Fluorescence Resonance Energy Transfer (FRET) Indicates That Association with the Type I Ryanodine Receptor (RyR1) Causes Reorientation of Multiple Cytoplasmic Domains of the Dihydropyridine Receptor (DHPR) α_{1S} Subunit

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§ The abbreviations used are: EC, excitation-contraction; DHPR, dihydropyridine receptor; RyR1, type 1 ryanodine receptor; SR, sarcoplasmic reticulum; AID, α_{1S} interaction domain.

Background: In skeletal muscle, DHPR cytoplasmic domains are thought to couple membrane depolarization to Ca^{2+} release via RyR1.

Results: The presence of RyR1 alters FRET between donor/acceptor pairs in cytoplasmic domains of the DHPR α_{1S} subunit.

Conclusion: Interaction with RyR1 causes rearrangement of α_{1S} cytoplasmic domains.

Significance: Multiple cytoplasmic domains of α_{1S} may be involved in the interaction with RyR1.

In skeletal muscle, excitation-contraction (EC) coupling depends upon junctions between the plasma membrane and sarcoplasmic reticulum (SR) (1). At these junctions, dihydropyridine receptors (DHPRs), which are heteromeric proteins, serve as voltage sensors that respond to depolarization by triggering the release of Ca^{2+} from the SR via the type 1 ryanodine receptor (RyR1), a homotetrameric protein (2–4). In the junctional membrane, DHPRs are arrayed in groups of four (“tetrad”) as a consequence of physical links with RyR1 (5, 6). The existence of these links and the fact that skeletal-type EC coupling does not require the entry of external Ca^{2+} have led to the idea that conformational changes of the linking regions are responsible for the activation of RyR1 (7, 8). However, the identity of the linking regions has remained obscure in large measure because the interaction takes place between two separate membrane systems and depends upon voltage across the plasma membrane.

A variety of approaches has been used to try to identify the sites of linkage between the DHPR and RyR1, including binding assays in vitro and functional analysis of muscle cells after expression of proteins having altered sequence in specific regions. In the case of RyR1, this approach has shown that skeletal-type EC coupling depends on multiple regions far separated in the primary sequence (9, 10). However, these studies have been hampered by the relative lack of information about the tertiary structure of RyR1. There has been somewhat more progress for the DHPR because of its smaller size and its better understood domain structure. The principle subunit, α_{1S}, has four homology repeats, which form the voltage-sensing and ion permeation structures. The N and C termini and the loops linking the repeats are all cytoplasmic (11). Two loops of particular importance are the I–II loop, which is known to bind the auxiliary β_{1a} subunit (12), and the III–IV loop, which is critical for both tetrad formation (13) and skeletal-type EC coupling (14–17). The β_{1a} subunit is a soluble protein which binds to α_{1S} and promotes its membrane trafficking (18). Moreover, β_{1a} is covalent in importance with the α_{1S} II–III loop for tetrad formation (19) and skeletal EC coupling (20, 21). Among the possible explanations for these results is that β_{1a} and/or the II–III loop bind to RyR1, perhaps in close proximity to one another. There
is also evidence suggestive of binding interactions between other DHPR cytoplasmic domains and RyR1. These include the α_{1S} III-IV loop, which is known to modulate EC coupling (22, 23), and the C terminus, a segment of which binds to RyR1 in vitro (24), and which becomes partially occluded in the presence of RyR1 in vivo (25, 26).

The evidence that multiple cytoplasmic domains of the DHPR may interact with RyR1 makes it important to determine the spatial interrelationships between these cytoplasmic domains within living muscle cells and whether these interrelationships are altered by the presence of RyR1. In the current study, we have investigated this issue by constructing cDNAs encoding α_{1S} subunits doubly tagged with fluorescent proteins, in which a FRET donor (CyPet) was placed at one cytoplasmic site and a FRET acceptor (YPet) was situated at a second site. These constructs were expressed in skeletal myotubes produced from dysgenic (RyR1 null) and dysgenic (α_{1S} null) mice. After expression in dysgenic myotubes, energy transfer indicated proximity (<10 nm separation) between N- and C termini and between the I-III loop and both termini. By contrast, the I-II loop produced weak FRET with the II-III loop and none with the N or C termini. After expression in dysgenic myotubes, the presence of RyR1 appeared to cause a substantial reorientation of the domains, including a shift of the I-III loop toward the C terminus and a shift of the I-II loop toward both the II-III loop and N terminus. Because the I-II loop is associated with β_{1S}, the decreased proximity between the I-II and II-III loops may indicate that β_{1S} and the II-III loop form a unified structure that interacts with RyR1 to promote tetrad formation and skeletal-type EC coupling.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs for the Expression of Fluorescently Labeled α_{1S}**—The backbone of the mammalian expression vectors pECPF and pEYFP (Clontech, Palo Alto, CA) was used to design plasmids for expression of fluorescently tagged rabbit α_{1S} (GenBankTM accession no. M23919). Site-directed mutagenesis (Stratagene, La Jolla, CA) was used to generate unique restriction sites at defined positions within the α_{1S} cDNA sequence, which were used to introduce the sequences encoding the FRET-optimized fluorescent proteins CyPet and/or YPet (27). The labeling positions were as follows.

- **CyPet-α_{1S}-YPet** and **YPet-α_{1S}-CyPet**—One fluorescent protein was attached to the first residue of the α_{1S} N terminus, separated by the 12-residue linker RSRAQASNSAVD. The second fluorescent protein was attached to the last residue of α_{1S} truncated at residue 1636, separated by the four-residue linker PVAT.

- **CyPet or YPet within the α_{1S} I-II Loop**—The fluorescent protein was inserted with its N terminus immediately after residue 406 of the α_{1S} I-II loop, and its C terminus connected via the 3-residue linker RLN to α_{1S} residues 407–1860.

- **CyPet or YPet within the α_{1S} II-III Loop**—The fluorescent protein sequence was inserted into the II-III loop between α_{1S} residues 726 and 727, connected via a four-residue linker (PVAT) and 18-residue linker (RSRAQASNSAVDGTAGPV), respectively.

**CyPet or YPet within the α_{1S} III-IV Loop**—The fluorescent protein sequence was inserted into the III-IV loop between α_{1S} residues 1096 and 1097 via a single leucine and a three-residue linker (SAR), respectively.

- **YPet-α_{1S}-CyPet (α_{1S} tandem)**—The first α_{1S} was attached via its C terminus, which had been truncated at residue 1636, to a three-residue linker (GVD), which was attached to the N terminus of the second α_{1S} which was C-terminally truncated at residue 1636. YPet was attached to the N terminus of the first α_{1S} via a 12-residue linker (SRAQASNSAVD) and CyPet was attached to the C terminus of the second α_{1S} via a four-residue linker (PVAT).

**Primary Skeletal Muscle Cell Culture and Transfection**—Myoblasts were prepared from newborn dysgenic mice, homozygous for absence of α_{1S} (28), or newborn dysgenic mice, homozygous for absence of RyR1 (29) as described (30). The myoblasts were grown for 6–7 days in a humidified 37 °C incubator with 5% CO2 in Dulbecco’s modified eagle medium (E15-009, PAA, Coelbe, Germany), supplemented with 10% fetal bovine serum/10% horse serum (Biochrom, Berlin, Germany). This medium was then replaced with differentiation medium (DMEM supplemented with 2% horse serum). Two to four days following the shift to differentiation medium, single nuclei were microinjected with plasmid cDNA (100 ng/μl in water).

**Microscopy and Measurements of FRET**—An important consideration for the studies reported here is that fluorescently tagged α_{1S} constructs expressed in myotubes are concentrated in numerous discrete foci of small size (=1 μm). Thus, methods for analysis of FRET, which require multiple scans, have the drawback that the measurements will be compromised if movement of the foci occurs in the time interval between the initial and final scans, an interval that can be substantial. For example, the acceptor photobleaching method requires the measurement of cyan fluorescence before after the bleaching of yellow and typically requires several seconds or more. Thus, we selected to employ a method in which an index of FRET could be obtained without multiple scans. Specifically, myotubes were examined 24–48 h after microinjection using a FV1000 confocal laser-scanning microscope (Olympus) under 60× magnification. For the measurement of FRET, an area of 512 × 512 pixels was selected to include both a part of the myotube to be analyzed and an adjacent region, which was cell-free. Excitation was at 440 nm, and emission was measured simultaneously via two photomultipliers, one of which was equipped with a 465–495 nm band pass filter (cyan channel, intensity I_c), and the other photomultiplier was equipped with a 535–565 nm band pass filter (yellow channel, intensity I_y). The photomultiplier gains, offsets, and pinhole diameters were kept the same for all experiments. Even when both YPet and CyPet are present, only CyPet contributes to I_c because YPet does not emit in the 465–495 nm range. However, I_y contains three contributions, emission from CyPet in the 535–565 nm range (I_{τcy}), emission from YPet, which is directly excited by 440 nm (I_{τyp}), and YPet emission, which occurs as a consequence of FRET (I_{τFRET}).

\[
I_y = I_{τcy} + I_{τyp} + I_{τFRET} \tag{1}
\]
Correction for $I_{V(Y)}$ is straightforward because the magnitude of $I_{V(Y)}$ is a constant fraction of $I_C$. To determine this fraction, we measured $I_C$ and $I_{V(Y)}$ in cells expressing $\alpha_{1S}$ labeled only with a single CyPet and excited at 440 nm. This yielded $I_{V(Y)}/I_C = 0.28 \pm 0.02$ ($n = 25$). Thus, for constructs labeled with both CyPet and YPet, we calculated the following in Equation 2:

$$I_T^* = I_{V(Y)} + I_{Y(FRET)} - 0.28 \cdot I_C$$  \hspace{1cm} (Eq. 2)

In principle, it would also have been possible to correct for $I_{V(Y)}$ (emission from YPet excited at 440 nm) by scanning each cell twice, once with 440 nm excitation and once with 515 nm excitation (which does not excite CyPet). However, because 440 nm is near the absorption minimum for YPet, we chose for simplicity to calculate Equation 3 as an indication of FRET.

$$I_T^*/I_C$$  \hspace{1cm} (Eq. 3)

The calculation of $I_T^*/I_C$ was restricted to the punctate foci to which labeled $\alpha_{1S}$ constructs localize in myotubes. First, the background intensity measured in a cell-free region was subtracted from all pixels of the respective scan. Then, by means of a custom made Microsoft Excel macro, the image was "masked" with an adjustable threshold, such that all areas below this threshold were excluded from analysis, and only areas exceeding the threshold (including the punctate foci) were included. The macro iteratively incremented the cut-off intensity, resulting in a successively decreasing number of included pixels. For each value of the threshold, the macro calculated $I_T^* = I_T - 0.28 \cdot I_C$, and the ratio $I_T^*/I_C$. During this iterative process, $I_T^*/I_C$ approached a quasi-constant level as the threshold was increased and the contribution of regions outside the punctate foci was reduced. Subsequently, $I_T^*/I_C$ abruptly became unstable when the cut-off threshold became so high that the small size of the analyzed areas caused large inhomogeneities in pixel levels between the cyan and yellow channels. The values of $I_T^*/I_C$ reported under "Results" (see Figs. 3 and 4) are from the approximately constant-level phase as revealed by the analysis macro. All data are reported as mean ± S.D. The unpaired Student’s t test was used to compare the $I_T^*/I_C$ ratios calculated for a given construct expressed in dyspedic or dysgenic myotubes.

**Electrically Evoked Contractions**—The ability of fluorescently labeled $\alpha_{1S}$ constructs to support EC coupling was tested by determining whether myotubes expressing the construct and bathed in rodent Ringer’s (146 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 11 mM glucose, pH 7.40, with NaOH) contracted in response to a 10-ms, 100-V pulse applied via an extracellular pipette filled with 150 mM NaCl.

**Measurement of L-type Currents**—The whole cell patch clamp method was used to measure macroscopic Ca$^{2+}$ currents (31). Borosilicate glass pipettes were polished to a final resistance of ~2.0–3.0 MΩ when filled with intracellular solution containing the following: 140 mM CsAsp, 5 mM MgCl$_2$, 10 mM Cs$_2$EGTA, 10 mM HEPES, pH 7.4, with CsOH. The external bath solution contained the following: 10 mM CaCl$_2$, 145 mM tetraethylammonium-Cl, 0.003 mM tetrodotoxin, 0.1 mM N-Benzyl-p-toluene sulfonamide, 10 mM HEPES, pH 7.4 with tetraethylammonium-OH. To isolate L-type currents, voltage was stepped from the holding potential (−80 mV) to −20 mV for 1 s to inactivate endogenous T-type current and then repolarized to −50 mV for 50 ms, depolarized to varying test potentials for 200–400 ms, repolarized to −50 mV for 100 ms, and then returned to the holding potential. Test currents were corrected for linear components of leak and capacitive current by digitally scaling and subtracting the average of 11 control currents elicited by a hyperpolarizing step from −80 to −110 mV. Cell capacitance was determined by integration of a transient from −80 to −70 mV and was used to normalize current amplitudes (pA/pF). Peak currents as a function of test potential were fitted according to Equation 4,

$$I = G_{max} \cdot (V - V_{rev})/(1 + \exp[-(V - V_{1/2})/k_G])$$  \hspace{1cm} (Eq. 4)

where $I$ is the peak inward Ca$^{2+}$ current measured at the potential $V$, $V_{rev}$ is the reversal potential, and $k_G$ is a slope factor.

**RESULTS**

**Sites within $\alpha_{1S}$ Cytoplasmic Domains Suitable for Labeling with Fluorescent Protein**—The goal of this study was to determine whether association with RyR1 causes rearrangement of the cytoplasmic domains of $\alpha_{1S}$, as indicated by altered FRET between donor/acceptor pairs inserted at various cytoplasmic sites. A necessary requirement for these constructs was that they target to plasma membrane/SR junctions in both dyspedic (RyR1 null) and dysgenic (null) myotubes and that they function as both L-type Ca$^{2+}$ channels and voltage sensors for EC coupling after expression in dysgenic myotubes. Correct targeting was assessed by the presence of discrete, fluorescent foci (−0.3–1 μm in diameter) located close to or at the cell surface. Focal extracellular stimulation was used to test for contractions as an indicator of EC coupling, and whole-cell voltage clamp measurements were used to test for L-type Ca$^{2+}$ current.

As a first step toward double labeling $\alpha_{1S}$ with fluorescent proteins, we tested constructs with single additions to the cytoplasmic domains. As expected from previous work (25), a construct in which the fluorescent protein was attached to the N terminus, produced fluorescent puncta in both dyspedic and dysgenic myotubes, and was able to produce L-type Ca$^{2+}$ current and support EC coupling in dysgenic myotubes (Fig. 1a). The effects of insertion into the I-II loop depended on whether this was upstream or downstream of the AID (residues 357–374), at which high affinity binding of β$_{1S}$ occurs (32). A construct with upstream insertion (between residues 350 and 351) failed to target in dyspedic myotubes and did not restore Ca$^{2+}$ channel and EC coupling functions in dysgenic myotubes, although targeting in dysgenic myotubes still appeared to occur (Fig. 1b). Insertion downstream of the AID, between residues 406 and 407, did not interfere with either targeting or function (Fig. 1c). Previous work (33) had shown targeting to be unaffected, and function to be only partially reduced, after expression in dysgenic myotubes of a construct in which a single fluorescent protein had been inserted into the II-III loop between residues 726 and 727, which is near the N terminus of a domain (residues 720–765) known to be critical for EC coupling (16). Here, we also found that targeting and function in dysgenic myotubes were preserved after insertion of fluorescent protein...
between residues 726 and 727, and additionally observed that constructs with this insertion displayed junctional targeting in dyspedic myotubes (Fig. 1d). We did not test insertions further downstream in the II-III loop because the previous work had shown that signaling interactions with RyR1 were ablated by insertions either between residues 760 and 761, or between residues 785 and 786 (33). In the III-IV loop, we found that insertion at a point near the middle (between residues 1096 and 1097) abolished targeting in both dyspedic and dysgenic myotubes and also eliminated EC coupling (Fig. 1e). Given the relatively short length of the III-IV loop (53 residues), we did not test other insertion sites. Based on the results illustrated in Fig. 1, the N terminus, the I-II loop downstream of the AID, and the II-III loop at the beginning of the domain critical for EC coupling were identified as potential sites for double labeling of α1S.

Previous work had shown that attachment of either a CFP-YFP tandem (25), or the biotin acceptor domain (26), to a C-terminally truncated α1S (at residue 1667) did not interfere with function after expression in dysgenic myotubes, and we found similar results for truncation and attachment of YPet at residue 1666 (data not shown). Additionally, we observed that constructs C-terminally tagged with YPet at residue 1666 and N-terminally tagged with CyPet (CyPet-α1S1666-YPet) produced co-localized cyan and yellow puncta after expression in dysgenic myotubes (Fig. 2a, upper row). Co-localized puncta were also observed after expression of CyPet-α1S1666-YPet in dyspedic myotubes, but this was superimposed on a substantial background of diffuse yellow fluorescence (Fig. 2a, bottom row). Because post-translational cleavage of full-length α1S (1873 residues) has been reported to occur at residue 1664 (34), this diffuse yellow fluorescence could have been a consequence of the proteolytic liberation of YPet from a fraction of the CyPet-α1S1666-YPet molecules. Thus, we tested the construct CyPet-α1S1636-YPet in which YPet had been attached to the C terminus truncated at residue 1636, upstream of the putative cleavage site. This construct produced co-localized cyan and yellow foci in both dysgenic and dyspedic myotubes (Fig. 2b).

On the basis of these results, all the C-terminally tagged α1S constructs used for the FRET measurements in this study were truncated at residue 1636. All other α1S constructs did not have this truncation and their sequence ended at residue 1860 (25).

**Doubly Labeled α1S Constructs**—On the basis of the results illustrated in Figs. 1 and 2, there were potentially six constructs of α1S that could be doubly tagged; these are illustrated in Fig. 3, between residues 726 and 727, and additionally observed that constructs with this insertion displayed junctional targeting in dyspedic myotubes (Fig. 1d). We did not test insertions further downstream in the II-III loop because the previous work had shown that signaling interactions with RyR1 were ablated by insertions either between residues 760 and 761, or between residues 785 and 786 (33). In the III-IV loop, we found that insertion at a point near the middle (between residues 1096 and 1097) abolished targeting in both dyspedic and dysgenic myotubes and also eliminated EC coupling (Fig. 1e). Given the relatively short length of the III-IV loop (53 residues), we did not test other insertion sites. Based on the results illustrated in Fig. 1, the N terminus, the I-II loop downstream of the AID, and the II-III loop at the beginning of the domain critical for EC coupling were identified as potential sites for double labeling of α1S.

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Figure 2. Removal of the distal half of the α₁₅ C terminus preserves α₁₅ function and prevents partial loss of C-terminally attached fluorescent protein in dyspedic myotubes. Co-localized cyan and yellow puncta were observed in dyspedic myotubes after expression of α₁₅ that was N-terminally tagged with CyPet and C-terminally tagged with YPet at residue 1666 (a). However, when this construct was expressed in dyspedic myotubes, there was a substantial amount of diffuse yellow fluorescence, in addition to co-localized yellow and cyan puncta. The diffuse yellow fluorescence was eliminated when the YPet tag was attached to a further truncated C terminus at residue 1636, leaving only co-localized cyan/yellow puncta in both dysgenic (dysg.) and dyspedic (dysp.) myotubes (b). Bars, 5 μm.

α₁₅S(1666) constructs illustrated in Fig. 3 were able to restore L-type Ca²⁺ current and electrically evoked contractions in dysgenic myotubes, although the fraction of contracting cells was lower than reported for α₁₅ constructs tagged only on the C terminus (33). Based on the previous work in which both electrically evoked contractions and whole-cell Ca²⁺ transients were measured (cf. Table 2 of Ref. 33), this indicates that these doubly labeled constructs restored excitation-contraction coupling of reduced efficiency, with the result that the amount of released Ca²⁺ was subthreshold for movement in a significant fraction of the expressing myotubes. Thus, all cells in which the doubly tagged constructs produced discrete fluorescent foci were analyzed for FRET.

The Presence of RyR1 Alters FRET between α₁₅ Cytoplasmic Domains—Both functional and structural evidence strongly support the idea that α₁₅ and RyR1 are linked to one another either directly or via intervening proteins. On first principles, one would expect that these functional and structural interactions would involve cytoplasmic domains of α₁₅. Thus, as already stated, the main goal of the current work was to test whether the presence of RyR1 causes spatial reorganization of the cytoplasmic domains as indicated by altered FRET between fluorescent probes attached to these domains. For this, we calculated the FRET index $I_{F}/I_{C}$ for each of the doubly tagged constructs expressed in dysgenic myotubes (indicated by asterisks in the targeting column). Only fluorescent puncta were used for analysis. The column lists the fraction of cells that contracted (contr.), upon electrical stimulation (indicated as the number of responding cells over the number of cells tested) with the number of spontaneously contracting cells (spont.) indicated in parentheses. For each of the constructs, the average current density (pA/pF ± S.D.) is shown together with calibration bars corresponding to 2 nA and 50 ms. The quotient $I_{F}/I_{C}$ is a measure of the degree of energy transfer from CyPet to YPet (see text for details).
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CyPet (indicated by the dashed, horizontal line). Third, RyR1 caused the emergence of FRET between the I-II loop and N terminus (Fig. 4a) and caused increased FRET between the II-III loop and N terminus (Fig. 4b). Fourth, RyR1 caused increased FRET between the I-II and II-III loops (Fig. 4d) and decreased FRET between the II-III loop and C terminus (Fig. 4f). Overall, these results suggest that the presence of RyR1 causes an appreciable rearrangement of the α₁S cytoplasmic domains.

**DISCUSSION**

In this work, we have described measurements of FRET on intact muscle expressing α₁S constructs in which the fluorescent proteins CyPet and YPet were attached to pairs of cytoplasmic domains. We found that junctional targeting, L-type Ca²⁺ current, and EC coupling were all preserved for doubly labeled constructs with the fluorescent proteins attached at the N terminus, I-II loop (residue 406, 33 residues downstream of the AID), II-III loop at residue 726 (i.e. within the domain that spans residues 720–765 and is critical for EC coupling), and the C terminus truncated at residue 1636 (Figs. 1–3). When these constructs were expressed in dyspedic myotubes, which lack RyR1, appreciable FRET occurred between the N and C termini, and from the II-III loop to the N terminus, the C terminus, and (to a lesser extent) the I-II loop (Fig. 4). After expression in dysgenic myotubes, in which RyR1 is present, FRET from the N to C terminus was unchanged, from the II-III loop was increased to the N terminus and decreased to the C terminus; for the I-II loop, FRET became measurable to the N terminus and increased to the II-III loop (Fig. 4). Thus, the presence of RyR1 appeared to cause a widespread rearrangement of the cytoplasmic domains of α₁S.

Although the changes in FRET are strongly suggestive that the presence of RyR1 causes a reorientation of the α₁S cytoplasmic domains, a more specific interpretation in terms of interdomain distances depends upon a number of assumptions that are difficult to test directly. For example, FRET depends not only on distance but also on the donor:acceptor ratio (CyPet: YPet), which we have assumed to be 1:1 for our constructs. However, the “effective” stoichiometry depends upon the extent of maturation (35) of each of the fluorophores and on their local environment (e.g. dielectric constant), which could be differentially affected by the presence or absence of RyR1 and thus affect measured FRET. The presence of RyR1 could also alter FRET by affecting the orientation of the donor and acceptor dipoles with respect to one another (36). In addition to these “generic” concerns, which apply to most studies employing FRET, an issue of specific importance for our studies is the extent to which intermolecular FRET, between neighboring DHPRs, might have contributed to the measured FRET values. It seems likely that the FRET measured in the absence of RyR1 was only intramolecular because DHPRs appear to be randomly oriented in dyspedic myotubes (37). In the presence of RyR1, however, the ordered array of DHPR tetramers (37, 38) could have resulted in an additional, intermolecular component that would have increased total FRET. In principle, this might account for the RyR1-associated increase in FRET between the I-II loop and both the N terminus (Fig. 4a) and II-III loop (Fig.
RyR1 Reorganizes the Cytoplasmic Interface of $\alpha_{1S}$

![Diagram](image)

**FIGURE 5.** Model of how spatial reorientations of cytoplasmic $\alpha_{1S}$ domains caused by association with RyR1 could account for the observed changes in $I_p/I_c$. Shown is a potential arrangement of $\alpha_{1S}$ cytoplasmic domains, viewed from the intracellular side, in the absence (left) and presence (right) of RyR1. The circles are $\sim 50 \text{ Å}$ in radius and the extent of overlap correlates with the extent of FRET ($I_p/I_c$, Fig. 3) in the absence (red) or presence (blue) of RyR1 (see text for additional details).

4b). Relevant to this issue, earlier experiments examined the spatial interrelationships of $\beta_{1a}$ subunits in DHPR tetrad arrays by analyzing constructs expressed in $\alpha$ null myotubes. These experiments failed to detect either intermolecular FRET (39) or bimolecular fluorescence complementation (40) between $\beta_{1a}$ subunits in adjacent DHPRs. Thus, it was suggested that the adjacent $\beta_{1a}$ subunits were separated from one another by $>10$ nm (see Fig. 8 in Ref. 40). If this suggested arrangement is correct, then the I-II loops of $\alpha_{1S}$ also would likely be positioned at a distance $>10$ nm from the $\alpha_{1S}$ cytoplasmic domains of adjacent DHPRs.

Under the assumption that the FRET we measured was produced intramolecularly and is an indication of proximity, our results can be summarized by the diagram in Fig. 5, which represents a cytoplasmic view of $\alpha_{1S}$. Because the practical limit for measurable FRET is a donor-acceptor distance of $\sim 100 \text{ Å}$ (36), the diagram illustrates each of the labeled domains surrounded by an $\sim 50 \text{ Å}$ circle and the extent of FRET between any two domains is indicated by the overlap between the circles surrounding those domains. In the absence of RyR1 (overlapping areas indicated in red), the N and C termini are close enough to one another to produce an $I_p/I_c$ ratio of 0.54, and, to a slightly lesser degree, the II-III loop is close to both the N and C terminus ($I_p/I_c$ ratios of 0.47 and 0.50, respectively). There is less proximity between the I-II and II-III loops ($I_p/I_c = 0.33$, where 0.25 corresponds to the absence of FRET), and the I-II loop is sufficiently far from both the N and C terminus that there was no detectable FRET. The presence of RyR1 causes a significant rearrangement of both the I-II and II-III loops (Fig. 5, overlapping areas indicated in blue). In particular, the II-III loop appears to move away from the C terminus ($I_p/I_c$ decreases to 0.39) and toward the N terminus ($I_p/I_c$ increases to 0.60), and the I-II loop moves toward the II-III loop and the N terminus ($I_p/I_c$ increases to 0.48 with respect to both). Thus, the overall consequence is that the cytoplasmic domains appear to assume a more compact arrangement when RyR1 is present.

Obviously, Fig. 5 presents an extremely simplified view of the cytoplasmic domains because each of them spans many residues (N terminus, residues 1–51; I-II loop, residues 335–432; II-III loop, residues 662–799; full-length C terminus, residues 1382–1873). Virtually nothing is known about the folding of these domains, but their dimensions would be substantial even if they were folded as compact globular structures (diameters from $\sim 20$ to $\sim 50 \text{ Å}$). Thus, one would expect that the interdomain distances inferred from FRET would be strongly depend-
ent in the membrane of β1 null zebrafish muscle cells, it is not arrayed as tetramers (19), indicating that α15 null does not bind to RyR1 in the absence of the β subunit. Conversely, in α15 null myotubes, fluorescently tagged β1a does not bind to RyR1 (18) and is freely diffusible (39). Thus, it appears that even though there must be protein-protein contacts linking the DHPR and RyR1, identifying these contacts may not be feasible using isolated components of the DHPR complex. For example, one could imagine that a high affinity interaction required the simultaneous binding of β1a and the α15 II-III loop to RyR1 domains, which form a single binding pocket in three dimensions. If so, it might explain why the FRET results suggest a decreased separation between the I-II loop (to which β1a is attached) and the II-III loop when RyR1 is present (Fig. 4). Interestingly, the larger separation between the I-II and II-III loops in the absence of RyR1 may be represented in the structure of the isolated DHPR determined by electron cryo-microscopy (49), in which β1a appears to be deflected laterally with respect to α15.

The approach we have employed here, of measuring FRET for doubly tagged α15 constructs expressed in myotubes, is useful because it provides spatial information about functional DHPRs in plasma membrane/SR junctions. However, as already discussed above, this information is necessarily somewhat imprecise. Nonetheless, this information provides obvious directions for future experiments. For example, it will be important to determine whether the interdomain FRET changes in response to depolarization and whether such changes correlate with activation of Ca2+ release via RyR1. Similarly, it will be of interest to determine whether these FRET signals are altered by application of pharmacological agents (e.g. ryanodine, dihydropyridines) or by mutations of α15 and RyR1 that are associated with human muscular disorders.

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