Uncoupling of *in vivo* torque production from EMG in mouse muscles injured by eccentric contractions

Gordon L. Warren, Christopher P. Ingalls, Shree J. Shah and R. B. Armstrong

Muscle Biology Laboratory, Texas A&M University, College Station, TX 77843, USA

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1. The main objective of this study was to determine whether eccentric contraction-induced muscle injury causes impaired plasmalemmal action potential conduction, which could explain the injury-induced excitation–contraction coupling failure. Mice were chronically implanted with stimulating electrodes on the left common peroneal nerve and with electromyographic (EMG) electrodes on the left tibialis anterior (TA) muscle. The left anterior crural muscles of anaesthetized mice were stimulated to perform 150 eccentric (ECC) ($n = 12$ mice) or 150 concentric (CON) ($n = 11$ mice) contractions. Isometric torque, EMG root mean square (RMS) and M-wave mean and median frequencies were measured before, immediately after, and at 1, 3, 5 and 14 days after the protocols. In parallel experiments, nicotinic acetylcholine receptor (AChR) concentration was measured in TA muscles to determine whether the excitation failure elicited a denervation-like response.

2. Immediately after the ECC protocol, torque was reduced by 47–89%, while RMS was reduced by 9–21%; the RMS decrement was not different from that observed for the CON protocol, which did not elicit large torque deficits. One day later, both ECC and CON RMS had returned to baseline values and did not change over the next 2 weeks. However, torque production by the ECC group showed a slow recovery over that time and was still depressed by 12–30% after 2 weeks. M-wave mean and median frequencies were not affected by performance of either protocol.

3. AChR concentration was elevated by 79 and 368% at 3 and 5 days, respectively, after the ECC protocol; AChR concentration had returned to control levels 2 weeks after the protocol. At the time of peak AChR concentration in the ECC protocol muscles (i.e. 5 days), AChR concentration in CON protocol muscles was not different from the control level.

4. In conclusion, these data demonstrate no major role for impaired plasmalemmal action potential conduction in the excitation–contraction coupling failure induced by eccentric contractions. Additionally, a muscle injured by eccentric contractions shows a response in AChR concentration similar to a transiently denervated muscle.

Traditionally, most of the strength loss induced by eccentric contractions (i.e. contractions in which a muscle is lengthened while it is activated) has been ascribed to damage to or loss of force-bearing structures within the muscle. Recent data from both *in vitro* and *in vivo* mouse injury models indicate that this is not the case and that a failure in the excitation–contraction (EC) coupling process is primarily responsible for the force deficit (Warren *et al.* 1993, 1994; Balnave & Allen, 1995; Ingalls *et al.* 1998*a*,*b*). Up to and including the 5th day after initiation of the injury, most (i.e. 57–75%) of the force deficit is due to a reduction in tetanic free cytosolic Ca^{2+} concentration (Balnave & Allen, 1995; Ingalls *et al.* 1998*b*). The reduction in tetanic free cytosolic Ca^{2+} concentration is not attributed to impaired sarcoplasmic reticulum (SR) function, at least not in the first 24 h following injury initiation. In mouse extensor digitorum longus (EDL) muscles that experienced a ~50% reduction in maximal isometric tetanic force (P_o) at 0–24 h post-injury, the rates of ATP-induced Ca^{2+} uptake and $AgNO_3$-induced Ca^{2+} release in SR fractions were minimally affected (Ingalls *et al.* 1998*b*). We concluded that the site of EC coupling failure must lie above the level of the SR Ca^{2+} release channel in the EC coupling pathway.

Impaired action potential conduction by the plasmalemma would seem to be a probable cause of the EC coupling failure. In muscles composed predominantly of fast-twitch fibres (i.e. the fibre type most easily injured by eccentric contractions (Fridén *et al.* 1983; Lieber *et al.* 1991; Warren *et al.* 1994)), there is a minimal safety margin with regard to action potential overshoot; as little as a 5 mV reduction in overshoot can lead to a reduction in force (Fuglevand, 1995). Also, there is ample evidence for damage to the plasmalemma

following performance of eccentric contractions. Intracellular proteins (e.g. creatine kinase, myoglobin) leak out of injured fibres (Clarkson *et al.* 1986; Jones *et al.* 1986; Warren *et al.* 1995) while exogenous markers are able to enter the fibres (McNeil & Khakee, 1992; Warren *et al.* 1995). There is a preliminary report of a 13 mV membrane depolarization occurring in muscle fibres immediately after injury induced by eccentric contractions (McBride *et al.* 1994). These observations suggest that resting membrane conductances were altered and that the fibres' capacity to conduct action potentials may have been impaired. However, we previously reported resting membrane potentials to be unchanged in injured fibres showing EC coupling failure (Warren *et al.* 1993).

The microelectrode technique, the means for measuring membrane potentials in the studies of McBride *et al.* (1994) and Warren *et al.* (1993), has three shortcomings that limits its usefulness in these type of studies. First, the technique is slow enough to preclude measurements on a large number of fibres in a given muscle (e.g. ~3% of the fibres in a mouse soleus muscle can be sampled in 1 h (Warren *et al.* 1993)). Second, sampling is limited to the superficial fibres because of changes in reference potential and electrode capacitance as the microelectrode penetrates deeper muscle fibres (Purves, 1981). Third, action potential measurement in an intact muscle in the absence of a paralysing agent (e.g. butanedione monoxime) is difficult, and when paralysing agents are used, their effects on the electrical properties of the plasmalemma are subject to question.

In the present study, we sought to bypass the limitations of the microelectrode technique by measuring the muscle electromyographic (EMG) signal during a bout of eccentric contractions and in the days following initiation of the injury. Because the muscles were maximally recruited via electrical stimulation of the motor nerve, any observed decrement in the EMG root mean square (RMS) would be interpreted as an impairment of plasmalemmal action potential conduction (or alternatively as a failure of the neuromuscular junction (NMJ) and/or motor nerve). The results indicate that there was not an impairment of plasmalemmal function because in the injured muscles, we observed a dissociation between electrical activity and force production (i.e. very large, prolonged force reductions with small, transient reductions in EMG RMS and no changes in M-wave spectral properties).

It is known that an electrically silent muscle (e.g. during denervation or chronic NMJ blockade) shows an increase in nicotinic acetylcholine receptor (AChR) density resulting from increased extrajunctional AChR expression (Grinnell, 1995). In such models, it is not known whether the increased AChR expression is due to absence of muscle electrical activity itself or the contractile inactivity that results. In the present study, we used a model in which muscle fibre electrical activity was probably normal while contractile activity was markedly reduced, so the relative importance of electrical and contractile activity in determining AChR expression could be investigated. Thus a secondary objective was to determine whether AChR concentration was altered as a result of the eccentric contractions.

METHODS

Animals

Female ICR mice ($n = 78$) obtained from Harlan Laboratories were used. The mice were 2–4 months old and weighed 31·5 ± 2·5 g at the time of the contraction protocols. They were housed with a 12 h light–12 h dark photoperiod at the American Association of Laboratory Animal Care-accredited Laboratory Animal Research and Resources facility at Texas A&M University. For surgical implantation of the stimulating nerve cuff on the common peroneal nerve, the mice were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg kg^{-1}). For surgical implantation of the EMG electrodes on the tibialis anterior (TA) muscle, the mice were anaesthetized with an intraperitoneal injection of fentanyl (0·33 mg kg^{-1}), droperidol (16·7 mg kg^{-1}) and diazepam (5 mg kg^{-1}). This latter anaesthetic regimen was also used when contractile and EMG measurements were made. Supplementary injections of the respective anaesthetic regimens were given when indicated by the return of pedal or palpebral reflexes. In the experiments using a bolus injection of succinylcholine or tubocurarine, the extent of NMJ blockade was monitored throughout the experiments and taken into consideration when evaluating pedal or palpebral reflexes. Following the final experimental protocol, the mice were killed with an overdose of sodium pentobarbitone (200 mg kg^{-1}). All animal care and use procedures met guidelines set by the American Physiological Society and were approved by the Institutional Animal Care and Use Committee.

Experimental procedures

Surgical procedures. While the mouse was under surgical anaesthesia (see above), a stimulating nerve cuff was implanted on the left common peroneal nerve as described previously (Warren *et al.* 1998). Briefly, the nerve cuff was constructed from two Teflon-coated, multi-stranded 90% platinum (Pt)–10% iridium (Ir) wires (0·15 mm diameter) (Medwire-Sigmund Cohn Corp. 10Ir9/49T; Mt Vernon, NY, USA). An incision was made through the biceps femoris muscle and the two loops formed from 2·5 mm segments of deinsulated Pt–Ir wire were placed around the common peroneal nerve. The proximal end of the nerve cuff was externalized in the dorsal cervical region.

Twenty-five days after implantation of the stimulating nerve cuff, maximal isometric torque produced about the ankle by the anterior crural muscles was measured in the anaesthetized mouse (see above) using the servomotor system described previously (Lowe *et al.* 1995; Ingalls *et al.* 1996). The EDL and TA muscles produce 11 and 89%, respectively, of the measured torque in this model (Lowe *et al.* 1995). TA muscle EMG electrodes were implanted if torque production exceeded 2·54 N mm; this minimum torque level was selected based on observations that 95% of all female ICR mice in the weight range studied produce this torque or greater when using the fentanyl/droperidol/diazepam anaesthetic regimen (Warren *et al.* 1998).

TA muscle EMG electrodes were implanted in the anaesthetized mouse (see above) as described previously (Warren *et al.* 1998). Briefly, after making a skin incision running longitudinally along the anterior lower leg, a 23 gauge needle was passed just beneath the fascial sheath covering the TA muscle at its midbelly. The needle was passed in a direction perpendicular to the superficial

fibre longitudinal orientation. The deinsulated end of a Pt–Ir wire was passed through the needle and the needle withdrawn, leaving a 3 mm segment of deinsulated wire beneath the fascia. A second length of wire was routed underneath the fascia in a direction parallel to the first wire but offset in the distal direction by 2 mm. The electrode wire spacing theoretically permitted sampling of EMG activity from the full thickness of the TA muscle beneath the electrodes (Basmajian & De Luca, 1985). The wires were secured to tissue adjacent to the TA muscle and the proximal ends of the wires were externalized in the dorsal cervical region. Using these surgical techniques, we have demonstrated good reliability for measurement of maximal isometric torque and RMS when measured six times over a 14 day period (Warren *et al.* 1998).

Contraction protocols. Contraction protocols were initiated 5 days after surgical implantation of the EMG electrodes. The anaesthetized mouse (see above) was positioned in the servomotor system as described previously (Lowe *et al.* 1995; Ingalls *et al.* 1996). Peak torque production was then optimized by varying stimulation voltage in a series of 5–8 isometric contractions; the stimulations were 45–60 s apart and consisted of 200 ms trains of 75 μs biphasic pulses at 300 Hz. If RMS did not equal or exceed 0·75 mV during these optimization contractions, the mouse was removed from the study. Next, a series of 12 isometric contractions was done. These contractions were 200 ms long with 45 s between contractions; the stimulation frequencies used were 20, 40, 60, 80, 100, 125, 150, 200, 250, 300, 350 and 400 Hz and the contractions were done in order of increasing stimulation frequency. From these data, the relationships of stimulation frequency to torque and RMS were determined. M-wave spectral properties were determined from the 40 Hz stimulation in this series of contractions.

The left anterior crural muscles were then stimulated to perform a series of either 150 eccentric (ECC) ($n = 12$ mice) or 150 concentric (CON) ($n = 11$ mice) contractions. The eccentric contractions were done as described previously (i.e. from 20 deg of ankle dorsiflexion to 20 deg of ankle plantarflexion at an angular velocity of 2000 deg s^{-1}; this movement was preceded by a 100 ms isometric stimulation, so the contraction lasted a total of 120 ms) (Lowe *et al.* 1995; Ingalls *et al.* 1998*b*). The concentric contractions were done similarly but in the reverse direction (i.e. from 20 deg of plantarflexion to 20 deg of dorsiflexion). There were 10 s between contractions except for every tenth contraction, which took an additional 12 s while the data were written to disk. Beginning 3 min after the end of the ECC or CON protocol, the series of 12 isometric contractions (i.e. 20–400 Hz stimulations) was repeated; this series of contractions was also done by each of the mice at 1, 3, 5 and 14 days after the ECC and CON protocols.

EMG. While the mouse was under anaesthesia (see above), the proximal ends of the TA muscle EMG electrodes were externalized and cleaned with acetone. A Pt–Ir wire acutely implanted beneath the skin in the abdominal region served as the reference electrode. Electrode impedance at 30 Hz was then measured; impedance remained constant over the 14 day period at 10–15 kΩ. The EMG signal was amplified 1000 times and passed through a bandpass filter with low and high cut-off frequency settings of 10 and 3000 Hz, respectively (Grass Instruments model P-15; Quincy, MA, USA). The amplified EMG signal was sampled at 5 kHz by the computer used for acquisition of the torque and ankle angle position data. The EMG signal was subsequently passed through a lowpass (i.e. 2000 Hz) digital filter (Keithley-Metrabyte VTX, version 1.1) to remove aliasing in the 2000–3000 Hz range; any DC offset was also removed at this time. Figure 1 shows typical anterior crural muscle torque and TA muscle EMG signals acquired during 300 Hz isometric stimulations, both before and after an ECC protocol.

Analysis of the electrically evoked myoelectric signal was done using the recommended amplitude (i.e. RMS) and frequency (i.e. mean and median frequencies) parameters (Merletti & Knaflitz, 1992). For the contractions during the ECC and CON protocols, RMS was calculated only for the 100 ms isometric contraction immediately preceding the 20 ms isokinetic contraction. M-wave mean and median frequencies were determined from the EMG signal acquired during the 200 ms, 40 Hz isometric stimulations. The eight M-waves, each a 10 ms long epoch, were averaged followed by application of a Hamming window. The data were then zero-padded before running a 2048-point fast Fourier transform. Median frequency was calculated from the power density spectrum as the frequency at or below which 50 % of the points in the spectrum lay. The 40 Hz stimulations were chosen for M-wave analyses because: (1) there was sufficient time between stimulation pulses to ensure return of the EMG signal to baseline in between pulses, and (2) in the injured muscles, one of the largest decreases in RMS (i.e. 19 %) occurred at this frequency.

Torque–RMS relationship. Three sets of experiments were performed to determine the torque–RMS relationship in anaesthetized mice (see above) that were chronically implanted with the stimulating nerve cuff and EMG electrodes. Isometric torque production from the anterior crural muscles and RMS from the TA muscle were altered either by systematic step increases in stimulation voltage or by administration of the NMJ blockers, succinylcholine or tubocurarine. The rationale for these experiments was to determine whether small changes in RMS could elicit relatively large changes in torque.

In the experiments employing step increases in stimulation voltage ($n = 8$ mice), isometric contractions (200 ms train of 75 μs pulses at 300 Hz) were elicited every 45 s starting at a stimulation voltage that resulted in the first signs of an EMG signal. The contractions were stopped when additional increases in stimulation voltage failed to produce any further increase in torque. Typically, 15–18 contractions were done.

In the NMJ blocker experiments, an intraperitoneal injection of either succinylcholine chloride (3·0 mg kg^{-1}; $n = 4$ mice) or *d*-tubocurarine chloride (0·4 mg kg^{-1}; $n = 4$ mice) was given. Isometric torque and RMS were measured at 1 min intervals for 20 min, and then at 2 min intervals for 20 min or until the time RMS had recovered. In these experiments, an anaesthesia chamber was used to keep the inspired O_2 at ~100 %. In a given animal, NMJ blockade resulted in peak RMS reduction of 43–71 %.

AChR binding assays. In this portion of the study, the mice were not chronically implanted with either the stimulating nerve cuff or the TA muscle EMG electrodes. The ECC and CON protocols were performed on anaesthetized mice (see above) using percutaneous nerve stimulation (Lowe *et al.* 1995; Ingalls *et al.* 1998*b*). After completion of the contraction protocols, the mice were allowed to recover in their cages, until the time they were killed and the TA muscles excised. For mice undergoing the ECC protocol, experimental and contralateral TA muscles were excised immediately before ($n = 6$) and after ($n = 7$) the protocol, and at 1 day ($n = 6$), 3 days ($n = 9$), 5 days ($n = 7$) and 14 days ($n = 6$) after the protocol. The muscles were frozen in liquid N_2 and stored at −80 °C until the time of the AChR assay. For mice undergoing the CON protocol ($n = 6$), experimental and contralateral TA muscles were excised at 5 days after the protocol, i.e. the time of peak AChR concentration observed for the muscles that had undergone the ECC protocol.

AChR concentration was determined in the TA muscles using a slight modification of the ^{125}I-labelled α-bungarotoxin binding

assay described by Kim *et al.* (1995). All steps were carried out at 4 °C except where stated otherwise. Muscles (~50 mg wet weight) were minced and homogenized in 500 μl of 10 mM phosphate buffer (pH 7·4) containing 1 mM EDTA, 2 mM benzamidine hydrochloride, 0·1 mM phenylmethylsulfonyl fluoride, 0·05% bacitracin and 0·02% sodium azide. The homogenate was then centrifuged at 16 000 *g* for 38 min. The supernatant was discarded and the pellet re-homogenized in 750 μl of the buffer described above along with 2% Triton X-100. The homogenate was incubated overnight on an orbital shaker followed by centrifugation at 16 000 *g* for 63 min. Supernatant aliquots (100 μl) were mixed with 100 μl of 5·0 nM ^{125}I-α-bungarotoxin (specific activity of 15·0–16·4 Ci g^{-1}; New England Nuclear, Boston, MA, USA) in phosphate buffer and then incubated for 90 min on an orbital shaker at room temperature (23–25 °C). Non-specific binding was determined by pre-incubation of the supernatant sample with 4·17 μM of unlabelled α-bungarotoxin for 15 min. Assays were run in triplicate for samples incubated with ^{125}I-α-bungarotoxin alone and for those incubated with unlabelled α-bungarotoxin and ^{125}I-α-bungarotoxin together. Unbound ^{125}I-α-bungarotoxin was separated from bound ^{125}I-α-bungarotoxin by vacuum filtration through glass filters (Whatman GF/B; Clifton, NJ, USA); the filters were pretreated with 0·3% polyethylenimine for $\geqslant$ 5 h at 4 °C. The filters were then counted using a gamma counter (Packard Auto-Gamma 5780 or Beckman Gamma 4000). AChR concentration was expressed as specific ^{125}I-α-bungarotoxin binding in femtomoles per milligram of supernatant protein, with supernatant protein content being determined using the bicinchonic acid method.

Statistical analyses

For analysis of the torque and RMS data collected during the ECC and CON protocols, the effects of protocol type (CON *vs.* ECC) and contraction number (1, 10, 20, . . . 150) were evaluated using a two-way ANOVA with repeated measures on contraction number. For analysis of the torque–frequency and RMS–frequency relationships, the effects of protocol type, time (i.e. pre- and immediately post-protocol and at 1, 3, 5 and 14 days), and stimulation frequency

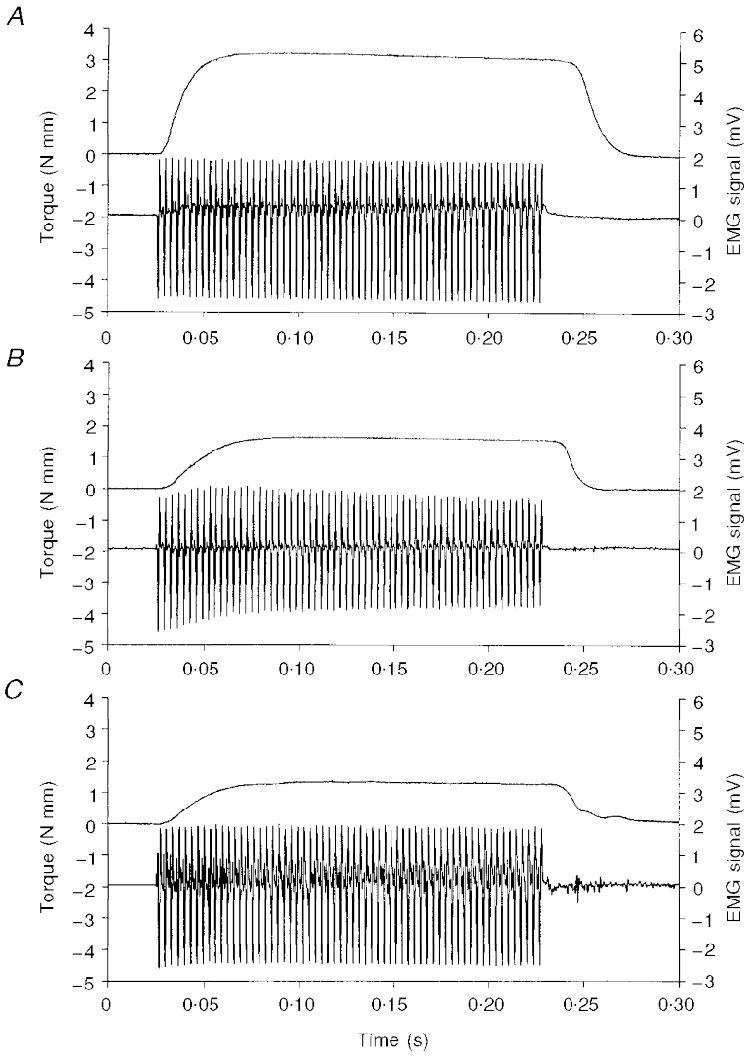

Figure 1. Torque and EMG recordings typical of those acquired during a 200 ms, 300 Hz isometric stimulation of the anterior crural muscles

Recordings shown are those acquired before (*A*), immediately after (*B*), and 1 day after (*C*) performance of the 150 eccentric contractions protocol. In this example, torque and RMS were reduced by 48 and 18%, respectively, immediately after the protocol. One day after the protocol, torque was reduced by 58% compared with baseline, while RMS was 7% greater than baseline.

(i.e. 20, 40, . . . 400 Hz) were analysed using a three-way ANOVA with repeated measures on time and stimulation frequency. The effects of protocol type and time on M-wave spectral properties were analysed using a two-way ANOVA with repeated measures on time. When significant main effect interactions were found, significant differences between means were determined using single degree of freedom contrasts.

For analysis of the AChR concentration data, the five groups of muscles that had undergone the ECC protocol were compared with control muscles using a one-way ANOVA. *Post hoc* tests were done using Student–Newman–Keuls comparisons. Muscles that had undergone the CON protocol were compared with control muscles using Student's unpaired t test. A repeated measures analysis was not used for the AChR concentration data because all experimental muscles could not be paired with their contralateral control muscles.

Systat 7.0 software (SPSS Science Inc., Chicago, IL, USA) was used for the statistical analyses, and an α level of 0·05 was used for all tests. The values reported in the Results section are means ± S.E.M.

RESULTS

Torque. Peak torque declined by 43% from the first to the last contraction in the ECC protocol, whereas peak torque did not change significantly during the CON protocol (Fig. 2). Immediately after the ECC protocol, isometric torques were significantly decreased at all stimulation frequencies with the greatest relative deficits (i.e. 83–89%) occurring at stimulation frequencies ⩽125 Hz (Fig. 3); at stimulation frequencies ⩾250 Hz, the percentage decreases in isometric torque were 47–49%. In the 14 days following the ECC protocol, isometric torque showed a progressive recovery, but even at 14 days post-protocol, torques at all stimulation frequencies were lower than baseline levels by 12–30%.

Immediately after the CON protocol, isometric torques measured at stimulation frequencies ⩾250 Hz were increased by 5–7% while torques at ⩽100 Hz were decreased by 18–25% (Fig. 3). Isometric torques measured at all stimulation frequencies were not different from baseline levels 1 day after the CON protocol and did not change until 14 days after the CON protocol. Fourteen days after the CON protocol, isometric torques measured at stimulation frequencies ⩾125 Hz were significantly greater than baseline levels by 10–11%.

EMG. RMS decreased over the 150 contractions by 10 and 3%, respectively, for the ECC and CON protocols (Fig. 2); there was no significant difference in the decrease between the two protocols ($P = 0{\cdot}42$). Immediately after the contraction protocols, RMS values were decreased (at all stimulation frequencies except 20 and 200 Hz) by 9–21% and 0–6% for the ECC and CON protocols, respectively (Fig. 4); however, the decreases observed for the ECC protocol were not significantly different from those observed for the CON protocol ($P = 0{\cdot}61$). One day after both the ECC and CON protocols, RMS values measured at all stimulation frequencies were not significantly different from

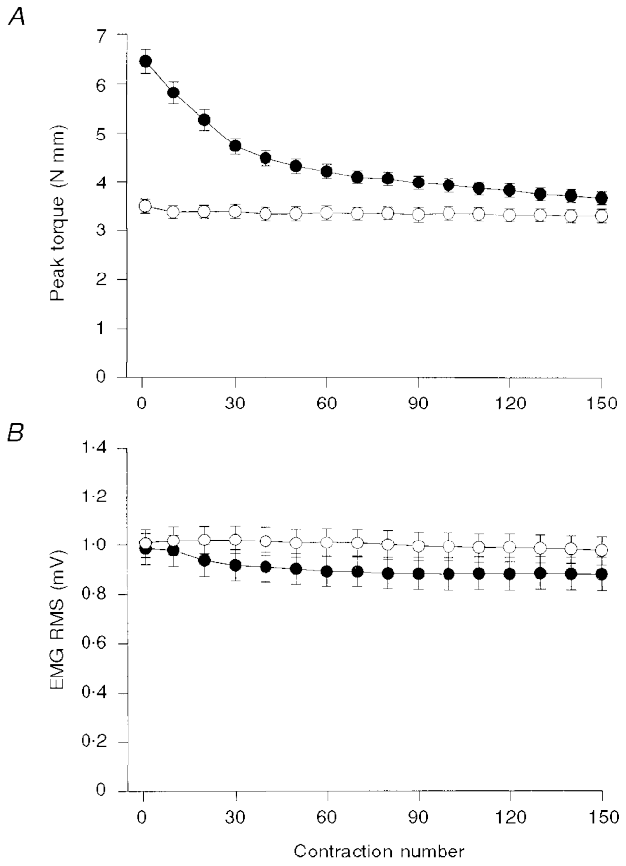

Figure 2. Mean (± S.E.M.) peak torque (*A*) and EMG RMS (*B*) produced during the two 150 contraction protocols

Values for the eccentric (ECC) and concentric (CON) protocols are indicated by the filled and open circles, respectively. ECC peak torque decreased over the bout ($P \leqslant 0{\cdot}0001$), while CON peak torque did not change ($P = 0{\cdot}28$). RMS decreased significantly over the bouts ($P = 0{\cdot}0007$), but there was no difference in the decreases between the ECC and CON protocols ($P = 0{\cdot}42$).

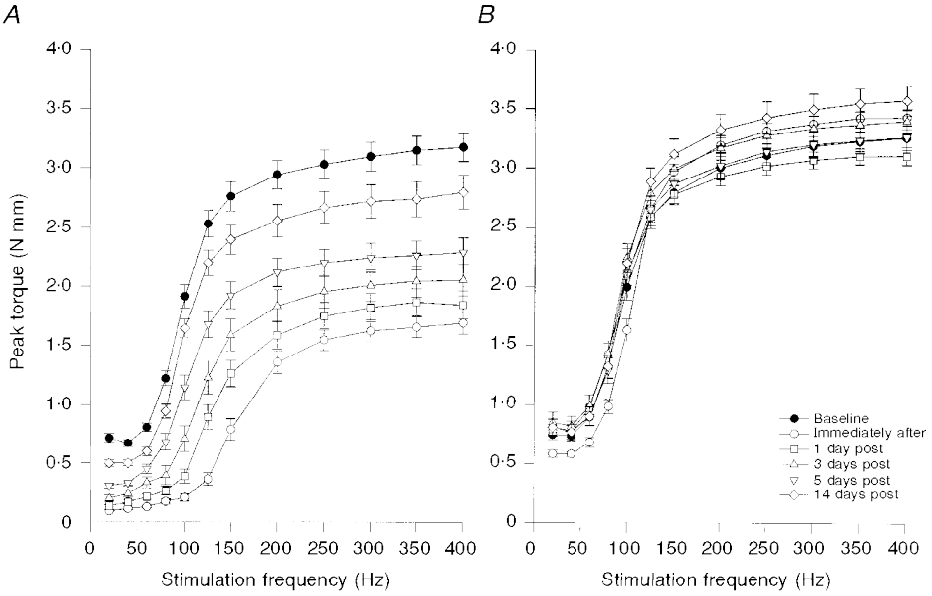

Figure 3. Mean (± S.E.M.) peak isometric torque as a function of stimulation frequency measured before (i.e. baseline), immediately after, and at 1, 3, 5 and 14 days after the ECC (*A*) and CON (*B*) protocols

For the ECC protocol muscles, torques at all frequencies were decreased below baseline values at all times following the protocol ($P \leqslant 0{\cdot}03$). For the CON muscles immediately after the protocol, torques were decreased below baseline at stimulation frequencies $\leqslant 100$ Hz ($P \leqslant 0{\cdot}006$), while torques were above baseline at frequencies $\geqslant 250$ Hz ($P \leqslant 0{\cdot}04$). For the CON protocol muscles, torques at all frequencies were not different from baseline at 1, 3, or 5 days post-protocol; by 14 days post-protocol, torques at $\geqslant 125$ Hz were increased above baseline ($P \leqslant 0{\cdot}01$).

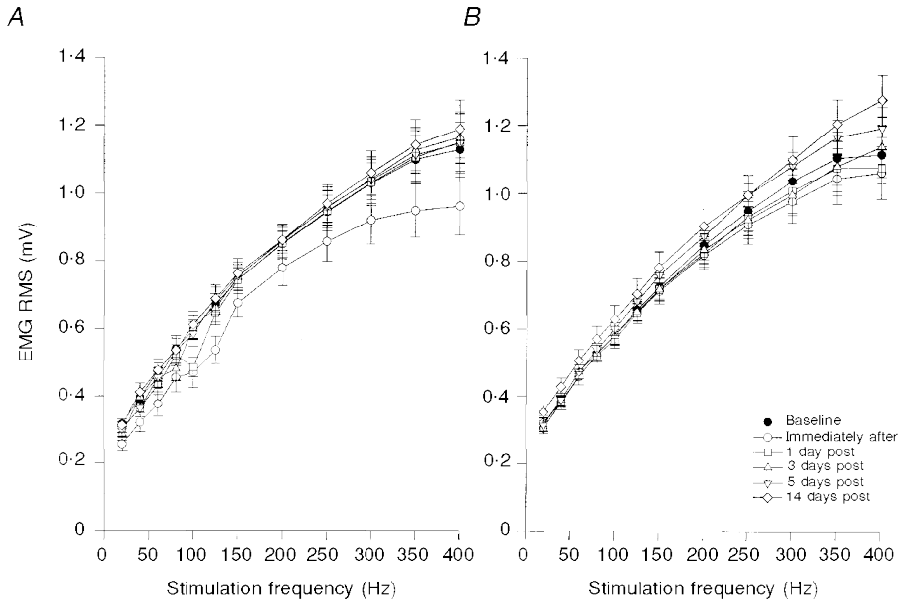

Figure 4. Mean (± S.E.M.) EMG RMS as a function of stimulation frequency measured before (i.e. baseline), immediately after, and at 1, 3, 5 and 14 days after the ECC (*A*) and CON (*B*) protocols

Immediately after the protocols, RMS was decreased below baseline at all stimulation frequencies except 20 and 200 Hz, but there was no difference in the decreases between the ECC and CON protocols ($P = 0{\cdot}61$). For both protocols, RMS at all frequencies was not different from baseline at 1, 3, 5 or 14 days post-protocol.

Table 1. Mean ± S.E.M. M-wave mean and median frequencies of the tibialis anterior muscle before and after the 150 contraction protocols

	Baseline	Immediately after	1 day post	3 days post	5 days post	14 days post
ECC protocol						
Mean frequency (Hz)	590 ± 36	616 ± 38	585 ± 30	589 ± 27	554 ± 37	570 ± 47
Median frequency (Hz)	570 ± 35	604 ± 42	570 ± 31	579 ± 38	530 ± 44	540 ± 45
CON protocol						
Mean frequency (Hz)	588 ± 40	571 ± 31	568 ± 37	587 ± 41	539 ± 29	538 ± 49
Median frequency (Hz)	546 ± 33	544 ± 30	544 ± 38	583 ± 45	508 ± 34	513 ± 52

For both measures, there were no significant effects of protocol ($P \geqslant 0{\cdot}56$), time ($P \geqslant 0{\cdot}17$), or protocol × time interaction ($P \geqslant 0{\cdot}94$).

baseline levels. Furthermore, for both protocols, RMS measured at all stimulation frequencies did not deviate from baseline levels over the remaining 13 days of the study.

Mean and median frequencies of the averaged M-wave acquired during the 40 Hz isometric stimulations were not acutely affected by performance of the ECC or CON protocols, nor did the values change over the 2 weeks following the protocols (Table 1). Figure 5 depicts M-waves typical of those acquired before and immediately after performance of an ECC protocol; note that despite a marked reduction in M-wave amplitude after the ECC protocol, M-wave duration was not prolonged.

Torque–RMS relationship. We explored the torque–RMS relationship because the RMS decrease observed immediately after the protocols tended to be greater for the ECC protocol compared with the CON protocol. We questioned whether the small 'non-significant' RMS difference between the protocols could elicit the large 'significant' torque difference observed between the protocols. This could happen if the relationship between isometric torque and EMG RMS was not proportional and/or was non-linear. Figure 6 shows the relationship of isometric torque to RMS in control mice determined from manipulation of stimulation voltage or administration of NMJ blockers. The slopes of the normalized torque to normalized RMS relationships varied between 1·00 and 1·14. Thus it is unlikely that the 'small' 11% RMS deficit measured at 300 Hz immediately after the ECC protocol could account for the 'large' 48% torque deficit observed at that time.

AChR concentration. In this part of the study, the ECC and CON protocols were conducted using percutaneous stimulation of the common peroneal nerve. However, the changes in contractile function were similar to those observed when the chronic stimulating nerve cuff was used. Maximal isometric torque was decreased by 52 and 48% immediately after the ECC protocol for the percutaneous and nerve cuff stimulation, respectively. For both types of stimulation, maximal isometric torque was not adversely affected immediately after the CON protocol (i.e. 1% decrease *vs.* 5% increase for percutaneous and nerve cuff stimulation, respectively).

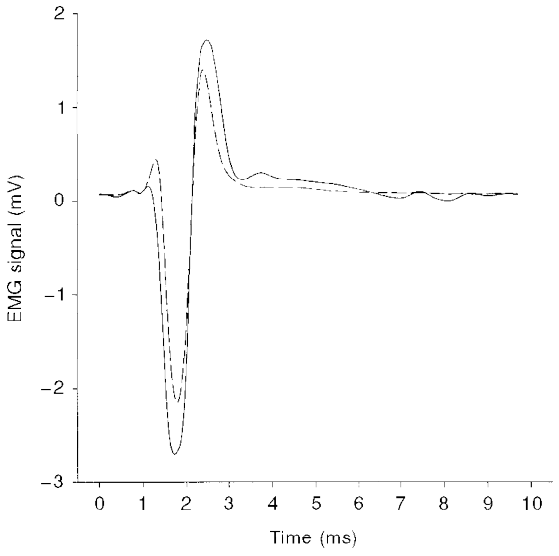

Figure 5. Averaged M-waves typical of those acquired during 40 Hz isometric stimulations both before (continuous line) and immediately after (dashed line) performance of an ECC protocol

Each curve is a spline fit of the 50 data points from that epoch. In this example, RMS is reduced by 30% immediately after the ECC protocol, but there is no evidence for a prolongation of the waveform (e.g. median frequency actually increased from 488 to 585 Hz).

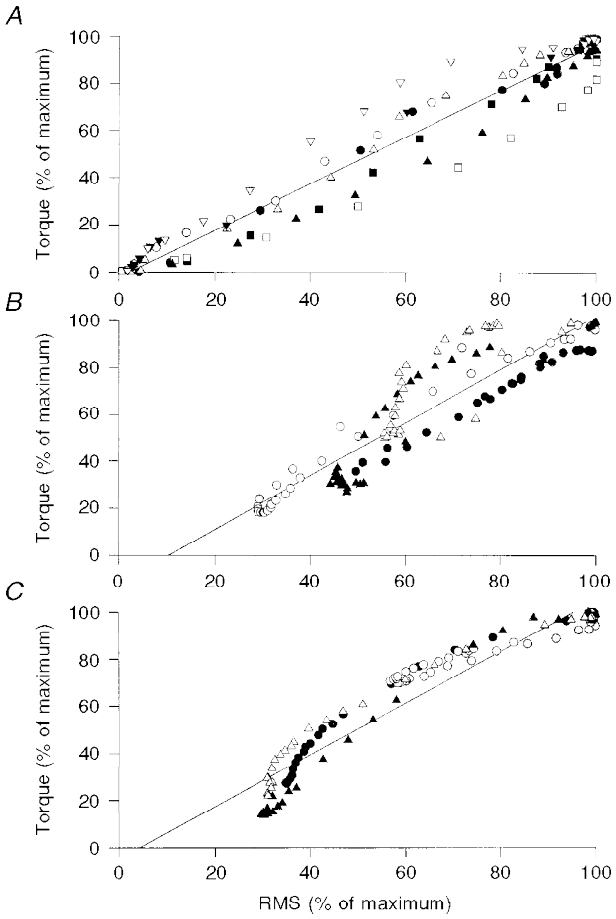

Figure 6. Relationship between normalized isometric torque produced by the anterior crural muscles and normalized tibialis anterior muscle EMG RMS as determined via systematic step increases in stimulation voltage (*A*), administration of succinylcholine (*B*), and administration of *d*-tubocurarine (*C*)

In *A*, *B* and *C*, a given symbol type represents data from one mouse. The linear regression lines determined from the pooled data are shown; the slopes of the regression lines equal 1·00 ($r = 0{\cdot}98$), 1·14 ($r = 0{\cdot}92$) and 1·10 ($r = 0{\cdot}96$) for *A*, *B* and *C*, respectively.

For the muscles that had undergone the ECC protocol, AChR concentration as determined via ^{125}I-α-bungarotoxin binding was unchanged before and up to 1 day after the protocol (Fig. 7). However, at 3 and 5 days post-protocol, AChR concentration was increased by 79 and 368%, respectively. By 14 days after the ECC protocol, AChR concentration was not different from control levels.

For the muscles excised 5 days after the CON protocol, AChR concentration was not different from that of the contralateral control muscles (i.e. 46·1 ± 3·8 *vs.* 42·2 ± 2·9 fmol mg^{-1}, respectively; $P = 0{\cdot}43$). The average AChR concentration observed in these control muscles was notably higher than that observed for the control muscles excised from the mice that underwent the ECC protocol (i.e. 42·2 ±

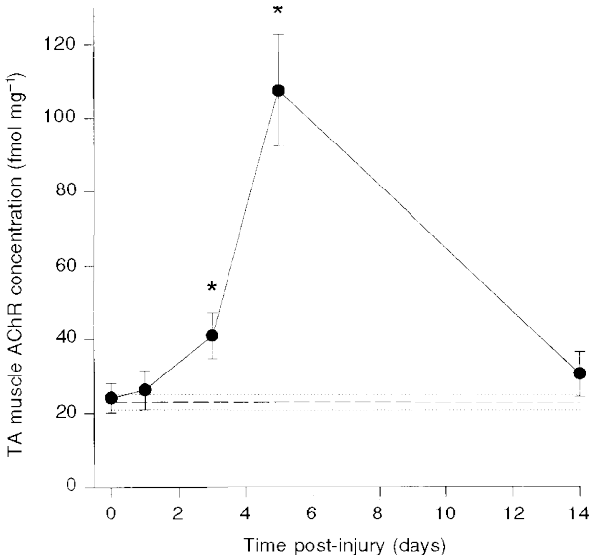

Figure 7. Mean (± S.E.M.) nicotinic acetylcholine receptor (AChR) concentration measured in control tibialis anterior muscles and in muscles immediately after (0 days) and at 1, 3, 5 and 14 days after performing the ECC protocol

AChR concentration is expressed as specific ^{125}I-α-bungarotoxin binding in femtomoles per milligram of supernatant protein. The dashed and dotted lines represent the mean values and S.E.M., respectively, for the control muscles. The control muscles include muscles that were excised immediately prior to when the ECC protocol would have been performed plus the muscles contralateral to the muscles that performed the ECC protocol. * Time points significantly different from the control group.

2·9 *vs.* 23·0 ± 2·0 fmol mg^{-1}). We attribute this difference to the fact that the AChR binding assays for the ECC and CON protocol muscles were conducted at different times, by different personnel, and using different instrumentation. The difference we observed between the two groups of control muscles is consistent with the within-laboratory variation reported in the literature for rat gastrocnemius muscle AChR concentration (i.e. 5·7 *vs.* 30 fmol mg^{-1} from Kim *et al.* (1995) and Ward & Martyn (1993), respectively).

DISCUSSION

These data demonstrate the lack of a major role for impaired plasmalemmal action potential conduction in eccentric contraction-induced EC uncoupling. Presumably, injury-induced reductions in action potential amplitude would have been reflected in a reduced EMG RMS. However, the decreases observed in RMS immediately after the ECC protocol (i.e. 9–21 %) were relatively small compared with the torque deficits observed at the same time (i.e. 47–89 %). Furthermore, the ECC protocol RMS decrements observed immediately after the protocol were not significantly different from those observed for the CON protocol. However, the CON protocol did not produce large contractile deficits. Within 24 h of completing the ECC protocol, RMS values were not different from baseline, but torque values were still depressed by 41–81 %. Thus we estimated that the RMS deficits could at best explain 8–24 % of the torque deficits observed immediately after the ECC protocol, but none of the torque deficits at later times.

It is possible that changes in action potential amplitude could have been dissociated from the observed RMS changes both in magnitude and temporally if the action potential waveform had changed as well (e.g. percentage decreases in action potential amplitude could have exceeded those observed in RMS if action potential duration was prolonged). However, we saw no evidence for an injury-induced alteration in action potential waveform as indicated by the M-wave spectral analyses (see Table 1 and Fig. 5). Longer duration action potentials would have contributed lower frequency components to the EMG spectrum, which would have resulted in lower mean and median frequencies. We observed no injury-induced change in either spectral measure.

We had some concerns that the methodology used may have masked significant alterations in the plasmalemmal electrical properties resulting from the ECC protocol, and that these alterations may have in fact had functional consequences. We considered the possibility that the EMG electrode implantation may have damaged superficial fibres and that the electrical properties of these fibres were altered. There is evidence that this occurred to a small degree. Maximal isometric tetanic torque measured just prior to performance of the contraction protocols was 5 % lower ($P = 0{\cdot}03$) than that measured just before the TA muscle EMG electrodes were implanted. However, while damage to superficial fibres may have occurred, the EMG sampling volume should have encompassed the entire muscle thickness and not just the underlying fibres (Basmajian & De Luca, 1985). Furthermore, the CON protocol controlled for any effect of superficial fibre injury on the EMG signal.

We also considered the possibility that the recorded EMG signal was contaminated with stimulation artifact. There are two observations that argue against this concern. First, succinylcholine (3 mg kg^{-1}) and tubocurarine (0·4 mg kg^{-1}) were able to reduce RMS by over 59 % on average, an observation that one would not expect if the EMG signal was composed predominantly of stimulation artifact. Higher dosages of the NMJ blockers were tried but resulted in death, so those data are not included. Second, in the experiments in which stimulation voltage was altered to determine the torque–RMS relationship, RMS values plateaued after a certain threshold voltage was attained; no effect on RMS was observed even when stimulation voltage was $\geqslant 3$ times the threshold voltage (see also Fig. 5 in Warren *et al.* 1998).

Finally, we considered the possibility that the relationship between torque and RMS may not be proportional (and/or non-linear) and thus could have caused us to underestimate the effect of the small observed decreases in RMS on torque. The close to one-to-one torque–RMS relationships observed in the experiments using NMJ blockers and step increases in stimulation voltage argue against this possibility. We do acknowledge that the means by which RMS is reduced during stimulation voltage manipulation and NMJ blockade (i.e. impaired transmission at the nerve or NMJ with minimal change in muscle fibre action potential amplitude) would probably be different from what one might expect in injured fibres (i.e. reduced action potential amplitude in the muscle fibres with normal nerve and NMJ function).

Our observation of a minimal role for impaired plasmalemmal action potential conduction is in general agreement with previous data regarding EC coupling failure in the eccentric contraction injury model. First, as mentioned earlier, we have observed resting membrane potentials to be unchanged in muscles injured by eccentric contractions (Warren *et al.* 1993) and assumed that the action potential conducting properties of the plasmalemma and t-tubules were also unaltered. In those muscles, virtually all of the P_o deficit could be attributed to an EC coupling failure. Second, we have observed that potassium-induced contracture force is reduced to the same extent as P_o in muscles injured by eccentric contractions (Ingalls *et al.* 1998*b*). This observation would indicate that the defect in the EC coupling pathway lies at or below the level of the voltage sensor in the t-tubule. However, the potassium contracture experiments were carried out on whole mouse EDL muscles, and force developed under those conditions is highly dependent on the rapid activation/inactivation properties of the voltage sensor in conjunction with the relatively slow diffusion of potassium within the extracellular space of the muscle fibres. An alternative explanation for the potassium contracture data and EMG results of the present study would be an

injury-induced detubulation (i.e. sealing-off) of the t-tubules, as occurs following osmotic shock (Fraser *et al.* 1998). A sealing-off of the t-tubules would be predicted to cause a reduced potassium contracture force with minimal changes in the plasmalemmal action potential conduction properties. However, our observations of injury-induced changes in cell membrane integrity would argue against a sealing-off of the t-tubular system (Warren *et al.* 1995). In that study, fluorescent markers placed in the bathing medium following the injury protocol still had access to the t-tubular membrane and lumen, although there were indications of transient damage deep within the t-tubular system.

When the above data are considered in the light of our observations of unaltered intrinsic SR function until 3 days post-injury (Ingalls *et al.* 1998*b*), we believe the main EC coupling failure site is localized between the t-tubular voltage sensor and the SR Ca^{2+} release channel. Specifically, the failing process may be that of sensing the membrane depolarization by the voltage sensor and/or the transduction of that signal to the SR Ca^{2+} release channel.

It is remarkable that EMG RMS and M-wave spectral properties are maintained in the days following injury induction when phagocytic cell infiltration and muscle fibre remodelling are occurring. In the mouse EDL muscle injured using the same ECC protocol, we previously found plasmalemmal integrity, as indicated by lactate dehydrogenase release, to be maximally impaired at 1–2 days post-injury (Lowe *et al.* 1995). We also found phagocytic cell infiltration to be apparent by 24 h post-injury and to progressively increase until at least 5 days post-injury. Protein degradation reached its maximal rate at 2–5 days post-injury.

We interpret these previous observations in the light of the data of the present study to indicate that: (1) the degenerative processes are occurring exclusively in a small population of necrotizing fibres, and that the loss of those fibres has no measurable effect on the EMG signal; or (2) the degenerative processes are restricted to a relatively small volume within injured fibres and the membrane electrical properties are maintained as the affected fibre portions undergo the degeneration/regeneration processes. Supporting the first possibility is our observation that $\leqslant 5\%$ of the fibres show histological damage despite contractile deficits of $\geqslant 50\%$ (Lowe *et al.* 1995). Arguing against the first possibility is our observation that fibre number is not diminished at 5 or 14 days post-injury (Ingalls *et al.* 1998*a*).

A surprising finding in the present study was the marked increases in AChR concentration that followed performance of the ECC protocol. Both the magnitude and timing for the increase in AChR concentration that we observed appear similar to that observed following denervation. In congruence with our observations, AChR concentration in rat hindlimb muscle following denervation begins to increase by 2 days and has increased 5- to 6-fold at 4–5 days (Miledi & Potter, 1971; Almon & Appel, 1976). However, peak AChR levels are not attained following denervation until 1–3 weeks, at which time they have increased 18- to 28-fold (Miledi & Potter, 1971; Almon & Appel, 1976). Unlike the rapid decline that we observed in AChR concentration from 5 to 14 days post-injury, the decline to normal levels following denervation is prolonged (i.e. ~2 months) (Fambrough, 1979). We hypothesize that the rapid decline in AChR concentration after 5 days post-injury was due to the more rapid recovery of contractile activity compared with the recovery observed following denervation.

It is known that there is postsynaptic regulation of AChR density based on studies using chronic NMJ blockade and direct electrical stimulation of denervated muscle (Saltpeter & Loring, 1985; Grinnell, 1995). In those studies, it was not possible to determine whether the attenuated muscle fibre electrical activity *per se* or the resulting reduction in SR Ca^{2+} release or mechanical activity was responsible for the increased AChR expression. In our injury model, it appears that the AChR concentration increase is due to diminished SR Ca^{2+} release or mechanical activity and not to depressed electrical activity. This conclusion is consistent with observations made in muscle cell cultures that a cytosolic Ca^{2+} influx via L-type Ca^{2+} channels and/or SR Ca^{2+} release channels downregulates extrajunctional AChR expression (Huang *et al.* 1994; Adams & Goldman, 1998). Because eccentric contraction-induced injury causes a prolonged (i.e. $\geqslant 6$ h) elevation in the resting free cytosolic Ca^{2+} concentration (Balnave & Allen, 1995; Ingalls *et al.* 1998*b*), the AChR downregulation is probably not linked to the free cytosolic Ca^{2+} level *per se*.

In conclusion, these data demonstrate an uncoupling of force production from electrical activity in muscles injured by eccentric contractions. These data plus those of our previous work indicate that the failure site in the EC coupling pathway lies below the plasmalemma, but above the SR Ca^{2+} release channel. The disturbed relation of electrical activity to force production is also associated with a marked but relatively brief rise in AChR concentration, similar to a muscle that had transiently lost its innervation.

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Corresponding author

G. L. Warren: Department of Physical Therapy, Georgia State University, Atlanta, GA 30303-3083, USA.

Email: phtglw@langate.gsu.edu