Regions of ryanodine receptors that influence activation by the dihydropyridine receptor β_{1a} subunit

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Abstract

**Background:** Although excitation-contraction (EC) coupling in skeletal muscle relies on physical activation of the skeletal ryanodine receptor (RyR1) Ca^{2+} release channel by dihydropyridine receptors (DHPRs), the activation pathway between the DHPR and RyR1 remains unknown. However, the pathway includes the DHPR β_{1a} subunit which is integral to EC coupling and activates RyR1. In this manuscript, we explore the isoform specificity of β_{1a} activation of RyRs and the β_{1a} binding site on RyR1.

**Methods:** We used lipid bilayers to measure single channel currents and whole cell patch clamp to measure L-type Ca^{2+} currents and Ca^{2+} transients in myotubes.

**Results:** We demonstrate that both skeletal RyR1 and cardiac RyR2 channels in phospholipid bilayers are activated by 10–100 nM of the β_{1a} subunit. Activation of RyR2 by 10 nM β_{1a} was less than that of RyR1, suggesting a reduced affinity of RyR2 for β_{1a}. A reduction in activation was also observed when 10 nM β_{1a} was added to the alternatively spliced (ASI(−)) isoform of RyR1, which lacks ASI residues (A3481-Q3485). It is notable that the equivalent region of RyR2 also lacks four of five ASI residues, suggesting that the absence of these residues may contribute to the reduced 10 nM β_{1a} activation observed for both RyR2 and ASI(−)RyR1 compared to ASI(+)RyR1. We also investigated the influence of a polybasic motif (PBM) of RyR1 (K3495KKRRDGR3502) that is located immediately downstream from the ASI residues and has been implicated in EC coupling. We confirmed that neutralizing the basic residues in the PBM (RyR1 K-Q) results in an ~50 % reduction in Ca^{2+} transient amplitude following expression in RyR1-null (dyspedic) myotubes and that the PBM is also required for β_{1a} subunit activation of RyR1 channels in lipid bilayers. These results suggest that the removal of β_{1a} subunit interaction with the PBM in RyR1 could contribute directly to ~50 % of the Ca^{2+} release generated during skeletal EC coupling.

**Conclusions:** We conclude that the β_{1a} subunit likely binds to a region that is largely conserved in RyR1 and RyR2 and that this region is influenced by the presence of the ASI residues and the PBM in RyR1.

**Keywords:** Excitation-contraction coupling, Dihydropyridine receptor β_{1a} subunit, Ryanodine receptor isoforms, Skeletal muscle, Cardiac muscle
Background

Contraction in skeletal and cardiac muscle depends on Ca\(^{2+}\) release from the intracellular sarcoplasmic reticulum (SR) Ca\(^{2+}\) store through ryanodine receptor (RyR) Ca\(^{2+}\) release channels embedded in the SR membrane. This Ca\(^{2+}\) release is crucial to excitation-contraction (EC) coupling. During EC coupling, cardiac RyRs (RyR2) are activated by an influx of extracellular Ca\(^{2+}\) through depolarization-activated dihydropyridine receptor (DHPR) L-type channels located in the surface and transverse-tubule membranes. In contrast, EC coupling in skeletal muscle is independent of extracellular Ca\(^{2+}\), apparently requiring a physical interaction between skeletal isoforms of the RyR (RyR1) and DHPR [1, 2]. However, despite exhaustive investigation, the physical components of this interaction still remain unclear [3, 4] and are investigated in this manuscript.

It is well established that the skeletal isoforms of both the membrane spanning \(\alpha_{15}\) subunit and the cytoplasmic \(\beta_{1a}\) subunit of the DHPR heterotetramer are essential for skeletal EC coupling [5, 6]. The \(\alpha_{15}\) subunit contains the voltage sensor for EC coupling [7, 8] and the “critical” region for skeletal EC coupling (residues L720-764/5) in its intracellular II-III loop [9–11]. The \(\beta_{1a}\) subunit is responsible for the targeting of the DHPR to the triad and assembly into tetrads that are closely aligned with RyR1 in the SR [12–14]. There is also evidence that the \(\beta_{1a}\) subunit also plays an active role in the EC coupling process. The \(\beta_{1a}\) subunit directly activates RyR1 channels incorporated into lipid bilayers and enhances voltage-activated Ca\(^{2+}\) release in skeletal muscle fibers [5, 15, 16]. The C-terminal region of \(\beta_{1a}\) (V490-M524) supports \(\beta_{1a}\) binding to RyR1 in vitro and influences voltage-induced Ca\(^{2+}\) release in mouse myotubes [15, 17, 18]. A peptide corresponding to the same residues mimics full length \(\beta_{1a}\) subunit activation of RyR1 channels in lipid bilayers [15] and a truncated peptide of the same region enhances voltage-induced Ca\(^{2+}\) release to the same degree as the full length \(\beta_{1a}\) subunit in intact adult mouse muscle fibers [16, 19]. Furthermore, overexpression of a \(\beta\) subunit interacting protein, Rem, in adult mouse skeletal muscle fibers was recently shown to reduce voltage-induced Ca\(^{2+}\) transients by ~65% without substantially altering \(\alpha_{15}\) subunit membrane targeting or intramembrane gating charge movement or SR Ca\(^{2+}\) store content [20]. This suggests that the DHPR-RyR1 interaction may be uncoupled by virtue of direct interference of \(\beta_{1a}\) subunit. Residues in RyR1 that influence binding to the \(\beta_{1a}\) subunit have also been identified. The M3201-W3661 fragment of RyR1 binds to \(\beta_{1a}\) and the strength of binding is substantially reduced by replacing the six basic residues in a polybasic motif (PBM; K3495KKRRDGR3502) with glutamines [18]. Replacement of the same six residues with glutamines in the full-length RyR1 protein substantially reduces depolarization-dependent Ca\(^{2+}\) release [18]. The in vitro studies indicate a high-affinity interaction between the isolated RyR1 and the \(\beta_{1a}\) subunit that is influenced by the PBM. However, the basic residues unlikely bind directly to the hydrophobic residues in the \(\beta_{1a}\) C-terminus, although they could contribute to the overall conformation of the binding domain [21]. Similarly, it is unlikely that basic residue binding to the hydrophobic residues could contribute to EC coupling, although both basic residues and hydrophobic residues in the \(\beta_{1a}\) C-terminus influence EC coupling [16, 19].

The fact that skeletal DHPR and RyR isoforms are critical for skeletal-type EC coupling [22–26] suggests that isoform-specific regions of these proteins enable unique interactions in skeletal muscle. Also, in the context of isoform dependence, we reported that an alternatively spliced region of RyR1 (A3481-Q3485), located close to the PBM, is significant in setting the gain of EC coupling [27]. It is notable that RyR2 lacks the equivalent sequence to the ASI residues in ASI(+)/RyR1 and, in this respect, more closely resembles the ASI(−)/RyR1 isoform. Therefore, here we examined the RyR isoform dependence of the in vitro interaction with the \(\beta_{1a}\) subunit. We use the RyR isoforms as tools to explore regions of the RyR1 that influence its interaction with the C-tail of the \(\beta_{1a}\) subunit. Interactions between RyR2 and the cardiac \(\beta\) subunit were not examined as they have no physiological significance, and there is little sequence homology between the C-terminal tails of the cardiac and skeletal \(\beta\) isoforms [12–14].

The results indicate that while \(\beta_{1a}\) activates RyR1 and RyR2 isolated from the skeletal muscle and the heart and activates recombinant ASI(−)/RyR1 and ASI(+)/RyR1, \(\beta_{1a}\) activation of RyR2 and ASI(−)/RyR1 requires higher \(\beta_{1a}\) concentrations than that required to activate RyR1 or ASI(+)/RyR1. In addition, we show that neutralization of the basic residues in the RyR1 PBM abolishes \(\beta_{1a}\) activation of RyR1 in lipid bilayers and confirm that this also markedly reduces voltage-dependent Ca\(^{2+}\) release in skeletal myotubes. Together, the results reinforce the conclusion that \(\beta_{1a}\) binding to RyR1 contributes to EC coupling and suggest that the region encompassing the adjacent ASI residues and PBM is a determinant of \(\beta_{1a}\) binding to and regulation of RyR1.

Methods

The work was approved by The Australian National University Animal Experimentation Ethics Committee (Australian Capital Territory, Australia) and by the University Committee on Animal Resources at the University of Rochester (New York, USA).

Preparation of RyR1 ASI (−) and K-Q cDNA

The ASI(−)/RyR1 variant was introduced into rabbit RyR1 cDNA (accession #X15750) using two-step site-directed mutagenesis as described previously [28]. The K-Q mutant
(K3495KKRRGDR3502) was similarly introduced into a rabbit RyR1 cDNA by two-step site-directed mutagenesis in the following manner: using a BsiWI/BamHI subclone of RyR1, residues R3498Q and R3499Q were introduced via mutagenesis to create a double mutation (R3498Q/R3499Q). This mutant was used as a template to introduce a third mutation R3502Q. Finally, glutamine substitutions for residues K3495, K3496, and K3497 were introduced into the triple mutated plasmid to generate the PBM mutant Q3495QQQGDQ3502 (K-Q mutant). The entire PCR-modified cDNA portion of the BsiWI/BamHI mutant subclone was confirmed by sequence analysis and then cloned back into full-length RyR1.

**Preparation of SR vesicles**

Skeletal muscle SR vesicles were prepared from back and leg muscles (fast twitch skeletal muscle) from New Zealand white rabbits [29–31] and cardiac SR vesicles collected from sheep hearts [32, 33]. Vesicles were stored at −70 °C.

**Transfection and preparation of microsomal protein**

Microsomal vesicles were collected from HEK293 transfected with recombinant rabbit RyR1 ASI(+), ASI(−), or K-Q RyR1 mutant cDNAs in mammalian expression vector (pCIneo) as described previously [28] with minor modifications. HEK cells were grown in 175-mm flasks at 37 °C, 5 % CO2 in 10 % fetal calf serum in MEM. At 50–60 % confluence, cells were transfected with 80 μg cDNA in a phosphate buffer solution (125 mM CaCl2, 70 mM NaH2PO4, 140 mM NaCl, 76 mM HEPES, 7 mM Na2HPO4, pH 7.2) using a calcium phosphate precipitation method. Cells were maintained for 48 h and then harvested in phosphate buffer (137 mM NaCl; 7 mM Na2HPO4; 2.5 mM NaH2PO4; H2O; and, 2 mM EGTA, pH 7.4). The resulting pellet was resuspended in homogenizing buffer (300 mM sucrose, 5 mM imidazole, 1× complete EDTA-free protease inhibitor cocktail, pH 7.4), homogenized and centrifuged at 11,600 × g for 20 min. The resulting pellet was resuspended in homogenizing buffer, further homogenized and centrifuged at 91,943 × g for 2 h. The pellet was resuspended in homogenizing buffer, homogenized, and then briefly sonicated. The microsomal mixture was separated into 15 μL aliquots and stored at −70 °C.

**Preparation and injection of dyspedic myotubes**

Primary cultures of myotubes were obtained from skeletal myoblasts isolated from newborn RyR1-null (dyspedic) mice as previously described [34, 35]. Four to 6 days after initial plating of myoblasts, nuclei of dyspedic myotubes were microinjected with cDNAs encoding CD8 (0.1 μg/μl) and the appropriate RyR1 expression plasmid (0.5 μg/μl) [36]. Expressing myotubes were identified 2–4 days after cDNA microinjection by incubation with CD8 antibody beads (Dynabeads, Dynal USA). All animals were housed in a pathogen-free area at the University of Rochester and experiments performed in accordance with procedures reviewed and approved by the local University Committees on Animal Resources.

**Preparation of β1a subunit**

The β1a protein was expressed in transformed Escherichia coli BL21(DE3) and purified as described previously [15]. The proteins were dialyzed against a phosphate buffer (50 mM Na2PO4, 300 mM NaCl, pH 8) and stored at −70 °C.

**Single-channel recording and analysis**

Channels from cardiac, skeletal, or HEK293 microsomal vesicles were incorporated into lipid bilayers with solutions containing (mM): cis (20 CsCl, 230 CsCH3O2S, 10 TES, and 1 CaCl2) and trans (20 CsCl, 30 CsCH3O2S, 10 mM TES, and 1 CaCl2), pH 7.2. After RyR incorporation, 200 mM CsMS was added to the trans solution for symmetrical [Cs+]. BAPTA was added to the cis solution as determined with a Ca2+ electrode to achieve 10 μM Ca2+, and 2 mM ATP was added. Bilayer potential, \( V_{cis} \), was switched between −40 and +40 mV. Channel activity under each condition was analyzed over 180 s using the program Channel 2 (developed by P. W. Gage and M. Smith). Threshold levels for channel opening were set to exclude baseline noise at ~20 % of the maximum single-channel conductance and open probability \( p_o \), mean open time \( \tau_o \), and closed open time \( \tau_c \) measured. Dwell-time distributions for each channel were obtained using the log-bin method [37–39]. Event frequency (probability) was plotted against equally spaced bins (on a logarithmic scale) for open or closed durations (seven bins per decade). The time constants are indicated by the frequency peaks. The area under each peak indicates the fraction of single-channel open or closed events falling into each time constant component.

**Simultaneous measurements of macroscopic Ca2+ currents and transients in myotubes**

The whole-cell patch clamp technique was used to simultaneously measure voltage-gated L-type Ca2+ currents (L currents) and Ca2+ transients in expressing myotubes [36]. Patch clamp experiments were conducted using an external solution consisting of (in millimolar): 145 TEA-Cl, 10 CaCl2, and 10 HEPES, pH 7.4 with TEA-OH and an internal pipette solution consisting of (in millimolar): 145 Cs-aspartate, 10 CsCl, 0.1 Cs2-EGTA, 1.2 MgCl2, 5 Mg-ATP, 0.2 K3-fluo-4, and 10 HEPES, pH 7.4 with CsOH. Peak L-current magnitude was normalized to cell capacitance (pA/pF), plotted as a function of the membrane potential (I-V curves in Fig. 6c), and fitted according to:
\[ I = G_{\text{max}} \cdot (V_m - V_{\text{rev}}) / (1 + \exp}\left(\frac{(V_{G1/2} - V_m)}{k_G}\right) \]

where \( G_{\text{max}} \) is the maximal L-channel conductance, \( V_m \) is test potential, \( V_{\text{rev}} \) is the L-channel reversal potential, \( V_{G1/2} \) is the potential for half-maximal activation of \( G_{\text{max}} \), and \( k_G \) is a slope factor. Relative changes in fluo-4 fluorescence (\( \Delta F/F \)) were measured at the end of each 200-ms depolarization, plotted as a function of the membrane potential, and fitted according to:

\[ \Delta F/F = (\Delta F / F_{\text{max}}) / \left(1 + \exp\left(\frac{(V_{F1/2} - V_m)}{k_F}\right)\right) \]

where \( \Delta F/F_{\text{max}} \) is the maximal fluorescence change, \( V_{F1/2} \) is the potential for half-maximal activation of \( \Delta F/F \) and \( k_F \) is a slope factor. The bell-shaped voltage dependence of \( \Delta F/F \) measurements obtained in RyR1 K-Q mutant-expressing myotubes were fitted according to the following equation:

\[ \Delta F/F = ((\Delta F / F)_{\text{max}}(V_m - V_{\text{rev}})/k') / (1 + \exp\left(\frac{(V_{F1/2} - V_m)}{k_F}\right)) \]

where \( (\Delta F/F)_{\text{max}}, V_m, V_{\text{rev}}, V_{F1/2} \) and \( k_F \) have their usual meanings. The additional variable \( k' \) is a scaling factor that varies with \( (\Delta F/F)_{\text{max}} \) [40, 41]. The maximal rate of voltage-gated SR Ca\(^{2+}\) release was approximated from the peak of the first derivative of the fluo-4 fluorescence trace (\( dF/dt \)) elicited during the test depolarization at 30 mV. Pooled current-voltage (\( I-V \)) and fluorescence-voltage (\( \Delta F/F-V \)) data in Table 1 are expressed as mean ± SEM.

**Immunofluorescence labeling**

RyR-null (*dyspedic*) myotubes expressing either WT RyR or RyR K-Q mutant that were plated on glass coverslips were fixed and immunostained with a mouse monoclonal anti-RyR antibody (34C, 1:10; Developmental Studies Hybridoma Bank) and a sheep polyclonal anti-DHPR antibody (1:200; Upstate Biotechnology) overnight at 4 °C as previously described [41]. On the following day, coverslips were washed with PBS three times each for 5 min and then incubated for 1 h at room temperature in blocking buffer containing a 1:500 dilution of Alexa Fluor 488--labeled donkey anti-mouse IgG (Molecular Probes) and 1:500 dilution of rhodamine-labeled donkey anti-sheep IgG (Jackson Immunoresearch Laboratories Inc.) and washed with PBS (three times for 5 min each). Coverslips were mounted on glass slides and images obtained using a Nikon Eclipse-C1 confocal microscope (Nikon Instruments Inc.) and a 40× oil objective. All confocal images were sampled at a spatial resolution (pixel diameter) of 100 nm.

**Statistics**

Average data are given as the mean ± SEM. Statistical significance was evaluated by a paired or unpaired two-way Student’s t-test or analysis of variance (ANOVA) with Fisher’s post hoc test, as appropriate. The numbers of observations (\( N \)) are given in the figure legends. To reduce the effects of variability in control single-channel activity parameters (\( P_{oC}, T_{oC}, T_{oB} \)) and to evaluate parameters after \( \beta_{1a} \) subunit (\( P_{oB}, T_{oB}, T_{oB} \)) addition, data were expressed as the difference between the logarithmic values, i.e., \( \log_{10} P_o = \log_{10} P_{oB} - \log_{10} P_{oC} \). The difference from control was assessed with a paired \( t \)-test applied to \( \log_{10} P_{oB} \) and \( \log_{10} P_{oC} \). Variance in \( P_o \) parameter values was assessed with an unpaired \( t \)-test. A \( p \) value of <0.05 was considered significant.

**Results**

Ability of the \( \beta_{1a} \) subunit to activate different RyR isoforms

**The \( \beta_{1a} \) subunit activates RyR1 and RyR2 channels**

As we reported previously [15], when added to the cytoplasmic cis chamber, the full-length \( \beta_{1a} \) subunit increases the activity of native RyR1 channels incorporated into planar lipid bilayers (Fig. 1a). Both 10- or 100-nM concentrations of \( \beta_{1a} \) subunit maximally activate RyR1 channels in the presence of 10 \( \mu \)M Ca\(^{2+}\) and 2 mM Na\(_3\) ATP [15]. The records in Fig. 1b show that RyR2 channel activity also increases upon cytoplasmic exposure to 10 nM \( \beta_{1a} \) subunit, but in contrast to RyR1, greater activation of RyR2 is observed with 100 nM \( \beta_{1a} \). On average, addition of 10 nM or 100 nM \( \beta_{1a} \) to the cis solution significantly increased the relative \( P_o \) of RyR2 by 1.8-fold and 2.6-fold, respectively (Fig. 2a, left). Data is presented as average relative \( P_o \) which is the average of the logarithm to the base 10 of \( P_o \) of each individual channel in the

**Table 1** Parameters of fitted \( I-V \) and \( \Delta F/F-V \) curves

| \( I-V \) data | \( \Delta F/F-V \) data |
|----------------|-----------------|
| \( G_{\text{max}} \) (\( \mu \)S/\( \mu \)F) | \( k \) (mV) | \( V_{\text{half}} \) (mV) | \( V_{\text{rev}} \) (mV) |
| \( \Delta F/F_{\text{max}} \) | \( k \) (mV) | \( V_{\text{half}} \) (mV) |
| WT RyR1 (\( n = 12 \)) | 264 ± 16 | 5.4 ± 0.4 | 10.5 ± 1.8 | 71 ± 1.8 | 3.4 ± 0.7 | 3.9 ± 0.5 | -4.7 ± 1.6 |
| RyR1 K-Q (\( n = 10 \)) | 201 ± 17* | 5.7 ± 0.3 | 11.5 ± 1.7 | 70 ± 2.1 | 1.6 ± 0.3* | 4.1 ± 0.4 | -2.5 ± 1.7 |

Maximal L-channel conductance (\( G_{\text{max}} \)), the potential for half-maximal \( G_{\text{max}} \) (\( V_{\text{half}} \)), slope factor (\( k \)), and reversal potential (\( V_{\text{rev}} \)). Values presented as mean ± SEM for \( I-V \) data presented in Fig. 6c. Maximal Ca\(^{2+}\) transient (\( \Delta F/F_{\text{max}} \)), the potential at half maximal fluorescence (\( V_{\text{half}} \)) and slope factor (\( k \)). Values presented as mean ± SE for (\( \Delta F/F-V \)) data presented in Fig. 6d.

* \( p < 0.05 \) vs WT RyR1-expressing dyspedic myotubes.
The action of $\beta_{1a}$ on single-channel gating parameters (Fig. 2b, c) reflected the changes in $P_o$ (Fig. 2a, left and right). Both RyR1 and RyR2 activity increased with 10 and 100 nM $\beta_{1a}$ as a result of increases in mean channel open time and an abbreviation of mean channel closed time (Fig. 2b, c). There was also a trend towards a greater increase in mean open time in RyR2 with the higher $\beta_{1a}$ concentration that is consistent with the greater RyR2 open probability in the presence of 100 nM $\beta_{1a}$. In contrast, RyR1 mean open time was similar at both $\beta_{1a}$ concentrations. Mean closed times were similarly reduced for both RyR isoforms by 10 and 100 nM $\beta_{1a}$.

The effects of $\beta_{1a}$ on the open ($T_o$) and closed ($T_c$) time constant components and the relative distribution of events between time constants is presented in Figs. 3 and 4. Open events in RyR1 and RyR2 channels were well described by the sum of three time constants of $\sim 1$ (tcp1), $\sim 10$ (tcp2), and $\sim 100$ ms (tcp3) (Fig. 3). Closed times were also characterized by three time constants of $\sim 1$ (tcc1), $\sim 10$ (tcc2), and $\sim 100$ ms (tcc3) (Fig. 3). Figure 4 shows plots of the average probability of open (Fig. 4a, b, upper plots) and closed (Fig. 4a, b, lower plots) events as a function of the average time constant in the absence (control) and presence of either 10 or 100 nM $\beta_{1a}$. Neither the time constants nor the relative probability of events for each time constant varied significantly ($p = 0.12–0.99$) between +40 and −40 mV and thus were combined in the average data. Both 10 and 100 nM concentrations of $\beta_{1a}$ subunit decreased the fraction of RyR2 openings in $T_o$ by $18.7 \pm 1.8 \%$ (p = 0.003) and $16.3 \pm 2.0 \%$ (p = 0.012), respectively (Fig. 4b). There was a corresponding increase in the fraction of events for the longer open time constant components at both $\beta_{1a}$ concentrations (Fig. 4b). In contrast to RyR2, the maximal increases in RyR1 activity after exposure to 10 or 100 nM $\beta_{1a}$ subunit were reflected in a reduction in the fraction of RyR1 open events in tcp1 and increases in events in the longer time constant group (tcp2) at both $\beta_{1a}$ concentrations (Fig. 4a). The closed time constant distributions in RyR2 and RyR1 were also altered by both 10 and 100 nM $\beta_{1a}$, albeit in slightly different ways. There was an apparent transfer of $14.9 \pm 3.1 \%$ of closed events in RyR2 from tcp1 to tcp2 with 10 nM $\beta_{1a}$ and $13.8 \pm 4.7 \%$ with 100 nM $\beta_{1a}$ (Fig. 4b). In contrast, for RyR1, there were fewer long closed events in tcp3 and more short closed events in tcp1 with both 10 and 100 nM $\beta_{1a}$ than in control (Fig. 4a).

Overall, the results indicate that 10 and 100 nM $\beta_{1a}$ increase both RyR1 and RyR2 activity but with a reduced activation of RyR2 by 10 nM $\beta_{1a}$. The dwell-time distributions indicate subtle differences between RyR1 and RyR2 in the effects of $\beta_{1a}$ in redistribution between the presence of $\beta_{1a}$, relative to the logarithm of the $P_o$ of its internal control activity measured before application of $\beta_{1a}$. Use of relative $P_o$ eliminates any effect of the normal variability between individual RyR channels [39, 42]. The logarithm is used to reveal the extent of variation of the effects of $\beta_{1a}$. The relative changes are relative to the logarithm of the $P_o$ of its internal control activity measured before application of $\beta_{1a}$. Use of relative $P_o$ eliminates any effect of the normal variability between individual RyR channels [39, 42]. The logarithm is used to reveal the extent of variation of the effects of $\beta_{1a}$. The average of $P_o$ parameter values are also shown to indicate relative level of each parameter (Fig. 2a–c, right), however, the relative changes should be used as the most accurate indicator of effects of $\beta_{1a}$ on RyRs. The effects on RyR2 channel activity were similar at +40 and −40 mV (relative $P_o$ with 10 nM $\beta_{1a}$ increasing by ~2-fold at +40 mV and ~1.7-fold at −40 mV), and these values were combined in the average data in Fig. 2a. It has been established that the activation of RyR1 by $\beta_{1a}$ is maximal at 10 nM and does not increase between 10 and 1000 nM [15]. Therefore, the reduced efficacy of 10 nM $\beta_{1a}$ on RyR2 suggests that affinity of RyR2 for $\beta_{1a}$ is lower than that of RyR1.
different time constant components. In particular, $\beta_{1a}$ induced a significant increase in events in the longest open time constant component in RyR2 but not RyR1 activity, while significantly reducing the number of events in the longest closed time constant component of RyR1 but not RyR2 activity.

The alternatively spliced ASI residues impact the functional interaction between $\beta_{1a}$ and RyR1

There is a curious similarity between the cardiac RyR2 isoform and the ASI($-$) splice variant of RyR1 in that both lack ASI residues. This may be relevant to the effect of $\beta_{1a}$ on RyR1 and its contribution to EC coupling as we have shown that the presence of the alternatively spliced ASI residues influences the gain of EC coupling in skeletal myotubes [27] and modulates RyR1 activity in vitro [28]. Therefore, we determined the impact of the alternatively spliced ASI residues on the activation of RyR1 by $\beta_{1a}$. Recombinant ASI($-$)-RyR1 and ASI($+$)-RyR1 constructs [28, 43] were incorporated into lipid bilayers and the actions of the $\beta_{1a}$ subunit on channel activity examined (Fig. 5). The ASI($+$) isoform is the adult isoform of RyR1 and its sequence is equivalent

Fig. 2 $\beta_{1a}$ subunit increases RyR1 and RyR2 channel activity in lipid bilayers. Single-channel gating parameters of RyR1 and RyR2 in response to 10 or 100 nM $\beta_{1a}$ subunit. a (left) Average relative $P_o$ ($\log_{10}P_o$) is the average of the differences between the logarithm of $P_o$ following addition of $\beta_{1a}$ subunit ($\log_{10}P_{oB}$) and the logarithm of the control $P_o$ ($\log_{10}P_{oC}$), where $P_{oC}$ was measured before $\beta_{1a}$ subunit addition. b (left) Average relative mean open time ($\log_{10}$ rel $T_o$). c (left) Average relative mean closed time ($\log_{10}$ rel $T_c$) were calculated in the same way as the average $\log_{10}P_o$ (above). a-c (right) The average single-channel parameter values are shown right of the corresponding relative values. a-c Single-channel parameters were calculated from ~180 s of channel activity (at +40 and −40 mV). Data are shown for 0 nM $\beta_{1a}$ (black bar), 10 nM $\beta_{1a}$ subunit (dark shade bar), and 100 nM $\beta_{1a}$ subunit (light shade bar), when examined. Error bars indicate ± SEM., n = 7–15 experiments/bar. *p < 0.05 vs control determined using paired (left) or un-paired (right) Student’s t-test, *p < 0.05 vs 10 nM $\beta_{1a}$ subunit with RyR2 determined by ANOVA.
to the adult rabbit RyR1 used in the previous section and to the cloned wild type (WT) rabbit RyR1 sequence described in the following section. It is notable in the single-channel activity, as shown in Fig. 5a, b (and in Fig. 7 below), that the recombinant channels (both ASI(-)RyR1 and ASI(+)RyR1) display strong sub-conductance (or sub-state) activity, with long channel openings to levels at ~50 % of the maximal conductance. Channel activity was measured as usual ("Methods" section) with an open threshold set at ~20 % of the maximum single-channel conductance to exclude baseline noise but to include sub-conductance openings to levels >20 % of the maximum. It is important to note that similar amounts of sub-conductance activity were seen in HEK293-expressed WT and ASI(−) compared in Fig. 5 and in WT and RyR1 K-Q channels compared in Fig. 8. Similarly, the smaller amounts of sub-conductance activity were comparable in RyR1 and RyR2 isolated from muscle tissue and compared in Fig. 1. In each case, sub-state activity was similar in constructs being compared.

Subconductance activity has been associated with full or partial depletion of FKBP12 from RyRs [29, 30]. Densitometry measurements of immunoprobed RyR and FKBP following co-immunoprecipitation of the RyR1 complex indicate a 65 % reduction ($p = 0.014$) in FKBP bound to RyR1 in recombinant ASI(+) RyR1 when compared to native RyR1 isolated from muscle. Thus, the sub-conductance activity observed for the recombinant

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**Fig. 3** Effects of $\beta_{1a}$ subunit on the distribution of representative RyR channel open and closed dwell times. Exponential open and closed time constants determined for RyR1 (a–c) and RyR2 (d–f). Open and closed times were collected into logged bins and the square root of the relative frequency of events (probability $1/2$) was plotted against the logarithm of open (open circles) or closed times (filled circles) in milliseconds. Examples are shown for the data from representative individual channels under control (a, d) and after exposure to 10 nM $\beta_{1a}$ subunit (b, e) and then 100 nM $\beta_{1a}$ subunit (c, f). The solid lines represent the fit of multiple exponentials to the data. The individual open time constants ($\tau_{o1}$, $\tau_{o2}$, and $\tau_{o3}$) and individual closed time constants ($\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$) are indicated by arrows.
channels is consistent with reduced FKBP12 expression in HEK293 cells and reduced amounts associated with the recombinant RyR1 channels.

Cytoplasmic addition of 10 nM $\beta_{1a}$ to ASI(+)RyR1 channels produced a significant ~4.4-fold increase in relative $P_o$ and a significantly smaller ~2.3-fold increase in relative $P_o$ of ASI(-)RyR1 (Fig. 5c). There was no significant difference between the degree of activation of the two RyR1 splice variants following application of 50 nM $\beta_{1a}$ (Fig. 5c), so that the efficacy of 10 nM $\beta_{1a}$ on ASI(-)RyR1 and RyR2 that lack the ASI sequence to application of 10 nM $\beta_{1a}$ is significantly reduced compared with RyR proteins that contain the ASI sequence, i.e., ASI(+)RyR1 or adult RyR1 isolated from rabbit skeletal muscle.

The impact of the polybasic K3495-R3502 residues on EC coupling and $\beta_{1a}$ activation of RyR1

The RyR1 polybasic motif facilitates EC coupling in expressing dyspedic myotubes

The PMB (residues K3495-R3502) in RyR1, located immediately downstream from the ASI region (A3481-Q3485), has been implicated in $\beta_{1a}$ binding to RyR1 and EC coupling [18]. To assess the effect of the PMB on the interaction between $\beta_{1a}$ and RyR1 channels in bilayers, a mutant of RyR1 in which all six polybasic residues were substituted with glutamines (RyR1 K-Q) was constructed. The functional effects of the RyR1 K-Q mutant on voltage-gated SR Ca$^{2+}$ release and DHPR L-type currents were confirmed following expression in dyspedic myotubes [18].

Depolarization-dependent Ca$^{2+}$ release was measured simultaneously with DHPR L-type Ca$^{2+}$ currents (Fig. 6a, b). Peak L-current density (Fig. 6c) and maximal DHPR Ca$^{2+}$ conductance ($G_{max}$) were significantly reduced in RyR1 K-Q mutant-expressing myotubes compared to WT RyR1-expressing myotubes (Table 1). Consistent with an earlier report [18], maximal voltage-induced SR Ca$^{2+}$ release was also significantly reduced in RyR1 K-Q mutant-expressing myotubes (Fig. 6d and Table 1). In addition, the maximum rate of depolarization-induced Ca$^{2+}$ release (approximated from the peak of the first derivative of the fluo-4 fluorescence trace elicited during a test depolarization at 30 mV) was significantly reduced in RyR1 K-Q-expressing myotubes compared to WT RyR1-

![Fig. 4 Effects of $\beta_{1a}$ subunit on the distribution of RyR channel open and closed dwell times. The probability of open and closed events falling into each time constant is plotted against the respective time constant. The open ($\tau_o$, top graphs) and closed ($\tau_c$, bottom graphs) time constants and the probability of events in each time constant component were calculated from ~180 s of single channel activity (at +40 and −40 mV). Data is shown for a RyR1 and b RyR2 before (open circle) and after addition of 10 nM $\beta_{1a}$ subunit (open triangle) or 100 nM $\beta_{1a}$ subunit (open square), n = 6–12 channel traces. Error bars indicate ± SEM. The individual open time constants ($\tau_{o1}$, $\tau_{o2}$, and $\tau_{o3}$) and individual closed time constants ($\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$) are indicated on the top and bottom graphs, respectively. *p < 0.05 vs the probability of events in each time constant in control with 10 nM $\beta_{1a}$ subunit, determined by ANOVA. #p < 0.05 vs the probability of events in each time constant in control with 100 nM $\beta_{1a}$ subunit, determined by ANOVA.]

Rebbeck et al. Skeletal Muscle (2015) 5:23 Page 8 of 15
expressing myotubes (Fig. 6e). These findings indicate that the RyR1 K-Q mutation substantially reduces voltage-induced SR Ca\(^{2+}\) release, with a small effect on maximal L-channel conductance. It should be noted that the reduced Ca\(^{2+}\) release is unlikely to result from reduced expression of RyR1 K-Q as it was previously shown that peak 4-chloro-m-cresol stimulated SR Ca\(^{2+}\) release was similar in WT RyR1- and RyR1 K-Q-expressing myotubes [18]. In addition, we found that WT RyR1 and RyR1 K-Q exhibited a similar punctate pattern and DHPR co-localization in expressing myotubes, consistent with similar levels of WT and K-Q expression and junctional localization (Additional file 1: Figure S1 and Additional file 2).
The small reduction in $G_{\text{max}}$ is unlikely to fully account for the large reduction in voltage-induced SR Ca$^{2+}$ release observed in RyR1 K-Q-expressing myotubes (Fig. 6d). This is supported by the sigmoidal voltage dependence of peak Ca$^{2+}$ release, a feature of skeletal-type EC coupling demonstrating that Ca$^{2+}$ release is independent of Ca$^{2+}$ influx. The reduction in depolarization-induced DHPR currents and SR Ca$^{2+}$ release could have resulted from poor targeting of the RyR1 K-Q mutant to the triad junction. However, double immunofluorescence labeling of RyR1 and the DHPR $\alpha_1$ subunit in expressing myotubes indicates that the DHPR and RyR1 proteins similarly co-localized as indicated by the yellow puncta in the overlays shown in Additional file 1. Therefore, compared to WT RyR1, the efficacy of voltage-induced
SR Ca\(^{2+}\) release is reduced in RyR1 K-Q-expressing myotubes.

**The polybasic motif in RyR1 is required for \(\beta_{1a}\) activation of RyR1**

We explored the possibility that the reduction in efficiency of depolarization-induced Ca\(^{2+}\) release was due to an effect of the K-Q substitution on gating properties of the RyR channel or to its response to cytoplasmic [Ca\(^{2+}\)] or ATP. RyR1 K-Q mutant channels exhibited unitary conductance of 222.3 ± 18.5 pS at +40 and 247.3 ± 33.1 pS at −40 mV, similar (\(p=0.07–0.78\)) to that of WT RyR1 (311.9 ± 25.2 pS at +40 and 236.5 ± 12.5 pS at −40 mV), or ~220 pS as previously reported for WT RyR1 expressed in HEK293 cells under the recording conditions used in this study [44].

The effects of cytoplasmic Ca\(^{2+}\) and ATP were similar (\(p=0.356–0.894\)) between +40 and −40 mV, and the data were combined. A decrease in cis free [Ca\(^{2+}\)] from 1 mM to 10 \(\mu\)M caused a 1.7-fold increase in WT RyR1 \(P_o\) and a similar 1.6-fold increase in RyR1 K-Q \(P_o\) (log\(_{10}\) rel \(P_o\) of 0.22 ± 0.06 \(p=0.013\) and 0.20 ± 0.09 \(p=0.048\), respectively, \(n=7\) for each). Similar increases in \(P_o\) with a decrease in cis free [Ca\(^{2+}\)] from 1 mM to 10 \(\mu\)M have been reported previously for recombinant WT RyR1 channels in lipid bilayers [44] and for \(^{3}H\)ryanodine binding to RyR1 [33]. Addition of 2 mM Na\(_2\) ATP to the cis solution increased WT RyR1 activity by 2.2-fold and RyR1 K-Q activity by 2.5-fold (log\(_{10}\) rel \(P_o\) of 0.34 ± 0.12 \(p=0.032\) and 0.40 ± 0.14 \(p=0.037\), respectively, \(n=7\) for each). As observed for recombinant WT RyR1, prominent sub-state activity was also observed for recombinant RyR1 K-Q channels (Fig. 7a, b). The similar conductance, sub-state activity, and regulation by Ca\(^{2+}\) and ATP between WT RyR1 and RyR1 K-Q channels indicate that the K-Q mutation does not markedly alter RyR1 function in the absence of the \(\beta_{1a}\) subunit.

As before (Figs. 1, 2, 3, 4 and 5), cis addition of 100 nM \(\beta_{1a}\) significantly increased WT RyR1 channel activity (Fig. 7a). In marked contrast, the activity of RyR1 K-Q channels was unaffected by addition of 100 nM \(\beta_{1a}\) (Fig. 7a). As effects of the \(\beta_{1a}\) subunit on WT or RyR1 K-Q channels did not differ (\(p=0.677–0.991\)) between +40 and −40 mV, values at these two potentials were again combined in the average data. On average, addition of 10 or 100 nM \(\beta_{1a}\) significantly increased WT RyR1 relative \(P_o\) by 1.8- and 1.9-fold, respectively (Fig. 8a), due to a significant increase in mean open time (Fig. 8b) and decrease in mean closed time (Fig. 8c). On the other hand, neither the relative \(P_o\) nor the mean open or closed times of RyR1 K-Q channels were significantly altered by addition of either 10 or 100 nM \(\beta_{1a}\) subunit (Fig. 8a–c). Thus, the ability of the \(\beta_{1a}\) subunit to activate RyR1 was abolished by neutralizing the polybasic residues within the K4395-R3502 region, indicating that the PBM is required for the functional effect of \(\beta_{1a}\) subunit on RyR1 activity.

**Discussion**

The results presented here provide novel insight into the regions of RyR1 that influence the action of the \(\beta_{1a}\) subunit on RyR1 activity and have implications for the
role of the β₁a subunit in skeletal muscle EC coupling. Our results demonstrate that the functional effect of 100 nM β₁a subunit is conserved between RyR1 and RyR2, although the activation by 10 nM β₁a was lower in RyR2 than in RyR1. Interestingly, a difference was also observed for the activation of ASI(−)RyR1 and ASI(+)RyR1 isoforms by 10 nM β₁a, in that the lower concentration of β₁a was also less effective in activating ASI(−)RyR1 than ASI(+)RyR1. In contrast to the maintained, although different, activation of the two RyR isoforms by the β₁a subunit, neutralization of the PBM in RyR1 abolished β₁a activation of RyR1. One interpretation of this finding is that the ∼50 % reduction in depolarization-dependent Ca²⁺ release results from disruption of direct β₁a activation of RyR1 during EC coupling.

The action of β₁a subunit on RyR1 and RyR2 channel activity is largely conserved

The activation of RyR1 and RyR2 by β₁a suggests that the β₁a binding site is conserved across these RyR isoforms. The small concentration-dependent differences between effects on RyR1 and RyR2 suggest minor differences in either the binding residues or the binding pocket that reduces the affinity of β₁a for RyR2 (and ASI(−)RyR1). It is difficult to identify specific sequences that could account for the different affinities for β₁a as there is a 13.2 % (>600 residues) sequence disparity between RyR1 and RyR2 isoforms, according to a CLUSTALW multiple alignment [45] of rabbit RyR1 [Swiss-Prot: P11716.1] and rabbit RyR2 [Swiss-Prot: P30957.3]. Given that the string of positive residues is reduced from six to five, this variation is unlikely to account for the
observed concentration-dependent difference between $\beta_{1a}$ modulation of the two isoforms, although such a possibility cannot be fully excluded. Interestingly, except for one additional positive charge in the RyR1, the PBM is conserved in RyR2 (RyR1: K3495KRR_ _ R3502 and RyR2: K3452MKRK_ _R3459) and thus is unlikely to account for the observed concentration-dependent difference between $\beta_{1a}$ modulation of the two isoforms. However, just upstream from the PBM, four of the five ASI residues in RyR1 (A3481-Q3485) are missing from the rabbit (and predicted pig) RyR2 sequence. It may be significant that the lack of ASI residues in full-length ASI(−)RyR1 reduces the efficacy of 10 nM $\beta_{1a}$-mediated activation. Thus, it is plausible that the difference between $\beta_{1a}$ modulation of RyR1 and RyR2 is partially due to the presence or absence of the ASI residues, respectively. The conservation of the modulatory effect of $\beta_{1a}$ on RyR1 and RyR2 does not reflect the in vivo studies showing that RyR2 is unable to replace RyR1 in skeletal muscle EC coupling [25, 46]. However, the lack of skeletal muscle EC coupling in RyR2-expressing dyspedic myotubes is most likely due to the fact that DHPR tetramers are not restored in RyR2-expressing dyspedic myotubes [46], indicating that $\beta_{1a}$ is unable to correctly align DHPRs with RyR2 in order to ensure a direct interaction between the two proteins. It is also possible that the II-III loop critical region is unable to engage with RyR2 through $\beta_{1a}$.

The importance of the RyR1 polybasic motif for $\beta_{1a}$ subunit increase in RyR1 channel activity

The role of the RyR1 PBM in the $\beta_{1a}$-mediated increase in channel activity was assessed from the response of recombinant RyR1 K-Q channels in bilayers to the addition of the $\beta_{1a}$ subunit. RyR1 K-Q and WT RyR1 channel conductance and regulation by cytoplasmic modulators were similar, indicating that RyR1 K-Q channels function normally. However, RyR1 K-Q channel activity was unaltered by the $\beta_{1a}$ subunit. Therefore, the reduction in voltage-gated Ca$^{2+}$ release observed in RyR1 K-Q-expressing myotubes is likely to reflect a specific effect of the polybasic residues on $\beta_{1a}$ subunit regulation of RyR1 channel activity during EC coupling rather than a general effect on RyR1 channel function. However, we cannot rule out the possibility that modest differences in RyR1 expression contribute to the reduced L-channel conductance and voltage-gated Ca$^{2+}$ release in K-Q-expressing myotubes, although this seems unlikely given previous reports of 4-chloro-m-cresol stimulated SR Ca$^{2+}$ release in myotubes RyR1 K-Q [18] and the data in Additional file 1.

Given that the PBM in the larger M3201-W3661 fragment of RyR1 is required for pull down of the $\beta_{1a}$ subunit [18], it is likely that the lack of an effect of $\beta_{1a}$ on RyR1 K-Q channels is due to the inability of $\beta_{1a}$ to bind to the PBM mutant channel. Alternatively, the PBM may be important for maintaining RyR1 in a conformation permissive for $\beta_{1a}$ binding, rather than directly contributing to binding, as the RyR1 basic residues would be unlikely to interact with the hydrophobic residues in the $\beta_{1a}$ C-terminal domain (L496, L500, and W503) previously shown to bind RyR1 [21]. In addition, although the PBM is implicated in ASI-mediated inter-domain inhibition of RyR1 [27, 43], the structure of this motif is not altered by substituting three of the six basic residues with alanine residues [27]. Thus, neutralization of the PBM more likely disrupts the inter-domain interaction rather than changes the intrinsic structure of the ASI-polybasic region. In this case, disruption of the RyR1 PBM inter-domain interaction may alter an essential conformation of the $\beta_{1a}$ binding site or prevent $\beta_{1a}$ access to its binding site on RyR1.

The $\beta_{1a}$ subunit is unlikely to be the sole signaling conduit between the DHPR and RyR1 during EC coupling. Consistent with this, expression of the RyR1 K-Q mutant in dyspedic myotubes partially restored sigmoidal, depolarization-dependent Ca$^{2+}$ release even though $\beta_{1a}$ modulation of RyR1 in bilayers was abolished. In addition, previous studies have also shown that truncation of $\beta_{1a}$ C-terminal residues, essential for $\beta_{1a}$ modulation of RyR1, also reduces but does not abolish depolarization-induced Ca$^{2+}$ release [17, 40], an outcome that was also observed in adult skeletal muscle fibers that overexpressed a $\beta_{1a}$ subunit interacting protein, Rem [20]. Finally, alanine substitution of $\beta_{1a}$ subunit hydrophobic triplet residues (L496, L500, and W503) only partially reduces depolarization-induced Ca$^{2+}$ release in $\beta_{1a}$ null myotubes [14], despite this mutation fully abolishing $\beta_{1a}$ modulation of RyR1 activity in vitro [21].

The role of the RyR1 ASI residues in $\beta_{1a}$ subunit increase in RyR1 channel activity

It is curious that 10 nM $\beta_{1a}$ subunit increased ASI(−)RyR1 activity less than ASI(+)RyR1 given that EC coupling is enhanced in dyspedic myotubes that express ASI(−)RyR1 relative to ASI(+)RyR1 [27]. The greater activation of ASI(+)RyR1 by 10 nM $\beta_{1a}$ is consistent with effects reported previously of agonists of RyR1, including caffeine and 4-chloro-m-cresol [27, 28]. Thus, the increased gain of EC coupling observed for ASI(−)RyR1 may not reflect a contribution of the $\beta_{1a}$ subunit to EC coupling. However, it is possible that activation of RyR1 by agonist binding includes a common mechanism for activation by agonists that differs from that involved in EC coupling. As it is likely that more than one interaction between RyR1 and the DHPR is involved in EC coupling, the combined result of these interactions may produce different effects on the two alternatively spliced...
variants such that ASI(−)RyR1 channels are activated more strongly by depolarization than ASI(+)RyR1 channels.

The possibility that the ASI region is involved in an inhibitory inter-domain interaction was previously investigated using peptides corresponding to the ASI region from T3471–G3500 [43]. The peptide corresponding to the ASI(−) sequence was more effective in activating ASI(−)RyR1 than ASI(+)RyR1. Together with the finding that ASI(−)RyR1 channels were generally less active than ASI(+)RyR1 channels, these findings suggest that stronger inhibitory inter-domain interactions may exist in ASI(−)RyR1. It is possible then that the triggering mechanism activated during EC coupling disrupts this inhibitory inter-domain interaction giving rise to greater activation of ASI(−)RyR1. This disruption may not occur with RyR1 agonist binding and indeed a stronger inhibitory inter-domain interaction in ASI(−)RyR1 may even oppose activation by β1a and other agonists, allowing for these triggers to more strongly activate ASI(+)RyR1 channels.

Conclusions

The results presented in this study suggest that a functional β1a interaction is conserved between RyR1 and RyR2 and that β1a activation of RyRs is regulated by the presence of the ASI residues. Importantly, we also show that the PBM residues are essential for direct activation of RyR1 by β1a subunit in vitro. This suggests that the ~50% reduction in Ca2+ release during EC coupling in dyspedic myotubes expressing RyR1 with a neutralized PBM is due to removal of β1a activation of RyR1, and hence, that other DHP-RyR1 coupling elements (e.g., II-III loop critical domain) contribute to transmission of the remaining Ca2+ release during EC coupling.

Additional files

Additional file 1: Figure S1. Description of data: a figure with two parts showing immune-fluorescent labeling of DHPR and RyR1 in myotubes.

Additional file 2: A legend to additional Figure S1.

Abbreviations

ASI: alternatively splicing region I; DHPR: dihydropyridine receptor; EC: excitation-contraction; PBM: polybasic motif; RyR1: skeletal ryanodine receptor; RyR2: cardiac ryanodine receptor; SR: sarcoplasmic reticulum.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

All authors participated in study design, data interpretation, and preparation and critical revision of the manuscript for important intellectual content. RR contributed to the design of the native RyR1 and RyR2 experiments and to the recombinant WT RyR1 and RyR1 K-Q. She carried out lipid bilayer experiments and analysis of data and expressed and purified RyR1 K-Q and WT RyR1 constructs. HW contributed to the design of the ASI(+) and ASI(−) experiments, expressed and isolated channel protein, and carried out single-channel recording and analysis with recombinant ASI(+) and ASI(−) RyR1. LG designed the RyR1 K-Q construct and carried out simultaneous measurements of macroscopic Ca2+ currents and Ca2+ transients in myotubes and immunofluorescence labeling of myotubes and data analysis. MGC and PGB provided major input into the design and data interpretation. PGB also contributed to recombinant protein expression and purification. NB performed the experiments and analyzed the data showing the presence and level of FKBP12 expression in HEK cells. RD participated in the design of the study, particularly to RyR1 K-Q construct design, measurements of macroscopic Ca2+ currents, and Ca2+ transients in myotubes and immunofluorescence labeling of myotubes, and contributed to the analysis and interpretation of the data. AD contributed to the concepts, design and coordination of all aspects of the experiments, interpretation of the data, and coordination of manuscript preparation and submission. All authors read and approved the final manuscript.

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