Changes in contractile properties of skeletal muscle during developmentally programmed atrophy and death

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Received 19 October 2000; accepted in final form 8 January 2002

Schwartz, Lawrence M., and Robert L. Ruff. Changes in contractile properties of skeletal muscle during developmentally programmed atrophy and death. Am J Physiol Cell Physiol 282: C1270–C1277, 2002.—Skeletal muscle atrophy and death are protracted processes that accompany aging and pathological insults in mammals. The intersegmental muscles (ISMs) from the tobacco hawkmoth Manduca sexta are composed of giant fibers that undergo distinct hormonally-regulated programs of atrophy and death at the end of metamorphosis. Atrophy occurs during the 3 days preceding adult emergence and results in a 40% reduction of mass, whereas death takes place during the subsequent 30 h and results in the complete loss of the fibers. There are no significant changes in tetanic force or calcium sensitivity in skinned fiber preparations during atrophy. However, the size of caffeine-induced contractions fell by about 50%. With the onset of the death phase, dramatic reductions occur in ISM: tetanic force, twitch amplitude, resting potential, caffeine-induced contractions, calcium sensitivity, and Hill coefficients. Several lines of evidence suggest that ISM atrophy is caused by an increase in protein turnover without significant modification of fiber organization. In contrast, ISM death is accompanied by disorganization of the contractile apparatus and concomitant loss of contractile function.

Manduca; apoptosis; calcium; degeneration; sarcopenia

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suggest that muscle fiber atrophy and death are distinct processes. Although there are dramatic changes in muscle mass during atrophy, there are few concomitant changes in the physiological properties of the cells. In contrast, death results in both a loss of muscle mass and a profound reduction in physiological function.

MATERIALS AND METHODS

Animals. Manduca sexta were reared and staged as previously described (49). Animals were dissected under saline to expose the lateral ISMs (49). The saline had the following composition (in mM): 9 NaCl, 34 KCl, 4.2 CaCl2, 6 MgCl2, 172 dextrose, and 25 sodium phosphate buffer, pH 6.7. ISMs were collected at four times: 1) on day 15 of pupal/adult development, which was 3 days before adult emergence, 2) before emergence on day 18 of pupal/adult development, 3) at 4 h after emergence on day 18, and 4) at 15 h after emergence on day 18 of pupal/adult development. All experiments were performed at room temperature (22 ± 1°C).

Tension measurements. Single ISM fibres were dissected from the fifth abdominal segment and attached to a custom-built isometric tension transducer modeled on the design of Hellam and Podolsky (15). To minimize possible changes during an experiment, only 1 fiber was used from each animal, and 5–20 fibers were examined for each time point. Intact fibers were stimulated with extracellular electrodes. When tension data was analyzed, the force of contraction was normalized for fiber cross-sectional area. At the outset of the experiment, the diameter of each muscle fiber was measured with a microscope positioned over the recording apparatus. Mechanically skinned fibers (see below) had an essentially circular cross section, and their area was calculated accordingly. When examined histologically, intact fibers had an elliptical cross section, with the minor axis measuring 0.505 times the major axis. Therefore, contractile force for the intact fibers was normalized to fiber cross-sectional area by dividing the force by \( \pi(diameter)^2(0.505)/4 \).

We studied ISM fibers obtained at each of the four collection times. We compared data from the different collection times by using analysis of variance (ANOVA). Alpha was set at 0.05, and two-tailed tests were employed for all analyses. Subsets of data were studied with ANOVA to determine whether there were any significant differences among the groups of fibers studied at the four different collection times. When significant interactions were present, post hoc comparisons between different groups were made by using Tukey's honestly significant difference test for pairwise comparisons, with Scheffé's S method when more than two means were compared. The data presented satisfied normality criteria (32).

In some experiments ISM fibers were mechanically skinned by splitting the fibers longitudinally with dental picks. Skinned fibers were individually attached to a tension transducer and bathed in solutions containing differing levels of buffered free calcium, following the standard notation that pCa = \(-\log [Ca^{2+}]\). The skinned fiber activating solutions contained (in mM) 1.0 free Mg2+, 4.0 MgATP, 135 K+, 15 creatine phosphate, and 12 EGTA. The concentration of the pH buffer MOPS was varied to keep the ionic strength of the activating solutions at 200 mM. The concentration of MOPS for each integral pCa used was (in mM) 26 (pCa 4.0), 28 (pCa 5.0), 32 (pCa 6.0), 44 (pCa 7.0), and 50 (pCa 8.0). The major anion was propionate, and the pH was adjusted to 7.0. Solutions were mixed at one time, and 20 U/ml creatine kinase was added to the activating solutions at the time of an experiment. Binding constants of EGTA and ATP with hydroxyl, calcium, and magnesium ions were adjusted for ionic strength and temperature (25, 42). The concentration of free calcium (pCa 8.0 to pCa 4.0) and other solution components were calculated as previously described (25, 42).

Each skinned fiber was secured between the flaps of an aluminum foil clip. The clips were slipped over stainless steel hooks attached to a micromanipulator and a custom-made force transducer (25). The micromanipulator was used to adjust each fiber to its rest length. The transducer had a resonant frequency of 50 Hz, a compliance of 0.4 \(\mu\)m/mg, and a sensitivity of 12 mV/mg and was linear over the range of 0–500 mg. To elicit tension, a fiber was lowered into one of a set of Plexiglas wells containing 2.0 ml of relaxing (pCa 8.0) or activating (pCa <7.0) solution. The fiber could be transferred between wells in <1 s.

Data for the force measurement at each pCa were normalized to the maximum tension and plotted vs. pCa. A nonlinear least-squares fit of the Hill equation (16) was calculated from the data from the second tension trial of each fiber. The equation tension/maximum tension = \([Ca^{2+}]^{nH}/[K^{H} + [Ca^{2+}]^{nH}]\), where K is the calcium concentration associated with half-maximal tension and \(nH\) is the Hill coefficient, describes the steepness of the relationship between tension and calcium concentration. To minimize artifactual reduction of the slope when pooling data from several different fibers, \(nH\) was calculated from the tension-pCa relationships by shifting individual fiber tension pCa curves along with the pCa axis so that the pCa for 50% tension for all fibers coincided. These data were then fit with the Hill equation to determine \(nH\), the slope parameter. To control for possible variability in the solutions or recording apparatus, chemically skinned rabbit adductor magnus muscle was tested at the beginning of each experiment because the calcium sensitivity of this muscle is well known and does not vary between rabbits (9). Consequently, these muscle fibers provided a biological calibration for both the transducer and the activating solutions. Rabbit adductor magnus muscles were skinned by incubating muscles for 1 wk at \(-20°C in 50% glycerin at pCa 8.0. To test the effects of caffeine, intact ISM fibers were attached to a tension transducer and exposed to 25 mM caffeine in Manduca saline (49).

Electrophysiological measurements. Abdomens were removed from animals at various times relative to adult emergence, rinsed with saline, cut middorsally, and eviscerated. The abdomens were pinned over a hole in the center of a wax-filled Plexiglas recording chamber by using a thin bead of high-vacuum silicon grease (Dow Corning) to create a watertight seal. The spiracles were aerated through the hole under the preparation. Because the rigidity of the overlying pupal cuticle prevents spiracle aeration, reliable recordings could not be obtained with preparations before day 18 of pupal/adult development. The chamber was then flooded with saline, and the preparation was used for <30 min. Resting potentials were recorded with glass microelectrodes (7–12 MΩ) filled with 3 M KCl. Between 20 and 34 fibers were examined at each stage from at least three different individual animals.

RESULTS

Contractile properties of intact fibers. The contractile properties of intact ISM fibers were tested at various stages beginning on day 15 of pupal/adult development and ending 15 h after adult emergence on day 18. Data from day 15 represented the period before the onset of atrophy, data from early on day 18 represented the end of muscle hypertrophy, and data from late on day 18 represented the period of muscle atrophy. Data from day 18 were used to determine all physiological parameters. To elicit tension, a fiber was lowered into one of a set of Plexiglas wells containing 2.0 ml of 90% relaxing (pCa 8.0) or activating (pCa <7.0) solution. The fiber could be transferred between wells in <1 s.

When tension data was analyzed, the force of contraction was normalized for fiber cross-sectional area. At the outset of the experiment, the diameter of each muscle fiber was measured with a microscope positioned over the recording apparatus. Mechanically skinned fibers (see below) had an essentially circular cross section, and their area was calculated accordingly. When examined histologically, intact fibers had an elliptical cross section, with the minor axis measuring 0.505 times the major axis. Therefore, contractile force for the intact fibers was normalized to fiber cross-sectional area by dividing the force by \( \pi(diameter)^2(0.505)/4 \).
of the atrophy phase, and data from 4 and 15 h after emergence represented the active period of cell death. Individual intact muscle fibers were dissected free from the animal and attached to an isotonic tension transducer. When stimulated with 10-ms depolarizing pulses delivered via extracellular bipolar electrodes, the fibers produced discrete twitches (data not shown). Between days 15 and 18, there was a 1.6-fold increase in the normalized individual twitch amplitude displayed by the ISM (Fig. 1A). By 4 h after emergence, the twitch amplitude had increased 2.1-fold relative to that on day 15. However, by 15 h after emergence, when the fibers were well into the degeneration phase, the fibers produced barely discernible twitches.

Stimulation of the fibers with 10-ms pulses at 15 Hz resulted in fused tetanus (data not shown). To correct for variability between cells, we stimulated ISM fibers from different developmental stages at 30 Hz. When tetanic force was normalized for cross-sectional area, the force generated by fibers on day 15 was the same as that for muscles on day 18, even though the ISM had lost 40% of their mass during this period (Fig. 1B). With the onset of degeneration following emergence, ISM force declined rapidly so that by 15 h after emergence it had fallen by 78%. This represented an actual weakening of the fibers and not just a reflection of reduced fiber size, because the force was normalized for the cross-sectional area of the fibers (see MATERIALS AND METHODS).

The physiological trigger for skeletal muscle contraction is the release of calcium from the sarcoplasmic reticulum (34). Calcium release can be initiated by electrical depolarization of the surface membrane, as was performed above, or by pharmacological means, such as exposure to caffeine (36). Exposure to 25 mM caffeine generated large tension transients that peaked after ~3 s (Fig. 2A). Despite the continued presence of caffeine, the fibers relaxed to baseline levels within 30 s. The responsiveness of ISM fibers to caffeine changed during atrophy and degeneration. On day 18, before emergence, the peak tensions generated by caffeine were only 61% of those on day 15 (Fig. 2B).

![Fig. 1. Changes in tension generated by individual intact intersegmental muscle (ISM) fibers. Tension was normalized to the fiber cross-sectional area. Data were collected on day 15 of pupal/adult development, on day 18 before emergence, and on day 18, 4 and 15 h postemergence (PE). A: twitch tension measured after the fibers were stimulated with bipolar electrodes with single 10-ms pulses. B: amplitude of tetanic contraction in individual fibers stimulated with 10-ms pulses at 30 Hz with bipolar electrodes.](image1)

![Fig. 2. Contractions induced by 25 mM caffeine in individual intact ISM fibers. A: recording from a single fiber exposed to 25 mM caffeine in Manduca saline starting at the downward arrow. Time of caffeine withdrawal is noted by the upward arrow. The small bumps in the record are artifacts due to vibration of the apparatus when solutions were changed. B: peak amplitude of caffeine-induced contraction in individual fibers. Data were collected on day 15 of pupal/adult development, on day 18 before emergence, and on day 18, 4 and 15 h postemergence. Significance was determined relative to the preemergence time point on day 18.](image2)
By 15 h after emergence, caffeine-induced contractions were only 10% of the value on day 15.

Contractile properties of skinned fibers. The reduction in ISM responsiveness to caffeine during development could have resulted from changes in either the physiological behavior of the sarcoplasmic reticulum or the ability of the contractile apparatus to respond to free calcium. To address this question, individual fibers were mechanically skinned and attached to a tension transducer. Fibers were bathed in solutions containing different pCa levels to determine their tension-calcium relationships. Figure 3 shows representative records for ISM fibers from day 15, day 18 preemergence, and 18 h after emergence. For the day 15 fiber (Fig. 3A), tension was initiated at pCa 5.6 (2.5 μM Ca$^{2+}$). At pCa 5.4 there was a slight increase in tension, with maximal tension elicited at pCa 5.2. Exposure to pCa 5.0 did not generate more tension than pCa 5.2. Upon return to pCa 8.0, the fiber relaxed to baseline tension. For the day 18 fiber (Fig. 3B), tension was again initiated in response to pCa 5.6. However, this fiber generated a more graded response than the day 15 fiber to increased levels of free calcium ions and did not achieve maximal tension until pCa 5.0. Again, upon return to pCa 8.0, the fiber relaxed to baseline level. By 18 h after emergence, the ISM were quite far along in the degeneration process. Fibers from this stage did not generate tension until exposed to pCa 5.2 (6.3 μM). They continued to display slow incremental increases in tension with each elevation of free calcium ions up to pCa 4.0 (100 μM). Upon return to pCa 8.0 buffer, the fiber relaxed but did not achieve the baseline level of tension.

These data suggest that as the ISM undergo atrophy and degeneration, they become progressively less sensitive to free calcium ions. To quantify this observation, we generated force-pCa profiles for ISM fibers from several developmental stages ranging from day 15 to 18 h after emergence (Fig. 4). For each stage, the force-pCa records were normalized and pooled as described in MATERIALS AND METHODS. On day 15 of development, ISM fibers rapidly initiated contraction in response to elevations in the concentration of free calcium, with 50% tension achieved at pCa 5.69 (2.04 μM) ± 0.02 (n = 20) (Fig. 4). On day 18, before emergence, 50% tension was generated at pCa 5.33 (2.95 μM) ± 0.08 (n = 6). At 4 h after emergence, this value had increased to pCa 5.31 (4.90 μM) ± 0.02 (n = 5). By 18 h after emergence, 50% tension was not achieved until pCa 4.89 (12.88 μM) ± 0.16 (n = 6). Therefore, during the 3-day atrophy period (days 15–18 preemergence), ISM fibers required almost 1.5 times more free calcium to reach the midpoint in their force-pCa curve. During the subsequent 18 h, the fibers became progressively less sensitive to calcium and required more than six times the levels free calcium to generate 50% tension relative to day 15 fibers.

Not only did the fibers display an increase in the amount of calcium required for 50% tension generation, but the nature of their responses to calcium also changed. Day 15 ISM fibers displayed a steep relationship between the percent tension generated and the pCa. This group of fibers had a Hill coefficient of 5.71 (Fig. 4). The Hill coefficient for tension-pCa relationship just before emergence was 6.11, which was similar to the value for day 15. After emergence as ISM degeneration progressed, the slope of the force-pCa curves became progressively more shallow, with Hill coeffi-

![Fig. 3. Individual tension-pCa records for skinned ISM fibers. Individual fibers were mechanically skinned and attached to a tension transducer.](image)

![Fig. 4. Changes in force-pCa relationships in skinned ISMs at various times relative to adult emergence. Curves were normalized for comparison by defining the maximal tension for each fiber as 100% and shifting the curves for all fibers at a given stage so that they shared the same pCa value for 50% tension. ISM fibers were sampled for each tension-pCa relationship at the following times of pupal/adult development: day 15 (A), day 18 just before emergence (B), 3 h postemergence (C), 13 h postemergence (D), and 18 h postemergence (E).](image)
cients of 4.21 at 4 h after emergence and 2.93 at 15 h after emergence.

Resting potentials. Before adult emergence, the ISMs maintained a resting potential of approximately −70 mV (Fig. 5). This potential was dependent on aeration of the spiracles, because the cells rapidly depolarized to about −30 mV when air to the spiracles was withheld (data not shown). With use of the Nernst equation, a resting potential of −30 mV roughly coincides with the potassium equilibrium potential (−22 mV), assuming an internal potassium concentration of 84 mM (19).

Late on day 18, coincident with adult emergence, the resting potential began to decline at a rate of ~2.5 mV/h (Fig. 5). This reduction in membrane potential paralleled the loss of muscle mass (49). At 15 h after emergence, the muscles still retained a resting potential even though they were no longer contractile in response to tactile stimulation (data not shown) and had lost ~50% of their mass. Therefore, despite extensive degradation of the contractile apparatus, sarcolemmal integrity was maintained until this time.

DISCUSSION

Muscle is a very dynamic tissue that can rapidly undergo anabolism or catabolism to grow or atrophy, respectively. The ability to shuttle amino acids in and out of muscle has obvious benefits for the organism, because muscle can serve as an amino acid reservoir. The underlying pathways for synthesis and degradation of muscle protein are carefully regulated (43, 52). The underlying pathways for synthesis and degradation of muscle protein are carefully regulated (43, 52). The patterns of gene expression in the day 15 ISMs look much like that of earlier stages. The levels of actin and myosin heavy chain transcripts are at the same high level observed earlier in development (45). However, the levels of polyubiquitin mRNA and ubiquitin-protein conjugates are slightly elevated at this stage relative to levels on day 14 (13, 47). Activation of the ubiquitin pathway suggest that whereas rates of protein synthesis may remain relatively constant, more rapid turnover of proteins may facilitate the global loss of protein that begins at this stage of development with the onset of the atrophy program (44).

Preemergence on day 18 of adult/pupal development. Adult emergence occurs late on day 18 of pupal/adult development. The ISMs provide the major motive force that powers the peristaltic waves of abdominal contractions that help propel the adult moth out of the overlying pupal cuticle. In this study we found that by early day 18, before the ISMs have initiated the death program, the muscles have lost ~40% of their mass relative to day 15 (Fig. 5). During this period the strength of tetanic contraction was reduced due to the gross loss of contractile proteins. However, when tensions were normalized to fiber cross-sectional area, tetanic force did not change between day 15 and 4 h after emergence (Fig. 1). This finding suggests that the reduction in muscle mass observed during the atrophy phase re-
reflects a generalized enhancement of protein turnover, rather than the selective targeting of specific contractile proteins. This hypothesis is supported by the observation that de novo expression of actin and myosin heavy chains remains at the same level during atrophy (45).

There are modest changes in the twitch responses of ISM fibers between day 15 and 4 h after emergence. When normalized to cross-sectional area, the amplitude of twitch tensions increased (Fig. 1), whereas the magnitude of caffeine-induced contraction decreased. The increased strength of individual twitches may reflect the reduced fiber volume that accompanies atrophy, which in turn might facilitate faster diffusion rates of calcium between the sarcoplasmic reticulum and the contractile proteins. Putative increases in this diffusion rate may not impact on tetanic tension. Prior studies indicated that the sarcoplasmic reticulum and transverse tubular system undergo only slight swelling just before emergence (1, 29).

At a molecular level, dramatic changes in the patterns of gene expression precede the initiation of cell death. RNA stability is significantly reduced in the cytoplasm of day 17 ISMs relative to other stages of development (5). This may function to facilitate the rapid accumulation of newly transcribed death-associated RNAs relative to the high background of housekeeping transcripts that were present in the cells. Late on day 17/early on day 18, the ISMs become committed to die. Whereas most transcripts are retained at steady-state levels, some genes are repressed, such as actin and myosin heavy chain genes (45). Concurrently, there is an exponential increase in the levels of several other transcripts, including polyubiquitin (47), apolipoprotein III (51), proteasome subunits (31, 50), DALP (death-associated LIM-only protein) (18); SCLP (small cytoplasmic leucine-rich repeat protein) (22), Acheron (D. Sun, C. Valavanis, and L. M. Schwartz, unpublished observations), and several uncharacterized transcripts (46). If animals are treated on day 17 with either 20E (49) or inhibitors of RNA or protein synthesis (27, 46), these changes in gene expression do not take place and the muscles do not initiate death. Once these changes in gene expression take place however, death becomes inevitable and cannot be inhibited pharmacologically.

Postemergence. The death program is initiated essentially coincident with adult emergence. This can be seen in terms of the structure, function, and biochemical properties of the muscles. Coincident with emergence, the muscles begin to lose mass at a rate of ~4%/h. Concomitantly, there is a slow, progressive depolarization of the fibers at a rate of ~2.5 mV/h. By 24 after emergence, reliable resting potentials can no longer be recorded (Fig. 5). These data are similar to those of earlier reports (29), except that the use of aerated preparations appears to provide a more accurate measure of the resting potential.

At the RNA level, the abundance of specific transcripts is essentially unchanged from earlier in the day. However, the initiation of the death program appears to employ a trigger that enhances the rate of translation for the death-associated transcripts. For example, polyubiquitin mRNA is rapidly translated, resulting in an exponential increase in the level of ubiquitin protein (13). This facilitates ubiquitin-protein conjugation and subsequent proteosome-dependent proteolysis. The ubiquitin/proteosome pathway appears to play the major role in enhancing the degradation of ISM proteins during death (7, 13, 20).

Although there is little change in the organization of the contractile apparatus during the atrophy phase, the postemergence period is marked by profound sarcomer disruption (29). Whole filaments rapidly disappear with a preferential loss of thick relative to thin filaments (1). During this same period, mitochondria are lost and the T-tubule system swells (1, 29).

Not surprisingly, there are physiological consequences that accompany these dramatic changes in ISM structure (see Fig. 6). The fibers rapidly weaken, even when force is normalized to cross-sectional area.

![Fig. 6. Summary data from the present study and previous work (13, 46) are schematized to facilitate correlations. Curves indicate relative changes in each parameter and are not intended to represent actual levels. The schematized curves for tetanic force, twitch tension, caffeine-induced contraction, pCa producing half-maximal tension, and the Hill coefficient are based on data from the present study. Data for ubiquitin-dependent proteolysis are from Haas et al. (13). Note: technical limitations do not allow resting potential measurements to be collected on day 15 animals that are comparable to those obtained with day 18 and older animals, so the dashed line is speculative.](http://ajpcell.physiology.org/)
This is true for twitches, tetanus, and caffeine-induced contractions. It has been suggested that calcium ions may be extruded from dying ISMs and accumulate in the lumen of the swollen T-tubule system (2). If so, this may account for the reduced ability of the ISMs to generate tension in response to either depolarization or caffeine exposure. There are also definitive defects in the ability of the contractile apparatus to respond to free calcium, independent of its source. In skinned fiber preparations, higher levels of free calcium are required to initiate contraction and responses to elevations in pCa are graded. During this phase, the Hill coefficient is greatly reduced, suggesting reduced cooperativity in calcium ion binding.

There are some parallels between the patterns of muscle atrophy and cell death seen in Manduca sexta and those observed in some pathological conditions in mammals. Moderate parenteral doses of glucocorticoids induce reversible muscle atrophy in rats that is reminiscent of the changes that occur during atrophy (21). For example, steroid-treated rat muscle undergoes a reduction in the absolute strength of both twitch and tetanic contractions. However, when strength is normalized to cross-sectional area, there is no net force reduction (42). The calcium sensitivity of the contractile proteins in skinned fiber preparations from rats subjected to glucocorticoid-induced atrophy were altered only slightly compared with controls (24), results similar to our observations with atrophying ISMs (Fig. 4). Pharmacologically large doses of glucocorticoids or systemic sepsis can trigger degeneration of mammalian skeletal muscle fibers that is associated with marked loss of contractile proteins (40, 41) and muscle fiber depolarization (38, 39), changes observed in ISM fibers during the post-adult emergence period (Fig. 5). Several triggers of skeletal muscle atrophy in mammals involve enhanced ubiquitin-dependent proteolysis (6, 33). In fact, two ubiquitin E3 ligases have recently been identified that are induced in all models of mouse skeletal muscle atrophy tested (4, 12). The data from these studies suggest that whereas a variety of manipulations result in atrophy, enhanced protein turnover mediated by the ubiquitin-proteasome pathway represents a final common event. Nevertheless, these results do not preclude the possibility that other proteolytic pathways may also participate in atrophy, such as the calpains (54). Although it is not known whether the calpains are involved in ISM atrophy, they do participate in muscle atrophy in other arthropods (35).

In summary, the ISMs provide a simple model system for the study of developmentally regulated skeletal muscle atrophy and death. These two phases represent distinct developmental programs that are sequentially induced by physiological triggers. Atrophy appears to be mediated by a subtle increase in protein turnover relative to synthesis and is not accompanied by significant changes in either the structure or function of the muscle, despite a 40% loss of the mass in just 3 days. In contrast, the ISM death program involves the induction of stage-specific transcripts and catastrophic alterations in the contractile apparatus that lead to reduced physiological capabilities. At present few studies have examined changes that accompany natural, nonpathological muscle cell death in mammals. Hopefully the insights gained from analysis of the ISMs may identify key regulatory components that play subtle but important roles in sarcopenia and other disorders of muscle.

We thank Laura Bolles for technical assistance, Dr. Walter Stühmer for the computer analysis of the pCa data, and David Tharp for drawing Fig. 6.

L. M. Schwartz was supported by National Institutes of Health grants. R. L. Ruff was supported by the Office of Medical Research and Rehabilitation Research and Development of the Research and Development Service of the Department of Veterans Affairs.

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