ACETYLCHOLINESTERASE: A USEFUL MARKER FOR THE ISOLATION OF SARCOLEMMA FROM THE BIVALVE (MODIOLUS DEMISSUS DEMISSUS) MYOCARDIUM

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SUMMARY

The presence of cholinesterase activity in M. demissus hearts was demonstrated by light- and electron-microscopic histochemistry and by enzymic assay. The enzyme proved to be acetylcholinesterase (AChE) since acetyltiocholine was the preferred substrate, and eserine or BW284c51 inhibited the enzyme activity, while isoOMPA was without effect. The AChE was localized and uniformly distributed along the cell surface membranes of the cardiac muscle cells. A fraction 8-fold enriched in AChE was isolated from pooled ventricles by a combination of differential and sucrose density gradient centrifugation. This sarcolemmal fraction contained little mitochondrial contamination as determined by electron microscopy and by succinate cytochrome c reductase activity. In addition, this fraction stained uniformly for AChE, indicating that it was free of other membrane types (for example sarcoplasmic reticulum which did not stain for AChE). Therefore, this fraction contained purified cell surface membrane free of contamination by other membranous organelles.

INTRODUCTION

Cell volume regulation by means of amino acid permeability change has been studied in detail in the bivalve (Modiolus demissus demissus (Dillwyn)) myocardium (Pierce & Greenberg, 1972, 1973, 1976). These studies have led to the hypothesis of a relationship between amino acid permeability control and membrane-bound adenosinetriphosphatase (ATPase) activity. In order to characterize that enzyme and test that hypothesis, a cell membrane fraction was required which was largely free of other cellular components known to contain divalent ATPase activity. Since preliminary experiments indicated that the sodium-potassium ATPase usually utilized as a marker for cell membrane separations was inadequate for that purpose in M. demissus cardiac tissue, another marker was sought.

The validity of AChE as a marker for cell surface membrane must be carefully evaluated. For example, some studies have found AChE as a surface membrane enzyme in vertebrate skeletal muscle (Severson, Drummond & Sulakhe, 1972; Kracke, Beringer & Koening, 1975). Other studies have indicated that in at least some verte-
brate skeletal muscle preparations AChE is only loosely attached to the membrane and, therefore, in this tissue the use of AChE as a surface membrane marker is questionable (Betz & Sakmann, 1973). While many workers have discarded AChE as an appropriate surface membrane marker for vertebrate fast muscles, that by no means a priori disqualifies AChE as a marker in other preparations. Indeed, in both mammalian brain (Sellinger & Borens, 1969; Salvaterra, Mahler & Moore, 1975) and red blood cells (Mitchell, Mitchell & Hanahan, 1965; Steck, 1974) AChE is clearly a cell surface membrane enzyme. Therefore, since the presence of a cholinesterase (AChE) in *M. demissus* ventricles has been demonstrated both by manometric measurement (Smith & Glick, 1939) and by pharmacological techniques (Greenberg, 1969) and since the ultrastructural cytochemistry for AChE visualization has been well described (Friedenberg & Seligman, 1972; Karnovsky, 1964; Koelle, 1963; Tsuji, 1974), we tested the suitability of AChE as a sarcolemmal marker in the *M. demissus* cardiac cell.

The requirements for a cell membrane marker include the following: (1) the marker must be limited to the cell membrane only; (2) the marker must be specifically located in the cell type to be studied; (3) the marker must be stable to permit good recovery during fractionation (DePierre & Karnovsky, 1973). An additional requirement should be that the marker be uniformly distributed in the cell membrane, thereby allowing a representative fraction to be isolated.

The following study indicates that AChE fits all the criteria for a cell surface membrane marker. Furthermore, a pure cell membrane fraction has been produced from the *M. demissus* myocardial cells using AChE as a marker. Some of these results were previously communicated to the American Society of Zoologists (Watts & Pierce, 1976).

**MATERIALS AND METHODS**

**Animals**

Specimens of *Modiolus demissus* were collected at Assateague Island, Maryland and maintained in aquaria containing aerated Instant Ocean (Aquarium Systems Inc., Eastlake, Ohio) (osmotic concentration: 950–1000 mosm) at 14 °C. Animals were kept under these conditions at least 2 weeks and not more than 2 months prior to use.

**Cholinesterase localization: electron microscopy**

Ventricles were removed from mussels and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.0) containing 0.4 M sucrose for 1 h (4 °C). After fixation, the ventricles were placed in glutaraldehyde-phosphate buffer without sucrose for 30 min and then stained for ChE by the method of Karnovsky & Roots (1964) as modified and demonstrated by Dr J. Rash (University of Maryland Medical School, Baltimore, Md.). Two staining solutions were utilized. Stain solution A contained 1.65 mM acetylthiocholine iodide (ATC), 10 mM citrate, and 6 mM CuSO₄ in 0.15 M phosphate buffer (pH 7.0) with 1.7% glutaraldehyde. Solution B consisted of 1% OsO₄ and 2.5 mM potassium ferricyanide in 0.05 M phosphate buffer (pH 7.0). The fixed ventricles were incubated at room temperature in solution A for 3 h with fresh stain added after 1.5 h. Next, the tissue was rinsed in 0.2 M phosphate buffer (pH 7.0) and incubated in solution B for 1 h. Following this last incubation, the tissue was rinsed in buffer, dehydrated in an ethanol series, and embedded in Epon 812. Thin sections were made using a glass or diamond knife on an ultramicrotome (Porter-Blum MT2-B, Sorvall Instruments, Newtown, Conn.). The sections were mounted on no. 500 mesh copper grids, and some sections were then further
AChE: bivalve myocardial cell membrane marker

stained with uranyl acetate and lead citrate (Venable & Coggshall, 1965). Finally, the sections were examined and photographed with an electron microscope (Hitachi HU-12).

To ensure that the observed reaction product was due to ChE activity, samples of the tissues were stained under the following conditions: (a) incubation in stain solutions lacking the substrate, ATC; (b) pre-incubation for 1 h in eserine sulphate (10^{-4} M) followed by staining in the usual solutions described above with eserine (10^{-5} M) included in the staining solutions; (c) stained sections were examined without uranyl acetate and lead citrate counterstain. In addition, rat skeletal muscle motor end plates, a classical AChE stain preparation (Hunt, 1966), were processed through the staining procedure to ensure that the stain solutions were functioning properly.

Histological preparation: photomacrography

The AChE staining pattern was also observed in intact M. demissus ventricles, again using rat skeletal muscle as a positive control for the staining reaction. Ventricles and the rat muscles were incubated unfixed in solution A for 1–2 h and then transferred to solution A plus 0.5 mM potassium ferricyanide for 1 h. Muscles were then photographed with dark-field optics and a bellows extension.

ChE localization in cryostat sections

Since there is some evidence that ATC does not penetrate well into intracellular regions (Friedenberg & Seligman, 1972), cryostat sections were examined for ChE activity according to the following procedure. Ventricles were removed and fixed as described above, then rinsed in the phosphate buffer solution for 30 min and frozen in blocks of OCT embedding compound (Ames Co., Elkhart, Ind.) by immersion in liquid nitrogen. Sections were cut at 50 μm on a freezing microtome, mounted on plastic coverslips, rinsed in buffer and stained with the ChE procedure for electron microscopy described above. The sections were then dehydrated and embedded in Epon 812. Thin sections were taken within the first 1–2 μm of the outermost surface of the cryostat section and examined without counterstain with the electron microscope.
Tissue preparation for ChE assay and localization

Nine ventricles were homogenized in 3 ml of artificial seawater (ASW) (Wilkens, 1972). The homogenate was centrifuged (1060 g for 30 min) and the supernatant saved for ChE essay. Subcellular fractions were then prepared from a group of 175 ventricles (approximately 2.5 g wet weight) (Fig. 1). The tissue was homogenized for 2-5 min (Sorvall Omnimixer at top speed) in 20 ml ASW in an icebath. This was followed by 20 passes with a motor-driven teflon Potter-Elvehjem homogenizer (0.11-0.15 mm clearance) (model S63C, TRI-R Instruments, Rockville Center, New York, at setting 6) also in an icebath. The homogenate was centrifuged at 10866 g for 30 min (4 °C). The supernatant was saved and the pellet resuspended in 0-10 ml ASW, homogenized and recentrifuged at 10866 g for 30 min (4 °C). This pellet (P1 hereinafter) was saved and both supernatants were combined and further centrifuged at 153400 g for 90 min (4 °C) in an ultracentrifuge (Beckman L-4 with SW41 rotor, Beckman Instruments Inc., Spinco Div., Palo Alto, Cal.). The supernatant obtained from this centrifugation is called the soluble fraction hereinafter and the pellet labelled P2. P2 was then resuspended in 2 ml of 8 % sucrose, layered on a continuous 25-45 % (w/v) sucrose gradient and centrifuged at 106500 g for 120 min (4 °C) in the ultracentrifuge (SW41 rotor). Following centrifugation, the gradient was fractionated from the top with a fraction collector (ISCO model 328, Instrument Specialities Co., Lincoln, Nebraska) equipped with a u.v. monitor (ISCO UA-5) set at 280 nm. The peaks indicated by the monitor were collected as separate fractions and diluted with 0.1 M phosphate buffer (pH 8.0). These fractions were pelleted at 208000 g for 60 min and the sucrose discarded. Each pellet was resuspended with an appropriate amount of 0.1 M phosphate buffer (pH 8.0) and ChE activity determined as described below. In addition, some isolated subcellular fractions were prepared and stained in suspension for ChE.

ChE assay

ChE was assayed by the method of Ellman, Courtney, Andres & Featherstone (1961) as described by Roop & Greenberg (1976): 10-125 μg protein were added to paired 1-cm cuvettes containing 2.9 ml of 0.1 M phosphate buffer (pH 8.0), 0.1 ml of 0.1 M 5,5-dithio-2-(2-nitrobenzoic acid) (DTNB) (Nutritional Biochemical Corp., Cleveland, Ohio) in 0.1 M phosphate buffer (pH 8.0) in the ultracentrifuge (SW41 rotor). Following centrifugation, the gradient was fractionated from the top with a fraction collector (ISCO model 328, Instrument Specialities Co., Lincoln, Nebraska) equipped with a u.v. monitor (ISCO UA-5) set at 280 nm. The peaks indicated by the monitor were collected as separate fractions and diluted with 0.1 M phosphate buffer (pH 8.0) and ChE activity determined as described below. In addition, some isolated subcellular fractions were prepared and stained in suspension for ChE.

Localization of mitochondrial membranes: succinate cytochrome c reductase assay

The subcellular fractions obtained by the above procedures were resuspended in 5 mM potassium phosphate buffer (pH 7.4) to disrupt the mitochondria. Succinate cytochrome c reductase activity was determined in the fractions using the method of Fishbein & Stowell (1968): 6-70 μg protein in 50 μl were added to microcuvettes (1-cm path length) containing 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0.06 ml of 20 mM NaCN, 0.165 ml distilled water and 0.05 ml of 1 % cytochrome c (Sigma grade III). The reaction was initiated with 0.175 ml of 0.1 M disodium succinate while blanks received distilled water. The reaction rate was measured using a double beam spectrophotometer (Varian model 635) at 548 nm as cyto-
Fig. 2. Cholinesterase stain in rat skeletal muscle. Stain product is confined to specific areas along the muscle (arrows). Photomicrograph, × 227.

Fig. 3. Electron micrograph of sections of stained areas (from Fig. 2) showing localization of cholinesterase activity at the motor end plate region. × 53750, bar = 315 nm.
chrome c was reduced. A 3-min incubation at 24 °C was linear with protein and time. Specific activity in the reaction volume was calculated using the extinction coefficient (reduced-oxidized) of 18.5 × 10^3 M^-1 cm^-1 for cytochrome c.

Statistical analysis

Bartlett’s test was used to test for homogeneity of variance between groups. Analysis of Variance test was performed to determine if treatment effects were significant and the Student-Newman-Keuls test (Sokal & Rohlf, 1969) was used to test individual treatment effects. A probability of \( P < 0.05 \) was accepted for significance. Where necessary, the data were log transformed to obtain homogeneity of variance and transformed data were further analysed. In these cases antilog means and 95% confidence limits are reported.

RESULTS

ChE staining: light and electron microscopy

The vertebrate skeletal muscle preparations (Fig. 2) exhibited ChE stain confined to specific regions of the cell. Electron-microscopic examination of these stained regions showed typical motor end plate profiles containing dense stain deposits (Fig. 3). In contrast, the *M. demissus* ventricle showed a diffuse ChE staining pattern (Fig. 4). The stain reaction product was uniformly distributed over wide regions of the trabeculae lining the inner cardiac surface. While distribution of the enzyme may be patchy on a microscale, no discrete staining regions appear and during the staining procedure the entire tissue darkens uniformly, unlike vertebrate skeletal muscle, where the stain appears in isolated areas. Little staining occurred on the surface of the pericardial epithelium of the ventricle. Electron-microscopic examination of stained cardiac sections (Fig. 5) showed dense ChE reaction product deposits along the cell surface membrane. Intracellular enzyme staining was never observed with this technique. In addition, the thin sections taken at the outer surface of a cryostat section of the ventricle also exhibited staining associated only with the cell surface membrane (Fig. 6). No staining occurred in the sarcoplasmic reticulum or other intracellular organelle membranes. Examination of the stained membranes at high magnification revealed stain deposits on the outer sarcolemma surface (Fig. 7). In a lightly stained preparation, the stain deposits appear to be very regularly dispersed along the entire outer leaflet of each surface membrane (Fig. 8). Stain deposits from ChE activity were never observed in tissues treated with the staining procedure but lacking ATC (Fig. 9) nor in tissue stained in the presence of eserine (10^-4 M) (Fig. 10). Further-
AChE: bivalve myocardial cell membrane marker
more, ChE staining occurred in the absence of uranyl acetate and lead citrate (Fig. 6), indicating that the stain deposits were the result of enzyme activity rather than from possible chemical interaction of the components of the reaction mixture with these counter stains. Thus, the stain reaction product observed in *M. demissus* ventricles is due to the enzymic activity of a ChE which is uniformly distributed along the surface membrane only.

### ChE assay: effects of substrates and inhibitors

Homogenate ChE activity was measured with acetylthiocholine (ATC), propionylthiocholine (PTC) and butyrylthiocholine (BTC) substrates (Table 1). The order of substrate preference was ATC > PTC > BTC with enzyme activity in the presence of BTC barely detectable. Differences between each substrate were significant (*P* < 0.05). The effects of specific inhibitors were studied with ATC as substrate (Table 2). Eserine (10⁻⁶ M) and BW284C51 (10⁻⁵ M) significantly inhibited (P < 0.05) 99 and 97% of ChE activity respectively, while iso-OMPA (10⁻⁵ M) had no significant effect. These results indicate that the enzyme was AChE.

### Subcellular fractions: marker enzymes and electron microscopy

The P₁ fraction obtained from the differential centrifugation steps described above (Fig. 1) yielded a 50% increase of AChE specific activity compared to homogenate activity (Fig. 11). The subsequent sucrose gradient subfractionation of P₁ resulted in 3 discrete absorbance peaks (280 nm) within the gradient (Fig. 12) which were collected as separate fractions. The least-dense fraction (F₁) contained an 8-fold enrichment of AChE over that of the homogenate (Fig. 11). In addition, the F₁ fraction

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**Table 1. Substrate specificity of cholinesterase from whole cell homogenates of *M. demissus* ventricle**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity, <em>μmol SH/mg protein/min</em></th>
<th>95% confidence interval</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylthiocholine</td>
<td>0.30*</td>
<td>0.23 - 0.39</td>
<td>100</td>
</tr>
<tr>
<td>Propionylthiocholine</td>
<td>0.11*</td>
<td>0.07 - 0.20</td>
<td>37</td>
</tr>
<tr>
<td>Butyrylthiocholine</td>
<td>0.004*</td>
<td>0.003 - 0.005</td>
<td>1</td>
</tr>
</tbody>
</table>

* Mean of 3 separate preparations.

Means are significantly different (*P* < 0.05).

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**Fig. 7.** High magnification of *M. demissus* cardiac sarcolemma stained for cholinesterase activity. While diffusion of reaction product usually obliterates the extracellular space with the staining protocol used here, the stain distribution occurring in areas of adjacent membrane divergence (arrows) indicates reaction product formation on both membranes. × 200,000, bar = 50 nm.

**Fig. 8.** A lightly stained *M. demissus* preparation showing very uniform reaction product formation along both outer leaflets of adjacent myocardial cells. × 240,000, bar = 50 nm.
exhibited low succinate cytochrome c reductase activity when compared with that of the homogenate or the P₁ fraction, indicating little mitochondrial contamination (Fig. 11). The mitochondrial membranes appear to be removed in the P₁ fraction. Finally, the F₁ and F₁₁ fractions were stained for AChE and examined with the electron microscope. The F₁ fraction consisted of trilaminar membrane vesicles 0.18 μm in diameter, almost all of which show dense deposits of ChE reaction product associated with the membrane (Fig. 13). In contrast, F₁₁ contained only a few stained vesicles interspersed among a large number of unstained vesicles (Fig. 14).

Table 2. Comparative effects of anticholinesterase agents on acetylcholinesterase activity from whole cell homogenates of M. demissus ventricle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity, μmol SH/mg protein/min</th>
<th>95% confidence interval, L₁ – L₂</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.21*</td>
<td>0.19 – 0.23</td>
<td>100</td>
</tr>
<tr>
<td>iso-OMPA</td>
<td>0.21*</td>
<td>0.19 – 0.23</td>
<td>100</td>
</tr>
<tr>
<td>B.W. 28451</td>
<td>0.007</td>
<td>0.005 – 0.009</td>
<td>3</td>
</tr>
<tr>
<td>Eserine</td>
<td>0.002</td>
<td>0.0018 – 0.0020</td>
<td>1</td>
</tr>
</tbody>
</table>

* Mean of 3 separate preparations.

Means are not significantly different (P > 0.05).

DISCUSSION

Acetylcholinesterase from M. demissus myocardial cells fulfills all the characteristics required of a cell membrane marker. First, the ultrastructural localization of AChE in both intact and cryostat-sectioned M. demissus cardiac cells demonstrated that AChE was found along the cell surface membrane only; intracellular stain deposits were never observed. Second, cytochemical demonstration of AChE in whole-mount preparations showed that AChE was uniformly distributed over wide regions of the cell surface. Furthermore, the AChE was non-junctional in the mussel ventricle, which was not unexpected since only sparse innervation of the bivalve ventricle has been demonstrated (Greenberg & Roop, 1977). Third, the major cell type present in M. demissus ventricle which contained AChE was muscle cell. Therefore, the cell membrane isolated from this tissue using AChE as a marker represented primarily sarcolemma. Finally, 90% of the AChE found in the initial whole cell homogenate was recovered in all of the fractions produced by the subfractionation technique, indicating that the enzyme was relatively stable to the purification procedure. Although we cannot specify whether or not the AChE is actually an integral part of the surface membrane.

Fig. 9. Inhibition of cholinesterase stain in the M. demissus ventricle by omission of the substrate ATC. No cholinesterase stain deposits were observed. × 73500, bar = 100 nm.

Fig. 10. Cholinesterase stain deposits were never observed in the M. demissus ventricles when eserine (10⁻⁴ M) was included in the staining procedure. × 34000, bar = 250 nm.
membrane or only superficially attached to the outer leaflet, the actual relationship is not of importance here. Both the biochemically measured activity and the AChE stain accompanies a portion of membrane which our purification procedure isolates. These results indicate that AChE has potential as a cell membrane marker in systems where it does not dissociate from the cell surface membrane and the marker criteria are

![Figure 11](image1.png)

**Fig. 11.** Specific activity of acetylcholinesterase (stippled bars) and succinate cytochrome c reductase (open bars) in subcellular fractions of *M. demissus* ventricles. See text for definition of fraction symbols. The bars represent mean activity (± S.E.M.) for 3 separate experiments (acetylcholinesterase) and mean activity for 2 separate experiments (succinate cytochrome c reductase).

![Figure 12](image2.png)

**Fig. 12.** A typical scan (280 nm) of the sucrose density gradient fractions showing the F₁, F₃, and F₅ peaks. Density 1.11 g/cc indicates the top of the gradient.

![Figure 13](image3.png)

**Fig. 13.** The F₁ (cell membrane) fraction stained for cholinesterase activity. The fraction consists of trilaminar vesicles almost all of which show dense stain deposits of cholinesterase reaction product. × 90,000, bar = 100 nm.

![Figure 14](image4.png)

**Fig. 14.** The F₃ fraction stained for cholinesterase activity. The F₅ fraction is a mixture of stained (arrow) and unstained vesicles. × 78,000, bar = 100 nm.
AChE: bivalve myocardial cell membrane marker
satisfied. Furthermore, AChE is relatively simple to assay, and may be useful in tissues where other more commonly used markers are low in activity or not found with uniform distribution.

The cell membrane fraction obtained from the *M. demissus* myocardial cells was free of other membrane types and contained 3-5% of the total AChE contained in the initial homogenate. This percentage yield is comparable to other plasma membrane fractions which, in general, contain 1-10% of theoretical (DePierre & Karnovsky, 1972). Furthermore, the F₁ fraction was 8-fold enriched in cell membrane as measured by increase in specific activity of AChE over that of the homogenate. This enrichment is also similar to the purification of cell membrane enriched fractions obtained in other tissues using various cell membrane markers (Neville, 1976). The F₁ fraction sedimented near the interface of the loading solution and the sucrose gradient, indicating that the density of cell membranes was approximately 1.11 g/cc. Similar density values have been obtained for cell membrane from other muscle cells under similar conditions (Kidwai, Radcliffe, Duchon & Daniel, 1971a, b; Wheeldon & Gan, 1971; Barr et al. 1974; Rufeger, Tellhelm & Kroker, 1974). Recognizable mitochondrial fragments were not observed by electron-microscopic examination of the F₁ fraction and the activity of succinate cytochrome c reductase was very low in the F₁ fraction compared to the activities in the homogenate and low-speed fraction. Direct measurement of F₁ contamination by sarcoplasmic reticulum was not possible since there are no accepted sarcoplasmic reticulum markers (Kidwai et al. 1971a). However, since the AChE staining reaction never occurred in the sarcoplasmic reticulum and the AChE stain in the cell membrane fraction (F₁) was homogeneous, significant contamination by sarcoplasmic reticulum is unlikely.

The threshold for ACh excitation of *M. demissus* heartbeat is high (10⁻⁴ M) and is greatly potentiated by eserine (Greenberg, 1969; Wilkens & Greenberg, 1973). In addition, both the order of substrate preference (ATC > PTC > BTC) and inhibitor studies (inhibition by BW284c51, no inhibition by iso-OMPA) show that ChE in *M. demissus* ventricle was indeed AChE. These characteristics are similar to those previously reported for *Crassostrea virginica* myocardium (Roop & Greenberg, 1967, 1976; Greenberg & Roop, 1977) and further substantiate the observation that the high threshold to ACh in hearts of *Pterimorphia* (Ostreidae, Mytilidae) is coincident with high AChE specific activity while lower ACh thresholds in hearts of *Heterodonta* (Veneridae, Cardiidae, Mactridae (Welsh & Taub, 1948)) is coincident with a BuChE which only slowly degrades ACh (Greenberg & Roop, 1977).

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AChE: bivalve myocardial cell membrane marker

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