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## Regions of the skeletal muscle dihydropyridine receptor critical for excitation–contraction coupling

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It is thought that in skeletal muscle excitation–contraction (EC) coupling, the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum is controlled by the dihydropyridine (DHP) receptor in the transverse tubular membrane, where it serves as the voltage sensor<sup>1–3</sup>. We have shown previously<sup>4</sup> that injection of an expression plasmid carrying the skeletal muscle DHP receptor complementary DNA<sup>3</sup> restores EC coupling and L-type calcium current that are missing in skeletal muscle myotubes from mutant mice with muscular dysgenesis<sup>5–9</sup>. This restored coupling resembles normal skeletal muscle EC coupling<sup>4</sup>, which does not require entry of extracellular  $\text{Ca}^{2+}$  (refs 10, 11). By contrast, injection into dysgenic myotubes of an expression plasmid carrying the cardiac DHP receptor cDNA<sup>12</sup> produces L-type calcium current and cardiac-type EC coupling<sup>13</sup>, which does require entry of extracellular  $\text{Ca}^{2+}$  (refs 14–16). To identify the regions responsible for this important functional difference between the two structurally similar DHP receptors, we have expressed various chimaeric DHP receptor cDNAs in dysgenic myotubes. The results obtained indicate that the putative cytoplasmic region between repeats II and III of the skeletal muscle DHP receptor<sup>3</sup> is an important determinant of skeletal-type EC coupling.

The main differences in primary structure between the skeletal muscle<sup>3</sup> and cardiac<sup>12</sup> DHP receptors reside in the large, putative cytoplasmic regions, that is, the amino- and carboxy-terminal regions as well as the regions linking repeats I and II (I–II loop) and repeats II and III (II–III loop). To test whether these regions of the skeletal muscle DHP receptor are required for skeletal-type EC coupling, we constructed five different expression plasmids carrying chimaeric DHP receptor cDNAs (Fig. 1). These constructs encode the cardiac DHP receptor as the basic structure in which the large, putative cytoplasmic regions are replaced by the corresponding regions of the skeletal muscle DHP receptor. The cardiac DHP receptor was chosen as the starting structure because the L-type calcium current expressed in myotubes injected with the entirely cardiac DHP receptor cDNA (pCARD1) is about sixfold larger than that expressed in myotubes injected with the entirely skeletal muscle DHP receptor cDNA (pCAC6)<sup>13</sup>. A large L-type current is helpful in identifying cells expressing a chimaeric DHP receptor that fails to mediate skeletal-type EC coupling.

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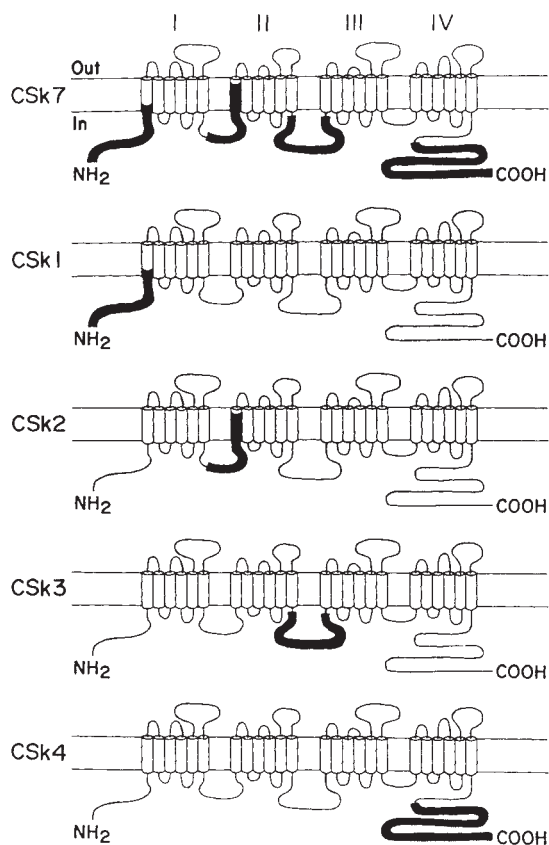


FIG. 1 Schematic representation of the structures of chimaeric DHP receptors (CSk7 and CSk1–4 from top to bottom) composed of cardiac and skeletal muscle sequences. For each chimaeric DHP receptor, the four units of homology (repeats I–IV) are displayed linearly and the six putative transmembrane segments (S1 to S6 from left to right) in each repeat are shown by cylinders. The darkly shaded areas indicate the regions of the cardiac DHP receptor that have been replaced by the corresponding portions of the skeletal muscle DHP receptor. Note that the junctional sequences common to the two DHP receptors are not darkly shaded and that the exchanged portion of segment S1 of repeat II is very similar in amino acid sequence between the two DHP receptors (see below). The compositions of the individual chimaeric DHP receptors are as follows (C and Sk, cardiac and skeletal muscle DHP receptor, respectively; numbers in parentheses, amino-acid numbers<sup>3,12</sup>; the junctional sequences common to the two DHP receptors are represented by amino-acid numbers of the cardiac DHP receptor). CSk7: Sk(1–55), C(159–464), Sk(364–448), C(571–787), Sk(666–791), C(923–1,634) and Sk(1,510–1,873). CSk1: Sk(1–55) and C(159–2,171). CSk2: C(1–464), Sk(364–448) and C(571–2,171). CSk3: C(1–787), Sk(666–791) and C(923–2,171). CSk4: C(1–1,634) and Sk(1,510–1,873).

METHODS. The expression plasmids (pCSk7 and pCSk1–4) carrying the cDNAs encoding the individual chimaeric DHP receptors were constructed by inserting the corresponding cDNAs into the *Hind*III site of the plasmid pKCRH2 (ref. 17). The chimaeric DHP receptor cDNAs are composed of the following restriction fragments derived from the plasmids pCAC6 (ref. 4) and pCARD1 (ref. 12) (C and Sk in parentheses denote the origin of the fragments and the restriction endonuclease sites are identified by numbers<sup>3,12</sup> in parentheses indicating the 5'-terminal nucleotide generated by cleavage). pCSk1: 213-base-pair (bp) *Hind*III (Sk 5'-terminal linker)–*Bst*XI (Sk 196) and 6.4-kilobase-pair (kb) *Bst*XI (C 505)–*Hind*III (C 3'-terminal linker); there is one base difference at the *Bst*XI site between the cardiac and skeletal muscle DHP receptors, which causes no amino-acid difference. pCSk2: 1.6-kb *Hind*III (C 5'-terminal linker)–*Pvu*II (C 1,378), *Pvu*II (Sk 1,075)–*Hgi*AI (Sk 1,359) and 5.2-kb *Hgi*AI (C 1,725)–*Hind*III (C 3'-terminal linker). pCSk3: 2.5-kb *Hind*III (C 5'-terminal linker)–*Xmn*I (C 2,330), *Xmn*I (Sk 1,964)–*Hinc*II (Sk 2,389) and 4.1-kb *Hinc*II (C 2,782)–*Hind*III (C 3'-terminal linker). pCSk4: 5.1-kb *Hind*III (C 5'-terminal linker)–*Bgl*II (C 4,863) and 1.2-kb *Bgl*II (Sk 4,488)–*Hind*III (Sk 3'-terminal linker). pCSk7: 665-bp *Hind*III–*Sac*I from pCSk1, 1.2-kb *Sac*I–*Eco*RI from pCSk2, 1.7-kb *Eco*RI–*Aat*II from pCSk3 and 2.1-kb *Aat*II–*Hind*III from pCSk4.

Dysgenic myotubes injected with each of the five chimaeric plasmids (pCSk7 and pCSk1-4) did indeed display electrically evoked contractions; the number of responsive myotubes relative to the number of tested myotubes was comparable to that reported previously<sup>13</sup> for pCAC6- or pCARD1-injected myotubes. Although not systematically monitored, spontaneous contractions were also observed in some myotubes injected with each of the chimaeric plasmids. Myotubes that had been injected with a chimaeric plasmid and observed to contract all showed L-type calcium current (Fig. 2c-g). The L-type currents induced by the chimaeric plasmids displayed a rapid rate of activation, which was very similar to that in pCARD1-injected myotubes (Fig. 2b) and much faster than that in pCAC6-injected myotubes (Fig. 2a). Two additional similarities were found between the calcium currents produced by the chimaeric plasmids and by pCARD1, as exemplified by a pCSk3-injected myotube (Fig. 3). First, substitution of Ba<sup>2+</sup> for Ca<sup>2+</sup> caused an increase in peak current that was comparable to the response of L-type current in myotubes expressing pCARD1 but larger than that in myotubes expressing pCAC6 (ref. 13). Second, with Ca<sup>2+</sup> as the charge carrier, the potential that activated peak L-type current in myotubes expressing the chimaeric cDNAs or pCARD1 (ref. 13) was 20–40 mV more negative than that in myotubes expressing pCAC6 (refs 4, 13). Thus, properties characteristic of the cardiac L-type channel are maintained even when its major putative cytoplasmic regions are replaced by their skeletal muscle counterparts.

Examination of electrically evoked contractions demonstrated that dysgenic myotubes expressing pCAC6, as reported previously<sup>4,13</sup>, displayed skeletal-type EC coupling. Thus, for pCAC6-expressing myotubes, contraction was observed both in Ca<sup>2+</sup>-free Ringer's solution (Fig. 4a, 2) and in 0.5 mM Cd<sup>2+</sup>-containing Ringer's solution (Fig. 4a, 3) as well as in normal Ringer's (Fig. 4a, 1). In contrast, myotubes expressing

pCARD1 showed cardiac-type EC coupling (Fig. 4b; see also ref. 13). Dysgenic myotubes expressing pCSk7, which encodes the chimaeric DHP receptor with all the major putative cytoplasmic regions replaced by their skeletal muscle counterparts, exhibited EC coupling (Fig. 4c) similar to that produced by pCAC6. Thus, switching these regions of the DHP receptor from cardiac to skeletal muscle changed the character of EC coupling from cardiac-type (Fig. 4b) to skeletal-type (Fig. 4a).

We next examined whether replacement of a single putative cytoplasmic region is sufficient to produce this change. Dysgenic myotubes expressing either pCSk1, which encodes the chimaera with only the amino-terminal region replaced (Fig. 4d), or pCSk4, which encodes the chimaera with only the carboxy-terminal region replaced (Fig. 4g), displayed cardiac-type EC coupling. In contrast, myotubes expressing pCSk3, which encodes the chimaera with only the II-III loop replaced, showed skeletal-type EC coupling (Fig. 4f). Altogether, 42 myotubes expressing pCSk3, as well as 20 expressing pCSk7 and 25 expressing pCAC6, were tested and all were found to display skeletal-type EC coupling. That myotubes expressing pCSk3 or pCSk7 exhibited weaker contraction in Ca<sup>2+</sup>-free or Cd<sup>2+</sup>-containing Ringer's than in normal Ringer's (Fig. 4c, f) can be accounted for by entry of extracellular Ca<sup>2+</sup> contributing to the contraction of these myotubes in normal Ringer's. When tested under identical conditions (extracellularly applied stimulus with a duration of 5 ms), myotubes expressing pCSk2, which encodes the chimaera with only the I-II loop replaced, showed cardiac-type EC coupling. When the stimulus duration was increased to 20 ms, however, weak contractions were observed in some myotubes expressing pCSk2 both in Ca<sup>2+</sup>-free (9 of 23 tested) and in Cd<sup>2+</sup>-containing (14 of 20 tested) Ringer's. These weak contractions, barely resolvable by the contraction-monitoring apparatus, are evident as a brief, small, upward deflection at the beginning of the trace (Fig. 4e, 2 and 3). Even

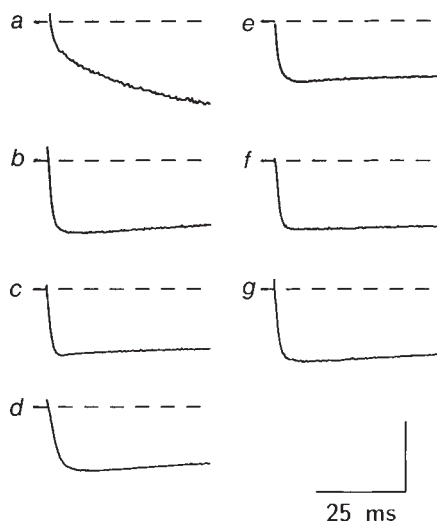


FIG. 2 Comparison of calcium currents in dysgenic myotubes expressing pCAC6 (a), pCARD1 (b), pCSk7 (c), pCSk1 (d), pCSk2 (e), pCSk3 (f) or pCSk4 (g). Calcium currents were measured using the whole-cell variant of the patch-clamp technique<sup>18</sup>. For all the currents illustrated, the holding potential was  $-80$  mV and the test potential was  $+30$  mV. Vertical calibration corresponds to 1.5 nA (a), 15 nA (b) or 10 nA (c-g). The experimental procedures used were essentially the same as described previously<sup>4,13</sup>. The linear cell capacitance ( $C$ ) for each cell was as follows: a, Cell AJ01,  $C=500$  pF; b, Cell CA37,  $C=450$  pF; c, Cell CA87,  $C=335$  pF; d, Cell CB11,  $C=490$  pF; e, Cell CA91,  $C=565$  pF; f, Cell CA33,  $C=250$  pF; g, Cell CA78,  $C=560$  pF. The pipette solution contained: 140 mM caesium-aspartate, 5 mM MgCl<sub>2</sub>, 10 mM caesium-EGTA, 10 mM HEPES buffer (pH 7.4 with CsOH). The bathing solution contained: 10 mM Ca<sup>2+</sup>, 145 mM tetraethylammonium ion, 165 mM Cl<sup>-</sup>, 0.003 mM tetrodotoxin, 10 mM HEPES buffer (pH 7.4 with CsOH). Temperature 20–22 °C.

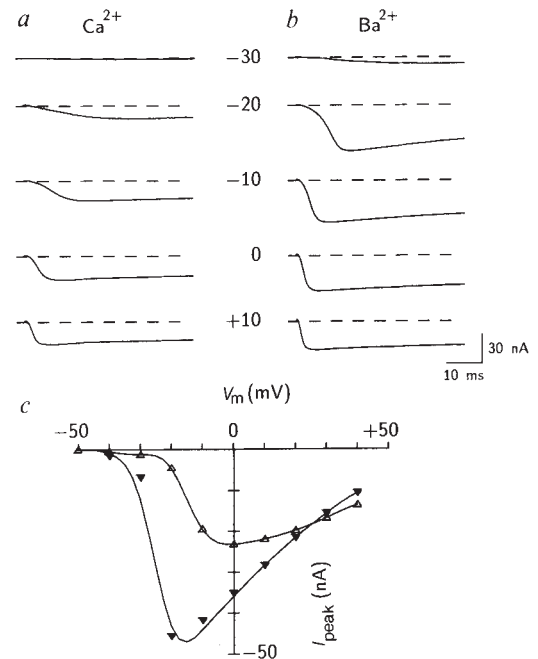


FIG. 3 L-type currents carried by Ca<sup>2+</sup> and Ba<sup>2+</sup> in a dysgenic myotube expressing pCSk3. a, b, Currents evoked by stepping from the holding potential ( $-80$  mV) to the test potential  $V_m$  (in mV) indicated next to each trace, with either 10 mM Ca<sup>2+</sup> (a) or 10 mM Ba<sup>2+</sup> (b) as the charge carrier. c, Peak current ( $I_{peak}$ ) for these records plotted as a function of test potential (open symbols, Ca<sup>2+</sup>; closed symbols, Ba<sup>2+</sup>). The composition of the bathing solution containing 10 mM Ca<sup>2+</sup> is described in Fig. 2 legend. The bathing solution containing 10 mM Ba<sup>2+</sup> was identical, except for equimolar substitution of Ba<sup>2+</sup> for Ca<sup>2+</sup>. The data are from cell CA35,  $C=780$  pF.

with the longer stimulus of 20-ms duration, myotubes expressing pCsk1 ( $n=15$ ), pCsk4 ( $n=13$ ) or pCARD1 ( $n=15$ ) never showed contractions in  $\text{Ca}^{2+}$ -free or  $\text{Cd}^{2+}$ -containing Ringer's.

In addition to using extracellular stimulation, we also examined contraction using the whole-cell patch-clamp technique, where only 0.1 mM EGTA was present in the solution filling the patch-pipette<sup>13</sup>. In  $\text{Ca}^{2+}$ -free, 10 mM  $\text{Mg}^{2+}$ -containing bathing solution, stepping from the holding potential (-80 mV) to +10 mV for 20 ms caused contraction of all the tested myotubes expressing pCsk3 ( $n=9$ ) or pCAC6 ( $n=3$ ). Under identical test conditions, three of 12 myotubes expressing pCsk2 contracted, but the other nine myotubes did not contract even when the duration of the stimulus was increased to 400 ms. Myotubes expressing pCsk1 ( $n=7$ ), pCsk4 ( $n=3$ ) or pCARD1 ( $n=9$ ) did not contract in the  $\text{Ca}^{2+}$ -free test solution even with a 400-ms stimulus. In conclusion, pCsk3 produces effective skeletal-type EC coupling. pCsk2 is also able to produce skeletal-type EC coupling, although the efficiency is so low that the amount of  $\text{Ca}^{2+}$  released from the sarcoplasmic reticulum exceeds the threshold for contraction in only a fraction of cells.

Our results indicate that the II-III loop of the skeletal muscle DHP receptor is a major determinant site for skeletal-type EC coupling, that the I-II loop is possibly less important and that the amino- and carboxy-terminal regions are probably unimpor-

tant. Alternatively, the weaker skeletal-type EC coupling observed with pCsk2 than with pCsk3 may mean that the I-II and the II-III loop are both critical, but that the I-II loop is more functionally interchangeable between the cardiac and skeletal muscle DHP receptors than is the II-III loop. Similarly, it is possible that the putative cytoplasmic region linking repeats III and IV, which is highly conserved between the two DHP receptors, is also important in skeletal-type EC coupling. The finding that expression of pCsk3 and pCsk7 produces skeletal-type EC coupling but rapidly activating, cardiac-type calcium current shows that the slow activation of the skeletal muscle L-type channel is not an obligatory consequence of the DHP receptor serving as the voltage sensor for EC coupling. This finding also suggests that the putative membrane-spanning and adjacent regions of the DHP receptor, forming the channel and its entrances, are important in determining the properties of L-type calcium current. □

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## Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs

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**THE skeletal muscle dihydropyridine (DHP) receptor is essential in excitation-contraction (EC) coupling<sup>1-4</sup>. The receptor is postulated to be the voltage sensor giving rise to the intramembrane current, termed charge movement<sup>5</sup>. We have now tested this hypothesis using myotubes from mice with the muscular dysgenesis mutation, which alters the skeletal muscle DHP receptor gene and prevents its expression<sup>3,4,6</sup>. Our results indicate that charge movement is deficient in dysgenic myotubes but is fully restored following injection of an expression plasmid carrying the rabbit skeletal muscle DHP receptor complementary DNA, strongly supporting the hypothesis that the DHP receptor is the voltage sensor for EC coupling in skeletal muscle. Additionally, our data obtained for normal and chimaeric DHP receptor constructs demonstrate that DHP receptors with widely differing abilities to function as calcium**

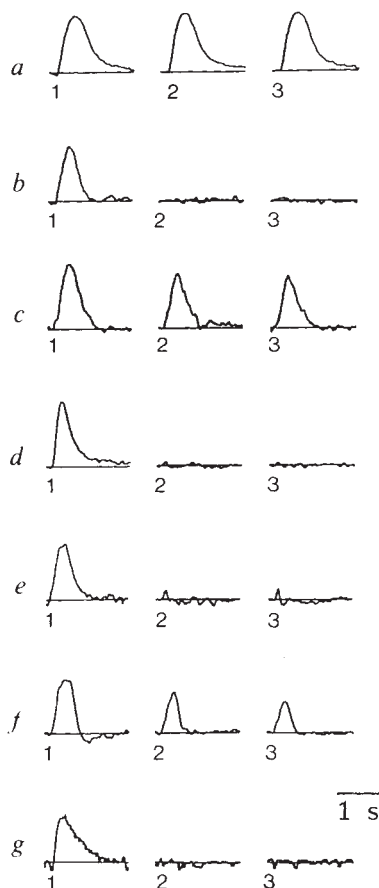


FIG. 4 Comparison of electrically evoked contractions in dysgenic myotubes expressing pCAC6 (a), pCARD1 (b), pCsk7 (c), pCsk1 (d), pCsk2 (e), pCsk3 (f) or pCsk4 (g). Contractions were recorded initially in normal rodent Ringer's (1) and subsequently in a test solution that was either  $\text{Ca}^{2+}$ -free (2) or 0.5 mM  $\text{Cd}^{2+}$ -containing (3) Ringer's. A period in normal Ringer's sufficient for full recovery was interspersed between exposures to the two test solutions. The methods for extracellular electrical stimulation and optical recording of contraction were as described previously<sup>4,13</sup>. The stimulus duration was 5 ms for all the traces, except 20 ms for e, 2 and e, 3. The normal rodent Ringer's contained: 146 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES buffer (pH 7.4 with NaOH). The  $\text{Ca}^{2+}$ -free Ringer's was made by equimolar substitution of  $\text{Mg}^{2+}$  for  $\text{Ca}^{2+}$  in the normal rodent Ringer's. Horizontal calibration, 1 s. Vertical scale, arbitrary units.