Effects of inserting fluorescent proteins into the $\alpha_{1S}$ II–III loop: insights into excitation–contraction coupling

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In skeletal muscle, intermolecular communication between the 1,4-dihydropyridine receptor (DHPR) and RYR1 is bidirectional: orthograde coupling (skeletal excitation–contraction coupling) is observed as depolarization-induced $Ca^{2+}$ release via RYR1, and retrograde coupling is manifested by increased L-type $Ca^{2+}$ current via DHPR. A critical domain (residues 720–765) of the DHPR $\alpha_{1S}$ II–III loop plays an important but poorly understood role in bidirectional coupling with RYR1. In this study, we examine the consequences of fluorescent protein insertion into different positions within the $\alpha_{1S}$ II–III loop. In four constructs, a cyan fluorescent protein (CFP)–yellow fluorescent protein (YFP) tandem was introduced in place of residues 672–685 (the peptide A region). All four constructs supported efficient bidirectional coupling as determined by the measurement of L-type current and myoplasmic $Ca^{2+}$ transients. In contrast, insertion of a CFP–YFP tandem within the N-terminal portion of the critical domain (between residues 726 and 727) abolished bidirectional signaling. Bidirectional coupling was partially preserved when only a single YFP was inserted between residues 726 and 727. However, insertion of YFP near the C-terminal boundary of the critical domain (between residues 760 and 761) or in the conserved C-terminal portion of the $\alpha_{1S}$ II–III loop (between residues 785 and 786) eliminated bidirectional coupling. None of the fluorescent protein insertions, even those that interfered with signaling, significantly altered membrane expression or targeting. Thus, bidirectional signaling is ablated by insertions at two different sites in the C-terminal portion of the $\alpha_{1S}$ II–III loop. Significantly, our results indicate that the conserved portion of the $\alpha_{1S}$ II–III loop C terminal to the critical domain plays an important role in bidirectional coupling either by conveying conformational changes to the critical domain from other regions of the DHPR or by serving as a site of interaction with other junctional proteins such as RYR1.

INTRODUCTION

The skeletal muscle L-type $Ca^{2+}$ channel (1,4-dihydropyridine receptor [DHPR]) serves as the voltage sensor for excitation–contraction (EC) coupling (Tanabe et al., 1988), activating $Ca^{2+}$ release from the SR via RYR1 in response to depolarization of the plasma membrane. The interaction with RYR1 also increases the magnitude of the L-type $Ca^{2+}$ current produced by DHPR (Nakai et al., 1996; Grabner et al., 1999; Avila and Dirksen, 2000; Avila et al., 2001; Ahern et al., 2003; Sheridan et al., 2006). Neither of these interactions depends on any structural correlate of the functional interaction between DHPR and RYR1 (Block et al., 1988; Takekura et al., 1994, 2004; Protasi et al., 2002; Sheridan et al., 2006). Specifically, intramembranous particles in the plasma membrane, which appear to represent DHPRs, are arranged into groups of four (tetrads) with spacing that places them in register with the four subunits of every other RYR1. Moreover, the distance between each DHPR within the tetrad (~19 nm) is decreased (~2 nm) by application of a high concentration of ryanodine (Paolini et al., 2004), which locks RYR1 in an inactivated, nonconducting state (Buck et al., 1992; Zimányi et al., 1992). Thus, it seems quite certain that protein–protein interactions link DHPR and RYR1.

Two basic strategies have been used in the search to identify the protein–protein interactions that may couple the DHPR and RYR1. One approach has been biochemical analysis of isolated proteins. With this approach, it has been shown that specific regions of the DHPR bind to, or affect the function of, RYR1 and that specific regions of RYR1 bind to DHPR. This approach has revealed that segments of the $\alpha_{1S}$ II–III loop (Leong and
MacLennan, 1998a), α1S III–IV loop (Leong and MacLennan, 1998b), and the proximal α1S C terminus (Sencer et al., 2001) bind to fragments of RYR1 and that segments of RYR1 bind to DHPR subunits (Sencer et al., 2001; Cheng et al., 2005). An important limitation of such biochemical approaches is that they may reveal interactions between segments of proteins that do not interact within living cells. Moreover, the interactions revealed by these biochemical approaches have often been difficult to reconcile with results obtained by functional analyses of myotubes after expression of cDNAs encoding wild-type or engineered DHPRs or RYRs (Beam and Horowicz, 2004). For the functional analysis of the DHPR, the focus has been on the α1S and β1S subunits because bidirectional signaling is little affected by knockout/knockdown of the γ1 (Freise et al., 2000; Ursu et al., 2001) or α2δ-1 (Obermair et al., 2005, 2008; Garcia et al., 2007; Gach et al., 2008; Tuluc et al., 2009) subunits. Expression of cDNAs in myotubes null for endogenous β1 has revealed that the C terminus of β1 is important for EC coupling (Beurg et al., 1999; Sheridan et al., 2003a,b, 2004). Likewise, expression of cDNAs in dysgenic (α1S null) myotubes has shown that EC coupling is critically dependent on the α1S II–III loop (see next paragraph) and is also influenced by the α1S III–IV loop (Weiss et al., 2004; Bannister et al., 2008b).

The α1S II–III loop substituted into the corresponding region of the cardiac DHPR α1C subunit confers skeletal-type coupling (Tanabe et al., 1990; Carbonneau et al., 2005), and, conversely, substitution into an α1S backbone with the II–III loop of α1C (SkLC; Grabner et al., 1999) or α1M (SkLM; Wilkens et al., 2001; Kugler et al., 2004b) abolishes bidirectional signaling. However, bidirectional signaling is restored when a critical domain of the α1S II–III loop (residues 720–765; Nakai et al., 1998b) is reintroduced within SkLC or SkLM (Grabner et al., 1999; Wilkens et al., 2001; Kugler et al., 2004b). Within the critical domain, a central region (α1S residues 737–751) contains the binding site (residues 737–744) for mAb 1A (Kugler et al., 2004a) and an adjacent cluster of negative charges (residues 744–751). Within this central region, mutation of Ala739, Phe741, Pro742, or Asp744 to its corresponding α1C residue reduces both skeletal-type EC coupling and the binding of mAb 1A, and the secondary structure of residues 744–751 also appears to be important for skeletal-type EC coupling (Kugler et al., 2004a,b). On the basis of these results, it has been proposed that some portion of α1S residues 737–751 may bind to and activate RYR1 in response to conformational changes of other regions of the DHPR (Kugler et al., 2004b).

If conformationally driven interactions between regions of the α1S II–III loop and RYR1 are important for bidirectional signaling, it is important to understand how those interactions are coupled to other regions of the DHPR. Previous work (Ahern et al., 2001b) has shown that skeletal-type EC coupling is preserved after expression of a one-piece α1S construct lacking residues 671–690 (the peptide As-20 region; El-Hayek and Ikemoto, 1998). Moreover, two-fragment α1S constructs created by deletion of residues 671–690 (Flucher et al., 2002) or 671–700 (Ahern et al., 2001a) also supported robust EC coupling. Although these results indicate that coupling does not require the presence of the peptide A region or the connection of the critical domain to α1S repeat II via the peptide backbone, they do not exclude a modulatory role for this segment of the α1S II–III loop, which has been recently reported to bind in vitro to a fragment of RYR1 (residues 1,085–1,208; Cui et al., 2009; Tae et al., 2009).

It is important to note that the α1S II–III loop region extending from within the C-terminal portion of the critical domain to α1S repeat III has not been adequately tested because this region of the loop is overall considerably conserved between α1S and the corresponding regions of α1C and α1M (Wilkens et al., 2001). To examine more systematically the importance of different portions of the α1S II–III loop, we introduced perturbations at distinct sites within the loop. In particular, we introduced substantial extra mass (one or two fluorescent proteins) at various sites. The rationale for this approach is that one would expect that sites able to accommodate this insertion without affecting function could neither directly interact with other junctional proteins nor undergo large conformational changes during EC coupling.

We have found that bidirectional coupling is not affected by insertion of a tandem of fluorescent proteins (aggregate mass of ≈56 kD) in place of α1S residues 672–685, adding support to the prevailing view that regions of the α1S II–III loop flanking this insertion site do not undergo important conformational changes and that the critical domain does not interact with more proximal portions of the loop. We found that insertions located more toward the C-terminal end of the loop had a much larger impact on bidirectional signaling. Thus, bidirectional coupling was totally abolished by insertion of the tandem within the critical domain’s N-terminal boundary (between α1S residues 726 and 727) but only partially ablated by insertion of a single fluorescent protein at this site. However, bidirectional signaling was essentially ablated by the insertion of a single yellow fluorescent protein (YFP) near the C-terminal boundary of the critical domain (between α1S residues 760 and 761). This also occurred when a single YFP was inserted between α1S residues 785 and 786, which is C terminal to the critical domain and well conserved between α1S, α1C, and α1M. Control experiments indicated that these insertions did not interfere with membrane expression or targeting to peripheral junctions. Thus, our results raise the possibility that the signaling functions of the critical domain depend on its linkage to α1S repeat III via the C-terminal portion of the α1S II–III loop. Alternatively, this conserved region of the α1S II–III loop may be an important site of protein–protein interaction required for signaling.
The constructions of YFP-α15 (671-CFP-YFP-686), and α15 (671-CFP-YFP) + (686) α15 were previously described in Papadopoulos et al. (2004). All residue numbers refer to the amino sequence of rabbit α15 (GenBank/EMBL/DDJ accession no. X05921).

α15(671-CFP) and (YFP-686)α15, α15(671-CFP) and (YFP-686)α15 encode, respectively, (a) α15 residues 1–671 followed by a 1-residue linker and by cyan fluorescent protein (CFP) and (b) YFP followed by a 12-residue linker and by α15 residues 686–1,860. To make α15(671-CFP), the sequence encoding α15 residues 1–671 (2,039 bp) was excised from α15(671-CFP)-YFP with HindIII and KpnI and ligated into the polylinker of the mammalian expression vector pECFP-N1 (Clontech Laboratories, Inc.) cleaved by HindIII and KpnI (4,706-bp fragment). To make (YFP-686)α15, the sequence encompassing the region encoding YFP (769 bp) was excised from pEYFP-C1 (Clontech Laboratories, Inc.), which had been modified to bring the HindIII site into frame for the subsequent cloning procedure. Specifically, an XhoI–SalI fragment of the pEYFP-N1 polylinker encompassing the HindIII site was reversed by ligation into the compatible NheI and HindIII, which are sites of (686)α15 (Papadopoulos et al., 2004) opened by NheI and HindIII (7,843 bp).

α15(671) and (CFP-YFP-686)α15, α15(671) and (CFP-YFP-686)α15 encode, respectively, (a) α15 residues 1–671 followed by a 12-residue linker and (b) the CFP-YFP tandem (separated by a 23-residue linker) followed by a 12-residue linker and by α15 residues 686–1,860. To make untagged α15(671), the sequence encoding α15 residues 2,039 bp was removed from α15(671)-CFP (see previous paragraph) with HindIII and KpnI and inserted into the backbone (3,973 bp) of untagged (686)α15, from which the sequence encoding (686)α15 had been excised by digestion with HindIII and KpnI. To make (CFP-YFP-686)α15, the sequence encoding CFP (794 bp) was excised from pECFP-C1 (Clontech Laboratories, Inc.) with NheI and Xmal and ligated into (YFP-686)α15 (see previous paragraph) cleaved with NheI and AgeI (8,243 bp). Xmal and AgeI cleavage sites are compatible for relegation.

α15(726-CFP-YFP-727), α15(726-CFP-YFP-727) encodes (in order) (a) α15 residues 1–726, (b) a four-residue linker, (c) CFP-YFP, (d) a 21-residue linker, and (e) α15 residues 727–1,860. A 574-bp fragment encoding the CFP–YFP tandem and the aforementioned linkers were subsequently ligated into the untagged α15 expression vector (Papadopoulos et al., 2004) in which an Agel recognition sequence was introduced between the triplets encoding Ghur256 and Ser257 via site-directed PCR mutagenesis (QuickChange; Agilent Technologies).

α15(726-CFP-YFP-727), α15(726-CFP-YFP-727) encodes (in order) (a) α15 residues 1–726, (b) a four-residue linker, (c) YFP, (d) a 21-residue linker, and (e) α15 residues 727–1,860. A 786-bp sequence encoding YFP was cloned into a YFP-1S expression vector in which an GGT triplet (encoding Gly) had been introduced to form a unique Agel restriction site between the triplets encoding Thr726 and Asn727. Restriction digests and sequencing were used to verify each cDNA construct.

Expression of cDNA
All procedures involving mice were approved by the University of Colorado Denver Institutional Animal Care and Use Committee. Primary cultures of phenotypically normal (+/+ or +/−mdg) or dysgenic (mdg/mdg) myotubes were prepared from newborn mice as described previously (Beam and Franzini-Armstrong, 1997). For electrophysiological experiments, myoblasts were plated into 35-mm plastic culture dishes (Falcon) coated with entactin–collagen IV–laminin (Millipore). Myoblasts destined for immunocytochemistry were plated into 35-mm culture dishes with entactin–collagen IV–laminin–coated glass coverslip bottoms (MatTek). Cultures were grown for 6–7 d in a humidified 37°C incubator with 5% CO2 in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% fetal bovine serum/10% horse serum (Hyclone Laboratories). This medium was then replaced with differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum), 2–4 d after the shift to differentiation medium, single nuclei were microinjected with cDNA. For one-piece constructs (α15(671-CFP-YFP-686), α15(726-CFP-YFP-727), α15(760-YFP-761), or α15(785-YFP-786)), myotubes to be used in electrophysiological experiments were injected with 100 ng/μl cDNA, and myotubes to be immunostained were injected with 60 ng/μl cDNA. For electrophysiology on two-piece constructs, the injection solution contained 60 ng/μl α15(671) hemichannel cDNA and 100 ng/μl (686)α15 hemichannel cDNA. Only myotubes exhibiting YFP fluorescence were used in experiments.

Measurement of ionic currents
For electrophysiological experiments, myotubes were examined 2 d after injection. Pipettes were fabricated from borosilicate glass and had resistances of ~2.0 MΩ when filled with internal solution, which consisted of 140 mM Cs-aspartate, 10 mM Cs 2-EGTA, 5 mM MgCl2, and 10 mM HEPES, pH 7.4, with CsOH. The standard external solution contained 145 mM TEA-Cl, 10 mM CaCl2, 0.003 mM tetrodotoxin, and 10 mM HEPES, pH 7.4, with TEA-OH. In some experiments, 10 mM MgCl2 was substituted for 10 mM CaCl2 in the external solution. Linear capacitative and leakage currents were determined by averaging the currents elicited by 11 30-mV hyperpolarizing pulses from a holding potential of ~80 mV. Test currents were generated for linear components of leak and capacitative current by digital scaling and subtraction of this average control current. Electronic compensation was used to reduce
the data given, the calculated average voltage error was <5 mV. Significant differences between Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. See Materials and methods for fits. For all of

Uninjected dysgenic pulse to

Ionic currents were filtered at 2 kHz and digitized at 10 kHz.

constant for charging the linear cell capacitance (usually to <0.5 ms).

Changes in intracellular Ca\(^{2+}\) were recorded with Fluo-3 (Invitrogen).

(Unless otherwise noted, the peak value of the fluorescence change

t represents the change in peak fluorescence from baseline during

\[ F/F = \frac{[F/F]_{\text{max}}}{1 + \exp \left\{ - \frac{(V - V_c)/b}{k_b} \right\}}, \] (3)

where \(\Delta F/F\) is the maximal fluorescence change and \(V_c\) and \(b\) are fit parameters (Wilkens and Beam, 2003).

Measurement of charge movements

For measurement of intracellular charge movements, ionic currents were blocked by the addition of 0.5 mM CaCl\(_2\) + 0.1 mM LaCl\(_3\) to the standard extracellular recording solution. All charge movements were corrected for linear cell capacitance and leakage currents using a \(-P/S\) subtraction protocol (Bannister et al., 2008a,b). Filtering was at 2 kHz (eight-pole Bessel filter; Frequency Devices, Inc.), and digitization was at 20 kHz. Voltage clamp command pulses were exponentially rounded with a time constant of 50–500 µs, and the prepulse protocol (Adams et al., 1990) was used to reduce the contribution of gating currents from voltage-gated Na\(^+\) channels and T-type Ca\(^{2+}\) channels. The integral of the ON transient (\(Q_{\text{on}}\)) for each test potential (\(V\)) was fitted according to

\[ Q_{\text{on}} = \frac{Q_{\text{max}}}{1 + \exp \left[ - \frac{(V - V_{c})}{b} \right]}, \] (4)

where \(Q_{\text{max}}\) is the maximal \(Q_{\text{on}}\), \(V_c\) is the potential causing half the maximal change in fluorescence, and \(b\) is a slope parameter.

### Measurement of intracellular Ca\(^{2+}\) transients

Changes in intracellular Ca\(^{2+}\) were recorded with Fluo-3 (Invitrogen). The salt form of the dye was added to the standard internal solution for a final concentration of 200 µM. After entry into the whole cell configuration, a waiting period of >5 min was used to allow the dye to diffuse into the cell interior. A 100-W mercury illuminator and a set of fluorescein filters were used to excite the dye present in a small rectangular region of the voltage-clamped myotube. A computer-controlled shutter was used to block illumination in the intervals between test pulses. Fluorescence emission was measured by means of a fluorometer apparatus (Biomedical Instrumentation Group, University of Pennsylvania). The average background fluorescence was quantified before bath immersion of the patch pipette. Fluorescence data are expressed as the total change in fluorescence (\(\Delta F/F\)), where \(\Delta F\) represents the change in peak fluorescence from baseline during the test pulse and \(F\) is the fluorescence immediately before the test pulse minus the average background (non–Fluo-3) fluorescence. Unless otherwise noted, the peak value of the fluorescence change (\(\Delta F/F\)) for each test potential (\(V\)) was fitted according to

\[ \Delta F = \frac{[\Delta F/F]_{\text{max}}}{1 + \exp \left[ - \frac{(V - V_{c})/b}{k_b} \right]}, \] (2)

where \(\Delta F/F\) is the maximal fluorescence change, \(V_c\) is the potential causing half the maximal change in fluorescence, and \(k_b\) is a slope parameter. In the cases of \(\alpha_{15}(760\text{-YFP}-761)\) and \(\alpha_{15}(785\text{-YFP}-786)\), the \(\Delta F/F\) relationship was fit by

Where \(\alpha_{15}\)F data from Bannister and Beam (2005) are included for comparison.

\(^{a}\)F test.

\(^{b}\)P < 0.001 by \(t\) test.

\(^{c}\)P < 0.01 by \(t\) test.

\(^{d}\)P < 0.05 by \(t\) test.

### Measurement of charge movements

For measurement of intracellular charge movements, ionic currents were blocked by the addition of 0.5 mM CaCl\(_2\) + 0.1 mM LaCl\(_3\) to the standard extracellular recording solution. All charge movements were corrected for linear cell capacitance and leakage currents using a \(-P/S\) subtraction protocol (Bannister et al., 2008a,b). Filtering was at 2 kHz (eight-pole Bessel filter; Frequency Devices, Inc.), and digitization was at 20 kHz. Voltage clamp command pulses were exponentially rounded with a time constant of 50–500 µs, and the prepulse protocol (Adams et al., 1990) was used to reduce the contribution of gating currents from voltage-gated Na\(^+\) channels and T-type Ca\(^{2+}\) channels. The integral of the ON transient (\(Q_{\text{on}}\)) for each test potential (\(V\)) was fitted according to

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where \(Q_{\text{max}}\) is the maximal \(Q_{\text{on}}\), \(V_c\) is the potential causing movement of half the maximal charge, and \(k_b\) is a slope parameter.

### Electrically evoked contractions

Contractions were elicited by 20-ms, 100-V stimuli applied via an extracellular pipette that contained 150 mM NaCl and was placed near intact myotubes expressing constructs of interest. The myotubes were bathed in Rodent Ringer’s solution (146 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 11 mM glucose, pH 7.4, with NaOH). Contractions were assayed by the

### Table 1

| Construct       | \(G_{\text{max}}\) | \(G_{\text{max}}/Q\) | \(Q_{\text{max}}\) | \(Q_{\text{max}}/Q\) |
|-----------------|--------------------|----------------------|------------------|----------------------|
| \(\alpha_{15}\)F | \(G_{\text{max}}\) | \(G_{\text{max}}/Q\) | \(Q_{\text{max}}\) | \(Q_{\text{max}}/Q\) |
| \(\alpha_{15}(671\text{-CFP}-\text{YFP}) + (686)\alpha_{15}\) | \(140 \pm 5 \) (37) | \(31.2 \pm 0.8\) | \(8.1 \pm 0.2\) | \(6.0 \pm 0.4 \) (8) |
| \(\alpha_{15}(671\text{-CFP}) + (YFP)\alpha_{15}\) | \(144 \pm 5 \) (20) | \(56.0 \pm 0.9\) | \(7.4 \pm 0.3\) | \(4.2 \pm 0.8 \) (7) |
| \(\alpha_{15}(726\text{-CFP}) + (727)\alpha_{15}\) | \(164 \pm 5 \) (18) | \(35.5 \pm 1.1\) | \(7.8 \pm 0.3\) | \(3.5 \pm 1.4 \) (4) |
| \(\alpha_{15}(785\text{-CFP}-786) + (686)\alpha_{15}\) | \(182 \pm 11 \) (33) | \(39.9 \pm 1.1\) | \(7.3 \pm 0.3\) | \(4.4 \pm 1.1 \) (5) |
| \(\alpha_{15}(726\text{-CFP}-727)\) | \(67 \pm 5 \) (30) | \(38.5 \pm 1.8\) | \(10.4 \pm 0.7\) | \(6.3 \pm 0.8 \) (7) |
| \(\alpha_{15}(726\text{-YFP}-727)\) | \(116 \pm 11 \) (15) | \(53.1 \pm 2.2\) | \(9.7 \pm 0.7\) | \(5.4 \pm 0.9 \) (6) |
| \(\alpha_{15}(760\text{-CFP}) + (761)\alpha_{15}\) | \(102 \pm 10 \) (12) | \(34.1 \pm 0.9\) | \(9.2 \pm 0.7\) | \(6.9 \pm 0.6 \) (6) |
| \(\alpha_{15}(785\text{-YFP}-786)\) | \(72 \pm 10 \) (12) | \(38.9 \pm 3.4\) | \(9.5 \pm 1.7\) | \(8.0 \pm 1.6 \) (5) |

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. See Materials and methods for fits. For all of the data given, the calculated average voltage error was <5 mV. Significant differences between \(\alpha_{15}(671\text{-CFP}-\text{YFP}) + (686)\alpha_{15}\) and the other individual one-piece constructs (i.e., \(\alpha_{15}(726\text{-CFP}-\text{YFP}), \alpha_{15}(726\text{-YFP}-727), \alpha_{15}(760\text{-YFP}-761), \) and \(\alpha_{15}(785\text{-YFP}-786)\)) are indicated.

\(^{a}\)\(J_{\text{CFP}}\) construct conductance and intramembrane charge movement
movement of an identifiable portion of a myotube across the visual field.

**Immunohistochemistry**

2 d after the injection, myotubes were washed twice in Ca\(^{2+}\)/Mg\(^{2+}\)-free Ringer’s solution (146 mM NaCl, 5 mM KCl, 10 mM HEPES, and 11 mM glucose, pH 7.4, with NaOH) and fixed in 4% paraformaldehyde solution in PBS (Sigma-Aldrich) for 20 min. Myotubes were then permeabilized with 0.1% Triton X-100/PBS for 30 min. After another PBS wash, nonspecific reactivity was blocked by application of 1% BSA/PBS for 2 h. The primary antibody (mouse anti-\(\alpha_{1S}\), 1:2,000; Thermo Fisher Scientific; also referred to as mAb 1A) was applied overnight at room temperature (~25°C) in a dark, humid environment. The next day, myotubes were again washed with 1% BSA/PBS. The secondary antibody (Alexa Fluor 568–conjugated goat anti-mouse IgG, 1:4,000; Invitrogen) was applied in the dark for 1 h at room temperature. Excess secondary antibody was removed with three 1% BSA/PBS washes. Finally, immunostained myotubes were rinsed with PBS.

**Confocal microscopy**

Immunostained myotubes were examined in PBS using a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss, Inc.). An area of 500–2,500 \(\mu\)m\(^2\) was selected from the field of view (63×1.4 NA oil immersion objective), which included the myotube and also an adjacent noncellular region for measurement of background fluorescence. YFP was excited with the 488-nm line of an argon laser (30-mW maximum output, operated at 50% or 6.3 A), and Alexa Fluor 568 was excited with a separate sweep of the 543-nm line from a HeNe laser (1-mW maximum output, operated at 100%), which were directed to the cell via a 488/543-nm dual dichroic mirror. The emitted YFP fluorescence was directed to a photomultiplier equipped with a 505–530 band-pass filter (Chroma Technology Corp.). For Alexa Fluor 568, the emitted fluorescence was directed to a photomultiplier equipped with a 560-nm long-pass filter. Confocal fluorescence intensity data 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.

**Analysis**

For calculation of \(G_{\text{max}}/Q^n\) ratios, \(G_{\text{max}}\) was obtained by Eq. 1, whereas \(Q^n\) was derived by subtracting the average maximal charge movement of dyogenic myotubes (\(Q_{\text{max}}\)) from the average total \(Q_{\text{max}}\) where \(Q_{\text{max}} = 1.0\ nC/\mu F\ (n = 6;\ Table I)\). Figures were made using the software program SigmaPlot (version 7.0 or 11.0; SSPS Inc.). All data are presented as mean ± SEM. Statistical comparisons were made by ANOVA or by unpaired, two-tailed \(t\) test (as appropriate), with \(P < 0.05\) considered significant.

**Online supplemental material**

Fig. S1 confirms that \(\alpha_{1S}(726-YFP-727)\) partially supports skeletal-type EC coupling. Myoplasmic Ca\(^{2+}\) transients and I-type currents were recorded with 10 mM Mn\(^{2+}\) substituted for 10 mM Ca\(^{2+}\) in the bath solution. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200910241/DC1.

**RESULTS**

The overall aim of this study was to determine how perturbing the DHPR \(\alpha_{1S}\) II–III loop with inserted fluorescent proteins affects bidirectional interactions with RYR1. These constructs are illustrated in Fig. 1 and represent insertions into the peptide A region (Fig. 1, A–D), into the N-terminal portion of the critical domain (Fig. 1, E and F), near the C-terminal edge of the critical domain (Fig. 1 G), and the conserved segment of the \(\alpha_{1S}\) II–III loop that intervenes between the critical domain and repeat III (Fig. 1 H).

**Insertions into the peptide A region**

Previous work monitored the fluorescence resonance energy transfer efficiency of an ~56-kD CFP–YFP tandem inserted in place of the peptide A region of the \(\alpha_{1S}\) II–III loop (residues 671–686) as an indicator of spatial environment (Papadopoulos et al., 2004). The fluorescence resonance energy transfer efficiency showed a small dependence on whether or not RYR1 was present when CFP–YFP replaced \(\alpha_{1S}\) residues 672–685 in a one-piece construct (Fig. 1 A) but no dependence when the tandem

![Figure 1. Schematic diagrams of the fluorescent protein \(\alpha_{1S}\) fusion constructs.](image-url)
was fused to the C terminus of an α₁S I–II hemichannel (after α₁S residue 671) and was coexpressed with an α₁S III–IV hemichannel (beginning at α₁S residue 686; Fig. 1B). Thus, in this study, we examined these two constructs in more detail as well as two additional constructs centered on this location. In α₁S(671) + (CFP-YFP-686)α₁S, the division was made before the tandem (Fig. 1C), and in α₁S(671-CFP) + (YFP-686)α₁S, the tandem was split, and the fluorescent proteins resided separately on each hemidomain (Fig. 1D).

L-type Ca²⁺ currents for the four sets of constructs after expression in dysgenic myotubes are shown in Fig. 2. The one-piece construct α₁S(671-CFP-YFP-686) produced large-amplitude L-type Ca²⁺ currents (−6.6 ± 0.5 pA/pF at 50 mV; n = 33) with an I-V relationship (V₅₀ = 33.9 ± 1.1 mV; Fig. 2A) that were similar to those reported previously for other fluorescent protein–tagged DHPRs (Table I; Grabner et al., 1998; Wilkens et al., 2001; Flucher et al., 2002; Papadopoulos et al., 2004; Bannister and Beam, 2005; Bannister et al., 2008b). The construct pairs α₁S(671-CFP-YFP) + (686)α₁S and α₁S(671-CFP) + (YFP-686)α₁S produced currents similar to those of α₁S(671-CFP-YFP-686) in both magnitude (−6.2 ± 0.6 pA/pF [n = 20] and −5.8 ± 0.8 pA/pF [n = 18], respectively; P > 0.05, ANOVA) and voltage dependence (P > 0.05, ANOVA; Table I). The construct pair α₁S(671) + (CFP-YFP-686)α₁S produced currents with a peak current density (−4.5 ± 0.5 pA/pF at 50 mV; n = 20) slightly lower relative to the other two hemichannel combinations (P > 0.05, ANOVA; Fig. 2C), despite having similar membrane expression (as assessed by intramembrane charge movement; P > 0.05, ANOVA; Table I). However, it is possible that some of the charge movement for the two-piece constructs was produced by α₁S I–II hemidomains unpartnered with α₁S III–IV hemidomains (Ahern et al., 2001a; Flucher et al., 2002). Dysgenic myotubes expressing each of the four sets of constructs with the CFP–YFP tandem introduced in place of the peptide A region produced contractions in response to extracellular electrical stimuli (Table II).

Depolarization-triggered myoplasmic Ca²⁺ transients for these constructs are shown in Fig. 3. α₁S(671-CFP-YFP-686) triggered robust Ca²⁺ transients ([ΔF/F]₀ = 0.69 ± 0.12; n = 7) with an amplitude that had a sigmoidal dependence on test potential (Table II and Fig. 3A), a signature characteristic of skeletal-type EC coupling (García and Beam, 1994; García et al., 1994). Likewise, α₁S(671-CFP-YFP) + (686)α₁S ([ΔF/F]₀ = 0.61 ± 0.08; n = 9) and α₁S(671-CFP) + (YFP-686)α₁S ([ΔF/F]₀ = 0.93 ± 0.14; n = 7) also served as effective voltage sensors for EC coupling (Table II and Fig. 3, B and D). α₁S(671) + (CFP-YFP-686)α₁S had a slightly reduced ability to trigger myoplasmic Ca²⁺ release ([ΔF/F]₀ = 0.48 ± 0.07; n = 6; P < 0.05, ANOVA; Fig. 3 C and Table II). This slight reduction in ability to mediate EC coupling mirrored the reduced L-type current produced by α₁S(671) + (CFP-YFP-686)α₁S (Fig. 2 C). However, the main conclusion that can be drawn from the experiments illustrated in Figs. 2 and 3 is that introduction of the CFP–YFP tandem...
in place of $\alpha_{15}$ residues 672–685 had little or no effect on the ability of DHPR to conduct L-type Ca$^{2+}$ current or to support EC coupling.

We next probed the effects of insertions at more C-terminal sites of the loop (Fig. 1, E–H). One obvious

Figure 3. EC coupling is little affected by replacement of $\alpha_{15}$ residues 672–685 with a CFP–YFP tandem. $\Delta F/F$–V relationships are shown for dysgenic myotubes expressing $\alpha_{15}(671$-CFP-YFP-686) (A), $\alpha_{15}(671$-CFP-YFP) + (686)$\alpha_{15}$ (B), $\alpha_{15}(671$) + (CFP-YFP-686)$\alpha_{15}$ (C), and $\alpha_{15}(671$-CFP) + (YFP-686)$\alpha_{15}$ (D). Transients were elicited at 0.1 Hz by 200-ms test potentials ranging from −20 through 80 mV in 10-mV increments after a prepulse protocol (Adams et al., 1990). The smooth $\Delta F/F$–V curves are plotted according to Eq. 2, with the best fit parameters for each plot presented in Table II. Representative transient families (test potentials of −20, 0, 20, 40, and 60 mV) are shown for each construct in the insets. Vertical scale bar, 0.5 $\Delta F/F$; horizontal scale bar, 25 ms. Error bars represent ±SEM.

| Construct | $[\Delta F/F]_{\text{max}}$ | $V_c$ | $k_F$ | Contracting cells/number tested |
|-----------|-----------------|-------|------|---------------------------------|
| $\alpha_{15}$YFP$^a$ | 0.58 ± 0.09 (15) | 6.0 ± 1.6 | 4.7 ± 0.4 | 41/47 |
| $\alpha_{15}(671$-CFP-YFP) + (686)$\alpha_{15}$ | 0.61 ± 0.08 (9) | 0.4 ± 1.5 | 3.7 ± 0.4 | 9/11$^b$ |
| $\alpha_{15}(671$) + (CFP-YFP-686)$\alpha_{15}$ | 0.48 ± 0.07 (6) | 7.1 ± 2.0 | 3.7 ± 0.4 | 12/15 |
| $\alpha_{15}(671$-CFP) + (YFP-686)$\alpha_{15}$ | 0.93 ± 0.14 (7) | 7.8 ± 1.5 | 4.1 ± 0.4 | 10/25 |
| $\alpha_{15}(671$-CFP-YFP-686) | 0.69 ± 0.12 (7) | −0.5 ± 2.7 | 4.9 ± 1.1 | 26/32 |
| $\alpha_{15}(726$-CFP-YFP-727) | No fit (12) | No fit | No fit | 0/52 |
| $\alpha_{15}(726$-YFP-727) | 0.19 ± 0.08 (7)$^c$ | 11.9 ± 3.2$^d$ | 10.0 ± 2.8$^d$ | 5/56 |
| $\alpha_{15}(726$-YFP-727) (10 mM Mg$^{2+}$ external) | 0.29 ± 0.17 (4) | 4.4 ± 6.0 | 9.1 ± 2.0 | ND |
| $\alpha_{15}(760$-YFP-761) | See legend (6) | See legend | See legend | 0/72 |
| $\alpha_{15}(785$-YFP-786) | No fit (3) | No fit | No fit | 0/29 |
| Uninjected dysgenic myotubes | No fit (8) | No fit | No fit | 0/100 |

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. All $\Delta F/F$–V data given were fit with Eq. 2 except for $\alpha_{15}(760$-YFP-761). The $\Delta F/F$–V relationship for $\alpha_{15}(760$YFP-761) was best fit by the Gaussian function $\Delta F/F = [\Delta F/F]_{\text{max}} \times \exp\{-0.5 (V/V_c)^2\}$, where $[\Delta F/F]_{\text{max}} = 0.1$, $V_c = 49.2$ mV, and $b = 25.7$ mV (Eq. 3). “No fit” indicates that the $\Delta F/F$–V relationship could not be well fit by either Eq. 2 or Eq. 3 for the number of myotubes indicated. Significant differences between $\alpha_{15}(671$-CFP-YFP-686) and the other individual one-piece constructs that were fit by Eq. 2 (i.e., $\alpha_{15}(726$-YFP-727) are indicated.

$^a$ $\alpha_{15}$YFP data from Bannister and Beam (2005) are included for comparison.

$^b$ $\alpha_{15}(671$-CFP-YFP) + (686)$\alpha_{15}$ contraction data were obtained from Papadopoulos et al. (2004).

$^c$ P < 0.005 by $t$ test.

$^d$ P < 0.05 by $t$ test.
Thus, we sought to determine whether bidirectional signaling would be affected by larger structural perturbations within this region. In contrast to insertion in the peptide A region, insertion of the CFP–YFP tandem between \( \alpha_{1S}(726-\text{CFP-YFP-727}) \), essentially eliminated bidirectional interactions with RYR1 (Fig. 5 A). Thus, \( \alpha_{1S}(726-\text{CFP-YFP-727}) \) produced currents that were much smaller than those of \( \alpha_{1S}(671-\text{CFP-YFP-686}) \) (Fig. 5 B and Table I) despite producing similar intramembrane charge movements (Fig. 4, A and B; and Table I). Furthermore, \( \alpha_{1S}(726-\text{CFP-YFP-727}) \) triggered only barely detectable \( \text{Ca}^{2+} \) transients in the 12 myotubes examined (Fig. 5 C). Likewise, neither spontaneous nor evoked contractions were observed in dysgenic myotubes expressing \( \alpha_{1S}(726-\text{CFP-YFP-727}) \) (\( n = 52 \); Table II).

To determine the effects of introducing a smaller mass at this site, we constructed and characterized an \( \alpha_{1S} \) in which a single YFP (\( \sim 27 \text{kD} \)) was inserted after residue 726 (\( \alpha_{1S}(726-\text{YFP-727}) \)). In contrast to \( \alpha_{1S}(726-\text{CFP-YFP-727}) \), partial bidirectional signaling occurred for...
These results indicate that bidirectional coupling was partially restored by α₁S(726-YFP-727), although both orthograde and retrograde coupling were impaired.

Bidirectional coupling with RYR1 is disrupted in α₁S(760-YFP-761)

With the knowledge that the triad junction can accommodate insertion of a single YFP near the N-terminal boundary of the critical domain, we next tested whether introduction of YFP near the C-terminal boundary of this region, α₁S(760-YFP-761), would also partially spare bidirectional signaling. This construct produced small Ca²⁺ currents and transients (Fig. 6 A). On average, α₁S(760-YFP-761) produced Ca²⁺ currents that were about half the magnitude of those for α₁S(671-CFP-YFP-686) (Fig. 6 B). However, α₁S(760-YFP-761) produced somewhat larger charge movements than the other clones examined (Fig. 4 and Table I). If this increased membrane expression is taken into account, the magnitude of the currents produced by α₁S(760-YFP-761) differed little from those of α₁S(726-CFP-YFP-727) (Fig. 5 D), although this signaling was compromised compared with α₁S(671-CFP-YFP-686) (Fig. 5 E and Table I). Moreover, α₁S(762-YFP-727) supported modest myoplasmic Ca²⁺ transients ([ΔF/F]ₘₐₓ = 0.19 ± 0.08; n = 7; Fig. 5 F and Table II), which displayed a sigmoidal voltage dependence that was positively shifted (10–20 mV) relative to α₁S(671-CFP-YFP-686). A small number of evoked contractions were observed in dysgenic myotubes expressing α₁S(726-YFP-727) (5 of 56 myotubes tested; Table II).

Although the sigmoidal voltage dependence of the Ca²⁺ transients (Fig. 5 F) suggested that the modest Ca²⁺ release observed for α₁S(726-YFP-727) was skeletal type, we further tested the nature of this release by equimolar substitution of Mg²⁺ for Ca²⁺ in the external solution. Substitution of Mg²⁺ for Ca²⁺ had little effect on either the magnitude ([ΔF/F]ₘₐₓ = 0.29 ± 0.17; n = 4; P > 0.05, t test) or the sigmoidal voltage dependence of SR Ca²⁺ release (Fig. S1).
Depolarization-elicited Ca\(^{2+}\) transients were detectable in dysgenic myotubes expressing \(\alpha_{1S}(760\text{-}YFP\text{-}761)\) ([\(\Delta F/F\)]\(_{max} = 0.10 \pm 0.02; n = 6, \text{Fig. 6 A}]). However, these small transients appeared to be a consequence of Ca\(^{2+}\) entry via the L-type current because the \(\Delta F/F-V\) relationship (Fig. 6 C) did not display the sigmoidal shape expected for skeletal-type EC coupling but instead mirrored the peak I-V relationship (Fig. 6 B). No spontaneous or evoked contractions were observed in dysgenic myotubes expressing \(\alpha_{1S}(760\text{-}YFP\text{-}761)\) (\(n = 72; \text{Table II}\)). Thus, it appears that both orthograde and retrograde signaling are largely ablated by insertion of YFP between \(\alpha_{1S}\) II–III loop residues 760 and 761.

Bidirectional coupling with RYR1 is also disrupted in \(\alpha_{1S}(785\text{-}YFP\text{-}786)\)

An important limitation of the analysis of chimeras of \(\alpha_{1C}\) or \(\alpha_{1M}\) with \(\alpha_{1S}\) is that the region corresponding to \(\alpha_{1S}\) residues 773–799 is quite well conserved between the three \(\alpha_1\) subunits (Wilkens et al., 2001). Thus, the chimeras are not informative about the potential importance of this region. Therefore, we directly probed this region by inserting YFP between \(\alpha_{1S}\) residues 785 and 786 (\(\alpha_{1S}(785\text{-}YFP\text{-}786)\)). Dysgenic myotubes expressing \(\alpha_{1S}(785\text{-}YFP\text{-}786)\) produced minimal Ca\(^{2+}\) currents and transients (Fig. 7 A). Thus, current density for \(\alpha_{1S}(785\text{-}YFP\text{-}786)\) was very low (\(-1.4 \pm 0.4\) pA/pF at 40 mV; \(n = 12\); Fig. 7 B), although it produced charge movements (\(Q_{max} = 5.2 \pm 0.8 \text{nC/\(\mu\)F}; n = 5\); Fig. 4 E) that were similar to those of the other one-piece constructs that supported bidirectional signaling (Fig. 4, A and C; and Table I). The \(G_{max}/Q'\) ratio for \(\alpha_{1S}(785\text{-}YFP\text{-}786)\) was similar to that of \(\alpha_{1S}(726\text{-}CFP\text{-}YFP\text{-}727)\) and \(\alpha_{1S}(760\text{-}YFP\text{-}761)\) (Table I).

In addition to eliminating retrograde coupling, insertion of YFP between \(\alpha_{1S}\) residues 785 and 786 also abolished orthograde coupling, as Ca\(^{2+}\) transients were nearly undetectable (Fig. 7 C). No spontaneous or evoked contractions were observed in dysgenic myotubes expressing \(\alpha_{1S}(785\text{-}YFP\text{-}786)\) (\(n = 29; \text{Table II}\)). These results indicate that insertion of a single fluorescent protein between \(\alpha_{1S}\) residues 785 and 786 disrupts bidirectional coupling.

Insertion of fluorescent proteins does not impede antibody binding to the critical domain

Within the critical domain, \(\alpha_{1S}\) residues 737–744 are recognized by mAb 1A, and mutations within this region were shown to have parallel effects on the immunohistochemical binding of the antibody and on orthograde coupling (Kugler et al., 2004a). On the basis of this result, it was hypothesized that the appropriate conformation of \(\alpha_{1S}\) residues 737–744 is essential for communication between DHPR and RYR1. Thus, we used the strategy
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Illustrated in Fig. 8 A to test whether mAb 1A could recognize its epitope in the constructs with impaired bidirectional coupling (i.e., α1S(726-CFP-YFP-727), α1S(726-YFP-727), α1S(760-YFP-761), and α1S(785-YFP-786); Figs. 5–7). Fig. 8 B shows for YFP-α1S that the yellow fluorescence (middle, green) and mAb 1A staining (top, red) colocalize in discrete puncta (bottom, yellow). Similarly colocalized puncta of yellow fluorescence and mAb 1A staining were also observed for α1S(726-CFP-YFP-727), α1S(726-YFP-727), α1S(760-YFP-761), and α1S(785-YFP-786) (Fig. 8, C–F). Such puncta were completely absent in dysgenic myotubes not injected with α1S cDNAs (unpublished data). Furthermore, in control experiments in which the primary antibody was omitted, red puncta were absent despite the persistence of puncta generated by YFP fluorescence (unpublished data). Previous work has demonstrated that DHPR puncta in myotubes colocalize with RYR1 (Takekura et al., 2004). Thus, the data in Fig. 8 support the idea that each of the fluorescent protein α1S constructs was efficiently targeted to plasma membrane junctions with the SR. In addition, these data demonstrate that the introduction of either a single YFP or a CFP–YFP tandem did not severely disrupt the conformation of the antibody epitope within the critical domain.

**DISCUSSION**

In this study, we have evaluated the effects of fluorescent protein insertion as a tool for obtaining information about domains of the α1S II–III loop (residues 662–799) that are essential for bidirectional coupling with RYR1. The insertions were at four sites: (1) replacing residues 672–685 within the peptide A region, (2) between residues 726 and 727 near the N-terminal boundary of the critical domain (residues 720–765), (3) between residues 760 and 761 near the C-terminal boundary of the critical domain, and (4) between residues 785 and 786 in the conserved C-terminal region of the α1S II–III loop. Bidirectional coupling between DHPR and RYR1 displayed a differential sensitivity to insertions at these four sites. This coupling was unaffected by insertion of a large (~56 kD) CFP–YFP tandem in place of the peptide A region but was ablated by the same insertion between α1S residues 726 and 727. Bidirectional coupling was partially spared by insertion of only a single fluorescent protein (YFP) between α1S residues 726 and 727 but totally eliminated by insertion either between α1S residues 760 and 761 or between residues 785 and 786.

Each of the α1S tandem constructs in which the CFP–YFP tandem was substituted for α1S residues 672–685 (Fig. 1, A–D) functioned normally both as a voltage-gated Ca\(^{2+}\) channel and as a voltage sensor for EC coupling (Figs. 2 and 3) regardless of whether the α1S construct was expressed as a combination of two hemidomains (α1S repeats I–II and repeats III–IV) or as an intact channel. This finding is in agreement with several previous studies demonstrating that EC coupling is unaffected by scrambling, deleting, or substituting unrelated sequence for the peptide A region of the α1S II–III loop (Proenza et al., 2000b; Ahern et al., 2001a,b; Wilkens et al., 2001; Flucher et al., 2002; Lorenzon et al., 2004; Papadopoulos 2004).
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Significantly impacted bidirectional signaling. To evaluate these effects, it is useful to consider the landmarks of the critical domain. This region was initially characterized as 1S residues 720–765 on the basis of functional analysis of chimeras between 1S and 1C (Nakai et al., 1998b). The top portion of Fig. 9 compares the sequence of these 46 1S residues (720–765) with the corresponding residues of 1C (851–896) and 1M (712–756). Subsequent work identified a slightly smaller region of 31 1S residues that was sufficient to restore full bidirectional signaling when inserted into a II–III loop otherwise having a 1M sequence (chimera SkLMS31; Kugler et al., 2004b). Fig. 9 illustrates the position of this slightly smaller segment of the critical domain and also compares the sequences of 1S, 1C, and 1M that extend from the C-terminal edge of the critical domain to the beginning of repeat IIS1.

Insertion of the tandem within the N-terminal portion of the critical domain abolished bidirectional signaling (Fig. 5, A–C). This loss of signaling did not appear to be a consequence of loss of membrane expression because charge movements for 1S(726-CFP-YFP-727) were not...
The absence of bidirectional signaling for $\alpha_{1S}(726$-CFP-YFP-727) and $\alpha_{1S}(760$-YFP-761), $\alpha_{1S}(760$-YFP-761), and $\alpha_{1S}(785$-YFP-786) (Fig. 8, C–F).

Compared with the N-terminal edge of the critical domain, the C-terminal edge appeared more sensitive to introduction of a single fluorescent protein. Thus, weak bidirectional signaling was present for the construct $\alpha_{1S}(726$-YFP-727) but was completely absent for the construct $\alpha_{1S}(760$-YFP-761). It should be noted that the YFP connecting linkers were substantially shorter for the insertion between residues 760/761 than for the insertion between residues 726/727 (see Materials and methods). Although the different linker length could have contributed to the greater effect of YFP insertion at residues 760/761 than at residues 726/727, it seems unlikely to account for the much greater effect of YFP insertion at residues 785/786 compared with residues 726/727 because the linker lengths were nearly identical at the two positions. Thus, it seems reasonable to conclude that bidirectional signaling is much more sensitive to perturbation at residues 785/786 than at residues 726/727.

The suppression of bidirectional signaling produced by inserting fluorescent proteins within the N- or C-terminal portions of the critical domain could, in principle, result from disruption of the tertiary structure of this entire region. However, the structure of this region did not appear to be severely altered because mAb 1A was able to recognize its epitope (at residues 737–744) within the critical domain of the constructs $\alpha_{1S}(726$-CFP-YFP-727), $\alpha_{1S}(726$-YFP-727), $\alpha_{1S}(760$-YFP-761), and $\alpha_{1S}(785$-YFP-786) (Fig. 8, C–F).
that voltage sensor movement in response to depolarization promotes a conformation of the α1S II–III loop that favors the bound state of this region of the critical domain and, thus, both SR Ca\(^{2+}\) release and retrograde signaling. If this model were correct, the addition of the extra mass of fluorescent protein near either the N- or C-terminal boundaries of the critical domain could slow the on-rate for binding, shift the equilibrium toward the unbound state, and thus reduce the magnitude of bidirectional coupling. This kinetic model would explain why the CFP–YFP tandem with its greater mass had a larger effect when inserted after residue 726 than did YFP alone. A second hypothesis (occlusion model) is that the presence of the fluorescent proteins sterically occludes entry of α1S II–III loop residues into a binding pocket. According to this model, the binding of residues 727–760 would be completely occluded by the presence of the CFP–YFP tandem between residues 726–727 but only partially occluded by a single YFP at this position.

Either the kinetic or occlusion model could be made compatible with the observation that bidirectional coupling was totally absent for α1S(760-YFP-761). With the kinetic model, for example, it could be postulated that the C-terminal segment of the α1S II–III loop (roughly from residue 760 to repeat III) is essential for coupling between voltage sensor movement and the active conformation of the critical domain and that this coupling is drastically slowed by the presence of fluorescent protein between α1S residues 760 and 761. Alternatively, the YFP placed between α1S residues 760 and 761 might directly interfere with the binding of the adjacent critical domain residues to other junctional proteins.

Whether or not these aforementioned specific hypotheses have validity, it is important to recognize that the region of the α1S II–III loop downstream from the critical domain is well conserved between α1S, α1C, and α1M (Fig. 9, bottom), which means that its importance has not been previously tested by chimeras between these channels. In this regard, the ablation of both orthograde and retrograde coupling by introduction of YFP between α1S residues 785 and 786 (Fig. 7) clearly indicates that the integrity of this region is vital for skeletal-type EC coupling. It remains to be determined whether this domain functions as a conduit for intramolecular communication between the voltage sensor and the critical domain or whether it serves as a site for intermolecular interactions that support bidirectional communication between DHPR and RyR1.

In addition to functional evidence for the importance of the critical domain of the α1S II–III loop, yeast two-hybrid assays have revealed a weak interaction between the critical domain and a segment of RyR1 (residues 1,837–2,168; Proenza et al., 2002). Freeze-fracture electron microscopy also provides structural evidence that the critical domain of the α1S II–III loop is important for linking DHPRs to RyR1. In particular, the arrangement of DHPRs into tetrads depends on the presence of RyR1 in the SR at sites of junction with the plasma membrane (Protasi et al., 1998, 2000, 2002). Tetrads are not formed upon expression in dysgenic myotubes of a chimera consisting of α1S with a II–III loop having an α1C sequence (SKLC), but tetrads are formed when the critical domain portion of the loop of SKLC is converted back to an α1S sequence (Takekura et al., 2004). Interestingly, however, relatively good tetrad formation was observed for a chimera (SKLM) consisting of α1S with a II–III loop having an α1M sequence (Takekura et al., 2004), even though this construct did not support skeletal-type EC coupling (Wilkens et al., 2001).

Ahern et al. (2001b) found that a one-piece α1S construct lacking the critical domain did not mediate skeletal-type EC coupling, but a construct lacking both the critical domain and the peptide A region did support weak coupling, producing maximal transients of ~15% of the amplitude of wild-type α1S. Thus, in addition to the critical domain, other regions of the DHPR have some ability to participate in the interactions that support EC coupling. The organization of SkLM into tetrads (Takekura et al., 2004) also implies that yet-to-be-identified regions of the DHPR must participate in the interactions linking the DHPR to RyR1. Our present results (Figs. 2–7) raise the possibility that one of these yet-to-be-identified regions is the portion of the α1S II–III loop that is C-terminal to the critical domain (Fig. 9, bottom).

In addition to the critical domain and C-terminal region of the α1S II–III loop, other DHPR domains are also possible sites involved in coupling to RyR1. For example, the C-terminal region of β1, strongly influences bidirectional signaling (Beurg et al., 1999; Sheridan et al., 2003a,b, 2004; García et al., 2005). β1 also facilitates tetrad formation, as the β1-null zebrafish mutant relaxed lacks the characteristic orthogonal DHPR arrays typically observed in freeze-fracture replicas of normal skeletal muscle (Schredelseker et al., 2005, 2008). Other possible sites that may interact with RyR1 are the α1S III–IV loop (Leong and MacLennan, 1998b; but see Bannister et al., 2008b) and proximal portions of the C terminus of α1S (Slavik et al., 1997; Flucher et al., 2000; Proenza et al., 2000a; Sencer et al., 2001; Lorenzon et al., 2004; Papadopoulos et al., 2004; Lorenzon and Beam, 2007).

Overall, the effects of inserting fluorescent proteins into the α1S II–III loop are consistent with the idea that binding of the α1S II–III loop critical domain to other junctional proteins is important for bidirectional signaling. Our current results expand our knowledge of the mechanism of bidirectional coupling by identifying the importance of the conserved region of the α1S II–III loop that connects the critical domain to α1S repeat III. However, our results do not exclude roles for other regions of the DHPR that have been implicated as involved in coupling with RyR1. Indeed, it seems quite likely that several cytoplasmic regions of the DHPR, in addition to
those already implicated, participate in interactions that positively or negatively influence coupling between the DHPR and RYR1 (Bannister, 2007). However, we are now in a position to exclude at least a couple of regions from likely involvement. One such region is the N terminus of α1S, which can be largely deleted without affecting function (Bannister and Beam, 2005). Based on previous results (Proenza et al., 2000b; Ahern et al., 2001a,b; Flucher et al., 2002; Lorenzon et al., 2004; Papadopoulos et al., 2004; Lorenzon and Beam, 2007) and those described in this study, it also seems possible to exclude the involvement of peptide A and adjacent regions of the N-terminal portion of the α1S II–III loop as an essential trigger for EC coupling.

The fact that gating of RYR1 is responsive to voltage across the plasma membrane means that the functional state of the DHPR must control the conformation of at least some of the cytoplasmic DHPR regions important for coupling. Regions of this sort may be difficult to define by standard biochemical or structural approaches. Thus, it is likely to continue to be of value to use the kinds of approaches described here to probe the importance of other cytoplasmic domains of the DHPR.

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