The EF-hand Ca\textsuperscript{2+} Binding Domain Is Not Required for Cytosolic Ca\textsuperscript{2+} Activation of the Cardiac Ryanodine Receptor*  
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Activation of the cardiac ryanodine receptor (RyR2) by elevating cytosolic Ca\textsuperscript{2+} is a central step in the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, but the molecular basis of RyR2 activation by cytosolic Ca\textsuperscript{2+} is poorly defined. It has been proposed recently that the putative Ca\textsuperscript{2+} binding domain encompassing a pair of EF-hand motifs (EF1 and EF2) in the skeletal muscle ryanodine receptor (RyR1) functions as a Ca\textsuperscript{2+} sensor that regulates the gating of RyR1. Although the role of the EF-hand domain in RyR1 function has been studied extensively, little is known about the functional significance of the corresponding EF-hand domain in RyR2. Here we investigate the effect of mutations in the EF-hand motifs on the Ca\textsuperscript{2+} activation of RyR2. We found that mutations in the EF-hand motifs or deletion of the entire EF-hand domain did not affect the Ca\textsuperscript{2+}-dependent activation of \textsuperscript{[3H]}ryanodine binding or the cytosolic Ca\textsuperscript{2+} activation of RyR2. On the other hand, deletion of the EF-hand domain markedly suppressed the luminal Ca\textsuperscript{2+} activation of RyR2 and spontaneous Ca\textsuperscript{2+} release in HEK293 cells during store Ca\textsuperscript{2+} overload or store overload-induced Ca\textsuperscript{2+} release (SOICR). Furthermore, mutations in the EF2 motif, but not EF1 motif, of RyR2 raised the threshold for SOICR termination, whereas deletion of the EF-hand domain of RyR2 increased both the activation and termination thresholds for SOICR. These results indicate that, although the EF-hand domain is not required for RyR2 activation by cytosolic Ca\textsuperscript{2+}, it plays an important role in luminal Ca\textsuperscript{2+} activation and SOICR.

Contraction of cardiac muscle cells is initiated via a mechanism known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR)\textsuperscript{4} (1, 2). In this process, membrane depolarization opens the voltage-dependent L-type Ca\textsuperscript{2+} channel in the sarcolemmal membrane, leading to a small influx of Ca\textsuperscript{2+} from the extracellular space. This Ca\textsuperscript{2+} entry then activates the cardiac Ca\textsuperscript{2+} release channel (ryanodine receptor type 2, RyR2) in the sarcoplasmic reticulum membrane, resulting in a large Ca\textsuperscript{2+} efflux from the sarcoplasmic reticulum. Therefore, activation of the RyR2 channel by elevating cytosolic Ca\textsuperscript{2+} is an essential step in the process of CICR. RyR2 also plays a critical role in the pathogenesis of cardiac arrhythmias and cardiomyopathies (2, 3). Despite its imperative role in CICR, the molecular basis of RyR2 activation by cytosolic Ca\textsuperscript{2+} remains poorly understood.

Purified single RyR2 channels incorporated into lipid bilayers are activated by submicromolar cytosolic Ca\textsuperscript{2+} (4–6), indicating that the RyR2 channel contains high-affinity cytosolic Ca\textsuperscript{2+}-activating sites or Ca\textsuperscript{2+} sensors. However, the identity and nature of these putative RyR2 cytosolic Ca\textsuperscript{2+} sensors have yet to be defined. We have shown previously that mutating residue Glu-3987 in RyR2 (E3987A) reduced the sensitivity of RyR2 to cytosolic Ca\textsuperscript{2+} activation \textasciitilde1000-fold (6). Similarly, mutating residue Glu-3885 (E3885A) in RyR3 (corresponding to E3987A in RyR2) dramatically reduced the cytosolic Ca\textsuperscript{2+} sensitivity of RyR3 \textasciitilde10,000-fold (7). These observations suggest that residue Glu-3987 is an essential element of the RyR2 cytosolic Ca\textsuperscript{2+}-sensing mechanism. On the other hand, Xiong et al. (8) have identified two EF-hand Ca\textsuperscript{2+} binding motifs (EF1 and EF2, residues 4081–4127) that resemble those of the C-lobe of calmodulin (CaM) (9). Ca\textsuperscript{2+} binding studies have shown that these two EF-hands bind Ca\textsuperscript{2+} with millimolar affinities, suggesting that they may be involved in Ca\textsuperscript{2+}-dependent inactivation of the channel because single RyR channels are inactivated by millimolar Ca\textsuperscript{2+} (8). Consistent with this view, Fessenden et al. (10) have also found that mutating the EF1 sequence reduced the sensitivity of RyR1 to Ca\textsuperscript{2+}-dependent inactivation \textasciitilde2-fold but increased the sensitivity of RyR1 to Ca\textsuperscript{2+}-dependent activation \textasciitilde2-fold. Interestingly, mutating the EF2 sequence abolished high-affinity \textsuperscript{[3H]}ryanodine binding, but single EF2 mutant channels remained sensitive to cytosolic Ca\textsuperscript{2+} activation. Similarly, Gomez and Yamaguchi (11) have also shown that the EF-hand Ca\textsuperscript{2+} binding domain in RyR1 is involved in Ca\textsuperscript{2+}-dependent inactivation. Furthermore, a peptide that encompasses the EF1 and EF2 motifs has been found to bind to the intact RyR1 channel and altered the Ca\textsuperscript{2+} dependence of \textsuperscript{[3H]}ryanodine binding (12). It activated \textsuperscript{[3H]}ryanodine binding to RyR1 at low Ca\textsuperscript{2+} concentrations, inhibited it at intermediate Ca\textsuperscript{2+} concentrations, and inhibited it at high Ca\textsuperscript{2+} concentrations.
prevented Ca\(^{2+}\)-dependent inactivation of the channel at high Ca\(^{2+}\) concentrations. Taken together, these findings suggest that the EF-hand Ca\(^{2+}\) binding domain is involved in Ca\(^{2+}\) regulation of RyR1.

Recently, the three-dimensional structure of RyR1 has been solved at near-atomic resolutions by using cryo-electron microscopy and single particle analysis (13–15). These high-resolution structures have provided unprecedented insights into the structure-function relationship of Ca\(^{2+}\) regulation of RyR. The EF-hand Ca\(^{2+}\) binding domain is located in the central domain. This central domain interacts with the C-terminal domain believed to be involved in channel gating (13–15). On the basis of the three-dimensional structure of RyR1, it has been proposed that the paired EF-hand Ca\(^{2+}\) binding motifs in RyR1 may function as a Ca\(^{2+}\) sensor or conformational switch important for the activation of RyR1 by cytosolic Ca\(^{2+}\) and, therefore, the mechanism of CICR (13, 14). However, the role of these Ca\(^{2+}\) binding motifs in cytosolic Ca\(^{2+}\) activation of RyR2 is unknown. In this study, we mutated the EF1 and EF2 motifs in RyR2 and assessed their effect on the Ca\(^{2+}\) activation of RyR2. We found that mutating either the EF-hand motifs or deleting the entire EF-hand domain did not alter the Ca\(^{2+}\) dependence of \(^{[\text{H}]\text{yanodine binding or the activation of a single RyR2 channel by cytosolic Ca}^{2+}\). Interestingly, we found that mutating the EF2 motif, but not EF1 motif, increased the termination threshold for store overload-induced Ca\(^{2+}\) release (SOICR), whereas deletion of the entire EF-hand domain diminished the sensitivity of single RyR2 channels to luminal Ca\(^{2+}\) activation and the propensity for SOICR and increased both the activation and termination thresholds for SOICR. Our data indicate that the EF-hand Ca\(^{2+}\) binding domain is important for luminal Ca\(^{2+}\) activation of the RyR2 channel and SOICR but not essential for cytosolic Ca\(^{2+}\) activation of RyR2.

**Experimental Procedures**

**Generation of Mutations in the EF-hand Ca\(^{2+}\) Binding Domain of RyR2**—The EF-hand mutations and deletion in mouse RyR2 were generated by the overlap extension method using PCR (16, 17). Briefly, a SalI and a MluI restriction site were first introduced into the full-length mouse RyR2 cDNA in pBluescript vector at positions 11816 and 12340, respectively, without altering the amino acid sequence. A SalI/MluI fragment containing EF-hand mutants or deletion was obtained by overlapping PCR and used to replace the corresponding WT fragment in the full-length RyR2 cDNA in pBluescript, which was then subcloned into pcDNA5. All point mutations and deletions were confirmed by DNA sequencing.

**Generation of Stable, Inducible Cell Lines Expressing RyR2 WT and Mutants**—Stable, inducible HEK293 cell lines expressing RyR2 WT, D4036A/D4038A, E4071A/D4073A, and EF-hand deletion (EF-Del) were generated using the Flp-In T-REx core kit (Invitrogen) (18). Briefly, Flp-In T-REx HEK293 cells were co-transfected with the inducible expression vector pcDNA5/FRT/TO containing the RyR2 mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the Ca\(^{2+}\) phosphate precipitation method. The transfected cells were washed with PBS, 137 mm NaCl, 8 mm Na\(_2\)HPO\(_4\), 1.5 mm KH\(_2\)PO\(_4\), and 2.7 mm KCl (pH 7.4) 24 h after transfection, followed by a change into fresh medium for 24 h. The cells were then washed again with PBS, harvested, and plated onto new dishes. After the cells had attached (~4 h), the growth medium was replaced with a selection medium containing 200 μg/ml hygromycin (Invitrogen). The selection medium was changed every 3–4 days until the desired number of cells were grown. The hygromycin-resistant cells were pooled, aliquoted (1 ml), and stored at ~80°C. These positive cells are believed to be isogenic because the integration of RyR2 cDNA is mediated by the Flp recombinase at a single flippase recognition target site.

**Western Blotting**—HEK293 cells grown for 24 h after transfection with RyR2 WT and EF-hand mutant cDNAs were washed with PBS plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 8 min at 700 × g in an International Equipment Company Centra-CL2 centrifuge. The cells were then washed with PBS without EDTA and centrifuged again at 700 × g for 8 min. The PBS-washed cells were solubilized in lysis buffer containing 25 mM Tris, 50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soy bean phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mixture (1 mm benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotonin, and 0.5 mM PMSF). This mixture was incubated on ice for 1 h. Cell lysate was obtained by centrifuging twice at 16,000 × g in a microcentrifuge at 4°C for 30 min to remove unsolubilized materials. The RyR2 WT and mutant proteins were subjected to SDS/PAGE (6% gel) (19) and transferred onto nitrocellulose membranes at 90 V for 1.5 h at 4°C in the presence of 0.01% SDS (20). The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween 20 and 5% (w/v) nonfat dried skim milk powder. The blocked membrane was incubated with anti-RyR antibody (34C, 1:1000 dilution) and then incubated with secondary (anti-mouse IgG (heavy and light)) antibodies conjugated to horseradish peroxidase (1:20,000 dilution). After washing for 5 min three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce. The intensity of each band was determined from its intensity profile obtained by ImageQuant LAS 4000 (GE Healthcare Life Sciences), analyzed by using the ImageJ software, and normalized to β-actin (21).

**Single-cell Ca\(^{2+}\) Imaging (Cytosolic Ca\(^{2+}\) Measurements)**—Cytosolic Ca\(^{2+}\) levels in stable, inducible HEK293 cells expressing RyR2 WT or EF-hand mutants were monitored using single-cell Ca\(^{2+}\) imaging and the fluorescent Ca\(^{2+}\) indicator dye Fura-2/AM as described previously (22–24). Briefly, cells grown on glass coverslips for 8–18 h after induction (as indicated) by 1 μg/ml tetracycline (Sigma) were loaded with 5 μM Fura-2/AM in KRH buffer (125 mM NaCl, 5 mM KCl, 6 mM glucose, 1.2 mM MgCl\(_2\), and 25 mM Hepes (pH 7.4)) plus 0.02% pluronic F-127 and 0.1 mg/ml BSA for 20 min at room temperature (23°C). The coverslips were then mounted in a perfusion chamber (Warner Instruments) on an inverted microscope (Nikon TE2000-S). The cells were perfused continuously with KRH buffer containing increasing extracellular Ca\(^{2+}\) concentrations (0, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mM). Caffeine (10 mM) was applied at the end of each experiment to confirm the expression of active RyR2 channels. Time-lapse images (0.25 frame/s) were captured and analyzed with Compix Simple Imaging Software.
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PCO 6 software. Fluorescence intensities were measured from regions of interest centered on individual cells. Only cells that responded to caffeine were analyzed. The filters used for Fura-2/AM imaging were λexcitation = 340 ± 26 nm and 387 ± 11 nm and λemission = 510 ± 84 nm with a dichroic mirror (410 nm).

Single-cell Ca\(^{2+}\) Imaging (Luminal Ca\(^{2+}\) Measurements)—Luminal Ca\(^{2+}\) levels in HEK293 cells expressing RyR2 WT or EF-hand mutants were measured using single-cell Ca\(^{2+}\) imaging and the FRET-based ER luminal Ca\(^{2+}\)-sensitive chameleone protein D1ER, as described previously (25, 26). The cells were grown to 95% confluence in a 75-cm\(^2\) flask, dissociated with PBS, and plated in 100-mm-diameter tissue culture dishes at ~10% confluence 18–20 h before transfection with D1ER cDNA using the Ca\(^{2+}\) phosphate precipitation method. After transfection for 24 h, the growth medium was changed to induction medium containing 1 μg/ml tetracycline. In intact cell studies, after induction for ~22 h, the cells were perfused continuously with KRH buffer containing various concentrations of CaCl\(_2\) (0, 1, and 2 mm) and tetracaine (1 mm) to estimate the store capacity or caffeine (20 μM) to estimate the minimum store level by depleting the ER Ca\(^{2+}\) stores at room temperature (23 °C). Images were captured with Compix Simple PCI 6 software every 2 s using an inverted microscope (Nikon TE2000-S) equipped with an S-Fluo ×20/0.75 objective. The filters used for D1ER imaging were λexcitation = 436 ± 20 nm for cyan fluorescent protein and 500 ± 20 nm for yellow fluorescent protein and λemission = 465 ± 30 nm for cyan fluorescent protein and 535 ± 30 nm for yellow fluorescent protein with a dichroic mirror (500 nm). The amount of FRET was determined from the ratio of the light emission at 535 and 465 nm.

\[^{3}H\]Ryano dine Binding—The cells were grown to 95% confluence in a 75-cm\(^2\) flask, dissociated with PBS, and plated in 100-mm tissue culture dishes at ~10% confluence 18–20 h before transfection with RyR2 WT and EF-hand mutant cDNAs. After transfection for 24 h, the cells were harvested and lysed in lysis buffer containing 25 mm Tris, 50 mm Hepes (pH 7.4), 137 mm NaCl, 1% CHAPS, 0.5% egg phosphatidylcholine, 2.5 mm DTT, and protease inhibitor mixture (1 mm benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml apro tinin, and 0.5 mM PMSF) on ice for 60 min. Cell lysate was obtained after removing the unsolubilized materials by centrifugation twice in a microcentrifuge at 4 °C for 30 min each. Equilibrium \[^{3}H\]ryanodine binding to cell lysates was performed as described previously (6) with some modifications. \[^{3}H\]Ryanodine binding was carried out in a total volume of 300 μl of binding solution containing 30 μl of cell lysate, 100 mm KCl, 25 mm Tris, 50 mm Hepes (pH 7.4), 5 nm \[^{3}H\]ryanodine, and CaCl\(_2\) to set free [Ca\(^{2+}\)] from pCa 9.89 to pCa 4 and a protease inhibitor mixture at 37 °C for 20 min. The Ca\(^{2+}\)/EGTA ratio was calculated using the computer program of Fabiato and Fabiato (27). The binding mixture was diluted with 5 ml of ice-cold washing buffer containing 25 mm Tris (pH 8.0), and 250 mm KCl and filtered immediately through Whatman GF/B filters presoaked with 1% polyethylenimine. The filters were washed three times, and the radioactivity associated with the filters was determined by liquid scintillation counting. Nonspecific binding was determined by measuring \[^{3}H\]ryanodine binding in the presence of 50 μM unlabeled ryanodine. All binding assays were done in duplicate.

Sucrose Density Gradient Purification of Recombinant RyR2 Proteins—RyR2 WT or EF-hand mutant cell lysates (2.5 ml) layered on top of a 10.5-ml (7.5–25%, w/v) linear sucrose gradient containing 25 mm Tris, 50 mm Hepes (pH 7.4), 0.3 mM NaCl, 0.1 mm CaCl\(_2\), 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 a Beckman SW-41 rotor at 4 °C for 17 h. Fractions with a relatively small volume (0.7 ml each) were collected to maximize the concentration of RyR protein and, at the same time, minimize the loss of RyR2 protein after pooling the selected fractions. Peak fractions containing RyR proteins, as determined by immunoblotting, were pooled, aliquoted, and stored at −80 °C.

Single-channel Recordings—Recombinant RyR2 WT and EF-hand mutant channels were purified from cell lysate prepared from HEK293 cells transfected with the RyR2 WT or EF-hand mutant cDNA by sucrose density gradient centrifugation as described previously (6, 23). Heart phosphatidylethanolamine (50%) and brain phosphatidylserine (50%) (Avanti Polar Lipids), dissolved in chloroform, were combined, dried under nitrogen gas, and resuspended in 30 μl of n-decane at a concentration of 12 mg lipid/ml. Bilayers were formed across a 250-μm hole in a Delrin partition separating two chambers. The trans chamber (800 μl) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments, Austin, TX). 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 unless indicated otherwise. A 4-μl aliquot (~1 μg of protein) of the sucrose density gradient-purified recombinant RyR2 WT or the EF-hand mutant channels was added to the cis chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca\(^{2+}\)\(^{2+}\). The chamber to which the addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic side of the Ca\(^{2+}\) release channel. The direction of single-channel currents was always measured from the luminal to the cytosolic side of the channel unless mentioned otherwise. Recordings were filtered at 2500 Hz. Data analyses were carried out using the pclamp 8.1 software package (Axon Instruments). Free Ca\(^{2+}\) concentrations were calculated using the computer program of Fabiato and Fabiato (27).

Statistical Analysis—All values shown are mean ± S.E. unless indicated otherwise. To test for differences between two groups, we used unpaired Student’s t tests (two-tailed). p < 0.05 was considered to be statistically significant.

Results
Effect of Mutations in the EF-hand Ca\(^{2+}\) Binding Motifs on the Ca\(^{2+}\) Dependence of \[^{3}H\]Ryano dine Binding to RyR2—Amino acid residues 4036–4082 in RyR2, corresponding to residues 4081–4127 in RyR1, encompass two EF-hand Ca\(^{2+}\) binding motifs (EF1 and EF2) (8, 10). The sequences of EF1 and EF2 in RyRs share a high degree of homology with that of the C-lobe of CaM (Fig. 1A). To assess the role of these EF-hands in the Ca\(^{2+}\)-dependent activation of RyR2, we determined the effect of mutations in EF1 and EF2 Ca\(^{2+}\) binding motifs on the
Ca\textsuperscript{2+} dependence of \[^3H\]ryanodine binding. Because ryanodine only binds to the open state of RyRs, a \[^3H\]ryanodine binding assay has been used widely to monitor the activity of RyR. As shown in Fig. 1, mutating single or double residues in the EF1 Ca\textsuperscript{2+} binding motif (D4036A, D4036A/D4038A, or S4044A/D4047A) (Fig. 1A) did not alter the EC\textsubscript{50} of Ca\textsuperscript{2+}-dependent activation of \[^3H\]ryanodine binding to RyR2 (Fig. 1B). Similarly, mutating single, double, or triple residues in the EF2 Ca\textsuperscript{2+} binding motif (E4071A, E4071A/D4073A, or D4079A/E4081A/E4082A) (Fig. 1A) did not affect the EC\textsubscript{50} of Ca\textsuperscript{2+}-dependent activation of \[^3H\]ryanodine binding (Fig. 1C). The expression levels of the RyR2 WT and EF-hand mutants are shown in Fig. 1, D and E. The D4036A and D4036A/D4038A mutations slightly reduced the expression...
level of RyR2, whereas the E4071A and E4071A/D4073A mutations slightly increased it. The S4044A/D4047A and D4079A/E4081A/E4082A mutations had no significant effect on RyR2 expression. Collectively, these data suggest that the CaM C-lobe-like EF-hand Ca$^{2+}$/H11001 binding motifs in RyR2 are not essential for Ca$^{2+}$/H11001 activation of the channel.

**The Effect of Mutations in the EF-hand Ca$^{2+}$/H11001 Binding Motifs on the Cytosolic Ca$^{2+}$/H11001 Activation of Single RyR2 Channels**—To directly determine the role of the EF-hand Ca$^{2+}$/H11001 binding motifs in the cytosolic Ca$^{2+}$/H11001 activation of RyR2, we assessed the response of RyR2 WT and the EF1 and EF2 mutants to various cytosolic Ca$^{2+}$/H11001 concentrations using single-channel recordings in planar lipid bilayers. If the EF1 and EF2 motifs were critical for cytosolic Ca$^{2+}$/H11001 activation, then one would expect that mutating these EF-hand motifs would diminish cytosolic Ca$^{2+}$/H11001 activation of RyR2. However, this was not the case. As shown in Fig. 2, mutating the EF1 (D4036A/D4038A) or EF2 (E4071A/D4073A) Ca$^{2+}$/H11001 binding motif did not diminish the activation of single RyR2 channels by cytosolic Ca$^{2+}$/H11001. The response of single D4036A/D4038A or E4071A/D4073A mutant channels to cytosolic Ca$^{2+}$/H11001 was similar to that of single RyR2 WT channels (Fig. 2). Therefore, our data indicate that the EF-hand Ca$^{2+}$/H11001 binding motifs are not critical for cytosolic Ca$^{2+}$/H11001 activation of single RyR2 channels.

**Deletion of the Entire EF-hand Domain Has No Significant Effect on Cytosolic Ca$^{2+}$/H11001 Activation of RyR2**—To determine whether the EF-hand Ca$^{2+}$/H11001 binding domain is required for cytosolic Ca$^{2+}$/H11001 activation of RyR2, we deleted the entire EF-hand domain encompassing both EF1 and EF2 and assessed the effect of this deletion on Ca$^{2+}$/H11001-dependent activation of RyR2. As shown in Fig. 3, the Ca$^{2+}$/H11001 dependence of [$^{3}$H]ryanodine binding to the EF-hand deletion mutant (EF-Del) is similar to that of [$^{3}$H]ryanodine binding to the RyR2 WT. We also performed single-channel analysis of the EF-Del mutant. As shown in Fig. 4, the response of single EF-Del mutant channels to cytosolic Ca$^{2+}$/H11001 activation was similar to that of single RyR2 WT channels. Therefore, the EF-hand Ca$^{2+}$/H11001 binding domain is not required for the cytosolic Ca$^{2+}$/H11001 activation of RyR2.

**Deletion of the Entire EF-hand Domain Suppresses Luminal Ca$^{2+}$/H11001 Activation of RyR2**—We next determined the effect of the EF-Del mutation on the sensitivity of single RyR2 channels to luminal Ca$^{2+}$/H11001 activation. Single RyR2 WT or EF-Del mutant channels were incorporated into a lipid bilayer. The cytosolic Ca$^{2+}$/H11001 concentration was kept at ~45 nM. The luminal Ca$^{2+}$/H11001 concentration was then increased stepwise from 45 nM to 40 mM by additions of aliquots of CaCl$_2$. As shown in Fig. 5, in the presence of 45 nM cytosolic Ca$^{2+}$/H11001 and 2.5 mM ATP, single RyR2 WT channels were activated markedly by luminal Ca$^{2+}$/H11001 (1–40
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The Effect of Mutations in the EF-hand Domain on Spontaneous Ca$^{2+}$ Release in HEK293 Cells—To further investigate the role of the EF-hand domain in SOICR, we determined the activation and termination thresholds for SOICR in HEK293 cells expressing the RyR2 WT or EF-hand mutants. The SOICR activation and termination thresholds were assessed by monitoring the ER luminal Ca$^{2+}$ dynamics using a FRET-based ER luminal Ca$^{2+}$-sensing protein, D1ER (25, 26). As shown in Fig. 7, increasing the extracellular Ca$^{2+}$ concentration from 0 to 2 mM induced spontaneous ER Ca$^{2+}$ oscillations in RyR2 WT-expressing HEK293 cells (shown as downward deflections of the FRET signal), as reported previously (26, 28, 29). SOICR occurred when the ER luminal Ca$^{2+}$ content reached a threshold level (F$\text{SOICR}$) and terminated when the ER luminal Ca$^{2+}$ content decreased to another threshold level (F$\text{terminal}$) (Fig. 7A). Consistent with their effect on SOICR propensity, we found that the D4036A/D4038A or E4071A/D4073A mutation did not significantly alter the activation threshold for SOICR (D4036A/D4038A, 92.8% ± 0.3%; E4071A/D4073A, 94.4% ± 0.3%; WT, 93.2% ± 0.7%). However, the E4071A/D4073A mutation in EF2, but not the D4036A/D4038A mutation in EF1, increased the termination threshold for SOICR (D4036A/D4038A, 60.9% ± 1.8% versus WT, 60.6% ± 2.3% and E4071A/D4073A, 66.9% ± 1.0% versus WT, 60.6% ± 2.3%; p < 0.05). The fractional Ca$^{2+}$ release in E4071A/D4073A

FIGURE 3. Deletion of the entire EF-hand domain does not alter the Ca$^{2+}$ dependence of [H]$^{3}$ryanodine binding to RyR2. A, amino acid sequence of the RyR2 EF-hand domain encompassing the EF1 and EF2 Ca$^{2+}$ binding motifs. B, [H]$^{3}$ryanodine binding to cell lysate prepared from HEK293 cells expressing the RyR2 WT or the EF-Del was carried out in symmetrical recording solution containing 250 mM KCl and 25 mM Hepes (pH 7.4). The cytosolic Ca$^{2+}$ concentration was increased from 45 nM to various levels by addition of aliquots of CaCl$_2$ solution. To, mean open time; Tc, mean closed time. B, the relationships between open probability and pCa of single RyR2-WT (solid circles) and the EF-Del mutant (open squares). The data points shown are mean ± S.E. (n = 6). C, HEK293 cells were transfected with RyR2 WT and the EF-Del mutant from the same amount of transfected cell lysates using anti-RyR antibody (34c) and anti-β-actin antibody. The expression levels of RyR2 WT and the EF-Del mutant were normalized to that of β-actin. Data shown are mean ± S.E. (n = 3). *, p < 0.05 versus WT.

FIGURE 4. Deletion of the EF-hand domain has no effect on the cytosolic Ca$^{2+}$ activation of single RyR2 channels. A, single-channel activities of EF-Del were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM Hepes (pH 7.4). The cytosolic Ca$^{2+}$ concentration was increased from 45 nM to various levels by addition of aliquots of CaCl$_2$ solution. To, mean open time; Tc, mean closed time. B, the relationships between open probability and pCa of single RyR2-WT and five EF-Del single channels.

The Effect of Mutations in the EF-hand Domain on the Threshold for SOICR Activation and Termination—To further investigate the role of the EF-hand domain in SOICR, we determined the activation and termination thresholds for SOICR in HEK293 cells expressing the RyR2 WT or EF-hand mutants. The SOICR activation and termination thresholds were assessed by monitoring the ER luminal Ca$^{2+}$ dynamics using a FRET-based ER luminal Ca$^{2+}$-sensing protein, D1ER (25, 26). As shown in Fig. 7, increasing the extracellular Ca$^{2+}$ concentration from 0 to 2 mM induced spontaneous ER Ca$^{2+}$ oscillations in RyR2 WT-expressing HEK293 cells (shown as downward deflections of the FRET signal), as reported previously (26, 28, 29). SOICR occurred when the ER luminal Ca$^{2+}$ content reached a threshold level (F$\text{SOICR}$) and terminated when the ER luminal Ca$^{2+}$ content decreased to another threshold level (F$\text{terminal}$) (Fig. 7A). Consistent with their effect on SOICR propensity, we found that the D4036A/D4038A or E4071A/D4073A mutation did not significantly alter the activation threshold for SOICR (D4036A/D4038A, 92.8% ± 0.3%; E4071A/D4073A, 94.4% ± 0.3%; WT, 93.2% ± 0.7%). However, the E4071A/D4073A mutation in EF2, but not the D4036A/D4038A mutation in EF1, increased the termination threshold for SOICR (D4036A/D4038A, 60.9% ± 1.8% versus WT, 60.6% ± 2.3% and E4071A/D4073A, 66.9% ± 1.0% versus WT, 60.6% ± 2.3%; p < 0.05). The fractional Ca$^{2+}$ release in E4071A/D4073A

mm) (Fig. 5, A and C). However, under the same conditions, single EF-Del mutant channels were hardly activated by luminal Ca$^{2+}$ (Fig. 5, B and C). Therefore, the EF-Del mutation dramatically decreases the sensitivity of single RyR2 channels to activation by luminal Ca$^{2+}$.

The Effect of Mutations in the EF-hand Domain on Spontaneous Ca$^{2+}$ Release in HEK293 Cells—RyR2-mediated Ca$^{2+}$ release can occur spontaneously during store Ca$^{2+}$ overload (18, 22–24). To determine whether the EF hand Ca$^{2+}$ binding motifs play a role in this spontaneous Ca$^{2+}$ release, also known as SOICR, we assessed the effect of the EF-hand mutations on SOICR. SOICR was induced in HEK293 cells expressing the RyR2 WT or the EF-Del by elevating the extracellular Ca$^{2+}$ concentration (0–2 mM) as described previously (22, 23). The resultant SOICR was then monitored by single-cell Ca$^{2+}$ imaging. As shown in Fig. 6, the D4036A/D4038A or E4071A/D4073A mutation had no significant effect on the propensity for SOICR (Fig. 6, A, B, C, and E). On the other hand, deletion of the EF-hand domain substantially suppressed SOICR (Fig. 6, D and E). Therefore, the EF-hand Ca$^{2+}$ binding domain is an important determinant of SOICR.
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(28.2% ± 0.7%, \( p < 0.05 \)) but not D4036A/D4038A (32.0% ± 1.5%) mutant cells was also significantly different from that of WT cells (32.7% ± 0.16%) (Fig. 7, D–F). It should be noted that there was no significant difference in the store capacity (\( F_{\text{max}} - F_{\text{min}} \)) between RyR2-WT and the EF-hand mutant cells (Fig. 7G).

To be able to determine the SOICR activation and termination thresholds in EF-Del mutant cells that show little SOICR, we enhanced the occurrence of SOICR in these cells by applying low concentrations of caffeine. We have shown previously that caffeine sensitizes RyR2 to SOICR (30, 31). As shown in Fig. 8, HEK293 cells expressing the EF-Del mutant displayed SOICR in the presence of 0.5 mM caffeine. Importantly, the activation and termination thresholds for SOICR in HEK293 cells expressing the EF-Del mutant were significantly higher than those in WT-expressing HEK293 cells under the same conditions (i.e. in the presence of 0.5 mM caffeine). The activation thresholds were 77.8% ± 1.3% for the WT and 94.0% ± 1.2% for EF-Del (\( p < 0.001 \)). The termination thresholds were 46.5% ± 1.4% for the WT and 55.9% ± 0.8% for EF-Del (\( p < 0.001 \)) (Fig. 8, A–D). The fractional \( \text{Ca}^{2+} \) release in EF-Del cells (38.1% ± 1.1%) was also significantly higher than in WT cells (31.3% ± 0.6%) (\( p < 0.001 \)) (Fig. 8F). There was no significant difference in store capacity between RyR2 WT and EF-Del mutant cells (Fig. 8F). It should be noted that SOICR did not occur in control HEK293 cells expressing no RyR2 and that SOICR was not affected by the IP3R inhibitor xestospongin C (28), indicating that SOICR is mediated by RyR2. Therefore, these data indicate that the EF-hand \( \text{Ca}^{2+} \) binding domain of RyR2 plays an important role in the activation and termination of SOICR.

Discussion

The activation of RyR2 by cytosolic \( \text{Ca}^{2+} \) is a critical step in the mechanism of CICR that underlies excitation-contraction coupling in cardiac muscle (1, 2). Despite its significant role in excitation-contraction coupling, the molecular basis of RyR2 activation by cytosolic \( \text{Ca}^{2+} \) remains largely unknown. On the basis of the near-atomic resolution three-dimensional structure of RyR1, it has been proposed recently that the EF-hand \( \text{Ca}^{2+} \) binding domain (Fig. 9) encompasses a \( \text{Ca}^{2+} \) sensor or conformational switch that is important for the activation of RyR1 by cytosolic \( \text{Ca}^{2+} \) (13–15). However, the significance of the EF-hand domain in the cytosolic \( \text{Ca}^{2+} \) activation of the RyR2 channel is unclear. In this study, we assessed the role of the RyR2 EF-hand domain in the \( \text{Ca}^{2+} \) activation of the channel. Our data indicate that the EF-hand \( \text{Ca}^{2+} \) binding domain is not required for cytosolic \( \text{Ca}^{2+} \) activation of the RyR2 channel.

The role of the EF-hand \( \text{Ca}^{2+} \) binding domain in the function of the RyR1 channel has been investigated previously (8, 10–12). Disruption of the EF1 or EF2 \( \text{Ca}^{2+} \) binding motif did not abolish the \( \text{Ca}^{2+} \) activation of single RyR1 channels or the \( \text{Ca}^{2+} \) dependence of \([^{3}\text{H}]\text{ryanodine binding. Consistent with} \)
these observations, we found that mutating residues that are critical for \( \text{Ca}^{2+} \) binding in the EF1 or EF2 motif or deleting the entire EF-hand \( \text{Ca}^{2+} \) binding domain of RyR2 encompassing both EF1 and EF2 did not affect the \( \text{Ca}^{2+} \) dependence of \([\text{H}]\text{ryanodine binding or the sensitivity of the single RyR2 channel to cytosolic } \text{Ca}^{2+} \) activation. Therefore, the findings from the RyR1 and RyR2 studies demonstrate that the EF-hand \( \text{Ca}^{2+} \) binding domain is not critical for the activation of RyR by cytosolic \( \text{Ca}^{2+} \).

Where then is the \( \text{Ca}^{2+} \) sensor that mediates the activation of RyR2 by cytosolic \( \text{Ca}^{2+} \)? We have shown previously that residue Glu-3987 in RyR2 is a critical element of the cytosolic \( \text{Ca}^{2+} \) activation mechanism (6). Interestingly, on the basis of the recently solved three-dimensional structure of RyR1, this Glu-3987 residue (corresponding to Glu-4032 in RyR1) is one of a cluster of negatively charged residues that may potentially contribute to the formation of a \( \text{Ca}^{2+} \) binding pocket (15). This putative \( \text{Ca}^{2+} \) binding pocket is located in the central domain next to the COOH-terminal domain that is directly connected to the S6 helix (the gate) of the channel (Fig. 9) (15). Interestingly, the corresponding glutamate residue (Glu-2100) in the inositol 1,4,5-trisphosphate receptor (IP3R1) has also been shown to play a critical role in cytosolic \( \text{Ca}^{2+} \) activation of the IP3R1 channel (32, 33). Furthermore, the three-dimensional structure of the domain that encompasses this critical glutamate residue is highly conserved in RyR1 and IP3R1 (34). On the other hand, the corresponding EF-hand motifs are absent in IP3R1 (34). Therefore, it appears that cytosolic \( \text{Ca}^{2+} \) activation of RyR1 and IP3R1 does not involve EF-hand \( \text{Ca}^{2+} \) binding motifs. Further comprehensive and detailed studies will be needed to determine whether the cluster of negatively charged residues near Glu-3987 in RyR2 plays any roles in RyR2 activation by cytosolic \( \text{Ca}^{2+} \).

Although the EF-hand \( \text{Ca}^{2+} \) binding domain is not required for RyR2 activation by cytosolic \( \text{Ca}^{2+} \), it plays an important role in RyR2 activation by luminal \( \text{Ca}^{2+} \) and spontaneous \( \text{Ca}^{2+} \) release upon store \( \text{Ca}^{2+} \) overload (or SOICR). We found that deletion of the RyR2 EF-hand domain dramatically suppressed luminal \( \text{Ca}^{2+} \) activation of single RyR2 channels and SOICR. Furthermore, mutations in EF2 but not in EF1 raised the threshold for SOICR termination, whereas deletion of the EF-hand domain increased both the thresholds for SOICR activation and termination. These data indicate that the EF-hand domain is involved in SOICR. However, how the EF-hand domain regulates SOICR is unknown. It has been suggested that the EF-hand \( \text{Ca}^{2+} \) binding domain (residues 4064–4210 in RyR1) and regulate the response of the channel to \( \text{Ca}^{2+} \) and calmodulin (12). We have shown recently that calmodulin modulates the termination of SOICR by increasing the SOICR termination threshold in a \( \text{Ca}^{2+} \)-dependent manner (12, 35). Therefore, it is conceivable that the EF-hand \( \text{Ca}^{2+} \) binding domain may regulate the activation and termination of SOICR by interfering with the action of calmodulin. Alternatively, because the EF-hand \( \text{Ca}^{2+} \) binding domain is located close to the COOH-terminal domain that regulates the gating of the channel (Fig. 9), deletion of the entire EF-hand domain may result in a large conformational/structural change in this region. Therefore, the impaired luminal \( \text{Ca}^{2+} \) regulation of RyR2 and SOICR observed in the EF-hand deletion mutant could be due to alterations in the secondary structure of RyR2 as a result of the deletion. Clearly, future studies are required to understand the roles of the EF-hand \( \text{Ca}^{2+} \) binding domain in the function of the RyR channel.
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**FIGURE 7.** The effect of EF-hand mutations on SOICR activation and termination thresholds. A–C, stable, inducible HEK293 cell lines expressing RyR2 WT (A), D4036A/D4038A (B), and E4071A/D4073A (C) were transfected with the FRET-based ER luminal Ca\(^{2+}\)-sensing protein D1ER 48 h before single-cell FRET imaging. Expression of the RyR2 WT and mutants was induced 24 h before imaging. The cells were perfused with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0–2 mM) to induce SOICR. This was followed by the addition of 1.0 mM tetracaine to inhibit SOICR and then 20 mM caffeine to deplete the ER Ca\(^{2+}\) stores.

D and E, FRET recordings from representative cells (total 48–84 cells each) are shown. To minimize the influence of cyan fluorescent protein/yellow fluorescent protein cross-talk, we used relative FRET measurements to calculate the activation threshold (D) and termination threshold (E) using the equations shown in A. \(F_{\text{SOICR}}\) indicates the FRET level at which SOICR occurs, whereas \(F_{\text{termi}}\) represents the FRET level at which SOICR terminates. \(F_{\text{max}}\) is defined as the FRET level after tetracaine treatment. \(F_{\text{min}}\) is defined as the FRET level after caffeine treatment. The data shown are mean ± S.E. (n = 3).

**FIGURE 8.** Deletion of the EF-hand domain increases the SOICR activation and termination thresholds. A and B, HEK293 cells expressing RyR2 WT (A) and the EF-Del mutant (B) were transfected with the FRET-based ER luminal Ca\(^{2+}\)-sensing protein D1ER, and the expression of RyR2 WT and mutants was induced 24 h before imaging. The cells were perfused with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0–2 mM) and 0.5 mM caffeine to induce SOICR. This was followed by the addition of 1.0 mM tetracaine to inhibit SOICR and then 20 mM caffeine to deplete the ER Ca\(^{2+}\) stores. Note that a low dose of caffeine (0.5 mM) was used to increase SOICR and the number of oscillating cells in the EF-Del mutant cells. C–F, we used relative FRET measurements to calculate the activation threshold (C), termination threshold (D), the fractional Ca\(^{2+}\) release (E), and the store capacity (F). Single-cell luminal Ca\(^{2+}\) recordings from representative WT cells (92 cells) and EF-Del mutant (74 cells) are shown. The data shown are mean ± S.E. (n = 5). *, \(p < 0.001\) versus WT.
We have shown previously that altered SOICR is a common defect of RyR2 mutations associated with cardiac arrhythmias and cardiomyopathies (3). For instance, RyR2 mutations linked to catecholaminergic polymorphic ventricular tachycardia have been shown to reduce the threshold for SOICR activation (22, 23), whereas RyR2 mutations linked to idiopathic ventricular fibrillation increase the SOICR activation threshold (36). Furthermore, RyR2 mutations linked to hypertrophic cardiomyopathies or dilated cardiomyopathies affect the threshold of SOICR termination (28). The role of the EF-hand domain in the pathogenesis of cardiac arrhythmias and cardiomyopathies is currently unknown. Given the role of SOICR in disease and the potential role of the EF-hand domain in SOICR, it is likely that naturally occurring mutations in the EF-hand domain would be associated with cardiac abnormalities. Consistent with this view, the EF-hand domain encompasses a mutation, E4076K, that is associated with catecholaminergic polymorphic ventricular tachycardia (37). However, the effect of this E4076K mutation on RyR2 function has yet to be defined.

It should be noted that HEK293 cells do not express many cardiospecific proteins. Therefore, the HEK293 cell heterologous expression system was employed in this study to investigate the intrinsic properties of structure and function of the RyR2 channel. Our data show that the EF-hand domain is not required for the cytosolic Ca\(^{2+}\) activation of isolated, HEK293 cell-expressed RyR2 channels. The role of the EF-hand domain in the cytosolic Ca\(^{2+}\) activation of RyR2 in the context of cardiac cell environment has yet to be determined.

In summary, we show that the EF-hand Ca\(^{2+}\) binding domain of RyR2 does not mediate the activation of the RyR2 channel by cytosolic Ca\(^{2+}\). On the other hand, the EF-hand Ca\(^{2+}\) binding domain is important for luminal Ca\(^{2+}\) activation of RyR2 and the activation and termination of store overload-triggered spontaneous Ca\(^{2+}\) release or SOICR. The molecular mechanism by which the EF-hand domain regulates RyR2 and Ca\(^{2+}\) release has yet to be determined.

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