Acetylcholinesterase from the Motor Nerve Terminal Accumulates on the Synaptic Basal Lamina of the Myofiber

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Abstract. Acetylcholinesterase (AChE) in skeletal muscle is concentrated at neuromuscular junctions, where it is found in the synaptic cleft between muscle and nerve, associated with the synaptic portion of the myofiber basal lamina. This raises the question of whether the synaptic enzyme is produced by muscle, nerve, or both. Studies on denervated and regenerating muscles have shown that myofibers can produce synaptic AChE, and that the motor nerve may play an indirect role, inducing myofibers to produce synaptic AChE. The aim of this study was to determine whether some of the AChE which is known to be made and transported by the motor nerve contributes directly to AChE in the synaptic cleft. Frog muscles were surgically damaged in a way that caused degeneration and permanent removal of all myofibers from their basal lamina sheaths. Concomitantly, AChE activity was irreversibly blocked. Motor axons remained intact, and their terminals persisted at almost all the synaptic sites on the basal lamina in the absence of myofibers. 1 mo after the operation, the innervated sheaths were stained for AChE activity. Despite the absence of myofibers, new AChE appeared in an arborized pattern, characteristic of neuromuscular junctions, and its reaction product was concentrated adjacent to the nerve terminals, obscuring synaptic basal lamina. AChE activity did not appear in the absence of nerve terminals. We concluded therefore, that the newly formed AChE at the synaptic sites had been produced by the persisting axon terminals, indicating that the motor nerve is capable of producing some of the synaptic AChE at neuromuscular junctions. The newly formed AChE remained adherent to basal lamina sheaths after degeneration of the terminals, and was solubilized by collagenase, indicating that the AChE provided by nerve had become incorporated into the basal lamina as at normal neuromuscular junctions.

ACETYLCHOLINESTERASE (AChE,[1] EC 3.1.1.7) in skeletal muscle is highly concentrated at the neuromuscular junctions, where it terminates synaptic transmission by catalyzing hydrolysis of acetylcholine (20). A substantial fraction of this enzyme is attached to the myofiber's basal lamina in the synaptic cleft (reviewed in 6, 28, 35, 38). In frog muscle, much of the AChE remains associated with synaptic basal lamina, even after removal in vivo of both nerve terminals and myofibers from the synaptic sites (2, 30). The external location of AChE, in the cleft between the myofiber and its opposing nerve terminal, raises the question of whether synaptic AChE is produced by muscle, by nerve, or by both. Several studies show that after denervation, AChE is markedly reduced at the synaptic sites of rat muscles (9, 11, 17, 42). However, the myofibers can be induced to produce AChE, which accumulates at the denervated synaptic sites; this can be achieved by either introducing ectopic innervation at a distal region, removed from the original sites of innervation (16, 44) or by direct electrical stimulation of the muscles (25, 26). Our studies on denervated regenerating frog muscles have demonstrated that the regenerating myofibers, in the absence of nerve and stimulation, produce AChE that becomes concentrated on the surface of these myofibers and is associated with the basal lamina at the original synaptic sites (3). Muscle cells in culture may form sites of accumulation of AChE in the absence of neurons (31, 36, 43). These various results lead to the conclusion that the myofibers produce synaptic AChE, and that the nerve plays an indirect role: by inducing activity in the myofibers, it triggers them to provide the synaptic enzyme. In addition, the nerve may provide cue molecules that become incorporated into the synaptic portion of the myofiber basal lamina, and direct the formation of the postsynaptic specializations in the regenerating myofibers, such as the accumulation of AChE (see above) as well as acetylcholine receptors and postjunctional folds (27, 29, 34). Despite considerable speculation that the motor nerve terminal may contribute components to the synaptic cleft material at the neuro-

A brief account of some of these experiments was presented in abstract form (*Soc. Neurosci. Abstr.* 1987. (3:1211.)).

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

muscular junction no direct experimental evidence for such contribution has been obtained. The purpose of the present study was to determine whether the motor nerve, which is known to contain and transport AChE (7, 8, 13) may contribute directly to the AChE of the neuromuscular junction. We examined frog muscle preparations from which all myofibers had been removed in vivo, leaving behind their basal laminae, with intact axons and nerve terminals still attached at most of the original synaptic sites. We demonstrate that these nerve terminals produce synaptic AChE and that the enzyme becomes a component of the synaptic basal lamina as is the case for neuromuscular junctions. These results represent the first direct experimental evidence that the nerve terminal may indeed provide components of the synaptic cleft material.

Materials and Methods

Muscle Damage

The thin paired cutaneous pectoris muscles of the frog (*Rana pipiens*) were used in these studies. The muscles, which are situated just beneath the skin of the frog's thorax, were exposed for surgery in frogs anesthetized with MS-222 (tricaine methane sulfonate) (Sigma Chemical Co., St. Louis, MO). Surgery was performed using a Wild M8 (Wild Heerbrugg Ltd., Heerbrugg, Switzerland) dissecting stereoscope. We followed surgical procedure developed by Yao and McMahan. *Soc. Neurosci. Abstr.* 1984. 10:1085 (Fig. 1): muscles were cut across on both sides of the middle third of the muscle, where all the neuromuscular junctions are concentrated (thus referred to as the "junctional region" of the muscle). Some of the noninnervated regions were removed and discarded. Further short incisions were made carefully across the myofibers in between nerve arbors so as not to cause damage to axonal branches and their terminal arbors. Immediately after surgery, the operated muscles were treated with anticholinesterase drugs to block all original AChE (detailed description follows). The skin incisions were then sutured shut. This procedure caused the degeneration and removal in vivo of all myofibers from their basal lamina sheaths, leaving behind the axons, their nerve terminals, and synaptic basal lamina sheaths. X-ray irradiation of the frogs on the three subsequent days after surgery (2,400 rad/dose) prevented regeneration of the myofibers. Nerve terminals persisted in such preparations at almost all synaptic sites for a year, and showed no discernible differences in structural features from terminals at intact neuromuscular junctions (45). These preparations are referred to below as "innervated sheaths" of muscle. The surgical approach which we used to obtain innervated sheaths of myofibers was best suited to our studies because, unlike the other procedures (e.g., 37, 41), it produced preparations in which most of the original synaptic sites in the muscle were preserved and retained on the surface of the empty sheaths, and the nerve terminals remained at almost all sites for the duration of the experiment, which ensured a maximal effect and a clear result.

Staining for AChE

We used Karnovsky's staining method (19) to observe the distribution of surface AChE. For normal frog 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 0.12 mM acetylthiocholine. For innervated sheath preparations, the concentration of acetylthiocholine was increased 20-fold. The dissected preparations were routinely pinned out in frog Ringer's solution (3), fixed for 25 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 (Ladd Research Industries, Inc., Burlington, VT). Since the muscle preparations were <100 μm thick, the 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 acetate. Incubation of the whole dissected muscle preparation in Karnovsky's staining medium under the conditions employed revealed cell surface but not cytoplasmic AChE (24).

To inactivate all original cholinesterase irreversibly in the synaptic portion of the basal lamina and elsewhere, so that it would not be detected histochemically in the innervated "muscle ghost" preparation, we treated the exposed muscles following surgery with Ringer's containing 5 mM diisopropylfluorophosphate (DFP; Sigma Chemical Co.) or 10 mM methanesulfonyl fluoride (MSF; Aldrich Chemical Co., Milwaukee, WI) (21, 39). So as to maximize the accessibility of the cutaneous pectoris muscles to the blocking reagents while protecting the neighboring tissues, the muscles that during the operation had been disconnected at their origin and insertion (Fig. 1), were further separated from the underlying muscles and a piece of parafilm was inserted underneath them, so as to shield the inner tissues. The drug solution was applied directly over the muscle and cotton wool soaked with the solution was maintained over the muscle for 45 min at room temperature. The solution was reapplied to the muscle every few minutes, and was replaced every 15 min with a fresh dilution of the drug. After the incubation period, the muscles were extensively rinsed in Ringer's, and after removal of the parafilm, were properly aligned in their bed before suturing. (No staining for AChE could be detected by light or electron microscopy when the muscles were examined immediately after the blocking treatment, see Fig. 4 *a*.)

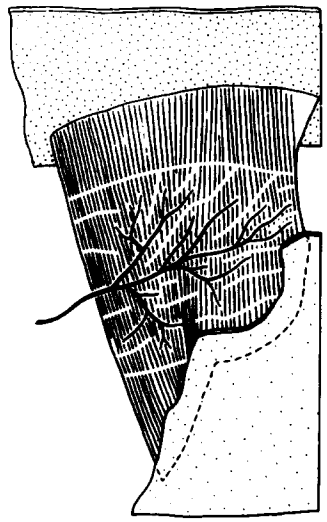

Figure 1. Sketch of the surgical procedure. The cutaneous pectoris muscle was exposed and incisions (*white lines*) were made across the myofibers, separating the innervated and noninnervated regions, and between the nerve arbors, avoiding damage to axons and their branches. The muscle was then treated with anticholinesterase drugs to block irreversibly all original AChE (details in text).

Results

Reappearance of AChE at Synaptic Sites in the Absence of Myofibers

4 wk after damaging the muscle fibers of the cutaneous pectoris muscles, all cellular fragments of the muscle fibers discernible by EM had already been phagocytosed and removed. Yet, most of the basal lamina sheaths of the myofibers were preserved and nearly all synaptic sites on the sheaths were occupied by nerve terminals (45). Accordingly, 1 mo after we had damaged the muscles and treated them with DFP or MSF to block the original AChE, we removed the innervated sheaths from the frogs, pinned them out, and fixed and stained them for AChE. In all 30 preparations (10 DFP-treated and 20 MSF-treated preparations) that composed the experimental set, we found by light microscopy, patches of AChE stain ~5 μm wide and up to several tens of micrometers long, associated with each of hundreds of terminal axonal branches. In each of the innervated sheath preparations, the patches of stain were arranged in a way that clearly resembled the arborized pattern of stain at normal neuromuscular junctions (Fig. 2, *a* and *b*). All the AChE patches were confined to the junctional region of the muscles. The axons could be traced directly to the patches of stain, leaving no doubt that these patches were coextensive with the terminal arbors of the axons. The specific double-

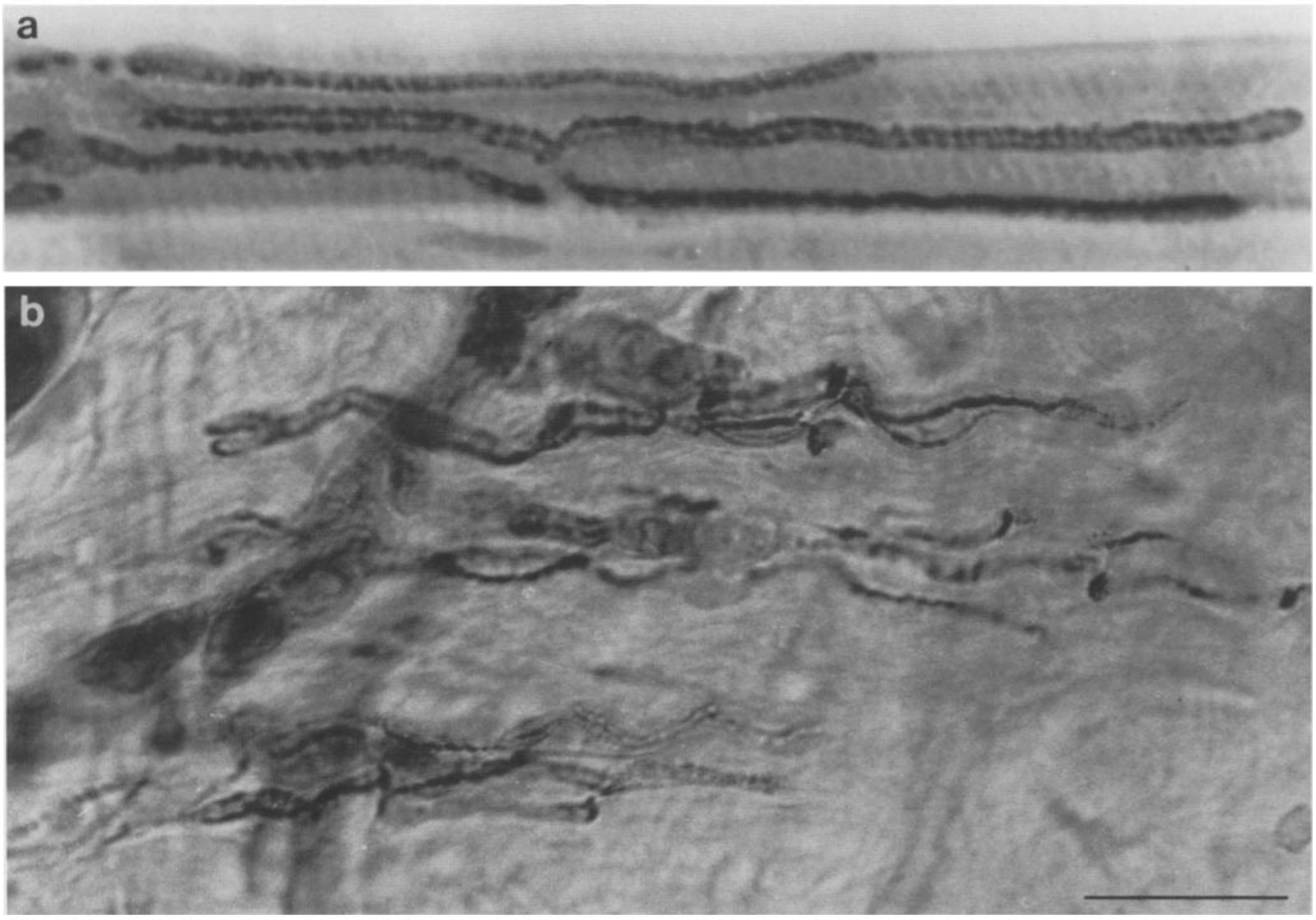

Figure 2. Distribution of surface AChE that reappeared at the innervated sheath preparations in the absence of myofibers is much like the distribution of AChE in normal muscle fibers. (*a*) Light micrograph of a neuromuscular junction in a whole mount of a normal muscle. Stain for AChE activity is concentrated along the terminal arborizations of the axon. (*b*) Innervated basal lamina sheaths, 30 d after permanent removal of myofibers and irreversible inactivation of original AChE. New AChE was produced and the stain for its activity appears at the terminal arborizations as in normal neuromuscular junctions. Bar, 30 μm.

staining procedure of neuronal arbors (by nitroblue tetrazolium [NBT]) and cholinesterase activity (23) confirmed, at the light microscope level, the colocalization of terminal branches with the elongated cholinesterase-stained patches (not shown).

We used EM to document that the AChE stain of the innervated sheath preparations was indeed concentrated at the synaptic basal lamina sites adjacent to the nerve terminals. We made cross-sections through the innervated regions of four innervated preparations, taken 30 d after surgery and treatment with MSF. For each muscle, we examined 30–35 sites containing nerve terminals. Out of a total of 127 nerve terminals, all had crystals of the enzymatic reaction product adjacent to them. One such site is illustrated in Fig. 3 *b*.

Karnovsky's method can stain both true AChE and pseudocholinesterase (19). The following experiments show that most if not all of the staining we observed was due to true AChE. First, three normal and three innervated ghost preparations were incubated for 1 h in Ringer's solution containing 0.1 mM tetraisopropylpyrophosphoramide (iso-OMPA) (Sigma Chemical Co.), a specific inhibitor of pseudocholinesterase. The preparations were then incubated for 10 min, our routine staining period, in staining medium that also contained iso-OMPA. When examined in whole muscles by light microscopy or in sectioned muscles by EM, the amount of stain at synaptic sites was similar to that in muscles not treated with iso-OMPA (Figs. 1 and 2). Secondly, three normal and three innervated sheaths were incubated for 1 h in Ringer's solution containing 1 μM 1,5-bis[4-allyldimethylammoniumphenyl]pentane-3-one dibromide (BW284c51) (Burroughs Wellcome Co., Research Triangle Park, NC), 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. EM 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 preparations which had not been treated with the AChE inhibitor (e.g., Fig. 3 *b*).

In the following way, we checked to ensure that both DFP and MSF had blocked the activity of the original AChE at the synaptic sites. 10 muscles were treated with AChE histochemical staining solution immediately after damaging and exposing them to anticholinesterases (5 DFP- and 5 MSF-

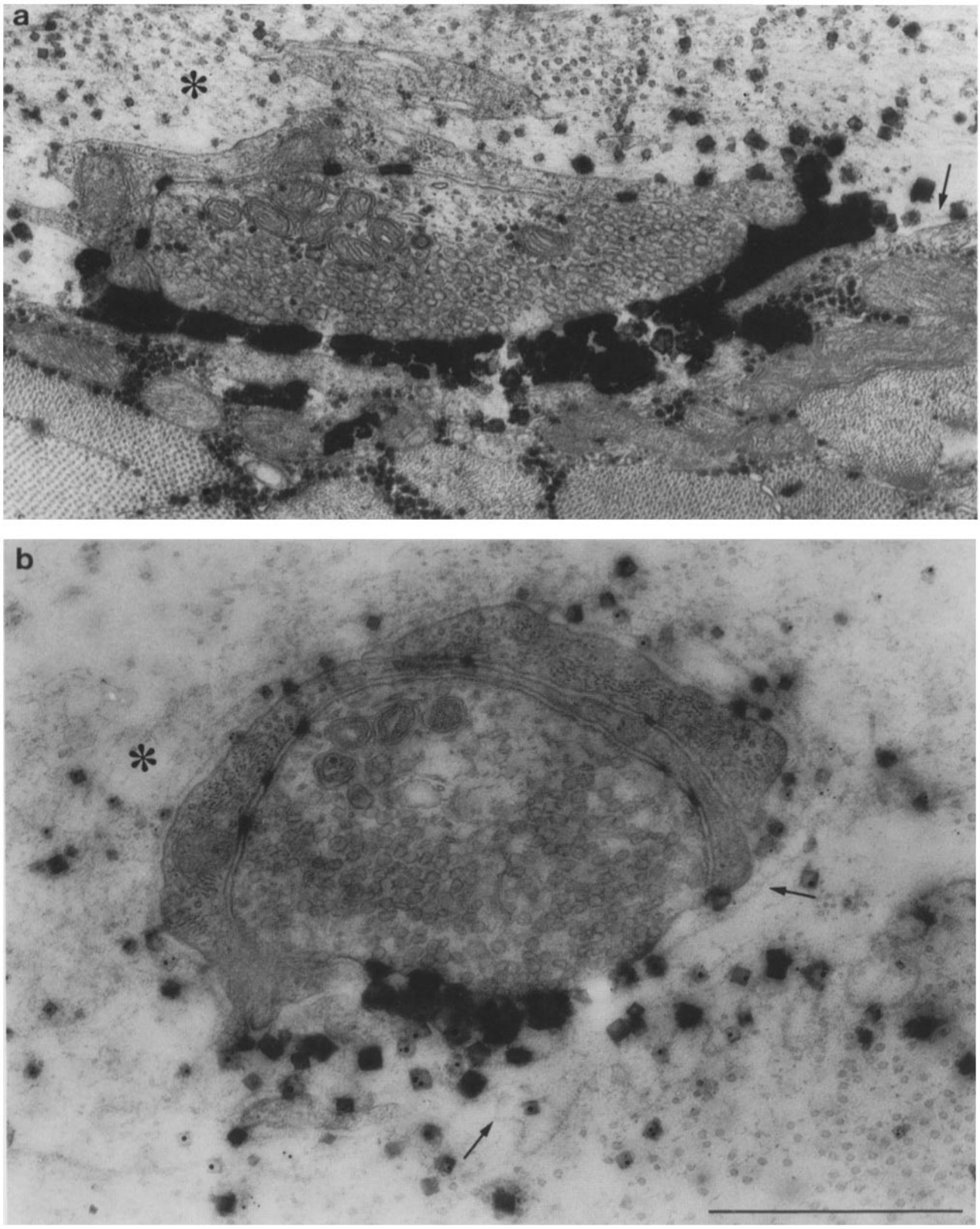

Figure 3. New AChE reaccumulates on the surface of the nerve terminals adherent to the synaptic portion of the myofiber's basal lamina, in the absence of myofibers. Electron micrographs of cross sections through muscle preparations histochemically stained for AChE (whole mounts shown in Fig. 2). (*a*) Neuromuscular junction in a normal muscle. Crystals of AChE intensive stain are concentrated in the synaptic cleft and junctional folds, obscuring completely the synaptic portion of the myofiber's basal lamina (*arrow*). (*b*) Innervated basal lamina sheaths 30 d after surgery and irreversible inactivation of all original AChE, and after new AChE has been produced. The myofiber's basal lamina (*arrows*) persists after the removal of the myofiber but has collapsed and folded in its absence (right arrow is inside the vacated

treated muscles); we observed no staining in the whole mounts using light microscopy or in cross-sections through junctions using EM (Fig. 4 *a*).

Acetylcholinesterase Accumulation at Synaptic Sites Is Dependent on the Presence of Nerve Terminals

To demonstrate that the presence of the nerve terminals was required for the appearance of the accumulations of AChE at the synaptic sites in the absence of myofibers we carried out the following experiment: during the original damage and blocking procedure, the frogs were also chronically denervated. The nerve to the cutaneous pectoris muscle was sectioned at the muscle border and the second spinal nerve, from which it branched was cut at the spinal cord and a 1-cm stretch of nerve was evulsed. 1 mo later, these preparations were devoid of any cellular components at most of their synaptic sites: there were neither myofibers, nerve terminals, nor Schwann cells. The whole mounts of the empty basal lamina sheaths still showed no AChE staining. We examined by EM 78 synaptic sites in cross-sections through 4 of these preparations. The synaptic sites were identified by the presence of Schwann cell basal lamina and its extracellular coat, which can serve as an extracellular matrix marker for the synaptic sites at frog muscles: this particulate material, ~2 μm thick, is found on the surface of the myofiber only at synaptic sites, where it persists also after removal of the cellular components (3, 22). Because of its thickness and persistence after damage, the Schwann cell coat provided us with a convenient marker for the original synaptic sites, in the absence of any cellular synaptic tracer. Only at some vacated synaptic sites could a few crystals of stain be detected (Fig. 4 *b*), but in no case was the staining nearly as intense as at nerve terminal sites. This result indicates that in the absence of nerve terminal and its Schwann cell no AChE appeared de novo at the synaptic site. It excludes the possibility that spontaneous reactivation of the original blocked AChE may account for any significant reappearance of AChE activity.

We further showed that the presence of Schwann cell at the synaptic site did not lead to the appearance of AChE there. We searched cross-sections of the denervated sheath preparations described above, at 30 d after damage, blocking, and denervation. We identified seven synaptic sites, which possessed neither myofibers nor nerve terminals but contained cellular processes of Schwann cells. These were processes of Schwann cells that remained at the denervated presynaptic side after the removal of nerve terminals (37). The amount of stain associated with the basal lamina at the sites containing Schwann cell processes was no greater than the background level seen in the absence of any cells (Fig. 4 *b*), indicating that the Schwann cells do not make any significant contribution to synaptic AChE.

In addition, in a few of our preparations of innervated sheaths, we noticed during the electron microscopic examination of the cross-sections, synaptic sites that became denervated probably due to unintentional damage during surgery. As before, the sites were identified by the occurrence of the Schwann cell extracellular coat. In our search, 11 sites on the vacated myofibers' basal laminae were overlaid with Schwann cell coat but had no terminal or Schwann cell present. 10 of these sites had no stain on them. The only site that had a few crystals also had a preterminal axonal profile in its vicinity, indicating that possibly the axon had reinnervated that site and retracted from it before our examination; other studies had shown that terminals of injured axons that reinnervate synaptic sites on basal lamina in the absence of myofibers, do not persist there, but retract after a peak of maximal occupancy at 3–4 wk (37; Yao, Y.-M., and U. J. McMahan. 1985. *Soc. Neurosci. Abstr.* 11:947).

Together, our results demonstrate that most, if not all, AChE detected at the synaptic sites on innervated sheaths after removal of myofibers and treatment with cholinesterase inhibitors is supplied de novo by the nerve terminals.

Localization

As at intact neuromuscular junctions, the AChE stain, which appeared at innervated synaptic sites in the absence of myofibers, was highly concentrated on the synaptic surface of the nerve terminal, obscuring the synaptic portion of the myofiber basal lamina (Fig. 3, *a* and *b*). This raised the possibility that AChE provided by the terminals had been incorporated in the basal lamina as at normal neuromuscular junctions. Alternatively, the enzyme that produced the stain could have been a component of the nerve terminal plasma membrane. The presence of presynaptic membrane-bound AChE in the related electric organ synapse was reported (e.g., 38). The results from two sorts of experiments presented below, indicate that at least some of the newly formed AChE in the innervated basal lamina sheaths of frog muscle is localized on the synaptic basal lamina.

First, the innervated sheath preparations were damaged a second time in situ after the new AChE had been produced, and were examined later to see whether the enzyme remained associated with synaptic basal lamina also in the absence of the presynaptic components. At 30 d after muscle damage and treatment with an irreversible AChE inhibitor (MSF), the innervated sheath preparations (three pairs) were denervated by sectioning the second spinal nerve and evulsing a segment of the brachial nerve, including its branch to the cutaneous pectoris. The frogs were dissected for analysis 3 wk later. The innervated sheath preparations, now devoid of both myofibers and nerve terminals with their Schwann cells, were stained for AChE. The denuded basal lamina sheaths displayed numerous arborizations of stain (Fig. 5 *a*) in the junctional region of the muscle that resembled those at neuromuscular junctions, or on innervated empty sheaths (Fig. 2). The enzyme remained concentrated at the synaptic basal lamina, although almost all cellular debris (including plasma membranes) detectable by electron microscopy had been removed (Fig. 5 *b*). Since all of the original AChE activity had been blocked by MSF, the enzyme that gave rise to the stain on the empty sheaths could have been produced only by the nerve terminals, which remained at the synaptic

sheath). The nerve terminal with the Schwann cell and its characteristic particulate coat of extracellular matrix (*) remains adherent to the synaptic basal lamina. AChE stain is highly concentrated on the surface of the nerve terminal where it is positioned against the persisting myofiber basal lamina, obscuring that part of the sheaths. The stain is less intense than at the normal junction. Bar, 1 μm.

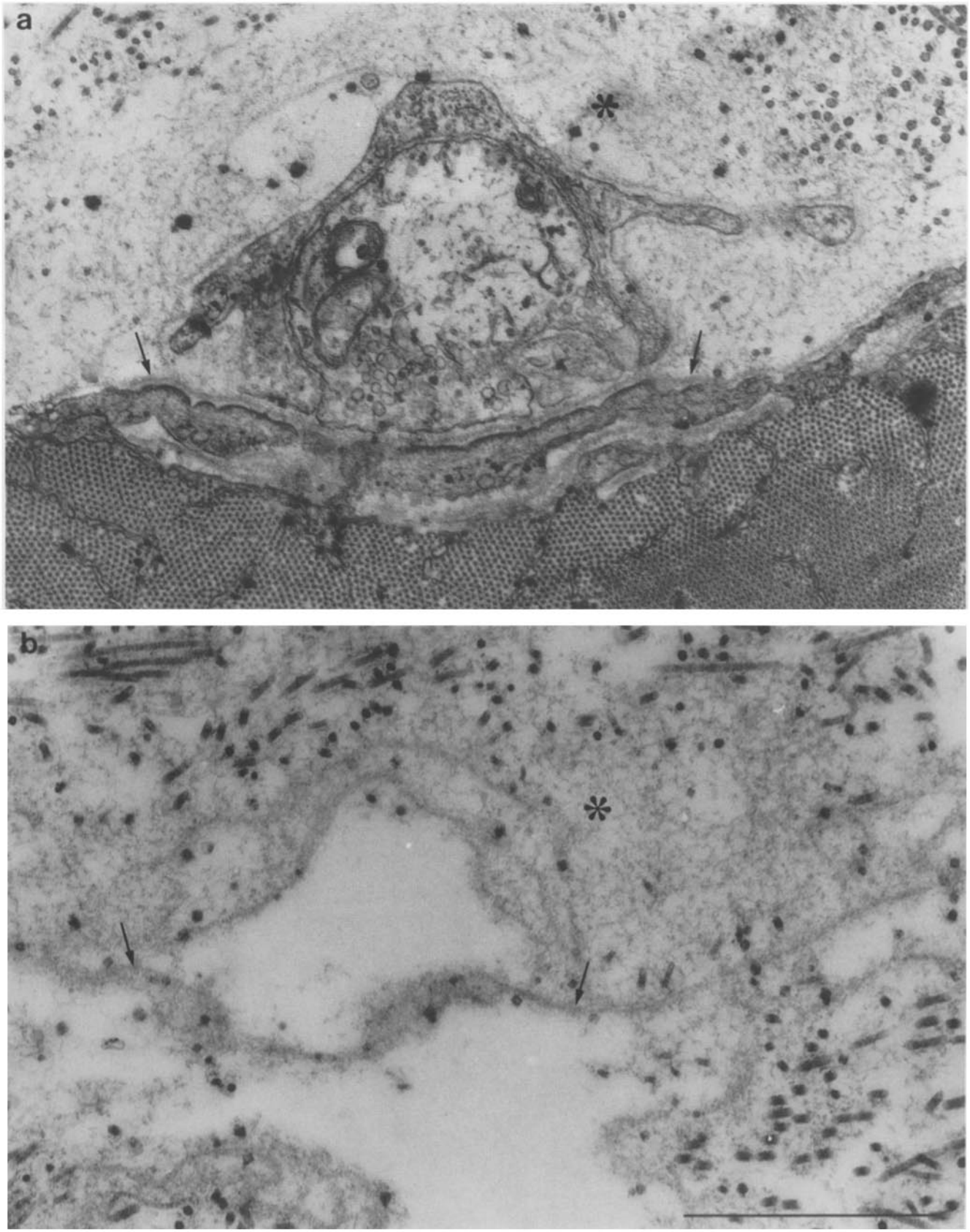

Figure 4. Treatment of the damaged muscles with AChE inhibitors blocks all the activity of the original basal lamina AChE detected by the histochemical procedure and no AChE reappears in the absence of innervation. (*a*) A synaptic site on a muscle fiber immediately after damage and inhibition with DFP. Staining for AChE was almost completely eliminated. The nerve terminal, Schwann cell with its extracellular coat (*) and basal lamina are intact. (*b*) The synaptic site on the myofiber's basal lamina sheath (*arrows*) 1 mo after muscle damage, enzyme inhibition with DFP and x-ray irradiation. The muscle was also chronically denervated at the time of damage. The synaptic site

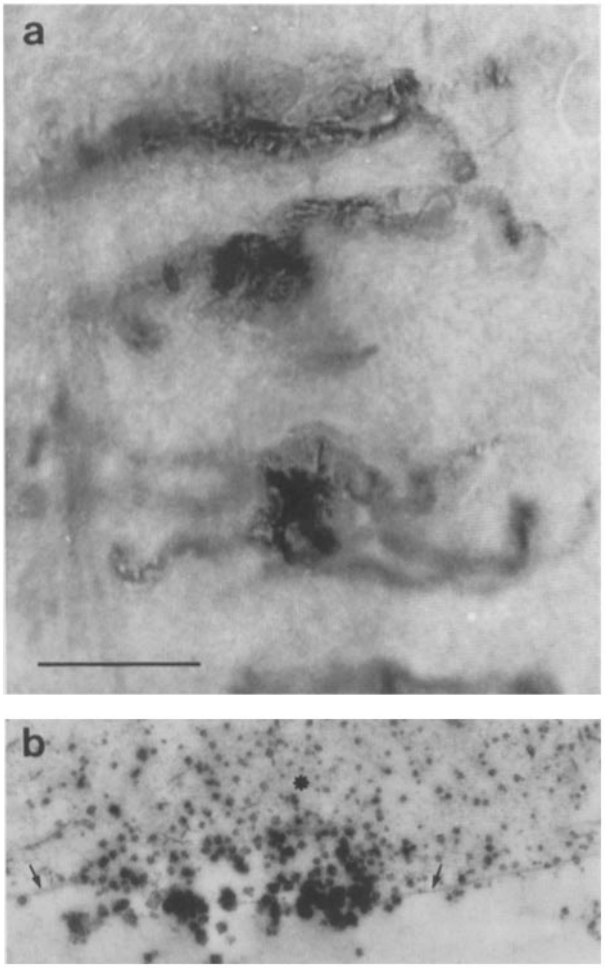

Figure 5. AChE produced by nerve is tightly adherent to the synaptic basal lamina of the original myofibers. At 30 d after removal of myofibers and inactivation of original AChE by MSF, when new AChE had been produced and accumulated at nerve terminal sites, the muscle was denervated. 3 wk later, after nerve terminals and Schwann cells had been removed, the preparation was stained for AChE and examined. (*a*) Light micrograph of the junctional region of the damaged muscle viewed in whole mount. Arborizations of AChE stain on the empty basal lamina sheath are similar to the arborizations at normal muscle or innervated sheaths (compare with Fig. 2). (*b*) Electron micrograph of a cross section through a stained arbor (shown in *a*). AChE remains concentrated at the synaptic portion of the vacated myofiber's basal lamina (*arrows*). This enzyme must have been produced by the nerve terminal previously occupying the synaptic site, marked by the persisting extracellular matrix of the Schwann cell (*, as in Figs. 3 and 4). Bars: (*a*) 30 μm, (*b*) 1 μm.

sites for a month after the initial damage that had caused the myofibers to degenerate.

Second, four muscles that had had their myofibers removed in situ, were dissected out at 30 d after muscle damage and treatment with the irreversible inhibitor MSF, pinned out in a dish and incubated for 2.5 h at 37°C in

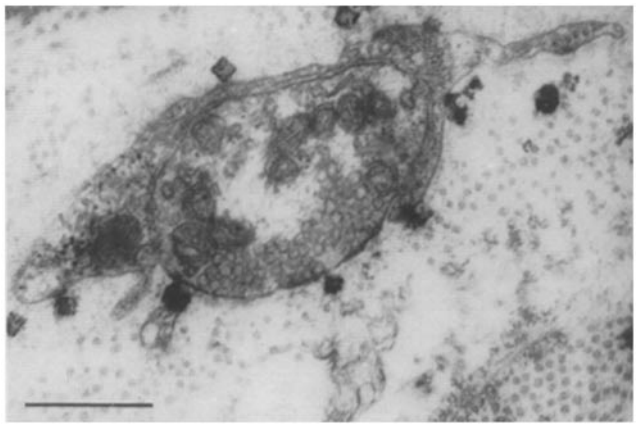

Figure 6. Surface AChE produced by nerve terminals in the absence of the myofibers is removed by collagenase treatment. Electron micrograph of a nerve terminal in an innervated sheath preparation removed from the frog 30 d after muscle damage and inhibition of original AChE. Before fixing the preparation and staining it for the newly produced AChE, it was treated for 150 min with collagenase in Ringer's solution at 37°C. The preparation was then stained four times longer than required to obtain intense staining of AChE in similar untreated preparations. No AChE staining was observed in whole muscle by light microscopy, and only few scattered crystals are evident in electron micrographs of cross-sections, indicating that much of the enzyme was solubilized. Nerve terminals appear structurally unchanged but the extracellular matrix structures including basal laminae are highly disorganized. Bar, 0.5 μm.

Ringer's solution containing purified collagenase form III (13 μg/ml, 800 U/ml), reconstituted and devoid of any detectable nonspecific protease activities (Advanced Biofactures Co., Lynbrook, NY; e.g., 14). Under these conditions collagenase is known to degrade myofiber basal lamina and extracellular matrix and thereby to remove AChE. It should not affect nerve terminals or their membranes (5, 18). We had to limit the length of incubation since, unlike muscles, the innervated sheath preparations which lack the myofibers, are fragile and would fall apart as a result of more extensive exposure. Yet, when we stained the collagenase-treated preparations for AChE and examined them by light microscopy, we observed no reaction product even when we incubated them in staining medium more than three times longer than required to reveal the arborizations of the newly formed AChE in control preparations that had not been treated with collagenase. EM examination of such preparations revealed only a few small crystals of stain associated with nerve terminal sites (Fig. 6). This result indicates that much of the AChE which had been produced by the nerve terminals at the synaptic sites is held in place by a collagenase-sensitive interaction.

Discussion

To determine whether the motor nerve is capable of producing some of the synaptic AChE in vivo, we designed an experimental system, in which, after surgery, the myofibers

is marked by the vacated space previously occupied by the nerve terminal and by the persisting basal lamina and extracellular matrix coat of the Schwann cell (*). Only a few small crystals of stain are associated with the synaptic site. The square crystals of the reaction product are distinguished from the round cross-sections of the collagen fibrils that are abundant in the extracellular material. 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 (30). Bar, 1 μm.

were removed in situ, so that their possible contribution to the enzyme at the neuromuscular junction could be completely eliminated. In this procedure, all the AChE originally present was irreversibly blocked, so as not to show up in the course of our experimental protocol. The motor axons and their branches were preserved and nearly all the nerve terminals persisted at the original synaptic sites on the myofiber basal lamina sheaths which were left behind despite the disappearance of the myofibers themselves. We used a stain for cholinesterase activity to view the appearance and distribution of surface enzyme, and we showed that new AChE, produced by the nerve terminals, accumulated at the synaptic sites.

Our conclusion that synaptic AChE from presynaptic origin appeared de novo in the innervated sheaths is based on the following observations: First, in whole mounts examined by light microscopy, the stain was in the junctional region of the innervated sheaths, concentrated along the terminal arborizations of the nerve, in narrow elongate parallel patches resembling the characteristic pattern displayed by normal neuromuscular junctions. Second, in electron micrographs of cross-sectioned innervated sheaths, nearly all the nerve terminal profiles were decorated by intense cholinesterase stain, which was concentrated at the persisting myofiber basal lamina in contact with the terminal. Third, the cholinesterase staining on the innervated sheaths was inhibited by a specific inhibitor of AChE. Fourth, in preparations from which nerve terminals and presynaptic elements had also been permanently removed at the time of muscle damage no stain could be detected.

Several lines of evidence lead further to the conclusion that the motor nerve terminals were the source of the new synaptic basal lamina AChE. The terminal was the only cellular component that was in direct apposition to and in contact with the synaptic portion of the myofiber basal lamina throughout the whole course of the experiment. The reaction product of the newly formed AChE was highly concentrated within this portion of the basal lamina. Only a few crystals were scattered elsewhere, as is also the case at intact neuromuscular junctions, possibly due to diffusion before capture. The contribution of the Schwann cell, if any, was surely very limited: (*a*) As at the intact junction, no more than a background level of crystals was found around the Schwann cells and their surrounding basal lamina. (*b*) It was demonstrated that in denervated frog muscles or sheaths preparations, from which the nerve had been chronically disconnected, the Schwann cells occupied the synaptic sites on the basal lamina during the first weeks after denervation, after nerve terminals had been phagocytosed and removed (24, 37). But despite the prolonged persistence of Schwann cells (24), the cholinesterase level at sites of denervation was drastically diminished (33). (*c*) In fact, in the denervated sheath preparations we describe, even 4–5 wk after damage to both myofibers and nerve and inactivation of the original AChE, the Schwann cells have not yet vacated all synaptic sites. But even in these sites no AChE activity reappeared, proving that the Schwann cells on their own could not produce synaptic AChE. It is thus reasonable to conclude that in the innervated basal lamina sheaths, the nerve terminals situated at the synaptic sites were the source of the synaptic AChE. It should be noted that nerve terminals innervating synaptic basal lamina in the absence of myofibers were morphologically differentiated and contained vesicular structures (37, 45), and showed active vesicle recycling (15, 41). A further confirmation is obtained from our recent studies showing that some of the molecular forms of AChE which are externalized in such preparations are carried by fast anterograde axonal transport (Anglister, L., and B. Haesaert, manuscript in preparation).

We found, in addition, that the AChE that had accumulated at sites of innervation on the vacated basal lamina sheaths persisted on the synaptic basal lamina after the presynaptic cellular components were damaged and removed. We also found that the newly provided AChE was solubilized by collagenase treatment. Similar findings on undamaged or regenerating muscles have been used to indicate that much of the enzyme at normal or regenerating neuromuscular junctions is a component of synaptic basal lamina (3, 5, 14, 18, 40). Thus, much of the enzyme that was provided by the presynaptic terminal in the absence of myofibers, and accumulated at the synaptic sites apparently became associated with the basal lamina. That collagenase solubilized so much of the surface enzyme may not only be because these molecules are associated with the extracellular matrix, but also perhaps because they involve their own collagen-like subunit component in their associations (1). Our own preliminary biochemical studies indicate that there is a contribution of asymmetric AChE forms to surface enzyme (4). It should be noted, however, that there might also be a contribution of nerve membrane-associated AChE. This contribution cannot be quantitatively significant since after the removal in situ of the cellular presynaptic components there is practically no change in the appearance of the synaptic AChE.

Although AChE supplied by nerve terminals in the absence of myofibers accumulated at synaptic sites on the myofiber basal lamina, the arborizations of stain in the innervated sheath preparations were generally fainter than those in normal junctions (such differences are not evident in Fig. 2). Despite the better accessibility to staining reagents of the sites in the vacated innervated sheath preparations than of those in the myofibers-containing normal muscle, we had to use 20-fold the concentration of the substrate for the same staining period so as to obtain an intensity of staining that was still weaker than that obtained at intact junctions (Fig. 3, *a* and *b*). Thus, under the conditions we used, less AChE appeared at the innervated sites in the absence of the muscle than was present at the original intact junctions. This may indicate that the nerve could supply only a fraction of the synaptic enzyme. It is also possible that more AChE would be supplied in the presence of the postsynaptic myofiber and a functional synapse. Additional factors, such as a possible damage and loss of requisite components from the synaptic basal lamina, caused by the degradation and removal of the myofibers, may also account for the low accumulation of AChE in it. Moreover, it is possible that many of the sites available for AChE on the persisting basal lamina are occupied by the DFP/MSF-blocked original AChE, leaving only a few sites free to attach AChE supplied de novo by the nerve. It should be noted in this context that arborizations of AChE stain that appear on regenerating frog myofibers in the absence of innervation (3) are of even fainter intensity than those we observed on the innervated sheaths under the same staining conditions. It is thus difficult, under these con-

ditions, to make further conclusions regarding the amount of synaptic enzyme that may be supplied by nerve terminals.

Our results demonstrate that the motor nerve provides synaptic basal lamina AChE in the absence of myofibers, but it is not yet known to what extent the nerve contributes to the synaptic enzyme in the presence of the muscle cells, at the intact junction. Although spinal motoneurons in culture produce and secrete AChE (e.g., 32), an immunocytochemical study of heterologous nerve-muscle cocultures (from chicken and rat), showed that the AChE that appeared at the motoneuron-myotube contacts was of muscle origin (12). However, this may not represent the situation in vivo, in a homologous synapse and at the amphibian neuromuscular junction. Thus, at the intact junction the motor nerve may play at least three roles in the formation of synaptic AChE. First, the nerve can directly provide some of the synaptic enzyme, as we show that it does in the absence of myofibers. Second, it triggers the accumulation of AChE at synaptic sites by inducing electrical activity in the postsynaptic myofibers (10, 25, 44). Third, it can mark the synaptic sites by incorporating special components into the synaptic portion of the myofibers' basal lamina that direct the accumulation of AChE, as well as the formation of other synaptic specializations. One such component, agrin, was identified (3, 27, 34, 43).

In conclusion, at the intact neuromuscular junction both muscle and nerve may contribute AChE molecules into the synaptic cleft. It is most likely that their function is coordinated, in terms of concentrations and molecular forms of the enzyme, and its organization within the extracellular matrix layers composing the synaptic basal lamina. Experiments to determine the individual contribution of nerve and muscle to the AChE at the intact neuromuscular junction are in progress.

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