Synaptic Activity and Connective Tissue Remodeling in Denervated Frog Muscle

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Abstract. Denervation of skeletal muscle results in dramatic remodeling of the cellular and molecular composition of the muscle connective tissue. This remodeling is concentrated in muscle near neuromuscular junctions and involves the accumulation of interstitial cells and several extracellular matrix molecules. Given the role of extracellular matrix in neurite outgrowth and synaptogenesis, we predict that this remodeling of the junctional connective tissue directly influences the regeneration of the neuromuscular junction. As one step toward understanding the role of this denervation-induced remodeling in synapse formation, we have begun to look for the signals that are involved in initiating the junctional accumulations of interstitial cells and matrix molecules. Here, the role of muscle inactivity as a signal was examined. The distributions of interstitial cells, fibronectin, and tenascin were determined in muscles inactivated by presynaptic blockade of muscle activity with tetrodotoxin. We found that blockade of muscle activity for up to 4 wk produced neither the junctional accumulation of interstitial cells nor the junctional concentrations of tenascin and fibronectin normally present in denervated frog muscle. In contrast, the muscle inactivity induced the extrajunctional appearance of two synapse-specific molecules, the acetylcholine receptor and a muscle fiber antigen, mAb 3B6. These results demonstrate that the remodeling of the junctional connective tissue in response to nerve injury is a unique response of muscle to denervation in that it is initiated by a mechanism that is independent of muscle activity. Thus connective tissue remodeling in denervated skeletal muscle may be induced by signals released from or associated with the nerve other than the evoked release of neurotransmitter.

DENERVATION of skeletal muscle results in striking changes in the cellular and molecular composition of the muscle connective tissue. Interstitial cells accumulate selectively in junctional regions of skeletal muscles, near neuromuscular junctions, after damage to motor axons (6, 16, 45). Experimental evidence suggests that these interstitial cells are fibroblasts, cells that produce extracellular matrix (6). The redistribution of several extracellular matrix molecules is coincident with the accumulation of interstitial cells; fibronectin, tenascin, and heparan sulfate proteoglycan become concentrated in junctional regions of denervated muscle (16, 46). The hypothesis that connective tissue remodeling in junctional regions of denervated muscle may influence the reinnervation of muscle is suggested by evidence that several extracellular matrix molecules are involved in aspects of synaptogenesis (for review see reference 44). Neurite outgrowth and elongation in vitro is promoted by laminin and fibronectin (26, 30, 42). In vivo, neurons regenerate axons along the laminin-rich Schwann cell basal lamina sheaths to reinnervate muscle fibers (21, 24). The assembly of postsynaptic specializations, clusters of acetylcholine receptors (AChR)[1] and acetylcholinesterase, is initiated in vivo by molecules in the muscle fiber basal lamina (1, 31) and in vitro by the matrix molecule, agrin (15, 35, 39, 47, 48).

One step toward determining the role of the junctional interstitial cells in the formation of neuromuscular junctions is the identification of the signals that initiate the connective tissue remodeling following denervation. The level of muscle activity is one variable altered by denervation and is known to influence the structure and molecular organization of the muscle. For example, in normal innervated muscle, muscle fibers are sensitive to acetylcholine (ACh) at neuromuscular junctions; AChRs are concentrated there (25). In contrast, muscle fibers made inactive by either denervation or blockade of synaptic transmission display dramatically increased sensitivity to ACh in extrajunctional muscle regions due to insertion of AChRs throughout the muscle fiber membrane (3, 13, 28, 29, 32). This development of extrajunctional sensitivity to ACh in inactive mammalian muscles can be in-

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1. *Abbreviations used in this paper*: ACh, acetylcholine; AChR, acetylcholine receptor; TTX, tetrodotoxin; MEPP, miniature endplate potential.

hibited or reversed by restoration of muscle activity with electrical stimulation (12, 23, 29).

The experiments presented here address the role of muscle activity in initiating the remodeling of the junctional connective tissue in denervated frog muscle. Muscles were made inactive by presynaptic blockade of synaptic activity with tetrodotoxin (TTX), a poison that blocks voltage-sensitive sodium channels (33). This pharmacological blockade of muscle activity is referred to as functional denervation. We have determined that denervation-like remodeling of the junctional connective tissue does not occur in functionally denervated muscles. There was no junctional accumulation of interstitial cells; the interstitial cell densities of functionally denervated muscles were indistinguishable from those of normal innervated muscles. Further, consistent with the hypothesis that the accumulated interstitial cells are responsible for matrix remodeling after denervation, denervation-like accumulations of the extracellular matrix molecules tenascin and fibronectin did not appear in inactive muscles. In contrast, muscle inactivity did induce known denervation responses in frog muscle fibers; sensitivity to ACh and the synaptic muscle antigen mAb 3B6 were detected in extrajunctional regions of functionally denervated muscle fibers. These data demonstrate for the first time an activity dependence of denervation responses of frog muscle and suggest that muscle inactivity does not initiate the junctional connective tissue remodeling in denervated muscle. Thus, unlike the activity-dependent responses of denervated muscle fibers, the connective tissue response to denervation is mediated by a mechanism that is independent of muscle activity.

Materials and Methods

Muscles

Each muscle fiber of the paired cutaneous pectoris muscle of the frog, *Rana pipiens*, is singly innervated in its center third by the cutaneous pectoris nerve, resulting in clearly defined junctional and extrajunctional regions (6). Due to the arrangement of muscle fibers, the anterior tibialis muscle has neuromuscular junctions all along its length so that each cross section contains a group of muscle fibers sectioned through the junctional region.

Denervation

The innervation to the cutaneous pectoris was severed bilaterally either at the level of the forelimb and entrance of the nerve into the muscle or, for longer periods of denervation, as the brachial nerve exited the vertebral column (27). The anterior tibialis muscle was denervated unilaterally by severing the seventh, eighth and ninth spinal nerves proximal to their fusion to form the sciatic nerve.

General Procedures for Light Microscopy

Cutaneous pectoris muscles were dissected in Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $NaH_2PO_4H_2O$, pH 7.2), fixed for 30 min in 0.8% glutaraldehyde in 0.09 M phosphate buffer, stained for cholinesterase, refixed for 1 h in 1% osmium tetroxide in 0.09 M phosphate buffer, dehydrated in ethanol, and embedded flat in a mixture of LX-112 and araldite (6). 1 μm cross sections of junctional and extrajunctional muscle regions were stained with toluidine blue.

Presynaptic Blockade of Muscle Activity

Cutaneous pectoris nerve activity was blocked for 2 wk by implantation of a unidirectional 1.5 mm time-release pellet containing 9 μg TTX (Innovative Research of America, Toledo, OH). The 9 μg dose of TTX was chosen since nerve activity was restored prematurely at a lower dose (4.5 μg TTX) and the activity blockade with a higher dose (13.5 μg TTX) resulted in decreased survival and was so extensive as to prevent successful stimulation of the nerve distal to the pellet site. Frogs were anesthetized by immersion in 0.15% tricaine menthanesulfonate in water (pH 7.0) and a TTX or placebo pellet was placed on the cutaneous pectoris nerve at least 1 mm from its entrance to the muscle. The skin incision was closed with suture. Nerve activity was blocked at the earliest time at which the blockade of activity was assessed, 48 h after pellet implantation. For longer periods of activity blockade (3–4 wk), the pellet was removed and replaced after 2 wk. Frogs fully recovered from pellet implantation within 24 h and resumed normal levels of activity.

The effectiveness of the blockade of synaptic transmission by TTX and the integrity of the cutaneous pectoris nerve were assessed at the time of dissection both in situ and after the muscle was removed. To determine if synaptic transmission was blocked, the nerve was electrically stimulated (1 Hz, 10 ms duration, 0.1–20 V) first proximal and then distal to the pellet and the cutaneous pectoris was observed for evidence of contractions. TTX-treated nerves were challenged with voltage pulses 3–10 times greater than those needed in control preparations to elicit contractions (1–2 V). Muscles were included in the analysis only if muscle contractions were elicited by stimulation of the nerve distal but not proximal to the TTX pellet. Further, muscles for interstitial cell density determination and electrophysiological recording were fixed, embedded in a plastic wafer as described above, and examined in whole mount for the presence of degenerating axons. Disruption of the myelin sheath was considered an indicator of axonal damage when assessing the integrity of individual axons (Fig. 1). Innervated (control, TTX, and placebo-treated) preparations that displayed damage to more than one axon were excluded from the analysis.

Determination of Interstitial Cell Density

Interstitial cell density was quantitated in junctional and extrajunctional sections of each muscle as previously described (6). Briefly, the number of interstitial cell nuclei/muscle fiber was determined for at least five fields in each section and a mean density was obtained. For junctional sections, a field contained several muscle fibers with cholinesterase-stained synaptic sites. Cell density was determined without knowledge of the type of preparation (control, TTX, and placebo-treated).

Length of Neuromuscular Junctions

Cutaneous pectoris muscles were fixed, stained for cholinesterase, and embedded flat as described above. The linear extent of individual neuromuscular junctions, marked by cholinesterase stain, was measured with an eyepiece micrometer at 200×.

Immunohistochemistry

Cutaneous pectoris muscles were dissected in sylgard-coated petri dishes and the borders of junctional regions were marked with pins. To reduce the damage due to thawing during mounting, muscles were sandwiched between other frog muscles and were frozen in liquid nitrogen. Junctional

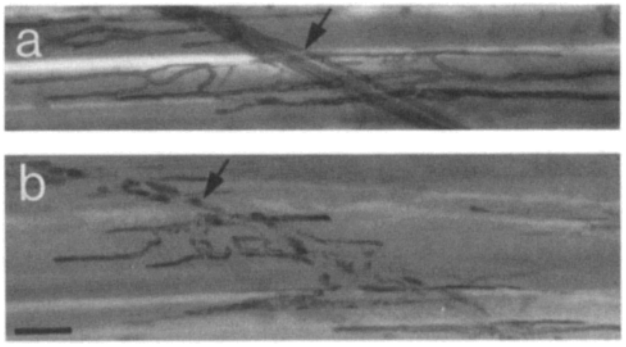


Figure 1. The integrity of myelin sheaths (*arrows*) can be assessed in whole mount preparations of cutaneous pectoris muscles. (*a*) Innervated muscle. (*b*) 2 wk denervated muscle. Myelin is visualized by staining with osmium. Neuromuscular junctions are marked by staining for cholinesterase. Bar, 50 μm.

regions of muscles were cut out and mounted with Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN). Anterior tibialis muscles were dissected, frozen, and a block of muscle was mounted in a similar fashion. Ten micrometer cross sections were collected on gelatin-coated slides and stored at −20°C. Sections were fixed for 5 min in 1% formalin in PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.0 mM Na_2HPO_4), washed for 5 min in 0.1% Triton-X 100 in PBS (PBST), incubated for 10 min in blocking solution (3% bovine serum albumin, 0.2% Tween 20, 5% goat serum in PBS), and incubated for 1 h at room temperature in primary antibody. Sections were then washed for 30 min in PBST, incubated for 1 h in fluorochrome-labeled secondary antibodies, and washed for 30 min in PBST. Primary and secondary antibodies were diluted in blocking solution prior to incubation. Rhodamine-labeled α-bungarotoxin (Molecular Probes, Eugene, OR) was added to the secondary incubation to mark neuromuscular junctions. Coverslips were mounted with Citifluor mountant medium AF1 (City University, London).

Antibodies

The primary antibodies directed against the extracellular matrix molecule fibronectin were a rabbit polyclonal directed against *Xenopus* fibronectin (kindly provided by R. Hynes, Massachusetts Institute of Technology, Cambridge, MA) and a mouse IgM mAb 2G3, that recognizes an epitope of *Rana pipien* cellular fibronectin (Connor, E. A., unpublished results). Tenascin was labeled with a mouse IgG mAb, M1-B4, obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Science (Johns Hopkins University School of Medicine, Baltimore, MD) and the Department of Biology (University of Iowa, Iowa City, IA) under contract N01-HD-2-43144 from the National Institute of Child Health and Human Development. In addition, mAb 3B6, a mouse IgM that stains an intracellular muscle epitope at the frog neuromuscular junction was used (7). Secondary antibodies were labeled with FITC and included goat anti-rabbit IgG, goat anti-mouse IgM, and affinity-purified goat anti-mouse IgG (Cappel Research Products, Durham, NC).

Iontophoresis of Acetylcholine

Cutaneous pectoris muscles were dissected in a Ringer's solution (110 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4). Determination of ACh sensitivity was similar to the method of del Castillo and Katz (11). Muscle fibers in the lateral portion of the muscle were impaled about 1 mm from the main nerve trunk; there neuromuscular junctions were consistently found. The junctional location of the recording electrode was confirmed in innervated and activity-blocked muscles by the detection of miniature endplate potentials (MEPPs). In general, membrane potentials of muscle fibers included in the analysis ranged from −80 to −90 mV though recordings were made at −60 mV in a few functionally denervated fibers. ACh pulses were delivered at the site of the recording electrode by iontophoretic micropipets (3 M ACh chloride; Sigma Chemical Co., St. Louis, MO). ACh release currents were normally limited to 100 nA with a 10 ms duration and produced ACh potentials 1–5 mV in amplitude. Longer pulse durations (up to 50 ms) were used in extrajunctional regions of some functionally denervated muscle fibers. After the first impalement and application of ACh, electrodes were moved a measured distance (50–300 mm) along the muscle fiber where the same fiber was reimpaled and again pulsed with ACh. This was repeated along the muscle fiber until no response was recorded. In some instances, impalement and iontophoresis were in clear extrajunctional muscle regions; the impalement sites were greater than 4 mm from the entrance of the nerve into the muscle and at least 2 mm from the insertion of the fibers into the skin. In addition, neither axons nor cholinesterase stain were detected near these extrajunctional recording sites. Voltage responses of muscle fibers were recorded on a storage oscilloscope and photographed. ACh sensitivity was computed as the ratio of amplitude of the ACh potential (mV)/charge of ACh current (nC) (32). Data are presented as mean ± SD.

Results

Muscle Activity and Interstitial Cell Density

A first set of experiments determined that pellet implantation itself did not induce or block the junctional accumulation of interstitial cells. The interstitial cell density in placebo-treated innervated preparations was not different from that in normal muscles (Fig. 2). Further, after 2 wk of denervation, equivalent junctional accumulations of interstitial cells resulted in muscles with or without placebo pellets.

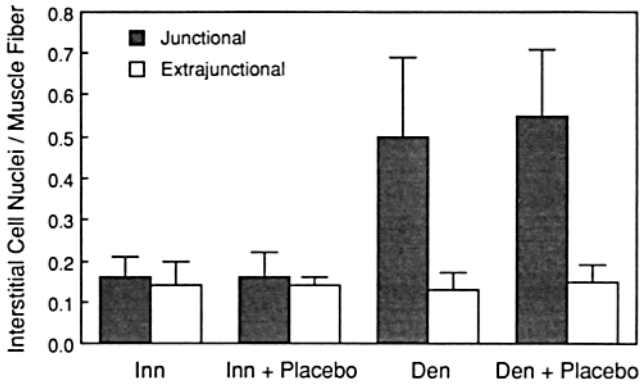


Figure 2. The distribution of interstitial cell nuclei in innervated and denervated muscle is not altered by a 2 wk implantation of placebo pellets. The number of muscles in each group (n) are innervated (11), innervated + placebo (6), 2 wk denervated (8), and 2 wk denervated + placebo (8). Data are shown as the mean ± SD.

Whereas true denervation resulted in a striking accumulation of interstitial cell nuclei in junctional regions of denervated muscles as previously described (6), we found no junctional accumulation of interstitial cells in muscles functionally denervated for 2–4 wk (Figs. 3 and 4). Instead, the connective tissue morphology of junctional regions of functionally denervated muscles closely resembled that of control innervated muscles; there were few interstitial cell nuclei in the endomysium and perimysium of either type of muscles. This distribution of interstitial cells was maintained in muscles functionally denervated for up to 4 wk, indicating that the onset of the interstitial cell accumulation was not simply delayed in functionally denervated preparations. Further, overall interstitial cell density was unaltered by muscle inactivity; the density of interstitial cells in extrajunctional muscle regions of 4 wk functionally denervated muscles (0.16 ± 0.07 SD, n = 5 muscles) was not different from control innervated values (0.15 ± 0.06, n = 18). The failure of interstitial cells to accumulate in functionally denervated muscles was not due to a direct effect of TTX since the interstitial cell density was not different in control muscles after 2 wk of denervation (0.50 ± 0.19 SD, n = 7 muscles) when compared to muscles after 2 wk of denervation and TTX treatment (0.66 ± 0.15, n = 6). These data demonstrate that the junctional accumulation of interstitial cells in denervated frog muscle is not regulated by muscle activity. Thus, denervation must provide other signals that induce the interstitial cells to proliferate and accumulate near denervated synaptic sites (6).

Muscle Activity and Distribution of Matrix Molecules

Following denervation of rat skeletal muscle, the muscle extracellular matrix is remodeled in that fibronectin, tenascin and heparan sulfate proteoglycan become concentrated in junctional regions (46). Evidence that interstitial cells isolated from denervated rat muscle produce these matrix molecules suggests that the interstitial cells are responsible for the junctional matrix remodeling in denervated muscle (16). We have found a similar junctional accumulation of the matrix

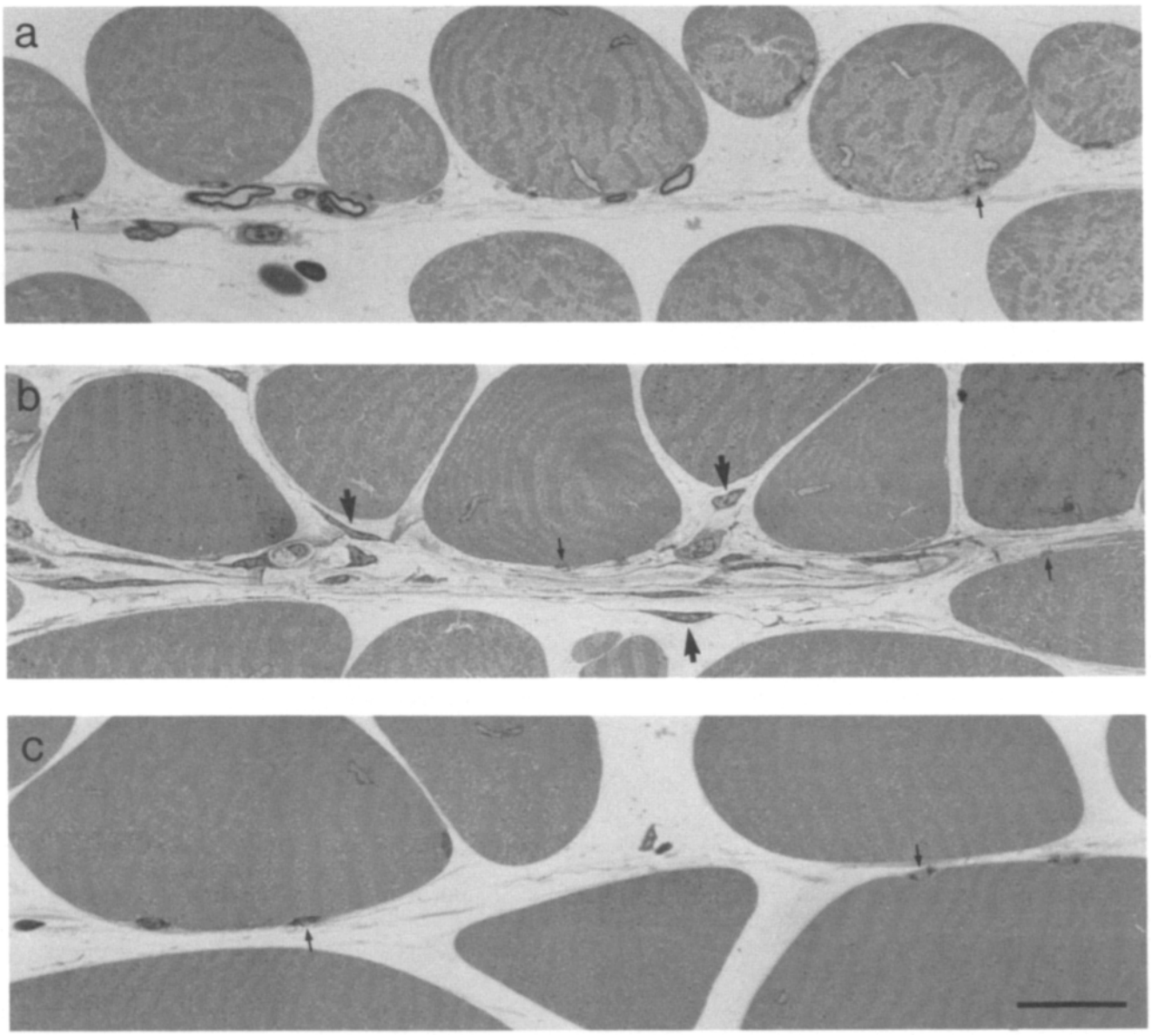
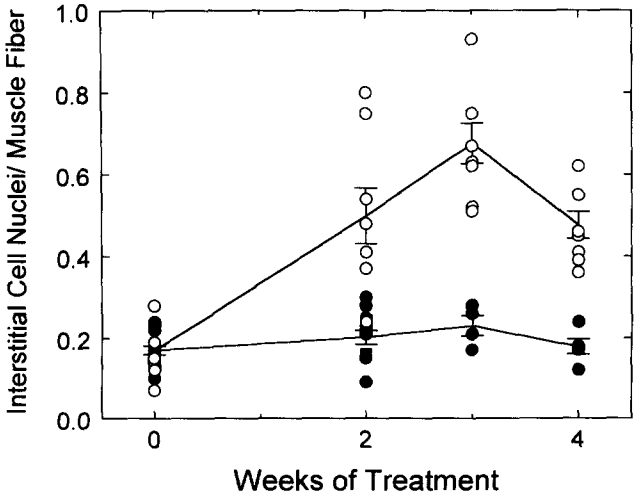


Figure 3. Presynaptic blockade of synaptic activity in innervated muscles did not result in a junctional accumulation of interstitial cells (*large arrows*). 1 μm cross sections of junctional regions of (*a*) innervated, (*b*) 4 wk denervated, and (*c*) 4 wk functionally denervated cutaneous pectoris muscles are stained with toluidine blue. Sites of neuromuscular junctions are marked with cholinesterase stain (*small arrows*). Bar, 20 μm.



molecules, tenascin, and fibronectin, in frog muscle in response to denervation. In normal innervated muscles of *Rana pipiens*, there is limited staining with tenascin antibodies (tendons, not shown, and an occasional neuromuscular junction) while fibronectin staining is distributed in both junctional and extrajunctional muscle connective tissue (Fig.

Figure 4. Muscle inactivity produced by denervation results in a junctional accumulation of interstitial cells whereas presynaptic blockade of muscle activity does not. The junctional interstitial cell density is shown for denervated muscles (*open circles*), innervated muscles inactivated by TTX (*closed circles*), and their respective controls (0 wk of treatment). Values for individual muscles are shown as well as the mean ± SEM (*lines*) for each group. Four muscles from four frogs were the minimum number of muscles examined for any group.

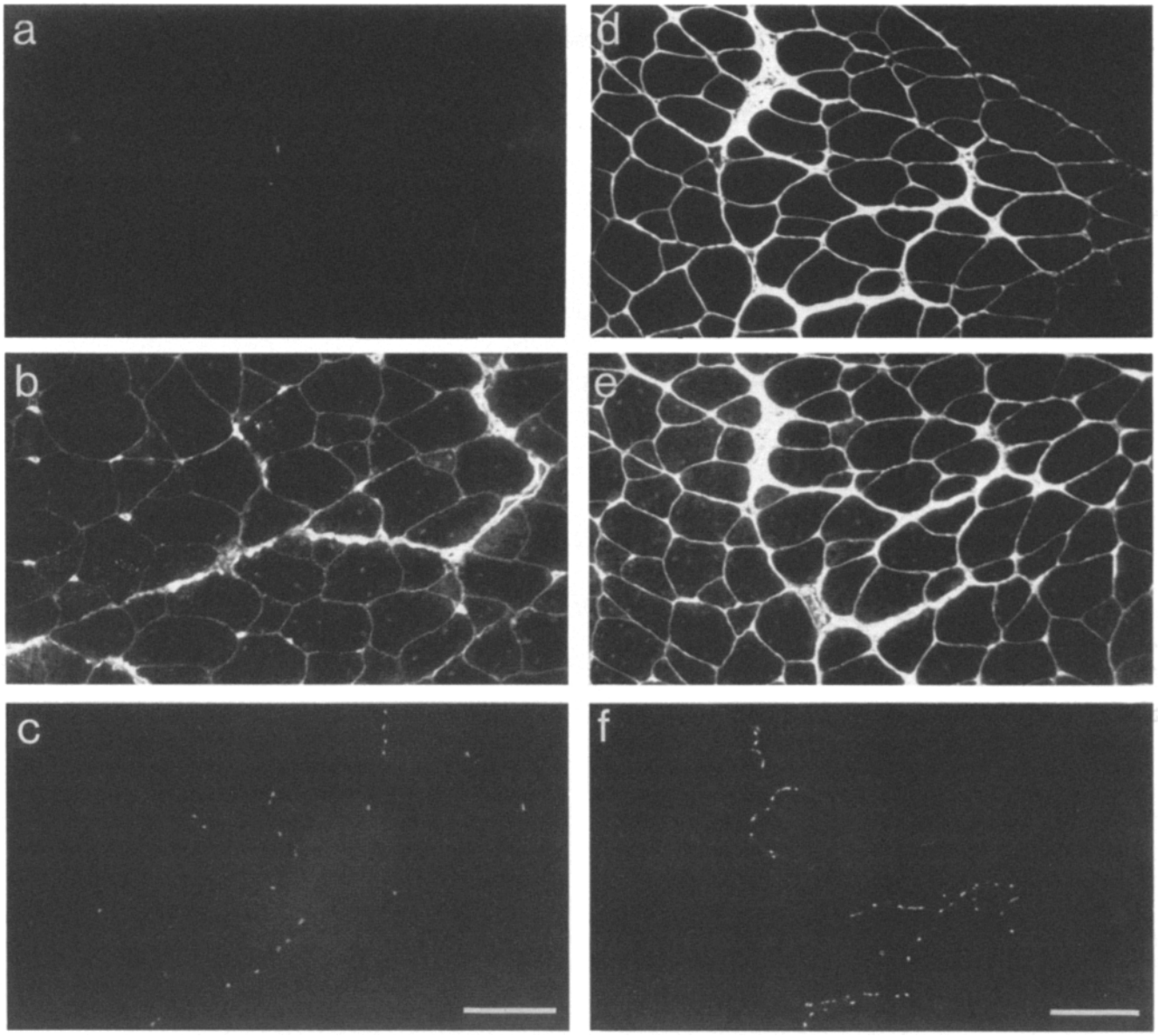


Figure 5. The junctional distribution of tenascin and fibronectin in anterior tibialis muscles is altered by denervation. Semi-consecutive cryostat sections of innervated (*a–c*) and 2 wk denervated muscle (*d–f*) were stained for indirect immunofluorescence with antibodies specific for either tenascin (*a* and *d*) or fibronectin (*b* and *e*). Staining of neuromuscular junctions with rhodamine-labeled α-bungarotoxin (*c* and *f*) marks the location of muscle junctional regions. Bars: (*a–c*) 100 μm; (*d–f*) 150 μm.

5). The distributions of tenascin and fibronectin immunoreactivity are strikingly altered by denervation; both tenascin and fibronectin are highly concentrated in the junctional connective tissue of 2 wk denervated muscles (Fig. 5). To determine the role of muscle activity in this remodeling of the muscle extracellular matrix, we examined the distribution of tenascin and fibronectin in muscles functionally denervated for up to 4 wk.

The distribution of the matrix molecules tenascin and fibronectin was not altered by functional denervation. In four muscles (four frogs) functionally denervated for 4 wk, denervation-like junctional accumulations of tenascin stain were not observed (Fig. 6). Similarly, there was no such redistribution of tenascin after either 2 or 3 wk of muscle inactivation (data not shown). The pattern of tenascin stain in functionally denervated muscles was not however identical to that in control innervated muscles. In 4 wk functionally denervated muscles, structures in the muscle interstitial space, presumably nerve bundles, occasionally displayed tenascin immunoreactivity and the staining of synaptic sites was more prevalent. This tenascin immunoreactivity was very limited and not equivalent to the induction of tenascin by denervation. The pattern of fibronectin stain in 2-4 wk functionally denervated muscles resembled controls; there was limited fibronectin stain in both junctional and extrajunctional regions (Fig. 6). These results indicate that the interstitial accumulations of the matrix molecules tenascin and fibronectin in junctional regions after denervation are not regulated by the level of muscle activity. These results are consistent with the hypothesis that the interstitial cells are responsible for the matrix remodeling observed after denervation.

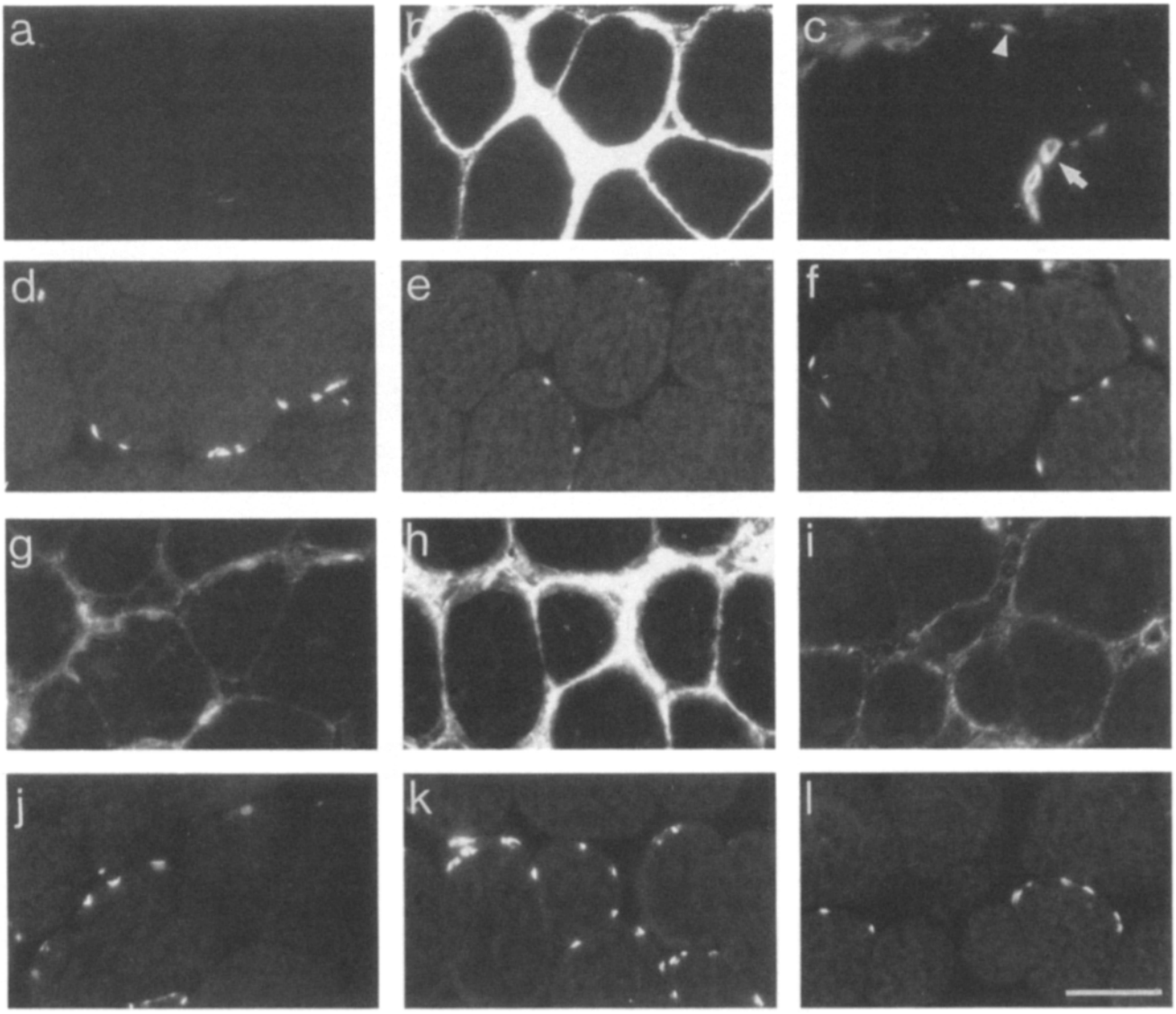


Figure 6. The distribution of the extracellular matrix molecules tenascin and fibronectin in muscles inactivated by denervation or tetrodotoxin. Semi-consecutive cryostat sections of junctional regions of cutaneous pectoris muscles were stained for indirect immunofluorescence with antibodies specific for tenascin (*a–c*) and fibronectin (*g–i*). Rhodamine-labeled α-bungarotoxin (*d–f* and *j–l*) marks the location of neuromuscular junctions. (*a*, *d*, *g*, and *j*) Innervated muscle. (*b*, *e*, *h*, and *k*) 4 wk denervated muscle. (*c*, *f*, *i*, and *l*) Innervated muscle inactivated by TTX for 4 wk. (*c*) Some connective tissue structures (*arrow*) and neuromuscular junctions (*arrowhead*) display tenascin immunoreactivity. Bar, 40 μm.

The failure of blockade of synaptic activity to induce remodeling of the muscle connective tissue may be due to either ineffective blockade of synaptic transmission or a failure of frog muscle to display activity-dependent denervation responses. Previous evidence of induction of denervation responses by muscle inactivity comes from mammalian preparations (3, 4, 9, 12, 14, 18, 23, 29, 37, 41, 50). The effectiveness of the TTX blockade of muscle activity was tested by electrical stimulation of the cutaneous pectoris nerve both proximal and distal to the TTX pellet. Only muscle preparations that contracted with distal nerve stimulation and not proximal stimulation were included in the analysis. As further evidence of the effectiveness of the TTX blockade and the activity dependence of frog muscle denervation responses, we examined the distribution of two synaptic molecules in functionally denervated muscles.

Muscle Activity and Acetylcholine Sensitivity

We first determined the distribution of AChR in functionally denervated muscle fibers by assaying ACh sensitivity. In mammalian muscle, extrajunctional ACh sensitivity is one of several denervation responses known to be induced by blockade of muscle activity (12, 23, 29). Though extrajunctional ACh sensitivity has been demonstrated in denervated amphibian muscle (13, 32), its activity dependence has not been documented. We used iontophoretic application of ACh to map the ACh sensitive membrane on individual muscle fibers from innervated, denervated, and functionally denervated muscles.

Normal Muscle. At neuromuscular junctions, located by the presence of MEPPs, an ACh pulse produced a transient membrane depolarization, an ACh potential, of 1–5 mV with

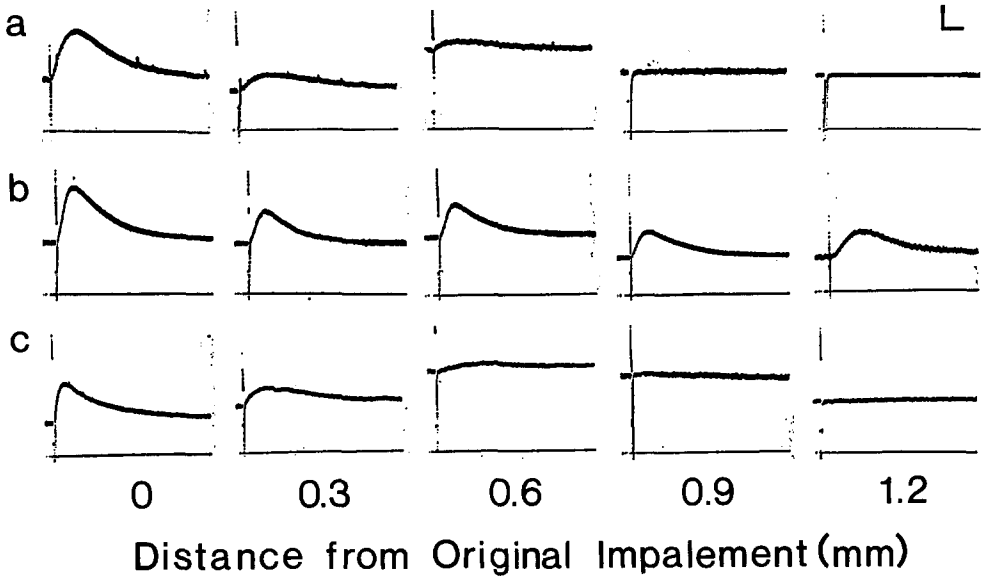


Figure 7. Voltage responses of an (*a*) innervated, (*b*) 2 wk denervated, and (*c*) 2 wk activity-blocked muscle fiber to iontophoretically applied ACh. ACh sensitivity was tested by moving the ACh pipette and recording electrode along the muscle fiber in steps of 0.3 mm from the original impalement site (endplate). Upper trace, records of ACh potentials. Lower trace, records of releasing current through ACh pipette. ACh pulse duration is 10 ms. Horizontal bar, 300 ms; vertical bar, 2 mV or 50 nA.

a rising phase of 100–300 ms (Fig. 7 *a*). Occasionally no ACh potentials were detected though MEPPs were present and it was assumed that the neuromuscular junction was on the opposite surface of the muscle fiber. In normal innervated muscle fibers, ACh potentials (maximal sensitivity: 9.6 ± 3.2 mV/nC, $n = 7$ muscle fibers) were detected for an average distance of 0.4 mm (± 0.17, $n = 7$) from the original impalement site (Fig. 8 *a*). The measured lengths of ACh-sensitive membrane were consistent with neuromuscular junction lengths determined by cholinesterase stain; neuromuscular junctions ranged from 0.2–1.2 mm in length with an average length of 0.6 mm (± 0.19 SD, $n = 150$ muscle fibers).

Denervated Muscle. 2 wk after denervation, ACh potentials were detected at greater distances along muscle fibers, up to 5 mm from the original impalement site. Though the absence of MEPPs prevented the direct determination of the position of neuromuscular junctions, original impalement sites in denervated muscle were likely at endplates since the recordings were made in the same endplate-rich areas in both denervated and innervated muscles. In addition, the maximal ACh sensitivity at denervated junctional regions (12.7 ± 5.3 mV/nC, $n = 5$ muscle fibers) was similar to that of innervated muscles. Unlike innervated muscle fibers where ACh sensitivity dropped off less than a millimeter from the impalement site, ACh sensitivity remained elevated in extrajunctional regions of denervated muscle fibers (Fig. 7 *b*). Extrajunctional ACh sensitivity was about 1/10 of the sensitivity at the neuromuscular junction (Fig. 8 *b*), a finding consistent with previous reports (13, 32).

Functionally Denervated Muscle. After 2 wk of blockade of synaptic activity by TTX, the distribution of ACh sensitivity was strikingly similar to that of normal innervated muscles (Fig. 7 *c*). The presence of MEPPs at neuromuscular junctions indicated that the nerve was intact. ACh sensitivity at endplates (10.2 ± 3.0 mV/nC, $n = 6$ muscle fibers) was similar to that of innervated and 2 wk denervated muscle fibers. The ACh sensitivity remained localized to the neuromuscular junction; ACh potentials were detected on average 0.51 mm (± 0.12, $n = 6$ muscle fibers) from the first impalement site (Fig. 8 *c*). In one 2 wk TTX preparation, extrajunctional sensitivity to ACh was detected but the lack of MEPPs and inspection of the muscle after fixation revealed that the nerve had degenerated and the muscle was denervated.

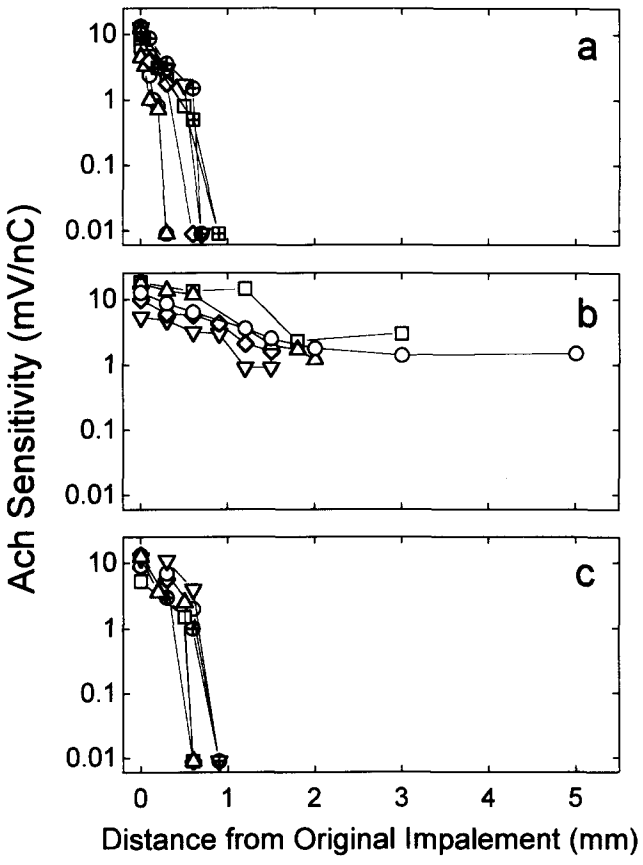


Figure 8. Spatial distribution of ACh sensitivity (*mV/nC*) in (*a*) innervated (seven muscle fibers, four frogs), (*b*) 2 wk denervated (five muscle fibers, three frogs) or (*c*) 2 wk activity-blocked (six muscle fibers, three frogs) muscle fibers. Distance is the length along an individual muscle fiber from the original impalement site (zero distance).

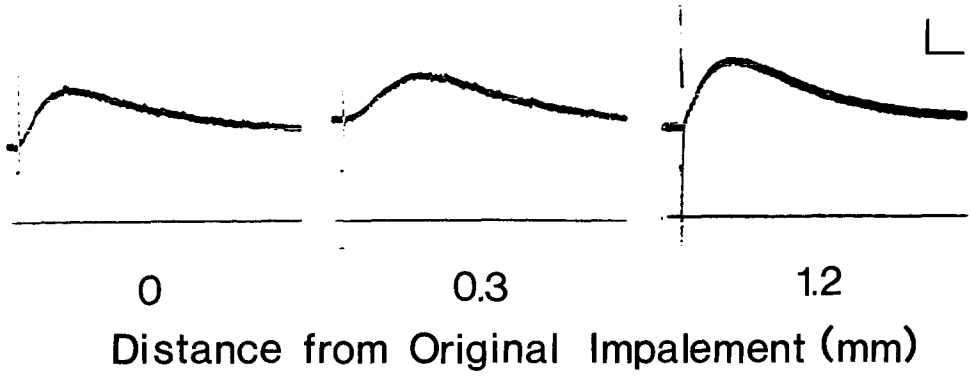


Figure 9. ACh potentials recorded from an individual muscle fiber after 3 wk of muscle inactivity. The first ACh potential was recorded at the neuromuscular junction. ACh potentials were recorded as far as 1.2 mm from the original recording site, in a clearly extrajunctional region of the muscle fiber. Upper trace, records of ACh potentials. Lower trace, records of releasing current through ACh pipette. ACh pulse duration is 10 ms. Horizontal bar, 250 ms; vertical bar, 1.5 mV or 38 nA.

When the period of muscle activity blockade was extended to 3 wk, sensitivity to ACh was detected in extrajunctional muscle regions (Fig. 9). In four muscle fibers from two frogs ACh potentials were recorded several millimeters from cholinesterase-stained neuromuscular junctions. There was no evidence of axon damage in these preparations. ACh pulses of similar size and duration failed to induce ACh potentials in the same extrajunctional positions in a normal innervated muscle. The extrajunctional ACh sensitivity of muscle fibers after 3 wk of functional inactivity (3.8 ± 2.3, $n = 3$ muscle fibers, two frogs) was similar to that of muscle fibers denervated for 2 wk (Fig. 8). In two other functionally denervated muscle fibers, ACh potentials were produced only in response to very long ACh pulses (90–150 ms); these were not considered positive responses.

Muscle Activity and Distribution of a Muscle Fiber Antigen

In normal muscle fibers, the muscle antigen recognized by mAb 3B6 is concentrated at the neuromuscular junction (7). After denervation, the mAb 3B6 antigen appears in extrajunctional regions of the muscle fiber perimeter as well as the cytoplasm. The distribution of mAb 3B6 staining was examined in muscles functionally denervated with TTX (Fig. 10). After 2 wk of activity blockade, mAb 3B6 stain remained concentrated at neuromuscular junctions of most muscle fibers with only limited nonsynaptic stain on a few muscle fibers (data not shown). After 3–4 wk of activity blockade, a denervated pattern of mAb 3B6 stain was de-

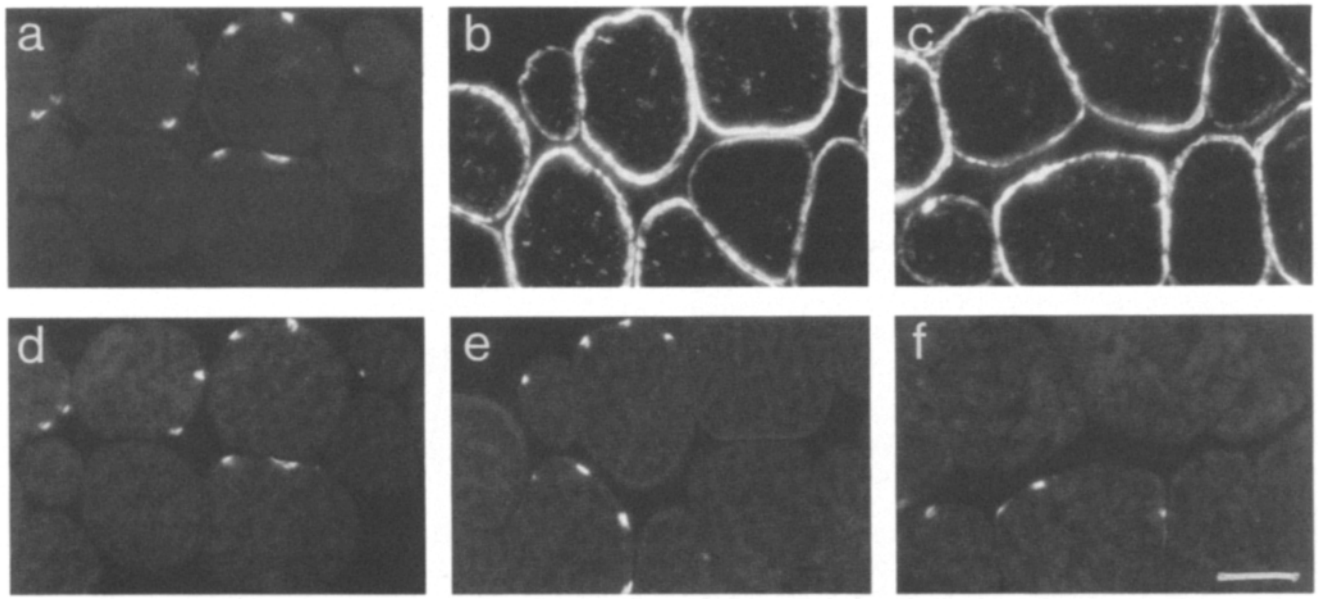


Figure 10. The synaptic muscle antigen mAb 3B6 appears in nonsynaptic regions of the muscle fibers after loss of muscle activity. The junctional distribution of mAb 3B6 is shown for an (*a*) innervated, (*b*) 4 wk denervated, and (*c*) 4 wk functionally denervated muscle. Rhodamine-labeled α-bungarotoxin (*d–f*) marks the location of neuromuscular junctions. Bar, 40 μm.

tected; there was a significant level of mAb 3B6 staining in extrajunctional regions of muscle fibers (Fig. 10).

These data demonstrate that the presynaptic blockade of synaptic activity was effective and sufficient to induce known denervation responses of muscle fibers, extrajunctional appearance of ACh sensitivity and the mAb 3B6 antigen. This is the first demonstration that a denervation response of frog muscle can be induced by blockade of muscle activity. Further, these results suggest that denervation responses of frog muscle fibers are not initiated solely by muscle inactivity since the development of extrajunctional ACh sensitivity and mAb 3B6 immunoreactivity was slowed in muscles made inactive by TTX when compared to denervated muscles.

Discussion

The experiments presented here examined whether loss of muscle activity was a sufficient signal to generate the cellular and molecular connective tissue remodeling normally observed in junctional regions of denervated frog skeletal muscle. The activity of innervated muscles was modified by presynaptic blockade of synaptic transmission with TTX. In muscles functionally denervated for up to 4 wk no denervation-like accumulations of either interstitial cells or the matrix molecules tenascin and fibronectin were detected. In contrast, similar periods of inactivity were sufficient to induce the redistribution of two synaptic molecules, AChR and the mAb 3B6 antigen. These results indicate that remodeling of the junctional connective tissue in response to denervation is initiated by a mechanism independent of muscle activity.

We first established the effectiveness of the blockade of muscle activity by TTX. For inclusion in our analysis, a muscle inactivated by TTX had to meet two criteria. First, muscle had to be functionally denervated at the time of dissection. This was determined by stimulation of cutaneous pectoris nerve proximal to the location of the TTX pellet. In all but two preparations treated with 9 μg of TTX, the muscles failed to contract in response to stimulation of the nerve. Second, the cutaneous pectoris nerve had to be intact and undamaged by the pellet. This was assessed at the time of dissection by evoking muscle contractions in response to nerve stimulation distal to the TTX pellet. Since it was not possible to determine that all muscle fibers responded to stimulation, the nerve integrity was assessed further in all preparations, except those for frozen section, by embedding the muscle in plastic and examining it in whole mount. Due to the thin nature of the cutaneous pectoris muscle it was possible to examine individual axons for disruption of myelin sheaths. Seven muscles were excluded from analysis because a number of axons appeared damaged. The detection of MEPPs in functionally denervated muscles further confirmed that innervation was intact. Also, the absence of a denervated pattern of matrix remodeling in functionally denervated muscles indicated that the innervation to muscles examined by frozen section was intact. Thus in the muscle preparations analyzed, muscle activity was effectively blocked and the innervating nerve was undamaged.

Further evidence for the effectiveness of the activity blockade was the induction of denervation responses in inactive muscle fibers. We demonstrated for the first time a role for muscle activity in inducing denervation responses of frog muscle. Several denervation responses of mammalian muscle, including atrophy (20); the appearance of AChRs and neural cell adhesion molecule in extrajunctional regions of the muscle membrane (3, 9, 12, 23, 29, 41); fibrillations (37); increased expression of Thy-1 (4); and altered expression of the genes coding for voltage-dependent sodium channels (50), the α, β, δ, and γ AChR subunits (18), MyoD, and myogenin (14) have been shown to be induced in innervated muscle by activity blockade or reversed in denervated muscle by restoration of activity. We have documented a role for muscle activity in inducing the denervation-like redistribution of two synaptic molecules in frog muscle, AChR and the mAb 3B6 antigen. Extrajunctional sensitivity to ACh was detected in muscle fibers functionally denervated for 3 wk. The detection of MEPPs and/or the presence of intact axons confirmed that the extrajunctional sensitivity to ACh was not a result of axon damage. The extrajunctional ACh sensitivity of denervated frog muscle fibers was similar to previously determined values (13) and comparable sensitivities were obtained from extrajunctional regions of functionally denervated muscle fibers. Loss of muscle activity also resulted in the extrajunctional redistribution of the synaptic antigen mAb 3B6. Its distribution resembles that of AChR in that mAb 3B6 is localized at endplates of innervated muscle fibers but is found extrajunctionally in denervated muscle fibers (7). Thus both AChR and mAb 3B6 appeared in extrajunctional regions of innervated muscle fibers functionally denervated by tetrodotoxin.

One striking feature of this muscle remodeling was that the redistribution of these synaptic molecules occurred more rapidly when induced by denervation than by presynaptic blockade of muscle activity. While extrajunctional ACh potentials were recorded in muscle fibers denervated for 2 wk, 3 wk of functional denervation were required to detect extrajunctional ACh sensitivity. The extrajunctional appearance of the mAb 3B6 antigen was similarly slowed; 3–4 wk of functional inactivity were required to produce the 2 wk denervation response (7). The development of extrajunctional ACh sensitivity in mammalian muscle occurs with a similar time course when induced by either denervation or blockade of synaptic transmission (3, 29). Some membrane properties of muscle fibers however are more effectively altered by denervation than by presynaptic blockade of muscle activity including the onset of increased AChR turnover (2). Thus denervation responses of both frog and mammalian muscle fibers are induced by muscle inactivity, however additional signals are likely to contribute as well. Proposed activity-independent signals include loss of spontaneous vesicular release and products of nerve degeneration (2, 23, 32).

We determined the role of muscle inactivity as a signal in initiating connective tissue remodeling at synaptic sites. The junctional interstitial cell accumulation can be detected one week after nerve damage with a maximal response at 2–3 wk of denervation (6). Given that the onset of this denervation response may be slowed in functionally denervated muscles, we examined interstitial cell density after 2–4 wk of inactivity. Presynaptic blockade of muscle activity in innervated muscles for 2–4 wk had no effect on the distribution of interstitial cells in junctional and extrajunctional regions of muscle. Marked junctional accumulations of interstitial cells were detected in muscles made inactive for similar periods of time by denervation. TTX itself did not prevent the ac-

cumulation of interstitial cells since there were junctional accumulations in denervated muscles exposed to TTX. Taken together, these results indicate that the junctional accumulation of interstitial cells in denervated muscle is not initiated by the loss of muscle activity. This is consistent with the observation of Murray and Robbins (34) that blockade of synaptic transmission by tetrodotoxin did not induce a generalized mitotic response in mouse muscle.

An additional consequence of denervation in frog as in mammalian muscle is that tenascin and fibronectin stain become concentrated in the interstitial spaces of junctional regions of muscle. This denervation-like remodeling of the extracellular matrix did not occur in junctional regions of muscles functionally denervated for up to 4 wk. These results indicate that, like the junctional interstitial cell accumulation, molecular remodeling of the muscle matrix is not induced by blockade of muscle activity. Further, these data support the hypothesis that the interstitial cells are responsible for the molecular remodeling of the extracellular matrix in denervated muscle. Interstitial cells isolated from rat muscle produce fibronectin, tenascin, neural cell adhesion molecule, and heparan sulfate proteoglycan in vitro (16). Though a denervation-like accumulation of tenascin was not present in functionally denervated muscles, there was an altered pattern of tenascin immunoreactivity when compared to control innervated muscles. Structures within the connective tissue, presumably nerves, and neuromuscular junctions were more frequently stained by antibodies directed against tenascin in functionally denervated muscles. This result is consistent with the observation that Schwann cells and cells of the perineurium produce tenascin after nerve injury (10), and suggests that this response may be dependent on nerve activity. Thus the production of tenascin in denervated muscle may well arise from different cell sources; Schwann cells, skeletal muscle and fibroblasts produce tenascin (10, 16). We propose that the majority of interstitial tenascin in denervated muscles is contributed by the accumulated interstitial cells and is induced only by denervation and not loss of synaptic activity. Some modification of the extracellular matrix in junctional regions of muscle is known to occur with loss of muscle activity. The appearance of neural cell adhesion molecule in the interstitial space, as well as on the muscle fiber surface, is induced by presynaptic blockade of muscle activity with tetrodotoxin (9). Also in rat, the level of tenascin immunoreactivity was increased in functionally denervated muscles, however it was not associated with junctional regions of the muscle (46). Thus denervation may result in the generation of activity-independent signals that induce the remodeling of the junctional connective tissue and act in concert with muscle inactivation to elicit the full denervation response of muscle.

We conclude that the cellular and molecular remodeling of the junctional connective tissue in denervated frog muscle is not mediated by the level of muscle activity. What signal then, other than loss of muscle activity, may be responsible for initiating this connective tissue remodeling after denervation? Previous experiments revealed that the junctional accumulation of interstitial cells involves mitosis (6, 16). Thus, damage to motor axons could result in either the production of a positive mitogenic signal that initiates the interstitial cell proliferation and matrix remodeling or the cessation or removal of a negative signal that inhibits interstitial cell proliferation in innervated muscle. Possible cells or structures that may generate the signal include axonal degeneration products, the terminal Schwann cell, the intact nerve terminal, or the muscle fiber. Previously we determined that axonal debris is not the signal; reinnervation of a muscle following nerve crush inhibited the interstitial cell accumulation though axonal debris was present (6). Products of axonal degeneration however have been thought to induce denervation responses on muscle fibers (23), and include molecules such as nerve growth factor, interleukin-1, and apolipoproteins produced following nerve injury (43). The restriction of the connective tissue remodeling to the vicinity of synaptic sites suggests a localized site for signal generation, and thus the terminal Schwann cell, at the neuromuscular junction, is a likely candidate. Though recent evidence indicates that terminal Schwann cells respond to evoked activity of axons (17, 22, 38), the experiments presented here exclude the possibility of the signal originating in terminal Schwann cells as a consequence of loss of activation. However, phagocytosis of axons by terminal Schwann cells could induce the production of a signal by these cells and its restriction to the junctional regions of muscle. Terminal Schwann cells at denervated neuromuscular junctions are known to extend elaborate processes (40). In addition, nonglial cells such as macrophages may be involved in the signaling mechanism in response to their phagocytosis of degenerating axons (36). The axon itself remains a candidate given the inhibition of the interstitial cell accumulation by reinnervation of the muscle. Though evoked vesicular release is blocked by TTX, spontaneous release remains intact and the products of release could act on terminal Schwann cells or muscle fibers. Finally, the muscle fiber may be involved in signaling by an activity-independent mechanism. Insulin-like growth factors, one of several factors released by denervated muscle fibers (19), have been shown to induce some degree of connective tissue remodeling (5).

Clearly, further experiments are needed to elucidate the mechanism by which connective tissue remodeling is initiated after denervation. Such information will aid in our determination of the role of the interstitial cells in the regeneration of the neuromuscular junction. The reconfiguration of the junctional matrix after denervation may serve as a favorable substrate for regenerating axons (8, 19). We have discovered that interstitial cells are also concentrated in junctional regions of developing muscles during the period of synaptogenesis (Connor, E. A., manuscript in preparation). The similarity in the cellular composition of connective tissue of developing and denervated muscle suggests a role of the interstitial cells in the development of the neuromuscular junction. Further, there is evidence that interstitial cells near developing neuromuscular junctions are different from those in extrajunctional regions in that they, along with Schwann cells and perineurial cells, express a particular transgene during embryonic and early postnatal life (49). To fully understand the process of synaptogenesis in developing and regenerating systems it is necessary to determine the contribution of the interstitial cells and the matrix molecules that they produce.

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