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MECHANICAL FACTORS IN THE INITIATION OF ECCENTRIC CONTRACTION-INDUCED INJURY IN RAT SOLEUS MUSCLE

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SUMMARY

1. Mechanical factor(s) associated with the initiation of eccentric contraction-induced muscle injury were investigated in isolated rat soleus muscles ($n = 180$; 42 protocols with 4–6 muscles per protocol). Five eccentric contractions were performed with 4 min between contractions. Three levels of peak eccentric contraction force (100, 125 and 150% of pre-injury maximal isometric tetanic tension, P_0), length change (0·1, 0·2 and 0·3 muscle length, L_0) and lengthening velocity (0·5, 1·0 and 1·5 L_0/s) were utilized. Force was varied with stimulation frequency (10–150 Hz). The eccentric contractions were initiated at muscle lengths of 0·85 or 0·90 L_0. Following the fifth eccentric contraction, the muscle was incubated in Krebs–Ringer buffer for 60 min. Peak isometric twitch tension (P_T), P_0, maximal rate of tension development ($+dP/dt$), maximal rate of relaxation ($-dP/dt$), and creatine kinase (CK) release were measured prior to the five eccentric contractions and at 15 min intervals during the incubation period. Total muscle [Ca^{2+}] was measured after 60 min incubation.

2. The mean ($\pm$S.E.M.) initial decline in P_0 for the muscles performing the most injurious protocol was $13{\cdot}6 \pm 4{\cdot}8$% ($n = 6$); P_0 in control muscles immediately following performance of five isometric contractions was elevated $1{\cdot}2 \pm 1{\cdot}0$% ($n = 8$). These means were different at probability, $p = 0{\cdot}005$. Mean [ATP] in muscles immediately following the isometric control and most injurious protocols, respectively, were $16{\cdot}30 \pm 1{\cdot}49$ and $19{\cdot}84 \pm 1{\cdot}38$ μmol/g dry wt ($p = 0{\cdot}229$).

3. Decrements in P_0, P_T, $+dP/dt$, and $-dP/dt$ immediately after the injury protocol were related most closely to the peak forces produced during the eccentric contractions; greater initial declines in P_0, $+dP/dt$ and $-dP/dt$ were also observed at higher lengthening velocities independent of peak force. Slow declines in P_0 and $-dP/dt$ during the 60 min incubation following the injury protocol were greatest for muscles performing contractions at the longer initial length. CK release was independent of all mechanical factors with the exception of lengthening velocity. CK activity at 45 and 60 min into the incubation period was greater for muscles lengthened at the highest velocity used (1·5 L_0/s). Mean total muscle [Ca^{2+}] for muscles performing the eccentric contractions was elevated by 38% over isometric

control muscles but the elevation was unrelated to any of the four mechanical factors.

4. These data support the hypothesis that eccentric contraction-induced injury is initiated by mechanical factors, with muscle tension playing the dominant role. They also demonstrate that specific mechanical factors differentially affect the various injury criteria, i.e. reductions in contractile performance were most related to produced forces, and CK release was most related to lengthening velocity.

INTRODUCTION

Exercise-induced muscle fibre microinjury has been hypothesized to be initiated by disruption of the force-generating and/or -transmitting structures and loss of sarcolemma integrity followed by a calcium overload phase resulting in an influx of extracellular calcium (Ca^{2+}) that activates several intrinsic degradative pathways (Armstrong, 1990; Armstrong, Warren & Warren, 1991). The specific event that serves to initiate exercise-induced muscle fibre injury is not known. It is generally recognized that this type of injury is associated with eccentric contractions (for review, see Armstrong, 1984; Ebbeling & Clarkson, 1989; Stauber, 1989); some have hypothesized that one or more mechanical aspects of the eccentric contraction that distinguish it from isometric or concentric contractions may be responsible for initiation of the injury. For example, the degree of eccentric contraction-induced injury has been reported to be related to the specific tension produced during the contraction (Katz, 1939; McCully & Faulkner, 1985, 1986), which can be twice that produced during an isometric or concentric contraction (Woledge, Curtin & Homsher, 1985). It has also been reported that the degree of injury produced during eccentric contraction is related to the muscle length prior to the contraction (Katz, 1939; Newham, Jones, Ghosh & Aurora, 1988). There are, however, no previous investigations of eccentric contraction-induced injury that have systematically evaluated the role of multiple mechanical factors in inducing damage. Also, it is not clear from the earlier studies whether the produced tension and initial length influence the initiation of the injury process or exert their effects on subsequent stages of the injury process.

The main objective of this study was to determine the mechanical factor(s) associated with the initiation of eccentric contraction-induced muscle injury by manipulating four primary factors (peak eccentric contraction force, initial muscle length, length change, and lengthening velocity). By using an *in vitro* rat soleus muscle model, the confounding effects of phagocytic processes on the initial and early events in the injury process were eliminated. Also, the number of eccentric contractions used in the injury protocol was kept to a minimum in order to minimize metabolic costs and the potential contribution of autogenetic processes to the injury during performance of the injury protocol.

The primary criterion used to quantify injury was a reduction in maximal isometric tension (P_0), which has previously been shown to occur in muscles injured by eccentric contractions (McCully & Faulkner, 1985; Warren, Jenkins, Packer, Witt & Armstrong, 1992). Reduction in peak isometric twitch tension (P_T) and maximal rates of tetanic tension development ($+dP/dt$) and relaxation ($-dP/dt$) were also measured. Reductions in P_0 also result from metabolic deficiencies, but decreases in

muscle function in the present study were attributed to injury because the muscles did not recover over time following the injury protocol.

A second objective was to provide insight into the site of structural failure. It was hypothesized that the sarcolemma would be a site of failure and that this failure would lead to elevations in muscle [Ca^{2+}] and loss of creatine kinase (CK) activity. There is evidence to suggest that most of the energy lost during a stretch–shortening cycle is absorbed in the sarcolemma (Tidball, 1986; Tidball & Daniel, 1986) and that most of the passive tension at longer muscle fibre lengths (> 140–150% of resting muscle length, L_0) is borne by the sarcolemma (Casella, 1951; Higuchi & Umazume, 1986; Rapoport, 1972).

METHODS

Animals

Untrained female Sprague–Dawley rats were used in this study. The age of the rats was 28±4 (mean±S.D.) days, and their body mass was 48·2±3·9 g. The rats were housed individually at 23 °C with a 12 h dark–light cycle. Food was restricted so as to limit daily body mass gains to 1–2 g; food restriction was begun at 22 days of age. The rats were anaesthetized with sodium pentobarbitone (65 mg/kg I.P.) with supplemental doses administered as required. The animal care procedures and experimental protocol employed met the guidelines set by the American Physiological Society and were approved by the Institutional Animal Care and Use Committee at The University of Georgia.

Study I: role of mechanical factors

In vitro muscle preparation

Gastrocnemius and plantaris muscles were carefully removed exposing the soleus muscle. Soleus muscle length (L_0) was measured during maximal dorsi- and plantar flexion of the ankle; L_0 was taken as the average of these two values. Soleus L_0 determined by this procedure coincides with the value determined by maximization of twitch tension. Mean (±S.D.) L_0 for all muscles was 15·00±0·69 mm. Mean blotted dry mass of the muscles at the end of the respective experiments was 16·87±2·82 mg. Muscle mass ranged from 8·91 to 26·01 mg, thus meeting the criterion of Segal & Faulkner (1985) for maintaining viability of an *in vitro* soleus muscle preparation at 37 °C.

The muscle was mounted in a 7 ml glass chamber and maintained at 37 °C with a thermoregulated water-jacket. The glass chamber contained a Krebs–Ringer bicarbonate buffer (pH 7·5) with 144 mM Na^+, 129 mM Cl^-, 6 mM K^+, 1 mM Mg^{2+}, 1 mM SO_4^{2-}, 1 mM PO_4^{2-}, 25 mM HCO_3^-, 2·5 mM Ca^{2+}, 10 mM glucose, and 0·10 U/ml insulin and was equilibrated with a 95% O_2 5% CO_2 gas. The distal tendon was attached by silk suture to a stainless-steel support, while the proximal tendon was attached by suture to the lever arm of a position feedback servomotor (Cambridge Technology model 300B; Cambridge, MA, USA). Length and force outputs from the Cambridge Technology unit were sampled at 500–1000 Hz using a Metrabyte DAS-16 interface board (Taunton, MA, USA) and a 80286-16 MHz or 80386-33 MHz microcomputer. Muscle length in the glass chamber was set using a micrometer and a sighting telescope mounted to a micrometer stage.

The isolated muscles were stimulated with 36 gauge platinum wire electrodes using a Grass S48 stimulator and SIU5 isolation unit (Quincy, MA, USA). The electrodes ran parallel to the muscle and were located ~ 1 mm from the muscle surface. Isometric twitch stimulation was performed using a 0·5 ms 150 V square-wave pulse. Isometric tetanic stimulation was performed using a 400 ms train of 0·5 ms 150 V pulses at 130 Hz.

Pre-injury measurements

The general experimental protocol, including set-up and pre-injury measurements, injury protocol, and incubation period, is presented in Fig. 1. Within 15 min of entering the Krebs–Ringer buffer, the muscle performed two isometric twitches, separated by 1 min. Mean (±S.D.) peak tension (P_T) on the second twitch was 86·7±14·8 mN. One minute after the second twitch, the muscle performed an isometric tetanus; P_0, $+dP/dt$, and $-dP/dt$ were measured. The mean P_0 was 328·8±48·4 mN. Mean P_0 normalized to muscle fibre cross-sectional area was 22·19±2·53 N/cm^2. This normalization assumed that muscle fibre length was 71% of L_0 (Claflin & Faulkner, 1985). The mean $+dP/dt$ and $-dP/dt$ were 7·141±1·231 and 6·320±1·109 N/s, respectively.

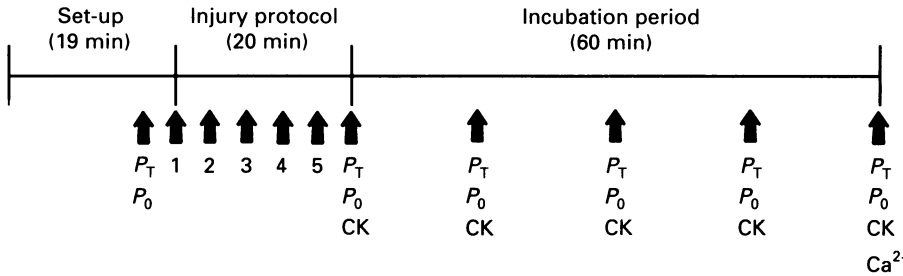

Fig. 1. Schematic diagram indicating the ordering of the protocol and experimental measurements (isometric twitch, P_T; isometric tetanus, P_0; creatine kinase assay, CK; and total muscle $[Ca^{2+}]$ assay, Ca^{2+}).

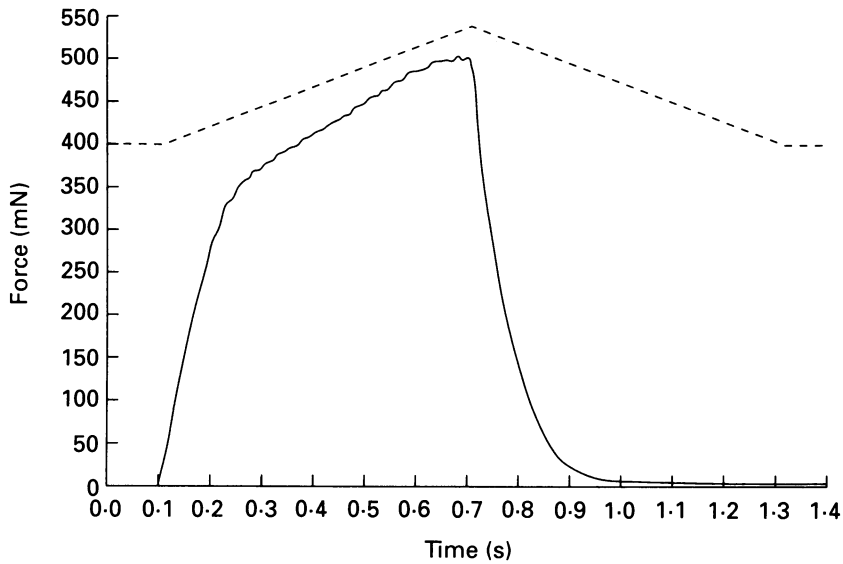

Fig. 2. Representative force and muscle length tracing from one eccentric contraction in a typical injury protocol. The top dashed curve is unitless with respect to the y-axis and shows the muscle length change (velocity = 0·5 L_0/s; length change = 0·30 L_0; initial length = 0·90 L_0). The continuous curve is muscle force (peak force = 150 % P_0) attained with a stimulation frequency of 60 Hz. In this contraction, stimulation commenced at 0·1 s and ended at 0·7 s.

Muscle injury protocol

Immediately following the isometric tetanus, muscle length was adjusted to the eccentric contraction starting length. The muscle was then stretched passively using the same length change and lengthening velocity as used during the following eccentric contractions. Peak passive tension ranged from 2·4 to 273·6 mN (0·7–111·3 % of pre-injury P_0). Length change and lengthening velocity were controlled by computer using Laboratory Technologies Notebook data acquisition/process control software (Wilmington, MA, USA) and the Metrabyte DAS-16 interface board. A constant velocity movement was achieved by applying a ramping DC voltage change into the length input port of the Cambridge Technology unit.

The injury protocol, which consisted of five eccentric contractions, was then performed; the first began 4 min after the isometric tetanus. Figure 2 shows a typical force tracing obtained during an eccentric contraction. The muscle was stimulated only during the lengthening phase of the

contraction. At the end of the stimulation, the muscle was allowed to shorten to the starting length at the same velocity used in the lengthening phase of the contraction. There was a 4 min rest interval between contractions. The 4 min interval was estimated to be more than adequate for complete O_2 consumption recovery using a Q_{10} (temperature coefficient) of 2·0 and the rate constant determined for mouse soleus (0·6 min at 20 °C) (Crow & Kushmerick, 1982). Mechanical measures

TABLE 1. Range of eccentric contraction mechanical measures for individual muscles (n = 180)

Negative work	56·6–694·7	mJ/g protein
Work absorbed by muscle	18·7–597·7	mJ/g protein
Negative power	0·259–3·100	W/g protein
Tension–time integral	70·6–1620·6	mN m s/g protein
Passive tension	0·7–111·3	% pre-injury P_0
Peak active tension	15·3–148·6	% pre-injury P_0
Peak active tension (normalized to fraction of maximal thick–thin filament overlap)	47·7–428·7	% pre-injury P_0 per fraction of maximal overlap

Values are means of the five eccentric contractions for an individual muscle. Negative work equals total force integrated over length change during the lengthening phase. Work absorbed by muscle equals negative work minus total force integrated over length change during the passive shortening phase. Negative power equals negative work divided by stimulus duration. To convert from protein mass to muscle mass, multiply by 5·87 (S.D. = 0·88).

acquired during the eccentric contractions include work done on the muscle (i.e. negative work), work absorbed by the muscle (i.e. negative work minus the work done by the muscle on the lever arm during the subsequent shortening phase), negative power (i.e. negative work divided by stimulus duration), tension–time integral, and peak active tension (i.e. peak tension attained during the first eccentric contraction minus the peak tension attained during the preceding passive stretch). Work was calculated from total force integrated over length change. In addition, the peak active tension normalized to the fraction of maximal thick–thin filament overlap at the final muscle length was estimated. Thick–thin filament overlap was estimated from the soleus length–active tension curve determined in preliminary experiments. Table 1 provides the range of these six mechanical measures observed in this study.

A factorial experimental design was employed in this study. Four factors (peak tension on the first eccentric contraction, lengthening velocity, length change, and initial length) were used. Three levels of peak tension (100, 125 and 150% of pre-injury P_0), lengthening velocity (0·5, 1·0 and 1·5 L_0/s), and length change (0·1, 0·2 and 0·3 L_0) were used. Contractions were initiated at a muscle length of 0·85 or 0·90 L_0. Peak force during the first eccentric contraction was controlled by varying stimulation frequency (range, 10–150 Hz). Actual peak force values closely approximated target values; the mean ($\pm$S.E.M.) actual peak force values for the three levels were 100·4 $\pm$ 1·3, 125·9 $\pm$ 0·9 and 152·4 $\pm$ 1·3% P_0. Because we were not aware of data for *in vivo* lengthening velocities in rat soleus muscle, lengthening velocities were chosen to be comparable to those reported for cat soleus muscle during treadmill running (0·9–1·7 L_0/s) (Goslow, Reinking & Stuart, 1973; Walmsley, Hodgson & Burke, 1978). The levels of initial length and length change were chosen to maintain muscle length within the normal anatomical range (78–122% of L_0 as determined in preliminary experiments).

Because a peak eccentric contraction force of 150% P_0 could only be attained during length changes of 0·30 L_0, the experimental design was not completely balanced. Thus, there were forty-two protocols with four experimental muscles per protocol except for the protocols requiring peak eccentric contraction tensions of 150% P_0. In these six protocols, there were six experimental muscles per protocol. The total number of muscles used in Study I was 180. The order in which the protocols were performed was completely randomized with respect to peak eccentric contraction force, lengthening velocity, and length change but not with respect to initial length. All protocols utilizing an initial length of 0·85 L_0 were performed first.

Incubation period

Immediately following the fifth eccentric contraction, muscle length was reset to L_0. The muscle performed an isometric twitch 4 min after the fifth contraction. One minute later, an isometric tetanus was performed. In between the twitch and tetanus, a 50–100 μl sample of the Krebs–Ringer buffer was taken and assayed for CK activity at 25 °C using a commercial kit (Sigma 45-5; St Louis, MO, USA). This sequence of events (twitch, buffer sampling and tetanus) was repeated four more times at 15 min intervals and constituted the incubation period. At the end of the 60 min incubation period, the muscle was freed from visible fat and connective tissue, blotted dry, weighed, and then frozen in liquid nitrogen. The muscle was stored at −80 °C until protein and total muscle [Ca^{2+}] analyses were performed.

Muscle analyses

Each muscle was homogenized in 2 ml of ice-cold 0·033 M phosphate buffer (pH 7·4) using 2 ml glass tissue grinders or a mechanical homogenizer (Biospec Tissue Tearor; Bartlesville, OK, USA). Total protein content was measured using the Lowry method (Sigma Protein Assay Kit, procedure No. P5656). Total muscle [Ca^{2+}] was determined using a Ca^{2+} analyser (Precision Systems Calcette; Natick, MA, USA). This analyser incorporates a fluorometric titration method to measure [Ca^{2+}] in aqueous solutions. A 0·5 ml sample of the muscle homogenate was adjusted to pH 5 using 10% acetic acid. The sample was then centrifuged at 11 600 *g* for 5 min and the supernatant assayed in triplicate for [Ca^{2+}]. Total muscle [Ca^{2+}] values were not obtained on the protocols using an initial muscle length of 0·85 L_0 because of Ca^{2+} contamination occurring in the homogenization of muscles when using the glass tissue grinders.

The test–retest reliability of the Ca^{2+} analyser was determined on six muscle homogenates on three separate days. The muscle homogenate [Ca^{2+}] values spanned the range previously seen in this laboratory (0·4–2·2 p.p.m.). The intraclass correlation coefficient for the three trials was 0·99. In addition, the six samples were assayed using plasma emission analysis (Mark II Jarrell-Ash 965; Franklin, MA, USA). The mean ($\pm$S.D.) difference between the two [Ca^{2+}] measurement techniques was 0·05$\pm$0·04 p.p.m. with the Calcette giving the higher value on average.

Two muscles that performed the most injurious protocol (i.e. defined as the protocol eliciting the greatest decrement in P_0; peak eccentric contraction force = 150% P_0; lengthening velocity = 1·5 L_0/s; length change = 0·30 L_0, initial length = 0·90 L_0; stimulus duration = 200 ms; stimulation frequency = 35 Hz) and two unstimulated muscles were prepared for transmission electron microscopic analysis. Following the 60 min incubation period, the muscles were placed in one-half strength Karnovsky's fixative for 20 min at room temperature. Each of the muscles was then teased into eight fibre bundles and fixed for another 2·5 h at room temperature. The fibre bundles were then postfixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series, and embedded in Epon/Araldite. Thin sections were cut on a microtome (Sorvall MT-2; Norwalk, CT, USA), stained with uranyl acetate and lead citrate, and viewed under an electron microscope (JEOL 100CX II; Tokyo, Japan); thirty to fifty sections from each muscle were examined.

Study II: assessment of metabolic status

The purpose of this study was to determine if the decrements in muscle function following the muscle injury protocol were associated with a reduction in [ATP]. An attenuation in muscle [ATP] immediately after the injury protocol would support a possible metabolic rather than mechanical etiology for the reduction in P_0. The most injurious protocol was performed on eight muscles. Four minutes after the fifth eccentric contraction, the muscles were quickly frozen using tongs cooled to the temperature of liquid nitrogen and then stored at −80 °C. The muscles were freeze-dried overnight and then assayed for [ATP] using the technique of Lowry & Passonneau (1972).

Muscles performing the most injurious protocol were compared against sixteen control muscles that performed five isometric contractions (initial length = 0·90 L_0; stimulus duration = 200 ms; stimulation frequency = 130 Hz) instead of the five eccentric contractions. Eight of the sixteen control muscles were quick frozen 4 min after the fifth isometric contraction and assayed for [ATP]. The other eight control muscles performed the isometric contraction protocol and were incubated for 60 min. The measurements (mechanical measurements, CK release, and total muscle [Ca^{2+}]) made on these control muscles were the same as described in Study I. Unstimulated contralateral muscles for each of the experimental muscles (both eccentric and isometric control) were quick frozen *in situ* and assayed for [ATP].

Statistical analyses

Stepwise regression analyses were employed to determine which combination of mechanical factors accounted for the most variance in each of the muscle function measures. The α-levels selected for a factor to be entered into and removed from a regression equation were 0·10 and 0·05, respectively. The independent variables included in the analyses were peak tension on the first eccentric contraction, lengthening velocity, length change, initial length, final length, negative work, work absorbed by the muscle, negative power, and tension–time integral. The values used for negative work, work absorbed by the muscle, negative power, and tension–time integral were the means of the five eccentric contractions. First- and second-order terms for peak tension on the first eccentric contraction, lengthening velocity, length change, and final length were used.

The significance of differences among mechanical factor level means for the muscle function measures was evaluated using one-way ANOVA. When significant differences were found, Student–Neuman–Keuls *post hoc* tests were performed to determine means that were significantly different. CK release over the incubation period and the change in the muscle function measures from 15 to 60 min during the incubation period were evaluated using one-way ANOVA with repeated measures and polynomial transformation. Means of experimental measures for muscles performing the most injurious protocol were compared against means for the isometric control muscles using Student t tests. Change score values (i.e. value at one point in time minus value at second point in time divided by the pre-injury value) were utilized for the stepwise regression, one-way ANOVA without repeated measures, *post hoc* tests, and t tests.

Because the variance in peak eccentric contraction tension and work absorbed by the muscle explained a significant proportion of the variance in several muscle function measures, change-point linear regression (Jones & Molitoris, 1984) was employed to determine the levels of these two factors above which a marked decrement in muscle function occurred. With the exception of the change-point linear regression, all analyses were performed using PC-SAS (SAS Institute; Cary, NC, USA). Change-point linear regression was performed using a BASIC program provided by Jones & Molitoris (1984). An α-level of 0·05 was used for all tests of significance. Values reported in the results are means $\pm$ standard errors.

RESULTS

Mechanical injury markers

There were three relatively distinct phases in the response of the mechanical injury markers (P_0, P_T, $-dP/dt$ and $+dP/dt$) over the 60 min incubation period. The first phase, termed the initial decline, refers to the change in muscle function from pre-injury to 0 min into the incubation. From 0 to 15 min into the incubation, there was a partial recovery in several measures of muscle function; this phase is termed the recovery phase. From 15 to 60 min into the incubation, muscle function deteriorated at a relatively slow rate; this phase is termed the slow decline. Figure 3 illustrates the three phases observed for P_0. The response of the injury markers in each of the three phases was independent of muscle mass, rat age or body mass.

Initial decline

Table 2 contains the results of the stepwise regression analyses for the initial declines in P_0, P_T, $+dP/dt$ and $-dP/dt$. The factors negative work, negative power and final length did not explain a significant proportion of the variance in the initial decline of P_0, P_T, $+dP/dt$ or $-dP/dt$ in this phase or any other phase, so they are not included in the table.

Peak total tension during the first eccentric contraction was the dominant factor in explaining the initial declines in the four measures (Table 2). As shown in Fig. 3,

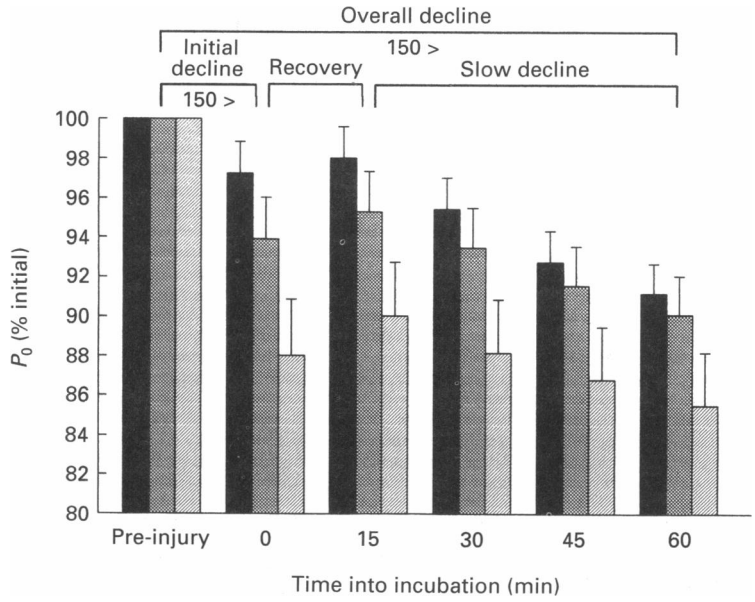

Fig. 3. The effect of altering peak eccentric contraction force on the percentage changes in maximal isometric tetanic force (P_0) following the injury protocol. Mean initial pre-injury values were: 100% P_0 (filled bar), 336·1 mN; 125% P_0 (cross-hatched bar), 327·1 mN; 150% P_0 (hatched bar), 318·0 mN. Error bars are equal to 1 S.E.M. The subdivision of the overall decline in P_0 into three phases is indicated above the graph. The numbers below the horizontal lines (i.e. '150 >') represent factor levels that were significantly different from other factor levels for percentage changes in P_0 (e.g. the reduction in P_0 from pre-injury to 0 min into the incubation was greater for muscles performing different contractions eliciting 150% P_0 than for muscles performing 100% or 125% P_0 contractions).

TABLE 2. Results of the stepwise regression analyses on the percentage initial decline (pre-injury to 0 min post-injury) of the measured variables

	Partial coefficients of determination for							
Variable	Peak total tension	Initial length	Length change	Lengthening velocity	Work absorbed by muscle	Tension–time integral	Model r^2	p
P_0	0·1112*	—	—	0·0763*	—	—	0·1875	0·0001
P_T	0·3003*	—	0·0304*	—	—	0·0229	0·3536	0·0001
$+dP/dt$	0·1391*	—	—	0·0176	0·0534	0·0408	0·2509	0·0001
$-dP/dt$	0·0358*	—	—	0·0429*	—	—	0·0787	0·0007

* Values are partial coefficients of determination for second-order terms. Dashes indicate that the given factor did not significantly contribute to the variance in initial declines for the respective variables.

muscles performing 150% P_0 eccentric contractions exhibited a greater initial decline in P_0 (12·1% *vs.* 2·5–5·9%). The results from the change-point regression analyses indicate that the initial decline in P_0 was independent of the peak eccentric contraction force level for peak eccentric contraction forces eliciting less than

112·9 % of pre-injury P_0. For peak eccentric contraction forces exceeding 112·9 % of pre-injury P_0, the initial decline in P_0 was linearly related to the peak eccentric contraction force; above the threshold, P_0 declined by 2·7 % for every 10 % P_0 increase in peak eccentric contraction force. The peak active tension (total tension − passive

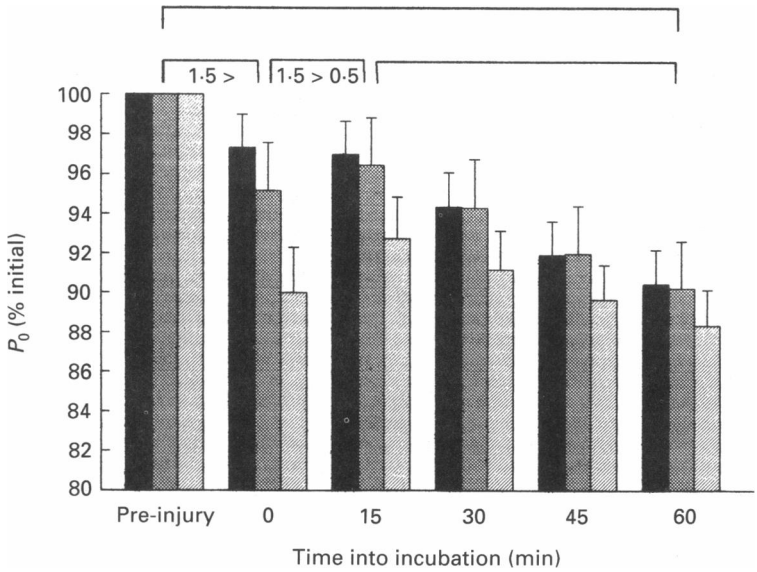

Fig. 4. The effect of altering lengthening velocity on the percentage changes in maximal isometric tetanic force (P_0) following the injury protocol. Mean initial pre-injury values were: 0·5 L_0/s (filled bar), 335·3 mN; 1·0 L_0/s (cross-hatched bar), 318·6 mN; 1·5 L_0/s (hatched bar), 332·5 mN. Numbers below horizontal lines are the same as described for Fig. 3.

tension) produced in the first eccentric contraction was unrelated to the reduction in P_0 ($r^2 = 0{\cdot}019$, $p = 0{\cdot}063$). However, when peak active tension was normalized to the degree of overlap at the final muscle length, the relationship was significant ($r^2 = 0{\cdot}063$, $p = 0{\cdot}0007$). There was also a weak relation between the peak tension elicited during the passive stretch and the initial decline in P_0 ($r^2 = 0{\cdot}027$, $p = 0{\cdot}028$).

The initial declines in P_T for muscles performing 150, 125 and 100 % P_0 eccentric contractions were 21·5, 8·0 and 0·9 %, respectively (all significantly different from each other). The initial decline in $+dP/dt$ for the muscles performing 150 % P_0 contractions (11·7 %) was significantly greater than that for the lower two force levels (0·2–4·1 %). The initial decline in $-dP/dt$ for the muscles performing 150 % P_0 contractions (10·4 %) was significantly greater than that for the muscles performing the 100 % P_0 contractions (4·3 %).

Lengthening velocity, tension–time integral, length change, and work absorbed by the muscle explained a lesser proportion of the variance in the initial decline of the mechanical measures (Table 2). Initial length had no effect on the initial decline of any of the four variables. Figure 4 depicts the effect of lengthening velocity on the initial decline in P_0. Muscles lengthened at a velocity of 1·5 L_0/s exhibited a greater

initial decline in P_0 than did muscles lengthened at the two slower velocities (9·6 *vs.* 2·7–5·0 %). The highest lengthening velocity also elicited a greater initial decline in $-dP/dt$ (9·8 *vs.* 4·1–5·0 %). The initial declines in $+dP/dt$ for muscles lengthened at 1·0 and 1·5 L_0/s (5·4–5·7 %) were significantly greater than that for muscles lengthened at 0·5 L_0/s (1·2 %).

TABLE 3. Results of the stepwise regression analyses on the percentage recovery (0–15 min incubation) of the measured variables

	Partial coefficients of determination for							
Variable	Peak total tension	Initial length	Length change	Lengthening velocity	Work absorbed by muscle	Tension–time integral	Model r^2	p
P_0	—	0·0940	—	0·0650	—	—	0·1589	0·0001
P_T	—	—	0·0568	—	—	—	0·0568	0·0013
$+dP/dt$	—	0·0328	—	0·0484	—	—	0·0812	0·0006
$-dP/dt$	—	0·1379	—	0·0235*	—	—	0·1614	0·0001

* Values are partial coefficients of determination for second-order terms. Dashes indicate that the given factor did not significantly contribute to the variance in recovery for the respective variables.

The initial decline in P_T and $+dP/dt$ was less for the eccentric contractions with the highest tension–time integrals. If all other variables in the regression equation were held constant, the protocol eliciting the lowest mean tension–time integral (0·094 N m s/g protein) would be predicted to have a 10·5 % greater reduction in P_T than that predicted for the protocol eliciting the highest mean tension–time integral (1·376 N m s/g protein). For $+dP/dt$, the reduction would be predicted to be 36·2 % greater for the lower mean tension–time integral.

Greater initial declines in $+dP/dt$ were observed in protocols with greater amounts of work absorption by the muscle. If all other variables in the regression equation were held constant, the protocol eliciting the highest mean work absorption (0·502 J/g protein) would be predicted to have a 24·8 % greater reduction in $+dP/dt$ than that predicted for the protocol eliciting the lowest mean work absorption (0·030 J/g protein).

Recovery

Initial length and lengthening velocity were the dominant factors in explaining the recovery (from 0 to 15 min of the incubation period) of muscle function (Table 3). Figure 4 illustrates the effect of lengthening velocity on P_0 during the recovery phase. The recovery in P_0 for muscles lengthened at 1·5 L_0/s was 2·7 %. Muscles lengthened at 0·5 L_0/s experienced a slight decrease in P_0 (0·1 %). The recovery in $-dP/dt$ was greater for the muscles lengthened at 1·5 L_0/s (3·2 to 93 % of pre-injury levels) compared to the two slower velocities (0·2–0·9 to 95–96 % of pre-injury levels). The recovery in $+dP/dt$ was smaller for the muscles lengthened at 0·5 L_0/s (4·8 to 103 % of pre-injury levels) compared to the two faster velocities (7·5–8·2 to 101–103 % of pre-injury levels).

Muscles that performed eccentric contractions at an initial muscle length of 0·85 L_0 exhibited a 2·7% increase in P_0 during this phase while muscles that performed contractions at 0·90 L_0 exhibited a 0·1% decrease in P_0 (Fig. 5). The muscles that began contractions at the shorter muscle length exhibited a 4·0% increase (to 98%

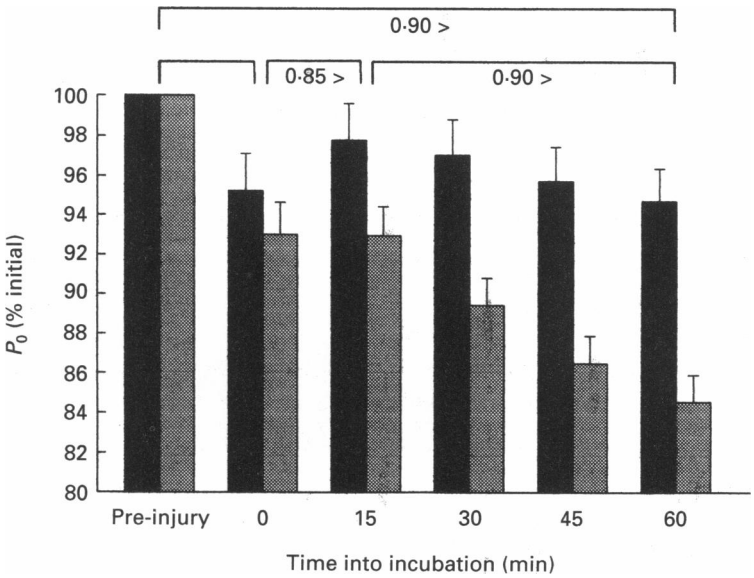

Fig. 5. The effect of altering initial muscle length on the percentage changes in maximal isometric tetanic force (P_0) following the injury protocol. Mean initial pre-injury values were: 0·85 L_0 (filled bar), 325·4 mN; 0·90 L_0 (cross-hatched bar), 325·9 mN. Numbers below horizontal lines are the same as described for Fig. 3.

of pre-injury levels) in $-dP/dt$, while muscles performing contractions at the longer length exhibited a 1·1% decrease (to 91% of pre-injury levels). For $+dP/dt$, the increases were 8·0% (to 105% of pre-injury levels) and 5·7% (to 104% of pre-injury levels) for muscles beginning contractions at 0·85 and 0·90 L_0, respectively.

Length change played a minor role in accounting for the recovery in P_T. Muscles performing length changes of 0·1 L_0 exhibited increases in P_T of 3·2% (to 101% of pre-injury levels) compared with increases of 6·3–6·5% (to 93–99% of pre-injury levels) for the 0·2 and 0·3 L_0 length changes.

Slow decline

The factor that accounted for most of the variance in the slow decline (i.e. change from 15 to 60 min into the incubation) in P_0 and $-dP/dt$ was initial length. As shown in Fig. 5, the slow decline in P_0 for muscles with initial lengths of 0·90 L_0 was 2·7-fold greater than for muscles with initial lengths of 0·85 L_0 (8·4% *vs.* 3·1%). The slow decline in $-dP/dt$ was greater for muscles with initial lengths of 0·90 L_0 (10·9 to 81% of pre-injury levels) compared with that for muscles with initial lengths of 0·85 L_0 (4·9 to 93% of pre-injury levels). The slow decline observed in P_T and $+dP/dt$ was independent of any mechanical factor.

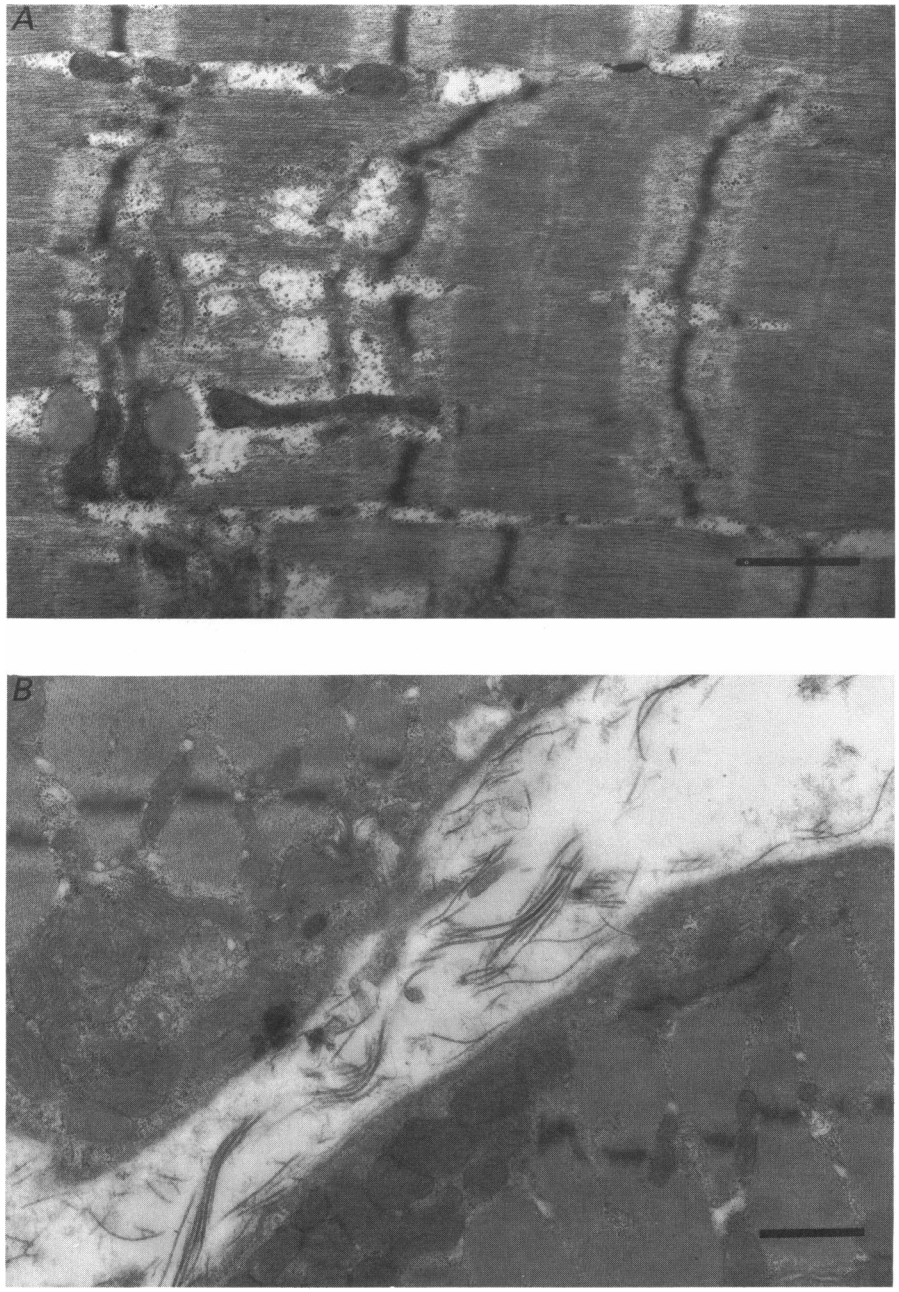

Fig. 6. *A*, transmission electron micrograph illustrating sarcomeric dissolution and Z-line streaming in a muscle that performed the most injurious protocol. The scale bar represents 1 μm. *B*, transmission electron micrograph illustrating basement membrane disruption in a muscle that performed the most injurious protocol. The scale bar represents 1 μm.

Comparison against isometric control muscles

The mean initial decline in P_0 for the six muscles performing the most injurious protocol equalled 13·6±4·8%. For the eight control muscles that performed isometric contractions, the mean P_0 at 0 min into the incubation period was elevated by 1·2±1·0% over the mean baseline P_0. The difference in the means was significant at $p = 0{\cdot}005$. The overall decline in P_0 after the 60 min incubation was also greater ($p = 0{\cdot}007$) for the muscles performing the most injurious protocol (18·3±1·8%) than for the control muscles (7·2±2·2%).

The greater initial decline in P_0 for the most injurious protocol could not be explained by a lower [ATP]. The mean [ATP] for the muscles performing the most injurious protocol and the isometric control muscles were 19·84±1·38 and 16·30±1·49 μmol/g dry weight, respectively. These means were not significantly different ($p = 0{\cdot}229$). The mean [ATP] differences (i.e. experimental muscle [ATP] minus unstimulated contralateral muscle [ATP]) for the muscles performing the most injurious protocol and the isometric control muscles were +2·21±0·73 and −0·55±1·12 μmol/g dry weight, respectively. These two means were not significantly different at $p = 0{\cdot}063$.

The greater initial and overall decline in P_0 for the muscles performing the most injurious protocol can most likely be attributed to structural defects occurring in the muscles. The electron micrographs in Fig. 6 show the loss of force-generating (i.e. sarcomere dissolution) and force-transmitting (i.e. Z-line streaming and disruption of the basement membrane) structures. These lesions could be found in each of the thirty to fifty thin sections cut from the two injured muscles, although there were regions that appeared normal. Such defects were not observed in two unstimulated muscles.

CK release

CK release was independent of all mechanical factors with the exception of lengthening velocity. Figure 7 depicts the effect of lengthening velocity on CK release during the incubation period. The downward trend in CK activity from 0 to 30 min is attributed to inactivation of the enzyme in the Krebs–Ringer buffer; in separate experiments we have found the rate of CK inactivation to be ~ 30% per hour. The CK activity at 45 and 60 min was significantly higher for muscles lengthening at 1·5 L_0/s compared to the muscles lengthening at 0·5 L_0/s. An unexpected finding was that the slow decline in P_0 was inversely related to the CK activity at 60 min ($p = 0{\cdot}001$). Mean CK activity for the muscles performing the most injurious protocol was 39–145% higher than that for the isometric control muscles, but the difference was not statistically significant at any time during the incubation ($p \geqslant 0{\cdot}102$).

Total muscle $[Ca^{2+}]$

Negative power was the only mechanical factor that could account for a significant proportion of the variance in total muscle $[Ca^{2+}]$ ($r^2 = 0{\cdot}063$, $p = 0{\cdot}018$). The injury protocol eliciting the lowest mean negative power (0·425 W/g protein) would be predicted to have a 37·2% lower $[Ca^{2+}]$ than that predicted for the protocol eliciting the highest mean negative power (2·349 W/g protein), if all other variables in the regression equation were held constant. No relation was found between total muscle

[Ca^{2+}] and the slow decline in any of the mechanical injury markers or between total muscle [Ca^{2+}] and CK release.

The mean total muscle [Ca^{2+}] for the muscles performing the most injurious protocol and the isometric control muscles were $15{\cdot}24 \pm 1{\cdot}48$ and $13{\cdot}23 \pm 1{\cdot}74$ μmol/g

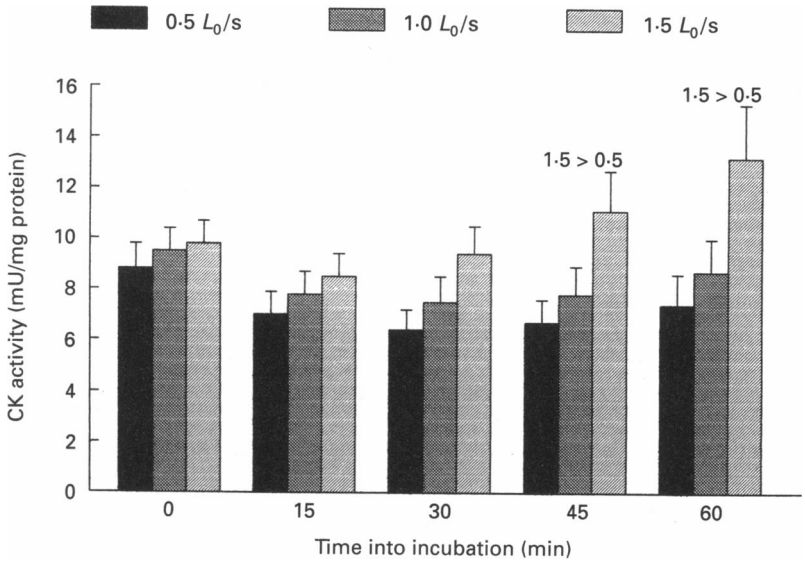

Fig. 7. The effect of altering lengthening velocity on the creatine kinase (CK) activity in the incubation medium. Error bars are equal to 1 S.E.M. Muscles lengthened at 1·5 L_0/s had significantly greater mean CK activity at 45 and 60 min than did muscles lengthened at 0·5 L_0.

protein, respectively. The difference in the two means was not statistically significant ($p = 0{\cdot}208$). The mean total muscle [Ca^{2+}] for the ninety Study I muscles in which [Ca^{2+}] measurements were made was $18{\cdot}20 \pm 0{\cdot}71$ μmol/g protein and was significantly higher than the control muscle mean ($p = 0{\cdot}022$). This difference in total muscle [Ca^{2+}] could not be attributed to a greater extracellular volume in the Study I muscles since the protein content-to-wet weight ratios were identical for the Study I and isometric control muscles.

DISCUSSION

This study supports the hypothesis that eccentric contraction-induced muscle injury has a mechanical etiology. Systematically varying four primary mechanical factors during the eccentric contractions (i.e. peak force, initial length, length change, and lengthening velocity) resulted in graded levels of injury in the muscles. The primary injury criteria used in this study were measures of muscle performance immediately after the injury protocol (P_0, P_T, $+dP/dt$ and $-dP/dt$). The mechanical factor that most affected muscle performance was peak total force produced in the muscles during the eccentric contractions. Katz (1939) and McCully & Faulkner

(1986) previously reported that the degree of injury resulting from eccentric contractions was directly related to the forces produced during the contractions; the present findings extend those observations by demonstrating that peak eccentric force, independent of lengthening velocity, initial (or final) length or length change, is associated with initiating the injury process.

These results have important practical implications. They demonstrate that a relatively small number of high force eccentric contractions can cause significant reductions in muscle performance. The reductions are immediate and the muscles do not recover over the short term. These are important considerations for muscular performance in the workplace and sports field. In this study, the highest forces produced during the eccentric contraction protocol were 150% P_0. Muscles are capable of producing up to 200% P_0 during lengthening contractions (Woledge *et al.* 1985; Armstrong *et al.* 1991), however, and the present results would suggest contractions producing these higher tensions may cause even greater damage to the muscles.

Although the present findings provide compelling evidence that the initial event in the injury process is mechanical in nature, they do not prove the hypothesis. For example, it is possible that during the first eccentric contraction of the five contraction injury protocol, some metabolic event (e.g. elevated $[H^+]$, $[P_i]$) initiated the injury sequence and the mechanical factors simply modulated subsequent autogenetic processes during the remaining 20 min of the injury protocol. However, indirect evidence indicates that the initiating event and the mechanisms underlying the decrements in muscle function were not metabolic in nature. First, it has been firmly established that eccentric contractions require lower energy expenditure than isometric or concentric contractions (Woledge *et al.* 1985; Armstrong *et al.* 1991). In this study, there were immediate reductions in P_0 (and other mechanical variables) following eccentric contractions, but not after isometric contractions. Furthermore, the reduction in muscle function immediately after completion of the injury protocol was not associated with reduction in [ATP]. In this study, the absolute [ATP] was lower following the control isometric contraction protocol than after the eccentric contraction protocol. Finally, and perhaps most importantly, P_0 did not recover in the 60 min following the injury protocol, which would have been expected if the initial reductions in muscle function resulted from metabolic fatigue.

The initial decrements in P_0, P_T, $+dP/dt$ and $-dP/dt$ are consistent with the hypothesis that the first event in the injury sequence was a loss of contractile and/or of non-contractile components common to both the series and parallel elastic elements. This interpretation is based on the observation that the initial decrease in P_0 was related to both the active force normalized for thick–thin filament overlap produced during the first eccentric contraction and to the passive tension produced during the stretch, respectively. From the present results, it is not possible to distinguish between loss of contractile elements and loss of series elements involved in transmission of forces produced by the contractile structures. The electron microscopic observations support both possibilities, i.e. there is evidence of loss of sarcomeres and series components (e.g. Z-lines and basal laminar structures).

Because the initial decline in P_0 became progressively greater as peak eccentric contraction tension exceeded 113% P_0, it is feasible that the failure of a critical

component (contractile or series) was due to excessive tensile stress in accordance with the normal stress theory mechanism of failure. This theory states that a component will fail when it is subjected to tensile stress that surpasses its yield strength. According to this postulate, the yield or tensile strength of the weakest component in the soleus muscles in this study was 113% P_0. This force threshold for failure is in congruence with the findings of Tidball & Chan (1989). They reported that the breaking stress of a muscle fibre is ~ 20% greater than that produced in a maximally activated fibre during an isometric contraction. In their study, the site of failure was found to be the myotendinous junction. Whether or not the myotendinous junction was the site of failure in the present study is unknown, although the abundant evidence of disruption of sarcomeres in the belly of the muscle does not seem compatible with this hypothesis. Failure of the component by a materials fatigue mechanism, as opposed to the normal stress mechanism discussed above, cannot be ruled out. The protocol in this study used five eccentric contractions, and it is not known after which contraction the injury was initiated. During performance of the most injurious injury protocol ($n = 6$ muscles), there was a relatively steady decline in the force produced in each of the five eccentric contractions (first, 151 ± 3·6% P_0; second, 149·9 ± 3·8% P_0; third, 145·7 ± 2·8% P_0; fourth, 141·9 ± 3·9% P_0; fifth, 139·6 ± 5·4% P_0), suggesting the injury was progressively manifested through the protocol.

Higher lengthening velocities in this study were associated with greater initial declines in P_0, $+dP/dt$ and $-dP/dt$ and the recovery (change from 0 to 15 min into the incubation) for these three mechanical measures was greatest for the same muscles. The recovery was complete for $+dP/dt$ but not for $-dP/dt$ or P_0. This transient effect of lengthening velocity on $+dP/dt$, $-dP/dt$ and P_0 is consistent with a temporary reduction in the rate of cross-bridge turnover and/or force production per cross-bridge. This proposed transient effect of velocity on cross-bridge properties is analogous to that proposed to explain the effect of velocity on isometric force production immediately following shortening or lengthening (Woledge *et al.* 1985).

Although the initial declines in $+dP/dt$ and $-dP/dt$ are consistent with an alteration in cross-bridge properties, they could also be attributed to a temporary disturbance in muscle fibre Ca^{2+} homeostasis. A temporary decrement in the rate of SR (sarcoplasmic reticulum) Ca^{2+} release could explain the velocity-induced transient reduction in $+dP/dt$. Similarly, the lengthening velocity-dependent reduction in $-dP/dt$ could be caused by a reduction in the rate of Ca^{2+} reuptake by the SR. However, if this was so, one would expect an increase in P_T and not a decrease (Taylor, Abresch, Lieberman, Fowler & Portwood, 1984). A decrease in P_T while there was a reduction in the rate of SR Ca^{2+} reuptake is plausible only if there was a concomitant large reduction in muscle stiffness.

The slow decline phase that occurred during the 60 min incubation was consistent with the hypothesis that there is a focal loss of Ca^{2+} homeostasis at the site of fibre injury. The mechanical factor most closely associated with the slow decline in muscle performance was initial muscle length. Morgan (1990) hypothesized that a greater degree of sarcomere length inhomogeneity exists in a fibre beginning an eccentric contraction at longer fibre lengths. He predicted that under these conditions there is a greater chance for extreme lengthening occurring in the longer, weaker sarcomeres,

and that parallel structures (e.g. sarcolemma and SR) adjacent to these over-lengthened sarcomeres may be disrupted. In the present study, the effect of initial muscle length on contractile performance was not evident until $\geqslant 15$ min into the incubation. The delayed effect of initial length is not inconsistent with the predictions of Morgan (1990), or with the hypothesis that a focal loss of Ca^{2+} homeostasis follows initiation of injury, because it would take a finite time for elevations in cytosolic $[Ca^{2+}]$ to exceed the fibre's Ca^{2+} buffering capacity and activate lipolytic and proteolytic pathways.

There is indirect evidence for an association between initial length and elevations in cytosolic $[Ca^{2+}]$ in the present study. First, the slow decline in $-dP/dt$ during the post-injury incubation was much greater for muscles beginning eccentric contractions at the longer initial muscle length (0·90 L_0). This suggests that the effective resequestration of Ca^{2+} by SR (or transport through sarcolemma) became progressively impaired over time in these muscles. Secondly, there was a rise in resting tension over time, peaking at 15 min into the incubation. Pre-injury resting tension for all muscles that performed eccentric contractions was $4{\cdot}80 \pm 0{\cdot}10$ mN; resting tensions at 0, 15, 30 and 60 min following eccentric contractions were $6{\cdot}38 \pm 0{\cdot}29$, $6{\cdot}97 \pm 0{\cdot}39$, $6{\cdot}38 \pm 0{\cdot}29$ and $4{\cdot}02 \pm 0{\cdot}20$ mN. These observations also imply a loss of free intracellular Ca^{2+} homeostasis. The greater slow decline in P_0 observed for muscles beginning contractions at 0·90 L_0 may then have resulted from greater activation of Ca^{2+}-mediated proteolytic pathways leading to degradation of contractile and non-contractile proteins (Baracos, Greenberg & Goldberg, 1984).

The findings do not support the hypothesis that the longer initial length elicited a greater loss of sarcolemma integrity. Neither elevations in total muscle $[Ca^{2+}]$ nor losses of CK from the muscles were related to initial length. Arguing for the disruption of sarcolemma is the sarcolemmal damage evident in electron micrographs (Fig. 6) and the observation that total muscle $[Ca^{2+}]$ was elevated in muscles performing eccentric contractions. Also, others have reported a dissociation of CK release from the histological evidence of injury (Manfredi, Fielding, O'Reilly, Meredith, Lee & Evans, 1991; Van Der Meulen, Kuippers & Drukker, 1991). Sarcolemmal disruptions could be varied in size and/or smaller than the CK molecule (MW 88000).

Finally, some caution must be exercised in interpreting the findings on initial length effects. First, the order in which the forty-two injury protocols was performed may have confounded the effect of initial muscle length on the injury sequence. Second, there was a slow decline in P_0 during the incubation period even for the control muscles that performed the isometric contraction protocol. The rate of the slow decline in these muscles was only slightly less than that observed in those performing the eccentric contraction protocols yielding the least amount of injury. This was probably due to a greater than normal extracellular-to-intracellular Ca^{2+} gradient in the preparation that was used, leading to a high baseline influx of extracellular Ca^{2+} and to a subsequent activation of autogenetic degradative pathways. The ionic $[Ca^{2+}]$ in the Krebs–Ringer buffer was 2·5 mM compared with a normal serum ionic $[Ca^{2+}]$ value of 1·1–1·3 mM. In subsequent experiments using a buffer with 1·25 mM Ca^{2+}, the slow decline in muscle function during the incubation has been eliminated in both control and injured muscles.

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