR2 channels in the presence of 2 mM Mg\(^{2+}\) plus 2 mM caffeine are shown in Panel C. The holding potential was +20 mV in both A and B. Openings are upward. The dash line indicates the Ca\(^{2+}\) response of ryanodine-modified E3987A mutant channel in the presence of Mg\(^{2+}\), but in the absence of caffeine,
Fig. 7. Effect of caffeine on intracellular Ca\(^{2+}\) release in transfected HEK293 cells

HEK293 cells were transfected with wt RyR2 (a, b), E3987A mutant RyR2 (c,d), wt RyR3 (e,f), and E3885A mutant RyR3 (g,h) cDNAs. Transfected cells were pretreated with (b,d,f,h) or without (a,c,e,g) 500 µM ryanodine for 2 hr before assaying for caffeine-induced Ca\(^{2+}\) release. Fluorescence intensity was monitored continuously before and after addition of caffeine (2 mM) indicated by the letter c. Decreases in fluorescence immediately after addition of caffeine were due to fluorescence quenching by caffeine. Note that pretreatment of ryanodine abolished caffeine-induced Ca\(^{2+}\) release in wt RyR2 or wt RyR3 transfected HEK293 cells, whereas, ryanodine pretreatment restored caffeine-sensitive Ca\(^{2+}\) release in HEK293 cells transfected with the Ca\(^{2+}\) sensing mutant E3987A RyR2 or mutant E3885A RyR3. Traces shown are from a representative experiment that was repeated three times. Similar results were observed.

Fig. 8. Effect of high concentration of ryanodine on single E3987A mutant RyR2 channels

A single E3987A mutant RyR2 channel was activated by 400 µM Ca\(^{2+}\) plus 2 mM caffeine (a). Subsequent addition of 500 µM ryanodine modified the channel into a long-lived open state with reduced single channel conductance, which was followed by a complete and irreversible block (b). A total of six mutant channels were tested for their sensitivity to ryanodine blockade. Four channels were blocked by 500 µM ryanodine within 25 seconds, and two were...
blocked within 120 seconds. The single channel shown was incorporated into the bilayer with its cytoplasmic side facing the cis chamber, based on its sensitivity to EGTA and Ca\(^{2+}\). All additions were made to the cis chamber. The holding potential was +20 mV. Openings are upward.

**Fig. 9.** Effect of high concentration of ryanodine on the dissociation of \([^3H]ryanodine\) bound to the wt and E3987A mutant RyR2

[^3H]ryanodine binding to cell lysates prepared from HEK293 cells transfected with wt (A,C) and E3987A mutant (B,D) RyR2 cDNAs was carried out as described in the text. The amounts of \([^3H]ryanodine\) that remained bound to the wt RyR2 (A) or E3987A mutant RyR2 proteins (B) after incubating in the diluting buffer with (open squares) or without (open circles) 200 µM unlabelled ryanodine for 0-240 minutes were determined by membrane filtration. Panels C and D show plots of ln(Bt/Bo) versus time for wt (C) and E3987A mutant (D) RyR2 in the presence or absence of unlabelled ryanodine. B_t is the specific binding at time t, and B_0 is the specific binding at time 0. The k_{off} values were determined according to the equation k_{off} x t = ln(B_t/Bo). The k_{off} values for wt and mutant E3987A in the absence of unlabelled ryanodine were determined by using data points obtained at 0-120 min, therefore representing the initial rate of dissociation.
mRyR2 (wt)

**a** Control \((128 \text{ nM Ca}^{2+})\) - 20 mV

\[
\begin{align*}
\text{Po} &= 0.005 & \text{To} &= 1.01 \text{ ms} & \text{Tc} &= 171 \text{ ms} \\
\end{align*}
\]

**b** + 17 \(\mu\text{M ryanodine}\)

\[
\begin{align*}
\text{Po} &= 0.95 & \text{To} &= 33.8 \text{ ms} & \text{Tc} &= 1.45 \text{ ms} \\
\end{align*}
\]

**c** + 0.43 mM EGTA

\[
\begin{align*}
\text{Po} &= 0.88 & \text{To} &= 7.22 \text{ ms} & \text{Tc} &= 1.05 \text{ ms} \\
\end{align*}
\]

**d** + 2 mM MgCl\(_2\)

\[
\begin{align*}
\text{Po} &= 0.03 & \text{To} &= 1.38 \text{ ms} & \text{Tc} &= 37.6 \text{ ms} \\
\end{align*}
\]

**e** + 2 mM caffeine

\[
\begin{align*}
\text{Po} &= 0.10 & \text{To} &= 2.30 \text{ ms} & \text{Tc} &= 17.1 \text{ ms} \\
\end{align*}
\]

**f** + 2 mM ATP

\[
\begin{align*}
\text{Po} &= 0.72 & \text{To} &= 6.11 \text{ ms} & \text{Tc} &= 2.19 \text{ ms} \\
\end{align*}
\]
A  m RyR2 (wt) (+2 mM MgCl₂ + 17 μM ryanodine)
  a 282 nM CaCl₂
  
  Po = 0.91  To = 8.12 ms  Tc = 1.03 ms

  b 150 nM CaCl₂
  
  Po = 0.54  To = 4.73 ms  Tc = 4.34 ms

  c 84 nM CaCl₂
  
  Po = 0.06  To = 2.03 ms  Tc = 30.2 ms

  d 49 nM CaCl₂
  
  Po = 0.003  To = 0.54 ms  Tc = 84.4 ms

B  m RyR2 (wt) (+2 mM MgCl₂)
  a 2.71 μM CaCl₂
  
  Po = 0.006  To = 0.95 ms  Tc = 132 ms

  b 50 μM CaCl₂
  
  Po = 0.19  To = 1.18 ms  Tc = 4.58 ms

  c 100 μM CaCl₂
  
  Po = 0.41  To = 1.30 ms  Tc = 1.83 ms

  d 700 μM CaCl₂
  
  Po = 0.76  To = 1.76 ms  Tc = 0.70 ms

  e 2.2 mM CaCl₂
  
  Po = 0.71  To = 1.39 ms  Tc = 0.69 ms

  f 7.2 mM CaCl₂
  
  Po = 0.21  To = 0.56 ms  Tc = 1.49 ms

C  m RyR2 (wt) + 2 mM MgCl₂

  Po = 0.8
  To = 80 ms
  Tc = 20 pA

  + ryanodine

  - ryanodine

  pCa 8 7 6 5 4 3 2 1
A  mRyR2 (wt) (+17 μM ryanodine)

a  64 nM CaCl₂  
   +20 mV
   \( P_o = 0.95 \)  \( T_o = 29.3 \) ms  \( T_c = 1.71 \) ms

b  11 nM CaCl₂
   \( P_o = 0.52 \)  \( T_o = 2.32 \) ms  \( T_c = 1.8 \) ms

c  0.32 nM CaCl₂
   \( P_o = 0.29 \)  \( T_o = 0.71 \) ms  \( T_c = 1.45 \) ms

B  mRyR2 (wt) (+17 μM ryanodine)

a  2.7 nM CaCl₂
   +20 mV
   \( P_o = 0.99 \)  \( T_o = 281 \) ms  \( T_c = 1.00 \) ms

b  0.87 nM CaCl₂
   \( P_o = 0.93 \)  \( T_o = 257 \) ms  \( T_c = 3.2 \) ms

C  mRyR2(wt)

\[ P^o \]

+ryanodine

-ryanodine

\[ pCa \]

11 10 9 8 7 6 5
E3987A

a 200$\mu$M CaCl$_2$ +20 mV
  $Po= 0.001$  $To= 0.50$ ms  $Tc= 506$ ms

b 6.67 $\mu$M CaCl$_2$ + 60 $\mu$M ryanodine
  $Po= 0.95$  $To= 18.0$ ms  $Tc= 1.09$ ms

c 0.64 $\mu$M CaCl$_2$ + 60 $\mu$M ryanodine
  $Po= 0.60$  $To= 3.36$ ms  $Tc= 2.30$ ms

d 0.38 $\mu$M CaCl$_2$ + 60 $\mu$M ryanodine
  $Po= 0.18$  $To= 1.19$ ms  $Tc= 4.74$ ms

e 85 nM CaCl$_2$ + 60 $\mu$M ryanodine
  $Po= 0.014$  $To= 0.50$ ms  $Tc= 18.5$ ms

f E3987A

\[ \text{Po} \]
\[ \text{pCa} \]

\[ \frac{100 \text{ ms}}{20 \text{ pA}} \]

\[ +\text{ryanodine} \]
\[ -\text{ryanodine} \]
E3987A (+33 μM ryanodine + 2 mM MgCl₂)

a 400 μM CaCl₂ +20 mV
   Po = 0.92  To = 9.87 ms  Tc = 1.01 ms

b 200 μM CaCl₂
   Po = 0.66  To = 4.11 ms  Tc = 2.16 ms

c 100 μM CaCl₂
   Po = 0.31  To = 2.15 ms  Tc = 4.61 ms

d 6.7 μM CaCl₂
   Po = 0.03  To = 0.91 ms  Tc = 24.1 ms

e E3987A (+ryanodine) 20 pA

\[
\begin{align*}
\text{Po} & \quad 0.8 \\
\text{pCa} & \quad 5 \quad 6 \quad 7 \quad 8
\end{align*}
\]

\(-\text{MgCl}_2\)  \(+\text{MgCl}_2\)
A E3987A (+2 mM MgCl$_2$ + 2 mM caffeine +25 μM ryanodine)

a 100 μM CaCl$_2$ +20mV

Po = 0.99  To = 248 ms  Tc = 0.72 ms

b 0.52 μM CaCl$_2$

Po = 0.60  To = 4.85 ms  Tc = 3.60 ms

c 0.39 μM CaCl$_2$

100 ms

Po = 0.19  To = 2.14 ms  Tc = 9.11 ms

20 pA

d 0.23 μM CaCl$_2$

Po = 0.006  To = 0.83 ms  Tc = 105 ms

B E3987A (+2 mM MgCl$_2$ + 2 mM caffeine)

a 200 μM CaCl$_2$ +20 mV

Po = 0.005  To = 0.79 ms  Tc = 106 ms

b 950 μM CaCl$_2$

Po = 0.01  To = 0.72 ms  Tc = 42.3 ms

c 1.7 mM CaCl$_2$

Po = 0.03  To = 0.74 ms  Tc = 17.3 ms

C

![Graph showing Po vs. pCa with different conditions](image)

- caffeine
+ ryanodine
- caffeine
+ ryanodine

-ryanodine

Po

8 7 6 5 4 3 2 1 0 0

pCa
E3987A

a + 400 μM CaCl₂ + 2 mM caffeine

b + 500 μM ryanodine
Ryanodine sensitizes the Ca2+ release channel (ryanodine receptor) to Ca2+ activation
Haruko Masumiya, Pin Li, Lin Zhang and S.R. Wayne Chen

*J. Biol. Chem.* published online August 15, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M106557200](http://doi.org/10.1074/jbc.M106557200)

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