Globular and Asymmetric Acetylcholinesterase in Frog Muscle Basal Lamina Sheaths

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Abstract. After denervation in vivo, the frog *cutaneus pectoris* muscle can be led to degenerate by sectioning the muscle fibers on both sides of the region rich in motor endplate, leaving, 2 wk later, a muscle bridge containing the basal lamina (BL) sheaths of the muscle fibers (28). This preparation still contains various tissue remnants and some acetylcholine receptor–containing membranes. A further mild extraction by Triton X-100, a nonionic detergent, gives a pure BL sheath preparation, devoid of acetylcholine receptors. At the electron microscope level, this latter preparation is essentially composed of the muscle BL with no attached plasmic membrane and cellular component originating from Schwann cells or macrophages. Acetylcholinesterase is still present in high amounts in this BL sheath preparation. In both preparations, five major molecular forms (18, 14, 11, 6, and 3.5 S) can be identified that have either an asymmetric or a globular character. Their relative amount is found to be very similar in the BL and in the motor endplate–rich region of control muscle. Thus, observations show that all acetylcholinesterase forms can be accumulated in frog muscle BL.

In normally innervated vertebrate skeletal muscle, acetylcholinesterase (AChE,[1] EC 3.1.1.7) is concentrated at the motor endplate. Some of the frog junctional enzyme is localized in muscle synaptic basal lamina (BL) (20). Moreover, the asymmetric forms of AChE, composed of tetrameric protomers and a collagen-like multistranded tail (4, 6, 9, 25; see reference 19 for a review), have been shown to interact after extraction with typical BL components such as glycosaminoglycans (3), fibrous material (33), fibronectin (11), or heparan sulfate proteoglycan and laminin (29, 32). In mouse muscle, the asymmetric forms of AChE have been shown to be part of the synaptic BL (7) and may play a crucial role in cholinergic neurotransmission (14, 16, 30). The frog BL sheath preparation allows a direct approach for the determination of the cellular distribution of the AChE forms between either cytoplasmic or plasmic and external cell layers. We show in this report that all AChE molecular forms, and not exclusively asymmetric forms, are contained in or associated with BL of the motor endplate–rich (MEP-r) region of frog muscle.

Materials and Methods

Frogs and BL Preparation

Experiments were performed on the paired *cutaneus pectoris* muscles of adult male frog, *Rana temporaria* L. (18–30 g body weight). The in vivo experimental procedure of Sanes et al. (28) was used to obtain muscle BL in the MEP region after degeneration and phagocytosis of muscle cell components and nerve terminals. On day 1, after tricaine methane-sulfonate (MS 222 Sandoz, 1p1000, Sandoz Ltd., Basel) anesthesia, the nerve to the right *cutaneus pectoris* was transected near the muscle, and a 4–7-mm nerve segment was excised. Two rectangular slabs were removed from muscle on each side of the intramuscular nerve trunk, leaving a bridge (1.5 mm wide) of damaged muscle fibers corresponding to the MEP-r region. On day 3, frogs were x-ray irradiated (100 kV, 8 mA; filtration, 1.7 mm Al; total dose, 1,600 rad with 127 rad/min; from source to animal thorax, 30 cm) upon a restricted surface of the experimental hemithorax. After the operation, the frogs were kept in water tanks at room temperature (19–22°C). Bridges of BL and contralateral MEP-r regions were biopsied for analysis 15 d after surgery.

Pure BL Preparation

We purified the above described BL bridges, extracting non-BL components using a simple incubation in a detergent medium (1% Triton X-100, 0.001 M EGTA, and 0.01 Tris-HCl, pH 7.2) with intermittent shaking at 20°C for 75 min followed by three washes in Ringer medium.

Extraction and Analysis of AChE and Its Multiple Molecular Forms

After rinsing in Ringer's solution, pooled muscle samples (4 to 8 MEP-r regions, BL, or purified BL sheaths) were extracted at 4°C with a glass-glass conical hand homogenizer in either a one- or a two-step procedure. In the one-step method AChE was solubilized in a standard medium (1% Triton X-100, 1 M NaCl, 0.001 M EGTA, and 0.01 M Tris-HCl, pH 7.2) with or without antiproteases (21). In the two-step procedure, the first extraction was carried out in a detergent medium (1% Triton X-100, 0.001 M EGTA, 0.01 M Tris-HCl, pH 7.2). The second extraction was performed on the resulting pellet in a high salt medium (1 M NaCl, 0.001 M EGTA, 0.01 M Tris-HCl, pH 7.2). All centrifugations in these procedures were carried out at 27,000 g_{max} at 4°C, and tissue extracts or exudates were immediately analyzed.

AChE activity was estimated by the method of Ellman et al. (10) using acetylthiocholine (ActhCh) as a substrate. The specific reversible AChE inhib-

[1] *Abbreviations used in this paper:* ACh, acetylcholine; AChE, acetylcholinesterase; AChR, ACh receptor; ActhCh, acetylthiocholine; α-Bg-Tx, α-bungarotoxin, BL, basal lamina(e); MEP, motor endplate; MEP-r, motor endplate rich; MPT, methylphosphorothiolate derivative.

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itor BW 284 C 51 (Burroughs-Wellcome, Research Triangle Park, NC) was sometimes used to assess enzyme specificity. Analysis of AChE forms was performed on a 5–20% continuous sucrose gradient (26). Peak areas were determined only for the major forms (3.5, 6, 11, 14, and 18 S), without correction for the contributions of other minor forms, using a Kontron computer (Kontron Bioelectronique, Velizy) and a program correcting for spontaneous nonenzymatic ActhCh hydrolysis (8).

Collagenase Treatment

Pure collagenase (Advance Biofactures Corp., Lynbrook, NY) was added (500 IU/ml) to aliquots of muscle extracts in a high salt medium (1% Triton X-100, 1 M NaCl, 1 mM $CaCl_2$, 50 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.2). Collagenolytic action was obtained by 30-min incubation at 20°C immediately before sedimentation analysis (3).

AChE Irreversible Inhibition In Vivo by a Methylphosphorothiolate Derivative (MPT)

To evaluate the recovery of enzymes in MEP-r regions and BL, a sublethal dose (100 μg/kg) of MPT, known as a specific irreversible and cell-permeant inhibitor of AChE (32, 13), was injected into the subcutaneous lympathic sac of experimental frogs 3 d after surgery. The animals were kept for various periods for recovery.

Acetylcholine Receptor Analysis by Binding of ^{125}I-labeled α-Bungarotoxin (^{125}I-α-BgTx)

MEP-r regions and BL preparations were incubated in a culture medium (24) containing ^{125}I-α-BgTx (15–20 Ci/μg, New England Nuclear, Boston, MA) at 0.2 μg/ml for 20 min at 20°C. Then the samples were washed twice with the incubation medium and twice with a Ringer's solution. After extraction in the standard medium, a specific irreversible toxin-AChR complex sediments as a main 9 S peak (24).

Electron Microscopy

Conventional electron microscopy was performed as described by Pinçon-Raymond and Rieger (23).

Results

Presence of All AChE Forms in Muscle MEP-r Regions and BL Preparations

Globular and Asymmetric Molecular Forms in Normal Muscle: Action of Collagenase. In extracts of *cutaneus pectoris* muscle obtained with the standard medium, eserine (5×10^{-4} M), which inhibits both AChE and butyrylcholinesterase, inhibited at least 95% of the hydrolysis of ActhCh. 5 μM BW 284 C 51, the selective inhibitor of AChE, inhibits this hydrolysis by ~90%, and 5 μM of isooctamethyl pyrophosphoramide (Sigma), a specific inhibitor of butyrylcholinesterase, 15%. Thus, under standard conditions most of ActhCh hydrolysis is due to specific AChE. The sedimentation analysis of the extracts on continuous sucrose gradients reveals a complex profile reflecting the AChE polymorphism (Fig. 1). We identify five major forms of AChE: 3.5, 6, 11, 14, and 18 S.

After treatment of the muscle extracts by collagenase, at 20°C, there is no change in total AChE activity. The collagenase treatment at 20°C provokes an increase of the sedimentation coefficients of the two heavy forms, from 14 and 18 S to 16.5 and 20.5 S, respectively, (Fig. 2). Incubation of samples without collagenase at 20°C, or heat-inactivated collagenase (80°C for 10 min) does not lead to any change in the sedimentation profiles.

After dialysis of the standard medium muscle extract against a low ionic strength medium (0.001 M EGTA, 0.01 M Tris-HCl, pH 7.2) 3 h, at 4°C, the sedimentation in a low

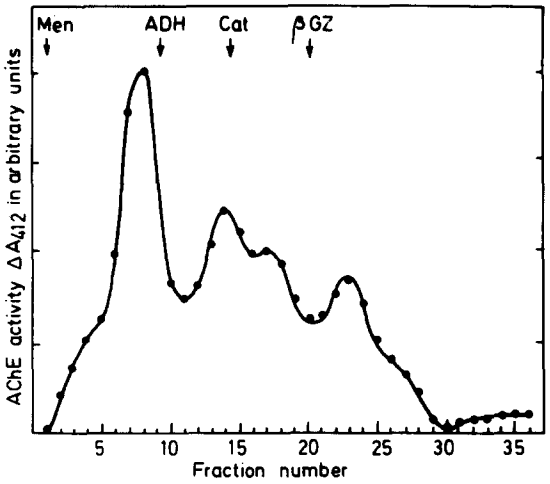


Figure 1. Sedimentation analysis of AChE extracted from intact frog *cutaneus pectoris* muscle. Extraction in standard (detergent-high salt) medium, centrifugation, and enzyme assay were done as described in Materials and Methods. Here and in Figs. 2 and 4: *Men*, meniscus. Protein markers are β-galactosidase (*βGZ*), catalase (*Cat*), and alcohol dehydrogenase (*ADH*).

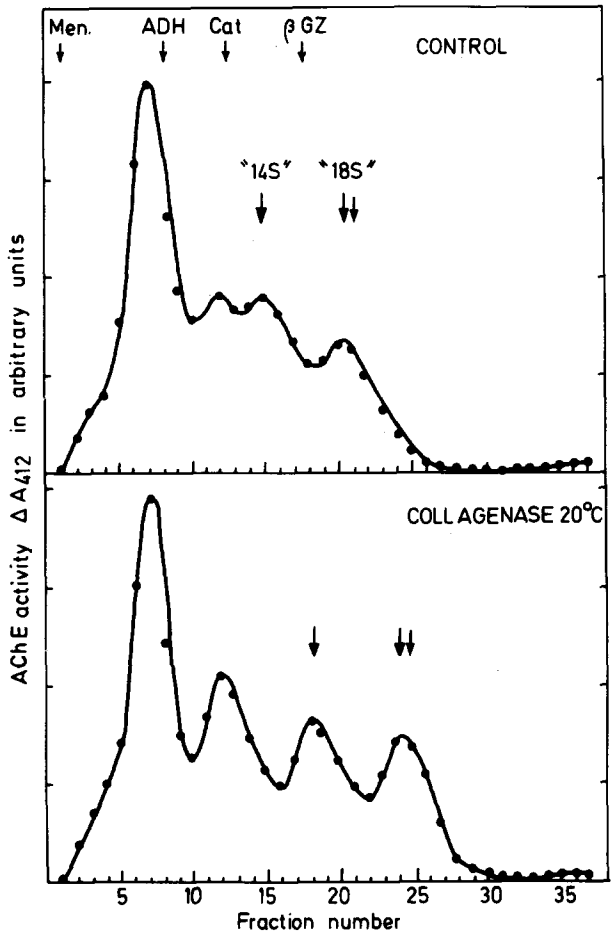


Figure 2. Sedimentation analysis of AChE of *cutaneus pectoris* muscle extract after collagenase treatment at 20°C. Extraction (detergent-high salt medium without EGTA), collagenase treatment, and centrifugation were done as described in Materials and Methods. 14 S becomes 16.5 S after collagenase (single arrow). 18 S becomes 20.5 S after collagenase (double arrows).

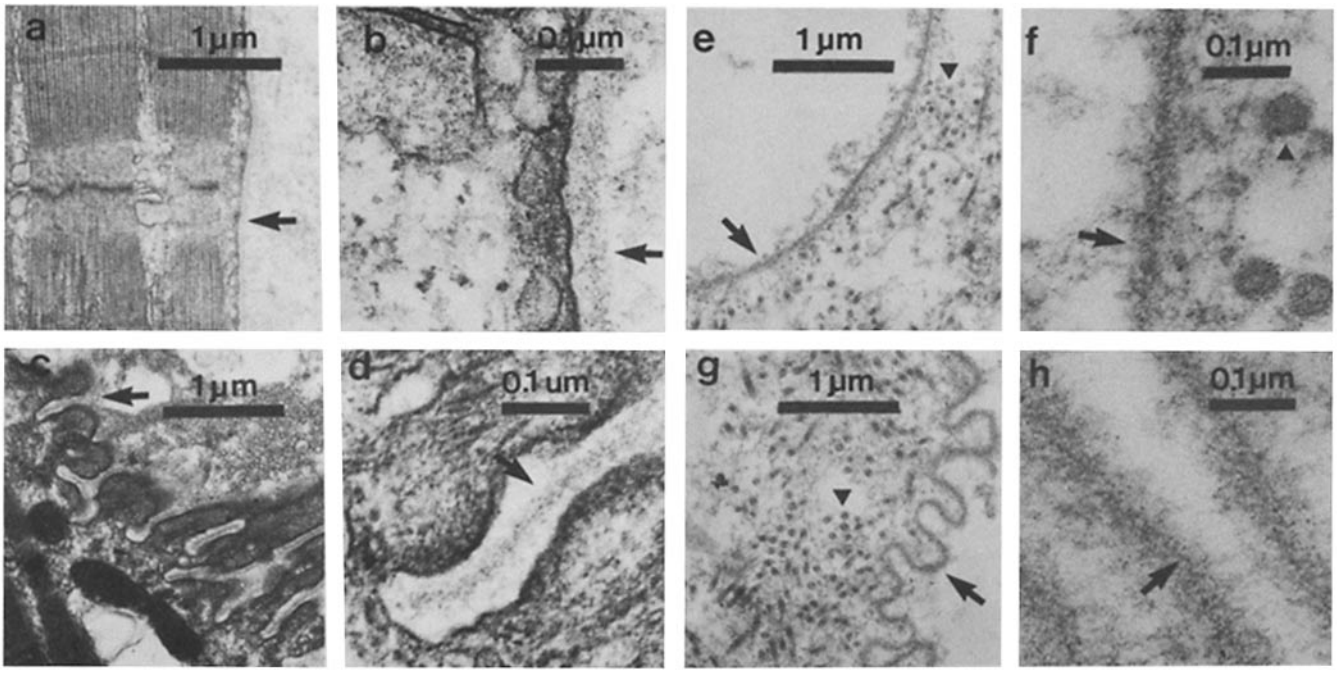
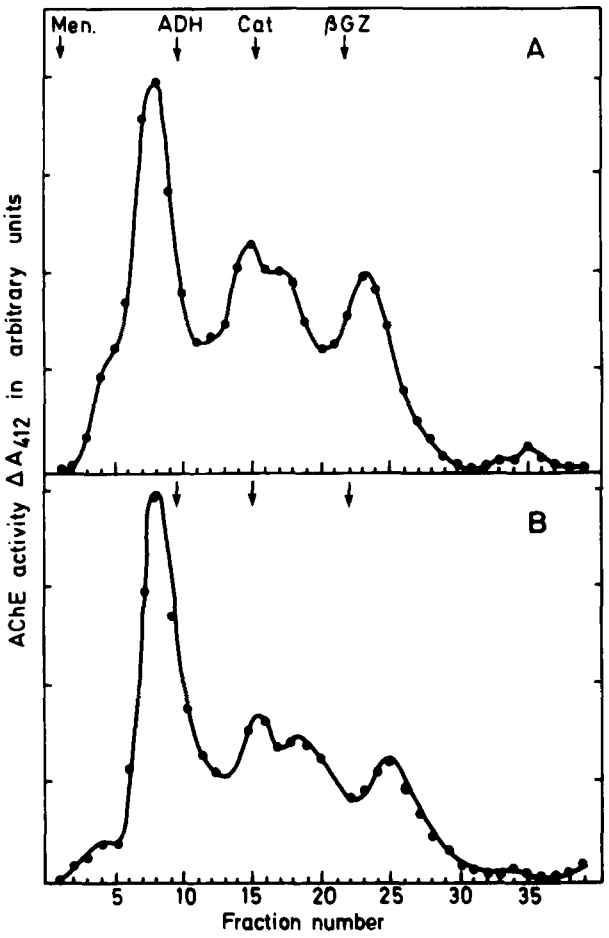


Figure 3. Electron microscopy of non-endplate- and endplate-containing sites on normal myofibers and damaged myofibers. Control muscle (*a–d*) BL, 15 d after lesion (*e–h*). Non-endplate plasmic membrane and BL are shown. Note the dense central part of the BL (arrow in *h*); (*a*) low and (*b*) high magnification. Junctional folds and synaptic BL in normal muscle; (*c*) low and (*d*) high magnification. Extrasynaptic BL; (*e*) low and (*f*) high magnification. Synaptic BL; (*g*) low and (*h*) high magnification. Arrowhead, collagen fibrils.



salt gradient shows no discrete peaks at 14 and 18 S, but only the slowly sedimenting forms (3.5, 6, and 11 S). Sedimentation of dialyzed extracts on high ionic strength sucrose gradients gives a normal distribution of AChE forms (data not shown). Thus, there is reversible aggregation of AChE heavy forms at low ionic strength. The collagenase sensitivity and low salt aggregation of frog muscle AChE heavy forms 14 and 18 S, suggest that these molecules have an asymmetric collagen-like tail and the same basic head-tail structure as in other vertebrates (6, 19, 25).

AChE Forms in BL Preparation. The procedure of Sanes et al. (28) gives a biological preparation of myofiber ghosts, which still contains the BL of the original myofiber in the MEP-r regions. An electron microscope study (Fig. 3; see also Fig. 6) shows, 15 d after surgery, that empty BL co-exists with surrounding degenerating Schwann cell processes, fibroblasts, nerve terminal debris, and collagen fibrils. Sometimes, the BL contains small mononucleated cells, probably macrophages and muscle satellite cells (Fig. 6*a*). Small fragments of plasmic membranes are occasionally seen attached to the inner surface of the BL. Most of the basal lamina does not show any important folded part except in a restricted region in which BL infolding (Fig. 3*g*) is of the size and periodicity of the junctional folds of postsynaptic membrane in intact muscle (Fig. 3*c*), as already observed in an independent study by Sanes and Chiu (27).

The quantitative analysis of AcChR estimated by ^{125}I-α-

Figure 4. Sedimentation analysis of AChE from control MEP-r region and BL. (*A*) Control muscle. (*B*) BL 15 d after lesion. Extraction was in standard (detergent-high salt) medium in the presence of protease inhibitors. The two curves are quantitatively directly comparable, although sedimentation distances were slightly higher in experiment *B*.

BgTx binding (Table I) shows that the total α-BgTx binding in the BL was ~3% of that found in the normal MEP-r region.

AChE was extracted from control MEP-r regions or experimental BL bridges in a one-step standard medium procedure. The presence or the absence of protease inhibitors did not change the relative proportion of AChE forms. Fig. 4 shows that all AChE forms are found in the BL, with the same S values as in normal muscle. Table II shows that there is no drastic change in their relative proportion, except for a significant decrease of the 3.5 S (Student's t-test, $P < 0.01$) and 18 S ($P < 0.05$) forms. Table III gives the total and specific activities of the main molecular forms of AChE in the MEP-r regions of the muscle and in the BL preparation. A rather important proportion of activity, ~20–50% relative to that of the intact MEP-r regions, remains in the BL. An important decrease of protein content (by a factor of 5) of the BL preparation is responsible for a significant increase ($P < 0.01$) of specific AChE activity of each form, except for the 3.5 S form, which remains at about the same level.

After irreversible inhibition in vivo by MPT of all preexisting enzyme, AChE recovery was compared in MEP-r and BL tissues, 3, 52, and 216 h later (Fig. 5). After 52 h, no recovery was observed in the BL sheath preparation. Less than 2.5% of initial activity was recovered between 52 and 216 h. The

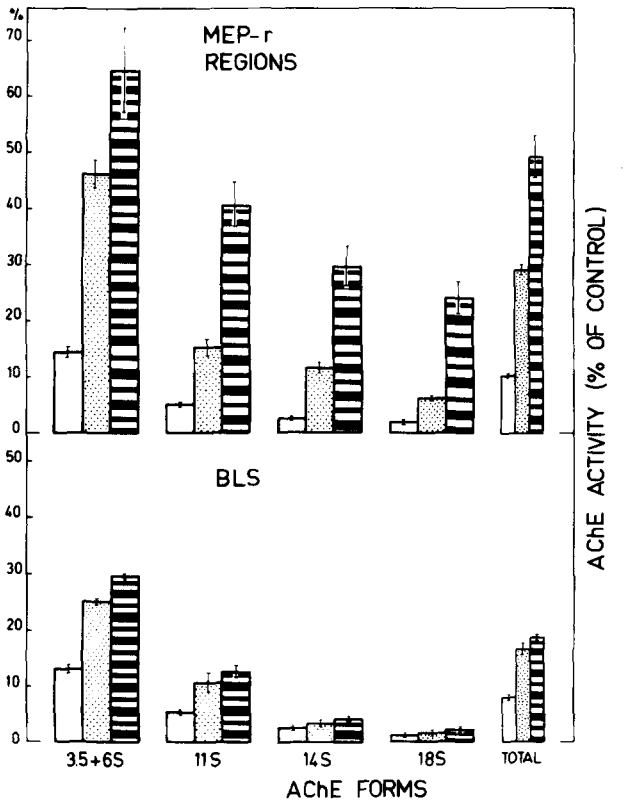


Figure 5. Recovery of AChE molecular forms after in vivo MPT-irreversible inhibition in *cutaneus pectoris* MEP-r region and BL 15 d after lesion. Data shown are means ± SEM (error bars) from three independent experiments given as percentage of control, untreated samples. Open bars represent recovery after 3 h, stippled bars after 52 h, and striped bars after 216 h.

Table I. Distribution of α-BgTx Binding Sites (AChR) in Total Extracts of Cutaneus Pectoris MEP-r Region and of BL Preparations

	Total ^{125}I-α-BgTx*		
	fmol/MEP-r region or BL	Recovery	Specific binding per mg protein
			fmol/mg
Contralateral MEP-r ($n = 8$)	4.3 ± 0.23	100	14.7 ± 0.92
BL 15 d ($n = 3$)	0.13 ± 0.019	3.0	4.0 ± 0.07

Results are the mean ± SEM of n independent experiments performed with pools of from five to seven MEP-r or BL.

* Specific toxin binding (after gradient analysis).

Table II. Relative Proportions of the Major Molecular Forms of AChE in Intact MEP-r Regions and BL Preparations

	3.5 S (3.5 ± 0.11)*	6 S (6.0 ± 0.13)	11 S (11.2 ± 0.25)	14 S (13.8 ± 0.31)	18 S (18.5 ± 0.39)
Contralateral MEP-r ($n = 12$), (*% total activity*)	9.2 ± 0.79‡	36.1 ± 1.84	17.5 ± 0.71	16.6 ± 1.26	17.9 ± 0.68
BL 15 d ($n = 5$) (*% total activity*)	4.1 ± 0.44	42.1 ± 1.26	16.8 ± 0.61	17.9 ± 0.72	15.3 ± 0.68

* Sedimentation coefficients given in heading of the columns are current designations. Measured values are the means (± SEM) in Svedberg units of 18 independent runs of entire *cutaneus pectoris* muscle or MEP-r region extracts.

‡ Relative proportions given as a percentage of total recovered AChE from the gradient ± SEM for n independent experiments.

Table III. Total and Specific Activity of the Major Forms of AChE in Intact MEP-r Regions and BL Preparations

	3.5 S	6 S	11 S	14 S	18 S
Total activity*					
MEP-r ($n = 12$)	2.8 ± 0.27	11.0 ± 0.43	5.4 ± 0.33	5.2 ± 0.46	5.5 ± 0.30
BL ($n = 5$)	0.5 ± 0.07	5.6 ± 0.53	2.2 ± 0.16	2.3 ± 0.18	2.0 ± 0.19
Ratios BL/MEP-r	17.8%	50.9%	40.7%	44.2%	36.4%
Student's t-test	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Specific activity‡					
MEP-r ($n = 12$)	12.8 ± 1.12	49.6 ± 3.31	23.3 ± 1.32	23.1 ± 2.01	24.9 ± 1.23
BL 15 d ($n = 5$)	16.0 ± 1.72	166.2 ± 10.32	66.0 ± 1.58	70.1 ± 1.93	60.1 ± 2.69
Ratios BL/MEP-r	125%	335%	283%	303%	241%
Student's t-test	$P > 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$

Results are means ± SEM for n independent experiments.

* Total activity is expressed in micromoles hydrolyzed ActhCh per minute per MEP-r region or BL bridge.

‡ Specific activity is expressed in micromoles hydrolyzed ActhCh per minute per milligram of protein.

asymmetric forms (14 and 18 S) represented ~5% only of the activity recovered after 216 h.

Purification of BL Sheaths: Pure BL Sheaths Still Contain Asymmetric AChE Forms

We performed a mild extraction of the BL bridges by simple incubation in the detergent medium. AChE staining (15) rather similar to that observed in the BL bridges (22) is still present after this detergent treatment (data not shown). This mild extraction eliminates completely mononucleated cells and cellular debris that contaminates the original BL bridges (Fig. 6). Detailed ultrastructural studies demonstrate that there is only one remaining organized structure, the muscle BL (Fig. 7). However, some ultrastructural aspects of the purified BLs are modified. The dense, centrally located part of the BL has disappeared. The AChR, which remained in the crude BL preparation, is mostly solubilized by the mild detergent treatment: ~91% of the total α-BgTx binding capacity of the BL bridges (and 85% of that of the MEP-r regions) is recovered in the exudate.

AChE is only partially released from the bridges by the detergent exposure. The loss of individual forms in the exudates ranges between 30 and 50% of their original content in these tissues (Table IV). All forms are still present in the pure BL sheaths, as shown in Fig. 8. We found that the total amounts of asymmetric and globular forms that remain in both MEP-r regions and pure BL bridges are very similar (50 to 70%, Table IV).

Discussion

McMahan and associates (20) have shown that most synaptic AChE in frog muscle is associated with muscle BL. An important feature of BL AChE emerges from our work: all major globular and asymmetric AChE forms are present in the external layers of the frog muscle MEP-r regions. The BL preparation is obtained in vivo after muscle damage, leaving a bridge of BL ghosts that contains the old sites of the MEP.

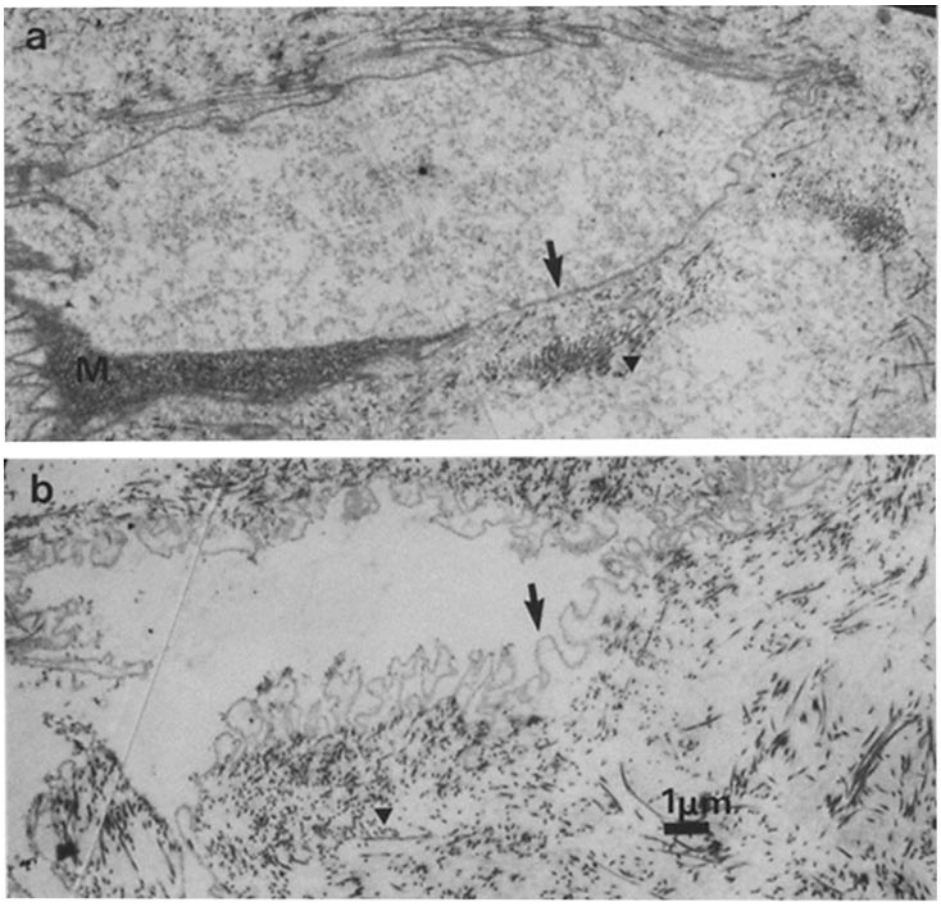


Figure 6. Effect of a mild exposure to detergent on the BL bridges: purification of BL. Low power micrograph (*a*) before and (*b*) after treatment. (*M*, macrophage. Arrowhead, collagen fibrils. Arrow, BL). Amorphous material and macrophage cell processes have been removed by exposure to detergent.

Table IV. Fraction of Major AChE Forms Released from MEP-r Regions and BL Bridges by a Mild Detergent Treatment

	Molecular forms							
	3.5 S + 6 S		11 S		14 S		18 S	
	Total activity*	Recovery‡	Total activity	Recovery	Total activity	Recovery	Total activity	Recovery
MEP-r	3.0 ± 0.37	32.7 ± 4.19	2.3 ± 0.02	50.7 ± 2.61	1.2 ± 0.25	37.4 ± 2.69	1.9 ± 0.17	44.4 ± 1.39
BL bridges	1.6 ± 0.15	37.0 ± 1.94	0.9 ± 0.01	41.7 ± 0.62	1.3 ± 0.12	46.7 ± 0.79	1.9 ± 0.02	49.0 ± 2.09

* Micromoles hydrolyzed ActhCh per minute per MEP-r or BL ± SEM of three independent experiments.

‡ Percentage of total activity recovered for each AChE peak in the mild detergent treatment compared with its total AChE activity, determined by three successive extractions: mild exposure to detergent, detergent extraction, and high salt extraction of the resulting pellet.

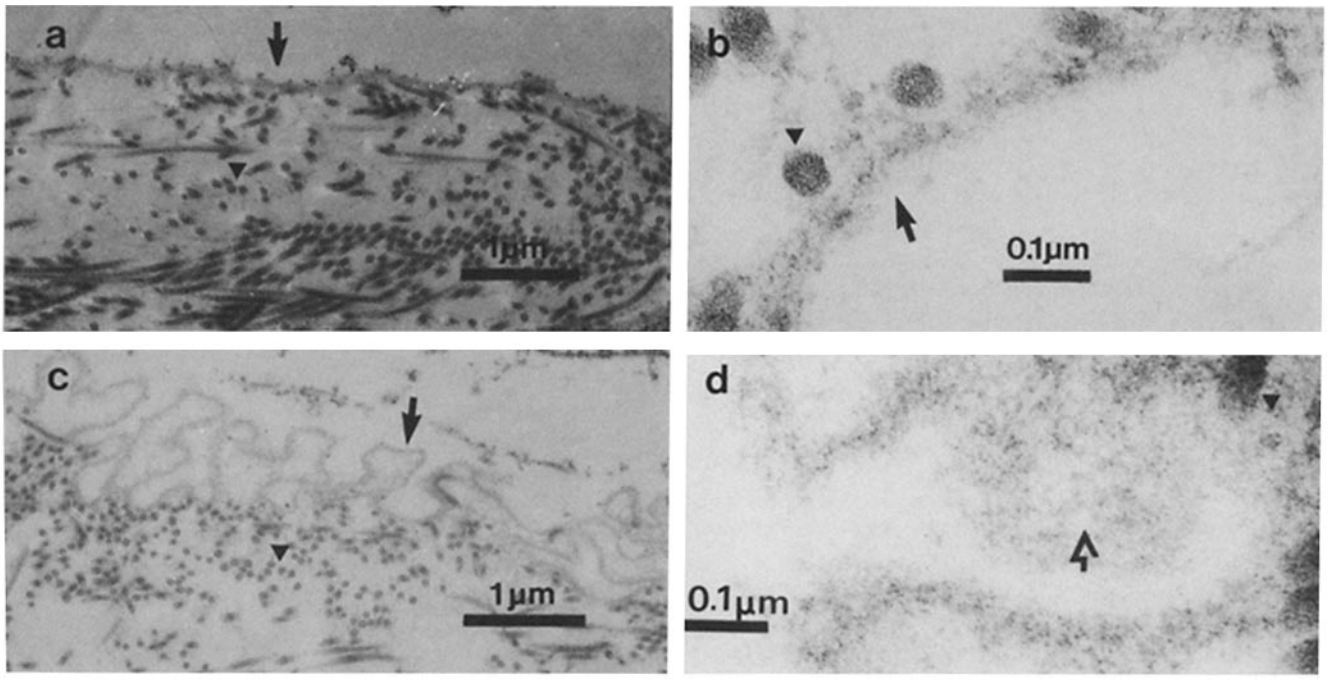


Figure 7. Ultrastructural aspects of pure BL sheaths. (*a* and *b*) Extrasynaptic BL sheaths. (*c* and *d*) Synaptic BL sheaths. (Arrowhead, collagen fibrils; arrow, BL sheaths.) In the pure BL sheath preparation, all cellular debris have been removed but the BL sheaths remain roughly organized as in native BL, with the marked exception of the disappearance of the electron dense central part of BL (⇳).

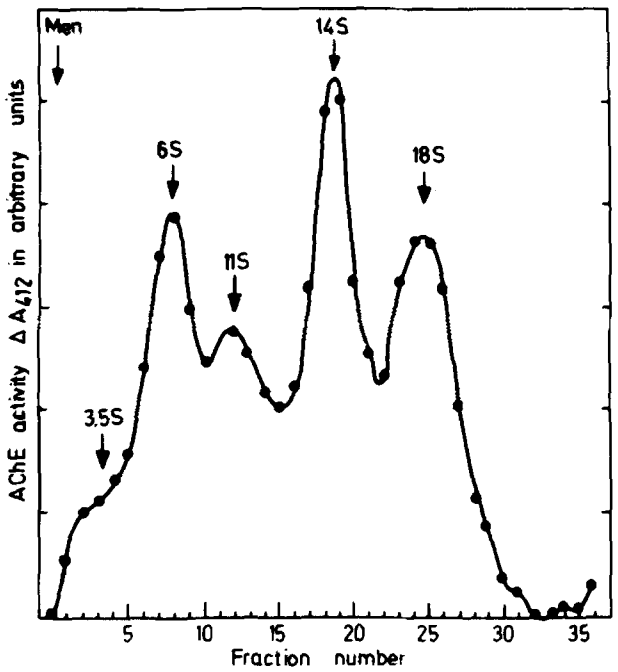


Figure 8. Sedimentation analysis of AChE extracted from pure BL sheaths. All conditions and symbols as in Fig. 1.

Thus, the BL preparation is enriched in synaptic BL, although the enrichment is not very important. In intact *cutaneus pectoris* muscle we estimated that the synaptic area corresponds to 0.1% of the total myofiber surface, as compared with 1 or 2% in the experimental bridge. We are thus not dealing with synaptic BL only but with a mixture of synaptic and extrasynaptic elements. The presence of asymmetric forms of AChE in muscle BL is directly demonstrated in this report. Until recently, only indirect evidence for a BL location was available: (*a*) the collagenous nature of the tail part of AChE and its aggregation properties (3) suggested a possible attachment to acidic components such as those found in BL; (*b*) asymmetric AChE is generally found in an external location, as shown with the use of reversible and irreversible, permeant or nonpermeant inhibitors (5, 12, 17, 18, 34). Globular AChE forms are also found in the frog BL preparation in contrast to mouse muscle and almost in the same proportions as in normal, intact muscle. Both globular and asymmetric forms were not recovered in significant amounts after irreversible inactivation by an organophosphorus compound, MPT. Thus, the AChE forms present in frog BL are not synthesized by contaminating or invasive cells or do not have an extraterritorial origin. Globular forms of AChE are found in BL ghosts later, 24 d after surgery and 15 d after the muscle is damaged. The same proportions of these forms have been found earlier, 7 d after surgery. Thus, the globular forms are probably not degradation products of the original asymmetric forms present in the BL of intact muscle, unless transitory proteolysis is activated during the first week after damage. Recent results from McMahan's group (1) differ from ours: only a 4.5 S globular form of AChE was found in the BL preparation obtained 30 d after damaging of the muscles of *Rana pipiens* frogs. The use of frogs of different origin (*R. pipiens* and *R. temporaria*) or even simply different extraction conditions may explain these differences.

As already pointed out by Anglister and McMahan (1), the presence of globular forms in the BL preparation suggests that AChE does not need to have a tail structure to remain associated with the BL. Moreover, the presence of globular forms of AChE in intact BL raises the possibility of a functional role for these forms at a junctional as well as at an extrajunctional location. Such a role has already been suggested at the nerve-muscle junction of chicken slow muscle,

which lacks asymmetric 20 S AChE (2).

Our results concerning the presence of all forms of AChE in BL raise the question as to how these forms are inserted into the BL structure, the physicochemical nature of their association with other BL molecules (collagen type IV, fibronectin, laminin, polyanionic components), and their precise physiological role at these extracellular synaptic locations.

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