

The Influence of Basal Lamina on the Accumulation of Acetylcholine Receptors at Synaptic Sites in Regenerating Muscle

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ABSTRACT If skeletal muscles are damaged in ways that spare the basal lamina sheaths of the muscle fibers, new myofibers develop within the sheaths and neuromuscular junctions form at the original synaptic sites on them. At the regenerated neuromuscular junctions, as at the original ones, the muscle fiber plasma membrane is characterized by infoldings and a high concentration of acetylcholine receptors (AChRs). The aim of this study was to determine whether or not the synaptic portion of the myofiber basal lamina sheath plays a direct role in the formation of the subsynaptic apparatus on regenerating myofibers, a question raised by the results of earlier experiments. The junctional region of the frog cutaneous pectoris muscle was crushed or frozen, which resulted in disintegration and phagocytosis of all cells at the synapse but left intact much of the myofiber basal lamina. Reinnervation was prevented. When new myofibers developed within the basal lamina sheaths, patches of AChRs and infoldings formed preferentially at sites where the myofiber membrane was apposed to the synaptic region of the sheaths. Processes from unidentified cells gradually came to lie on the presynaptic side of the basal lamina at a small fraction of the synaptic sites, but there was no discernible correlation between their presence and the effectiveness of synaptic sites in accumulating AChRs. We therefore conclude that molecules stably attached to the myofiber basal lamina at synaptic sites direct the formation of subsynaptic apparatus in regenerating myofibers. An analysis of the distribution of AChR clusters at synaptic sites indicated that they formed as a result of myofiber-basal lamina interactions that occurred at numerous places along the synaptic basal lamina, that their presence was not dependent on the formation of plasma membrane infoldings, and that the concentration of receptors within clusters could be as great as the AChR concentration at normal neuromuscular junctions.

The basal lamina that ensheaths each myofiber in skeletal muscles is similar to the basal lamina associated with cells of other tissues (34, 49, 51, 56, 61). It is ~20 nm thick and it is separated from the external surface of the myofiber plasma membrane by a narrow gap that is bridged by fine strands of material. It consists of collagen and contains or has associated with it proteoglycans and glycoproteins such as fibronectin and laminin. At the neuromuscular junction the basal lamina of the myofiber is situated in the 50-nm cleft between the axon terminal and muscle cell where it is nearly equidistant from the pre- and postsynaptic membranes. The synaptic

portion of the basal lamina has associated with it antigens not found in nonsynaptic regions of the sheath (50).

In muscle, as in other tissues, basal lamina plays an important role in regeneration (37, 49, 56). After damage to muscles and their motor nerves, myofibers and axons degenerate and are phagocytized but the basal lamina sheaths of myofibers survive. New myofibers regenerate within the basal lamina sheaths of the original myofibers, regenerating axons grow to original synaptic sites on the sheaths, and neuromuscular junctions are formed that look and perform much like the original ones. Thus, in regenerating muscles the basal lamina

sheaths of original myofibers provide scaffolding for restoration of the muscles and their neuromuscular junctions. The basal lamina is not merely an inert guide for the regenerating cells, however. Molecules associated with the basal lamina play a role in the differentiation of the neuromuscular junction by directing the formation of active zones in the regenerating axon terminals (49).

The experiments we describe concern the question of whether or not the basal lamina of original myofibers also plays a role in the differentiation of the subsynaptic apparatus in regenerating myofibers. At normal neuromuscular junctions, which occupy <0.1% of the myofiber surface, the density of acetylcholine receptors (AChRs)¹ directly beneath axon terminals is more than 1,000 times greater than elsewhere on the muscle cell (14, 21, 33, 38). The subsynaptic membrane is also characterized by a regular array of infoldings. Earlier studies from this laboratory (15), now repeated in part in another (4), revealed that the presence of axon terminals is not required for the development of the subsynaptic apparatus on regenerating myofibers. In muscles that had been damaged and denervated, myofibers were allowed to regenerate within the basal lamina sheaths of original myofibers but reinnervation was deliberately prevented. Despite the absence of nerve terminals, AChRs preferentially aggregated at original synaptic sites on the basal lamina sheaths. The plasma membrane at the original synaptic sites also had invaginations that resembled the junctional folds in the subsynaptic membrane of normal muscles. Surviving components of the synaptic site that could have directed the organization of AChRs and the formation of folds were either the synaptic portion of *basal lamina* of the original muscle cell, or *Schwann cells*, which cap normal nerve terminals and respond to denervation by engulfing them, after which they occupy for a time the position of the nerve terminals on the presynaptic side of the basal lamina sheath and release acetylcholine (8, 9, 20).

In the studies presented here, the region of innervation in chronically denervated muscles was briefly frozen or was crushed, resulting in disintegration and phagocytosis of *all* cellular components of the neuromuscular junction—myofiber, nerve terminal, and Schwann cell—while leaving intact large portions of the myofiber basal lamina sheath. When new myofibers regenerated within the basal lamina sheaths of the original fibers, AChRs accumulated and folds formed in the myofiber membrane selectively at original synaptic sites on the sheaths. Processes of unidentified cells gradually appeared on the presynaptic side of the basal lamina at some of the synaptic sites but there was no correlation between their presence and the effectiveness of synaptic sites in organizing AChRs. These findings lead to the conclusion that factors that direct the organization of the subsynaptic apparatus in regenerating myofibers are associated with the myofiber basal lamina sheath. Additional findings on the distribution of AChRs and infoldings at the synaptic sites provide information on how the basal lamina induces these specializations. Brief accounts of some of the experiments have appeared elsewhere (41, 42, 48).

MATERIALS AND METHODS

Experiments were performed on 5-cm long male frogs (*Rana pipiens*, northern variety). The frogs were housed at room temperature (19–22°C) and were fed

¹ *Abbreviations used in this paper.* AChR, acetylcholine receptor; α -BGT, α -bungarotoxin; ChE, cholinesterase; HRP, horseradish peroxidase.

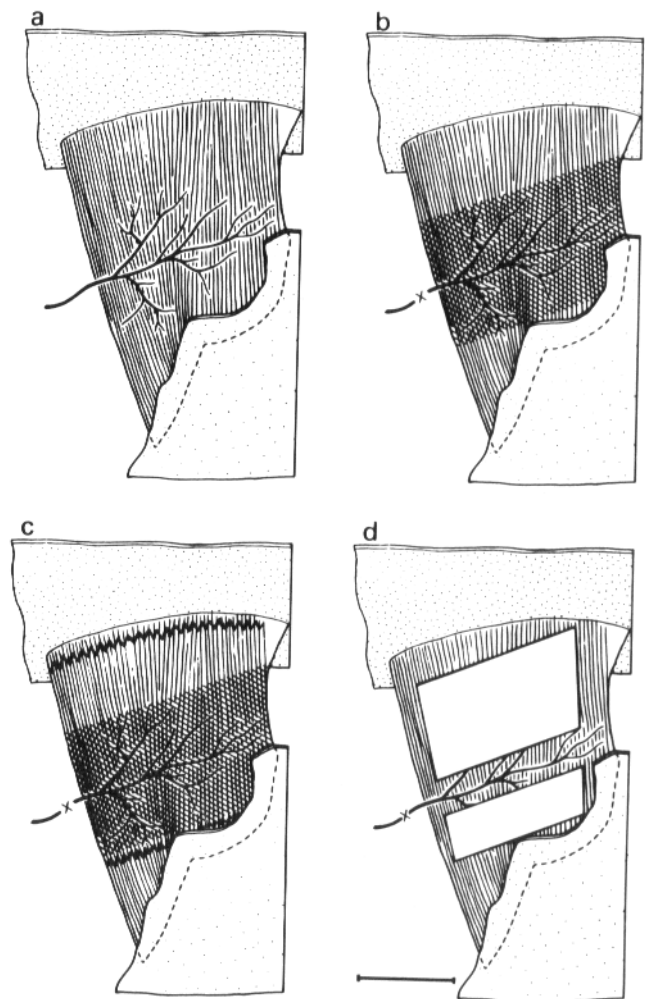


FIGURE 1 The cutaneous pectoris muscle, its nerve, and the sorts of damage used in this study. (a) Normal muscle. (b) Muscle damaged in situ by freezing its region of innervation (crosshatched area). (c) Muscle damaged by cutting (serrated lines) it from its origin and insertion, placing it in a Petri dish, and crushing the region of innervation. The muscle was then replaced in its bed within the frog. (d) Muscle damaged in situ by cutting and removing a rectangular slab from each side of the region of innervation. Bar, 3 mm.

crickets daily.

We used the thin, paired cutaneous pectoris muscles (Fig. 1a) which are situated just beneath the skin of the frog's chest. In preparation for histological procedures, muscles were dissected in frog Ringer's solution (116 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 10 mM dextrose, 1 mM NaH₂PO₄, pH 7.2).

Operations

Frogs were anesthetized by immersion in 0.1% tricaine methane sulfonate (Finquel, Ayerst Laboratories, NY), and both cutaneous pectoris muscles were exposed. The muscles were denervated by resecting 0.5–1 cm of nerve at their lateral borders and were damaged. The skin overlying the muscles was then sewn shut, and the animals were allowed to recover for up to 39 d. To prevent reinnervation of the damaged muscles throughout the course of the experiments, 1–2 wk after the first operation a second operation was performed in which a 1-cm length of the second spinal nerve was removed near the vertebral column.

Muscles were damaged in one of the following ways:

FREEZING (FIG. 1b): A brass block cooled in liquid nitrogen was placed on the muscle. The width of the block face that contacted the muscle was 1.0 mm and the length was sufficient to cover the muscle's breadth. The block, held with a hemostat, was applied with enough pressure to indent the underlying pectoral muscles, which were also frozen. Almost immediately, ice crystals formed in the cutaneous pectoris muscles for up to 1 mm to either side of the block. When the muscle thawed it contracted in the region of the freeze; it was

returned to near normal length by pulling on the skin at the muscle's cutaneous insertion. Our routine for freezing the region of innervation was to position the cold block so that it would cover the main nerve and most of its primary branches and hold it in place for 30 s. The block was replaced in liquid nitrogen and the muscle was allowed to thaw. The process was then repeated twice over the next 4–5 min at positions on each side of the main nerve. The frozen area included all of the neuromuscular junctions but a 1–2-mm wide band at the origin and insertion of the muscle was spared.

CRUSHING (FIG. 1c): The muscle was cut at its origin and insertion and pinned out in a Sylgard-coated Petri dish containing Ringer's solution. Under a dissecting microscope, the fire-polished edge of a glass slide was pressed into the muscle with enough force to indent the Sylgard. The crushed region of the muscle instantly became transparent. The slide was 1-mm thick and the length of the edge used for the crush was greater than the breadth of the muscle. The entire junctional region and a short distance beyond was damaged by a series of 5–7 overlapping crushes. The sequence was repeated twice more and then the muscle was replaced in its bed within the frog. Within 5 min the muscle had adhered to the matrix of the bed so that it did not become distorted during suturing of the skin.

CUTTING (FIG. 1d): The muscle was cut near its origin and insertion and on each side of the junctional region. Most of the extrajunctional region of the muscle was then removed and discarded while the 1–2-mm wide junctional region remained in place.

FREEZING AND CUTTING: The muscle was frozen as described in "Freezing." The extrajunctional region of the muscle was then removed and discarded as described in "Cutting."

General Procedures for Light and Electron Microscopy

Muscles were fixed with 1% glutaraldehyde in 0.09 M phosphate buffer for 30 min, refixed with 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol, rinsed in propylene oxide, and embedded in a mixture of Epon and Araldite.

For embedding, the tissue stood in the plastic overnight. A single muscle was then sandwiched between glass slides which had been coated with a silicone-based releasing agent (Release Agent Dry Lubricant MS-122; Miller-Stephenson Chem Co., Los Angeles, CA) and polished with a paper towel to prevent the glass from sticking tightly to the Epon-Araldite when it hardened (25). The sandwich was then placed in an oven for 1–1½ h at 80°C. When the slides had cooled one was popped free by forcing a razor blade between it and the plastic. Thus the muscle was embedded flat in a wafer of plastic <1 mm thick and supported by a slide. Since the muscle itself is ~100 µm thick all of the myofibers could be seen clearly with a light microscope up to × 1,000 magnification, and specific regions of the muscle could be cut out and mounted for sectioning. Thin sections were stained with uranyl acetate and lead citrate (54).

In most preparations the synaptic sites were marked with cholinesterase (ChE) stain; Karnovsky's histochemical staining method was applied after glutaraldehyde and before osmium tetroxide fixation (31, 35). If the ChE-stained preparations were to be used for studies involving autoradiography (see below), they were allowed to stand in Ringer's overnight before treatment with osmium tetroxide. Failure to include the overnight wash often resulted in the absence of expected grains in the autoradiographic emulsion.

Localization of Acetylcholine Receptors Utilizing ¹²⁵I and Horseradish Peroxidase-Coupled α-Bungarotoxin

AChRs were labeled with α-bungarotoxin (α-BGT) conjugated either to ¹²⁵I or to horseradish peroxidase (HRP). The toxin is a low molecular weight protein that binds tightly and specifically to AChRs of skeletal muscle (6, 17, 43). Our procedures for labeling receptors with the α-BGT conjugates in the cutaneous pectoris muscles have been described previously (15). A brief account is presented here.

¹²⁵I-α-BGT: Muscles were incubated in 4 × 10⁻⁸ M ¹²⁵I-α-BGT (New England Nuclear, Boston, MA; 100 Ci/mmol) in frog Ringer's for 2 h at room temperature (19–20°C), washed in Ringer's for 1 h, and fixed in glutaraldehyde. They were then stained for ChE to mark synaptic sites, washed overnight in Ringer's, postfixed in osmium tetroxide, dehydrated, and embedded. Transverse sections (1.0 µm thick) were mounted on slides, and coated with liquid emulsion (Kodak NTB-2, Rochester, NY), which was exposed for 4–7 d at 4°C before it was developed and fixed. The sections were then washed (30 min), allowed to dry overnight, stained with toluidine blue, and covered with coverslips mounted with glycerol.

To examine in detail the distribution of grains produced by ¹²⁵I-α-BGT, we

made camera lucida drawings of the outline of muscle fibers (or their basal lamina ghosts), and marked on the drawings the extent of the perimeter stained with ChE and the position of autoradiographic silver grains. The magnification of the drawings was × 1,000. In normal muscle the grains were concentrated over the ChE-stained sites and the extrasynaptic grain density was indistinguishable from background. We found that 50% of the grains at synaptic spots fell within 3 µm of the patch of ChE stain. At 50–100 such patches in each muscle, both the length of the patch and the number of grains falling within a window whose border was 3 µm from the patch were determined. Because of the occurrence of ChE patches with no grains, the mean value of grain density for each muscle was calculated as the total length of ChE-stained surface analyzed divided by the total number of grains counted for that muscle. The length of ChE spots was measured with a digitalized planimeter. Typical values of grain density in the window at synaptic sites in normal muscles were ~0.25 grain/µm².

To account for variations of specific activity, exposure, and development of the autoradiographs, as well as of the physiological state of the animals, 2–10 muscles from unoperated frogs, fed and housed together with the experimental ones, were analyzed in each experiment. The values of synaptic site grain density from these muscles were averaged to give a mean "normal" value for each experiment and all values from operated muscles were divided by this to give normalized values that could be compared from experiment to experiment. These normalized values, expressed as "percent of normal" are used throughout the text.

In regenerating muscle, the extrajunctional grain density was greater than background, but grains were again concentrated over sites marked by ChE stain. Extrajunctional grain density was determined from sections through extrajunctional regions of muscle by dividing the number of grains falling within 3 µm of the myofiber surface by the length of the perimeter.

HRP-α-BGT: HRP (Worthington Biochemical Corp., Freehold, NJ) was coupled to α-BGT (Boehringer-Mannheim, Indianapolis, IN) with glutaraldehyde as described by Vogel et al. (55). Acetylcholine receptors were labeled by incubating muscles with 10⁻⁷ M HRP-α-BGT for 1 h at room temperature. Muscles were then washed in several changes in Ringer's for 10 min, fixed in glutaraldehyde, washed in 0.13 M cacodylate buffer (pH 7.3) for 5 min, and then incubated for 2 h at room temperature in 0.05% 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.02% H₂O₂ in cacodylate buffer. For light microscopy the muscle was dehydrated, cleared in xylene, mounted whole in Permount (Fischer Scientific Co., Fair Lawn, NJ), and viewed with bright field optics. Muscles prepared for light microscopy were also treated with 0.5% CoCl₂ in cacodylate buffer (15–30 min; reference 1), after glutaraldehyde fixation and before treatment with diaminobenzidine and H₂O, to enhance HRP stain. For electron microscopy muscles were immersed in osmium tetroxide (1% in 90 mM sodium phosphate, pH 7.0), stained en bloc with uranyl acetate (0.5% in 50 mM sodium maleate, pH 5.2), and embedded in Epon-Araldite. Thin sections were stained with lead citrate (54).

Freeze Fracture

Glutaraldehyde fixed muscles were treated with 25% glycerol in Ringer's for 30 min. Pieces were cut out and mounted between two gold alloy planchets made to fit a Balzers double replica device. The pieces were then frozen by immersion for 10 s in liquid propane maintained at liquid nitrogen temperature. They were fractured with the double replica device in a Balzers freeze etch apparatus at -110°C (Balzers, Hudson, NH). Platinum-carbon replicas, determined to be 25Å thick by a quartz crystal monitor, were made with the gun angle at 45° to the tissue surface. After thawing, the replicas were coated with a thin film of 1% collodion in amyl acetate (60) to prevent them from shattering during subsequent handling. The tissue was then dissolved in household bleach. After washing repeatedly the cleaned replicas were picked up on Formvar-coated single-slot grids. To remove the collodion the grids were placed in amyl acetate for 2 h.

RESULTS

The structure of the frog's neuromuscular junction and the location of several molecular components involved in synaptic transmission have been described in detail elsewhere (e.g., see reference 41 for review); the arrangement of constituents germane to this study is as follows. The axon branch that innervates each muscle fiber terminates in an arborization of processes which are 1–3 µm diam and up to several hundred micrometers long (Fig. 2a). Each terminal process lies within a shallow gutter on the myofiber surface and is

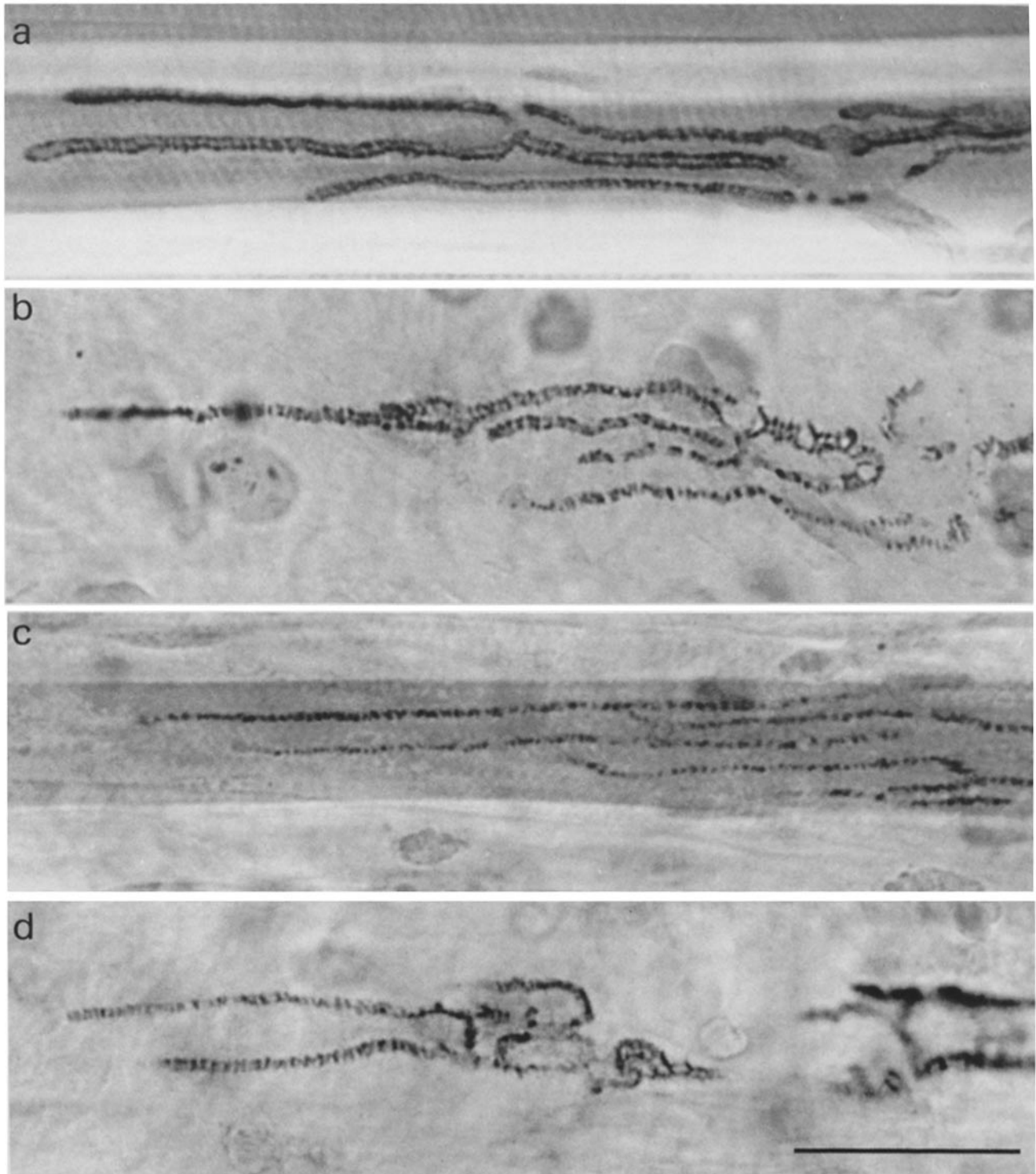
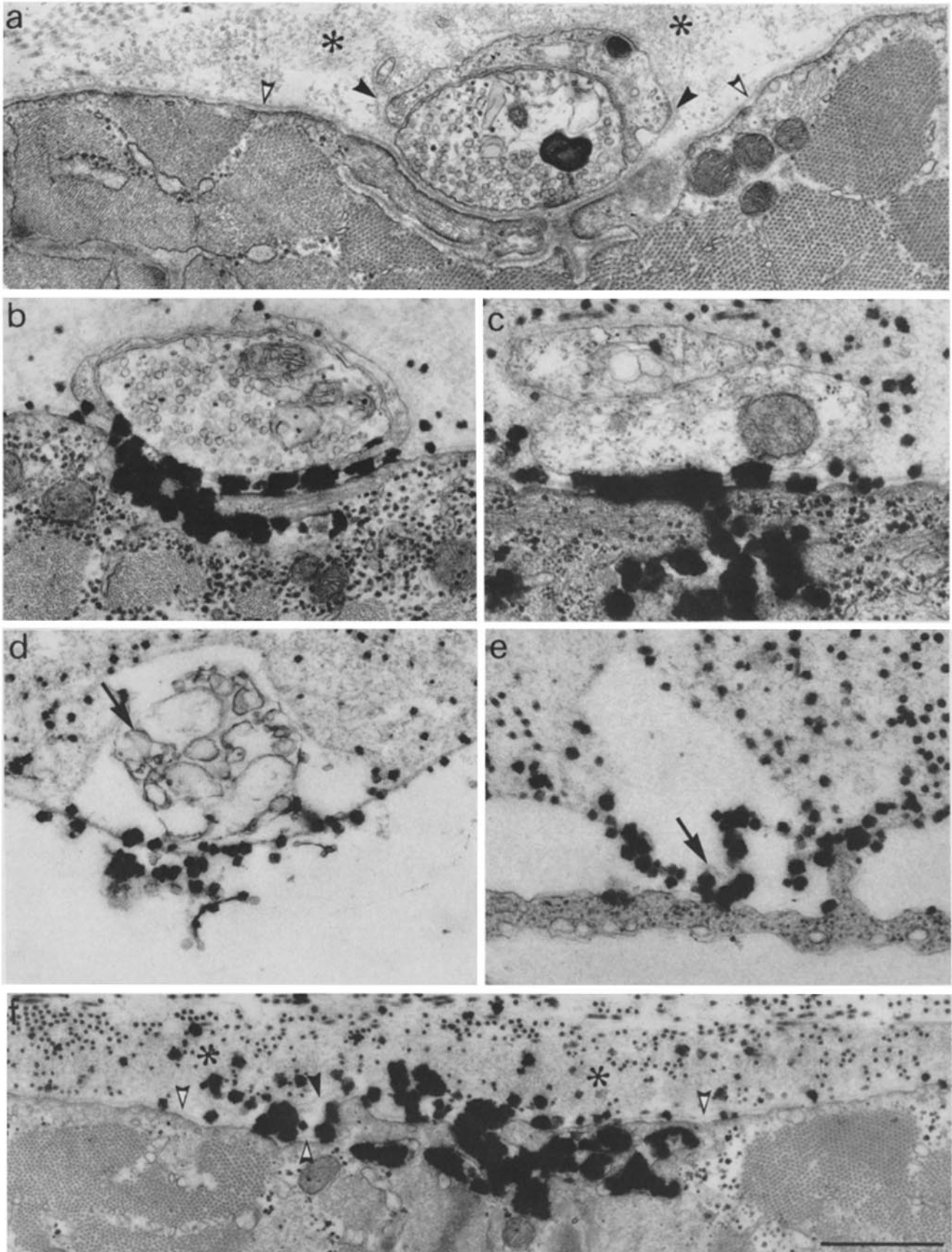


FIGURE 2 Cholinesterase stain marks the synaptic sites in muscles after damage by freezing. (a) Normal muscle. Each branch of the stained arborization is associated with an axon terminal branch (see Fig. 3 b). The narrow bands of stain within and orthogonal to each branch are due to the presence of junctional folds. Arborizations similar to those in normal muscles are present in basal lamina sheaths (b) 10 d after damage before new myofibers have formed, (c) at 30 d after damage when new myofibers have developed, or (d) at 30 d after damage if myofiber regeneration is prevented. Bar, 30 μ m.

FIGURE 3 In muscles damaged by cutting them at the boundary between junctional and extrajunctional regions, Schwann cells persist at synaptic sites on the myofiber basal lamina. Freeze damage to the region of innervation results in disintegration and removal of all cells from synaptic sites. New myofibers develop within the basal lamina sheaths of the original myofibers. (a) Normal neuromuscular junction. A vesicle-filled nerve terminal lies opposite a junctional fold in the myofiber and is capped by a Schwann cell. The basal lamina sheath (open arrowheads) of the myofiber in the synaptic cleft sends a projection into the junctional fold. The Schwann cell basal lamina (closed arrowheads) is characterized by a thick, loosely associated, particulate coat (asterisks). (b) Normal neuromuscular junction. ChE stain fills the synaptic cleft and obscures the basal lamina. (c) ChE-



stained synaptic site in a muscle damaged 30 d previously by cutting it at the boundaries between the junctional and extrajunctional regions. Schwann cell processes are situated opposite the regenerated myofiber. (*d* and *e*) ChE-stained synaptic sites 10 d after freezing muscles. The sites have disintegrated remnants (arrow) of the original synaptic cells. (*f*) ChE-stained synaptic site 30 d after freeze-damage, when a new myofiber had regenerated and there were no remnants of original cells at the synaptic site. Bar: (a and *f*) 1 μm ; (b–e) 0.6 μm .

~50 nm from the subsynaptic membrane. In general, the axon terminals run parallel to the muscle cell's long axis. Accordingly, when muscles are cross-sectioned, as illustrated in Fig. 3, profiles of terminals are ovoid and they occupy only a small fraction of the myofiber's perimeter. The muscle fiber plasma membrane just opposite an axon terminal is depressed at ~1- μ m intervals as in skeletal muscles of other vertebrates. In the frog, the mouths of the junctional folds are narrow and elongate; the long axis is orthogonal to the long axis of the axon terminal (Fig. 2*a*). The myofiber basal lamina sheath separates the axon terminal from the myofiber and projects into each of the junctional folds (Fig. 3*a*). Each terminal is capped by a Schwann cell process (Fig. 3, *a* and *b*). The Schwann cell also has a basal lamina and it is continuous with that of the myofiber at the edge of the junction. Associated with Schwann cell basal lamina is a coat of particulate material up to 2 μ m thick (41) that distinguishes the Schwann basal lamina from the basal lamina of the myofibers (Fig. 3*a*). Acetylcholinesterase, like AChRs, is concentrated along the path of each terminal branch (Fig. 2*a* and 3*b*). Unlike AChRs, which are integral proteins of the myofiber plasma membrane, at least some of the acetylcholinesterase is external to the membrane and tightly bound to the basal lamina (5, 24, 40).

Muscle fibers in the cutaneous pectoris muscle extend the full length of the muscle; thus, when the entire width of the muscle is damaged, as was done in this study, all of the muscle's myofibers are affected. The neuromuscular junctions are confined to a 3–4-mm wide zone across the middle one third of the muscle, which we refer to here as the junctional region (Fig. 1*a*). The extrajunctional regions of the muscle comprise a 3-mm wide region on each side of the junctional region.

The types of muscle preparations we used and their purpose is as follows:

(*a*) Normal muscles.

(*b*) Muscles in which the junctional region was briefly frozen or (*c*) crushed (Figs. 1, *b* and *c*). These procedures resulted in disintegration and phagocytosis of all original cells at the synaptic sites on myofiber basal lamina sheaths and development of new myofibers within the sheaths by 10–11 d after damage. The new myofibers were formed from mononucleated cells that migrated into the junctional region after it had been damaged. Such mononucleated cells are probably the muscle satellite cells that normally are situated within the myofiber basal lamina sheath (10, 53), those in extrajunctional regions having escaped damage. The body of this work documents that cells of the synaptic sites are damaged and removed after freezing or crushing the junctional region of the muscle and that AChRs in the membrane of new myofibers accumulate preferentially at the original synaptic sites on the sheaths.

(*d*) Muscles that were damaged simply by cutting the myofibers at the border of the junctional and extrajunctional region (Fig. 1*d*). This procedure resulted in degeneration of the original myofibers and development of new myofibers in the junctional region, but unlike the situation in frozen and crushed muscles, the Schwann cells (and muscle satellite cells in the junctional region) were spared (Fig. 3*c*). This preparation was described in previous studies (15, 37, 49) where it was shown that AChRs in the membrane of regenerating myofibers accumulate at synaptic sites on the basal lamina in the absence of nerve terminals. Relevant results from those

studies are presented again here. We used this preparation to compare the extent of AChR accumulation on regenerating myofibers when Schwann cells were undamaged with the extent of accumulation when the Schwann cells were deliberately removed by freezing the junctional region.

(*e*) Muscles whose junctional region was frozen and then separated from the extra-junctional regions of the muscle by cutting along the boundary between the two, which prevented development of new myofibers within the basal lamina sheaths for more than 1 mo. This preparation enabled us to examine the fate of AChRs that remain attached to basal lamina after removal of myofibers.

In all of the experiments, muscles were denervated at the time they were damaged and reinnervation was prevented.

Cholinesterase Stain as a Marker for Synaptic Sites on Basal Lamina after Muscle Damage

We used ChE stain to identify synaptic sites on basal lamina during degeneration of original myofibers and regeneration of new ones. As observed in muscles damaged by cutting them at the edge of the junctional region (15, 37, 49), ChE remained attached to the synaptic basal lamina for more than a month after freezing or crushing the junctional region. Fig. 2, *b–d* shows typical ChE staining patterns from the junctional region of freeze-damaged muscles during periods of myofiber degeneration and regeneration and when myofiber regeneration was prevented for 1 mo. In all cases they are similar to those in normal muscles (Fig. 2*a*). In general, the intensity of staining was somewhat diminished in damaged muscles and individual branches were less continuous than normal, but the arborization characteristic of normal neuromuscular junctions was always apparent. 350–500 arborizations were observed in each regenerating muscle that was damaged by freezing or crushing, which was in the range of arborizations found in normal muscles.

The regenerated muscles that had been damaged by freezing or crushing had patches of ChE stain other than the junctional arborizations. Never found in normal muscles, the nonjunctional patches, as illustrated in Fig. 4*a*, were usually ovoid having an average length of 25 μ m (SD 9) and an average width of 5–10 μ m, and they were scattered randomly (see below) along the length of the myofibers. The number of patches observed in 17 whole-mounted muscles, 4–5 wk after damage, ranged from less than 10 in some muscles to nearly 100 in others, which is much less than the number of junctional arborizations. (However, very small nonjunctional patches of ChE stain deep within the muscle might not have been detected in whole mounts.) Although the nonjunctional patches of ChE stain differed markedly from the junctional arborizations in size, shape, and distribution, they shared certain features. Like junctional arborizations, the nonjunctional patches of cholinesterase stain had an irregular staining pattern when viewed by light microscopy (Fig. 4*a*); electron microscopy of the patches revealed that the irregularity was due to infoldings of the myofiber plasma membrane (Fig. 4*b*) which also occur at junctional arborizations (see below). Moreover, we will show below that the myofiber membrane at the nonjunctional patches has a dense accumulation of AChRs as at junctional arborizations.

Much of our study on the removal of cells from synaptic sites on basal lamina after damage and of the accumulation of AChRs at these sites during regeneration was made on

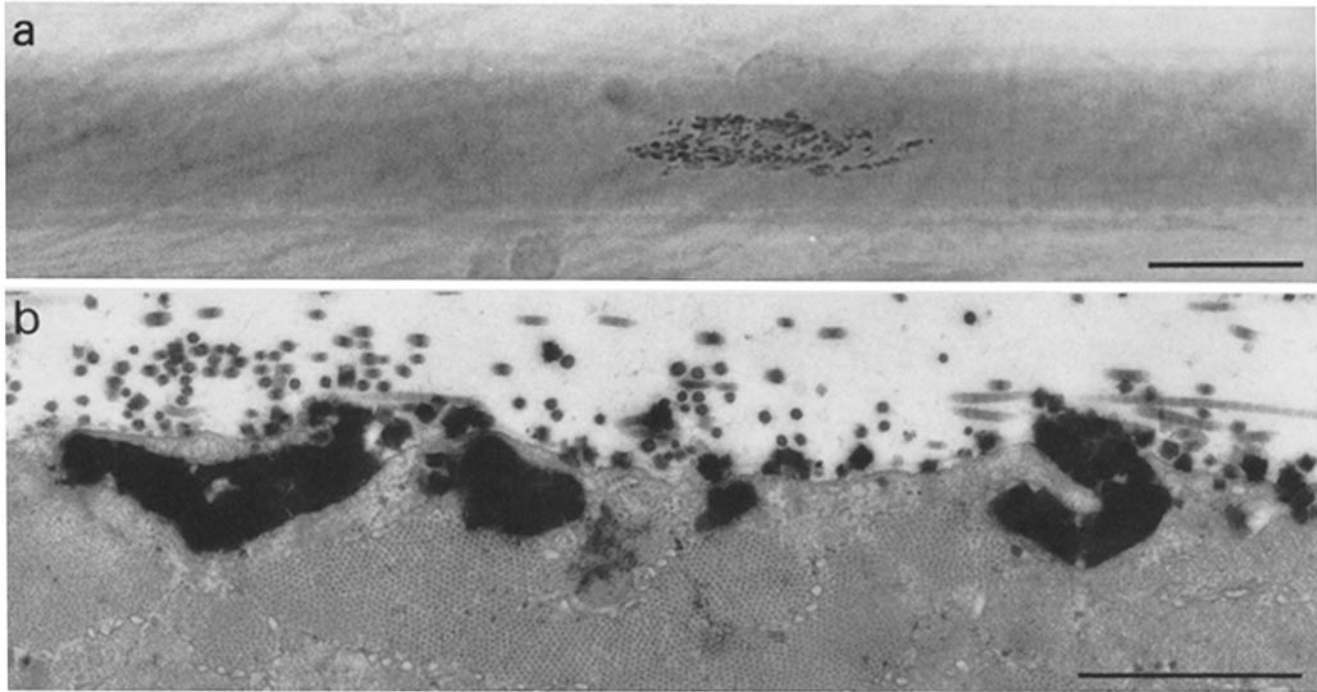


FIGURE 4 Nonjunctional ChE patch on a regenerated muscle fiber 30 d after freeze damage. (a) Whole mount. Bar, 40 μm . (b) Cross section through same patch seen in a revealing that the patch has an infolded membrane. Bar, 1 μm .

cross sections through junctional regions of muscles in which ChE stain was used to mark the synaptic sites. Each section contained several hundred muscle fibers and had a total of 50–100 ChE-stained spots, some fraction of which may have been nonjunctional patches present in the junctional region. To estimate this fraction, we examined the distribution of junctional and nonjunctional ChE staining in the following way. First we determined in whole mounts of 12 muscles, prepared 4–5 wk after freeze damage, the number of nonjunctional patches per unit area in junctional and extrajunctional regions. This density was highly variable both within and between muscles, but was not significantly different in the two regions. We therefore used the frequency of ChE spots in sections through the extrajunctional region to estimate the number of nonjunctional spots in the junctional region. From sections of 17 freeze-damaged muscles removed 4–5 wk after damage, we found the frequency of ChE spots per myofiber in the extrajunctional region to be 3% (SD 4%) of that in the junctional region. In five crush-damaged muscles prepared 35 d after damage and studied in this way, the ratio was <1%. We thus conclude that >95% of the ChE spots seen in sections through the junctional region of regenerating muscles used for this study mark original synaptic sites on myofiber basal lamina.

Disintegration and Removal of Cells from Synaptic Sites on the Myofiber Basal Lamina

Damaging the junctional region of muscles by freezing or crushing it resulted in disintegration of all cells at the synaptic site on the basal lamina within a few hours. We examined by electron microscopy ChE-stained spots from muscles that had been crushed, replaced in the frog, and then removed 4 h later. The muscles were incubated for 3 h in cationic ferritin (Polysciences, Inc., Warrington, PA; 10 mg/ml Ringer's) before fixation. Cationic ferritin is ~12 nm diam (47) and

normally enters cells only by endocytosis. Thus, when inside cells it is contained in membrane-bounded compartments. Its presence within the cytoplasmic ground substance after muscle damage would be indicative of disrupted plasma membranes. Synaptic sites from a normal muscle and a crushed muscle that were processed in parallel are shown in Fig. 5, *a* and *b*. Of 100 synaptic sites from two normal muscles treated with ferritin, the cellular components were structurally similar to those in untreated muscles; in the few terminal profiles where intracellular ferritin was observed, it was in one or two vesicles. At all of the more than 200 synaptic sites examined in four crushed muscles, there was clear evidence of cellular disintegration. On the presynaptic side of the basal lamina, disrupted and disorganized membrane profiles were present, and, when evident, the cytoplasmic ground substance was patchy. In no case could we distinguish the Schwann cell from the nerve terminal or identify the boundaries between them. In all cases there was a high density of ferritin particles in the area once occupied by the intact nerve terminal and Schwann cell. The particles were not confined to membrane compartments confirming that the plasma membranes had been disrupted. Further evidence that crushing the junctional region had disrupted Schwann cells was that each of 12 Schwann cell nuclei we could identify contained ferritin particles, a situation never found in the muscles from normal animals. All muscle fibers in these preparations also had disrupted plasma and cytoplasmic membranes and ferritin had invaded the cytoplasmic ground substance (Fig. 5*b*).

Similar findings have been made in denervated and freeze-damaged muscles by D. R. Edginton, D. P. Kuffler, and U. J. McMahan (manuscript in preparation). Using the ferritin treatment protocol presented above, they observed the same signs of cell disintegration we have described on both pre- and postsynaptic sides of the basal lamina at all of several hundred synaptic sites examined.

By 30 d after freezing or crushing the muscles, all fragments

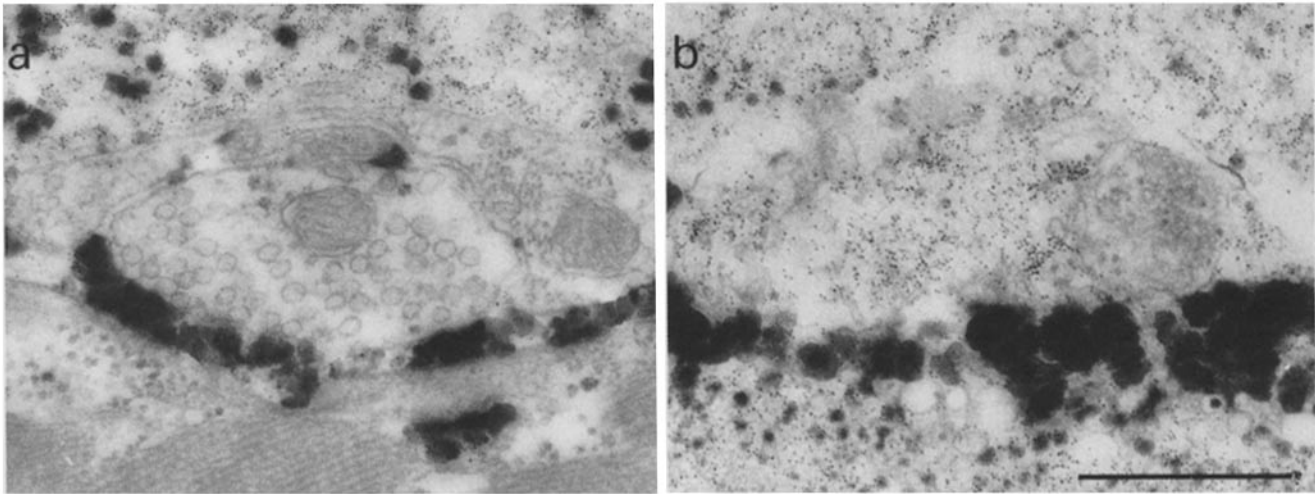
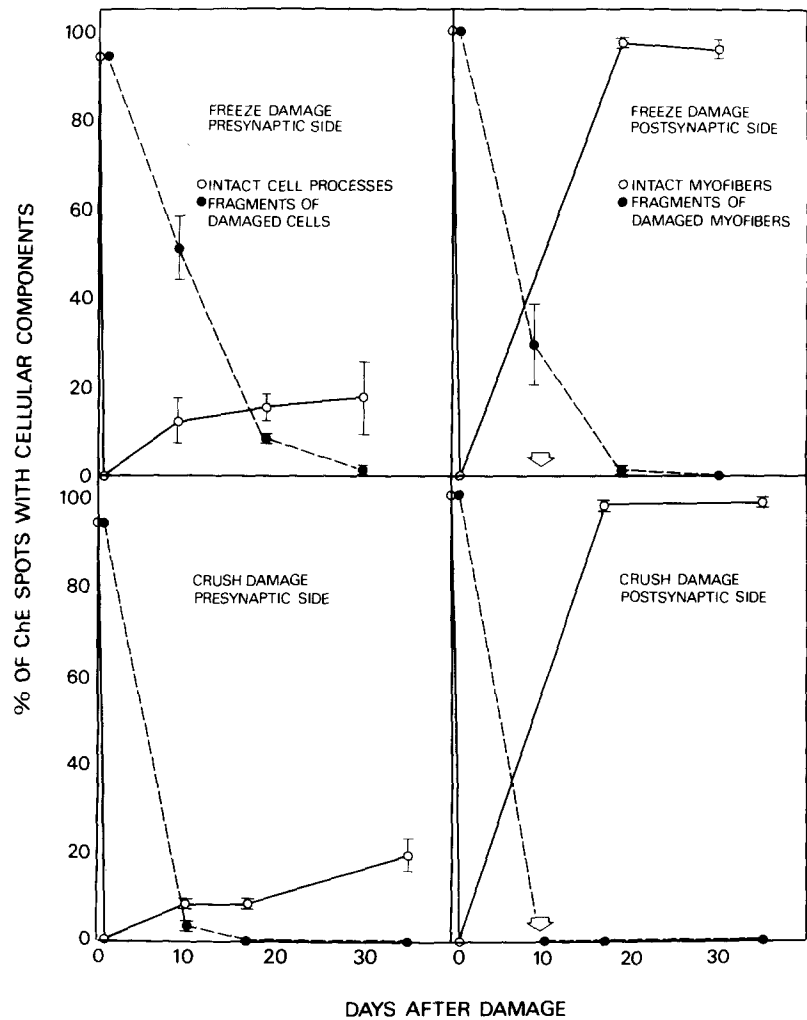


FIGURE 5 Crushing the region of innervation results in disintegration of all original cells at the synaptic site. (a) Normal neuromuscular junction. (b) Neuromuscular junction removed from frog 4 h after muscle was crushed. Both normal and crushed muscles were incubated in Ringer's containing cationic ferritin for 3 h before fixation and staining for ChE. In the crushed muscle the plasma and cytoplasmic membranes are disrupted and disorganized, the boundary between the Schwann cell and nerve terminal is not evident, and ferritin has invaded the cytoplasm of all cells confirming that the plasma membranes are no longer diffusion barriers. Bar, 0.5 μm .

FIGURE 6 Time course for removal of disintegrated cells from synaptic sites on the basal lamina and reappearance of new cells at these sites. Synaptic sites in all preparations were marked with ChE stain. Each point for damaged muscles represents at least 40 cross-sectioned sites from each of three freeze-damaged or 10 crush-damaged muscles. Error bars, SEM. Points for normal muscles taken from reference 49 and points for 3 h after damage are based on the finding that all cells were disrupted (see text) at this time. Arrows indicate the time when regenerating muscle fibers were first seen in a few of the basal lamina sheaths. The percentage of sheaths occupied by myofibers at this time could not be accurately determined because our method of identifying myofibers by the presence of clusters of thick and thin filaments was not reliable during early stages of development. For determining the percentage of sites with intact processes on the presynaptic side of the basal lamina after damage, we scored as positive any ChE spot that had a process within the Schwann cell basal lamina sheath. At some ChE spots the Schwann cell basal lamina was not evident or could not be clearly identified; in these cases we scored as positive sites those that had a process within 2 μm of the ChE stain.



of damaged axon terminals, Schwann cells, and myofibers discernible in the electron microscope had been removed from nearly all synaptic sites (Figs. 3 *f* and 6).

Regeneration of Muscle Fibers and the Reappearance of Live Cell Processes on the Presynaptic Side of the Synaptic Basal Lamina

Regenerating myofibers, identified by organized arrays of thick and thin filaments (Fig. 3*f*), were first observed within a few basal lamina sheaths 10–11 d after freezing or crushing the muscles. By 17–19 d, 98% of the basal lamina sheaths having ChE-stained spots, as observed in cross sections through the junctional region, contained regenerating myofibers (Fig. 6). Since nearly all these spots marked original synaptic sites (see above), the sheaths were those of the original myofibers. At this time, the myofibers nearly filled the basal

lamina sheaths and the myofiber plasma membrane lay within a few tens of nanometers of the basal lamina throughout much of the synaptic region. A similar proportion of original basal lamina sheaths were occupied by new myofibers at 30 d after damage (Fig. 6).

At 9–10 d after damage, while many synaptic sites still had debris of the original cells (Figs. 3, *d* and *e*, and 6), ~10% of the sites had intact profiles of cellular processes on the presynaptic side of the basal lamina (Figs. 6 and 7). In three freeze-damaged muscles examined in detail, the intact processes at half (8/16) of the synaptic sites that had them were traced through holes in the basal lamina to mononucleated cell bodies situated on its postsynaptic side (Fig. 7*a*). Holes in the basal lamina were also seen at some of the synaptic sites in crushed muscles 10 d after damage and these too were often occupied by cellular processes. Thus, while freezing and crushing the muscle causes disintegration and removal of the

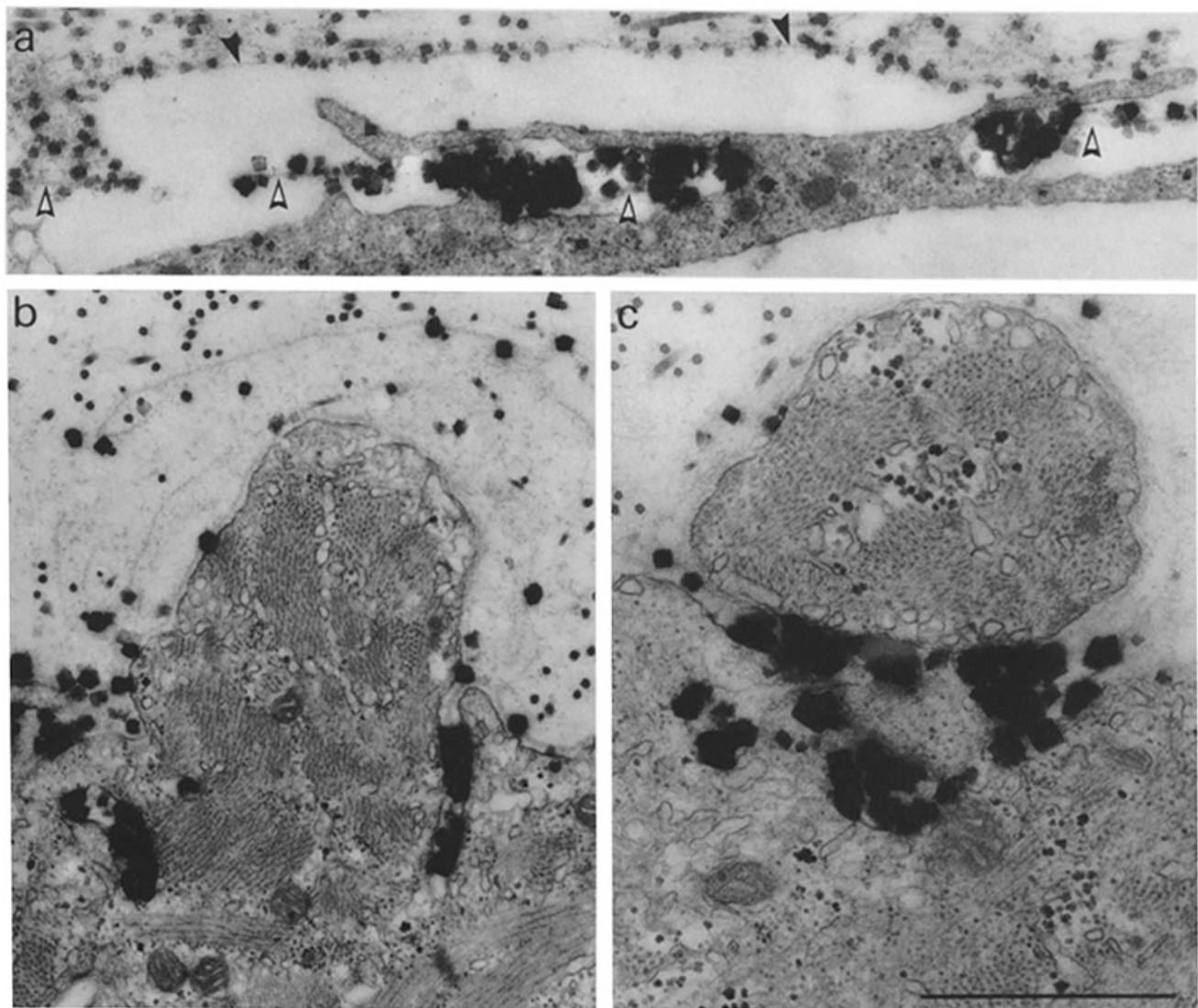


FIGURE 7 Cellular processes that come to occupy the presynaptic side of the basal lamina are often from cell bodies situated on the postsynaptic side. (a) 10 d after freezing, a mononucleated cell (nucleus not shown) on the postsynaptic side of the myofiber basal lamina extends processes through holes in the ChE-stained synaptic portion of the myofiber basal lamina (open arrowheads). Schwann cell basal lamina, closed arrowheads. (b) 19 d after freezing, a finger of a muscle fiber extends through a hole in the ChE-stained synaptic basal lamina. (c) 19 d after freezing, a profile of a muscle fiber, characterized by clusters of thick and thin filaments, occupies the position of a nerve terminal on the presynaptic side of ChE-stained basal lamina, just opposite a myofiber. Bar, 1 μ m.

original cellular components of the synapse, it also results in the formation of holes in the synaptic basal lamina through which cellular processes can pass.

At 17–30 d after freeze or crush damage, when new myofibers occupied nearly all basal lamina sheaths (Fig. 6), the synaptic sites with intact processes on the presynaptic side of the basal lamina increased to ~15–20% of normal. In three freeze-damaged muscles examined in detail 19 d after freezing, about half (18/34) of the “presynaptic” processes were traced through holes in the basal lamina to myofibers lying on the postsynaptic side of the sheath. Usually these processes were without cytoplasmic specialization but in some cases, they contained well defined clusters of thick and thin filaments characteristic of the body of the muscle fiber (Fig. 7*b*). Some presynaptic profiles not connected to muscle fibers on the postsynaptic side of the basal lamina also had these features clearly identifying them as portions of myofibers (Fig. 7*c*). Thus, while intact cellular processes come to occupy the presynaptic side of the basal lamina at a small fraction of the synaptic sites during the period when new myofibers are developing, at many, if not all, of these sites they are processes of the muscle cells themselves.

AChRs Accumulate at Synaptic Sites on the Basal Lamina in Regenerating Muscle

To study quantitatively the distribution of AChRs on regenerated muscle fibers, we labeled the receptors by incubating isolated muscles with ^{125}I - α -BGT. The location of bound ^{125}I - α -BGT in cross sections of muscle was determined by light microscopic autoradiography and was related in the same sections to the position of synaptic sites as marked by ChE stain (see Materials and Methods).

FREEZE-DAMAGED MUSCLES

We conducted seven experiments, involving a total of 90 muscles, in which we compared the density of autoradiographic silver grains at ChE spots in normal muscles with that at ChE spots in freeze-damaged muscles at various times after damage. The results are summarized in Figs. 8 and 9. During the first 8–11 days after damage, while the original cellular components were being removed but before most of the new myofibers had begun to develop, the mean grain density for ChE spots fell to ~10% of its normal value (mean of five experiments involving 23 muscles 10.4%, SD 3.3%). After 4–6 wk, when the new myofibers had developed, the grain density at ChE spots was on average ~50% of that at synaptic spots in normal muscles although it varied considerably from experiment to experiment (mean of six experiments involving 27 muscles 53.3%, SD 23.6%).

A substantial fraction of the mean grain density at ChE spots 4–6 wk after freezing resulted from a selective accumulation of AChRs in the plasma membrane where new myofibers made contact with the original synaptic sites on the basal lamina sheaths. The density of AChRs that accumulated at the synaptic sites was on average 10–15-fold greater than the AChR density in extrajunctional regions. We reached these conclusions after accounting for several factors in the regenerating muscles that could have contributed to the value for the mean grain density at synaptic sites. The importance of each of these factors was examined in detail in the two most extensive experiments of our series, and is described in the following paragraphs and Table I.

EMULSION BACKGROUND: The density of grains in the emulsion not over sections of muscle was 1–2/1,000 μm^2 . In both experiments this represented 0.6% of the grain density at normal synaptic sites. The contribution of this background has been subtracted from all the values in Table I.

NONJUNCTIONAL CHE SPOTS: As already mentioned, ChE spots that were clearly nonjunctional were present in both junctional and extrajunctional regions of regenerated muscles at equal frequencies. When determined from cross sections through the extrajunctional region, this frequency, expressed as ChE spots per muscle fiber cross section, varied considerably from muscle to muscle. When the results from all the muscles in each of the two experiments were pooled, the frequencies were 20/3,238 and 1/1,519 and represented 5.4 and 0.4% of the frequency of ChE spots in the junctional region.

The average length of cross-sectioned nonjunctional ChE spots in the extrajunctional region (8.4 μm , SD 6.4 μm) was more than three times that of spots in the junctional region (2.7 μm , SD 1.4 μm) as expected from the dimensions seen in whole mounts (see above). The grain density associated with the nonjunctional spots was close to that at neuromuscular junctions in normal muscles included in the same experiment (86%) and thus appreciably higher than the average observed in the junctional region of damaged muscles.

To allow for nonjunctional spots when estimating the mean grain density at synaptic sites, we used the frequency of ChE spots in the extrajunctional sample to calculate the expected number of nonjunctional spots included in the sample from the junctional region. We then removed that number of the most heavily labeled spots from our sample and used the remaining data to calculate the grain density at junctional sites. The effect of this was much greater in one of the experiments than in the other, as seen in Table I.

UNIFORMLY DISTRIBUTED AChRs: A higher than normal density of AChRs is present uniformly over the surface of regenerated frog muscle fibers, as in immature and denervated intact fibers (14). In our experiments the density of grains in extrajunctional regions of regenerated muscles (excluding clusters present at ChE-stained spots) was at least five times greater than in normal muscles, where it was indistinguishable from the emulsion background. It represented ~2% of the density at normal synaptic sites. A somewhat higher value for regenerated muscles (5–6% of that at normal junctions) was obtained in early experiments (e.g., references 41 and 42) where extrajunctional grain densities were measured in ChE-free portions of muscle membrane from junctional regions. In those experiments, grains produced by ^{125}I -associated with AChRs at nearby ChE spots may have caused the higher values.

RESIDUAL AChRs: The next question was whether all of the grains at synaptic sites were generated by ^{125}I - α -BGT bound to AChRs in the plasma membrane of the new myofiber or whether some were generated by toxin-binding sites that were not eliminated during the phagocytosis of muscle fibers. As indicated above, ~10% of the normal density of grains was present at ChE spots on basal lamina sheaths 8–11 d after freezing. Since new muscle fibers with their own AChRs begin to develop about this time, we could not follow the fate of these residual toxin-binding sites in regenerated muscles. To determine if such sites could persist for 1 mo after damage in the absence of myofibers, we blocked regeneration in several freeze-damaged muscles by cutting the

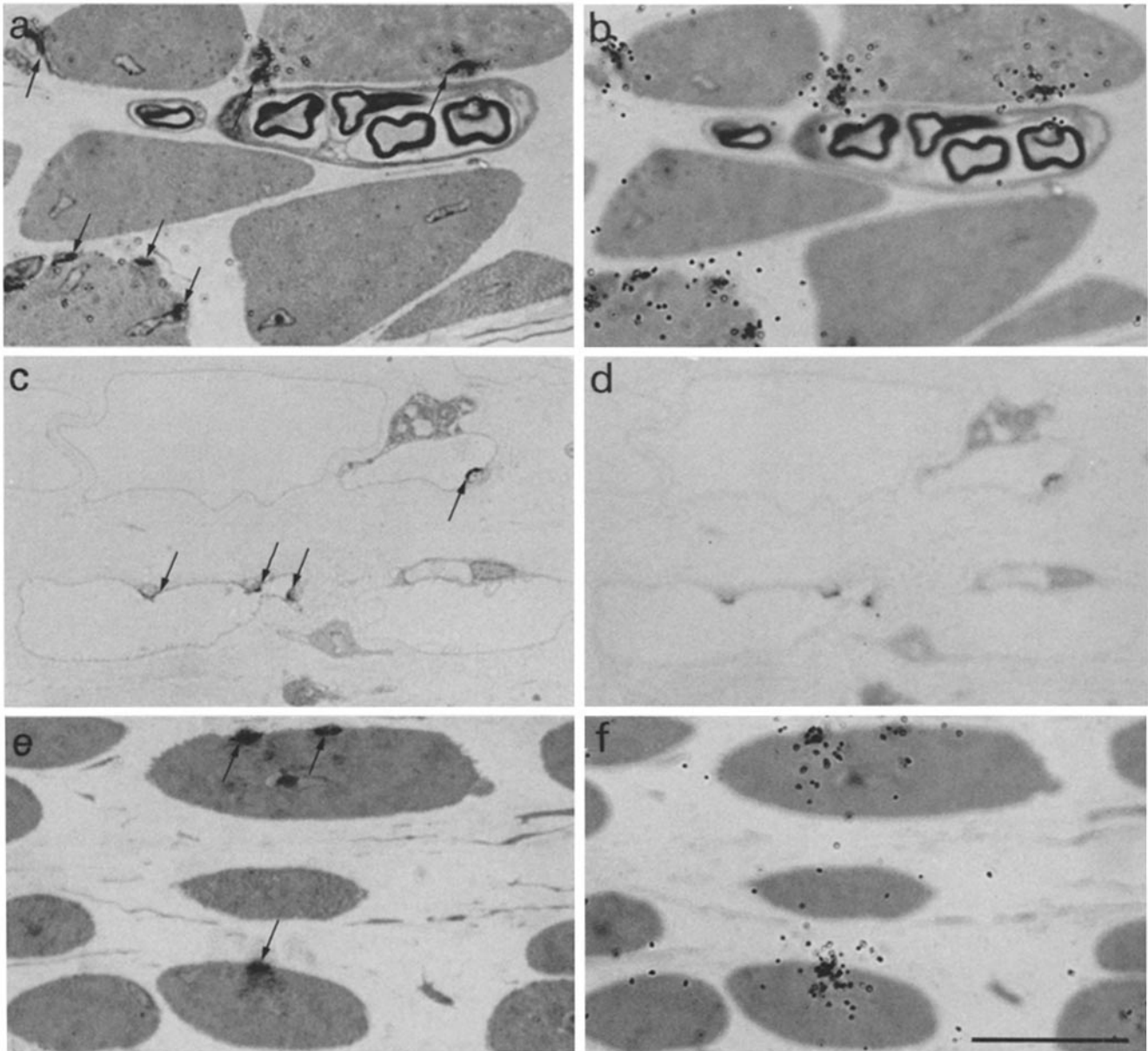


FIGURE 8 ^{125}I - α -BGT binding at synaptic sites decreases upon damaging muscles by freezing and increases after myofiber regeneration. (a and b) Normal muscles. (c and d) Vacated basal lamina sheaths 10 d after damage. (e and f) Denervated regenerated myofibers 30 d after damage. Synaptic sites are marked with ChE stain (arrows in a, c, and e, myofibers in focus), and ^{125}I - α -BGT binding sites on the same cross sections are marked by autoradiographic grains (b, d, and f, emulsion in focus). Bar, 20 μm .

muscles at the edge of the frozen region, thus preventing invasion of the damaged region by myogenic cells. In these muscles, examined 34 d after damage, the density of grains at ChE spots (mean of three muscles, 12% normal, SD 3%) was almost the same as at ChE spots observed at 11 d in a similarly prepared set of muscles (mean of five muscles, 7%, SD 3%) and in muscles routinely frozen but not cut (mean of nine muscles, 9%, SD 4%). Although we do not know whether the residual toxin-binding sites that produced these grains persist when muscles regenerate, the presence of such sites after 1 mo in unregenerated muscles warranted subtracting the grain density at 8–11 d from that in 4–6-wk regenerated muscles to obtain the net increase at synaptic spots.

When the contributions of all the factors described were subtracted from the observed grain density at ChE spots in

regenerated muscles in the two experiments analyzed in Table I, the calculated net increase in grain density at synaptic spots was five and 20 times greater than in the surrounding extra-junctional region. The main difference between the results of the two experiments was in the size of this increase rather than in the identified “background” factors. On this basis we can make a conservative estimate of the net increase in our entire sets of experiments by subtracting a combined background of 20% of normal (the larger value from the two experiments analyzed in Table I) from the mean of all the experiments (53%, see above). Thus our best estimate is that the density of AChRs within regenerating myofiber plasma membrane at synaptic sites on the basal lamina is on average about one third of that at normal neuromuscular junctions. This is some 10–15 times greater than the AChR density in

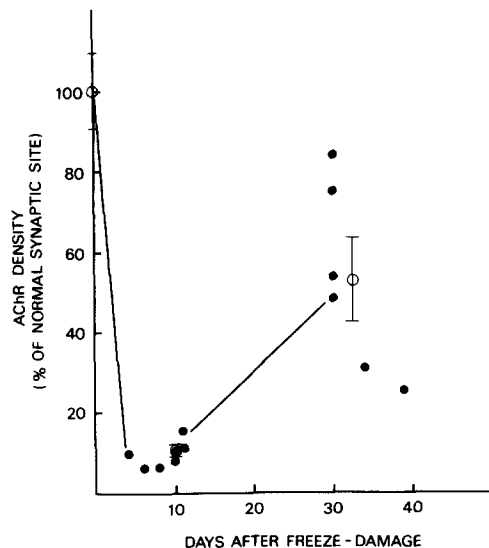


FIGURE 9 Density of AChRs at ChE-stained sites during degeneration and regeneration of myofibers after freezing. The binding of ^{125}I - α -BGT to AChRs at ChE-stained sites on the myofiber basal lamina was determined by autoradiography (see Materials and Methods). Values are expressed as a fraction of the density of grains present at synaptic sites in normal muscles in the same experiment. For each muscle the density was determined from analysis of 50–100 sites. Each point shows the mean of values from 2–10 muscles analyzed in a single experiment. The circles with error bars show the mean \pm SEM of the pooled results from normal muscles and those muscles 8–11 d and 4–6 wk after freezing.

TABLE I

Factors Contributing to the Net Grain Density at Synaptic Spots in Regenerated Muscles 4–6 wk after Freeze Damage

	Exp. 6		Exp. 7	
	%N	Balance	%N	Balance
Observed grain density at ChE spots	31.2	31.2	47.9	47.9
Nonjunctional spots	7.7	23.5	2.6	45.3
Uniform extrajunctional AChRs	1.9	21.6	1.8	43.5
Residual (8–11 d after damage)	11.1	10.5	9.1	34.4

For each experiment the first column shows the contribution of the factor indicated and the second shows the balance of the observed grain density after subtracting that contribution. All values are expressed as a fraction of the mean grain density observed at synaptic sites in normal muscles analyzed in the same experiments (%N). The number of muscles used in the experiments were as follows (exp. 6, exp. 7): normal (8, 8); residual (4, 8); 4–6 wk after damage (7, 6).

the extrajunctional region, a ratio that is significantly different from 1 (Student's *t* test comparing mean ratio of six experiments with 1, $P < 0.01$).

MEMBRANE FOLDING: Our determinations of grain densities at ChE spots were made from light microscope camera lucida drawings of the perimeter of myofibers on which we had marked the extent of ChE spots and the position of grains. While many of the irregularities of the myofiber surface can be seen with the light microscope, especially narrow folds would not be detected. This raises the question of whether the increase in grain density at junctional ChE spots between 10 and 30 d is due to an increase in density of AChRs per unit area of plasma membrane or to extensive folding of the myofiber membrane in the region of the synaptic sites. We examined this problem by determining with

the electron microscope the true extent of membrane folding in synaptic and extrasynaptic sites and comparing this with what we observed in the light microscope. Electron micrographs of regenerated muscle fibers in four muscles were made at the same magnification as that used for making camera lucida drawings of autoradiograms. The perimeter of the myofibers was traced and measured as it would have been in the camera lucida studies. Then, using a magnifying lens, we traced and measured the true perimeter of the myofibers, which included folds whose width was below the resolving power of the light microscope. The ratio of true length of myofiber plasma membrane to the length measured by light microscopy defined an index of folding. In our sample of 40 muscle fibers, (10 from each of the four muscles, which had a total of 60 ChE spots) the folding index was 1.13 (SEM 0.05) for ChE-free membrane and 1.28 (SEM 0.03) for ChE spots. Thus the extent of folding undetected with the light microscope was only 13% greater at ChE spots than elsewhere on the muscle fiber surface.

We conclude that the 10–15-fold net increase in grain density at synaptic spots on regenerating myofibrils is almost entirely due to an increase in the number of AChRs per unit area of muscle cell surface.

CRUSH-DAMAGED MUSCLES

We performed an experiment, involving 70 muscles, in which changes in AChR distribution were studied in muscles damaged by crushing rather than freezing (see Materials and Methods). The results of this experiment (Fig. 10) were similar to those just described. At 10 d after damage, when phagocytosis was essentially complete and new myotubes were first detected, partially occupying only a few basal lamina sheaths (Fig. 6), the mean grain density at ChE sites was 5% of normal. At 35 d, when nearly all the sheaths were almost fully occupied by myofibers, the mean density was 33% of normal. In the regenerated muscles in these experiments, ChE-labeled spots with clusters of autoradiographic grains were not found in the extrajunctional region. Thus, the net increase in AChR density at synaptic sites, calculated as in the previous section, was 26% of normal, a value not significantly different from that in the freeze-damage experiments.

We also examined crush-damaged muscles 17 d after damage (Fig. 10). At this time new myofibers were already present in nearly all the sheaths and the mean density of grains at synaptic sites was 47% of normal. Thus, the accumulation of AChRs at synaptic sites must occur within 1 wk after new myofibers form.

AChR Aggregation at Synaptic Sites Is Not Correlated with the Presence of Cell Processes on the Presynaptic Side of the Basal Lamina

Freezing or crushing muscles resulted in disintegration of all original cells at synaptic sites but 1 mo after damage, when new myofibers had developed, on the average 15–20% of the synaptic sites again had cellular processes on the presynaptic side of the sheath. At many of these sites (more than half in at least some preparations, see above) the processes were myofiber fingers that projected through holes in the basal lamina to take a presynaptic position. Since we could not account for the source of all the “presynaptic” processes, some of which could have belonged to Schwann cells that had

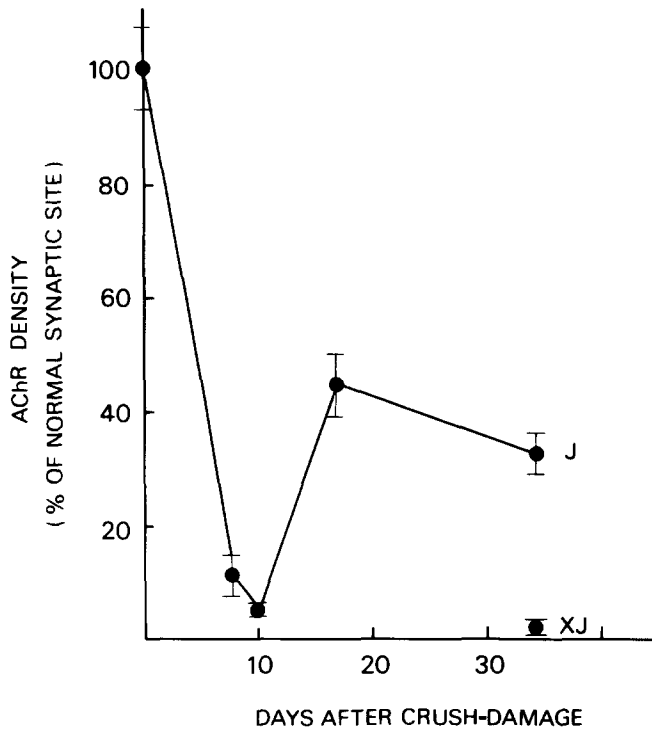


FIGURE 10 Density of AChRs at ChE-stained sites during degeneration and regeneration of crush-damaged muscles. AChR density at junctional ChE spots (J) determined as in Fig. 9. The values are the mean \pm SEM of 6–10 muscles. The uniform extrajunctional AChR density (XJ) at 35 d is also shown.

migrated from nerve bundles in undamaged regions of the muscle, and since the processes were the only structures at the synaptic site other than the basal lamina that could have caused the aggregation of AChRs, we searched for a correlation between the percentage of a muscle's synaptic sites that had processes, determined by electron microscopy, and the effectiveness of the synaptic sites in accumulating AChRs, determined by ^{125}I - α -BGT autoradiography as described above. The results of two sorts of experiments indicate that there is no obvious correlation.

First, we plotted the mean AChR density for each of the twenty 17-d and 35-d muscles that had regenerated after crush damage against the fraction of each muscle's synaptic sites that were occupied by one or more presynaptic processes (Fig. 11). The relationship between the two factors was highly variable and the correlation coefficient (<0.2) was not significantly different from zero.

Second, we ran in parallel a set of freeze-damaged muscles and a set of muscles that had been damaged by cutting the myofibers at the boundary between the junctional and extra-junctional region, which spared Schwann cells. In six cut muscles removed 5 d after damage, just prior to the regeneration of new myofibers, 77% (mean of six muscles, SD 10%) of the synaptic sites had cellular processes on the presynaptic side of the basal lamina. After 30 d, 63% (mean of five muscles, SD 10%) of the synaptic sites had presynaptic processes (Fig. 3c). Thus, we were able to compare the mean AChR density at synaptic sites in 30-d regenerated muscles that had more than half of the synaptic sites occupied by presynaptic processes during the entire period of regeneration with that in muscles that gradually came to have only 15–20% of the synaptic sites occupied by such processes (see

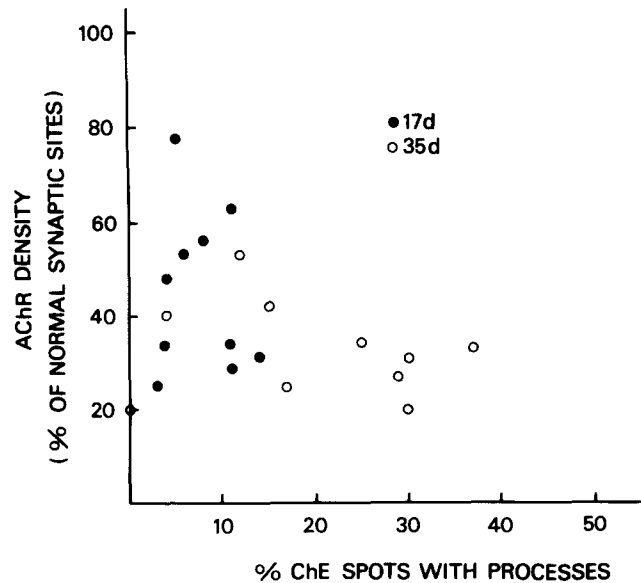


FIGURE 11 Lack of correlation between the presence of "presynaptic" processes and the extent of AChR accumulation. The extent of AChR accumulation was determined on muscles 17 d (dots) and 35 d (circles) after crush damage as in Fig. 10. The fraction of sites with processes on the presynaptic side of the basal lamina was determined with the electron microscope by examining thin sections adjacent to thick sections used for autoradiography. For each muscle, $>50\%$ of the ChE-stained spots studied for AChR localization were also examined for the presence of cellular processes.

above). The mean AChR density for both sets was nearly the same (48% of normal for freeze-damaged muscles and 38% of normal for the cut muscles), again indicating that there is no correlation between the presence of presynaptic processes and the accumulation of AChRs. (Other experiments [15] have shown that the density of AChRs at the synaptic sites in regenerated cut-damaged muscles can reach near normal levels which can also occur in freeze-damaged muscles as shown here [Fig. 9].)

The Distribution of AChRs and the Presence of Folds in the Area of Myofiber Membrane That Underlies the Synaptic Basal Lamina

The observation that AChRs accumulate in the plasma membrane of regenerating myofibers where it lies adjacent to the synaptic basal lamina raises the problem of how the receptors are arranged in the "subs synaptic membrane." In normal muscles AChRs are nearly uniformly distributed along the synaptic basal lamina having the highest concentration at the crests and upper sides of the junctional folds (2, 38). The set of experiments we now describe revealed that the AChRs in the membrane of regenerated myofibers are usually not evenly distributed along the synaptic basal lamina but, rather, they are often situated in scattered clusters. They also showed that the area of myofiber membrane adjacent to synaptic basal lamina is infolded and that infolding of the myofiber membrane is selectively localized to this region as it is to synaptic sites in normal muscle.

HRP- α -BGT LABELING: A convenient way of seeing the distribution of AChRs in whole regenerated muscles is to label the receptors with HRP-conjugated α -BGT and then to stain for HRP. In freeze-damaged preparations treated in this way, we saw HRP-stained arborizations similar in size and shape

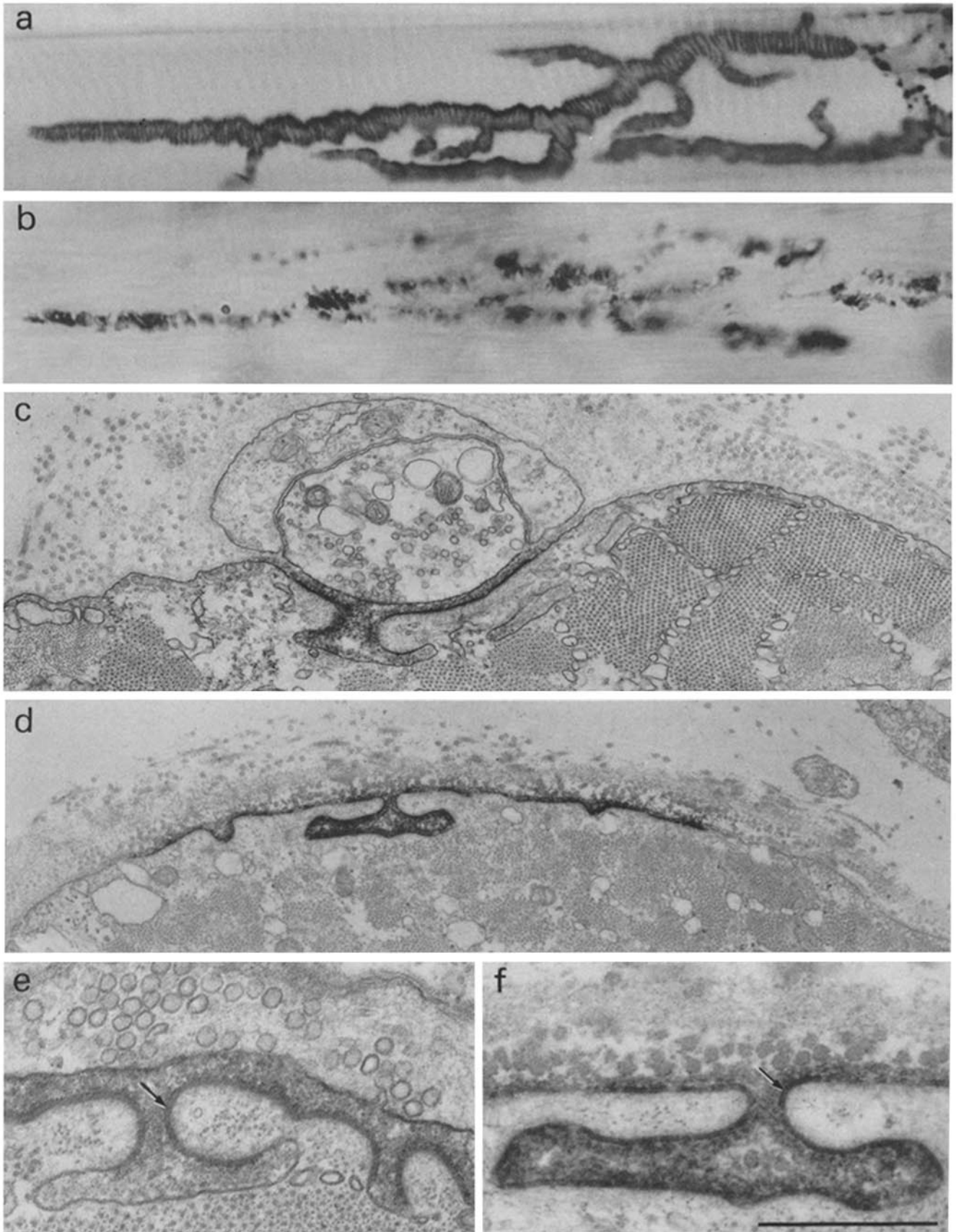


FIGURE 12 AChRs in muscles that have regenerated after freeze damage are situated in patches distributed along the synaptic basal lamina. The myofiber membrane at these patches is often folded. AChRs were labeled with HRP- α -BGT. (a) Synaptic arborizations from a normal muscle; whole mount. Bands of stain within and orthogonal to each branch are due to junctional folds. (b) Arborization from a 30-d regenerated muscle; whole mount. Staining along each branch is patchy. Some patches have bands indicative of folds. (c) Cross-sectioned normal neuromuscular junction with folds. (d) Cross-sectioned patch of AChRs on

to those of neuromuscular junctions in normal muscles (Fig. 12, *a* and *b*). The arborizations were confined to the junctional region of the muscles and when we used Nomarski differential interference contrast optics (see reference 39 for technical details) to visualize the empty basal lamina sheaths of axons, the sheaths were traced directly to the arborizations, leaving no doubt that the stained arborizations were at synaptic sites on the myofiber basal lamina. As expected from our ^{125}I - α -BGT studies, a few ovoid patches of stain (not shown) similar in size to the nonjunctional ChE patches described above were observed in both junctional and extrajunctional regions of the muscles.

At nearly all of the arborizations we examined in regenerated muscles, the staining differed from normal (Fig. 12, *a* and *b*). In many cases one or two branches were stained throughout their length, as in normal muscles, but other branches of the same arborization were defined by a series of small, ovoid (1–5 μm , long axis) patches which were separated from each other by several micrometers. In other examples, each branch of the entire arborization consisted of a series of isolated patches.

Within the HRP- α -BGT-stained patches, there were narrow, densely stained bands (Fig. 12*b*). Such bands in normal muscle result from the presence of junctional folds (Fig. 12, *a* and *b*). We confirmed that the myofiber membrane at HRP-stained sites in regenerating muscles is selectively folded by examining the stained sites with the electron microscope (Fig. 12, *c–f*). In electron micrographs of HRP-stained sites, an easily detectable dense leaflet of stain lined the external surface of the myofiber plasma membrane as at normal neuromuscular junctions. At 48 of 67 stained sites sampled there were one or more indentations of the plasma membrane. The dimensions of some of the folds were similar to those in normal muscles (Fig. 12*d*), but others were more shallow, and in some cases the mouths of the folds were more open than normal ones. Unstained regions of myofiber plasma membrane, which in our micrographs accounted for >90% of the total plasma membrane, were relatively smooth, having few folds comparable in width or depth (scored by eye).

FREEZE-FRACTURE STUDIES: The studies with HRP- α -BGT reveal that AChRs are situated in clusters at original synaptic sites on the basal lamina. Because of the limits of resolution of the HRP staining method, those studies do not establish whether or not the receptor clusters are actually within the plasma membrane of the regenerated myofiber or are clusters of AChRs that remain associated with the basal lamina after removal of the original myofibers (see above).

An alternate way of viewing the location of AChRs and folds in normal muscles is by freeze-fracturing the muscles and examining replicas of the interior of the plasma membranes. Freeze-fracture studies on normal muscle have shown that the P-face of the fractured membrane at the neuromuscular junction contains densely packed particles ~9nm diam, which, based on several sorts of evidence (e.g., references 28 and 30), are considered to be AChRs. The mouths of junctional folds are also evident in such replicas.

When we examined replicas of freeze-fractured regenerating

muscles that had been damaged by freezing, we found areas of myofiber plasma membrane that were characterized by several linear arrays of folds as at neuromuscular junctions (Fig. 13*a*). Unlike folds at normal neuromuscular junctions, those in regenerating muscles were often not deep enough to have been cross fractured and their mouths were often irregular in shape. As in normal muscles, the mouths generally ran at right angles to the long axis of the muscle fiber.

We confirmed that each series of folds within an array was adjacent to an original synaptic site on the basal lamina by freeze-fracturing regenerating muscles (30 d after freeze damage) that had been stained for ChE. In replicas from three muscles, we identified 15 myofibers with one or more series of folds. In every series, crystals of ChE stain were present in the extracellular matrix of cross-fractured folds and indented the plasma membrane between folds (Fig. 13*d*). The crystals of ChE stain were rarely found >1–2 μm beyond the edge of a series of folds but they often extended several tens of micrometers beyond the end of a series indicating that folds had not formed along the entire synaptic basal lamina (Fig. 14).

In addition to folds, there were also in regenerated muscles clusters of particles similar to AChR particles at normal neuromuscular junctions (Figs. 13, *b–d*, 14, and 15, *a* and *b*). The clusters, seen only on the P-face of the myofiber membrane, were highly variable in size and shape; on average they were 0.4 μm^2 in area. Within the clusters the mean density of particles was ~1,000/ μm^2 (see below), but the density in many patches was 4,000/ μm^2 . The density of AChR particles in our preparations of normal frog neuromuscular junctions ranged between 3,000 and 4,000/ μm^2 (others have reported densities as great as 6,000/ μm^2 ; reference 27) which is less than one half the density of AChRs estimated from ^{125}I - α -BGT autoradiographic studies (38). The inability to detect in normal muscles the density of AChR particles predicted by autoradiography is probably a result of inadequacies in freeze-fracture technology (26, 28). Thus, the density of AChRs in the most dense clusters on the regenerating myofibers may be much >4,000/ μm^2 and similar to the density of AChRs found at normal neuromuscular junctions.

It was clear from scanning many replicas that particle clusters were selectively localized to the vicinity of the folds and that they were often separated from each other by a distance of several micrometers. Fig. 13, *a* and *b* shows the remarkably close fit of particle clusters to several series of folds in a replica from a single muscle fiber.

We established the relationship of particle clusters to folds in 13 other muscle fibers (from three muscles) in the following way. We first made montages (magnification $\times 11,000$) of micrographs of the replica from each muscle fiber. The total length of the arrays of folds was on the average 170 μm , SD 131 μm), about one half the length of the total arborization of normal motor axon terminals in the cutaneous pectoris muscles (35). We then drew a reference line down the middle of each series of folds and outlined each cluster of particles in the montage. We did not use ChE staining for this experiment because the indentations in the membrane made by the

a 30-d regenerated myofiber with folds similar to normal. A thick particulate band of extracellular material similar to that which characteristically coats Schwann cell basal lamina (see Fig. 3, *a* and *f*) lies adjacent to the stained patch. (*e* and *f*) Stain is distributed throughout the basal lamina at synaptic sites in both normal (*e*) and regenerated (*f*) muscles. A dense leaflet (arrows) of stain that characteristically lines the external surface at the myofiber membrane at neuromuscular junctions is also present on regenerated myofibers. Bar: (*a* and *b*) 20 μm ; (*c* and *d*) 1.4 μm ; (*e* and *f*) 0.5 μm .

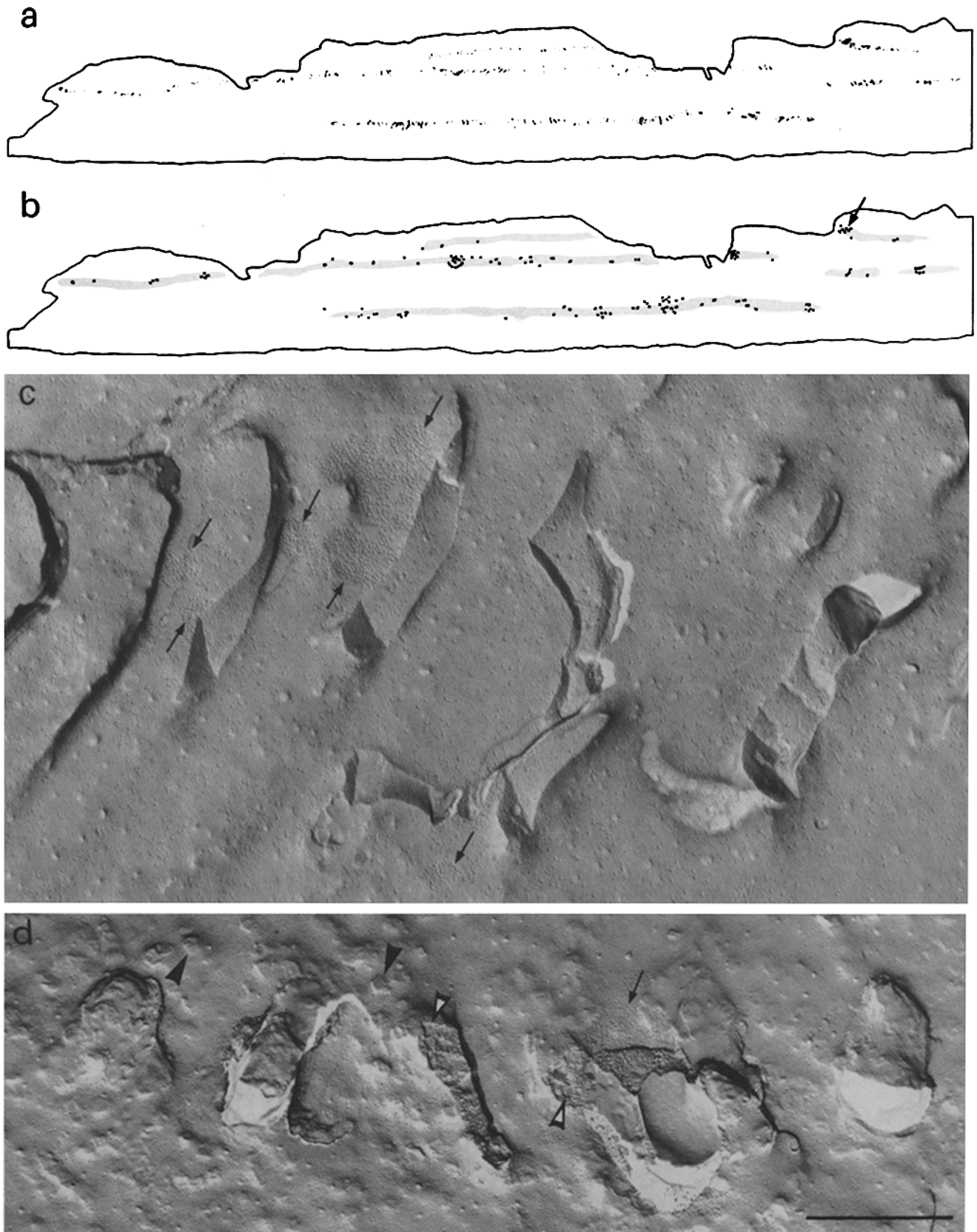


FIGURE 13 AChR particle clusters in freeze-fractured membranes of regenerated myofibers are situated at original synaptic sites on the basal lamina. 30 d after freeze-damage. (a) Tracing of a replica of a myofiber plasma membrane with arrays of membrane infoldings which are known to be situated opposite synaptic basal lamina (see below and text). (b) Same replica as in a. The position of each series of folds is indicated here by the shaded band and the position of AChR particle clusters in the myofiber membrane is indicated by dots. In a few instances dots were slightly shifted to prevent overlap. (c) Part of a fold series with associated particle clusters (arrows) from region at arrow in b. (d) Replica of myofiber plasma membrane from ChE-stained preparation. Crystals of stain in folds (open arrowheads) and indenting the plasma membrane (closed arrowheads) are evident indicating that this area of plasma membrane lies opposite the synaptic portion of the myofiber basal lamina. A particle cluster is at arrow. Bar: (a and b) 50 μm ; (c) 0.8 μm ; (d) 1.1 μm .

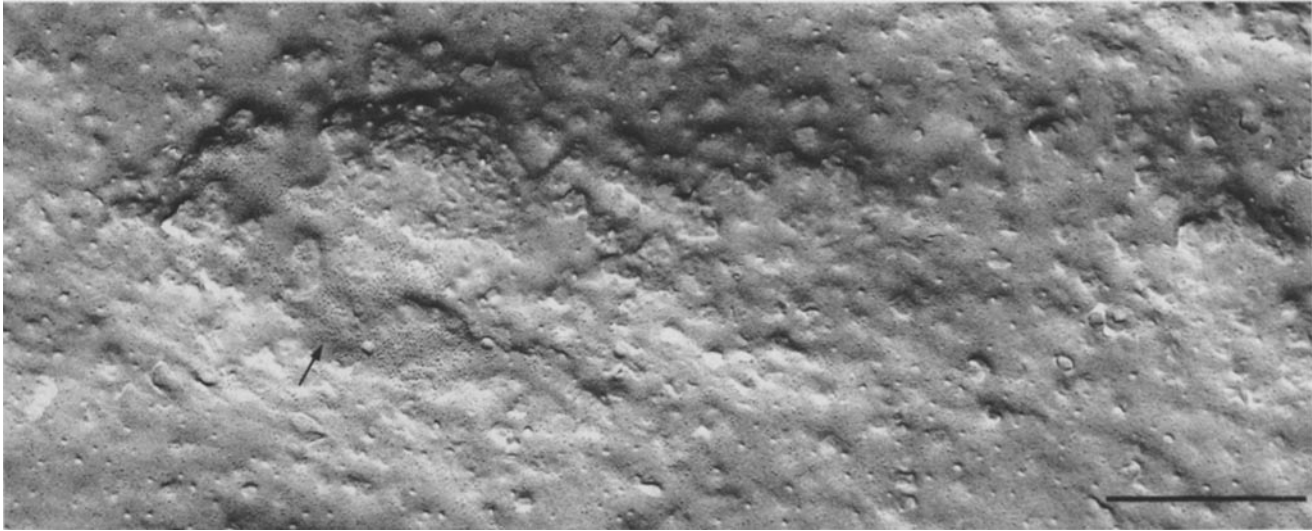


FIGURE 14 The formation of AChR-particle clusters at synaptic sites on the basal lamina does not require the immediate presence of membrane infoldings. The indentations in the plasma membrane of the regenerating myofiber (30 d after freeze damage) were produced by crystals of ChE stain used to mark synaptic sites in this preparation. Arrow marks a particle cluster near the end of a "stained" area. This stained area was part of an arborization; elsewhere in the arborization, beginning 45 μm away, there were plasma membrane infoldings as shown in Fig. 13. Bar, 1 μm .

crystals of stain tended to obscure AChR particle clusters; thus the line through the center of the folds, which were often a fraction of the length of normal folds, only approximated the true midline of the synaptic region of the basal lamina. Each outlined particle cluster contained at least 50 particles (mean 486, SD 459) and the average density of particles within the clusters (mean of 59 clusters, $1,093/\mu\text{m}^2$, SD $272/\mu\text{m}^2$) was four to five times greater than the mean density of particles outside the clusters, whether measured adjacent to folds (mean of 59 areas, $210/\mu\text{m}^2$, SD $118 \mu\text{m}^2$) or in extrajunctional regions of muscle (mean of 20 areas, $226/\mu\text{m}^2$, SD $67/\mu\text{m}^2$). (For these measurements we counted all particles regardless of size. However, it was our impression that had we limited our analysis to the relatively large $\sim 9\text{-nm}$ particles characteristic of AChRs, this ratio would be even greater.) When we measured the maximum distance of each cluster from the central reference line, we found that nearly 90% of all the clusters were entirely contained within a region extending 4 μm (true scale) to either side of the line, i.e., the region occupied by the folds plus $\sim 2 \mu\text{m}$ on each side of it. The density of clusters, whether expressed as the number of clusters per unit area or as the total area occupied by the clusters per unit area was more than 10 times higher within 4 μm of the center of the folds than in the immediately adjacent region of the replica (Fig. 16) and more than 100 times higher than on replicas from the extrajunctional region of 13 myofibers.

It is probable that many, if not all, of the clusters we observed just beyond the 4- μm limit from the center of the folds occurred in regions of myofiber membrane that were apposed to a stretch of synaptic basal lamina but lacked folds. In fact, in replicas of ChE-stained regenerated muscles, we encountered several examples of clusters that were associated with a region of stain several tens of micrometers beyond the end of a series of folds (Fig. 14).

In the course of our freeze-fracture studies, we examined replicas of several hundred muscle fibers and among them we recognized nearly 50 with synaptic sites characterized by arrays of folds and small clusters of particles as just described. We also observed in the membrane (P-face) of each of four

muscle fibers ovoid patches of particles similar to those at synaptic sites but much larger in area (Fig. 15, *c* and *d*). The clusters ranged from 7–10 μm along their short axis. They were observed in replicas from both junctional and extrajunctional regions of muscles. Like the clusters at synaptic sites they were associated with folds (Fig. 15 *c*). However, the folds were not lined up in series (Fig. 15 *d*). Based on their size, their relationship to folds, their distribution, and their frequency, these large clusters of particles must correspond to the clusters of AChRs associated with nonjunctional ChE patches described above.

In sum, our findings on freeze-fractured regenerated muscles complement those from the studies in which we used ^{125}I - and HRP- α -BGT. By labeling AChRs with ^{125}I - α -BGT we observed that AChRs in the plasma membrane of the developing myofibers accumulate in the narrow regions where the myofiber lies adjacent to the synaptic basal lamina. HRP- α -BGT labeling enabled us to view the distribution of AChRs within the region of the synaptic basal lamina and revealed that AChRs are often concentrated in clusters that are scattered along the synaptic site. If the AChRs in the clusters are components of the developing myofiber membrane and not residue of the original myofiber that remains attached to the basal lamina, one might expect to find in freeze-fracture preparations scattered clusters of AChR particles in the myofiber membrane at the synaptic sites. In fact, clusters of particles similar to AChR particles in normal muscle were clearly apparent in the replicas and they were selectively localized to the region of plasma membrane that was associated with the synaptic basal lamina. Our observations with α -BGT derivatives also predict that we should have encountered in the freeze-fracture replicas a few large, ovoid nonjunctional patches of AChR particles associated with plasma membrane infoldings, and we did.

DISCUSSION

We find that a high density of AChRs and an array of ordered folds form in the plasma membrane of regenerating myofibers

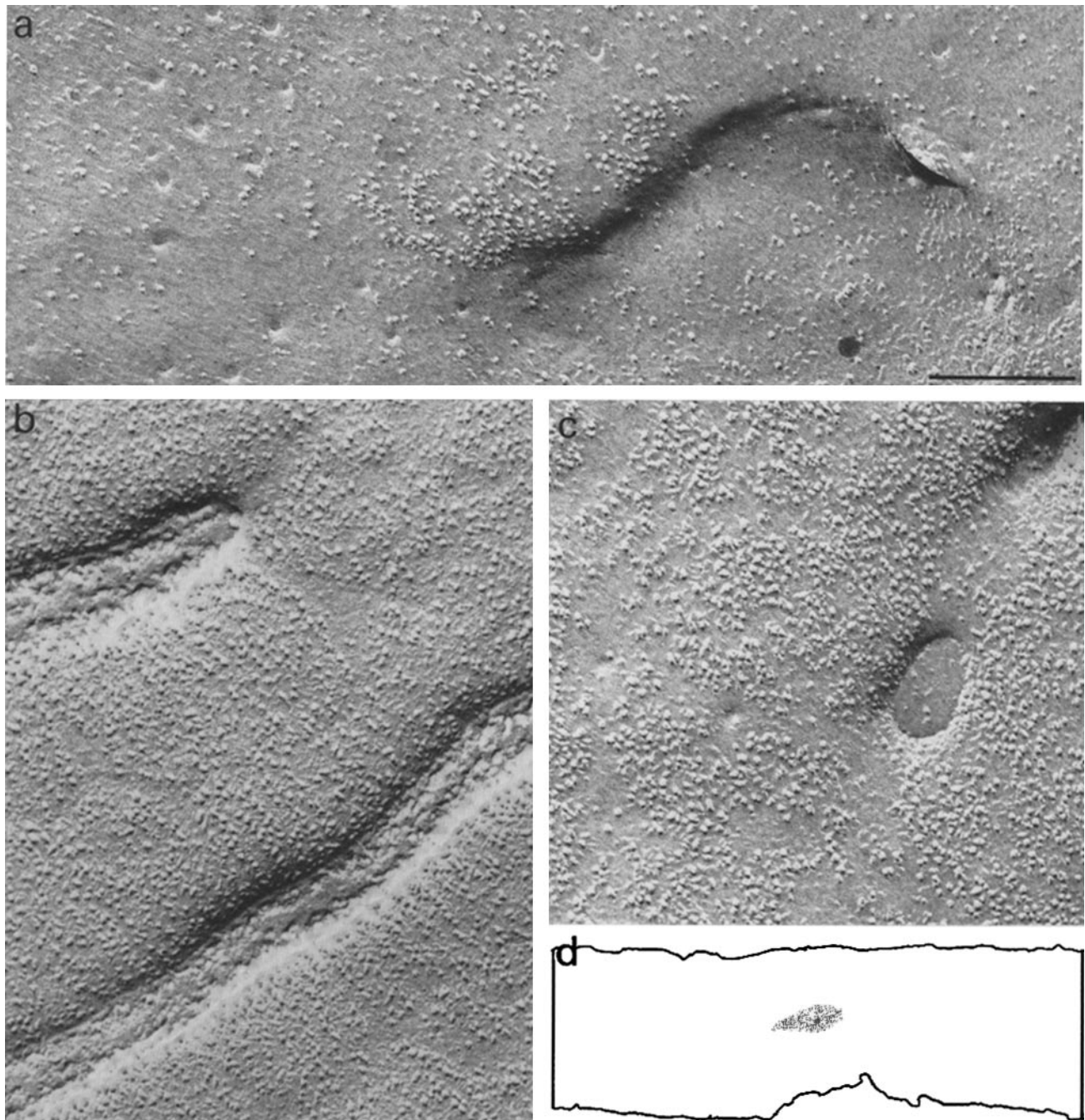


FIGURE 15 Particles that cluster in regenerating muscles are similar in size and shape to AChR particles in normal muscles. (a) Myofiber membrane at the synaptic site on basal lamina in 30-d freeze-damaged muscle. (b) Myofiber membrane at neuromuscular junction. (c) Myofiber membrane from extrajunctional region of 30-d freeze-damaged muscle. This nonjunctional patch of particles is associated with folds. (d) Tracing of the folds in the nonjunctional patch for comparison with the distribution of folds in the junctional arborization of Fig. 13 a; unlike those in junctional arborizations, folds in nonjunctional patches are not characteristically arranged in series. Bar: (a-c) 0.25 μm ; (d) 42 μm .

selectively at sites where the myofibers lie apposed to the synaptic basal lamina of the original muscle fibers, even after all the cellular components of the original neuromuscular junction have been destroyed. We conclude that the synaptic basal lamina directs the formation of these synaptic specializations.

Our evidence that all cells at synaptic sites in the frozen or crushed muscles disintegrated after damage is based on observations made with the electron microscope. At each of hundreds of synaptic sites fixed 7-8 h after damage, the

plasma membrane and the membranes of most organelles were severely disrupted and disorganized. Nerve terminals and their Schwann cell caps did not possess distinguishing characteristics and there was no obvious boundary between them. Ferritin in the Ringer's solution that bathed the damaged preparations entered the cytoplasmic ground substance of all cellular components of the synapse confirming that the plasma membranes were no longer diffusion barriers. There is no reason to believe that any of the cells at any of the synaptic sites ever recovered.

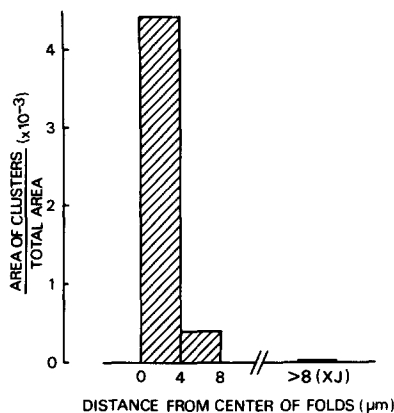


FIGURE 16 Particle clusters are selectively localized to original synaptic sites on regenerated myofibers 1 mo after freeze damage. The area occupied by particle clusters is expressed as a fraction of the total area analyzed either close to ($\leq 4 \mu\text{m}$) or farther from ($4\text{--}8 \mu\text{m}$) the center of the array of folds at original synaptic sites (see text for further details) or

from extrajunctional regions of regenerated muscles. Data were gathered from freeze-fracture replicas of a total of 192 clusters in the junctional region of 13 muscle fibers and from six clusters ($<1 \mu\text{m}$ diam) in the extrajunctional region of 12 muscle fibers. The total areas analyzed were $36,350 \mu\text{m}^2$ in the junctional region and $23,250 \mu\text{m}^2$ in the extrajunctional region.

Damaging muscles by freezing them or crushing them also resulted in holes in the synaptic basal lamina. Despite these disruptions much of the synaptic basal lamina when viewed by electron microscopy was intact. Accordingly, the pattern of cholinesterase staining in basal lamina, when viewed in whole mounts by light microscopy, was nearly the same as normal even 1 mo after damage.

Removal of fragments of the original cells by phagocytes required ~ 2 wk in crushed muscles; in frozen muscles it took somewhat longer. By 10 d after either type of damage, the average density of AChRs at synaptic sites, determined by labeling with $^{125}\text{I}\text{-}\alpha\text{-BGT}$, had been reduced to 10% of normal. Because in regeneration experiments new myofibers began to develop at this time and accumulate their own AChRs at the synaptic sites, we could not be certain that all of the original AChRs were eventually removed. However, in experiments where we prevented muscle from regenerating, $\sim 10\%$ of the normal $^{125}\text{I}\text{-}\alpha\text{-BGT}$ binding persisted at synaptic sites for at least 1 mo. This finding suggests that at least some AChRs in normal muscles are firmly attached to the basal lamina and retain that attachment after phagocytosis of damaged muscle fibers. Morphological studies on normal muscles involving techniques that reveal certain components of the basal lamina with great clarity have shown that filaments continuous with the basal lamina are closely apposed to AChRs (28). Such filaments may well be attachment sites for AChRs.

AChRs on regenerating myofibers preferentially accumulate at synaptic sites on the basal lamina within 1 wk after myofibers form in crush-damaged preparations. High concentrations of AChRs and membrane infoldings were present at synaptic sites in both crushed and frozen muscles within 3 wk. These synaptic specializations were situated in clusters that were distributed in an arborized pattern similar to that in normal muscles. Patches of myofiber membrane having a high concentration of AChRs, and infoldings and ChE (newly formed) were also situated opposite nonjunctional regions of the basal lamina sheath. They were apparently distributed randomly on the myofiber surface, and were far less frequent and generally much larger than those at synaptic sites. It is now well known that nonjunctional patches of synaptic specializations occur in muscle fibers under a variety of experimental conditions, both in vivo (12, 17, 32, 62) and in vitro

(3, 17, 22, 44, 46, 58). Our finding that nonjunctional patches of AChRs and folds in regenerating frog myofibers were not distributed in any specific pattern reveals that the muscle fiber is not programmed to lay out these sorts of specializations in the arborized pattern that is formed at the synaptic sites on the basal lamina sheaths. Accordingly, the formation of the junctional arborizations is probably not initiated by events that occur at a single point at or near the synaptic sites. More likely, the arborizations of AChRs are the result of myofiber-basal lamina interactions that take place at numerous points along the arborized pattern of synaptic basal lamina.

Even though our methods of damaging the muscle allowed myofibers to regenerate within the basal lamina sheaths of the original myofibers in the absence of nerve terminals and original junctional Schwann cells, cellular processes were again present on the presynaptic side of the basal lamina at an average of 15–20% of the synaptic sites by 30 d after damage. Many of the processes were from myofibers that had projected through holes in the synaptic basal lamina, but the source of others could not be established. Some of the unidentified processes could have belonged to Schwann cells that had been associated with axons in the intramuscular nerve bundles and, having escaped damage, used the empty basal lamina of the junctional Schwann cells as a guide to migrate to the synaptic sites. We did not examine the effects of crushing muscles on the Schwann cells of intramuscular nerve bundles. However, studies on freeze-damaged muscles using the same methods described here indicate that this form of damage killed all Schwann cells in the major nerve bundles of the frozen region as at the neuromuscular junctions (D. R. Edgington, D. P. Kuffler, and U. J. McMahan, manuscript in preparation). Whatever the source of the presynaptic cellular processes at the synaptic sites, there was no correlation in crush-damaged muscles between the number of synaptic sites that had processes, which varied widely from muscle to muscle, and the extent of AChR accumulation at these sites. Even when we damaged muscles in ways that spared 75% of the original Schwann cells, the synaptic sites were no more effective in organizing AChRs on regenerating myofibers than those of muscles where all of the original Schwann cells had been killed by freeze damage. These findings leave little doubt that it is the synaptic portion of the basal lamina itself that contains, or has stably associated with it, the molecules that interact with the myofiber to cause the aggregation of AChRs.

The influence of synaptic basal lamina in regenerating muscle on the accumulation of AChRs varied considerably from experiment to experiment and from muscle to muscle within individual experiments. This variation was observed regardless of the type of muscle damage. It could not be traced to seasonal changes, which are known to influence certain aspects of frog neuromuscular physiology (13, 36, 45, 59). However, even in experiments where the synaptic sites on basal lamina were least effective, the mean density of AChRs at synaptic sites was nearly five times greater than the mean uniform extrajunctional density; where basal lamina was most effective it was 30–40 times greater. The effectiveness of the synaptic site in organizing AChRs is probably dependent on several conditions including the stage of development of the regenerating myofiber, the distance between the myofiber and the basal lamina, and the amount of the AChR-aggregating signal that is present. Our freeze-fracture and HRP- α -BGT studies revealed that the AChRs that accumulate at synaptic sites can be concentrated in patches widely scattered along

the synaptic basal lamina, which demonstrates that even on a given muscle fiber the conditions for AChR organization are not uniformly met throughout the entire synaptic site. Some of these patches, when viewed in freeze fracture replicas, had the same density of AChR particles as at synaptic sites in normal muscles. Moreover, the density of ^{125}I - α -BGT binding sites at synaptic sites was in some cases as great in regenerated as in normal muscles. Thus, while the density of AChRs that accumulate along the synaptic basal lamina of a particular muscle fiber can vary widely from region to region, at some regions the synaptic sites may be as effective in organizing AChRs as the nerve terminal was during normal development.

The regular pattern of infoldings in the plasma membrane of regenerated myofibers at sites of contact with synaptic basal lamina was unlike anything observed in extrajunctional regions and was similar to that at normal neuromuscular junctions, although the individual folds were often more shallow and less regular in shape. The folds may form by a mechanical interaction between the plasma membrane and the tongues of basal lamina that formerly projected into the junctional folds of the original myofibers. There were long stretches of myofiber membrane apposed to synaptic basal lamina that were devoid of folds. Thus, as in the case of AChR accumulation, the requirements for the formation of the synaptic specialization were not met uniformly throughout the synaptic basal lamina. During the normal development of neuromuscular junctions, AChR clusters form prior to extensive membrane folding (16, 18, 19, 29). In our preparations of regenerating muscles, dense clusters of AChR particles seen by freeze-fracture methods were often scattered between and alongside folds. However some particle clusters occurred in the stretches of "subs synaptic" membrane devoid of folds. Thus AChR accumulation at the synaptic sites in the regenerating muscles, as in developing muscles, did not require the immediate presence of folds.

The studies presented here complement earlier ones carried out in this laboratory showing that the synaptic basal lamina on myofibers directs the formation of active zones in regenerating nerve terminals (49). Thus the basal lamina contains molecules that can influence the differentiation of both pre- and postsynaptic structures at regenerating neuromuscular junctions. The formation of the neuromuscular junction in normal development and its maintenance in adult muscles require communication between the nerve terminal and the myofiber (e.g., references 2, 7, 52, 61, 62). It may well be that the molecules in the basal lamina that direct synaptic differentiation during regeneration are produced as a part of this normal communication process. An alternate possibility is that the active molecules are not normally involved in synaptogenesis but are only active after muscle damage. Establishing the cellular source of these molecules, their role, if any, during normal synaptic development and/or maintenance, the nature of their association with other basal lamina molecules, how they direct differentiation, and how they in turn are regulated would be greatly aided by identifying the molecules and making specific markers for them. Studies aimed at identifying the molecules in the synaptic basal lamina responsible for organizing the AChRs are in progress (23, 48, 57).

We are grateful to Deborah Stairs for excellent assistance with photography and to Drs. Steven Burden and Peter Sargent who partici-

pated in initial experiments.

This study was supported by National Institutes of Health grant NS 14506 and a Muscular Dystrophy Association of America Senior Investigator Fellowship to C. R. Slater.

Received for publication 26 September 1983, and in not significantly revised form 28 December 1983.

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