Effects of Muscle Immobilization at Different Lengths on Tetrodotoxin-Induced Disuse Atrophy

Anne-Caroline Dupont Salter, *Member, IEEE*, Frances J. R. Richmond, and Gerald E. Loeb, *Member, IEEE*

***Abstract*—Previous studies have shown that immobilization causes muscle atrophy and that the rate of atrophy depends on the length at which the muscle is immobilized. However, most studies have been carried out in neurologically intact animals that were capable of generating at least some voluntary muscle activation. In this study, tetrodotoxin was applied chronically to the rat sciatic nerve to produce complete paralysis of distal muscles for seven days, and the ankle was immobilized to hold the muscles at long or short lengths. Paralysis without immobilization resulted in relative weight losses of 36% for soleus, 19% for tibialis anterior (TA), and 17% for lateral gastrocnemius (LG) muscles. Casting the ankle in plantarflexion stretched TA and reduced its weight loss to 10%. Soleus and LG were shortened by this intervention and had increased losses of 43% and 28%, respectively. Fixing the limb in dorsiflexion resulted in a posture similar to that adopted by the unrestrained rats and had no significant effect on the amount of muscle atrophy compared to that in unrestrained paralyzed animals.**

***Index Terms*—Atrophy, disuse, muscle length, rats.**

I. Introduction

MUSCLES can respond to changes in their patterns of use by changing almost every aspect of their physiology, morphometry, and histology. This plasticity provides the basis for a wide range of training regimes used in competitive sports and clinical rehabilitation. The most profound changes occur following denervation, which may result in almost complete atrophy of contractile tissue. Denervation eliminates both electrical activation and any chemotrophic effects that might arise from the motor endplates themselves, which degenerate rapidly after motor axons are cut. The denervated limb may also adopt unusual postures and experience unusual loading conditions, which could affect the ongoing disuse atrophy. In order to separate the effects of denervation from changes in muscle activation, most studies have relied on indirect means to reduce the recruitment of otherwise intact motor units.

Casting or pinning of the joints crossed by the muscle has been carried out on the premise that the animal will stop using muscles that then produce no useful motion of the limb. Muscles immobilized at short lengths have generally atrophied more than muscles immobilized under neutral or lengthened conditions [1]–[7]. However, the question remains whether the difference results from the length at which muscles are held or from differences in the residual activity of the immobilized muscles. Fournier *et al.* [6] recorded the electromyogram (EMG) activity in immobilized muscles and found it to be generally reduced but still correlated with muscle length, as might be expected from the residual presence of the tonic stretch reflex.

Suspending the rear of the animal by its tail assumes that the animal will stop using muscles when they cease to contribute to postural support [8], [9]. This approach controls neither muscle length nor EMG activity, so it is not surprising that EMG activity in such muscles is more variable and may even be higher than normal [10].

Reducing the central excitatory drive to the motoneurons can be accomplished by surgically cutting the spinal cord. The clinical course of similar injuries in humans, however, includes variable development of spasticity [11], because a decreased inhibitory drive is provided from the brain [11] and other plastic changes occur in the segmental circuitry [12], [13].

Even when heroic measures are taken to isolate motoneurons from reflexive as well as descending inputs by combining dorsal rhizotomy with spinal hemisection, the ipsilateral muscles show little or no atrophy [14]. Full section of the spinal cord may be more effective than hemisection, but the relative effects have yet to be characterized systematically. Further, this approach imposes a large burden of care because the bowel and bladder control of the animals is impaired [15].

A more direct approach to blocking muscle activation without denervation is to block nerve conduction with tetrodotoxin (TTX). TTX blocks sodium channels of the axons, and, therefore, prevents the action potentials from reaching the motor endplate. The motoneurons and endplates remain intact and undamaged [16], axonal transport appears to be relatively normal and the blockade is complete but reversible after the source of TTX is removed [17]–[19]. Since the nerve is still present but quiet, this is similar to acute spinal cord injury (below the level of the injury) or stroke. In order to study the effects of muscle length on the development of disuse atrophy, we combined chronic TTX blockade with immobilization by casting the ankle in various postures. We focused on the earliest stages of muscle atrophy, before compensatory changes in tendon length or sarcomere numbers are likely to occur. We selected three ankle muscles that differ in their biomechanical relationships and fiber-type compositions to identify other factors that might contribute to the responses of muscles to disuse.

Manuscript received March 19, 2002; revised May 15, 2003.

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Digital Object Identifier 10.1109/TNSRE.2003.817675

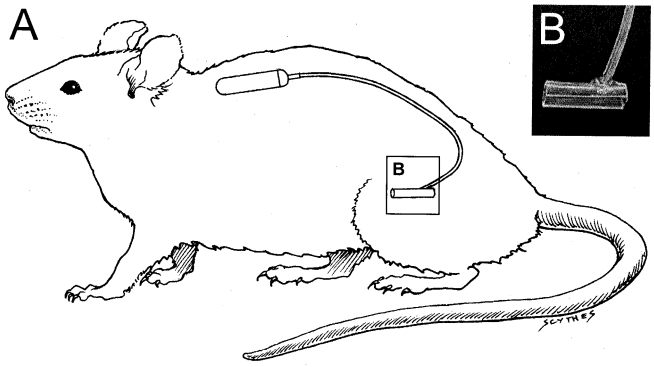

Fig. 1. Rat with mini-osmotic pump and nerve cuff. (A) Placement of pump-and-nerve-cuff system. (B) Picture of nerve cuff.

II. Methods

A. Surgical Implantation of TTX Cuffs

Female Sprague–Dawley rats (Charles River, Toronto, ON, Canada) weighing between 200 and 250 g were housed in groups of two or three, provided with rat chow and water ad libitum, and kept on a 12 h light/dark cycle. Each rat was anesthetized with ketamine (70 mg/kg body weight) and xylazine (5 mg/kg body weight) intraperitonally; additional doses of ketamine were administered as needed. An incision was made on the lateral side of the left thigh of each animal and the biceps femoris muscle was incised to expose the sciatic nerve. The sciatic nerve was freed from surrounding tissues and implanted with a nerve cuff connected to an osmotically driven drug-delivery system. To produce these implanted systems, mini-osmotic pumps (Alza Corporation, Palo Alto, CA) were filled with 100-μl TTX solution (350 μg/ml in normal saline) (Sigma–Aldrich, Oakville, ON, Canada) and attached to a tube-and-nerve-cuff assembly, made in-house as a modification of the design used by Michel and Gardiner [8]. The tube and cuff system was made from silastic tubing (Dow Corning, tube: 0.025 in- I.D. $\times$ 0.047-in O.D.; cuff: 0.078-in I.D. $\times$ 0.125-in O.D.; Fig. 1). The pump delivered the TTX solution constantly to the left sciatic nerve of the rat via the cuff at a rate of 0.5 μl/h for seven days. The nerve cuff was closed by looping four sutures (3-0 silk) around it. The TTX-containing pump was passed subcutaneously to a second incision made between the scapulae of the animal's back. The pump was positioned under the skin caudal to the incision on the back of the animal (Fig. 1). The biceps femoris muscle and both skin openings were reapproximated with sutures. Lidocaine cream was applied around the sutures immediately following surgeries and for the next 2–3 days to minimize sensations that might elicit grooming of the sites. Paralysis of the left ankle muscles developed within 24 h after surgery. The presence of paralysis was evaluated daily in all rats by monitoring the loss of toe-spreading and pinch reflexes [8], [19].

The rats were divided randomly into four groups:

1) sham-operated control group in which a cuff but no pump was implanted;
2) TTX-exposed group in which the ankle was unrestrained;
3) TTX-exposed group in which the ankle was immobilized in a plantarflexed position;
4) TTX-exposed group in which the ankle was immobilized in a dorsiflexed position.

Sham-operated Controls: In six rats, cuffs with tubes but no osmotic pump were implanted to ensure that surgery alone did not result in paralysis or muscle atrophy. Muscles on nonoperated and operated sides were later found to differ in their average weights by less than 1%.

Ankle Unrestrained (TTX-C rats): In 17 rats, TTX cuffs were implanted but no restraint was placed on the paralyzed limb. The animals were kept for seven or 14 days. They were free to move around their cages, and they displayed only a small limp on the side of the paralysis.

Ankle Plantarflexed (TTX-P rats): In 9 animals, the paralyzed hindlimb was immobilized with the ankle in a plantarflexed position of about 165° by applying a plaster cast around the foot and distal leg. Care was taken to ensure that the cast did not interfere with the circulation or damage the foot. Nail polish was applied on the cured cast as a marker to indicate whether the rat was chewing on the cast. The polish also tended to discourage chewing of the cast. Two to three inspections of the foot were carried out daily to assess tissue integrity. In the occasional circumstance when we found redness or swelling of the toes, the foot was massaged to promote venous return into the leg and the cast was trimmed back for a short distance around the foot.

Ankle Dorsiflexed (TTX-D rats): In an initial three rats, the ankle on the side of the implant was immobilized in a dorsiflexed position a plaster cast. However, casting in a fully dorsiflexed position was found to be difficult because the thick plaster of the cast prevented the foot from flexing as far as we intended. Further, the combination of the cast and dorsiflexed position tended to restrict blood flow to and from the foot. Therefore, in six animals, a stainless steel wire suture was threaded through the skin and looped around the tibia and the metatarsal bones of the foot. The ends of the wire were tied together on the lateral side of the hindlimb of the rat, to hold the ankle at an angle of approximately 15°. Care was taken to curl the wire ends and then coat them with wax to soften sharp edges that might injure adjacent tissues.

B. Video Recording

One randomly-selected paralyzed rat (TTX-C) and one normal, nonparalyzed rat were videotaped for 24 h with an infrared camera and a time-lapse recorder (1 frame per 1.4 s) in order to monitor the postures and motor activity of the animals. Each animal was housed in a clear plastic cage and was given a plexiglass tube instead of its regular opaque housing tube so that its activity was always visible to the camera. Three mirrors were placed around the cage in order to monitor rats in different positions. The hip, knee, and ankle joints of both hindlimbs were marked using indelible black ink to approximate the joint centers. The environment was kept on the normal 12-h day-night cycle. The video recordings were analyzed frame-by-frame and the time spent in different activities (sitting, standing, walking, grooming, stretching, sleeping) and different postures (sitting, stretching, standing)

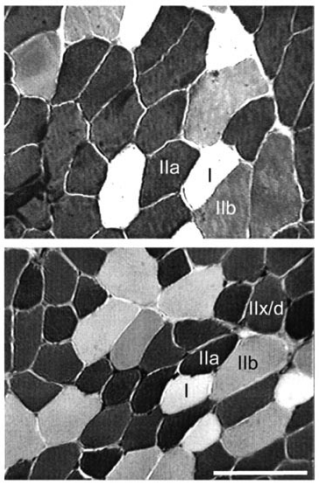

Fig. 2. Alkaline ATPase staining of rat muscle fibers. Bar indicates 100 ìm (for both). (Top) Three fiber types are visible: I, IIa, and IIb (Bottom) Four fiber types are visible: I, IIa, IIx/d, and IIb.

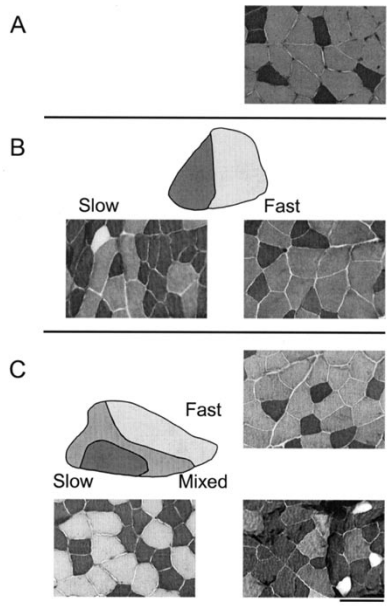

Fig. 3. Fiber composition of studied muscles. (A) Soleus. (B) TA. (C) LG.

was documented. The range of joint angles adopted in each posture was estimated by measuring the angle of the long bones with respect to the mark representing the joint center.

C. Muscle Weight and Histology

After seven days of paralysis, animals were anesthetized with sodium pentobarbital injected intraperitonally. The soleus, tibialis anterior, and gastrocnemius muscles were removed from both the paralyzed and the control sides of the animal. Lateral and medial gastrocnemius were separated by cutting along the line suggested by the centrally placed tendon. The rats were then killed with an overdose of sodium pentobarbital injected into the heart. The belly of each muscle was mounted on a cryostat chuck in a recorded orientation. The muscle pieces were coated in talcum powder and frozen in liquid nitrogen. Blocks were stored at minus 70°C in liquid nitrogen until sections of 12–16 μm from the belly of each muscle were cut in a cryostat and mounted on glass slides. Adjacent sections were stained with Hematoxylin and Eosin and for ATPase activity following alkaline preincubation (pH 10.4) [20]. This method was found previously to produce reliable differentiation of fast glycolytic (IIb—pale), fast oxidative-glycolytic (IIa—dark), and slow oxidative (I—white) fiber types in rats (Fig. 2) [21], [22]. This is in contrast to the typical pattern seen in feline muscles with the same methods, in which the intensity of coloration is higher in Type IIb than IIa fibers. Fiber type IIx/d are difficult to distinguish from IIa fibers using alkaline ATPase methods [23]–[27]. On some slides, we could see an intermediate stain intensity between the dark IIa fibers and the pale IIb fibers; these were assumed to be type IIx/d fibers because they had sizes intermediate between IIa and IIb fiber sizes [24], [26], [27] and had intermediate staining characteristics [23]–[27]. However, because these fibers were not always distinguishable from IIa fibers, we grouped IIx/d and IIa fiber subpopulations (Fig. 2). Muscles that were damaged during dissection or histological processing were not evaluated quantitatively. As well, data from muscles were eliminated when ATPase staining did not discriminate three fiber types across the whole muscle cross-section.

D. Statistical Analysis

Differences in muscle weights, fiber-type proportions, and fiber cross-sectional areas (CSAs) were evaluated by comparing matched muscles removed from the two sides of the animal. Although previous studies have suggested that the contralateral unparalyzed limb might hypertrophy somewhat to compensate for the paralysis [19], contralateral control muscles exhibited ratios of muscle weight to body weight that matched closely the values obtained from sham-operated rats in this study. Paired T-tests were used to compare data points from control and experimental muscles of groups of animals. Data from groups of rats subjected to different treatments were compared using analyses of variance (ANOVAs) followed by student T-tests with significance measured at $p < 0.05$ when data had a normal distribution. The Mann–Whitney rank-sum test was used when data were not distributed normally.

E. Muscle Fiber CSAs

Single muscle fibers were typed according to their profiles after ATPase staining. Their CSAs were measured using ImagePro software (Media Cybernetics, Silver Spring, MD). All of the muscles studied here had a mixture of fiber types whose responses to the experimental intervention might have been expected to differ [28], [29]. In order to assess the level of atrophy in such mixed muscles, it was necessary to develop a strategy for analysis of muscle cross-sections. One of the muscles, soleus, was relatively simple to study because it was composed of a relatively uniform mix of type I and IIa fibers in proportions of approximately 85%: 15%, as reported elsewhere [30], [Fig. 3(a)]. The other two muscles, TA and LG, had a nonuniform distribution of fiber types. In TA, we divided the muscle for purposes of analysis into two regions, a superficial “fast” region accounting for about 55% of the muscle and a deep, relatively “slow” region comprising the remaining 45%. The fast region was found to contain on average of 74% (s.d., 10%) type-IIb fibers and 26%

(s.d., 10%) type-IIa fibers in control muscles, whereas the slow region contained about 3% (s.d., 2%) type-I fibers, 32% (s.d., 8%) type IIb, and 65% (s.d., 8%) type-IIa fibers [Fig. 3(b)]. LG was divided into three regions characterized as "fast," "mixed," and "slow," which accounted for 42%, 39% and 19% of the muscle, respectively [Fig. 3(c)]. The fast region was found, on average, to be made up of 75% (s.d., 12%) type-IIb fibers and 25% (s.d., 12%) type-IIa fibers. The slow region was made up of 42% (s.d., 5%) type-I fibers and 58% (s.d., 5%) type-IIa fibers. The mixed region was more variable in composition, but on average was composed of 10% (s.d., 6%) type-I fibers, 35% (s.d., 12%) type-IIb fibers, and 55% (s.d., 13%) type-IIa fibers [Fig. 3(c)]. To assess fiber-type proportions, a randomly-chosen sample of each muscle area, containing a minimum of 100 cells, was examined; all cells in that area were categorized as type I, IIa, or IIb and the numbers of each type of cells were calculated. The fiber-type proportions were not found to change significantly after seven or 14 days of paralysis, nor did they change after seven days of paralysis and immobilization. When analyzing CSAs for fibers in these muscles, samples of no less than ten fibers of each type were measured in each muscle region. Estimates of atrophy in each fiber type were reported as the percentage reduction in averaged CSA of the paralyzed fibers with respect to that of contralateral control fibers in each region. To obtain an overall estimate of fiber atrophy for the whole muscle, the averaged values of atrophy for each fiber type were multiplied by the fractional contribution of that type in the region and then summed. Values for each region were then multiplied by the fractional contribution of the region to the CSA of the whole muscle and summed.

III. Results

A. Systemic and Behavioral Observations

Animals implanted with TTX cuffs lost, on average, 2% (± 5) and 6% (± 5) of their weight by the end of the seven- and 14-day paralysis periods, respectively. Sham-operated rats showed no significant change in weight over the seven-day period.

Paralysis and important action potential blockade were confirmed with the following observations:

1) absence of withdrawal reflex when toes are pinched;
2) absence of adverse reaction when toes are pinched (confirms that sensory conduction is affected);
3) absence of toe spreading reflex when foot is unloaded;
4) absence of ankle extension reflex when ankle is flexed by an investigator.

Video analysis showed that both the paralyzed and nonparalyzed rats spent most of their time (paralyzed, 90% of time; nonparalyzed, 83% of time) sitting with all four limbs on the floor and the hindlimbs tucked under the body with both ankles and knees strongly dorsiflexed [Fig. 4(a)]. In this posture, the animals would groom, sit, and sleep. Both rats spent small periods of time standing on their hindlimbs to drink and reach food placed on the wire top of the cage (paralyzed rat, 5% of time; nonparalyzed rat, 12% of time). In this posture, the rat held its ankles at an angle of about 55° [Fig. 4(b)]. Both rat groups spent little time with one or both hindlimbs extended and the ankle plantarflexed beyond 75° (1% of time for both paralyzed and nonparalyzed animals) [Fig. 4(c)]. The rest of the time (4%) was spent in nonclassified positions because the joint angles were changing as rats moved about the cage.

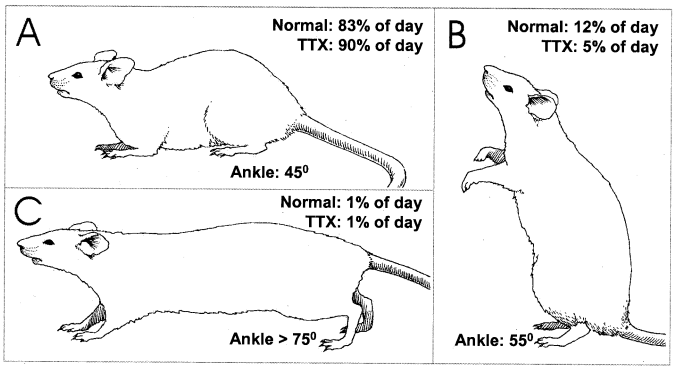

Fig. 4. Ankle angles adopted by rat during a 24-h cycle.

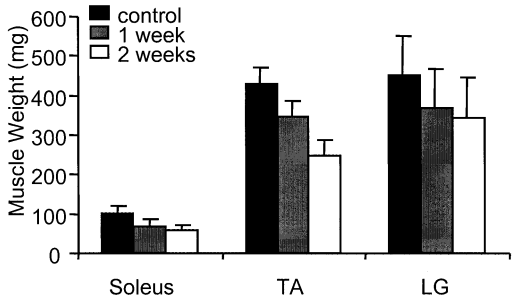

Fig. 5. Muscle weights in control animals and unrestrained animals after one and two weeks paralysis.

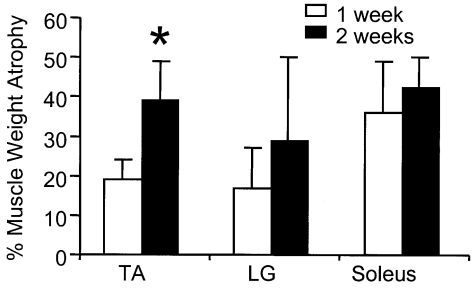

Fig. 6. Changes in muscle weight, expressed as percentages of weight of the contralateral matched muscles in unrestrained animals after one and two weeks of paralysis. $*$ indicates significant difference from 1 week atrophy.

B. Muscular Changes

1) Paralyzed and Unrestrained Animals: Paralysis for seven days resulted in a marked loss of muscle weight (Fig. 5). Changes in TA and LG were similar (Fig. 6): TA lost $19 \pm 5\%$ of its weight and LG lost $17 \pm 10\%$ compared to contralateral controls. Paralyzed soleus muscles were significantly more atrophied than TA and LG, losing an average of $36 \pm 13\%$ of their weight compared to contralateral controls. After paralysis for 14 days, some muscles underwent further weight reduction: TA lost $39 \pm 10\%$ ($p < 0.05$ with TA at seven days); LG lost $29 \pm 21\%$ (nonsignificant (NS) difference with LG at seven days); soleus lost $42 \pm 8\%$ (NS difference with soleus at seven

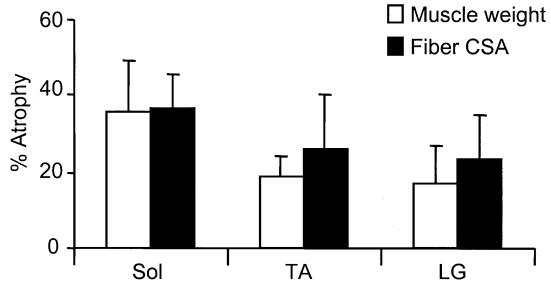

Fig. 7. Relative changes in muscle weight and cross-sectional areas of fibers (expressed as percent values) in unrestrained animals paralyzed for one week.

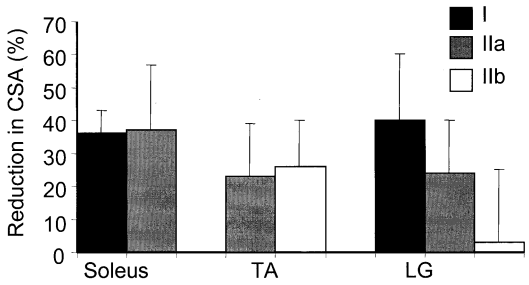

Fig. 8. Changes in cross-sectional area of different fiber types after paralysis for one week.

days). A larger variation in measured weights was observed for LG than for soleus and TA in all groups (Fig. 5). This increased variability was not associated with a similarly increased level of variability in measures of fiber CSA (see the following and Fig. 7), and was suspected to arise from the dissection required to separate LG from MG.

Changes in fiber CSAs were consistent with the decrease in muscle weight. In unrestrained animals, paralyzed fibers decreased in CSA by 26 ± 14% for TA, 23 ± 12% for LG, and 37 ± 9% for soleus after one week (Fig. 7). Individual fiber cross-sectional areas were typically quite variable even within a single fiber type however. After one week, type IIa and IIb fibers in unrestrained TA showed similar reductions in CSA of 23 ± 16% and 26 ± 14%, respectively. Similarly, in soleus, type I- and type-IIa fibers showed a similar reduction in CSA of 36 ± 7% and 37 ± 20%, respectively (Fig. 8). In some LG muscles, however, type-IIb fibers appeared to atrophy little (3 ± 22%), compared to type IIa- (24 ± 16%) and type-I fibers (40 ± 20%). As a consequence, atrophy was on average greater in core regions where slow fibers predominated.

Fiber CSAs generally decreased more for all fiber types in all muscles after two weeks verses one week. In TA, type-IIa fibers appeared to atrophy less (29 ± 10%) than type-IIb fibers (43 ± 7%) ($p < 0.005$). In LG, IIb and IIa fibers showed less atrophy (IIb, 23±17%; IIa, 26±16%) than type-I fibers (44±9%). In soleus after two weeks of atrophy, both fiber types showed similar large reductions in CSA (43 ± 8% and 39 ± 22%, respectively).

2) Paralyzed and Immobilized Animals:

a) Plantarflexed position: When the paralyzed limb was immobilized in a plantarflexed position, the stretched TA atrophied less than when it was unrestrained (10 ± 4% versus 19 ± 5%; $p < 0.002$ and the shortened LG atrophied more

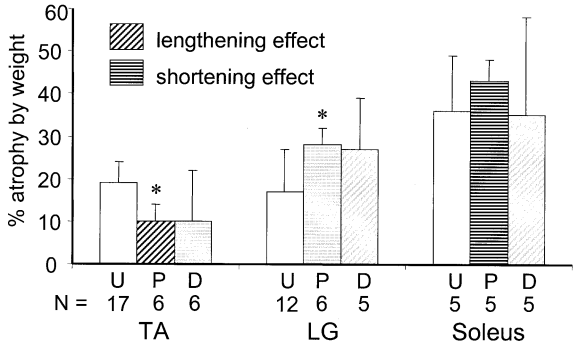

Fig. 9. Changes in muscle weight, expressed as percentages of weight of the contralateral matched muscles when ankle was unrestrained (U), plantarflexed (P), or dorsiflexed (D). * indicates significant difference from unrestrained ($p < 0.05$). the darkness of the hatch indicates the magnitude of the change in position from unrestrained position; dark: large change; pale: small change (see text for details).

(28 ± 4% versus 17 ± 10%; $p < 0.005$). The level of atrophy in shortened soleus, however, was not significantly different than that in paralyzed, unrestrained soleus (43±5% shortened versus 36 ± 13% unrestrained) (Fig. 9).

Changes in fiber CSAs showed a similar trend to changes in muscle weight. The average decrease in the CSA of stretched TA fibers was significantly smaller than that of paralyzed but unrestrained fibers (4±14% versus 26±14%, significant difference: $p < 0.01$). However, the average CSA of shortened fibers in LG and soleus did not change significantly from that of comparable fibers in paralyzed, unrestrained muscles (LG: 23 ± 9% versus 23±12%; SOL, 35±8% versus 37±9%). When changes in the CSA's of different fiber types are compared, patterns are similar to those seen in unrestrained paralyzed muscles. In LG, fibers were affected differentially, and the severity of fiber atrophy was ordered from most severe in type-I fibers (33 ± 15%) to least severe in IIb fibers (IIa: 27 ± 10%; IIb: 21 ± 10%). In stretched TA, IIa and type-IIb fibers atrophied by -1 ± 19% and 11 ± 13% respectively. In shortened soleus, type IIa and type-I fibers atrophied by 35 ± 6% and 36 ± 22%, respectively.

b) Dorsiflexed Position: When the paralyzed limb was immobilized in a dorsiflexed position, no significant changes were seen in the degree of atrophy in any of the studied muscles, when compared to the nonparalyzed group. The stretched LG lost 27 ± 12% (versus 17 ± 10%; NS) of its weight. The shortened TA lost 10 ± 12% (versus 19 ± 5%) and the stretched soleus lost 35 ± 23% (versus 32 ± 13%). A similar pattern of change was apparent from an analysis of fiber CSA's. Fibers in the stretched LG atrophied more than fibers in paralyzed unrestrained muscles (42 ± 4 versus 23 ± 12, $p < 0.0002$), consistent with trends for change in relative weights. These differences were distributed quite evenly across the fiber types (I: 42 ± 10%; IIa: 41 ± 7%; IIb: 35 ± 5%). However, neither the shortened fibers of TA nor the stretched fibers of soleus atrophied to a degree significantly different from fibers in paralyzed, nonimmobilized muscles (TA: 25 ± 8 versus 26 ± 14; SOL: 37 ± 5 versus 37 ± 9). In TA, type IIa and IIb fibers atrophied to a similar extent (IIa: 28 ± 10% versus IIb: 26 ± 10%), but in soleus, type-I fibers were somewhat

less atrophic than IIa fibers (I: $34 \pm 5\%$ versus IIa: $51 \pm 14\%$, $p < 0.02$).

c) Effects of Immobilization: Analysis of variance followed by student T-tests showed differences in muscle weight loss for immobilization in the shortened position for LG and in the stretched position for TA. For soleus, immobilization in either position had no effect on the rate of atrophy

IV. Discussion and Conclusion

We studied the effects of immobilizing muscles at different length on the atrophy of muscles whose neural activity had been blocked with TTX. This methodology circumvents the confounding effects of uncertain activity and length in prior studies that analyzed either the atrophy of paralyzed muscles without controlling muscle lengths or the atrophy of muscles in which muscle length or loading were modified to reduce natural recruitment in muscles with an intact motor supply. Comparisons with the literature are further complicated, however, by differences in fiber type and in skeletal attachments and usage patterns of the various muscles that have been studied.

Experimental methods to study atrophy differ also in whether they use the contralateral limb as a control. We measured atrophy as a percentage decrease in two measures of muscle morphometry (weight and cross-sectional area) compared to the same muscle in the contralateral limb of each animal. This assumes that the experimental manipulation (paralysis and/or casting) has no effect on the contralateral muscles. Michel and colleagues [19] have reported that muscles contralateral to those paralyzed with TTX appear to increase their weights faster than sham operated equivalents, as might be expected if unparalyzed muscles were compensating for the paralysis by working harder and becoming larger. The slow extensor, soleus, was most affected and, increased its weight by 23% relative to sham-operated controls, whereas the faster muscle, plantaris, showed a relative weight gain of only 6% [19]. In the present study, no such effects were seen in the paralyzed-unrestrained or paralyzed-immobilized animals, even though some rats carried the extra weight of a cast.

A. Effects of Paralysis on Unrestrained Muscles

As a baseline against which to compare casted, paralyzed rats, the effects of TTX blockade were studied first in animals in which the paralyzed limb was free to move passively. Under these conditions, muscle weights decreased by amounts comparable to decreases reported elsewhere in the available literature. As expected, more atrophy was apparent after 14 than seven days, but the rate of atrophy was found to decrease during the second week. The decrease in the rate of atrophy over time has previously been well documented as a logarithmic relationship that starts to plateau at around four weeks [8], [18], [19], [31]–[33]. At the seven-day point, the slowest muscle, soleus, was found to be most affected by the paralysis. Its weight loss of 36% was somewhat larger than the average weight loss of 26% reported by Michel *et al.* [34] under similar conditions of TTX application, and also larger than the 17%–19% atrophy seen here in the faster muscles, TA and LG. This large amount of atrophy was also reflected by the $37 \pm 9\%$ decrease in cross-sectional area of soleus fibers after TTX application. Differences in the degree of atrophy between fast and slow muscles was also observed at the seven-day time point when fiber CSAs were evaluated as a secondary measure of atrophy. However, by 14 days, the degree of weight loss in all muscles became more similar. The average weight loss of 42% in soleus closely approximated previously reported weight losses ranging from 41%-47% after 14 days of TTX paralysis [18], [19], [31]. The weight losses of 29%–39%, seen in TA and LG were slightly smaller than losses of between 42%–54% reported for paralyzed plantaris and medial gastrocnemius muscles previously [8], [18], [19], [33].

B. Effects of Casting on Muscle Length

A decrease in the mass of a muscle is usually assumed to reflect a reduction in the physiological cross-sectional area of its muscle fibers. If muscle fibers were to change only their length, however, this also would result in a change of muscle mass. Under normal conditions of use, muscles adapt to changes in mean length by adding or subtracting sarcomeres at the ends of their muscle fibers [35] and [36]. This has the tendency to normalize the length of the individual sarcomeres toward values closer to their optimal length for active force production. This mechanism presumably accounts for normal growth and development of the musculoskeletal system, for adaptive recovery following injuries or surgical interventions that change the joint moments of muscles and tendons, and for functional adaptation to postural shifts such as the shortening of the triceps surae muscles in women who habitually wear high-heeled shoes. In the experiments presented here, the weights and computed total cross-sectional areas of muscle fibers agreed closely, regardless of whether the muscles had been set at short or long lengths. This suggests that the one-week duration of these experiments was insufficient for significant length changes to take place. For longer duration experiments, this effect should be, but generally has not been, considered.

In all of the adaptations to length described previously, the muscles were at least somewhat active and producing mechanical work. It was not clear whether the responses of completely paralyzed muscles to length changes would be similar or not. Any mechanisms for responding to changed muscle length either by changing muscle fiber length or cross-sectional area may operate differently in paralyzed muscles. In our experiments on paralyzed muscles, the only significant effects of length on muscle mass occurred in plantarflexion. Stretched TA had less atrophy than unrestrained TA, and shortened LG had more atrophy than unrestrained LG. This is consistent with the general notion that shortening exacerbates disuse atrophy and lengthening may have protective effects (but see the following). Unfortunately, attempts to reverse these length changes with extreme dorsiflexion produced no significant effects, perhaps because the dorsiflexed posture is not much different from the unrestrained posture and also because of the large variability in

these data. Soleus had essentially the same large amount of atrophy (about 40%) under all three length conditions, perhaps suggesting that the severe atrophy of this normally tonically active slow muscle in the face of complete paralysis reflects a saturated process no longer sensitive to muscle length.

C. Interpretation of Length Effects in Unparalyzed Muscles

Many investigators have studied the effect of immobilizing unparalyzed animal limbs by pinning, casting, and bracing joints in an effort to shorten or lengthen muscles. Results from these studies have suggested that lengthening can confer a protective effect against atrophy [1], [3], [5], [6]. Stretched muscles have even been reported to hypertrophy [2], [3], [7], [37] after survival periods of 6–8 days. However, experiments conducted for a longer time (4–9 weeks) have shown that this effect is transitory. Stretched muscles later lose weight, annulling the effects of the hypertrophy and eventually showing overall atrophy from preimmobilization weight values [5], [6], [37], [38].

It has not been clear from previous studies why nonparalyzed stretched muscles lose less weight than shortened ones. At least three mechanisms might potentially operate individually or in concert. First, lengthened muscles may have had greater electrical and contractile activity, at least in part because segmental reflexes are enhanced in stretched muscles [3] before sarcomeres have been added to readjust muscle length to the new posture. These reflexes will evoke isometric contractions that are likely to be more forceful in stretched than shortened muscles even if the level of electrical activity were to be matched because stretched muscles are typically at more advantageous positions on their length-tension curves [39], [40]. Consistently heightened electrical activity may also change intracellular Ca^{2+} concentrations. Such changes are known to have long-term effects on the levels of signaling proteins such as calcineurin, implicated in the control of muscle fiber size and type [16], [41], [42]. The prediction that electrical activity plays a role in the differential effects of stretch and shortening seems supported by the results of the study reported here. For example, paralyzed, lengthened TA was found to atrophy by 10% over seven days, whereas unparalyzed, lengthened TA was reported previously to lose no weight under similar conditions of study [2]. Paralyzed lengthened soleus was found to atrophy by $35 \pm 23\%$ over seven days in this study, whereas unparalyzed, lengthened soleus was found by Goldspink [3] to hypertrophy by 22%.

At the same time as stretched or shortened muscles are responding to changed electrical signals, they are also responding more directly to the change in length by adding or removing sarcomeres in series. Williams and Goldspink, [36] and [43] showed that immobilized murine soleus muscles add or lose sarcomeres and show changes in sarcomere lengths as early as one week after immobilization. As might also be expected, shortening shifts the length/tension curve to the left, whereas lengthening shifts it to the right (longer lengths). In rats, too, the passive and active length/tension curves of the soleus were found to move to the left after the muscle had been shortened by plantarflexion for 42 days [44]. Changes in the number of sarcomeres of chronically shortened and lengthened muscles are also seen in cats after four weeks of immobilization. Values return to normal within four weeks after cast removal [35]. This process might be expected to have some impact on the eventual muscle weight, because an increase in muscle length due to sarcomere addition would increase the volume of the muscle even if the cross-sectional area of the muscle were to remain constant [5]. In the present study, lengthened muscles were held at lengths at least 10% (range: 10%–30%) greater than shortened muscles. If the accumulation or loss of proteins and other building blocks for new sarcomeres were to be largely accomplished by the end of two weeks, we might expect that stretched muscles should weigh at least 10% more than shortened muscles, even if no other changes were to take place.

The last and most commonly discussed effect is one that is related to the muscle length itself. In the past studies, the evidence for such a direct, length-related effect was not easily divorced from the effects that might be attributed to differences in EMG activity at the different lengths. In present experiments, evidence for a length-related effect seemed compelling when experiments in which limbs were plantarflexed were considered in isolation. However, experiments in which limbs were dorsiflexed were found to exhibit little change. The large variability of the responses in this population is interesting, and may reflect the methodological problems in controlling joint angles at the knee and strongly flexed ankle.

D. Dynamics of Muscle Atrophy

Muscles probably have several distinct mechanisms to respond to changes in activity and length by changing fiber cross-sectional area and length and eventually histochemical and contractile properties. Our initial experiments in unrestrained animals, like those of others, demonstrate that muscle changes continue to develop over the first two weeks and longer [19], [45]. Experiments conducted over longer durations might produce larger changes for which the variability in the data would be less problematic. They would, however, introduce additional questions such as how to correct mass and cross-sectional area for fiber-length changes, how to deal with possible contractures of the endomysial connective tissue and whether the various adaptive responses plateau at different times in different fiber types. In order to address these issues, additional experimental procedures would have to be introduced to control the length of the muscle when frozen for histology and to correct for the length of the sarcomeres in the frozen muscle. The effects of changing muscle length may also have highly variable effects on slow versus fast muscles or pinnate versus parallel-fibered muscles, which have not been considered when experimental interventions are evaluated in only one muscle at a time.

ACKNOWLEDGMENT

The authors thank Dr. R. Michel and S. Dunn for their expert help with the surgical technique and this animal model in general, and Dr. D. Parry for his expertise with muscle cell types. As well, the authors owe a great debt to J. Creasy for her technical assistance and expertise with animals. The authors also want to thank E. A.-Y. E. Chung, A. Conliffe, C. Cridland, and J. Manson for their help in the lab.

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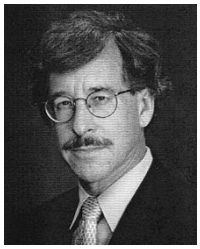

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