Functional Equivalence of Dihydropyridine Receptor α1S and β1a Subunits in Excitation-Contraction Coupling in Skeletal Muscle

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Abstract

Molecular understanding of the mechanism of excitation-contraction (EC) coupling in skeletal muscle has been made possible by cultured myotube models lacking specific dihydropyridine receptor (DHPR) subunits and ryanodine receptor type 1 (RyR1) isoforms. Transient expression of missing cDNAs in mutant myotubes leads to a rapid recovery, within days, of various Ca^{2+} current and EC coupling phenotypes. These myotube models have thus permitted structure-function analysis of EC coupling domains present in the DHPR controlling the opening of RyR1. The purpose of this brief review is to highlight advances made by this laboratory towards understanding the contribution of domains present in the DHPR β1a subunit to EC coupling signaling. The evidence indicates that at least two subunits in the DHPR, namely α1S and β1a, control the signal transmitted to RyR1 during EC coupling.

Key words: b1 KO myotubes, Ca^{2+} transients, cDNA expression, confocal imaging, excitation-contraction coupling, L-type Ca^{2+} channel, skeletal muscle.

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EC coupling is a fast process in which a single action potential induces a massive increase in cyotosolic Ca^{2+} resulting in the mechanical activation of the muscle cell. Events are controlled by two key components, namely the voltage-gated L-type Ca^{2+} channel formed by the DHPR, and the SR Ca^{2+} release channel formed by RyR1 [13]. The well-established paradigmis that in response to depolarization, the DHPR produces a signal that briefly opens the RyR1 channel leading to the release of SR-stored Ca^{2+} [51]. EC coupling take place at specialized junctions between transverse tubules (T-tubules) and SR membranes. At these junctions, 4 DHPRs in tetrad arrangement, interact with a single tetrameric RyR1 channel [26]. The close physical proximity of the DHPR and RyR1 channels suggests that interactions between the two complexes are extensive. One manifestation of this is the fact that signals exchanged by the DHPR and RyR1 are bi-directional and have functional consequences for both channels. In the DHPR to RyR1 (orthograde) direction, tetrads of DHPRs lined-up with the foot structure of RyR1 function as surrogate "gating particles" which, after acquiring the correct conformation, open the RyR1 channel [52]. In the RyR1 to DHPR (retrograde) direction, the RyR1 behaves as a surrogate "DHPR subunit" essential for gating transitions that open the L-type Ca^{2+} channel [7, 39].

In molecular terms, the skeletal DHPR is a ~430 kDa heteropentamer composed of α1S, β1a, α2-δ1, and γ1 subunits. The DHPR α1 subunit belongs to the superfamily of voltage-gated channel proteins with four internal repeats. Electrical charges in the S4 segments in each repeat move outward in response to membrane depolarization, and this movement is coupled to the opening of both the L-type and RyR1 channels. β subunits are ~65 kDa proteins that bind strongly and to the cytosolic loop between repeats I and II of α1 via a conserved ~30-residue β interaction domain or BID [20, 48]; Fig. 1). β subunits modulate the kinetics of activation and inactivation of the Ca^{2+} current (Berrou et al., 2001, [50, 64]) and strengthen the coupling between S4 charge movements and pore opening [32, 41, 42]. The molecular structure and function of the α2-δ1, and γ1 subunits of the skeletal DHPR is far less defined [1, 6, 30]. The hypothesis that the DHPR and RyR1 complexes are mechanically coupled has gained support for more than a decade, and has been extensively reviewed [19, 25, 37, 38, 53, 60]. Protein-protein interactions between the pore subunit of the DHPR (α1S) and RyR1 are extensive [33, 34, 49, 54]. 3-D reconstructions of skeletal DHPR particles and models of DHPR tetrads suggest that the cytosolic loops of the pore subunit are in intimate contact with the foot structure of RyR1 [57, 65]. The α1S-RyR1 topology is further constrained by

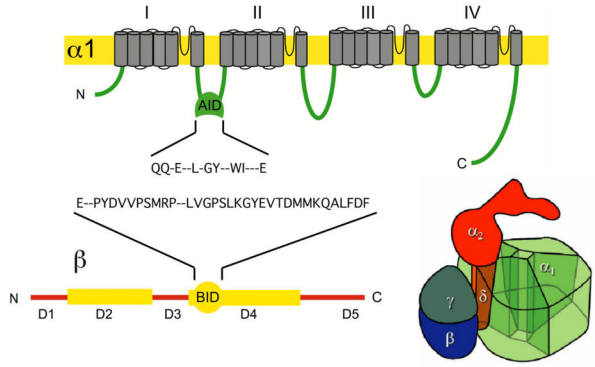

Figure 1. Conserved residues in the alpha interaction domain (AID) of the Ca^{2+} channel α1 pore subunuit and the beta interaction domain (BID) of the associated β subunit critical for α1/β binding and Ca^{2+} current expression (from De Waard et al., 1996). b subunits consist of two separate blocks of conserved residues (yellow) flanked by divergent sequences (red) in the linker region connecting the conserved domains (D3), amino terminus (D1), and carboxyl terminus (D5) regions (see Perez-Reyez and Schneider, 1994 for sequence line-ups). Subunit arrangement of the skeletal DHPR according to cryo electron microscopy shows that the β subunit is located to the side of the pore subunit facing the cytoplasm [65].

FRET measurements which place the N and C termini of α1S facing the inside of the tetrad [44]. Protein-protein interactions between the β1a subunit of the DHPR and RyR1 are also present [14]. 3-D reconstructions of skeletal DHPR particles place the β1a subunit to the side of a 10 nm disc outlined by α1S [65], or tucked under the cytosolic face of α1S [57]. In either case, a physical contact between β1a and RyR1 is almost certain. Studies in dysgenic myotubes lacking a functional α1S protein conducted a decade or more ago ([27, 61]; reviewed below) led to the widespread assumption that the α1S II-III loop may be solely responsible for opening RyR1. This view needs to now be challenged for two fundamental reasons. In a strict sense, the observations in dysgenic myotubes cannot exclude the participation of other DHPR subunits because non-pore subunits of the DHPR are constitutively expressed in the dysgenic myotube [6]. Hence, if EC coupling domains were to be present in other subunits of the skeletal DHPR, they would have escaped detection in the dysgenic myotube by virtue of being constitutively present. Second, the availability of gene knockout (KO) myotubes generated by gene targeting techniques in mice has permitted molecular studies of DHPR β subunits that parallel those of α1 subunits in dysgenic myotubes. What is most surprising about the β subunit studies [5, 8, 9, 10, 55, 56] is that the phenotypes produced by molecular manipulations of β subunits in β1 KO myotybes are strikingly similar to those described earlier by molecular manipulations of α1S subunits in dysgenic myotubes. Such phenotypic parallels (described below) suggest to us that the DHPR β subunit, much like the α1S pore subunit, plays a key role in signal transmission to RyR1.

Dysgenic and β1 KO myotubes as expression platforms for DHPR α1 and β subunits.

Tanabe et al. [8] utilized the α1S-null dysgenic myotube to show that the α1S pore subunit was essential for EC coupling signaling. In the dysgenic myotube, a base deletion leads to truncation and degradation of α1S and loss of voltage-activated Ca^{2+} transients. Transfection of cultured dysgenic myotubes with α1S cDNA leads to de novo synthesis of α1S subunits [2, 4], concentration of the expressed DHPR in EC coupling junctions (Takekura et al., 1994), and recovery of skeletal type EC coupling [27]. This expression system permitted studies in which the missing α1S Ca^{2+} pore isoform was replaced by α1C, the cardiac/brain pore variant [62]. The Ca^{2+} fluorescence vs. voltage curve expressed by α1C in dysgenic myotubes had a maximum at ~+30 mV followed by a continuous decrease at more positive potentials. The biphasic nature of this relationship, along with a dependence of Ca^{2+} transients on external Ca^{2+} , showed that Ca^{2+} dependent EC coupling could be implemented in skeletal myotubes by a hybrid DHPR composed of a cardiac pore subunit and the non-pore subunits expressed endogenously in the dysgenic myotube. The expression of chimeras of α1S and α1C were later used to demonstrate that a domain within the cytosolic loop linking repeats II and III of α1S, the 720-765 region, harbored a unique signal essential for skeletal type EC coupling [40].

Gene targeting techniques have substantially increased the number of myotube models that can be used as expression platforms for EC coupling studies. Gregg et al. [6] described a knockout (KO) of the mouse β1 gene, encoding DHPR β isoforms expressed in skeletal muscle (β1a) and brain (β1b, β1c) [47]. The β1 KO mutation, like the dysgenic mutation, is perinatally lethal due to the absence of EC coupling in the skeletal musculature. β1 KO myotubes fail to contract in response to electrical stimulation despite the presence of normal action potentials, Ca^{2+} storage capacity, and caffeine-sensitive Ca^{2+} release. However, β1 KO cells lack L-type Ca^{2+} current, and depolarization does not produce Ca^{2+} transients [29, 58, 59]. Expression of the skeletal muscle β1a isoform in cultured β1 KO myotubes resulted in the recovery of the wild type L-type Ca^{2+} current density, the intramembrane charge movement density, and the amplitude and voltage dependence of Ca^{2+} transients [8]. In contrast, expression of the cardiac/brain β2a variant recovered L-type Ca^{2+} currents, but the amplitude of depolarization-activated Ca^{2+} transients was drastically depressed. Since the interaction between the DHPR α1 and β subunits is essential for

cell surface expression [12, 17], the loss of EC coupling in the β1 KO myotube could be exclusively due to the loss of DHPR voltage sensors from the cell surface.

Alternatively, domains of the DHPR β1a subunit, similar to elements present in the α1S subunit, might be directly involved in activation of RyR1 channels. To examine the role of DHPR β1a in skeletal-type EC coupling, we chimerized β1a and the cardiac/brain β2a variant and mapped the domain(s) required for functional recovery of skeletal-type EC coupling in cultured β1 KO myotubes [9, 10, 55]. DHPR β subunits share two conserved central regions amounting to more than half of the total peptide sequence (domains D2 and D4), a non-conserved linker between the two conserved domains (D3), a non-conserved amino terminus (D1), and a non-conserved carboxyl terminus (D5) ([46, 53]; Fig. 1). We tested the participation of D1, D3, and D5 of β1a in the recovery of Ca^{2+} conductance, charge movements, and EC coupling in β1 KO skeletal myotubes [10, 55]. Deletion of the D5 region of β1a (470-524) drastically reduced the amplitude of voltage-evoked Ca^{2+} transients without affecting the density of DHPR charge movements. On the basis of this result, we have implicated the D5 region of β1a specifically in skeletal-type EC coupling [55]. The identification of D5 was also important because it showed that domains of β1a that participate in EC coupling differ from the BID domain, present in D4. Hence it is clear that in the β subunit, the trafficking function controlled by the BID domain [12, 17] and the EC coupling function controlled by D5 have entirely different molecular underpinnings.

Skeletal-type excitation-contraction coupling in the absence of molecular determinants identified in the α1S II-III loop

The α1S II-III loop, under the influence of membrane depolarization, has been suggested to trigger the release of Ca^{2+} from the SR [27, 62]. A prediction of this model is that the II-III loop must contain molecular motifs essential for activation of the RyR1 channel. A search for different regions of the II-III loop that affect RyR1 channel activity was conducted using recombinant and synthetic peptides covering the entire II-III loop peptide sequence [15, 22, 23, 31, 35, 43]. The strongest stimulation of RyR1 channels in vitro was produced by a synthetic peptide corresponding to residues α1S 671-690. A second peptide, 724-760, had low stimulatory activity, but inhibited the stimulatory activity of the 671-690 peptide when both synthetic peptides were presented to RyR1-containing skeletal SR vesicles [23]. These biochemical studies suggest that the 671-690 region of the II-III loop could be the structural element of the II-III loop that triggers EC coupling in the muscle cell [24]. A second region of the II-III loop critical for EC coupling was identified in dysgenic myotubes by functional expression of chimeric cDNAs for DHPR pore subunits [40]. This study showed that a chimera consisting of a cardiac α1C subunit with α1S 720-765 replacing the homologous cardiac region expressed voltage-evoked Ca^{2+} transients of near-normal amplitude. The narrower α1S 725-742 region recovered a slightly lower than normal Ca^{2+} transient. Furthermore, the Ca^{2+} transients in each case persisted in the presence of Ca^{2+} channel blockers, indicating recovery of skeletal-type EC coupling by the chimeras. Thus the two screening strategies, namely peptide analysis in vitro [23] and chimera analysis in situ [40], arrived at different conclusions regarding the regions of the II-III loop essential for EC coupling function.

Is the α1S II-III loop necessary and sufficient for triggering EC coupling? This question was addressed by Ahern et al. [3] using a deletion strategy. Residues 671-690 and 720-765 are the only two regions of the II-III loop that have been reported to screen positively for EC coupling-related functions, albeit by different techniques. We thus investigated if voltage-evoked Ca^{2+} transients were expressed when both regions were removed from the II-III loop.

Surprisingly, Δ671-690/Δ720-765 expressed Ca^{2+} transients that increased with depolarization and saturated at a maximum amplitude ~20% of control (Fig. 2). Analysis of the voltage-dependence of the Ca^{2+} transient showed that neither the midpoint potential nor the steepness of the fluorescence vs. voltage relationship was affected by this double deletion. These results provide a clear indication that the skeletal DHPR has a latent EC coupling activity that is unmasked by simultaneous removal of residues 671-690 and 720-765. In molecular terms, the 720-765 region could be a conformation-sensitive region of the II-III loop stabilized by strong residue-residue interactions with other regions of the II-III loop. Thus, the loss of EC coupling produced by removal of residues 720-765 could be the result of a conformation change which is partially reverted by a second conformational change in the II-III loop produced by removal of residues 671-690.

The mechanism by which EC coupling is eliminated by removal of the α1S 720-765 region and recovered by removal of 720-765 and 671-690 at the same time is reminiscent of the electrostatic interactions that hold the voltage sensor of the Shaker K + channel in a functional state [45]. This study showed that some charge neutralization mutations in the S4 transmembrane segment block protein folding. In these cases, channel function can be rescued by a second charge neutralization in S2 or S3. The functional rescue by double mutations suggested that S4 forms a strong network of local electrostatic charges with other transmembrane domains. The observation in the Shaker K + channel may be directly applicable to the II-III loop, which contains several loci of positive and negative charges within the 671-690 and 720-765 regions, and also outside these two regions. A study of charge-charge interactions in the II-III loop by mutagenesis and functional expression is clearly re-

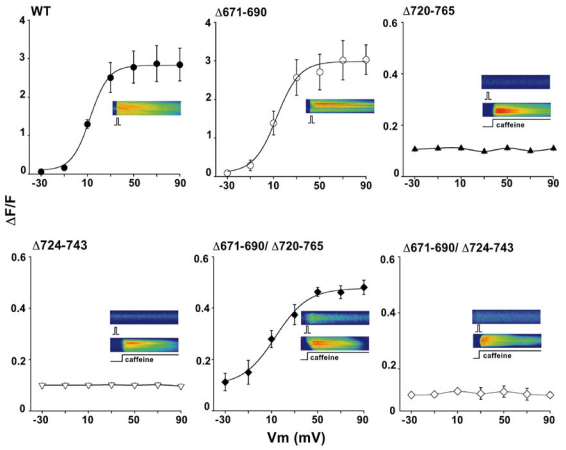

Figure 2. Ca^{2+} transients expressed by α1S II-III loop deletions in dysgenic myotubes [3]. The insets show confocal line-scan images of fluo-4 fluorescence in response to a 50-ms depolarization to +90 mV from a holding potential of -40 mV. The line-scan duration (x-axis) was 2.05 seconds and the cell dimension (y-axis) varied with myotube. In some panels, a second line-scan shows a Ca^{2+} transient after perfusion with external solution containing 10 mM caffeine. α1S lacking the two identified II-III loop domains involved in EC coupling (Δ671-691/Δ720-765) recovered a significant fraction of the Ca^{2+} fluorescence signal. Controls utilizing pore mutations that rendered α1S unable to conduct Ca^{2+} showed that the recovered EC coupling was strictly skeletal-type.

quired to provide an explanation consistent with all results.

A clear implication of the work of Ahern et al. [3] is that the 720-765 region of α1S, instead of a signaling domain, could be a conformation-sensitive "permissive" region controlling protein folding. Disruption of proper protein folding by deletion of residues in this region could impair EC coupling signals generated elsewhere in the DHPR. Alternatively, α1S 720-765 may be one of several regions involved in bi-directional signaling between the DHPR and RyR1. The latter explanation is highly attractive since it also implies that different DHPR-RyR1 coupling domains could trigger SR Ca^{2+} release perhaps independently of each other. Regardless of the final explanation, the deletion experiments leave no doubt that the α1S II-III loop, although necessary, is not sufficient for triggering EC coupling. Therefore other regions of the DHPR must participate directly in opening RyR1.

Molecular determinants of skeletal-type EC coupling present in the DHPR β1a subunit.

Are there EC coupling signals generated elsewhere in the DHPR, including in the β1a subunit that could account for EC coupling in the absence of α1S II-III loop domains? Studies in β1 KO myotubes by Sheridan et al. [55] showed that serial truncation of the carboxyl terminus of DHPR β1a modifies EC coupling, transforming it from a process controlled by voltage to a much weaker coupling process controlled by the Ca^{2+} current. This observation is significant since prior to this work, only exchanges of α1C for α1S were known to produce Ca^{2+} dependent EC coupling in skeletal myotubes. Hence, manipulations of α1S and β1a subunits each independently produced similar outcomes. As a function of carboxyl terminus truncation length (Fig 3), there was an overall decrease in the amplitude of Ca^{2+} transients with evident changes in the shape of the Ca^{2+} fluorescence vs. voltage relationship and the kinetics of the Ca^{2+} transient.

For the most severe truncations, we also observed a dependence of Ca^{2+} transients on external Ca^{2+} . These observations are consistent with the emergence of Ca^{2+} dependent EC coupling, whereby Ca^{2+} entering the cell via the DHPR induces SR Ca^{2+} release, presumably by Ca^{2+} dependent activation of RyR1 and possibly RyR3.

We have speculated that a Ca^{2+} dependent mechanism of EC coupling in a skeletal myotube could originate by weakening of the physical docking between the DHPR and RyR1. Physical docking occurs between DHPR tetrads and every other tetrameric RyR1 complex, and is essential for normal transmission of the voltage signal [26]. A strongly-docked system with a β subunit that

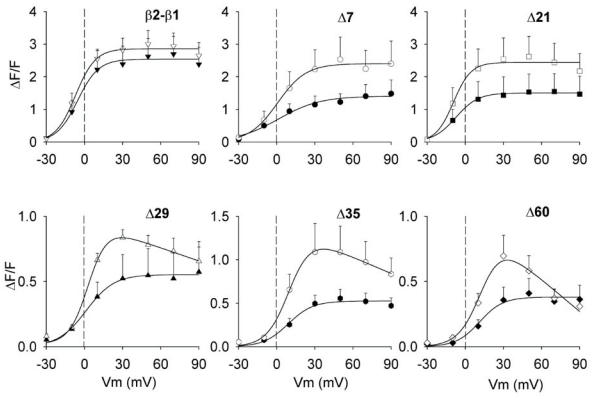

Figure 3. Sigmoidal and bell-shaped voltage dependencies of Ca^{2+} transients in β1 KO myotubes expressing truncated β variants [55]. Ca^{2+} transients were triggered by depolarization of 50 ms (filled symbols) and 200 ms (empty symbols) from a holding potential of -40 mV. Constructs are identified by the number of deleted residues counting from the carboxyl terminus. β2-β1 correspond to a parent chimera with full-length carboxyl terminus and full skeletal-type phenotype from which the serial truncations were made. The longer depolarization increases Ca^{2+} entry into the cell which promotes Ca^{2+} - dependent SR Ca^{2+} release and bell-shaped fluorescence vs. voltage relationships in the truncated mutants.

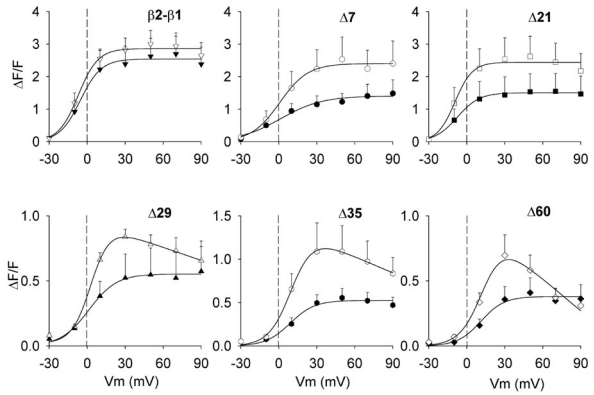

Figure 4. Summary of voltage-dependence of Ca^{2+} transient in dysgenic mdg myotubes [3, 16] and in β1 KO myotubes [55, 56]. Cytosolic fluo-4 Ca^{2+} fluorescence acquired by confocal microscopy and voltage-clamp are shown for dysgenic mdg myotubes expressing the skeletal DHPR pore variant α1S, the cardiac variant α1C, and α1S lacking the two identified II-III loop domains involved in EC coupling (Δ671-691/Δ720-765). Fluorescence curves are shown for β1 KO myotubes expressing the skeletal DHPR variant β1a, the cardiac variant β2a, and the skeletal variant lacking the carboxyl terminus domain (Δ60). Red traces according to the y-scale on the right.

has an intact carboxyl terminus could be essential for translating voltage changes in the DHPR into a signal recognized by RyR1. A weakly-docked system imposed by the presence of severely truncated β variants could permit the Ca^{2+} current to intervene as EC coupling signal as long as the DHPR and RyR1 complexes remain co-localized.

It is also possible that the partial elimination of the carboxyl terminus of β somehow leads to an increase in the Ca^{2+} sensitivity of RyR1 (i.e., increased exposure of Ca^{2+} activation sites in RyR1) and excessive Ca^{2+} feedback on RyR1. Interestingly, spontaneous Ca^{2+} sparks in β1 KO myotubes were found to be significantly wider and longer in duration than their counterparts in normal or dysgenic (α1S null) myotubes [18]. Thus, the properties of clusters of RyR1 channels can be modified by the absence of the β subunit. Distinguishing between these two possibilities, elucidating the molecular mechanisms underlying the switch from voltage to Ca^{2+} dependent EC coupling, and investigating the way in which Ca^{2+} and voltage signals might interact to produce either sigmoidal or bell-shaped Ca^{2+} transient vs. voltage relationships are all areas that deserve close scrutiny in future experiments.

The systematic truncation approach provided clues on at least one structural motif present in the D5 region of β1a potentially responsible for the changes in EC coupling. Ca^{2+} dependent EC coupling was clearly noticeable in the severely truncated Δ29 and Δ60 constructs and much less in the Δ21 and Δ7 constructs (Fig. 3). This could be inferred from the exaggerated curvature of the fluorescence vs. voltage plots for Δ29 and Δ60 and by the dependence of the Ca^{2+} transients generated by these variants on external Ca^{2+}. Inspection of the D5 region indicates that the last 17 to 38 residues of the β1a tail contain a hydrophobic "quasi" heptad repeat, namely L478(n)-V(n+7)-V(n+14)-L(n+22)-L(n+28), present in Δ21 but almost entirely removed in Δ29. Heptad repeats are known to be important in protein-protein interactions responsible for gating [28, 36]. A conserved heptad repeat is present in voltage-gated K + , Na + , and L-type Ca^{2+} channels in the S4-S5 cytosolic linker immediately following the S4 charges. In the Shaker K + channel, alanine substitutions in the heptad repeat leads to large positive shifts in the conductance vs. voltage curve [11, 36]. These conductance shifts suggest that the S4 heptad repeat might be critical for the tight coupling that exists in voltage gated channels between movements of the voltage sensor and the opening of the pore. Perhaps the heptad repeat in the carboxyl terminus of the β subunit could affect the coupling between charge movements in the DHPR voltage sensor and opening of the RyR1 channel.

Functional equivalence of α1S and β1a subunits.

Drastic changes in EC coupling were reported when α1S, the skeletal pore isoform, was replaced by α1C, the cardiac pore isoform, in the cellular context of a primary dysgenic α1S-null skeletal myotube [62]. The Ca^{2+} fluorescence vs. voltage curve expressed by α1C in dysgenic myotubes had a maximum at ~+30 mV followed by a continuous decrease at more positive potentials. The biphasic nature of this relationship, together with a dependence of Ca^{2+} transients on external Ca^{2+} , showed that Ca^{2+} dependent EC coupling could be implemented in skeletal myotubes by a hybrid DHPR composed of a cardiac pore subunit and skeletal non-pore subunits. Likewise, elimination of the D5 region of DHPR β1a, in the context of a skeletal myotube lacking β1 but expressing wild-type α1S, changed the EC coupling trigger signal in a manner similar to the change observed when α1C replaced α1S (Fig. 4). The similar outcome of these two sets of experiments, namely α1S replacement by α1C vs. β1a replacement by β2a, can only mean that both subunits have entirely analogous function and that most likely, the α1S/β1a pair, and not either subunit alone, controls the EC coupling signal in skeletal myotubes. This suggestion is strongly supported by the fact that the minimal structural and functional unit of the L-type Ca^{2+} channel is the α1/β pair [20, 63].

It is also important to point out that although the fluorescence signal generated by Δ671-690/Δ720-765 is small, this result cannot be taken as an argument favoring the notion that the bulk of the EC coupling is initiated by the II-III loop and that a minor component is initiated outside of the II-III loop. Fig. 4 shows that ~80% of the EC coupling fluorescence signal can also

be eliminated, under conditions of normal charge movement, by deletions of the DHPR β1a subunit carboxyl terminus in the context of an otherwise wild-type DHPR. Hence the output signal leaving the DHPR receives contributions from more than one subunit and, by nature, is non-additive. The relative contribution of different DHPR domains to the EC coupling signal transmitted to RyR1 is a challenging issue that needs to be addressed in the future.

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