Localization in the II-III Loop of the Dihydropyridine Receptor of a Sequence Critical for Excitation-Contraction Coupling*

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Skeletal and cardiac muscles express distinct isoforms of the dihydropyridine receptor (DHPR), a type of voltage-gated Ca\(^{2+}\) channel that is important for excitation-contraction (EC) coupling. However, entry of Ca\(^{2+}\) through the channel is not required for skeletal muscle-type EC coupling. Previous work (Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T., and Numa, S. (1990) Nature 346, 567-569) revealed that the loop between repeats II and III (II-III loop) is an important determinant of skeletal-type EC coupling. In the present study we have further dissected the regions of the II-III loop critical for skeletal-type EC coupling by expression of cDNAs in dysgenic myotubes. Because Ser\(^{687}\) of the skeletal II-III loop has been reported to be rapidly phosphorylated in vitro, we substituted this serine with alanine, the corresponding cardiac residue. This alanine-substituted skeletal DHPR retained the ability to mediate skeletal-type EC coupling. Weak skeletal-type EC coupling was produced by a chimeric DHPR, which was entirely cardiac except for a small amount of skeletal sequence (residues 725–742) in the II-III loop. Skeletal-type coupling was stronger when both residues 725–742 and adjacent residues were skeletal (e.g. a chimera containing skeletal residues 711–765). However, residues 725–742 appeared to be critical because skeletal-type coupling was not produced either by a chimera with skeletal residues 711–732 or by one with skeletal residues 734–765.

Dihydropyridine receptors (DHPRs)\(^1\) in skeletal and cardiac muscle are closely related proteins encoded by two different genes (1, 2). In both muscle types, DHPRs serve dual functions (2–6) as voltage-gated, L-type Ca\(^{2+}\) channels and as a trigger for excitation-contraction (EC) coupling, which controls the release of Ca\(^{2+}\) through ryanodine receptors (RyRs) (7) in the sarcoplasmic reticulum. However, the mechanism of EC coupling is different in cardiac and skeletal muscle. In cardiac muscle, depolarization causes opening of L-type Ca\(^{2+}\) channels, and the resulting entry of extracellular Ca\(^{2+}\) triggers RyRs to release Ca\(^{2+}\) (6). In skeletal muscle, EC coupling does not require Ca\(^{2+}\) entry (8); rather, depolarization causes some other kind of signal to be transmitted from DHPRs to RyRs. To identify regions that are critical for this skeletal-type EC coupling, we have previously used the approach of constructing cDNAs encoding chimeras of the skeletal and cardiac DHPRs (3). Expression of these chimeric DHPRs in dysgenic myotubes, which lack a functional gene for the skeletal DHPR (9), revealed that the putative cytoplasmic region between repeats II and III (II-III loop, amino acids 666–791) of the skeletal DHPR is an important determinant of skeletal-type EC coupling (3).

Comparison of the skeletal and cardiac DHPRs reveals differences scattered throughout the II-III loops. One important difference is the presence of a site (Ser\(^{687}\)) in the skeletal II-III loop, which is phosphorylated by cyclic AMP-dependent protein kinase (PKA) (1, 10) and is lacking in the cardiac loop (2). Another important difference is the presence of a 12-amino acid insertion in the N-terminal half of the cardiac II-III loop. To determine the importance of these and other amino acid differences for skeletal-type EC coupling, we have now examined a skeletal DHPR in which Ser\(^{687}\) was mutated to alanine and also analyzed chimeric DHPRs in which skeletal sequence was substituted for successively smaller portions of the II-III loop of the cardiac DHPR. We found that removal of the serine phosphorylation site did not alter the function of the skeletal DHPR and that a small region slightly toward the C-terminal half from the midpoint of the II-III loop was critical for the ability of chimeric DHPRs to mediate skeletal-type EC coupling. The results are discussed in light of work from other laboratories in which the function of isolated RyRs was assayed during exposure to peptide fragments corresponding to various regions of the skeletal and cardiac DHPRs (11–15).

EXPERIMENTAL PROCEDURES

Chimeric DHPRs—In the descriptions below, Sk and Ca indicate the sequence derived from the skeletal muscle (1) and cardiac (2) DHPRs, respectively. The chimeric DHPRs all have an amino acid sequence of Cal-X\(_1\)-Sk(X\(_2\)-X\(_3\)-Sk) (X\(_4\)-2171), which is abbreviated X\(_1\)X\(_2\)X\(_3\)X\(_4\) in the following list: CSk3(3), 787/666–791/923; CSk31, 787/666–709/830; CSk32, 800/711–791/923; CSk33, 830/711–765/897; CsK34, 903/773–791/923; CSk39, 830/711–732/864; CSk40, 864/734–765/897; CSK53, 850/720–765/897; CSk54, 855/725–791/923; CSK56, 830/711–742/874; CSk58, 855/725–742/874 (if a region flanking the Ca/Sk joining site is identical in the cardiac and skeletal DHPRs, the amino acid numbers given are those for the cardiac sequence).

The expression plasmids encoding the chimeric DHPRs were constructed using PCR-amplified fragments, already existing restriction

1 The abbreviations used are: DHPR, dihydropyridine receptor; EC, excitation-contraction; II-III loop, putative cytoplasmic region between repeats II and III of the DHPR; RyR, ryanodine receptor; PCR, polymerase chain reaction; PKA, cyclic AMP-dependent protein kinase; pF, picofarads.
sites, and new restriction sites introduced by site-directed mutagenesis (16) and PCR; the nucleotide changes used to introduce the new restriction sites were selected so as not to alter the amino acid sequence present in the cDNA inserts of the expression plasmids pCAC6 (4) and pCARD1 (2, 17), which were used as the starting materials. All fragments amplified by PCR were confirmed by sequencing. In the following constructions, an asterisk and the letter "p" are used to designate nucleotides within restriction sites introduced by site-directed mutagenesis and PCR, respectively (nucleotide substitutions are given in brackets): pCSk31, XmnI-Styl (Sk 1964–2127) from pCAD1 and StyI-XmnI (Ca 2348–2330) from pCARD1; pCSk32, StyI-HindII (Sk 2127–2389) from pCAD6 and HindII-Styl (Ca 2792–2487) from pCARD1; pCSk33, StyI-AfII (Sk 2127–2297* (C2298T, A2301G)) from pCSk32 and AfII-Styl (Ca 2690–2487) from pCARD1; pCSk34, AfII-HindII (Sk 2297* (C2298T, A2301G)–2389) from pCAD6 and HindII-AfII (Ca 2782–2690) from pCARD1; pCSk39, StyI-BamHI (Sk 2127–2200* (C2202T)) from pCAD6 and BamHI-Styl (Ca 2593* (A2593G, G2594A)–2487) from pCARD1; pCSk40, BamHI-AfII (Ca 2200* (C2202T)–2297* (C2298T, A2301G)) from pCAD6 and AfII-BamHI (Ca 2690–2593* (A2593G, G2594A)) from pCARD1; pCSk53, EcoRI-HindIII (Ca 2215–2459p [A2551C, C2553T]) fragment from pCARD1, HindIII-AfII (Sk 2156p (C2160T)–2297* (C2298T, A2301G)) from pCSk33, and AfII-EcoRI (Ca 2690–2215) from pCAD6, pCSk54, EcoRI-BstBI (Ca 2215–2256p (C2256T, C2256G, G2571A)) from pCARD1 and BstBI-EcoRI (Sk 2256–2297) from pCSk32; pCSk55, BstBI-AfII (Ca 2927–2389) from pCSk33 and Ca 2690–2690 from pCARD1, which were joined by PCR and AfII-BstBI (Ca 2690–Sk 2175) from pCSk33; pCSk58, BstBI-ClaI (Sk 2175–Ca 258) from pCSk56 and ClaI-BstBI (Ca 258–Sk 2175) from pCSk54. To construct pCAD6-S687A867A, a mutation (T2059G) was introduced into pCAD6 by PCR.

Functional Analysis of Chimeric DHPRs—Primary cultures of dysgenic myotubes were prepared and injected with plasmid DNA as described previously (4). Cells expressing the injected plasmid were identified by confraction in response to extracellular stimulation (4). The whole-cell patch clamp technique (18) was used in combination with a photomultiplier system to allow the simultaneous measurement of membrane currents and Ca2+ transients 1–4 days after plasmid injection. The patch pipette contained (mM): 140 Cs-aspartate, 5 MgCl2, 10 Ca2+EGTA (20 mM free Ca2+) and La3+, 0.2 pentapotassium-Flu-3 (Molecular Probes, Eugene, OR), and 10 HEPES (pH 7.4 with 1 M KCl and 150 mM choline). The bath solution contained (mM): 145 tetraethylammonium+, 165 Cl−, 10 HEPES (pH 7.4 with CsOH), 0.003 tetrodotoxin, and either 10 Ca2+ (to support Ca2+ current) or 2 Ca2+, 8 Mg2+, 0.5 Cd2+, and 0.3 La3+ (to block Ca2+ current). The voltage clamp command sequence was step from the holding potential (~80 mV) to ~50 mV for 25–30 ms, to the test potential for 15 ms, to ~50 mV for 25–30 ms, and back to the holding potential. The parameters Gm, Vm, Vh, and k* were determined from the slow calcium current as described previously (19). Because there was large variability in magnitude of the calcium transients, the transients were not analyzed quantitatively, except to determine the "strength" of skeletal-type EC coupling, which was defined for each chimeric as the ratio of the number of cells with a measurable Ca2+ transient in Ca2+/La3+ solution to the number of cells with a transient in 10 mM Ca2+-containing solution. Additionally, traces selected for illustration are representative in terms of time course but not necessarily in terms of magnitude. Temperature was 20–22°C.

RESULTS

Fig. 1 schematically illustrates the skeletal muscle and cardiac DHPRs, designated CAC6 (4) and CARD1 (2, 17), respectively, and the chimeric DHPR, CSk3 (3), which has a skeletal II-III loop but otherwise has cardiac sequence. The skeletal II-III loop is 138 amino acids in length, whereas the cardiac II-III loop is 147 amino acids in length (1, 2). In the skeletal muscle II-III loop, Ser2297 is phosphorylated in vitro by PKA (10); alanine occurs at the corresponding position (residue 609) of the cardiac II-III loop. To test for the importance of this PKA site, we constructed a mutant skeletal DHPR (S687A) in which Ser2297 was replaced by an alanine.

Intracellular Ca2+ transients in response to depolarization were recorded in dysgenic myotubes expressing CAC6, S687A, CARD1, and CSk3 with the cells bathed first in a solution containing 10 mM Ca2+ (Fig. 1, center column) and then in a solution containing Ca2+ plus La3+ to block the entry of extracellular Ca2+ (Fig. 1, right-hand column). Blocking the entry of extracellular Ca2+ abolished the intracellular Ca2+ transient for constructs mediating cardiac-type EC coupling (CARD1 trace). For constructs mediating skeletal-type EC coupling (e.g., CAC6), the Ca2+ transient was still present (although decreased in amplitude) after Ca2+ entry had been blocked by the addition of Cd2+ and La3+. The decreased amplitude of the Ca2+ transient (i.e. ∆F/Φ) has been previously described for normal myotubes exposed to inorganic Ca2+ channel blockers (20). It probably results from deleterious effects of Cd2+ and La3+, which cause an essentially irreversible increase in resting fluorescence (Φ).

In agreement with previous results (3), skeletal-type EC coupling was observed in dysgenic myotubes expressing the chimera CSk3, in which the II-III loop of the cardiac DHPR was replaced with the corresponding skeletal sequence (Fig. 1). The presence of a PKA phosphorylation site did not appear to be critical, because S687A was able to mediate skeletal-type EC coupling (all 7 myotubes tested). Moreover, the magnitude and voltage dependence of the slow Ca2+ current for S687A (Gmax = 54.4 ± 44.6 nanosiemens/nanofarad; Φ1/2 = 27.6 ± 9.5 mV; k′ = 7.9 ± 2.2 mV; n = 6) were similar to those of the slow Ca2+ current in dysgenic myotubes expressing CAC6 (19).

To localize the region of the II-III loop most important for skeletal-type EC coupling, we constructed new chimeric DHPRs, using CSk3 as the parent structure. These chimeras had cardiac sequence except for a small amount of skeletal sequence within the II-III loop. Schematics of the II-III loops of the chimeras and representative Ca2+ transients observed after expression in dysgenic myotubes are illustrated in Fig. 2. The initial chimeras examined were CSk31 and CSk32, in which either the N- or C-terminal half, respectively, of the II-III loop of CSk3 was replaced with the corresponding cardiac sequence. CSk32, but not CSk31, produced a Ca2+ transient after block of the Ca2+ current by Cd2+ and La3+ (Fig. 2), indicating the
importance of the C-terminal half of the II-III loop. Thus, we next examined CSk33 and CSk34, in which complementary regions of the skeletal portion of CSk32 were converted to cardiac sequence. CSk33 supported skeletal-type EC coupling, whereas CSk34 did not. Thus, the middle portion of the II-III loop, indicated by the vertical dashed lines in Fig. 2, is important for skeletal-type EC coupling. Successively larger segments of the N-terminal half of this region were converted to cardiac sequence in the constructs CSk53, CSk54, and CSk40 and of the C-terminal half in the constructs CSk56 and CSk39. Of the constructs with less skeletal sequence at the N-terminal half, CSk53 and CSk54 produced skeletal-type EC coupling, but CSk40 did not. Of the constructs with less skeletal sequence at the C-terminal half, CSk56 produced skeletal-type coupling but CSk39 did not. Because there appeared to be insufficient skeletal sequence in CSk39 or CSk40 to support skeletal-type coupling, it suggested that the region of overlap between the next two larger constructs mediating skeletal-type EC coupling (CSk54 and CSk56) might be essential. Thus, we made CSk58, which has skeletal muscle sequence corresponding to this overlap region (indicated by vertical dotted lines in Fig. 2 (skeletal residues 725–742)). CSk58 was able to mediate skeletal-type EC coupling, but the coupling was weak in that a detectable Ca\(^{2+}\) transient was present after block of Ca\(^{2+}\) current in only 3 of 12 cells expressing CSk58 compared with 8 of 8 cells expressing CSk33 (see legend to Fig. 2).

**DISCUSSION**

In this study, we identified a small region (CSk58, skeletal residues 725–742) of the DHPR II-III loop that is critical for skeletal-type EC coupling. This region is located a little toward the C-terminal half from the middle of the II-III loop and includes 5 conservative and 7 non-conservative amino acid changes from the corresponding portion of the cardiac loop (Fig. 3). Chimeras that included only the N-terminal half (CSk39) or C-terminal half (CSk40) of the CSk58 region were unable to mediate skeletal-type EC coupling. Although an intact CSk58 region appeared to be necessary for skeletal-type EC coupling, this coupling was considerably weaker than that for chimeras containing additional skeletal residues on either side of the CSk58 region. Ser\(^{645}\) in the skeletal II-III loop, which is phosphorylated by PKA, is not included in the CSk58 region, and a purely skeletal DHPR with the mutation S687A retained the ability to mediate skeletal-type EC coupling (Fig. 1). The cardiac II-III loop contains an inserted segment of 12 amino acids (cardiac residues 831–842) corresponding to a site between skeletal residues 710 and 711 (Fig. 3). The presence of this insertion in CSk53, CSk54, and CSk58 did not prevent them from mediating skeletal-type EC coupling (Fig. 2).

A simple way to account for our results would be to postulate that skeletal-type EC coupling involves a direct interaction between the RyR and the II-III loop of the DHPR, with loop residues 725–742 being especially important. For example, these amino acids might constitute an “agonist,” which assumes the right configuration to bind to (and activate) the RyR only when the sarcolemma is depolarized. Unfortunately, the experiments with chimeric DHPRs do not provide a direct test of this hypothesis. An approach that has been used by other investigators to probe interactions between DHPRs and RyRs is to create peptides corresponding to various parts of the DHPR sequence and then to test for effects on RyRs by applying the peptides either to triad/SR vesicular preparations (activation monitored as an increase in \(^{3}H\)|ryanodine binding or Ca\(^{2+}\) efflux) or to RyRs reconstituted in artificial planar bilayer...
ers (activation monitored by an increase in open probability). As discussed below, it is difficult to construct a consistent framework that can account for both the results of application of the constructed peptides and our results on expression of DHPR cDNAs in dysgenic myotubes.

Lu et al. (11) found that [3H]ryanodine binding to skeletal SR vesicles is increased by both a skeletal and a cardiac II-III loop peptide (SDCL and CDCL, respectively). This result seems difficult to reconcile with the observation that CSk3 (the chimera with a skeletal II-III loop) restores skeletal-type EC coupling whereas CARD1 (purely cardiac DHPR) does not (Fig. 1 (3, 4, 17). Lu et al. (12) subsequently found that phosphorylated SDCL specifically binds to, but does not activate, the skeletal RyR and that mutation of Ser687 to alanine abolishes the ability of the SDCL to activate the skeletal RyR. This latter observation contrasts with our finding that both wild-type and S687A mutant skeletal DHPRs are able to mediate skeletal-type EC coupling (Fig. 1). More recently, Leong and MacLennan (13) found that a 37-amino acid segment of the skeletal RyR binds to the skeletal II-III loop and that this binding is mostly lost when Lys877 and Lys882 of the skeletal II-III loop are mutated to glutamates, the corresponding cardiac residues. This contrasts with our observation that the skeletal versus cardiac identity of this portion of the II-III loop had no effect on the ability to mediate skeletal-type EC coupling.

To localize regions that might be critical for EC coupling, Lu et al. (12) divided the II-III loop into three smaller peptides, termed F1 (Glu668–Glu726), F2 (Pro708–Leu766), and F3 (Lys735–Leu791). The peptide F1, which represents only about the N-terminal half of the SDCL (Fig. 3), was found both to increase [3H]ryanodine binding to the skeletal RyR and to displace binding of the phosphorylated SDCL, whereas peptides F2 and F3 caused little or no increase in [3H]ryanodine binding and did not detectably displace the binding of the phosphorylated SDCL (12). Using somewhat smaller peptides corresponding to four distinct regions, El-Hayek et al. (14) also found that an N-terminal portion of the II-III loop causes activation of the RyR. Specifically, they found that “peptide A” (Thr677–Leu690; see Fig. 3) caused Ca2+ efflux from, and enhanced [3H]ryanodine binding to, triad-enriched microsomes of skeletal muscle. Peptides corresponding to portions of the II-III loop closer to the C-terminal half, including a “peptide C1” (Phe725–Gly743), which approximately overlaps the skeletal sequence of our CSk58 chimera (see Fig. 3), did not increase [3H]ryanodine binding or Ca2+ efflux. Although not causing activation, “peptide C” (Glu754–Pro760), which roughly overlaps the skeletal sequence in our CSk53 chimera, moderately antagonized the actions of peptide A. Thus, it may be that two portions of the DHPR II-III loop correspond to the A and C peptide regions of the skeletal RyR and that this binding is mediated by a region closer to the C-terminus, which was shown not to affect whether chimeric DHPRs could mediate skeletal-type EC coupling (3).

Just as there are important limitations on experiments with chimeric DHPRs (i.e. the inability to test for direct interactions between the DHPR and RyR), there are also significant weaknesses in the experiments with isolated peptides. For example, the isolated peptides may assume conformations different from those of the corresponding regions of the DHPR in living cells. Equally or more important, the isolated peptides may act at sites on the RyR that are different from those actually involved in EC coupling. By expressing cDNAs encoding engineered RyRs in dysgenic myotubes in which the RyR-1 gene is disrupted (21, 22), it may be possible to identify regions of the RyR critical for skeletal-type EC coupling. In turn, this will make it possible to determine whether one or more of the isolated peptides acts at sites coincident with those identified by the expression of RyR cDNAs. Thus, progress in our understanding of EC coupling is likely to continue benefiting from analysis of DHPR/RyR interactions using both isolated proteins and expression of cDNAs in dysgenic and dyspedic myotubes.

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