Mapping Sites of Potential Proximity between the Dihydropyridine Receptor and RyR1 in Muscle Using a Cyan Fluorescent Protein-Yellow Fluorescent Protein Tandem as a Fluorescence Resonance Energy Transfer Probe

Excitation-contraction coupling in skeletal muscle involves conformational coupling between the dihydropyridine receptor (DHPR) and the type 1 ryanodine receptor (RyR1) at junctions between the plasma membrane and sarcoplasmic reticulum. In an attempt to find which regions of these proteins are in close proximity to one another, we have constructed a tandem of cyan and yellow fluorescent proteins (CFP and YFP, respectively) linked by a 23-residue spacer, and measured the fluorescence resonance energy transfer (FRET) of the tandem either in free solution or after attachment to sites of the \( \alpha_{1S} \) and \( \beta_{1a} \) subunits of the DHPR. For all of the sites examined, attachment of the CFP-YFP tandem did not impair function of the DHPR as a Ca\(^{2+}\) channel or voltage sensor for excitation-contraction coupling. The free tandem displayed a 27.5% FRET efficiency, which decreased significantly after attachment to the DHPR subunits. At several sites examined for both \( \alpha_{1S} \) (N-terminal, proximal II–III loop of a fragment construct) and \( \beta_{1a} \) (C-terminal), the FRET efficiency was similar after expression in either dystogenic (\( \alpha_{1S}\)null) or dysgenic (RyR1-null) myotubes. However, compared with dystogenic myotubes, the FRET efficiency in dystrophic myotubes increased from 9.9 to 16.7% for CFP-YFP attached to the tubes, the FRET efficiency in dyspedic myotubes (RyR1-null) myotubes. However, compared with dystogenic myotubes, the FRET efficiency in dystrophic myotubes increased from 9.9 to 16.7% for CFP-YFP attached to the N-terminal of \( \beta_{1a} \) and from 9.5 to 16.8% for CFP-YFP at the C-terminal of \( \alpha_{1S} \). Thus, the tandem reporter suggests that the C terminus of \( \alpha_{1S} \) and the N terminus of \( \beta_{1a} \) may be in close proximity to the ryanodine receptor.

A variety of evidence indicates that excitation-contraction (EC)\(^1\) coupling in skeletal muscle involves conformational coupling between dihydropyridine receptors in the plasma membrane and type 1 ryanodine receptors (RyR1) in the sarcoplasmic reticulum (SR). The dihydropyridine receptor (DHPR) is a voltage-gated calcium channel consisting of a principal \( \alpha_{1S} \) subunit and the auxiliary subunits \( \alpha_{2-\delta} \), \( \beta_{1a} \), and \( \gamma \). The ryanodine receptor is a calcium release channel composed of four identical subunits (4). One argument for conformational coupling between the DHPR and RyR1 is that depolarization of the plasma membrane elicits calcium release from the SR without the requirement for entry of extracellular Ca\(^{2+}\) (5). In addition to this orthograde signal from the DHPR to RyR1, there is also a retrograde signal whereby RyR1 increases the magnitude of the Ca\(^{2+}\) current via the DHPR (6). Morphological evidence strongly supports the idea that this bidirectional signaling involves physical links (direct or indirect) between DHPRs and RyRs in skeletal muscle. In particular, freeze-fracture images of the plasma membrane at junctions with the SR reveal clusters of intramembranous particles that appear to represent DHPRs arranged in groups of four (tetrads) such that each DHPR is apposed to a subunit of an RyR (7). Additionally, this arrangement of DHPRs requires the presence of RyR1 because the arrangement of clustered particles into tetrads does not occur in dyspedic (RyR1-lacking) myotubes (8).

In an attempt to determine whether, and at what sites, interactions occur between the DHPR and RyR1, a number of different approaches have been utilized. This work has yielded no consistent picture. As one example, application of peptides corresponding to \( \alpha_{1S}(II–III) \) loop residues 671–690 was found to activate isolated RyRs (9–12), but scrambled or deleting these residues did not interfere with the ability of \( \alpha_{1S} \) constructs to restore skeletal-type EC coupling in dystrophic myotubes (13–16). As another example, biochemical studies found that the \( \alpha_{1S}(II–III) \) and (III–IV) loops interact with residues 954–1112 of RyR1 but not with the corresponding RyR2 residues (17). However, analysis of RyR chimeras revealed that replacing RyR1 residues 1–1634 with those of RyR2 has little effect on bidirectional signaling (18). Other biochemical studies showed an interaction between RyR1 residues 3609 and 3643 and the proximal portion (1393–1527) of the \( \alpha_{1S} \) C-terminal (19). However, if this interaction actually occurs in vivo, it cannot be a sole determinant of skeletal-type EC coupling because replacing the II–III loop of \( \alpha_{1S} \) with that of \( \alpha_{1C} \) is sufficient to convert EC coupling from cardiac-type (Ca\(^{2+}\)-entry dependent) to skeletal-type, independent of the identity (cardiac versus skeletal) of the C-terminal (20). Indeed, a subset of \( \alpha_{1S} \) residues (720–765) in the II-III loop have been shown to affect isolated RyRs (9–12), but scrambling or deleting these residues did not interfere with the ability of \( \alpha_{1S} \) constructs to restore skeletal-type EC coupling in dystrophic myotubes (13–16). As another example, biochemical studies found that the \( \alpha_{1S}(II–III) \) and (III–IV) loops interact with residues 954–1112 of RyR1 but not with the corresponding RyR2 residues (17). However, analysis of RyR chimeras revealed that replacing RyR1 residues 1–1634 with those of RyR2 has little effect on bidirectional signaling (18). Other biochemical studies showed an interaction between RyR1 residues 3609 and 3643 and the proximal portion (1393–1527) of the \( \alpha_{1S} \) C-terminal (19). However, if this interaction actually occurs in vivo, it cannot be a sole determinant of skeletal-type EC coupling because replacing the II–III loop of \( \alpha_{1S} \) with that of \( \alpha_{1C} \) is sufficient to convert EC coupling from cardiac-type (Ca\(^{2+}\)-entry dependent) to skeletal-type, independent of the identity (cardiac versus skeletal) of the C-terminal (20). Indeed, a subset of \( \alpha_{1S} \) residues (720–765) in the II–III loop have been shown to be both critical for bidirectional signaling (21) and to interact very weakly with RyR1 residues 1837–2168 in yeast two-hybrid studies (22). However, this interaction also seems unlikely to be the sole determinant of skeletal-type coupling because (i)

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strong coupling is mediated in dyspedic myotubes by a chimera in which RyR1 residues 1645–2154 are replaced by RyR2 residues 1637–2118 (22), and (ii) weak skeletal-type coupling can be produced in dysgenic myotubes by an enzyme lacking residues 720–765 as long as a segment of upstream sequence (671–690, peptide A) is also removed (15). In addition to the enzyme loop, a role for the C-terminal has been suggested from analyses of cDNA constructs expressed in 1a-null myotubes (23, 24). Moreover, preliminary biochemical studies have suggested that 1a binds to RyR1 in vitro (25).

In evaluating the various results described above, one must take into account the limitations of each of the approaches. In the case of the biochemical and peptide experiments, it is possible that regions of the DHPR and RyR are artificially brought into contact with one another in a manner that cannot occur in vivo. In the case of the expression of cDNAs encoding DHPRs and RyRs, the observation that function is altered by changes introduced into a segment of primary sequence does not reveal whether that segment actually represents a site of intermolecular contact. Thus, we have undertaken a new approach in the attempt to obtain information about the molecular architecture of DHPRs and RyRs within plasma membrane-SR junctions of living muscle cells. This approach makes use of fluorescent resonant energy transfer (FRET) between donor and acceptor fluorophores (ECFP and EYFP in the present experiments) (26). FRET is an appropriate tool for analyzing the disposition of donors and acceptors that are separated by distances (~10 nm or less) that are relevant for interacting proteins in vivo (27). In addition to being very sensitive to donor-acceptor separation, FRET efficiency is also affected by the relative orientation of the donor and acceptor moieties (28). We have engineered cDNAs in which a CFP-YFP tandem has been incorporated at varying positions of 1S and 1a. This approach allows one to determine whether the FRET differs for the CFP-YFP tandem attached to these sites from the FRET of the free CFP-YFP tandem. At all sites examined, FRET efficiency was reduced when the tandem was attached to the DHPR subunits within junctional membranes. Comparison of FRET efficiency after expression in dysgenic and dyspedic myotubes allowed determination of whether RyR1 influenced the environment of the attachment sites. Interestingly, the presence of RyR1 did not affect the FRET signal of the CFP-YFP tandem attached to the C-terminal of 1a, the N-terminal of 1S, or the II–III loop of two-fragment 1S constructs. By contrast, the FRET efficiency of the tandem attached either to the N-terminal of 1a or the truncated C-terminal of 1S was significantly reduced by the presence of RyR1. Thus, the tandem reporter suggests that RyR1 may be in close proximity to these two sites of the DHPR. In the accompanying article (29), we describe another approach for probing the topology of junctions.

MATERIALS AND METHODS

cDNA Constructs

**CFP-YFP Tandem**—Fig. 1 shows the CFP-YFP constructs used in the experiments described here. The mammalian expression vectors pECFP-C1 and pEYFP-N1 (Clontech, Palo Alto, CA) were cut at unique

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**Fig. 1. Schematic diagrams of the CFP-YFP fusion proteins used in these experiments.** The position of the CFP-YFP tandem is indicated for each construct, in relationship to the native 1S and 1a subunits (shown top left). The linker sequences connecting CFP and YFP to one another, and linking the tandem to 1S or 1a, are indicated by wavy lines, with the number of linker residues as indicated.
gestion with KpnI to remove the sequence coding for the distal part of 686
the 126-bp fragment of pEYFP-C1 that had been digested with the N1) to create constructs encoding the N- and C-terminal labeled fusion proteins YFP-α1s and α1s-YFP, respectively (these constructs contained a KpnI site 6 and 10 nucleotides, respectively, downstream from the end of the α1s coding sequence). Restriction digests and sequencing were used to verify these constructs.

Unlabeled α1s—to provide an unlabeled α1s construct with an expression level comparable with that of the fluorescently labeled subunits, α1s-YFP was digested with KpnI and MfeI (unique sites), and the small fragment containing the YFP sequence was discarded. The large fragment containing the α1s coding sequence was cloned into pCMV-Tag2B (Stratagene, CA) at nucleotide 5581 (immediately after amino acid 1860 of α1s, where 1873 is full-length). The KpnII fragment containing the α1s coding sequence was inserted into the multiple cloning site of the pCMV-Tag2B construct (the CFP-YFP tandem sequence) at nucleotide 1501 (immediately after amino acid 120 of CFP). The CFP-YFP tandem was then opened with AgeI and DraIII, which cut both at this introduced site and a 5' AgeI site positioned near the myotube. The resulting YFP-α1s-CFP-YFP-α1s construct is the slope factor.

The α1s–CFP–YFP labeled rabbit skeletal muscle α1s (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 α1s coding sequence was then ligated to EcoRI/Sall-digested pEYFP-C1 or pEYFP-N1 to obtain the intermediate constructs YFP-α1s and β1s-CFP-YFP. The β1s-CFP-YFP was made by cutting YFP-β1s with AgeI and ligating it with the CFP-YFP tandem fragment containing the α1s-CFP tandem sequence (QuikChange, Stratagene, La Jolla, CA) at nucleotide 5581 (immediately after amino acid 1860 of α1s, where 1873 is full-length).

Unlabeled α1s–Subunit—to provide an unlabeled α1s construct with an expression level comparable with that of the fluorescently labeled subunits, α1s-YFP was digested with AgeI and DraIII cuts and then ligating the fragment containing the α1s sequence to a fragment coding for the CFP-YFP tandem sequence, obtained by digestion of the CFP-YFP tandem with the same enzymes. Correct orientation of the sequences was checked with appropriate restriction digests.

Cell Culture and cDNA Microinjection

Primary cultures of dysgenic (31) and dyspedic (8) and β1s-null (23) myotubes were prepared from newborn mice as described previously (32). Briefly, myoblasts were plated into 35-mm culture dishes with collagen coated glass coverslips (MatTek, Ashland, MA) and grown for 6–7 days in a humidified 37 °C incubator at 5% CO2. The culture medium, Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, was then replaced by differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum), and after 2–4 days single nuclei of myotubes were microinjected with a small amount of plasmid DNA in water. The DNA concentration in the injection solution was 10 ng/μl for both the CFP-YFP tandem and the β1s constructs, and 50–80 ng/μl for the α1s constructs. These same concentrations were used for each of the individual plasmids when combinations of plasmids were injected. For the measurement of Ca2+ currents, the injected cDNA concentration was 20 ng/μl for all the β1s constructs and 100 ng/μl for all the α1s constructs except for the two fragment construct: 60 ng/μl for α1s-II–CFP–YFP, and 100 ng/μl for α1s (III–IV). Myotubes expressing unlabeled α1s or β1s were usually identified by co-injection of 10 or 2 ng/μl, respectively, of a cDNA expression plasmid encoding YFP.

The ability of the α1s and β1s constructs to support EC coupling in dysgenic or β1s-null myotubes, respectively, was assayed by the presence of spontaneous contractions and/or evoked contractions. To test for evoked contractions, the myotubes were placed in physiological saline (containing in mM: 140 NaCl, 1.5 KCl, 1 MgCl2, 2.5 CaCl2, 11 glucose, 10 HEPES, pH 7.4, with NaOH) and stimulated via 100-V, 30-ms pulses applied via a patch pipette that contained physiological saline and was positioned near the myotube.

Characterization of Ca2+ Currents

Macroscopic Ca2+ currents were measured with the whole cell patch clamp method (33). Patch pipettes of borosilicate glass had resistances of 2–3.5 MΩ when filled with an intracellular solution containing (mM): 140 CsCl, 2.5 MgCl2, 10 Cs2EGTA, and 10 HEPES, pH 7.4, with CsOH. For the external bath solution containing (mM): 10 NaCl, 145 tetraethylammonium-C1, 0.003 tetrodotoxin, and 10 HEPES, pH 7.4, with tetraethylammonium-OH. To measure the L-type current, myotubes were stepped from the holding potential (−80 mV) to −20 mV for 1 s (to inactivate endogenous T-type current), repolarized to −50 mV for 50–100 ms, depolarized to test potentials (Vtest) for 200 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 to −110 mV delivered from the holding potential prior to each test pulse. Cell capacitance was determined by integration of a transient from −80 to −70 mV using Clampfit 8.0 (Axon Instruments, Foster City, CA). Current was used to normalize current amplitudes (pA/ Pf). Current–voltage (I–V) curves were fitted using the following Boltzmann expression,

$$I = G_{max} \cdot \frac{(V - V_{rev})}{1 + exp\left(-\frac{V - V_{1/2}}{k_z}\right)}$$

(1)

where I is the current for the test potential V, Vrev is the reversal potential, Gmax is the maximum Ca2+ channel conductance, V1/2 is the half-maximal activation potential, and k_z is the slope factor.
shown of the cyan intensity for the free CFP-YFP tandem before (pre, left) and after (post, right) photobleaching of YFP. Prior to photobleaching, the proximity of the YFP to CFP allows it to serve as an acceptor for non-radioactive energy transfer (FRET) from CFP. After photobleach, YFP is no longer able to serve as an acceptor causing the cyan fluorescence to increase. The magnitude of this increase, $I_{\text{CFPpost}} - I_{\text{CFPpre}} / I_{\text{CFPpre}}$, suggests that the CFP and YFP moieties of the free tandem are separated by <10 nm. Bar, 5 μm.

Measurements of FRET

Intact fluorescent myotubes were examined 24–48 h after cDNA microinjection using the confocal laser scanning microscope LSM 510 META (Zeiss, Thornwood, NY). With physiological saline, spontaneous contractions occurred in a large fraction of dyogenic myotubes expressing the αn1 constructs and in βn-null myotubes expressing βn1 constructs. Because such contractions would interfere with analysis of FRET, the contractions were eliminated by bathing the cells in a Ca²⁺/Mg²⁺-free saline (containing in mM: 155 NaCl, 5 KCl, 11 glucose, 10 HEPES, pH 7.4, with NaOH). Based on measurements of steady-state inactivation of sodium currents (100-ms prepulses), this solution appeared to shift the effective transmembrane potential by at least +25 mV (not shown).

An area of 500 to 2500 μm² was selected from the field of view (×63 oil objective, 1.4 NA), which included the part of the myotube to be analyzed and also an adjacent, non-cellular region for measurement of background fluorescence. CFP and YFP were excited with separate 458- and 514-nm lines, respectively, of an argon laser (30-milliwatt maximum output, operated at 50% or 6.3A) directed to the cell via a 458/514 nm dual dichroic mirror. The emitted fluorescence was split via a 515-nm long-pass, and for CFP was directed to a photomultiplier equipped with a 465–495-nm bandpass filter (Chroma, Rockingham, VT) and for YFP was directed to a photomultiplier equipped with a 530-nm long-pass filter. With this arrangement, there was no cross-talk between the CFP and YFP fluorescence because CFP is not excited at 514 nm and because YFP does not emit in the 465–495 nm range. Confocal fluorescence intensity data ($I_{\text{CFPpre}}$ and $I_{\text{YFPpre}}$) were recorded as the average of four line scans per pixel and digitized at 8-bits, with photomultiplier gain adjusted such that maximum pixel intensities were no more than ~70% saturated (particularly for CFP, which increased in intensity after photobleaching of YFP). Repeated scans (20–60) with 514 nm at maximum laser intensity (20–100-fold greater than for image capture) were used to photobleach YFP, which required ~30–120 s at maximal scan rates. After completion of YFP bleaching, fluorescence intensity ($I_{\text{CFPpost}}$ and $I_{\text{YFPpost}}$) was measured using the identical parameters as before bleaching. FRET efficiency ($E$) in percent (%) was calculated as,

$$E = \left( \frac{I_{\text{CFPpost}} - I_{\text{CFPpre}}}{I_{\text{CFPpre}}} \right) \times 100\%$$  \hspace{1cm} \text{(Eq. 2)}

where $I_{\text{CFPpre}}$ and $I_{\text{CFPpost}}$ are the background corrected CFP fluorescence intensities before and after photobleaching YFP, respectively.

RESULTS

FRET Efficiency of the Free CFP-YFP Tandem—Fig. 2 illustrates the method used to measure FRET efficiency, as applied to the CFP-YFP tandem expressed in a normal myotube. The
Tethered CFP-YFP as a FRET Probe of DHPR-RyR Proximity

Excitation-contraction coupling was assayed as electrically evoked contractions (see “Materials and Methods”) after cDNA expression in dysgenic or β1-null (asterisks) myotubes. The number of contracting myotubes over the total number tested is given for each of the constructs. FRET efficiency was calculated according to Equation 2 for the designated constructs expressed in dysgenic or dyspedic myotubes, as indicated. Data are given as mean ± S.D., with the numbers of cells indicated in parentheses.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Restoration of EC coupling</th>
<th>FRET efficiency</th>
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</thead>
<tbody>
<tr>
<td>Controls</td>
<td>CFP-YFP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CFP-YFP</td>
<td>27.5 ± 2.5 (11)</td>
</tr>
<tr>
<td>α1S</td>
<td>CFP-YFP</td>
<td>12.6 ± 2.7 (15)</td>
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<td>α1S(CFP-YFP)</td>
<td>15.1 ± 1.7 (14)</td>
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<td></td>
<td>α1S(II)-CFP-YFP + α1S(III-IV)</td>
<td>14.8 ± 3.8 (9)</td>
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<td></td>
<td>α1S(II)-CFP-YFP,α1S(III-IV)</td>
<td>8.7 ± 2.6 (8)</td>
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<tr>
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<td>α1Sshort-CFP-YFP</td>
<td>16.8 ± 2.6 (9)</td>
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<tr>
<td>β1a</td>
<td>β1a-CFP-YFP</td>
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<tr>
<td></td>
<td>β1a-CFP-YFP + α1S</td>
<td>15.4 ± 3.2 (13)</td>
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<tr>
<td></td>
<td>CFP-YFP-β1a</td>
<td>21.2 ± 2.7 (8)</td>
</tr>
<tr>
<td></td>
<td>CFP-YFP-β1a + α1S</td>
<td>16.7 ± 3.9 (13)</td>
</tr>
</tbody>
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Fig. 3. Discrete foci of fluorescence are present in myotubes expressing CFP-YFP-α1S. Confocal images of yellow fluorescence are shown of dysgenic (a and b) and dyspedic (c) myotubes expressing the construct CFP-YFP-α1S. For the dysgenic myotube, an x-y scan close to the surface (a) and a scan centered between the upper and lower surfaces (b) are shown. Note that the abundant fluorescent puncta (~0.5–2 μm in diameter) in the surface scan are often aligned in rows (arrows). In the central scan, nuclei (n) are present and most of the fluorescent puncta are located close to the lateral margins, consistent with localization in or near the surface membrane. Fluorescent puncta in dyspedic myotubes (c, x-y scan close to the surface) are similar to those for the same construct in dysgenic myotubes. Bar, 5 μm.

Table 1

FRET efficiency of the CFP-YFP tandem attached to sites of the DHPR, and the ability of the tandem-tagged DHPRs to restore excitation-contraction coupling

Excitation-contraction coupling was assayed as electrically evoked contractions (see “Materials and Methods”) after cDNA expression in dysgenic or β1-null (asterisks) myotubes. The number of contracting myotubes over the total number tested is given for each of the constructs. FRET efficiency was calculated according to Equation 2 for the designated constructs expressed in dysgenic or dyspedic myotubes, as indicated. Data are given as mean ± S.D., with the numbers of cells indicated in parentheses.
optical section close to the surface of the myotube, both the yellow (Fig. 3a) and cyan fluorescence (not shown) were present in discrete, irregularly shaped foci that were about 1–2 μm in size. The preferential association of these fluorescent foci with the surface was also evident in an optical section taken roughly midway between the lower and upper surfaces of the myotube (Fig. 3b), although some fluorescent foci together with diffuse fluorescence were present in the interior of the cell. The prevalence of discrete foci within the interior varied somewhat between myotubes, perhaps as a function of maturation. Additionally, the diffuse interior fluorescence could be relatively intense at regions close to the injected nucleus. Overall, the size and distribution of the fluorescent foci was similar to that of the small clusters of DHPRs and RyRs as revealed by immunostaining of wild-type myotubes (34). Thus the fluorescent foci seem likely to represent groupings of DHPRs at presumptive contacts between the surface membrane and SR and/or between nascent transverse tubules and the SR. However, some of the fluorescent foci may also have represented DHPRs being delivered to the plasma membrane. Fig. 3c shows that a dysgenic myotube expressing CFP-YFP-α1S displayed fluorescent foci similar to those of dysgenic myotubes expressing the same construct (e.g. Fig. 3, a and b). The presence of such fluorescent foci in a dysgenic myotube are consistent with previous results that demonstrate both that junctions form between the plasma membrane and SR, and that DHPRs target to those junctions, in muscle cells lacking RyR1 (35).

In addition to placement at the N-terminal of α1S, the CFP-YFP tandem was also placed within the II–III loop and at the C-terminal. In the latter case, the tandem was placed in a construct (α1S-short-CFP-YFP) that terminated at α1S residue 1667 (the native coding sequence terminates at residue 1873) to avoid the potential proteolytic cleavage that is believed to occur within α1S residues 1685–1689 (36). Truncation of the C-terminal after residue 1662 does not alter the function of unlabeled α1S as a calcium channel or voltage sensor for EC coupling (37). In the case of the II–III loop, two different constructs were examined. One of these, α1S(I–II)-CFP-YFP + α1S(III–IV), which separated the α1S sequence into two separate fragments, was based on previous work (15, 16) showing that similar two-fragment constructs (without the tandem) can complement one another to form DHPRs that function as both Ca2+ channels and voltage sensors for EC coupling. The second construct (α1S(I–II)-CFP-YFP-III–IV) corresponded to a fusion of α1S(I–II)-CFP-YFP and α1S(III–IV) into a single protein. For both the single and double fragment constructs, α1S residues 672–685, which correspond roughly to the “peptide A” region (9), were deleted entirely and the CFP-YFP tandem was placed appreciably upstream of residues 720–765 that have been found to be critical for bidirectional signaling (21).

Expression in both dysgenic and dyspedic myotubes of α1Sshort-CFP-YFP, of α1S(I–II)-CFP-YFP + α1S(III–IV), or of α1S(I–II)-CFP-YFP-III–IV gave rise to the formation of small fluorescent patches that were similar to those illustrated in Fig. 3 for CFP-YFP-α1S. As mentioned above, the size and distribution of these fluorescent patches is similar to those previously reported for DHPRs in junctions between the plasma membrane and SR, as revealed by immunostaining (34). Moreover, all the tagged α1S constructs were able to restore EC coupling as indicated by contractions in response to...
focal, electrical stimulation (Table I). Although not quantified, spontaneous contractions were also frequently observed in dysgenic myotubes expressing these constructs. Thus, the presence of the CFP-YFP tandem did not appear to grossly interfere with either the targeting or function of $\alpha_{1S}$. However, it should be noted that in myotubes expressing $\alpha_{1S}$-CFP-YFP, a variable amount of bright fluorescence was observed in the vicinity of the injected nucleus (those regions were omitted in FRET measurements). This fluorescence likely represented $\alpha_{1S}$-CFP-YFP backed up in the biosynthetic pathway, although measurements of charge movement indicate that similar two-repeat $\alpha_{1S}$ constructs are able to reach the plasma membrane (16, 38). However, the delivery of $\alpha_{1S}$ (I-II) to the surface may be less efficient because such constructs do not target to junctional membranes without accomplishment by $\alpha_{1S}$ (III-IV) (16).

**CFP-YFP-labeled $\beta_{1a}$ Interacts with $\alpha_{1S}$—** The CFP-YFP tandem was attached to either the N- or C-terminal of $\beta_{1a}$ to produce CFP-YFP-$\beta_{1a}$ and $\beta_{1a}$-CFP-YFP, respectively. Under all conditions examined, the two constructs produced a similar pattern of fluorescence; typical examples are illustrated for $\beta_{1a}$-CFP-YFP in Fig. 4. After expression in dysgenic myotubes lacking $\alpha_{1S}$, the CFP-YFP-labeled $\beta_{1a}$ produced a diffuse intracellular fluorescence similar to that of the free CFP-YFP tandem (Fig. 2), except that the tandem-labeled constructs were excluded from the nuclei (Fig. 4, left). By contrast, multiple fluorescent foci were present when the tandem-tagged constructs were expressed in cells that contained an $\alpha_{1S}$ subunit; i.e. co-expressed with $\alpha_{1S}$ in dysgenic myotubes, or expressed in either RyR1-null (dyspedic) or $\beta_{1a}$-null myotubes, as shown in Fig. 4. The diffuse fluorescence of CFP-YFP-tagged $\alpha_{1S}$ in cells lacking $\alpha_{1S}$ and punctate fluorescence in cells having $\alpha_{1S}$ is consistent with previous work using green fluorescent protein-tagged $\beta_{1a}$ and immunolabeling with antibodies directed toward $\alpha_{1S}$ and the ryanodine receptor (39). Thus the diffuse fluorescence probably reflects tagged $\beta_{1a}$ subunits that are free within the myoplasm, whereas the foci represent DHPRs that contain both $\alpha_{1S}$ and the tagged $\beta_{1a}$ and have been inserted into junctional membranes. In support of this idea, when the YFP was completely photobleached within a subregion (a stripe of

**FIG. 6. Use of a binary mask to measure FRET efficiencies within fluorescent foci.** Confocal fluorescence images are shown of pre-bleach yellow fluorescence ($I_{YFP_{pre}}$) and of cyan fluorescence measured both before ($I_{CFP_{pre}}$) and after ($I_{CFP_{post}}$) bleaching of YFP of a dysgenic in which $\beta_{1a}$-CFP-YFP was co-expressed with unlabeled $\alpha_{1S}$. Note that in both the yellow and cyan images, that there are both bright foci (representing junctionally targeted $\beta_{1a}$-CFP-YFP) and substantial diffuse fluorescence (presumably representing cytoplasmic $\beta_{1a}$-CFP-YFP not associated with $\alpha_{1S}$). Comparison of the $I_{YFP_{pre}}$ and $I_{YFP_{post}}$ images reveals that bleaching of yellow caused increased cyan fluorescence in both the punctate and diffuse regions. To measure the change in fluorescence intensity only with the puncta, the three-dimensional software package (Zeiss) was used to create a binary mask. Specifically, the software was used to display the adjacent images of both the original $I_{YFP_{pre}}$ image and the mask, in which the elements of the mask were set to either zero (shown as black) or unity (shown as white) depending on whether they fell below or above a user-adjusted threshold. The threshold was adjusted by eye until very nearly all of the diffuse fluorescence appeared as black in the mask and the foci appeared as white regions within the mask. Subsequently pixel intensities were measured in the $I_{CFP_{pre}}$ and $I_{CFP_{post}}$ images only for those portions of the mask having the value unity (i.e. the white portions of the mask), as is shown by the images labeled $I_{CFP_{pre,mask}}$ and $I_{CFP_{post,mask}}$. This binary mask method was used for all constructs that targeted to junctional membranes and thus produced foci of fluorescence.
Fig. 7. Comparison of the FRET efficiency of the free CFP-YFP tandem with the FRET efficiencies of the tandem after attachment to the β1a subunit expressed under varying conditions. Compared with free tandem, FRET efficiency was reduced after attachment to either the N- or C-terminal of cytoplasmic β1a. A substantial further reduction for both the N- and C-terminal tandems occurred for junctionally inserted β1a (i.e. constructs expressed in dysgenic myotubes). For the β1a targeted to junctions that did not contain RyR1 (expression in dyspedic myotubes which contain endogenous α1S), the efficiency for the C-terminal tandem (β1a-CFP-YFP) is similar to that found for junctions containing RyR1 (expression with α1S in dysgenic myotubes). However, for the tandem at the N-terminal (CFP-YFP-β1a), the FRET efficiency was significantly higher in junctions lacking RyR1. Error bars indicate ± S.D. Single and double asterisks represent a significant difference of p < 0.01 and p < 0.001, respectively.

The CFP-YFP-tagged α1S and β1a Constructs Form Functional Calcium Channels—To determine whether attachment of the CFP-YFP tandem affected calcium channel function, the patch clamp technique was used to measure whole cell currents in dysgenic myotubes expressing the tagged α1S constructs and in β1a-null myotubes expressing the tagged β1a constructs. Fig. 5 illustrates peak current-voltage relationships for the various constructs. As shown in Fig. 5A, the amplitude and voltage dependence of calcium currents produced by either CFP-YFP-α1S or α1Sshort-CFP-YFP were similar to those of unlabeled α1S. Interestingly, peak currents were slightly larger for both the one- and two-fragment α1S constructs containing CFP-YFP in the II–III loop (Fig. 5B), although the voltage dependence was similar to that of the untagged α1S. Both CFP-YFP-β1a and β1a-CFP-YFP produced currents with magnitude and voltage dependence like those of unlabeled β1a (Fig. 5C). The currents for all the β1a constructs were larger, and had a steeper, left-shifted voltage dependence, compared with those of the α1S constructs. The kinetics of the currents produced by the CFP-YFP-tagged α1S and β1a subunits did not differ obviously from those of the untagged subunits (not shown).

Quantification of FRET in Fluorescent Spots—For all cases in which the CFP-YFP-tagged constructs were localized in junctional membranes, the analysis of FRET efficiency required a means of measuring pixel intensities only within fluorescent foci rather than from entire myotubes, because the latter would have caused the averages to be dominated by the large areas between the foci. This was of special concern in the case of the CFP-YFP-tagged β1a constructs, because there could be a substantial fraction of these constructs free within the cytoplasm. Because the fluorescent foci have an irregular shape and lack clearly defined boundaries, the approach taken was to use the pre-bleach yellow image (I_{CFPpre}) to create a binary mask having the value of unity for all yellow pixels above an adjustable threshold intensity and a value of zero below the threshold (Fig. 6). The threshold was adjusted so that all low intensity pixels outside the fluorescent puncta vanished, leaving a binary mask that was congruent with the majority of the obvious foci of yellow fluorescence. The cyan intensity values (I_{CFPpre} and I_{CFPpost}) were then measured only for those pixels having the value of unity within the binary mask (Fig. 6). Additional details are provided under “Materials and Methods.” FRET efficiencies for all the various constructs examined are summarized in Table I.

FRET Efficiency Is Altered by Junctional Insertion of β1a—Fig. 7 compares the FRET efficiencies of the free CFP-YFP tandem with the FRET efficiencies of the tandem attached to β1a. Compared with the free tandem, the tandem attached to either the N- or C-terminal of cytoplasmic β1a had a significantly (p < 0.001) reduced FRET efficiency (see Table I for values). These FRET efficiencies appear to truly reflect energy transfer between the linked CFP and YFP moieties because no measurable FRET was observed for dysgenic myotubes injected with isomolar mixtures (10 ng/μl each) of cDNAs for CFP-β1a (or β1a-CFP) and YFP-β1a (not shown). After co-expression with unlabeled α1S in dysgenic myotubes, the FRET efficiency was much lower both for tandem at the N-terminal and C-terminal, compared with cytoplasmic β1a (Fig. 7). These reduced FRET efficiencies suggest that membrane association and/or the presence of other proteins within the junctions alter the environment of the N- and C-terminals of β1a.

RyR1 Alters the Environment of the N-terminal of β1a but Not That of the C-terminal—To determine whether the presence of RyR1 contributed to the reduced FRET efficiencies of the CFP-YFP tandem attached to β1a in junctions, the tandem-tagged constructs were expressed in dysgenic myotubes. As shown in Fig. 4, tandem-tagged β1a subunits target to junctions in dyspedic myotubes, despite the lack of RyR1. For β1a-CFP-YFP, the FRET efficiency in dysgenic myotubes did not differ from that in dysgenic myotubes in which α1S was co-expressed (Fig. 7). However, for CFP-YFP-β1a, the FRET efficiency in dyspedic myotubes was significantly greater (by 69%) compared with the same construct co-expressed with α1S in dysgenic myotubes. Thus, the presence of RyR1 causes a large reduction in FRET efficiency for the tandem at the C-terminal of β1a but has no effect on the efficiency of the tandem at the N-terminal, suggesting that RyR1 may be in closer proximity to the N-terminal than to the C-terminal.

RyR1 Alters the Environment of the C-terminal of α1S but Has Little Effect on the Proximal Portion of the II–III Loop—Fig. 8 summarizes the FRET efficiencies for the CFP-YFP tandem attached to sites of α1S. In all instances, the efficiency was lower for tandem attached to α1S than for the free tandem. We have no way of determining how much of this decrease was attributable to attachment per se and how much was attributable to the targeting of the constructs to junctions, because all of the tandem-tagged α1S constructs targeted to junctions.

However, comparison of efficiencies after expression in dyspedic myotubes allowed a determination of which sites were
affected by the presence of RyR1. For the N-terminal (CFP-YFP-α1S), there was no difference in efficiency between dysgenic and dyspedic myotubes. This result suggests that there may not be close proximity between RyR1 and the N-terminal of α1S. The similar FRET efficiency of CFP-YFP-α1S in dysgenic and dyspedic myotubes also suggests that the FRET signal is dominated by energy transfer within tandems rather than between tandems attached to adjacent α1S subunits. Specifically, intermolecular FRET might be expected to decrease in dyspedic myotubes where, unlike dysgenic myotubes, the tagged α1S subunits would not organize into tetrads. As already just mentioned, no such decrease was observed.

As for the N-terminal, tandem in the II–III loop of two fragment constructs (α1S(I–II)-CFP-YFP + α1S(III–IV)) produced similar FRET in dysgenic and dyspedic myotubes (Fig. 8), consistent with the idea that RyR1 does not closely approach the part of the loop (residue 671) to which the tandem was attached. However, this portion of the loop may be indirectly affected by association of RyR1 with α1S because the FRET of tandem at the same position within the loop of a single fragment construct (α1S(I–II)-CFP-YFP(III–IV)) actually displayed a slightly decreased efficiency in dyspedic myotubes compared with dysgenic myotubes. Whatever the interpretation, the tandem clearly exists in a different environment within the intact II–III loop than within the loop of two-fragment constructs. In the case of tandem on the (shortened) C-terminal of α1S, FRET efficiency was significantly greater in dyspedic myotubes compared with dysgenic myotubes. Thus, RyR1 may be in close proximity to the C-terminal.

**DISCUSSION**

By measuring the FRET efficiency of a CFP-YFP tandem either free or fused to sites of the DHPR, we have examined how the environment of these sites changes when the DHPR is inserted into plasma membrane at junctions with the SR. For β1a in the cytoplasm (i.e. expressed in the absence of α1S), the FRET efficiency of the tandem attached to either the N- or C-terminal (CFP-YFP-β1a, C-terminal) was lower than that of the unattached CFP-YFP tandem. An additional decrease in FRET efficiency occurred when either CFP-YFP-β1a or CFP-YFP-β1a-CFP-YFP was inserted into junctions (by co-expression with α1S). Thus, the environment of N- and C-terminals of β1a is affected by proximity to the membrane and/or by the presence of junctional proteins. For the N-terminal, a portion of this decreased FRET efficiency was attributable to RyR1. In all cases for α1S (N-terminal, C-terminal, and within the II–III loop), the FRET efficiency of the attached CFP-YFP tandem was lower than that of the free CFP-YFP tandem. However, it was not possible to determine how much of this decreased efficiency was simply a consequence of attaching CFP-YFP to the DHPR and RyR1 as suggested by the FRET efficiencies of the CFP-YFP tandem attached to sites of α1S and β1a. The grey circles indicate the sites of attachment of the tandem. Horizontal arrows indicate that the FRET efficiency was similar whether or not RyR1 was present, the downward arrows indicate that FRET efficiency was lower in the presence of RyR1 and the upward arrow indicates that the efficiency was higher in the presence of RyR1. For the II–III loop, the numbers 1 and 2 indicate the results for the single- and double-fragment constructs, respectively.

**FIG. 8. Comparison of the FRET efficiency of the free CFP-YFP tandem with the efficiencies of the tandem attached to sites of α1S.** For all sites of attachment, the FRET efficiency was reduced compared with that of the free tandem (note that all constructs targeted to junctions). The presence or absence of RyR1 (expression in dysgenic or dyspedic myotubes, respectively) did not influence the FRET efficiency either for the N-terminal (CFP-YFP-α1S) or for the II–III loop examined with two-fragment constructs (α1S(I–II)-CFP-YFP + α1S(III–IV)). However, the absence of RyR1 led to a decreased FRET efficiency for tandem in the II–III loop of the single fragment construct (α1S(I–II)-CFP-YFP(III–IV)) and an increased efficiency for tandem at the C-terminal (α1Sshort-CFP-YFP). Error bars indicate ± S.D. Single and double asterisks represent a significant difference of \( p < 0.01 \) and \( p < 0.001 \), respectively.
these sites independent of junctional targeting (because all the constructs targeted to junctions). The FRET efficiency for the α1S N-terminal was unaffected by the presence of RyR1, whereas that of the C-terminal was substantially higher when RyR1 was absent. For the α1S(II–III) loop, the influence of RyR1 depended on the construct examined. When α1S was expressed as two fragments divided at the II–III loop (α1S residues 1–671–CFP-YFP co-expressed with α1S residues 686–1860), the efficiency was unaffected by RyR1. However, the presence of RyR1 increased the FRET efficiency when the tandem was inserted into the II–III loop of a single fragment construct, α1S(1–671)–CFP-YFP(686–1860).

Fig. 9 summarizes the effects of RyR1 on FRET efficiency at the sites examined for α1S and β1A. For those regions that display RyR1-dependent FRET efficiencies, a number of different mechanisms could be postulated. The possibility portrayed in Fig. 9 is that the decreases in FRET efficiency occurred because RyR1 interacts with nearby sites of the α1S and β1A subunits of DHPR and thus closely approaches the attached CFP-YFP tandem. If the postulated interaction between RyR1 and β1A actually occurs, it must require α1S. In particular, β1A expressed without α1S showed only a diffuse distribution (Fig. 4), whereas significant binding to RyR1 should have resulted in a punctate distribution. With respect to mechanism, a close approach between RyR1 and the tandem could produce a decrease in FRET efficiency by increasing the separation between the two fluorophores, by restricting their dipole to a less favorable orientation with respect to one another, or by shifting the spectral properties of one or both fluorophores (e.g., by a change in local pH). There is also the possibility that association with RyR1 at one site could affect the environment of a far-removed second site by, for example, inducing a conformational change at the second site. This might explain why the presence of RyR1 increased the FRET efficiency of the CFP-YFP tandem within the II–III loop of the one-piece α1S construct. In this construct, the tandem was introduced —50 residues upstream from the critical domain (720–765; Ref. 40), which may interact with RyR1 (22). Thus, the conformational change affecting the CFP-YFP tandem might be propagated via the loop segment connecting the tandem to this critical domain. Such an explanation is attractive because the CFP-YFP tandem in the loop of the two-fragment α1S construct showed no dependence of FRET efficiency on the presence of RyR1. In any case, the effect of RyR1 on FRET efficiency of the tandem in the II–III loop of the onefragment α1S construct seems unlikely to be of great functional significance, because there were no obvious differences between the one-and two-fragment α1S constructs with regard to behaviors as calcium channels and voltage sensors for EC coupling.

One very important effect of RyR1 is to organize DHPRs into tetramers, and this tetradic organization would influence not only arrangement of DHPRs with respect to one another but also with respect to other junctional proteins, either of which could alter the FRET efficiency of attached CFP-YFP tandem. However, whether or not the effects are direct or longer range, one can still say the changes in FRET reveal regions whose environment is affected by association of DHPRs with RyR1. The accompanying article (29) describes a completely independent approach, avidin accessibility, to gauge the distances between sites of the DHPR and other junctional proteins.

Independent of the exact physical interpretation of the CFP-YFP FRET signals, our results demonstrate that functional triad junctions can accommodate the presence of substantial additional mass (~56 kDa) placed 11–15 residues away from the sites illustrated in Fig. 1. Of course, it is possible that the structure of the DHPR, RyR, or other junctional proteins becomes altered to accommodate these fluorescent proteins (each of which occupies a cylindrical space 4.2 nm in height and 2.4 nm diameter; Ref. 41), but it seems unlikely that such structural alterations could occur in domains of functional importance. In this regard, it is very interesting that the single particle reconstruction of RyR1 shows pits and holes that are large enough (~5 nm) to accommodate the CFP-YFP tandem. For RyR2, single particle analysis of frozen-hydrated material has revealed the three-dimensional localization of green fluorescent protein added at different positions within the linear sequence (42, 43). Similar information would be of great value for the fluorescent proteins introduced into the β1A and α1S subunits of the DHPR. This information would place an important limit on how DHPRs can be oriented with respect to the RyR1, which in turn would provide clues about regions of potential interaction between the two proteins. Another important goal for the future will be to determine whether the environment of the CFP-YFP tandem at some sites changes dynamically during conformational changes important for EC coupling.

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REFERENCES


Tethered CFP-YFP as a FRET Probe of DHPR-RyR Proximity
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