The role of proteases in excitation-contraction coupling failure in muscular dystrophy

Davi A. G. Mázała,1 Robert W. Grange,2 and Eva R. Chin1

1Department of Kinesiology, School of Public Health, University of Maryland, College Park, Maryland; and 2Department of Human Nutrition, Foods and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, Virginia

Submitted 3 September 2013; accepted in final form 7 October 2014

Mázała DA, Grange RW, Chin ER. The role of proteases in excitation-contraction coupling failure in muscular dystrophy. Am J Physiol Cell Physiol 308: C33–C40, 2015. First published October 8, 2014; doi:10.1152/ajpcell.00267.2013.—Duchenne muscular dystrophy (DMD) is one of the most frequent types of muscular dystrophy. Alterations in intracellular calcium (Ca2+) handling are thought to contribute to the disease severity in DMD, possibly due to the activation of Ca2+-activated proteases. The purpose of this study was twofold: 1) to determine whether prolonged excitation-contraction (E-C) coupling disruption following repeated contractions is greater in animals lacking both dystrophin and utrophin (mdx/Utr−/−) compared with mice lacking only dystrophin (mdx); and 2) to assess whether protease inhibition can prevent E-C coupling failure following repeated tetani in these dystrophic mouse models. Excitation-contraction coupling was assessed using Fura-2 ratio, as an index of intracellular free Ca2+ concentration, in response to electrical stimulation of single muscle fibers from the flexor digitorum brevis muscle. Resting Fura-2 ratio was higher in dystrophic compared with control (Con) fibers, but peak Fura-2 ratios during stimulation were similar in dystrophic and Con fibers. One hour after a series of repeated tetani, peak Fura-2 ratios were reduced by 30 ± 5.6%, 23 ± 2%, and 36 ± 3.1% in mdx, mdx/Utr−/−, and mdx/Utr−/−, respectively, with the greatest reduction in mdx/Utr−/− fibers (P < 0.05). Protease inhibition attenuated this decrease in peak Fura-2 ratio. These data indicate that E-C coupling impairment after repeated contractions is greatest in fibers lacking both dystrophin and utrophin and that prevention of protease activation can mitigate the prolonged E-C coupling impairment. These data further suggest that acute protease inhibition may be useful in reducing muscle weakness in DMD.

Duchenne muscular dystrophy; intracellular Ca2+; excitation-contraction coupling; protease(s)

DUCHENNE MUSCULAR DYSTROPHY (DMD) is one of the most frequently occurring and devastating types of muscular dystrophy (1). Skeletal muscles of patients with DMD lack dystrophin (26), a protein that contributes to membrane stability during muscle contractions (6, 43). The absence of dystrophin increases the susceptibility of muscle to stretch-induced damage (60) and disrupts intracellular signaling pathways (34). Disruptions in membrane integrity and/or intracellular homeostasis (e.g., increased oxidative stress) result in increased intracellular free calcium concentration ([Ca2+]i) in dystrophic muscle (28) and alterations in Ca2+-dependent signaling. In particular, elevated resting Ca2+ levels may contribute to increased activation of the Ca2+-activated proteases (e.g., calpains) and the ubiquitin proteasome pathway and thus may accelerate proteolysis in response to contractile activity.

Repetitive tetanic contractions result in muscle fatigue. Force recovery after repeated contractions occurs in two phases: a rapid phase that is related to metabolic recovery (15) and a slow phase related to the elevation in [Ca2+]i, during contractions (14, 15, 33). In an in vitro single muscle fiber model, slow recovery from fatigue was shown to be due to prolonged failure of mechanisms of excitation-contraction (E-C) coupling, leading to reduced Ca2+ release. This prolonged reduction in E-C coupling can also be induced without contractions by exposing muscle fibers to high Ca2+ concentrations (33, 54).

Activation of calpains is thought to be a mechanism contributing to the prolonged disruption of E-C coupling following repetitive tetanic contractions (33, 54). Elevations in [Ca2+]i in the physiological range of muscle contractions (0.5–3.0 μM) can lead to activation of the two major calpains, calpain-3 and μ-calpain (7, 40, 41). Calpains can then degrade proteins involved in E-C coupling such as ryanodine receptor (22) or junctophilin-1 (39). Contraction-induced impairments of E-C coupling are also observed in dystrophic muscle, with greater impairments in dystrophic branched fibers compared with control and “nonbranched” dystrophic fibers (24). The dysfunctional Ca2+ handling combined with evidence of increased proteolytic activity in dystrophic muscle (20) suggests a potential role for calpains in DMD disease progression (38, 52).

Studies that investigate underlying mechanisms (20, 60) and potential treatments (17, 46) for DMD have largely been conducted in mdx mice. However, under normal physiological conditions, the diaphragm is the only muscle in mdx mice demonstrating similar disease progression to DMD patients, with progressive degeneration and detectable muscle weakness (50). Other muscles appear to be protected because utrophin, a protein with a similar function in muscle to dystrophin, is upregulated in these animals (37, 55). Thus, a double mutant model lacking both dystrophin and utrophin (mdx/Utr−/−) is thought to be more suitable for studies of the intracellular mechanisms altered in DMD (23). In response to repeated tetanic contractions, muscle fibers from the mdx/Utr−/− mice had a greater decay in Ca2+ transients compared with the mdx and control mice (9), suggesting greater E-C coupling impairment with repeated contractions in fibers lacking both dystrophin and utrophin. However, it is not known whether the prolonged reductions in E-C coupling following muscle fatigue, which contribute to muscle weakness, are exacerbated in the muscle fibers from the mdx/Utr−/− mice.

The evidence for poor [Ca2+]i handling during repeated muscle contractions in dystrophic muscle and the impaired E-C coupling due to protease (e.g., calpain) activation with repeated tetani suggest that proteases play a role in prolonged E-C coupling impairment in dystrophic muscle. Owing to the more severe disease pathology in the mdx/Utr−/− mice [i.e., reduced
lifespan, early onset of damage in the diaphragm, joint contractures, and kyphosis (18)], we hypothesized that prolonged E-C coupling disruption following repeated contractions would be greater in muscles that lack both dystrophin and utrophin. We also hypothesized that protease inhibition would attenuate the decrease in E-C coupling failure following repeated contractions in both dystrophic animal models.

METHODS

Ethical approval. All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

Animals. Three control (Con) mice (C57BL/10ScSn) were obtained from Jackson Laboratories. Three mdx/Utr-/- mice (two females and one male) were obtained from Dr. Diego Fraidenraich (University of Medicine and Dentistry, New Jersey) and in collaboration with Dr. Robert Grange (Virginia Tech University). Colonies of mdx/Utr-/- mice were bred at the University of Maryland Central Animal Research Facility according to previously published breeding schemes (18). Once females appeared pregnant, they were removed from the males and housed separately until pups were born and weaned. Offspring were weaned at age 21 days, sorted by sex, and then genotyped. Genotyping of animals was performed using a primer sequence and polymerase chain reaction conditions generously provided by Dr. Dawn A. Lowe (University of Minnesota, Department of Physical Medicine & Rehabilitation). It was expected that 25% of the pups born would be mdx/Utr+/-. (hereinafter referred to as mdx) and 25% would be mdx/Utr ----, while the other 50% would be mdx/Utr +/-. However, on the basis of offspring from several generations, the percentages of mice per genotype was greater for mdx/Utr+/-(~60%), than mdx (~30%) and mdx/Utr--/-(~10%). This is consistent with the findings of others (Dr. Robert Grange, personal communication) and indicates some in utero loss of the mdx/Utr+/- mice. Moreover, not all of the animals born lacking both dystrophin and utrophin survived for 2 mo. At approximately 2 mo of age, mice were used for assessment of E-C coupling in single muscle fibers. This age was chosen on the basis of the shortened lifespan (100% lethality by 90–100 days) of the mdx/Utr---- mice (18) and previous reports by Capote et al. (9), which is a comparative data set. All mice were kept in the same room (typical ambient conditions 20.9% O2 and 22 ± 1°C) and had the same access to food and water, bedding, and light cycle (12:12-h light-dark) until used for analyses. At time of use, animals were euthanized by cervical dislocation and tissues were immediately after dissection from the flexor digitorum brevis (FDB) muscle.

Single muscle fiber isolation. The FDB single muscle fibers were obtained by collagenase digestion with type 2 collagenase (Worthington) in minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) according to the protocol described by Bischoff (5). After incubation at 37°C in 95% O2-5% CO2, single muscle fibers were obtained by trituration. Subsequently, fibers were maintained in MEM solution with 10% FBS at 37°C, 95% O2-5% CO2 until used for E-C coupling assessment. All single muscle fibers were evaluated at room temperature.

Intracellular Ca2+ measurements. One day after dissection, fibers were loaded with Fura-2AM for 15 min. The Fura-2 ratio was assessed in response to varying stimuli (see protocol below) as an index of [Ca2+], and the E-C coupling response. Fibers loaded with Fura-2AM were placed in a stimulation chamber containing parallel electrodes that was positioned on a Nikon TiU microscope stage. Intracellular Ca2+ levels were assessed by the Fura-2 fluorescence ratio using an IonOptix Hyperswitch system with dual excitation, single emission filter set for Fura-2 (excitation 340 nm and 380 nm; emission 510 nm). Signals were captured and analyzed using the IonWizard software (IonOptix).

Single muscle fiber stimulation protocol. Muscle fibers were continuously perfused with a stimulating Tyrode solution (units in mM: 121.0 NaCl, 5.0 KCl, 1.8 CaCl2, 0.5 MgCl2, 0.4 NaH2PO4, 24.0 NaHCO3, and 5.5 glucose) with 0.2% FBS (13). This solution was bubbled with 95% O2-5% CO2 to maintain a pH of 7.3 (13). The muscle fibers used for assessment were selected on the basis of three criteria: 1) normal shape (nonbranched); 2) initial response to 15-Hz stimuli; and 3) normal contractile behavior when exposed to six 100-Hz tetani (2 s between each). Fibers that elicited robust contractions in response to the 100-Hz stimuli were deemed suitable for repeated tetanic contractions to induce E-C coupling failure and thus used for subsequent analyses. This represented ~90% of Con fibers and 60%, 50%, and 20% of mdx, mdx/Utr-/-, and mdx/Utr--/-- fibers, respectively. Although branched fibers show greater deficits in Ca2+ release in response to electrical stimulation (10, 36), we chose nonbranched fibers because of the high rate of failure for fibers to complete the entire stimulation protocol (described below) and the inability to image specific regions of the branched fibers (i.e., branch bifurcation vs. trunk region).

Global Fura-2 ratio was assessed in muscle fibers using trains of stimuli at 10, 30, 50, 70, 100, 120, and 150 Hz for 350 ms. Fibers rested 1 min between frequencies and then rested for 10 min before assessment at all frequencies again. After the first set of stimuli, fibers were either perfused with the calpain inhibitor ALLN (N-acetyl-l-leucyl-l-leucyl-l-norleucinal; EMD Biosciences) at 1 μM in ethanol (EtOH) or the vehicle ETOH (Veh) to test for acute exposure effects. Subsequently, fibers remained at 10 min before being stimulated by repeated 100-Hz tetani to induce E-C coupling failure. Fibers were evaluated again 1 h after repeated tetani at the same seven stimulation frequencies to evaluate the prolonged decrease in E-C coupling. Fibers that did not continue to respond to stimuli during this protocol were counted as “fibers not responding to stimuli” and the percentage of fibers failing to respond relative to total fibers assessed were recorded.

Peak Fura-2 ratios at each frequency were determined by the average ratio in the last 100 ms of the 350-ms tetanus, when Ca2+ levels (i.e., failing fibers) at each frequency were completed to determine the number of fibers failing to maintain peak Ca2+ levels (i.e., failing fibers) at each stimulation frequency. The number of failing fibers, relative to the number of fibers assessed (% failing), was also determined for each genotype.

Assessment of E-C coupling failure. Fibers were exposed to intermittent 100-Hz tetani (350-ms duration) starting at 1 contraction every 4 s for 2 min, then 1 contraction every 3 s for 2 min, then 1 contraction every 2 s for 2 min, and then 1 contraction every s for 2 min (14). We used an indirect assessment of muscle fatigue, the time for Fura-2 signal to decline, because we were not able to measure force production in our single muscle fiber preparation. Using this same protocol for repeated tetanic contractions, it was previously shown that the peak of fatigue, defined as a 70% decrease in force, corresponded with a 50% reduction in [Ca2+], and induced prolonged impairments in E-C coupling (14, 57). Thus, we used time to 50% decline in Fura-2 peak as the criterion for “fatigue” and induction of E-C coupling failure, and discontinued the repetitive 100-Hz tetani at that point. One hour after induction of E-C coupling failure, single fiber Fura-2 ratios were reassessed in the same fiber using the same stimulation protocol (10, 30, 50, 70, 100, 120, and 150 Hz) to determine the prolonged decrease in E-C coupling as an index of muscle weakness (15).

Determining the role of proteases in prolonged E-C coupling failure. To evaluate the role of proteases in the prolonged decrease in E-C coupling, muscle fibers were continuously perfused with a protease inhibitor (ALLN) or Veh after the first set of stimulation frequencies and throughout the entire protocol. Changes in Fura-2
Assessment of maximal calpain activity in dystrophic muscle. Extensor digitorum longus and plantaris muscles were used to determine maximum calpain activity. On the basis of the sample size needed to achieve 80% power to detect a $P < 0.05$ difference ($n = 7$), we used muscle from the cohort of mice used in the present study combined with samples obtained in a similar fashion from subsequent studies that were stored at $-80°C$. Maximal calpain activity was determined using a calpain assay kit (Calbiochem catalog no. QIA120) as described by the manufacturer. Muscles were homogenized in lysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM MgCl$_2$, 0.1% Triton X-100, 20% glycerol) containing 1 mM DTT and protease inhibitor cocktail (complete mini EDTA-free Protease Inhibitor Cocktail, Roche). The cytosolic fraction was obtained by centrifugation (20,000 g for 5 min). Calpain distribution in Con muscle is diffused throughout the cytoplasm and slightly enriched in the cell membrane (49). Further, calpain localization changes slightly in dystrophic mice at different stages of the disease. At the age we evaluated the animals (~8 wk old), most of the calpain is localized in the cytoplasmic portion of the muscle (49). Maximal calpain activity was determined by the amount of AMC released by its cleavage from the substrate Suc-LLVY-AMC in the presence of tissue lysate. Several studies have used a similar protocol to assess maximal calpain activity in muscle homogenates (8, 11, 31). AMC cleavage was measured using a fluorescence plate reader (PerkinElmer Wallac Victor 1420) with excitation of 340 nm and emission of 460 nm. Calpain activity is expressed as $\mu$mol AMC per gram (g) tissue. Muscle lysates were incubated with either activation (10 mM Ca$^{2+}$) or inhibition (5 mM BAPTA) buffer, and calpain activity was determined as the difference between the activation and inhibition buffers. To assess the efficacy of ALLN to inhibit calpain specifically, we also evaluated calpain activity in muscle lysates with activation buffer in the presence of 1 $\mu$M ALLN (15 min of exposure).

Statistical analysis. SPSS software (version 18.0; IBM, Somers, NY) was used to calculate between and within genotype differences in Fura-2 ratio for resting Fura-2 and peak Fura-2 at each stimulation frequency, time to E-C coupling failure, increase in resting Fura-2 ratio during repeated tetani, Fura-2 ratios at each frequency pre- vs. post-E-C coupling failure, and maximal calpain activity. One-way analysis of variance (ANOVA) was used for group comparisons, with Scheffé’s test for post hoc analyses. For changes in Fura-2 ratio pre- vs. post-E-C coupling failure, a paired $t$-test was performed. For comparing maximum calpain activity between Con and dystrophic genotypes, and between +/− ALLN treatment in vitro, unpaired $t$-tests were used. To compare the percentage of failed fibers between groups, a $\chi^2$-test was used. The statistical significance was accepted at $P < 0.05$. Values shown represent means ± SE as specified.

RESULTS

Animals and single muscle fibers. The characteristics of animals and number of fibers analyzed in the current study are shown in Table 1. Body mass of mdx/Utr$^{-/-}$ mice was significantly lower than the other genotypes ($P < 0.05$), consistent with previous reports (9, 18, 23). The diameter of fibers was also significantly smaller for mdx/Utr$^{-/-}$ mice compared with fibers from all other genotypes ($P < 0.05$; see Table 1). There were no differences in fiber length between groups (data not shown).

Ca$^{2+}$ handling. Resting Fura-2 ratio, representing resting [Ca$^{2+}$], was higher in all dystrophic phenotypes compared with Con fibers ($P < 0.05$; Table 1) with no differences between mdx, mdx/Utr$^{+/−}$, and mdx/Utr$^{-/-}$. The peak Fura-2 ratio during stimulation was similar in all fibers from the various genotypes across all stimulation frequencies (data not shown). Acute (10 min) exposure to ALLN or Veh did not alter the Fura-2 levels in response to stimulation at any of the frequencies measured (data not shown).

E-C coupling failure. Excitation-contraction coupling failure was induced by subjecting single muscle fibers to repeated 100-Hz tetani with gradually decreasing rest intervals between tetani until Fura-2 peak reached 50% of the initial level. A representative trace for the change in Fura-2 ratio with repeated tetani from an mdx fiber is shown in Fig. 1. There were no differences in time to E-C coupling failure (i.e., 50% initial Fura-2 peak) between fibers from Con and dystrophic mice (Table 2). During repeated tetani there is a characteristic increase in resting [Ca$^{2+}$] (56). In all groups, there was an increase in resting Fura-2 ratio at the end of the contractions, but the magnitude of increase in resting Fura-2 ratio in response to repeated tetani was not different between Con and dystrophic fibers (Table 2). Therefore, all fibers were exposed to the same number of tetani (~90 tetani) and the same magnitude of increase in resting Ca$^{2+}$ during the repeated tetani.

Although all fibers from the Con and dystrophic mice were exposed to the same number of tetani, there was a large discrepancy in the ability of fibers to respond to the test contractions following the 1 h of recovery. After repeated tetani, eight of eleven fibers (73%) from mdx/Utr$^{-/-}$ mice were either unable to respond to stimuli ($n = 6$) or demonstrated abnormal Ca$^{2+}$ transients ($n = 2$) (Table 3). While there were fibers from both mdx and mdx/Utr$^{+/−}$ mice that either failed to maintain the Fura-2 peak during the 350-ms stimulus or did not respond to stimuli after the repeated tetani, the % failing fibers was highest for mdx/Utr$^{-/-}$ mice (Table 3; $P < 0.05$). These data indicate that mdx/Utr$^{-/-}$ muscle fibers are more severely impaired by repetitive contractile activity.

Effect of protease inhibition on E-C coupling failure. There were no differences in time to reach 50% initial [Ca$^{2+}$], between treatment conditions (Veh and ALLN) for fibers from Con and dystrophic mice (Table 2). Also, there were no differences in resting Fura-2 ratio increase during repeated contractions in Veh compared with ALLN treated fibers for

Table 1. Body mass and single muscle fiber characteristics for each genotype

<table>
<thead>
<tr>
<th></th>
<th>No. of Mice</th>
<th>Body Mass, g</th>
<th>No. of Fibers</th>
<th>Fiber Diameter, μm</th>
<th>Resting Fura-2 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>3</td>
<td>26 ± 0.3</td>
<td>13</td>
<td>31 ± 1.7</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>mdx</td>
<td>5</td>
<td>28 ± 2.5</td>
<td>23</td>
<td>31 ± 1.4</td>
<td>0.37 ± 0.03†</td>
</tr>
<tr>
<td>mdx/Utr$^{+/−}$</td>
<td>5</td>
<td>28 ± 1.2</td>
<td>28</td>
<td>30 ± 1.5</td>
<td>0.37 ± 0.03†</td>
</tr>
<tr>
<td>mdx/Utr$^{-/-}$</td>
<td>4</td>
<td>14 ± 3.0*</td>
<td>21</td>
<td>23 ± 1.6*</td>
<td>0.36 ± 0.02†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *$P < 0.05$ vs. control (Con), mdx, and mdx/Utr$^{+/−}$; †$P < 0.05$ vs. Con.
any of the genotypes (Table 2). Thus, protease inhibition did not alter the level of [Ca$^{2+}$], that enzymes such as proteases were exposed to during the repeated tetani.

As shown in Fig. 2, there were no differences in peak Fura-2 ratios pre vs. post repeated tetani for Con fibers exposed to Veh or ALLN (Fig. 2, A and B). This outcome indicates that there was no long-lasting decrease in E-C coupling in response to repeated tetanic contractions in nondystrophic fibers and no effect of ALLN treatment. In contrast, there were differential effects of the repeated tetani on peak Fura-2 ratio in dystrophic fibers in the absence and presence of ALLN. In fibers from mdx and mdx/Utr$^{-/-}$ there was an average 30 ± 5.6% and 23 ± 2.0% decrease, respectively, in peak Fura-2 ratio, in response to the tetani when treated with Veh (Fig. 2, C and E; P < 0.05). This decrease, however, was not observed in fibers treated with ALLN (Fig. 2, D and F). Fibers from mdx/Utr$^{-/-}$ (Veh) had lower Fura-2 ratios after repeated tetani at some but not all stimulation frequencies (100 and 120 Hz, P < 0.05; Fig. 2G).

On average, Fura-2 ratio decreased 36 ± 3.1% across all stimulation frequencies in Veh-treated fibers from mdx/Utr$^{-/-}$ mice (P < 0.01), a greater decrease compared with mdx and mdx/Utr$^{-/-}$ (P < 0.05), indicating greater E-C coupling failure in fibers lacking both dystrophin and utrophin. This deficit was not observed in fibers treated with ALLN (Fig. 2H). Given that only 5 out of 11 (45%) of mdx/Utr$^{-/-}$ fibers responded to stimuli after repeated tetani, this assessment underestimates the true impairment of E-C coupling failure in these mice.

**Maximal calpain activity in control and dystrophic muscles.** To assess the potential for elevations in [Ca$^{2+}$], to activate calpain-dependent proteolysis, maximal calpain activity was measured ex vivo in muscle homogenates. Maximal calpain activity under maximal Ca$^{2+}$-activating conditions was significantly higher in mdx (2.8-fold), mdx/Utr$^{-/-}$ (2.1-fold), and mdx/Utr$^{-/-}$ (2.9-fold) compared with Con muscle (P < 0.05) (Fig. 3A). We also confirmed that ALLN, under the conditions used in the single fibers (1 μM and 15 min of exposure), could inhibit maximal calpain activity (95% decrease) in dystrophic muscle ex vivo (Fig. 3B, P < 0.05).

**DISCUSSION**

We examined the changes in E-C coupling in response to repeated tetanic contractions in single muscle fibers from three mouse models of DMD. We found that peak Ca$^{2+}$ in response to electrical stimulation was not different between fibers from dystrophic and Con mice before repeated tetani but that intracellular Ca$^{2+}$ was reduced in response to electrical activation after the repeated tetani in dystrophic mice, indicating E-C coupling failure. This pattern was observed in fibers from mdx, mdx/Utr$^{-/-}$, and mdx/Utr$^{-/-}$ mice but not in Con fibers. In fibers lacking both dystrophin and utrophin, there was a lower probability of the fibers being able to respond to electrical activation after repetitive contractions. The impairment appears to be due to protease activation because the decrease was mitigated in the presence of a protease inhibitor. Overall, these data suggest that repetitive activation of dystrophic muscle leads to protease activation and contributes to prolonged muscle weakness due to E-C coupling impairment after a bout of activity.

Although the primary cause of DMD is the lack of dystrophin (26), many of the downstream mechanisms contributing to the muscle pathology have not been fully elucidated. Weakening of the muscle membrane is one of the mechanisms that contribute to disease severity (4). It is known that dystrophic muscle has greater susceptibility to damage during eccentric contractions compared with normal muscle (60). Another mechanism that contributes to disease severity is related to intracellular modifications within dystrophic muscle. Studies have shown that muscles lacking dystrophin have increased oxidative stress, decreased antioxidant capacity, and poor Ca$^{2+}$ handling (21, 27, 44). A novel finding from this investigation is related to the role of proteases in the prolonged E-C coupling defects that would lead to muscle weakness in dystrophic muscle. Dystrophin-deficient fibers treated with a pan-protease inhibitor could maintain stimulation-induced Ca$^{2+}$ peaks after repeated contractions, whereas fibers treated with vehicle showed prolonged reductions in peak Ca$^{2+}$ after a bout of repeated tetani (Fig. 2).

**Ca$^{2+}$ handling in dystrophic vs. normal muscle.** In the current study, we show that elevations in resting [Ca$^{2+}$], are similar in all three models of muscular dystrophy (Table 1).
These data suggest that the lack of utrophin does not augment the increase in [Ca\(^{2+}\)], found in muscle fibers that lack only dystrophin. This outcome is consistent with previous reports of increased intracellular free Ca\(^{2+}\) concentration in fibers from mdx mice (27, 58, 59). However, other studies reported no difference in resting [Ca\(^{2+}\)], between Con fibers and muscle fibers from dystrophic mdx mice (16, 19, 35, 36, 42).

We reported no differences in peak tetanic Ca\(^{2+}\) at any of the stimulation frequencies assessed between Con, mdx, mdx/Utr\(^{+/+}\), and mdx/Utr\(^{-/-}\) fibers prior to repeated tetani, suggesting that there are no differences in E-C coupling and Ca\(^{2+}\) release mechanisms under normal physiological conditions. Previous studies evaluating differences in peak Ca\(^{2+}\) between mdx and Con fibers have shown inconsistent results, with some studies reporting no differences between mdx and Con muscle (16, 25, 51, 52), and others showing lower Ca\(^{2+}\) release in dystrophic muscle (27, 36, 58, 59). Capote et al. (9) reported that both mdx and mdx/Utr\(^{-/-}\) fibers have peak Ca\(^{2+}\) release that is reduced compared with Con fibers, but that there is no further reduction in the absence of utrophin (i.e., peak Ca\(^{2+}\) release from mdx/Utr\(^{-/-}\) fibers was similar to that from mdx fibers). In the current study we did not observe these differences in peak Ca\(^{2+}\) between fibers lacking dystrophin or both dystrophin and utrophin and Con fibers. This discrepancy may be due to a number of technical differences, such as the type of muscle fiber preparation, the dye form used to assess intracellular Ca\(^{2+}\), and/or the resting tension of the fibers during assessment of Ca\(^{2+}\) transients.

**Ca\(^{2+}\)-induced disruption of excitation-contraction coupling.**

Previous studies have demonstrated the role of increased intracellular Ca\(^{2+}\) during repeated muscle contractions in the prolonged reduction in E-C coupling following a bout of activity (14, 15, 39). This inability to release Ca\(^{2+}\) in response to membrane depolarization is also seen in skinned muscle fibers exposed to high Ca\(^{2+}\) concentrations (33, 53, 54) and was shown to be due, at least in part, to calpain activation (41, 53, 54). Recently, Murphy et al. (39) showed that increases in [Ca\(^{2+}\)], in intact muscle fibers via repeated contractions in the presence of caffeine leads to E-C coupling disruption due to selective proteolysis of junctophilin-1 (39). The role of junctophilin-1 in the proper formation and maintenance of mature skeletal muscle triad junction (29, 30), these data suggest that junctophilin proteolysis contributes to prolonged impairments in E-C coupling. The proteolysis of junctophilin-1 and corresponding autolytic activation of calpain-1 (39) further suggest that Ca\(^{2+}\)-activated proteases contribute to prolonged disruption of the skeletal muscle triads and thus E-C coupling failure. Although we did not measure proteolysis of these target proteins in the current study, our data are consistent with this mechanism of E-C coupling failure being due to protease activation.

---

**Table 3. Summary of fibers unable to respond to stimuli or demonstrating failing peaks**

<table>
<thead>
<tr>
<th></th>
<th>Fibers Failing to Maintain Peak</th>
<th>Fibers Not Responding to Stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Fatigue</td>
<td>After Fatigue</td>
</tr>
<tr>
<td>Con</td>
<td>0/13 (0%)</td>
<td>1/13 (8%)</td>
</tr>
<tr>
<td>mdx</td>
<td>8/23 (35%)</td>
<td>2/13 (15%)</td>
</tr>
<tr>
<td>mdx/Utr(^{+/+})</td>
<td>11/28 (39%)</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>mdx/Utr(^{-/-})</td>
<td>9/21 (43%)</td>
<td>2/11 (18%)</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Con, mdx, and mdx/Utr\(^{+/+}\).

---

**Fig. 2.** Peak Fura-2 ratio in single muscle fibers before and after inducing excitation-contraction coupling failure with repetitive tetanic contractions. Peak Fura-2 ratios are shown across the range of stimulation frequencies assessed before and 1 h after recovery from repeated tetani. A and B: vehicle-treated fibers (n = 5; A) and ALLN-treated fibers (n = 5; B) from Control (Con) mice. C and D: vehicle-treated fibers (n = 6; C) and ALLN-treated fibers (n = 6; D) from mdx mice. E and F: vehicle-treated fibers (n = 5; E) and ALLN-treated fibers (n = 9; F) from mdx/Utr\(^{+/+}\) mice. G and H: vehicle-treated fibers (n = 2; G) and ALLN-treated fibers (n = 3; H) from mdx/Utr\(^{-/-}\) mice. ALLN, N-acetyl-l-leucyl-l-leucyl-l-norleucinal. Data shown are means ± SE. †P < 0.10; *P < 0.05.
In the current study, we assessed intracellular Ca\(^{2+}\) in nonbranched fibers. Others have shown that force production and peak intracellular Ca\(^{2+}\) are reduced and that susceptibility to damage is increased in branched compared with nonbranched dystrophic fibers (10). Furthermore, when specific regions within a fiber are assessed using confocal microscopy, greater reductions in electrically induced Ca\(^{2+}\) transients are observed in the branch of the bifurcated fiber compared with the linear trunk region of the same fiber (36). Our imaging technique only allowed us to measure average intracellular Ca\(^{2+}\) across a fiber and therefore we were not able to address differences in branched vs. unbranched regions. Therefore, we focused on the unbranched fibers. As a result, our findings likely underestimate the magnitude of change in E-C coupling failure that may occur in dystrophic muscle in vivo where there are both branched and unbranched fibers.

In the current study we have only measured the average intracellular Ca\(^{2+}\) in response to electrical activation. We have not directly assessed whether a ryanodine receptor agonist (e.g., caffeine) could override the reduction in Ca\(^{2+}\) transient nor have we assessed the role of Ca\(^{2+}\) stores in this reduction. Thus, we cannot infer that the cause was a true impairment in electrical activation of Ca\(^{2+}\) release from sarcoplasmic reticulum Ca\(^{2+}\) stores. Rather our data show only a deficit in the Ca\(^{2+}\) transient in response to electrical activation after repetitive stimuli and that this reduction is mitigated with a protease inhibitor in all three models of muscular dystrophy.

**Effects of protease inhibition on E-C coupling in dystrophic muscle.** Calpains are nonlysosomal Ca\(^{2+}\)-activated proteases that, when activated, participate in the breakdown of muscle proteins. We hypothesized that impaired intracellular Ca\(^{2+}\) regulation in dystrophic muscle would be exacerbated during repeated tetani and this would contribute to greater E-C coupling disruption in single muscle fibers lacking both dystrophin and utrophin. In dystrophic muscles, there was a 2.1- to 2.9-fold increase in maximal calpain activity measured ex vivo. This, combined with the finding that ALLN eliminated the reduction in peak Ca\(^{2+}\) following tetanic contractions in single fibers from the dystrophic mice, supports the notion that proteases play an important role in prolonged E-C coupling failure. Since ALLN is also a potent inhibitor of other proteases it is possible that other proteolytic enzymes contributed to the E-C coupling impairment. The primary target of ALLN is calpain-1; however, calpain-2 and cathepsin B are also inhibited by ALLN with similar potency, while cathepsin L is inhibited at a greater potency (~400-fold more potent for cathepsin L). We did confirm in our ex vivo assay that ALLN effectively inhibits calpain activity in muscle tissue lysates (Fig. 3B).

The repeated tetani that reduce tetanic [Ca\(^{2+}\)], after contraction are expected to increase calpain activity and result in prolonged reductions in muscle force (i.e., muscle weakness) (14, 57) in dystrophic muscle. Further, bouts of contractile activity are expected to increase calpain activity to a similar extent in both dystrophin-null (mdx) and dystrophin/utrophin-null (mdx/Utr\(^{-/-}\)) fibers. A limitation of the current study is that we did not measure the acute increase in calpain activity with stimulation. However, previous studies have shown increased calpain activity of ~26% during exercise (2) and 13–25% (45) with resistive loading of the diaphragm and thus we expect a similar increase with the repeated contractions used in this study. Given that ALLN blunted the prolonged E-C coupling failure, our data suggest that a pan-protease inhibitor would protect against the contractile-induced impairments in Ca\(^{2+}\) release (and thus muscle weakness) in dystrophic muscle by preventing this acute increase in calpain activity. In the present study we have not identified the specific targets of calpain/protease activation. While this is a limitation of the present study, others have shown that elevations in Ca\(^{2+}\) are associated with key calpain-induced alterations of proteins involved in E-C coupling (e.g., ryanodine receptors, junctophilin-1) (22, 39). Thus, alterations in these proteins may explain the calpain-dependent impairment in E-C coupling and the protection we observed with ALLN.

Studies evaluating the effectiveness of calpain inhibitors for improving muscle function in the mdx mouse model in vivo appear controversial but overall provide proof of concept for calpain inhibition attenuating muscle damage and improving muscle function (3, 8, 32, 47, 48). While some studies show a decrease in necrosis and regenerating areas (central nucleated fibers) within the muscle (3, 48), others show no differences in regenerating areas or in muscle necrosis (8, 47). In mdx mice treated for 30 days with daily intramuscular injections of the calpain inhibitor leupeptin, there was a decrease in muscle fibrosis and necrosis (3). However, with long-term (6 mo) leupeptin treatment by intraperitoneal (ip) injections there was no attenuation of fibrosis or necrosis; moreover, these mice had an increase in µ-calpain and proteasome activity (47). Thus, short-term rather than long-term chronic treatment may be beneficial. Mode of administration (direct muscle vs. ip injec-
protease activity. A potentially more effective and less toxic calpain inhibitor, C101, did not reduce muscle necrotic area when animals were treated with daily injections from 8 to 12 wk of age (47). The compound C101 was also examined in a canine model of DMD and although it showed some efficacy in maintaining muscle strength in cranial pelvic limb muscles, it failed to maintain strength in posterior pelvic limb muscle (12). Moreover, C101 did not attenuate the decrease in force following eccentric contractions or mitigate the muscle necrosis observed in the dystrophic dogs (12). Others have shown that inhibition of both Ca\(^{2+}\)-dependent ubiquitin proteasome as well as calpain inhibition using a small-molecule inhibitor is more beneficial in reducing muscle damage (8). Combined with data from the current study, broader spectrum protease inhibitors targeting other proteolytic enzymes may be most effective in mitigating muscle damage, impaired contractile function, and prolonged E-C coupling defects in dystrophic muscle. Future studies need to address in vivo dosing strategies to optimize the benefits of calpain and other protease/proteasome inhibition acutely after repetitive contractile activity while not suppressing the beneficial role of proteolysis in normal turnover of muscle proteins.

In conclusion, this study is the first to demonstrate the role of proteases in the prolonged E-C coupling failure following repeated muscle contractions in multiple dystrophic mouse models. Dystrophic single muscle fibers are less likely to respond to electrical stimulation after fatigue-inducing contractions, and this failure is greater in fibers lacking both dystrophin and utrophin. However, the prolonged E-C coupling failure can be prevented by a potent pan-protease inhibitor. Protease inhibition may therefore be beneficial in protecting against prolonged weakness in dystrophic muscle after an acute bout of activity, rather than as a long-term treatment to reduce protease activity.

ACKNOWLEDGMENTS

We thank Dr. Diego Fraidenraich and Joel Schneider (University of Medicine and Dentistry, New Jersey) for advice on breeding and maintaining the mdx/Utr\(^{iso}\) colony and establishing the genotyping protocols. We also thank Dapeng Chen and Samuel Andrew English (Department of Kinesiology, University of Maryland) for technical contributions to the project.

GRANTS

This work was supported by the University of Maryland, College Park new investigator funds (to E. R. Chin). Additional grant support was provided by the Department of Kinesiology Graduate Research Initiative Project (GRIP) and the National Institute on Aging Predoctoral Training Grant T32AG-00268 (to D. A. G. Mážala).

DISCLOSURES

E. R. Chin is the Founder and Chief Scientific Officer of MyoTherapeutics. No other conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

22. Gilchrist JS, Wang KK, Katz S, Belcastro AN. Calcium-activated neutral protease effects upon skeletal muscle sarcoplasmic reticulum.


