

A Major Portion of Synaptic Basal Lamina Acetylcholinesterase Is Detached by High Salt- and Heparin-containing Buffers from Rat Diaphragm Muscle and *Torpedo* Electric Organ*

(Received for publication, December 6, 1996, and in revised form, December 3, 1997)

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Collagen-tailed asymmetric acetylcholinesterase (AChE) forms are believed to be anchored to the synaptic basal lamina via electrostatic interactions involving proteoglycans. However, it was recently found that in avian and rat muscles, high ionic strength or polyanionic buffers could not detach AChE from cell-surface clusters and that these buffers solubilized intracellular non-junctional asymmetric AChE rather than synaptic forms of the enzyme. In the present study, asymmetric AChE forms were specifically solubilized by ionic buffers from synaptic basal lamina-enriched fractions, largely devoid of intracellular material, obtained from the electric organ of *Torpedo californica* and the end plate regions of rat diaphragm muscle. Furthermore, foci of AChE activity were seen to diminish in size, number, and staining intensity when the rat synaptic basal lamina-enriched preparations were treated with the extraction buffers. In the case of *Torpedo*, almost all the AChE activity was removed from the pure basal lamina sheets. We therefore conclude that a major portion of extracellular collagen-tailed AChE is extractable from rat and *Torpedo* synaptic basal lamina by high ionic strength and heparin buffers, although some non-extractable AChE activity remains associated with the junctional regions.

The enzyme acetylcholinesterase (AChE)¹ plays a key role in cholinergic neurotransmission (1). Its predominant form at the neuromuscular junction is the collagen-tailed asymmetric form, A₁₂, which is located on the extracellular surface positioned for the hydrolysis of acetylcholine. Most of this junctional AChE is associated with the basal lamina (BL), located between the nerve ending and the muscle plasma membrane (2, 3), and can be removed from the cell surface of muscle tissue (4, 5) and mouse myotubes (6) by treatment with collagenase, indicating that the collagenic tail of the enzyme is involved in its anchorage to the BL (7, 8). Although the precise mechanisms by which asymmetric AChE forms are anchored to the BL remain elusive (9), there is compelling evidence to suggest

that heparan sulfate proteoglycans (HSPGs) or related proteoglycans are involved (7, 10). This evidence includes the recent finding that A₁₂ has two heparin-binding consensus sequences in its collagenic tail (11). Asymmetric AChE forms have a high binding affinity for BL components, particularly HSPGs (12) which are themselves major constituents of basement membranes (13, 14). Heparin and heparan sulfate have also been shown to release asymmetric AChE activity from rat muscle end plate regions (15) and BL sheets purified from the electric organ of *Discopyge* (16). The demonstration that A₁₂ could bind and be selectively eluted from heparin-agarose columns, whereas non-collagenous forms and A₁₂ after collagenase treatment could not, proved the direct interaction of A₁₂ with heparin *in vitro* (11, 17). Direct interactions with heparin/heparan sulfate moieties *in vivo* have also been demonstrated. Asymmetric AChE forms were shown to bind the surface of HSPG-rich cells, such as mouse myotubes and CHO-K1 cells, in a saturable and time-dependent manner, whereas pretreatment of these cells with heparitinase almost abolished this binding. In addition, the binding of A₁₂ AChE was reduced by 80% in Chinese hamster ovary clone 606, a mutant expressing under-sulfated HSPGs (10). Similarly, purified BL sheets from electric organ were found to release only asymmetric AChE forms when treated with heparitinase but not chondroitinase ABC (16). Finally, the assumption that HSPGs were likely involved in the intracellular assembly, transport, and cell surface deposition of asymmetric AChE is supported by the finding that a mutant variant of rat PC12 neuronal cells lacking HSPGs expressed a predominantly internal distribution of asymmetric AChE in contrast to normal PC12 cells in which almost all asymmetric AChE was extracellular (18, 19).

Despite the overwhelming evidence supporting the notion that asymmetric AChE forms are associated with the BL through electrostatic bonds, it was recently suggested that the enzyme displaced by high ionic strength and heparin buffers was in fact non-junctional and originated in intracellular compartments (20, 21). In quail muscle fibers and myotube cultures, these ionic buffers did not disaggregate previously formed surface AChE clusters, as detected by immunofluorescence techniques; however, the presence of heparin impeded their formation. Hence the authors formulated the hypothesis that only the newly secreted pool of asymmetric AChE was capable of binding heparin, as a transient event prior to its permanent, covalent association with the BL (21).

Our aims were to study the solubilization properties of junctional AChE first by quantifying the extraction capacity of high salt- and heparin-containing buffers, and second by evaluating the effect of these buffers on the AChE activity associated with BL-enriched preparations derived from rat muscle end plate regions and *Torpedo californica* electric organ. Our results

* This work was supported by Grants 1940694 and 1971240 from FONDECYT (to N. C. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: AChE, acetylcholinesterase; BL, basal lamina; HSPG, heparan sulfate proteoglycan; LDH, lactate dehydrogenase; iso-OMPA, tetraisopropyl pyrophosphoramidate.

strongly suggest that both high salt and heparin buffers solubilize a major portion of extracellular collagen-tailed AChE and demonstrate the capacity of these buffers to dissolve AChE-rich regions from neuromuscular junctions, as detected by histochemical staining.

EXPERIMENTAL PROCEDURES

Preparation of BL-enriched Fractions—The tissues used were frozen samples of *T. californica* electric organ, obtained from Pacific Bio-Marine (Venice, CA), and end plate regions of rat diaphragm muscle. The latter were obtained from anesthetized female Sprague-Dawley rats (200–250 g) by removing the diaphragm muscles together with the surrounding ribs and dissecting 2-mm strips of end plate regions (1 mm on either side of the phrenic nerve) (2, 15). The end plate strips were maintained in ice-cold saline solution (0.9% NaCl). The tissues were then homogenized for 5 min using a glass-to-glass homogenizer at full speed, in 1:10 (w/v) detergent buffer: 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, and protease inhibitors (1 mM *N*-ethylmaleimide, 1 mM benzamide, 0.1 mg/ml bacitracin, 100 mM caproic acid, 5 mM EDTA) (22). The homogenates were centrifuged at 15,000 rpm for 25 min in a fixed rotor using a Kubota KR-20000T centrifuge. The supernatants were then removed and assayed for activity, and the pellets were resuspended and rehomogenized as above. This wash cycle was repeated a total of 5 times, removing the majority of AChE globular forms and yielding a fibrous BL-enriched preparation for both rat muscle end plate tissue and *Torpedo* electric organ (23, 24). All procedures were carried out at 4 °C. Aliquots were taken from the initial tissue homogenates for activity assays and from the BL-enriched preparations for activity and histochemical analyses.

Quantifying Residual Intracellular Material in the BL-enriched Preparations—Lactic dehydrogenase (LDH) was used as a quantitative marker for intracellular contamination of the BL-enriched preparations, before and after undergoing extractions. LDH activity was measured using the Promega kit according to the manufacturer's instructions, and the percentage of remnant activity was calculated from the activity measured in the initial tissue homogenates (100%). Sialyltransferase activity, a marker of the Golgi apparatus (25), was used in some experiments to assess the presence of Golgi vesicles, and therefore intracellular contaminants, in our BL fractions.

Solubilization of Asymmetric AChE Activity from the BL—The BL-enriched preparations were divided into 3 groups, and duplicate samples for each group were homogenized for 5 min in 1:10 (w/v) of one of the following buffers: control buffer, 10 mM Tris-HCl, pH 7.4, containing several protease inhibitors (as above); heparin extraction buffer, 1 mg/ml heparin in control buffer; or high salt extraction buffer, 1 M NaCl in control buffer. The homogenates were then centrifuged at 15,000 rpm for 25 min and the supernatants removed, and the extraction procedure was repeated. All procedures were carried out at 4 °C. Aliquots were taken from the extraction supernatants for activity assays and sedimentation analyses. The post-extraction pellets were kept at 4 °C for subsequent histochemical studies and collagenase digestion.

Perfusion of Asymmetric AChE Activity from Rat Diaphragm Muscle Strips—Adult rat diaphragm muscle was dissected and cut into thin strips, pinned, and perfused for 1 h with high salt borate extraction buffer, in the presence or absence of heparin, as described by Rossi and Rotundo (20). After washing with phosphate-buffered saline, the muscle fibers were incubated with the histochemical reaction buffer of Karnovsky and Roots (26). In addition, the AChE activity released by these buffers was assayed in parallel experiments.

Sedimentation Analysis of AChE Forms—The molecular forms of AChE present in the tissue homogenates and BL-enriched fractions, and those solubilized from the latter, were resolved by sedimentation analysis on 5–20% linear sucrose gradients, as described previously (6, 27). To release all the molecular forms present in the homogenate samples, initial homogenates and BL preparations were homogenized in detergent buffer containing 1 M NaCl for the purpose of sedimentation analysis.

AChE Activity Assay—AChE activity was measured by the method of Ellman *et al.* (28). All incubations were carried out at 37 °C for rat muscle samples and 25 °C for *Torpedo* electric organ, in a 1-ml reaction mixture containing 100 mM sodium phosphate buffer, pH 7.0, 0.3 mM dithionitrobenzoic acid, and 0.75 mM acetylthiocholine iodide. 10 mM tetraisopropyl pyrophosphoramide (iso-OMPA) was also included as a specific inhibitor of butyrylcholinesterase activity. Absorbance was read at 412 nm in a Shimadzu UV-150–02 double-beam spectrophotometer.

Collagenase Digestion of the BL Preparations following Ionic Extractions—Collagenase digestions were carried out as described by Younkin

et al. (29), to quantitatively determine the AChE activity extracted from the BL preparations as a function of the total AChE present. In short, the BL pellets were incubated following either control, high salt, or heparin extractions, at 25 °C for 4 h with 0.1 mg/ml collagenase (Sigma, type V) in 1:10 (w/v) of a buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM *N*-ethylmaleimide, 2 mM benzamide, 10 mM CaCl₂, but devoid of EDTA. The reaction was stopped when the samples were centrifuged, and the supernatants were finally assayed for AChE activity.

Preparation of Rat End Plate Muscle Honeycomb Ghosts—Rat muscle end plate honeycomb ghost preparations were obtained using a method similar to that of Sanes and Hall (23), omitting the salt extraction step. End plate regions were dissected as before from diaphragm muscles and were maintained in ice-cold saline solution (0.9% NaCl) before being thinly cut into transverse sections and washed twice for 1 h with detergent buffer as follows: 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, and protease inhibitors. The end plate strips were then divided into three groups and incubated overnight at 4 °C with 1:25 (w/v) of detergent buffer alone, detergent buffer containing 1 M NaCl, or detergent buffer plus 1 mg/ml heparin. The resultant honeycomb ghosts were then analyzed for residual AChE activity by histochemical staining, and solubilized AChE activity was also assayed in the preparation buffers.

Visualization of Surface End Plate AChE Activity by Histochemical Staining—AChE activity was detected by histochemical staining in the BL-enriched fractions of both rat muscle end plates and *Torpedo* electric organ, in the semi-intact perfused rat muscle fibers and in the rat muscle end plate honeycomb ghost preparations, before and after their particular extractions. For this, the method of Karnovsky and Roots (26) was employed, using 2.2 mg/ml acetylthiocholine iodide, 0.71 M malate buffer, pH 6.0, 0.1 M sodium citrate, 30 mM copper sulfate, 5 mM potassium ferricyanide, and 3% neutral formol. All incubations were carried out at 4 °C, and all samples were processed simultaneously to make their staining intensities comparable. In the case of the BL-enriched preparations, samples weighed ~3 mg. For the honeycomb ghosts, multiple section samples were stained for each group. All samples were incubated for 20 min with iso-OMPA (10 mM), prior to the histochemical reaction, to inhibit butyrylcholinesterase activity. Control samples were incubated with 10 mM iso-OMPA plus either 50 mM methanesulfonyl fluoride or 10 μM BW 284c51 dibromide to inhibit cholinesterase activity or specifically AChE activity, respectively. Samples were then viewed under the phase-contrast light microscope.

Purification of Asymmetric AChE from *Torpedo* Electric Organ—Affinity chromatography using an acridine-agarose column was used to purify the collagen-tailed form of AChE, as described previously (30). Both specific activity (4,000 units/mg protein) and staining intensities following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (a single band of 67 kDa) were used to verify purity. Sucrose sedimentation analysis was subsequently used to confirm that the purified protein corresponded to the asymmetric A₁₂ form of AChE.

Association of *Torpedo* AChE with Rat Myotubes—Rat muscle primary cultures were prepared from the hindlimb muscles of an 18-day-old rat embryo and were maintained as described by Koenig (31). Approximately 3 × 10⁵ cells were plated onto 35-mm plastic tissue culture dishes coated with gelatin. The myoblasts reached confluence 24 h after plating, and the onset of fusion and large myotube formation began at 48 h. Most of the myotubes presented spontaneous contractile activity around the 5th day in culture. The medium was then removed, and the myotubes were washed three times with Dulbecco's modified Eagle's medium containing 0.1 M NaCl and 0.5 mg/ml bovine serum albumin. The myotubes were then incubated for 1 h at 4 °C with 50 milliunits of AChE enzyme, purified from *Torpedo* electric organ, in the presence or absence of 1 mg/ml heparin. The cells were then washed twice with 2:5 Dulbecco's modified Eagle's medium/phosphate-buffered saline and incubated for 15 min at 4 °C with phosphate-buffered saline buffer containing 2 mg/ml bovine serum albumin and 2 mg/ml heparin. The cell surface-associated AChE activity solubilized by this medium was then assayed. For the determination of total cell surface-bound AChE, an additional incubation with the same medium containing 1 M NaCl instead of heparin was performed.

RESULTS

Asymmetric AChE Is Associated with BL-enriched Preparations—Since it was recently reported that neither high salt- nor heparin-containing buffers detached asymmetric collagen-tailed forms of AChE from the BL of the vertebrate neuromuscular junction (20, 21), we wanted to re-evaluate the solubilization properties of the asymmetric forms present in BL-enriched preparations, derived from both diaphragm muscle

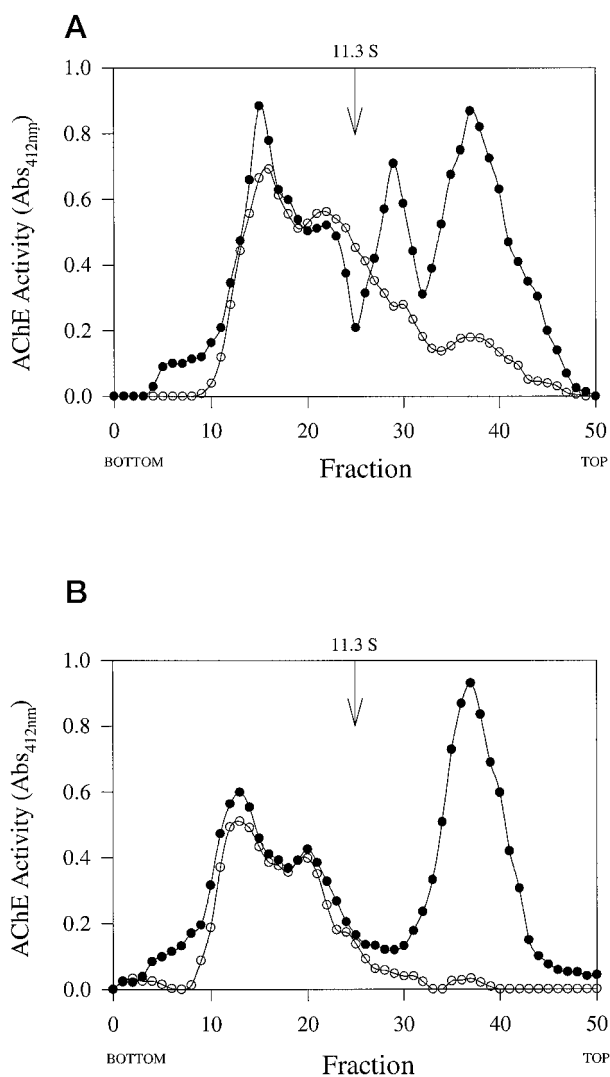


FIG. 1. Molecular forms of AChE solubilized from the end plate regions of rat diaphragm muscle and from *Torpedo* electric organ. *A* corresponds to rat muscle end plates, and *B* corresponds to the *Torpedo* electric organ, in which ● represents the initial tissue homogenate and ○ represents the BL-enriched fraction. For the purpose of sedimentation analysis, fresh (rat) or frozen (*Torpedo*) tissue samples were homogenized in a high salt detergent buffer (1:10, w/v) as follows: 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 1% Triton X-100, and protease inhibitors. By having submitted the rest of the tissue to 5 homogenization-centrifugation cycles with detergent buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, protease inhibitors) to remove the majority of globular forms, the resultant BL-enriched fractions were also solubilized in high salt detergent buffer for sucrose sedimentation analysis. The arrow indicates migration of the catalase marker at 11.3 S.

end plates as well as *Torpedo* electric organ. Several homogenization-centrifugation cycles in the presence of Triton X-100 detergent were used to remove most of the hydrophobic and soluble proteins and yield BL-enriched preparations for both tissues. The AChE forms present in these BL-rich fractions were compared with those present in the corresponding initial homogenates by velocity sedimentation analysis (Fig. 1). Characteristic sedimentation profiles were obtained for both rat diaphragm (Fig. 1A) and *Torpedo* electric organ (Fig. 1B) homogenates, including asymmetric (A_{12} and A_8) as well as globular forms (G_1 and G_4 for rat and G_2 for *Torpedo*). In contrast, the asymmetric AChE forms were greatly enriched in the BL preparations as seen by the absence of the peaks corresponding to the globular forms. When the *Torpedo* BL preparations were examined under the electron microscope, the typical cytoarchitecture of *Torpedo* basement membranes was apparent and



FIG. 2. Basal lamina sheets are the major constituents of the extracellular matrix material purified from *Torpedo* electric organ. Typical electron micrograph of basement membranes obtained after extensive detergent extraction of electric organ tissue. The sample was fixed in Karnovsky's fixative, post-fixed, stained with uranyl acetate, and finally prepared for electron microscopy. (Magnification, $\times 70,000$.)

predominant (Fig. 2). The presence of asymmetric AChE associated with these BL sheets was an important confirmation that the enzyme was in fact anchored to such extracellular structures.

Remnant Cytoplasmic Material in the BL-enriched Preparations—To discard the possibility that intracellular asymmetric AChE, destined to be exported to the BL, may be contaminating our BL fractions (despite exhaustive washes with detergent buffer), we analyzed such preparations for the presence of two intracellular markers. First, LDH activity was used as an indicator of remnant-soluble cytoplasmic material. After repeated Triton X-100 extractions, some residual LDH activity ($\sim 6\%$ of the total) was detectable in the final rat muscle end plate BL-enriched preparation (Fig. 3). Furthermore, two subsequent extraction steps with either high salt or heparin solubilized half of this remnant activity (see inset of Fig. 3), indicating that a residual intracellular material was probably trapped within or retained electrostatically by the BL sheets. Similar results were obtained for *Torpedo* electric organ preparations. These results indicate that asymmetric AChE is not trapped in vesicles as a soluble entity. Second, the absence of sialyltransferase activity, a Golgi marker, in both the rat and *Torpedo* BL-enriched preparations (data not shown), makes it unlikely that intracellular asymmetric AChE remained associated, as a contaminant, to the BL fractions used in these studies.

High Salt- and Heparin-containing Buffers Solubilized Extracellular Asymmetric AChE from the BL Preparations of Rat Muscle End Plates and *Torpedo* Electric Organ, and from Rat Muscle Honeycomb Ghost Preparations, but Not from Perfused Rat Muscle Strips—Having identified the source of collagen-tailed AChE as predominantly BL, a study of the solubilization properties of these forms was carried out at short extraction times. First, samples of purified BL material were submitted to two 5-min homogenization-centrifugation cycles with either high salt- or heparin-containing buffers. Both AChE activity and the molecular forms solubilized were then determined. As Fig. 4 shows, only asymmetric forms of AChE were released by either high salt or heparin from both rat (Fig. 4A) and *Torpedo* (Fig. 4B) BL preparations. Additional experiments were carried out using sectioned rat muscle end plates treated with detergent so as to generate honeycomb ghost preparations (23) with structurally integral junctional regions. The AChE activity released by the high salt- or heparin-containing buffers used for the ghost preparation was assayed; heparin was able to solubilize 50% of that released by NaCl (data not shown).

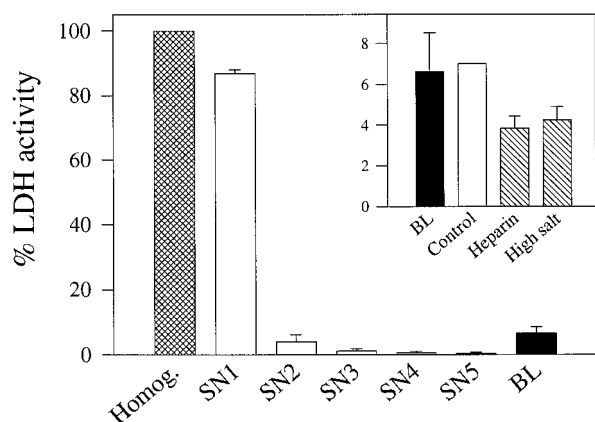


FIG. 3. A small portion of cytoplasmic material remained associated with the extracellular matrix fractions. LDH activity was measured in the initial rat muscle end plate homogenate (*Homog.*), the five supernatant fractions of the preparative wash cycle (*SN1–5*), and the final BL-enriched fraction (*BL*). The latter was also analyzed following control, heparin, and high salt extractions (*inset*). LDH activity was determined using the Promega kit according to the manufacturer's instructions.

Similarly, in rat muscle end plate BL, heparin extracted approximately 40% of the activity solubilized by high salt, as shown in Fig. 5A, whereas in *Torpedo* electric organ, both buffers solubilized similar quantities (80–90%) of asymmetric AChE (Fig. 5B). The total fraction of asymmetric AChE solubilized by heparin was much greater in *Torpedo* electric organ than in rat muscle end plate regions (80% compared with 30%) (Table I), suggesting that a larger population of heparin-extractable asymmetric AChE exists in the former. When high salt and heparin-containing buffers were used to perfuse rat diaphragm muscle strips for 1 h, no AChE activity was detectable in the activity assays carried out on these buffers (data not shown).

A Minor Portion of Junctional AChE Persists in the BL-enriched Preparations following Extractions with High Salt- or Heparin-containing Buffers—A population of collagen-tailed AChE associated with the BL persists for long periods after frog muscle denervation (3). In rat diaphragm and gracilis muscles, most of the asymmetric AChE activity is extracellular, of which only 15–25% is considered to be a non-extractable fraction (29, 32). However, a recent report suggested that all junctional asymmetric AChE formed part of this non-extractable pool, given that high salt and heparin buffers seemed unable to disrupt or diminish the number of cell-surface AChE clusters in quail myotube cells or end plate regions of avian muscles (20, 21). Therefore in the present study, we sought to quantify the extractable as well as the non-extractable fraction of BL-associated AChE activity and ascertain whether a change in the quantity and distribution in extracellular AChE-rich regions could be detected following high salt or heparin extractions. First, collagenase digestion after high salt extractions indicated that the non-extractable pool of asymmetric AChE in rat end plate regions accounted for 23% of the total BL-associated asymmetric AChE (Fig. 5A, see also Table I), in agreement with Younkin *et al.* (29). Moreover, in *Torpedo* electric organ, a minor portion of junctional AChE remained after high salt or heparin extractions, accounting for 9% of non-extractable enzyme (Fig. 5B).

Second, AChE activity was visualized by histochemical staining before and after extractions on the BL-enriched fractions obtained from rat and *Torpedo* tissues. In the former, the morphological appearance, size, and organization of the histochemically stained structures seen in Fig. 6 revealed the presence of numerous isolated and well preserved motor end plates

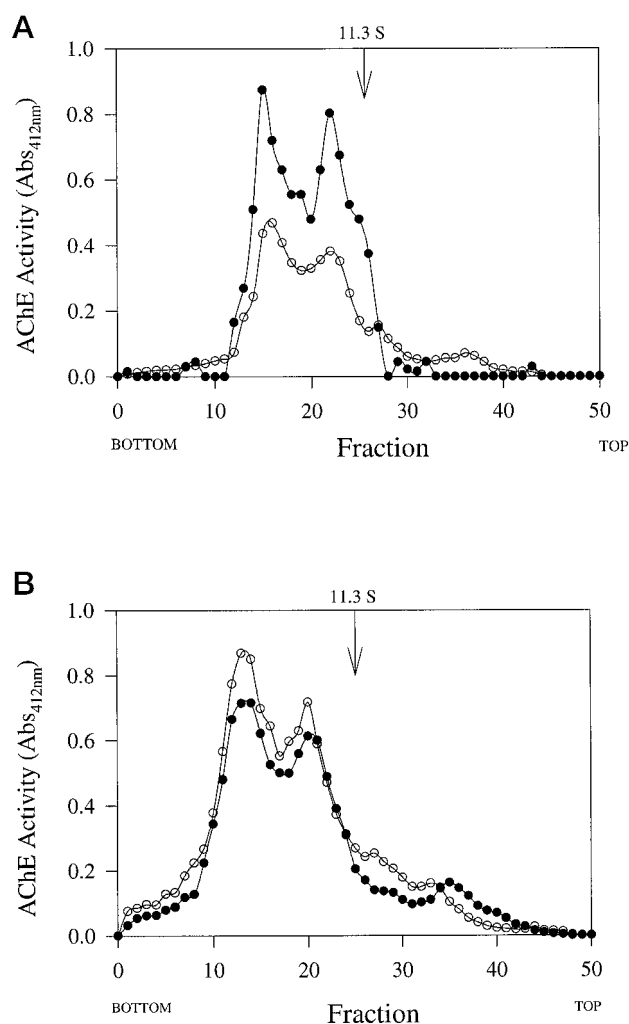


FIG. 4. Solubilization of the molecular forms of AChE from the rat and *Torpedo* BL-enriched fractions. A corresponds to rat muscle end plates, and B corresponds to *Torpedo* electric organ, in which ● represents the AChE forms solubilized by 1 M NaCl and ○ represents those released by 1 mg/ml heparin from the BL-enriched fractions. The latter were homogenized for 5 min in a buffer containing 10 mM Tris-HCl, pH 7.4, protease inhibitors, and either 1 mg/ml heparin or 1 M NaCl. Following centrifugation, the extraction procedure was repeated, and the molecular AChE forms solubilized by the first extraction were analyzed on a 5–20% sucrose gradient. Two distinct peaks were obtained for both heparin and NaCl, corresponding to the asymmetric forms A_{12} and A_8 . Absorbance values represent the proportion of AChE activity extracted by each buffer with respect to the total extractable activity, determined by collagenase digestion, as described for Table I. The arrow indicates migration of the catalase marker at 11.3 S.

with their synaptic gutters. These neuromuscular junctions, rich in AChE activity, were also clearly visible in the rat muscle BL fractions prior to high salt and heparin extractions (Fig. 7A). Following high salt treatment, most of the activity in these end plates disappeared (Fig. 7B). Heparin extractions also produced a reduction in the size and staining intensities of the end plates detected (Fig. 7C). Control experiments in the presence of inhibitors specific for AChE did not show end plate hydrolytic activity (Fig. 7D). Histochemistry carried out on the BL sheets purified from *Torpedo* electric organ demonstrated that these samples contained isolated surfaces highly enriched in AChE activity (Fig. 8A). On the other hand, the same preparations treated with either high salt (Fig. 8B) or heparin (Fig. 8C) showed only minimal residual AChE activity in both cases. Controls treated with AChE inhibitors showed no activity (Fig. 8D). In the experiments using rat muscle end plate honeycomb ghost preparations, histochemical staining of AChE revealed

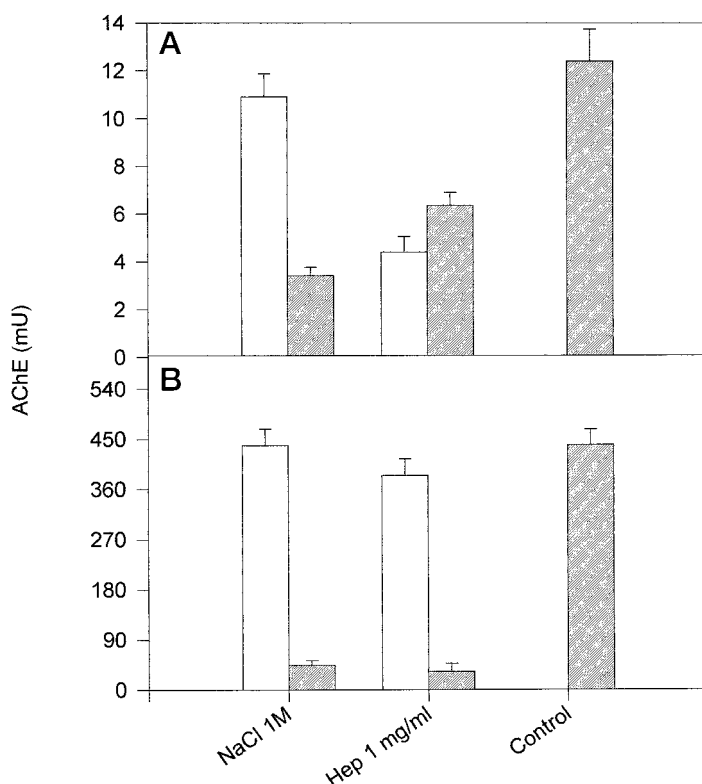


FIG. 5. AChE activity released from the rat and *Torpedo* BL-enriched fractions by ionic extractions and collagenase treatment. Rat (A) and *Torpedo* (B) BL preparations were submitted to control and ionic extractions, as described earlier, using Tris-HCl (10 mM), heparin (1 mg/ml), or NaCl (1 M) buffers (white bars). Following centrifugation, the BL pellets were treated with collagenase (Sigma type V) for 4 h at 25 °C to solubilize the remnant AChE activity resistant to extraction (gray bars). Each value is the mean \pm S.D. of three experiments carried out in duplicate.

TABLE I
Solubilization of asymmetric AChE from rat and *Torpedo* basal lamina-enriched fractions

Extractions were carried out by homogenizing BL-enriched preparations in Tris-HCl buffer (control) and either 1 M NaCl or 1 mg/ml heparin. The samples were then centrifuged at 15,000 rpm for 25 min, and the procedure was repeated. The activity in both supernatants was then measured and summed. Each value is the mean \pm S.D. of three representative experiments done in duplicate, and the values given for collagenase correspond to the AChE fraction remnant following the high salt extractions. The number in parentheses corresponds to the percentage of AChE activity solubilized by each procedure. The activity released by collagenase treatment of the control samples was used to calculate the total extractable activity (100%) associated with the BL preparations.

Extraction	Heparin	NaCl	Collagenase
		<i>milliunits</i>	
Rat BL	4.4 \pm 0.66 (30%)	10.9 \pm 0.98 (76%)	3.4 \pm 0.35 (23%)
<i>Torpedo</i> BL	385 \pm 20.2 (80%)	439 \pm 30.1 (91%)	44.3 \pm 7.5 (9%)

an array of end plate regions in these muscle ghosts (Fig. 9A). However, when these samples were prepared in the presence of high salt, most of the AChE activity was removed from the end plate ghosts (Fig. 9B), whereas less activity was removed when heparin was present. Almost no AChE activity was visible in control samples incubated with 20 μ M BW284c51 and 10 mM iso-OMPA (Fig. 9C). In contrast, when rat diaphragm muscle strips were perfused with a mixture of high salt and heparin buffers, no variation in AChE histochemical staining intensity

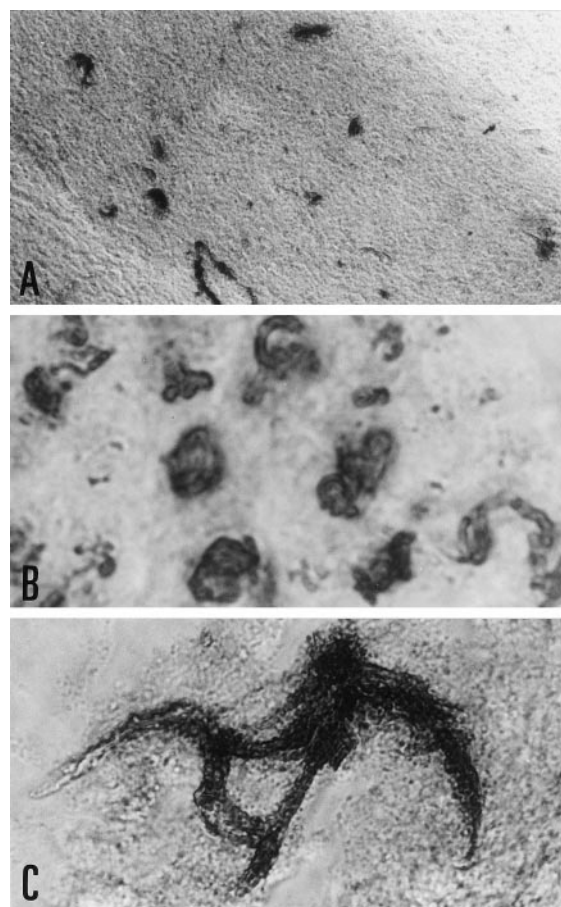


FIG. 6. Visualization of isolated motor end plates in the rat muscle BL-enriched preparation. Histochemical localization and phase-contrast light microscopy were used to confirm the presence of end plate-associated AChE activity in the BL-enriched fraction of rat diaphragm muscle. Low ($\times 100$) (A), medium ($\times 300$) (B), and high field ($\times 800$) (C) views show isolated motor end plates with their apparent postsynaptic folds. Histochemical staining was carried out using the method of Karnovsky and Roots (26), prior to which samples were incubated with 10 mM iso-OMPA for the specific inhibition of butyrylcholinesterase activity.

was seen between the control and treated samples (data not shown). Together, these results clearly show that most of the junctional AChE is readily detached by either high salt or heparin from rat or *Torpedo* BL preparations as well as muscle end plate ghosts, which retain the basic cytoskeletal architecture of the muscle, but not from semi-intact rat muscle strips.

Association of Torpedo AChE to Rat Primary Culture Myotubes—Despite the fact that rat muscle and *Torpedo* electric organ are phylogenetically distant, the anchorage mechanisms of the collagen-tailed enzyme may not be fundamentally different in both systems. To determine the extent to which AChE association in these systems was compatible, a study of the binding properties of asymmetric AChE, purified from *Torpedo*, was carried out using rat primary muscle cultures. Fig. 10 shows that *Torpedo* AChE was capable of associating with rat myotubes in a reversible manner, as demonstrated by its subsequent detachment from the cell surface using a heparin-containing buffer. Of the total AChE activity associated (3.65 milliunits/ml), heparin was able to release approximately 70% (2.6 milliunits/ml). However, incubation with AChE in the presence of heparin eliminated the capacity of the enzyme to bind to the myotube cell surface. When fibroblasts were used instead of myotubes, no association was observed with or without heparin co-incubation (Fig. 10). These results suggest that specific AChE-binding sites are present on the cell surface of

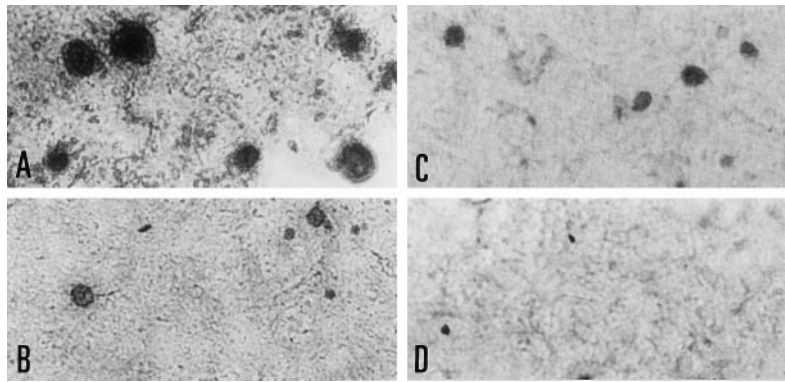


FIG. 7. Residual activity of AChE seen in the rat muscle end plate BL-enriched fractions following high salt and heparin extractions. Histochemical localization of BL-associated AChE activity (26) as viewed under the phase-contrast microscope is shown. A, rat muscle BL-enriched fraction prior to heparin or high salt treatment; B, following heparin extractions; C, NaCl extractions. Samples weighed ~3 mg and were processed simultaneously to make their staining intensities as comparable and quantitative as possible. All samples used were incubated with 10 mM iso-OMPA prior to the histochemical reaction. D, no AChE activity was visible in control samples incubated with 10 mM iso-OMPA and 50 mM methanesulfonyl fluoride for the inhibition of all cholinesterase activity. (Magnification, $\times 200$.)

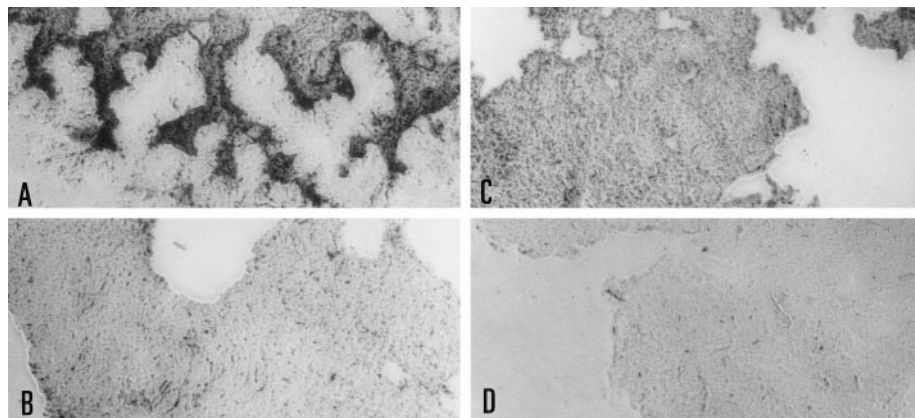


FIG. 8. A minor residual AChE activity is present in the basal lamina sheets of *Torpedo* electric organ following high salt and heparin extractions. Histochemical localization of BL-associated AChE activity as viewed under the phase-contrast microscope is shown. The histochemical reaction was carried out according to the method of Karnovsky and Roots (26). A, *Torpedo* basal lamina sheets prior to heparin or high salt treatment; B, following heparin extractions; and C, NaCl extractions. D, almost no AChE activity was visible in control samples incubated with 10 mM iso-OMPA and 50 mM methanesulfonyl fluoride. Samples weighed ~3 mg and were processed simultaneously to make their staining intensities as comparable and quantitative as possible. All samples were incubated with iso-OMPA (10 mM) prior to histochemistry. (Magnification, $\times 600$.)

rat myotubes which are absent in fibroblasts. Moreover, such sites are also recognized by *Torpedo* asymmetric AChE, implying that the anchorage of the collagen-tailed form of AChE involves either similar extracellular molecules or similar interaction mechanisms in both species. Whether or not this association occurs in clusters over the myotube surface is a matter that deserves further study.

DISCUSSION

Prevailing opinion indicates that the collagen-tailed forms of AChE are anchored to the synaptic BL through electrostatic interactions with polyanionic components such as HSPGs (9, 10). This widely accepted hypothesis was later reinforced by the identification of two heparin-binding domains in the collagenous tail of the enzyme (11, 33). The objective of the present study was to determine directly whether the AChE molecules localized at the neuromuscular junction were quantitatively removed by high salt and heparin extractions, in view of the results of Rossi and Rotundo (20) in which perfusions with these buffers were unable to detach clustered cell-surface AChE from quail and rat muscle fibers. Furthermore, these authors suggested that the ability of heparin to solubilize asymmetric AChE, as reported for other systems, stemmed from its apparent ability to release mainly intracellular and non-junctional AChE molecules (20, 21). However, our bio-

chemical and histochemical results demonstrate that these conventional ionic buffers do in fact specifically detach a major portion of asymmetric AChE from purified synaptic BL devoid of intracellular material and derived from two of the most studied neuromuscular junction systems, rat muscle end plates and *Torpedo* electric organ.

In view of this apparent discrepancy, we wanted first to confirm the extracellular source of asymmetric AChE prior to its extraction. Asymmetric AChE occupies less than 0.1% of the total surface area of the BL at the rat neuromuscular synapse, yet its density increases severalfold specifically at junctional regions (34). In contrast, the electric organ of *Torpedo* is a tissue in which the synaptic junctions are overdeveloped (35, 36). Therefore, the use of both these tissues in the present study minimized the possibility that extra-junctional asymmetric AChE was being solubilized during the extractions. Furthermore, several end plates rich in junctional AChE were observed in the rat and *Torpedo* BL-enriched preparations prior to extraction. Treatment with high salt or heparin buffers reduced both the number, size, and staining intensities of these regions. The studies of Rossi and Rotundo (21) nevertheless demonstrated that neither high salt- nor heparin-containing buffers were able to reduce the number of immunoreactive AChE clusters on the surface of quail myotubes or quail muscle

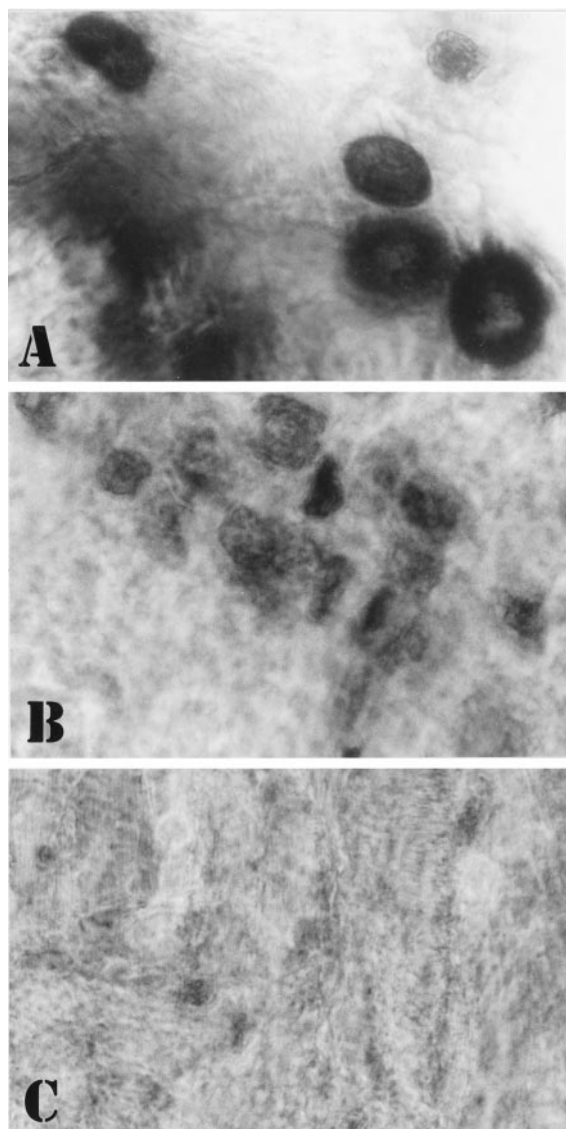


FIG. 9. High salt treatment of honeycomb ghosts derived from rat muscle end plate regions released an important portion of junctional AChE activity. Honeycomb ghosts were prepared from sectioned rat muscle end plates by incubating the sections with detergent buffer overnight, either in the presence or absence of 1 M NaCl. Remnant end plate AChE activity was then stained histochemically and viewed by phase-contrast light microscopy. *A*, AChE activity was visible as large, densely stained end plate regions in the ghost preparations (*control*). *B*, honeycomb ghosts prepared in the presence of high salt, to extract asymmetric AChE, contained only small dispersed foci of histochemical activity, which displayed lower staining intensities than their control counterparts. *C*, no AChE activity was detected in honeycomb ghosts incubated with 20 μ M BW284c51 dibromide and 10 mM iso-OMPA. (Magnification, $\times 400$.)

fibers. Yet by incubating the myotubes in the presence of heparin, newly synthesized asymmetric AChE accumulated in the medium and not as cell-surface clusters. The authors concluded that only a transient cell-surface interaction occurred in avian cell cultures between newly formed asymmetric AChE and heparin or HSPGs. However, in the present study, the use of purified BL, as seen under the electron microscope, eliminated the possibility that newly synthesized asymmetric AChE and its subsequent externalization to the cell surface could account for the enzyme detached during the extraction processes.

It should also be noted that in quail skeletal muscle only 40% of total asymmetric AChE is BL-associated, with the remainder being intracellular (37). Therefore, the subcellular localization

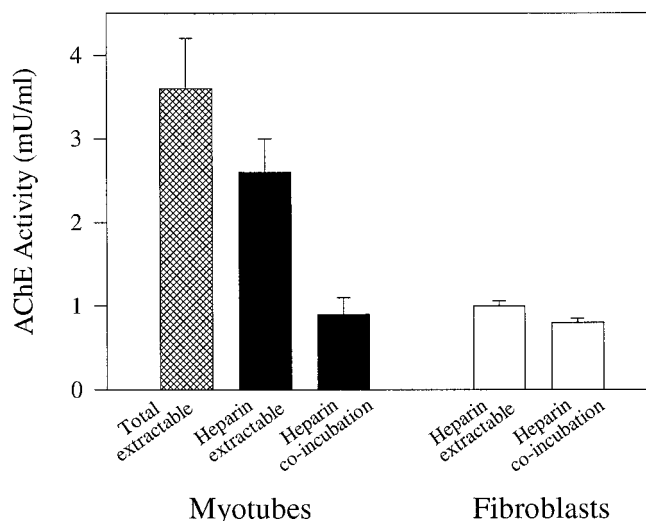


FIG. 10. Association of purified *Torpedo* asymmetric AChE with rat myotubes. Fifty milliunits of asymmetric AChE, purified from *Torpedo* electric organ, were incubated for 15 min with 5-day-old rat myotubes in 35-mm culture dishes, in the presence or absence of 2 mg/ml heparin. Fibroblasts obtained from the same muscle primary cultures were used as controls and submitted to the same treatments. Cell surface-associated AChE was then released by incubating the cells with 2 mg/ml heparin buffer, and the activity solubilized was determined for cells incubated with AChE in the presence or absence of heparin (labeled as *Heparin co-incubation* and *Heparin extractable*, respectively). Total cell surface-associated AChE (*Total extractable*) was the total amount of activity solubilized from the myotubes by 2 mg/ml heparin followed by 1 M NaCl buffer. Asymmetric AChE associated only with rat myotubes, in a manner that was inhibited by heparin. No significant association of asymmetric AChE was detected in fibroblasts. Values represent the average of three experiments done in duplicate.

of collagen-tailed AChE in avian muscle differs from most of the other systems studied, as virtually all findings agree that the majority of asymmetric AChE is extracellular. For instance, studies performed in rat diaphragm muscle *in vivo* showed that 22% of asymmetric AChE forms were intracellular in rat diaphragm end plate regions (29), 30% in rat gracilis muscle (32), and 20% in mouse C_2 myotubes (38). Moreover, there is further evidence to support the notion that AChE attachment in the avian system may be unusual. For instance, chondroitinase ABC or AC was found to detach asymmetric AChE from chicken muscle (39) but not from mouse C_2 myotube cultures or from *Discopyge* electric organ, in which only heparitinase released the enzyme (10, 16).

Altogether, the above evidence clearly indicates that differences in species' extractability of asymmetric AChE, extracellular to intracellular ratios and anchorage mechanisms, are likely to account for some of the differences obtained in the present study and the work of Rossi and Rotundo (20, 21). However, there is also a fundamental difference in the sample preparation and extraction methods used, subcellular fractionation *versus* intact muscle fibers and homogenization *versus* perfusion. This difference could explain the apparent controversy of different results obtained in the same species (Ref. 20, Fig. 7). For this reason, we carefully repeated the experiment of Rossi and Rotundo (20) in which rat muscle strips were perfused and extracted for 1 h with buffers containing high salt and heparin. In our experiment using rat diaphragm, we obtained the same results as Rossi and Rotundo with rat gastrocnemius muscle (Ref. 20, Fig. 7), whereby these high ionic buffers extracted no or little AChE activity from the muscle fibers. This was visualized using both the same histochemical method as Rossi and Rotundo (20, 21) and also by activity assays. Considering that in both rat muscle honeycombs and

purified synaptic BL, we successfully solubilized asymmetric AChE but were unable to do so in muscle strips, we believe that perfusion of a semi-intact muscle sample is not the most appropriate method for the extraction of synaptic molecules such as AChE.

A non-extractable population of asymmetric AChE has been identified previously, which can only be released by collagenase treatment (29, 32). This non-extractable pool of AChE, in rat diaphragm end plates, accounts for 21% of the total asymmetric forms which in these regions exist principally as extracellular enzyme (29). However, the ultrastructure of this tissue may present a greater resistance to the extraction procedure than that of *Torpedo* electric organ, as in the latter there seems to be a larger pool of heparin-sensitive collagen-tailed AChE. Although some asymmetric AChE seems to be covalently associated with the synaptic BL, the majority of the extracellular enzyme is extractable using high salt- and heparin-containing buffers, indicating that electrostatic interactions must be involved in its anchorage at the neuromuscular junction.

The cell surface AChE binding studies, carried out using *Torpedo* enzyme and rodent muscle cultures, proved to be effective heterologous systems for such studies. Indeed, Rotundo *et al.* (40) recently carried out transplant experiments, in which frog neuromuscular synapses were incubated with purified quail asymmetric AChE, and binding was observed. These authors even suggest the presence of specific collagen-tailed AChE-binding sites at the synaptic BL of the vertebrate neuromuscular junction. In our case, the localization of the AChE bound to the myotube surface was not investigated. However, bound AChE would most likely appear as clustered foci of activity but would nonetheless present a "non-junctional" distribution due to the absence of neuronal stimuli. Therefore, the formation of AChE clusters in myotube cell cultures does not imply the presence of "junctional" AChE (as in *Torpedo* electric organ and muscle end plates), which is defined by the existence of the neuromuscular junction and requires the presence of motor neurons. For this reason, we suggest caution in the use of the term junctional when referring to the clustering of AChE on the surface of cultured myotubes. The fact that heparin could be used to release the asymmetric AChE bound to the myotube surface or block its association in our *in vitro* studies suggests first that HSPGs are involved in the binding of the *Torpedo* enzyme to the rodent muscle cells, and second that these proteoglycans may be universally recognized in such anchorage mechanisms. These possibilities are reinforced by the finding that HSPGs are indeed present in the extracellular matrix of rodent myotubes (41, 42), and it would be interesting to study the focalization of AChE exogenously added to myotube cultures, both in the presence and absence of motor neurons.

In conclusion, we have established that a major portion of junctional collagen-tailed AChE is in fact associated with the BL through electrostatic interactions. Extracellular asymmetric AChE foci can be disrupted by high salt- or heparin-containing buffers. These results are entirely consistent with previous work regarding the anchorage mechanism(s) of collagen-

tailed AChE to the BL (10, 16) and with the current notion that HSPGs may interact with the heparin-binding domains present in the collagenic tail of the enzyme (11). Further studies using synthetic peptides, derived from the collagenic region, as well as molecular modeling and docking, should help us to elucidate the exact mechanisms involved in the anchorage of asymmetric AChE to the BL at the neuromuscular junction.

Acknowledgments—We thank Dr. David J. Carey and Dr. Enrique Brandan for helpful comments on the manuscript and to Dr. Ariel Orellana for assistance in the sialyltransferase assays.

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CELL BIOLOGY AND METABOLISM:
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Eliseo O. Campos, Rebeca Aldunate, Jorge
Garrido and Níbaldo C. Inestrosa
J. Biol. Chem. 1998, 273:4258-4265.
doi: 10.1074/jbc.273.7.4258

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