Organization of Calcium Channel $\beta_{1a}$ Subunits in Triad Junctions in Skeletal Muscle

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In skeletal muscle, dihydropyridine receptors (DHPRs) in the plasma membrane interact with the type 1 ryanodine receptor (RyR1) at junctions with the sarcoplasmic reticulum. This interaction organizes junctional DHPRs into groups of four termed tetrads. In addition to the principle $\alpha_{1S}$ subunit, the $\beta_{1a}$ subunit of the DHPR is also important for the interaction with RyR1. To probe this interaction, we measured fluorescence resonance energy transfer (FRET) of $\beta_{1a}$ subunits labeled with cyan fluorescent protein (CFP) and/or yellow fluorescent protein (YFP). Expressed in dysgenic myotubes (1S-null) myotubes, YFP-$\beta_{1a}$-CFP and CFP-$\beta_{1a}$-YFP were diffusely distributed in the cytoplasm and highly mobile as indicated by fluorescence recovery after photobleaching. Thus, $\beta_{1a}$ does not appear to bind to other cellular proteins in the absence of $\alpha_{1S}$. FRET efficiencies for these cytoplasmic $\beta_{1a}$ subunits were ~6–7%, consistent with the idea that <10 nm separates the N and C termini. After coexpression with unlabeled $\alpha_{1S}$ (in dysgenic or $\beta_{1a}$-null myotubes), both constructs produced discrete fluorescent puncta, which correspond to assembled DHPRs in junctions and that did not recover after photobleaching. In $\beta_{1a}$-null myotubes, FRET efficiencies of doubly labeled $\beta_{1a}$ in puncta were similar to those of the same constructs diffusely distributed in the cytoplasm and appeared to arise intramolecularly, since no FRET was measured when mixtures of singly labeled $\beta_{1a}$ (CFP or YFP at the N or C terminus) were expressed in $\beta_{1a}$-null myotubes. Thus, DHPRs in tetrads may be arranged such that the N and C termini of adjacent $\beta_{1a}$ subunits are located >10 nm from one another.

The DHPR consists of a principal $\alpha_{1S}$ subunit, which contains the ion-conducting pathway and voltage-sensing structures, together with auxiliary $\beta_{1a}$, $\alpha_{1S}$-I–II, and $\gamma_{1S}$ subunits (for review, see Ref. 2). A significant body of evidence indicates that the cytoplasmic loop connecting $\alpha_{1S}$ homology repeats II and III (the II–III loop) plays an important role in conformational coupling between the DHPR and RyR1. The auxiliary $\beta_{1a}$ subunit appears to be similarly important. One essential function of all calcium channel $\beta$ subunits (currently known to be encoded by four different genes) is that of facilitating trafficking to the plasma membrane, which occurs largely as a consequence of $\beta$ binding to the $\alpha_{1}$ I–II loop. Consequently, knock-out of the gene encoding the predominant form in skeletal muscle ($\beta_{1}$) causes a nearly complete absence of DHPRs in the plasma membrane (3) and a resultant loss of EC coupling (4). Significantly, truncating or altering the sequence of the $\beta_{1a}$ C-terminal preserves membrane expression but suppresses EC coupling (5, 6), indicating that this region is important for communication between the DHPR and RyR1.

All $\beta$ subunits can be subdivided into five regions: highly variable N- and C-terminal regions flanking a core segment with three regions. Crystal structures have been recently reported for this core segment (7–9), which confers the principal functional properties on full-length $\beta$ subunits. Within the core segment, the first region is highly conserved among $\beta$ subunits and is homologous to canonical Src homology 3 domains. The third region of the core segment is also highly conserved and is guanylate kinase-like. The Src homology 3- and guanylate kinase-like domains are linked by a more variable, flexible loop so that the core segment as a whole shares structural features with the membrane-associated guanylate kinases, a protein family that organizes signaling components near membranes (10).

Based on sequence alignment of $\beta_{1a}$ with $\beta_{1s}$ (GenBank™ numbers: M25514 and M80545), and on the crystal structure of the $\beta_{2a}$ core segment, leucine 74 and threonine 463 of $\beta_{1a}$ are likely separated by 42 Å. However there is little basis for predicting the structure of the $\beta_{1a}$ segments N-terminal (73 residues) and C-terminal (61 residues) to the core. Thus, one goal of the present experiments was to obtain a rough idea about the separation of the N and C termini of $\beta_{1a}$ and about whether or not there were large changes in the relative arrangements of the N and C termini as a consequence of the binding of $\beta_{1a}$ to $\alpha_{1S}$. A second goal was to obtain information about the arrangement of $\beta_{1a}$ subunits within tetrads. Toward these ends, we measured fluorescence resonance energy transfer (FRET) with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) used as the donor and acceptor, respectively (11). The $\beta_{1a}$ subunits were singly labeled on either the N or C terminus with either CFP or YFP or doubly labeled with CFP on one terminal and YFP on the other. These fluorescently labeled $\beta_{1a}$ subunits were expressed in dysgenic ($\alpha_{1S}$-null) or $\beta_{1a}$-null myotubes. We found that the $\beta_{1a}$ subunit was incorporated into fluorescent puncta at the cell surface (indicative of junctional targeting) only when the $\alpha_{1S}$ subunit was also present. Based on intramolecular FRET, it appears that the N

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2 The abbreviations used are: EC, excitation-contraction; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; SR, sarcoplasmic reticulum.
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FIGURE 1. Schematic diagrams of the β_{1a} constructs used in these experiments. The position of the fluorescent protein, CFP and/or YFP, is indicated for each construct, in relationship to the native β_{1a} subunit. The linker sequences connecting CFP and YFP to the β_{1a} subunit are indicated by wavy lines labeled with the number of linker residues.

and C termini of β_{1a} are <10 nm apart. Additionally, the absence of intermolecular FRET between singly labeled subunits is consistent with the idea that the N and C termini of β_{1a} subunits are directed away from the center of tetrads.

MATERIALS AND METHODS

Generation of the Constructs Expressed in Myotubes—Fig. 1 illustrates the β_{1a} constructs that were used for this study. The coding sequence for rabbit skeletal muscle β_{1a} (GenBank™ number M25514) was isolated by using PCR to introduce EcoRI and Sall sites immediately before the ATG start codon and immediately after the last codon, respectively. The EcoRI-Sall segment containing the β_{1a} coding sequence was then ligated to EcoRI/Sall-digested pEYFP-C1, pEYFP-N1, pECFP-C1, or pECFP-N1 (Clontech, Palo Alto, CA) to obtain the constructs YFP-β_{1a}, β_{1a}-YFP, CFP-β_{1a} and β_{1a}-CFP, respectively. To construct CFP-β_{1a}-YFP, CFP-β_{1a} and β_{1a}-YFP were individually digested with MfeI, and the 5903-bp fragment obtained from CFP-β_{1a} and the 1128-bp fragment from β_{1a}-YFP were ligated together. For the YFP-β_{1a}-CFP construct, YFP-β_{1a} and β_{1a}-CFP were individually digested with MfeI, and the 5903-bp fragment from YFP-β_{1a} and the 1128-bp fragment from β_{1a}-CFP were ligated together. Unlabeled β_{1a} was obtained by digesting β_{1a}-YFP with Acc651 and BsrGI, discarding the small fragment containing the YFP sequence, and re-ligating the large fragment containing the β_{1a} coding sequence. Unlabeled α_{15} was obtained as described previously (12).

Cell Culture and cDNA Microinjection—Primary cultures of dysgenic muscles (13) and β_{1a}-null (14) myotubes were prepared from newborn mice as described previously (15). Briefly, myoblasts were plated into 35-mm culture dishes (MatTek, Ashland, MA) with glass coverslip bottoms that had been coated with ECL (Upstate Biotechnology, Lake Placid, NY) and were cultured in DMEM (Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 3 Mg-ATP, and 0.6 GTP, pH 7.4 with tetrathylammonium-OH). The external bath solution contained (mM): 10 CaCl_2, 145 tetrathylammonium-Cl, 0.003 tetrodotoxin, 0.02 N-benzyl-p-toluene sulfonamide, and 10 HEPES, pH = 7.4 with tetrathylammonium-OH. To measure L-type current, myotubes were stepped from the holding potential (−80 mV) to −30 mV for 1 s (to inactivate endogenous T-type current), repolarized to −50 mV for 30–50 ms, depolarized to varying test potentials (V_{test}) for 200 ms, and then returned to the holding potential. Cell capacitance was determined by integration of the capacity transient resulting from 30-mV hyperpolarizations from the holding potential and was used to normalize current amplitudes (pA/pF) obtained from different myotubes. Current-voltage curves were fitted using the Boltzmann expression: I = G_{max} [1/1 + exp({V - V_{rev} - V_{0.5}})/k_{G}]], where I is the current for the test potential V, V_{rev} is the reversal potential, G_{max} is the maximum Ca^{2+} channel conductance, V_{0.5} is the half-maximal activation potential and k_{G} is the slope factor.

Measurement of FRET and Fluorescence Recovery after Photobleaching—Measurement of FRET was as described previously (12). Briefly, intact fluorescent myotubes were examined 48 h after cDNA microinjection using an LSM 510 META laser scanning confocal microscope (Zeiss, Thornwood, NY). In a typical experiment, an image of the cell region of interest was taken using standard spectroscopic settings: CFP and YFP were excited with separate sweeps of the 458 and 514 nm lines, respectively, of an argon laser (30 milliwatt maximum output, operated at 50% or 6.3 A) attenuated to 10 and 2%, respectively, and directed to the cell via a 458/514 nm dual dichroic mirror. The emitted fluorescence was split via a 515 nm dichroic mirror and for CFP was directed to a photomultiplier equipped with a 465–495 nm bandpass filter (Chroma, Rockingham, Vermont) and for YFP was directed to a photomultiplier equipped with a 530-nm long-pass filter. Confocal fluorescence intensity data (I_{CFPpost} and I_{YFPpost}) were recorded as the average of four line scans per pixel and digitized at 8 bits. Repeated scans (20–60) with unattenuated 514 nm illumination were used to photo-bleach YFP, which required ~30–120 s at maximal scan rates. After completion of YFP bleaching, fluorescence intensity (I_{CFPpost} and I_{YFPpost}) was measured using the identical parameters as before bleaching. FRET efficiency (E) in percent was calculated as E = (I_{CFPpost} −
RESULTS

$\beta_{1a}$ Subunit Constructs Are Targeted to the Membrane in the Presence of $\alpha_{1S}$ and Restore EC Coupling and L-type $Ca^{2+}$ Current—To analyze the arrangement of $\beta_{1a}$ subunits at plasma membrane/SR junctions, both singly labeled (CFP-$\beta_{1a}$-YFP, $\beta_{1a}$-CFP, and $\beta_{1a}$-YFP) and doubly labeled (CFP-$\beta_{1a}$-YFP and YFP-$\beta_{1a}$-CFP) constructs were examined. Confocal microscopy and functional measurements were used to verify that these fusions of one or two fluorescent proteins to the $\beta_{1a}$ subunit did not affect targeting or the ability of the DHPR to function as a voltage sensor for EC coupling. Fig. 2 illustrates confocal fluorescence sections of myotubes expressing CFP-$\beta_{1a}$-YFP. After expression in dysgenic myotubes (lacking $\alpha_{1S}$), CFP-$\beta_{1a}$-YFP was diffusely distributed within the cytoplasm of the cell (Fig. 2a). When the same construct was coexpressed with unlabeled $\alpha_{1S}$ in dysgenic myotubes, it was arranged in fluorescent puncta, many of which were close to the surface of the cell (Fig. 2b). Similar, fluorescent puncta were also observed when CFP-$\beta_{1a}$-YFP was expressed in $\beta_{1a}$-null myotubes, which endogenously express $\alpha_{1S}$. Bar, 5 μm.

$I_{C\text{FPre}}/I_{C\text{FPpost}} \times 100\%$, where $I_{C\text{FPre}}$ and $I_{C\text{FPpost}}$ are the background-corrected CFP fluorescence intensities before and after photobleaching YFP, respectively. Data are reported as mean ± S.D. Statistical significance was tested with an unpaired Student’s t test.

To measure the recovery of yellow fluorescence after photobleaching of YFP, myotubes were first imaged for cyan and yellow fluorescence as already described above. Within this image, a smaller region-of-interest was designated for photobleaching of YFP, which was accomplished with repeated scans of non-attenuated 514 nm excitation. Complete bleaching required about 70 s (similar sized regions of interest were used for all these experiments). Images of cyan and yellow fluorescence were then obtained as above every ~30 s, beginning immediately after, and continuing until ~400 s after, completion of the bleaching.

All of the singly labeled $\beta_{1a}$ constructs had the same type of expression pattern as shown in Fig. 2 for doubly labeled $\beta_{1a}$. Moreover, when mixes of singly labeled $\beta_{1a}$ constructs (CFP-$\beta_{1a}$ + $\beta_{1a}$-YFP, CFP-$\beta_{1a}$ + YFP - $\beta_{1a}$, or $\beta_{1a}$-CFP + $\beta_{1a}$-YFP) were expressed in $\beta_{1a}$-null cells, colocalized puncta of cyan and yellow fluorescence were observed (Fig. 3). Thus, the clusters of DHPRs, which produce the visible puncta, appeared to contain the CFP- and YFP-tagged $\beta_{1a}$ with approximately equal probability.

Previously, we demonstrated that the presence of a CFP-YFP tandem on either the N or C terminus of $\beta_{1a}$ did not affect function of the DHPR as either a voltage sensor for EC coupling or as an L-type $Ca^{2+}$ channel (12). To determine whether the addition of fluorescent proteins to both the N and C termini affected function of $\beta_{1a}$, we measured evoked contractions and L-type $Ca^{2+}$ currents in $\beta_{1a}$-null myotubes expressing CFP-$\beta_{1a}$-YFP. Such myotubes produced robust contractions in response to focal extracellular stimulation (Fig. 4A and Table 1) and also produced large, whole-cell L-type $Ca^{2+}$ currents (Fig. 4, B and C). Thus, the function of $\beta_{1a}$ did not appear to be altered by the attachment of fluorescent proteins.

Photobleaching of Labeled $\beta_{1a}$ Subunits—To further compare labeled $\beta_{1a}$ subunits in the absence and presence of $\alpha_{1S}$, subregions of cells were
subjected to intense illumination at 514 nm to bleach YFP. Fig. 5 illustrates this kind of experiment for YFP-β₁a-CFP expressed in either a dysgenic myotube without α₁S (A) or coexpressed in a dysgenic myotube together with unlabeled α₁S (B). In the absence of α₁S, both the yellow and cyan fluorescence had a diffuse appearance. The β₁a subunits producing this diffuse pattern appeared to be freely mobile, since immediately after the bleaching episode (total duration of 70 s), there was a partial loss of yellow fluorescence outside the portion of the myotube exposed to bleaching illumination, and within the bleached region the intensity of yellow fluorescence gradually increased as the distance to the non-bleached portion of the myotube decreased (Fig. 5A, panel b). Moreover, by 400 s after the end of the bleaching episode, there was a restoration of uniform pattern of yellow fluorescence observed prior to bleaching (Fig. 5A, panel c). The time course of the yellow fluorescence intensity in Fig. 5C clearly shows recovery within the bleached region relative to the non-bleached region, while the cyan fluorescence remains relatively stable. Note the small increase in cyan fluorescence immediately after the bleaching of YFP, indicative of the FRET from CFP to YFP prior to bleaching. These results appear to indicate that there is negligible binding of the labeled β₁a subunits to cellular structures. By contrast, the majority of β₁a subunits in the presence of α₁S appeared to be immobile because of anchoring within junctional membranes. Thus, there was a sharp demarcation in yellow fluorescence between bleached and non-bleached regions of the myotube and no loss of yellow fluorescence in the non-bleached regions (Fig. 5B, panel b). Furthermore, even 400 s after the completion of bleaching (Fig. 5B, panel c), there was essentially no recovery of yellow fluorescence within puncta inside the bleached region, although a small amount of diffuse fluorescence did recover within the bleached region. The corresponding time course of YFP fluorescence intensity within (YELLOW 2) as well as outside (YELLOW 1) the bleached area is shown in Fig. 5D. The small continuous decrease of YELLOW 1 can be attributed to bleaching due to repetitive scanning whereas the slow recovery of YELLOW 2 may reflect diffusional invasion of β₁a subunits not associated with α₁S subunits. Results similar to those illustrated in Fig. 5A were obtained from a total of 6 dysgenic myotubes without α₁S and similar to those illustrated in Fig. 5B from a total of 3 dysgenic myotubes plus unlabeled α₁S and a total of 2 β₁-null myotubes.

**Measurement of Intra- and Intermolecular FRET**—For doubly labeled β₁a, FRET efficiencies were compared for subunits diffusely distributed in the cytoplasm with the efficiencies for subunits associated with α₁S subunits in plasma membrane/SR junctions (Fig. 6). Diffuse cytoplasmic distribution was studied by expression in dysgenic myotubes without α₁S (see Fig. 2A). For CFP-β₁a-YFP and YFP-β₁a-CFP in the cytoplasm, the FRET efficiencies were 6.9 ± 1.8 and 5.9 ± 1.9, respectively. For the doubly labeled subunits targeted to junctional membranes, the FRET efficiency varied depending on the conditions of expression. When coexpressed in dysgenic myotubes with unlabeled α₁S (see Fig. 2B) the FRET efficiencies were reduced compared with those of the same constructs in the cytoplasm, being 3.4 ± 1.1 and 3.3 ± 1.6% for CFP-β₁a-YFP and YFP-β₁a-CFP, respectively. Two issues complicate the interpretation of this result. First, the measured FRET efficiency for doubly labeled β₁a subunits in junctions could contain contributions from both intra- and intermolecular FRET. Second, dysgenic myotubes endogenously express β₁a subunits, which could have reduced intermolecular FRET. To investigate the latter, the doubly labeled constructs were expressed in β₁-null myotubes (see Fig. 2C). Under these conditions, the FRET efficiencies were 7.2 ± 2.8 and 5.6 ± 2.6% for CFP-β₁a-YFP and YFP-β₁a-CFP, respectively. Thus, FRET efficiencies for doubly labeled β₁a subunits in junctions were higher in β₁-null myotubes (Fig. 6; iii) than in dysgenic myotubes (Fig. 6; ii). To confirm that this difference was a consequence of the presence of unlabeled β₁a, FRET efficiencies were measured for the doubly labeled constructs coexpressed with unlabeled β₁a in β₁-null myotubes, which yielded FRET efficiencies of 3.1 ± 0.9% for CFP-β₁a-YFP and 3.0 ± 1.9% for YFP-β₁a-CFP (Fig. 6, iv). Thus, the presence of unlabeled β₁a under two conditions (dysgenic myotubes + unlabeled α₁S; Fig. 6; ii; and β₁-null myotubes + unlabeled β₁a; Fig. 6; iv) yielded FRET efficiencies about half those of the doubly labeled, junctionally targeted constructs in the absence of unlabeled β₁a (Fig. 6, iii).

One way to explain the results in Fig. 6 is to suppose that (i) intramolecular FRET is lower for doubly labeled β₁a in junctions compared with that in cytoplasm and (ii) the measured FRET efficiency for doubly labeled β₁a in junctions contains contributions from both intra- and intermolecular FRET. Accordingly, the presence of unlabeled β₁a would reduce the contribution of the intermolecular component. If this explanation were correct, one would expect to be able to measure intermolecular FRET with singly labeled constructs. This was tested by coexpression in β₁-null myotubes of 1:1 mixtures of CFP-β₁a + YFP-β₁a, β₁a-CFP + β₁a-YFP, or CFP-β₁a + β₁a-YFP. For none of these mixtures was detectable FRET observed (Table 2), which argues against a contribution of intermolecular FRET for the doubly labeled β₁a constructs in β₁-null myotubes.

**DISCUSSION**

The four major findings of the present work are as follows. First, the addition of fluorescent protein to the N terminus, C terminus, or both termini of β₁a does not interfere with its targeting or function. In the

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**TABLE 1**

| Constructs | Restoration of EC coupling |
|------------|---------------------------|
| CFP-β₁a-YFP | 10/12 |
| YFP-β₁a-CFP | 16/20 |
| YFP-β₁a | 16/20 |
| β₁a-YFP | 15/18 |
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FIGURE 5. Photobleaching of yellow fluorescence associated with YFP-β₁₅-CFP. Images of cyan and yellow fluorescence of YFP-β₁₅-CFP expressed in dysgenic myotubes without (A) or with (B) unlabeled α₁S are shown. The images were acquired immediately before (panel a), immediately after (panel b), and ~400 s after (panel c) the bleaching. The dotted red rectangle indicates the region of yellow fluorescence bleaching (see “Materials and Methods” for details). Note that the yellow fluorescence shows strong recovery in rectangle of time, normalized by the prebleach values.

After expression in dysgenic myotubes lacking α₁S (in β₁₅-null myotubes or coexpressed with α₁S in dysgenic myotubes), the β₁₅ subunits are incorporated into DHPRs that are present in punctate regions of the plasma membrane that form junctions with the SR. The DHPRs containing these labeled β₁₅ subunits retain the ability to mediate excitation-contraction coupling. Second, in the absence of α₁S, there were no foci of β₁₅ subunit indicative of binding to other cellular structures and these β₁₅ subunits appeared to be freely diffusible in the cytoplasm. Third, in the cytoplasm: the 6–7% intramolecular FRET efficiency of the doubly labeled constructs (CFP-β₁₅-YFP or YFP-β₁₅-CFP) expressed in dysgenic myotubes indicates that the N and C termini of cytoplasmic β₁₅ subunits are likely separated by <10 nm. Fourth, within junctions: there was no measurable intermolecular FRET for 1:1 mixtures of CFP-β₁₅ + YFP-β₁₅, β₁₅-CFP + β₁₅-YFP, or CFP-β₁₅ + β₁₅-YFP expressed in β₁₅-null myotubes. This result is consistent with the idea that then N and C termini of adjacent β₁₅ subunits are >10 nm apart, which would occur if they were oriented away from the center of tetrads.

After expression in dysgenic myotubes lacking α₁S, doubly labeled β₁₅ was diffusely distributed (Figs. 2a and 5A). Such a diffuse distribution would seem to indicate that in the absence of α₁S the β₁₅ subunits do not bind to immobile cellular structures. Consistent with this idea, yellow fluorescence at the ends of dysgenic myotubes showed substantial recovery within several minutes following the selective bleaching of YFP within YFP-β₁₅-CFP or CFP-β₁₅-YFP. The observation here that doubly labeled β₁₅ is diffusely distributed in the absence of α₁S is consistent with earlier results showing a diffuse distribution after expression in dysgenic

FIGURE 6. Average FRET efficiencies for doubly labeled β₁₅ expressed in dysgenic (i, ii) or β₁₅-null (iii, iv) myotubes. The doubly labeled β₁₅ constructs were expressed in dysgenic myotubes either without (i) or with (ii) unlabeled α₁S, and in β₁₅-null myotubes either without (iii) or with (iv) unlabeled α₁S. The labeled β₁₅ subunits had a cytoplasmic distribution in the absence of α₁S (i) and a punctate (junctional) distribution in the presence of exogenous (ii) or endogenous (iii, iv) α₁S. Error bars indicate ± S.D. For each of the conditions (i–iv), FRET efficiencies were not significantly different between CFP-β₁₅-YFP and YFP-β₁₅-CFP (p > 0.24). FRET efficiencies were significantly different between conditions i and ii (p < 0.0036) and between conditions iii and iv (p < 0.043).
myotubes of either β1a subunits tagged with a CFP-YFP tandem (12) or with GFP (16). However, small, weak puncta on a diffuse background were observed following immunostaining for endogenous β1a in dysgenic myotubes, from which it was concluded that the isolated β1a subunits could bind to some component of plasma membrane/SR junctions (14). One possible explanation for the discrepancy between the results with immunostaining and expressed fluorescent protein-labeled β1a is that immunostaining puncta in dysgenic myotubes reflect nonspecific sites of anti-β1a antibody binding. Alternatively, the presence of fluorescent protein might interfere with the ability of isolated β1a to bind to other cellular proteins, although if this occurred it would be difficult to explain why fluorescent protein labeling had no observable effect on β1a function (12, 16).

Within junctions, the FRET efficiency (E) for the doubly labeled β1a was 6–7% in β1a-null myotubes (where no unlabeled β1a was present) and decreased to about 3% when unlabeled β1a was present (expression in dysgenic myotubes or expression together with unlabeled β1a in β1a-null myotubes). The interpretation of this result requires one to consider the possibility that measured FRET efficiency within junctions ("jun") contained contributions from both intra- and intermolecular FRET (E_{jun} = a_{jun} + e), where a and e are the intra- and intermolecular FRET efficiencies, respectively. For doubly labeled β1a in the cytoplasm ("cyt"), only intramolecular FRET would occur (E_{cyt} = a_{cyt}). One could then imagine a number of possibilities for why the presence of unlabeled β1a would cause a decrease in E.

One possibility is that the only effect of unlabeled β1a was to reduce the contribution of intermolecular FRET in junctions (because the unlabeled β1a reduced the number of adjacent doubly labeled β1a subunits within tetrads). If this were the case, one would have to conclude that e ≥4% and that a_{jun} ≤3%, which is substantially less than a_{cyt} (6–7%). This change in intramolecular FRET could be a consequence of a conformational change in β1a that occurred upon binding to α1S (for example, an increase in separation between the N and C termini). However, x-ray crystallography demonstrates that the structure of the β subunit core is little affected by binding to the AID of the α1-I-II loop (9). Moreover, this interpretation suffers from the weakness that intermolecular FRET was not detected for combinations of singly labeled β1a subunits (Table 2).

A second possibility is that e = 0 and that the conformation of the doubly labeled β1a subunit depends upon whether or not its neighboring β1a subunit had attached fluorescent proteins. Specifically, one could imagine that the presence of two fluorescent proteins on all four β1a subunits within a tetrad produces "molecular crowding" that results in a decreased distance between the N and C termini of each of the individual β1a subunits. The presence of one or more unlabeled β1a subunits could then allow a relaxation of the doubly labeled β1a subunits to the native conformation for β1a subunits bound to α1S. An argument against molecular crowding is that previous work has shown that addition of two fluorescent proteins per β1a subunit (tagging with CFP-YFP tandems) has no discernible effect on DHPR function as channel or voltage sensor for EC coupling (12).

A third possibility is that e = 0, the observed FRET signal from puncta is entirely intramolecular, and the effect of unlabeled β1a can be attributed to errors in measurement of cyan intensity. In particular, the contrast between the cyan fluorescence signal and cellular autofluorescence can sometimes be low. The presence of unlabeled β1a within the puncta would decrease the cyan fluorescence signal and cause a further loss of contrast. Because the autofluorescence is unaffected by bleaching of YFP, its presence would cause an underestimate of FRET efficiency, which would become worse as the contrast between cyan fluorescence and autofluorescence decreased as a result of unlabeled β1a.

Whatever the explanation for the effect of unlabeled β1a on the FRET signal produced by doubly labeled β1a, it remains the case that we were unable to measure directly any intermolecular FRET from N to N terminus, from C to C terminus or from N to C terminus of adjacent β1a subunits in tetrads. Taken together with the presence of N- to C-terminal intramolecular FRET for cytoplasmic and junctional β1a, our results are consistent with the model presented in Fig. 7. Individual, β1a subunits are sufficiently compact (<10 nm) that FRET occurs between fluorescent proteins on the N and C termini. In the absence of α1S, the β1a subunits do not appear to bind to other cellular structures and are randomly distributed in the cytoplasm at an average distance too large to allow intermolecular FRET (Fig. 7A). In the presence of α1S, the β1a subunits are assembled into DHPRs, which are organized into groups of four (tetrads), where each tetrad is associated with a single RyR1 (Fig. 7B). Within tetrads, intramolecular FRET occurs within individual doubly labeled β1a subunits, but adjacent subunits are separated by too great a distance to allow FRET. The specific model illustrated in Fig. 7B is based on the arrangement proposed by Wolf et al. (17), but our data are also consistent with the tetradic arrangement of DHPRs suggested by Paolini et al. (18). Independent of any particular arrangement of DHPRs into tetrads, our data are most easily explained by the hypothesis that the N and C termini of β1a lie outside a 10-nm diameter circle at the center of each tetrad. An important goal of future work will be to refine

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**TABLE 2**

| Cell type | Constructs | FRET efficiency mean ± S.D. |
|-----------|------------|-----------------------------|
| Dysgenic  | CFP-β1a-YFP | 6.9 ± 1.8 (8)               |
|           | YFP-β1a-CFP | 5.9 ± 1.9 (9)               |
|           | CFP-β1a-YFP + α1S | 3.4 ± 1.1 (6)           |
|           | YFP-β1a-CFP + α1S | 3.3 ± 1.6 (12)          |
| β1a-null | CFP-β1a-YFP | 7 ± 2.8 (7)                 |
|           | YFP-β1a-CFP | 5.6 ± 2.6 (12)              |
|           | CFP-β1a-YFP + β1a | 3.1 ± 0.9 (5)            |
|           | YFP-β1a-CFP + β1a | 3 ± 1.9 (6)              |
|           | β1a-CFP + YFP-β1a | 0 (4)                    |
|           | CFP-β1a + β1a-YFP | 0 (5)                    |
|           | CFP-β1a + β1a-YFP | 0 (6)                    |

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**FIGURE 7. Schematic model of β1a subunit structure and localization.** The β1a subunits are indicated by dark gray ovals, with CFP and YFP indicated by the small, cyan and yellow ovals. In the absence of α1S (A), the β1a subunits are not bound to other cellular proteins and are thus diffusely distributed in the cytoplasm at an average distance too large for intermolecular FRET to occur from one β1a subunit to another. Within a single β1a subunit, the N- and C-terminal fluorescent proteins are separated by <10 nm and thus support intramolecular FRET. In the presence of α1S (B), the β1a subunits are present within the multiprotein complex that constitutes the DHPR (light gray oval), which assemble into a group of four (a tetrad) in association with RyR1 (outlined by the black square). The view shown is looking from the extracellular space in toward the SR and is based on the model of Wolf et al. (17) with respect to the general size and shape of the entire DHPR complex, the arrangement of the DHPRs within a tetrad, and approximate position, size, and shape of the β1a subunit with the DHPR. According to this model, the β1a subunits within tetrads are too far separated to support intermolecular FRET. The dotted circle has a diameter of 10 nm, the practical maximum separation consistent with measurable FRET.
our knowledge of the spatial orientation of defined DHPR regions with respect to tetrads and with respect to defined regions of RyR1.

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