

Molecular Basis of Ca²⁺ Activation of the Mouse Cardiac Ca²⁺ Release Channel (Ryanodine Receptor)

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ABSTRACT Activation of the cardiac ryanodine receptor (RyR2) by Ca²⁺ is an essential step in excitation-contraction coupling in heart muscle. However, little is known about the molecular basis of activation of RyR2 by Ca²⁺. In this study, we investigated the role in Ca²⁺ sensing of the conserved glutamate 3987 located in the predicted transmembrane segment M2 of the mouse RyR2. Single point mutation of this conserved glutamate to alanine (E3987A) reduced markedly the sensitivity of the channel to activation by Ca²⁺, as measured by using single-channel recordings in planar lipid bilayers and by [³H]ryanodine binding assay. However, this mutation did not alter the affinity of [³H]ryanodine binding and the single-channel conductance. In addition, the E3987A mutant channel was activated by caffeine and ATP, was inhibited by Mg²⁺, and was modified by ryanodine in a fashion similar to that of the wild-type channel. Coexpression of the wild-type and mutant E3987A RyR2 proteins in HEK293 cells produced individual single channels with intermediate sensitivities to activating Ca²⁺. These results are consistent with the view that glutamate 3987 is a major determinant of Ca²⁺ sensitivity to activation of the mouse RyR2 channel, and that Ca²⁺ sensing by RyR2 involves the cooperative action between ryanodine receptor monomers. The results of this study also provide initial insights into the structural and functional properties of the mouse RyR2, which should be useful for studying RyR2 function and regulation in genetically modified mouse models.

KEY WORDS: excitation-contraction coupling • Ca²⁺-induced Ca²⁺ release • Ca²⁺ sensing • sarcoplasmic reticulum • planar lipid bilayers

INTRODUCTION

Excitation-contraction (E-C)* coupling in heart muscle is believed to occur via a mechanism known as Ca²⁺-induced Ca²⁺ release (CICR; Endo, 1977; Fabiato, 1985; Ebashi, 1991). Upon depolarization of the transverse tubular membrane, the voltage-dependent Ca²⁺ channel or the dihydropyridine receptor (DHPR) is activated, resulting in a small influx of Ca²⁺. This Ca²⁺ entry is thought to trigger the opening of the Ca²⁺ release channel (ryanodine receptor [RyR]), leading to a large Ca²⁺ release from the sarcoplasmic reticulum (SR) and subsequent muscle contraction (Coronado et al., 1994; Meissner, 1994; Ogawa, 1994; Sorrentino, 1995; Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). Thus, RyR acts like a Ca²⁺ amplifier, sensing and amplifying the Ca²⁺ signal from DHPR. To achieve efficient and stable Ca²⁺ amplification, the RyR Ca²⁺ amplifying system must possess a unique Ca²⁺-sensing element that is able to detect small local Ca²⁺ signals from DHPR and

to avoid being further activated by the much larger output signal from its own system, the SR Ca²⁺ release. The sensitivity or the threshold of RyR to Ca²⁺ activation, therefore, is an important parameter in determining the gain and stability of E-C coupling (Cheng et al., 1996; Cannell and Soeller, 1997; Stern et al., 1999). Alterations in the sensitivity of RyR to Ca²⁺ activation have been implicated in diseases including malignant hyperthermia and heart failure (Mickelson and Louis, 1996; Loke and MacLennan, 1998; Marx et al., 2000).

Despite the central role of Ca²⁺ activation of RyR in CICR and in cardiac E-C coupling, the molecular mechanism of Ca²⁺ sensing by RyR has been elusive. Three RyR isoforms (RyR1, RyR2, and RyR3) have been identified and cloned, and their responses to Ca²⁺ have been investigated (Coronado et al., 1994; Meissner, 1994; Ogawa, 1994; Sorrentino, 1995; Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). It has been shown that, in muscle cells, RyR3 exhibits much lower sensitivity to activation by Ca²⁺ than RyR1, and that RyR2 has the highest sensitivity to Ca²⁺ activation (Takeshima et al., 1995; Yamazawa et al., 1996). On the other hand, single-channel studies have demonstrated that, under the same conditions, detergent-solubilized and sucrose gradient-purified single recombinant RyR1 and RyR3 channels exhibit a similar threshold to Ca²⁺ activation (Chen et al., 1997a,b). Similar sensitivities to Ca²⁺ activation of the recombinant RyR1 and RyR2 expressed in HEK293

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*Abbreviations used in this paper: CICR, Ca²⁺-induced Ca²⁺ release; DHPR, dihydropyridine receptor; E-C coupling, excitation-contraction coupling; Po, open probability; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; wt, wild-type.

cells also have been shown by using [³H]ryanodine binding assay (Du et al., 1998). Thus, these observations suggest that although the sensitivity to Ca²⁺ activation of different RyR isoforms may be modulated differently in cells, the major determinant of their intrinsic Ca²⁺ sensitivities is most probably conserved.

Activation of RyR by Ca²⁺ is believed to result from binding of Ca²⁺ ions to the high affinity Ca²⁺ binding sites in the channel protein (Meissner et al., 1986, 1997). The locations of these high affinity Ca²⁺ activation sites have not been identified. Most mutations associated with malignant hyperthermia and central core disease have been found in the NH₂-terminal and central region of the skeletal muscle RyR (RyR1; Mickelson and Louis, 1996; Loke and MacLennan, 1998). Some of these mutations have been shown to alter the sensitivity to caffeine and Ca²⁺ (Mickelson and Louis, 1996; Tong et al., 1997; Loke and MacLennan, 1998). However, the major determinant for Ca²⁺ activation is most likely to be located within the COOH-terminal ~1,000 amino acid residues, rather than in the NH₂-terminal region of RyR, since a truncated RyR1 lacking ~4,000 residues from the NH₂ terminus retains Ca²⁺ activation (Bhat et al., 1997). Further deletion studies have also precluded residues 4,274–4,535 as the site for Ca²⁺ activation, as deletion of this region led to an increase in sensitivity of the channel to caffeine and Ca²⁺ (Du et al., 2000). Thus, the Ca²⁺ activation sites most probably lie within the NH₂-terminal quarter or the COOH-terminal half of the last ~1,000 residues of RyR.

Site-specific mutational studies have provided new insights into the molecular determinant of Ca²⁺ activation (Chen et al., 1998). We have recently demonstrated that a single substitution of alanine for glutamate 3885 (E3885A), located in the putative transmembrane segment M2 of RyR3, reduces the sensitivity to Ca²⁺ activation by ~10,000-fold. It should be noted that the corresponding M2 sequence (Zorzato et al., 1990) in RyR1 is located within the NH₂ terminus of the truncated RyR1 that maintains Ca²⁺ activation (Bhat et al., 1997). Coexpression of the wild-type and E3885A mutant RyR3 proteins resulted in synthesis of single channels with intermediate sensitivities to Ca²⁺ activation. These observations have led us to postulate that the glutamates at position 3885 of each RyR3 monomer contribute to the formation of the RyR3 Ca²⁺ sensor (Chen et al., 1998).

In line with the importance of this conserved glutamate in channel function and regulation, mutation of the corresponding glutamate 4032 to alanine (E4032A) in RyR1 abolished caffeine response and [³H]ryanodine binding (Du and MacLennan, 1998). However, it is not known whether the E4032A mutation alters the sensitivity of RyR1 to activating Ca²⁺ and/or directly affects high affinity ryanodine binding. The ap-

parently different impact of this mutation on RyR1 as compared with that on RyR3 raises a question of whether the role of this conserved glutamate is isoform-dependent. Therefore, it is important to determine whether the corresponding glutamate in another ryanodine receptor isoform, RyR2, plays any roles in Ca²⁺ activation. In the present investigation, we examined the role in Ca²⁺ sensing of the corresponding glutamate 3987 in the mouse RyR2. We cloned and expressed the wild-type and E3987A mutant RyR2 cDNA in HEK293 cells. Functional properties, in particular the Ca²⁺ response of both the wild-type and E3987A mutant RyR2 channels, were characterized by using single-channel recordings in planar lipid bilayers and by [³H]ryanodine binding assay. Our results show that mutation E3987A markedly reduces the sensitivity of the mouse RyR2 channel to activating Ca²⁺, supporting the view that this conserved glutamate is a key determinant of the RyR2 Ca²⁺ sensor. This study also reveals single-channel properties of the mouse cardiac ryanodine receptor.

MATERIALS AND METHODS

Materials

Anti-RyR mAb 34C was obtained from Affinity BioReagents Inc. Brain phosphatidylserine was obtained from Avanti Polar Lipid. Egg phosphatidylcholine was purchased from Sigma-Aldrich. Synthetic 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) were from Northern Lipids.

Cloning and Expression of the Mouse RyR2 cDNA and Construction of the Mutant Construct

Cloning of the mouse RyR2 cDNA and expression of RyR in HEK293 cells have been described previously (Chen et al., 1997b; Zhao et al., 1999; see Fig. 1). Point mutation of glutamate 3987 into alanine (E3987A) in mouse RyR2 was performed by the overlapping extension method (Ho et al., 1989) using PCR. A SalI and a MluI restriction site were first introduced into the full-length mouse RyR2 cDNA at position 11816 and 12340, respectively, without altering the amino acid sequence. This mouse RyR2 cDNA (SalI⁺, MluI⁺) was then used as a template for PCR. The “outer” two oligonucleotides used are as follows: forward, 5'-GAGCAGGGGCAGCGTAAC-3' and reverse, 5'-GAAGGTCTGCAGACGGTGCATTG-3'. The oligonucleotides for the E3987A mutation are as follows: forward, 5'-TGTCTATGTTAGCAGGTAATGTGGT-3' and reverse, 5'-ACCACATTACCTGCTAACATAGACA-3'. The sequence of the PCR product was confirmed by DNA sequencing. The SalI-MluI fragment was removed from the PCR product and was used to replace the corresponding wild-type (wt) fragment in the full-length RyR2 cDNA in pBluescript, which was subcloned subsequently into the expression plasmid pCDNA3.

Preparation of Cell Lysate from Transfected HEK293 Cells and Heavy Sarcoplasmic Reticulum from Canine Heart Muscle

HEK293 cells grown for 24–26 h after transfection using Ca²⁺ phosphate precipitation were washed three times with PBS (137

mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, and 2.7 mM KCl) plus 2.5 mM EDTA, and were harvested in the same solution by centrifugation. Cells from 15 tissue culture dishes (100 mm in diameter) were solubilized in 2.5 ml 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 benzamide, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 mM PMSF) on ice for 1 h. Cell lysate was obtained after removing the unsolubilized materials by centrifugation in microcentrifuge at 4°C for 30 min. Heavy SR was isolated from canine cardiac muscle according to the method described previously (Chamberlain et al., 1983). 2 mg of canine SR was solubilized in 2.5 ml lysis buffer plus a protease inhibitor mix, and unsolubilized materials were removed by centrifugation in microcentrifuge.

Sucrose Density Gradient Purification of Recombinant RyR2 and Canine RyR2 Proteins

2.5 ml of cell lysate or solubilized canine cardiac SR membranes (2 mg in 2.5 ml) was layered on top of a 10.5-ml (7.5–25%, wt/wt) linear sucrose gradient containing 25 mM Tris, 50 mM HEPES, pH 7.4, 0.3 M NaCl, 0.1 mM CaCl₂, 0.3 mM EGTA, 0.25 mM PMSF, 4 μg/ml leupeptin, 5 mM DTT, 0.3% CHAPS, and 0.16% synthetic phosphatidylcholine. The gradient was centrifuged at 29,000 rpm in Beckman SW-41 rotor at 4°C for 17 h. Fractions of 0.7 ml each were collected. Peak fractions containing RyR proteins, as determined by immunoblotting, were pooled, aliquoted, and stored at -80°C.

Single-channel Recordings

Recombinant wt and E3987A mutant RyR2 and canine RyR2 proteins solubilized and purified by sucrose density gradient centrifugation were used for single-channel recordings as described previously (Chen et al., 1997b; Zhao et al., 1999) with some modifications. Brain phosphatidylserine and synthetic DOPE, dissolved in chloroform, were combined, in a 1:5 ratio (wt/wt), dried under nitrogen gas, and suspended in 30 μl of n-decane at a concentration of 15 mg of 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 recordings. A 2–4-μl aliquot of the sucrose density gradient-purified recombinant wild-type or mutant mouse RyR2 proteins or native canine cardiac ryanodine receptor was added to the cis chamber. Unless otherwise indicated, spontaneous channel activity was always tested for sensitivity to EGTA and/or 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 1,500 Hz using a low-pass Bessel filter (Frequency Devices), digitized at 20 kHz. 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 (1979).

[³H]Ryanodine Binding

Equilibrium [³H]ryanodine binding to cell lysate was performed as described previously (Du et al., 1998) with some modifica-

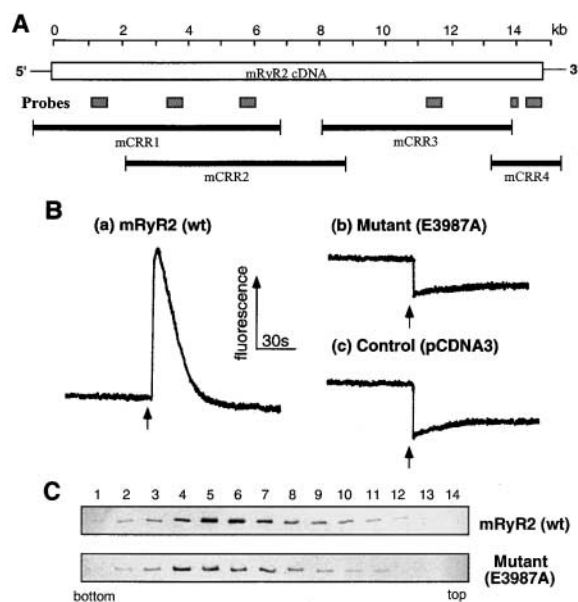


FIGURE 1. Cloning and functional expression of the mouse RyR2 cDNA. (A) The strategy and outline for the cloning of cDNA encoding the mouse RyR2. Four overlapping clones (mCRR1-mCRR4, solid boxes) covering the entire coding region of the mouse RyR2 cDNA (open box) were obtained by screening a mouse cardiac cDNA library using six short PCR fragments as probes. (B) Caffeine-induced Ca²⁺ release in HEK293 cells transfected with 6 μg wt RyR2 cDNA (mRyR2 (wt)) (B, panel a), 6 μg mutant (E3987A) cDNA (B, panel b), or 6 μg control (pCDNA3) DNA (B, panel c). Fluorescence intensity of fluo-3 loaded HEK293 cells was monitored continuously before and after addition of 2 mM caffeine (arrows). Decreases in fluorescence upon additions of caffeine are due to fluorescence quenching by caffeine. (C) Immunoblotting of sucrose density gradient fractions of wt or E3987A mutant RyR2 proteins. An aliquot of 10 μl each sucrose gradient fraction was used for immunoblotting. The wt and mutant RyR2 proteins were detected by using the anti-RyR mAb 34C. The top (right) and bottom (left) of the gradient were labeled.

tions. [³H]ryanodine binding was performed in a total volume of 300-μl binding solution containing 30 μl of cell lysate, 500 mM KCl, 25 mM Tris, 50 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.7 mM CaCl₂, 0.1–100 nM [³H]ryanodine, and the protease inhibitor mix at 37°C for 2 h. The binding mix was diluted with 5 ml ice-cold washing buffer containing 25 mM Tris, pH 8.0, and 250 mM KCl, and was immediately filtered through 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 20 μM unlabeled ryanodine. All binding assays were done in duplicate.

Ca²⁺ Release Measurements

Free cytosolic Ca²⁺ concentration in transfected HEK293 cells was measured using the fluorescence Ca²⁺ indicator dye fluo-3-AM as described previously (Chen et al., 1997b) with some modifications. Cells grown for 18–20 h after transfection were washed four times with PBS and incubated 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) without MgCl₂ and

CaCl₂ at room temperature for 40 min and at 37°C for 40 min. After being detached from culture dishes by pipeting, cells were collected by centrifugation at 2,500 rpm for 2 min in a Beckman TH-4 rotor. Cell pellets were washed twice with KRH buffer and loaded with 10 μM fluo-3 in KRH buffer plus 0.1 mg/ml BSA and 250 μM sulfinpyrazone at room temperature for 60 min, followed by washing 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 (final volume) 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 (SLM Instruments).

RESULTS

Cloning and Functional Expression of the Mouse Cardiac Ryanodine Receptor cDNA

Fig. 1 A outlines the strategy for the cloning of the mouse cardiac ryanodine receptor (RyR2) cDNA. The mouse RyR2 cDNA encodes a 565-kD protein composed of 4,967 amino acids. The deduced amino acid sequence of the mouse RyR2 shares 97% identity with that of the rabbit (Nakai et al., 1990; Otsu et al., 1990) and human RyR2 (Tunwell et al., 1996). To investigate the single-channel properties of the mouse RyR2 and the role in Ca²⁺ sensing of the conserved glutamate 3987, we constructed full-length cDNAs encoding the wild-type (wt) and glutamate 3987 to alanine mutant (E3987A) and expressed the constructs in HEK293 cells. Caffeine-induced Ca²⁺ release was readily detected in HEK293 cells transfected with the full-length wt mouse RyR2 cDNA, but not in cells transfected with the E3987A mutant RyR2 cDNA or vector (pCDNA3) DNA ($n = 5$; Fig. 1 B). However, the E3987A mutant RyR2 protein was expressed in HEK293 cells and exhibited sedimentation profile similar to that of the wt (Fig. 1 C), suggesting that the lack of caffeine response of the E3987A mutant is unlikely to be due to defects in expression.

Mutation E3987A Altered the Ca²⁺ Response of Single Mouse RyR2 Channels

To further understand the defect in mutant E3987A, we incorporated the wt and E3987A mutant proteins into planar lipid bilayers and determined the open probability (Po) of single channels at a wide range of Ca²⁺ concentrations. As shown in Fig. 2 A, a single wt channel was activated by Ca²⁺ at ~100 nM and was inactivated at ~10 mM. At Ca²⁺ concentrations between ~1 and 2,000 μM, the channel was maximally activated, resulting in a bell-shaped Ca²⁺ response curve (Fig. 2 C). Curve fitting of the wt Ca²⁺ response using the Hill equation yielded an EC₅₀ of 0.26 μM and a Hill coefficient of 3.0 for activation by Ca²⁺ ($n = 22$) and an IC₅₀ of 2.1 mM and a Hill coefficient of 1.3 for inactivation by Ca²⁺ ($n = 19$). The extent of inactivation of single wt channels by high concentrations of Ca²⁺ was found to vary from channel to channel. Some channels were inacti-

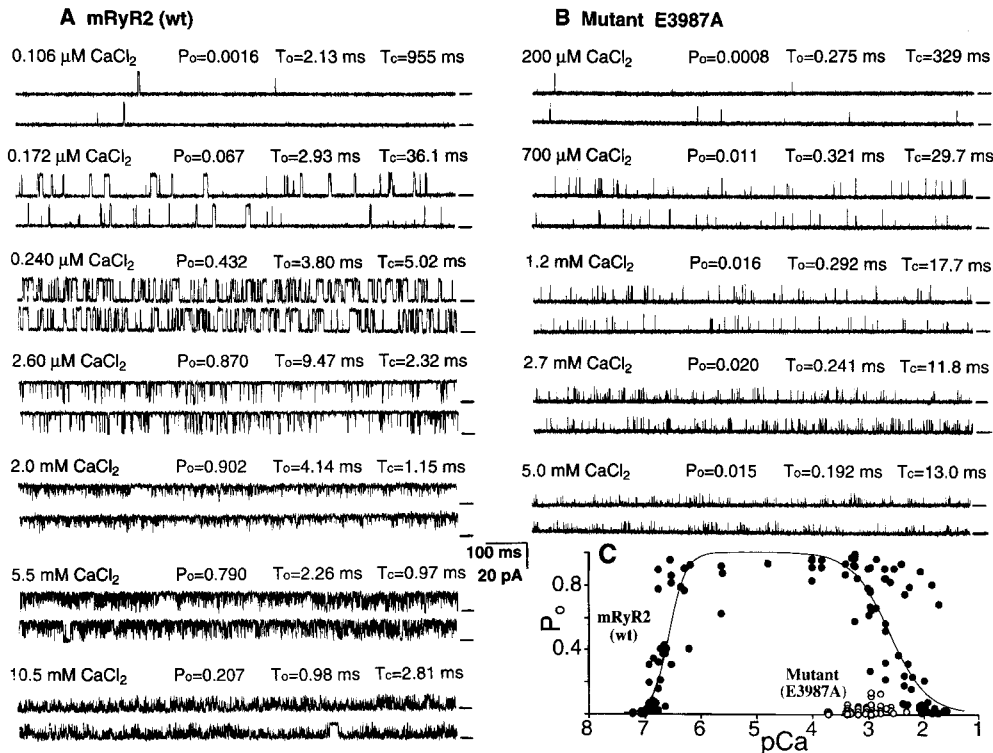
vated, whereas the others remained highly active at high Ca²⁺ concentrations (Fig. 2 C). In contrast, the E3987A mutant channels required several hundreds of micromolar Ca²⁺ for activation and hardly responded to increasing Ca²⁺ concentrations (Fig. 2 B). The maximum Po of most E3987A mutant channels activated by a wide range of Ca²⁺ concentrations was <0.05 (Fig. 2 C). In addition, opening events of the E3987A mutant channels were extremely brief. The mean open time of single E3987A mutant channels is ~10-fold shorter than that of the wt channels (Fig. 2, A and B). It appears that some opening events were too brief to be resolved completely under our recording conditions (Fig. 2 B). Thus, the Po of the E3987A mutant channels activated by Ca²⁺ alone may have been underestimated. Nevertheless, these data indicate that mutation E3987A severely impairs the threshold, maximum extent, and kinetics of Ca²⁺ activation of the mouse RyR2.

Ligand Gating Properties of Single wt and E3987A Mutant RyR2 Channels

We next examined the response of wt and E3987A mutant RyR2 channels to various modulators. Consistent with the results of [³H]ryanodine binding studies reported previously (Zhao et al., 1999), single wt channels were activated by ATP and caffeine, and were inhibited by Mg²⁺ ($n = 5$; Fig. 3 A). Similarly, single E3987A mutant channels were also activated by ATP and caffeine, and were inhibited by Mg²⁺ ($n = 5$; Fig. 3 B). It should be noted that the Ca²⁺ concentrations in the control conditions for wt (~200 nM) and mutant (~2 mM) channels differ considerably because of their differences in Ca²⁺ activation (Fig. 2). Hence, the extent of activation or inhibition by a ligand of the wt and mutant channels may not be compared quantitatively. It should also be noted that Mg²⁺ could inhibit Ca²⁺-activated wt or mutant E3987A channels in the absence of ATP and caffeine (not shown). Both the wt and E3987A mutant channels were modified in a similar fashion by ryanodine, which shifted the channel into a state with high Po and reduced conductance (Fig. 3 A, panel e, and Fig. 3 B, panel f). The single-channel conductance of the E3987A mutant channel is 793 ± 2.8 pS ($n = 4$), similar to that of the wt channels (~800 pS). Thus, the E3987A mutation does not change the single-channel conductance and does not cause gross alterations in channel function.

Mutant E3987A Retained High Affinity [³H]Ryanodine Binding

Different from mutant E3987A in RyR2, the corresponding mutant E4032A in RyR1 showed no response to either caffeine or ryanodine and lacked high affinity [³H]ryanodine binding. It has been suggested that the E4032A mutation may affect ryanodine binding di-



the cytoplasmic side of the channel (cis chamber). The relationships between P_o and Ca^{2+} concentrations of single wt (solid circles) and E3987A mutant (open circles) channels are shown in C. Data points shown are individual P_o measurements from 22 single wt channels and 16 single E3987A mutant channels. Curve shown represents fit of data from single wt channels using the Hill equation. Data points at Ca^{2+} concentrations >1 mM from three single wt channels that did not show significant Ca^{2+} -dependent inactivation were not included in the fitting for Ca^{2+} inactivation. The parameters of the fitting are indicated in the text. Note that the sensitivities of single wt channels to activation by Ca^{2+} are similar, with a threshold for activation ~ 100 nM, whereas the sensitivities to inactivation by Ca^{2+} vary significantly among single wt channels. The threshold for activation of single E3987A mutant channels by Ca^{2+} is ~ 0.2 mM. The maximum P_o of the Ca^{2+} -activated E3987A mutant channels ranges from 0.01 to 13%. The open probability (P_o), arithmetic mean open time (T_o), and the arithmetic mean closed time (T_c) at each Ca^{2+} level 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 for both the wt and E3987A mutant channels was $+20$ mV.

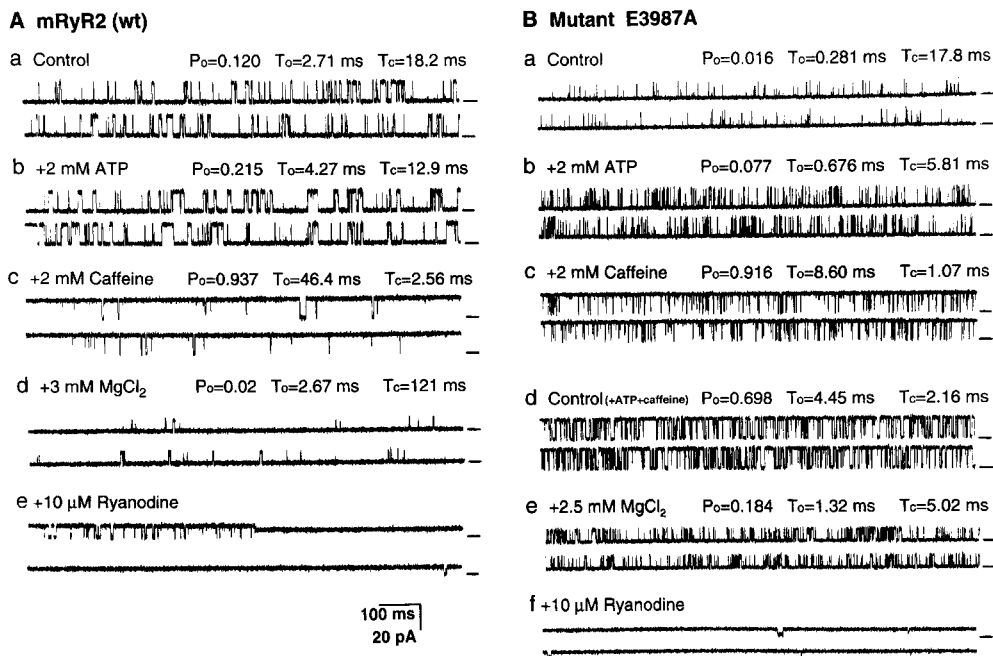


FIGURE 2. Ca^{2+} response of single wt and E3987A mutant RyR2 channels. Single-channel activities of the wt (mRyR2(wt)) (A) and the E3987A mutant (B) were recorded in a symmetrical recording solution containing 250 mM KCl, 25 mM Hepes, pH 7.4, and varying concentrations of cytoplasmic free Ca^{2+} . The orientation of each single channel detected in the bilayer was determined at the beginning of each experiment by addition of an aliquot of EGTA solution to either the cis or the trans chamber. 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 A and B were inhibited by addition of 0.1 mM EGTA to the cis chamber, indicating that the cytoplasmic side of the incorporated channel was facing the cis chamber. All subsequent additions were made to

FIGURE 3. Ligand gating properties of single wt (A) and E3987A mutant (B) RyR2 channels. Single-channel recordings were performed as described in the legend to Fig. 2. (A) Control a was performed in the presence of 171 nM cytoplasmic free Ca^{2+} . Subsequent additions of ATP, caffeine, Mg^{2+} , and ryanodine were made to the cytoplasmic side of the same channel. (B) The concentration of free Ca^{2+} in control a is 1.7 mM, and the recording solution in control d contains 1.7 mM Ca^{2+} , 0.5 mM EGTA, 2 mM ATP, and 4 mM caffeine. Current recordings in a–c are from the same channel, whereas those in d–f are from a different channel. Base lines are indicated. The holding potential was $+20$ mV for all recordings shown.

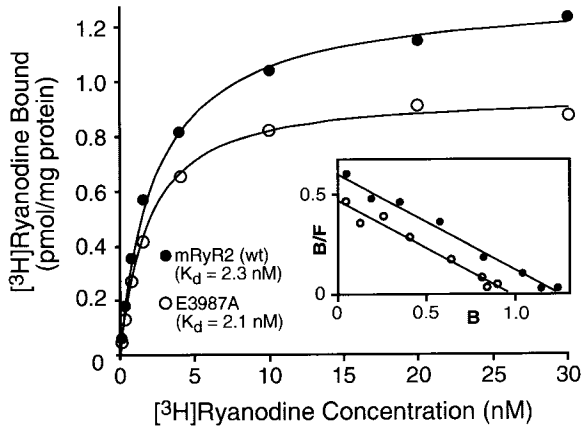


FIGURE 4. Mutation E3987A does not alter the affinity of $[^3\text{H}]$ ryanodine binding to RyR2. $[^3\text{H}]$ ryanodine binding to cell lysate prepared from HEK293 cells transfected with wt or mutant E3987A cDNA was performed in the presence of 25 mM Tris, 50 mM HEPES, pH 7.4, 500 mM KCl, CaCl_2 (0.2 mM for wt and 1.0 mM for mutant E3987A), 0.1–30 nM $[^3\text{H}]$ ryanodine, and various protease inhibitors at 37°C for 2 h. Data shown are from a representative experiment, which has been repeated three to four times. Inset shows a Scatchard plot.

rectly (Du and MacLennan, 1998). The characteristic functional response to ryanodine of single E3987A mutant RyR2 channel shown in Fig. 3 indicates that the functional high affinity ryanodine binding site is retained in the mutant channel. However, it is possible that the E3987A mutation could affect the properties of ryanodine binding in addition to Ca^{2+} activation. To test this possibility, we determined the equilibrium dissociation constant of $[^3\text{H}]$ ryanodine binding to mutant E3987A in the presence of 1 mM Ca^{2+} (Fig. 4). Scatchard analysis showed that the E3987A mutant RyR2 exhibited high affinity $[^3\text{H}]$ ryanodine binding with a K_d of 2.1 ± 0.39 nM and a B_{max} of 0.76 ± 0.15 pmol/mg ($n = 5$), similar to the K_d of 2.3 ± 0.63 nM and B_{max} of 1.24 ± 0.26 pmol/mg ($n = 4$) of the wt RyR2. High affinity $[^3\text{H}]$ ryanodine binding to the corresponding mutant E3885A in RyR3 was also detected (data not shown). Therefore, mutation of this conserved glutamate does not alter the affinity of $[^3\text{H}]$ ryanodine binding to RyR2.

Mutation E3987A Markedly Reduced the Sensitivity of Mouse RyR2 Channels to Activating Ca^{2+}

As seen in Fig. 3 B, single E3987A mutant channels could be fully activated by Ca^{2+} in the presence of ATP

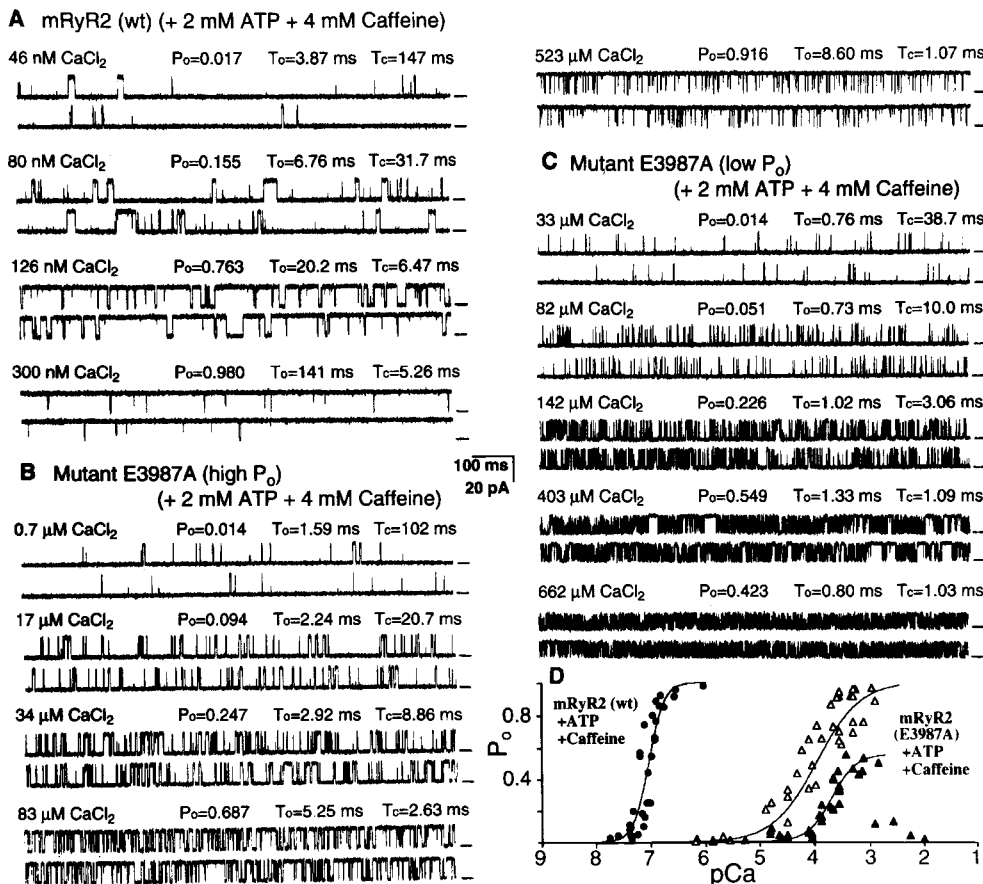


FIGURE 5. Mutation E3987A reduced dramatically the sensitivity of single mouse RyR2 channel to Ca^{2+} activation. The Ca^{2+} responses of single wt (A) and E3987A mutant (B and C) channels were determined in the presence of 2 mM ATP and 4 mM caffeine. Other conditions for recordings were the same as described in Fig. 2. According to their maximum P_o , single E3987A mutant channels could be divided into two groups with high (B) and low P_o (C). The P_o -pCa relationships of the wt and E3987A mutant channels are shown in D. Data points shown are individual P_o measurements from five single wt channels (solid circles), five E3987A mutant channels with high P_o (open triangles), and five E3987A mutant channels with low P_o (solid triangles). Lines represent curve fits using the Hill equation. The yielded parameters of fitting are indicated in the text. The holding potential for all recordings shown was +20 mV. Base lines are indicated.

and caffeine. This property allows us to quantify the relative Ca^{2+} sensitivity of the E3987A mutant channels at the single-channel level. As shown in Fig. 5 A, in the presence of 2 mM ATP and 4 mM caffeine, a single wt channel was activated by ~ 50 nM Ca^{2+} and reached maximum activation at ~ 300 nM Ca^{2+} . The Ca^{2+} response of single wt channels under these conditions could be described by an EC_{50} of 93 nM and a Hill coefficient of 3.1 ($n = 5$; Fig. 5 D). On the other hand, under the same conditions, single mutant E3987A channels were activated by submicromolar Ca^{2+} and reached maximum activation at ~ 500 μM (Fig. 5, B and C). The difference in the extent of maximum activation was observed among single E3987A mutant channels. Some mutant channels could be fully activated, whereas others showed maximum activation of $\sim 50\%$ (Fig. 5, B–D). This discrepancy is most likely attributable to different extents of Ca^{2+} inactivation, as seen among single wt channels (Fig. 2 C). The Ca^{2+} responses of the high and low Po E3987A mutant channels were analyzed by using the Hill equation. These analyses yielded an EC_{50} of 109 μM and a Hill coefficient of 1.1 for Ca^{2+} activation of the high Po E3987A mutant channels ($n = 5$), and an EC_{50} of 246 μM and a Hill coefficient of 1.63 for Ca^{2+} activation of the low Po mutant channels ($n = 5$). Hence, single E3987A mutant channels exhibited $\sim 1,000$ – $3,000$ -fold reduction in Ca^{2+} sensitivity to activation as compared with single wt RyR2 channels. We have previously shown that the corresponding mutation E3885A in the RyR3 isoform reduced the Ca^{2+} sensitivity of single RyR3 channels to activation in lipid bilayers by $>10,000$ -fold (Chen et al., 1998), which is 3–10 times more severe in reducing the Ca^{2+} sensitivity than does the E3987A mutation in the RyR2 isoform. This observed variance might reflect differences in the exact composition of the Ca^{2+} sensor or differences in the Ca^{2+} activation pathway between the RyR2 and RyR3 isoform.

Marked reduction in the sensitivity of the E3987A mutant channel to Ca^{2+} activation could be also demonstrated in the absence of ATP and caffeine by using $[^3\text{H}]$ ryanodine binding assay. Although mutant E3987A exhibited a binding affinity and maximum binding capacity similar to those of the wt (Fig. 4), they differ considerably in the Ca^{2+} dependence of $[^3\text{H}]$ ryanodine binding. $[^3\text{H}]$ ryanodine binding to wt proteins was activated by Ca^{2+} with an EC_{50} of 0.22 ± 0.03 μM and a Hill coefficient of 2.6 ± 0.34 ($n = 6$), whereas activation by Ca^{2+} of $[^3\text{H}]$ ryanodine binding to mutant E3987A proteins could be described by an EC_{50} of 59 ± 14 μM and a Hill coefficient of 1.3 ± 0.3 ($n = 6$; Fig. 6). Thus, the E3987A mutation resulted in ~ 270 -fold reduction in Ca^{2+} sensitivity to activation of $[^3\text{H}]$ ryanodine binding. This estimated reduction in Ca^{2+} sensitivity differs by ~ 4 – 10 -fold from that estimated by single-channel mea-

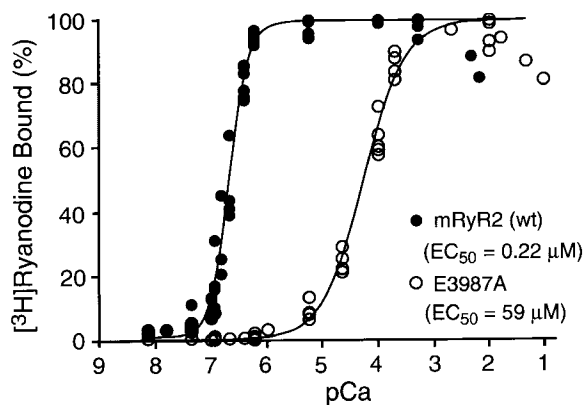


FIGURE 6. Ca^{2+} dependence of $[^3\text{H}]$ ryanodine binding to wt and mutant E3987A proteins. $[^3\text{H}]$ ryanodine binding to cell lysate prepared from HEK293 cells transfected with wt or mutant E3987A cDNA was performed at various concentrations of Ca^{2+} , as described in MATERIALS AND METHODS. The concentration of $[^3\text{H}]$ ryanodine used was 10 nM. Data shown are individual measurements from six separate binding experiments.

surements. The Ca^{2+} sensitivity of the wt channels estimated by single-channel measurements (0.26 μM) is similar to that (0.22 μM) estimated by $[^3\text{H}]$ ryanodine binding analysis (Figs. 2 and 6). On the other hand, the relative Ca^{2+} sensitivity to activation of single E3987A mutant channels measured in lipid bilayers may have been underestimated due to the influence of Ca^{2+} -dependent inhibition (Fig. 5 D). As a result, the differences in Ca^{2+} sensitivity between single wt and E3987A mutant channels measured in lipid bilayers may have been overestimated. It is of interest to note that significant Ca^{2+} -dependent inhibition of $[^3\text{H}]$ ryanodine binding was not detected at Ca^{2+} concentrations as high as 1 M (Fig. 6). The lack of Ca^{2+} -dependent inhibition of $[^3\text{H}]$ ryanodine binding to rabbit RyR2 proteins also has been reported previously (Du and MacLennan, 1999). With little influence by Ca^{2+} -dependent inhibition, $[^3\text{H}]$ ryanodine binding assay may provide more accurate estimates of the sensitivity of the mutant channels to activation by Ca^{2+} , especially at high Ca^{2+} concentrations.

Coexpression of wt and E3987A Mutant RyR2 Proteins Produced Single Channels with Intermediate Ca^{2+} Sensitivities

To investigate the role of subunit interaction in Ca^{2+} activation of the tetrameric RyR channel, we coexpressed the wt and E3987A mutant proteins in HEK293 cells and determined the Ca^{2+} response of each single channel detected in lipid bilayers (Fig. 7). A total of 19 single channels were observed and characterized. Based on their responses to Ca^{2+} , these single channels could be divided into five groups. Group I (6/19) exhibited a Ca^{2+} response similar to that of the wt, with an EC_{50} of 0.40 μM and a Hill coefficient of 2.8 for acti-

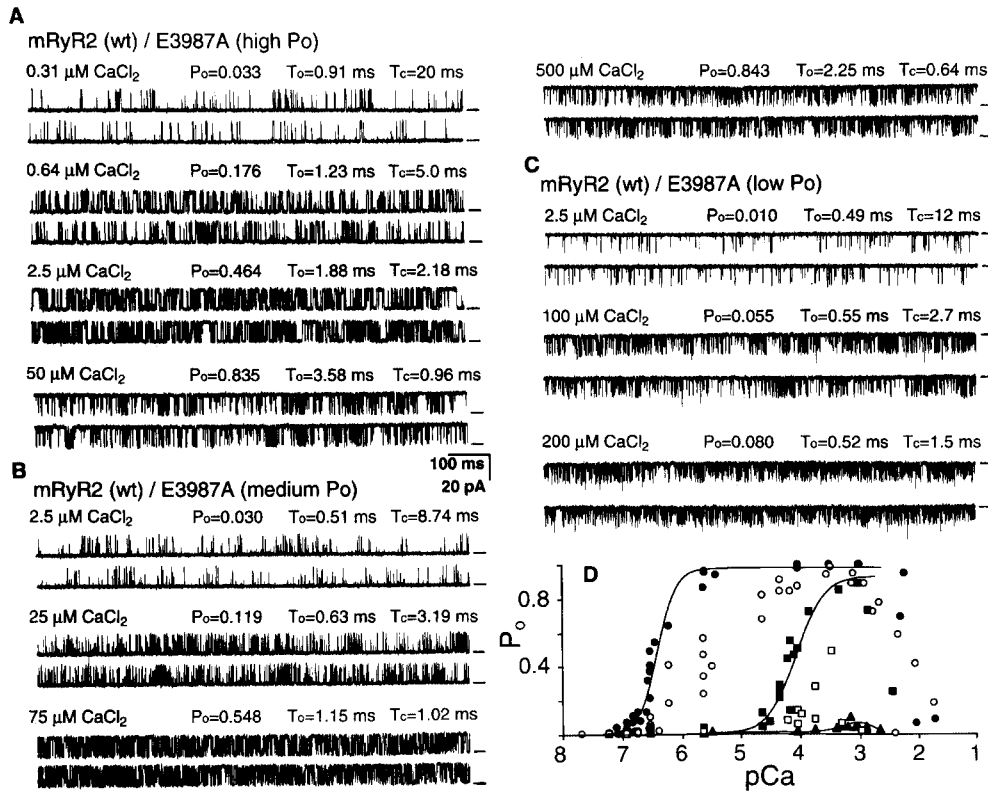


FIGURE 7. Ca^{2+} responses of single channels produced by coexpression of the wt and E3987A mutant RyR2 proteins in HEK293 cells. HEK293 cells were transfected with an equal amount (6 μg) of wt and E3987A mutant RyR2 cDNA. The Ca^{2+} responses of single hybrid channels with high Po (A), medium Po (B), and low Po (C) were determined as described in the legend to Fig. 2. Single-channel activities shown in A and B were inhibited by addition of 0.1 mM EGTA to the cis chamber, indicating that the cytoplasmic side of the incorporated channel was facing the cis chamber. On the other hand, single-channel activities shown in C were inhibited by addition of 0.1 mM EGTA to the trans chamber, indicating that the cytoplasmic side of the incorporated channel was facing the trans chamber. To measure currents in the same direction, from the luminal to

the cytoplasmic side of the channel, a +20-mV holding potential was applied in A and B and -20 mV in C. The P_o - $p\text{Ca}$ relationships of single hybrid channels are shown in D. Data points represent individual measurements. According to their Ca^{2+} sensitivities to activation, single hybrid channels could be divided into at least five groups (I-V). Group I (solid circles) and group II (solid triangles) displayed Ca^{2+} sensitivity to activation similar to that of the wt and E3987A mutant channel, respectively. Group III (solid squares), group IV (open circles), and group V (open squares) showed sensitivities to Ca^{2+} activation in between those of the wt and E3987A mutant channels. These different Ca^{2+} sensitivities most probably resulted from hybrid channels with different compositions of wt and E3987A mutant subunits. Note that two single channels are present in recordings shown in C and P_o indicates the average open probability.

vation by Ca^{2+} (Fig. 7 D, solid circles). This group of single channels most probably corresponds to the wt homotetramer. On the other hand, single channels in group II (2/19) were hardly activated by Ca^{2+} , resembling the E3987A mutant homotetramer (Fig. 7 D, solid triangles). Group III (medium P_o ; 4/19) displayed a Ca^{2+} response clearly different from that of the wt homotetramer and E3987A mutant homotetramer, exhibiting an EC_{50} of 85 μM and a Hill coefficient of 2.0 for activation by Ca^{2+} (Fig. 7, B and D, solid squares). Since the E3987A mutant protein forms a functional channel, presumably a homotetramer, it is most likely that the E3987A mutant protein is capable of forming a heterotetrameric channel with the wt protein. If so, group III single channels most probably represents hybrid channels formed by the wt and E3987A mutant subunits. The Ca^{2+} response of group IV (high Po) single channels (5/19; open circles) was found to be in between or similar to those of group I (wt homotetramer) and group III (presumably wt/mutant hybrid; Fig. 7, A and D). Group V (low Po; 2/19) showed Ca^{2+} response in between or similar to those of the

group III (presumably wt/mutant hybrid) and group II (E3987A mutant homotetramer) (Fig. 7, C and D, open squares). Because of the overlap in Ca^{2+} response with the wt homotetramer or with the mutant homotetramer, we are not certain that single channels in groups IV and V are all wt/mutant hybrid channels. Nevertheless, it is clear from Fig. 7 D that coexpression of the wt and E3987A mutant proteins produced single channels displaying sensitivities to activating Ca^{2+} in between those of the wt and E3987A mutant RyR2 channels. These results also indicate that the sensitivity to activation of the RyR2 Ca^{2+} sensor may depend on the cooperative interaction among RyR monomers.

Single Mouse RyR2 Channels Exhibited Ca^{2+} Response Similar to that of Single Canine RyR2 Channels

The mouse heart is quite different from that of other mammalian species. The most distinctive feature of the mouse heart is its fast heart rate (500–600 beats per minute; Hamilton and Ianuzzo, 1991). It has been suggested that the contractile and Ca^{2+} regulating systems

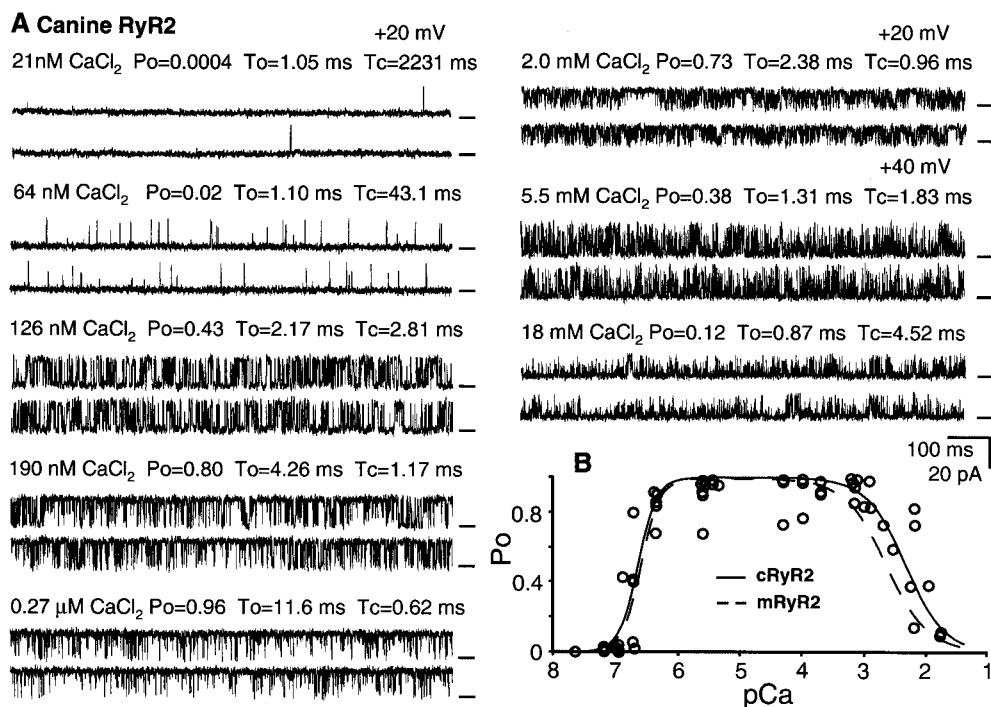


FIGURE 8. Single mouse and canine RyR2 channels share similar Ca²⁺ response. Single-channel activities of the canine RyR2 were recorded as described in MATERIALS AND METHODS and in the legend to Fig. 2. Single-channel activities shown in A were inhibited by the addition of 0.1 mM EGTA to the cis chamber, indicating that the cytoplasmic side of the incorporated channels was facing the cis chamber. All subsequent additions were made to the cytoplasmic side of the channel (cis chamber). Current traces (21 nM–0.27 μM) shown on the left and those (2–18 mM) shown on the right were from separate channels. The relationships between Po and Ca²⁺ concentrations of single canine (open circles, solid line) and mouse (dashed line, from Fig. 2) RyR2 channels are

shown in B. Data points shown are individual Po measurements from nine single canine RyR2 channels. The holding potential was +20 mV for Ca²⁺ concentrations at 21 nM, 64 nM, 126 nM, 190 nM, 0.27 μM, and 2 mM, and +40 mV for Ca²⁺ concentrations at 5.5 and 18 mM. The open probability (Po), arithmetic mean open time (To) and the arithmetic mean closed time (Tc) at each Ca²⁺ level are indicated on the top of each panel. A short line to the right of each current trace indicates the base line.

in the mouse heart must possess unique properties to accommodate such a high frequency operation (Hamilton and Ianuzzo, 1991). For example, mouse heart muscle contains a large amount of SR, which may help to speed up muscle relaxation (Forbes et al., 1985). In this context, it would be of interest to examine whether the Ca²⁺ response of the mouse RyR2 channel differs from that of RyR2 from other mammalian species, such as dog which has much slower heart rate (Hamilton and Ianuzzo, 1991). Fig. 8 shows the Ca²⁺ response of single canine RyR2 channels and comparison with that of single mouse RyR2 channels. Under the same conditions as for mouse RyR2, single canine RyR2 channels were activated by Ca²⁺ with an EC₅₀ of 0.24 μM and a Hill coefficient of 2.8, and were inactivated by Ca²⁺ with an IC₅₀ of 4.8 mM and a Hill coefficient of 1.3. These parameters are very similar to those observed with single mouse RyR2 channels (Figs. 2 and 8 B). Thus, the intrinsic Ca²⁺ dependence of single mouse and canine RyR2 channels in lipid bilayers appears to be similar.

DISCUSSION

The Role of the Conserved Glutamate in Ca²⁺ Activation of Different RyR Isoforms

In earlier studies, we have provided evidence that

glutamate 3885 is a major determinant of the sensitivity of RyR3 to activation by Ca²⁺ (Chen et al., 1998). Considering the highly conserved nature of the glutamate and its flanking residues in the M2 sequence, one would expect that the corresponding glutamate in other RyR isoforms would play a similar role in Ca²⁺ sensing. In a recent study, the corresponding glutamate 4032 in RyR1 was mutated into alanine (E4032A). Unlike the E3885A mutant RyR3, the E4032A mutant RyR1 expressed in HEK293 cells showed neither caffeine response nor [³H]ryanodine binding, even under the conditions that would fully activate the E3885A mutant RyR3 channel (Du and MacLennan, 1998). Although these observations support the view that glutamate 4032 is essential for RyR1 channel function and regulation, the role of this glutamate in Ca²⁺ activation of RyR1 is unclear. These observations also raise an important question of whether this conserved glutamate is involved in Ca²⁺ activation of RyR isoforms other than RyR3.

In view of the essential role of Ca²⁺ activation of RyR2 in CICR and in E-C coupling, we set out to investigate the molecular basis of activation of RyR2 by Ca²⁺ and to determine whether the Ca²⁺-sensing role of this conserved glutamate is maintained in the RyR2 isoform. To this end, we have cloned the cDNA encoding the mouse RyR2 and made the corresponding muta-

tion E3987A. Single-channel properties of the wt and E3987A mutant RyR2 were assessed and compared. Our results demonstrate that the E3987A mutant RyR2 channels exhibit marked reduction in the sensitivity to activating Ca^{2+} , while retaining similar single-channel conductance, high affinity [^3H]ryanodine binding, and responses to ATP, caffeine, Mg^{2+} , and ryanodine. These properties of the E3987A mutant RyR2 are very similar to those of the E3885A mutant RyR3, indicating that the specific role of glutamate 3987 in Ca^{2+} sensing is conserved in the RyR2 isoform.

The reasons for the lack of activity of the E4032A mutant RyR1 are not known. One possible explanation for the observed discrepancy between the E4032A mutant RyR1 and E3987A mutant RyR2 or E3885A mutant RyR3 may lie, in part, in different sensitivities of these mutant channels to inactivation by high Ca^{2+} concentrations. RyR1 is known to be 10–20 times more sensitive to inactivation by Ca^{2+} than RyR2 or RyR3 (Chen et al., 1997b; Laver et al., 1995). Robust Ca^{2+} inactivation in RyR1 may prevent the E4032A mutant channel from being considerably activated at high Ca^{2+} concentrations even in the presence of channel activators such as ATP and caffeine.

As to the E3987A mutant RyR2, Ca^{2+} activation is partially overlapped with Ca^{2+} inactivation due to the much lower sensitivity of RyR2 to inactivating Ca^{2+} . Residual activity of the E3987A mutant channel could be detected at submillimolar Ca^{2+} , although its Ca^{2+} response was significantly suppressed at higher Ca^{2+} concentrations (Fig. 2, B and C). This suppression could be alleviated by the addition of ATP and caffeine (Fig. 5). ATP and caffeine are known to be able to increase the sensitivity to Ca^{2+} activation and decrease the sensitivity to Ca^{2+} inactivation of the channel (Meissner et al., 1997). These effects of ATP and caffeine would effectively reduce the overlap between Ca^{2+} activation and Ca^{2+} inactivation of the E3987A mutant channel so much that the mutant channel could now be activated considerably by Ca^{2+} before Ca^{2+} inactivation could occur. Conversely, because of the robust Ca^{2+} inactivation in RyR1, significant overlap between Ca^{2+} activation and Ca^{2+} inactivation may still exist in the E4032A mutant RyR1 channel in the presence of ATP and caffeine.

The sensitivity to Ca^{2+} inactivation differs also among single recombinant mouse RyR2 channels (Fig. 2 C). Some single recombinant mouse RyR2 channels displayed no significant level of Ca^{2+} inactivation. Heterogeneity in Ca^{2+} inactivation of single native rabbit RyR2 channels has also been observed (Copello et al., 1997). The molecular basis for the heterogeneity is not known. Calmodulin has been shown recently to mediate Ca^{2+} -dependent inactivation of the L-type Ca^{2+} channel, the inositol 1,4,5-trisphosphate receptor, and the RyR1 isoform (Tripathy et al., 1995; Lee et al., 1999;

Michikawa et al., 1999; Peterson et al., 1999; Qin et al., 1999; Zuhlke et al., 1999; Rodney et al., 2000). However, the significance of CaM in Ca^{2+} -dependent inactivation of RyR2 remains to be determined.

Possible Mechanisms of Ca^{2+} Sensing by RyR

It is clear from the results of our present and previous studies that the absolutely conserved glutamate located in the putative transmembrane sequence M2 is a key residue in determining the sensitivity of RyR to activation by Ca^{2+} . However, the molecular basis of how this glutamate is involved in Ca^{2+} sensing remains to be understood. One possibility is that each RyR subunit has one Ca^{2+} sensor and that the conserved glutamate contributes to the formation of the Ca^{2+} sensor in each subunit. Mutation of this glutamate would decrease the sensitivity of each sensor to Ca^{2+} activation and probably alter the cooperativity between sensors, thus, reducing the overall sensitivity and cooperativity of the tetrameric RyR channel to Ca^{2+} activation. This multi-sensor model is analogous to that proposed for Ca^{2+} activation of Ca^{2+} -activated potassium channels in which activation of all four Ca^{2+} sensors is necessary for channel opening and the steeply cooperative channel gating would arise from the cooperative interaction between sensors (Fanger et al., 1999; Keen et al., 1999)

Alternatively, each subunit may contribute partially to the formation of a single Ca^{2+} sensor in the tetrameric RyR channel, and the conserved glutamates of each subunit may be located in close proximity and act cooperatively to form a major part of the Ca^{2+} sensor. The sensor could be composed of two or more cooperative Ca^{2+} binding sites. Mutation of this glutamate would affect both the sensitivity and cooperativity of the sensor to activating Ca^{2+} . This single-sensor model is reminiscent of the Ca^{2+} binding sites in the SR Ca^{2+} pump in which amino acid residues from four transmembrane segments contribute to the formation of two Ca^{2+} binding sites (MacLennan et al., 1998; Toyoshima et al., 2000). Both the multi- and single-sensor models are, apparently, compatible with the observed results. Further studies are required for distinguishing these models. For example, localization of the Ca^{2+} sensor(s) in the 3-D architecture of RyR would provide some essential clues to the molecular mechanisms of Ca^{2+} sensing.

Channel Properties of the Mouse RyR2

Most information on RyR2 channel properties has primarily come from studies using sheep, canine, or rabbit hearts. Little is known about the channel properties of RyR2 from the mouse heart. This is due, in part, to the limited amount of mouse RyR2 proteins that could be isolated and used for either biochemical or electro-

physiological analyses. In the present study, we were able to express the mouse RyR2 cDNA in HEK293 cells and functionally characterize the recombinant mouse RyR2 protein at the single-channel level. We show that recombinant mouse RyR2 channels can be activated by ATP and caffeine, inhibited by Mg^{2+} , and modified by ryanodine. The Ca^{2+} response of the mouse RyR2 channels is biphasic, being activated by Ca^{2+} at low concentrations and inhibited by Ca^{2+} at high concentrations, similar to that of the canine RyR2 channels. Further mutational studies should lead to a better understanding of the structure and function relationships of the mouse RyR2 channel. This kind of knowledge should be useful for manipulating specific properties of the mouse RyR2 channel such as Ca^{2+} sensing and ion conduction and assessing the physiological significance of these properties in cardiac function via genetic engineering of the mouse RyR2 gene.

We would like to thank Lin Zhang and Xiaoli Li for invaluable technical assistance, 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. Chen. S.R.W. Chen is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

Submitted: 23 March 2001

Revised: 21 May 2001

Accepted: 22 May 2001

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