Triadins Modulate Intracellular Ca\(^{2+}\) Homeostasis but Are Not Essential for Excitation-Contraction Coupling in Skeletal Muscle\(^*\)**

To unmask the role of triadin in skeletal muscle we engineered pan-triadin-null mice by removing the first exon of the triadin gene. This resulted in a total lack of triadin expression in both skeletal and cardiac muscle. Triadin knockout was not embryonic or birth-lethal, and null mice presented no obvious functional phenotype. Western blot analysis of sarcoplasmic reticulum (SR) proteins in skeletal muscle showed that the absence of triadin expression was associated with down-regulation of junctophilin-1, junctin, and calsequestrin but resulted in absence of triadin expression.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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3 The abbreviations used are: DHPR, dihydropyridine receptor; RyR1, Ryanodine receptor 1; Csq, calsequestrin; CRU, calcium release unit; SR, sarcoplasmic reticulum; jSR, junctional SR; EC, excitation contraction; FDB, flexor digitorum brevis; TA, tibialis anterior; SoI, soleus; EDL, extensor digitorum longus; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase.
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Generation of Triadin-null Mice—A mouse genomic DNA library was screened with a rabbit triadin cDNA fragment to yield a 14-kb DNA fragment containing the complete Exon-1 and a partial fragment of Intron-1 of Trdn gene. A targeting construct was designed by flanking the entire Exon-1 and its putative promoter regulatory region with two loxP sites. The floxed Exon was then inserted into pTKLNCL vector (kindly provided by Dr. Rick Mortensen), downstream of the cytochrome deaminase/neomycin expression cassette (Fig. 1A). The vector was electroporated into 129/SvJ ES cells, and the recombined cells were then microinjected into C57BL/6N blastocysts to generate chimeric mice. Heterozygous Trdn+/loxP/+ mice, which lack both Exon-1 and the CD-Neo selection cassette, were then interbred to obtain homozygous Trdn-null mice. Genotyping was performed by PCR and Southern blotting analysis using genomic DNA (Fig. 1B).

Cell Culture and Ca2⁺ Imaging—Primary myotubes from triadin-null and wild-type mice were isolated according to the method of Rando and Blau (27). The myoblasts were grown and differentiated as described previously (28). Calcium imaging was performed 4–5 days after differentiation, in myotubes loaded with 2 μM Fluo-4 AM (Molecular Probes, OR). The cells were imaged in imaging buffer (mM: 125 NaCl, 5 KCl, 2 CaCl₂, 1.2 MgSO₄, 6 glucose, and 25 HEPES, pH 7.4) at 490–500 nm using a DG4 multiwavelength light source with a Stanford Photonics 12 bit digital intensified charge-coupled device, and the data were displayed and analyzed using QED imaging software (QED Software, Pittsburgh PA). Depolarization-initiated Ca²⁺ release was tested by exposing the cells to increased voltage steps using electrical field stimulation in imaging buffer without CaCl₂ and supplemented with 0.5 mM CdCl₂ and 0.1 mM LaCl₃. Chemical depolarization was achieved by a 10-s exposure to high K⁺ buffer (28), supplemented with or without 2 mM Ca²⁺ plus Cd²⁺ and La³⁺, using a multivalve perfusion system (AutoMate Scientific, Inc., Oakland, CA).

SR Ca²⁺ Loading Content Determination—Relative SR Ca²⁺ content levels of myotubes and flexor digitorum brevis (FDB) fibers were estimated from the magnitude of the Ca²⁺ release induced by 40 mM caffeine in Fluo-4-AM-loaded cells. Caffeine was supplemented with 1 μM thapsigargin and 0.5 mM Cd²⁺/0.1 mM La³⁺ to block the SERCA pump and avoid Ca²⁺ entry from the extracellular medium, respectively. Single muscle fibers from FDB muscle were enzymatically dissociated with collagenase as described previously (29) and were imaged at 1 fps in the presence of 100 μM n-benzyl-p-toluenesulfonamide to prevent muscle contraction. Total SR calcium content was expressed as the area under the curve of the Ca²⁺ release transient induced by 70- to 90-s exposure to the caffeine mixture.

Resting Free Ca²⁺ Measurements—Determination of myoplasmic resting free Ca²⁺ in myotubes and intact muscles was performed with double-barreled Ca²⁺-selective microelectrodes assembled with ETH129 resin as described previously (28, 30).

Intact Adult Fiber Studies—Intact lumbricalis muscles (1.0–2.0 × 0.2–0.3 mm) from the hind foot were dissected in ice-cold Krebs-Henseleit solution (mM: 119 NaCl, 4.6 KCl, 1.8 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 glucose) containing 30 mM 2,3-butanedione monoxime (Sigma, St. Louis, MO), mounted in the Guth muscle research apparatus and loaded with 5 μM Fura 2-AM (Molecular Probes, Eugene, OR) in an oxygenated Krebs-Henseleit solution containing 0.5% cremophore for 1 h, as described previously (31). Fura-2 fluorescence, expressed as a 340 nm/380 nm excitation ratio, was collected with a 4-ms resolution timeframe.

Membrane Vesicle Preparation and Immunoblotting—Microsomal vesicles were prepared from lower limb and individual muscles (tibialis anterior (TA), soleus (Sol), and extensor digitorum longus (EDL)) of wild-type and triadin-null mice, 6–8 weeks old. Muscles were homogenated in a Polytron cell disruptor in 5 mM imidazole, pH 7.4, 300 mM sucrose supple-
FIGURE 1. **Targeted disruption of Exon-1 of mouse triadin gene.** A, restriction map of mouse triadin gene showing the wild-type allele (top), targeting vector (first middle), recombinant allele before Cre recombination (second middle), and recombinant null allele after Cre recombination (bottom). The Exon-1 (filled boxes), cyto- 

tene resistance cassette (arrow), and loxp sites (open boxes) are indicated. Dashed lines indicate the backbone of the vector. Localization of hybridization probes is indicated by open arrows. B, BamHI; N, NdeI; P, Pvull; P*, deleted PvuII site. B, Southern blot analysis of mouse tail DNA from floxed intermates. Digestion with NdeI and hybridization with probe P1 (left panel) generated a 6.5- and a 4.3-kb fragment, for the wild-type and mutant allele, respectively. Digestion with BamHI and hybridization with probe P2 (middle panel) generated a 8.2-kb fragment and a 6.0-kb fragment, for the wild-type and mutant allele, respectively. Tail DNA from hetero- and homozygo 

gous triadin-null mice (right panel) was digested with PvuII/BamHI and then hybridized with probe P3. The wild-type allele is 5.5 kb, and the null allele is 6.7 kb. C, Western blot analysis of triadin expression in skeletal muscles. 40 μg/lane of microsomal vesicles from wild-type (+/+), triadin hetero (+/-), and homozygous (−/−) lower limb muscles were analyzed with triadin-specific antibodies GE 4.90. D, triadin expression pattern of cardiac (lanes a and b) and skeletal muscle (lanes c and d) from wild-type (+/+), and triadin-null (−/−) mice was compared with canine cardiac (lane e) and skeletal (lane f) muscle using the site-specific PA-TRN6 antibody. E, profile of triadin expression in fast and slow twitch muscle was analyzed in microsomal fractions (40 μg/lane) of EDL, TA, and Sol muscles from wild-type (+/+), and triadin-null (−/−) using PA-TRN6 antibody.

**Immunolabeling**—Sternomastoid, EDL, and soleus muscles were fixed with 3.5% glutaraldehyde in 0.1 m sodium cacodylate buffer, pH 7.2. Muscles were post-fixed in 2% OsO₄ in 0.1 m sodium cacodylate for 1 h at room temperature, stained en bloc with saturated uranyl acetate in 70% ethanol, and embedded in Epon. Ultrathin sections (70–90 nm) were stained with saturated uranyl acetate solution in 50% ethanol and Sato lead solution (34). The frequency of triads in different orientations was counted in images from longitudinal sections covering a 36-μm² area. Two or three images were taken for each fiber.

**RESULTS**

**Generation of Triadin-null Mice**—For the 5’ sequence the triadin 95 cDNA was used as probe to isolate the 5’ fragment of the triadin gene (*Trdn*) in a 129sv genomic DNA library and used to create a targeting construct. Because of the suspected role of triadin in EC coupling, a conditional knockout strategy was attempted. The targeting vector was designed to conditionally delete Exon-1, and its putative 5’-untranslated region, in a tissue-specific manner by “floxing” the exon and the Neo/CD selection cassette with three loxp sites (Fig. 1A). ES cells carrying the appropriately targeted allele were injected into C57Bl6 blastocysts and introduced into pseudo pregnant female mice. Germ line chimeric mice carrying the floxed alle-
les were backcrossed with C57BL6 EIIA cre mice in an attempt to either remove the Neo/CD selection cassette (to generate conditional knockouts) or to remove both Exon-1 and the Neo/CD selection cassette (to generate traditional knockouts). Progeny were screened using PCR (data not shown) and Southern blot (Fig. 1B). Despite several attempts it was never possible to obtain mice with the conditional allele. Heterozygous mice carrying the traditional knockout allele were then backcrossed, and surprisingly the homozygous mice (Trdn−/−) did not exhibit embryonic or birth lethality nor did they demonstrate any obvious gross functional phenotype.

Triadin-null Muscles Lack Expression of All CRU-related Triadin Isoforms—Analyses for expression of triadin isoforms were performed by immunoblot in crude membrane extracts from lower limb muscle using two different anti-triadin antibodies. Fig. 1C compares the triadin expression pattern of wild-type, heterozygous, and homozygous null muscles detected with MA3–297 antibody, specific for the 95-kDa isoform of triadin. As expected, skeletal muscle from heterozygous null mice (+/−) expressed significant amounts of the 95-kDa triadin isoform, although, to a lesser extent than wild-type (+/+). However, no detectable expression levels of this isoform were observed in membrane extracts from homozygous null muscle (−/−). The expression of the other triad-associated triadin isoform was further investigated by using a site-specific antibody (PA-TRN6) raised against residues 146–160 in the luminal domain of mouse cardiac and skeletal muscle triadins (25). In microsomal fractions from wild-type mouse skeletal muscle (Fig. 1D, lane c) and from canine skeletal muscle (Fig. 1D, lane f), this antibody detected two prominent bands of ~60 kDa and 95 kDa, corresponding to the higher molecular weight isoforms of triadin. No detectable levels of either isoform were evident in fractions from triadin-null mice (Fig. 1D, lane d). In addition this antibody did not detect either of the two lower molecular weight forms of triadin (35–40 kDa) that are primarily detected in cardiac ventricle (Fig. 1D, lane e). These two bands, although of different intensities, were readily observed in cardiac ventricular microsomal preparations from wild-type mice (Fig. 1D, lane a) but were absent in similar preparation from triadin-null animals (Fig. 1D, lane b), thus confirming that triadin-null skeletal and cardiac muscles lack any detectable expression levels of triadins.

Triadin expression was further separately tested in fast twitch (EDL and TA) and slow twitch (Sol) muscle. Immunoblot analysis of microsomal fractions from wild-type mice show that each muscle type had a slightly different triadin expression pattern: whereas EDL and Sol muscles primarily expressed the 60-kDa isoform and, to a lesser extent, the 95-kDa isoform of triadin, TA muscles express only the 60-kDa isoform (Fig. 1E). Unlike their wild-type counterparts triadin-null muscles showed no detectable expression levels of either triadin isoform. However, a weak band in the 75- to 80-kDa range was evident in all muscle types tested (Fig. 1, D and E). This band does not correlate with any known isoform of triadin and is assumed to be the result of nonspecific antibody cross-reactivity, because it was present in both wild-type and null mice.

Expression of Other Triadic Proteins in Triadin-null Skeletal Muscle—To address whether or not expression of triadin affected the expression pattern of other CRU proteins that are involved in the organization of the CRU, expression levels of RyR1, DHPR α1S, Csq, junctin, JP-1 and JP-2 were compared in wild-type and triadin-null EDL and soleus muscles (Fig. 2A). To quantify differences a densitometric analysis of the immunoblots was performed, and band densities were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase to correct for possible loading differences. Densitometric data show no significant differences in expression levels of DHPR α1S subunit or JP-2 between triadin-null and wild-type EDL or Sol muscles (Fig. 2, A and B, mean ± S.D., p > 0.05). However, a significant reduction in expression of JP-1 and Csq was detected in both EDL and Sol fibers in triadin-null mice. Expression of JP-1 was reduced to 76 ± 7% and 28 ± 4% of wild-type EDL and Sol fibers, respectively, and Csq was reduced to 76 ± 2% and 62 ± 15% of the wild-type EDL and Sol muscles, respectively (Fig. 2B). Expression of junctin and RyR1 was differentially affected in Sol and EDL muscles. Junctin was significantly down-regulated in EDL muscles (72 ± 2% of wild-type expression) but not in Sol fibers (99 ± 4% of wild-type expression, Fig. 2B), and RyR1 expression showed a slight but significant up-regulation in Sol (133 ± 8% of wild-type expression, Fig. 2B) but not in EDL (114 ± 4% of wild-type expression, Fig. 2B). Interestingly, unlike the adult muscles we detected no significant differences (p > 0.05) in Csq or junctin expression between wild-type and triadin-null myotubes (Fig. 2, A and B).

Immunolabeling for calsequestrin, junctin, and RyR1 showed no detectable changes in the position of these molecules in fast twitch (sternomastoid) muscles of null versus wild-type mice (Fig. 2C). In all cases these proteins were located at spots corresponding to the location of the triads in two transverse bands on either side of the Z lines (note: a positional shift of Csq, affecting only a small percentage of the molecules as was observed by electron microscopy (see below), would not be detectable in these confocal images). Unfortunately anti-triadin antibody GE 4.90, which was used for Western blots, gave nonspecific staining when used on p-formaldehyde fixed tissue in our attempts at triadin immunolabeling (data not shown).

Effect of the Absence of Triadin on the Organization of Junctional and Longitudinal SR—To evaluate the role of triadin on the subcellular architecture of skeletal muscle we analyzed the ultrastructure of junctional and free SR in wild-type and triadin-null fibers from fast twitch (sternomastoid, EDL) and slow twitch (Sol) muscles. The muscle ultrastructure from the null mice was basically unaltered in regards to the disposition and ordering of the myofibrils and cross-striation but showed subtle ultrastructural alterations in the triads and lateral SR that reveal a role for triadin both in retaining Csq within the jSR domains and, perhaps indirectly, in the arrangement of triads within the fiber.

Two structural alterations were observed in triadin-null muscles. One is a disorder in the orientation of the triads. Although located opposite to the edges of the A band as in wild type, some of the triads in null muscles lost their predominant transverse orientation, which is more or less perpendicular to the long axis of the fibers (Fig. 3A, asterisks) and acquired an
Groups of transversely and longitudinally oriented triads occupied adjacent domains within the same fibers, as shown in Fig. 3B (transverse triads, asterisks; longitudinal triads, double arrows). The frequency of longitudinally oriented triads varied in different muscles, and even in different areas of individual fibers, and was affected by the absence of triadin. In wild-type EDL and sternomastoid muscles, triads are almost exclusively transverse, although a small percentage of the areas counted showed a low frequency (5–15%) of longitudinal triads (Fig. 3 and supplemental Fig. S1). Wild-type soleus had no longitudinal triads. Triadin-null EDL and sternomastoid fibers show an increased frequency of areas with only a moderate frequency of triads (5–20%), and more than a third of the areas counted had a higher percentage of longitudinal triads (20 and 70%) than wild-type controls. In triadin-null soleus, on the other hand, longitudinal triads were present in very few areas and only at low frequency (Fig. 3B and supplemental Fig. S1). Average values and statistical analysis are shown in Table 1.

Triads of muscles from adult null and wild-type mice, whether longitudinally or transversely oriented, were undistinguishable from each other in regard to the structure of Csq. In all triads Csq appeared as a finely granular tightly aggregated meshwork within the terminal cisternae of the SR (Fig. 3). How-
ever, a detectable structural alteration of null muscles was the occasional presence of Csq in parts of the SR that are usually devoid of the protein. The misplaced Csq protein was found in tubes of the longitudinal SR that were dilated and located either in close proximity to the junctional SR, or at some distance from it, opposite the middle of either the A band or the I-Z-I region (Fig. 3, B–E, arrowheads). The randomness of this event is best shown in Fig. 3C, in which the SR tubes on the left of the Z line had the normal narrow diameter and an apparently empty lumen, whereas those on the right, even though continuous with the former, had a wider diameter and a visible granular content. Easily seen examples of displaced Csq are not frequent, and they were revealed only by intensive scrutiny of large areas of sectioned muscle. For that reason a quantitative assessment of the changes was not possible. It is likely that minor movements of Csq, below detection threshold by electron microscopy and also by immunolabeling (see above) are widespread but of small magnitude. The soleus muscle did not show this phenotype.

As expected, freeze-fracture analysis of the diaphragm in nine images from a triadin-null pup at E18 showed DHPRs grouped into tetrads indicating that in the absence of triadin, RyR1, and the DHPR maintained their appropriate link (supplementary Fig. S2). The finding is consistent with the Ca2⁺ imaging data that support near normal EC-coupling properties of the triadin-null muscles.

EC Coupling in Triadin-null Myotubes—The effect of triadin ablation on depolarization-initiated Ca²⁺ release was tested in Fluo4-loaded cultured myotubes from wild-type and triadin-null mice using high speed Ca²⁺ imaging in nominal Ca²⁺-free solutions and in Furaco-loaded adult lumbricalis muscles freshly dissected from null and wild-type mice. The presence or absence of triads in these myotubes (Fig. 4A, inset) was confirmed by immunoblot. Field stimulation of wild-type myotubes elicited transient elevations of intracellular calcium in the absence of extracellular Ca²⁺ (i.e. skeletal-type EC coupling). Subsequent addition of 40 mM caffeine induced an even larger Ca²⁺ release from internal stores. The same protocol elicited similar electrically stimulated and caffeine-induced transients in triadin-null myotubes, but both transients were smaller in amplitude (Fig. 4A) than seen in wild-type myotubes.

To probe for possible differences in the sensitivity of wild-type and triadin-null myotubes to depolarization we compared the ability of these cells to respond to increased depolarization steps induced by KCl. In addition to effectively clamping the membrane potential, K⁺ depolarization has the advantage of allowing prolonged plasmalemma depolarization (seconds) without deleterious effects on the integrity of the plasma membrane. As shown in Fig. 4B, both cell types respond to K⁺ in a concentration-dependent manner. However, triadin-null myotubes had a small but statistically significant reduction in their sensitivity to K⁺ depolarization (EC₅₀ 15.1 ± 0.4 mM versus 20.4 ± 0.7 mM for wild-type and nulls myotubes, respectively) and had Ca²⁺ transients that were smaller in amplitude than wild-type myotubes at all KCl concentrations (p < 0.05, Fig. 4C).

Calcium transients induced by 40 mM KCl, in the presence of either extracellular Ca²⁺ or calcium channel blockers, are compared in Fig. 5 (A and B). Triadin-null myotubes had Ca²⁺ transients that were on average 30% smaller in magnitude than those observed in wild-type cells. Normalization of the imaging data to the peak Ca²⁺ transient in each group shows that Ca²⁺ release and uptake kinetics were nearly identical (Fig. 5, C and D) both in the presence and in the absence of extracellular Ca²⁺.

Force Generation Studies in Intact Muscles—The contractile properties of skeletal muscles from hetero- and homozygous triadin-null mice were studied in intact lumbricalis muscles. Muscles were mounted in
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**FIGURE 5.** Comparison of the magnitude of K\(^{+}\)-induced Ca\(^{2+}\) transients of wild-type and triadin-null myotubes. Average magnitude of Ca\(^{2+}\) transients induced by 40 mM KCl in wild-type (black) and triadin-null (gray) myotubes. Transients were measured in the presence of 2 mM extracellular calcium (left panels) and in the absence of calcium plus Cd\(^{2+}\) and La\(^{3+}\) (right panels). Records were normalized by their peak Ca\(^{2+}\) amplitude (bottom panels) to allow for comparison of their Ca\(^{2+}\) transient kinetics. Data are presented as mean ± S.E. The horizontal scale bar indicates 10 s.

**FIGURE 6.** Force generation and Ca\(^{2+}\) release from skeletal muscle. Lumbaricalis muscles from wild-type, heterozygous (+/−), and homozygous (−/−) triadin-null mice were stretched in a Guth muscle apparatus until the maximum active twitch force was obtained and were allowed to equilibrate and develop a stable force for one hour before study. Upper, average normalized force and lower, Ca\(^{2+}\) transients amplitude, normalized by wild-type peak amplitude, in response to 1.0 Hz stimulation. Data are presented as mean ± S.E. (n = 4).

Guth muscle research apparatus and loaded with 5 μM Fura-2 for simultaneous measurement of force generation and global Ca\(^{2+}\) release in response to electrical stimulation. As expected, both heterozygous and homozygous muscle preparations had contractile responses evoked by electrical stimulation confirming our studies in myotubes. Analysis of the tension levels developed in response to single twitch revealed a large variation within each muscles group, which resulted in no recognizable differences in force generation between wild-type and triadin-null muscle. Normalization of the developed force (P/P0) by their peak values did not show any differences in kinetic of isometric force development between null and wild-type muscles (Fig. 6, upper). However, measurement of global Ca\(^{2+}\) release (Fura-2, 340/380 ratio) in response to single twitch revealed that triadin-null muscles had a significant reduction in the amplitude of their peak Ca\(^{2+}\) transient when compared with wild-type muscles (expressed as fraction of wild-type Ca\(^{2+}\) peak ratio, Fig. 6, lower).

**Effect of Triadin on SR Ca\(^{2+}\) Content**—Based in our Ca\(^{2+}\) imaging data, it appeared that both myotubes and adult fibers from triadin-null mice have smaller SR Ca\(^{2+}\) loading capacity than their wild-type counterparts. In an attempt to quantitate the level of SR calcium content we used 40 mM caffeine in the presence of 1 μM thapsigargin plus Cd\(^{2+}\) and La\(^{3+}\) to deplete SR stores in Fluo-4-loaded cells. Calcium release was measured in the presence of thapsigargin, Cd\(^{2+}\), and La\(^{3+}\) to avoid Ca\(^{2+}\) reloading into the SR stores and Ca\(^{2+}\) entry from the extracellular medium, respectively. Fig. 7A shows that under these conditions myotubes had a biphasic response to caffeine with a fast Ca\(^{2+}\) release component followed by a slower and sustained release event, most likely representing the release of free Ca\(^{2+}\) and Ca\(^{2+}\) bound to Csq, respectively. Although, wild-type and triadin-null cells display similar Ca\(^{2+}\) release kinetics, the amplitude of the fast Ca\(^{2+}\) release component in triadin-null myotubes was significantly decreased compared with wild-type cells. This difference in Ca\(^{2+}\) release very likely represents real differences in the SR calcium pool as both wild-type and triadin-null cells had identical sensitivity to caffeine (see Fig. 8). As a result, the quantification of the total Ca\(^{2+}\) released (area under the curve) showed that there was modest but significant reduction in total SR calcium content in triadin-null cells (15%, Fig. 7B). FDB fibers displayed a similar Ca\(^{2+}\) release pattern as myotubes but with a less prominent fast component and a more robust slow release phase (Fig. 7C). Unlike myotubes, however, the total Ca\(^{2+}\) released in triadin-null FDBs was much smaller (40% reduction in SR Ca\(^{2+}\) content) than wild-type fibers (Fig. 7D). The larger reduction in Ca\(^{2+}\) loading capacity observed in
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Resting Free Ca\(^{2+}\) Concentration in Triadin-null Myotubes—Because of the alleged role of Csq and intraluminal Ca\(^{2+}\) levels in modulating RyR1 activity and, therefore, Ca\(^{2+}\) homeostasis, it is not unlikely that the reduction in Csq expression observed in adult fibers but not in myotubes of triadin-null mice could affect Ca\(^{2+}\) regulation in myotubes and muscle fibers differently. To assess this possibility resting free Ca\(^{2+}\) concentration was measured in wild-type and triadin-null primary myotubes and TA muscles using calcium selective microelectrodes. As shown in Table 2, myoplasmic resting free Ca\(^{2+}\) in wild-type myotubes was 111 ± 2 nm, similar to the resting Ca\(^{2+}\) concentration previously reported in adult skeletal muscle fibers and RyR1 knock-out myotubes expressing recombinant RyR1 (28). By comparison, primary myotubes from triadin-null mice had a substantial increase (182 ± 3 nm) in myoplasmic resting free Ca\(^{2+}\). Likewise, in situ determinations of myoplasmic Ca\(^{2+}\) concentration in intact TA muscle from wild-type and triadin-null mice showed similar differences in resting Ca\(^{2+}\) concentrations as those seen in myotube preparations (see Table 2). However, triadin-null TA muscles displayed a seemingly lower resting calcium concentration than null myotubes. These results are consistent with our imaging data showing that triadin-null muscles have significantly less Sr Ca\(^{2+}\) loading capacity than myotubes and suggest that triadins may play a role in regulating myoplasmic Ca\(^{2+}\) homeostasis.

**Effects of Triadin Expression on RyR1 Ca\(^{2+}\) Release Channel Function**—Triadin/RyR1 interaction has been suggested to modulate RyR1 channels either directly or indirectly (20, 35). To evaluate the impact of the absence of triadin on RyR1 activity, \[^{3}H\]ryanodine binding was measured in crude SR membrane preparations in the presence of RyR1 agonists (caffeine and Ca\(^{2+}\)) and inhibitors (Ca\(^{2+}\) and Mg\(^{2+}\)). Fig. 8A shows that membrane preparations from both wild-type and triadin-null limb muscles had a classical bell-shaped dose response to Ca\(^{2+}\), in which sub-millimolar concentrations of Ca\(^{2+}\) increase RyR1 channel activity and millimolar Ca\(^{2+}\) concentrations inhibit it. Average EC\(_{50}\) values, calculated from the Ca\(^{2+}\) activation curve

### TABLE 2

| Condition           | Wild type | Null          | No. of determinations |
|---------------------|-----------|---------------|-----------------------|
|                     |           |               |                       |
| Myotubes            | 111 ± 2   | 182 ± 3       | 26/55                 |
| TA in situ          | 123 ± 2   | 157 ± 5       | 9/9                   |

*One-way analysis of variance (Tukey analysis), p < 0.001 in comparison to wild type.

triadin-null FDBs seems consistent with the sizable reduction of Csq expression observed in triadin-null adult muscle, which was not seen in myotubes (see Fig. 2, A and B).

**FIGURE 7.** Comparison of SR calcium loading content in myotubes and adult muscle fibers. SR calcium stores of Fluo-4 loaded primary myotubes (A) and isolated FDB fibers (C) were depleted with 40 mM caffeine in the presence of 1 μM thapsigargin, 0.5 mM Ca\(^{2+}\), 0.1 mM La\(^{3+}\), and nominally free (−7 μM) extracellular Ca\(^{2+}\). Total SR Ca\(^{2+}\) content was estimated from the amplitude of the cytosolic calcium transients (area under the curve, B and D). Data are presented as mean ± S.E. of 36–50 FDB fibers and 126–149 myotubes. *, p < 0.05; ***, p < 0.001.

**FIGURE 8.** \[^{3}H\]Ryanodine binding to wild-type and triadin-null skeletal muscle homogenates. Ca\(^{2+}\) dependence (A), caffeine activation (B), and Mg\(^{2+}\) inhibition curves (C) of specific \[^{3}H\]ryanodine binding to microsomal vesicles from wild-type (○) and triadin-null (●) mice skeletal muscles. Values are depicted as dose-response curves with each point representing mean ± S.D. for 5–10 measurements. EC\(_{50}\) and log IC\(_{50}\) values were determined as described under “Experimental Procedures.” Statistical analyses using analysis of variance yielded no significant differences for any condition tested.
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(0.1–100 μM Ca\(^{2+}\)) of three independent measurements done in duplicate, indicates that wild-type and triadin-null preparations had no significant difference in their sensitivity for Ca\(^{2+}\) activation (0.98 ± 0.09 μM and 0.79 ± 0.04 μM, for wild-type and null mice, respectively, \(p > 0.05\)) or Ca\(^{2+}\) inhibition. Caffeine (0.5–50 mM) caused an increase in channel activity in both wild-type and triadin-null membrane preparations, but there was no difference in their sensitivity to caffeine (Fig. 8C; \(E_{C50}\) 9.7 ± 1.2 mM and 9.3 ± 0.2 mM, respectively, \(n = 2\), \(p > 0.05\)). Similarly, Mg\(^{2+}\)–inhibited \([\text{H}]\)ryanodine binding in crude membrane preparations from both wild-type and null muscle in a concentration-dependent manner (Fig. 8B) with almost identical sensitivity (IC\(_{50}\) 0.54 ± 0.03 mM and 0.54 ± 0.04 mM, respectively, \(n = 2\), \(p > 0.05\)). Overall these results suggest that the absence of triadin expression does not significantly affect RyR1’s sensitivity to Ca\(^{2+}\) and Mg\(^{2+}\), a result consistent with Scatchard analysis of the data that show no changes in high affinity \([\text{H}]\)ryanodine binding between wild-type and triadin-null muscle preparations (see supplemental Fig. S3).

DISCUSSION

Direct biochemical evidence suggests that triadins have strong interactions with several key components of the CRU (12, 35–37), and several authors have suggested that they may be critical for skeletal muscle function (3, 19, 21, 23). In the present study we have addressed this by creating pan-triadin-null mice. Triadin-specific antibodies failed to detect the presence of skeletal (95 and 60 kDa) and cardiac-specific (32 and 40 kDa) isoforms of triadin in skeletal and cardiac muscles of these mice. The antibody used for these studies would be expected to cross-react with all triadin isoforms, because it was raised against a peptide specific for an intraluminal domain shared by all triad-localized triadin isoforms in skeletal (Trisk 95 and Trisk 51) and cardiac muscle (CT1: 32 kDa, CT2: 35 kDa, and CT3: 75 kDa) (25, 38). In addition this antibody would also be expected to bind to the recently reported non-triad-localized 49- and 32-kDa skeletal muscle-specific isoforms (23). Its specificity here was demonstrated by the fact that the same antibody was highly effective in detecting and differentiating between these isoforms in wild-type muscles and heart and confirming that triadin-1, the predominant cardiac isoform (25), was absent in cardiac muscle from null animals. Therefore, the fact that the null mice have a seemingly normal phenotype, reproducing, moving, and surviving normally suggests that if triadins have a role in muscle function, it is either a minor one, or one that can be taken over by some compensatory mechanism.

Direct testing of EC-coupling properties in wild-type versus null myotubes and adult muscle failed to demonstrate an obligatory role for triadin in regulating skeletal-type EC coupling. No changes in the kinetics of Ca\(^{2+}\) release were detectable, and the only significant change in Ca\(^{2+}\) release properties is a clear reduction in the magnitude of the calcium transient elicited by depolarization, which is 25–30% smaller than wild type in both myotubes and adult muscles. This result is in agreement with a recent study showing that the expression of triadin-binding deficient RyR1 in dyspedic myotubes resulted in electrically evoked Ca\(^{2+}\) transients with up to 50% smaller amplitude than wild-type RyR1 (39). Despite the reduction in Ca\(^{2+}\) release our data showed no clear differences in force development between wild-type and triadin-null muscles. This result, however, cannot rule out either the existence of a more subtle phenotype that could be masked by the large variation in our tension measurements or an altered phenotype that could only be exposed by heavy exercise or other forms of stress.

The role of triadins on skeletal EC coupling have been studied using several experimental approaches, including overexpression of different skeletal isoforms of triadin (19), expression of triadin binding-deficient RyRs in dyspedic myotubes (39), and knockdown of the expression of the 95-kDa isoform using siRNAs in C2C12 myotubes (40). All these studies suggest that triadin plays an important role in regulating skeletal EC coupling, because all of them demonstrated a significant reduction in the amplitude of the depolarization-evoked Ca\(^{2+}\) transient. In agreement with these studies here we show that total ablation of triadin expression resulted in a noticeable reduction in skeletal EC coupling gain, but the reduction was considerably smaller in magnitude than was reported in the former studies. Here the reduced Ca\(^{2+}\) release appears to be explained on the basis of a smaller SR Ca\(^{2+}\) pool. Interestingly, studies in myotubes expressing triadin binding-deficient RyRs reveal similar reduction in caffeine-induced Ca\(^{2+}\) transient amplitude (39, 41), suggesting the possibility that the lack of triadin interaction with RyR1 results in a similar reduction of the SR calcium pools as we report here. It is worth mentioning that, in all of the previous studies, the effect of triadin on EC coupling has been assayed after acute disruption of RyR1-triadin interaction, in some cases even with the endogenous triadin still present (19, 39). It is likely that our triadin-null myotubes and muscles have been exposed to a long term adaptation process that has allowed them to gradually compensate for the lack of triadins. This adaptation process may not take place during transient disruption experiments where the cells are prompted to respond to acute changes in protein expression. To what extent the short versus long term disruption of RyR1-triadin interaction can account for the discrepancies in severity of the observed phenotypes is unknown. However, from our data it is clear that ablation of the expression of all currently known isoforms of triadin does not prevent the occurrence of nearly normal EC coupling in mouse skeletal muscles.

Previous hypotheses for a role of triadin in controlling RyR activity, either by itself or as a mediator between Csq and RyR1, have come primarily from indirect evidence, based on the presumed disruption of the RyR-triadin or RyR-triadin-Csq association in vitro by antibodies (18, 35), peptides (35), triadin itself (20), and changes in ionic strength and/or intra-luminal calcium concentration (42). Our findings that absence of triadins has no apparent effect on \([\text{H}]\)ryanodine binding activity to isolated SR vesicles seems contrary to previous \([\text{H}]\)ryanodine binding and bilayer studies showing that triadin itself can inhibit RyRs activity (20) and to the effect that mutations in the RyR1 putative triadin binding site have on caffeine-induced Ca\(^{2+}\) transients (41). This discrepancy may be explained in part by the differences in experimental binding condition used in our study (crude membrane homogenates) and those in Ohkura’s study performed in CHAPS-purified RyR1 in the presence of soybean lecithin and dithiothreitol, two conditions known to
influence RyR channel behavior. In addition, pretreatment of triadin 95 with dithiothreitol in Ohkura’s study prevented the inhibitory effect of triadin on [3H]ryanodine binding, suggesting that functionally meaningful interactions between RyR1 and triadins may be highly dependent on the oxidation state of both proteins. Despite the fact that our [3H]ryanodine binding studies failed to detect any functional differences between wild-type and triadin-null muscles, the small but significant increase in the myoplasmic resting free Ca\(^{2+}\) levels observed in both null myotubes and adult muscles do suggest the possibility that there is an abnormally higher basal activity of RyR1 in triadin-null muscle.

An alternative possibility is that another regulatory protein with redundant or synergistic function with triadin could be functionally compensating for the lack of triadins in our membrane studies. Based on the major reshaping of the expression profile of several SR proteins observed in null muscles, this possibility seems likely. In this regard, junctin (26 kDa), which has a similar single membrane-spanning domain, a short cytoplasmic N terminus, and a longer luminal C-terminal tail that is highly conserved between both proteins (33, 43) and contains the joint putative interaction site (junctin with triadin and vice versa) and the interaction site with Csq and RyRs (43, 44), would be a likely candidate. In cardiac tissue junctin and triadin have been shown to have synergistic function in associating Csq with the junctional SR membrane (45) and to positively modulate the activity of RyR channels fused into lipid bilayers (46). However, this does not seem to be the case in the triadin-null mice. Our data show that, whereas in myotubes the ablation of triadin expression resulted in no significant changes in junctin expression in adult muscle fibers, it caused a significant reduction of junctin expression, revealing a rather negative compensation. These results were summed to the fact that triadin-null myotubes appear to have a higher RyR1 basal activity than adult fibers and seem to support an inhibitory role for 95- and/or 60-kDa triadin on RyR1 function. This is consistent with previous biochemical (1, 20, 42) and physiological data (19) and suggests that unlike cardiac muscle in skeletal muscle triadin and junctin may have opposite influences on Ca\(^{2+}\) release regulation. Thus a mismatch of the absence of triadin expression (a negative regulator) summed with an incomplete down-regulation of junctin (a positive regulator) could result in a net activating effect on CRU function. This in turn could be responsible for an augmented myoplasmic resting free Ca\(^{2+}\) such as we observed in both cultured myotubes and intact muscle. The facts, that junctin-knockout mice, like the triadin-null mice, do not seem to have a lethal phenotype (47) but siRNA knockdown of both junctin and triadin causes significantly impaired EC coupling in myotubes (40), seem to support the idea that both proteins have coordinated functions.

In view of the possible role of triadin and junctin in anchoring Csq within the junctional SR, it is somewhat surprising that movement of Csq away from the triad is rarely observed in the triadin-null muscles that not only totally lack triadin but also have a reduced amount of junctin. This may indicate that just a few anchoring sites may be sufficient to hold polymerized Csq in place. The occasional presence of Csq at sites over than triads has been detected in normal frog muscle (48), and thus it is likely that the same Csq displacement in triadin-null muscle does not have a strong functional effect. It was also interesting that any structural alterations seen in the triadin-null muscles were restricted only to fast twitch fibers. Somewhat similar structural alterations have been reported in both JP-1 (49, 50) and Csq1 (51) knockout mice, and the expression of both of these proteins is down-regulated in triadin-null mice. However, down-regulation of JP-1 and Csq1 cannot account for the differential response of fast muscles, because a comparable reduction in Csq and an even greater reduction in JP-1 were also observed in triadin-null Sol muscles that showed no ultrastructural alterations. A major difference between the two muscle types was the level of junctin expression. Unlike Sol muscle, which displayed unchanged expression levels of junctin, EDL muscles had a 30% reduction of junctin expression. Thus, it is possible that the deletion of triadin combined with the reduced expression of junctin could account for the mislocalization of Csq and in the structural organization of the jSR observed between these two muscle types.

Nonetheless, we cannot rule out that the altered orientation of triads may have a different origin. In this respect it is important to note that fast but not slow fibers seem to readily respond to a variety of stimuli (denervation and lack of Csq) with similar disarrangement of the triads (51, 52).

Overall, our data suggest that (i) 95- and 60-kDa triadin are indirectly involved in organizing the molecular complex of the triad, although they are not essential components; (ii) triadins are not required to support EC coupling in skeletal muscle; and (iii) triadins seem to play a negative regulatory role in RyR1 activity and thereby contribute to the modulation of global Ca\(^{2+}\) homeostasis as evidenced by an increased myoplasmic resting free Ca\(^{2+}\) in triadin-null muscles and myotubes.

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