The ry1<sup>53</sup> dyspedic mouse contains two disrupted alleles for ryanodine receptor type 1 (skeletal isoform of ryanodine receptor; Ry<sub>1</sub>R) resulting in perinatal death. In the present study, whole skeletal muscle homogenates and sucrose gradient-purified junctional sarcoplasmic reticulum from neonatal wild-type and dyspedic mice were assayed for biochemical and functional markers. Equilibrium binding experiments performed with 1–120 nM [3H]ryanodine reveal saturable high and low affinity binding to membrane preparations from wild-type mice, but not to preparations from dyspedic mice. Binding experiments performed with [3H]PN200 show a 2-fold reduction in [3H]PN200 binding capacity in dyspedic muscle, compared to age-matched wild-type muscle, with no change in receptor affinity. The presence or absence of proteins known to be critical for normal ryanodine receptor/Ca<sup>2+</sup> channel complex function was assessed by Western blot analysis. Results indicate that FKBP-12, DHPR<sub>1</sub>, triadin, calsequestrin, SERCA1 (sarcoplasmic reticulum Ca<sup>2+</sup> ATPase), and skeletal muscle myosin heavy chain are present in both dyspedic and wild-type muscle. Only wild-type membranes showed immunoreactivity toward Ry<sub>1</sub>R antibody. Neither dyspedic nor wild-type muscle mouse showed detectable immunoreactivity toward Ry<sub>2</sub>R or Ry<sub>3</sub>R antibodies, even after sucrose gradient purification of sarcoplasmic reticulum. These results indicate that proteins critical for ryanodine receptor function are expressed in dyspedic skeletal muscle in the absence of Ry<sub>1</sub>R, Ca<sup>2+</sup> transport measurements show that membranes from wild-type controls, but not dyspedic mice, release Ca<sup>2+</sup> upon exposure to ryanodice. Dyspedic muscle and cells derived from them serve as excellent homologous expression systems in which to study how Ry<sub>1</sub>R structure relates to function.

The dyspedic mouse contains two disrupted alleles (ry1<sup>53</sup> / ry1<sup>53</sup>) for ryanodine receptor type 1 (skeletal isoform of ryanodine receptor; Ry<sub>1</sub>R) resulting in a birth lethal defect. Skeletal muscle from dyspedic mice lack excitation-contraction (E-C) coupling (1)<sup>2</sup> but maintains many ultrastructural details of the triadic junction. Two important differences have been observed with thin section transmission and freeze fracture scanning microscopy of dyspedic muscle. First, dyspedic muscle lacks the regularly spaced array of junctional feet which span the gap between the t-tubule and SR membranes, significantly reducing the gap size (3).<sup>2</sup> Second, dyspedic fibers lack the tetradic arrangement of dihydropyridine receptors which is characteristic of normal fibers (3). It is not yet known if dyspedic muscle lacking Ry<sub>1</sub>R expression results in altered expression of other key triadic proteins involved in modulating SR Ca<sup>2+</sup> transport.

In addition to Ry<sub>1</sub>R, separate genes encode two other ryanodine receptors, namely the cardiac (Ry<sub>2</sub>R) and “brain” (Ry<sub>3</sub>R) isoforms (4–8). Ry<sub>2</sub>R is predominantly expressed in skeletal muscle and cerebellar Purkinje cells (9, 10). Ry<sub>3</sub>R is predominately expressed in cardiac tissue, where it functions as a Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release channel, but is also widely expressed in brain tissues (7, 9). In comparison, Ry<sub>3</sub>R is widely expressed at low levels in many non-muscle cells, in mammalian brain, and in very low levels in heart and skeletal muscle (11–13). However, its functional significance is presently unclear.

The development of Ry<sub>1</sub>R-null (dyspedic) mice (1)<sup>2</sup> represents a significant advance in the goal to understand the molecular mechanisms regulating the function of Ry<sub>1</sub>R. Recent studies have shown that dyspedic mice die perinatally and lack skeletal E-C coupling (1).<sup>2</sup> Dyspedic skeletal muscle fibers have also been found to have 30-fold less L-type Ca<sup>2+</sup> entry current than control fibers, despite the presence of comparable levels of immobilization-resistant charge movement. Significantly, both voltage-dependent Ca<sup>2+</sup> entry current and SR Ca<sup>2+</sup> release are concomitantly restored in cultured dyspedic myotubes by microinjection of Ry<sub>1</sub>R cDNA (14). Despite the loss of E-C coupling, dyspedic muscle fibers in culture exhibit small Ca<sup>2+</sup> fluxes in response to caffeine and adenosine nucleotide (14, 15). The pharmacological responses in cultured dyspedic muscle myotubes have been attributed to enhanced expression of Ry<sub>1</sub>R mRNA (15). However, there has been no direct evidence of Ry<sub>1</sub>R protein expression in dyspedic muscle.

The present study demonstrates for the first time that dyspedic mouse skeletal muscle expresses major elements of the triadic junction but lacks detectable ryanodine receptor protein and function.*

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§§ The abbreviations used are: Ry<sub>1</sub>R, ryanodine receptor, skeletal isoform; Ry<sub>2</sub>R, ryanodine receptor, cardiac isoform; Ry<sub>3</sub>R, ryanodine receptor, brain isoform; DHPR, dihydropyridine; DHPR, dihydropyridine receptor; E-C, excitation-contraction; ECL, enhanced chemiluminescence; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; FAGE, polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; t-tubule, transverse tubule; MOPS, 4-morpholinopropanesulfonic acid.

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pedic muscle 1) exhibits a 2-fold reduction in \[^{[3]H}PN200\] binding capacity with a concomitant change in immunoreactive DHPRα1 subunit expression, 2) lacks detectable levels of specific high or low affinity \[^{[3]H}ryanodine-binding sites, 3) lacks immunoreactivity relative to Rγ1, Rγ2, or Rγ3 antibody, and 4) lacks a ryanodine-sensitive Ca\(^{2+}\) efflux pathway across SR membranes, compared with skeletal muscle from wild-type littermates. However, the complement of other triadic proteins known to be critical for normal E-C coupling (triadin, FKBP-12 (12-kDa FK506-binding protein), salequestrin, SERCA1), and skeletal myosin heavy chain are shown to be expressed in dyspedic muscle.

**EXPERIMENTAL PROCEDURES**

Dyspedic Mice—Dyspedic mice were obtained as described elsewhere.\(^2\) Briefly, a 9-kilobase EcoR1-Thr111 genomic fragment which contains part of the skeletal Rγ1 gene, was screened from a 129/Sv strain by means of a 129/Sv genomic library and was used to construct a targeting vector. A neomycin-resistance gene (Neo) was used to disrupt transcription of the mRNA and select for homologous recombinants. A herpes simplex virus thymidine kinase gene was used to reduce the number of non-homologously targeted clones. Both were under the control of a phosphoglycerate kinase promoter and were inserted into the KpnI and EcoR1 sites, respectively, approximately 2.5 \(\times\) 10\(^5\) J1-ES of the sequence for were placed immediately before and after the targeting vector and subjected to double selection with G418 and FIAU. Homologously targeted ES clones were obtained from heterozygous matings and verified by PCR and FIAU. Homologously targeted ES clones were injected into blasto- cysts from C57BL/6 mice and transferred to pseudopregnant females. Chimeric males were bred to C57BL/6 females, and germline transmission was verified. Mutant mice homozygous for the Rγ1 targeted allele were obtained from heterozygous matings and verified by PCR and Southern blot.

**Tissue Preparation**—Crude and sucrose gradient-purified membrane fractions were prepared from wild-type (Rγ1+/+) and dyspedic (Rγ1-/-) neonates under identical conditions. Immediately after birth, wild-type animals were euthanized by cervical dislocation. Tissue from each sample (dyspedic and wild-type) was removed for PCR analysis. The legs were removed from each animal at the hip or shoulder, trimmed of fat, skinned, and frozen in liquid nitrogen. Wild-type or dyspedic tissues were then pooled and prepared in the following manner. Legs were thoroughly homogenized on ice using a Polytron at high speed in 50 mM Hepes, pH 7.1, 50 mM NaCl, 50 mM CaCl\(_2\), pH 7.1, and placed into vials containing 5 ml of scintillation mixture (Ready Safe, Beckman). Binding of radioligand to muscle membranes was determined by scintillation spectrometry. \(K_D\) and \(B_{max}\) values were derived from Scatchard analysis of the binding data.

Specific binding of \[^{[3]H}PN200\] (83 Ci/mmol, DuPont NEN) to the α1 subunit of the L-type Ca\(^{2+}\) channel (the dihydropyridine receptor) was measured in the presence of 140 mM NaCl, 15 mM KCl, 20 mM Hepes, pH 7.0, and wild-type or dyspedic skeletal muscle homogenates. The reaction was initiated by the addition of tissue and allowed to equilibrate for 30 min in dark. Paired nonspecific controls were measured in the presence of 10 μM nifedipine. Separation of bound and free ligand was performed as described for high affinity \[^{[3]H}ryanodine binding except that the filters were washed with 5 \(\times\) 2 ml of wash buffer. \(K_D\) and \(B_{max}\) values were derived from Scatchard analysis of the binding data.

**Electrophoresis and Immunoblot Analysis**—Constituent proteins from membrane preparations were resolved on 3–10% gradient, 4–20% gradient, or 7% isocratic gels by the method of Laemmli (20). Gels were either stained with silver (Silver Stain Plus, Bio-Rad) or stained with Coomassie blue (Bio-Rad). A whole membrane preparation was used for 1 h at 37°C in TTBS buffer (20 mM Tris-HCl, 500 mM NaCl, 0.5%Tween 20, pH 7.5) with the addition of 5% bovine serum albumin or 5% nonfat dry milk. Specific binding of the primary antibody of interest was performed by incubating the blots for 1 h at 37°C in TTBS buffer in the presence of 1% bovine serum albumin and antibody. Resulting immunoblots were labeled with horseradish peroxidase-conjugated goat anti-mouse (Sigma) or donkey anti-rabbit (Amersham) secondary antibody for 1 h at 37°C and then visualized using either colorimetric (TMB, Vector Laboratories) or chemiluminescent (ECL, Amersham) techniques. In some cases, exposed films from ECL were quantitated using a densitometer (model PC39310, Shimadzu). Nonspecific binding of secondary antibodies to membrane preparations was minimized by performing a dilution series in the absence of primary antibody. Antibodies were purchased or generously provided as follows: Ry1, (3A4C), generous gift of Dr. J. Sutko; Ry2 (C3–33), generous gift of Dr. G. Meissner; Ry3, generous gift of Dr. V. Sorrentino; DHPR/1 (MA3–920), Affinity BioReagents; triadin (11G12AS), generous gift of Dr. K. Campbell; FKBP-12, generous gift of Dr. M. Harding, Vertex Pharmaceuticals; SERCA1 (MA3–911), Affinity BioReagents; myosin heavy chain, Affinity BioReagents (1130-P; Biocytex biochemicals, San Diego, CA).

**Transport Measurements—Ca\(^{2+}\) flux measurements using microsomal membranes from wild-type or dyspedic mouse skeletal muscle were performed fluorometrically (SLM AB-2, SLM-Aminco). Briefly, 50 μg of skeletal muscle microsomal membranes were equilibrated to 37°C in a buffer consisting of 92 mM KCl, 7.5 mM NaP\(_2\)O\(_5\), 20 mM MOPS, pH 7.0, 0.01% NaN\(_3\), 2–10 μM Ca\(^{2+}\), and 0.5 μM fluo-3. A coupled enzyme system (20 μg/ml creatine phosphokinase and 5 μM phosphocreatine) was present to maintain ATP concentrations. Loading of Ca\(^{2+}\) was initiated by the addition of 1 mM MgATP. Transport of Ca\(^{2+}\) into or out of microsomal membranes was determined by following changes in the fluorescence intensity at 405 nm (excitation at 500 nm, emission at 405 nm) for 10 min. The presence of NaP\(_2\)O\(_5\), in the transport buffer maintained a linear dye response at added Ca\(^{2+}\) concentrations up to 8 μM. Ca\(^{2+}\) efflux was initiated by the addition of 20 or 200 μM ryanodine. Ca\(^{2+}\) accumulation by microsomal stores was verified by addition of 2 μg/ml 4-bromo-A23187. Linearity of fluo-3 emission with increasing Ca\(^{2+}\) concentration was verified after each experiment by adding known aliquots from a National Bureau of Standards Ca\(^{2+}\) stock.

**RESULTS AND DISCUSSION**

Silver Stain of Dyspedic and Wild-type Control Mouse Skeletal Muscle—Wild-type and dyspedic neonatal mouse skeletal muscle proteins, resolved on 3–10% Laemmli gels and visualized by silver stain, are shown in Fig. 1. Lanes containing either dyspedic (dys) or wild-type (w-t) preparations exhibit a similar pattern of staining and density of protein bands, with the major exception of a band in wild-type lanes corresponding

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in size to the RyR protomer found in junctional rabbit skeletal muscle SR (jasr lane, marked with arrow).

The Ry1R, Ry2R, and Ry3R Isoforms Are Not Detectable in Dyspedic Mouse Skeletal Muscle—The equilibrium binding of [3H]ryanodine to neonatal wild-type and dyspedic skeletal muscle microsomes was investigated under conditions that stabilize high affinity binding of the alkaloid to ryanodine receptors (21). Fig. 2 shows that membranes prepared from dyspedic skeletal muscle do not possess specific high affinity [3H]ryanodine-binding sites, whereas preparations of newborn wild-type muscle exhibit 148 ± 16 fmol/mg of high affinity [3H]ryanodine binding with a $K_d$ of 6.9 ± 1.3 nM (mean $B_{\text{max}}$ from three independent preparations, each performed in duplicate). Scatchard analysis of data from control muscle membranes is best fit using a two-site model, with a second site having lower affinity ($K_d = 20.3$ nM) and $B_{\text{max}}$ of 305 ± 94 fmol/mg of protein.

Experiments designed to measure the presence of binding sites having lower affinity were assessed using [3H]ryanodine concentrations from 250–1600 nM (specific activity 1.2 Ci/mmol) and revealed that while wild-type preparations were fully saturated, dyspedic preparations failed to show any specific binding (data not shown).

The binding data presented above suggest that dyspedic muscle either lacks general expression of ryanodine receptor isoforms or, if these proteins (e.g. Ry3R) are expressed, they fail to measurably recognize the alkaloid. We directly examined whether or not dyspedic muscle expresses any of the known ryanodine receptor isoforms by Western blot analysis. Proteins from wild-type and dyspedic skeletal muscles were resolved by SDS-PAGE and transferred overnight onto PVDF membranes as described under “Experimental Procedures.” Blots were probed using antibodies selective for either Ry1, Ry2, or Ry3 receptors. Specific antibody labeling was visualized using a highly sensitive chemiluminescent (ECL) technique.

Blots probed with a Ry2R-selective mouse monoclonal antibody (22) stain positive for the ~560-kDa protomer in a protein concentration-dependent manner with preparations from wild-type neonatal mice and with rabbit junctional SR (Fig. 3, top panel, w-t and jsr lanes). In comparison, dyspedic skeletal muscle preparations lack any detectable immunoreactivity with the Ry2R antibody (Fig. 3, top, dys lanes), as does rat cardiac SR (Fig. 3, top, crd lane).

As expected, Western blot analysis using a Ry3R-selective monoclonal antibody (23) shows an absence of reactivity with both dyspedic and wild-type skeletal muscle preparations (Fig. 3, middle panel, dys and w-t lanes). However, the antibody strongly recognizes the Ry3R protein found in rat cardiac preparations, which is included as a positive control (Fig. 3, middle, crd lane).

Western blot analysis using a RyR-selective polyclonal antibody (8) does not recognize any protein associated with either the dyspedic or wild-type membrane preparations (Fig. 3, bottom panel, dys and w-t lanes), nor does it recognize any proteins associated with rabbit fast skeletal muscle junctional SR (Fig. 3, bottom, jsr lanes). However, the antibody strongly recognizes the RyR protein found in avian pectoralis muscle, which possesses >80% sequence homology with the mammalian Ry3R “brain” isoform (Fig. 3, bottom, avi lanes). The anti-

![Figure 1](image1.png)

**Fig. 1.** SDS-PAGE of wild-type and dyspedic skeletal muscle proteins reveals the presence and absence of RyR, respectively, by silver stain. SDS-PAGE was performed with wild-type and dyspedic mouse skeletal muscle preparations according to the method of Laemmli using 3–10% gradient gels. Proteins were visualized using the Silver Stain Plus kit (Bio-Rad). jsr, 4 µg of rabbit skeletal junctional SR; dys, 4, 8, and 12 µg of dyspedic mouse microsomal membranes, respectively; w-t, 4, 8, and 12 µg of wild-type mouse microsomal membranes, respectively; std, protein standards. Molecular mass markers (indicated by arrows along left side of figure) are 170 kDa (reduced α2-macroglobulin), 116 kDa (β-galactosidase), 85 kDa (fructose 6-phosphate), 55 kDa (glutamate dehydrogenase), 39 kDa (aldolase), and 26 kDa (triosephosphate isomerase).

![Figure 2](image2.png)

**Fig. 2.** Dyspedic skeletal muscle membranes lack detectable high affinity binding sites for [3H]ryanodine. Specific binding of [3H]ryanodine to membrane preparations from wild-type and dyspedic mice were performed as described under “Experimental Procedures” in the presence of 1 nM [3H]ryanodine and 0.5–120 nM unlabeled ryanodine. Nonspecific binding was determined in the presence of 260 nM unlabeled ryanodine. Scatchard analysis (inset) of specific binding found in wild-type preparations was best fit by a two-site model. The high affinity site gave a $B_{\text{max}}$ of 148 ± 16 fmol/mg of protein and a $K_d$ of 6.9 ± 1.3 nM; the lower affinity site gave a $B_{\text{max}}$ of 305 ± 94 fmol/mg of protein and a $K_d$ of 21 ± 1.8 nM (pooled data from three independent experiments performed in duplicate). In contrast, specific ryanodine binding was not detected with dyspedic mouse skeletal muscle (three independent preparations).
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Constituent proteins from dyspedic and wild-type muscle membranes were resolved by SDS-PAGE using 3–10% gradient gels and transferred overnight onto PVDF membranes. Secondary antibody was visualized using enhanced chemiluminescent (ECL, Amersham) methods. Arrow along left side of blot indicates position of protein band of interest. Top panel, Ry1R Western blot. jsr, 5 μg of rabbit skeletal junctional SR; crd, 5 μg of rat cardiac SR; dys, left to right), 5, 10, and 15 μg of dyspedic muscle protein; w-t (left to right), 5, 10, and 15 μg of wild-type muscle protein. Middle panel, Ry2R Western blot. crd, 5 μg of rat cardiac SR; dys (left to right), 5, 10, and 15 μg of dyspedic muscle protein; crd (left to right), 5, 10, and 15 μg of wild-type muscle protein. Bottom panel, Ry3R Western blot. jsr, 10 μg of rabbit skeletal muscle protein; av, 15 μg of avian pectoralis SR; dys, 15 μg of dyspedic muscle protein; w-t, 15 μg of wild-type protein; tst, 20 μg of rat testicular protein.

Expression of Ry1R and Ry3R proteins was further investigated using SR membrane fractions purified by sucrose density gradient centrifugation. Gradient fractions isolated at the 38–45% sucrose interface and within the 45% sucrose layer were analyzed by Western blot using Ry1R- and Ry3R-selective antibodies. Fig. 4, top panel, shows that lanes containing either whole membrane fractions (memb), 38–45% sucrose gradient fractions (38–45%), or the 45% sucrose fraction (45%) react with the Ry1R-selective antibody only in preparations obtained from wild-type muscle, whereas dyspedic muscle preparations completely lack immunoreactivity toward this antibody. In comparison, no immunoreactive protein could be detected with the Ry3R polyclonal antibody in the purified fractions from either the wild-type or dyspedic muscle preparations (Fig. 4, bottom panel). Multiple bands labeled by the Ry3R-selective antibody in the 38–45% sucrose gradient lane of wild-type muscle (Fig. 4, top) are most likely proteolytic fragments of the ryanodine receptor with estimated to be 250,000 and 300,000, which is reflective of the fragmentation pattern of Ry1R induced by trypsin (24) or calpain (25) digestion. The body also recognizes Ry3R protein in rat testicular tissue (Fig. 3, bottom, tst lane), which has been shown to express very high levels of Ry3R mRNA (8). Sequential labeling experiments with wild-type protein; Western blot. bottom panel, either the wild-type or dyspedic muscle preparations (Fig. 4, dysmuscle protein; Western blot. Using enhanced chemiluminescent (ECL, Amersham) methods. overnights onto PVDF membranes. Secondary antibody was visualized

**Experi**mental **Procedures.** Purified membranes were transferred onto PVDF membranes and Western blot analysis performed as in Fig. 3. Arrow on left side of blot indicates position of protein band of interest. Top panel, Ry1R Western blot. Bottom panel, Ry1R Western blot. Lane markers are as follows: jsr, 1 μg of rabbit skeletal SR; av, 15 μg of avian pectoralis SR; memb, 15 μg of whole muscle homogenate from either dyspedic (dys) or wild-type (w-t) muscle; 38–45%, 15 μg of purified membranes from corresponding to pooled sucrose gradient fractions from either dyspedic or wild-type muscle; 45%, 15 μg of particulate fraction from bottom of 45% sucrose layer from either dyspedic or wild-type muscle. The multiple bands labeled in the 38–45% w-t lane are proteolytic fragments generated during the purification process.

**Expression** of Ry1R and Ry3R proteins in these preparations. Since dyspedic and wild-type muscle preparations were resolved by S...sponding to pooled sucrose gradient fractions from either dyspedic or wild-type muscle; 45%, 15 μg of particulate fraction from bottom of 45% sucrose layer from either dyspedic or wild-type muscle. The multiple bands labeled in the 38–45% w-t lane are proteolytic fragments generated during the purification process.

**Sedimentation** density of the vesicles could be related to the degree of proteolysis of Ry1R, especially since Ry1R fragmentation was not observed within the 45% sucrose gradient fraction.

The results presented here and elsewhere (1, 3, 15) clearly demonstrate that dyspedic muscle skeletal muscle does not express the skeletal isofrom (Ry1R) of the ryanodine receptor. In the present study, specific antibodies fail to detect the presence of Ry1R, Ry2R or, more importantly, Ry3R protein in the neonatal muscle, even when the highly sensitive ECL technique is used in conjunction with sucrose gradient purification of SR. Furthermore, analysis of high and low affinity [3H]ryanodine binding fails to support the presence of any known ryanodine receptor proteins in these preparations. Since dyspedic and wild-type skeletal muscle preparations are from newborn mice, SR volume (26) and frequency of SR/t-tubule junctions are much less than those found in adult mouse muscle (26–28). This morphological difference, along with results from the Western blots presented here, suggests that neonatal mouse skeletal muscle either does not express Ry1R protein or expresses it at levels below detection limits.

**The question** of whether dyspedic muscle expresses an alternate ryanodine receptor isofrom (Ry2R or Ry3R) is important, since responses to Ca²⁺, caffeine, and ryanodine have been observed in cultured dyspedic myotubes (1, 14, 15). However, the total Ca²⁺ released in response to these ligands was observed to be 10–15-fold less than that seen in normal myotubes, and the time course for release was significantly slower (1, 14). Furthermore, cultured dyspedic myotubes have been reported to lack responsiveness to a second addition of caffeine when ryanodine is introduced between caffeine additions (14, 15). Analysis of RNA from cultured myotubes using reverse transcription-polymerase chain reaction have revealed increased Ry3R mRNA expression in cultured dyspedic myotubes.
Based on these lines of evidence, the Ca$^{2+}$ fluxes seen in dyspedic muscle cells have been attributed to up-regulation of Ry$_\alpha$R protein as a consequence of Ry$_\alpha$R deletion (15). In support of this hypothesis, Conti et al. (12) recently described a differential distribution of the Ry$_\alpha$R gene product in various adult mammalian skeletal muscles using Western blot analysis and in situ hybridization. In that study, bovine hind limb, Type II rat skeletal muscle, and diaphragm from the mouse, rabbit, and cow were all found to differentially express Ry$_\alpha$R protein. Their results indicate that expression of Ry$_\alpha$R protein differs dramatically among muscle types within a single species and suggest that these differences may also be species-specific. Additionally, Ry$_\alpha$R protein expression was found to be concentrated to the terminal cisternae of SR in bovine diaphragm muscle. However, the significance of Ry$_\alpha$R expression in skeletal muscle remains unclear. In the Ry$_\alpha$-null mouse, the protein does not appear to be essential for E-C coupling or normal muscle development (12), but the maximum Ca$^{2+}$-induced Ca$^{2+}$ release response obtained at high Ca$^{2+}$ in permeabilized muscle fibers is reduced by $\sim$25% (12).

**DHPR Protein Expression Is Diminished in Dyspedic Mouse Skeletal Muscle**—The functional importance of DHP receptors in E-C coupling has been amply demonstrated in studies of the dysgenic mouse, which has been shown to lack expression of DHPR$_\alpha$1 subunit protein and DHPR function (29). Like dysgenic muscle (30), dyspedic muscle lack tetrads found in normal skeletal muscle. When cultured dysgenic myotubes are transfected with cDNA coding for the DHPR$_\alpha$1 subunit, E-C coupling is restored and the DHP receptors align as tetrads revealing the same structures as seen in wild-type muscle (31). Electron microscopic studies of dysgenic and dyspedic skeletal muscle have revealed that neither Ry$_\alpha$R nor DHPR$_\alpha$1 expression is the signal required for close apposition of the t-tubule and SR membranes and the subsequent co-localization of the remaining triadic cytoskeletal elements (3). Interestingly, while disruption of Ry$_\alpha$R gene expression does not preclude the close proximity of the triadic membranes, it does prevent DHPR tetrad formation (3).

Recently, Nakai et al. (14) reported that whole cell L-type Ca$^{2+}$ currents in cultured dyspedic myotubes are reduced $\sim$30-fold when compared to wild-type controls. Micronuclear injection of Ry$_\alpha$R cDNA into dyspedic myotubes reconstituted L-type inward Ca$^{2+}$ current to $\sim$40% of control myotubes and restored E-C coupling with no concomitant change in immobilization-resistant charge movement ($Q_{\text{max}}$). Their results indicate that E-C coupling is restored in dyspedic myotubes upon expression of Ry$_\alpha$R, and that Ry$_\alpha$R expression does not significantly alter the level of expression and/or targeting of DHPR$_\alpha$1 subunit protein to the surface membrane, but rather is important in conveying reciprocal regulation for DHPR Ca$^{2+}$ channel function. However, since $Q_{\text{max}}$ is normalized to membrane surface area, the absolute change in the amount of DHPR expression in dyspedic muscle remains unclear.

To more directly ascertain levels of DHPR expression in dyspedic skeletal muscle, the specific binding of [3H]PN200 (0.06–5.0 nM) is compared in whole membrane preparations from dyspedic and wild-type skeletal muscle. Fig. 5A shows that compared to those from wild-type muscle, the density of specific [3H]PN200-binding sites is reduced $\sim$50% in dyspedic muscle preparations without a significant change in $K_d$ (Fig. 5B). Three independent experiments using preparations from different animals reveal that the density of [3H]PN200-binding sites is consistently reduced in the dyspedic preparations compared to wild-type ($168 \pm 7$ fmol/mg of protein and $331 \pm 29$ fmol/mg of protein, respectively). Western blot analysis with a DHPR$_\alpha$1-selective antibody (32) reveals that while both dyspedic and wild-type muscle preparations exhibit immunoreactivity at $\sim$170 kDa, dyspedic preparations stain to a lesser degree compared to wild-type lanes containing equal amounts of whole membrane-bound protein (Fig. 6, **DHPR$_\alpha$1 blot**). Consistent with the [3H]PN200 binding data shown in Fig. 5, densitometric analysis of ECL radiograms reveals that the immunoreactive band at $\sim$170 kDa corresponding to DHPR$_\alpha$1 is approximately 50% less dense in the dyspedic preparations, regardless of the amount of protein loaded on the gel (data not shown).

The observation of reduced DHPR$_\alpha$1 expression in dyspedic skeletal muscle compared to age-matched wild-type muscle could reflect either 1) a lower density of DHP receptors within the t-tubule membrane, or 2) a decrease in t-tubule surface area, both of which would lower the total DHPR capacity of dyspedic membranes. It is unlikely that DHP receptor density is reduced in t-tubule membranes, since $Q_{\text{max}}$ is normalized to membrane surface area and Nakai et al. reported no significant difference in $Q_{\text{max}}$ between dyspedic and wild-type myotubes (14). It is more likely that the lower density of [3H]PN200-
membranes. Secondary antibody was visualized using either chemiluminescent (ECL, Amersham) or colorimetric (TMB, Vector Laboratories) methods. Arrow on left side of each blot indicates position of protein band of interest. **DHPRa1 Western blot, jsr, 10 μg of rabbit skeletal SR; std, protein standard from overexposed blot showing position of 170-kDa marker; dys, 5, 10, and 15 μg of dyspedic membranes; w-t, 5, 10, and 15 μg of wild-type membranes. FKBP-12 Western blot, jsr, 5 μg of rabbit skeletal junctional SR; dys, 15 μg of dyspedic membranes; w-t, 15 μg of wild-type membranes. Triadin Western blot, jsr, 1 μg of rabbit skeletal junctional SR; dys, 10 and 15 μg of dyspedic membranes; w-t, 10 and 15 μg of wild-type membranes. Calsequestrin blot (visualized with Stains All), dys, 10 μg of dyspedic membranes; w-t, 10 μg of wild-type membranes; jsr, 1 μg of rabbit skeletal junctional SR. SERCA1 Western blot, jsr, 1 μg of rabbit skeletal junctional SR; dys, 10 μg of dyspedic membranes; jsr, 10 μg of wild-type membranes; w-t, 10 μg of wild-type membranes. Myosin Western blot, jsr, 5 μg of rabbit skeletal junctional SR; w-t, 10 μg of wild-type membranes; dys, 10 μg of dyspedic membranes. Blot is visualized colorimetrically (TMB, Vector Laboratories).**

Fig. 6. **Dyspedic skeletal muscle expresses key triadic proteins.** Constituent proteins from wild-type and dyspedic mouse membranes were resolved by Laemmli SDS-PAGE using 3–10% gradient, 4–20% gradient, or 7% isocratic gels and transferred overnight onto PVDF membranes. Triadic Proteins Critical for E-C Coupling Are Expressed in Dyspedic Mouse Skeletal Muscle—Whether or not Ry1R deletion alters the expression of other key triadic proteins normally found in skeletal muscle has not been addressed. In addition to DHPRa1, several proteins localized at the muscle triad have been shown to directly or indirectly modulate the function of Ry1R. Therefore, the presence of FKBP-12, triadin, calsequestrin, SERCA1, and myosin heavy chain in dyspedic mouse skeletal muscle membranes was examined by Western blot analysis.

A high affinity interaction between Ry1R and FKBP-12 (12-kDa FK506-binding protein) has been shown to be essential for stabilizing the full conductance gating behavior of the SR channel (33–35). Blots stained with a monoclonal antibody directed against FKBP-12 reveal the presence of this protein (M<sub>r</sub> ~14,000) in both dyspedic and wild-type muscle membranes (Fig. 6, **FKBP-12 blot, dys and w-t lanes**). Interestingly, the amount of FKBP-12 associated with dyspedic membrane preparations was consistently observed to be greater than the corresponding wild-type preparations. The underlying reason for this increase in FKBP-12 in dyspedic membranes is unknown. Whether FKBP-12 is elevated in dyspedic muscle membranes as a direct result of loss of muscle function or is associated with infiltration of immunocompetent cells or erythrocytes (36) remains to be determined. However, the latter possibility is unlikely since microscopic examination of muscle does not reveal evidence of an inflammatory response or increased numbers of red cells in dyspedic muscle. In agreement with previous reports (33, 37), purified rabbit junctional SR preparations contain FKBP-12 (Fig. 6, **FKBP-12 blot, jsr lane**).

[^3]: C. Franzini-Armstrong, personal communication.
Triadin, a 95-kDa protein initially identified by Caswell et al. (38) and cloned by Campbell and co-workers (39), appears to form an association with RyR and calsequestrin (40–42). Blots probed with a monoclonal antibody directed toward triadin (39) and visualized by ECL, reveal the presence of this protein in both dyspedic and wild-type skeletal muscle (Fig. 6, triadin blot, dys and w-t lanes, respectively), as well as in rabbit junctional SR (Fig. 6, triadin blot, jsr lane). The low density of labeling seen in the dys and w-t lanes, compared to the junctional SR lane, reflects the lower density of this protein found in the whole muscle preparation.

In addition to its role in enhancing the Ca\(^{2+}\) loading capacity of SR, calsequestrin appears to elicit a signal that is communicated to RyR during Ca\(^{2+}\) release (43). The presence of calsequestrin in dyspedic and wild-type skeletal muscle was probed using Stains All as described by Campbell et al. (44). Proteins were resolved on 3–10% Laemmli gels and stained for 48 h with Stains All, followed by de-staining for 1–2 h to resolve an intensely blue band at M\(_r\) ~60,000. The calsequestrin gel shown in Fig. 6 reveals the presence of a blue band corresponding to the location of calsequestrin in lanes containing either rabbit junctional SR (jsr), dyspedic (dys), or wild-type (w-t) muscle proteins. Note that the lane containing rabbit junctional SR stains a comparatively broad band, reflecting the higher density of this protein found in the purified preparation.

The presence of two additional proteins critical for normal muscle function was probed by Western blot analysis. The SERCA1 and myosin blots in Fig. 6 show that antibodies selective for SERCA1 (2) and myosin heavy chain recognize their respective targets in both wild-type and dyspedic membrane preparations. Since the relative density of protein labeling by these antibodies reflects differences in protein content within these preparations, SERCA1 stains to a much higher degree in purified junctional SR preparations (SERCA1 blot, jsr lane) than in the whole muscle preparations (SERCA1 blot, dys and w-t lanes). In comparison, antibody specific for myosin heavy chain reveals a higher density of myosin in the dyspedic and wild-type preparations (myosin blot, dys and w-t lanes, respectively) since these are whole membrane preparations.

Results presented here using Western blot analysis reveal that FKBP-12, triadin, and calsequestrin are all expressed in dyspedic mouse skeletal muscle (Fig. 6, dys and w-t lanes, respectively) since these are whole membrane preparations. Since the relative density of protein labeling by these antibodies reflects differences in protein content within these preparations, SERCA1 stains to a much higher degree in purified junctional SR preparations (SERCA1 blot, jsr lane) than in the whole muscle preparations (SERCA1 blot, dys and w-t lanes). In comparison, antibody specific for myosin heavy chain reveals a higher density of myosin in the dyspedic and wild-type preparations (myosin blot, dys and w-t lanes, respectively) since these are whole membrane preparations.

Results presented here using Western blot analysis reveal that FKBP-12, triadin, and calsequestrin are all expressed in dyspedic mouse skeletal muscle preparations. Interestingly, while FKBP-12 expression is increased and DHPR expression is decreased in dyspedic mouse skeletal muscle as compared to wild-type membranes, the pattern of expression of key triadic proteins remains and suggests that the molecular components required to form a functional triadic complex will also be present in myogenic cell lines produced using the RyR1 gene targeting approach.

**Dyspedic Mouse Skeletal Muscle Microsomes Do Not Exhibit Ryanodine-induced Ca\(^{2+}\) Release**—[\(^{3}H\)]Ryanodine binding and Western blot analysis reveal a lack of ryanodine receptor expression in dyspedic muscle. As a functional correlate, Ca\(^{2+}\) flux measurements were performed and ryanodine-induced Ca\(^{2+}\) release was assayed using microsomal membranes from dyspedic and wild-type muscle. Ca\(^{2+}\) transport across isolated microsomal membranes was assessed fluorometrically with the dye fluo-3. In the presence of 7.5 \(\mu\)M sodium pyrophosphate in the buffer, Ca\(^{2+}\) additions of 1–8 \(\mu\)M produced linear responses from the dye (Fig. 7A). Addition of MgATP in the presence of a regenerating system initiates active accumulation of Ca\(^{2+}\) into membrane vesicles (Fig. 7, B and C, insets). Under the experimental conditions used, wild-type and dyspedic membranes could be loaded with similar amounts of Ca\(^{2+}\) (100 and 74 nmol/mg of protein, respectively). Addition of 20 \(\mu\)M ryanodine to Ca\(^{2+}\)-loaded microsomes from wild-type muscle results in release of approximately 40% of the intravesicular Ca\(^{2+}\), and this effect is fully blocked by pretreatment with 10 \(\mu\)M ruthenium red (Fig. 7B, traces a and b, respectively). Ryanodine-induced Ca\(^{2+}\) release from control microsomes can also be...
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block by prior addition of 1 μM neomycin (data not shown). Addition of 200 μM ryanodine to wild-type vesicles elicited a biphasic effect on Ca^{2+} transport, initially inducing Ca^{2+} release followed by Ca^{2+} reaccumulation (Fig. 7B, trace c). This result is in agreement with results commonly obtained with rabbit junctional SR and reflects a sequential action of ryanodine-sensitive Ca^{2+} effluxes. The absence of a transient response to 200 μM ryanodine in the dyspedic preparation is further evidence that these muscle microsomes do not contain measurable ryanodine-sensitive Ca^{2+} effluxes.

The results presented above indicate that while dyspedic mouse skeletal muscle does not express Ry, it does express the balance of the major triadic elements critical for E-C coupling. These findings, along with the Ca^{2+} flux measurements presented above, indicate that the mouse model is an ideal system with which to examine Ca^{2+} regulation in skeletal muscle using a homologous expression system and transgenic approaches.

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