

# Triad Formation: Organization and Function of the Sarcoplasmic Reticulum Calcium Release Channel and Triadin in Normal and Dysgenic Muscle In Vitro

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**Abstract.** Excitation–contraction (E–C) coupling is thought to involve close interactions between the calcium release channel (ryanodine receptor; RyR) of the sarcoplasmic reticulum (SR) and the dihydropyridine receptor (DHPR)  $\alpha_1$  subunit in the T-tubule membrane. Triadin, a 95-kD protein isolated from heavy SR, binds both the RyR and DHPR and may thus participate in E–C coupling or in interactions responsible for the formation of SR/T-tubule junctions. Immunofluorescence labeling of normal mouse myotubes shows that the RyR and triadin co-aggregate with the DHPR in punctate clusters upon formation of functional junctions. Dysgenic myotubes with a deficiency in the  $\alpha_1$  subunit of the DHPR show reduced expression and clustering of RyR and triadin; however, both proteins are still capable of forming clusters and attaining mature cross-striated distributions. Thus, the

molecular organization of the RyR and triadin in the terminal cisternae of SR as well as its association with the T-tubules are independent of interactions with the DHPR  $\alpha_1$  subunit. Analysis of calcium transients in dysgenic myotubes with fluorescent calcium indicators reveals spontaneous and caffeine-induced calcium release from intracellular stores similar to those of normal muscle; however, depolarization-induced calcium release is absent. Thus, characteristic calcium release properties of the RyR do not require interactions with the DHPR; neither do they require the normal organization of the RyR in the terminal SR cisternae. In hybrids of dysgenic myotubes fused with normal cells, both action potential-induced calcium transients and the normal clustered organization of the RyR are restored in regions expressing the DHPR  $\alpha_1$  subunit.

**T**HE triad is the junction between the transverse tubules (T-tubules)<sup>1</sup> and the sarcoplasmic reticulum (SR) of skeletal muscle. In accordance with its function in excitation–contraction (E–C) coupling both membrane compartments are composed of a highly specialized set of proteins. T-tubules are enriched in voltage-gated  $\text{Ca}^{2+}$  channels (also called dihydropyridine receptors, DHPR) (Fosset et al., 1983; Borsotto et al., 1985; Flockerzi et al., 1986; Striessnig et al., 1986), which are believed to serve

as voltage sensors in E–C coupling (Rios and Brum, 1987). The terminal cisternae of the SR is the calcium storage and release compartment. It contains the calcium release channel (called the ryanodine receptor, RyR) (Inui et al., 1987; Lai et al., 1988) in its membrane and calsequestrin, a calcium-binding protein, in its lumen (Meissner et al., 1973; Jorgensen et al., 1983).

The DHPR is composed of five subunits ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) (Takahashi et al., 1987). The  $\alpha_1$  subunit forms the ion channel and is the putative voltage sensor in E–C coupling. Functions of the other subunits are unknown. The DHPR has been localized in the junctional T-tubule membranes (Jorgensen et al., 1989; Flucher et al., 1990). Freeze–fracture of junctional T-tubules reveals rows of integral membrane particles arranged in tetrads (Block et al., 1988). Based on the localization of the DHPR and on the observation that the junctional tetrads are missing in dysgenic mouse muscle that has a deficiency in the DHPR  $\alpha_1$  sub-

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1. *Abbreviations used in this paper:* DHPR, dihydropyridine receptors; E–C, excitation–contraction; FLT, fast localized transient; HS, horse serum; KLH, keyhole limpet hemocyanine; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; mdg, muscular dysgenesis; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubules, transverse tubules.

unit, the tetrads are believed to represent groups of four DHPRs (Franzini-Armstrong et al., 1991). The orientation and spacing of the tetrads matches the disposition of the junctional feet, which span the gap between the SR and the T-tubules in regular intervals. The feet correspond to the large cytoplasmic domains of homotetramers of RyR (Wagenknecht et al., 1989). The matching disposition of DHPR and the RyR in the apposing membranes of the triad is indicative of their close interaction.

The rapid transduction of membrane depolarization into calcium release from SR in vertebrate twitch muscles necessitates the proximity of the voltage sensor (DHPR) and the calcium release channel (RyR). Physical coupling of the DHPR and the RyR has been proposed as the mechanism of E-C coupling in skeletal muscle (Schneider and Chandler, 1973). A revised version of this model is today widely accepted; however, it still awaits experimental confirmation (for review see Fleischer and Inui, 1989; Ríos et al., 1991). This model assumes that upon depolarization of the T-tubule membrane, the charge movement in the voltage sensor is directly transduced into a conformational change in the RyR, which in turn opens its calcium pore. In addition to the proposed role in E-C coupling, direct interactions between the DHPR and RyR may be involved in the formation of the triad during development (Yuan et al., 1991). In this case specific binding of the two membrane proteins may either play a role in the association of T-tubules and SR, or may create a diffusion-trap mechanism responsible for the concentration of DHPRs and RyR at contact sites between the membrane compartments. The concomitant expression of the DHPR and RyR during development is consistent with this idea (Yuan et al., 1991). However, direct evidence for binding of RyR and DHPR is lacking.

Triadin is a 95-kD integral membrane protein, isolated from heavy SR fractions, that binds both the RyR and the DHPR (Brandt et al., 1990; Kim et al., 1990). It is located on the junctional face of the terminal cisternae (Caswell et al., 1991). Thus, triadin is a candidate for mediating interactions between the RyR and DHPR and could play a role in the development or function of the triad.

The dysgenic mutation has become a valuable tool in the study of E-C coupling and triad formation (for review see Adams and Beam, 1990; Flucher, 1992). Muscular dysgenesis (*mdg*) in the mouse is a lethal recessive mutation (Pai, 1965; Bowden-Essien, 1972) of the skeletal muscle DHPR  $\alpha_1$  subunit (Tanabi et al., 1988) resulting from a single nucleotide deletion at position 4010 of the DHPR transcript (Chaudhari, 1992). Homozygous dysgenic muscle lacks E-C coupling (Powell and Fambrough, 1973; Klaus et al., 1983), L type calcium conductance (Beam et al., 1986; Rieger et al., 1987) as well as the charge movement associated with voltage sensing (Adams et al., 1990). The  $\alpha_1$  subunit of the DHPR is missing from dysgenic muscle (Knudson et al., 1989) and the targeting and organization of the  $\alpha_2$  subunit is impaired (Flucher et al., 1991b). De novo expression of the  $\alpha_1$  subunit from normal non-muscle cells fused with dysgenic myotubes restores the functions as well as the structural organization of the E-C coupling apparatus (Courbin et al., 1989; Rieger et al., 1987; Flucher et al., 1991b, 1992).

We have investigated the interactions of the RyR and tri-

adin with the DHPR by examining the effects of the deficiency in the DHPR on the organization and function of SR proteins in developing dysgenic muscle *in vitro*. The results of this study indicate that the DHPR  $\alpha_1$  subunit is not required for the normal organization of the RyR and triadin in the terminal cisternae of SR. Further, important calcium release properties of SR, such as spontaneous and caffeine-induced calcium transients are essentially unaffected by the lack of E-C coupling in dysgenic myotubes.

## Materials and Methods

### Animals

Newborn and embryonic mice, both homozygous dysgenic mutants, *mdg/mdg*, and their normal littermates, *+/mdg?* (*+/+* or *+/mdg?*) were obtained by dated pregnancies of heterozygous matings. No morphological, histological, or physiological differences between control *+/+* of the strain or *+mdg?* mice have been reported. Sprague-Dawley rats (Charles River) were mated overnight and dated pregnancies were assessed by remains of vaginal plugs. 15-d-old embryonic rats were used to obtain spinal cord cells.

### Cell Cultures

Primary muscle cultures were prepared from 18-d-old embryonic or newborn *mdg/mdg* or *+mdg?* mice as previously described (Klaus et al., 1983; Courbin et al., 1989; Flucher et al., 1991b). Briefly, hindlimb muscle was digested in a solution of 0.125% trypsin and 0.05% pancreatin in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HBSS. The cell suspension was filtered and centrifuged. The pellet was resuspended in plating medium containing three parts complete medium, consisting of DME with 10% horse serum (HS), 10% FBS (both GIBCO-BRL, Gaithersburg, MD), and 2% chick embryo extract, to one part conditioned medium obtained from 5-d-old muscle cultures. After preplating to enrich for myoblasts, dysgenic cultures were plated onto carbon-coated coverslips with 0.1% gelatin at  $5 \times 10^3$  cells per 13-mm coverslip or  $1 \times 10^4$  per 25-mm coverslip. Once myotubes had formed and cultures were near confluency, the cultures were treated with  $10 \mu\text{M}$  Ara-C, (Sigma Immunochemicals, St. Louis, MO) to prevent fibroblastic overgrowth. Cultures were maintained in contraction medium (DME, 10% HS, 1.25% chick embryo extract) in a humidified incubator at  $37^\circ\text{C}$  for 2-3 wk before fixing and immunolabeling or were kept quiescent by incubation in medium containing 12 mM  $\text{K}^+$  until use. Cultures typically contained a heterogeneous population of myotubes of different developmental stages. Contractility was achieved after about one week and the number of myotubes forming cross-striated myofibrils increased with time in culture. Both normal and dysgenic myotubes reached a degree of differentiation equivalent to the myofiber stage *in vivo*, although fewer myotubes did so in dysgenic cultures (Flucher et al., 1992).

To achieve restoration of E-C coupling in dysgenic myotubes, rat fibroblasts were added to dysgenic myoblasts at the onset of fusion, 3-5 d after initial plating. The fibroblasts were derived from a spontaneous transformation within a culture prepared from newborn rat sciatic nerve sheath. These cells were maintained in DME, 10% HS, 5% FBS. Cells were added to dysgenic cultures at  $2 \times 10^4$  cells per 35-mm dish. "Rescued" cultures were fixed 11 to 18 d after the addition of fibroblasts. Co-cultures were treated with Ara-C and maintained in contraction medium as above.

### Immunofluorescence Labeling of Cultured Muscle Cells

The co-cultures used in the "rescue" experiments were incubated in  $10 \mu\text{g/ml}$  Hoechst nuclear dye (No. 33342; Polysciences, Inc., Warrington, PA) for 45 min at room temperature and rinsed several times in PBS, pH 7.3, before fixation. All cultures were fixed in 100% methanol at  $-20^\circ\text{C}$  for 10 min, rehydrated by plunging into PBS at room temperature and then rinsed in several changes of PBS. Subsequently, the cultures were incubated with 10% normal goat serum in PBS containing 0.1% BSA (PBS/BSA) for 30 min or longer and the incubated in primary antibodies for at least 2 h at room temperature or overnight at  $4^\circ\text{C}$ . After washing in five changes of PBS/BSA the cultures were incubated in fluorochrome-conjugated secondary antibodies for 1-2 h at room temperature and washed again. Finally, they

were mounted in 90% glycerol, 0.1 M Tris, pH 8.0, with 5 mg/ml *p*-phenylene diamine to retard photobleaching.

The semiquantitative analysis of labeling patterns was performed by screening double labeled coverslips with a 40× objective on a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Thornwood, NY) and scoring all segments of myotubes visible at one time in the field. If clustered distribution of both antigens was observed, regardless of the degree of maturation (random or cross-striated organization), the myotube was rated as "clustered." If no clusters could be seen in a myotube segment, it was rated as "diffuse." The latter includes diffuse distribution of fluorescence without discernable substructure and diffuse distribution in fine linear structures presumably representing SR. In dysgenic myotubes double labeled with anti-RyR and anti-DHPR the scores are based on the RyR label only.

## Antibodies

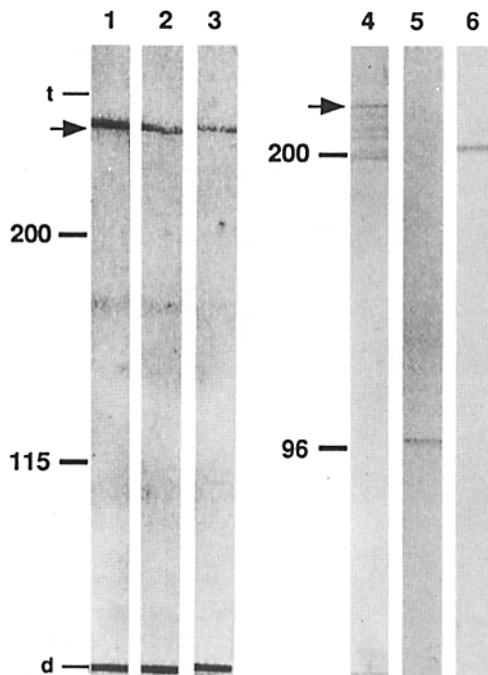
**RyR Antibody.** A sequence-specific antibody (anti-RyR #5) was prepared against a synthetic peptide corresponding to a known RyR amino acid sequence from rabbit skeletal muscle (22 amino acids, 1333-1353, [C-ENLRRSAGGWGEAEGGKEGTA]) (Takeshima et al., 1989). A cysteine was added to the NH<sub>2</sub>-terminal end so that it could be linked to keyhole limpet hemocyanine (KLH) via  $\epsilon$ -amino lysines using the bifunctional agent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). The antigen was injected into rabbits using the protocol of Gonatas et al. (1987). Affinity-purified antibody was prepared from the antiserum (McGray et al., 1989) and was characterized on a Western blot of isolated terminal cisternae of SR. The terminal cisternae was separated by SDS-PAGE on a 6% resolving gel and the protein was transferred to a Immobilon-P membrane (0.45  $\mu$ m PVDF) for 16 h at 150 mA constant current. The membrane was blocked with nonfat milk protein and strips were probed with RyR antibody #5. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody. (Western blot courtesy of Dr. A. Timerman, Vanderbilt, Nashville, TN.) The antiserum to peptide #5 (which has only 18% sequence identity with the heart RyR) is specific for rabbit skeletal muscle RyR from terminal cisternae of SR (Saito et al., 1984) and does not cross-react with cardiac RyR (Fig. 1). In contrast, the antibody to peptide #8 (20 amino acids, 4681-4700), which has 86% sequence identity with the heart receptor strongly reacts with cardiac RyR by Western Blot analysis (not shown).

Other primary antibodies used in this study were: a mouse mAb GE 4.90 specific to triadin used at a concentration of 15  $\mu$ g/ml (Caswell et al., 1991); mouse mAb 1A (specific for the  $\alpha_1$  subunit of the DHPR) (Morton and Froehner, 1987) used at a concentration of 0.1  $\mu$ M IgG; and monoclonal mouse antibody 5D2 specific to the fast isoform of Ca<sup>2+</sup>-ATPase used at 5  $\mu$ g/ml (Kaprielian and Fambrough, 1987). As secondary antibodies in immunofluorescence staining, rhodamine-conjugated goat anti-mouse IgG (BCA/Cappel Products, Organon Technika) and fluorescein-conjugated goat anti-rabbit IgG (Miles Scientific) were used at dilutions of 1:500 and 1:400, respectively. For specificity of antibodies, see Table I.

The primary antibodies were analyzed on Western blots of a crude membrane preparation of mouse skeletal muscle. Leg muscle was homogenized in a Polytron homogenizer and subsequently in a glass-teflon homogenizer. The homogenate was centrifuged at 500 *g* for 10 min at 4°C and then the supernatant was centrifuged at 200,000 *g* for 2 h. The pellet was resuspended in 2% SDS, separated on a 4–20% gradient SDS-PAGE gel and transferred onto a 0.22  $\mu$ m nitrocellulose membrane. The membrane was blocked with BSA, incubated in the primary antibodies and subsequently in alkaline phosphatase-conjugated secondary antibodies (Fig. 1, lanes 4–6).

## Calcium Imaging and Measurements

Recordings of intracellular free calcium concentrations were performed as described in Flucher and Andrews (1992), but with a dual-emission pho-

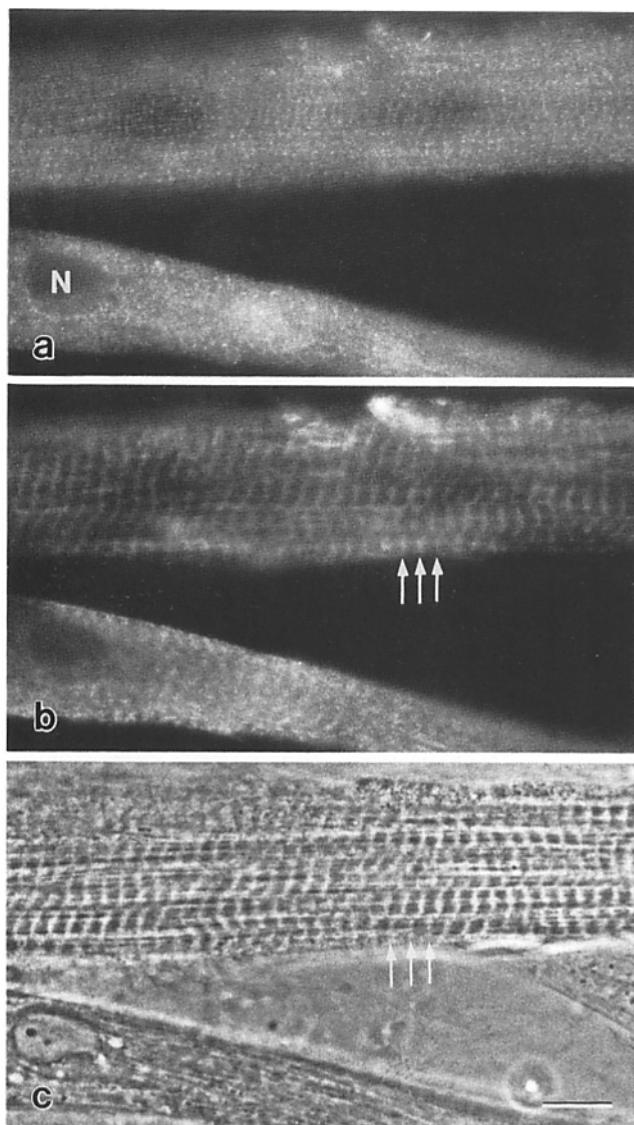


**Figure 1.** Western blot analysis of primary antibodies. The affinity-purified antibody to the calcium release channel peptide #5 (see Materials and Methods) was analyzed on rabbit skeletal muscle terminal cisternae at dilutions of 1:1,000, 1:5,000, and 1:10,000 (lanes 1, 2, and 3, respectively). The arrow indicates positive reactivity to the RyR in the immunoblot. Molecular weight markers were determined by the position of the calcium pump protein (115 kD) and myosin (200 kD) as visualized by amido black staining. The top (*t*) of the resolving gel and the dye front (*d*) are indicated. Antibodies #5 (anti-RyR), GE 4.90 (anti-triadin), and 1A (anti-DHPR) were further analyzed on a crude membrane preparation of mouse muscle (lanes 4, 5, and 6, respectively). Anti-RyR reacted with a major band at about 400 kD (arrow) and with several minor bands just below (probably proteolytic fragments). Anti-triadin and anti-DHPR each labeled a single band at approximately 96 kD and 200 kD, respectively. Molecular weights were determined by high molecular weight standards on adjacent lanes.

tometry system. Cultures plated on 25-mm round coverslips were rinsed once in serum-free DME without phenol red (GIBCO-BRL) and then incubated in the fluorescent calcium indicator indo-1 (5  $\mu$ M) or in fluo-3 AM (5  $\mu$ M) together with fura-red (10  $\mu$ M) or SNARF-1 (0.5  $\mu$ M) plus 0.1% Pluronic F-127 (all Molecular Probes, Eugene, OR) in the same medium for 30 min at room temperature. After another rinse the coverslips were transferred into the observation chamber (Leiden dish) and mounted in a holder or a micro-incubation chamber (Medical Systems, Greenvale, NY) on the stage of a Zeiss Axiovert inverted microscope. The observation chambers were modified to accommodate at least three infusion needles to drain and fill the chamber with medium or experimental solutions. Two needles on opposite sides of the chamber were also connected to the output of a Grass stimulator for the electrical field stimulation. A 1-ms pulse of

**Table I.** Primary Antibodies Used in the Present Study

Antigen	Type	Code	Reference
RyR (peptide 1333-1353)	Rabbit (affinity purified)	#5	present study
Triadin	Mouse monoclonal	GE 4.90	Caswell et al., 1991
DHPR, $\alpha_1$ subunit	Mouse monoclonal	1A	Morton and Froehner, 1987
Ca <sup>2+</sup> -ATPase (fast isoform)	Mouse monoclonal	5D2	Kaprielian and Fambrough, 1987



**Figure 2.** Double immunofluorescence labeling of the RyR and the  $\text{Ca}^{2+}$ -ATPase in skeletal myotubes. The RyR (*a*) is localized in clusters which are aligned in transverse double rows in well cross-striated myotubes (upper myotube), as seen with phase contrast optics (*c*), and randomly distributed in less differentiated (lower) myotubes.  $\text{Ca}^{2+}$ -ATPase (*b*), a general SR marker, labels in a cross-banded pattern. Cross striations of  $\text{Ca}^{2+}$ -ATPase are prominent in the cross-striated myotube seen in phase contrast but are also present in the less differentiated myotube. The  $\text{Ca}^{2+}$ -ATPase cross-bands correspond to accumulations of SR at the I bands (arrows) which contain no detectable amounts of RyR. The RyR clusters are localized on both sides of the SR bands, at the A-I border, which is the site of the triads in mature muscle. N, nucleus. Bar, 10  $\mu\text{m}$ .

15–20 V was sufficient to stimulate an action potential in electrically active cells. Change of experimental solutions was accomplished by complete aspiration of the solution, immediately followed by loading through another infusion needle.

Fluorescent calcium imaging and measurements were carried out with epifluorescence using a narrow-band 488 nm excitation filter for fluo-3 and 365 nm for indo-1. The illumination from a 100 W mercury arc lamp was attenuated with a 3% neutral density filter and the illuminated area was restricted by closing the field diaphragm to just outside the field of observation. The fluorescence signals emitted during a calcium transient were

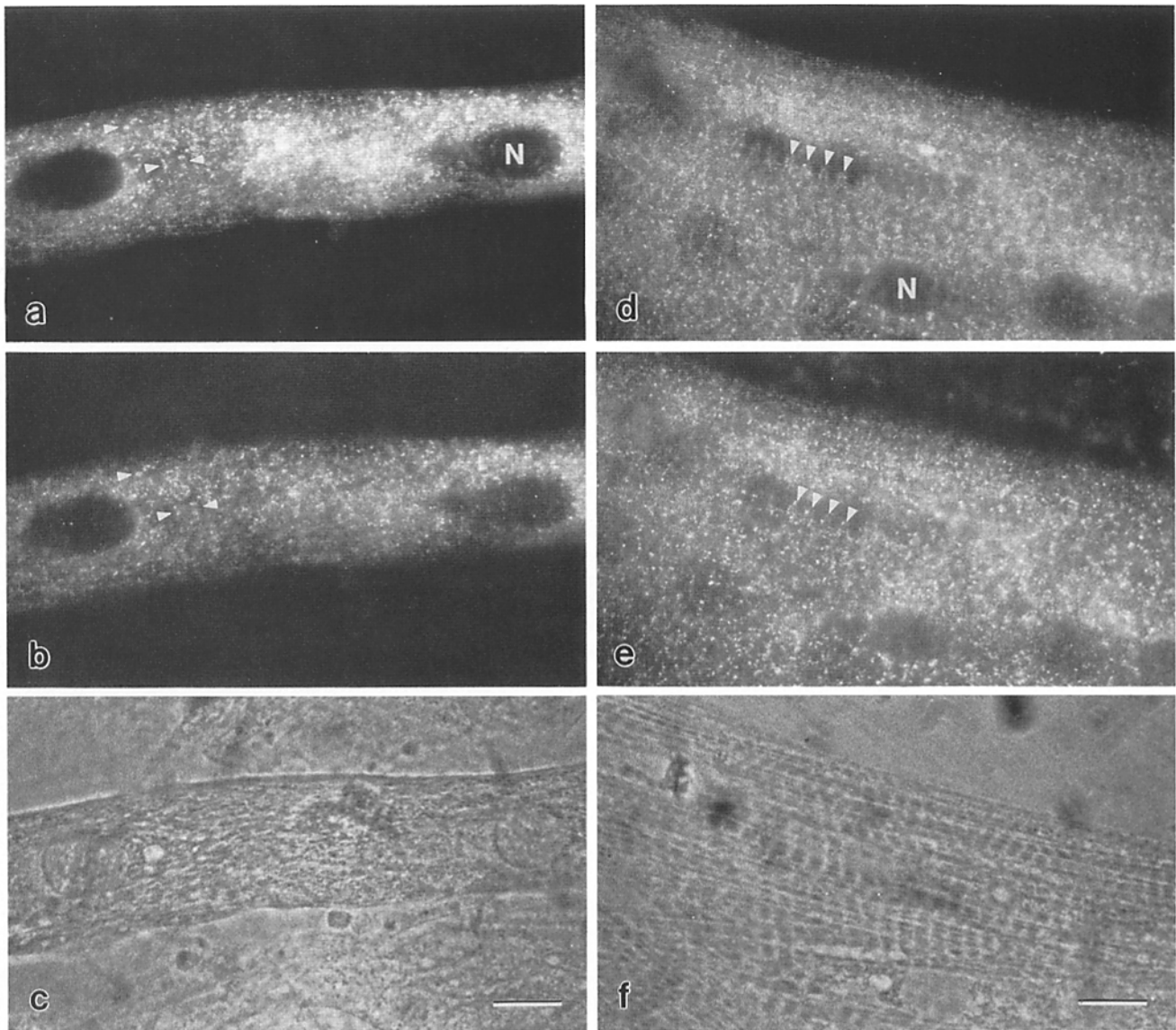
strong enough to be seen with the naked eye through the eyepieces, which facilitated the screening of cultures and the selection of sample myotubes. Images were intensified with a multichannel plate intensifier (Video Scope International, Washington, DC), captured at video rate with a CCD camera (Pulnix America Inc., Sunnyvale, CA) and recorded on a 3/4-in tape recorder (Sony U-matic; Sony, Montvale, NJ). Video sequences were subsequently processed and analyzed with the Image-1/AT (Universal Imaging Corp., West Chester, PA) image processing unit. Fluorescent point measurements were performed with a Photon Technology International (PTI, S. Brunswick, NJ) dual photometer system. The variable measuring window was set to a width corresponding to a field of  $30 \times 30 \mu\text{m}$  on the specimen. The following dichroic mirrors and barrier filters (Omega Optical, Brattleboro, VT) were used to select the appropriate emission wavelength for the various indicators: 590 nm dichroic, 530 nm for fluo-3, and 610 nm for SNARF-1 or 660 nm for fura-red; 440 nm dichroic, 405 and 495 nm for indo-1. Both channels were recorded at a sampling rate of 200 Hz and the ratio of the two emission wavelengths was calculated using the PTI *Oscar* software. Dual-emission ratios from cells simultaneously loaded with two indicators corresponded well to the ratios obtained with the dual-emission dye indo-1. However, the dye combinations containing fluo-3 were superior in sensitivity and signal-to-noise ratio. Furthermore, fluo-3 as the primary calcium indicator allowed screening of the cultures by eye as well as video recording in parallel with microfluorometry.

## Results

### *Distribution of the Ryanodine Receptor and Triadin in Normal Myotubes*

Primary cultures from normal mouse skeletal muscle labeled with an anti-peptide antibody specific to the skeletal muscle ryanodine receptor showed intense labeling of multi-nucleated myotubes (Fig. 2). The labeling pattern was punctate. In myotubes with little organization of the sarcomeres, as determined by phase contrast optics, the punctate label was randomly distributed (Fig. 2 *a*, lower myotube). In myotubes with cross-striated organization the immunolabel was arranged in double rows of dots (Fig. 2 *a*, upper myotube), similar to those patterns observed with other triad-specific antibodies in comparable cultures and in tissue sections (Flucher et al., 1990, 1991b). Double labeling with an antibody to the fast isoform of the skeletal muscle  $\text{Ca}^{2+}$ -ATPase (Fig. 2 *b*) showed that the double rows of punctate RyR label are localized on both sides of the  $\text{Ca}^{2+}$ -ATPase cross bands. The  $\text{Ca}^{2+}$ -ATPase antibody labeled the whole SR uniformly, and the cross bands correspond to SR accumulations in the region of the A band. The RyR labeling pattern is consistent with the known location of triads in mouse myotubes (at the A/I borders) of that developmental stage. The randomly distributed RyR label in less organized myotubes may correspond to early junctions (diads, triads, inverted triads) between SR and T-tubules with no apparent relation to the myofibrils. Distinct labeling patterns of RyR and  $\text{Ca}^{2+}$ -ATPase were observed in myotubes throughout development, indicating that the RyR in the SR is always clustered in specific domains. In no case was the RyR seen in a non-punctate, diffuse distribution pattern throughout the SR as will be noted below in dysgenic myotubes (see Fig. 5 *a*).

Double labeling of the RyR and the  $\alpha_1$  subunit of the skeletal muscle dihydropyridine receptor (DHPR), which is located in the junctional T-tubule membrane, resulted in very similar labeling patterns of both proteins (Fig. 3). The location, size and relative fluorescent intensity of the punctate DHPR label matched that of the RyR both in poorly organized (Fig. 3, *a-c*) and in cross-striated (Fig. 3, *d-f*) myotubes. In 97% of analyzed normal myotubes ( $n = 132$ ),



**Figure 3.** Double immunofluorescence labeling of the RyR and the DHPR  $\alpha_1$  subunit in normal myotubes. Phase contrast images show a myotube without cross-striated organization of the myofibrils (c) and a cross-striated myotube (f). Clusters of RyR (a and d) and DHPRs (b and e) are colocalized in the less differentiated myotubes (a–c) and in cross-striated myotubes (d–f) (examples indicated by arrowheads). The high coincidence of RyR and DHPR clusters indicates that the sites of RyR clustering represent junctions between the SR and T tubules. N, nuclei. Bars, 10  $\mu\text{m}$ .

RyR and DHPR labels were punctate and colocalized. This high correspondence of the RyR and DHPR labels in cultured myotubes indicates that the RyR clusters resemble junctions between SR and T-tubules. Individual RyR clusters without their counterpart in the T-tubule membrane were observed only rarely in normal myotubes.

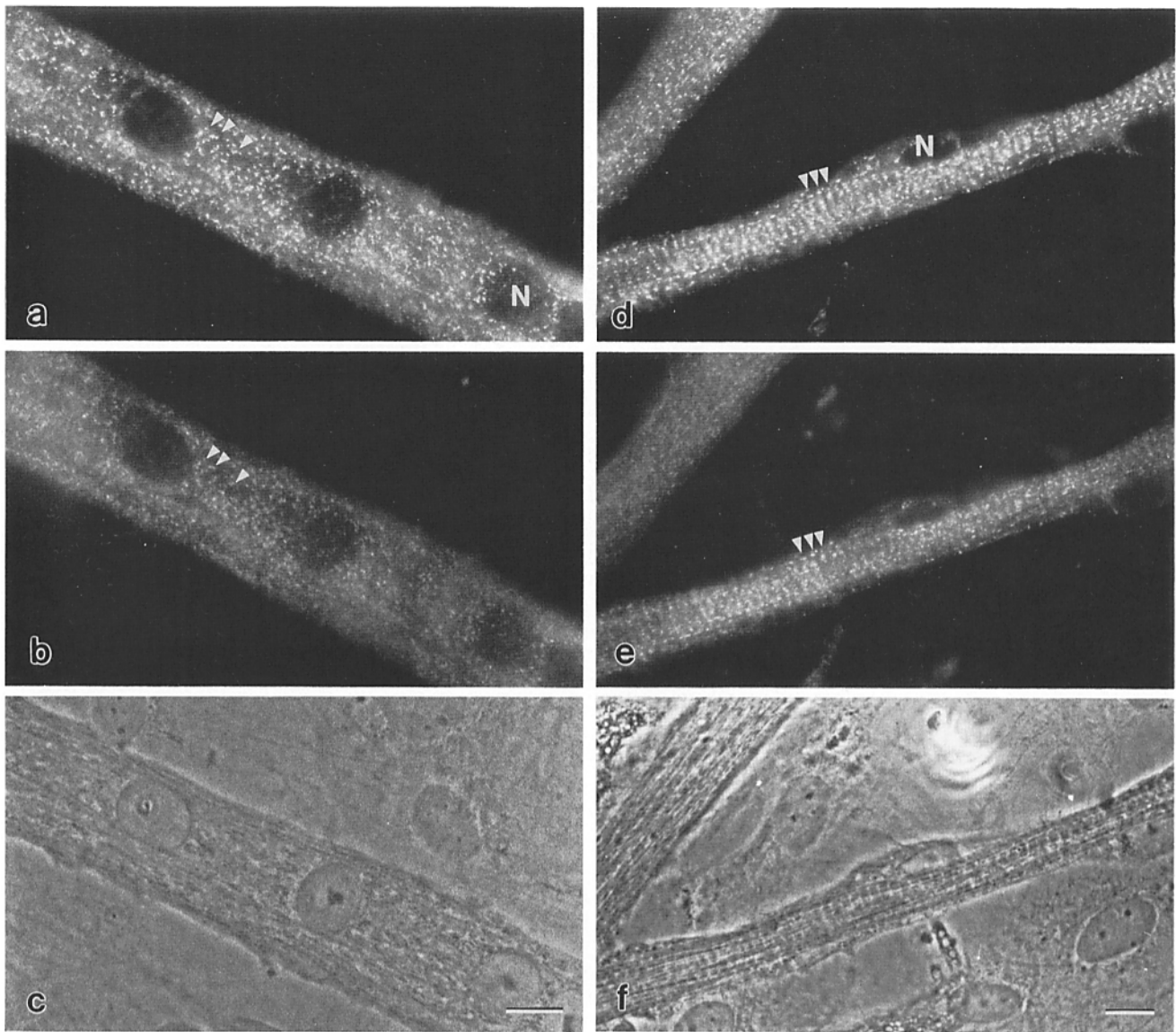
Double labeling of normal myotubes with the RyR antibody and a mAb specific to triadin also resulted in very similar distribution patterns of both proteins (Fig. 4). Triadin, an integral membrane protein from heavy SR, was localized in randomly distributed clusters in myotubes with little organization of the sarcomeres (Fig. 4, a–c) and in well-aligned double rows of clusters in well differentiated myotubes (Fig. 4, d–f). Like the RyR, triadin was at no stage observed diffusely distributed throughout the SR. Although the RyR label was generally brighter than triadin label, the punctate

distribution of both labels was colocalized in 96% of analyzed myotubes ( $n=203$ ). The excellent correspondence of RyR and triadin distribution patterns in developing myotubes indicates that both membrane proteins are constituents of the SR/T-tubule junctions from the early stages of development. Rare examples of cells were observed that only labeled with the RyR antibody and not with triadin or in which the labeling patterns for RyR and triadin were distinct. However, since these cases were infrequent and the labeling patterns inconsistent, we do not believe that these represented normal developmental states of myotubes.

#### **Distribution of the Ryanodine Receptor and Triadin in Dysgenic Myotubes**

Myotubes grown from skeletal muscle of homozygous dysgenic mice (*mdg/mdg*) lack the  $\alpha_1$  subunit of the DHPR.



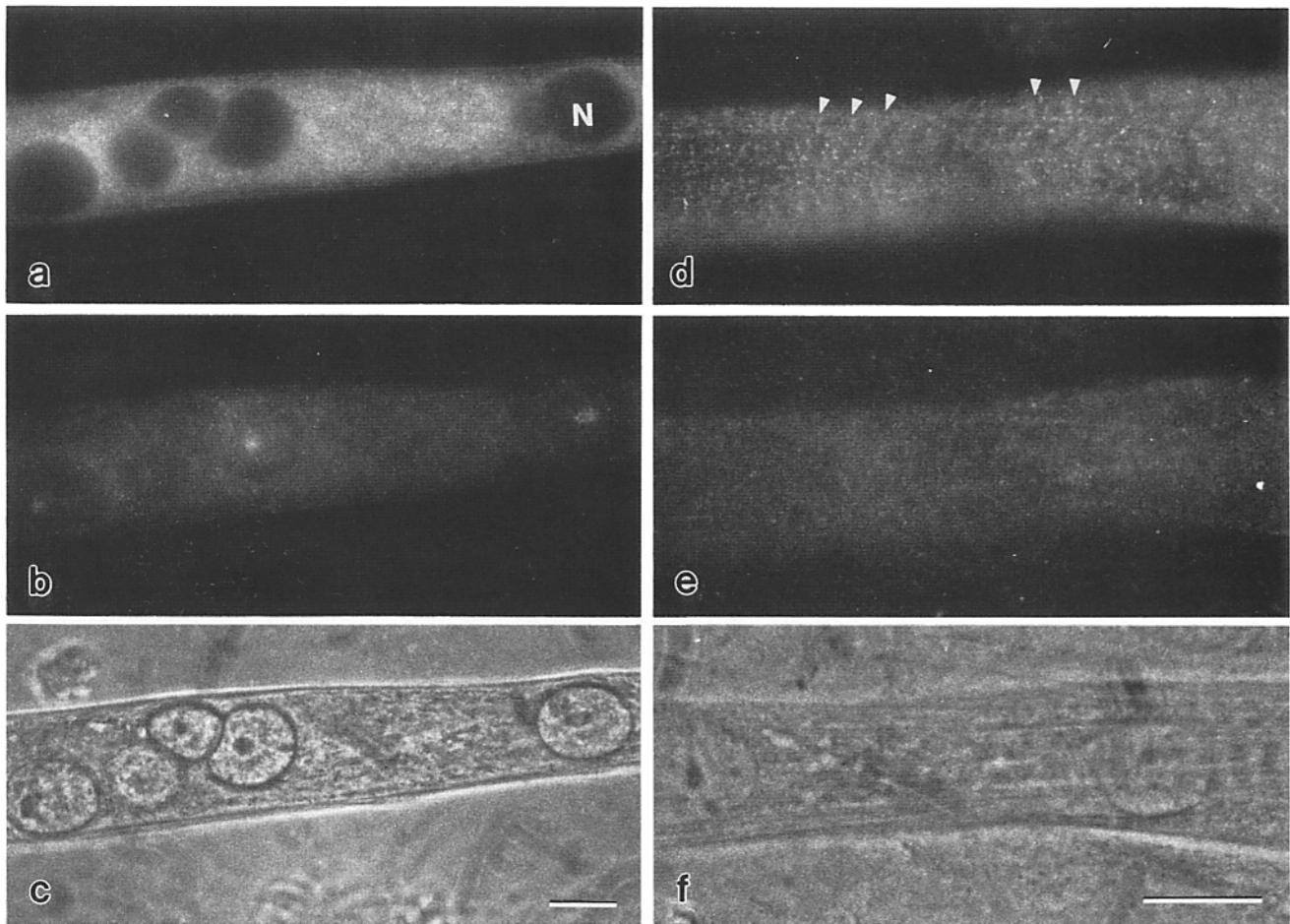


**Figure 4.** Double immunofluorescence labeling of the RyR and triadin in normal myotubes. Triadin labeling (*b* and *e*) results in a punctate distribution pattern in myotubes with and without cross-striated organization of the myofibrils (*f* and *c*, respectively). Clusters of RyR (*a* and *d*) and triadin (*b* and *e*) are colocalized in myotubes of all stages (examples indicated by arrowheads), showing the close spatial and developmental association of triadin and the RyR in junctional SR. *N*, nuclei. Bars, 10  $\mu\text{m}$ .

Double labeling with anti-RyR and anti-DHPR showed that the RyR is expressed in dysgenic myotubes whereas the DHPR is lacking (Fig. 5). The distribution patterns of RyR in dysgenic myotubes were different from those found in normal myotubes and varied considerably. In the majority of dysgenic myotubes—79% in a sample of 542 analyzed segments—RyR immunolabel was diffuse, not punctate and weaker than in normals (Fig. 5 *a*; cf. Fig. 4 *a*). The distribution within a myotube was often uneven. However, 21% of the analyzed myotubes showed punctate RyR distribution (Figs. 5 *d* and 6 *a* and *d*), but usually at lower density than in normal myotubes (cf. Fig. 4, *a* and *e*). Since the  $\alpha_1$  subunit of the DHPR is not expressed, it is unclear if these randomly distributed RyR clusters in dysgenic myotubes correspond to SR/T-tubule junctions. In some well-differentiated dysgenic myotubes, however, the RyR clusters were ar-

ranged in clear double rows (Figs. 5 *d* and 7 *a*), indistinguishable from normal distribution patterns. Even in those cases DHPR label was absent (Fig. 5 *e*). Thus, the double rows of RyR clusters very likely represent SR/T-tubule junctions in the absence of the DHPR  $\alpha_1$  subunit, corresponding to triads observed in electronmicroscopic preparations of dysgenic muscle *in vivo* (Armstrong, 1991).

Double labeling of dysgenic myotubes with RyR and triadin antibodies revealed that triadin is expressed in the mutant myotubes and that its distribution patterns are highly variable (Fig. 6). In myotubes with clustered RyR distribution triadin followed the distribution of RyR (Figs. 6 *b* and 7 *b*). In 25% of 150 analyzed myotubes triadin immunolabel was punctate and colocalized with RyR label. Triadin- and RyR-positive clusters were found randomly distributed or in highly regular, cross-striated arrays (Fig. 7). Apart from the



**Figure 5.** Double immunofluorescence labeling of the RyR and the DHPR  $\alpha_1$  subunit in dysgenic (*mdg/mdg*) myotubes. The DHPR  $\alpha_1$  subunit is lacking from all dysgenic myotubes (*b* and *e*). RyR is expressed, but occurs in variable distribution patterns (*a* and *d*). The RyR is often diffusely distributed in the myotube (*a*) or is found in punctate distribution patterns (*d*). Occasionally, normal double rows of RyR clusters are seen in cross-striated myotubes (*d*, arrowheads), indicating the potential of dysgenic muscle for normal molecular organization of the RyR in the SR. *c* and *f* are corresponding phase contrast images. *N*, nucleus. Bar, 10  $\mu\text{m}$ .

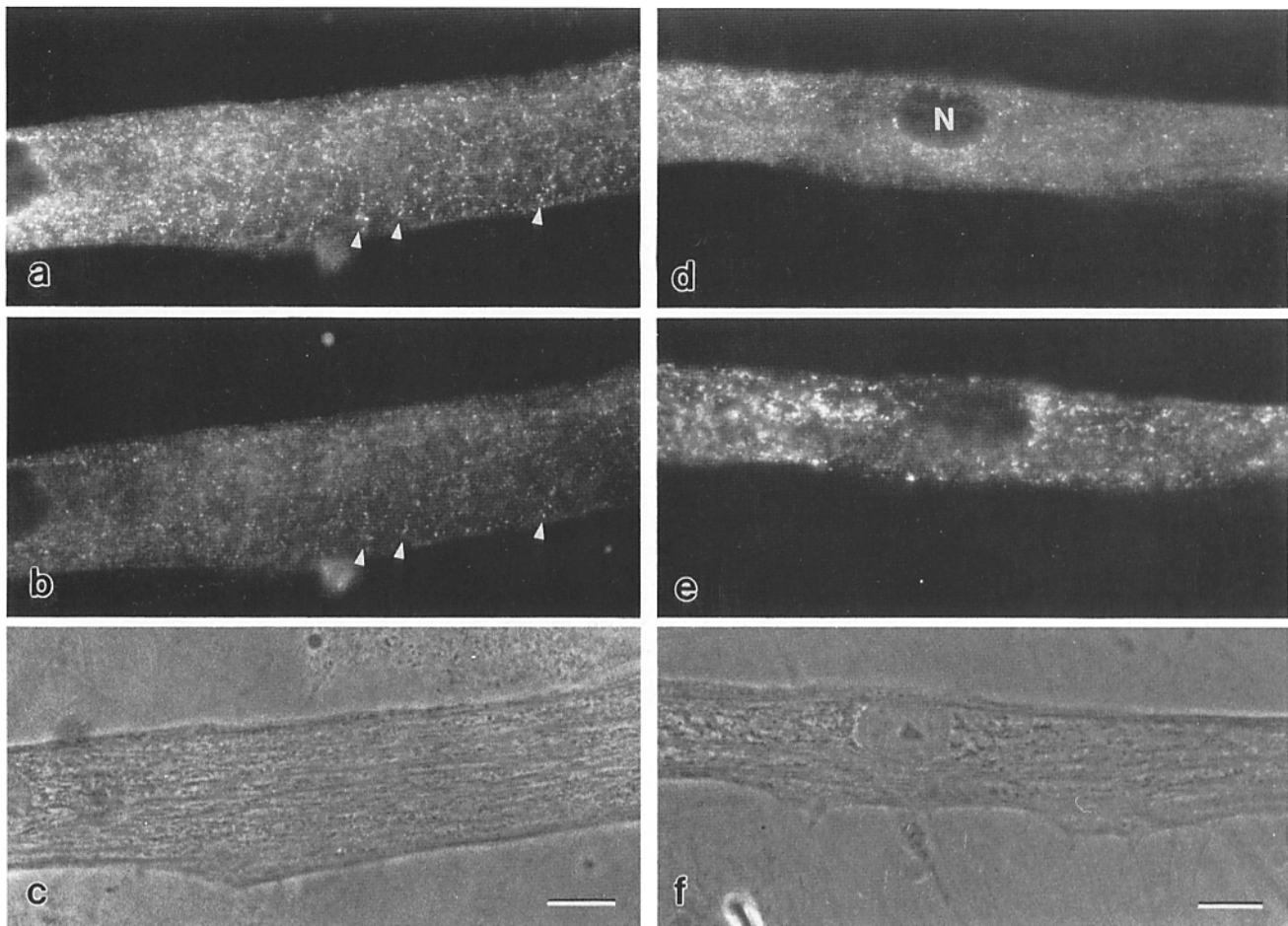
clusters, triadin did not closely follow the distribution of RyR. In myotubes with little RyR label, triadin was unevenly distributed (sometimes more abundant than RyR) but it did not form normal clusters (Fig. 6 *e*). Thus, triadin appears to depend on the RyR for its proper organization in the junctional SR; alternatively, the organization of both membrane proteins may be accomplished by a common mechanism.

#### **Calcium Release from SR of Dysgenic Myotubes**

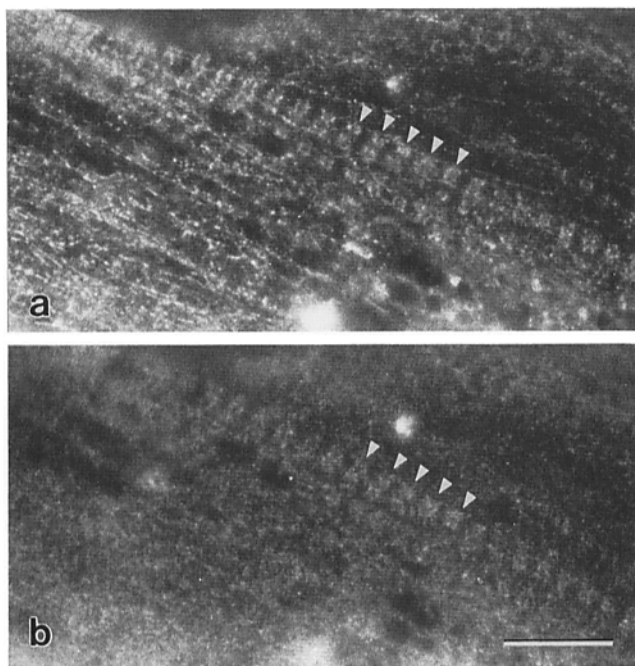
To characterize the properties of the RyR uncoupled from the DHPR in the dysgenic system, the release of calcium from the intracellular stores (SR) was monitored with fluorescent calcium indicators. Normal mouse myotubes in culture show three types of calcium transients (Fig. 8, *a-d*) (see also Flucher and Andrews, 1993): (*a*) a brief focal release event without considerable spread or propagation (fast localized transient, FLT) occurs frequently in quiescent (non-contracting) myotubes but ceases soon after the developmental onset of spontaneous contractions; (*b*) spontaneous or electrically induced calcium transients that occur synchronously throughout a myotube and are associated with contractions (action potential-induced transients); and (*c*) a slowly

propagated calcium wave that can be induced by low concentrations of caffeine (2 mM) or non-physiological culture conditions (pH, osmolarity). In addition, the action potential-induced transient is prolonged in the presence of 2 mM caffeine. All transients can be sustained in calcium-free medium, indicating that they represent calcium release from internal stores. The caffeine sensitivity of action potential-induced transients and the slowly propagated calcium wave suggest that the ryanodine-sensitive calcium release channel (RyR) underlies these transients.

In dysgenic cultures calcium transients could not be induced by electrical stimulation. However, FLT's as well as propagated calcium waves were frequently observed (Fig. 8, *h* and *i*). Thus, normal association of the RyR and triadin—as inferred from their distribution patterns—are not sufficient to restore E-C coupling by themselves. Conversely, FLT's and calcium waves proved to be independent of the presence of DHPR and E-C coupling. Since FLT's and caffeine-induced calcium waves could be observed in the majority of dysgenic myotubes while only a small portion reached the apparently normal clustered organization of the RyR, the generation of these two types of calcium transients



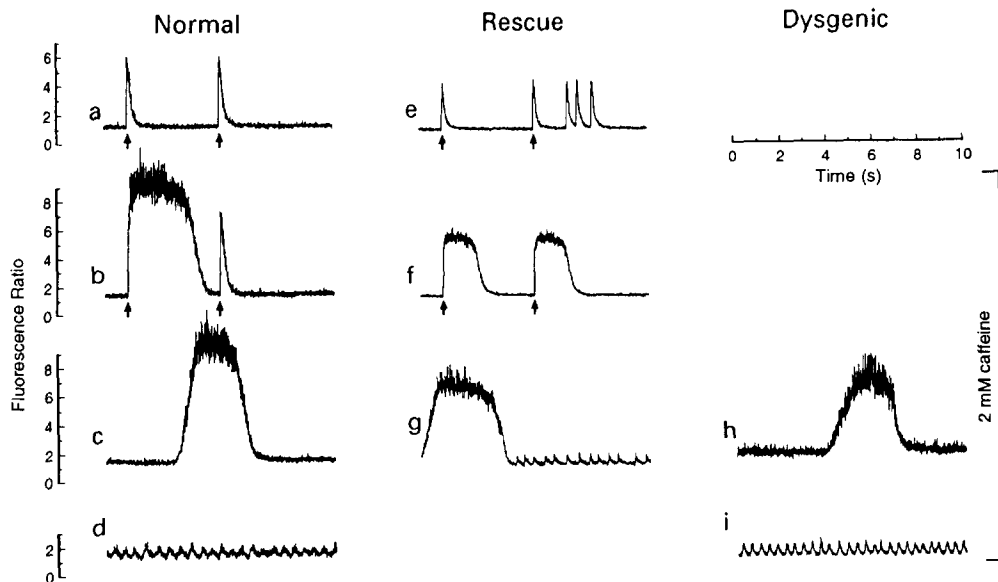
**Figure 6.** Double immunofluorescence labeling of the RyR and triadin in dysgenic myotubes. In myotubes with clustered RyR distribution (a), triadin is generally colocalized with the RyR clusters (b, examples indicated by arrowheads), regardless of the degree of structural differentiation of the myotube. In dysgenic myotubes expressing little RyR (d), triadin shows irregular distribution patterns distinct from RyR label (e). These distribution patterns suggest that RyR is required for the correct distribution of triadin. c and f are corresponding phase contrast images. N, nucleus. Bar, 10  $\mu$ m.



is not dependent on the ability of the RyR to form clusters. Interestingly, FLTs were observed more frequently in dysgenic than in normal myotubes and calcium waves were sometimes obtained without addition of caffeine. This could result directly from deficient calcium regulation in dysgenic muscle due to the lack of the DHP-sensitive calcium channel, or it could reflect the altered disposition of the calcium release system as seen by the aberrant distribution of the RyR. A comparison of resting calcium levels in normal and dysgenic myotubes did not show any significant differences in the two culture systems (not shown). Generally, resting calcium levels varied more in quiescent myotubes than in ac-

**Figure 7.** Double labeling of RyR (a) and triadin (b) in a dysgenic myotube. A region of a dysgenic myotube shows colocalization of RyR and triadin in cross-striated double rows of clusters (arrowheads), presumably representing junctions between SR and T-tubules in their mature location in the sarcomere. In regions where RyR labels linear structures (lower left), triadin labeling patterns are less defined and different from the RyR distribution. Bar, 10  $\mu$ m.





**Figure 8.** Calcium transients in normal, dysgenic, and rescued mouse myotubes. Myotubes were simultaneously loaded with the fluorescent calcium indicators fluo-3 and fura-red and analyzed with dual channel microfluorometry. The traces are representative ratios from single simultaneous fluo-3 and fura-red recordings. Normal myotubes (a–d) respond to electrical stimulation (arrows) with a transient increase of cytoplasmic free  $[Ca^{2+}]$ . In the presence of 2 mM caffeine, action potential-induced  $Ca^{2+}$  transients are increased in amplitude and prolonged (b); however, prolonged release activity is blocked immediately after a preceding transient (second transient in b). Inactive normal myotubes in 2 mM caffeine give rise to propagated  $Ca^{2+}$  waves (c) (a–c are recordings from a single measuring point). Immature myotubes with and without caffeine give rise to fast localized  $Ca^{2+}$  transients (d). Dysgenic myotubes rescued with normal rat fibroblasts (e–g) show similar action potential-induced and spontaneous  $Ca^{2+}$  transients (e and f are from the same measuring point). The myotube in e contracted spontaneously after the second stimulated twitch. g was recorded at the border of a rescued domain, the small transients following the  $Ca^{2+}$  wave represent weak spontaneous contractions (compare e) in this area. Dysgenic myotubes (h–i) generate caffeine-induced  $Ca^{2+}$  waves and fast localized transients, but electrical stimulation did not induce  $Ca^{2+}$  transients. Both  $Ca^{2+}$  waves and fast localized transients were qualitatively similar to those in normal myotubes. 10-s traces recorded at 5 ms/sample.

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tive myotubes. This was also reflected by a higher variability of calcium levels in the inherently quiescent dysgenic cultures.

### Restoration of E-C Coupling and the Normal Organization of RyR

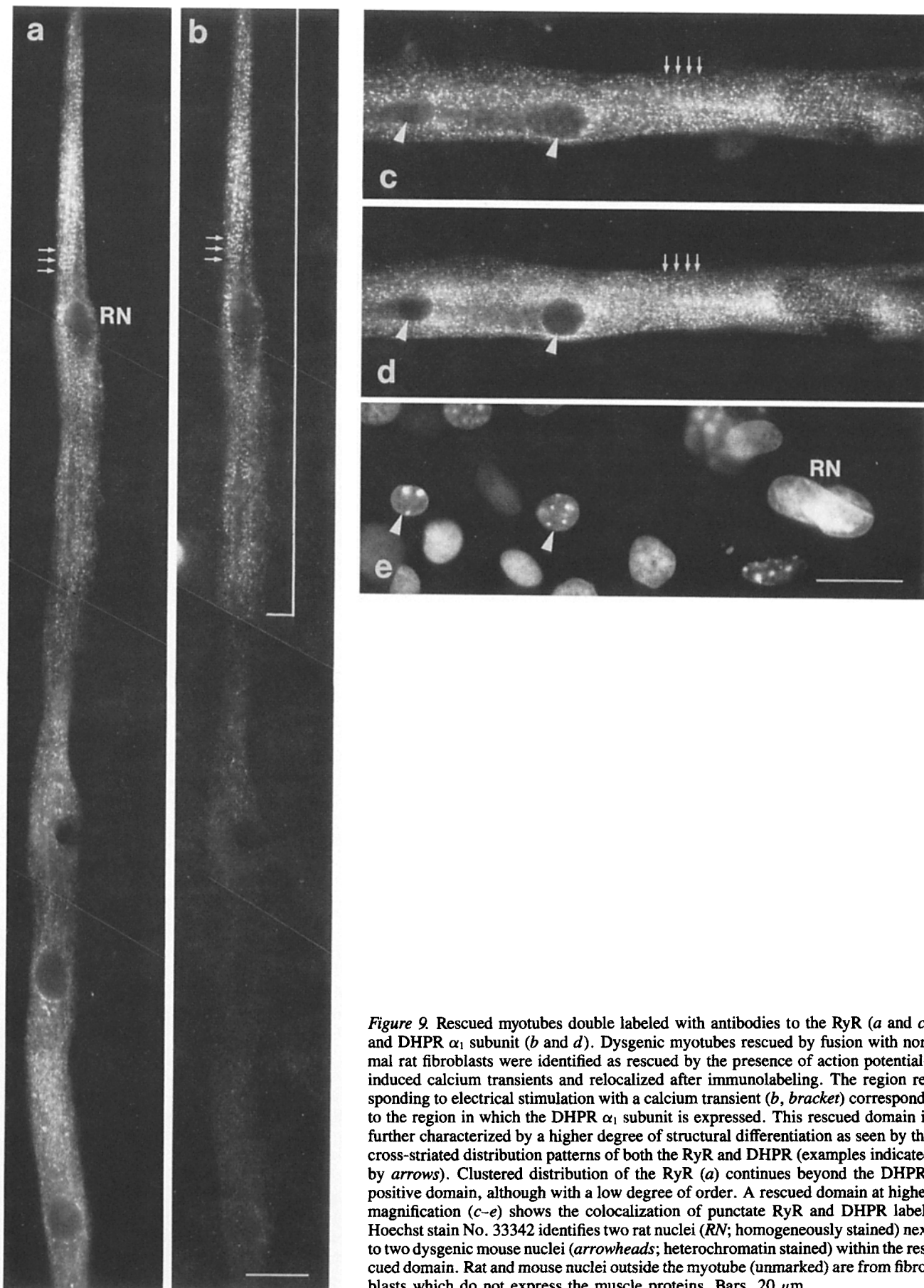
Fusion of normal rat fibroblasts with dysgenic myotubes locally restores the expression and normal distribution of the DHPR  $\alpha_1$  subunit as well as spontaneous contractility. Cocultures were loaded with fluorescent calcium indicators and calcium transients were monitored. Many myotubes behaved like dysgenic cultures, generating FLT and calcium waves but not responding to electrical stimulation. However, in some myotubes action potential-induced calcium transients could be stimulated, or were found accompanying spontaneous contractile activity. These calcium transients were similar in shape and caffeine-sensitivity to action potential-induced transients in normal cultures (Fig. 8, e–g). The region of a myotube responsive to stimulation was locally restricted; however, the size of the rescued calcium transients varied greatly. In some myotubes the intensity of the action potential-induced calcium transients tapered off near the border of the rescued domain (Fig. 8 g), in other myotubes the calcium transient ended abruptly. Occasionally, action potential-induced transients gave rise to calcium waves at the border of the rescued domain (Fig. 8 g).

Rescued myotubes identified by means of calcium indicators were relocated after fixation and immunolabeling and then examined for the expression and distribution patterns of the DHPR and the RyR (Fig. 9). Rescued domains always

contained at least one normal rat nucleus, identified with the nuclear dye Hoechst No. 33342 (Fig. 9 e). The region showing action potential-induced calcium transients coincided with the region of DHPR expression. RyR clusters were colocalized with DHPR clusters in the rescued domain, but expression of RyR continued outside the DHPR-positive domain. Within rescued domains the degree of organization was generally higher and cross-striated arranged RyR clusters were frequently observed. These results suggest that expression of the DHPR or restoration of contractile activity promotes the differentiation of the SR/T-tubule system and probably of the entire contractile apparatus as well.

### Discussion

We have compared the expression and distribution patterns of two integral membrane proteins of the SR—the RyR and triadin—in normal and dysgenic muscle *in vitro*. The study shows that during normal development RyR and triadin are colocalized in clusters, presumably at SR/T-tubule junctions, which become arranged in regular cross-striated arrays. In dysgenic myotubes both SR proteins are expressed but frequently fail to cluster. However, RyR in dysgenic myotubes still possess the capacity to cluster and to become organized into cross-striated arrays, despite the lack of the DHPR  $\alpha_1$  subunit. Parallel investigation of the calcium release properties in normal and dysgenic myotubes shows that although E-C coupling is lost in muscular dysgenesis, spontaneous and caffeine-induced calcium release from SR are unaffected by the generally poor organization of the RyR.



**Figure 9.** Rescued myotubes double labeled with antibodies to the RyR (*a* and *c*) and DHPR  $\alpha_1$  subunit (*b* and *d*). Dysgenic myotubes rescued by fusion with normal rat fibroblasts were identified as rescued by the presence of action potential-induced calcium transients and relocalized after immunolabeling. The region responding to electrical stimulation with a calcium transient (*b*, *bracket*) corresponds to the region in which the DHPR  $\alpha_1$  subunit is expressed. This rescued domain is further characterized by a higher degree of structural differentiation as seen by the cross-striated distribution patterns of both the RyR and DHPR (examples indicated by *arrows*). Clustered distribution of the RyR (*a*) continues beyond the DHPR-positive domain, although with a low degree of order. A rescued domain at higher magnification (*c-e*) shows the colocalization of punctate RyR and DHPR label. Hoechst stain No. 33342 identifies two rat nuclei (RN; homogeneously stained) next to two dysgenic mouse nuclei (*arrowheads*; heterochromatin stained) within the rescued domain. Rat and mouse nuclei outside the myotube (unmarked) are from fibroblasts which do not express the muscle proteins. Bars, 20  $\mu\text{m}$ .

### **Organization of the RyR and Triadin during Normal Development**

In normal myotubes developing in culture, the RyRs appear concentrated in clusters in the SR, unlike the Ca<sup>2+</sup>-ATPase, which is first expressed in terminally differentiated myoblasts and is evenly distributed throughout the SR (Jorgensen et al., 1977; Flucher, B. E., unpublished results). Thus, the RyR is either initially expressed in concentrations below detectability and then becomes aggregated, or it is inserted into the SR in high concentrations and prevented from diffusion after insertion. The RyR-rich membrane domains may represent junctional SR which will subsequently differentiate into the terminal SR cisternae. A single mechanism may be responsible for the initial anchoring of the RyR as well as for the maintenance of the specialized composition of junctional SR in the mature triad. The finding that the RyR clusters colocalize with the DHPR of the junctional T-tubule membrane suggests that the process of RyR aggregation or insertion is closely associated with the formation of the early SR/T-tubule junctions. Electron microscopic studies provide supporting evidence for the formation of junctions during this period of development (Daniels, M. P., and B. E. Flucher, unpublished results). Colocalization of the RyR and DHPR has previously been observed in developing rabbit muscle in vivo (Yuan et al., 1991). Based on this finding a model has been proposed by which RyR/DHPR complexes form between transport vesicles of SR and T-tubule membranes before their insertion into the T-system. However, this appears not to be the case in our system, because cultured mouse and rat myotubes form an elaborate T-tubule system before or simultaneously with the expression of RyR and DHPR (Flucher et al., 1991a,b).

The developmental expression of triadin in normal mouse myotubes corresponds closely to that of the RyR. Furthermore, both proteins are invariably colocalized with one another in clusters, suggesting participation of triadin in newly formed SR/T-tubule junctions. The triadin distribution patterns found in myotubes as well as its colocalization with the RyR are in agreement with its distribution in developing rat muscle in vivo (D. Ferguson, personal communication). The simultaneous expression of two specific integral membrane proteins of the terminal SR cisternae are indicative of the differentiation of the junctional SR membranes at this developmental stage. It will be interesting to see if a luminal protein specifically localized in the mature terminal cisternae, e.g., calsequestrin, becomes enriched in the junctional domain at the same stage. The close developmental and morphological association of triadin and the RyR supports the suggested physical association of the RyR and triadin in the triad and its potential involvement in E-C coupling (Kim et al., 1990). Since triadin binds to the RyR as well as the DHPR, triadin may also serve in linking the terminal SR cisternae to the T-tubules during development. Such a function is consistent with the simultaneous clustering of triadin, the RyR, and the DHPR.

### **Consequences of the Lack of DHPR $\alpha_1$ in Dysgenic Myotubes on Triad Formations**

It has yet to be determined which proteins are critically involved in interactions leading to the formation of the triad. The dysgenic system allows us to address this question with

respect to the involvement of the DHPR in triad formation. If the  $\alpha_1$  subunit of the DHPR is required for the association of SR and T-tubules and for the molecular organization of the junctional SR membranes, then SR/T-tubule junctions should not form in dysgenic myotubes; neither should the constituents of the terminal cisternae assume their normal disposition. Although in many dysgenic myotubes triads seem to be missing (Banker, 1977; Pinçon-Raymond et al., 1985; Courbin et al., 1989) and the RyR as well as triadin fail to form clusters (present study), we regularly found examples of dysgenic myotubes that achieved an apparently normal molecular and structural organization of the SR proteins under investigation. The RyR and triadin colocalized in clusters which were in some cases arranged in transverse double rows, apparently positioned in their mature location at the A-I borders. We have previously shown that T-tubules could be organized in similar regular arrays in normal and dysgenic myotubes (Flucher et al., 1991b, 1992). Further, triads with discernible feet were observed in dysgenic muscle in vivo (Franzini-Armstrong, 1991). Thus, it is likely that the RyR/triadin-positive clusters described here represent junctions between SR and T-tubules that do not contain the skeletal muscle DHPR  $\alpha_1$  subunit.

A low level of expression of the DHPR  $\alpha_1$  subunit could not be ruled out by biochemical studies on dysgenic mice (Tanabe et al., 1988; Chaudhari, 1992; but see Knudson et al., 1989). Thus, residual DHPRs could conceivably account for the formation of a small number of triads containing both RyR and DHPR. However, in double labeling experiments we did not detect any clusters of DHPRs colocalized with the RyR clusters, and calcium recordings further confirmed that all dysgenic myotubes lack E-C coupling. Even if undetectable levels of DHPR existed in these presumptive triads, they could not account for the expected association of every (or every other) foot with a tetrad of DHPRs (Block et al., 1988). Thus, it appears that the skeletal DHPR  $\alpha_1$  subunit plays no critical role in the molecular specialization of the terminal SR cisternae. Consequently, the junctional SR membrane appears to possess the potential for self-assembly, including the determination of the regular spacing of the junctional feet. Nevertheless, it remains possible that the normal organization of DHPR into junctional tetrads in the T-tubule membrane depends on interactions with the RyR.

The formation of triads is undoubtedly reduced in dysgenic muscle, indicating a role of the DHPR (direct or indirect) in triad formation. However, RyR clusters are still observed in myotubes in which the DHPR is undetectable. These could represent junctions which do not require the  $\alpha_1$  subunit of the DHPR or they could appear as a consequence of the low-level expression of an alternative isoform not detectable by the antibody used in the present study. Dysgenic myotubes express a DHP-sensitive calcium current (Adams and Beam, 1989) as well as low levels of mRNA for the cardiac DHPR  $\alpha_1$  subunit (Chaudhari and Beam, 1993). However, the mRNA levels for the cardiac isoform diminish within 5 d in culture, whereas we observed putative junctions in 2–3-wk-old cultures. Other constituents of the T-tubules which may be potential candidates for interactions with the SR include the DHPR  $\alpha_2/\delta$ ,  $\beta$ , and  $\gamma$  subunits (Takahashi et al., 1987; Jorgensen et al., 1989; Flucher et al., 1990). To date we have no information as to whether the  $\beta$  or  $\gamma$  subunits are targeted correctly into the T-tubules in

the absence of the  $\alpha_1$  subunit. However, the  $\alpha_2$  subunit, and consequently the  $\delta$  subunit, fail to aggregate in T-tubules of dysgenic myotubes and are instead mistargeted to the plasma membrane (Flucher et al., 1991b). Therefore the  $\alpha_2/\delta$  complex is an unlikely candidate for involvement in the connection of SR and T-tubules. A possible role for the membrane cytoskeleton in triad formation must be considered. However, until now only one member of this class of proteins has been identified in the triad, namely ankyrin, and it is not known if ankyrin is associated with the SR or T-tubules (Flucher et al., 1990).

Triadin does not require the DHPR  $\alpha$  subunits for its anchoring in the terminal cisternae. Therefore, its specific binding to the RyR may underlie its location in the triad. Indeed, in those myotubes where RyR was severely underexpressed, triadin was found in a very uneven distribution, whereas in myotubes that expressed the RyR, the distribution of triadin closely resembled that of the RyR. Thus, triadin appears to depend on the RyR or on a common anchoring mechanism for its own arrangement.

If SR proteins are capable of organizing normally in the absence of DHPR, why should many dysgenic myotubes fail to do so? The lack of proper compartmentalization of the SR cannot arise from a general arrest in development since the poor organization observed in dysgenic myotubes is never seen even in early stages of development in normal myotubes. Dysgenic cultures appear to have reduced levels of RyR expression. This is consistent with results from an immunoblot study showing a strong reduction of RyR and calsequestrin levels in dysgenic muscle *in vivo* and to a lesser degree in dysgenic myotubes *in vitro* (Knudson et al., 1989), as well as with reduced [ $^3$ H]ryanodine-binding in dysgenic muscle *in vivo* (Pinçon-Raymond et al., 1990). Moreover, the mRNA levels of several muscle-specific proteins fail to show their normal developmental increase between E15 and birth in dysgenic muscle (Chaudhari and Beam, 1989). Thus, direct or indirect effects of the dysgenic mutation appear to impair a regulatory signal of muscle development. It has been repeatedly observed that the rescue of E-C coupling by fusion of normal cells with dysgenic myotubes is accompanied by an improvement of overall differentiation in the rescued domain (Courbin et al., 1989; Flucher et al., 1991b; and present study). Thus, contractile activity and the associated ion fluxes by itself might promote differentiation of myotubes and its lack may lead to the slowing down of developmental processes. However, the disruption of normal RyR distribution can commonly be seen at less differentiated stages (before the onset of activity), which should not be influenced by contractile activity. Alternatively, a change in calcium homeostasis may lead to the described adverse effects on E-C coupling proteins. This possibility is further supported by the observations that dysgenic myotubes show fast localized calcium transients more frequently than normal myotubes and that calcium waves are often generated without the addition of caffeine.

### ***SR Calcium Release in Dysgenic Myotubes***

Dysgenic muscle has long been known to lack E-C coupling, and we know now that this deficiency is due to the lack of the voltage sensor (Tanabe et al., 1988; Adams et al., 1990). Occasionally, dysgenic myotubes achieved an apparently nor-

mal organization in the protein composition and disposition of the terminal SR cisternae. However, without *de novo* expression of DHPRs this high degree of organization did not restore E-C coupling. Only when expression of the DHPR was restored in hybrids of dysgenic myotubes and normal non-muscle cells could contractions be observed. The calcium transients accompanying electrically stimulated contractions in rescued hybrid myotubes were similar to action potential-induced calcium transients in normal myotubes. The size of the rescued domain generating action potential-induced transients corresponded to the region where the DHPRs were expressed, thus demonstrating the close relationship of DHPR expression and the rescue of E-C coupling.

It was of further interest to determine the properties of uncoupled RyR in an otherwise intact system. In addition to action potential-induced calcium release from the SR, normal myotubes generate spontaneous FLT<sub>s</sub> and caffeine-induced propagated calcium waves in the absence of extracellular calcium (Flucher and Andrews, 1993). FLT<sub>s</sub> and calcium waves were also frequently observed in dysgenic myotubes and their properties were unchanged compared to normal myotubes. This observation supports the proposition that both types of transients represent release of calcium from the intracellular stores of the SR, with the RyR being the prime candidate for the underlying release channel. Furthermore, it shows that the calcium stores in dysgenic muscle are functional and that during early stages of myogenesis, dysgenic myotubes experience the same fluctuations in cytoplasmic calcium levels as normal myotubes. The gating mechanism for FLT<sub>s</sub> is unknown, whereas calcium waves are thought to represent calcium-induced calcium release (Jaffe, 1991; Lechleiter and Clapham, 1992; but see Allbritton et al., 1992). Our observation that both these transients can be supported by the calcium release apparatus of dysgenic myotubes indicates that the spontaneous and calcium-induced opening of the RyR as well as its caffeine sensitivity are properties of the release channel that are not under the control of the voltage sensor.

In the present study we demonstrated that: (a) the RyR and triadin appear together in specific domains of the SR upon formation of junctions with T-tubules; (b) that the normal composition and arrangement of junctional SR can occur in dysgenic myotubes; and (c) that the lack of the DHPR in dysgenic myotubes eliminates E-C coupling without interfering with other gating modes of the RyR or with its drug sensitivity. These findings provide strong evidence for the autonomous molecular organization of the junctional SR membrane as well as for an independent function of the calcium storage and release system apart from E-C coupling.

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