Ca\textsuperscript{2+} Inactivation Sites Are Located in the COOH-terminal Quarter of Recombinant Rabbit Skeletal Muscle Ca\textsuperscript{2+} Release Channels (Ryanodine Receptors)*

Guo Guang Du‡ and David H. MacLennan§

From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario M5G 1L6, Canada

Ca\textsuperscript{2+} activation of skeletal (RyR1) and cardiac (RyR2) muscle Ca\textsuperscript{2+} release channels (ryanodine receptors) occurs with EC\textsubscript{50} values of about 1 \(\mu\text{M}\). Ca\textsuperscript{2+} inactivation occurs with an IC\textsubscript{50} value of about 3.7 \text{mM} for RyR1, but RyR2 shows little inactivation, even at >100 \text{mM} Ca\textsuperscript{2+}. In an attempt to localize the low affinity Ca\textsuperscript{2+} binding sites responsible for Ca\textsuperscript{2+} inactivation in RyR1, chimeric RyR1/RyR2 molecules were constructed. Because \(\text{[3H]}\)ryanodine binds only to open channels, and because channel opening and closing are Ca\textsuperscript{2+}-dependent, the Ca\textsuperscript{2+} dependence of \(\text{[3H]}\)ryanodine binding was used as an indirect measurement of Ca\textsuperscript{2+} release channel opening and closing. IC\textsubscript{50} values for \(\text{[3H]}\)ryanodine binding suggested that Ca\textsuperscript{2+} affinity for the low affinity Ca\textsuperscript{2+} inactivation sites was unchanged in a chimera in which a glutamate-rich sequence (amino acids 1743–1964) in RyR1 was replaced with the corresponding, less acidic sequence from RyR2. Ca\textsuperscript{2+} affinity (IC\textsubscript{50}) for low affinity Ca\textsuperscript{2+} inactivation sites was intermediate in RyR1/RyR2 chimeras containing RyR2 amino acids 3726–4186 (RF9), 4187–4628 (RF10), or 4629–5037 (RF11), was closer to RyR2 values in RyR1 chimeras with three other sequences (3726–4186 (F9), 4187–4628 (F10), and 4629–5037 (F11)) in RyR1 were replaced separately and in combinations. These data suggest that multiple low affinity Ca\textsuperscript{2+} binding sites or multiple components of a low affinity Ca\textsuperscript{2+} binding site are located between amino acids 3726 and 5037 and that their effects on Ca\textsuperscript{2+} inactivation of the release channel are cooperative. Measurement of Ca\textsuperscript{2+} activation of \(\text{[3H]}\)ryanodine binding showed that chimeras RF10, RF9/10, and RF9/10/11 were more sensitive to Ca\textsuperscript{2+} than was either RyR1 or RyR2. Measurement of caffeine activation of Ca\textsuperscript{2+} release in vivo showed that chimeras RF9, RF10, RF9/10, RF9/10/11, and RF9/10/11 were more sensitive to caffeine than wild-type RyR1. These results suggest that Ca\textsuperscript{2+} and caffeine activation sites also involve COOH-terminal sequences in RyR1 and RyR2.

Ca\textsuperscript{2+} release channels from the sarcoplasmic reticulum of skeletal and cardiac muscle (ryanodine receptors, RyRs) are modulated by endogenous and exogenous modulators such as ATP, Ca\textsuperscript{2+}, calmodulin, Mg\textsuperscript{2+}, ruthenium red, and ryanodine (1, 2). The Ca\textsuperscript{2+} release channels from both skeletal (RyR1) and cardiac (RyR2) muscles are activated by micromolar Ca\textsuperscript{2+}, but only RyR1 is inactivated by millimolar Ca\textsuperscript{2+} (1–8). \(\text{[3H]}\)Ryanodine binds only to activated Ca\textsuperscript{2+} release channels, making \(\text{[3H]}\)ryanodine binding a useful assay for the activation state of the channels (1, 2). High concentrations of Ca\textsuperscript{2+}, which inhibit \(\text{[3H]}\)ryanodine binding to CHAPS-solubilized recombinant RyR1, do not inhibit \(\text{[3H]}\)ryanodine binding to CHAPS-solubilized recombinant RyR2 under identical conditions (9). These results suggest that RyR1 has high affinity Ca\textsuperscript{2+} binding sites for Ca\textsuperscript{2+} activation and low affinity Ca\textsuperscript{2+} binding sites for inactivation of channel function, whereas RyR2 has only high affinity sites for Ca\textsuperscript{2+} activation (1, 7, 9).

The location of the low affinity Ca\textsuperscript{2+} binding site(s) in RyR1 is not known. A glutamate-rich sequence lying between residues 1872 and 1923 (D3) is a potential low affinity Ca\textsuperscript{2+} binding site, based on the amino acid sequence deduced from a cDNA sequence (10). This potential Ca\textsuperscript{2+} binding site is also one of the three most divergent sequences between RyR1 and RyR2, which include RyR1 amino acids 1342–1403 (D2) and 4254–4631 (D1) (11). Three regions in the COOH terminus of RyR1 and two regions in the middle of RyR2 score highly as potential EF-hand structures for high affinity Ca\textsuperscript{2+} binding (10, 12–14). Two potential EF-hand sequences detected in lobster RyR1 were shown to have homology with similar sequences in mammalian RyR1 and RyR2 and were proposed to be involved in Ca\textsuperscript{2+} inactivation (15).

We have explored the question of whether the glutamate-rich region (D3) and the COOH-terminal region are responsible for Ca\textsuperscript{2+} inactivation in RyR1 by constructing a series of RyR1/RyR2 chimeras in which the glutamate-rich D3 sequence and three other sequences (3726–4186 (F9), 4187–4628 (F10), and 4629–5037 (F11)) in RyR1 were replaced separately and in groups by the corresponding sequences in RyR2. We tested the Ca\textsuperscript{2+} dependence of high affinity \(\text{[3H]}\)ryanodine binding to the chimeras, and we measured \textit{in vivo} Ca\textsuperscript{2+} release induced by caffeine in Ca\textsuperscript{2+} photometry. We found that the low affinity Ca\textsuperscript{2+} inactivation site is not affected by exchange of the D3 sequence but that Ca\textsuperscript{2+} inactivation is affected to different degrees by multiple exchanges of fragments at the COOH terminus of RyR1. Our results suggest that multiple Ca\textsuperscript{2+} inactivation sites or multiple components of a single Ca\textsuperscript{2+} inactivation site in RyR1 are located between amino acids 3726 and 5037.
EXPERIMENTAL PROCEDURES

Materials—Pfu DNA polymerase, restriction endonucleases, and other DNA-modifying enzymes were from Stratagene, Roche Molecular Biochemicals, New England Biolabs, Promega, and Amersham Pharmacia Biotech; Fura-2 acetoxy methyl ester was from Molecular Probes; caffeine and protease inhibitors were from Sigma; [3H]ryanodine was from NEN Life Sciences Products; unlabelled ryanodine was from Calbiochem-Nova Biosciences; and phosphatidylcholine was from Avanti Polar Lipids. The expression vector pcDNA3.I(-) was from Invitrogen. Monoclonal antibody 34C (mAb 34C) was a kind gift from Dr. Judith Airey (22). All other reagents were of reagent grade or highest grade available.

Construction of Full-length Chimeric RyR cDNA for Expression—The methods for expression of cDNAs encoding rabbit skeletal muscle RyR1 and cardiac muscle RyR2 were described previously (9, 23). The boundaries used in construction of chimeric RYR cDNAs from RYR1 and RYR2 are outlined in Fig. 1. The three regions most divergent in amino acid sequence between RyR1 and RyR2 are labeled as D1–D3 (11) in Fig. 1. Case 9, lying between NdeI and NheI in a modified RYR1 cDNA (25) and encoding amino acids 3726–4186, is designated F9; part of case 10, lying between NheI and XclI in a modified RYR1 cDNA (25) and encoding amino acids 4186–4628, which encompass the D1 region, is designated F10; the remainder of case 10 plus cassette 11, lying between XclI and HindIII and encoding amino acids 4629–5037, is designated F11. RYR2 fragments were amplified using a Pfu polymerase-based polymerase chain reaction in which restriction endonuclease sites were introduced at each end (NdeI-NheI for F9, NheI-XclI for F10, XclI-HindIII for F11). These fragments were inserted into their respective positions in pBS-RF9 to form pBS-RF9, pBS-RF10, and pBS-RF11. The chimeric constructs were subcloned into pcDNA3.I(-) with XbaI and HindIII to obtain pcDNA-RF9, pcDNA-RF10, and pcDNA-RF11. To obtain RF9/10 and RF10/11, the F10 fragment from pBS-RF10 was excised and inserted into pBS-RF9 or pBS-RF11 with NheI-XclI, and the full-length chimeric cDNAs were inserted into pcDNA3.I(-) with XbaI and HindIII to form pcDNA-RF9/10 and pcDNA-RF10/11. To obtain RF9/11, which is a natural, complete NdeI restriction endonuclease site at nucleotide 11170 in RYR1 and 11072 in RYR2 cDNA, we exchanged the RYR1 sequence between amino acids 3726 and 5037 with the corresponding RYR2 sequence (amino acids 3692 to 4969) to construct chimera RF9/10/11. The NdeI restriction endonuclease site that is present in RYR1 at nucleotide 11170 and an NdeI restriction endonuclease site at nucleotide 11072 in RYR1 and RYR2 cDNA. We exchanged the RYR1 sequence between amino acids 3726 and 5037 with the corresponding RYR2 sequence (amino acids 3692 to 4969) to construct chimera RF9/10/11. The NdeI (11072–Clal) fragment from plasmid pBS-CRR (9), encoding amino acid residues 3692 and 4969, was excised and inserted into pBS-RF11 cleaved with NdeI-Clal. The entire chimera was then cleaved with XbaI and NheI and inserted into the XbaI site of pcDNA3.I(-) to form pcDNA-RF9/10/11. The construction of the chimera involving the D3 region was carried out in RF9 cDNA cassette 5 (MluI-SpeI). The sequence in RF9 between MluI and Eco47III, encoding amino acids 1743–1964 (designated as F5a) was replaced with the corresponding nucleotide sequence encoding amino acids 1734–1931 from RYR2. The fragment was amplified by a Pfu DNA polymerase-based polymerase chain reaction, with the introduction of MluI and Eco47III restriction endonuclease sites on the ends. The chimera cassette 5 was inserted into the corresponding region of pBS-RF11 to form pBS-RF12F5a with MluI and SpeI, and the entire chimeric cDNA sequence was then excised and inserted into pcDNA3.I(-) with XbaI and HindIII. These chimeric inserts were confirmed by DNA sequencing and restriction enzyme digestion mapping.

Cell Culture and DNA Transfection—Culture of HEK-293 cells and cDNA transfection by the calcium phosphate precipitation method (26) was carried out as described previously (24).

Fluorescence Measurements—A microfluorimetry system (Photon Technologies, Inc.) was used to monitor the Fura-2 acetoxy methyl ester fluorescence changes in transiently transfected or nontransfected HEK-293 cells, as described previously (9).

Solubilization of Transfected HEK-293 Cells and Measurement of [3H]Ryanodine Binding—Transfected HEK-293 cells grown in 100-mm Petri dishes were solubilized with 1% CHAPS and 5 mM MgCl2, 0.2 mM EGTA, 50 mM HEPES, pH 7.1, a protease inhibitor mixture (9), and various concentrations of [3H]ryanodine. Nonspecific binding was determined using a 1000-fold excess of unlabelled ryanodine. After 2 h at 37 °C, the 0.25-nl samples were diluted with 1 ml of ice-cold washing buffer composed of 25 mM HEPES, pH 7.1, and 0.25 mM KCl and placed on Whatman GF/B membrane filters pre-soaked with 1% polyethyleneimine in washing buffer. Filters were washed three times with 6 ml of washing buffer. The radioactivity remaining on the filters was determined by liquid scintillation counting to determine the amount of [3H]ryanodine bound to the filter. All binding assays were carried out in duplicate. To assess the effects of caffeine on high affinity ryanodine binding, protocols were modified by removal of ATP from the binding buffer and addition of different concentrations of Ca2+ with 2.5 mM [3H]ryanodine. Free Ca2+ was calculated using the apparent binding constants described by Fabiato (27). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immuno blotting—About 30 μg of proteins from cells lysed with CHAPS were separated by 5% SDS-polyacrylamide gel electrophoresis (28). RyR1 epitopes were detected by immuno blotting (29), as described previously (23).

Protein Assay—Protein concentration was determined by dye binding using bovine serum albumin as a standard (30).

Data Analysis—All data were analyzed with Microcal Origin software (Microcal Software Ltd., Northampton, MA). Scatchard analysis was used to determine the dissociation constant (Kd) and maximal binding capacity (Bmax) from equilibrium binding data. EC50 or IC50 values were obtained by fitting the curves with an equation for logistic dose response. Data are expressed as mean ± S.E. An unpaired Student's t test was used for evaluation of the mean values between groups. A value of p < 0.05 was considered to be statistically significant.

RESULTS

Transient Expression of Chimeric RyR cDNAs—In earlier studies, we showed that the curve of Ca2+ dependence for [3H]ryanodine binding to recombinant RyR1 is bell-shaped, indicating that [3H]ryanodine binding is sensitive to both Ca2+ activation and inactivation of Ca2+ release channel function. By contrast, the curve of Ca2+ dependence of [3H]ryanodine binding for recombinant RyR2 did not display a falling phase, indicating that [3H]ryanodine binding is sensitive to Ca2+ activation but resistant to Ca2+ inactivation of Ca2+ release channel function (9). Because we were interested in the structural basis for this difference in Ca2+ inactivation, we constructed several chimeric RyR proteins by replacing the glutamate-rich D3 sequence and different lengths of the COOH terminus of RyR1 with the corresponding sequences from RyR2 (Fig. 1). These constructs were then expressed transiently in HEK-293 cells, and the Ca2+ dependence of [3H]ryanodine binding (9) was used as an indirect measurement of Ca2+ activation and inactivation. In addition, caffeine-induced Ca2+ release was measured by in vivo Ca2+ photometry applied to HEK-293 cells transfected with the chimeric proteins.

Immunostaining of CHAPS-solubilized cell lysates, using mAb 34C against an epitope located in RyR1 amino acids 2756–2803 (29), was used to detect the expression of RyR proteins in transfected HEK-293 cells. Fig. 2 shows the absence of RyR immunostaining in pcDNA-transfected cells. Monoclonal antibody 34C stained both RyR1 and RyR2, but the similar level of staining of RyR1 and RyR2 does not reflect a difference in their levels of expression, probably because of slight differences in the epitope that binds mAb 34C. Because the chimeric proteins all retained the RyR1 epitope, immunostaining with mAb 34C can be used as a measure of RyR expression. Immunostaining demonstrated that chimeras RF10, RF11, RF9/10, RF10/11, and RF9/10/11 were all expressed at levels up to 1.5-fold higher than RyR1.

These studies illustrate the fact that RyR1 and RyR2 are not expressed with equal efficiency in mammalian cells. The studies of chimeras show that F10 and F11 are the key sequences concerned with high levels of expression in mammalian cells (Fig. 2). It is not clear whether the differences in expression are transcriptional, translational, or post-translational. It is of interest, however, that bacteria-expressing plasmids containing RyR2 cDNA grow very slowly and that the yield of DNA is low, whereas bacteria-expressing plasmids containing RyR1 cDNA grow much faster, yielding more DNA.
RyR2 moved in SDS gels with a slightly higher mobility than did RyR1. The mobility of chimeras RF10, RF9/10, RF10/11, and RF9/10/11 was also higher than that of RyR1. Thus there is a correlation between the presence of the F10 amino acid sequence and high mobility of the expressed protein.

Fluorescence Measurements of Caffeine-induced Ca2+ Release in Vivo—We used Fura-2 fluorescence to measure the properties of caffeine-induced Ca2+ release in the chimeric proteins expressed in HEK-293 cells (9). No significant Ca2+ release occurred with caffeine up to 30 mM in pcDNA-transfected cells (9), but caffeine-induced Ca2+ release was observed in transfected cells. The peak fluorescence amplitude was measured following incremental application of 0.03 to 30 mM caffeine and normalized to the peak amplitude for maximal Ca2+ release induced by 30 mM caffeine. EC50 values for the chimeras were then calculated by fitting the caffeine dose-response curves with an equation for logistic dose response. As described previously (9), EC50 values measuring the caffeine sensitivity of Ca2+ release were higher for recombinant RyR2 than for recombinant RyR1 (Fig. 3). Dose-response curves for RyR1, RyR2, and chimeric RyR1/RyR2 proteins are shown in Fig. 3, and EC50 values are summarized in the inset to Fig. 3.

Chimeras involving the D3 sequence had EC50 values for caffeine that were similar to that of RyR1. However, chimeras RF9, RF10, RF9/10, RF10/11, and RF9/10/11 had lower caffeine EC50 values, indicating that RyR1 chimeras containing RyR2 sequences F9 and F10 were more sensitive to caffeine than was wild-type RyR1. The Hill coefficient for RyR1 was 1.9, whereas the Hill coefficient for RyR2 was 1.2. The Hill coefficients for all chimeras except RF5a resembled that for RyR2 (inset to Fig. 3), indicating that each of the three RyR2 sequences can partially suppress the cooperative interactions that occur in RyR1.

High Affinity Equilibrium Binding of [3H]Ryanodine to Chimeric RyRs—We measured the equilibrium properties of [3H]ryanodine binding to the chimeric RyRs to determine whether the high affinity ryanodine binding site was preserved in these chimeras. We also used [3H]ryanodine binding to determine expression levels, because 1 mol of a tetrameric RyR molecule binds 1 mol of ryanodine with high affinity (9, 23, 31). Scatchard analysis showed a single binding site in all of the chimeras (Fig. 4). Kd values for these chimeras were similar to those for wild-type RyR2 and RyR1 (Ref. 9 and the inset to Fig. 4), ranging from 1.6 to 2.6 nM. These data indicate that the high affinity binding site for ryanodine is normal in all the chimeras. Bmax values ranged from 0.46 to 2.0 pmol/mg of protein in the chimeras (Fig. 4 and inset), reflecting different expression levels. These results show that RF10 and RF11 expression was increased 4- to 5-fold over RyR1 expression, RF9/10 expression was increased 3-fold, and RF10/11 and RF9/10/11 expression was increased 10-fold, confirming results from immunoblotting. As reported previously (9), there was no significant binding in lysates isolated from pcDNA-transfected HEK-293 cells.

Ca2+ Activation of High Affinity [3H]Ryanodine Binding to Chimeric RyRs—Dose-response curves for Ca2+ activation and inactivation of [3H]ryanodine binding to wild-type RyR1 and RyR2 and to chimeras are shown in Fig. 5. At Ca2+ concentrations below pCa 7, there was little binding of [3H]ryanodine to the recombinant RyR proteins. [3H]Ryanodine binding was activated by increasing Ca2+ concentrations, with maximal binding occurring between pCa 5.7 and pCa 4. EC50 values, expressed as pCa, for wild-type RyR1 and RyR2 were similar, and EC50 values for chimeras RF9, RF11, RF10/11, and RF5a did not differ from those of either RyR1 or RyR2 (Fig. 5 and Table I). However, in chimeras RF10, RF9/10, and RF9/10/11, activation of [3H]ryanodine binding was observed with lower Ca2+ concentrations, and EC50 values were significantly higher than those for wild-type RyR1 and RyR2 (Fig. 5 and Table I). These results suggest that chimeras RF10, RF9/10, and RF9/10/11 are more sensitive to Ca2+ activation.

Ca2+ Inactivation of High Affinity [3H]Ryanodine Binding to Chimeric RyRs—Ca2+ inactivation was studied indirectly through measurement of the inhibition of [3H]ryanodine binding by elevated Ca2+. IC50 values, expressed in terms of pCa, are also presented in Table I and illustrated in Fig. 5. The difference in IC50 between RyR1 (pCa 2.43) and RyR2 (pCa 0.12) was very large. The IC50 for chimera RF5a in which the D3 sequence of RyR2 replaced the corresponding region in RyR1 did not differ from wild-type RyR1. This experiment, together with a previously published analysis of deletions (32), effectively rules out any involvement of the D3 sequence in Ca2+ inactivation.

The pattern of Ca2+ inactivation of [3H]ryanodine binding was different, however, for chimeric mutants at the COOH terminus. IC50 values were reduced, when compared with
RyR1, for each of the chimeras RF9 (pCa 1.46), RF10 (pCa 1.56), and RF11 (pCa 1.83) in which individual RyR1 sequences were replaced with RyR2 sequences. The IC_{50} values were reduced still further for chimeras RF9/10 (pCa 0.26) and RF10/11 (pCa 0.24) in which two adjacent sequences were replaced. In the chimera in which all three RyR1 COOH-terminal sequences were replaced with the corresponding RyR2 sequences, the IC_{50} value was not significantly different from wild-type RyR2 (pCa 0.14).

The slopes for the curves of inactivation could be divided into two groups. For RyR1 and chimeras RF9, RF10, RF11, and RF5a, the slope of Ca^{2+} inactivation ranged between 0.8 and 1.1. For RyR2 and chimeras RF9/10, RF10/11, and RF9/10/11, slopes of inactivation ranged between 0.1 and 0.4. These data indicate that each of the three sequences tested made a contribution to Ca^{2+} inactivation. This may occur because several low affinity Ca^{2+} binding sites exist that act cooperatively, or because a single low affinity Ca^{2+} binding site exists that is composed of elements contained in several sequences throughout the COOH-terminal region.

Our studies did not permit analysis of the topology of the Ca^{2+} inactivation sites, because they were carried out in the presence of detergent. We examined the question of whether similar results would be obtained if we measured [3H]ryanodine binding to microsomal vesicles. We recognized, however, that the conditions of the experiment (2 h at 37 °C) would lead to significant equilibration of Ca^{2+} across the membrane. [3H]Ryanodine binding to microsomal vesicles from recombinant RyR1, RyR2, RF10, RF11, and RF5a, the slope of Ca^{2+} inactivation ranged between 0.8 and 1.1. For RyR2 and chimeras RF9/10, RF10/11, and RF9/10/11, slopes of inactivation ranged between 0.1 and 0.4. These data indicate that each of the three sequences tested made a contribution to Ca^{2+} inactivation. This may occur because several low affinity Ca^{2+} binding sites exist that act cooperatively, or because a single low affinity Ca^{2+} binding site exists that is composed of elements contained in several sequences throughout the COOH-terminal region.

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Ca\textsuperscript{2+} Inactivation in Chimeric Ryanodine Receptors

**DISCUSSION**

Ca\textsuperscript{2+} Inactivation—As reported previously, \[^{3}H\]ryanodine binding by RyR1 is decreased at Ca\textsuperscript{2+} concentrations higher than pCa 4 but remains elevated up to pCa 1.5 in RyR2 (Ref. 9 and Fig. 5, A and B), indicating that RyR2 is not sensitive to Ca\textsuperscript{2+} inactivation and does not have the low affinity Ca\textsuperscript{2+} binding site(s) responsible for inactivation at high concentrations of Ca\textsuperscript{2+} (1, 7). We have attempted to characterize and locate these low affinity Ca\textsuperscript{2+} binding sites in RyR1 by constructing chimeric RyRs in which various sequences are replaced by the corresponding sequence in RyR2. Because it is widely recognized that the inhibition of \[^{3}H\]ryanodine binding...
by high concentrations of Ca\(^{2+}\) is a consequence of occupation of the low affinity, inactivating Ca\(^{2+}\) binding sites (1, 2), we have used \(^{3}H\)ryanodine binding as an indirect assay of Ca\(^{2+}\) inactivation of the channel to show that these sites are located in the COOH-terminal end of RyR1.

Our studies of RyR1/RyR2 chimeras have allowed us to map the low affinity Ca\(^{2+}\) binding sites to three adjacent regions lying between amino acids 3726 and 5037 at the COOH terminus of RyR1. This mapping is in agreement with earlier observations that there are multiple Ca\(^{2+}\) binding sites and that these sites are located in the COOH terminus of RyR1 (19, 33). These sites are probably interactive because replacement of one or even two of these sequences did not result in full insensitivity to high concentrations of Ca\(^{2+}\). We could not, however, rule out the possibility that a single low affinity Ca\(^{2+}\) binding site in the tetrameric molecule is made up of multiple components from these three fragments. Among these three fragments, it is evident that the F9 and F10 fragments in RyR1 contribute most strongly to Ca\(^{2+}\) inactivation (Fig. 5).

Earlier studies have shown that several Ca\(^{2+}\) binding sites exist in the COOH-terminal sequences. A \(^4^{39}\)Ca\(^{2+}\) and ruthenium red binding fragment, composed of amino acids 3657–3776 (18), overlaps partially with F9, composed of amino acids 3726–4186. Two EF-hand Ca\(^{2+}\) binding sites have been identified in lobster RyR, and these correspond to RyR1 amino acids 4073–4135 (15). In F10, composed of amino acids 4187–4628, three sequences, amino acids 4246–4267, 4382–4417, and 4478–4512, bind \(^4^{39}\)Ca\(^{2+}\) and ruthenium red (16–18). In F11, composed of amino acids 4629–5037, amino acids 4765–5037 bind \(^4^{39}\)Ca\(^{2+}\) and ruthenium red (18). Most of these sequences are hydrophilic, even though F11 has several hydrophobic sequences. Antibodies against the sequence between amino acids 4478 and 4512 bind to both denatured and native forms of the ryanodine receptor, indicating that the sequence containing the Ca\(^{2+}\) binding site is detectable on the surface and is hydrophilic. Because these sequences bind Ca\(^{2+}\) in the millimolar range and because RyR1 is activated by micromolar Ca\(^{2+}\) and inhibited by millimolar Ca\(^{2+}\), these sequences are likely to form low affinity Ca\(^{2+}\) binding sites and may play a role in the Ca\(^{2+}\) inactivation of RyR1.

The properties of low affinity Ca\(^{2+}\) binding sites in RyR1 have been investigated using terbium (Tb\(^{3+}\)) fluorescence (33). As a Ca\(^{2+}\) analog, Tb\(^{3+}\) replaces Ca\(^{2+}\) in both the high affinity and low affinity binding sites. At low concentrations, Tb\(^{3+}\) activates single channels in lipid bilayers. At higher concentrations, Tb\(^{3+}\) binds to two or more low affinity Ca\(^{2+}\) binding sites and causes an inhibition of \(^{3}H\)ryanodine binding, a strongly enhanced green fluorescence at 543 nm, and an inhibition of single channel activity, indicating that Tb\(^{3+}\) binding to the low affinity Ca\(^{2+}\) sites results in conformational changes in the protein and in closure of the channel. This knowledge has not yet led to the identification of the low affinity Ca\(^{2+}\) binding sites.

A possible location of the low affinity Ca\(^{2+}\) binding sites has been deduced from studies using dicyclohexylcarbodiimide (19), a carboxyl modifying compound, which binds to hydrophobic sequences, usually those forming transmembrane helices. Because dicyclohexylcarbodiimide modifies both low affinity and high affinity Ca\(^{2+}\) binding sites, these sites are likely to be located in the COOH terminus of RyR1, where the transmembrane sequences are concentrated (19). Single-channel recordings in planar lipid bilayers showed that an increase in luminal Ca\(^{2+}\) from micromolar to millimolar decreased channel activities, suggesting that luminal Ca\(^{2+}\) could pass through the Ca\(^{2+}\) release channel and then regulate the channel (34). From these studies, it was concluded that the Ca\(^{2+}\) inactivation sites were very close to the Ca\(^{2+}\) release site, implying that the inactivation sites are those sequences located around the aqueous pore of the channel.

The D3 sequence between amino acids 1872 and 1923 in RyR1 has been proposed as a possible low affinity Ca\(^{2+}\) binding site (10). However, constructs in which D3 and surrounding sequences were deleted formed channels that retained Ca\(^{2+}\) inactivation (32). Replacement of the D3 sequence in RyR1 with the corresponding sequence in RyR2 in this study did not alter the Ca\(^{2+}\) dependence of \(^{3}H\)ryanodine binding from that of wild-type RyR1. These data, when taken together, strongly argue that the D3 sequence is not the Ca\(^{2+}\) inactivation site.

**Ca\(^{2+}\) and Caffeine Activation**—In this and a previous study (9), we demonstrated that recombinant RyR2 is more sensitive to caffeine-induced Ca\(^{2+}\) release than is recombinant RyR1, although both had a similar sensitivity to Ca\(^{2+}\) activation of \(^{3}H\)ryanodine binding. Similar observations have been made in studies of skeletal and cardiac muscle sarcoplasmic reticulum (1, 6–8, 35). The replacement of F9 and F10 in the COOH terminus of RyR1 with the corresponding sequence from RyR2 in this study did not alter the Ca\(^{2+}\) dependence of \(^{3}H\)ryanodine binding from that of wild-type RyR1. These data, when taken together, strongly argue that the D3 sequence is not the Ca\(^{2+}\) inactivation site.

### Table I

Ca\(^{2+}\) activation (EC\(_{50}\)) and Ca\(^{2+}\) inactivation (IC\(_{50}\)) for Ca\(^{2+}\) dependence of \(^{3}H\)ryanodine binding to RyR1, RyR2, and their chimeras

Values were obtained by fitting of the curves, from 3–4 separate experiments for each construct, as shown in Fig. 5, with the logistic dose-response equation for EC\(_{50}\) and IC\(_{50}\). Part of the data for RyR1 and RyR2 has been shown elsewhere (9).

| Construct   | EC\(_{50}\) pCa | Slope | IC\(_{50}\) pCa | Slope |
|-------------|-----------------|-------|-----------------|-------|
| RyR1        | 6.21 ± 0.08     | 2.2 ± 0.6 | 2.43 ± 0.13     | 1.1 ± 0.2 |
| RyR2        | 6.14 ± 0.12     | 2.6 ± 0.3 | 0.12 ± 0.08\(^a\) | 0.2 ± 0.1 |
| RF9         | 6.37 ± 0.03     | 1.7 ± 0.3 | 1.46 ± 0.14\(^a\) | 0.8 ± 0.1 |
| RF10        | 6.70 ± 0.05\(^a\) | 1.6 ± 0.1 | 1.56 ± 0.09\(^a\) | 1.1 ± 0.3 |
| RF11        | 6.33 ± 0.02     | 1.6 ± 0.03 | 1.83 ± 0.15\(^a\) | 0.8 ± 0.2 |
| RF9/10      | 6.59 ± 0.07\(^a\) | 1.9 ± 0.04 | 0.26 ± 0.26\(^a\) | 0.3 ± 0.3 |
| RF10/11     | 6.31 ± 0.10     | 1.8 ± 0.2 | 0.24 ± 0.14\(^a\) | 0.4 ± 0.2 |
| RF9/10/11   | 6.51 ± 0.09\(^a\) | 2.4 ± 0.3 | 0.14 ± 0.14\(^a\) | 0.1 ± 0.1 |
| RF5a        | 6.40 ± 0.20     | 1.9 ± 0.2 | 2.14 ± 0.14     | 1.1 ± 0.1 |

\(^a\) p < 0.05 when compared with RyR1.
4478–4512, inside F10, increased the Ca$^{2+}$ sensitivity for activation of the Ca$^{2+}$ release channel in a planar bilayer system by approximately 1 order of magnitude. Because Ca$^{2+}$ was still able to activate the antibody-Ca$^{2+}$ release channel complex, it did not appear to act directly against the high affinity Ca$^{2+}$ site. We proposed that the antibody was acting against a sequence adjacent to the Ca$^{2+}$ binding site or that it activated a distant Ca$^{2+}$ binding site through allosteric interactions (16). It is conceivable that this antibody bound to a Ca$^{2+}$ inactivation site, which, we now believe, lies in this region, resulting in activation of the channel or in conformational changes favoring activation of the channel. Further knowledge of the composition of the activation and inactivation sites will help to clarify the way in which these sites act to regulate the channel.

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