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Neurons segregate clusters of membrane-bound acetylcholinesterase along their neurites

(cultured sympathetic neurons/monoclonal antibodies/hydrophobic tetramer)

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ABSTRACT **Immunocytochemical studies with a monoclonal antibody show that acetylcholinesterase (AcChoEase; EC 3.1.1.7) is distributed in clusters along the fibers of cultured sympathetic neurons but is essentially absent from cell bodies. Although tissue-cultured sympathetic neurons synthesize several oligomeric forms of AcChoEase, only the hydrophobic globular (G4) form of AcChoEase is present within these clusters. This G4 form is asymmetrically distributed within neurons and is transported preferentially into nerve fibers following its synthesis in the cell bodies. Thus G4 is found in clusters on neurons and is readily distinguishable from the hydrophilic forms on the surfaces of myotubes. The association of a specialized form of AcChoEase in densities on neurons in culture indicates that neurons and myotubes have distinct mechanisms for localizing AcChoEase molecules on their surfaces and suggests that these two types of electrically excitable cells have different requirements for organizing synaptic components on their plasma membranes.**

Despite the fluid nature of the lipid bilayer many membrane-bound macromolecules are known to be restricted within select domains on the cell surface. In the nervous system, this molecular compartmentalization is of particular importance for formation of the numerous, discreet, synaptic contacts made between neurons and their target cells. Contained within these synapses are densities of integral and peripheral membrane proteins whose function is to transmit electrical and chemical signals between the two cells as well as to maintain the appropriate sites of contact. Therefore, one important question concerns the strategies used by electrically excitable cells in the organization of identified synaptic macromolecules on their surfaces. The synthesis and localization of these synaptic components can be studied using tissue-cultured cells. For example, skeletal myotubes in culture produce large clusters, or "hotspots," of acetylcholine receptors (1) and acetylcholinesterase (AcChoEase; EC 3.1.1.7) (2, 3) on their surfaces. Myotubes synthesize, assemble, and externalize three oligomeric forms of AcChoEase, but only one of these, the asymmetric AcChoEase form (A12), predominates in hotspots (2). A fourth form, a monomer, is exclusively intracellular and appears to be the precursor of the multimeric forms. The A12 form is comprised of three hydrophilic tetramers covalently attached to a collagen-like tail and is believed to be localized on the cell surface by association of its collagenous tail with components of the basal lamina surrounding the muscle fiber (4, 5).

Neurons in culture also synthesize, assemble, and release a complement of AcChoEase forms that, by velocity sedimentation, appear to be identical to those of myotubes (4, 5). Using a monoclonal antibody to chicken AcChoEase and fluorescence immunocytochemistry, we have found that the distribution of AcChoEase on the surfaces of sympathetic neurons in culture is distinctly nonuniform. These clusters of AcChoEase are distributed along nerve fibers and are essentially absent from cell bodies. As a first step to understanding the genesis of these accumulations of AcChoEase on neurons we have characterized the forms of AcChoEase synthesized and their distribution in and on the sympathetic neurons. One of these forms, a globular hydrophobic tetramer (G4), predominates in nerve fibers, where it is found in the plasma membrane. The remaining forms are either secreted or retained in the cell. This G4 form is easily distinguishable from the much larger A12 form that is localized on the surfaces of myotubes and can also be distinguished from a similar, but hydrophilic, tetramer (G4) present in myotubes by its aggregation in the absence of detergent. Furthermore, in the absence of detergents, the neuronal form of G4 aggregates preferentially with itself and does not interact with the more hydrophilic muscle form of G4. Interestingly, neurons have no basal laminae of their own, and those in the peripheral nervous system develop basal laminae by virtue of their association with Schwann cells (6). Thus neurons appear to have a strategy different from that of muscle cells for localizing putative synaptic forms of AcChoEase on their surfaces.

MATERIALS AND METHODS

Cell Culture and Immunocytochemistry. Cultures of dissociated neurons were made from the paravertebral sympathetic chains of 10- to 15-day chick embryos as described previously (7). Ganglionic explants were prepared by cutting sympathetic chains, treating the ganglia for 10 min at 37°C with 0.025% trypsin to disrupt the connective tissue capsule and then culturing them as for dissociated cells.

For immunocytochemical studies cultured cells were seeded on glass coverslips coated with poly(L-Lys) (20 μg/ml). After 1–2 days they were washed with 3 vol of Hanks' balanced salt solution (HBSS), incubated with an anti-chicken AcChoEase monoclonal antibody (mAb1A2) (8) for 30 min at 37°C, washed, incubated for 30 min at 37°C with fluorescein-conjugated rabbit anti-mouse antisera, and washed again. The anti-chicken AcChoEase antibody mAb-1A2 recognizes the catalytic subunits of all AcChoEase oligomeric forms found in nerves and muscle cells (8). Lectin receptors were localized on the cells by first incubating them with wheat germ agglutinin (WGA; 20 μg/ml) followed by fluorescein-conjugated anti-WGA IgG. Cells were viewed live or post-fixed with 3% paraformaldehyde and viewed with

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Abbreviations: AcChoEase, acetylcholinesterase; iPr_2P, diisopropylfluorophosphate; BW284c57, 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; WGA, wheat germ agglutinin.

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a Zeiss microscope equipped with phase and epifluorescence optics.

Characterization of AcChoEase Forms. Cultures were extracted with 20 mM borate buffer, pH 9/1 M NaCl/0.5% Triton X-100/0.5% bovine serum albumin containing protease inhibitors (extraction buffer) and analyzed by velocity sedimentation on 5–20% sucrose gradients containing 20 mM borate buffer, pH 9/1 M NaCl/1 mM EDTA with or without 0.5% Triton X-100 in a SW 50.1 rotor (8). Enzyme activity was measured by the method of Johnson and Russell (9) as previously described (10). Catalytically active cell surface AcChoEase was prepared by irreversibly inactivating the intracellular enzyme with diisopropylfluorophosphate (iPr_2P) while protecting the active sites of the extracellular enzyme with 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51), a membrane-impermeable, reversible inhibitor. Details of this procedure have been described extensively (11–13). Radiolabeled chicken brain AcChoEase was prepared by incubating the purified enzyme with sufficient ^3H-labeled iPr_2P to completely inactivate the enzyme (8).

Cosedimentation of Neuronal and Muscle AcChoEase. Tissue extracts were prepared from adult chicken brain or 16-day chicken embryo leg muscle by homogenizing the tissue in 10 vol of extraction buffer and centrifuging the homogenate for 30 min at 27,000 × *g*. Half of each tissue extract was treated with 10^{-4} M iPr_2P to completely inactivate esterase activity, and then all the treated extracts were dialyzed for 24 hr against four 1-liter vol of extraction buffer to remove unreacted iPr_2P prior to mixing. Additional details are given in Fig. 4 legend.

Reagents. Fluorescein-conjugated rabbit anti-mouse (Bethesda Research Laboratories); WGA and fluorescein-conjugated anti-WGA antisera (Sigma); [*acetyl*-1-^{14}C]acetylcholine iodide (New England Nuclear); and [^{3}H]iPr_2P (Amersham) were used as indicated.

RESULTS AND DISCUSSION

AcChoEase Is Localized in Clusters Restricted to Nerve Fibers. AcChoEase is found on the surfaces of tissue-cultured neurons where it is localized in clusters that are concentrated on nerve fibers (Fig. 1 *A* and *B*). These clusters are rarely seen on cell bodies and, when present, can often be traced to a passing fiber (Fig. 1 *C* and *D*). In addition, we find them associated with the lamellipodia and filopodia that attach the neuron to its substratum, as well as on growth cones. In cultures of ganglionic explants clusters are largely absent from cell bodies in the ganglia but are prominent in the halo of axons surrounding them (Fig. 1 *E* and *F*). This distribution of AcChoEase molecules stands in contrast to that of WGA receptors, visualized using fluorescein-conjugated antilectin IgG, which are distributed uniformly on cell bodies and nerve fibers (Fig. 1 *G* and *H*). Thus the distribution of AcChoEase in clusters does not appear to be an artifact resulting from varicosities or membranous infoldings on nerve fibers, nor does it appear to reflect restricted access of antibodies to neuronal cell bodies or their processes.

AcChoEase has been localized in the central and peripheral nervous systems using a variety of histochemical procedures based upon enzyme activity. These methods permeabilize the plasma membrane and, at the light microscopic level, reveal a uniform distribution of AcChoEase within neurons and skeletal muscle (4, 5, 14). Because all of our studies were done on nonpermeabilized living cells, the antibody did not gain access to intracellular AcChoEase and thus revealed only the distribution of surface enzyme molecules. However, multivalent cell ligands, such as lectins and antibodies, may alter the normal distribution of surface enzyme binding sites on the plasma membrane as well as stimulate their internalization into vesicles (15). Nevertheless, several lines of evidence suggest that AcChoEase is normally distributed in clusters on the cell surface and that these clusters are not the result of antibody-induced aggregation or internalization. (*i*) mAb1A2 appears to recognize a single antigenic determinant on each AcChoEase subunit and does not by itself cause aggregation of the enzyme in solution (R.L.R., unpublished observations). (*ii*) Nor does the multivalent second antibody used for indirect immunofluorescence induce the clusters because the clusters are still evident when monovalent Fab2 fragments are used. (*iii*) Incubation of these cells with antibodies at 4°C, which blocks redistribution of lectin receptors on cultured sympathetic neurons (15) and capping in lymphocytes (16), does not affect the distribution of AcChoEase clusters on nerve fibers. Unfortunately, fixation of neurons with glutaraldehyde or paraformaldehyde, which also blocks redistribution of membrane proteins (15, 16), inhibits binding of the monoclonal antibody to the cells. (*iv*) The AcChoEase clusters do not result from internalization of enzyme molecules. When lectin receptors are so internalized they appear at many levels of focus through the neurons, whereas the AcChoEase clusters are always in a single focal plane. Moreover, internalized lectin receptors are unavailable for antibody binding (15), which is obviously not so for the AcChoEase clusters. When these lectin receptors are labeled with WGA followed by rhodamine-conjugated rabbit anti-WGA (Fig. 1) they do not form clusters as do the labeled AcChoEase molecules.

Intracellular, Cell Surface, and Secreted Forms of AcChoEase in Nerves and Muscles. The major molecular forms of AcChoEase in nerves and muscle are characterized by their sedimentation coefficients. These oligomeric forms are structurally identical in neurons and muscle and include globular 5S monomers (G1), 7S dimers (G2), 11S tetramers (G4), and a 19S asymmetric form consisting of three tetramers attached to a three-stranded collagen-tail (A12) (Fig. 2*A*, for review see refs. 4 and 5). To determine the distribution of these forms in nerve fibers and cell bodies we separated sympathetic ganglia from nerve fibers by two methods. First, we removed chains of paravertebral sympathetic ganglia from chick embryos and cut the ganglia free of the interganglionic connectives. Second, we cultured explants of sympathetic ganglia that, after several days in culture, have an extensive halo of nerve fibers surrounding each ganglion. By microdissection we separated the halo of nerve fibers from the ganglia and analyzed the forms of AcChoEase in the two compartments. In both cases, all four forms of AcChoEase (A12, G4, G2, and G1) were demonstrable in nerve fibers and in cell bodies (Fig. 2). However, there was much more of the G4 form in the axons relative to the other globular forms (ratio of G4/G1+G2 is ≈3 in axons versus 0.5 in ganglia). Because the ganglia contain some nerve fibers, this ratio is likely an overestimate of the amount of G4 in neuronal cell bodies. This compartmentalization of G4 within axons probably results from its preferential transport into and along nerve fibers after its synthesis in the cell body (4, 5).

Our findings on the distribution of G4 within nerve fibers prompted us to further investigate the AcChoEase forms on the surface of neurons. To determine which molecular forms were located on the cell surface, cultures of dissociated ganglia were treated with the AcChoEase inhibitors BW284c51 and iPr_2P. Equilibration of cultures with BW284c51, a reversible membrane-impermeable inhibitor, protects the extracellular AcChoEase molecules from irreversible inactivation when the membrane-permeable inhibitor iPr_2P is added (11–13). This procedure irreversibly inhibits all intracellular enzyme molecules while protecting the catalytic sites of the surface AcChoEase, thus allowing its detection by enzymatic assay. After said treatment of neuronal cultures, analysis of the active enzyme by velocity sedimentation shows that the cell-surface enzyme consists

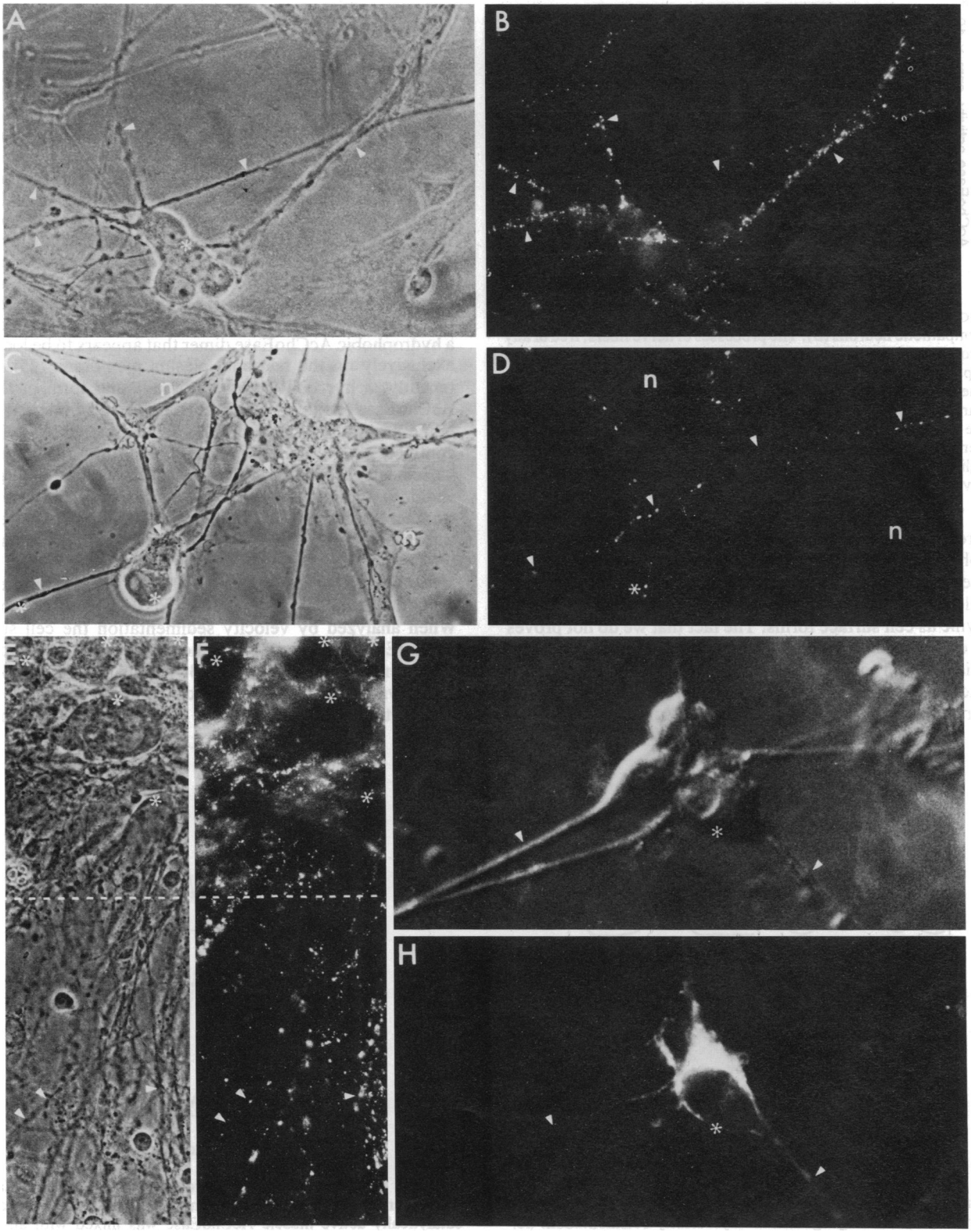

FIG. 1. Immunocytochemical localization of AcChoEase on sympathetic neurons in culture. (*A*) Phase photomicrograph of a small group of neurons (*). (*B*) Fluorescence photomicrograph of the same field as in *A*. Clusters of AcChoEase are visible outlining several, but not all, nerve fibers (arrows). The cell bodies have few clusters and are barely visible (*). (*C*) A single neuron (*) and several nonneuronal cells (n). (*D*) The nonneuronal cells (n) have little fluorescence above background. The cell body of the neuron (*) also has few AcChoEase clusters except for a trail made, *en passage*, by a nerve fiber (arrows). (*E*) A portion of an intact sympathetic ganglion in culture in which several cell bodies (*) are visible in the ganglion as well as a halo of axons extending from the ganglion. A broken line in this photomicrograph delineates the ganglion from the halo of axons. (*F*) When viewed with fluorescence illumination, the cell bodies in the ganglion are largely devoid of fluorescence (*), but the halo of axons has many fluorescent clusters on it. Some of the clusters can be traced to individual bundles of nerve fibers (arrows). (*G*) Photomicrograph taken with interference contrast optics of a small group of sympathetic neurons (*). (*H*) The distribution of WGA receptors is uniform on these neurons and appears in a ring pattern on the cell bodies (*) and uniformly along the nerve fibers (arrows). The diameters of neuronal cell bodies in these photomicrographs are ≈15–30 μm.

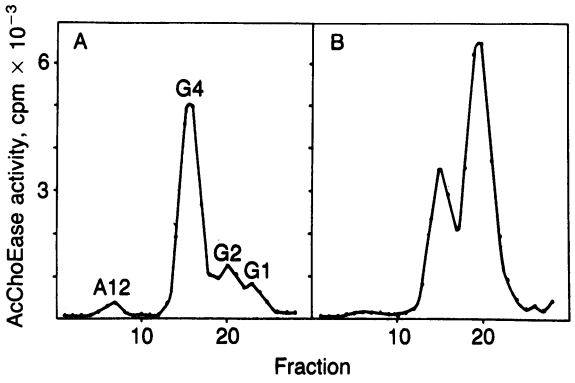

FIG. 2. Forms of AcChoEase in nerve fibers (*A*) and cell bodies of sympathetic neurons (*B*). The processes of nerve fibers, free of any neuronal somata, were separated by microdissection from the sympathetic ganglia isolated from 11-day chicken embryos. The tissue was extracted, analyzed by velocity sedimentation on 5–20% linear sucrose gradients and assayed for AcChoEase. The G4 form is the major form in nerve fibers. In the ganglia G4 is also present but lower M_r forms dominate. An undetermined fraction of the G4 in the ganglia is contributed by nerve fibers, and this amount of G4 is likely an overestimate of that in neuronal somata.

entirely of the G4 tetrameric form (Fig. 3*B*). It should be emphasized that if BW284c51 were penetrating the cells in these studies, thereby protecting intracellular enzyme, we would have seen the monomeric and dimeric forms of the enzyme as cell surface forms. The fact that we do not proves that only the cell surface AcChoEase was protected in these studies. Taken together these data suggest that the G4 form of AcChoEase is preferentially transported into nerve fibers where it appears in clusters on the cell surface.

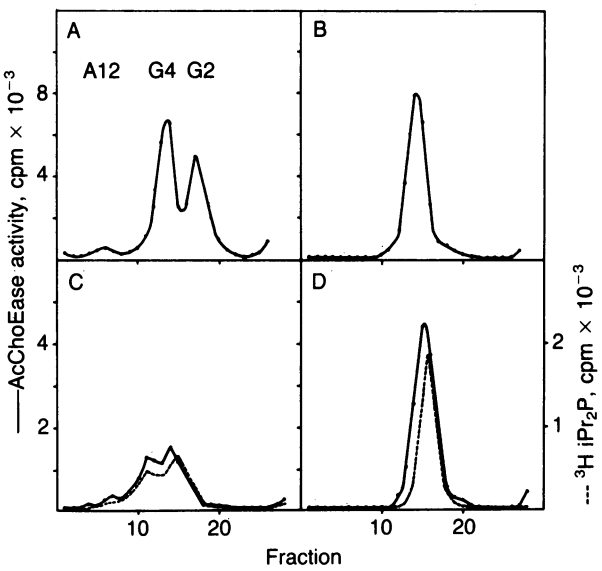

FIG. 3. Analysis of AcChoEase molecular forms in tissue-cultured sympathetic ganglion cells by velocity sedimentation. (*A*) Total cell-associated AcChoEase forms in sympathetic cell cultures; (*B*) molecular forms of AcChoEase on neuronal cell surface; (*C*) sedimentation on sucrose gradients in the absence of detergent (Triton X-100) of a mixture of catalytically active neuronal cell surface AcChoEase (——) and [^{3}H]iPr$_2$P-labeled chicken brain AcChoEase (---); (*D*) sedimentation of the same samples shown in (*C*) on gradients containing 0.5% Triton X-100. In the absence of detergent both the brain and neuronal AcChoEase tetramers aggregate and sediment in parallel. Letter designations above the peaks refer to the molecular forms according to the nomenclature proposed by Massoulie and Bon (4): G1, 5S monomer; G2, 7S dimer; G4, 11S tetramer; and A12, 19S asymmetric collagen-tailed AcChoEase.

The G4 Form of AcChoEase on Neurons Is a Hydrophobic Tetramer that Aggregates in the Absence of Detergent. Tissue-cultured neurons and muscle cells secrete AcChoEase molecules into the medium (17–20). The pattern and relative abundance of each secreted form from tissue-cultured sympathetic neurons is virtually identical to the cell-associated AcChoEase illustrated in Fig. 3*A* (data not shown). The clustered form of G4 AcChoEase in neurons could therefore arise from the pool of secreted enzyme molecules, possibly by binding to the nerve fiber surface, or it could arise from a pool of hydrophobic forms inserted directly into the plasma membrane. These hydrophobic membrane-bound forms of AcChoEase have been found on the surface of erythrocyte membranes (21, 22), in *Torpedo* electroplax (23, 24), and in the central nervous systems of several species (for review see refs. 4 and 5). Indeed, axons in *Torpedo* electroplax contain a hydrophobic AcChoEase dimer that appears to be localized exclusively on the neuronal plasma membrane (25). More recently, a hydrophobic membrane-bound form of the G4 AcChoEase tetramer, which appears to be an integral membrane protein, has been purified from chicken brain (8). In contrast, the tetrameric AcChoEase form from chicken muscle is hydrophilic (soluble in buffers without detergents) and does not interact with detergents (Fig. 4). To determine whether the hydrophobic or hydrophilic G4 AcChoEase form was associated with the clusters, cell surface AcChoEase was prepared as described above and mixed with purified brain AcChoEase that had been labeled at its active-site and catalytically inactivated with [^{3}H]iPr$_2$P (8) (Fig. 3 *C* and *D*). When analyzed by velocity sedimentation the cell surface AcChoEase from sympathetic neurons aggregates readily in the absence of detergents (Fig. 3*C*) indicating that it, like the G4 enzyme from brain, is a hydrophobic AcChoEase form (8). Furthermore, when tissue extracts containing the G4

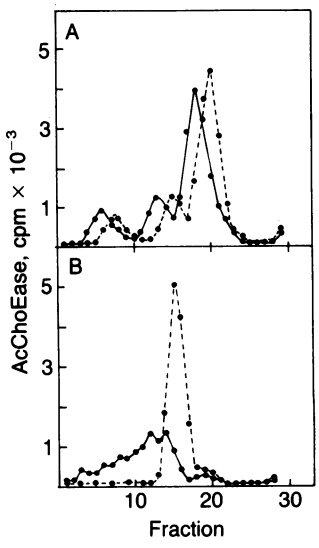

FIG. 4. Cosedimentation of neuronal and muscle AcChoEase in the presence or absence of detergents. A tissue extract containing catalytically active muscle AcChoEase was mixed with an equal volume of iPr$_2$P-inactivated brain extract and analyzed by velocity sedimentation in the presence (–●–) or absence (—●—) of 1% Triton X-100 (*A*). Conversely, a similar mixture containing catalytically active brain AcChoEase was mixed with inactivated muscle AcChoEase and analyzed in parallel (*B*). Without detergent only the G4 neuronal form of the enzyme aggregates to faster sedimenting forms as previously described (8), whereas the muscle AcChoEase forms remain unchanged. The markers alkaline phosphatase (6.1S) and β-galactosidase (16S) were included in each gradient in order to correct for differences in apparent sedimentation coefficients due to differences in the viscosity of the gradients with and without Triton X-100.

form from muscle and neurons were mixed and centrifuged in the absence of detergents, the neuronal hydrophobic forms aggregated preferentially, leaving the more hydrophilic muscle forms of G4 in solution (Fig. 4). We conclude that the cell surface AcChoEase on tissue-cultured sympathetic ganglion cells is a hydrophobic tetramer and that this particular form of the enzyme is localized in clusters on the cell surface.

SUMMARY AND CONCLUSIONS

Our studies provide direct evidence that membrane-bound AcChoEase molecules on sympathetic neurons are arranged in clusters that are restricted to nerve fibers. AcChoEase is also found in clusters on myotubes in culture (2, 3) where it codistributes, at least in part, with clusters of acetylcholine receptors (26). However, these clusters do not mature into synapses when the myotubes are innervated (26, 27). Nevertheless, recent findings showing that these densities on myotubes are induced or altered by neural cells (28–31) and increased by "factors" that themselves are localized at synapses (32) have convinced many that such densities are a valuable model for myoneural synapse formation. We do not know whether the clusters of AcChoEase on sympathetic neurons are involved in the formation of synapses or whether they correspond to structural specializations on mature axons *in vivo* that contain AcChoEase (33).

The distribution of AcChoEase is considerably different from that of a number of other membrane proteins that have been mapped on neurons in culture. For the most part, these and other proteins tend to be uniformly distributed (15, 34). We are aware of two other cell surface antigens that are distributed in clusters on neurons in culture, the G130 protein and the tetanus toxin binding sites (35, 36). In both cases the clusters are visible on cell bodies as well as on nerve fibers, which may be a clue that the antigen is normally uniform but has been redistributed by the antibody.

Major questions remain concerning the localization of membrane proteins on nerve cells. We do not know how the proteins become localized in clusters and how they are retained in them. With regard to the first issue several possibilities have been suggested including selective insertion of membrane proteins into clusters (37) and selective trapping of proteins in clusters following their insertion in the membrane (38). For AcChoEase in muscle cells, the A12 form of AcChoEase most likely interacts with molecules in the extracellular matrix to form stable aggregates on the basal lamina surrounding the cell surface (4, 5, 39). We can only speculate as to the mechanism by which the G4 form on neurons clusters within the plasma membrane. Perhaps the intracellular sorting that preferentially shunts G4 into nerve fibers, and selective insertion, is the key to the accumulation of G4 in the membranes of the fibers. Alternatively, it is also possible that the neuronal G4 could self-associate in the plane of the membrane.

At nerve-muscle synapses, AcChoEase is associated with the basal lamina (40) and can be solubilized, at least in part, by high salt-containing buffers (4, 5) or proteases (40–42). Sympathetic neurons in culture have none of the extracellular matrix components (e.g., laminin, fibronectin), detectable on myotubes by immunocytochemistry (S.T.C., unpublished observations) and the surface AcChoEase is an integral membrane protein. Moreover, the G4 form of AcChoEase is very hydrophobic relative to any other form of AcChoEase in muscle (R.L.R., unpublished work). These differences between AcChoEase molecules in neurons and muscle may reflect structural differences in the enzyme synthesized by the two types of cells and/or differences in the mechanisms of localizing synaptic components on their surfaces. Understanding the relationship of these structural differences to their compartmentalization in the cell could provide insight into how neurons segregate identified synaptic components on their plasma membranes and how these specialized domains develop into functional synaptic contacts.

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