

# Differences in Structure and Distribution of the Molecular Forms of Acetylcholinesterase

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**Abstract.** Two structurally distinct molecular forms of acetylcholinesterase are found in the electric organs of *Torpedo californica*. One form is dimensionally asymmetric and composed of heterologous subunits. The other form is hydrophobic and composed of homologous subunits. Sequence-specific antibodies were raised against a synthetic peptide corresponding to the COOH-terminal region (Lys<sup>560</sup>–Leu<sup>575</sup>) of the catalytic subunits of the asymmetric form of acetylcholinesterase. These antibodies reacted with the asymmetric form of acetylcholinesterase, but not with the hydrophobic form. These results confirm recent studies suggesting that the COOH-terminal domain of the asymmetric form differs from that of the hydrophobic form, and represent the first demonstration of antibodies selective for the catalytic subunits of the asymmetric form. In addition, the reactive epitope of a monoclonal antibody (4E7), previously shown to be selective for the hydrophobic form of acetylcholinesterase, has been identified as an N-linked complex carbohydrate, thus defining posttranslational differences between the

two forms. These two form-selective antibodies, as well as panselective polyclonal and monoclonal antibodies, were used in light and electron microscopic immunolocalization studies to investigate the distribution of the two forms of acetylcholinesterase in the electric organ of *Torpedo*. Both forms were localized almost exclusively to the innervated surface of the electrocytes. However, they were differentially distributed along the innervated surface. Specific asymmetric-form immunoreactivity was restricted to areas of synaptic apposition and to the invaginations of the postsynaptic membrane that form the synaptic gutters. In contrast, immunoreactivity attributable to the hydrophobic form was selectively found along the non-synaptic surface of the nerve terminals and was not observed in the synaptic cleft or in the invaginations of the postsynaptic membrane. This differential distribution suggests that the two forms of acetylcholinesterase may play different roles in regulating the local concentration of acetylcholine in the synapse.

**A**CETYLCHOLINESTERASE occurs in several structurally distinct forms that can be differentiated biochemically by the number and types of subunits they contain, the apparent molecular weight of their catalytic subunits, their sedimentation behavior, hydrophobicity, and ease of extraction (6–8, 12). Although much is also known about the kinetics of acetylcholinesterase catalysis, no functional or pharmacological differences between the catalytic subunits of the diverse structural forms have been identified. Thus the physiological significance of the structural polymorphism probably resides in differential cellular distribution of the individual enzyme forms. Several sensitive assays for acetylcholinesterase exist. However, investigation of the distribution and biosynthesis of the individual forms has required their extraction and identification on the basis of sedimentation constants or electrophoretic migration rates (14). To investigate structural differences and determine the relative distribution of the forms of acetylcholinesterase in greater detail, we have developed antibodies selective for the

different forms of acetylcholinesterase found in *Torpedo californica*.

The electric ray *Torpedo californica* contains two structurally distinct classes of acetylcholinesterase in approximately equal abundance. One form is a hydrophobic dimer of catalytic subunits that aggregate in the absence of detergent, but sediment at 5.6 S in the presence of neutral detergent (7). This form has been termed the hydrophobic, globular, or homologous form. The COOH-terminal amino acid of this form is posttranslationally modified with a novel glycopospholipid (4). The lipid can be removed with a phosphatidylinositol-specific phospholipase C, resulting in dimers that sediment at 5.6 S even in the absence of detergent. Immunocytochemical and subcellular fractionation studies suggest that the hydrophobic form of acetylcholinesterase preferentially associates with the presynaptic nerve terminals (5, 9). The second form of acetylcholinesterase is dimensionally asymmetric and composed of two (13 S) or three (17 S) tetramers of catalytic subunits linked by disulfide (6, 8) bonds to

a collagen-like tail and a noncollagenous subunit. This form has been termed the asymmetric or heterologous form. Subcellular fractionation studies and the collagen-like structure of the asymmetric form suggest that this form preferentially associates with the basal lamina (6, 8, 12). Mild treatment of the asymmetric form with trypsin releases fully active tetramers of catalytic subunits that sediment at 11 S (6).

The complete amino acid sequence of the catalytic subunits of the asymmetric form of acetylcholinesterase from *Torpedo californica* has been determined from sequencing the native protein and a cDNA clone (15). Protein sequencing of the hydrophobic form has revealed that NH<sub>2</sub>-terminal, active site and several other peptide sequences are identical in both forms (4, 11). However, removal of fatty acids from the hydrophobic form has allowed isolation and sequencing of a COOH-terminal peptide not found in the asymmetric form (4). The results suggest that both forms share an identical amino acid sequence through Thr<sup>535</sup>, where they diverge. The hydrophobic form continues for two amino acids (Ala-Cys), while the asymmetric form continues for 40 amino acids. Recent isolation of genomic clones from *Torpedo* has revealed the genomic organization of acetylcholinesterase (16, and unpublished results). It appears that both forms of acetylcholinesterase share the same two exons that code for the first 535 amino acids, and that their differences are the result of alternate splicing of third exons coding for their divergent COOH-terminal regions.

To generate antibodies selective for the asymmetric form, we prepared polyclonal antibodies to a synthetic peptide corresponding to a region found only in the COOH-terminal domain of the asymmetric form. In addition, we have identified the antigenic determinant of a monoclonal antibody (4E7) already shown to be selective for the hydrophobic form (3). Immunolabeling of cryosections of electric organ with antibodies reacting with sequences that are either common or unique to the two forms of acetylcholinesterase has allowed us to determine the differential distribution of acetylcholinesterase forms with respect to synaptic specializations. Several antibody probes were used, including: (a) COOH-terminal, sequence-specific rabbit polyclonal antibodies (CT) that selectively recognize the catalytic subunits of the asymmetric form; (b) a mouse monoclonal antibody (4F3) that selectively recognizes the collagen-like structural subunit of the asymmetric form; (c) a mouse monoclonal antibody (4E7) that selectively recognizes the catalytic subunits of the hydrophobic form; (d) rabbit polyclonal antibodies (80A and 80B) that recognize the catalytic subunits of both forms; and (e) a mouse monoclonal antibody (2C9) that recognizes a sequence common to the catalytic subunits of both forms.

## Materials and Methods

### Materials

The 5.6 S hydrophobic form of acetylcholinesterase and the 11 S tetramer of catalytic subunits derived from the asymmetric form of acetylcholinesterase were purified from *Torpedo californica* (6–8). They were labeled with <sup>3</sup>H-DFP (0.36 Ci/mmol), and reduced and alkylated as described previously (11). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Cambridge, MA). Glycopeptidase F (25,000 U/mg) endoglycosidase F (600 U/mg), and endoglycosidase H (40 U/mg), were obtained from

Boehringer Mannheim Diagnostics (Houston, TX). Pansorbin, a suspension of fixed protein A bearing *Staphylococcus aureus* cells, was obtained from Calbiochem-Behring Corp. (San Diego, CA). Monoclonal antibodies 4E7 and 4F3 were obtained in a previous study (3). Monoclonal antibodies 2C9 and 2C6 were obtained using similar procedures except that the antigen was DFP labeled, denatured, reduced, and alkylated. 11 S acetylcholinesterase. Monoclonal antibodies were purified from cell culture supernatants by precipitation with 50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, as described previously (3).

### Production of Site-directed Antibodies

A hexadecapeptide corresponding to the COOH-terminal amino acids (KNQFDHYSRHESCAEL, Lys<sup>560</sup>-Leu<sup>575</sup>) of the catalytic subunits of the asymmetric form of acetylcholinesterase was synthesized in the laboratory of Dr. Russell Doolittle (University of California, San Diego) by the Merrifield solid phase method (<sup>14</sup>C-labeled amino acids were included to give a specific activity of 7.8 mCi/mol). The authenticity of the peptide was determined by gas-phase sequencing on a protein sequencer (model 470A; Applied Biosystems Inc., Foster City, CA). The peptide was coupled to BSA by slowly adding glutaraldehyde (1 ml, 0.2%) to 2 ml of 100 mM sodium phosphate buffer (pH 7.5) containing  $1.5 \times 10^{-7}$  mol (10 mg) of BSA and  $7.6 \times 10^{-6}$  mol of peptide. The reaction was allowed to proceed for 30 min at 22°C, and then unreacted glutaraldehyde was quenched by the addition of 0.25 ml of 1.0 M glycine. The result of the coupling reaction was evaluated by SDS-PAGE. The reduced migration of the peptide-BSA conjugate corresponded to an average incorporation of 5–10 mol of peptide per mole of BSA. In addition, the peptide-BSA conjugate was excised from the gel, dissolved in 0.5 ml formic acid (80°C, 4 h), and neutralized by addition of 0.5 ml of 1.0 M Tris-HCl (pH 7.0). The radioactivity incorporated into the BSA was consistent with an average incorporation of 4–5 mol of peptide per mole of BSA.

Female white New Zealand rabbits (5–6 lbs) were immunized by injection in the isolated lymph nodes of the rear leg and intradermally down the back with 0.8 ml containing 0.4 ml peptide-BSA conjugate (1.54 mg/ml BSA) and 0.4 ml Freund's complete adjuvant. Booster immunizations were performed intradermally after 1 mo and 50 ml of blood was drawn 2 wk later. The serum was allowed to clot at 22°C, clarified by centrifugation, and frozen at -70°C in small aliquots.

### SDS-PAGE and Western Blots

Proteins were mixed with an equal volume of buffer containing 30 mM Tris-HCl (pH 6.8), 1.0% SDS, 5% glycerol, 10 mM DTT, 0.05 mg/ml bromophenol blue, and 0.05 mg/ml pyronin Y. Samples were boiled for 3 min and proteins were separated by discontinuous SDS-PAGE in 1.5-mm slab gels composed of a constant ratio of acrylamide and *N,N*-methylene-bis-acrylamide (37:1) polymerized with ammonium persulfate (0.75 mg/ml) and *N,N,N,N*-tetramethylethylenediamine (0.67  $\mu$ l/ml). The stacking gel was 3.3% acrylamide in 25 mM Tris-HCl (pH 6.8), 0.2% SDS, and the separating gel was either 8 or 10% acrylamide in 75 mM Tris-HCl (pH 8.6), 0.2% SDS. The gels were run in a slab gel apparatus (model SE 500; Bio-Rad Laboratories) at 120 V constant voltage in 25 mM Tris, 190 mM glycine (pH 8.6), 0.1% SDS. Proteins were detected in the gels by staining and destaining in the presence and absence, respectively, of Coomassie brilliant blue R (0.15 mg/ml) in 50% methanol, 10% acetic acid.

Electrophoretic transfer (50 V, 150 mA, 4°C, 10–16 h) of proteins from unstained gels to nitrocellulose was performed in a transphor unit (TE Series; Hoefer Scientific Instruments, San Francisco, CA) after soaking the gel in the transfer buffer (25 mM Tris, 190 mM glycine, pH 8.6, 20% methanol) for 30 min. Blotted proteins were detected by staining and destaining in 50% methanol, 10% acetic acid, in the presence or absence, respectively, of Amido black (0.1 mg/ml). Immunodetection of blotted proteins was performed using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) that uses a biotin-labeled goat anti-rabbit antibody and peroxidase-coupled avidin.

### Deglycosylation of Acetylcholinesterase

The deglycosylation reactions used <sup>3</sup>H-DFP-labeled acetylcholinesterase that had been desalted on a size exclusion column, and then lyophilized. <sup>3</sup>H-DFP-labeled acetylcholinesterase (1.09 mg) was resuspended in 50 mM sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 1 mM PMSF, 10  $\mu$ M pepstatin A, 0.5% NP-40, 0.5%  $\beta$ -mercaptoethanol, 0.1% SDS, and digested with endoglycosidase F (20 U) by incubation for 8 h at 37°C. <sup>3</sup>H-DFP-labeled acetylcholinesterase (1.76 mg) was deglycosylated

with glycopeptidase F (1.0 U) by incubation in 1.1 ml of 250 mM sodium phosphate buffer (pH 7.4) containing 10 mM EDTA, 1.0 mM PMSF, 10  $\mu$ M pepstatin A, 0.8% NP-40, 10 mM  $\beta$ -mercaptoethanol, 0.5% SDS, for 18 h at 37°C.  $^3$ H-DFP-labeled acetylcholinesterase (2 mg) was treated for 8 h at 37°C with endoglycosidase H (1.0 U) in 0.1 M sodium citrate buffer (pH 5.5) containing 1.0 mM PMSF, 10 mM pepstatin A, 2% SDS, and 1.0%  $\beta$ -mercaptoethanol.

### Immunoprecipitation of Acetylcholinesterase

$^3$ H-DFP-labeled acetylcholinesterase (0.4  $\mu$ g) was incubated with the indicated antibodies in 200  $\mu$ l of buffer A containing 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 0.02% NaN<sub>3</sub>, 0.01% Tween-20, and 0.1% BSA for 2 h at 37°C. Pansorbin was washed with buffer A containing 5 mM *b*-mercaptoethanol, and 0.5% NP-40, then washed with buffer A containing 0.05% NP-40, and finally resuspended to 2.5% (wt/vol) in buffer A. Antibodies were precipitated by the addition of 800  $\mu$ l of Pansorbin (15 min at 4°C). Precipitates were sedimented by centrifugation in a microfuge, the supernatants aspirated, and the pellets resuspended by incubation with 200  $\mu$ l of 2% SDS and 4 M urea at 90°C for 2 min. The Pansorbin was separated from the solubilized acetylcholinesterase by centrifugation in a microfuge, and the supernatant removed for determination of radioactivity. Precipitation of  $^3$ H-DFP-labeled acetylcholinesterase by the antibodies was compared to the maximal precipitation obtained by the addition of 1.0 ml of acetone rather than Pansorbin.

### Preparation of Tissue for Light and Electron Microscopy

The electric organs were removed from an adult male *Torpedo* and fixed in 4% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. The tissue was trimmed down to  $\sim 3 \times 2$ -mm pieces and cryoprotected in 1.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 30 min at 4°C followed by 2.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. Tissue was then mounted on aluminum specimen support pins so as to provide a cross section of the electrocytes and frozen in liquid propane cooled with liquid nitrogen.

Conventional electron microscopy was performed using small pieces of electric organ that were first fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS for 1 h, followed by 1% osmium tetroxide for 1 h. After dehydration with ethanol, the tissue was embedded in EPON-Araldite resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

### Immunofluorescent Localization of Acetylcholinesterase

Thick sections (2- $\mu$ m) of frozen tissue were cut on a ultracryomicrotome (model FC-4D; Reichert Scientific Instruments, Buffalo, NY) at -80°C and mounted on clean glass slides using a drop of 2.0 M sucrose in a platinum loop. Sections were reequilibrated with 0.1 M PBS followed by 0.1 M PBS with 0.05 M glycine for 5 min. To minimize nonspecific staining the sections were pretreated with 1% gelatin in PBS for 10 min followed by 2% normal goat sera (Organon Teknika-Cappel, Malvern, PA) in PBS for 10 min. Sections were incubated in specific antisera or normal rabbit or normal mouse sera for 30 min at room temperature. Dilutions of primary antibodies were 80B (100 $\times$ ), 2C9 (100 $\times$ ), 4F3 (50 $\times$ ), CT (100 $\times$ ), 4E7 (100 $\times$ ), and antireceptor antibodies (100 $\times$ ). After extensive washing in PBS, the sections were treated with either goat anti-rabbit rhodamine or goat anti-mouse fluorescein conjugate (Organon Teknika-Cappel) for 20 min. The sections were again washed extensively in PBS, covered with 90% glycerol in PBS, and placed under a coverslip. Sections were examined using a Leitz 63 $\times$  objective on a Zeiss universal microscope, and were photographed with Kodak T-Max 400 ASA film and a Nikon UFX camera system.

### Electron Microscopic Localization of Acetylcholinesterase

Thin cryosections (0.1 nm) or thick cryosections (0.5  $\mu$ m) were cut at -110°C and mounted on Formvar film carbon stabilized gold grids. The sections were treated much like the thick sections except after incubation in primary antisera the sections were immunolabeled with goat anti-rabbit or goat anti-mouse IgG conjugated to either 5, 10, 15, or 30 nm gold (Janssen Life Sciences Products, Piscataway, NJ) for 20 min. After extensive washing in PBS the sections were fixed in 1% glutaraldehyde/1% osmium

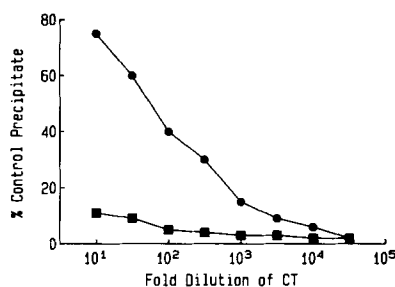
tetroxide in PBS for 3 min. Sections were washed in distilled water and counterstained in 2% aqueous uranyl acetate for 30 min. The sections were subsequently dehydrated in ethanol and embedded in a thin film of LR white acrylic resin (Ted Pella, Inc., Irvine, CA) and polymerized as previously described (20). Thin cryosections were viewed at 100 KeV with a JEOL 100CX electron microscope, and thick cryosections were viewed at 1 MeV with a JEM 1,000 high voltage electron microscope.

## Results

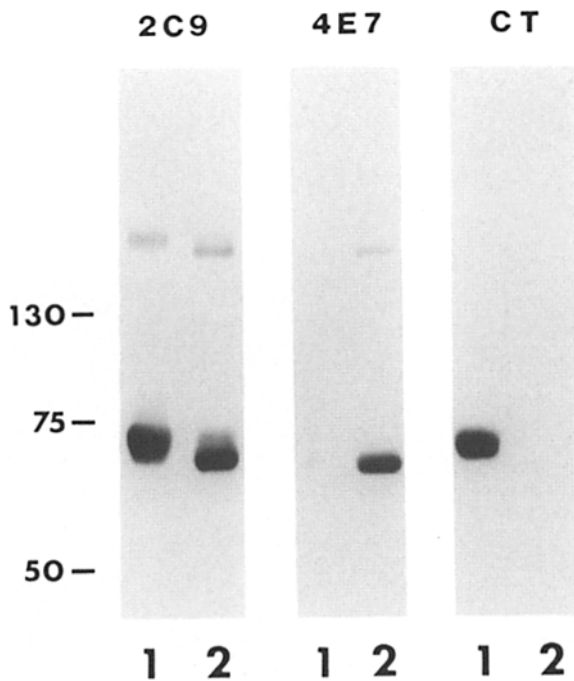
### Biochemical Characterization of Antibodies Used for Immunolocalization

Polyclonal antibodies raised against a synthetic peptide corresponding to the COOH-terminal sequence of the asymmetric form of acetylcholinesterase (CT) were tested for their ability to precipitate  $^3$ H-DFP-labeled acetylcholinesterase (Fig. 1). Antibodies raised against the COOH-terminal peptide precipitated the catalytic subunits of the asymmetric form of acetylcholinesterase (11 S enzyme), but did not precipitate the hydrophobic form of acetylcholinesterase (5.6 S enzyme).

The reactivity of antibodies 2C9, 4E7, and CT was determined by Western blots of the 11 S and 5.6 S forms of acetylcholinesterase (Fig. 2). The reduced monomers and irreducible dimers of the two forms can be distinguished by the slightly larger apparent molecular weight of the catalytic subunits of the 11 S form (7). The monoclonal antibody 2C9 reacted equally well with both forms. However, the monoclonal antibody 4E7 selectively recognized only the 5.6 S form of the enzyme. As was the case in the immunoprecipitation assay (Fig. 1), the polyclonal antibody CT selectively recognized only the 11 S form of acetylcholinesterase (Fig. 2). Although the CT antibodies didn't react as well with the irreducible dimer as with the monomer, reactivity with the irreducible dimer was observed when the concentration of 11 S or the CT antibodies was increased. The reactivity of the CT antibodies was completely blocked by earlier incubation of the antibodies with the peptide ( $2.8 \times 10^{-7}$  M) to which the rabbit was immunized, and was not affected by earlier incubation with the same concentration of a synthetic peptide



**Figure 1.** Immunoprecipitation of 11 S and 5.6 S acetylcholinesterase. Both forms of acetylcholinesterase were reacted with  $^3$ H-DFP, denatured, reduced, and alkylated as described in Materials and Methods. The ability of the COOH-terminal directed antibodies (CT) to precipitate 11 S (●) and 5.6 S (■) forms of acetylcholinesterase is plotted as a percentage of  $^3$ H-DFP-labeled acetylcholinesterase precipitated by acetone versus the fold dilution of CT.



**Figure 2.** Western blots of 11 S and 5.6 S acetylcholinesterase. Equal amounts (10  $\mu\text{g}/\text{lane}$ ) of the 11 S enzyme derived from the asymmetric form (lane 1) and the 5.6 S hydrophobic form (lane 2) were denatured in Lammeli buffer, including DTT (50 mM), by boiling for 2 min. Proteins were separated by SDS-PAGE on an 8% polyacrylamide gel, transferred to nitrocellulose, and reacted with the monoclonal (1:1,000 dilution) or polyclonal (1:100 dilution) antibodies as described in Materials and Methods. The positions of protein standards are indicated.

corresponding to the active-site region of the enzyme (data not shown).

Since the 5.6 S hydrophobic form of acetylcholinesterase is thought to contain only two amino acids not found in the 11 S asymmetric form (4, 16, 19), the basis of the selectivity of 4E7 was investigated further. Glycopeptidase F selectively cleaves N-linked carbohydrates between asparagine and the proximal N-acetylglucosamine. Digestion of  $^3\text{H}$ -DFP-labeled 5.6 S acetylcholinesterase with glycopeptidase F resulted in an almost complete loss of the ability of 4E7 to precipitate the enzyme (Fig. 3 A). Complete removal of N-linked carbohydrate was demonstrated by a decrease in the apparent molecular weight of 5.6 S acetylcholinesterase in SDS-PAGE after treatment with glycopeptidase F (Fig. 3 A, *inset*). The complexity of the carbohydrate was assessed further by digestion with endoglycosidase F and H. Digestion of  $^3\text{H}$ -DFP-labeled 5.6 S acetylcholinesterase with endoglycosidase F resulted in an almost complete loss of the ability of 4E7 to precipitate the enzyme (Fig. 3 B), while digestion with endoglycosidase H was without effect (Fig. 3 B, *inset*). These results suggest that the antigenic determinant of 4E7 is a complex-type N-linked carbohydrate. The ability of 2C9 to precipitate  $^3\text{H}$ -DFP-labeled acetylcholinesterase was not affected by similar treatments with these glycohydrolases (data not shown).

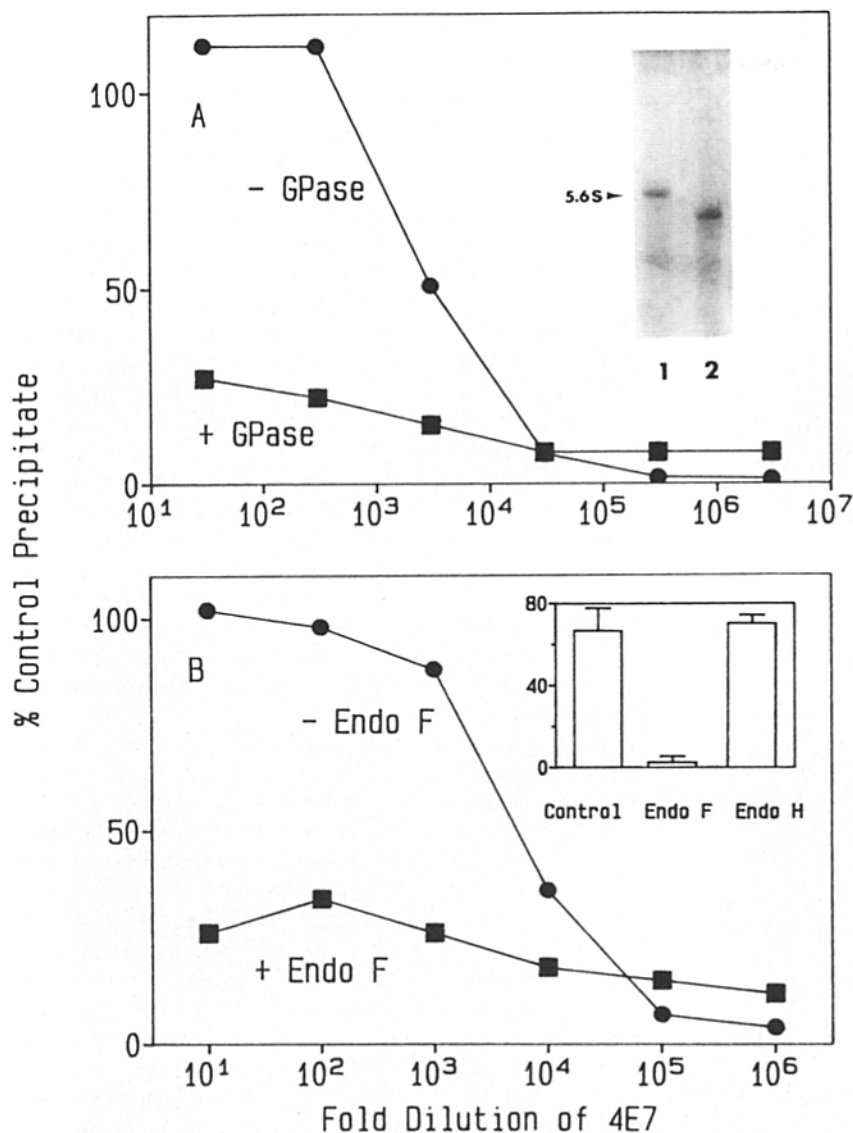
### Immunocytochemical Localization of Acetylcholinesterase

Several antibodies against acetylcholinesterase have been generated, characterized biochemically, and examined for their usefulness in immunocytochemical assays (Table I). Those with particularly interesting biochemical properties and relatively strong immunoreactivity, were used to investigate the distribution of the different forms of acetylcholinesterase in the electric organ of *Torpedo californica*. Antibodies chosen for further study included: (a) the COOH-terminal site-directed rabbit polyclonal antibodies (CT) that selectively recognize the catalytic subunits of the asymmetric form; (b) the mouse monoclonal antibody (4F3) that selectively recognizes the collagen-like structural subunit of the asymmetric form; (c) the mouse monoclonal antibody (4E7) that selectively recognizes the catalytic subunits of the hydrophobic form; (d) the rabbit polyclonal antibodies (80A and 80B) that recognize the catalytic subunits of both forms; and (e) the mouse monoclonal antibody (2C9) that recognizes a sequence in the catalytic subunits common to both forms. Polyclonal antibodies raised against nicotinic acetylcholine receptors purified from *Torpedo* (10) were used to compare the localization of acetylcholinesterase with the nicotinic receptor.

The ultrastructure of the *Torpedo* electric organ is well-characterized (14, 17). The ventral surface of the electrocytes is densely innervated by elongated presynaptic nerve terminals that lie in shallow trough-like depressions in the postsynaptic membrane. In addition, the postsynaptic membrane contains many finger-like tubular invaginations that occur at irregular intervals. Immunofluorescent localization of acetylcholinesterase by light microscopy using either the panselective polyclonal antibody 80B or the panselective monoclonal antibody 2C9 revealed that acetylcholinesterase-like immunoreactivity was confined almost exclusively to the ventral innervated surface of the electrocytes (Fig. 4, A and B). The finger-like invaginations of the postsynaptic membrane appeared to contain intense staining. However, reactivity was also evident across the entire ventral surface of the electrocytes.

The distribution of the form-selective antibodies was strikingly different from that of the panselective antibodies. Antibodies selective for the collagen-like subunit (4F3) and catalytic subunit (CT) of the asymmetric form revealed a somewhat punctate distribution, and this form of the enzyme appeared to be concentrated in the invaginations of the postsynaptic membrane (Fig. 4, C and E, respectively). In contrast, the monoclonal antibody selective for the hydrophobic form (4E7), revealed a distinctly different distribution (Fig. 4 D). At the innervated surface of the electrocyte the immunoreactivity of 4E7 appeared discontinuous and was not observed in the invaginations of the postsynaptic membrane. The antibody 4E7 also appeared to react moderately with an unknown antigen on the noninnervated surface of the electrocyte.

Higher magnification of tissue sections allowed the distribution of acetylcholinesterase immunoreactivity to be determined within the substructure of the synaptic specializations (Fig. 5). The panselective antibody 2C9 stained the nerve terminals, as well as the invaginations of the postsynaptic membrane (Fig. 5 A). The antibody selective for the hydro-



**Figure 3.** Effects of deglycosylation on the ability of 4E7 to precipitate 5.6 S acetylcholinesterase. <sup>3</sup>H-DFP-labeled 5.6 S acetylcholinesterase was treated with either glycopeptidase F (A) or endoglycosidase F (B) as described in Materials and Methods. The ability of 4E7 to precipitate control (●) or treated (■) enzyme is plotted as a percentage of <sup>3</sup>H-DFP-labeled acetylcholinesterase precipitated by acetone versus the fold dilution of 4E7. (Inset in A) Control (lane 1) and glycopeptidase F-treated (lane 2) 5.6 S enzyme were separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by silver staining. (Inset in B) The ability of 4E7 to precipitate control, endoglycosidase F-, or endoglycosidase H-treated 5.6 S enzyme is shown.

**Table I. Staining Intensity of Antibodies Used in Immunocytochemical Localization of Acetylcholinesterase in Torpedo Electric Organ**

Antigen	Name	Species	Poly/Mono	Subtype	Light	EMperox*	EMgold‡	Form§	Reference
11 S	80A	Rabbit	Polyclonal	—	+++	+++	++	Both	6,8
11 S	80B	Rabbit	Polyclonal	—	++	++	++	Both	6,8
17 S, 5.6 S	4E7	Mouse	Monoclonal	IgG2b	+++	++	+	5.6 S	3
17 S, 5.6 S	4F3	Mouse	Monoclonal	IgG1	++	+	+	17 S	3
11 S	2C9	Mouse	Monoclonal	IgG1	+++	ND	+++	Both	—
11 S	2C6	Mouse	Monoclonal	IgG1	+++	++	+	ND	—
Peptide	CT	Rabbit	Polyclonal	—	+++	++	++	17 S	—
Peptide¶	AS	Rabbit	Polyclonal	—	—	ND	ND	Both	—

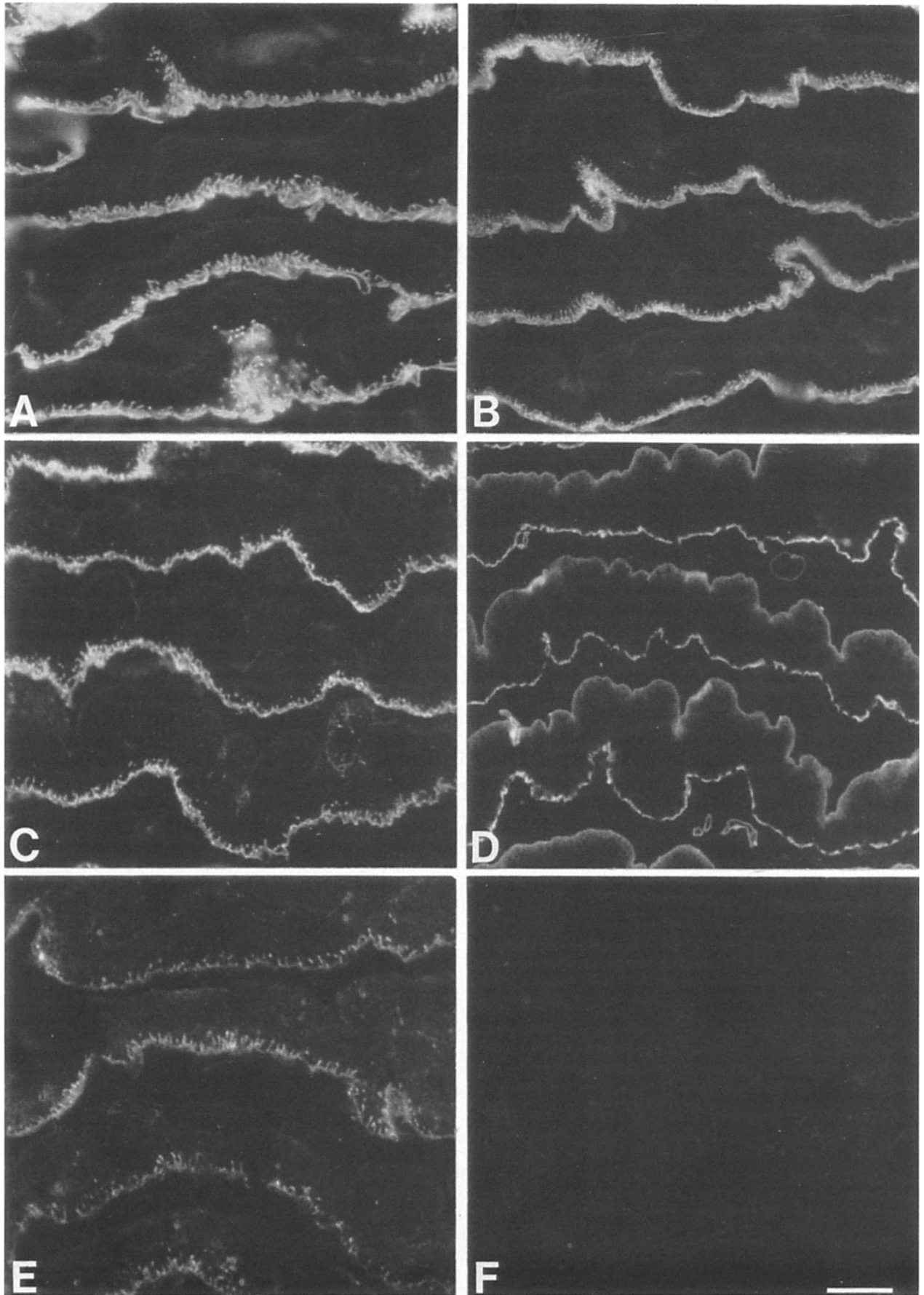
\* Results obtained with the electron microscope and peroxidase-labeled secondary antibodies.

‡ Results obtained with the electron microscope and gold-labeled secondary antibodies.

§ Molecular form of acetylcholinesterase (5.6 S, 17 S, or both) recognized by the antibody is indicated.

|| Synthetic peptide Lys<sup>260</sup>-Leu<sup>575</sup>, corresponding to the COOH-terminal amino acids of the catalytic subunit of the asymmetric form of acetylcholinesterase from *Torpedo californica*, was used as the antigen.

¶ The synthetic peptide Lys<sup>192</sup>-Arg<sup>216</sup>, corresponding to amino acids common to both forms of acetylcholinesterase from *Torpedo californica*, was used as the antigen.



phobic form (4E7) selectively stained nerve terminals (Fig. 5 B), while the antibodies selective for the asymmetric form (4F3 and CT) selectively stained the synaptic cleft and the invaginations of the postsynaptic membranes (Fig. 5, C and D, respectively). Single sections double-labeled with the monoclonal antibody 4E7 (Fig. 5 E) and polyclonal antibodies selective for the nicotinic acetylcholine receptor (Fig. 5 F) allowed the structures containing the hydrophobic form of acetylcholinesterase to be compared with those containing the receptor. Arrows indicate the positions of nerve terminals that react strongly with 4E7 (Fig. 5 E). Arrows at identical positions indicate that many of the nerve terminals can be seen to lie in shallow trough-like depressions in the postsynaptic membrane (Fig. 5 F).

Further resolution was obtained by electron microscopy. Conventionally stained and embedded sections of electric organ revealed elongated nerve terminals and the finger-like invaginations of the postsynaptic membrane (Fig. 6). In addition, thin Schwann cell processes could be observed covering the nonsynaptic surface of some, but not all, of the nerve terminals. Electron microscopy of immunogold-labeled ultrathin cryosections also revealed elongated nerve terminals cut in longitudinal (Fig. 7 A) or cross section (Fig. 7 B). The panselective monoclonal antibody (2C9) reacted with acetylcholinesterase-like antigens in the invaginations of the postsynaptic membrane, in the synaptic cleft, and on the nonsynaptic surface of the nerve terminals (Fig. 7 A, 5-nm gold, *arrowheads*). In the same section, however, the asymmetric form-selective polyclonal antibodies (CT) selectively stained the synaptic cleft and the invaginations of the postsynaptic membrane (Fig. 7 A, 10-nm gold, *arrows*). Immunoreactivity attributable to the asymmetric form was not detected on the nonsynaptic surface of the nerve terminals. In contrast, the hydrophobic form-selective monoclonal antibody (4E7) selectively stained the nerve terminals, and the distribution of gold particles was restricted to the nonsynaptic surface of the nerve terminals (Fig. 7 B, 5-nm gold). Immunoreactivity attributable to the hydrophobic form was not detected in the synaptic cleft or the invaginations of the postsynaptic membrane.

## Discussion

Since the various structural forms of acetylcholinesterase are kinetically and pharmacologically indistinguishable, their physiological significance may reside in their differential distribution. Studies of the distribution of the different structural forms of acetylcholinesterase have been severely limited by the necessity to isolate the different forms and characterize them biochemically. Nonetheless, there is evidence that suggests the two forms of acetylcholinesterase in *Torpedo* are differentially distributed. For instance, while the asymmetric form is presumably associated with the basal lamina based on its dissociation with collagenase, the hydrophobic form can be concentrated with highly purified synaptosomes prepared from *Torpedo* electric organ (9, 13). To al-

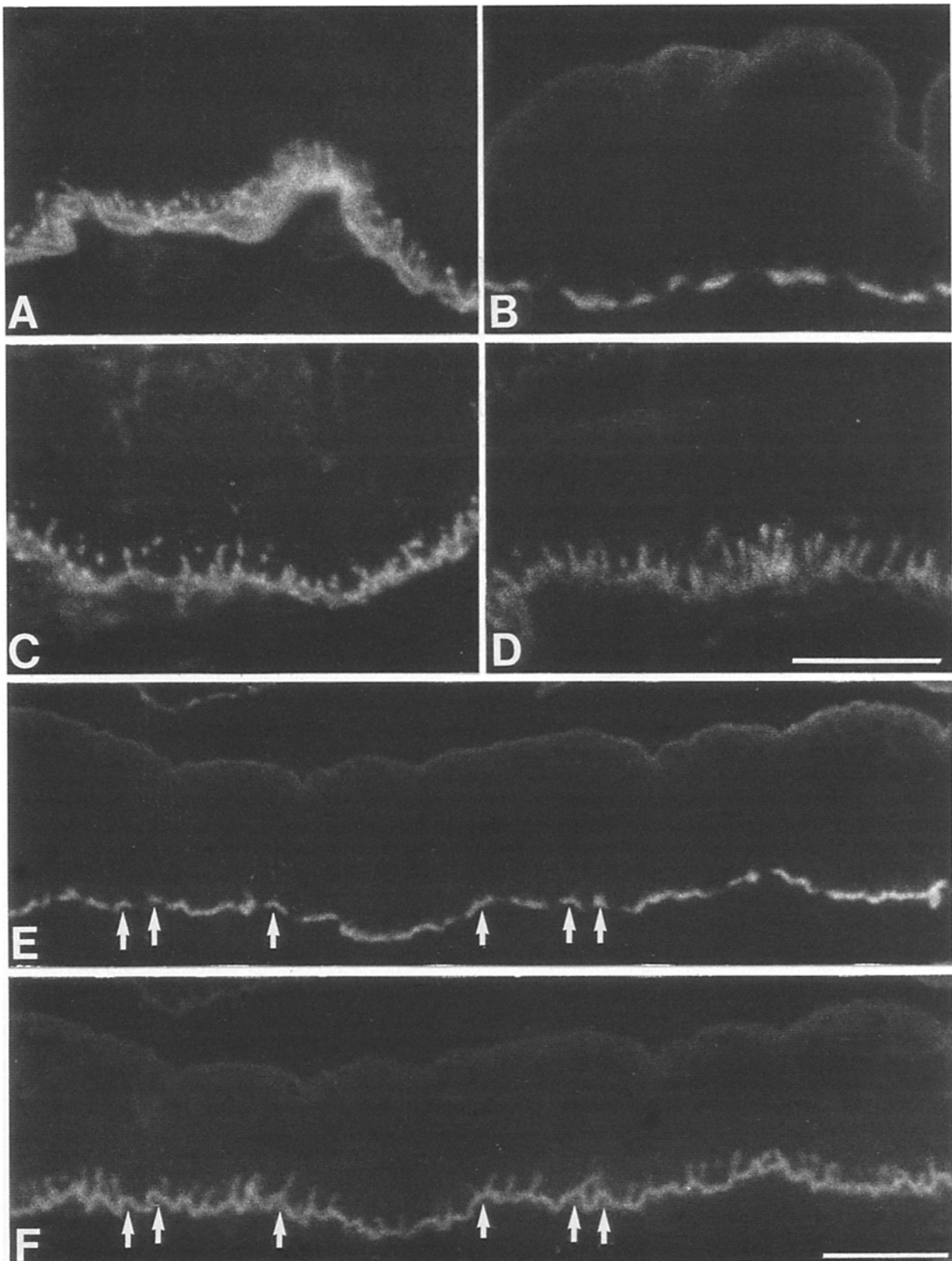
low more detailed investigation into the distribution of the different structural forms of acetylcholinesterase, we have undertaken the development and characterization of form-selective antibodies for use in high resolution in situ immunocytochemistry.

Several groups have raised polyclonal and monoclonal antibodies to acetylcholinesterase, and a comprehensive review of the existing antibodies has been compiled (2). Since the sequence of the first 535 amino acids is the same for both forms of the enzyme, it is not surprising that few form-selective monoclonal antibodies have been generated using the entire protein as an antigen. However, monoclonal antibodies that appear to react selectively with the hydrophobic form of acetylcholinesterase have been reported (1, 3, 5). In one case, purified synaptosomes from the electric organ of *Torpedo californica* were used as the antigen (5). A monoclonal antibody (*Tor* 23) was isolated, and shown to selectively precipitate the hydrophobic form of acetylcholinesterase and to selectively stain nerve terminals in *Torpedo* back muscle. However, the antigenic determinant of this antibody has yet to be identified. In another case, an IgM monoclonal antibody (Elec-39) selective for the hydrophobic form of *Torpedo* acetylcholinesterase was obtained by using the asymmetric form of the enzyme from *Electrophorus* electric organ as the antigen (1). This antibody was shown to react with several other proteins in addition to acetylcholinesterase, and the reactive epitope for this antibody was shown to be a carbohydrate sensitive to treatment by endoglycosidase F. This antibody, however, was not used for immunocytochemical localization of acetylcholinesterase.

Since the hydrophobic form contains only two amino acids not found in the asymmetric form, it seems likely that all hydrophobic form-selective antibodies recognize posttranslational modifications found only on this form. Therefore, we investigated the effects of several glycohydrolases on the ability of the hydrophobic-form selective antibody 4E7 to precipitate acetylcholinesterase. Deglycosylation with either glycopeptidase F or endoglycosidase F resulted in a complete loss of the ability of 4E7 to precipitate 5.6 S acetylcholinesterase, while treatment with endoglycosidase H was without effect. Therefore, the antigenic determinant for 4E7 appears to be a complex-type N-linked carbohydrate. Since 4E7 was the only antibody to react with the noninnervated surface of the electrocytes, the antigenic determinant for 4E7 appears to be shared by an unknown protein on the noninnervated surface of the electrocytes.

The position of the amino acid containing the carbohydrate identified by 4E7 has yet to be determined. However, both forms of acetylcholinesterase contain four identical potential sites for N-linked carbohydrate (asparagine residues 59, 416, 457, and 533). Peptides containing each of the four potential glycosylation sites have been isolated from the asymmetric form, and each of the four sites has been shown to be glycosylated (unpublished observations). Thus the selectivity of 4E7 likely results from a distinct complex-type carbohydrate found only on the hydrophobic form, rather than

**Figure 4.** Immunofluorescent localization of acetylcholinesterase in *Torpedo* electric organ. Tissue sections were prepared as described in Materials and Methods and were incubated with either (A) 80B, (B) 2C9, (C) 4F3, (D) 4E7, (E) CT, or (F) CT antibodies blocked by earlier incubation with the COOH-terminal peptide ( $2.8 \times 10^{-7}$  M). These primary antibodies were visualized with rhodamine or fluorescein-labeled secondary antibodies. Bar, 10  $\mu$ m.



**Figure 5.** High magnification of immunofluorescent staining. The primary antibodies were (A) 2C9, (B) 4E7, (C) 4F3, and (D) CT. Tissue sections were also double labeled with 4E7 (E) and antibodies selective for the nicotinic acetylcholine receptor (F). The same section is shown in both E and F, where the primary antibodies were visualized with fluorescein and Texas red-labeled secondary antibodies, respectively. The arrows (identical positions in both E and F) indicate nerve terminals stained by 4E7 (E) that lie in shallow troughs in the post-synaptic membrane (F). Bars, 10  $\mu\text{m}$ .





**Figure 6.** Electron micrograph of conventionally fixed and embedded electric organ. An elongated nerve terminal is shown. Some of the nonsynaptic surface of the nerve terminal is covered by a thin Schwann cell process (*arrowheads*), while most of the nonsynaptic surface of the nerve terminal is without the Schwann cell covering (*arrows*). Finger-like invaginations of the postsynaptic membrane are indicated with asterisks. Bar, 1  $\mu$ m.

from a unique site of glycosylation. Although the physiological significance of this difference in posttranslational glycosylation is not known, it is interesting to speculate that perhaps it allows for differential association with extracellular matrix components or cell-cell associations.

Antibodies raised against a synthetic peptide corresponding to the COOH-terminal 16 amino acids of the asymmetric form of acetylcholinesterase were shown to be selective for the asymmetric form by both Western blot and immunoprecipitation assays. These results provide further evidence for the divergent COOH termini of the two forms of acetylcholinesterase. In addition, they represent the first report of an antibody selective for the catalytic subunits of the asymmetric form. Since it is likely that the genomic organization of acetylcholinesterase is similar in different species, this method of generating asymmetric form-selective antibodies should prove successful in other species.

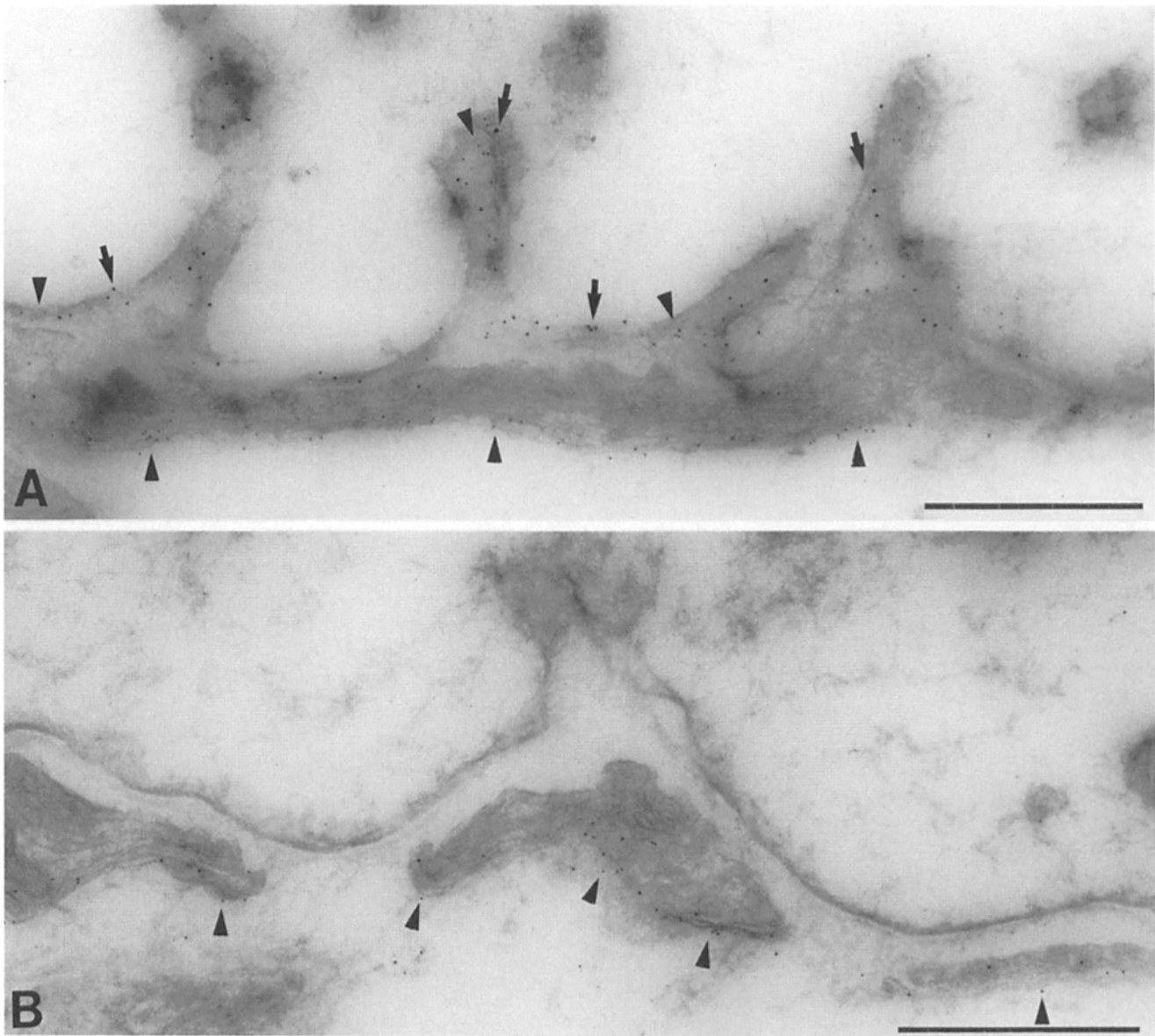
The generation of antibodies with defined antigenic determinants that are selective for the two forms of acetylcholinesterase has allowed us to investigate their differential distribution in the electric organ of *Torpedo californica*. At the light microscopic level panselective antibodies (80B and 2C9) revealed a relatively uniform labeling of the innervated surface of the electrocytes. The higher resolution provided by electron microscopy revealed that acetylcholinesterase-like immunoreactivity was located in the invaginations of the postsynaptic membrane, in the synaptic cleft, and on the nonsynaptic surface of the nerve terminals. In contrast, the distribution of the asymmetric form-selective antibodies was more punctate, and at the light microscopic level it appeared that the asymmetric form was concentrated in the synaptic cleft and in the invaginations of the postsynaptic membrane. Electron microscopy confirmed that the immunoreactivity attributable to the asymmetric form was restricted to the synaptic cleft and to the invaginations of the postsynaptic membrane. Similar results were obtained for antibodies directed against the collagen-like tail subunit (4F3) or the unique amino acid sequence found only in the catalytic subunits of the asymmetric form (CT).

Since acetylcholinesterase-like immunoreactivity was observed on the nonsynaptic surface of the nerve terminals, and

since the asymmetric form-selective antibodies did not react with the nonsynaptic surface of the nerve terminals, the acetylcholinesterase-like immunoreactivity found on the nonsynaptic surface of the nerve terminals presumably represented the hydrophobic form of the enzyme. This conclusion was confirmed by localization of the hydrophobic form-selective antibody 4E7. At the light microscopic level 4E7 appeared to react selectively with the nerve terminals. The greater resolution provided by electron microscopy revealed that 4E7 reacted exclusively with the nonsynaptic surface of the nerve terminals. Thus, by virtue of its inability to react with asymmetric form-selective antibodies and its ability to react with the panselective and hydrophobic form-selective antibodies, the acetylcholinesterase-like immunoreactivity on the nonsynaptic surface of the nerve terminals appears to represent the hydrophobic form of the enzyme.

Since all of the nerve terminals examined contained acetylcholinesterase-like immunoreactivity on their nonsynaptic surfaces (identified with either nonselective or hydrophobic form-selective antibodies), the enzyme is probably located on the neuronal membrane. However, some of the nerve terminals are covered by thin Schwann cell processes (14, 17). These thin Schwann cell processes were more difficult to resolve in the ultrathin cryosections used for immunocytochemistry than in the conventionally stained and embedded sections. Therefore, the possibility remains that some of the acetylcholinesterase-like immunoreactivity is associated with these thin Schwann cell processes rather than with the nerve terminals themselves.

Although both forms of acetylcholinesterase hydrolyze acetylcholine, their differential cellular localization suggests that they may have slightly different functions. Since the asymmetric form appeared concentrated in the extracellular space between the nerve terminal and the electrocyte, it may be the form primarily responsible for the rapid hydrolysis of acetylcholine in the synaptic cleft. In contrast, the hydrophobic form was located on the nonsynaptic surfaces of the nerve terminals, and may therefore be responsible for hydrolysis of acetylcholine that has diffused from the synaptic cleft. Selective manipulation of expression of the two forms of acetyl-



**Figure 7.** Electron micrographs of cryosections of electric organ. (A) Tissue section showing an elongated nerve terminal cut in longitudinal section, double-labeled with 2C9 and CT, and visualized with 5- and 10-nm colloidal gold-conjugated secondary antibodies, respectively. Arrows indicate 10-nm gold found only in the invaginations of the postsynaptic membrane and in the synaptic cleft, while arrowheads indicate 5-nm gold found in the invaginations of the postsynaptic membrane, in the synaptic cleft, and on the nonsynaptic surface of the nerve terminals. (B) Tissue section showing elongated nerve terminals cut in cross section, labeled with 4E7, and visualized with 5-nm gold. Arrowheads indicate the 5-nm gold found selectively on the nonsynaptic surface of the nerve terminals. Bars, 1  $\mu\text{m}$ .

cholinesterase may be required to fully assess the functional role of each form.

The present study extends our understanding of the cellular location of the two forms of acetylcholinesterase, and provides a more direct localization of these final gene products. However, the origin of synthesis of the two forms remains unknown. Synthesis of acetylcholinesterase could occur in the electrocytes or Schwann cells of the electric organ, or in the electromotor neurons of the electric lobe. *In vitro* translation of electric organ mRNA reveals two peptide precursors of the catalytic subunits (18), and RNase protection analysis of electric organ mRNA reveals the presence of two major mRNA species that diverge in the region corre-

sponding to the alternate third exon that gives rise to the two enzyme forms (16). These results support the hypothesis that both forms of the enzyme are synthesized by cells located in the electric organ. However, the cDNA abundance for the hydrophobic form is small when compared with the asymmetric form, supporting the hypothesis that the hydrophobic form is primarily synthesized in the electromotor neurons of the electric lobe (15, 19). A more complete understanding of the alternative mRNA processing resulting in the different mRNA that code for the two forms of acetylcholinesterase will ultimately allow the generation of selective probes for *in situ* hybridization studies. Such studies may shed light on the cellular origin of the two forms of acetylcholinesterase.

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