The dihydropyridine receptor (DHPR) in the skeletal muscle plasmalemna functions as both voltage-gated Ca\(^{2+}\) channel and voltage sensor for excitation-contraction (EC) coupling. As voltage sensor, the DHPR regulates intracellular Ca\(^{2+}\) release via the skeletal isoform of the ryanodine receptor (RyR-1). Interaction with RyR-1 also feeds back to increase the Ca\(^{2+}\) current mediated by the DHPR. To identify regions of the DHPR important for receiving this signal from RyR-1, we expressed in dysgenic myotubes a chimera (SkLC) having skeletal (Sk) DHPR sequence except for a cardiac (C) II-III loop (L). Tagging with green fluorescent protein (GFP) enabled identification of expressing myotubes. Dysgenic myotubes expressing GFP-SkLC or SkLC lacked EC coupling and had very small Ca\(^{2+}\) currents. Introducing a short skeletal segment (\(\alpha_{\text{IS}}\) residues 720–765) into the cardiac II-III loop (replacing \(\alpha_{\text{IC}}\) residues 851–896) of GFP-SkLC restored both EC coupling and Ca\(^{2+}\) current densities like those of the wild type skeletal DHPR. This 46-amino acid stretch of skeletal sequence was recently shown to be capable of transferring strong, skeletal-type EC coupling to an otherwise cardiac DHPR (Nakai, J., Tanabe, T., Konno, T., Adams, B., and Beam, K.G. (1998) \textit{J. Biol. Chem.} 273, 24983–24986). Thus, this segment of the skeletal II-III loop contains a motif required for both skeletal-type EC coupling and RyR-1-mediated enhancement of Ca\(^{2+}\) current.

Excitation-contraction (EC) coupling in skeletal muscle depends upon a functional interaction between dihydropyridine receptors (DHPRs) in the plasmalemna and ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). In skeletal muscle, the DHPR functions both as an L-type Ca\(^{2+}\) channel and as the voltage sensor, which, in response to plasmalemna depolarization, transmits a signal that causes RyR-1 (the skeletal RyR isoform) to release Ca\(^{2+}\) from the SR (1–3). The nature of the signal transmitted from the skeletal DHPR to RyR-1 is not yet understood, although there is strong evidence that skeletal-type EC coupling does not rely upon the entry of external Ca\(^{2+}\) (4).

An approach to identifying regions of the skeletal DHPR that are important for EC coupling has been to express cDNAs encoding chimeric DHPRs in dysgenic myotubes, which lack endogenous skeletal DHPRs. This work has shown that a purely cardiac DHPR expressed in dysgenic myotubes results in EC coupling which is cardiac type (dependent on entry of Ca\(^{2+}\)) (5), whereas skeletal-type EC coupling results from expression of a chimeric DHPR having cardiac sequence except for a skeletal II-III loop (6). More recently, it was shown that strong skeletal-type coupling could be produced by a chimeric DHPR that contained only a 46-amino acid skeletal segment within the II-III loop and weak skeletal-type coupling by a chimera containing only an 18-amino acid skeletal segment (7).

Analysis of myotubes from dyspedic mice, which lack RyR-1, has revealed that in addition to the orthograde (EC coupling) signal transmitted from the skeletal DHPR to RyR-1, there also appears to be a retrograde signal whereby RyR-1 increases the magnitude of the L-type Ca\(^{2+}\) current mediated by the DHPR. In particular, Ca\(^{2+}\) current density is very low in dyspedic myotubes even though the surface density of DHPRs appears to be essentially normal (8, 9). Expression of cDNA encoding RyR-1 causes the density of L-type current in dyspedic myotubes to increase toward normal (8). However, these experiments did not reveal whether the region of the skeletal DHPR that is crucial for orthograde coupling is also important for the RyR-1-mediated enhancement of DHPR Ca\(^{2+}\) current.

Here we describe experiments to identify regions of the skeletal DHPR that are critical for the ability of the DHPR to receive the retrograde (current-enhancing) signal from RyR-1. The results demonstrate that the II-III loop is critical for both orthograde and retrograde signaling. Within the II-III loop, the 46-amino acid segment found to be important for skeletal-type EC coupling is also important for transducing the retrograde signal from RyR-1.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric DHPRs**

Chimeras between the \(\alpha_{\text{IS}}\) subunits of the skeletal muscle DHPR (Sk (10)) and the cardiac muscle DHPR (C (11)) had amino acid composition (numbers in parentheses) as follows.

| Chimera       | Amino Acid Composition |
|---------------|------------------------|
| SkLC          | (Sk (1–654), C (777–927), Sk (797–1873)) |
| SkLCS46        | (Sk (1–654), C (777–850), Sk (720–765), C (897–927), Sk (797–1873)) |
| SkLCS18        | (Sk (1–654), C (777–855), Sk (725–742), C (874–927), Sk (797–1873)) |

The chimeras were constructed and inserted into mammalian expression vectors as described below (nucleotide numbers (nt) indicated in parentheses):

| Chimera       | Expression Vectors |
|---------------|--------------------|
| SkLC          | The EcoRI-XmnI fragment of Sk (nt 1007–1964) was coligated with the ligation product from the XmnI-HincII fragment of C (nt 2330–2782) plus the HincII-Xhol fragment of Sk (nt 2389–2654) into a mammalian expression vector. |

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the corresponding EcORI/XhoI restriction sites of a SacII-XhoI subclone of Sk (nt 86–2654) in pBluescript SK+ (Stratagene). Finally, the SacII- 
XhoI insert of the modified subclone (now carrying the cardiac II-III loop sequence) was ligated into the corresponding SacII/XhoI restriction sites of plasmid pCAC6, which contains the complete skeletal DHPR coding sequence inserts in normal orientation with the transcriptional start site of 21914  

\textbf{RESULTS}  

\textbf{Laser-scanning Confocal Microscopy}  

GFP-SkLC-expressing dysgenic myotubes cultured on 35-mm culture dishes were superfused with a normal rodent Ringer solution (145 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.4 with 1 M NaOH) and mounted under a glass coverslip. The culture dish was subsequently fastened upside-down on the stage of a Nikon inverted microscope. Fluorescing cells were analyzed using a Sarastro 2000 confocal laser-scanning microscope (Molecular Dynamics) with a Nikon 60× PlanApo oil immersion objective (numerical aperture 1.40) and the ImageSpaceTM software (Silicon Graphics Inc., Mt. View, CA). GFP excitation/emission was achieved with a filter set (485 nm/510 nm) designed for fluorescence detection. Images were 1024×1024 pixels with a pixel size of 0.11 μm. Step size between confocal sections was 2 μm. Images were processed using the Adobe Photoshop software (ADOBE Systems, Mountain View, CA).

\textbf{RESULTS}  

\textbf{The Skeletal Muscle DHPR II-III Loop Is Essential for Receiving the Retrograde (Current-enhancing) Signal—Previous} work showed that the cardiac DHPR expressed in dysgenic (DHPR-deficient) myotubes was unable to mediate orthograde signaling (i.e. skeletal EC coupling). However, a chimera with cardiac sequence except for a skeletal II-III loop (CSk3 (5)) could mediate orthograde signaling. More recently, it was found that the expression of RyR1 in dyspedic (RyR-deficient) myotubes increased the amplitude of slow L-type Ca$^{2+}$ current produced by the skeletal DHPR (8). To determine whether the II-III loop plays an important role in "receiving" this current-enhancing signal from RyR1, we constructed a chimeric DHPR (SkLC) that was the "inverse" of CSk3: a skeletal DHPR except for a cardiac sequence except for a skeletal II-III loop (CSk3 (5)).

Macrospheric Ca$^{2+}$ currents were measured using the whole-cell patch clamp technique (15). The patch pipettes (borosilicicate glass) had resistances of 1.5–1.9 MΩ when filled with an internal solution containing 140 mM cesium aspartate, 10 mM Cs$_2$EGTA, 5 mM MgCl$_2$, and 10 mM HEPES (pH 7.4 with CsOH). The composition of the external bath solution was 10 mM CaCl$_2$, 145 mM tetraethylammonium chloride, 3 μM tetrodotoxin, and 10 mM HEPES (pH 7.4 with tetraethylammonium hydroxide). Test pulses were preceded by a 1-s prepulse to −30 mV to inactivate endogenous T-type Ca$^{2+}$ currents (14). Test currents were corrected for the components of leakage and capacitative current by digitally scaling and subtracting the average of 10 preceding control currents, elicited by hyperpolarizing voltage steps (20–40 mV amplitude) applied from the holding potential of −80 mV. Ca$^{2+}$ currents were normalized by linear cell capacitance (expressed in pA/pF). After the recording of whole-cell Ca$^{2+}$ currents, 0.5 mM Cd$^{2+}$, and 0.1 mM La$^{3+}$ were added to the external bath solution to enable the recording of immobilization-resistant intramembrane charge movement (gating currents). The procedure for recording and calculating maximum charge movement densities and the prepulse protocol used was described in detail elsewhere (14, 16). To examine the effect of Ca$^{2+}$ release on sarcosylmen Ca$^{2+}$ current, Ca$^{2+}$ current and Ca$^{2+}$ transients were measured with the calcium indicator pKCRHR2-APCA (20). The Ca$^{2+}$ transients above for Ca$^{2+}$ currents and patch pipettes containing an internal solution composed of 145 mM cesium glutamate, 8 mM MgATP, 0.5 mM K$_2$-Fluo-3 (Molecular Probes, Eugene, OR), 2 mM CaCl$_2$, 10 mM EGTA, 10 mM HEPES, pH 7.4, with CsOH (10 EGTA solution) or 65 mM cesium glutamate, 5 mM MgCl$_2$, 0.5 mM K$_2$-Fluo-3, 40 mM BAPTA, 10 mM HEPES, pH 7.4, with CsOH, and the external solutions was 150 mM tetraethylammonium chloride, 10 mM HEPES, 5 mM CaCl$_2$, 1 mM MgCl$_2$, 1 μM tetrodotoxin (pH 7.2 with CsOH), and the external solutions was 150 mM tetraethylammonium chloride, 10 mM HEPES, 5 mM CaCl$_2$, 1 mM MgCl$_2$, 1 μM tetrodotoxin (pH 7.2 with tetraethylammonium hydroxide). For the measurement of Ca$^{2+}$ transients in dysgenic myotubes expressing chimeric DHPRs, the pipette contained 145 mM cesium glutamate, 8 mM MgATP, 0.5 mM K$_2$-Fluo-3, 0.1 mM EGTA, 2 mM CaCl$_2$, 10 mM HEPES (pH 7.2 with CsOH), and the external solutions was 150 mM tetraethylammonium chloride, 10 mM HEPES, 5 mM CaCl$_2$, 1 mM MgCl$_2$, 1 μM tetrodotoxin (pH 7.2 with tetraethylammonium hydroxide). For the measurement of Ca$^{2+}$ transients, it was not suitable to use the GFP-tagged constructs that had fluorescence excitation and emission wavelengths close to those of Fluo-3. Thus, cDNAs coding for SkLC, SkLCS46, and SkLCS46 were inserted into the expression plasmid pKCRHR2 (18) and were coexpressed with cDNA encoding the α subunit of the human surface antigen CD8 (19). Myotubes expressing the mutant channels were identified by a green beads coated with CDS antibodies as described previously (20). Transient changes in fluorescence (∆F) were normalized by the resting fluorescence (F). The maximum rate of change of ∆F/F was determined by fitting a line segment to the steepest portion of the transient. All recordings were made at room temperature (−20 °C) and data are reported as mean ± S.D.
smaller in amplitude than those present in GFP-αS-18-expressing myotubes (Fig. 2a). To allow comparisons between cells, peak current-voltage relationships were fitted (14) to yield a value of maximal Ca\(^{2+}\) conductance (G\(_{\text{max}}\)). The value of G\(_{\text{max}}\) for GFP-SkLC was significantly (p < 0.005) smaller than for GFP-αS-18 (Table I). This decrease in G\(_{\text{max}}\) for GFP-SkLC did not appear to be a consequence of a reduced number of DHPRs expressed on the surface membrane because values for maximal charge movement (Q\(_{\text{max}}\)) were similar (p > 0.05) for GFP-SkLC and GFP-αS-18 (Fig. 2, d and e; Table I). The ratio of Q\(_{\text{max}}\) to Q\(_{\text{max}}\) equals Q\(_{\text{max}}\) minus the average, endogenous charge in dysgenic myotubes; Ref. 14) for GFP-SkLC was less than half that for GFP-αS-18 (Table I). Thus, it appears that the presence of a cardiac II-III loop prevents GFP-SkLC from receiving the current-enhancing signal from RyR-1.

Identification of a Skeletal Segment in the II-III Loop Sufficient to Restore Both Skeletal-type EC Coupling and Enhance−Current—Nakai et al. (7) previously showed that substitution of a 46-amino acid segment of skeletal sequence into the II-III loop of an otherwise cardiac DHPR produced a chimera (CSk53; αS residues 720–765) capable of mediating strong, skeletal-type EC coupling upon expression in dysgenic myotubes. To determine whether this motif (Fig. 1b) is also sufficient to allow reception of the Ca\(^{2+}\) current-enhancing signal from RyR-1, we substituted this 46-residue segment into the cardiac II-III loop of GFP-SkLC to yield GFP-SkLCS\(_{46}\) or GFP-SkLCS\(_{18}\). Asterisks indicate identical amino acid residues, and dots show residues carrying the same (negative) charge.

The minimal sequence is contained within the 46-residue skeletal segment in the II-III loop of GFP-SkLCS\(_{18}\). However, this minimal segment is contained within the 46-residue skeletal segment of the GFP-SkLCS\(_{18}\), II-III loop.

**Skeletal-type EC Coupling Is Very Weak for SkLCS18**—The measurement of G\(_{\text{max}}\)/Q\(_{\text{max}}\) provides a quantitative assessment...
**TABLE I**

| Cell type/clone | G<sub>max</sub> | Q<sub>max</sub> | G<sub>max</sub>Q<sub>max</sub> |
|-----------------|----------------|---------------|------------------|
| **Dyspedic**    |                |               |                  |
| a SkLCS         | 22 ± 28 (28)   | 4.0 ± 1.4 (10)| 12               |
| b SkLCS<sub>R</sub> | 141 ± 80 (11)  | 6.4 ± 2.8 (10)| 36               |
| c SkLCS<sub>GFP</sub> | 154 ± 59 (15)* | 7.5 ± 3.1 (15)| 31               |
| d SkLCS<sub>GFP-SkLC</sub> | 65 ± 44 (16)/<sub>D</sub> | 6.8 ± 2.1 (16)| 15               |
| e SkLCS<sub>GFP-SkLC<sub>46</sub></sub> | 118 ± 43 (15)/<sub>D</sub> | 5.5 ± 2.1 (15)| 39               |
| f SkLCS<sub>GFP-SkLC<sub>18</sub></sub> | 42 ± 18 (9)/*  | 5.7 ± 1.2 (9) | 13               |
| **SkLCS<sub>46</sub>** |                |               |                  |
| a SkLCS        | 14 ± 10 (28)   | 4.4 ± 1.2 (10)| 12               |
| b SkLCS<sub>R</sub> | 105 ± 59 (15)/<sub>D</sub> | 7.5 ± 3.1 (15)| 31               |
| c SkLCS<sub>GFP-SKLC</sub> | 65 ± 44 (16)/<sub>D</sub> | 6.8 ± 2.1 (16)| 15               |
| d SkLCS<sub>GFP-SkLC<sub>46</sub></sub> | 188 ± 43 (15)/<sub>D</sub> | 5.5 ± 2.1 (15)| 39               |
| e SkLCS<sub>GFP-SkLC<sub>18</sub></sub> | 42 ± 18 (9)/*  | 5.7 ± 1.2 (9) | 13               |

*The holding potential was −90 mV, and cells were depolarized to the indicated test potentials following a prepulse protocol (14). Note that both the rate of change and maximal increase of ΔF/F are much smaller for SkLCS<sub>18</sub> than for SkLCS<sub>46</sub>. The vertical calibration bar (ΔF/F) corresponds to 5.0 for SkLCS<sub>46</sub> and 1.0 for SkLCS<sub>18</sub> and SkLCS. For the illustrated data, cell identity, linear cell capacitance (C), and immobilization-resistant charge movement (Q) at +70 mV were: a, cell C50, C = 524 pF, Q = 12.4 nC/pF; b, cell C69, C = 479 pF, Q = 10.2 nC/pF; c, cell C59, C = 363 pF, Q = 8.5 nC/pF.

**Fig. 3. Depolarization-induced Ca<sup>2+</sup> transients in dysgenic myotubes expressing DHPR chimeras SkLC (a), SkLCS<sub>46</sub> (b) and SkLCS<sub>18</sub> (c).**

The holding potential was −90 mV, and cells were depolarized to the indicated test potentials following a prepulse protocol (14). Note that both the rate of change and maximal increase of ΔF/F are much smaller for SkLCS<sub>18</sub> than for SkLCS<sub>46</sub>. The vertical calibration bar (ΔF/F) corresponds to 5.0 for SkLCS<sub>46</sub> and 1.0 for SkLCS<sub>18</sub> and SkLCS. For the illustrated data, cell identity, linear cell capacitance (C), and immobilization-resistant charge movement (Q) at +70 mV were: a, cell C50, C = 524 pF, Q = 12.4 nC/pF; b, cell C69, C = 479 pF, Q = 10.2 nC/pF; c, cell C59, C = 363 pF, Q = 8.5 nC/pF.

**Fig. 4. Ca<sup>2+</sup> currents in normal myotubes are only modestly affected by strong Ca<sup>2+</sup> buffering.** Whole-cell Ca<sup>2+</sup> currents (upper set of traces) and Ca<sup>2+</sup> transients (lower set of traces) were measured at the indicated times (min) after breaking into normal myotubes with a patch pipette that contained either 10 mM EGTA (a) or 40 mM BAPTA (b) as the predominant Ca<sup>2+</sup> buffer. The illustrated traces were obtained in response to a 200-ms depolarization to 40 mV (cell E21, I = 210 pA; access resistance 2.2 MΩ (a) or to 20 mV (cell D29, C = 174 pF, access resistance 2.3 MΩ (b)). The time calibration applies to all the currents and transients, the current calibration applies to both a and b, and the vertical scale for the Ca<sup>2+</sup> transients is in arbitrary fluorescence units that are identical for a and b.

**Fig. 4. Ca<sup>2+</sup> currents in normal myotubes are only modestly affected by strong Ca<sup>2+</sup> buffering.** Whole-cell Ca<sup>2+</sup> currents (upper set of traces) and Ca<sup>2+</sup> transients (lower set of traces) were measured at the indicated times (min) after breaking into normal myotubes with a patch pipette that contained either 10 mM EGTA (a) or 40 mM BAPTA (b) as the predominant Ca<sup>2+</sup> buffer. The illustrated traces were obtained in response to a 200-ms depolarization to 40 mV (cell E21, I = 210 pA; access resistance 2.2 MΩ (a) or to 20 mV (cell D29, C = 174 pF, access resistance 2.3 MΩ (b)). The time calibration applies to all the currents and transients, the current calibration applies to both a and b, and the vertical scale for the Ca<sup>2+</sup> transients is in arbitrary fluorescence units that are identical for a and b.

**Ca<sup>2+</sup> Release Is Not Responsible for Enhancement of Ca<sup>2+</sup> Current—**As described above, the chimeric DHPR constructs able to “receive” the Ca<sup>2+</sup> current-enhancing signal from RyR-1 were exactly the same as those able to “transmit” the EC coupling signal to RyR-1. Thus, it seemed possible that Ca<sup>2+</sup> released from RyR-1 (in response to the EC coupling signal) represented the feedback signal, causing the enhancement of Ca<sup>2+</sup> current. As a way of testing this possibility, we carried out simultaneous measurements of Ca<sup>2+</sup> currents and Ca<sup>2+</sup> transients in normal myotubes using two different pipette-filling solutions. One of these solutions (10 mM EGTA) contained 10 mM EGTA (to mimic the standard solution we used for measuring Ca<sup>2+</sup> currents) plus ATP to support Ca<sup>2+</sup> re-uptake into the SR. To ensure thorough dialysis, we selected only small myotubes (232 ± 76 pF, n = 9) with compact geometry and used low resistance patch pipettes (0.9 to 1.4 MΩ). For the cells analyzed, the uncompensated access resistance remained low (1.85 ± 0.33 MΩ, n = 9) after entry into whole-cell mode. Fig. 4 illustrates Ca<sup>2+</sup> currents and Ca<sup>2+</sup> transients evoked by constant amplitude depolarizations applied at the indicated times after breaking into a normal...
myotube with either 10 EGTA (a) or 40 BAPTA (b). Similar results were obtained for a total of 5 cells studied with 40 BAPTA and 4 cells with 10 EGTA. With 10 EGTA in the pipette, depolarization-evoked Ca$^{2+}$ release was sufficient to cause a transient increase in the fluorescence ($\Delta F$) of the indicator dye Fluo-3. Note that both $\Delta F$ and the base-line fluorescence ($F$) increased between 2.5 and 7.5 min after break-in with 10 EGTA, suggesting that during this time Fluo-3 was diffusing into the cell. Because both $\Delta F$ and $F$ remained stable at longer times, it appeared that 7.5 min was sufficient for equilibration between the pipette solution and the myoplasm. With 40 BAPTA in the pipette, depolarization failed to elicit a transient increase in fluorescence, and the base-line fluorescence remained very low, presumably because Ca$^{2+}$ was buffered so strongly that virtually all of the Fluo-3 entering the cell remained in the Ca$^{2+}$-free form. The absence of evoked fluorescence increases with 40 BAPTA indicates that there was effective buffering of Ca$^{2+}$ released from the SR (where Ca$^{2+}$ stores had likely been depleted). Therefore, the measurements with 40 BAPTA should give an indication of the behavior of Ca$^{2+}$ currents in myotubes where Ca$^{2+}$ transients near release sites were substantially suppressed.

As is evident in Figs. 4, a and b, Ca$^{2+}$ current amplitude ran down as a function of time after breaking into the cell with either 10 EGTA or 40 BAPTA. In fact, the rundown in these experiments (with small cells and low access resistance) was faster than that observed under the conditions we normally used for measurements of Ca$^{2+}$ currents (larger cells, higher access resistance). Several factors may have contributed to the more rapid rundown with the 40 BAPTA, including the much lower level of resting free Ca$^{2+}$ and the absence of ATP. However, because large Ca$^{2+}$ currents were still present at times when junctional Ca$^{2+}$ transients were effectively suppressed, it seems unlikely that Ca$^{2+}$ release represents the critical feedback signal whereby RyR-1 enhanced Ca$^{2+}$ current.

**GFP-SkLC Ca$^{2+}$ Channels Cluster in Punctate Foci—** By means of immunostaining, skeletal DHPRs in normal myotubes were shown to cluster in foci that colocalize with RyR clusters (21). Confocal microscopy also reveals focal clusters in living, dysgenic myotubes expressing GFP-tagged DHPRs (12). To determine whether the absence of either orthograde or retrograde signaling by GFP-SkLC (Figs. 2a and 3a, respectively) was a consequence of failure to co-localize with RyRs, we used confocal microscopy to determine whether or not focal clusters were present in dysgenic myotubes expressing this chimeric construct. As shown in Fig. 5, focal clusters were present in GFP-SkLC-expressing dysgenic myotubes. The pattern of distribution of these clusters does not appear qualitatively different from that of GFP-tagged DHPRs (12), which are capable of interacting with the RyRs of the SR.

**DISCUSSION**

We have found that replacing the II-III loop of the skeletal DHPR with the corresponding region of the cardiac DHPR causes the loss of two functions. This skeletal DHPR with a cardiac II-III loop (SkLC) can neither transmit the orthograde (EC coupling) signal to the skeletal ryanodine receptor (RyR-1) nor receive the retrograde (current enhancing signal) from RyR-1. Substitution of a 46-amino-acid segment of skeletal sequence into the cardiac loop of SkLC restores both orthograde and retrograde signaling.

**A Role for the $\beta_{1b}$ Subunit—** Because Ca$^{2+}$ currents are of small amplitude in skeletal muscle cells lacking RyR-1, we suggested in an earlier study that the small Ca$^{2+}$ currents observed after heterologous expression of skeletal DHPRs in nonmuscle cells might be a consequence of the absence of RyR-1 in these cells (8). Recently, however, it was shown that large Ca$^{2+}$ currents could be produced with skeletal DHPRs expressed in Xenopus oocytes if the $\beta_{1b}$ subunit was used instead of $\beta_{1a}$ (22), the predominant $\beta$ isoform in skeletal muscle (23). This work did not establish whether the $\beta_{1b}$ subunit simply increased expression of DHPRs in the oocyte plasmalemma or actually increased the current without changing the number of plasmalemmal DHPRs (as we have shown is the case for enhancement of current by RyR-1). Our preliminary experiments suggest that for DHPRs in their normal environment (muscle cells), expression of $\beta_{1b}$ does not overcome the loss of interaction with RyR-1. In particular, neither expression of $\beta_{1b}$ in dysgenic myotubes ($Q_{\text{max}}/Q_{\text{isoform}}$ ratio: 14 nS/pC; $n = 6$) nor co-expression of $\beta_{1b}$ together with GFP-SkLC in dysgenic myotubes ($Q_{\text{max}}/Q_{\text{isoform}}$ ratio: 12 nS/pC; $n = 4$) yielded values that were much different from the corresponding values obtained without $\beta_{1b}$ co-expression ($Q_{\text{max}}/Q_{\text{isoform}}$ ratios of 12 nS/pC and 15 nS/pC, respectively; see Table I).

**What is the Mechanism of Enhancement of Current?—** The mechanisms of orthograde and retrograde signaling between the skeletal DHPR and RyR-1 remain to be established. One possible explanation of retrograde signaling is that the Ca$^{2+}$ released during EC coupling feeds back onto the DHPR to enhance current. This hypothesis is consistent with the observation that precisely those chimeras that did not show enhancement of current were those that also lacked (SkLC) EC coupling. Furthermore, Feldmeyer et al. (24) have presented evidence that Ca$^{2+}$ release may modulate the Ca$^{2+}$ current in cut fibers from frog skeletal muscle, including the demonstration that prolonged...
exposure (>2.5 h) of the cut ends to 20 mM BAPTA or 1.8 mM ruthenium red caused the complete loss of current. Interestingly, the records showing the loss of Ca$^{2+}$ current (Figs. 2 and 3 of Ref. 24) appear to show a parallel loss of the nonlinear capacitance transients (charge movements), suggesting that there may have been disruption of the t-tubular system or a loss of the ability of the DHPR to undergo the voltage-driven conformational changes producing charge movement. Either of these kinds of changes would not have affected our analysis, which indicates that functional coupling of the DHPR to RyR-1 is associated with large differences in $G_{\text{max}}/Q_{\text{max}}$, the ratio of Ca$^{2+}$ conductance to charge movement. Furthermore, in contrast to the results on frog skeletal muscle (24), our experiments showed that large Ca$^{2+}$ currents were present in mouse myotubes in which Ca$^{2+}$ transients near release sites should have been largely suppressed by dialysis with 40 mM BAPTA (Fig. 4). Negligible effects on maximal Ca$^{2+}$ conductance have also been previously reported for dialysis of mouse myotubes with 1 mM ryanodine, 200 μM ruthenium red, or 20 mM BAPTA (25).

Results from work on DHPR chimeras (14) also argue against an essential role of Ca$^{2+}$ release in enhancement of current. In that study it was found that $G_{\text{max}}/Q_{\text{max}}$ was 55 nS/pC for CARD1 (the cardiac DHPR) and 157 nS/pC for CSk3 (the cardiac DHPR with a skeletal II-III loop). Thus, it appears that the presence of a skeletal II-III loop enhanced the current via a mechanism not strongly dependent on Ca$^{2+}$ release, because both CARD1 and CSk3 support depolarization-induced Ca$^{2+}$ release under the conditions used for measurement of Ca$^{2+}$ currents (7). Data from RyR-1/RyR-2 chimeras provide another argument that Ca$^{2+}$ release via skeletal-type EC coupling is not required for enhancement of current. In particular, expression in dyspedic (RyR-1 lacking) myotubes of the chimera R9 produced enhancement of Ca$^{2+}$ current but not restoration of skeletal-type EC coupling (26).

Finally, recent experiments show that Ca$^{2+}$ currents are enhanced in dyspedic myotubes after expression of a mutated ryanodine receptor, which releases almost no Ca$^{2+}$ in response to depolarization (27).

An alternative to the idea that the release of Ca$^{2+}$ from RyR-1 causes enhancement of current is to suppose that protein-protein interactions are responsible. Fig. 6 illustrates a model in which EC coupling involves transmission of a signal from the skeletal DHPR to RyR-1 via the II-III loop, and enhancement of current involves transmission of a retrograde signal from RyR-1 to the DHPR, again via the II-III loop (an intermediary protein coupling between the II-III loop and RyR-1 is another possibility). The nature of both the orthograde and retrograde signals remains unknown (for example, the retrograde signal might correspond to a covalent modification of the DHPR). However, in the illustrated model, interaction of RyR-1 with the II-III loop stabilizes the DHPR in a conformation (Fig. 6a), which increases single channel current and/or Po (channel open probability) compared with the conformation of the DHPR found in the absence of this interaction (Fig. 6b, no RyR-1; Fig. 6c, cardiac II-III loop). Both skeletal-type EC coupling and the enhancement of current are restored by introduction of a small segment of the skeletal II-III loop (Fig. 6d).

The importance of the II-III loop is emphasized by complementary gain-of-function and loss-of-function experiments. A gain of function (skeletal-type EC coupling) was shown with CSk3 in which the skeletal II-III loop was transplanted into the cardiac DHPR (5). As discussed above, these same experiments also suggest a second gain of function (enhancement of current) because $G_{\text{max}}/Q_{\text{max}}$ was ~3-fold larger for CSk3 than for CARD1 (14). The experiments reported here now demonstrate a loss of both functions (skeletal-type EC coupling, enhancement of current) when the cardiac II-III loop is transplanted into the skeletal DHPR (i.e. SkLC) and a restoration of both functions with SkLCS$_{\text{ag}}$. 

**Fig. 6. Model of DHPR-RyR-1 interactions incorporating results of the present and of previous (5, 8) studies.** Panel a shows the hypothetical DHPR-RyR-1 interaction that occurs in normal myotubes, in dysgenic myotubes injected with α$_{\text{ag}}$ cDNA (5), or in dyspedic myotubes injected with RyR-1 cDNA (8). The cytoplasmic II-III loop of the skeletal DHPR is critical for transmitting the signal controlling the release of Ca$^{2+}$ ions via RyR-1 (5) in the SR membrane. This skeletal-type EC coupling (Skeletal ECC) is not dependent on influx of extracellular Ca$^{2+}$. The II-III loop is also essential for receiving the current-enhancing signal from RyR-1 (Channel enhancement). Panel b depicts the situation as found in dyspedic muscle. Dyspedic myotubes lack RyR-1 but have intact DHPRs at a density comparable with normal myotubes (8). Dyspedic myotubes display no EC coupling and also show significantly reduced slow Ca$^{2+}$ current densities through the DHPR (8). In the absence of contact with RyR-1, the skeletal DHPR assumes a conformation (symbolized by the tilted cylinders representing homologous repeats I-IV) that produces reduced Ca$^{2+}$ current (symbolized by the smaller arrow). Panel c models the behavior of chimera GFP-SkLC expressed in dysgenic myotubes. The cardiac II-III loop (Cardiac loop) in an otherwise skeletal DHPR prevents the DHPR-RyR-1 interaction so that there is neither EC coupling nor appreciable Ca$^{2+}$ current. Panel d shows that the introduction of a short skeletal segment (α$_{\text{ag}}$ residues 720–765, symbolized by a bold line), which sufficed to transfer strong skeletal-type EC coupling to the cardiac DHPR (as in CSk53 described in Ref. 7), is also sufficient to restore wild-type Ca$^{2+}$ current densities (as in chimeras SkLCS$_{\text{ag}}$). Together, these observations suggest that these 46 amino acids of the skeletal II-III loop contain residues that are required for both strong skeletal-type EC coupling and RyR-1-mediated enhancement of skeletal Ca$^{2+}$ current.
For a model like the one in Fig. 6, how would one interpret the observation that orthograde and retrograde coupling are weak for SkLCS18? One possibility is that the great majority of SkLCS18 DHPRs and RyRs are simply not in physical contact because SkLCS18 lacks part of the required sequence. However, it seems very likely that SkLCS18 clusters into foci at sites where the plasmalemma forms junctions with RyR-containing regions of the SR, because even SkLC clusters into foci (Fig. 5). Of course, a demonstration of co-localization of DHPRs and RyRs at the light microscopic level does not imply direct physical contact. Suggestive evidence for direct physical contact between DHPRs and RyRs in skeletal muscle has been provided by freeze-fracture analysis. This analysis has shown that skeletal DHPRs appear to be organized in characteristic tetrads (thought to be four DHPRs, each of which is in contact with one of the four subunits of a RyR) (28). By contrast, cardiac DHPRs appear to be located close to, but not in contact with, RyRs, because tetrads are not observed in cardiac muscle (29). Thus, it will be important to carry out freeze-fracture analysis to determine whether or not tetrads are formed upon expression of Csk3, SkLC, SkLCS46, and SkLCS18. If very few tetrads are observed for SkLCS18, it would suggest that the expression of CSk3, SkLC, SkLCS46, and SkLCS18 induce a lower channel activity of a RyR-1 tetramer than do tetrads of SkLCS46.

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