Globular and Asymmetric Acetylcholinesterase in the Synaptic Basal Lamina of Skeletal Muscle

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Abstract. The aim of this study was to characterize the molecular forms of acetylcholinesterase (AChE) associated with the synaptic basal lamina at the neuromuscular junction. The observations were made on the neuromuscular junctions of cutaneous pectoris muscles of frog, *Rana pipiens*, which are similar to junctions of most other vertebrates including mammals, but are especially convenient for experimentation. By measuring relative AChE activity in junctional and extrajunctional regions of muscles after selective inactivation of extracellular AChE with echothiophate, or of intracellular AChE with DFP and 2-PAM, we found that >66% of the total AChE activity in the muscle was junction-specific, and that >50% of the junction-specific AChE was on the cell surface. More than 80% of the cell surface AChE was solubilized in high ionic strength detergent-free buffer, indicating that most, if not all, was a component of the synaptic basal lamina. Sedimentation analysis of that fraction indicated that while asymmetric forms (A_{12}, A_8) were abundant, globular forms sedimenting at 4–6 S (G_1 and G_2), composed >50% of the AChE. It was also found that when muscles were damaged in various ways that caused degeneration of axons and muscle fibers but left intact the basal lamina sheaths, the small globular forms persisted at the synaptic site for weeks after phagocytosis of cellular components; under certain damage conditions, the proportion of globular to asymmetric forms in the vacated basal lamina sheaths was as in normal junctions. While the asymmetric forms required high ionic strength for solubilization, the extracellular globular AChE could be extracted from the junctional regions of normal and damaged muscles by isotonic buffer. Some of the globular AChE appeared to be amphiphilic when examined in detergents, suggesting that it may form hydrophobic interactions, but most was non-amphiphilic consistent with the possibility that it forms weak electrostatic interactions. We conclude that the major form of AChE in frog synaptic basal lamina is globular and that its mode of association with the basal lamina differs from that of the asymmetric forms.

ACETYLCHOLINESTERASE (AChE)[1] (EC 3.1.1.7.) at the neuromuscular junction in skeletal muscle is highly concentrated in the portion of the muscle fiber's basal lamina that occupies the synaptic cleft, where it terminates synaptic transmission by hydrolyzing the neurotransmitter acetylcholine (e.g., McMahan et al., 1978; McMahan and Slater, 1984; Katz, 1966). Much, if not all, of the enzyme is adherent to the synaptic basal lamina, and readily detectable levels remain associated with it for weeks after degeneration of all cells at the neuromuscular junction.

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1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; 2-PAM, 1-methyl-2-hydroxy-iminomethylpyridinium.

Muscle contains several molecular forms of AChE which fall into two classes: asymmetric forms, which consist of a head, containing the catalytic subunits, attached to a non-catalytic collagen-like tail; and globular forms, which are monomers, dimers, and tetramers of catalytic polypeptides almost identical to the subunits in the head of the asymmetric forms (for reviews see Massoulié and Bon, 1982; Anglister and McMahan, 1984; Silman and Futerman, 1987; Rotundo, 1987; Toutant and Massoulié, 1988). Several lines of evidence indicate that at the neuromuscular junctions of rat muscles asymmetric forms are primarily extracellular (Hall, 1973; Vigny et al., 1976; Younkin et al., 1982; Fernandez et al., 1984). Because the tails are collagenous and because collagen, a known component of basal lamina, has binding sites for other basal lamina components (as well as for itself) (Timpl and Dziadek, 1986), it has been suggested that the tails help anchor the enzyme to the basal lamina (Anglister and Silman, 1978; Massoulié and Bon, 1982). Indeed, tailed

AChE forms in vitro have high affinity for proteoglycans, common constituents of basal laminae (Bon et al., 1978; Vigny et al., 1983; Grassi et al., 1983; Brandan and Inestrosa, 1984).

As part of a long-term study aimed at determining the mechanisms involved in the accumulation and turnover of AChE in the synaptic basal lamina (e.g., Anglister and McMahan, 1985; McMahan and Wallace, 1989; Anglister, 1991; Anglister et al., 1994), we undertook to characterize directly the molecular forms of the enzyme associated with the synaptic basal lamina. To this end we analyzed the forms of the enzyme in the junctional regions of the frog's paired cutaneous pectoris muscles under several different experimental conditions. The cutaneous pectoris muscles are advantageous for such studies because they are conveniently situated for surgical manipulations, can be pinned out intact in a petri dish for exposure to different reagents, and are two to four muscle fibers thick, thus permitting rapid and uniform penetration of reagents. Moreover, it has been demonstrated that AChE is stably bound to the synaptic basal lamina in these muscles (McMahan et al., 1978; Anglister and McMahan, 1984).

Our experiments were divided into two sets. In the first set, we exposed normal pinned-out muscles to AChE inhibitors using procedures that resulted in the selective inhibition of either intra- or extracellular enzyme. We then assayed for inhibited or active forms of the enzyme in extracts under conditions chosen to solubilize selectively extracellular matrix-bound enzyme, but not membrane-bound or intracellular enzyme. In the second set, we damaged muscles in vivo in ways that resulted in removal of cells at the synaptic site but left the synaptic basal lamina largely intact. This enabled us to analyze directly forms of AChE in the synaptic basal lamina in the absence of potential contribution of intracellular or membrane-bound enzyme. We also characterized the amphiphilic (or hydrophilic) properties of the AChE forms by analyzing their interactions with various detergents. Results of all experiments indicate that the frog synaptic basal lamina contains globular as well as asymmetric forms of AChE, with small globular species being predominant and largely non-amphiphilic, and that the strength of association of the asymmetric and globular forms with other basal lamina components differs. Some of our findings on damaged muscles have been briefly reported elsewhere (Anglister, L., and U. J. McMahan. 1983. *Soc. Neurosci. Abstr.* 9:540).

Materials and Methods

Biochemical and Analytical Techniques

Muscle Extracts and AChE Assay. Frogs (*Rana pipiens*), anesthetized by immersion in water containing 0.1% MS-222 (tricaine methane sulfonate; Sigma Chemical Co., St. Louis, MO), were perfused with Ringer's solution (50 ml, 116 mM NaCl, 20 mM KCl, 1.8 mM $CaCl_2$, 10 mM sucrose, 1 mM NaH_2PO_4, pH 7.2) and the cutaneous pectoris muscles (or basal laminae preparations, see below) were removed and pinned out in a Sylgard-coated Petri dish containing ice cold frog Ringer's solution. Altogether, the steps required less than 15 min. Virtually all the neuromuscular junctions in the cutaneous pectoris muscle are distributed across the middle third of the muscle (the innervated region), which is clearly distinguished by imaging nerves and myelinated axons with a dissecting microscope and transmitted illumination. We separated the innervated from the non-innervated parts of each muscle in less than 1 min by cutting across the muscle. When treatment of the muscles with inhibitors was required, it was done in frog Ringer's solution following dissection and prior to separation of innervated from non-innervated regions (for details see Results). To extract total AChE, the isolated preparations were homogenized (1 min) in a glass–glass conical homogenizer with ice-cold extraction buffer. The buffer contained 10 mM phosphate (pH 7.3), 2 M NaCl, 1% Triton X-100, 20 mM EDTA, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 20 U/ml aprotinin, 20 μg/ml pepstatin, 1 mM benzamidine, 1 mM *N*-ethylmaleimide, and 0.1 mg/ml bacitracin) (Sigma Chemical Co.). To extract only salt-soluble AChE, detergents were omitted, and the buffer contained high or low salt concentrations, as required. Protein was determined according to Bradford (1976). AChE activity was measured by a radiometric assay, monitoring the ^{3}H-acetate generated during acetylcholine hydrolysis (Johnson and Russell, 1975). The units of AChE activity (U) express picomoles of acetylcholine hydrolysed per hour, at room temperature. When comparing samples of normal muscles, specific activities (U/μg protein) were used. However, when normal muscles were compared with preparations of damaged muscles we used activities per whole muscle. This representation was considered preferable because much of the protein content of the muscles was lost during the process of degeneration and phagocytosis of cells that resulted in vacated basal lamina sheaths which, like other extracellular matrices, are relatively poor in protein but rich in proteoglycans and glycosaminoglycans (Timpl and Dziadek, 1986). Thus, a ratio of AChE activity to protein in these damaged preparations would not reflect the real decrease in AChE content that had occurred during degeneration.

Velocity Sedimentation Analysis. To determine sedimentation coefficient values of the different AChE molecular forms, samples were analyzed by sucrose gradient centrifugation, following the original procedure of Martin and Ames (1961): A sample containing AChE (50–70 μl) was layered on top of a 4.2 ml 5–20% linear gradient formed over a 0.2 ml 50% sucrose cushion, all made up in 10 mM phosphate buffer (pH 7.3), containing 1 M NaCl and 1% Triton X-100 (unless otherwise specified). To test the sensitivity of AChE forms to detergents (described under individual experiments), the samples were first incubated without or with appropriate detergents and then applied onto a gradient containing the respective detergent. Gradients were sedimented at 50 K rpm, for 8 h at 4°C, in a Beckman SW60Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). Each sample contained three enzyme markers: *Escherichia coli* β-galactosidase (16 S), horse liver catalase (11.3 S), and alkaline phosphatase (6.1 S) (Sigma Chemical Co.). 50–60 fractions were collected from each gradient and were tested for AChE by the radiometric assay (Johnson and Russell, 1975), and for the enzyme markers by the appropriate assays (Worthington Manual; Worthington, Freehold, NJ). To determine only true AChE, each sample was preincubated (20 min) with 0.1 mM iso-OMPA (tetra-isopropylpyrophosphoramide) (Sigma Chemical Co.). For specific inhibition of true AChE, samples were preincubated with and assayed in the presence of 1 μM BW284c51 (1,5-*bis* [4-allyldimethylammonium-phenyl] pentane-3-one dibromide) (Burroughs Wellcome Co., Research Triangle Park, NC) (Austin and Berry, 1953).

Analysis of Stokes Radii. Gel filtration was used to estimate the Stokes radii of AChE molecular forms. Chromatography was performed on Biogel A1.5 (1.5 cm × 60 cm, 15 ml/h). The Stokes radius was determined from the exclusion ratio of the sample, as compared to standard protein markers, as described by Bon et al. (1973, 1979). We used blue-Dextran 2000 (Sigma Chemical Co.) and potassium ferricyanide as markers for the void volume and the total included volume, respectively.

Operations

For all operations, frogs were anesthetized with MS-222 (as above). Cutaneous pectoris muscles, which lie directly beneath the skin of the frog's chest, were exposed by cutting and folding back a flap of skin (Sanes et al., 1978). Following surgery the skin incisions were closed with 7–0 suture. Frogs were housed at 20–22°C and were fed live crickets or house fly maggots three times a week.

Muscle-damaging Techniques

Freezing. A brass block cooled in liquid nitrogen was pressed for 15 s against the innervated region, i.e., the middle third of the muscle. After letting the muscle thaw, the freezing procedure was repeated two more times over the innervated region and three times on each of the non-innervated regions of the muscle. Freezing and thawing caused disintegration of all cellular components, myofibers, axon terminals, and the Schwann cells that cap the terminals of the neuromuscular junctions, but the basal lamina sheaths persisted (McMahan and Slater, 1984). Usually, extensive freezing and thawing blocks all myofiber regeneration because it kills the muscle satellite

cells which give rise to new myofibers. On occasion, however, satellite cells near the muscle's origin and insertion escape damage and some regeneration occurs. To completely block regeneration of all myofibers, the muscles were routinely cut at the boundaries of the frozen region, and the cut ends were frozen again.

Cutting. Most of the non-innervated region and some of the innervated region were cut out and discarded leaving in situ a 1–2-mm wide strip of muscle fiber segments, many of which were innervated, across the breadth of the muscle bed (Sanes et al., 1978; McMahan and Slater, 1984). Muscle fiber segments up to 2-mm-long completely degenerate while their basal lamina remains intact; segments greater than 2 mm often fail to degenerate completely. On occasion, to preserve virtually all junctional and extrajunctional basal lamina while causing degeneration of all muscle fibers, we cut the muscle by making numerous short incisions across the non-innervated area and carefully across the myofibers between the nerve arbors in the innervated area, so as not to cause direct damage to the nerve branches and terminal arbors and to components associated with them (Yao, 1988).

Crushing. The muscle was cut at its origin and insertion and pinned out in a Sylgard-coated petri dish containing frog Ringer's solution (see above). The muscle was viewed using a dissecting microscope, while the fire-polished edge of a glass-slide was pressed into the muscle, across the muscle fibers, with enough force to indent the Sylgard. The crushed region became transparent instantly. 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 area of the muscle was damaged by a series of overlapping crushes. The sequence was repeated twice more before replacing the muscle in its bed. The skin was sutured 5 min later, after the muscle had adhered to the underlying matrix.

Denervation. At the time of muscle damage, the nerve to the cutaneous pectoris was cut at the lateral edge of the muscle and a 1–2-cm long stretch of the nerve was removed central to the cut. One week later, a 1–2-cm segment of the second spinal nerve was excised near the vertebral column. This sequence prevented reinnervation of muscles throughout the course of study.

X-irradiation. To prevent regeneration of myofibers (after cut- or crush-damage), each of the surgical procedures described above was followed by x-irradiation of the frogs. Once on each of the first 3 d following surgery, the frogs were anesthetized and the thorax was exposed to irradiation (2,400 rads/dose) (Anglister and McMahan, 1985).

Light and Electron Microscopy

The cutaneus pectoris muscles, dissected and pinned out in Sylgard-coated petri dishes, were fixed with 1% glutaraldehyde in 0.09 M phosphate buffer (pH 7.2) for 30 min and stained for AChE activity according to Karnovsky (1964), using 0.08 mM acetylthiocholine for 10 min (Anglister and McMahan, 1985). Only extracellular AChE was stained by this procedure. The preparations were then refixed with 1% osmium tetroxide in the phosphate buffer (1 h), dehydrated in ethanol, rinsed in propylene oxide, and embedded in a mixture of Epon and Araldite (Ladd Res. Inds., Inc., Burlington, VT). For light microscopy, fixation in osmium tetroxide was omitted. Muscles were embedded flat in a wafer of plastic (<1-mm thick) supported by a slide, as described elsewhere (McMahan and Slater, 1984). In the wafers, muscle fibers and their neuromuscular junctions could be seen clearly with a light microscope. Specific regions of the muscle were cut out and sectioned. Thick sections (1 μm) were stained lightly with 0.25% Toluidine blue. Thin sections were counter stained with uranyl acetate and lead citrate for electron microscopy.

Results

Molecular Forms of AChE in Normal Muscle

To characterize the molecular forms of AChE concentrated at the neuromuscular junctions of frog muscle, *Rana pipiens*, the total enzyme (cytoplasmic, membrane-bound, and basal lamina-associated) was extracted from the junctional region (see Materials and Methods) of cutaneous pectoris muscles in high ionic strength buffer containing detergent, and analyzed by sucrose gradient centrifugation (Bon et al., 1979). Several molecular forms of AChE, distinguished by their sedimentation coefficients, were resolved: 17.5, 13.5, 10.5, and 4–6 S (Fig. 1 *a*). Nicolet and Rieger (1982) have observed similar forms in *Rana temporaria*. The non-innervated areas of the muscle contained the same forms, but the total AChE content per unit wet weight or relative to protein content was 5–10-fold (7.1 ± 2.7 SD, $n = 9$) less than the content in junctional regions: 1,100 AChE U/μg protein (±500 SD) for junctional segments, and only 150 U/μg protein (±75 SD) in nonjunctional segments. Thus, along frog myofibers, as in human muscles (Carson et al., 1979), AChE is highly concentrated at innervated regions, but not in a significantly different pattern of molecular forms from that in non-innervated regions.

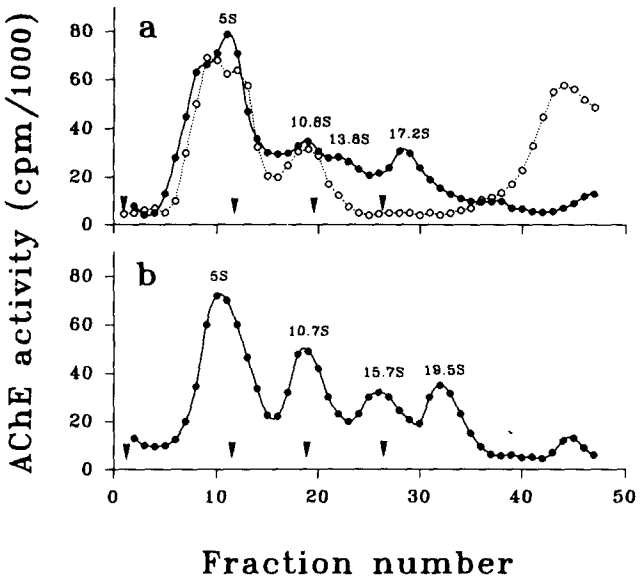


Figure 1. Frog cutaneous pectoris muscle contains globular and asymmetric collagen-tailed molecular forms of AChE. Samples of AChE extracted in buffer containing high salt concentration, detergent and protease inhibitors were analyzed by sucrose gradient sedimentation in buffers containing either high salt (●) or low salt (○) and sedimented at 54,000 rpm, 4°C for 5 h (low salt) or 6.5 h (high salt). AChE activity was measured by the radiometric assay and expressed in arbitrary units. (*a*) Normal muscle. (*b*) Normal muscle extract that had been incubated with collagenase (1 h, room temperature) before sedimentation. Arrowheads indicate the top of the gradient and migration of standard protein markers: alkaline phosphatase (6.1 S), catalase (11.3 S) and β-galactosidase (16 S) (from left to right).

Collagen-tailed forms of AChE are characterized by their capacity to aggregate reversibly at low ionic strength and display an increased sedimentation coefficient upon controlled digestion with collagenase (Johnson et al., 1977; Anglister and Silman, 1978; Bon and Massoulié, 1978). We therefore examined the forms of AChE from cutaneous pectoris muscle at high and at physiological ionic strength and after treatment with collagenase type III (15 U/ml, devoid of non-specific protease activity; Advanced Biofactures Co., Lynbrook, NY; e.g., Fernandez et al., 1984). As shown in Fig. 1 (*a* and *b*) the 17.5 and 13.5 S forms aggregated at physiological ionic strength and were converted to forms with higher sedimentation coefficients, of 19.5 and 15.5 S, respectively, by collagenase. The 10.5 and 4–6 S forms were unaffected by either of these treatments. Thus, as in muscles of other species, the

larger forms of AChE in frog muscles apparently have collagen-like tails, while most if not all of the smaller forms do not.

Little, if any, of the cholinesterase activity detected in the cutaneous pectoris muscle was due to pseudocholinesterase. More than 99% inhibition was observed in the presence of the true-AChE inhibitor BW284c51 (1 μM), while less than 2% inhibition was found when iso-OMPA (0.1 mM), a selective pseudocholinesterase inhibitor, was added to the samples (see Materials and Methods).

Extracellular AChE at Normal Neuromuscular Junctions

Inactivation of Extracellular AChE. One method we used for establishing the amount and molecular forms of AChE on the cell surface at the neuromuscular junctions was to treat whole pinned-out cutaneous pectoris muscles with echothiophate (phospholine, O,O-diethyl-S-[2-trimethylammonium-methyl] phosphorothiolate iodide; Ayerst Laboratories, New York), a charged organophosphorus inhibitor of AChE, which inhibits cell surface AChE selectively since it permeates only slowly through the cell membrane (e.g., Younkin et al., 1982). Accordingly, relative amounts of intra- and extracellular enzyme could be calculated by subtracting the level of uninhibited intracellular enzyme from total active enzyme in the muscles; total active enzyme was determined either from contralateral untreated muscles or from a set of treated muscles in which the inhibited extracellular enzyme had been reactivated with the specific AChE reactivator, 2-PAM (1-methyl-2-hydroxy-iminomethylpyridinium; Sigma Chemical Co.; 1 mM, 1 h, room temperature; see below) (Hobbiger, 1963). This approach has been successfully used to distinguish external from internal enzyme in various tissue culture preparations and mammalian muscles (Lazar and Vigny, 1980; Younkin et al., 1982; Fernandez et al., 1984). As shown in Fig. 2 (*inset*), there are two distinct phases of inhibition by echothiophate, a rapid phase which leads to inactivation of about 60% of the total AChE activity in less than 5 min, and a slow phase in which less than half of the residual activity is inhibited in 20 min. The rapid component is interpreted as inactivation of external enzyme, while the slow component is thought to be due to gradual penetration of the inhibitor into the cell and to subsequent inactivation of the intracellular pool (e.g., Younkin et al., 1982). Indeed, when frog muscles were homogenized before drug application, which removed permeability barriers and exposed the intracellular enzyme to inhibition, more than 98% of the activity was inhibited in less than 1 min (data not shown). Thus, with limited periods of exposure (4 min at 0°C) echothiophate can be used for frog muscles, as for muscles of other animals, to inactivate almost exclusively cell surface AChE. Altogether the findings indicate that >50% of the enzyme in the junctional region is on the cell surface.

To determine the molecular forms of the cell surface AChE and primarily those associated with basal lamina, we carried out the following experiments. The paired cutaneous pectoris muscles of six frogs were separated and one muscle of each frog was exposed to echothiophate (as above). The contralateral muscles served as untreated controls for determining total AChE. Both echothiophate-inactivated and untreated control muscles were then divided into innervated

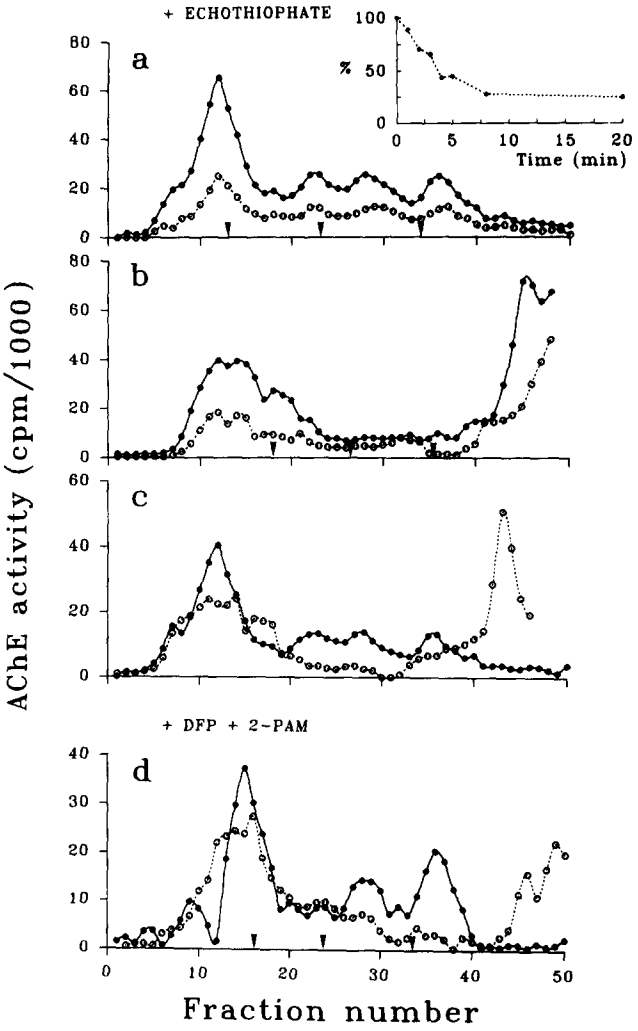


Figure 2. Molecular forms of extracellular AChE in the synaptic region of frog muscle. Sedimentation analysis was as described in Fig. 1. Muscles were treated with either echothiophate (*a–c*) or with a sequence of inhibition by DFP followed by 2-PAM reactivation (*d*). Inset describes the time course of inactivation of AChE in muscle at 0°C by 1 μM echothiophate: the active (uninhibited) AChE in each muscle was expressed as a percent of total AChE extracted from the control, untreated contralateral muscle of the same frog. Each point is the mean of 3–7 measurements. (*a*) Echothiophate-treated muscles, in which extracellular AChE was inactivated (○), and untreated contralateral muscles (●) were extracted and analyzed in sucrose gradients, all performed in buffer containing 1 M NaCl and no detergent (see text for details). (*b*) Muscles were treated as in *a* but extracted and analyzed in low salt buffer (0.1 M NaCl, no detergent). (*c*) External AChE obtained by subtracting the internal enzyme from total AChE in muscles, extracted and analyzed in high salt (●) or low salt (○) solutions, shown in *a* and *b*, respectively. (*d*) Muscles were first incubated with DFP to block all AChE activity, and then reactivated with 2-PAM to unmask the activity of external AChE. Extraction and sedimentation analysis were done either in high salt (●) or in low salt (○) buffers, as in *a* and *b*, revealing a profile of molecular forms of the external AChE similar to that obtained in *c*, with a major 4–6 S component solubilized in both high salt and low salt buffers. Arrowheads denote the sedimentation of standard markers, as in Fig. 1; position of markers in *c* is as in *a* for high salt and as in *b* for low salt curves.

and non-innervated regions, subjected to extraction in buffer containing a high salt concentration but no detergent, so as to minimize any solubilization of plasma membrane-bound AChE that might occur. The extracts were assayed for AChE activity and analyzed by sucrose gradient centrifugation. The amounts and forms of AChE derived from neuromuscular junctions (junction-specific AChE) were assessed by subtracting values measured for a given amount of protein in the non-innervated regions from those for an equal amount of protein in the innervated regions, assuming that the non-junctional AChE is practically constant throughout the length of each myofiber (see Hall, 1973). Fig. 2 *a* illustrates that echothiophate inactivated globular as well as tailed forms at the neuromuscular junctions. There was a marked decrease in the 4–6 S constituent. We, then, sought to determine the amount of 4–6 S extracellular AChE at the neuromuscular junctions in the following way: the active (intracellular) AChE was subtracted from total AChE, revealing the pool of extracellular AChE (Fig. 2 *c*), and the percentage of 4–6 S component in that pool was calculated. In three such experiments (2 muscles/experiment), the percentage of 4–6 S AChE was 55% (range, 45–65%) of the total.

Unlike the asymmetric forms, the extracellular 4–6 S AChE could be solubilized from neuromuscular junctions in isotonic Ringer's solution. Fig. 2 *b* illustrates the result of an echothiophate inhibition experiment identical to that described above and shown in Fig. 2 *a*, but in which the muscles were extracted and analyzed by sucrose gradient centrifugation at low ionic strength. Two major AChE components were resolved: a broad component sedimenting at 4–6 S and heavy material sedimenting to the bottom of the gradient, which may include both low salt aggregates of asymmetric forms and insoluble AChE associated with small tissue fragments. Both the 4–6 S AChE and the precipitating component were markedly inactivated by echothiophate, indicating that they had been extracellular.

Globular AChE forms, although extractable at low ionic strength, can be either nonamphiphilic or amphiphilic (Toutant and Massoulié, 1987). We analyzed the fraction of extracellular AChE extracted either at high or at low ionic strength and sedimenting as 4–6 S species, with respect to amphiphilic character, by examination of its interactions with different detergents. The detergents we used (cholate, Triton-X-100, and Brij 96) differ considerably in their aggregation numbers (listed in Sigma Chemical Co. catalogue), i.e., in the size of the micelles they form and in the number of detergent molecules bound to the amphiphilic (hydrophobic) protein. Accordingly cholate, which binds to an amphiphilic AChE with a low aggregation number (2–3), would have almost no effect on its sedimentation coefficient, while Triton X-100 and Brij 96, which form very large micelles, would reduce markedly the sedimentation of the protein (as in Futerman et al., 1985; Bon et al., 1988). Low salt extracts of echothiophate-inhibited muscles (see above) and contralateral control muscles were first spun to remove insoluble material (100,000 *g*, 30 min, 4°C). Aliquots of the supernatants were incubated with the three detergents and then analyzed in detergent-containing sucrose gradients. Extracellular AChE, extracted at low ionic strength and in the absence of detergents, migrated without detergents as a broad ~5 S component (Fig. 2 *c*). However, in the presence of cholate, while most of the activity was condensed at 5.2 S, an additional minor peak migrating at 6.8 S was well resolved; this minor component was not observed in Brij 96 or Triton X-100, where all AChE activity migrated at 4.9 or 5.3 S, respectively (not shown, but as in Fig. 3; see below). Consequently, most of the extracellular low salt soluble AChE, is a 5 S form, not sensitive to detergents, and thus non-amphiphilic. The detergent-sensitive 6.8 S AChE, resolved in cholate, is amphiphilic and resembles the hydrophobic AChE-dimer extracted from *Torpedo* electric organ (e.g., Futerman et al., 1985) and other sources (Toutant and Massoulié, 1987).

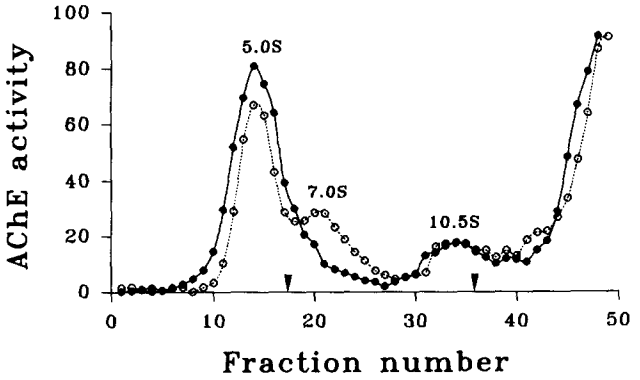


Figure 3. Non-amphiphilic and amphiphilic components in salt soluble 4–6 S AChE. Extracellular AChE in muscles pretreated in a sequence of DFP + 2-PAM (as in Fig. 2 *d*), solubilized in high salt buffer was analyzed by sucrose gradient sedimentation performed in low salt buffer containing either 0.5% Brij 96 (●) or 0.5% cholate (○). AChE activity is expressed as in Fig. 1. Arrowheads denote sedimentation of alkaline phosphatase (6.1 S) and catalase (11.3 *S*). The major 5 *s* component is not influenced by the detergents (compare with Fig. 2 *d*), and is therefore non-amphiphilic. The sedimentation of the 6.8 S AChE resolved in cholate is shifted in Brij, characteristic of amphiphilic AChE dimer, and cosediments with the 5 S component.

Inactivation of Intracellular AChE. We carried out a sequence of steps that produced, in contrast to the previous procedure, muscle preparations in which the intracellular enzyme was irreversibly inhibited but the extracellular AChE was active and could be directly examined. We first inactivated both extra- and intracellular AChE covalently, using the lipid-soluble organophosphorous reagent, diisopropylfluorophosphate (DFP; McIsaac and Koelle, 1959; Anglister and McMahan, 1985). The extracellular enzyme was subsequently reactivated with 2-PAM (see above), a quaternary oxime, with poor permeability through membranes (Hobbiger, 1963). Accordingly, 10 muscles were dissected in Ringer's solution and exposed to DFP (1 mM, 1 h, room temperature). This procedure inactivated >99% of total AChE activity in these muscles (Anglister and McMahan, 1985). The muscles were then rinsed extensively in Ringer's solution (five changes over 15 min, 4°C) and incubated with 2-PAM (0.1 mM, 45 min, room temperature). After washing the muscles to remove reagents, one muscle of each frog was extracted in buffer containing both high salt and detergent (1 M NaCl, 1% Triton X-100), so as to allow estimation of all extracellular AChE (i.e., membrane-bound and matrix-associated). Comparison of the amount of activity extracted from the neuromuscular junctions of these muscles (junction-

specific AChE determined as described above) with that extracted from the junctional regions of the untreated muscles revealed that 58% ± 8 (SD, $n = 7$) of the original activity had been restored by 2-PAM treatment, indicating that it was extracellular. To confirm that this partial restoration of activity was indeed due to selective reactivation of extracellular AChE, muscles were homogenized before being incubated with 2-PAM, under the same conditions used for the intact muscles. As expected, without the plasma membrane as a barrier, more than 90% of the original activity was restored (data not shown). Higher levels of reactivation could be obtained for such extracts if longer incubation times or higher concentrations of 2-PAM were used, but such conditions were avoided in intact muscles so as to minimize penetration of 2-PAM into the cells. As a confirmation that the 2-PAM–reactivated enzyme in intact junctions was indeed extracellular, we subjected the muscles, immediately after reactivation, to inhibition by echothiophate, using the same conditions for selective inhibition of extracellular AChE as used in the preceding section. Echothiophate reduced the junctional AChE activity in the 2-PAM–reactivated muscles in this experiment from 52% ± 7 (SD; three muscles) to less than 5% of the original activity, indicating that nearly all of the activity recovered was extracellular.

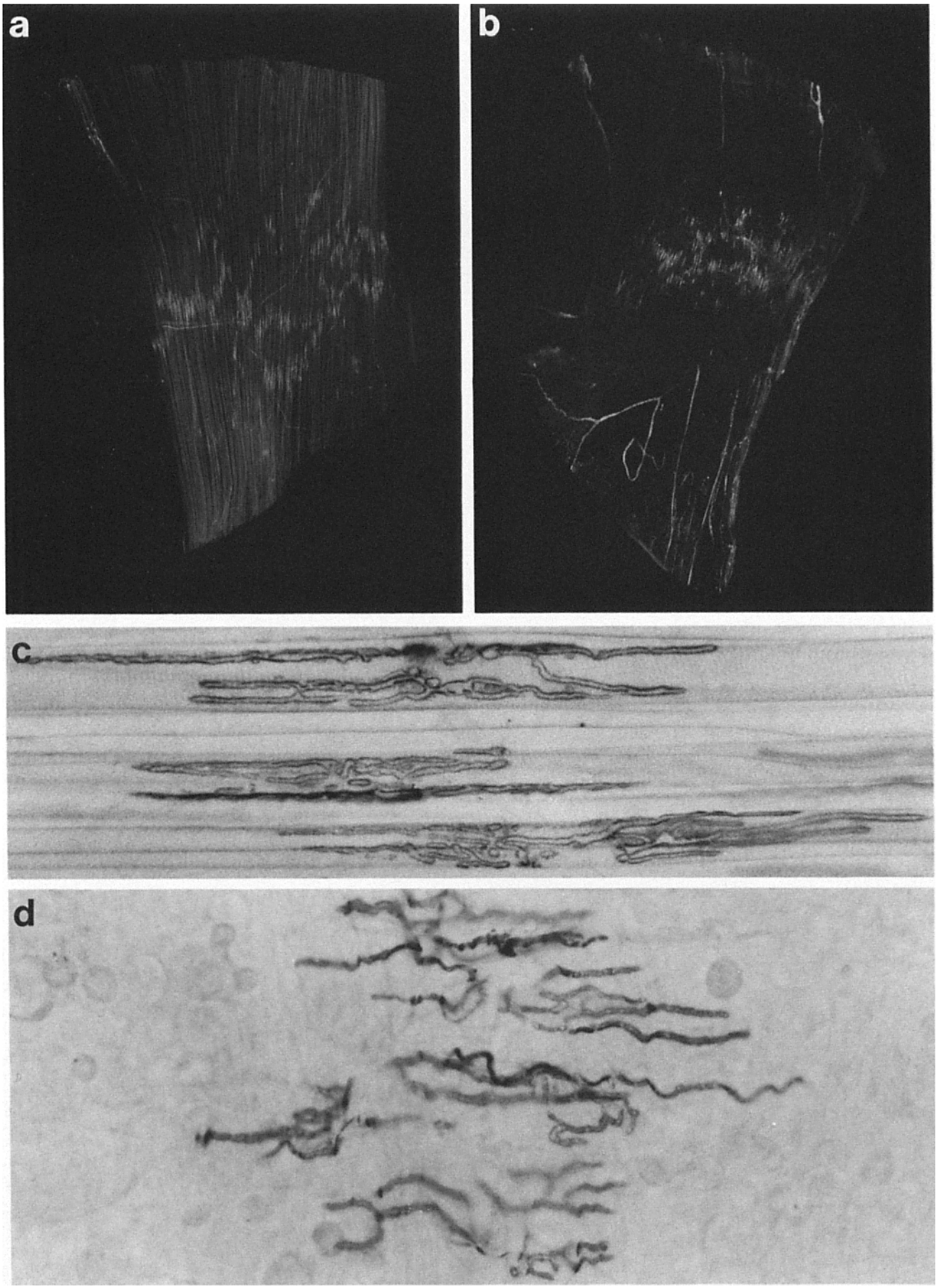
To identify the molecular forms of the extracellular AChE at the neuromuscular junctions that are associated with the extracellular matrix, we extracted 2-PAM–reactivated junctional enzyme in high ionic strength buffer, in the absence of detergents. In three experiments we found that high ionic strength buffer alone could solubilize 84% ± 3 (SD) of the total pool of extracellular enzyme in the junctional region which could be extracted by high salt in the presence of detergent (characterized above). Sucrose gradient–sedimentation analysis of the high salt extract of the 2-PAM–reactivated junctional enzyme showed that both asymmetric and small globular AChE forms were present. As illustrated in Fig. 2 *d*, the 4–6 S AChE accounted for 50% of the total activity. Moreover, the small globular component of the 2-PAM–reactivated enzyme was solubilized in Ringer's solution while the rest of the enzyme remained insoluble (Fig. 2 *d*).

The small globular components solubilized in either high or physiological salt concentrations were tested with respect to their amphipathic nature by analysis of their interaction with detergents in two ways. One way, as described above, was examination of shifts in sedimentation dependent upon the detergent used. All the activity of the small globular AChE extracted in high salt migrated at 5.0 S in Brij 96, whereas in cholate although more than 75% of the activity appeared at 5.3 S the rest migrated at 6.9 S (Fig. 3), indicating an interaction of the latter species with detergents. Migration in Triton-X-100 was as in Brij 96, and as in the absence of detergents (Fig. 2 *d*). Similar analysis of the small AChE species extracted at low ionic strength revealed a ratio of 5 to ~7 S of about 2:1. Another test for the amphipathic nature of the extracellular synaptic AChE solubilized in the absence of detergents was to analyze its partitioning in Triton-X-114 solution (TX114, Octylphenoxypoly-ethoxyethanol; Sigma Chemical Co.) (Bordier, 1981). After phase separation, integral membrane proteins, as well as hydrophobic proteins containing glycolipid anchors, are recovered in the detergent phase, while hydrophilic proteins are found in the aqueous phase (Etges et al., 1986). Only 25–30% of the activity of the junctional extracellular and low salt–soluble AChE was found in the TX114 detergent phase, while most of the activity partitioned into the aqueous phase (two experiments, eight muscles each). (As expected, the AChE in either phase migrated as 5.5 S species in gradients containing 1% Triton-X-100). In control experiments more than 90% of the hydrophobic G_2-AChE detergent-purified from *Torpedo* electric organ (containing a glycolipid anchor; Futerman et al., 1985) was recovered in the detergent phase. However, after degradation of the hydrophobic tail with PI-specific phospholipase-C, the hydrophilic G_2 product (same reference) shifted, as expected, to the aqueous phase. This experiment indicates that more than 70% of the small extracellular AChE in frog muscle does not bind detergents at all, or not as efficiently as integral membrane or glycolipid containing proteins.

These observations on extracellular junctional AChE studied in muscles where it was selectively reactivated following a total DFP block, yield data closely resembling those obtained in the echothiophate experiment, where extracellular AChE was inhibited selectively and could be analyzed only by the absence of its contribution to the total intracellular–extracellular pool (compare Fig. 2, *c* with *d*). Thus, the globular 4–6 S AChE is present at the neuromuscular junctions in frog muscle, where it comprises more than half of the extracellular AChE, and is extracted, together with the asymmetric forms, in high salt in the absence of detergent. Unlike the asymmetric forms, the 4–6 S AChE is soluble also in isotonic buffers. The major small globular AChE, a 5 S form, is non-amphiphilic, but an amphiphilic component is also present, and depending on the detergent used, migrates at 5 S in Brij 96 or Triton X-100, and at 6.8 S in cholate.

AChE Solubilized from Isolated Whole Muscles. The following experiment demonstrates that globular AChE can be released from pinned out whole muscles bathed in normal Ringer's solution. Paired muscles from five frogs were separated and pinned in different dishes. One muscle of each pair was repeatedly flushed with Ringer's solution, expelled from a micropipet over the junctional area for 2 h at room temperature, and then overnight at 4°C, while the control con-

Figure 4. The distribution of AChE in freeze–damaged muscles resembles the distribution of extracellular AChE in normal muscles. (*a* and *c*) Whole mounts of normal muscles stained for AChE. (*b* and *d*) Whole mounts of 30 d freeze–damaged muscles, stained for AChE, in which the cellular components have been phagocytized while myofiber basal lamina sheaths persist. The preparations in *a* and *b* were photographed by placing them in a photographic enlarger and projecting the image onto photographic paper. Accordingly, these are negative images: the white streaks that run parallel to the long axis of each muscle and occupy the middle third of the muscle are AChE outlines of the elongate synaptic sites characteristic of frog neuromuscular junctions. High magnification views of the synaptic sites were obtained using regular light microscopy (*c* and *d*). Shortening and folding of the synaptic sites in the freeze–damaged tissue is due to collapse and wrinkling of the basal lamina sheaths in the absence of muscle fibers. Bars: (*a* and *b*) 1 mm; (*c* and *d*) 30 μm.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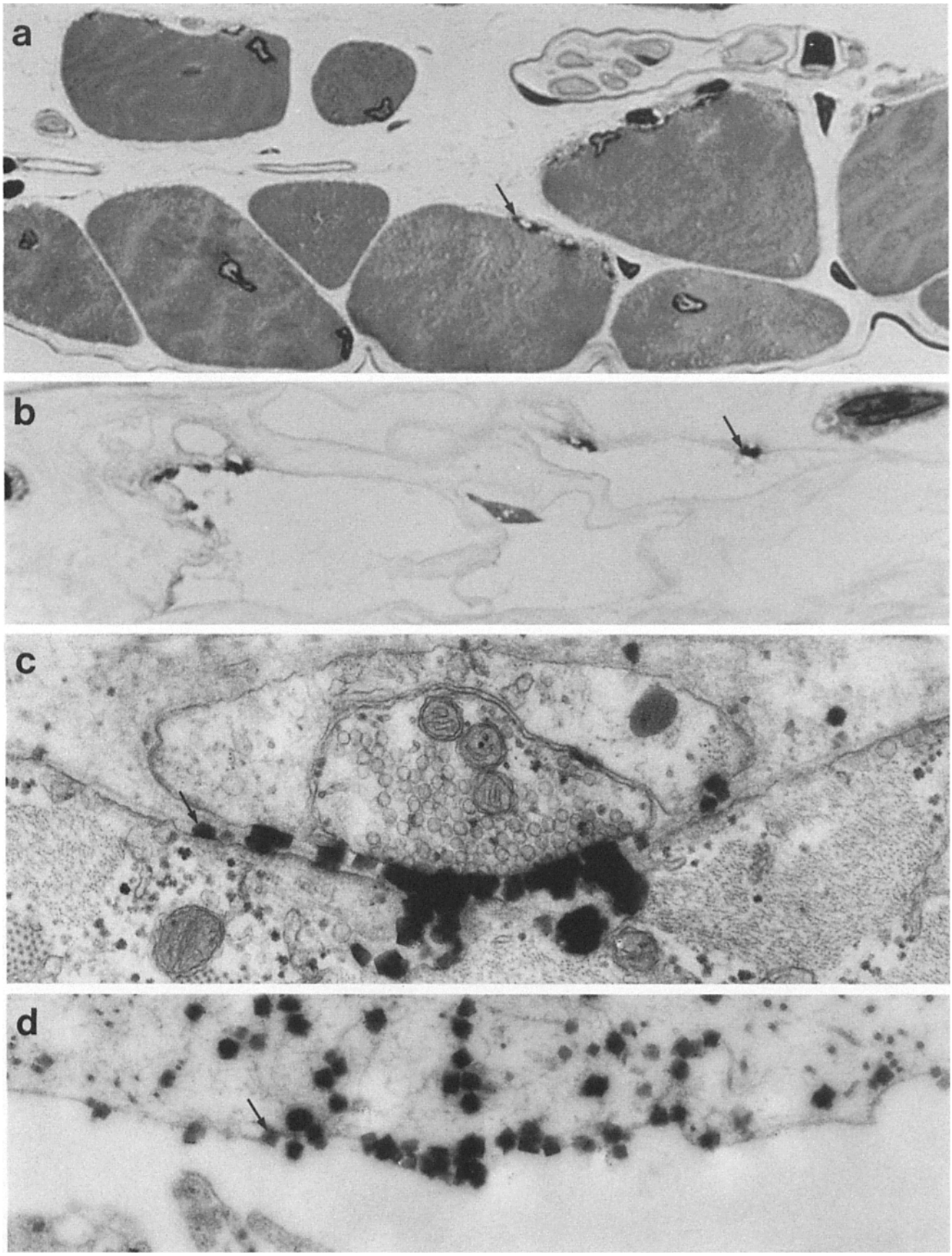
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tralateral muscle was left at 4°C throughout the entire period. The Ringer's solution of the washed muscle contained AChE activity that resolved as 4–6 S AChE upon sucrose gradient analysis, the pattern obtained being similar to that obtained for extracellular AChE in low ionic strength buffers (Fig. 2, *c* and *d*). There was no detectable damage to muscle fibers as observed by light microscopy in whole mounts; however, histochemical staining for AChE at neuromuscular junctions (see below) was slightly less intense than in control muscles (data not shown). These findings are consistent with the conclusion that the globular form is on the surface of the cells and can be dissociated in isotonic buffer.

Extracellular AChE at Damaged Neuromuscular Junctions

Freeze-Damage

The Distribution of AChE in Myofiber Basal Lamina Sheaths. Previous studies on frog cutaneous pectoris muscle have shown that by 30 d after freeze-damage, nearly all fragments of muscle fibers, axon terminals, and Schwann cells detectable in the electron microscope have been removed from the synaptic sites on the myofiber basal lamina; fragments of cells can be observed at less than 2% of cross-sectioned synaptic sites (McMahan and Slater, 1984). As shown in Figs. 4 and 5, the distribution of AChE in these muscles, despite the absence of cells, is nearly the same as normal. Stain for AChE activity as seen by light microscopy, was confined to the junctional region of the damaged muscles (Figs. 4, *a* and *b*, and 5, *a* and *b*) and was arranged in arborizations characteristic of normal neuromuscular junctions (Fig. 4, *c* and *d*). When viewed by electron microscopy, crystals of stain were concentrated in the synaptic portion of the basal lamina sheath (Fig. 5, *c* and *d*). This staining pattern was the same in muscles taken >6 mo after freeze-damage as it was after 1 mo.

AChE Activity. During the 30 d following freeze-damage, AChE activity in the cutaneous pectoris muscle declined markedly, in parallel to the phagocytosis of the disintegrated myofibers, axon terminals and Schwann cells (Fig. 6, *inset*). At 30 d, after most of the cellular components had disappeared (see Materials and Methods; also documented in the following), total AChE activity in homogenates of the junctional regions was 5% ± 0.7 (SEM, $n = 20$) of the total activity present in homogenates of the whole normal muscle. (Calculations evaluating the fraction of synaptic basal lamina AChE activity in normal junctions that was retained in the damaged sheaths are given in Discussion.) The junctional region contained almost all (>98%) of the AChE activity in the damaged sheaths. The amount of enzyme in the junctional region 30 d after freeze-damage remained nearly the same for several months. Thus, four months after damage, activity in the junctional region of the damaged muscles was still 3.5% ± 0.4 (SEM, $n = 7$) of that in normal muscle (Fig. 6, *inset*).

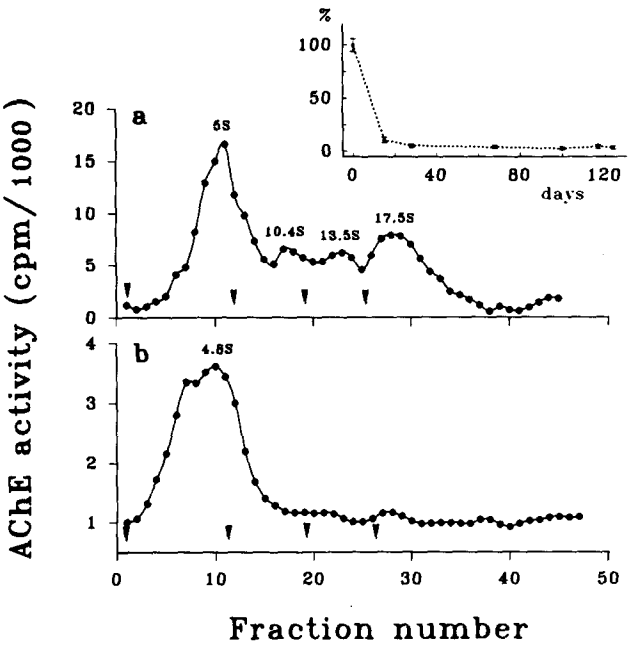

Figure 6. 4–6 S AChE remains attached to synaptic basal lamina after freeze-damage. Muscles that had been denervated and damaged by freezing in vivo were dissected out and AChE was extracted and analyzed. Inset illustrates the decline in total AChE activity in the damaged preparations (± SEM, results of 5–9 individual experiments, each combining 3–4 muscles) as a function of days after damage. Molecular forms were resolved by sucrose gradient sedimentation (5–20%) in buffer containing high salt concentrations and detergent (as in Fig. 1). (*a*) freeze-damaged muscle, 15 d after the operation, when degeneration was still taking place, indicating that despite the decrease in total AChE activity, several molecular forms could be resolved as in normal muscles (Figs. 1 *a*, 2 *a*). (*b*) Freeze-damaged muscle, 30 d after operation, when synaptic sites were clean of cellular components (illustrated in Fig. 5, *b* and *d*). Only a 4–6 S AChE component was retained in the sheaths. Arrowheads denote the top of the gradient and the migration of standard protein markers (as in Fig. 1).

Molecular Forms. At 2 wk after freeze-damage, homogenates of junctional region tissue were examined for their pattern of AChE molecular forms by sucrose gradient sedimentation. In spite of the loss in total AChE activity (Fig. 6, *inset*), the pattern of molecular forms revealed by the analysis resembled that obtained upon analysis of normal muscles (compare Figs. 6 *a* with 1 *a* and 2 *a*). This profile reflects a marked decrease in the content of each individual form (Fig. 7). During this time, degeneration was still taking place, and the preparations contained fragments of muscle fibers as well as invading phagocytic cells. This was documented by electron microscopy: cross-sections through junc-

Figure 5. AChE in freeze-damaged frog muscle remains associated with synaptic basal lamina in the absence of myofiber and nerve terminal. Light (*a* and *b*) and electron (*c* and *d*) micrographs of cross-sections through junctional area of normal (*a* and *c*) and freeze-damaged (*b* and *d*) frog muscles stained for AChE. In both normal muscles and in the vacated freeze-damaged preparations, crystals of reaction product are concentrated in the synaptic portion of the myofiber basal lamina sheaths (arrows). Bar: (*a* and *b*) 30 μm; (*c* and *d*) 1 μm.

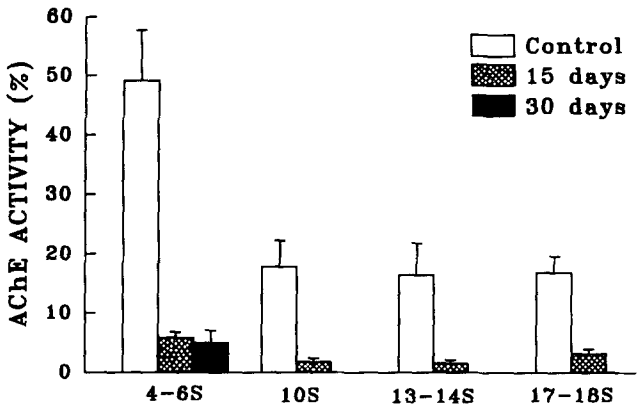

Figure 7. AChE molecular forms in freeze-damaged muscles. Data shown are means ± SEM (error bars) from 12–20 independent experiments given as percent of total AChE activity in undamaged control muscles. Bars represent control (*clear*), preparations dissected 15 d (*cross-hatched*, as in Fig. 6 *a*), and 30 d (*black*, as in Fig. 6 *b*) after freeze-damage. Only a 4–6 S AChE component could be detected at 30 d.

tional regions of the damaged muscle preparations, 2 wk after freeze-damage, were examined. Out of a total of 377 profiles of myofiber basal lamina envelopes checked in six muscle preparations (50–90 envelopes/muscle), only 72% ± 16 (SD) were wholly devoid of cells or cellular processes.

As expected, at 30 d after damage nearly all of the myofiber basal lamina sheaths were empty. Out of a total of 365 profiles of sheaths counted in cross-sections of eight damaged muscle preparations (50–80/muscle), the envelopes that contained remnants of cellular processes were less than 3% per muscle (±3 SD), indicating that degradation was essentially complete. In fact, less than 2% of the synaptic sites had remnants of cellular fragments. Preparations at this stage were analyzed for AChE molecular forms that had persisted at their synaptic sites; as reported above, AChE activity at extrajunctional regions was non-detectable based on histochemical and biochemical assays. As illustrated in Fig. 6 *b*, sucrose gradient sedimentation analysis revealed that almost all (97.7% ± 0.7 SEM, $n = 12$) of the AChE activity in the damaged muscle migrated with a sedimentation coefficient of 4–6 S. Only in a few preparations could we detect minute amounts of activity corresponding to forms of higher sedimentation coefficients (Fig. 6 *b*).

To further characterize the 4–6 S AChE, specifically with regard to shape and size so as to confirm that it indeed represents small globular species, we measured the Stokes radius of that form extracted from freeze-damaged preparations and of the 4–6 S component of normal muscles, which had been separated from the other forms by sucrose gradient centrifugation. Using exclusion chromatography (as in Bon et al., 1973, 1979; Anglister and Silman, 1978; details in Materials and Methods) an identical value of 5.0–5.5 nm was obtained for the Stokes radius of the 4–6 S species from both normal and damaged muscles. Thus, the 4–6 S form in damaged muscles is similar, at least in its physicochemical parameters, to the globular 4–6 S AChE in intact normal muscles of frog, and to the globular monomers and dimers (G_1, G_2), found in electric fish, birds, and mammals (Bon et al., 1979, 1988). The small globular AChE was further analyzed for amphipathic properties (i.e., for capacity to associate with detergent micelles, see below).

Our observation that extracts of freeze-damaged preparations contained almost exclusively small globular AChE, while extracts of normal muscles contained both globular and asymmetric forms, together with the earlier finding that asymmetric AChE is sensitive to proteolytic degradation (e.g., Anglister and Silman, 1978), raised the possibility that the globular forms in the damaged muscles were derived from asymmetric forms by proteases present in the damaged preparations, that were released during the extraction procedure despite the presence of several protease inhibitors in the extraction medium. To rule out this possibility, in two experiments we combined a segment of a normal muscle with two damaged muscles dissected 30 d after freeze-damage. The normal muscle segment and the damaged muscles contained similar amounts of total AChE activity, and were extracted together in buffer containing salt, detergent, and protease inhibitors, as routinely used in the extraction procedure (see under Materials and Methods). Sucrose gradient analysis of the mixed preparations resulted in a profile of AChE molecular forms which was identical to the summation of the individual sedimentation profiles of normal muscle and freeze-damaged muscles extracted separately (data not shown), indicating that tailed forms were not degraded to globular forms during extraction.

Solubility. Homogenization of the freeze-damaged muscles in Ringer's solution was sufficient to solubilize most of the enzyme in the empty sheaths. Of the total activity extracted by a phosphate buffer containing both high salt and detergent (1 M NaCl, 1% Triton X-100), 98% ± 1 ($n = 7$) could be solubilized in Ringer's solution. As indicated by the following experiment, even extensive washing of a pinned-out, freeze-damaged muscle with frog Ringer's solution resulted in solubilization of the enzyme: the pairs of muscles from three frogs were pinned out in six different dishes and maintained at 4°C. One muscle from each pair was washed by repeatedly and vigorously expelling frog Ringer's solution from a pipet over the junctional area of the muscle (for 3 min every 30 min, over a total period of 15 h). The other muscle served as control. The muscles were then stained for AChE. For each of the three pairs, the washed muscle stained much less than the contralateral unwashed control. Subsequent biochemical analysis showed that the Ringer's solution from the washed muscle contained 85% (range: 80–90%) of the activity extracted from the unwashed muscle by homogenization in Ringer's solution.

As mentioned earlier, globular AChE forms, extractable from normal muscles at low ionic strength in the absence of detergent, can be either amphiphilic or nonamphiphilic (e.g., Toutant and Massoulié, 1987; Bon et al., 1988). To further characterize the low salt soluble AChE in the basal lamina sheaths we studied its ability to associate with detergent micelles. As in the case of extracellular AChE at intact frog junctions, the AChE migrated at 5 S without detergents and in Brij 96 or Triton X-100, but an additional minor peak at 6.9 S was resolved in cholate, indicating the presence of a detergent-sensitive component (see previous sections). Thus, the AChE at the synaptic basal lamina is associated with other basal lamina components via weak hydrophilic and hydrophobic interactions.

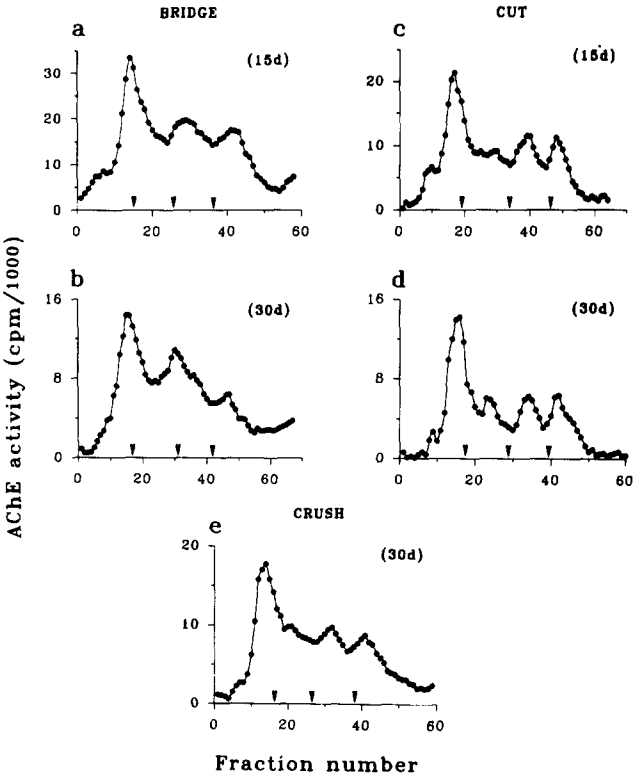

Figure 8. The 4–6 S AChE molecular form is the major form that remains attached to synaptic basal lamina after mechanical damage to muscles (cutting or crushing). Frog cutaneous pectoris muscles were damaged by cutting out slabs of muscle on each side of the innervated region (*bridge*, *a* and *b*), by cutting in between axonal branches and across myofibers (*cut*, *c* and *d*) or by crushing the whole area of the muscle (*crush*, *e*). Surgical procedures are described under Materials and Methods. The frogs were x-irradiated to prevent myofiber regeneration and were chronically denervated. The preparations were dissected out and AChE was extracted and analyzed by sucrose gradient centrifugation (as in Fig. 6) at 15 d (*a* and *c*), when degeneration was still occurring in vivo (see text), or at 30 d (*b*, *d*, and *e*), after degeneration was complete. In all cases, the 4–6 S form was the major component of AChE. Arrowheads denote the migration of standard markers (as in Fig. 1).

Cut-Damage

An alternative procedure for obtaining in vivo, myofiber- and nerve-free preparations of basal lamina sheaths with AChE maintained at synaptic sites, was to denervate and cut out slabs of the muscle, leaving a bridge of the junctional region (Sanes et al., 1978). Regeneration of the nerve and of the muscle was prevented. This procedure, unlike the freeze-damage used in the experiments described above, does not involve direct damage to the junctional region. Thus, although the axons and muscle fibers degenerate as a result of being cut, the Schwann cells originally capping the terminals at synaptic sites are spared and, at least for several days following denervation, they occupy the presynaptic side of the synaptic basal lamina after the phagocytosis of the nerve terminals and muscle fibers (Sanes et al., 1978). At 15 d after surgery, this preparation, like the freeze-damaged preparation described above, contained all forms of AChE (Fig. 8 *a*), as previously reported for *Rana temporaria* by Nicolet et al. (1986, 1987). Examination by electron microscopy of cross-sections through the preparations revealed that at this time 30% ± 17 (SD) of the profiles of myofiber basal lamina sheaths (307 in 6 preparations) still contained cells. This proportion was markedly reduced at 30 d subsequent to damage, when 10% ± 8 (SD) of the sheaths (340 in 6 muscles) had cellular processes associated with them. Sedimentation analysis revealed that, in contrast to the 30-d freeze-damaged preparations, these preparations still contained more than 10% of the total original AChE activity. Moreover, the number of molecular forms of AChE and the relative abundance of these forms resembled that found in the intact muscle (compare Figs. 8 *b* and 1 *a*) and that of the extracellular enzyme at the neuromuscular junctions (compare Figs. 8 *b* to 2, *c* and *d*), with the 4–6 S AChE being the predominant component. Similar results were obtained when the denervated and cut-damaged sheaths were prepared by cutting across the myofibers and between axonal branches, thus preserving almost fully the junctional and extrajunctional regions (Materials and Methods): all forms of AChE were present in the junctional region both at 15 and 30 d after damage (Fig. 8, *c* and *d*), and, as before, the small globular species were predominant. Thus, unlike synaptic basal lamina in denervated freeze-damaged muscles, which contained globular AChE and little, if any, asymmetric forms, the synaptic basal lamina in the denervated muscles damaged by cutting the myofibers between the nerve arbors and/or in the extrajunctional regions contained significant levels of both globular and asymmetric forms.

Crush-Damage

One explanation for the persistence of asymmetric forms in the synaptic basal lamina of denervated cut-damaged muscles, but not in freeze-damaged muscles, was that while denervation and cut-damage resulted in degeneration and phagocytosis of axons and myofibers as in freeze-damaged preparations, the Schwann cells that capped the axon terminals remained at the synaptic sites and provided factors that maintained levels of asymmetric forms. To test this possibility we carried out the following experiment: In two groups of five frogs each, muscles were cut out and damaged by crushing the whole area, thus causing, as in the case of freeze-damage, complete disintegration of all the cells in the junctional region (axon terminals, myofibers and Schwann cells). As in freeze- and cut-damaged preparations, AChE persisted in the synaptic basal lamina (McMahan and Slater, 1984). At 30 d, after all cellular components had been phagocytized, AChE was extracted and analyzed for its molecular forms. As for both freeze- and cut-damaged preparations, the major component of the synaptic basal lamina was the 4–6 S AChE. However, forms with all the other sedimentation coefficients (10, 13.5, and 17.5 S) were also resolved, in a distribution similar to that found in cut-damaged sheaths (compare Fig. 8, *e* to *b* and *d*), indicating that, in the absence of Schwann cells, as in their presence, synaptic basal lamina in mechanically damaged muscles contained asymmetric as well as globular forms and that their proportions were similar to those of intact muscles.

Discussion

When normal frog muscles were exposed to AChE inhibitors and subsequently treated in ways that permitted selective

analysis of cell surface AChE, both globular and asymmetric forms were detected, with small globular forms predominating. The small globular AChE was primarily non-amphiphilic and similar in physicochemical properties to globular monomers (G_1) but there was a small (less than 25%) contribution of amphiphilic dimers (G_2) (Bon et al., 1979, 1988; for review see Massoulié et al., 1993). The cell surface enzyme was highly concentrated in the junctional region of the intact muscle and was largely solubilized at high or low ionic strength buffers in the absence of detergent. When muscles and nerves were damaged so as to cause removal of cells and thus of intracellular and membrane-bound enzyme, while leaving basal lamina sheaths largely intact, both globular (non-amphiphilic and amphiphilic) and asymmetric forms were found to be concentrated in the junctional region of the damaged muscles. These observations, together with histochemical evidence showing that in both normal and damaged muscles AChE is highly concentrated in the basal lamina of the synaptic cleft, lead to the conclusion that synaptic basal lamina contains both globular and asymmetric forms of the enzyme.

Our findings on intact frog muscles indicate that >50% of the AChE at the muscles' junctional region is located on the cell surface and thus is available for transmitter hydrolysis. Some of the cell surface AChE may be embedded in the lipid bilayer of the plasma membranes of either the axon terminal or muscle fiber or both, via a glyco-phosphatidylinositol anchor or by other posttranslational modifications, as observed mainly for globular forms in other excitable tissues (e.g., Silman and Futerman, 1987; Eichler et al., 1992) or possibly by a hydrophobic polypeptide sequence (Massoulié et al., 1993). However, we show that most of the cell surface AChE in the frog muscles (>80%) does not require detergents for solubilization and should, therefore, contain the basal lamina AChE. We further demonstrate that about 60% of the cell surface enzyme that dissociates in salt solutions in the absence of detergents is small globular AChE, of which most is non-amphiphilic. Accordingly, globular forms comprise more than half of the AChE in the synaptic cleft, and at least half of the basal lamina AChE.

Both muscle fibers (Weinberg et al., 1981) and nerve terminals (Anglister, 1991) release AChE that is incorporated into the synaptic basal lamina. Both pre- and postsynaptic cells synthesize globular and asymmetric forms (Rotundo, 1987), and there is good evidence that both provide the asymmetric forms in the basal lamina (Weinberg and Hall, 1979; Anglister and Haesaert, 1991). Accordingly, it may well be that the globular forms in the basal lamina of the frog's neuromuscular junction are also secreted by both cell types. The observation of Jerdrzejczyk et al. (1984) that the only form of AChE present in chick tonic muscles is globular is consistent with the likelihood that at least some of the globular AChE at the frog neuromuscular junction is indeed secreted as such by either muscle or nerve or both. We find that the globular/asymmetric ratio of AChE in the basal lamina is approximately the same as in the muscle as a whole, which is consistent with the possibility that the exocytosis of both forms is jointly regulated.

Some of the globular AChE in the basal lamina might also be derived from the turnover of asymmetric forms. Since the basal lamina AChE has no obvious link to the plasma membrane, the mechanism of its removal during turnover might involve extracellular degradation rather than cellular internalization, causing the conversion of some of the asymmetric AChE to smaller globular forms. Indeed, certain proteases are known to degrade or remove the collagen-like tail while leaving catalytic subunits intact (e.g., Anglister and Silman, 1978). However, to date the reported degradation products of asymmetric forms are tetramers (G_4) (for review see Massoulié et al., 1993).

The possibility that globular forms in the basal lamina were the result of the proteolytic degradation of asymmetric forms during the extraction procedure is most unlikely for several reasons. First, in the present study, muscles were dissected rapidly, in ice-cold Ringer's solution; innervated regions were separated and immediately homogenized in ice-cold extraction buffer and analyzed. All extraction buffers contained a repertoire of protease inhibitors, in a combination successfully used in studies of AChE of mammalian and avian muscles, based on studies by Barnard and co-workers (Silman et al., 1978) who showed that use of a suitable cocktail of protease inhibitors, in conjunction with rapid dissection of fresh tissue appears to prevent proteolytic degradation almost completely (e.g., Jedrzejczyk et al., 1984). Second, the composition of forms in extracts of normal frog muscles, whether of total AChE, of extracellular or intracellular pools, or of damaged muscles, was found to be highly reproducible. Third, experiments which used the same procedures, but varying concentrations of protease inhibitors (not shown), did not reveal any significant proteolytic action on AChE forms in frog muscles. Fourth, since no asymmetric forms were detected in the freeze-damaged muscles, we were concerned whether in that unique situation, there might be a specific high proteolytic activity. In a "mixing" experiment, in which basal lamina sheaths were extracted together with a piece of normal muscle, the asymmetric forms present in the normal muscle were fully recovered in the combined extract. Thus, no potent proteolytic activity was exposed in the freeze-damaged preparation by the extraction procedure. If proteolysis is involved, the possibility that endogenous local proteolysis had occurred during the 30-d period of muscle and nerve degeneration, subsequent to freeze–damage (discussed above), seems much more plausible.

In considering the origin of the globular AChE in the synaptic basal lamina of damaged muscle, it may be argued that these forms may have been originally associated with the plasma membranes, despite their solublility in the absence of detergents, and that they had migrated following damage and removal of nerve and muscle cells to the adjacent basal lamina. This could explain in part the presence of an amphiphilic component in our salt extracts. However, the fact that little, if any, of the A_{12} or A_8 forms (or their putative G_4 degradation product) are retained in the freeze-damaged sheaths makes it unlikely that fresh forms external to this structure would be recruited. Furthermore, the data presented in Fig. 8, relating to patterns of AChE in basal lamina sheaths obtained after mechanical damage, all reveal percentages of small globular AChE similar to those present at the synapse in intact muscle. It is difficult to conceive how such proportions would be achieved by recruitment of exogenous forms in the course of degeneration. It is also worth noting that in order to detect the presence of residual plasma membrane in the damaged muscle preparations, we checked (by

^{125}I-α-Bungarotoxin binding assays) the freeze- and mechanically damaged sheaths for AChRs, which are highly concentrated at normal neuromuscular junctions, at levels severalfold higher than AChE (data not shown). No AChRs were detected indicating that this hydrophobic membrane protein did not migrate from the synaptic membranes to the basal lamina.

Our findings on freeze-damaged muscles, and on muscles damaged by cutting or crushing them, indicate that by 30 d after damage, when phagocytosis of the synaptic cells is nearly complete, AChE persists at the synaptic sites in the vacated basal lamina sheaths. Its amount is 5% and 10%, respectively, of the total amount of activity in whole intact muscle and it remains at such levels for months. The fraction of synaptic basal lamina AChE that persists after damage can be estimated as follows: our data on intact muscles reveal, on average, sevenfold more AChE per unit wet weight at the innervated region than in non-innervated area. Since only the middle one third of the cutaneous pectoris muscle is innervated, and the neuromuscular junctions occupy only a small fraction of that area, about 2/3 of the total AChE activity in the muscle is junction-specific. As discussed above 50% of the junction-specific AChE is extracellular, and out of that fraction 80% is basal lamina enzyme. Based on these values less than 25% of the AChE in the normal muscle is in the synaptic basal lamina and accordingly, in the freeze- and cut-damaged preparations, the persisting AChE constitutes 20 and 40%, respectively, of the AChE in the normal synaptic basal lamina. Thus, even though the enzyme is extracellular and stably associated with basal lamina, it and/or the basal lamina components to which it binds are apparently not resistant to the degradative events that accompany degeneration of the synaptic cells. It is well documented that a marked degradation of molecular components of extrajunctional myofiber basal lamina accompanies myofiber degeneration in mammalian muscles (e.g., Gulati et al., 1983).

One month after mechanical damage both globular and asymmetric forms were present in synaptic basal lamina, in ratios similar to those obtained in the salt extract of synaptic extracellular AChE of intact muscle, but 1 mo after freeze-damage we could reliably detect only globular forms in the synaptic basal lamina. One explanation for our detections of only small globular AChE in extracts of 30-d freeze-damaged muscles is that because 4–6 S AChE was originally present in basal lamina at a higher concentration than individual asymmetric AChE (e.g., Fig. 2 *c*) at 30 d after damage only globular AChE was abundant enough to be detected. Alternatively, the asymmetric forms may be more sensitive to the degeneration process than the globular forms. Consistent with this possibility is the fact that, based on the calculations presented above, about 40% of the detectable AChE in the freeze-damaged preparations should have been asymmetric.

We find that, unlike asymmetric forms which are solubilized in high salt buffer, globular AChE is largely extracted in low salt buffer. It is believed that interactions of asymmetric AChE with basal lamina are mainly electrostatic, and that accordingly, high salt disrupts these interactions between the collagen-like tail of the asymmetric forms and other basal lamina components (for review see Silman and Futerman, 1987). The low salt solubility of globular AChE indicates that it may be attached to basal lamina, for the most part, by weaker hydrophilic–electrostatic associations. The amphiphilic dimer may be attached by hydrophobic interactions instead of or as well as hydrophilic ones. Moreover, the globular AChE, whether amphiphilic or not, could be also mechanically entrapped in the basal lamina. For example, the diffusion of globular AChE in its physiological environment in vivo might be impeded by the apparent elaborate and dense glycoprotein jungle that composes the basal lamina (Sanes, 1982; Timpl and Dziadek, 1986). Since the small globular AChE may consist of the same catalytic subunits as asymmetric AChE (T-type; see Massoulié et al., 1993), this raises the possibility that both the catalytic subunits and collagen-like tails serve to keep the asymmetric forms in place in the basal lamina. The stronger combined association of the asymmetric forms may serve to restrict them to the immediate site of enzyme release, i.e., near the pre- and postsynaptic membranes, whereas the weaker associations of the globular forms may permit slow diffusion and thus a broader distribution. A difference in distribution might result in the two forms having different roles in regulating acetylcholine concentrations in the synaptic cleft. It will be of interest to learn whether globular AChE forms are also found in the basal lamina at mammalian neuromuscular junctions, as well as the asymmetric AChE (Hall, 1973; Younkin et al., 1982; Fernandez et al., 1984).

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