Contractile function is unaltered in diaphragm from mice lacking calcium release channel isoform 3

J. S. Clancy, H. Takeshima, S. L. Hamilton, and M. B. Reid. Contractile function is unaltered in diaphragm from mice lacking calcium release channel isoform 3. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1205–R1209, 1999.—Skeletal muscle expresses at least two isoforms of the calcium release channel in the sarcoplasmic reticulum (RyR1 and RyR3). Whereas the function of RyR1 is well defined, the physiological significance of RyR3 is unclear. Some authors have suggested that RyR3 participates in excitation-contraction coupling and that RyR3 may specifically confer resistance to fatigue. To test this hypothesis, we measured contractile function of diaphragm strips from adult RyR3-deficient mice (exon 2-targeted mutation) and their heterozygous and wild-type littersmates. In unfatigued diaphragm, there were no differences in isometric contractile function of diaphragm strips from adult RyR3-deficient mice (exon 2-targeted mutation) and their heterozygous and wild-type littersmates. In unfatigued diaphragm, there were no differences in isometric contractile properties (twitch characteristics, force-frequency relationships, maximal force) among the three groups. Our fatigue protocol (30 Hz, 0.25 duty cycle, 37°C) depressed force to 25% of the initial force; however, lack of RyR3 did not accelerate the decline in force production. The force-frequency relationship was shifted to higher frequencies and was depressed in fatigued diaphragm; lack of RyR3 did not exaggerate these changes. We therefore provide evidence that RyR3 deficiency does not alter contractile function of adult muscle before, during, or after fatigue.

Ryanodine-sensitive calcium channel isoform 3; fatigue; excitation-contraction coupling

There are three known mammalian isoforms of the ryanodine-sensitive calcium channel (RyR): RyR1, primarily expressed in skeletal muscle; RyR2, the cardiac isoform; and RyR3, which is found at low levels in many tissues, including skeletal muscle, without any preferential tissue association (6, 14, 20). All three RyR isoforms function as release channels of intracellular calcium stores (6, 20); however, the precise function of RyR3 in calcium homeostasis in the heart (17, 23).

In the terminal cisternae of the sarcoplasmic reticulum, cell depolarization is detected by the voltage sensor, and calcium is released from the sarcoplasmic reticulum, leading to contraction in a process known as excitation-contraction coupling (6, 20). Several lines of evidence suggest involvement of RyR3 in this process. First, RyR3 has been localized to the terminal cisternae of the sarcoplasmic reticulum (5). Second, differences in calcium regulation between RyR1 and RyR3 suggest that RyR3 might participate in excitation-contraction coupling via calcium-induced calcium release. For example, the open probability of RyR3, when fully activated by calcium, is five times higher than that of RyR1 (2, 3). In vivo, this type of regulation could significantly impact calcium release and contractile function. On the basis of differences in calcium regulation, the most popularly theorized role of RyR3 in excitation-contraction coupling is that calcium released via RyR1 activates RyR3 to amplify the calcium signal (termed calcium-induced calcium release) (1, 17–19, 22, 23). The pervasiveness of this theory was a primary stimulus for the present study.

At the tissue level the distribution of RyR3 among skeletal muscles appears to correlate with fatigue resistance. Among skeletal muscles examined, diaphragm has the most RyR3 (5). Diaphragm, the primary muscle of inspiration and a muscle that resists fatigue (11), contains 50 times more RyR3 than extensor digitorium longus, an easily fatigued muscle (5). Additionally, neonatal muscle expresses more RyR3 (1, 23) and resists fatigue more effectively than adult muscle (10, 12, 13, 16, 25). Thus an attractive theory of the specific functional role of RyR3 exists: RyR3 contributes to fatigue resistance (1, 5).

Fatigue is the failure of muscle to maintain mechanical output during repetitive contractions (reviewed in Ref. 9). This can be measured as a loss of force production. The loss of force is accompanied by depression of tetanic calcium transients and metabolic alterations, including an increase in free radicals, a decline in pH, an increase in free magnesium, and an increase in resting calcium concentration (9, 24, 26–28). Each of these perturbations is known to depress RyR1 function (8).

Regulatory differences between RyR1 and RyR3 support the idea that RyR3 could supplement calcium release when RyR1 function is depressed. For example, magnesium may differentially modulate the two isoforms in vivo. The concentration of free magnesium in fatigued muscle is approximately twice that in rested muscle, likely as a result of ATP breakdown (28). RyR1 and RyR3 differ in sensitivity to magnesium. In general, agents that stimulate calcium release increase [3H]ryanodine binding, whereas the opposite is generally true of agents that depress calcium release; therefore, [3H]ryanodine binding is frequently used as a rapid screen of channel effectors. Magnesium depresses [3H]ryanodine binding of RyR3 by only ~25%, whereas it depresses [3H]ryanodine binding of RyR1 by 90% (18). These findings suggest the possibility that RyR3 remains active when exposed to levels of magnesium that inhibit RyR1.

We, therefore, combined the popular model of calcium-induced calcium release with known differences in channel regulation to develop a working model. If RyR3 were resistant to a fatigue-generated metabolite, which
supplement calcium release and thereby maintain force. We evaluated this model using diaphragm strips from adult RyR3-deficient mice and their littermates by testing two specific hypotheses: 1) during fatigue, lack of RyR3 accelerates the decline in force production and 2) in fatigued muscle, lack of RyR3 exaggerates the functional decrement. Our results led us to reject our hypotheses and the working model.

**MATERIALS AND METHODS**

Maintenance of knockout animals. The National Institutes of Health Guide for the Care and Use of Laboratory Animals dictated conditions of all procedures, which were approved in advance by the Institutional Review Board of Baylor College of Medicine. The mutant mice lacking RyR3 were established by the targeted gene disruption technique by using J1 embryonic stem cells, as described previously (21). The RyR3-deficient mice carry a mixed genetic background of C57BL and 129 strains and a mutation to exon 2 of the RyR3 gene. A total of 26 6- to 8-wk-old offspring of heterozygous parents were used in these experiments: 11 were RyR3 deficient, 6 were wild type, and 9 were heterozygous. Animal genotype was determined by PCR analysis after collection of contractile data.

Diaphragm preparation. Animals were anesthetized by methoxyflurane inhalation, then killed by cervical dislocation. The entire diaphragm was rapidly excised and immersed in room-temperature Krebs-Ringer solution containing (mM) 137 NaCl, 5 KCl, 1 NaH2PO4, 24 NaHCO3, 2 CaCl2, and 1 MgSO4. The solution was maintained at room temperature and aerated with 95% O2-5% CO2. A diaphragm strip was cut parallel to fiber orientation, with care taken to leave the rib and central tendon connected. The rib was fixed to a metal bar with silk suture (size 5-0), and the tendon was similarly tied to a force transducer (model BG 100, Kulite Instruments, Leonia, NJ) mounted on a micrometer. Diaphragm strips were stimulated by field stimulation with platinum electrodes. In pilot studies, maximal stimulation of diaphragm strips occurred at 16 V. For these experiments we utilized a supramaximal voltage of 25 V. We adjusted muscle length to produce optimum twitch force.

Experimental protocol. The solution was then replaced with 37°C Krebs solution, and the temperature was controlled by a digital water bath throughout the remainder of the experiment. After a 30-min thermodilution period, we recorded twitch characteristics, including twitch force, time to peak force, and one-half relaxation time. We then determined the force-frequency relationship. Tetanic contractions were stimulated at 2-min intervals (500-ms train duration); between each intermediate frequency (15, 30, 50, 80, 120, 160, 250 Hz), a maximum tetanic contraction (Po, 300 Hz) was elicited to serve as a reference for changes in force over time.

Five minutes after completion of the force-frequency protocol, we induced fatigue by stimulating the muscle at 30 Hz using a 1:4 duty cycle (0.5 trains/s, 500-ms train duration). For the second train and every 30 s thereafter, a 300-Hz stimulation replaced the 30-Hz stimulation. The final force-frequency data were collected after 5 min of fatiguing contractions by increasing the stimulus frequency (15, 30, 50, 80, 120, 160, 300 Hz) every 2 s. After a 3-min recovery period, a final train of stimulation of 300 Hz was applied. We measured the forces developed during stimulated contractions and the force sustained between trains of electrical stimulation (unstimulated force).

Stripes were measured for muscle length at optimum and immediately trimmed of nonmuscle tissue and weighed. Force measurements were later normalized for functional cross section according to Close (4). PCR. Sections of tail (1–3 cm) were collected at the time of contractile studies and stored at −20°C. DNA was harvested by phenol-chloroform extraction. A solution containing PCR reagents (ExTaq Polymerase Kit, Takara) and appropriate primers (Keystone Labs, Camarillo, CA) was aliquoted into reaction tubes. Primers that annealed to wild-type sequences were 5'-ATGAAAGTTGACTCCAGTGCATTG-3' (P1) and 5'-TCCAGGAATCTCCTGATTAGG-3' (P2). A separate reaction combined P1 and P3, a primer that annealed within the exon 2 mutation: 5'-GCCACACCGGTACCTTAATGG-3'. Genomic DNA was added to a final concentration of 8 ng/µl. The reaction tubes were then subjected to 35 temperature cycles (0.5 min at 94°C, 1 min at 60°C, 1 min at 72°C). Reaction products were electrophoresed in a 1.5% agarose gel and detected by fluorescence of DNA-bound ethidium bromide.

Statistical analyses. Values are means ± SE. For differences in single measures among the three groups, we utilized a one-way ANOVA for parametric data or a Kruskal-Wallis ANOVA on ranks for nonparametric data. For recurrent measures, we used a two-way repeated-measures ANOVA (15).

**RESULTS**

Experimental model. Consistent with previous reports, the apparent phenotype of the RyR3-deficient mice was not grossly different from that of their littermates (21). Genotype was determined by PCR, as detailed in MATERIALS AND METHODS. Two sets of primers annealing to alleles as diagramed in Fig. 1, top, were used. Figure 1, bottom, shows representative PCR data from one animal of each genotype. The male-female distribution was similar among groups, with male

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**Fig. 1.** Products of PCR from 1 animal of each genotype; 2 reactions were run for each animal. Lane a, reactions using primers P1 and P2 (see MATERIALS AND METHODS), which anneal on either side of mutation. Presence of a normal allele results in a short product (~390 bp), whereas a mutant allele, with insertion of ~1.1-kb neomycin resistance gene, results in a longer product (~1,500 bp). Lane b, PCR products resulting from primers P1 and P3; P3 anneals within mutation of exon 2, producing a short (~390-bp) product. DNA of a wild-type animal yields no products in this reaction. Heterozygote lane a is expected to contain two products: 1,500 and 390 bp long. Shorter product was preferentially produced.
constituents as follows: 50% RyR3 deficient, 62.5% heterozygous, and 50% wild type. Body weight was not different among groups, averaging 20.5 ± 2.8 g. Average cross-sectional area of the diaphragm strips was 0.3 ± 0.1 mm² and was not different among groups.

Unfatigued contractile function. The absolute forces developed by unfatigued muscle were similar among groups. Table 1 shows the values obtained for Po, twitch force, time to peak force, and one-half relaxation time; there were no significant differences. The average twitch-to-tetanus ratios were not statistically different among groups: 0.22 ± 0.03 for RyR3-deficient, 0.23 ± 0.04 for heterozygous, and 0.20 ± 0.03 for wild-type mice. The force-frequency relationships of the unfatigued muscles are illustrated in Fig. 2. These relationships exhibit the characteristic sigmoidal shape. There were no differences among groups. During the force-frequency protocol, muscles from RyR3 knockout animals were as stable as those from wild-type controls, as evidenced by the maintenance of Po over time. Figure 3 shows the drop in maximum force over 30 min. In each group the total decrement averaged <10%.

Fatigue characteristics. Diaphragm strips from the three groups responded similarly to the fatigue protocol. Figure 4, top, shows the change in developed force during 30-Hz stimulation. Force initially fell at a rapid rate, then more gradually. This response was not different among groups. Changes in maximal force (300-Hz stimulation) also followed a similar pattern (Fig. 4, bottom). After 60 s of fatiguing contractions, we observed a progressive rise in the force measured between trains of electrical stimulation (unstimulated force); this force peaked at 150 s and remained elevated thereafter (Fig. 5). Changes in unstimulated force did not differ among groups.

Contractile function of fatigued muscle. Fatigue produced a rightward shift and depression in the force-frequency relationship. In Fig. 6 the three-group average of the force-frequency relationship before fatigue is shown, along with the fatigue-induced shift in each of the three groups; there was no difference among groups. At the conclusion of the contractile protocol, muscles were allowed to recover for 3 min. Neither recovery of Po nor drop in unstimulated force was different among groups. Po recovered to 56 ± 2% of prefatigue values and unstimulated force dropped to 0.7 ± 0.2 N/cm².

DISCUSSION

The diaphragm is peculiar among skeletal muscles. As the primary muscle of inspiration, it is crucial that the diaphragm be able to generate force sufficient for ventilation under a variety of conditions. Combined observations suggested a novel role for RyR3 in excitation-contraction coupling and fatigue resistance of the diaphragm (1).

We found no evidence that RyR3 is essential for normal contractile function. In unfatigued diaphragm from adult mice, contractile function was unchanged by a lack of RyR3. Our observations agree with those of Bertocchini et al. (1), whose data also indicated that RyR3 deficiency did not alter function in adult mouse diaphragm. However, this group did demonstrate a loss of function in diaphragm strips from neonatal, RyR3-deficient mice; the force-frequency relationship was shifted to higher frequencies than control diaphragm strips.

We also tested the postulate that RyR3 contributes to fatigue resistance. However, our studies do not support a role for RyR3 in resistance to fatigue. There was no divergence of developed or unstimulated force among groups. Bertocchini et al. (1) did not show data but mentioned that they observed no differences between

<table>
<thead>
<tr>
<th>Table 1. Isometric contractile properties</th>
<th>RyR3 Deficient</th>
<th>Heterozygous</th>
<th>Wild Type</th>
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<tbody>
<tr>
<td>Maximum force, N/cm²</td>
<td>24.0 ± 1.2</td>
<td>23.4 ± 1.5</td>
<td>20.6 ± 1.8</td>
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<tr>
<td>Twitch force, N/cm²</td>
<td>5.0 ± 0.3</td>
<td>5.2 ± 0.4</td>
<td>4.3 ± 0.6</td>
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<tr>
<td>Time to peak force, ms</td>
<td>14.7 ± 0.3</td>
<td>14.3 ± 0.3</td>
<td>14.3 ± 0.3</td>
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<tr>
<td>One-half relaxation time, ms</td>
<td>12.8 ± 0.8</td>
<td>12.2 ± 0.5</td>
<td>10.9 ± 0.6</td>
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Values are means ± SE. There were no significant differences among groups.
normal and RyR3-deficient mice in developed force during repetitive contractions.

Conclusion. As evidenced by this study, mice without RyR3 develop and mature to an end point of normal contractile function. Before we can conclude that RyR3 is not essential in skeletal muscle, the plasticity of the tissue must be taken into account. With its ability to adapt to a variety of conditions (unloading, stretch, increased endurance or strength demands) taken into consideration, it is possible that skeletal muscle simply adapts to the loss of RyR3. For example, absence of RyR3 could result in modification of some other calcium release channel (RyR1) to serve the lost function of RyR3. Evidence of such adaptation would support the relevance of a functional role of an RyR3-type channel while securing the conclusion that the RyR3 isoform itself is nonessential.

Perspectives

With the absolute requirement of diaphragm contractile function and the findings of Bertocchini et al. (1) taken into consideration, RyR3 might be essential only under specific conditions where the protein is highly expressed (young diaphragm) and contractile demand is increased. For example, if the neonates were subjected to premature birth or inspiratory loading, would RyR3-deficient mice experience greater mortality? To our knowledge, no group has examined this possibility. However, given the mounting evidence against functional contribution of RyR3 to calcium transients and contractile function, this postulate seems unlikely. An alternative hypothesis is that RyR3 is involved in calcium signaling of some type other than excitation-contraction coupling. For example, microdomains around RyR3 channels could include calcium-sensitive components involved in gene regulation. Is it possible that RyR3-deficient muscles are slower than normal to adapt to changes in contractile demand? A final possibility is that RyR3 in adult skeletal muscle is simply vestigial, leftover from a developmental process in which it was important.

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REFERENCES


