Morphology and Molecular Composition of Sarcoplasmic Reticulum Surface Junctions in the Absence of DHPR and RyR in Mouse Skeletal Muscle

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ABSTRACT Calcium release during excitation-contraction coupling of skeletal muscle cells is initiated by the functional interaction of the exterior membrane and the sarcoplasmic reticulum (SR), mediated by the "mechanical" coupling of ryanodine receptors (RyR) and dihydropyridine receptors (DHPR). RyR is the sarcoplasmic reticulum Ca^{2+} release channel and DHPR is an L-type calcium channel of exterior membranes (surface membrane and T tubules), which acts as the voltage sensor of excitation-contraction coupling. The two proteins communicate with each other at junctions between SR and exterior membranes called calcium release units and are associated with several proteins of which triadin and calsequestrin are the best characterized. Calcium release units are present in diaphragm muscles and hind limb derived primary cultures of double knock out mice lacking both DHPR and RyR. The junctions show coupling between exterior membranes and SR, and an apparently normal content and disposition of triadin and calsequestrin. Therefore SR-surface docking, targeting of triadin and calsequestrin to the junctional SR domains and the structural organization of the two latter proteins are not affected by lack of DHPR and RyR. Interestingly, simultaneous lack of the two major excitation-contraction coupling proteins results in decrease of calcium release units frequency in the diaphragm, compared with either single knockout mutation.

INTRODUCTION

In muscle cells depolarization of the cell membrane results in rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR) and subsequent contraction of the myofibrils. The series of events linking these two steps is called excitation-contraction (e-c) coupling. Specialized junctions (calcium release units (CRUs)), formed by a close apposition of the junctional SR (jSR) to either the plasmalemma or the transverse (T) tubules, constitute the sites of calcium release via the ryanodine receptor (RyR) or SR Ca^{2+} release channel. The apposition of exterior and interior membranes at CRUs allows functional and structural interaction between two essential contributors to e-c coupling: the voltage sensing, dihydropyridine sensitive, L-type Ca^{2+} channel (dihydropyridine receptors (DHPR)) of the cell membrane/T-tubules and the RyR (Franzini-Armstrong and Protasi, 1997). Within each CRU there may be either one or two functionally interacting clusters of RyRs and DHPRs called couplons (Stern et al., 1997). In skeletal muscle, conversion of the T-tubule depolarization into Ca^{2+} release from the SR is thought to occur via a micromechanical coupling between specific domains of type 1 RyR (RyR1) and of the II-III loop of the skeletal muscle specific α1subunit (α1s) of DHPR (Schneider and Chandler, 1973; Rios and Brum, 1987; Tanabe et al., 1990).

Mild detergent extraction of junctional SR cisternae derived from CRUs leaves behind a supramolecular complex of several proteins associated with RyRs (Caswell and Brunswick, 1984; Costello et al., 1986). Of these, calsequestrin, junctin, and triadin are best characterized and also have a structural signature defined below. Calsequestrin is a luminal SR protein (Jorgensen et al., 1983), which is specifically targeted to the jSR by its acidic carboxy-terminal end (Nori et al., 1997, 1999). It is visible as an electron dense network in the lumen of the SR (Meissner, 1975; Jones et al., 1998), and the network is connected to the luminal side of the SR membrane, predominantly in the region associated with feet by elongated links (Franzini-Armstrong et al., 1987). Junctin and triadin are two intrinsic membrane proteins of the SR membrane. Overexpression experiments have shown that junctin (Zhang et al., 2000) and triadin (Tijskens, Franzini-Armstrong, and Jones, unpublished observations) have similar roles in inducing tight periodic clustering of calsequestrin in proximity of the jSR membrane, perhaps in association with RyRs. Thus, although triadin and/or junctin cannot be directly visualized in thin section electron microscopy, their presence is unequivocally put in evidence by the clustering of calsequestrin.

The question arises whether the close linkage between DHPR and RyR not only allows the two proteins to interact functionally but also represents the structural framework that keeps the two membranes attached to each other and organizes other jSR proteins. Available null mutations that result in lack of either RyR1 or α1s subunit of DHPR have been used to determine the role of these two proteins in the function and formation of CRUs. Observations on a RyR1 knock out mutation ("dyspedic") and on a spontaneous mutation that results in a lack of α1s DHPR ("dysgenic")

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clearly demonstrate the functional importance of both proteins in e-c coupling. Muscle activation is not possible in the absence of either RyR1 or α1s DHPR (Takeshima et al., 1994; Buck et al., 1996; Beam et al., 1986; Knudson et al., 1989; Adams et al., 1990), but SR/T-tubule junctions are present in both types of mutations (Franzini-Armstrong et al., 1991; Takekura et al., 1995; Takekura and Franzini-Armstrong, 1999; Flucher et al., 1993). These findings demonstrate that the link between DHPR and RyR1 is not an essential prerequisite for the assembly of CRUs. However, based on the published results it is not clear whether or not the presence of at least one of the two proteins is necessary for the formation of the junction. That is, we do not know whether the RyR-DHPR link stabilizes the junction after its formation and whether either protein is necessary for the triadin/calsequestrin association with the jSR membrane.

The present study compares the structure of SR/T-tubule junctions and the frequency of couplons in the diaphragm muscle and primary cultures of skeletal muscle from wild-type, dyspedic, dysgenic, and new double knockout mutant mice that lack both RyR1 and α1s DHPR. We show that (necessarily nonfunctional) CRUs with a similar general architecture to wild type are formed in the double knock out, despite the lack of these two major e-c coupling components and that the luminal calsequestrin association with the jSR membrane is unaltered. Interestingly, we also find that the frequency of double knock out CRUs is reduced in diaphragm in vivo but seems to be unaffected in cultured primary myotubes.

MATERIALS AND METHODS

Double null mice breeding

Double null mice were obtained by breeding of previously described dyspedic (RyR null) and dysgenic (DHPR null) mice (Buck et al., 1996; Beam et al., 1986). Genotype was determined by polymerase chain reaction of tail DNA. The number of double mutant embryos obtained in these breedings was 5 of 145.

Preparation of primary cultures

Forelimbs and hind limbs muscle were removed from wild-type, dyspedic, dysgenic, and the same double knock out neonatal mice that were used for diaphragm electron microscopy. Satellite cells were selected by the method of Rando and Blau (1994). Briefly, cells were enzymatically dissociated from minced muscle by the addition of 2 ml/g of tissue of a solution of dispase (grade II, 2.4 U/ml, Boehringer Mannheim Corp., Indianapolis, IN) and collagenase (class II, 1% Boehringer Mannheim Corp.) supplemented with $CaCl_2$ to final concentration of 2.5 mM. The slurry was maintained at 37°C for 30 to 45 min and triturated every 15 min with a 5-ml plastic pipette and then passed through 80-μm nylon mesh (NITEX; Tetko Inc., Monterey Park, CA). The filtrate was spun at 350 $\times$ *g* to sediment the dissociated cells, the pellet resuspended in growth medium, and the suspension was plated on collagen-coated dishes. During the first several passages of the primary cultures, myoblasts were enriched by preplating (Richler and Yaffe, 1970).

The cells were expanded at 37°C in low glucose Dulbecco's modified Eagle medium (GIBCO, Invitrogen Corp., Grand Island, NY) containing 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and additional 2 mM L-glutamine (growth medium), 20 nM basic fibroblast growth factor (Promega, Madison, WI). After ~36/48 h the cells were replated in 35-mm dishes containing THERMANOX coverslips (Nunc Inc., Naperville, IL) coated with MATRIGEL (Collaborative Biomedical Products, Bedford, MA). When cells reached ~40% confluence, growth medium was replaced with differentiation medium (containing 5% heat inactivated horse serum instead 20% of fetal bovine serum with no bFGF) to induce differentiation. The medium was changed every day, and the cells were either fixed or imaged 4 to 5 d later.

Immunolabeling

The cells were fixed in methanol for a minimum of 20 min at −20°C, blocked in phosphate-buffered saline containing 1% bovine serum albumin and 10% goat serum for 1 h, incubated first with primary antibodies and then with secondary antibodies (cyanine 3-CY3 conjugated, Jackson ImmunoResearch Laboratories, Lexington, KY) respectively for 2 h and 1 h at room temperature. Code, specificity, working dilution, original reference, and the sources of primary antibodies are as follows: 34C, mouse monoclonal anti-RyR antibody not type-specific, 1:20 (Airey et al., 1990) (Developmental Studies Hybridoma Bank, The University of Iowa); 21A6, mouse monoclonal anti-α_1DHPR, 1:250 (Morton and Froehner, 1987) (Chemicon International, Temecula, CA); GE4.90, mouse monoclonal anti-triadin, 1:500 (Caswell et al., 1991) (gift of Dr. A.H. Caswell); CSQpAb (rabbit polyclonal, anti-dog cardiac calsequestrin, 1:500) (Jones et al., 1998). The specimens were viewed in an inverted fluorescence microscope Olympus IX70 with a 100X oil immersion lens (UplanFI 110X/1.30 n.a.).

Electron microscopy

Diaphragm muscles from one wild-type, one dysgenic, one dyspedic, and two double mutant mice at a late embryonic stage (18–19 d of gestation, E18–19) from the same litter were dissected and prefixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (Franzini-Armstrong et al., 1991) and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer. After an overnight en bloc stain with saturated uranyl acetate the specimens were dehydrated and embedded in Epon for thin sectioning. The diaphragm from one wild-type mouse at E 18 was from a different litter. Thin sections were examined in a Philips 410 electron microscope at 80 kV. Counting of couplons, measurements of the reference area (myofibril occupied area), and of the fiber diameter were performed on digitized micrographs using NIH image software.

RESULTS AND DISCUSSION

As all of the mutations are birth lethal, but allow embryonic muscle differentiation, we have focused on the late embryonic stage of the diaphragm, which is the most differentiated muscle at birth. Fig. 1, *A* through *I* compare the overall structure of diaphragm muscle fibers and the disposition of CRUs in double knock out and wild-type mice. CRUs are present in all muscles (some junctions marked with arrows, Fig. 1, *A* and *B*) and have the same general architecture: one or two dilated SR cisternae with a dense content are apposed to a T tubule that has an apparently empty lumen (Fig. 2). The random orientation of the junctions relative to the fiber

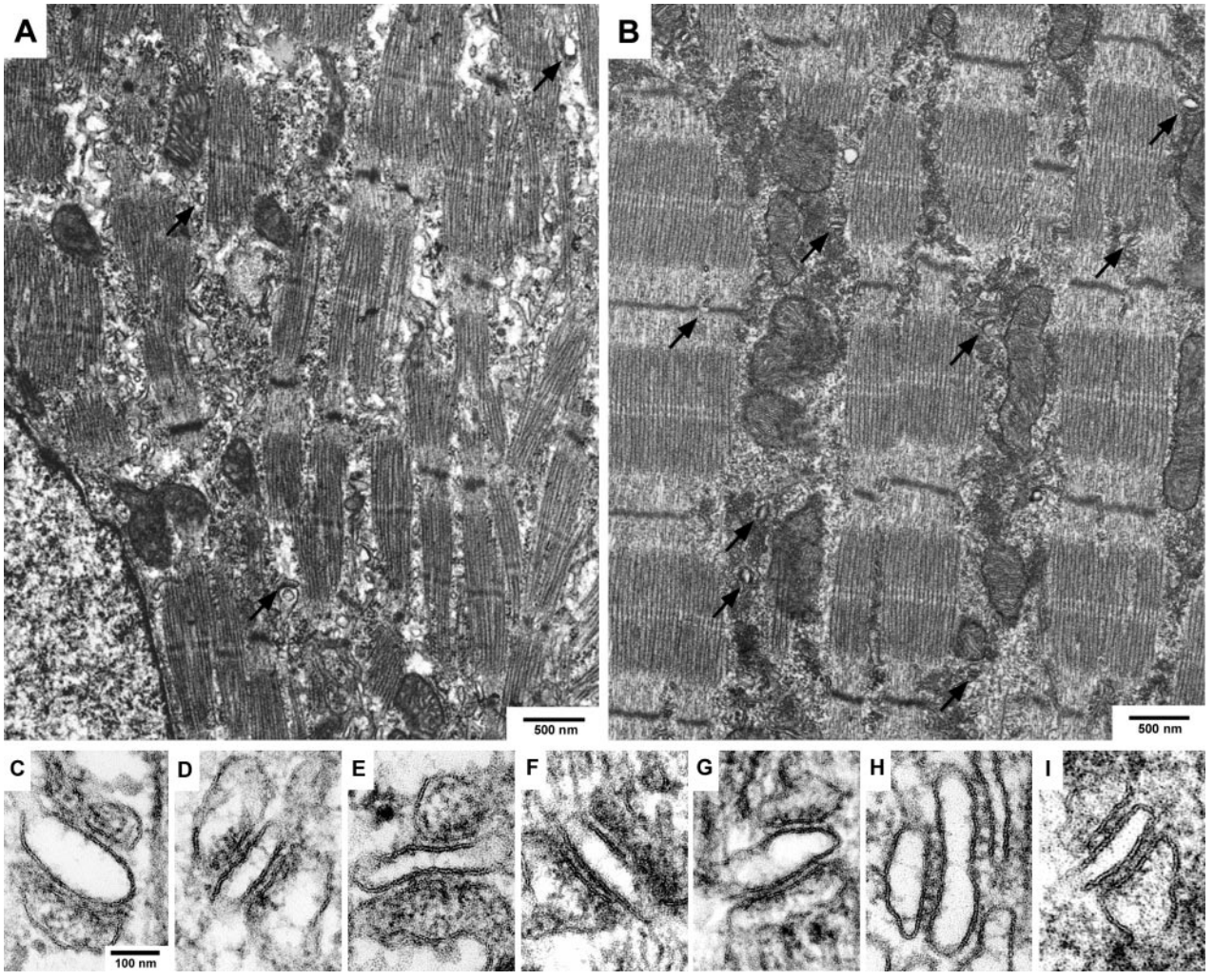

FIGURE 1 Overview of late embryonic (E18–19) diaphragm muscle of double knock out (*A*) and wild-type (*B*) mice and images of single CRUs of double knock out (*C–F*), dyspedic (*G*), dysgenic (*H*), and wild-type (*I*) mice. Muscle fibers in double knock out mutant mice (*A*) show smaller and less well aligned myofibrils than in wild type (*B*). Arrows point to junctional complexes (CRUs). The myofiber long axis in images (*C–I*) is vertical and the orientation of junctions relative to the fiber axis is varied at this developmental stage in all mutants as well as in wild-type mice.

long axis as show in Fig. 1 (*C–I*) is typical of diaphragm muscle at late embryonal age (Takekura et al., 2001).

Fig. 2 shows the structure of CRUs in wild-type (Fig. 2 *A*), dysgenic (missing DHPR, Fig. 2 *B*), dyspedic (missing RyR1; Fig. 2 *C*), and double knock out (missing both DHPR and RyR1, Fig. 2 *D*) fibers. The structure of the junctional SR cisternae and of the junctional gap between SR and T tubules are indistinguishable from each other in thin sections of normal and dysgenic CRUs (Fig. 2, *A* and *B*). In both types of muscles the apposed SR and T-tubule membranes are separated by a junctional gap distance of approximately 10 to 12 nm; and the gap is occupied by an evenly spaced row of feet, representing the cytoplasmic domains of RyRs (arrows) (see also Franzini-Armstrong et al., 1991). CRUs in dyspedic and double knock out mice (Fig. 2, *C* and *D*), on the other hand, show two clear differences from wild-type and dysgenic diaphragms: the junctional gap distance is smaller and no feet are visible in the gap between the two membranes. Thus, lack of RyR is clearly detectable in thin sections, whereas lack of DHPRs is not. The latter is put in evidence by freeze-fracture (Franzini-Armstrong et al., 1991), but this technique was not used in the present study.

To detect the presence of triadin and calsequestrin in CRUs and to confirm their interaction, we combined immunolocalization at the light microscope level with electron microscopy. This allowed us to detect the presence of triadin and calsequestrin in appropriately located foci by immunolabeling. Presence of calsequestrin and its clustering in the jSR cisternae are directly visible in the electron micrographs. For technical ease, immunolabeling was not done on diaphragm but was performed on myotubes derived from cultured primary myoblasts. Labeling for DHPR, RyR, calsequestrin (CSQ), and triadin shows that all four proteins form peripherally located foci, representing CRU sites, in

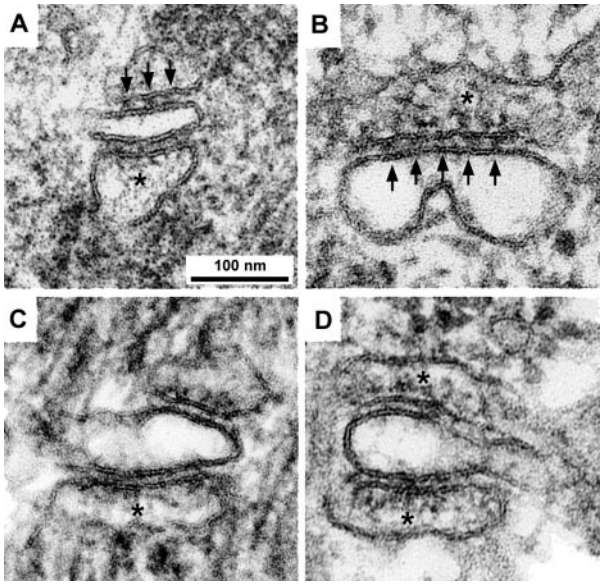

FIGURE 2 Images of single CRUs showing the presence of feet (on opposite side of the jSR membrane from the tips of *arrows*) in wild-type (*A*) and dysgenic mice (*B*) and their absence in dyspedic (*C*) and double knock out (*D*) mice. The junctional gap between the sarcoplasmic membrane and the membrane of the T-tubule is slightly narrower in dyspedic and double knock out mutants. Calsequestrin is visible in the SR sacs (*asterisk*) of wild-type and all mutant mice. Note: the CRUs in Fig. 2 are all presented in the same orientation, even though their positioning relative to the myofibrils varied in the original micrographs.

wild-type myotubes (Fig. 3, *A–D*; compare with Protasi et al., 1997, 1998, 2000). In the double mutant, peripheral foci are present at approximately equal density as in the wild-type myotubes and contain calsequestrin and triadin (Fig. 3, *G* and *H*) but lack α1s DHPR and RyR1 (Fig. 3, *E* and *F*).

In the electron micrographs, the content of the jSR cisternae is remarkably similar in wild type and in all three mutants (Fig. 2, asterisks). In all cases the electron density due to CSQ is present and it is also clearly periodically clustered in proximity of the junctional membrane, an effect traceable to the presence and appropriate localization of triadin. This confirms that CSQ and either triadin or junctin are present in the junctional SR and that CSQ is linked to at least one of them.

We conclude that not only SR-T tubule docking but also targeting of calsequestrin and triadin and the formation of a complex between them is independent of the presence of either RyRs or DHPR. This strengthens previous results from single mutations (Takekura et al., 1995; Takeshima et al., 1995; Takekura and Franzini-Armstrong, 1999; Flucher et al., 1993) and extends them to include the other junction-specific proteins. During normal differentiation of cardiac and skeletal muscle, SR docking and the presence of a luminal jSR densities due to CSQ clearly precede clustering of RyR (Protasi et al., 1996; Flucher and Franzini-Armstrong, 1996; Takekura et al., 2001), in agreement with the above observations. Thus junctophilin, the docking protein that is required for specific SR-surface membrane junction formation, acts in the absence of RyRs and DHPRs, a conclusion supported by the ability of this protein to induce junctions between the endoplasmic reticulum and the surface membrane in a nonmuscle cell (Takeshima et al., 2000; Ito et al., 2001).

Morphometry reveals an important difference in the abundance of SR/T-tubule junctions in the diaphragm between the wild type, the single null, and the double null mutations. Despite the fact that a limited sample size was available to obtain our data, the trend is very clear. Table 1 compares the frequency of couplons in the diaphragms of wild-type, dyspedic, dysgenic and double knock out mice, expressed as the number of couplons per myofibril-occupied area of a fiber section. This number is proportional to the ratio of couplons to myofibril-occupied fiber volume, assuming that the CRUs are of the same size on the average. The frequency of couplons in diaphragms of dyspedic and dysgenic mice is reduced to ~50% compared with the wild type, and the frequency of couplons in the two mutant muscle types are not different from each other. A considerable further reduction (again by ~50%) is seen between double null mice and the single null dyspedic/dysgenic mice.

The fiber cross-sectional area is not reduced in mutated versus normal fibers. The mean cross-sectional area is $135.0 \pm 58.6\ \mu m^2$, $n = 106$ (mean $\pm$ 1 SD, n = number of fibers) for wild-type fibers; $179.4 \pm 100.7\ \mu m^2$, $n = 92$ for dysgenic; $148.1 \pm 71.0\ \mu m^2$, $n = 54$ for dyspedic; and $187.1 \pm 98.6\ \mu m^2$, $n = 120$ for double null animals. It should be noted however that diaphragms of null animals show a considerable reduction in the total number of fibers relative to the wild-type diaphragm, probably indicating a high level of apoptosis, a phenomenon previously seen in dyspedic muscle (P.D. Allen and J. Sommer, unpublished observations). Therefore it is possible that the measured fiber diameters compare a population of primary and secondary fibers in the wild type versus only one generation of fibers in the mutated diaphragms.

In all null mutants the myofibrils fill most of the available space but show defects: changes in orientation and splitting (see Fig. 1 *A*). All three mutations result in muscle paralysis due to absence of excitation-contraction coupling, and all have similar overall fiber morphology with some myofibrillar defects. The lack of activity might be the cause of the observed defects as it was shown that muscles paralyzed with tetrodotoxin exhibit a similar disordered alignment of filaments (Houenou et al., 1990), indicating the importance of muscle activity for an adequate alignment of myofibrils. CRU frequency, on the other hand, is more strongly affected in the diaphragm of double null than in either single mutation. A possible explanation is that some movements of Ca^{2+} into the cytoplasm may occur in muscles with single mutations, either by leak through the RyR in the dysgenic

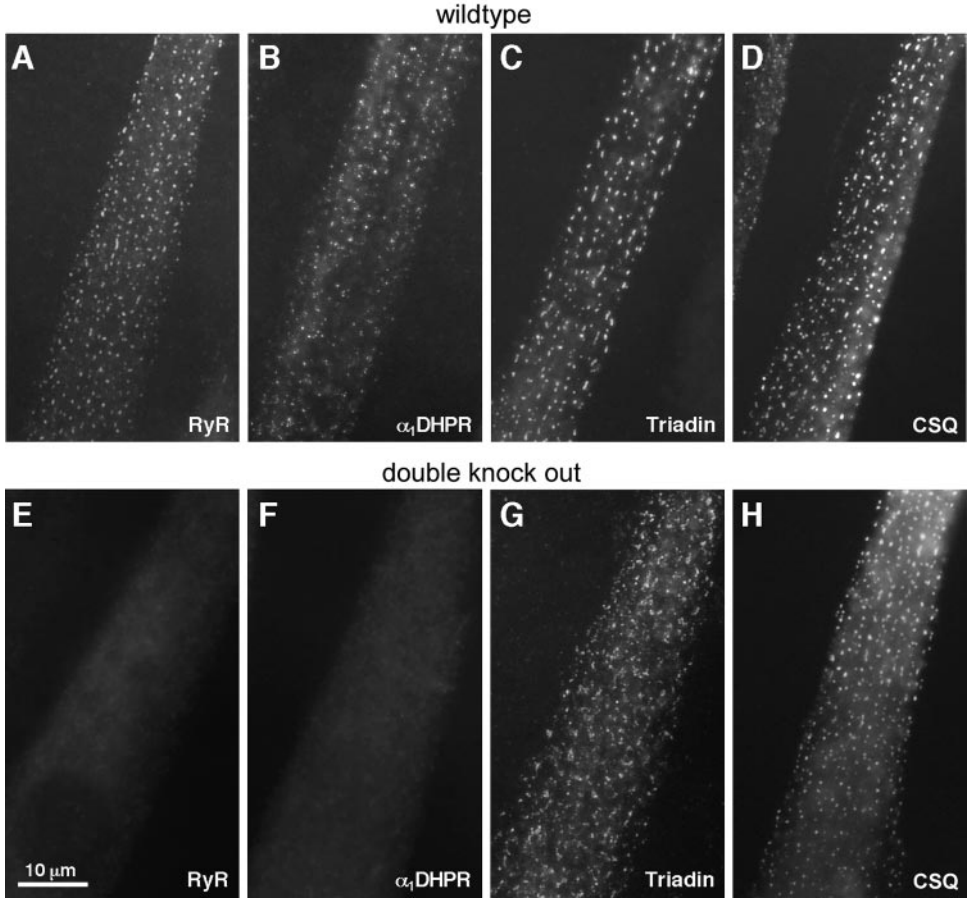

FIGURE 3 Immunolabeling images of cultured myotubes from wild-type (*A–D*) and double knock mice (*E* and *F*). Labeling for RyR and DHPR proves that neither is expressed in the mutants (*E* and *F*). Calsequestrin (CSQ) and triadin are located in distinct foci (*G* and *H*) in cells of in double knock out mice and show the same pattern as that of cells from wild-type mice.

mouse or through voltage gated activation of the DHPR in the dyspedic mouse. In the absence of either source of cytoplasmic Ca^{2+} movement, formation of the membrane system might be affected. Indeed, it is known that lack of functional DHPR causes altered transcription levels of certain genes during muscle cell development (Chaudari and Beam, 1997; Luo et al., 1996), and a further reduction in calcium movements are most likely to have more serious effects. The fact that only the diaphragm muscle showed this defect but both cultured myotubes (present study) and dyspedic hind limb muscle of the same age (Takekura et al., 1995) do not suggests that this effect is somehow related to the degree of development/differentiation. This is because the last two types of muscles are less differentiated than the diaphragm at this stage of development. Interestingly, fiber diameters in dyspedic leg muscles are also considerably smaller than in the wild type (Takekura et al., 1995), which was not found in diaphragm muscle. A lower degree of apoptosis in the leg muscle (again due to a lower degree of differentiation) could explain this fact as well.

TABLE 1. Frequency of couplons in wild-type and mutant mice diaphragm muscle

Mouse Type	Number of couplons/100 μm^2 of myofibril occupied area (Mean ± SD)
Wildtype	6.1 ± 2.2*
Dysgenic	3.6 ± 2.0*
Dyspedic	3.3 ± 1.8*
Double knock out	1.7 ± 1.1†

*One animal.
†Two animals.
Four different parts from each diaphragm were sectioned. 15–20 randomly chosen areas were counted per section (~20,000 μ^2m per animal in total).

The major conclusions of this work are that neither RyR nor DHPR, alone or separately, are necessary for T-SR docking and for the targeting and/or association of calsequestrin and triadin in the junctional SR. Both proteins however are needed for appropriate muscle development.

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