Acetylcholinesterase of mammalian neuromuscular junctions: Presence of tailed asymmetric acetylcholinesterase in synaptic basal lamina and sarcolemma

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ABSTRACT A sarcolemma-rich fraction can be isolated after subcellular fractionation of mouse intercostal muscles by sedimentation on a discontinuous sucrose gradient. The quantitative recovery of the acetylcholine receptor in this fraction is about 50%, which indicates the presence of a high proportion of postsynaptic membranes. Acetylcholinesterase (AcChoEase; EC 3.1.1.7) is found mainly in three different layers: the top layer, which contains soluble AcChoEase, the intermediate layer (fraction A), and the last, AcChoR-rich, layer (fraction C). The relative proportions of the molecular forms of AcChoEase are different in the three layers. The "16S" AcChoEase is in a higher proportion in both types of membrane fractions (A and C) compared to soluble AcChoEase. Both total AcChoEase and 16S AcChoEase are enriched in the A and C fractions. In the C fraction, the sequential use of homogenization in the presence of detergent and high ionic strength allows the "solubilization" of two distinct AcChoEase pools. One is detergent-soluble and mainly composed of slow-sedimenting forms; the other one is detergent-insoluble, high-ionic strength-soluble, and composed mainly of collagen-like, tailed, asymmetric (16S) AcChoEase. Thus, most of the asymmetric AcChoEase is specifically localized in the synaptic basal lamina of the mammalian muscle fiber. However, in the A fraction, most of the 16S AcChoEase found is solubilized by detergent alone, suggesting an association with mesosomal membranes. It may mean that at least some of the basal lamina-embedded 16S AcChoEase is preassembled intracellularly in the sarcoplasmic reticulum.

The 16S form of acetylcholinesterase (AcChoEase; EC 3.1.1.7) is highly concentrated in the regions of muscle containing endplates (1) and is absent or barely detectable after denervation (1, 2). Membrane fractionation experiments have been widely used to determine the cellular loci of the AcChoEase accumulation and localization. For example, muscle microsomal or sarcoplasmic reticulum, and also sarcolemmal fractions, have been prepared and analyzed for their enrichment in AcChoEase activity or content of various molecular forms. In particular, the sarcolemmal fraction isolated by McLaughlin et al. (3) was enriched for both 16S AcChoEase and extracellular matrix components (basal lamina). There is indirect evidence, essentially from studies on electric fish AcChoEase, that the asymmetric forms of AcChoEase, which are composed of tetrameric proteomers and a collagen-like multistranded tail (4-8), can interact with basal lamina components such as glycosaminoglycans of unknown nature (9), fibrous material (10), or fibronectin (11). In mammalian skeletal muscle, 16S AcChoEase has been found to be collagenase sensitive (12) and thus is probably homologous to the electric fish tailed enzyme. In view of its privileged localization in the region rich in motor endplates and its possible crucial role in cholinergic neurotransmission, it is of prime importance to determine the cellular structures with which it is associated and the interactions in which this complex macromolecular component is involved, in particular at the neuromuscular junction. In this paper, we report a muscle cell subfractionation that allows the isolation of a neuromuscular junction-enriched fraction, characterized by its specific content in pre- and postsynaptic proteins, namely choline acetyltransferase (ChoAcTase; acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) the acetylcholine receptor protein (AcChoR), and 16S AcChoEase. This preparation allows direct biochemical studies of various neuromuscular junction structures or protein components that were not possible previously. In particular, taking advantage of the detergent insolubility of synaptic basal lamina material, we have obtained direct proof that this material specifically contains most of the functional 16S AcChoEase.

MATERIAL AND METHODS

In each experiment we used 20 3- to 4-week-old mice of the C3H, Ca4+/Ca4+ strain (INSERM U153). The animals were sacrificed by cervical dislocation and the rib cage was immediately dissected out and freed from the superficial parathoracic muscles. All subsequent procedures were performed in a cold room (4-6°C). The tissues (intercostal muscles and ribs) were homogenized in Krebs/phosphate medium [5% (wt/vol); Brinkmann Polytron; 1 min] and the crude homogenate was filtered through four stainless steel filters (1,000-, 500-, 250-, 125-μm pore size). The filtrate was centrifuged (800 × g; 15 min; IEC centrifuge) and the pellet was resuspended in 48 ml of Krebs medium. Eight milliliters of this suspension (crude homogenate) was layered on top of a preformed discontinuous sucrose gradient (layers of 8 ml of 1.8 M, 1.4 M, 1 M, and 0.6 M sucrose). Sedimentation was for 150 min at 7,500 × g in a Beckman L8 ultracentrifuge with an SW 27 rotor. The main fractions obtained were, from the meniscus to the bottom: a clear supernatant ("soluble" fraction), three bands [A in the 0.6 M sucrose layer, B in the 1 M sucrose layer, and C (usually double—C1 and C2) in the 1.4 M sucrose layer], and a thick pellet. Each fraction was collected by aspiration, diluted 1:2 with Krebs medium, and centrifuged at 22,000 rpm for 35 min (L8 ultracentrifuge; SW 27 rotor). The supernatants were discarded and the pellets constituted the primary fractions.

Each primary fraction was then submitted to an extraction procedure either in a single step in a standard medium (1% Triton X-100/1 M NaCl/0.001 M EGTA/0.01 M Tris-HCl, pH 7.4) or in a stepwise manner in buffers of increasing ionic strength.

Abbreviations: AcChoEase, acetylcholinesterase; BtChoEase, butyrylcholinesterase; ChoAcTase, choline acetyltransferase; AcChoR, acetylcholine receptor; α-BgTx, α-bungarotoxin; ECM, extracellular matrix.
7.2) or in two successive steps, in the following order: the primary pellet was resuspended by vigorous shaking in 1 ml of a detergent medium, composed of 1% Triton X-100 and 0.01 M Tris-HCl, pH 7.2. After centrifugation the pellet was resuspended in high salt medium (1 ml of 1 M NaCl/0.001 M EGTA/0.01 M Tris-HCl, pH 7.2). The supernatants of both centrifugations were kept for further analysis. In a few experiments, the procedure was performed in the inverse order.

Cytotechnical Staining. A modification of the method of Koelle and Friedenwald (13) was used to stain motor endplate AcChoEase. Each primary fraction—A, B, and C—was dispersed into a 0.2 M Tris maleate/NaOH, pH 6.5/0.6% glutaraldehyde fixative and kept overnight. Then, after centrifugation, the pellets were suspended in the standard pH 5 incubation medium, which has acetylthiocholine iodide as a substrate. After two washings by centrifugation in the acetic acid/sodium acetate, pH 5.0, buffer used in the incubation, 3% potassium ferricyanide was used to resuspend the pellets and the brown coloration was allowed to develop for 15 min. Two additional washings were performed before mounting the preparation in pure glycerol for light microscopy.

AcChoR and Binding of 125I-Labeled α-Bungarotoxin (125I-α-BgTx). The intact rib cages were incubated in 10 ml of a culture medium (14) containing 125I-α-BgTx (15–20 Ci/μg; New England Nuclear; 1 Ci = 3.7 × 1010 Bq) at 0.2 μg/ml for 30 min at room temperature. Then, the tissues were washed twice with the culture medium and a third time with the Krebs medium. Finally, all the fractions were solubilized and subjected to sedimentation analysis (14).

AcChoEase and Butyrylcholinesterase and Their Multiple Molecular Forms. Total AcChoEase or butyrylcholinesterase (BuChoEase; cholinesterase; EC 3.1.1.8) activity was estimated by the method of Ellman et al., using acetylthiocholine or butyrylthiocholine iodide, respectively, as substrate (15). Analysis of the multiple forms of AcChoEase was performed on a 5–20% continuous sucrose gradient (16). The specific reversible AcChoEase inhibitor BW 284 C 51 (Burroughs Wellcome, Research Triangle Park, NC) or the irreversible BuChoEase inhibitor isooctamethyl pyrophosphoramide (Sigma) were sometimes used to assess enzyme specificities.

ChoAcTase. ChoAcTase activity was determined by the method of Fonnun (17), using [1-14C]acetyl-coenzyme A (Amer- shorn; 50 mCi/μmol), and is expressed as μmol of [14C]aceetylcholine synthesized per min. Protein concentrations were estimated by the method of Lowry et al. (18).

Collagenase Treatment. Pure collagenase (240 international units; Advance Biofactures, Lynbrook, NY) was added to 100-μl aliquots of high-salt extracts of the various subcellular fractions. Ca2+ concentrations were adjusted to 0.01 M. The collagenolytic action was allowed to occur at 37°C for 1 hr, and sedimentation analysis was started immediately.

RESULTS

Analysis of Primary Subcellular Fractions. Distribution of AcChoR. 125I-α-BgTx binding sites are highly concentrated in the C fractions. Table 1 shows that about 50% of the specific binding of 125I-α-BgTx is found in the C1 fraction. Only 6% is found in the supernatant fraction, and the rest of the specific binding is found in the C2 fraction (18%) and in the pellet (36%). It is in the C1 fraction that the specifically bound radioactivity per milligram of protein is at its highest value: more than 10 times higher than in the crude homogenate before fractionation (Table 1).

Evidence for isolated motor endplates in the AcChoR-rich fraction. Fig. 1 shows several aspects of the numerous subcellular structures that stain heavily after AcChoEase cyto-

<p>| Table 1. Distribution of α-BgTx binding sites (AcChoR) after subcellular fractionation of mouse intercostal muscles: Isolation of a junctional membrane-enriched fraction |
|---------------------------------|-----------------|-----------------|-----------|</p>
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total 125I-α-BgTx, cpm/g fresh tissue</th>
<th>Specific activity, cpm/mg protein</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>18,235 ± 551</td>
<td>151 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant (top of gradient)</td>
<td>1,096 ± 94</td>
<td>356 ± 31</td>
<td>6</td>
</tr>
<tr>
<td>Fraction A</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fraction B</td>
<td>NS</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fraction C1</td>
<td>9,027 ± 466</td>
<td>1,514 ± 79</td>
<td>49.5</td>
</tr>
<tr>
<td>Fraction C2</td>
<td>3,243 ± 302</td>
<td>702 ± 67</td>
<td>17.8</td>
</tr>
<tr>
<td>Pellet</td>
<td>6,588 ± 230</td>
<td>—</td>
<td>36.1</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM of three independent experiments. The fresh tissue is the bulk of the costal grids, weighed immediately after dissection, rib included. NS, not significant.

*a Specific binding (after gradient analysis).

chemistry. This staining is mainly due to AcChoEase, because it is not obtained in the presence of 10 μM BW 284 C 51, the specific inhibitor of AcChoEase. The morphological appearance of these subcellular structures (size and internal organization, with apparent postsynaptic folds in many of them) suggests the presence of numerous well-preserved motor endplates in fraction C1.

Distribution of ChoAcTase. The major part of muscle ChoAcTase activity is solubilized in the procedure and is found in the supernatant of the discontinuous gradient after fractionation (about 60% of total tissue ChoAcTase activity). Only 5% of total ChoAcTase is found in the isolated motor endplate-rich fraction C1. If we hypothesize that ChoAcTase is essentially localized in the nerve terminals, then only a small proportion of the presynaptic cytoplasm is recovered in the C1 fraction.

Fig. 1. Isolated motor endplates in a synaptic membrane-enriched subcellular fraction (C1 fraction). Cytochemical staining of AcChoEase (19). (a) Intact muscle fibers: region rich in motor endplates. (b) Suspension of isolated stained motor endplates (large field view). (c and d) Selected examples of isolated motor endplates, with their synaptic gutters.
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**Distribution of AcChoEase and its multiple molecular forms in the primary fractions.** AcChoEase activity is found in most of the fractions studied, but in variable amounts, and is also inequally distributed in its multiple molecular forms. The five main molecular forms of AcChoEase are identified by their sedimentation coefficients: 16 S, 12 S, 10 S, 6.5 S, and 4 S. Their sedimentation profiles are composed of three main peaks of activity (Fig. 2): "16S" (16 S and minor accompanying forms), "10S" (10 S and a small 12 S contribution), and "4S" (4 S and a minor 6.5 S contribution). The specific activity of total AcChoEase is about 10-fold higher in the C1 fraction than in the crude homogenate (Table 2), which represents a purification factor of the same order as for AcChoR in this fraction. The relative proportion of 16 S AcChoEase is significantly higher in the C1 fraction than in the crude homogenate before fractionation (Fig. 2) and is about 33% compared to 20% in the crude homogenate (Table 2). The AcChoEase activity found as 16 S AcChoEase in the C1 fraction represents 10% of 16 S AcChoEase activity in the crude homogenate. We found rather high total and specific AcChoEase activities in the A fraction, higher than in the crude homogenate or any other fraction. It is in the same fraction that 10 S AcChoEase is predominant (45% of total AcChoEase). The A fraction does not contain any significant amount of AcChoR or any cytochemically identifiable isolated motor endplates. The C2 fraction contains a higher relative proportion of 16 S AcChoEase than the C1 fraction (39%) but less AcChoR and less ChoAcTase activity.

**Sequential Solubilization of the Primary Subcellular Fractions by Detergent and High-Salt Media.** Extracellular Matrix Isolation and Association with Asymmetric AcChoEase. *Detergent treatment.* The treatment of the primary fractions by the detergent-containing (1% Triton X-100) and salt-free medium results in the total "solubilization" of specifically bound 125I-o-BgtTx in all fractions where it has been found, because it is quantitatively recovered in the supernatant of the low-speed centrifugation. Fig. 3A shows the "solubilization" obtained from the AcChoR-rich C1 fraction by the detergent treatment, and Table 3 shows the recovery of total AcChoEase and the low percentages of 16 S AcChoEase recovered in the supernatants from each fraction (about 14% in the C1 fraction).

**High-salt treatment of detergent-insoluble material.** The detergent-insoluble material can be "solubilized" by high-salt (1 M NaCl) media and is most probably composed of the extracellular matrix material of the muscle fibers. In the C1 fraction, which is highly enriched in AcChoR and 16 S AcChoEase, we are then dealing with extracellular matrix material mostly of synaptic origin. The high-salt treatment "solubilizes" a very high proportion of 16 S AcChoEase (Fig. 3B): No AcChoR is solubilized from the pellet of the detergent-treated C1 fraction by high concentrations of salt. Table 4 shows the relative proportions of the 16 S peak of AcChoEase, after extraction of the detergent-treated subcellular fractions by the high-salt medium; in the C1 fraction, 16 S AcChoEase represents 61.2 ± 2.1% of total AcChoEase recovered on the sucrose gradient. After a short exposure to pure collagenase at 37°C the high-salt-extracted 16 S AcChoEase is readily converted into a major 10 S form, without loss of AcChoEase activity (Fig. 3B), demonstrating that this species is a collagen-like tailed form of AcChoEase.

**BtChoEase, and particularly its 16 S molecular form, is barely**

### Table 1. Distribution of total AcChoEase and tailed symmetric 16 S AcChoEase after solubilization of the subcellular fractions in the detergent medium

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovery, %</th>
<th>Specific activity, ΔA₄₁₂/hr per mg protein</th>
<th>Specific activity, % of total AcChoEase</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>4.33 ± 0.06</td>
<td>6.5 ± 1.4</td>
<td>6.5 ± 1.8</td>
</tr>
<tr>
<td>A</td>
<td>42.2</td>
<td>25.5 ± 0.64</td>
<td>21.3 ± 4.1</td>
</tr>
<tr>
<td>B</td>
<td>1.5</td>
<td>3.3 ± 0.14</td>
<td>13.9 ± 5.3</td>
</tr>
<tr>
<td>C1</td>
<td>22.5</td>
<td>2.4 ± 0.33</td>
<td>13.9 ± 5.9</td>
</tr>
<tr>
<td>C2</td>
<td>24.2</td>
<td>6.5 ± 0.09</td>
<td>7.6 ± 2.3</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM of three independent experiments.

### Table 2. Distribution of total AcChoEase and its multiple molecular forms after subcellular fractionation of mouse intercostal muscles

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovery, %</th>
<th>ΔA₄₁₂/hr per mg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>100</td>
<td>0.28 ± 0.001</td>
</tr>
<tr>
<td>Supernatant</td>
<td>12.9</td>
<td>6.55 ± 0.37</td>
</tr>
<tr>
<td>Fraction A</td>
<td>40.2</td>
<td>17.49 ± 0.86</td>
</tr>
<tr>
<td>Fraction B</td>
<td>10.5</td>
<td>1.99 ± 0.07</td>
</tr>
<tr>
<td>Fraction C1</td>
<td>16.0</td>
<td>2.82 ± 0.06</td>
</tr>
<tr>
<td>Fraction C2</td>
<td>11.4</td>
<td>4.31 ± 0.06</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM of three independent experiments.

*One ΔA₄₁₂ unit corresponds to 73.5 μmol of hydrolyzed acetylthiocholine.*
detectable in the high-salt-soluble synaptic extracellular matrix (ECM) material (Fig. 4). About 42 ± 5% of the initial Bt-
ChoEase activity present in the crude homogenate is recovered in the C1 fraction. Only 11% of the BtChoEase of the C1 fraction can be "solubilized" by high salt, which results in a BtChoEase recovery of 4.5% of the total activity present in the crude homogenate. Thus, BtChoEase, and especially its very minor 16S component, is not accumulated in the synaptic ECM material.

**Increased extraction yield of total and 16S AcChoEase in the sequential double-step procedure, compared to the single-step procedure.** We consistently found more total AcChoEase extracted with detergent followed by high salt than with detergent and high salt added together. Table 5 shows that it is in the C1 fraction that both total AcChoEase and 16S AcChoEase are recovered in highly increased amounts in the double-step procedure. The two-step versus one-step ratios for 16S AcChoEase were 1.93, 1.34, and 4.95 for the crude homogenate, fraction A, and fraction C1, respectively.

**Hydrophilic and hydrophobic 16S AcChoEase.** It is of interest to estimate the relative amounts of 16S AcChoEase found in each "solubilization" step (detergent followed by high-salt treatments) compared to the sum of 16S AcChoEase solubilized in both steps. Table 6 shows that 16S AcChoEase may have two different solubility behaviors: in the C1 fraction, where 58% of the original crude homogenate "16S" AcChoEase is found, most of it is "solubilized" in the high-salt step (88%). Moreover, when the primary fraction C1 is first treated by high salt, then by detergent (inversion of the two steps of "solubilization"), the previous result holds: the percentage of 16S AcChoEase recovered in the high-salt extraction, when this step is performed first, is found to be 94 ± 10% (n = 2) for the fraction C1.

In contrast to the C1 fraction, the detergent step extracts most of the 16S AcChoEase contained in the A fraction. Thus, 16S AcChoEase must have a different subcellular location in the fractions C1 and A: the synaptic ECM of the isolated motor endplate in fraction C1 and the lipid-rich membranes from plasmalemma or sarcoplasmic reticulum in fraction A.

**DISCUSSION**

Our results constitute direct evidence for the association of tagged asymmetric AcChoEase with ECM material of mammalian muscle fiber. Moreover, we find tagged asymmetric AcChoEase in synaptic membrane-enriched subcellular fractions,
which suggests that it is probably concentrated in the muscle junctional basal lamina sheaths. However, we find that ECM is not the unique localization of tailed asymmetric AcChoEase.

A number of studies had previously shown that asymmetric forms of AcChoEase are highly concentrated in the motor endplate-rich regions of rat or mouse muscle (1, 2). The possibility, or likelihood, of the association of junctional AcChoEase with muscle basal lamina arose from the observation that collagenase solubilized most of AcChoEase activity found in the synaptic cleft (19). However, it was discovered that asymmetric AcChoEase was collagenase sensitive (5, 8, 9, 12) and is probably physicochemically homologous to the chick retina (20S) asymmetric AcChoEase, which has been proposed to be anchored into the cell plasma membrane (25). In contrast, Bt-ChoEase is nearly absent from ECM material. Our results call for a detailed biochemical study of the muscle ECM material, to explore further the fundamental aspects of the cellular biosynthesis, transport, and localization of AcChoEase.

### Table 5. Increased yield of total AcChoEase in the sequential extraction procedure

<table>
<thead>
<tr>
<th>Fraction</th>
<th>One-step*</th>
<th>First step†</th>
<th>Second step‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>154.3 ± 22.9</td>
<td>147.2 ± 39</td>
<td>108.5 ± 35.0</td>
</tr>
<tr>
<td>Fraction A</td>
<td>62.0 ± 6.5</td>
<td>62.1 ± 39</td>
<td>25.1 ± 0.9</td>
</tr>
<tr>
<td>Fraction C1</td>
<td>24.7 ± 0.3</td>
<td>33.1 ± 0.5</td>
<td>58.8 ± 8.9</td>
</tr>
</tbody>
</table>

Results are mean ± SEM.

* Detergent plus high salt (n = 3).
† Detergent (n = 3).
‡ High salt (n = 5).

posed mainly of 16S AcChoEase, but also of other minor peaks (18.5S and 12S), is thus embedded in the ECM material or in the portion of it that may be solubilized by high-salt media.

We found that both total AcChoEase and (especially) 16S AcChoEase are recovered in much higher yields when detergent and high-salt extractions are sequentially used rather than the single-step procedure, when detergent and high salt are both present in the extraction medium. We have no clear explanation for this phenomenon, which also occurs when high salt (with EGTA) is utilized first in the sequential procedure. It is interesting to note that both steps give nearly as much each as the single-step procedure, at least for the C1 fraction. Some protection or activation may occur in the sequential procedure that does not act with the simultaneous use of these extracting agents. Some of muscle asymmetric AcChoEase is found in a lighter, low density fraction (A) and is solubilized by detergent.

It clearly corresponds to a very distinct pool of 16S AcChoEase, which is associated not with the basal lamina but with some lipid membranes. These lipidic membranes may be either extrajunctional plasma membranes or, more probably, sarcoplasmic reticulum membranes, as recently suggested by electron microscopy. This lipid membrane-associated 16S AcChoEase is probably physicochemically homologous to the chick retina (20S) asymmetric AcChoEase, which has been proposed to be anchored into the cell plasma membrane (25). In contrast, Bt-ChoEase is nearly absent from ECM material. Our results call for a detailed biochemical study of the muscle ECM material, to explore further the fundamental aspects of the cellular biosynthesis, transport, and localization of AcChoEase.