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Evidence for a role of C-terminus in Ca^{2+} inactivation of skeletal muscle Ca^{2+} release channel (ryanodine receptor)

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Abstract Six chimeras of the skeletal muscle (RyR1) and cardiac muscle (RyR2) Ca^{2+} release channels (ryanodine receptors) previously used to identify RyR1 dihydropyridine receptor interactions [Nakai et al. (1998) J. Biol. Chem. 273, 13403] were expressed in HEK293 cells to assess their Ca^{2+} dependence in [^{3}H]ryanodine binding and single channel measurements. The results indicate that the C-terminal one-fourth has a major role in Ca^{2+} activation and inactivation of RyR1. Further, our results show that replacement of RyR1 regions with corresponding RyR2 regions can result in loss and/or reduction of [^{3}H]ryanodine binding affinity while maintaining channel activity.

Key words: Ca^{2+} release channel; Ryanodine receptor; Skeletal muscle; Cardiac muscle; HEK293 cell

1. Introduction

Ryanodine receptors (RyRs) are Ca^{2+} channels that control the Ca^{2+} levels of cells by releasing Ca^{2+} from the sarco/endoplasmic reticulum (SR), an intracellular storage compartment. Mammalian tissues express three RyR isoforms encoded by separate genes. RyR1 and RyR2 are primarily expressed in skeletal and cardiac muscle, respectively, and in brain and other tissues at lower levels [1–3]. RyR3 is present in diaphragm and slow twitch skeletal muscles, and other tissues at low levels. RyRs consist of four large subunits, each with a molecular mass of ~560 kDa, and four small 12 kDa FK506 binding proteins [1,3–5].

The two dominant mammalian RyR1 and RyR2 isoforms are highly related proteins, however, there are some important differences in the way the two channels are regulated. One of the major differences relates to the mechanism of in vivo activation. In cardiac muscle, surface membrane dihydropyridine-sensitive (L-type) Ca^{2+} channels (DHPRs) mediate the influx of a small amount of Ca^{2+} during an action potential that opens the high-conductance SR RyR2s [6]. In contrast, in mammalian skeletal muscle, voltage-sensing surface membrane DHPRs open closely apposed SR RyR1s through direct protein interactions [7]. A second major difference between RyR1 and RyR2 relates to their response to Ca^{2+}. Both isoforms show a characteristic biphasic Ca^{2+} dependence of channel opening, indicating high affinity activation and low affinity inactivation sites [4,5]. The affinity of the inactivation site for Ca^{2+} is about 10-fold lower for RyR2 than for RyR1. A second difference is that RyR2 is nearly fully activated by Ca^{2+}, whereas RyR1 can be only partially activated by Ca^{2+} in the absence of other channel effectors.

In the present study, several RyR1/RyR2 chimeras previously used to identify RyR1-DHPR interactions [8] were expressed in human embryonic kidney (HEK293) cells. The Ca^{2+} dependence of expressed proteins was assessed in [^{3}H]ryanodine binding and single channel measurements. Our results suggest that the C-terminus has a role in regulation of RyR1 by Ca^{2+}.

2. Materials and methods

2.1. Materials

HEK293 cells were obtained from the Tissue Culture Facility of Lineberger Cancer Center at the University of North Carolina, [^{3}H]ryanodine from Dupont NEN (Boston, MA), unlabeled ryanodine from Calbiochem (La Jolla, CA), and phospholipids from Avanti Polar Lipids (Birmingham, AL). All other chemicals were of analytical grade.

2.2. Expression of RyRs

Construction of full-length RyR1 [9] and RyR2 [10] cDNAs and RyR1/RyR2 chimeras [8] has been described. RyR cDNAs were cloned into pCIneo (Promega, Madison, WI) and transiently expressed in HEK293 cells using the Lipofectamine Plus (Gibco BRL, Grand Island, NY) method according to the manufacturer's instructions. Cells were maintained in DMEM-H medium containing 10% fetal bovine serum at 37°C in 5% CO_2 and plated the day before transfection. For each 10 cm tissue culture dish, 6 μg DNA and 30 μl Lipofectamine were used. Cells were harvested 42–46 h after transfection.

2.3. Membrane preparation

Cells were washed twice with 4 ml ice-cold PBS containing 1 mM EDTA and protease inhibitors (0.2 mM Pefabloc, 100 nM aprotinin, 50 μM leupeptin, 1 μM pepstatin, and 1 mM benzamidine), and harvested in the same solution by removal from the plates by scraping. Cells were collected by centrifugation, resuspended in the above solution and homogenized with a Tekmar Tissumizer for 5 s at a setting of 13 500 rpm. Cell homogenates were centrifuged for 1 h at 100 000 × *g*, resuspended in the above solution without EDTA, pelleted and finally taken up in a buffer containing 10 mM imidazole, pH 7.0, 0.3 M sucrose, 20 μM leupeptin and 0.2 mM Pefabloc, and stored at −80°C. Rabbit skeletal and cardiac muscle SR membranes enriched in Ca^{2+} release activities were prepared as described [11].

2.4. Purification and reconstitution

Skeletal and cardiac muscle SR membranes (1 mg protein/ml) and expressed RyRs from 2–4 culture dishes were solubilized for 10 min at room temperature in 1.5 ml of a buffer containing 5 mg/ml phosphatidylcholine and 1.45% (3-[3-cholamido-propyl)dimethylammonio]-1-propanesulfonate) (CHAPS), isolated by rate density centrifugation,

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Abbreviations: HEK, human embryonic kidney; E-C, excitation-contraction; RyR1, skeletal muscle Ca^{2+} release channel (ryanodine receptor); RyR2, cardiac muscle Ca^{2+} release channel (ryanodine receptor); SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; P_o, channel open probability

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and reconstituted into proteoliposomes by removal of CHAPS by dialysis [12].

2.5. [^{3}H]Ryanodine binding

Ryanodine is a plant alkaloid that binds with high affinity and specificity to RyRs. It is widely used as an indicator of channel activity due to its preferential binding to open channels [4,5]. Unless otherwise indicated, membranes of 1/12 culture dish were incubated with 2 nM [^{3}H]ryanodine at 24°C in 100 μl of 20 mM imidazole, pH 7.0, 0.25 M KCl, 0.15 M sucrose, 0.2 mM Pefabloc, 10 μM leupeptin, and the indicated Ca^{2+} concentrations. Non-specific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 20 h, aliquots of the samples were diluted with 20 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with 3×5 ml ice-cold 0.1 M KCl, 1 mM KPIPES, pH 7.0. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

2.6. Single channel recordings

Single channel measurements were performed by fusing proteoliposomes containing the purified RyRs with Mueller-Rudin-type bilayers containing 5:3:2 phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine (25 mg phospholipid per ml *n*-decane) [11]. The side of the bilayer to which the proteoliposomes were fused was defined as the *cis* side. The *trans* side was defined as ground. Purified channels were recorded in symmetric 0.25 M KCl or asymmetric 0.4 M *cis* KCl/0.02 M *trans* KCl media containing 20 mM KHEPES, pH 7.4. Additions were made to the *cis* bilayer chamber as indicated in the text because the large cytosolic regulatory region of native channels faced the *cis* (cytosolic) chamber in a majority (>98%) of the recordings [11]. Electrical signals were filtered at 2 kHz, digitized at 10 kHz and analyzed as described previously [11].

3. Results

RyR1 and RyR2 display several significant differences relating to their regulation by Ca^{2+} (see Secton 1). We rationalized that six RyR1/RyR2 chimeras previously constructed to identify skeletal muscle RyR-DHPR interactions [8] could be used to identify regions that are important for the regulation of RyRs by Ca^{2+}. In the six RyR1/RyR2 chimeras varying regions of RyR1 are replaced with corresponding regions of RyR2 (Fig. 1). Common to all six chimeras is a RyR2 3687–4968 amino acid C-terminal region. This region is predicted to encompass the transmembrane-spanning pore region as well as part of the large N-terminal cytoplasmic foot region of RyRs [4,5].

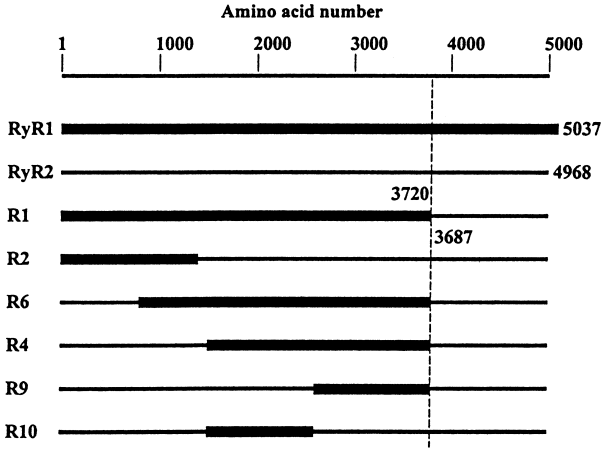

Fig. 1. Schematic representation of RyR1/RyR2 chimeras. RyR1 amino acid sequences are represented by thick lines and those of RyR2 by thin lines. Amino acid composition was as reported by Nakai et al. [8].

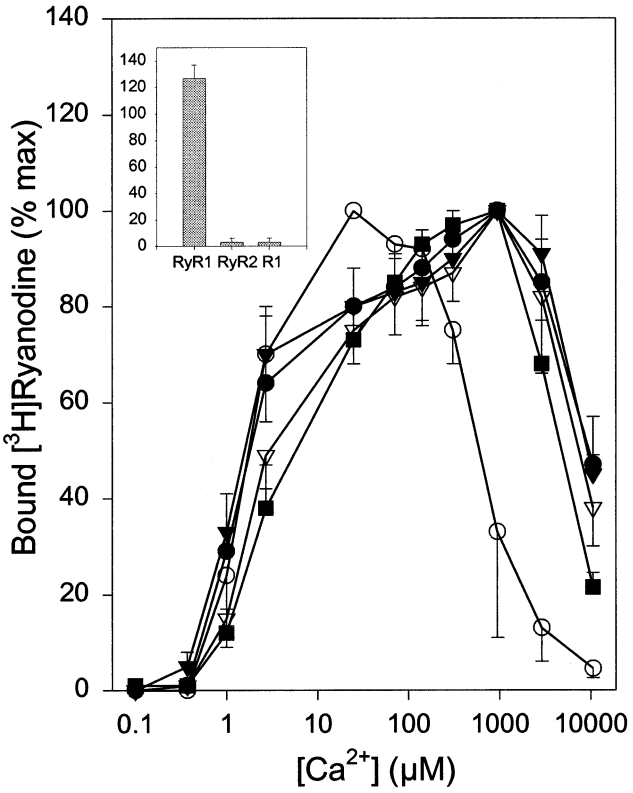

Fig. 2. [^{3}H]Ryanodine binding to recombinant full-length RyR1 and RyR2 and chimeras. Ca^{2+} dependence of specific [^{3}H]ryanodine binding to RyR1 (○), RyR2 (▽), R1 (●), R2 (▼) and R9 (■) was determined in 0.25 M KCl medium as described in Section 2. No specific [^{3}H]ryanodine binding was detected for cells transfected with the expression vector alone or R4, R6 or R10 cDNAs. Peak [^{3}H]ryanodine binding levels (100%) corresponded to 0.04 pmol/mg protein for RyR1 and 0.2–0.4 pmol/mg protein for RyR2, R1, R2 and R9. Inset: Specific [^{3}H]ryanodine binding to RyR1, RyR2 and R1 in 0.5 M choline-Cl medium containing 20 mM imidazole, pH 7.0, 4 nM [^{3}H]ryanodine, 5 mM choline AMP, 5 mM choline EGTA, 20 μM leupeptin, and 0.2 mM Pefabloc. Membranes were incubated for 44 h at 12°C. Data are expressed as % of maximum activity in KCl medium and are the means ± S.E.M. of 3–5 experiments.

3.1. [^{3}H]Ryanodine binding to RyR1/RyR2 chimeras

Response of RyR1/RyR2 chimeras to Ca^{2+} was investigated by determining the [^{3}H]ryanodine binding properties of cell membrane fractions and by carrying out single channel measurements with purified receptor preparations. HEK293 cells transfected with the full-length RyR1 and RyR2 cDNAs showed a biphasic Ca^{2+} dependence of [^{3}H]ryanodine binding typical of native receptors (Fig. 2). [^{3}H]Ryanodine binding to both receptors was negligible at 0.1 μM free Ca^{2+} and was half-maximal at ~5 μM free Ca^{2+}. Binding to RyR1 was maximal at ~20 μM free Ca^{2+} and to RyR2 at ~1 mM Ca^{2+}. As observed for native receptors [13], an ~10-fold higher Ca^{2+} concentration was required for inhibition of the recombinant RyR2 than recombinant RyR1. Cells transfected with the expression vector alone did not show specific high-affinity [^{3}H]ryanodine binding.

Membranes of cells transfected with R1, R2 and R9 chimeras showed a Ca^{2+} activation/inactivation profile similar to

the recombinant RyR2 (Fig. 2). Membranes of cells transfected with chimeras R4, R6 and R10 did not show specific high-affinity [^{3}H]ryanodine binding. Absence of [^{3}H]ryanodine binding was not due to lack of expression of the chimeras, as immunoblots indicated similar expression levels for all six chimeras (not shown).

Significant levels of [^{3}H]ryanodine binding were observed in choline-Cl media at low Ca^{2+} concentrations for skeletal but not cardiac muscle SR vesicles, which suggests that choline$^+$ is a weak Ca^{2+} agonist of RyR1 but not RyR2 [13]. The inset of Fig. 2 shows that membranes of cells expressing RyR1 bound [^{3}H]ryanodine in 0.5 M choline-Cl medium containing $< 10^{-8}$ M free Ca^{2+}, whereas cells expressing RyR2 did not. Cells transfected with R1 (Fig. 2, inset) and the other chimeras (not shown) did not show specific [^{3}H]ryanodine binding in choline media in the absence of Ca^{2+}. The results are in agreement with previous mutagenesis studies that have localized the Ca^{2+} activation site(s) to the C-terminal one-fourth of RyRs [14–16].

3.2. Single channel measurements

We also carried out single channel measurements because these provide a more direct means of determining the activity and Ca^{2+} dependence of the chimeras. We determined the single channel activities of two chimeras, R1 which showed Ca^{2+}-dependent [^{3}H]ryanodine binding and R4 which did not show specific [^{3}H]ryanodine binding. R2 and R9 showed a Ca^{2+} dependence of [^{3}H]ryanodine binding similar to R1 and were therefore not further studied. R6 and R10 were not characterized because single channel conductances or activities resembling those of native or recombinant RyRs were not observed.

Channel activity of purified R1 and R4 chimeras was recorded in KCl media with different additions in the *cis* (SR cytosolic) bilayer chamber. In 250 mM symmetrical KCl solution, R1 and R4 had a mean conductance of 800 ± 8 pS ($\pm$S.E.M., $n = 8$) and 769 pS ($n = 2$), respectively, which was essentially identical to that of native RyRs (not shown). In Fig. 3A, a single R1 channel was recorded in the presence of five different *cis* (SR cytosolic) Ca^{2+} concentrations. Infrequent, brief and sometimes not fully resolved channel openings were recorded at 1.1 μM free Ca^{2+}. Elevation of cytosolic Ca^{2+} to 11 μM greatly increased channel open probability (P_o). The channel was nearly fully activated at 100 μM Ca^{2+} and retained a high level of activity at 1 mM Ca^{2+}. A further increase to 10 mM Ca^{2+} reduced P_o to a low level, similar to that seen at 1.1 μM Ca^{2+}. Fig. 3B compares the Ca^{2+} dependence of R1 with native skeletal and cardiac muscle RyRs. The extent of activation by Ca^{2+} as well as the Ca^{2+} activation/inactivation profile of R1 were similar to RyR2. The results indicate that the C-terminus has a role in Ca^{2+} inactivation of RyR1. Furthermore, the data of Fig. 3 suggest that a reduction in the affinity of the inactivation sites for Ca^{2+} results in the formation of a RyR1/RyR2 chimera that can be nearly fully activated by Ca^{2+}.

Fig. 4 (upper trace) shows a single Ca^{2+}-activated R4 channel recorded in an asymmetric KCl solution. The lower trace of Fig. 4 shows that the R4 channel is sensitive to ryanodine. As observed for native cardiac ryanodine receptors [4,5], the addition of 10 μM *cis* ryanodine locked the R4 chimera in a sub-conductance state with occasional closings. R4 was modified by 10 μM ryanodine in two of two recordings. R4 showed

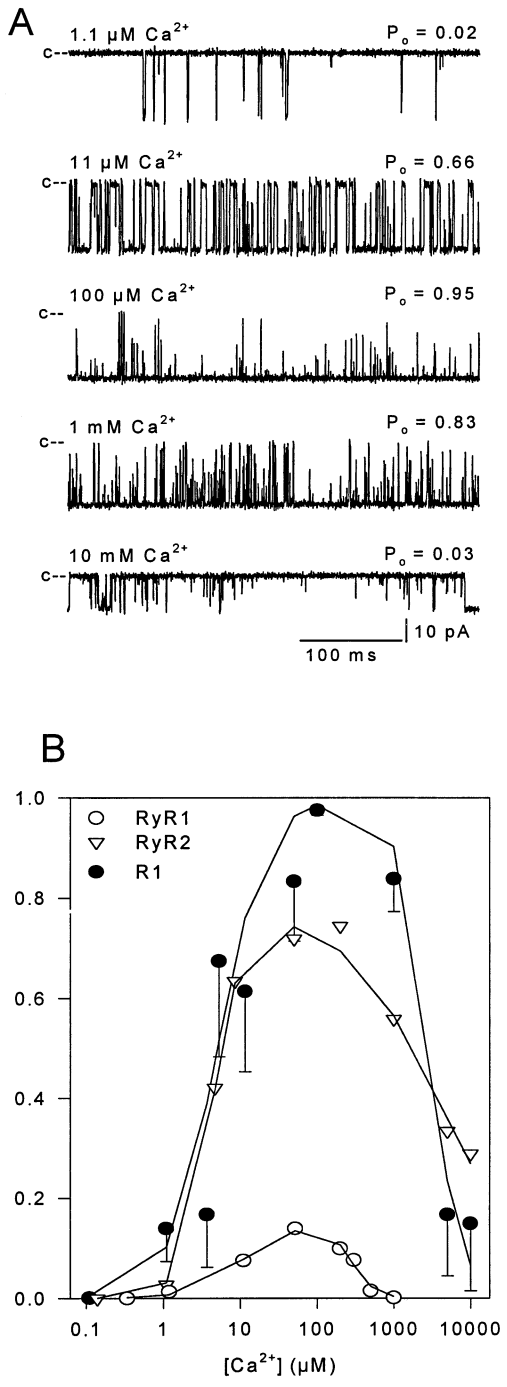

Fig. 3. Ca^{2+} dependence of single channel activities of R1 chimera. A: Proteoliposomes containing purified R1 were fused with a planar lipid bilayer. Currents of a single purified R1 channel, shown as downward deflections from closed level (c), were recorded at a holding potential of −35 mV in symmetrical 0.25 M KCl solution containing the indicated free cytosolic Ca^{2+} concentrations. The decrease in conductance at 10 mM Ca^{2+} was due to the blocking action of Ca^{2+}. B: Comparison of Ca^{2+} activation/inactivation profiles of R1 (●) with native RyR1 (○) and RyR2 (▽). Values for R1 are the mean ± S.E.M. of 4–7 experiments. Data for RyR1 and RyR2 are the averages of two experiments each. Solid lines were obtained by fitting data to a two-site (one activation, one inactivation) logistic function [13]. Derived Hill constants and coefficients for R1 are $K_a = 5.1$ μM Ca^{2+}, $n_a = 1.4$, $K_i = 2860$ μM Ca^{2+}, and $n_i = 2.1$.

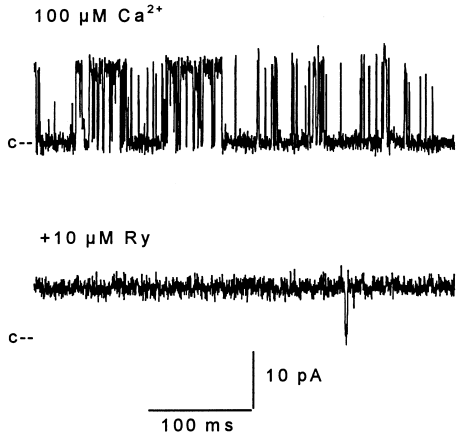

Fig. 4. Ryanodine modification of R4 chimera. Currents of a single purified R4 channel, shown as upward deflections from closed level (c), were recorded at a holding potential of −30 mV in an asymmetric 400 mM *cis* KCl/20 mM *trans* KCl solution with 100 μM free Ca^{2+} in the *cis* chamber before (upper trace) and 10 min after (lower trace) the addition of 10 μM ryanodine to the *cis* chamber.

cardiac-type activity being maximally activated at 1 mM Ca^{2+} in 250 mM KCl solution.

4. Discussion

Nakai et al. [8] expressed the six RyR1/RyR2 chimeras used in the present study in dyspedic myotubes that lack an endogenous RyR1. The results indicated that two regions of RyR1 are important for the reciprocal interaction with skeletal muscle surface membrane DHPR. Chimeras R1, R4, R6 and R10 all restored skeletal muscle excitation-contraction (E-C) coupling and enhanced surface membrane DHPR Ca^{2+} channel function. Chimera R9 enhanced surface membrane Ca^{2+} channel function without restoring skeletal-type E-C coupling. Chimera R2 neither restored skeletal muscle E-C coupling nor enhanced surface membrane Ca^{2+} channel function but it encoded a functional protein because it released Ca^{2+} in response to application of 0.1 mM caffeine to the myotubes.

In the present study, a heterologous expression system was used to identify protein regions that are important for the regulation of the RyR1/RyR2 chimeras by Ca^{2+}. Mutagenesis has been previously used to identify Ca^{2+} regulatory sites in RyRs. Three lines of evidence suggest that the C-terminal one-fourth of RyRs has a critical role in Ca^{2+} activation. First, antibodies raised against a negatively charged sequence (aa 4489–4499, PEPEPEPEPEPE) blocked Ca^{2+}-dependent activation of the channel [14]. Second, the mutation E3885A in RyR3 (equivalent to E4032A in RyR1) formed a functional channel with normal conductance but with greatly decreased Ca^{2+} sensitivity [15]. Third, a deletion mutant encoding the carboxy-terminal 1377 amino acids of RyR1 could be activated by μM Ca^{2+} [16]. In the present study, we used the weak RyR1 Ca^{2+} agonist $choline^{+}$ [13] to localize the Ca^{2+} activation region of RyR1. In agreement with the previous studies reporting a C-terminal localization of Ca^{2+} activation sites, we found that the weak RyR1 Ca^{2+} agonist $choline^{+}$ did not activate R1, R2 and R9 chimeras.

Less is known about the location of Ca^{2+} inactivation site(s). Unlike the full-length RyR1, truncated RyR1 (Δ1–3660) failed to close at high [Ca^{2+}], suggesting that the N-terminal foot structure has a role in Ca^{2+} inactivation [16]. At variance with this result is our finding that the chimeric construct R1 (RyR1(1–3720)/RyR2(3687–4968)) showed a reduced channel inactivation at elevated Ca^{2+} concentrations, similar to RyR2. A reduced channel inactivation at mM Ca^{2+} concentrations was also observed for R2 and R9. Common to these two chimeras and R1 is that the 3721–5037 amino acid C-terminal region in RyR1 is replaced by the corresponding region of RyR2. Our results therefore suggest that the C-terminus of RyR1 has a major role in Ca^{2+} inactivation. The reasons for the differences in the results of Bhat et al. [16] and those of the present study are not known but they may be due to restrictions inherent in using deletion mutants and chimeric constructs. A limitation of the present study is that the Ca^{2+} dependence of the reversed R1 chimera (RyR2(1–3686)/RyR1(3721–5037)) was not determined. We constructed a full-length reverse R1 cDNA, however, the expression levels were very low. Furthermore, immunoblotting with isoform-specific antibodies indicated the absence of a 560 kDa RyR band. Instead two proteolytic fragments were detected corresponding in size to the RyR2 N-terminal and RyR1 C-terminal regions (unpublished studies).

The biphasic Ca^{2+} dependence of RyR1 indicates the presence of two classes of Ca^{2+} binding sites, a high-affinity activation site and a low-affinity inactivation site. According to this model, removal of the Ca^{2+} inactivation site should result in an increased activation by Ca^{2+}. In support of this suggestion, we found that in single channel measurements R1, like RyR2, was nearly fully activated by Ca^{2+} alone.

An intriguing result was that replacement of RyR1 regions with corresponding RyR2 regions resulted in a loss of high-affinity [^{3}H]ryanodine binding in three of the six chimeras. The high-affinity [^{3}H]ryanodine binding site in RyR1 has been localized to a region [17,18] corresponding to the 3687–4968 amino acid C-terminal region of RyR2 that is common to the six chimeras. However, protein conformational changes mediated by the N-terminal portion have been shown to be important for ryanodine binding. In support of a long-range control are trypsin digestion studies in which the intact 30S RyR1 protein complex was degraded to a 15S complex. When the intact RyR1 complex bound [^{3}H]ryanodine before trypsin treatment, a 15S complex containing the C-terminus [17] retained [^{3}H]ryanodine binding, whereas the 15S complex by itself was incapable of binding [^{3}H]ryanodine [19]. The present study also indicates that protein conformational changes mediated by the N-terminal portion are important for ryanodine binding, as high-affinity [^{3}H]ryanodine binding was lost in R4, R6 and R10. However, it should also be noted that high-affinity [^{3}H]ryanodine binding and characteristic K^{+} conductance were observed for R1, which suggests a normally functioning channel with the exception of an altered Ca^{2+} dependence. A reduction of affinity rather than complete loss of specific [^{3}H]ryanodine binding was observed for R4 which was locked by 10 μM ryanodine in a sub-conductance state typical for RyRs. An important feature of the single channel method is that it can test ryanodine binding at micromolar to millimolar concentrations, whereas [^{3}H]ryanodine binding measurements with the recombinant RyRs were limited to nanomolar concentrations.

In conclusion, six RyR1/RyR2 chimeras were used to show that the C-terminal fourth plays an important role both in the

activation and inactivation of the RyR1 by Ca^{2+}. The results further suggest that replacement of RyR1 with RyR2 regions may result in long-range protein conformational changes. Our data point to one of these changes by showing a reduction in [^{3}H]ryanodine binding affinity for one chimera that maintained channel activity.

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