

Characterization of Ca²⁺-Dependent Protein-Protein Interactions within the Ca²⁺ Release Units of Cardiac Sarcoplasmic Reticulum

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In the heart, excitation-contraction (E-C) coupling is mediated by Ca²⁺ release from sarcoplasmic reticulum (SR) through the interactions of proteins forming the Ca²⁺ release unit (CRU). Among them, calsequestrin (CSQ) and histidine-rich Ca²⁺ binding protein (HRC) are known to bind the charged luminal region of triadin (TRN) and thus directly or indirectly regulate ryanodine receptor 2 (RyR2) activity. However, the mechanisms of CSQ and HRC mediated regulation of RyR2 activity through TRN have remained unclear. We first examined the minimal KEKE motif of TRN involved in the interactions with CSQ2, HRC and RyR2 using TRN deletion mutants and *in vitro* binding assays. The results showed that CSQ2, HRC and RyR2 share the same KEKE motif region on the distal part of TRN (aa 202-231). Second, *in vitro* binding assays were conducted to examine the Ca²⁺ dependence of protein-protein interactions (PPI). The results showed that TRN-HRC interaction had a bell-shaped Ca²⁺ dependence, which peaked at pCa4, whereas TRN-CSQ2 or TRN-RyR2 interaction did not show such Ca²⁺ dependence pattern. Third, competitive binding was conducted to examine whether CSQ2, HRC, or RyR2 affects the TRN-HRC or TRN-CSQ2 binding at pCa4. Among them, only CSQ2 or RyR2 competitively inhibited TRN-HRC binding, suggesting that HRC can confer functional refractoriness to CRU, which could be beneficial for reloading of Ca²⁺ into SR at intermediate Ca²⁺ concentrations.

INTRODUCTION

In the heart, pacemaker activities in the form of action potential depolarize the cell membranes leading to activation of dihydropyridine receptors (DHPs)/L-type Ca²⁺ channels, resulting in increased cytosolic Ca²⁺ concentration. The small amount of

fluxed Ca²⁺ could trigger Ca²⁺-induced calcium release (CICR), the amplified Ca²⁺ release from the SR through ryanodine receptor 2 (RyR2)/Ca²⁺ release channel (CRC) located in the SR membranes (Bers, 2002). This transitory elevation of cytosolic Ca²⁺ is responsible for activation of actin-myosin cross-bridges resulting in the generation of heartbeat.

Ca²⁺ release through RyR2/CRC is mediated by interactions between multiple proteins in a complex called calcium release unit (CRU) that includes triadin (TRN), calsequestrin (CSQ), junctin (JTN) (Gyorke et al., 2004), and histidine rich Ca²⁺ binding protein (HRC) (Fan et al., 2004). Both JTN and TRN may act as scaffold proteins anchoring CRU proteins such as CSQ and HRC (Goonasekera et al., 2007; Guo et al., 1996; Jones et al., 1995; Zhang et al., 1997) in the vicinity of RyR2. The SR luminal Ca²⁺ binding proteins, CSQ and HRC have effects on RyR2 through their Ca²⁺ sensitivity and interactions with TRN and JTN (Arvanitis et al., 2007; Boncompagni et al., 2012; Fan et al., 2004; Guo and Campbell, 1995; Kim et al., 2003; Park et al., 2012; Picello et al., 1992; Zhang et al., 1997). The interactions of CSQ or HRC with TRN are known to occur at highly conserved luminal domains within the structure of TRN. These domains have been characterized by the presence of alternating positively and negatively charged amino acids known as KEKE motif (Kobayashi et al., 2000; Lee et al., 2001; 2004b; Zhang et al., 1997).

The regulation of Ca²⁺ cycling by those CRU proteins is considered as an important area of investigation, since desynchronized Ca²⁺ release from SR (called Ca²⁺ leak) has been associated with arrhythmogenesis and heart failure (Arvanitis et al., 2007). Furthermore, the dynamically changing luminal Ca²⁺ levels in the SR could have critical influences on the interactions among the proteins in CRU, since the structural and functional defects of CRU are associated with aberrant Ca²⁺ homeostasis, as shown in myocardial hypertrophy, heart failure and ventricular arrhythmias (Hasenfuss et al., 1997; Kim et al., 2013; Lehnart et al., 2009; Postma et al., 2002; Priori and Napolitano, 2005). Hence, understanding the nature of protein-protein interactions (PPI) in CRU and its influence on RyR2 activities are critical for elucidating mechanistic insights of Ca²⁺ cycling in the pathological states of the heart.

Although the previous studies have shown evidence of the regulatory interactions between RyR and its protein partners within CRU, the dynamic interactions of RyR2 in the heart with the key luminal CRU proteins such as CSQ and HRC under different SR luminal Ca²⁺ levels remain to be investigated. In

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Received 15 October, 2015; revised 2 November, 2015; accepted 3 November, 2015; published online 15 December, 2015

Keywords: calsequestrin, histidine rich Ca²⁺ binding protein, and triadin, junctin, ryanodine receptor

eISSN: 0219-1032

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this study, we determine the minimal motif on the luminal part of TRN, which is critical for PPI of the CRU proteins, the characteristic of HRC-TRN binding and the nature of HRC-TRN binding with CSQ2 or RyR2, to provide new insights into the molecular events involved in the regulation of Ca²⁺ release from SR.

MATERIALS AND METHODS

Generation and purification of the recombinant proteins used for the present study

GST-protein fusion constructs were generated from mouse cardiac cDNAs using PCR. The PCR products were digested with restriction enzymes BamHI and XhoI, and subcloned into BamHI/XhoI cloning sites of pGEX 4T-1 vector (Amersham Biosciences). Clones with desired inserts were confirmed by direct sequencing, and the fusion proteins were subjected to translation as described previously (Zhang et al., 1997). The expression of GST fusion proteins were induced in *Escherichia coli* (*E. coli*) BL21 with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 12 h at 18°C. Cells were harvested by centrifugation, resuspended in phosphate buffered saline (PBS), lysed by ultrasonication on ice, and then incubated in 1% Triton X-100 for 30 min. The soluble fraction was obtained by centrifugation at

20,000 x g for 10 min at 4°C. The fusion proteins were immobilized by incubating 1 ml of the soluble *E. coli* fraction with 50 μl bead volume of glutathione-Sepharose 4B (Pharmacia) for 3 h and washed thrice with PBS. Primer sets used for generation of TRN mutants are as follows: 5'-CGC GGA TCC ATG ACT GAG ATC ACT GCT GAA-3', 5'-CCG CTC GAG AGG GGA ACT GAA TGT TGT CAC-3' for the cytoplasmic domain (aa 2-47); 5'-CGC GGA TCC GAT TTA GTG GAT TAT AAA AAC-3' and 5'-CCG CTC GAG TTA GTG TAT TTC TTT TCT TTT-3' for proximal TRN (aa 69-146); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG CTC TTT GTC ATC CTT CTT TCT-3' for TRN Del 1 (aa 147-254); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG TTT CAC TTT CTC CTG TTT TCC-3' for TRN Del 2 (aa 147-231); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG TTC AGT CTT TTC CTT AGT CTT-3' for TRN Del 3 (aa 147-215); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG TGC CAT CAT CTT CGT CTC TGG-3' for TRN Del 4 (aa 147-202); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG GTG AGT TGT TGT CTT CTC AGG-3' for TRN Del 5 (aa 147-186); 5'-CGC GGA TCC GAA AAG GCT GAA AAA GAG GAG-3' and 5'-CCG CTC GAG TCT GTG TGA

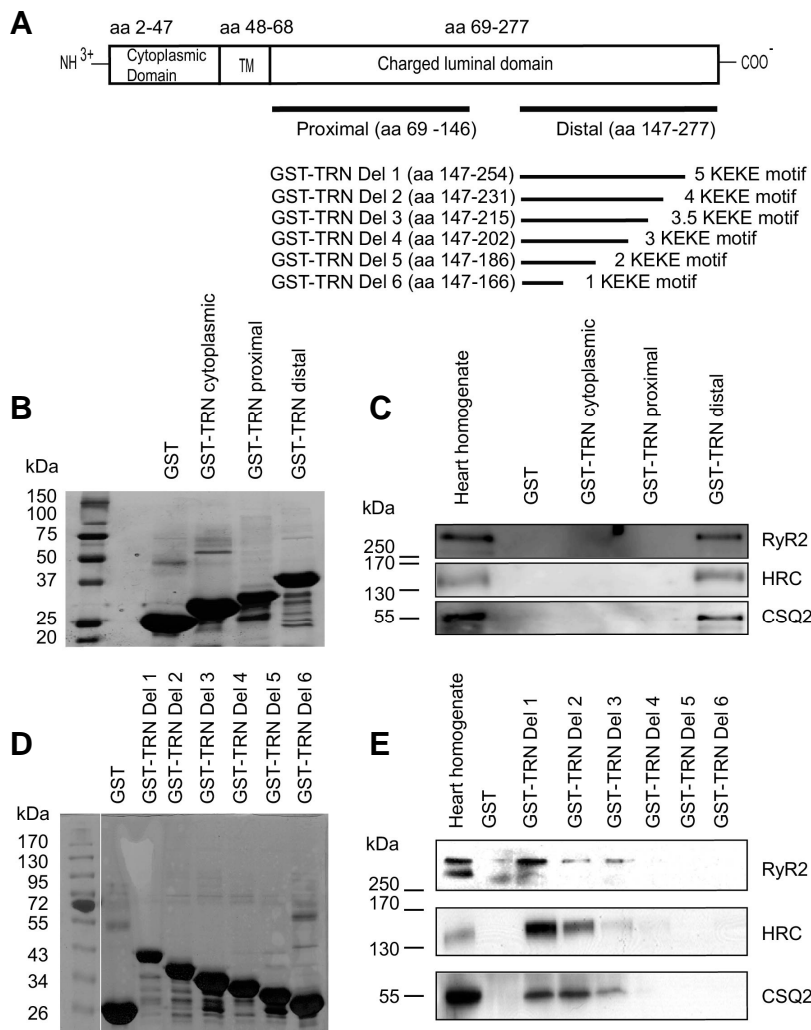


Fig. 1. Distal region of the luminal TRN interacts with RyR2, CSQ2 and HRC through the same KEKE motif. (A) Cardiac TRN1 constructs used in the study. Deletion mutants of cardiac TRN1 residues were fused to GST protein. (B) Coomassie-stained gel after protein purification of: GST, GST-TRN cytoplasmic, GST-TRN proximal, and GST-TRN distal regions. GST pull-down assay was performed using mouse heart homogenate. (C) RyR2, HRC and CSQ2 showed interactions with the distal TRN construct containing KEKE motifs. (D) Coomassie stained gel image of the distal TRN mutants containing KEKE motifs after protein purification. (E) The minimal regions for the interactions of RyR2, CSQ2, and HRC needed the presence of KEKE motif (aa 202-231) of TRN.

AGC TTT AGT TTG-3' for TRN Del 6 (aa 147-166).

GST pulldown assay and Western blot

GST pulldown assays were performed as described previously with minor modifications (Lee et al., 2004a). Briefly, the mouse heart homogenates were solubilized for 1 h on ice in buffer containing 2% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM sodium orthovanadate, 15 mM sodium fluoride, 1 mM DTT and protease inhibitor mixture. Solubilized proteins were obtained by centrifugation and diluted with 50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and protease inhibitor cocktail to reduce high salt and detergent concentration. Solubilized proteins were pre-cleared with 50% slurry of glutathione-sepharose 4B beads for 2 h at 4°C. The pre-cleared supernatant was incubated with equivalent amount of GST fusion proteins coupled to glutathione-Sepharose 4B beads for 12 h at 4°C. After the incubation, the fusion protein-Sepharose 4B complexes were collected by centrifugation and washed five times with wash buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.3% CHAPS. The bound proteins were eluted, and boiled in 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to SDS-PAGE, followed by immunoblotting with HRC, CSQ or RyR antibodies. Antibodies of HRC, CSQ2 and TRN (homemade rabbit polyclonal sera), RyR (ABR, MA3-925), western blot signal was detected by ImageQuant LAS 4000 mini (GE Healthcare Bio-Sciences AB) with a SuperSignal West Pico chemiluminescence kit (Thermo Fisher Scientific, Inc.). Western blot band intensities were measured using ImageJ software. Ca²⁺ dependent GST pulldown assay was performed after adjusting different concentrations of free Ca²⁺ as described previously (Zhang et al., 1997). In brief, the heart homogenate was prepared in the presence of 2 mM EGTA. Based on the maxchelator program, specific concentrations of CaCl₂ were added to the reaction solutions after homogenates were diluted. GST pulldown assay was performed as mentioned above.

Co-immunoprecipitation assay and competitive binding assay

Heart homogenate was used for co-immunoprecipitation (Co-IP). The solubilization step was identical to that of the GST-pulldown assay as described above. The solubilized samples were pre-cleared with protein A sepharose beads (Amersham) and subjected to incubation at 4°C overnight using appropriate antibody. Protein A sepharose beads were added and incubation was continued at 4°C for 2 h. Precipitates were washed four times with washing solution, eluted in SDS sample buffer and analyzed by western blot assay. Competition between RyR2, CSQ2, and HRC for binding to TRN was studied as described previously (Wyszynski et al., 1997). In brief, immunoprecipitation with triadin antibody was performed with increasing concentration of RyR2 peptide IEDPAGDEYIYRII or with CSQ2 peptide DEDNEDSDDDDDDDE, which have been known to interact with TRN (Lee et al., 2004b; Shin et al., 2000). Bound proteins were eluted and immunoblotted with HRC or CSQ antibody. These experiments were performed at free Ca²⁺ level of pCa4.

Statistics

All data are shown as mean ± SD. Statistical significance was analyzed by student's *t*-test or analysis of variance (ANOVA). A value of *P* < 0.05 was considered statistically significance.

RESULTS

RyR2, CSQ2 and HRC interact with the charged distal TRN

The KEKE motifs in the luminal region of TRN have been proposed to facilitate PPI with the different CRU proteins (canine cardiac CSQ at aa 210-224 of TRN, Kobayashi et al., 2000; rabbit skeletal RyR1 at aa 200-232 of TRN, Lee et al., 2004b; rabbit skeletal HRC at aa 204 -260 of TRN, Lee et al., 2001). Here, we evaluated mouse cardiac TRN interactions with RyR2, CSQ2 and HRC under the same experimental conditions to identify the minimal binding regions of TRN using mouse heart

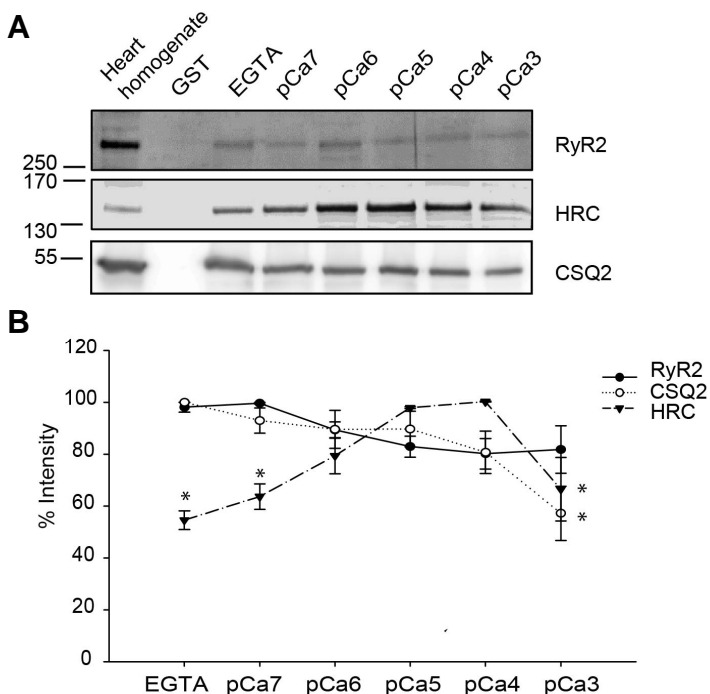


Fig. 2. Interaction of TRN with RyR2, HRC, and CSQ2 at increasing Ca²⁺ concentrations. (A) The distal TRN deletion mutant fused to GST protein was used for pull down assay and its interacting partners in the presence of increasing Ca²⁺ concentrations were studied. (B) Quantitative representation of the western blot results. (Mean ± SD, **P* value < 0.05, n = 3, the statistical significance was calculated with respect to the maximum interaction of the respective protein using one way ANOVA).

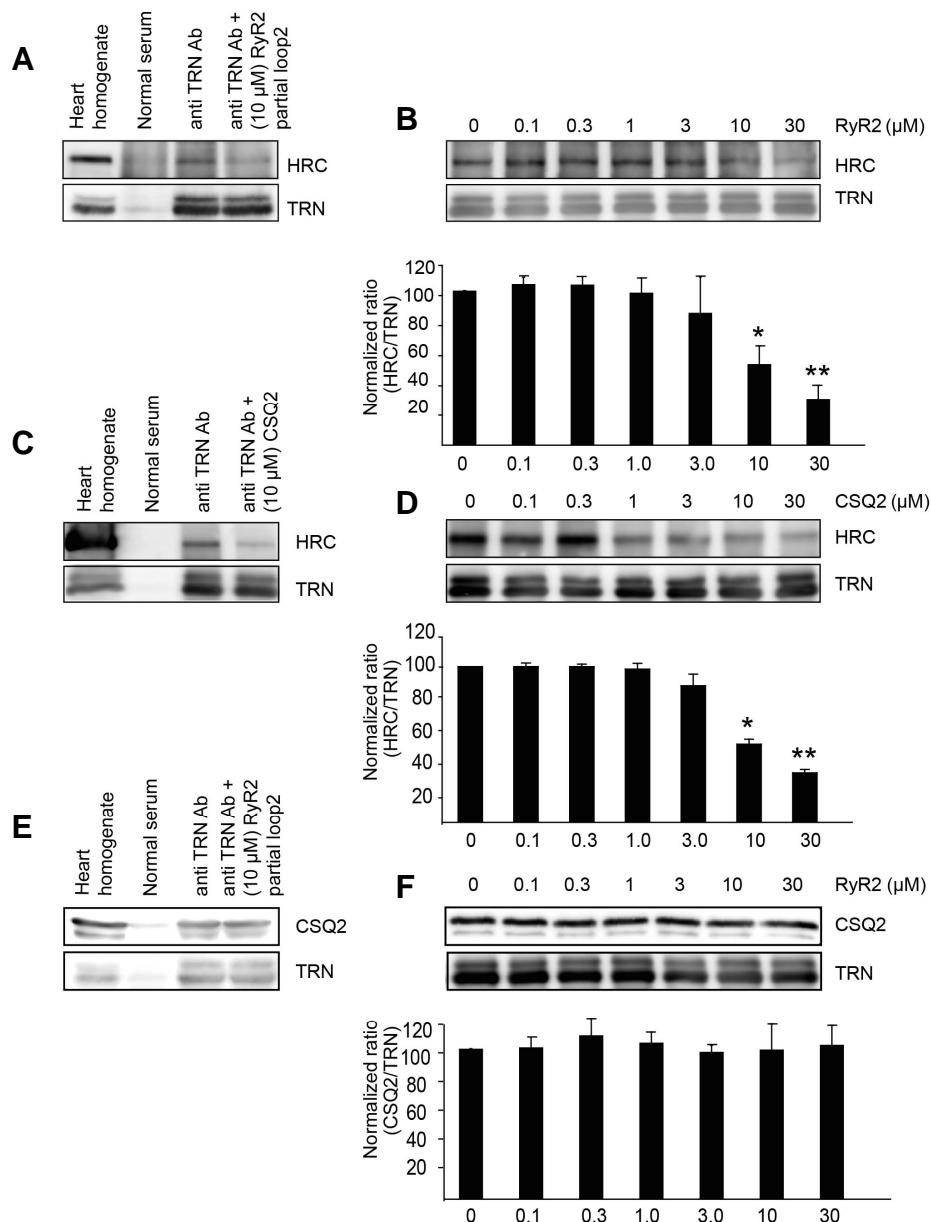


Fig. 3. HRC competes with RyR2 and CSQ2 to interact with TRN, whereas RyR2 did not show any competition with CSQ2 to interact with TRN at pCa4 (A) On immunoprecipitation with TRN antibody at pCa4 in the presence of synthesized RyR2 loop 2 (10 μM) peptide, HRC showed significantly reduced interaction with TRN. (B) HRC shows reduced interaction with TRN as the concentration of RyR2 loop 2 increased (0-30 μM). Quantitative representation of the Western blot results of HRC plotted as a ratio to total TRN as the RyR peptide concentration increased. (C) Immunoprecipitation with TRN antibody at pCa4 in the presence of synthesized CSQ2 peptide (10 μM). (D) HRC shows reduced interaction with TRN as the concentration of CSQ2 increased (0-30 μM). Quantitative representation of the Western blot results of HRC plotted as ratio to total TRN as CSQ2 peptide concentration increased. (E) Immunoprecipitation with TRN antibody at pCa4 in the presence of synthesized RyR2 loop 2 peptide (10 μM). CSQ2 showed no change in interaction to TRN. (F) No significant change was observed in the interaction of CSQ2 and TRN as the concentration of RyR2 increased (0-30 μM). Quantitative representation of the Western blot results of CSQ2 plotted as the ratio to total TRN as RyR2 peptide concentration increased. (Mean ± SD, *P value < 0.05, **P value < 0.01, n = 4).

samples. Three GST-tagged deletion mutants, cytosolic region of TRN (aa 2-47), the proximal region of luminal TRN (aa 69-146) and the distal region of luminal TRN (aa 147-277) were generated (Figs. 1A and 1B) and subjected to GST pull-down assay. The results showed that only the distal region of TRN1 containing five KEKE motifs interacted with RyR2, CSQ2 or HRC (Fig. 1C).

To further analyze the specific CRU protein-binding region in TRN, we generated GST-tagged deletion mutants of the KEKE motifs in TRN (Fig. 1D). We prepared different GST fusion proteins such as GST-TRN Del 1 (aa 147-254), GST-TRN Del 2 (aa 147-231), GST-TRN Del 3 (aa 147-215), GST-TRN Del 4 (aa 147-202), GST-TRN Del 5 (aa 147-186), and GST-TRN Del 6 (aa 147-166). The estimated approximate molecular sizes of the proteins were ~37.77, ~35.24, ~33.48, ~32.05, ~30.29, and ~28.09 kDa, respectively. Western blotting of the GST

pull-down samples with RyR, CSQ2, and HRC antibodies showed that GST-TRN Del1, GST-TRN Del2, and GST-TRN Del3 interacted with RyR, CSQ2, and HRC, whereas GST-TRN Del 4, GST-TRN Del 5, GST-TRN Del 6, or control GST did not show any interaction with the three proteins (Fig. 1E), suggesting that the KEKE motif region of TRN (aa 202-231) is involved in the interaction with the CRU proteins.

Ca²⁺-dependent interactions of TRN with RyR2, CSQ2, and HRC

In order to study the optimal Ca²⁺ concentrations required for binding between TRN-CSQ2, TRN-HRC, and TRN-RyR2 (Arvanitis et al., 2007; Lee et al., 2001), GST pull-down assays were conducted using the mouse cardiac distal TRN under increasing free Ca²⁺ concentrations followed by Western blot with specific antibodies for RyR, HRC, and CSQ2. The results

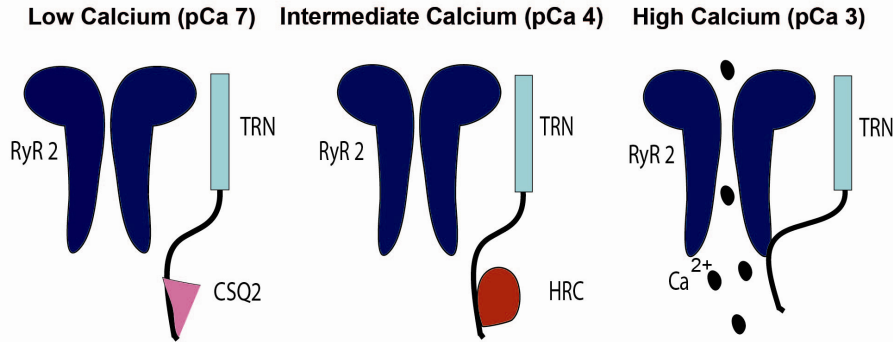


Fig. 4. A schematic diagram describing the effect of TRN, CSQ2, and HRC interaction with RyR2 and their response to luminal Ca²⁺. At low luminal Ca²⁺ concentration, CSQ2 bound to TRN might help in building Ca²⁺ concentration near the junctional membrane as a necessary condition for release of Ca²⁺ ions through RyR2 upon activation; RyR2 Ca²⁺ leak is prevented by CSQ2. As the luminal Ca²⁺ concentration reaches moderate levels, CSQ2/TRN/RyR2 complex interaction starts to attenuate. RyR2 inhibition by CSQ2 is gradually relieved enabling RyR2 leakage, but HRC bound to TRN stabilizes RyR2 from being leaky. At higher luminal Ca²⁺ concentrations HRC is detached from TRN allowing RyR2-TRN interaction resulting in increased RyR2 activity and Ca²⁺ release.

showed that HRC-TRN had a peak binding at pCa4 (called moderate Ca²⁺ concentration), but CSQ-TRN binding was maximum at low free Ca²⁺ level. On the other hand, RyR2-TRN binding showed no apparent Ca²⁺ dependence (Fig. 2), suggesting that the SR luminal Ca²⁺ can selectively affect the patterns of PPI depending on the types of CRU proteins.

HRC-TRN binding is competed with RyR2 or CSQ2

We performed a competitive binding assay to examine whether HRC-TRN binding is interfered by RyR or CSQ2. The study also could lead to the answers why the seemingly functionally redundant proteins, HRC and CSQ exist within the SR lumen. Since HRC showed a unique binding pattern with the maximum TRN binding ability at moderate Ca²⁺ levels, the competitive TRN-HRC binding experiments were conducted at pCa4 by varying concentrations of CSQ2 or RyR2 loop 2 peptide using immuno-precipitation assays with TRN. The results showed that HRC binding to TRN significantly decreased in the presence of RyR2 loop 2 or CSQ peptides in concentration range of 3-30 μM and 1-30 μM, respectively (Fig. 3). Taken together, the results indicate that HRC competes with RyR or CSQ to interact with TRN at the moderate free luminal Ca²⁺ concentrations.

CSQ2 does not compete with RyR2 to interact with TRN

Similar to the competitive binding assay as shown above, we performed a competitive binding assay to examine whether CSQ-TRN binding was interfered by RyR2 at moderate Ca²⁺ concentration (pCa4) (Figs. 3E and 3F). The result showed no obvious changes of CSQ-TRN binding by increasing concentrations of RyR2 loop 2, suggesting that there is no competition between CSQ2 and RyR2 to interact with TRN at moderate free luminal Ca²⁺ levels.

DISCUSSION

In the present study, we examined the binding properties of CRU proteins in the heart using deletion mutants and *in vitro* binding assays. The new findings of this study are: (1) CSQ, HRC, and RyR2 share the same binding sites on the KEKE motif region of the distal part of TRN (aa 202-231, Fig. 1); (2) HRC-TRN binding has a bell-shaped Ca²⁺-dependence peak at

pCa4 (moderate Ca²⁺) (Fig. 2); (3) HRC-TRN binding was competed by CSQ2 or RyR2 at pCa4, but CSQ2-TRN was not competed by RyR2 (Fig. 3); (4) Therefore, HRC, can confer functional refractoriness to CRU at pCa4, which is considered to be beneficial for reloading of Ca²⁺ into SR during the filling phase (Fig. 4).

Previous studies have reported that interactions of RyR/CRC with the luminal auxiliary proteins such as CSQ, HRC, TRN, and JTN are involved in the regulation of CRC in cardiac muscle (Knollmann, 2009; Lee et al., 2001). TRN has conserved domains such as KEKE motif, which could act as a nodal point for interactions with RyR, CSQ, and HRC (Guo and Campbell, 1995; Guo et al., 1996; Kobayashi et al., 2000; Lee et al., 2004b). Furthermore, a synthesized KEKE motif of TRN (TRN-KEKE motif) was found to enhance RyR1 channel activity (Wium et al., 2012), suggesting that the TRN-KEKE motif is an important regulator of RyR activity and also serves as the scaffold domain for interactions with the luminal auxiliary proteins. Here, we further investigated the presence of a critical interacting region on TRN for binding RyR2, CSQ2, and HRC in the heart. We found that the distal half of TRN (aa 147-277) containing KEKE motif was involved in PPI (Fig.1C), as was partially evidenced in previous reports (Kobayashi et al., 2000; Lee et al., 2004b). We further identified the specific KEKE motif(s) in the distal region that binds the three SR proteins (Fig.1E).

It has been reported that both TRN and junctin (JTN) are capable of interacting with CSQ and RyR, but only JTN has the ability to bind RyR and CSQ simultaneously, thus possessing a unique role in stabilization of RyR in a closed state at low luminal Ca²⁺ levels (Franzini-Armstrong et al., 2005; Jones et al., 1995; Zhang et al., 1997). We found that similar to JTN, significant fractions of CSQ and RyR2 are bound to TRN at low and intermediate luminal Ca²⁺ levels (Figs. 2-4) (Beard et al., 2005). Hence, the functional significance of TRN binding of CSQ in the luminal regions may be to anchor CSQ and facilitate building of Ca²⁺ micro-domain in the vicinity of RyR2 channels (Boncompagni et al., 2012; Gyorke et al., 2004).

HRC is a Ca²⁺ binding protein located in the lumen of SR, which may serve as a Ca²⁺ buffer, and interacts with TRN and SERCA2 in the lumen of SR. HRC is a bi-functional protein that can regulate activities of both SERCA2 and RyR2. The

affinity of HRC to SERCA2 could decrease, when the luminal Ca²⁺ concentration increases from pCa7 to pCa3 (Arvanitis et al., 2007). As shown in Fig. 2A, HRC binding to TRN showed a bell-shaped Ca²⁺ dependence, which peaked at pCa4, similar to the previous report (Sacchetto et al., 2001) (Fig. 2A). Recent animal- and cell-level studies using genetic manipulation techniques have shown that deletion or knockdown of HRC gene could increase the leakiness of Ca²⁺ through RyR2, suggesting that RyR2 activities are inhibited by HRC (Fan et al., 2004; Park et al., 2012). We predicted that HRC might have a regulatory role on RyR stability through its interaction with TRN. A recent CSQ2/HRC double knockout (KO) study suggests an interesting hypothesis that HRC may also act as a primer to enhance RyR2 activity at higher luminal Ca²⁺ concentrations [Liu et al., 2015 (in press)], which could support our data.

The model shown in Fig. 4 was constructed based on the results obtained in this study and some of previously published data (For simplicity, the roles of SERCA and JNT are not included).

At low Ca²⁺: pCa7: CSQ-TRN binding leads to maximum inhibition of RYR2 preventing Ca²⁺ leak. At intermediate Ca²⁺: pCa4: CSQ/TRN/RyR2 complex starts to dissociate and RyR2 inhibition by CSQ is gradually relieved as Ca²⁺ binding sites on CSQ become increasingly occupied with Ca²⁺ and CSQ-TRN bindings weaken. This event may be further facilitated by HRC binding to TRN conferring the refractoriness to RyR2. At high Ca²⁺: pCa3: HRC is dissociated from TRN-HRC complex, allowing RyR-TRN interaction, which increases RyR2 activity and facilitates Ca²⁺ release from SR.

ACKNOWLEDGMENTS

This work was supported by the 2015 GIST Systems Biology Infrastructure Establishment Grant and the NRF grant funded by the Korean Ministry of Science, ICT & Future Planning (NRF-2013M3A9A7046297).

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