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# Abnormal junctions between surface membrane and sarcoplasmic reticulum in skeletal muscle with a mutation targeted to the ryanodine receptor

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**ABSTRACT** **Junctions that mediate excitation–contraction (e-c) coupling are formed between the sarcoplasmic reticulum (SR) and either the surface membrane or the transverse (T) tubules in normal skeletal muscle. Two structural components of the junctions, the feet of the SR and the tetrads of T tubules, have been identified respectively as ryanodine receptors (RyRs, or SR calcium-release channels), and as groups of four dihydropyridine receptors (DHPRs, or voltage sensors of e-c coupling). A targeted mutation (*skrr$^{m1}$*) of the gene for skeletal muscle RyRs in mice results in the absence of e-c coupling in homozygous offspring of transgenic parents. The mutant gene is expected to produce no functional RyRs, and we have named the mutant mice "dyspedic" because they lack feet—the cytoplasmic domain of RyRs anchored in the SR membrane. We have examined the development of junctions in skeletal muscle fibers from normal and dyspedic embryos. Surprisingly, despite the absence of RyRs, junctions are formed in dyspedic myotubes, but the junctional gap between the SR and T tubule is narrow, presumably because the feet are missing. Tetrads are also absent from these junctions. The results confirm the identity of RyRs and feet and a major role for RyRs and tetrads in e-c coupling. Since junctions form in the absence of feet and tetrads, coupling of SR to surface membrane and T tubules appears to be mediated by additional proteins, distinct from either RyRs or DHPRs.**

Muscle contraction is initiated by depolarization of the surface membrane, the first event in the chain called excitation–contraction (e-c) coupling, which leads to contractile activation. A major and still unexplained step is transmission from the surface membrane to sarcoplasmic reticulum (SR), resulting in calcium release from the SR. Two distinct calcium channels play major roles in e-c coupling. One is the dihydropyridine receptor (DHPR), a voltage-gated calcium channel of surface and transverse (T) tubules (surface membrane invaginations), which acts as a voltage sensor. Charge movement in the DHPR is a primary event in e-c coupling, but calcium currents develop slowly and are not involved in the activation of contraction. The other channel is the ryanodine receptor (RyR), a large tetrameric protein that releases calcium from the SR. The two proteins interact at junctions between SR and surface membrane in developing muscle and between SR and T tubules in adult muscle (1). RyRs have been proposed as the calcium-release channels of the SR because of their high permeability to calcium (2). The role of DHPRs as essential voltage-sensing elements in e-c coupling has been proven with cultured myotubes from dysgenic mice, which carry a mutation of DHPRs and lack e-c coupling (3, 4).

Two structural components of SR/surface (including T tubules) junctions have been identified with these two calcium channels. The "feet," which span the SR/surface junctional gap, are the cytoplasmic domains of RyRs anchored in the SR membrane (2, 5, 6). Tetrads, the second structural component, are clusters of four proteins in surface membrane that are located in exact correspondence to the feet (6). Dysgenic myotubes are missing both DHPR protein and tetrads. Further, tetrads are restored by transfection of dysgenic myotubes with cDNA for DHPRs (7). These two findings clearly identify DHPRs as components of the tetrads. The matching disposition of tetrads and feet suggests that transmission of information from DHPR to RyRs may involve a direct molecular interaction between the two proteins (6, 8).

Homozygous transgenic mice resulting from a targeted mutation of the gene for RyRs (*skrr$^{m1}$*) lack e-c coupling and show developmental defects of the musculoskeletal system similar to those found in dysgenic mice (9). In both mutations, the embryos are not viable after birth. We have studied the effect of lack of RyRs on the development and structure of junctions. Abnormal junctions lacking feet and tetrads are formed in mutant myotubes. The concomitant absence of e-c coupling and of feet and tetrads is strong evidence for a role of these components in e-c coupling. It is also obvious that feet and tetrads are not needed for junction formation between SR and T tubules. Because of the absence of feet, we have named the mutant mice "dyspedic."

## MATERIALS AND METHODS

Targeted mutation of the skeletal muscle RyR was accomplished in mice as reported (9). Homozygous dyspedic embryos (*skrr$^{m1}$/skrr$^{m1}$*) and their heterozygous (+/*skrr$^{m1}$*) and wild-type (+/+) littermates were obtained at 15–21 days of gestation (E15–E21). Genotypes were determined by using PCR as described (9). As further controls, litters from wild-type (+/+) parents at E16, E17, and E18 were used.

Hindlimb muscles and diaphragm were fixed for 1 hr at room temperature in 3.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4, and subsequently stored at 4°C. For thin sectioning, muscles were postfixed in 2% $OsO_4$ in the same buffer, *en block*-stained with aqueous saturated uranyl acetate for 4 hr at 60°C, and embedded in Epon. Sections were stained with uranyl acetate and lead salts. For freeze-fracture, the muscles were cryoprotected in 30% (vol/vol) glycerol, frozen in propane, fractured and shadowed with platinum at 45° in a Balzer's 400 freeze-fracture, and examined in a Philips 400 electron microscope.

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Abbreviations: SR, sarcoplasmic reticulum; e-c, excitation–contraction; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; T tubules, transverse tubules.

The width of the junctional gap of SR/T junctions was measured by using a dissecting microscope fitted with an eyepiece micrometer, in micrographs from diaphragm and leg muscles at E21 at 61,000 to 85,000 magnification.

## RESULTS

Dyspedic myotubes are smaller than normal and overall muscle mass in mice is greatly reduced at birth, particularly in leg muscles. The most noticeable defect is limited development of the myofibrils, which often are split and have small-diameter, poorly defined outlines and misaligned striations (9). Similar characteristics are encountered in dysgenic myotubes (10). Muscles in heterozygous and wild-type embryos have indistinguishable structures and are thus described together as "normal."

**Junctions in Normal and Dyspedic Early Myotubes.** In the early stages of normal developing muscle, SR vesicles are found associated with the surface membrane and budding T tubules, and junction formation is visible at several stages. Some vesicles are "zippered" to the surface membrane by feet over a fraction of the junction (Fig. 1*a*). Other vesicles are zippered over the whole junction, but the arrays of feet are incomplete (Fig. 1*b*). Finally, some vesicles have a complete set of evenly spaced feet covering the entire junction (Fig. 1 *c* and *d*). Vesicles associated with budding T tubules are also zippered by arrays of feet, sometimes incomplete (Fig. 1*e*). In parts of junctions where no feet are present (e.g., at the left of the vesicle in Fig. 1*a*), short densities link the SR and surface membrane. As can be seen, most of the SR vesicles contain a protein, probably calsequestrin, which is periodically associated with the SR membrane at the junction. In contrast, caveolae and budding T tubules (asterisks) look empty.

In dyspedic myotubes (Fig. 1 *f–j*), SR vesicles have a configuration similar to their normal counterparts, but the junctions are incomplete (Fig. 1 *f–j*). The junctional gap is occupied by small irregular links between the two membranes, feet are absent, and the gap is much narrower on average than in the normal junctions. The protein contained within the SR has a normal association with the membrane.

**SR/T Junctions in Older Normal and Dyspedic Myotubes.** Internal junctions between SR and T tubules are formed at the same age in normal and dyspedic muscle fibers, but in the latter the junctions are less frequent. SR/T junctions are present throughout normal and dyspedic myotubes starting at E18 in the diaphragm, and at E19–E20 in leg muscles. Fig. 2*a* shows a myotube from leg muscle of a dyspedic embryo at E21. Despite poor development of the myofibrils, several SR/T junctions (arrows) are present. A normal myotube at this age would show larger and better aligned myofibrils and more frequent junctions.

The structure of SR/T junctions in normal and dyspedic myotubes differs in two significant details but has one similarity. First, in normal embryos, all SR/T junctions are fully zippered by complete arrays of feet (Fig. 2 *b–e*), while in dyspedic embryos, feet are missing and the junctional gap is occupied by thin irregularly placed strands (Fig. 2 *f–i*). Secondly, the gap in normal SR/T junction is wider than in dyspedic

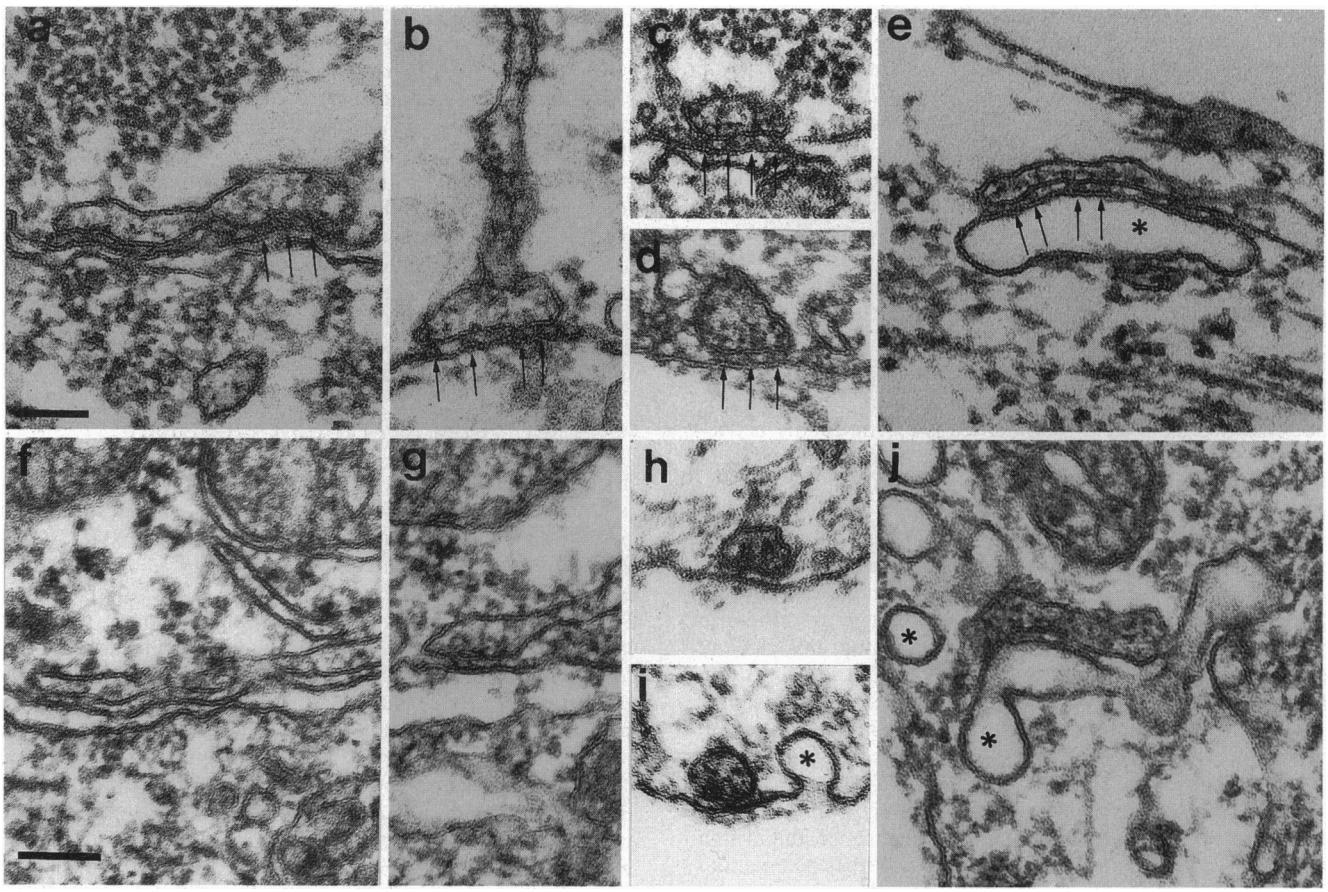


FIG. 1. SR/surface membrane (*a–d* and *f–i*) and SR/T junctions (*e* and *j*) in the diaphragm of wild-type (*a–e*) and dyspedic (*f–j*) mice. In developing junctions of normal myotubes, feet (arrows) occupy either a fraction of the junction (*a*) or most of it. In *a*, *b*, and *e*, arrays of feet are incomplete, since the spacing between them is not uniform. In *c* and *d*, spacing between the feet is uniform, and thus arrays are complete. The SR has a dense content, presumably calsequestrin, associated with the junctional membrane. In junctions from dyspedic muscle (*f–j*), the gap between SR and surface is less wide, and it is occupied by densities smaller than those of the feet. The SR has a dense content similar to that of normal muscle. *, Caveolae and T tubules. (*a–d*) E16 wild-type mice. (*e*) E15 wild-type mice. (*f–j*) E16 dyspedic ($skrr^{m1}/skrr^{m1}$) mice. (Bars = 0.1 μm.)

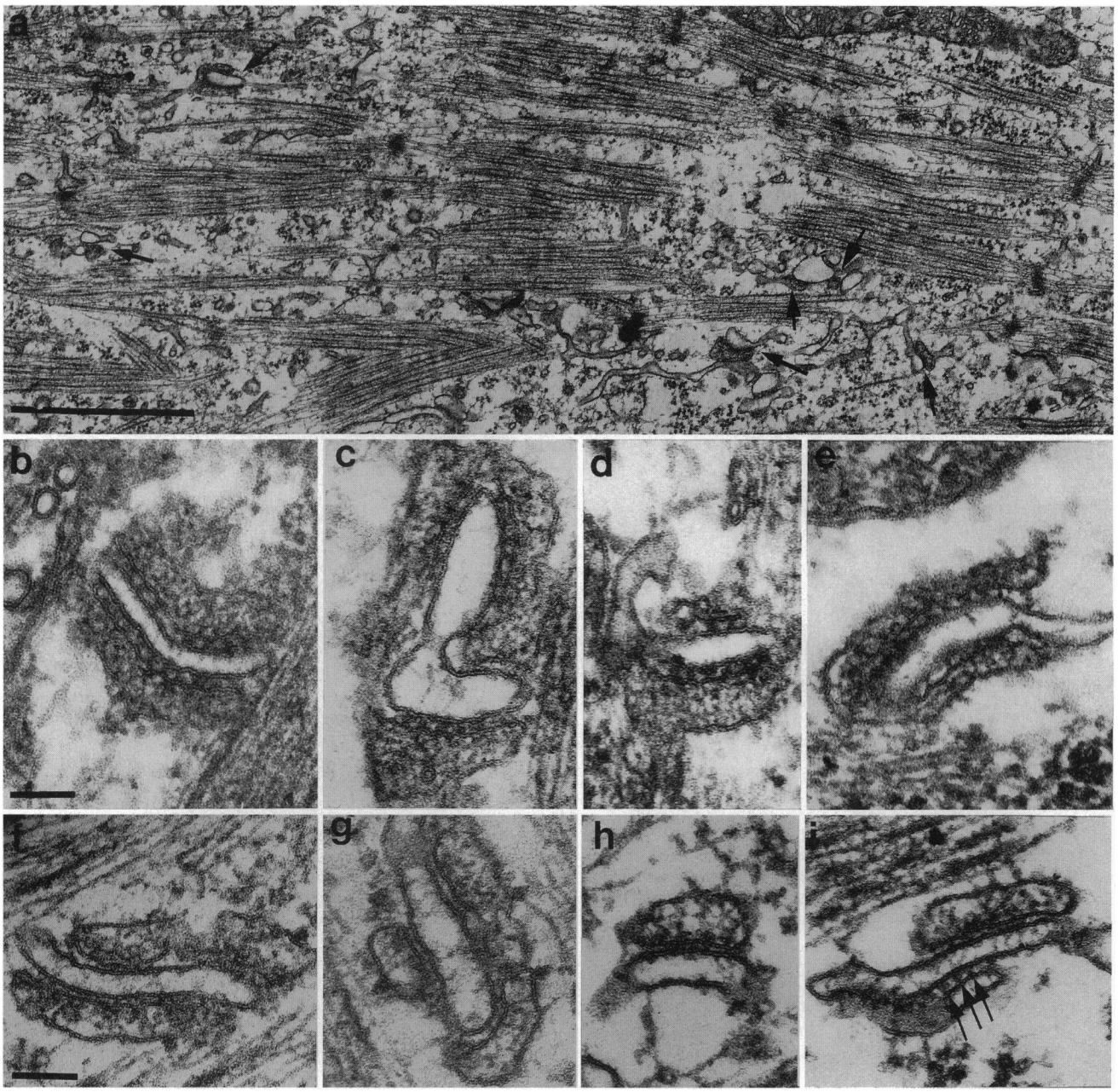


FIG. 2. (*a*) Myotube from the leg muscle of a dyspedic embryo at E21. Myofibrils are small and disordered. However, T tubules and SR/T junctions (arrows) are present. (Bar = 0.5 μm.) (*b–e*) Periodically disposed feet occupy the junctional gap between SR and T tubules, zippering the two membranes together, in normal diaphragm at E21. The protein within the SR, presumably calsequestrin, is clustered in proximity to the feet. (*b* and *d*) Wild-type mice. (*c* and *e*) Heterozygous (+/*skrr*$^{m1}$) mice. (Bar = 0.1 μm.) (*f–i*) Extensive SR/T junctions develop in diaphragm from dyspedic embryos at E21. However, their junctional gap is narrower than in normal myotubes (≈7 versus ≈12 nm) and assemblies of feet are not present, except, perhaps, in Fig. 2*i* (arrows). Note that the presumed calsequestrin is associated with the SR membrane as in the normal junctions. (Bar = 0.1 μm.)

junctions. The average gap in normal muscles is 11.9 ± 1.9 nm in heterozygous mice (mean ± 1 SD, $n$ = 186 measurements from 30 junctions) and 12.1 ± 1.6 nm in wild-type mice ($n$ = 196 from 33 junctions), while the gap in dyspedic junctions is 6.8 ± 1.9 nm ($n$ = 250, from 40 junctions). The difference between normal and dyspedic fibers is highly significant (Student's $t$ test, $P < 0.001$). In 1 dyspedic junction of the 190 observed, the gap was wider and three densities comparable in size to feet were present (Fig. 2*i*, arrows).

The one similarity between normal and dyspedic junctions is in the disposition of calsequestrin and its association with the SR membrane, which are apparently identical.

**Tetrads.** A tetrad is an easily recognizable group of four tetragonally arranged particles (Fig. 3*d Inset*). Tetrad arrays are visible in the surface membrane of normal young myotubes (Fig. 3 *a, b, d,* and *e*). We counted the number of myotube segments containing one or more tetrad arrays in freeze-fracture replicas of normal muscles (Table 1). In the diaphragm, the frequency of myotubes segments with tetrads is high at E15–E16, it declines at E17, and is fairly low at E18–E21. In leg muscles, the density is high at E18 and lower at E21 (Table 1). Thus, tetrad arrays decline in frequency with age, and in the diaphragm this decline coincides with the gradual development of T tubules (C.F.A. and B. E. Flucher, unpublished data; ref. 1).

Dyspedic myotubes, unlike normal ones, do not have tetrad arrays. We examined 256 segments, covering the range of ages at which tetrads arrays are present in the normal muscle and found none (Fig. 3 *c* and *f*; Table 1). However, in two fibers

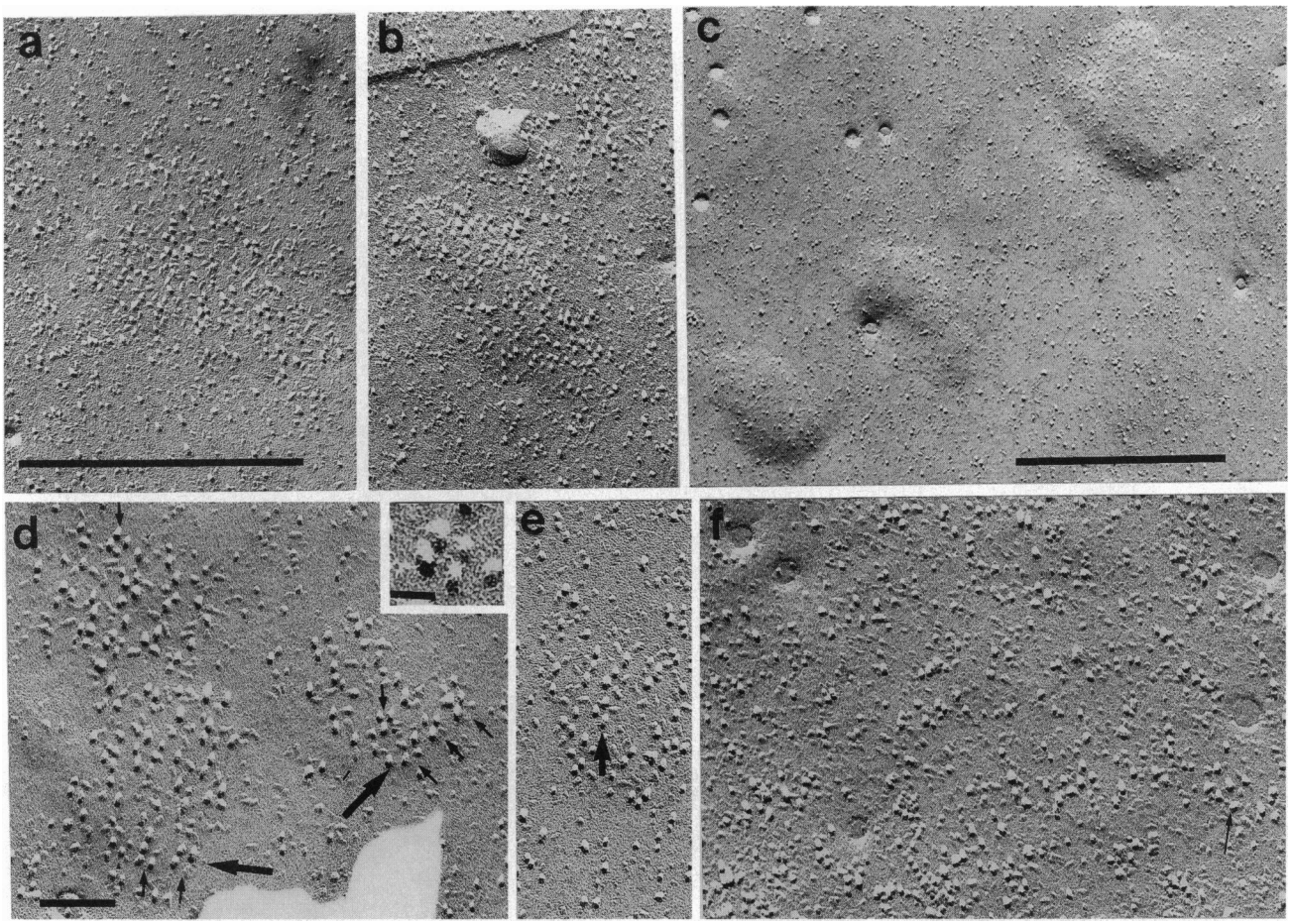


FIG. 3. Freeze-fracture of normal (*a, b, d,* and *e*) and dyspedic (*c* and *f*) myotubes. (*a–c*) Domed patches of surface membrane, which in normal myotubes (*a* and *b*) contain large particles and in dyspedic myotubes (*c*) have no distinguishing features. (*d* and *e*) Tetrads are identifiable in the domed patches of normal myotubes shown at a higher magnification (arrows indicate rows of tetrads, while smaller arrows indicate individual tetrads). (*d Inset*) A single tetrad in further detail. (*f*) In dyspedic myotubes, intramembrane particles do not form either tetrads or ordered arrays. (*a, b,* and *e*) E18 wild-type mouse leg muscle. (*c*) E15 dyspedic mouse diaphragm. (*d*) E15 wild-type mouse diaphragm. (*f*) E21 dyspedic mouse diaphragm. (Bar in *a* for *a* and *b* = 0.5 μm; in *c* = 0.5 μm; in *d* for *d–f* = 0.1 μm; in *d Inset* = 20 nm.)

from the leg muscle of a E21 dyspedic embryo, we did find three groups of particles (one in one fiber and two in another) that resembled tetrads. These were not part of an array.

## DISCUSSION

SR/surface and SR/T junctions in dyspedic muscle, regardless of the developmental stage, lack feet. This confirms that the targeted disruption of the RyR gene was successful (9) and strongly supports the identification of feet with RyRs (2, 5, 6).

In addition to lacking feet, dyspedic myotubes do not form arrays of tetrads in their plasmalemma, even though DHPRs are present in dyspedic muscle (9). Thus, the formation of tetrads and their disposition in ordered arrays depend on an interaction between DHPRs and RyRs.

The absence of e-c coupling in dyspedic muscle (9), despite the presence of T tubules and of SR/surface and SR/T junctions, confirms that tetrads (DHPRs) and feet (RyRs) are key elements in the activation of the muscle fiber. This is expected from the predicted roles of the two channels as, respectively, e-c coupling voltage sensors and SR calcium release channels. Block of e-c coupling also occurs in another mutation, which results in muscular dysgenesis, where DHPRs and tetrads are absent but feet are present (3, 4, 10).

The formation of SR/surface and SR/T junctions in dyspedic myotubes is contrary to the general assumption that feet hold the two membranes together. The narrow but fairly uniform gap in dyspedic junctions, in the absence of feet, suggests the presence of some yet unidentified protein capable of linking SR to surface membrane and T tubules. It is unlikely that the linking protein is a product of the mutated gene. From the targeting construct, it is expected that the peptide produced by the mutant gene is only 23 amino acids long, corresponding to the N terminus of the native RyR (9). This short segment would entirely lack the foot structure and indeed most of the molecule, and it is unlikely that it could insert in the SR membrane and span the junctional gap of dyspedic triads. Since the gap in the junctions of dyspedic myotubes is smaller than the feet, this also excludes the participation of either the cardiac or the full-length neural isoforms of RyRs in the junction (with the exception, perhaps of the single example shown in Fig. 2*i*).

The facts in the above paragraph suggest that a protein other than the RyRs allows docking of the SR to the surface membrane and T tubules. This is confirmed by the observation here and elsewhere that junctions without feet are transiently formed between SR and the surface membrane during early skeletal muscle development and in a skeletal muscle cell line (11–13). The identity of this "docking" protein is at present unknown.

Muscular dysgenesis and co-expression of RyRs and DHPRs in Chinese hamster ovary (CHO) cells provide further information relevant to the genesis of SR/surface and SR/T junctions. Dysgenesis is due to a single nucleotide deletion in the gene for the α1 subunit of DHPR (14), which results in the absence of the skeletal-type DHPR and of slow calcium

Table 1. Frequency of myotube segments with tetrads arrays in normal and dyspedic muscles

| Age | Muscle | Animal (no. of embryos) | Total no. of myotube segments | Segments with tetrads, % |
|---|---|---|---|---|
| E15 | Dia. | W(2) | 19 | 80 |
| E15 | Dia. | He(1) | 33 | 70 |
| E15 | Dia. | Ho(1) | 21 | 0 |
| E16 | Dia. | W(2) | 68 | 63 |
| E16 | Dia. | He(2) | 46 | 83 |
| E16 | Dia. | Ho(1) | 91 | 0 |
| E17 | Dia. | W(3) | 122 | 61 |
| E17 | Dia. | He(2) | 37 | 41 |
| E17 | Dia. | Ho(1) | 38 | 0 |
| E18 | Dia. | W(1) | 20 | 10 |
| E21 | Dia. | W(1) | 25 | 32 |
| E21 | Dia. | He(1) | 45 | 24 |
| E21 | Dia. | Ho(2) | 48 | 0 |
| E18 | Leg | W(1) | 40 | 95 |
| E21 | Leg | W(2) | 50 | 34 |
| E21 | Leg | Ho(2) | 58 | 0* |

Dia., diaphragm; W, wild-type; He, heterozygous; Ho, homozygous. *See text.

currents, charge movement, and e-c coupling (3). SR/T junctions containing ordered arrays of feet are found in dysgenic myotubes developing *in vivo* (15) in the absence of either the skeletal- or the cardiac-type DHPR (16). However, tetrads are missing, and the $\alpha_2$ subunit of DHPRs, which is expressed, is not targeted to the junctions (17). Thus, arrays of feet and an association between SR and T tubules can form in the absence of DHPRs and tetrads. DHPR clustering at junctions and tetrads are restored in parallel to e-c coupling in cells rescued either by fusion with normal cells or by transfection with cDNA for the DHPR (7, 17). In CHO cells coexpressing functional RyRs and DHPRs, RyRs assemble into arrays in the endoplasmic reticulum (ER) and DHPRs are in the surface membrane, but junctions between ER and surface membrane do not form, and DHPRs do not assemble into tetrads (H.T., H.T., S. Nishimura, M. Takahashi, T. Tanabe, V. Flockerzi, F. Hoffman, and C. Franzini-Armstrong, unpublished data).

Based on these observations from normal development, from dysgenic and dyspedic muscle, and from CHO cells transfected with e-c coupling proteins, we suggest three sequential steps in the formation of normal couplings. (*i*) Docking of SR and surface membrane, mediated by protein(s) to be identified. The junctional gap at this stage is narrow. (*ii*) Formation of arrays of feet, widening the junctional gap. (*iii*) Formation of tetrad arrays by association of DHPRs with RyRs, perhaps through some intermediate component (18). These three steps can be used to trace the formation of junctions in normal myotubes, dysgenic myotubes, and dyspedic myotubes and to explain the lack of junctions in transfected CHO cells. In normal development, all three steps occur, and tetrads arrays (step *iii*) form in tandem with arrays of feet (step *ii*). As a consequence, DHPRs and RyRs are colocalized in developing and differentiated muscle (17, 20), and tetrads and feet are both present at early stages of muscle development (11). During normal development, intermediate SR/surface junctions with incomplete arrays of feet show an intermediate step in the assembly process. In dysgenic muscle, docking and assembly of arrays of feet occur, but tetrads do not form for lack of DHPRs. In dyspedic muscle, docking occurs, but feet are not present, so the assembly of tetrads fails. Finally, in CHO cells cotransfected with cDNA for RyRs and DHPRs (17), docking fails, probably for lack of the needed docking protein(s), and this results in failure of DHPRs to group into tetrads, even though feet form arrays.

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