Dihydropyridine and ryanodine receptor binding after eccentric contractions in mouse skeletal muscle

Christopher P. Ingalls, Gordon L. Warren, Jia-Zheng Zhang, Susan L. Hamilton, and R. B. Armstrong. Dihydropyridine and ryanodine receptor binding after eccentric contractions in mouse skeletal muscle. J Appl Physiol 96: 1619–1625, 2004. First published December 12, 2003; 10.1152/japplphysiol.00084.2003.—The purpose of this study was to determine whether there are alterations in the dihydropyridine and/or ryanodine receptors that might explain the excitation-contraction uncoupling associated with eccentric contraction-induced skeletal muscle injury. The left anterior crural muscles (i.e., tibialis anterior, extensor digitorum longus, and extensor hallucis longus) of mice were injured in vivo by 150 eccentric contractions. Peak isometric tetanic torque of the anterior crural muscles was reduced ~45% immediately and 3 days after the eccentric contractions. Partial restoration of peak isometric tetanic and submaximal forces of injured extensor digitorum longus muscles by 10 mM caffeine indicated the presence of excitation-contraction uncoupling. Scatchard analysis of [1H]ryanodine binding indicated that the number of ryanodine receptor binding sites was not altered immediately postinjury but decreased 16% 3 days later. Dihydropyridine receptor binding sites increased ~20% immediately after and were elevated to the same extent 3 days after the injury protocol. Muscle injury did not alter the sensitivity of either receptor. These data suggest that a loss or altered sensitivity of the dihydropyridine and ryanodine receptors does not contribute to the excitation-contraction uncoupling immediately after contraction-induced muscle injury. We also concluded that the loss in ryanodine receptors 3 days after injury is not the primary cause of excitation-contraction uncoupling at that time.

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Voltage sensor; sarcoplasmic reticulum calcium release channel; injury

THE PERFORMANCE OF HIGH-FORCE lengthening (i.e., eccentric) contractions is known to induce an immediate and protracted decrease in skeletal muscle strength in humans (6, 16) and laboratory animals (17, 19, 21–23, 33, 34). Although disruptions in force-generating and/or -transmitting structures contribute to the decrease in skeletal muscle strength after eccentric contractions (17, 32, 33), failure of the excitation-contraction (E-C) coupling process has been shown to be a primary mechanism responsible for the strength loss in mouse skeletal muscle (2, 19, 33, 36). Specifically, our laboratory (19) and others (2) have shown that cytosolic Ca$$^{2+}$$ transients are reduced during electrical activation (i.e., tetanic contractions) but are unaffected during drug-induced activation (i.e., contractions) after (i.e., <3 days) eccentric contraction-induced injury in mouse skeletal muscle. On the basis of the relative changes in caffeine- and electrical stimulation-induced forces between injured and contralateral control muscles, we have estimated that E-C uncoupling accounts for ~75% of the maximal strength loss immediately and 3 days after the injury in the mouse extensor digitorum longus (EDL) muscle (19, 33).

Although the exact mechanism of E-C coupling in skeletal muscle is not completely understood, it is known that the dihydropyridine and ryanodine receptors are critical to the integrity of E-C coupling and normal skeletal muscle function (9, 27). Morphological studies indicate that each ryanodine receptor is physically linked to a cluster of four dihydropyridine receptors (9). It is hypothesized that voltage-dependent activation of the dihydropyridine receptor proteins triggers sarcoplasmic reticulum (SR) Ca$$^{2+}$$ release via directly linked ryanodine receptor channels (27). Presumably, activation of the directly linked ryanodine receptor then triggers adjacent unlinked ryanodine receptors, promoting further SR Ca$$^{2+}$$ release. Alteration of subunits of either receptor is known to result in altered radiolabeled ligand receptor binding and E-C coupling failure (4, 9, 10, 13). For example, mouse skeletal muscle lacking the β1-subunit of the dihydropyridine receptor exhibits marked reductions in the maximum density of dihydropyridine receptor binding sites (Bmax), decreases in the affinity of the receptor for ligands (Kd), and reduced intracellular Ca$$^{2+}$$ transients (30). In addition, ischemia, ischemic preconditioning, and ischemia-reperfusion are associated with reductions in ryanodine receptor Bmax and SR Ca$$^{2+}$$ release in cardiac muscle (41, 42). On the basis of several experimental techniques (i.e., contracture, Ca$$^{2+}$$ imaging, and electromyography), we have shown that the site of the exercise-induced E-C coupling failure is likely at or between the t tubule voltage sensor (i.e., dihydropyridine receptor) and SR Ca$$^{2+}$$ release channel (i.e., ryanodine receptor) in mouse skeletal muscle (19, 33, 34). Despite the critical role of the dihydropyridine and ryanodine receptors in E-C coupling in vertebrate skeletal muscle, very little is known about how these receptors are affected by eccentric contraction-induced muscle injury. Therefore, the purpose of this study was to test the hypothesis that the performance of eccentric contractions in vivo would disrupt dihydropyridine and ryanodine receptor ligand binding characteristics (i.e., Bmax and Kd) in mouse tibialis anterior (TA) muscle immediately after and 3 days after eccentric contraction-induced injury, which would implicate dysfunction of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of these receptors in E-C uncoupling and the resulting strength deficits.

MATERIALS AND METHODS

Animals. Female ICR mice (n = 32), 8–12 wk old, were used in the study. Their mean body mass was 31.6 ± 2.5 g (± SD). The mice were housed in groups of five or six animals per cage, supplied with food and water ad libitum, and maintained in a room at 20–22°C with a 12-h photoperiod. Mice were euthanized with an overdose of pentobarbital sodium. All animal care and use procedures were approved by the institutional animal care and use committee and met the guidelines set by the American Physiological Society.

Experimental design. Two studies were performed. In one study, 12 mice were used to evaluate the integrity of E-C coupling in EDL muscles immediately after (i.e., 0 days) and 3 days after the performance of 150 eccentric contractions in vivo. Force production of injured and contralateral control EDL muscles was measured during electrical stimulation with and without the presence of subcontracture concentrations of caffeine (i.e., 10 mM). Caffeine at this concentration acts directly on the SR to augment Ca2+ release during skeletal muscle activation (2, 15). Although this experimental approach has been used previously to demonstrate E-C uncoupling in mouse single muscle activation (2, 15), no studies have examined the effectiveness of subcontracture methods to indicate the presence of E-C uncoupling (8). This time interval was chosen because previous studies indicated that the two muscles share similar mechanisms (i.e., E-C uncoupling) and that the two muscles are almost exclusively fast-twitch (26; Warren GL, unpublished observations), exhibit similar force loss after eccentric contraction-induced injury. For example, observations indicating that the contribution of E-C uncoupling to strength deficit is the greatest in the first 3 days after eccentric contraction-induced muscle injury (19). Changes in the number of receptor binding sites (Bmax) and affinity (Ka) were determined by using [3H]ryanodine and [3H]PN200-110 binding to the ryanodine and dihydropyridine receptors, respectively, in the muscles. The TA muscle was used instead of the EDL muscle because there was insufficient EDL muscle mass to reliably study receptor-binding characteristics. Although E-C uncoupling has not been studied as extensively in the mouse TA muscle as in the EDL muscle owing to methodological limitations (e.g., diffusion limitations), we believe that the two muscles share similar mechanisms (i.e., E-C uncoupling) of force loss after eccentric contraction-induced injury. For example, high-to-low stimulation frequency ratios are significantly elevated (68–297%) over the first week in the anterior crural muscles after injury (reanalysis of the data in Fig. 3A from Ref. 34), which have been used to indicate the presence of E-C uncoupling (8). This time course is similar to that for E-C uncoupling in the EDL muscle (19).

Also, both muscles are almost exclusively fast-twitch (>95%) muscles (26; Warren GL, unpublished observations), exhibit similar functional deficits after the performance of 150 eccentric contractions in vivo and comparable recovery patterns (see Refs. 17, 19, 34), and experience similar reductions in myofibrillar protein content after injury (20). It was assumed that if eccentric contractions disrupted the dihydropyridine and/or ryanodine receptors, then receptor ligand binding characteristics (i.e., Bmax and Ka) should be altered in injured skeletal muscle.

In vivo muscle injury induction. The anterior crural muscles (TA, EDL, and extensor hallucis longus) from the left hindlimbs of mice were injured by use of 150 eccentric contractions as previously described (17, 19, 34). In this model, the TA muscle is responsible for nearly all (~89%) of the torque measured (22). Briefly, the anesthetized (0.33 mg/kg fentanyl citrate, 16.7 mg/kg droperidol, and 5.0 mg/kg diazepam) mouse was placed on a temperature (39°C)-controlled platform with the left foot secured to an aluminum “shoe” that is attached to the shaft of the servomotor (Aurora Scientific 300B), and the left knee was secured by a clamp. Sterilized platinum needle electrodes (25 gauge) were inserted through aseptically prepared skin for stimulation of the common peroneal nerve. Torque output from the Aurora Scientific servomotor system was measured with the use of a Keithley-Metrabyte DAS 1802AO interface board, TestPoint data-acquisition/process-control software (version 4.0; Capital Equipment), and computer. To optimize anterior crural muscle torque output, voltage and electrode placement were adjusted during a series of (<10) examined tetanic contractions (200-ms train of 0.1-ms pulses at 300 Hz). One minute after the last isometric tetanic contraction, the anterior crural muscles started the 150 eccentric-contraction (40° angular movement at 2,000/s starting from a 20° dorsiflexed position) injury protocol. In general, the time between eccentric contractions was 10 s. Two minutes after the last eccentric contraction, an isometric tetanic contraction was performed. In the caffeine study, isometric torque as a function of stimulation frequency was measured immediately before and after injury induction, as well as 3 days after the injury. Isometric contractions (200-ms train of 0.1-ms pulses) were performed every 45 s at the following stimulation frequencies: 20, 40, 60, 80, 100, 125, 150, 200, 250, and 300 Hz.

In vitro mechanical analysis. In the caffeine study, injured and contralateral control EDL muscles were injured at 0 and 3 days postinjury and studied at 25°C by use of an in vitro preparation as previously described (17, 19, 32, 37). Briefly, after dissecting free the TA muscle, the anatomic resting muscle length (L0) of the EDL muscle was measured with a micrometer by taking the average of the muscle’s minimum and maximum in situ lengths. EDL muscles were then mounted in a chamber containing a Krebs-Ringer bicarbonate buffer (pH 7.4) with (in mM) 144 Na+, 126.5 Cl-, 6 K+, 1 Mg2+, 1 SO42–, 1 PO43–, 25 HCO3–, 1.25 Ca2+, 0.17 leucine, 0.10 isoleucine, 0.20 valine, 10 glucose, and 10 g/liter gentamicin sulfate and 0.10 U/ml insulin (the buffer was equilibrated with 95% O2-5% CO2 gas). The distal tendon was attached by silk suture and cyanoacrylate adhesive to a fixed support, and the proximal tendon was attached to the lever arm of a servomotor system. Muscle length in the chamber was set at its anatomic L0 by adjusting resting tension to 4.4 mN, a force previously determined to correspond to the anatomic L0 in mouse EDL muscle (32). Isometric twitch (Pt; 0.2 ms pulse at 150 V) and subtetanic (200 ms train at 100 Hz) and tetanic (P0; 200 ms trains of 0.2 ms pulses at 180 Hz) contractions were initiated at 7, 8, and 12 min into the incubation, respectively. After the measurement of Pt, the EDL muscles were incubated in a Krebs-Ringer solution containing 10 mM caffeine for 5 min. Resting tension was measured during the caffeine incubation and remained unchanged. Pt, subtetanic, and P0 contractions were initiated at 5, 6, and 8 min into the caffeine incubation, respectively. To compare EDL and TA muscle subtetanic contractile responses, it was determined that the 100-Hz stimulation frequency of the noninjured TA muscle in vivo, with both frequencies resulting in contractile forces that are 93% of the isometric tetanic force (or torque) value.

Muscle homogenization and total protein analysis. In the receptor binding study, TA muscles were used to characterize dihydropyridine and ryanodine receptor binding properties. Pilot studies indicated that individual EDL muscles were too small to give reliable receptor binding results. Because of similarities in fiber type and strength loss at 0 and 3 days between the TA and EDL muscles, it was assumed that the degree of E-C uncoupling is comparable in the two muscles at these times.

Either immediately after the in vivo torque measurements or 3 days later, mice were anesthetized with pentobarbital sodium (50–80 mg/kg), and the left and right TA muscles were dissected out, washed, frozen in liquid nitrogen, and stored at −80°C. TA muscles were then homogenized on ice in 10 volumes of 100 mM NaCl, 50 mM MOPS (pH 7.4), 0.1 mM PMSF, 0.2 mM amino benzamidine, and 1 μg/ml.
Eccentric contractions caused significant reductions (48–88%) in isometric torques immediately after injury induction. Three days after injury induction, peak isometric torques were still significantly reduced from 45 to 68%. However, compared with torque values immediately after injury, isometric torques at stimulation frequencies below 250 Hz exhibited some recovery at 3 days postinjury, whereas isometric torques at stimulation frequencies above 200 Hz showed no significant recovery.

The mean wet weight of the contralateral control EDL muscles was 10.5 ± 0.2 mg. Wet weight of the injured EDL muscles was 6.4% greater than that of the control muscles. The resting length of the EDL muscles measured in the chamber (1.54 ± 0.01 cm) was not different from the anatomical $L_0$ (1.55 ± 0.01 cm) measured in situ, as previously shown (31). However, resting $L_o$ for the injured EDL muscles was 1.2% shorter than that for the contralateral control muscles.

The mean specific isometric tetanic force of the contralateral control EDL muscles was 27.0 ± 0.5 N/cm². The performance of eccentric contractions in vivo caused significant reductions in EDL muscle force production measured in vitro (Fig. 2). Compared with contralateral control muscles, peak tetanic, subtetanic, and twitch forces were all significantly reduced in EDL muscles immediately after performance of eccentric contractions (−52, −60, and −65%, respectively), and these force values were unchanged at 3 days (−45, −55, and −60%, respectively). Exposing injured EDL muscles to 10 mM caffeine before and after the injury bout are shown in Fig. 1. Eccentric contractions caused significant reductions (48–88%) in isometric torques immediately after injury induction. Three days after injury induction, peak isometric torques were still significantly reduced from 45 to 68%. However, compared with torque values immediately after injury, isometric torques at stimulation frequencies below 250 Hz exhibited some recovery at 3 days postinjury, whereas isometric torques at stimulation frequencies above 200 Hz showed no significant recovery.

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**RESULTS**

**Caffeine force potentiation study.** The peak torque produced by the anterior crural muscles at the ankle during the first eccentric contraction was 6.59 ± 0.22 N·mm, or 218% of the torque during the preinjury maximal isometric tetanic contraction. The relative decrease in peak eccentric torque from the first to the last eccentric contraction was 40.3 ± 2.1%. Peak isometric torques produced as a function of stimulation frequency before and after the injury bout are shown in Fig. 1. Eccentric contractions caused significant reductions (48–88%) in isometric torques immediately after injury induction. Three days after injury induction, peak isometric torques were still significantly reduced from 45 to 68%. However, compared with torque values immediately after injury, isometric torques at stimulation frequencies below 250 Hz exhibited some recovery at 3 days postinjury, whereas isometric torques at stimulation frequencies above 200 Hz showed no significant recovery.

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feine caused significant increases in peak force during tetanic (13–35%), subtetanic (30–59%), and twitch (76–84%) contractions immediately and 3 days after injury (Fig. 2). However, force production during tetanic and subtetanic contractions was unaffected by 10 mM caffeine in control muscles (Fig. 2). As expected, force production during twitch contractions was significantly potentiated (48–62%) by 10 mM caffeine in control muscles (Fig. 2). The ability of caffeine to significantly increase P0 in injured EDL muscles further supports our previous observation that E-C coupling failure contributes to the strength loss immediately and 3 days after the performance of eccentric contractions (19).

Receptor binding characteristics study. The peak torque produced by the anterior crural muscles at the ankle during the first eccentric contraction equaled 6.33 ± 0.17 N-mm, or 191% of the torque during the preinjury isometric contraction. The relative decrease in peak eccentric torque from the first to the last eccentric contractions was 44.8 ± 0.8%. The peak torque during the preinjury isometric tetanic contraction averaged 3.32 ± 0.10 N-mm. The peak torque during the postinjury isometric tetanic contraction was reduced 45.3 ± 1.3%.

Unlike the EDL muscle, there were no significant differences in wet weight between the injured (60.7 ± 1.1 mg) and contralateral control (59.3 ± 1.1 mg) TA muscles. However, total protein content in the injured TA muscles (10.9 ± 0.5 mg) was 10% less than in the contralateral control muscles (12.1 ± 0.5 mg) 3 days after injury induction.

Representative Scatchard analyses of ligand binding of [3H]ryanodine and [3H]PN200-110 to the ryanodine and dihydropyridine receptors, respectively, in injured and control TA muscles 3 days after injury are shown in Fig. 3. The mean and range of the correlation coefficients for [3H]ryanodine binding were 0.986 and 0.941–0.999, respectively. The mean and range of the correlation coefficients for [3H]PN200-110 binding were 0.978 and 0.904–0.999, respectively. The Bmax for [3H]ryanodine binding relative to muscle wet weight was unchanged immediately after injury but was reduced 13% 3 days after performance of eccentric contractions in the injured TA muscles compared with contralateral control muscles (Table 1). Because there are no differences in wet weight between injured and contralateral control TA muscles, the reduction in Bmax (expressed relative to wet weight) at 3 days reflects a decrease in the absolute number of ryanodine binding sites in the injured muscle. When Bmax for [3H]ryanodine binding was expressed relative to protein content, there were no differences between injured and contralateral control TA muscles, indicating that there was no preferential loss of the ryanodine receptor with the decrease in total protein content in injured TA muscle 3 days after injury. The Bmax for [3H]ryanodine binding expressed relative to protein content at 3 days was lower than

Table 1. Scatchard plot analysis of [3H]PN200-110 and [3H]ryanodine binding to injured and contralateral control tibialis anterior muscle homogenates

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<tr>
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<th>Control</th>
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<tr>
<td>[3H]PN200-110</td>
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<tr>
<td>Bmax, pmol/muscle</td>
<td>7.6±0.3* (n=9)</td>
<td>7.9±0.3* (n=9)</td>
<td>7.3±0.4* (n=8)</td>
<td>6.1±0.2* (n=8)</td>
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<tr>
<td>Bmax, pmol/g protein</td>
<td>727±28* (n=8)</td>
<td>700±32* (n=8)</td>
<td>613±49* (n=10)</td>
<td>557±34* (n=10)</td>
</tr>
<tr>
<td>Kd, nM</td>
<td>12.4±1.5* (n=8)</td>
<td>14.0±1.9* (n=8)</td>
<td>7.1±0.7* (n=10)</td>
<td>7.3±0.4* (n=10)</td>
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<tr>
<td>[3H]ryanodine</td>
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<tr>
<td>Bmax, pmol/muscle</td>
<td>6.5±0.7* (n=8)</td>
<td>8.0±0.7* (n=8)</td>
<td>7.1±0.8* (n=10)</td>
<td>8.2±1.1* (n=10)</td>
</tr>
<tr>
<td>Bmax, pmol/g protein</td>
<td>621±64* (n=8)</td>
<td>727±78* (n=8)</td>
<td>591±65* (n=10)</td>
<td>762±114* (n=10)</td>
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<tr>
<td>Kd, nM</td>
<td>1.9±0.2* (n=8)</td>
<td>2.3±0.1* (n=8)</td>
<td>2.1±0.1* (n=10)</td>
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Values are means ± SE. Bmax, maximum density of binding sites; Kd, affinity of receptor for ligands. Values with the same letter within the same row are not significantly different.
values immediately after injury induction in both control and injured muscles (Table 1). Likewise, the $K_d$ of [3H]ryanodine binding was not different between injured and contralateral control TA muscles, but 3-day values were lower than values immediately after injury induction in both control and injured muscles (Table 1). The reason for the difference in the affinity of [3H]ryanodine binding to the SR release channel between these two points is unknown. It is possible that differences in the drugs used to anesthetize mice (see Materials and Methods) between these time points may have influenced the sensitivity of ligand binding. Certain anesthetics are known to impair skeletal force production (18), as well as ligand-binding characteristics (11, 12).

In contrast with the [3H]ryanodine binding, the $B_{max}$ for [3H]PN200-110 binding relative to muscle wet weight and protein content was increased ~20% immediately and 3 days after performance of eccentric contractions in the injured TA muscles compared with contralateral control muscles (Table 1). There were no differences in the $K_d$ of [3H]PN200-110 binding between injured and contralateral control TA muscles. Also, the $K_d$ was not different between time points for either group.

The ratio of the $B_{max}$ for [3H]ryanodine binding to $B_{max}$ for [3H]PN200-110 binding was 1.2 ± 0.1 in control TA muscle. Most biochemical studies report a ratio of ryanodine-to-dihydropyridine receptors near 1 (ranging from 0.9 to 1.3) in mammalian fast-twitch muscle (1, 5, 7). There was no significant change in the ratio in injured TA muscles immediately after injury induction (1.09 ± 0.07), but the ratio decreased in injured TA muscles at 3 days (0.80 ± 0.1).

**DISCUSSION**

The primary objective of this study was to determine whether the performance of eccentric contractions would disrupt the affinity (i.e., $K_d$) and maximum number of binding sites (i.e., $B_{max}$) for dihydropyridine and ryanodine receptor ligands in mouse TA muscle immediately after and 3 days after eccentric contraction-induced injury, which presumably could contribute to the E-C uncoupling and strength deficits that have been observed. We hypothesized that, if eccentric contractions resulted in irreversible denaturation of either receptor, then $B_{max}$ values would be expected to decrease. Moreover, because ryanodine and dihydropyridine receptors are influenced by a number of ancillary proteins (e.g., FKBP12, calmodulin), disruptions in the ability of the receptors to bind these proteins may alter $K_d$; however, specific changes in $K_d$ would depend on the nature of the interaction of the specific protein with the receptor. The results suggest that neither E-C uncoupling nor strength losses in mouse muscles immediately after eccentric contraction-induced injury stem from disruptions in the integrity of either the ryanodine or dihydropyridine receptors. On the basis of the receptor binding experiments, neither the number ($B_{max}$) nor the sensitivity ($K_d$) of the ryanodine and dihydropyridine receptors was reduced immediately after injury induction (Table 1). The finding that [3H]ryanodine binding is not significantly altered immediately after injury agrees with our previous study showing that intrinsic SR Ca$^{2+}$ channel function is generally unaffected immediately after injury (19). When stimulated by drugs known to activate the SR Ca$^{2+}$ channel, the ability of the SR to release Ca$^{2+}$ was not significantly impaired immediately after injury when measured by using confocal laser scanning microscopic or fluorometric techniques, although small but significant reductions (6%) in SR Ca$^{2+}$ release were detected when measured with a Ca$^{2+}$ minielectrode (19). Previous work from our laboratory indicating that the neuromuscular junction (34), plasmalemma (34, 36), and t tubules (19) are not compromised after eccentric contractions suggests that the failure in E-C coupling occurs at or below the level of the voltage sensor. Therefore, our previous work and the present data suggest that the impaired SR Ca$^{2+}$ release observed immediately after injury during tetanic excitation (2, 3, 19, 36) results from a communication failure between the dihydropyridine and ryanodine receptors and not a failure of the receptors themselves.

In contrast to the response immediately after injury, a decrease (16%) in [3H]ryanodine receptor binding was observed 3 days after the injury to mouse anterior crural muscles. A loss of functional ryanodine receptors in the injured muscles may explain the reduction in the ability of 10 mM caffeine to increase force during tetanic and subtetanic contractions in injured EDL muscles 3 days after injury. Specifically, caffeine increased tetanic force 35% immediately after injury and only 13% at 3 days. Similarly, caffeine increased subtetanic forces 59% immediately after injury and only 30% at 3 days. Therefore, mouse skeletal muscle exhibits both reduced ryanodine receptor ligand binding (present study) and less effective Ca$^{2+}$ release from the SR Ca$^{2+}$ channel during contractile activation (with and without 10 mM caffeine exposure) 3 days after injury (present study, Ref. 19).

Although a loss in functional ryanodine receptors by 3 days postinjury could promote E-C uncoupling, it does not appear that this is the primary cause of the E-C coupling failure in the muscles. The main argument against a loss in ryanodine receptors as the primary contributor to E-C uncoupling is that it does not appear to affect anterior crural muscle force production. EDL and TA (i.e., anterior crural) muscle strength deficits are unchanged or improved 3 days after injury during tetanic activation (present study, Refs. 17, 19, 21, 34). Reductions in [3H]ryanodine receptor binding (present study) and intrinsic SR Ca$^{2+}$ release rate (19) at this time may have occurred in fibers that already had reduced force output and that were undergoing some degree of degeneration.

[3H]PN200-110 binding to dihydropyridine receptors was actually increased (20%) immediately and 3 days after injury. Whether the increase in [3H]PN200-110 binding reflects an absolute increase in receptor number or the uncovering of a previously “silent” fraction of receptors for binding is unknown. Uprogulation of dihydropyridine receptor gene expression has been reported in skeletal muscle after disruption of normal muscle excitation via denervation or tetrodotoxin treatment (24, 25, 28). Moreover, rapid (i.e., 30–60 min) down-regulation and subsequent upregulation of dihydropyridine and ryanodine receptors have been documented in cardiac muscle perfused with hypocalcemic and normocalcemic buffers, respectively (40). Although the normal function of the plasmalemma and neuromuscular junction does not appear to be impaired after eccentric contraction-induced injury in our model (34), we have previously reported transient increases in nicotinic acetylcholine receptor concentration in mouse TA muscle after eccentric contraction-induced injury (34). Conceivably, the loss in muscle protein observed between 5 and 28
days may stem from the transient denervation-like response of the muscle, as well as from the sarcomeric damage caused by the eccentric contractions. Nonetheless, it does not appear that a loss of dihydropyridine receptors or alteration in their binding capacity explains the disruption in E-C coupling and strength loss during the first several days after eccentric contraction-induced injury.

The exact site(s) of the E-C uncoupling in the injured muscles remains unknown. In previous work, we have provided indirect evidence that the lesion is at the interface of the dihydropyridine and ryanodine receptors, but this evidence has been deduced from eliminating other potential sites (i.e., neuromuscular junction, plasmalemma, t tubule, etc.). We continue to suspect that the lesion in E-C coupling in the injured muscles is at the interface between the two receptors. For example, disruptions in any one of the numerous ancillary proteins (e.g., triadin, calmodulin, FKBP12, junctin, calsequestrin) associated with the dihydropyridine and ryanodine receptors could contribute to the uncoupling (9).

There is evidence that the E-C uncoupling results from disruptions in the t-tubular membrane system. Previously, we have shown that t tubules become disrupted immediately after both eccentric and isometric contractions in mouse soleus muscle in vitro (35). However, we found no significant correlations between the degree of t-tubular membrane disruption and the reduction in maximal isometric force or release of lactate dehydrogenase (35). Recently, it has also been reported that membrane systems involved in E-C coupling are disrupted after eccentrically biased exercise in rat fast- and slow-twitch muscle fibers (31) and eccentric contractions in single mouse muscle fibers (39). However, neither study correlated the degree of the strength deficit with t tubule membrane disruption. Nevertheless, the pervasive and long-lasting disruption of membrane systems involved in E-C coupling (from immediately after injury to 10 days postinjury) in rat skeletal muscle after downhill running (31) is consistent with our observation that E-C uncoupling is immediate and long lasting (to at least 5 days postinjury) in mouse EDL muscle after the performance of eccentric contractions in vivo (19, 33).

The observation that 10 mM caffeine significantly increases $P_o$ (35%) and subtetanic force (59%) in injured EDL muscle, whereas it has no significant effect on $P_o$ and subtetanic (i.e., 100 Hz) force in contralateral control muscle, confirms previous findings that E-C coupling is impaired in mouse skeletal muscle after injury (2, 3, 19, 36). Estimates of the contribution of E-C uncoupling to the $P_o$ reduction in the first 3 days after injury (14–28%) are significantly lower in the present study than our previous estimate of 75% (19, 33). Estimates from the present study would be accurate if E-C uncoupling stemmed solely from defects in the ryanodine receptor and caffeine was capable of completely restoring normal tetanic Ca$^{2+}$ transients. However, data from Ca$^{2+}$ imaging (2, 19), caffeine-induced contracture force (19, 36), SR Ca$^{2+}$ release via spectrofluorimetry (19), and ligand receptor binding (present study) experiments all suggest that reduced tetanic Ca$^{2+}$ transients do not stem from defects in the ryanodine receptor immediately after injury. On the other hand, the contribution of E-C uncoupling to the $P_o$ deficit would be underestimated in the present study if the dihydropyridine receptor-induced activation of ryanodine receptors is compromised because 10 mM caffeine in mouse EDL muscle does not elicit any apparent SR Ca$^{2+}$ release or force production (i.e., based on resting muscle tension) independent of electrical activation. Therefore, defects in the E-C coupling pathway upstream of the ryanodine receptor and defects in force-generating and -transmitting structures presumably prevent caffeine from fully restoring peak force in injured EDL muscles.

In summary, mouse anterior crural muscle strength deficits after eccentric contraction-induced injury do not stem from disruptions in ryanodine and dihydropyridine receptor integrity. These data are not inconsistent with the hypothesis that communication failure between the dihydropyridine and ryanodine receptors is the cause of the E-C uncoupling in mouse skeletal muscle after the performance of eccentric contractions.

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