Basal Lamina Directs Acetylcholinesterase Accumulation at Synaptic Sites in Regenerating Muscle

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ABSTRACT   In skeletal muscles that have been damaged in ways which 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 fibers are characterized by junctional folds and accumulations of acetylcholine receptors and acetylcholinesterase (AChE). The formation of junctional folds and the accumulation of acetylcholine receptors is known to be directed by components of the synaptic portion of the myofiber basal lamina. The aim of this study was to determine whether or not the synaptic basal lamina contains molecules that direct the accumulation of AChE. We crushed frog muscles in a way that caused disintegration and phagocytosis of all cells at the neuromuscular junction, and at the same time, we irreversibly blocked AChE activity. New muscle fibers were allowed to regenerate within the basal lamina sheaths of the original muscle fibers but reinnervation of the muscles was deliberately prevented. We then stained for AChE activity and searched the surface of the new muscle fibers for deposits of enzyme they had produced. Despite the absence of innervation, AChE preferentially accumulated at points where the plasma membrane of the new muscle fibers was apposed to the regions of the basal lamina that had occupied the synaptic cleft at the neuromuscular junctions. We therefore conclude that molecules stably attached to the synaptic portion of myofiber basal lamina direct the accumulation of AChE at the original synaptic sites in regenerating muscle. Additional studies revealed that the AChE was solubilized by collagenase and that it remained adherent to basal lamina sheaths after degeneration of the new myofibers, indicating that it had become incorporated into the basal lamina, as at normal neuromuscular junctions.

The portion of a skeletal muscle fiber's basal lamina sheath that lies in the synaptic cleft of the neuromuscular junction is highly specialized. Positioned so that the neurotransmitter, acetylcholine, must pass through it to reach the acetylcholine receptors in the muscle fiber's plasma membrane, it is composed partly of a network of molecules common to extrasynaptic regions of the sheath and to basal lamina associated with epithelial cells throughout the body (13, 15, 27, 43). However, it also contains acetylcholinesterase (AChE),[1] a heparan sulfate proteoglycan, and one or more uncharacterized antigens which are absent or in much lower concentration in extrasynaptic basal lamina (e.g., 1, 14, 34, 44).

The synaptic portion of the myofiber basal lamina in frog

has also been shown to have components that direct the formation of plasma membrane and cytoplasmic specializations at regenerating neuromuscular junctions. In muscles damaged in ways that spare the basal lamina sheaths, new muscle fibers develop within the sheaths and regenerating axons grow to form new neuromuscular junctions at the original synaptic sites on them (30). The synaptic regions of the sheaths induce the formation of active zones in axon terminals and cause the aggregation of acetylcholine receptors and the formation of infoldings in the plasma membrane of the regenerating muscle fibers (9, 32, 45).

The pre- and postsynaptic cellular specializations that develop at regenerating neuromuscular junctions are strikingly similar to those at normal neuromuscular junctions, which raises the possibility that the basal lamina molecules that cause them to form are similar to the molecules that mediate

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[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; MSF, methanesulfonyl fluoride.

the formation of such specializations during synaptogenesis in the embryo. The same molecules may also play a role in maintaining the specializations in the normal adult. Indeed, basal lamina and other extracellular matrix constituents appear to be actively involved in the formation and maintenance of cell structure and organization in a variety of tissues (5, 17, 21, 50, 56). Thus, studies on the composition and function of the myofiber basal lamina at neuromuscular junctions are of interest not only because they are requisite for understanding mechanisms involved in synaptic transmission and the restoration of neuromuscular function after trauma, but also because they may provide insight to how synapses develop in the embryo and how cells in other organs interact with components of their extracellular matrix.

The experiments we describe have extended our knowledge of the role of synaptic basal lamina in regeneration of neuromuscular junctions in damaged frog muscles. We demonstrate that the synaptic basal lamina causes AChE produced by regenerating myofibers to accumulate at the original synaptic sites in the muscles and provide evidence that the newly formed enzyme is a component of basal lamina as at normal neuromuscular junctions. A brief account of some of the experiments appeared elsewhere (2).

MATERIALS AND METHODS

Muscle Damage: We used the thin, paired cutaneous pectoris muscles which are situated just beneath the skin of the frog's (*Rana pipiens*) thorax. The muscles were damaged by crushing their junctional region as described elsewhere (32). In brief, both muscles were removed from a frog anesthetized with MS-222 (tricaine methane sulfonate) (Ayerst Laboratories, New York, NY) and were pinned out in a petri dish containing Ringer's solution (116 mM NaCl, 20 mM KCl, 1.8 mM $CaCl_2$, 10 mM sucrose, 1 mM NaH_2PO_4, pH 7.2). The fire-polished edge of a glass histological slide was repeatedly pressed at overlapping positions into the middle third of the muscles, where all of the neuromuscular junctions are situated and which is thus referred to as the muscle's "junctional region." The damaged muscles were then replaced in their bed in the thorax and the skin incision was sutured shut. To prevent reinnervation, a 0.5–1-cm segment of each muscle's nerve was resected near the muscle and a 1-cm segment of each second spinal nerve was removed near the vertebral column.

Staining for AChE: We used Karnovsky's staining method (24) for observing the distribution of AChE on myofibers. For normal muscles the staining medium contained 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferricyanide, 32.5 mM maleate buffer (pH 6), and 0.08 mM acetylthiocholine. For regenerating muscles the concentration of acetylthiocholine was increased 20-fold. Muscles were routinely pinned out in frog Ringer's solution, fixed for 20 min in 1% glutaraldehyde (pH 7.2) buffered with 0.09 M phosphate, stained for 10 min for AChE, refixed for 1 h in osmium tetroxide, and embedded flat in a wafer of Epon and Araldite. Since the muscle is ~100 μm thick, muscle fibers and AChE stain could be clearly seen in the whole mount by light microscopy. Thin sections for electron microscopy were counterstained with lead citrate and uranyl actetate. Incubation of whole muscles in Karnovsky's staining medium under the conditions used here reveals cell surface but not cytoplasmic AChE (28).

To inactivate irreversibly (25, 48) all original cholinesterase in the synaptic portion of the basal lamina and elsewhere so that it would not be detected histochemically in the regenerated muscles, we incubated the muscles in Ringer containing 5 mM diisopropylfluorophosphate (DFP) (Sigma Chemical Co., St. Louis, MO) or 10 mM methanesulfonyl fluoride (MSF) (Aldrich Chemical Co., Milwaukee, WI) at room temperature for 20 min prior to crushing them.

Karnovsky's method can stain both true AChE and pseudocholinesterase (24). The following experiments showed that most, if not all, of the staining we observed was due to true AChE. First, three normal and three regenerated muscles were incubated for 1 h in Ringer's solution containing 0.1 mM iso-OMPA (tetraisopropylpyrophosphoramide) (Sigma Chemical Co.), a specific inhibitor of pseudocholinesterase. The muscles were then incubated for 10 min (our routine staining period) in staining medium which also contained iso-OMPA. When examined in whole muscles by light microscopy or in sectioned muscles by electron microscopy, the amount of stain at synaptic sites was similar to that in muscles not treated with iso-OMPA (Figs. 1 and 2). Second,

three normal and three regenerated muscles were incubated for 1 h in Ringer's solution containing 1 μM BW284c51 (1,5-bis[4-allyldimethylammonium-phenyl]pentane-3-one dibromide) (Wellcome Reagent Ltd., Beckenham, England), a specific inhibitor of AChE. The muscles were then incubated for 20 min (twice as long as our routine staining period) in staining medium containing BW284c51. We observed no stain in the whole muscles by light microscopy. Electron microscopy revealed only a few scattered crystals of stain in the vicinity of synaptic sites, far less than the amount of stain at synaptic sites in muscles not treated with the inhibitor for AChE.

RESULTS

Acetylcholinesterase Accumulates at Former Synaptic Sites

By 30 d after crushing the junctional region of cutaneous pectoris muscles, all electron microscopically discernible fragments of the original cellular components of the neuromuscular junctions—the muscle fiber and the axon terminal with its Schwann cell cap—have been phagocytized and nearly all of the basal lamina sheaths of the myofibers are occupied by new muscle fibers (32). Accordingly, 1 mo after we crush-damaged and permanently denervated the muscles and treated them with DFP or MSF to block the original AChE, we removed the muscles from the frog, pinned them out, and fixed and stained them for AChE. In all of the 20 muscles (10 DFP-treated and 10 MSF-treated muscles) that composed our experimental set, we found by light microscopy patches of stain ~5 μm wide and up to several tens of micrometers long associated with each of hundreds of regenerated myofibers; on many myofibers in each muscle the patches of stain were arranged in a way that clearly resembled the arborized pattern of stain at normal neuromuscular junctions (Fig. 1, *a* and *b*). Nearly all of the AChE patches were confined to the junctional region of the muscles. When we used Nomarski differential interference contrast optics to visualize the empty basal lamina sheaths of axons that had occupied the intramuscular nerve bundles (see reference 33 for technical details), the axon sheaths were traced directly to patches of stain, leaving no doubt that most of the patches were at the original synaptic sites on the muscle fiber basal lamina.

We used electron microscopy to document that most of the stain in the regenerated muscles was at the original synaptic sites on the myofiber basal lamina sheaths. To locate original synaptic sites, we used the conspicuous extracellular matrix coat of the Schwann cells that cap the nerve terminals. It has been pointed out in electron microscope studies that the Schwann cells that cap nerve terminals have basal lamina (7). The Schwann cell basal lamina in frog also has associated with it a coat of particulate material as illustrated in Fig. 2a (see also references 26, 32, 35). At normal neuromuscular junctions, the particulate coats range up to 2 μm in thickness and thus can be 40-fold greater in thickness than the basal lamina of Schwann cells or myofibers. Such a coat is never found associated with extrasynaptic myofiber basal lamina, and like the basal lamina of myofibers and Schwann cells, it persists for more than a month after damage to and phagocytosis of axon terminals, myofibers, and Schwann cells (26, 32). Because of its thickness and persistance after damage, the Schwann cell's coat provided us with a convenient marker for the former synaptic sites on the myofiber basal lamina in regenerating muscles. To determine whether AChE preferentially accumulated at the former synaptic sites, we first made cross sections through the junctional regions of three regenerating muscles taken 30 d after crush damage and treatment
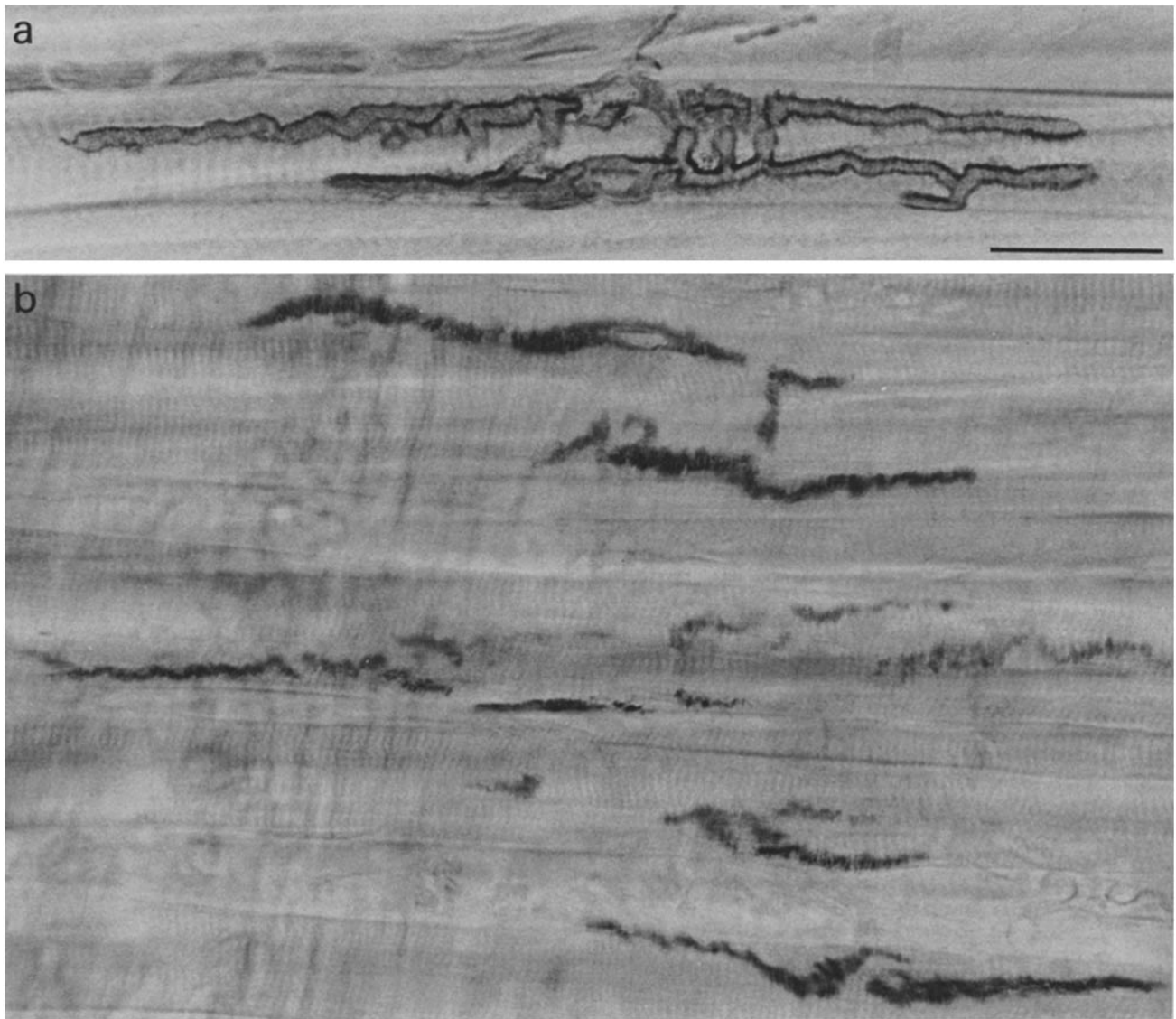
FIGURE 1   Distribution of cell surface AChE produced by regenerating myofibers that lack innervation is much like the distribution of the enzyme on the surface of normal muscle fibers. (a) Light micrograph of a neuromuscular junction in a whole mount of a normal muscle. Stain for AChE activity outlines the arborization of the axon terminal. (b) Former junctional region in a regenerated muscle 30 d after crushing the muscle, permanently denervating it, and irreversibly inhibiting the original enzyme with DFP. The new myofibers had developed within the basal lamina sheaths of the original myofibers. Stain for AChE activity is in thin elongate patches having an arrangement similar to the arborizations of stain at neuromuscular junctions. Bar, (a) 30 μm; (b) 40 μm.

with DFP, and then from each muscle we made low power electron micrographs which included the entire profile of muscle fibers having AChE spots. Our sample included 27–33 AChE spots from a total of 20 muscles fiber profiles for each muscle. An enlargement of one of these spots is illustrated in Fig. 2 b. Each spot was 5.9 ($\pm$ 3.7 [SD]) μm long (N = 90) and for each muscle fiber 11.3% ($\pm$ 6.8% [SD]) (N = 60) of its perimeter was occupied by AChE spots. Similarly, each Schwann cell coat (Fig. 2, b and c) on these myofibers overlay 6.4 μm ($\pm$ 3.2 [SD]) (N = 99) of the myofiber perimeter and for each myofiber 13.1% ($\pm$ 9.3% [SD]) (N = 60) of its perimeter was overlaid by coats. Despite the fact that the AChE spots and Schwann cell coats each occupied <15% of the myofiber perimeter, 86% of the spots were apposed to the coats. Indeed, for the AChE spots associated with coats, 99% ($\pm$ 2 [SD]) (N = 78) of the spot length was co-extensive with the coats. The high incidence of co-localiza-

tion of AChE spots with the coats of Schwann cell basal lamina clearly indicates that the AChE spots were preferentially situated at the original synaptic sites on the myofiber basal lamina; a random distribution of AChE spots would have resulted in only ~3% (instead of 86%) of the spots being co-extensive with the coats.

Other studies on crush-damaged muscles have revealed that infoldings form in the surface of regenerating myofibers preferentially at points where the myofibers are apposed to the former synaptic site on the myofiber basal lamina, and that such infoldings are similar to those found at normal neuromuscular junctions (32). Consistent with these findings, we observed that at 98% of the AChE patches just described the myofiber plasma membrane was infolded (Fig. 2, b and c).

We checked to make certain that the DFP and the MSF had blocked the activity of the original AChE at the synaptic sites in the following ways. First, we treated seven muscles
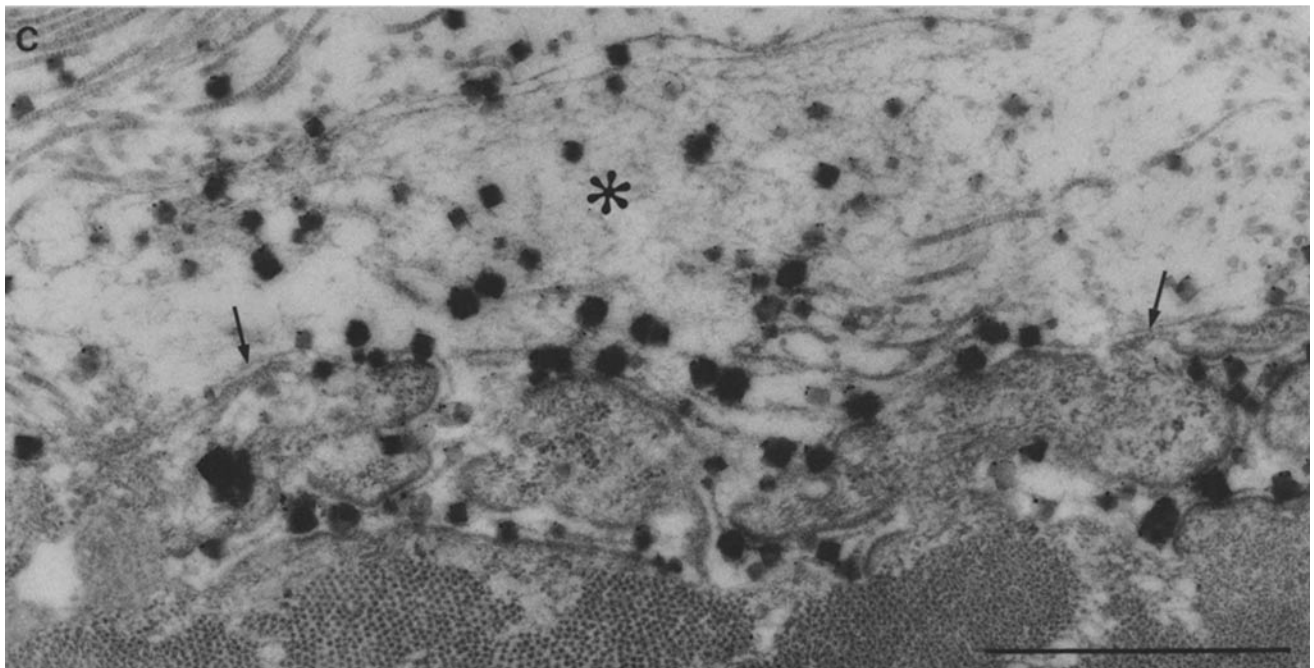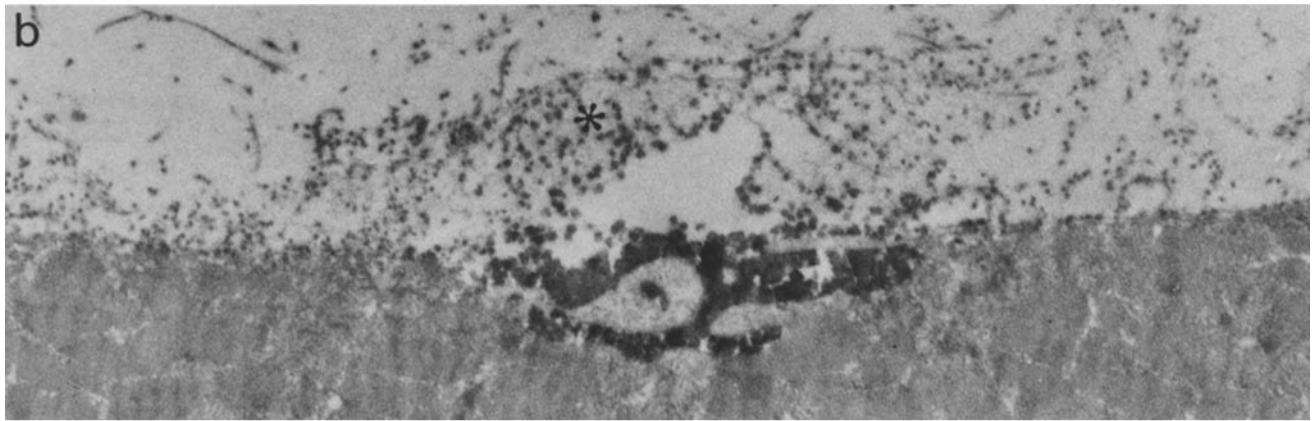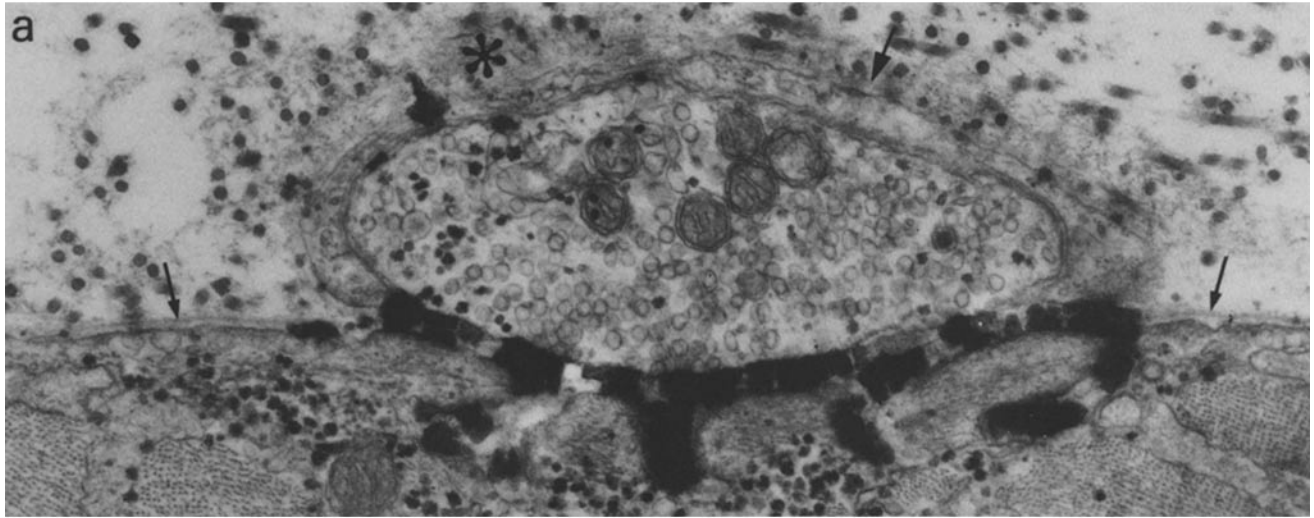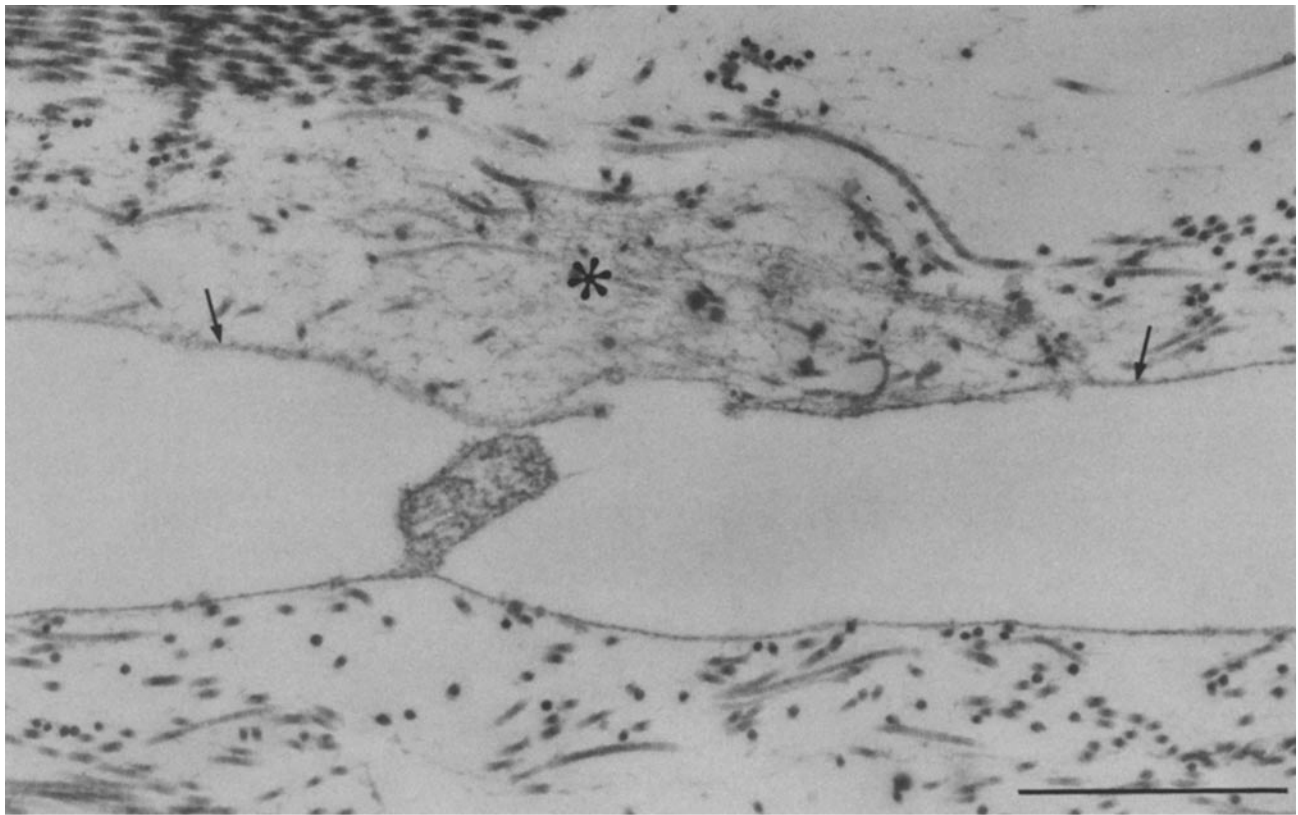
FIGURE 3 Treatment of crush-damaged muscles with AChE inhibitors blocks the activity of the original basal lamina AChE detected by our histochemical procedure. The synaptic site on a muscle fiber basal lamina sheath (arrows) 30 d after crush damage and enzyme inhibition with DFP is marked by the Schwann cell basal lamina coat (*). The muscle had been x-irradiated at the time of damage and DFP treatment to prevent regeneration of myofibers. Only a few small crystals of stain are associated with the synaptic site. Had the damaged muscle not been treated with DFP, staining of the synaptic site would have been nearly as great as at normal neuromuscular junctions (32). Bar, 1 μm.

with the AChE staining solution immediately after damaging them and exposing them to the DFP or MSF; we observed no staining in the whole mounts. Second, muscles were crush-damaged, treated with DFP ($N = 5$) or MSF ($N = 5$), and replaced in the frog's chest as usual. However, the frogs were x-irradiated once a day (2,400 rad) for 3 d after the operation to prevent muscle fiber regeneration (45). 1 mo later, whole mounts of the empty basal lamina sheaths still showed no AChE staining. We examined by electron microscopy 105 synaptic sites, identified by the presence of Schwann cell basal lamina and its coat, on myofiber basal lamina sheaths in three of these "muscles." At some synaptic sites there were a few crystals of stain (Fig. 3), but in no case was the staining nearly as great as that found at any of the synaptic sites in the

regenerating muscles. Finally, we noticed in whole mounts of a few damaged DFP-treated muscles that had not been x-irradiated there were small areas where the muscle fibers had failed to regenerate; we observed no AChE staining in the junctional region of these empty sheaths with the light microscope. Altogether these results demonstrate that most, if not all, AChE detected at the synaptic sites in muscles regenerating after crush damage and treatment with cholinesterase inhibitors is formed by the new muscle fibers, rather than being a residue of the original neuromuscular junction.

Accumulation Is Directed by Basal Lamina

We examined by electron microscopy cross sections

FIGURE 2 AChE produced by myofibers regenerating within the basal lamina sheaths of the original myofibers accumulates where the myofiber plasma membrane is apposed to the former synaptic sites on the sheaths. (a) Neuromuscular junction in a normal muscle. Crystals of AChE stain are concentrated in the synaptic cleft and junctional folds obscuring the myofiber basal lamina (small arrows) at these points. The Schwann cell basal lamina (large arrow) is continuous with a thick particulate coat (*) of extracellular matrix which distinguishes it from the myofiber basal lamina. (b) Regenerating myofiber 30 d after the muscle was crushed and permanently denervated, and the activity of the original enzyme had been blocked. The former synaptic site on the myofiber basal lamina is marked by the presence of the Schwann cell basal lamina and its coat (*). There is no cell within the Schwann cell basal lamina. The surface of the myofiber is infolded at the former synaptic site. The AChE stain is highly concentrated on the myofiber surface at the site and occupies the folds as at neuromuscular junctions. (c) Regenerating myofiber from a muscle treated as described in b but the staining time was shortened to reduce the amount of reaction product. The Schwann cell basal lamina coat (*) indicates that this field is at a former synaptic site. As at neuromuscular junctions, the myofiber surface is lined by basal lamina (arrows) and basal lamina is the sole occupant of the folds. Bar, (a and c) 1 μm; (b) 2 μm.

through the junctional regions of six AChE-stained regenerated muscles, taken 30 d after crush damage and treatment with DFP, to determine if there were cell processes on the presynaptic side of the myofiber basal lamina at sites that stained for AChE. 40–50 former synaptic sites, identified by their coat of Schwann cell basal lamina, were examined for each muscle. Profiles of "presynaptic" cell processes were observed at only 19% of the sites. Results from previous studies in frog muscles regenerating after crush damage indicate that such processes in most cases are muscle fiber fingers that have extended through holes in the synaptic basal lamina to take a presynaptic position (32). Indeed, in our sample of 105 former synaptic sites on myofiber basal lamina from crush-damaged muscles where myofiber regeneration had been prevented, as described in the preceding section, we observed presynaptic processes at only two (2%) of the sites. Since the only structure that was adjacent to muscle fibers at the vast majority (81%) of the synaptic sites in the regenerating muscles was the synaptic basal lamina (e.g., Fig. 2, b and c), we conclude that it is the basal lamina which directs the accumulation of AChE at synaptic sites.

Localization

As illustrated in Fig. 2, a and b, the relationship of the AChE stain to the regenerated myofibers was nearly the same as it was to normal ones; the stain was highly concentrated on the surface of the myofibers and it occupied the infoldings. This observation coupled with the fact that the surface of the myofibers, including the infoldings, was lined by basal lamina (Fig. 2c) raised the possibility that AChE produced by the regenerating myofibers had become associated with basal lamina as at normal neuromuscular junctions. Alternatively, the enzyme that generated the stain could have been a component of the myofiber plasma membrane; the results of several studies on the subcellular localization of AChE in normal muscles suggest that in addition to the enzyme in the basal lamina, there is also AChE which is integral to the muscle fiber plasma membrane (16, 31, 57). The findings from two sorts of experiments on our preparations of regenerating muscles indicate that at least some of the AChE that accumulates at original synaptic sites is a component of basal lamina.

First, four muscles that had regenerated for 30 d after crush damage and treatment with an irreversible AChE inhibitor (DFP, 3 muscles; MSF, 1 muscle) were pinned out and incubated for 4 h at 37°C in Ringer's solution containing 13 µg/ml (800 U/ml) purified bacterial collagenase (Biofactures, form III). Collagenase is known to remove basal lamina components, including AChE, from muscle (6, 20, 22, 49) without disrupting myofibers. When we stained the collagenase-treated regenerated muscles for AChE and examined them by light microscopy, we observed no reaction product, even when we incubated the muscles in staining medium more than 10 times longer than required to reveal the arborizations of the newly formed AChE in regenerated muscles not treated with collagenase. Electron microscopy on such preparations revealed only a few small crystals of stain at the synaptic sites (Fig. 4).

Second, regenerated muscles were damaged a second time and examined to see if the AChE they had produced remained associated with their basal lamina sheaths after degeneration and phagocytosis. 30 d after crush damage and incubation in DFP, four regenerated muscles were exposed and frozen in situ by repeatedly placing on them a brass block cooled in liquid nitrogen. Freezing the muscle in this way causes disintegration of muscle cells but leaves myofiber basal lamina sheaths largely intact (32). The frogs were x-irradiated to prevent myofiber regeneration and 1 mo later, when all cellular debris discernable with the electron microscope had been phagocytized, the muscles, now devoid of myofibers, were stained for AChE. The empty basal lamina sheaths displayed numerous arborizations of stain (Fig. 5) in the junctional region of the muscle similar to those at normal neuromuscular junctions. Since all of the original AChE activity had been blocked by DFP, the enzyme that gave rise to the stain on the empty sheaths could have been produced only by muscle fibers that had regenerated after the initial damage.

Nonjunctional AChE Patches

Previous histochemical studies on regenerating crush-damaged muscles (32) showed that such muscles have ovoid patches of cholinesterase which occur at very low frequencies in both junctional and extrajunctional regions. We also observed by light microscopy such "nonjunctional" patches of cholinesterase stain in whole regenerating muscles used for the studies described here (Fig. 6a). We note that the cholinesterase in the nonjunctional patches behaved the same way as the enzyme at former synaptic sites in experiments aimed at determining the type of cholinesterase and its subcellular
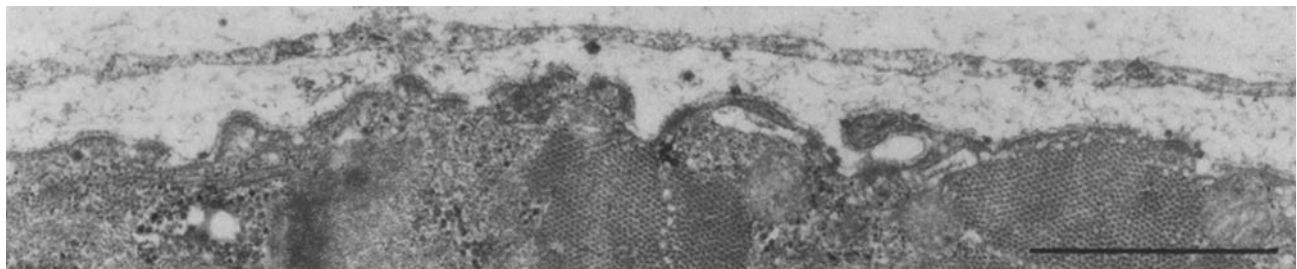

FIGURE 4   Cell surface AChE produced by regenerating myofibers is solubilized by collagenase treatment. Portion of a regenerating myofiber from the junctional region of a muscle removed from the frog 30 d after crush damage and inhibition of original AChE. Before fixing the muscle and staining it for the enzyme the new myofibers had produced, it was treated for 4 h with collagenase in Ringer's solution. No AChE staining was observed in whole muscles by light microscopy and, as shown here, there were only a few small crystals of stain evident in electron micrographs of cross sections. Much of the myofiber basal lamina is intact but other extracellular matrix constituents were highly disorganized; in no case could we identify with certainty Schwann cell basal lamina and its coat. The folds in the myofiber surface indicate that this portion of the muscle fiber is associated with a former synaptic site on myofiber basal lamina or is a place where a nonjunctional AChE patch had formed. Bar. 1 µm.
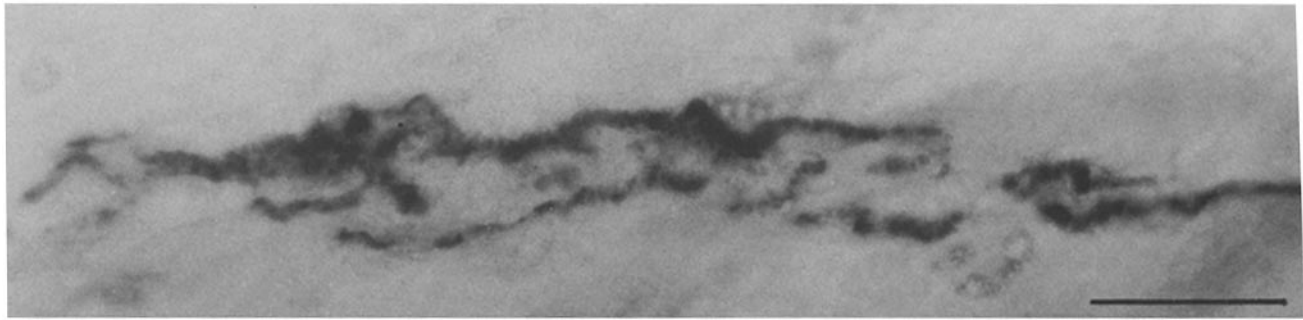
FIGURE 5 AChE produced by regenerating myofibers is tightly adherent to the synaptic basal lamina of the original myofibers. Arborization of AChE stain from the junctional region of a damaged muscle viewed in whole mount. The muscle had been crush-damaged and activity of the original AChE had been blocked by DFP. 30 d later, when myofibers had regenerated in the basal lamina sheaths of the original myofibers and produced new AChE that accumulated at the former synaptic sites on the sheaths, the muscle was freeze-damaged, resulting in disintegration and phagocytosis of the regenerated myofibers. Again the basal lamina sheaths of the original myofibers persisted. At the time of freeze damage, the muscle was x-irradiated to prevent the development of a third generation of myofibers. Accordingly, the arborization of stain is on an empty basal lamina sheath and the enzyme must have been produced by a regenerating myofiber. It is similar to the arborizations observed in the junctional regions of normal and regenerating muscles (compare with Fig. 1, a and b). Bar, 40 μm.
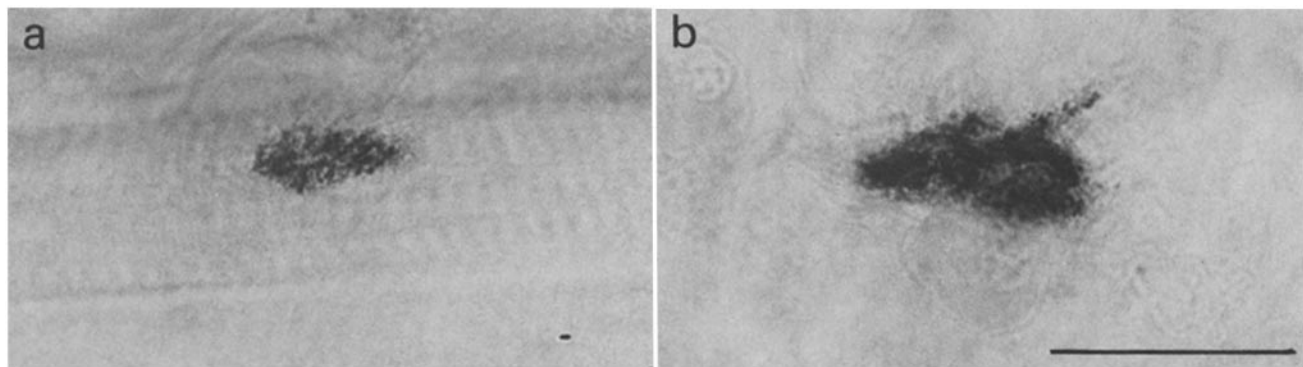

FIGURE 6 Nonjunctional patch of AChE on a muscle fiber in a regenerated muscle (a) and on an empty myofiber basal lamina sheath in a regenerated and then damaged muscle (b) viewed in whole mount. The muscle in b was treated as described in legend for Fig. 5. Bar, 20 μm.

location. Specifically, (a) we never saw nonjunctional patches of stain in our set of regenerated muscles that were treated with BW284c51, a specific inhibitor of true AChE (see Materials and Methods); (b) we did not find these patches in regenerated muscles that were treated with collagenase to remove basal lamina AChE; and, (c) in preparations where we removed regenerated myofibers from muscles by freeze damage as described above, the nonjunctional enzyme patches were clearly observed in extrajunctional regions of the empty basal lamina sheaths (Fig. 6 b). Thus, nonjunctional patches contain true AChE, which is associated with basal lamina.

DISCUSSION

There are several cell surface and cytoplasmic specializations common to normal and regenerated neuromuscular junctions. Of these, only active zones in the axon terminal and aggregates of AChE and acetylcholine receptors on the surface of the muscle fiber are known to be directly involved in synaptic transmission. Previous studies conducted in this laboratory have demonstrated that molecules in the synaptic portion of the muscle fiber's basal lamina sheath direct the formation of active zones in regenerating axon terminals and the aggregation of acetylcholine receptors in the plasma mem-

brane of regenerating myofibers (9, 32, 45). Here we complement these findings by showing that the synaptic basal lamina also causes the accumulation of AChE on the surface of regenerating myofibers.

Our conclusion that synaptic basal lamina directs the focal accumulation of AChE on regenerating myofibers is based on four principal observations. All were made on crush-damaged muscles where AChE activity of the damaged muscle fibers had been blocked, where new myofibers had regenerated within the basal lamina sheaths of the original myofibers, where reinnervation had been prevented, and where we used a stain for cholinesterase activity to view the distribution of cell surface enzyme produced by the new myofibers. First, in whole mounts examined by light microscopy, most of the stain was in the junctional region of the muscles where it was concentrated in narrow elongate patches laid out in patterns that unmistakably resembled the arborizations at normal neuromuscular junctions. Second, in electron micrographs of cross-sectioned regenerating muscle fibers, 86% of the patches of the cholinesterase stain on the myofiber surface were at identified former synaptic sites, points where the muscle fiber basal lamina was in apposition to the basal lamina of Schwann cells that had originally capped the axon terminals. Third, there were no cells or cell processes on the presynaptic side of the synaptic basal lamina at more than 80% of the stained

patches. Fourth, the cholinesterase staining on the regenerated myofibers was inhibited by a specific inhibitor of AChE activity.

We also found that the AChE that accumulates at the original synaptic sites on the basal lamina is solubilized by treating the regenerated muscles with collagenase and that the AChE persists at the synaptic sites after the regenerated muscle fibers are damaged and phagocytized. Similar findings on undamaged muscles have been taken to indicate that much of the enzyme at normal neuromuscular junctions is a component of the synaptic basal lamina (6, 16, 20, 49). Thus in regenerated muscles, as in normal ones, much of the enzyme that accumulates at former synaptic sites apparently becomes a component of the basal lamina.

Although AChE accumulates at the original synaptic sites on the myofiber basal lamina, the distribution of the enzyme in regenerating muscle differs from that in normal muscles in at least three ways. (a) In regenerated muscles, there were a few patches of AChE associated with nonjunctional regions of myofiber basal lamina. Nonjunctional patches of enzyme are found at the ends of myofibers (muscle–tendon junctions) in normal muscles (e.g., 31, 36), as they are in regenerating muscles (Anglister, L., and U. J. McMahan, unpublished observations), but in regenerated muscles they are also scattered over the muscle fiber surface, being found in equal numbers in junctional and extrajunctional regions (32). Such patches of AChE are often associated with an aggregation of acetylcholine receptors and infoldings in the myofiber plasma membrane. Nonjunctional patches of such synapse-like specializations have also been observed on the surface of cultured muscle cells grown in the absence of neurons (10, 37, 40, 46, 47, 55). The factors that cause them to form, both in vivo and in vitro, are unknown. Inestrosa et al. (23) have shown that on certain myotube cell lines nonjunctional patches of AChE are associated with basal lamina. We show here that the AChE in nonjunctional patches in regenerating muscles is solubilized by collagenase and that the nonjunctional AChE persists in the muscle after degeneration of the regenerated myofibers, indicating that it, too, is a component of basal lamina. (b) The arborizations of AChE stain at the original synaptic sites in regenerated muscles were generally less extensive and far more discontinuous than those at normal neuromuscular junctions. The effectiveness of synaptic basal lamina in causing the accumulation of AChE at any given point is probably dependent on several conditions, including the stage of development of the regenerating myofiber, the distance between the regenerating myofiber and basal lamina sheath of the original myofiber, and the amount of AChE-accumulating signal that is present. Moreover, holes are formed in the synaptic basal lamina of crush-damaged muscles (32). Any or all of these conditions could account for the differences in arborization continuity between regenerating and normal muscles. (c) The arborizations of stain in regenerated muscles were generally fainter than those in normal muscles, although such differences are not obvious in Fig. 1. Moreover, for the same staining period we had to use a 20-fold higher concentration of substrate to detect the enzyme in regenerating muscles than in normal muscles. Since the intensity of stain for a given staining period is dependent on the amount of enzyme present, the conditions described in b could account for the lowered staining intensity in the regenerated muscles. Accumulation of AChE may also be lower due to the absence of electromechanical activity in the regen-

erated (and denervated) muscles; such activity is known to be required for high levels of AChE at neuromuscular junctions (11, 29, 41, 42, 54), although the dependence of AChE accumulation on activity in amphibian muscles is apparently less than in muscles of other species (39, 55). Another alternative explanation is that there are a limited number of AChE binding sites in the basal lamina (see below), and many of these may already have been occupied by the original (DFP/MSF-inhibited) AChE.

In damaged mammalian muscles, as in damaged frog muscles, myofibers regenerate within the basal lamina sheaths of the original myofibers (4, 12, 18), but it is not yet known whether synaptic regions of myofiber basal lamina direct the formation of synaptic apparatus in the new myofibers. There is evidence, however, that nerve terminals need not be present in regenerating mammalian muscle in order for the postsynaptic apparatus, including aggregates of AChE and acetylcholine receptors, to form and that such apparatus can develop at or near original synaptic sites on the myofiber basal lamina (4). Moreover, it is now clear that axon terminals need not be present for AChE to accumulate at synaptic sites on undamaged mammalian myofibers (19, 29, 54). For example, if muscles are denervated, which results in a large and rapid decline in the amount of the enzyme present at the sites of the former neuromuscular junctions, subsequent innervation of the muscles at ectopic sites causes the reappearance of high concentrations of the enzyme at the original synaptic sites. In such experiments the only structures at the original synaptic sites, aside from the myofibers and the myofiber basal lamina, are the Schwann cells that originally capped the axon terminals. Accordingly, it may well be that the factors that direct the accumulation of AChE at the denervated synaptic sites in mammalian muscles are bound to the synaptic basal lamina and are similar to those that direct AChE accumulation in regenerating frog muscles.

How might synaptic basal lamina of original myofibers cause the AChE produced by regenerating myofibers to accumulate within it? The sequence of steps that lead to the assembly of basal lamina is not known for muscle fibers or any other cells. There is good evidence, however, that basal lamina molecules have specific and strong attachments to other basal lamina molecules, and that at least one type of basal lamina molecule, laminin, binds to specific integral plasma membrane proteins (51). Accordingly, among the possible mechanisms for the accumulation of basal lamina AChE are the following.

(a) Synaptic basal lamina may have a high concentration of molecules with sites that specifically bind AChE. Thus, even if the enzyme were secreted uniformly along the regenerating myofiber, it would accumulate preferentially in synaptic basal lamina. In fact, purified heparin and heparan sulfate proteoglycan bind tightly to certain forms of purified AChE (8, 52), and immunohistochemical studies have revealed that a heparan sulfate proteoglycan is highly concentrated in the synaptic basal lamina at neuromuscular junctions (1).

(b) The synaptic basal lamina might cause the aggregation of myofiber plasma membrane proteins that bind AChE. Then, if the AChE bound to the myofiber surface subsequently became stably associated with basal lamina, AChE would selectively accumulate in the synaptic basal lamina. It is not yet known whether extracellular AChE interacts with plasma membrane proteins of myofibers, but it is clear that

components of synaptic basal lamina can cause the aggregation of at least one type of plasma membrane protein, the acetylcholine receptor (9, 32).

(c) Components of the synaptic basal lamina might stimulate local secretion of AChE. Thus AChE would accumulate in regions where the regenerating myofiber comes in contact with synaptic basal lamina even if the affinity for AChE were uniform throughout the entire myofiber basal lamina sheath.

In vitro studies have revealed that basal lamina–rich fractions from *Torpedo* electric organ contain a factor that causes the formation of both acetylcholine receptor and AChE aggregates on the surface of myotubes (53). Monoclonal antibodies directed against this factor recognize molecules concentrated in or adjacent to the myofiber basal lamina at neuromuscular junctions in vivo (14). These observations, together with the findings that synaptic basal lamina induces the accumulation of AChE, as we show here, and acetylcholine receptors (9, 32) on regenerating myofibers in vivo, lead to the hypothesis that a single basal lamina molecule directs the formation of both specializations in regenerating muscles (53). Studies aimed at identifying the basal lamina molecules that cause the aggregation of AChE and acetylcholine receptors on regenerating myofibers and learning how they direct the formation of such aggregates are under way (3, 38).

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