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Occurrence of atypical Ca^{2+} transients in triadin-binding deficient-RYR1 mutants ☆

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Abstract

Triadin in the junctional sarcoplasmic reticulum (SR) of skeletal muscle cells has been suggested to interact with ryanodine receptor 1 (RYR1) via its KEKE motifs. Recently, we showed that amino acid residues D4878, D4907, and E4908 in RYR1 are critical for triadin-binding *in vitro* [J.M. Lee, S.H. Rho, D.W. Shin, C. Cho, W.J. Park, S.H. Eom, J. Ma, D.H. Kim, Negatively charged amino acids within the intraluminal loop of ryanodine receptor are involved in the interaction with triadin, J. Biol. Chem. 279 (2004) 6994–7000]. In order to test whether a disruption of the triadin-binding site(s) in RYR1 affects SR Ca^{2+} release, alanine-substituted single (D4878A, D4907A, and E4908A) and triple (RYR1-TM) mutants of D4878, D4907, and E4908 were expressed in RYR1-null myotubes. Co-immunoprecipitation experiments showed a 50–60% decrease of triadin brought down in the D4907A and RYR1-TM complexes compared to the triadin–wtRYR1 complex. Ca^{2+} imaging experiments using Fluo-4-AM showed atypical caffeine responses in myotubes expressing D4907A and RYR1-TM characterized by either a lack of or slower activation and faster inactivation of Ca^{2+} transients. The results suggest that disruption of interaction between triadin and RYR1 impairs RYR1 function and SR Ca^{2+} release.

Keywords: Ryanodine receptor; Excitation–contraction coupling; Caffeine; Calsequestrin

In mammalian skeletal muscle, depolarization of sarcolemma leads to activation of the voltage sensing dihydropyridine receptor (DHPR) and Ca^{2+} release from sarcoplasmic reticulum (SR) through mechanical coupling between DHPR and SR Ca^{2+} release channel (ryanodine receptor 1, RYR1) [1]. The homo-tetrameric RYR1 forms a functional Ca^{2+} release channel and each monomer is predicted to have 4–6 transmembrane segments [2–5]. RYR1 has a large cytoplasmic "foot" region and relatively small luminal loop regions that are predicted binding sites for accessory proteins such as triadin, junctin, and histidine-rich Ca^{2+}-binding protein [6,7].

Triadin has a short N-terminal cytoplasmic domain, a single transmembrane domain, and a long C-terminal domain containing multiple clusters of alternating lysine and glutamic acids (KEKE motifs) [8–10]. Triadin interacts with both RYR1 and calsequestrin (CSQ) via the KEKE motifs in its C-terminus [11,12]. Junctin has a similar structure and sequence to triadin and also binds to both RYR1 and CSQ [6,9]. CSQ is a high-capacity Ca^{2+} harboring protein in SR lumen and has been suggested to indirectly regulate RYR function by binding to triadin and junctin

☆ *Abbreviations:* RYR, ryanodine receptor; wt, wild type; RYR1-TM, triple mutant of RYR1 at D4878, D4907, and E4908; DHPR, dihydropyridine receptor; SR, sarcoplasmic reticulum; CRUs, Ca^{2+} releasing units; CSQ, calsequestrin; aa, amino acids; CPA, cyclopiazonic acid.

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[6,11,13,14]. However, the functional role of triadin in controlling Ca^{2+} release from SR has remained unclear.

Recently, by site-directed mutagenesis and *in vitro* binding assay, we found that three negatively charged residues, D4878, D4907, and E4908 of RYR1, could be critical for binding to a KEKE motif of triadin [15]. In the current study, to test whether a disruption of the triadin-binding sites in RYR1 affects the function of RYR1 *in situ*, we expressed either one of the three single mutants (D4878A, D4907A, and E4908A) or the three mutations together (RYR1-TM) in RYR1-null myotubes (1B5) [16] and examined the ability of the mutants to bind triadin in a co-immunoprecipitation experiment and to release Ca^{2+} following the addition of caffeine in a cellular Ca^{2+} imaging assay. The results suggest that D4907A and RYR1-TM mutants reduced triadin–RYR1 complex formation and/or stability, and caused an atypical pattern of caffeine-induced SR Ca^{2+} release.

Materials and methods

Construction of RyR1 mutants. RyR1 cDNA from rabbit skeletal muscle was mutated (single mutants, D4878A, D4907A or E4908A; a triple mutant at D4878, D4907, and E4908, RYR1-TM) by site-directed mutagenesis on a RyR1 subclone created via the introduction of RyR1 sequence extending from the natural *Cla*I and artificial *Hin*dIII sites, located at positions 14,305 and the end of full RyR1 sequence, respectively, and sub-cloned into pHSVprPUC (RYR1-pHSVprPUC) for viral packaging [17].

RYR1-null (1B5) myoblast culture and viral infection. As previously described [18], RYR1-null (1B5) myoblasts were cultured in 96-well plates with ultra-thin clear bottoms (Corning, Costar, NY) coated with Matri gel (BD Bioscience, Bradford, MA) at 37 °C in a 5% CO_2 incubator in low-glucose DMEM (growth medium) containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and additional 2 mM L-glutamine (materials were obtained from Gibco-BRL, Grand Island, NY). For differentiation, when myoblasts reached ~70% confluence, the growth medium was replaced with differentiation medium (containing 5% heat-inactivated horse serum instead of 20% FBS in the growth medium) and placed into an 18% CO_2 incubator for 6 days. Herpes simplex virus-1 (HSV-1) virions were packaged using each of the four RYR1-pHSVprPUC constructs as previously described [17], and 1B5 myotubes were infected with one of the four HSV-1-RYR1 virions after 6 days in differentiation medium at a concentration of 4×10^5 infectious units (IU)/ml (a multiplicity of infection was ~4). After 36–48 h, transduced 1B5 myotubes were imaged or disrupted to prepare whole cell lysates. Random selection of wells indicated that approximately 60% of virally infected myotubes expressed wild type (wt) or mutant RYR1s. Myotubes containing more than four nuclei showed approximately 90% transfection efficiency, and were selected for Ca^{2+} imaging experiments.

Immunohistochemistry. 1B5 myotubes were fixed in cold methanol (−20 °C) for 15 min and permeabilized with 0.05% Tween 20 in phosphate-buffered saline (PBS) for 1 min. After blocking myotubes with 5% normal goat serum (NGS) in PBS, myotubes were incubated with anti-RYR1 antibody (34C, Drs. J. Airey and J. Sutko, Developmental Studies Hybridoma Bank, Iowa City, IA, 1:25) for 1 h at 25 °C, washed with 5% NGS in PBS, and incubated with Cy-3-conjugated anti-mouse IgG (Affinity BioReagents, Golden, CO, 1:1000) for 1 h. Cy-3 fluorescence was visualized with a Nikon Diaphot microscope (Nikon, Melville, NY) with an epifluorescence attachment (510–560 nm excitation, 590 nm emission).

Preparation of whole cell lysates from 1B5 myotubes and co-immunoprecipitation experiment. As previously described [19], 1B5 myotubes were homogenized and incubated with a lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 100 μM $CaCl_2$, 1 mM Na_3VO_4, 10% glycerol, and protease inhibitors). After removing insoluble materials by a quick centrifuging, lysates (700 μg protein) were diluted with four volumes of Triton X-100-free lysis buffer and incubated with 25 μl of anti-RYR1 antibody for overnight at 4 °C followed by further incubation with protein G-Sepharose beads for 4 h. Beads were washed three times with Triton X-100-free lysis buffer to remove non-specifically bound proteins. The immune-complexes were subjected to SDS–PAGE and immunoblot analysis with anti-RYR1 or anti-triadin antibody.

Ca^{2+} imaging. 1B5 myotubes expressing wtRyR1 or one of the mutant RYR1s were loaded with 5 μM Fluo-4-AM (Molecular Probes, Eugene, OR) in an imaging buffer (125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 25 mM Hepes, 6 mM glucose, 1.2 mM $MgSO_4$, and 0.05% BSA, pH 7.4) at 37 °C for 45 min, then washed three times with the imaging buffer and transferred to an inverted stage microscope equipped with an Olympus Uaop/340 × 40 oil-immersion objective (NA 1.35) (Nikon Diaphot300; Melville, NY). The imaging buffer and caffeine were applied to the myotubes by a 16-channel multi-valve perfusion system (Automate Scientific, Oakland, CA). The myotubes were imaged by using a PTI delta-RAM (Photon Technology International, Lawrenceville, NJ) as the light source with a 12-bit digital intensified CCD camera (Stanford Photonics, Stanford, CA). Data were displayed and analyzed using QED Camera Plug-in package (QED Imaging, Pittsburgh, PA). After the Ca^{2+} imaging experiments, several representative wells of 96-well plates were subjected to immunohistochemistry with anti-RYR1 antibody to verify the expression of wt and mutant RYR1s in 1B5 myotubes.

Statistical analysis. Results are given as means ± SE with the number of experiments indicated in the figure legends. Significance of differences was analyzed by paired or unpaired *t*-test (GraphPad InStat, v2.04). Differences were considered to be significant when $P < 0.05$. Graphs were prepared using Origin v7.

Results

RYR1-D4878/D4907/E4908 mutant (RYR1-TM) showed reduced triadin-binding

According to the putative structural elements of pore-forming region in RYR1 (Supplementary data 1), three negatively charged residues, D4878, D4907, and E4908, previously identified as triadin-binding sites in RYR1 *in vitro* [15], would be localized in places near the putative pore helixes and the selectivity filter and all three residues would be predicted to face the luminal side of the SR.

To test whether a disruption of the RYR1 triadin-binding sites affects RYR1 function *in situ*, aa residues D4878, D4907, and E4908 were substituted by alanines (RYR1-TM). The cDNA was packaged into HSV-1 virions (HSV-1-RYR1-TM) as described in materials and methods and RYR1-null (1B5) myotubes [16] were infected with HSV-1-RYR1-TM at 6 days of differentiation. 1B5 myotubes infected with HSV-1 virions containing wild type RYR1 (wtRyR1) were used as positive control and uninfected 1B5 myotubes (no infection) were used as negative control. Like wtRYR1, the RYR1-TM construct was successfully expressed in 1B5 myotubes 24–36 h postinfection. Transduction with virions expressing either RYR1 construct caused no significant changes in cell size, shape or differentiation level (Fig. 1A). Immunohistochemistry with anti-RYR1 antibody to examine the expression pattern of wtRYR1 and RYR1-TM revealed that both were clustered in punctated foci, Ca^{2+} releasing units (CRUs) [20],

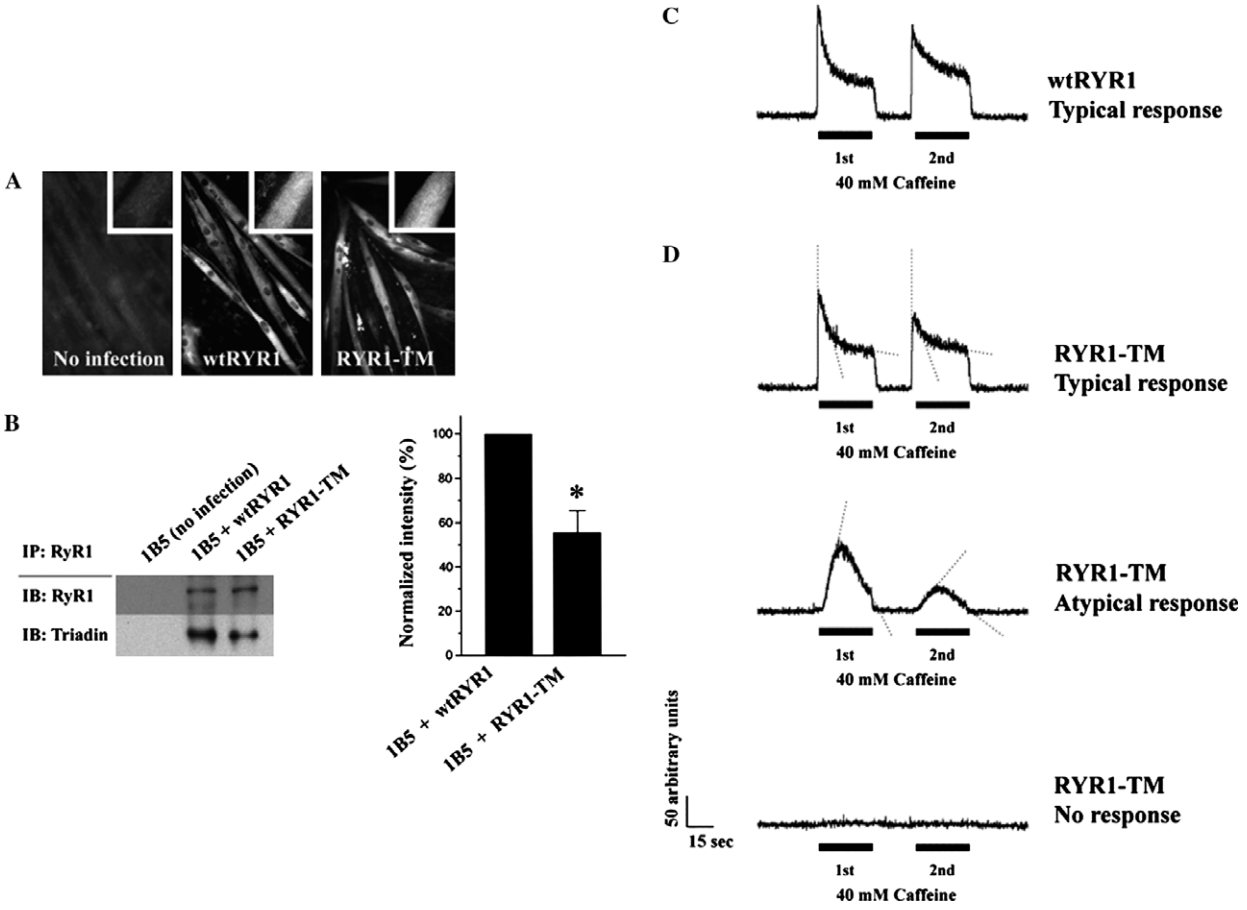

Fig. 1. Interaction between RYR1-TM and triadin, and atypical response of RYR1-TM to caffeine. (A) Expression profiles of wtRYR1 and RYR1-TM in 1B5 myotubes were probed by immunohistochemistry with anti-RYR1 antibody. Higher-magnification images in cross-sectional areas present the localization pattern of wtRYR1 and RYR1-TM. Uninfected 1B5 myotubes (no infection) were used as a negative control. (B) Whole cell lysate of 1B5 myotubes expressing wtRYR1 or RYR1-TM was subjected to co-immunoprecipitation experiment with anti-RYR1 antibody and the immune-complex was probed with anti-RYR1 or anti-triadin antibody. Normalized band intensity of co-immunoprecipitated triadin in RYR1-TM to that in wtRYR1 is presented as bar graphs on the right ($55.5 \pm 9.9\%$). *Significant difference compared to wtRYR1 ($P < 0.05$). The data are means ± SE of three independent experiments. (C) 1B5 myotubes expressing wtRYR1 or RYR1-TM were activated by 40 mM caffeine (two times with 20 s interval). wtRYR1 showed typical responses for caffeine. (D) Unlike wtRYR1, RYR1-TM responded to caffeine by three different modes: typical (upper), atypical (middle), and no response (bottom). The portion of each type of response is summarized in Table 1. Slopes for the activation and inactivation curves of typical and atypical responses are represented by dotted lines. Note that atypical responses have much slower component for activation and a single fast component for inhibition.

suggesting that RYR1-TM is successfully targeted and is part of the CRUs.

To examine the ability of RYR1-TM to bind triadin, whole cell lysates of 1B5 myotubes expressing wtRYR1 or RYR1-TM were subjected to co-immunoprecipitation experiment with anti-RYR1 antibody and the immune-complex was probed with anti-RYR1 or anti-triadin antibody (Fig. 1B). Immunoblot analysis indicated a significant reduction in the amount of triadin in RYR1-TM immunoprecipitates relative to wtRyR1 immunoprecipitates ($55.5 \pm 9.9\%$ of the wtRyR1 value). Control experiments indicated that the expression level of triadin/mg protein in myotubes expressing RYR1-TM was not significantly changed (see Supplementary data 2). The results suggest that the RYR1-TM mutation reduced triadin–RYR1 complex formation and/or stability, as suggested in our earlier *in vitro* study [15].

RYR1-TM showed atypical responses to caffeine

We next considered the possibility that disruption of the triadin-binding site in RyR1 affected SR Ca^{2+} release. We tested this directly by applying the RYR1 agonist caffeine to 1B5 myotubes expressing wtRYR1 or RYR1-TM, using Ca^{2+} indicator dye Fluo-4 (Fig. 1C and D). Colonies of 1B5 myotubes containing more than four nuclei and exhibiting a Ca^{2+} transient following the brief (1 s) addition of caffeine were selected. Myotubes were then treated twice with caffeine for 30 s at 20 s interval. Fig. 1C shows two typical Ca^{2+} transients for wtRYR1 obtained by addition of 40 mM caffeine. Approximately 90% of selected myotubes responded to 40 mM caffeine (Table 1).

RYR1-TM responded to caffeine in three modes, exhibiting a typical, atypical or no response (Fig. 1D). The upper panel in Fig. 1D shows two ‘‘typical’’ responses similar to

Table 1
RYR1-TM and D4907A showing atypical responses to caffeine

	wtRYR1		RYR1-TM		D4907A	
No. of cells/batches:	139/5		131/5		258/5	
Caffeine trials:	1st	2nd	1st	2nd	1st	2nd
No response (%)	6.73 ± 3.37	9.30 ± 2.84	29.32 ± 7.89*	35.14 ± 13.70*	21.47 ± 4.86*	23.27 ± 4.17*
Response (%)	93.27 ± 3.37	90.70 ± 2.84	70.68 ± 7.89*	64.86 ± 13.70*	78.53 ± 4.86*	76.73 ± 4.17*
Typical (%)	90.63 ± 5.98	82.29 ± 7.29	62.50 ± 4.17*	79.17 ± 11.50	72.61 ± 12.54*	80.56 ± 10.01
Atypical (%)	9.37 ± 5.98	17.71 ± 7.29	37.50 ± 4.17*	20.83 ± 11.50	27.39 ± 12.54*	19.44 ± 10.01

For each group (wtRYR1, RYR1-TM, and D4907A), the percentage of caffeine-responsive and unresponsive myotubes is given. Responsive myotubes are sub-divided into two types, typical and atypical response. Compared to typical responses, atypical responses are characterized by slower activation and faster inactivation.

* Significant difference compared to wtRyR1 ($P < 0.05$).

wtRYR1 (Fig. 1C). A fast phase of Ca^{2+} release and subsequent slower phase of cytosolic Ca^{2+} removal was followed by a fast removal phase that coincided with the removal of caffeine. The atypical responses are characterized by a slower activation phase and an almost complete removal of cytosolic Ca^{2+} in the presence of caffeine (Fig. 1D, middle panel). Approximately one-third ($37.50 \pm 4.17\%$) of myotubes expressing RYR1-TM showed an atypical response (Table 1). Atypical responses were also observed in wtRYR1, but with a 4-fold lower

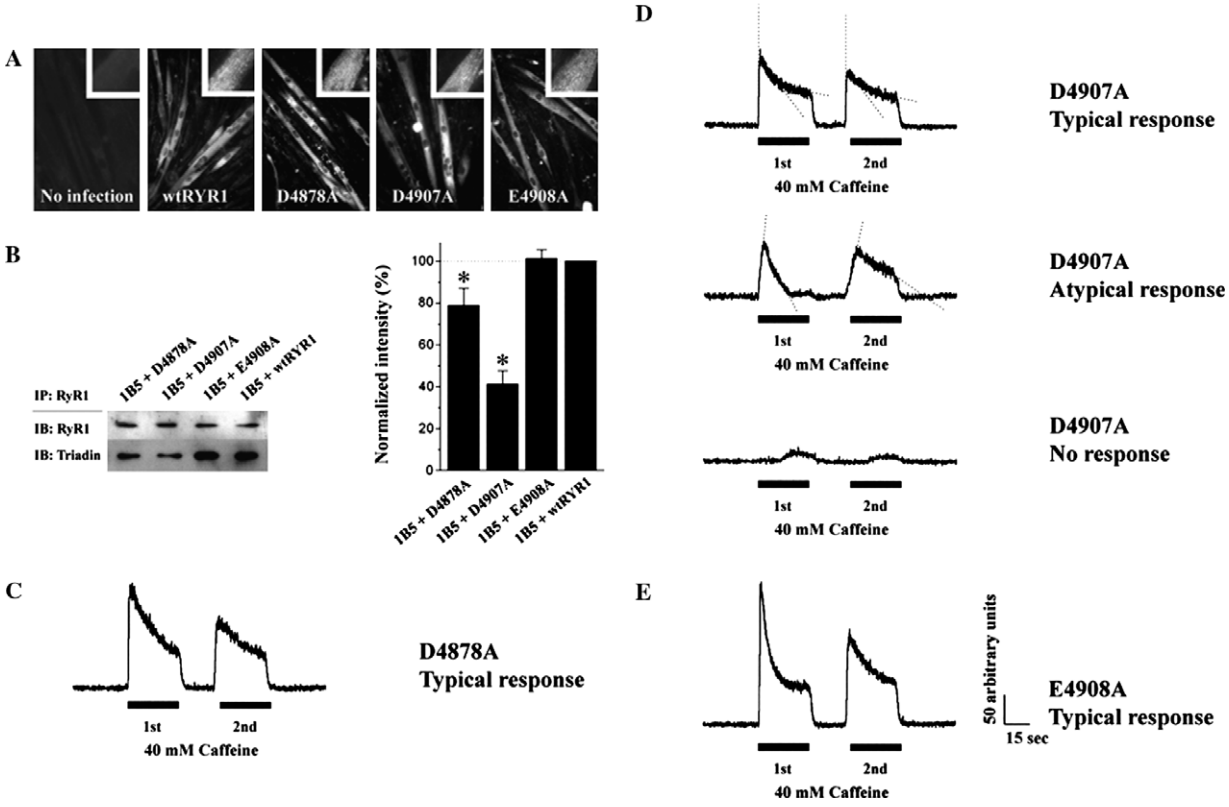

Fig. 2. Interaction between single mutant (D4878A, D4907A, and E4908A) and triadin, and atypical response of D4907A to caffeine. (A) Expression profiles of wtRYR1 and single mutants D4878A, D4907A, and E4908A in 1B5 myotubes were probed by immunohistochemistry with anti-RYR1 antibody. Higher-magnification images in cross-sectional areas present the localization pattern of wtRYR1 and single mutants. Uninfected 1B5 myotubes (No infection) were used as a negative control. (B) Whole cell lysate of 1B5 myotubes expressing each RYR1 was subjected to co-immunoprecipitation experiment with anti-RYR1 antibody and probed with anti-RYR1 or anti-triadin antibody. Normalized band intensity of co-immunoprecipitated triadin in each single mutant to that in wtRYR1 is presented as bar graphs on the right. *Significant difference compared to wtRyR1 ($P < 0.05$). The data are means ± SE of three independent experiments. (C) 1B5 myotubes expressing each single mutant were activated by 40 mM caffeine (two times with 20 s interval). D4878A showed typical responses for caffeine just like wtRYR1 in Fig. 1C. (D) Similar to RYR1-TM in Fig. 1D, D4907A responded to caffeine by three different modes: typical (upper), atypical (middle), and no response (bottom). The proportion of each type of responses in D4907A and RYR1-TM is compared in Table 1. Slopes for the activation and inactivation curves of typical and atypical responses are represented by dotted lines. Note that atypical responses have the same tendency in activation and inhibition curves: much slower component for activation and a single fast component for inhibition. (E) E4908A showed typical responses for caffeine.

frequency than in RYR1-TM (Table 1). In addition, 29.32 ± 7.89% of RYR1-TM did not respond to caffeine at all, which occurred with a much higher frequency than in wtRYR1 (6.73 ± 3.37%) (Table 1). Addition of 10 μM cyclopiazonic acid (CPA, a reversible SR Ca^{2+}-ATPase inhibitor) showed that this impaired Ca^{2+} release was not a result of lowered SR Ca^{2+} stores as there was no significant difference in the CPA response between wild type and mutant myotubes (data not shown).

RYR1-D4907 is the critical amino acid for triadin-binding and caffeine responses

To determine the role of each of the three mutations in RYR1-TM in contributing to the atypical caffeine responses and the ability to form triadin–RYR1 complexes, single mutants D4878A, D4907A or E4908A were examined with regard to their ability to bind triadin (Fig. 2A and B) and respond to caffeine (Fig. 2C–E). All three mutants were successfully expressed in 1B5 myotubes without a change in cell size, shape, differentiation level or targeting to CRUs (Fig. 2A). Co-immunoprecipitation experiment suggested greatly reduced amounts of triadin-binding to D4907A (41.3 ± 6.4% of the wtRYR1 value) (Fig. 2B), whereas D4878A showed a more modestly reduced binding (78.8 ± 8.2% of the wtRYR1 value) and E4908A bound triadin at a level comparable to that of wtRYR1.

Myotubes that expressed D4907A displayed the three types of caffeine response (typical, atypical, and no response) with almost the same frequency as in RYR1-TM expressing myotubes (Fig. 2D and Table 1). On the other hand, the caffeine response of the other two single mutants, D4878A and E4908A, was not different from that of wtRYR1 (Fig. 2C and E). Therefore it appears that the D4907 is the critical residue responsible for atypical caffeine responses observed in RYR1-TM expressing myotubes.

Discussion

In this study, we probed RYR1-triadin-binding site *in situ*, and its role in normal RyR1 function. We tested the effects of three amino acid substitutions (D4878, D4907, and E4908), previously shown to reduce *in vitro* triadin-binding to RYR1 [15], on their ability to bind triadin *in situ* and on their ability to support caffeine-induced Ca^{2+} release. Co-immunoprecipitation experiment and immunoblot analysis indicated significantly reduced amounts of triadin-binding in protein complexes isolated from myotubes expressing RYR1-TM and D4907A mutants. Furthermore, our results showed similar atypical Ca^{2+} transients in response to caffeine in myotubes expressing RYR1-TM and the single mutant (D4907A). Therefore, we conclude that the differences in triadin-binding and SR Ca^{2+} release observed in this study can be attributed to an alteration in a single amino acid (D4907).

Unlike our previous *in vitro* studies, we were unable to completely abolish triadin–RYR1 association *in situ* in any of the mutants tested. This may have been due to the presence of a weaker triadin–RYR1 interaction in the non-pore-forming region of RYR1. The cytoplasmic domain of triadin (aa residues, 18–46) was previously reported to interact with the cytosolic domain of RYR1 [21]. Use of various peptides corresponding to different parts of triadin also suggested that there are two different RYR1-binding sites in triadin, one with low and the other with high affinity [22]. Additionally, oxidation of hyper-reactive sulfhydryls in both RYR1 and triadin could be involved in stabilizing the triadin–RYR1 complex, leading to enhanced SR Ca^{2+} release [12].

We feel that it was unlikely that the altered caffeine responses were caused by a direct mutational effect on the RYR1 channel, because D4907A expressed in HEK293 cells, a triadin-free cell system, showed [^{3}H]ryanodine binding and single channel activities essentially identical to those of wild type RYR1 [23]. Thus we hypothesize that the decreased function resulted from the reduction of triadin–RYR1 interaction, with triadin in place. In support of this explanation, myotubes from a triadin-null mouse showed increased levels of resting free Ca^{2+} and spontaneous Ca^{2+} release activity compared to wild type myotubes [24]. A second possibility for the atypical caffeine response is that the D4907 mutation affected the interaction of RYR1 with CSQ. Although this may be the case, CSQ forms a multi-protein complex by binding to the RYR1-associated protein triadin [6,11,15] and, in single channel measurements, RYR1 was inhibited [25] or activated [26] by CSQ (using SR vesicles but not purified RYR1s), which suggests that the regulation of RYR1 by CSQ depends on the presence of RYR1-associated proteins such as triadin. We favor the explanation that a partial disruption of the interaction between triadin and RYR1 was responsible for the atypical caffeine responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.115.

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