J. Membrane Biol. 118, 1–9 (1990)

The Journal of
Membrane Biology

Topical Review

Association of Acetylcholinesterase with the Cell Surface

Nibaldo C. Inestrosa† and Alejandra Perelman
Molecular Neurobiology Unit, Department of Cell and Molecular Biology, Faculty of Biological Sciences, and †Joint Program with the Faculty of Medicine, Catholic University of Chile, Santiago, Chile

Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) serves a key physiological role in the operation of the cholinergic synapses by rapidly hydrolyzing the neurotransmitter acetylcholine and thereby terminating the chemical synaptic transmission (Kuffler, Martin & Nicholls, 1984). The process of synaptic transmission takes place within 1 msec (Rosenberry, 1979), demanding, therefore, a precise temporal and spatial integration of the functional components involved. Under physiological conditions apparently only some synaptic AChE molecules participate in the hydrolysis of acetylcholine, depending on their location at the endplate (Miledi, Molenaar & Polak, 1984). The study of AChE extends back to the early 20th century when Dale described a substance capable of cleaving choline esters. This preceded Loewi's (1926) identification of acetylcholine as the neurotransmitter of the cholinergic system as well as Nachmansohn's findings showing high concentration of AChE at the sites of nerve-muscle contacts (Marnay & Nachmansohn, 1938). In 1942, the fundamental role of AChE in cholinergic transmission was firmly established by Eccles, Katz and Kuffler. Since then, extensive information has accumulated regarding the basic chemistry and physiology of AChE, including important data on its purification, molecular forms, cellular distribution, catalytic properties, metabolism and regulation (Massoulié & Bon, 1982; Brimijoin, 1983; Ott, 1985; Quinn, 1987; Silman & Futerman, 1987; Inestrosa & Perelman, 1989). In 1979 Bon, Vigny and Massoulié introduced a nomenclature that permits the classification of the multiple molecular forms of AChE. Two classes were defined: the globular and asymmetric forms. *Globular forms* are monomers, dimers and tetramers of catalytic subunits (G_1, G_2, G_4) and *asymmetric forms* correspond to one, two or three tetramers associated with a collagen-like element or "tail" (A_4, A_8, A_{12}). In each case, the index indicates the number of catalytic subunits in the molecule. Asymmetric forms are associated with skeletal neuromuscular junctions (Hall, 1973; Vigny, Koenig & Rieger, 1976; Fernández, Inestrosa & Stiles, 1984) and with the electric organs of *Torpedo, Electrophorus* and *Discopyge* (Massoulié & Bon, 1982; Méndez et al., 1984). Globular forms of AChE not only occur in cells involved in cholinergic neurotransmission, but are also found in a variety of other neuronal and some non-neuronal cells as well (Rama-Sastry & Sadavongvivad, 1979). In addition to size isomers, there are solubility variants. Thus, globular forms may exist either as hydrophilic species (readily soluble in aqueous media) or as hydrophobic species. Such complex structural polymorphism, as well as the ubiquitous distribution of the enzyme throughout a wide range of vertebrate tissues, supports the current notion that individual AChE molecular forms may play distinct physiological roles in both cholinergic and noncholinergic systems.

We have chosen AChE to study the mechanisms by which proteins are localized and concentrated in specific regions of the cell surfaces. In fact, the distribution of different molecular forms is dramatically distinct within cells of an organism, the attachment of the enzyme to the surface membrane being accomplished by highly specialized modes of assembly and post-translational modifications for each given case. Hereafter the biochemical strategies used in the interaction of different molecular forms of AChE with the cell surface will be discussed.

Key Words cholinergic system · AChE molecular forms · membrane anchors · post-translational modification · cellular distribution

Asymmetric Forms of AChE Are Anchored to the Synaptic Basement Membranes

Asymmetric forms were first visualized in the electron microscope by Dudai, Herzberg and Silman, 1973. They appear as complex elongated structures in which a multi-subunit head, containing three tetramers of 12 catalytic subunits of 65–70 kD each, is connected to a triple-helical collagenous subunit (Cartaud et al., 1975; Rosenberry & Richardson, 1977; Anglister & Silman, 1978; Lee, Heinemann & Taylor, 1982).

The first indication that the functionally important AChE at the neuromuscular junction corresponds to an enzymatic form composed of collagen or attached to a collagenous surface membrane were the experiments carried out by Hall and Kelly in 1971. They showed that treatment of rat diaphragm muscle with collagenase released AChE, prolonging the falling phase of miniature endplate potentials. Later, the presence of asymmetric AChE forms at the neuromuscular junction was established by Hall (1973). Purified asymmetric AChE contains about 1% hydroxyproline while the 11S species contained, at most, barely detectable amounts (Anglister, Rogozinsky & Silman, 1976; Lwebuga-Mukasa, Lappi & Taylor, 1976). The above studies led Silman and Taylor to suggest that the tail subunits were responsible for the in vivo localization of AChE in the extracellular matrix (ECM). The tail consists of a pepsin-sensitive, noncollagenic domain and a pepsin-resistant domain which presents the spectroscopic properties of triple-helical collagen. The tail subunit contains, besides hydroxyproline, a high content of glycine and hydroxylysine. Most of the latter amino acid residues are linked to 2-O-α-D-glucopyranosyl-O-β-D-galactopyranose, which is characteristic of basement membrane collagens (Rosenberry, Barnett & Mays, 1980).

AChE activity is present in the synaptic basal lamina (McMahan, Sanes & Marshall, 1978); however, direct evidence for the association of the asymmetric AChE (A_{12}) with the ECM was provided years later, using C_2 mouse myotubes which possess a rich ECM (Silberstein, Inestrosa & Hall, 1982). For this purpose different AChE inhibitors were used to simultaneously protect the surface enzyme with BW284c51 dibromide and to inactivate the intracellular AChE with Di-isopropyl fluorophosphate (DFP). They were able to show that most of the A_{12} AChE was localized at the cell surface, from where it was removed by collagenase with the concomitant disappearance of A_{12} AChE patches from the cell surface (Inestrosa, Silberstein & Hall, 1982). In the middle of 1982, therefore, one important remaining question was "how is asymmetric AChE anchored to the ECM?" and specifically, "which was the ECM receptor for the enzyme?"

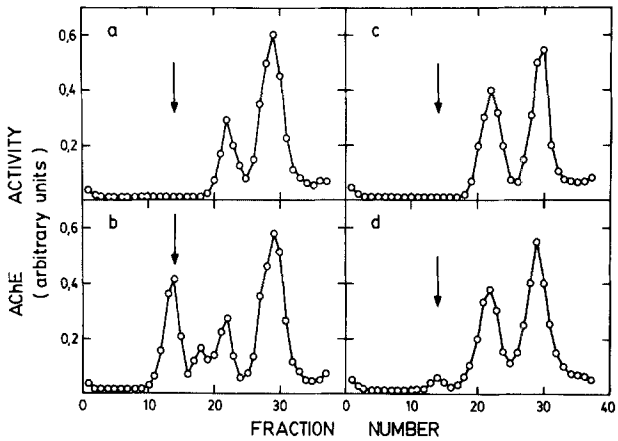

Fig. 1. Effect of heparin on the solubilization of asymmetric AChE. Shown are velocity sedimentation profiles of AChE molecular forms extracted from endplate (*a,b*) and nonendplate (*c,d*) regions of rat diaphragm muscle with 50 mM Tris-HCl (pH 7.8) and 0.5% Triton X-100 in the presence (*b,d*) or absence (*a,c*) of 2 mg/ml heparin. The arrow represents the position of the marker enzyme β-galactosidase (16.1S)

Heparan Sulfate Proteoglycans Are the Basement Membrane Receptors for the Asymmetric AChE

Early on, Bon, Cartaud and Massoulié (1978) suggested that glycosaminoglycans (GAGs)[1] of the chondroitin sulfate type were involved in the association of AChE to membranes of the electric organ of *Electrophorus*. The breakthrough came in 1983 when we showed that heparin was able to release AChE activity from endplate regions of rat diaphragm (Torres & Inestrosa, 1983). The effect of heparin was observed at low concentrations, and the half-maximal effect was obtained at 50 μg/ml. Neither chondroitin sulfate nor hyaluronic acid were able to release AChE from skeletal muscles. Analysis of the AChE molecular forms solubilized by heparin in 5–20% sucrose gradients showed that the GAG was able to release only asymmetric AChE from the endplate regions of rat diaphragm muscle (*compare* Fig. 1*a* with Fig. 1*b*). The observation that

[1] GAGs consist of unbranched polymers of disaccharide units that contain hexuronic acid and hexosamine, with a carboxilate and/or a sulfate ester, which provide the chains with linear arrays of anionic groups. On the basis of different disaccharide units there are seven types of GAGs. With the exception of hyaluronic acid, which lacks sulfate groups, GAG chains are linked to a protein core forming different types of proteoglycans (Höök et al., 1984).

heparin solubilizes A_{12} AChE from the neuromuscular junction clearly suggests that AChE possesses an "heparin-binding domain" on its structure, likely in its collagen-like tail, led us to study more in detail the interaction between AChE and heparin. Analysis of the AChE molecular forms solubilized by heparin in sucrose gradients containing 1 M NaCl showed the specific solubilization of the asymmetric A_{12} and A_8 AChE molecular forms. However, if the solubilized esterase was analyzed in low ionic strength, the majority of AChE activity sedimented at the bottom of the gradient, suggesting the presence of aggregated asymmetric AChE. Further experiments showed the existence of a polydisperse molecular aggregate sedimenting around 30S (Torres & Inestrosa, 1985). The data suggest that sulfated GAGs remove surface AChE by a mechanism involving the formation of a soluble complex which contains GAG and asymmetric AChE.

To prove the direct interaction of asymmetric AChE with heparin, we performed affinity chromatography in heparin-agarose columns and showed the specific esterase binding to the agarose gel. The interaction between AChE and heparin required an intact collagenous tail, as shown by the fact that binding is inhibited by pretreatment with collagenase (Brandan & Inestrosa, 1984). These results support the idea that heparin binds to a specific domain of the collagen-like tail of the asymmetric AChE (*see also* Vigny, Martin & Grotendorst, 1983).

The in vivo interaction between the asymmetric A_{12} AChE and heparin or heparan sulfate residues was studied by measuring the binding of AChE to intact endothelial cells rich in surface heparan sulfate proteoglycans. Asymmetric AChE was shown to bind to these cells with specific, time-dependent, and saturable kinetics (Brandan & Inestrosa, 1986). Another way to establish the interaction of asymmetric AChE and carbohydrate components of proteoglycans in the synaptic basement membranes is to use enzymes which specifically degrade GAGs. We incubated basement membranes purified from *Discopyge* electric organs with heparitinase or chondroitinase ABC. Increasing amounts of heparitinase, but not chondroitinase ABC, is able to release esterase from the basement membranes to the incubation medium (Brandan et al., 1985). The heparan sulfate hydrolase releases only collagen-tailed AChE forms. These results provide direct evidence for the in vivo interaction between asymmetric forms and heparan sulfate proteoglycan at the neuromuscular junction.

A different approach provides additional evidence for the relation between cell surface heparan sulfate proteoglycans and asymmetric AChE. A stable variant of the rat pheochromocytoma PC12 cell line, lacking a heparan sulfate proteoglycan on the cell surface, showed an atypical distribution of collagen-tailed AChE. In normal PC12 cells virtually all the asymmetric AChE form is on the external cell surface (Inestrosa et al., 1981), whereas in the variant most of the enzyme (60%) is in an internal compartment (Inestrosa et al., 1985). These findings suggest that heparan sulfate proteoglycan is probably involved in the intracellular assembly, transport, and surface deposition of asymmetric AChE. Further studies are needed in order to define the precise nature of proteoglycan-AChE interactions at the neuromuscular junction ECM.

The Dimeric G_2 Globular AChE is Associated with Plasma Membranes through a Phosphatidylinositol-Glycolipid

The amphiphilic dimeric G_2 AChE is a membrane-bound form that is solubilized in the presence of detergents and that for maximal recovery required extraction in the presence of sulfydryl alkylating agents (Rotundo & Fambrough, 1979; Toutant et al., 1990). The dimeric form is bound to the plasma membrane by a covalently linked, phosphatidylinositol (PI) containing glycophospholipid that behaves as an anchor domain (Futerman et al., 1985*a*). The involvement of PI in attaching proteins to membranes was first suspected when it was observed that a number of cell surface enzymes could be released from the membrane by treatment with PI-specific phopholipase C (PIPLC). This group of surface proteins includes alkaline phosphatase, 5'-nucleotidase, Thy-1, a 120-kDa neural cell adhesion molecule ($N\text{-}CAM_{120}$), the scrapie prion protein ($Pr\ P_{27\text{-}30}$) and the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Low, 1989). The amphiphilic dimeric AChE was shown to be solubilized from various sources such as rat and bovine erythrocytes, tegument of the worm *Schistosoma mansoni, Torpedo* electric organ, *Xenopus* skeletal muscle, rat liver, bovine chromaffin cells and human heart (Futerman et al., 1985*b*; Espinoza et al., 1988; Inestrosa et al., 1988; Perelman & Brandan, 1989; Prieto et al., 1989; González et al., 1990). The enzymes were released in a soluble nonaggregating hydrophilic form. Furthermore, the release of G_2 AChE involved neither cell lysis nor extensive PI hydrolysis. The fact that PIPLC can modify the hydrodynamic behavior of the detergent-solubilized G_2 AChE, and the finding of covalently bound inositol in the membrane-anchoring domain, confirmed the suggestion that PI is indeed involved in anchoring the G_2 form to the plasma membrane (Silman & Futerman, 1987). The hydrophobic domain of the G_2 AChE can be specifically labeled with the photoactivatable hydrophobic

reagent ^{125}I-trifluorophenyl-diazirine (^{125}I-TID), an affinity reagent which selectively labels the membrane hydrophobic domains of proteins, because it partitions into the hydrophobic phase of membranes or into detergent micelles and after photoactivation, covalently reacts with protein sequences in this phase (Brunner & Semenza, 1981). The labeled fragment was identified after proteolysis of *Torpedo* and human erythrocyte G_2 AChE (Stieger et al., 1984; Roberts & Rosenberry, 1986). Its molecular mass was established as 3 kDa in both cases.

The C-terminal structure proposed to link proteins to a PI molecule in the membrane is largely based on information derived from the VSG of *T. brucei* (Low & Saltiel, 1988). The data available for other proteins that use PI as a membrane-anchoring domain suggested that they are attached also by a similar structure. PI is attached via a glycosidic linkage to a glucosamine residue, which is unusual inasmuch as it is not N-acetylated. The glucosamine is attached to an oligoglycan, whose composition probably varies from protein to protein. This oligoglycan is attached to the C-terminal amino acid of the polypeptide chain via an ethanolamine residue (Low, 1989).

Not all the amphiphilic G_2 form is solubilized by PIPLC. In human and mouse erythrocytes, PIPLC is only partially able to solubilize AChE, whereas complete solubilization is obtained with AChE from bovine, porcine and rat erythrocytes (Roberts, Benedict & Rosenberry, 1987). The glycophospholipid membrane anchor of human erythrocyte AChE contains a novel inositol phospholipid, which by several techniques, including fast atom bombardment mass spectrometry, has been shown to be a plasmanyl-inositol, palmitoylated on the inositol ring (Roberts et al., 1988*a*,*b*). It appears therefore that palmitoylation of inositol inhibits the action of PIPLC, but does not interfere with digestion by anchor-specific phospholipase D (Toutant et al., 1989). Protection from PIPLC cleavage conferred by palmitoylation of the anchor inositol could provide a cellular mechanism for the regulation of phospholipid-induced protein release.

Concerning the biological meaning of the glycosyl-PI-anchored proteins, some studies suggest that the PI-anchor is involved in targeting glycoproteins to the apical surface membrane of polarized cells (Lisanti et al., 1988). Moreover, the distribution of glycosyl-PI-anchored proteins is differentially regulated by serum and insulin (Lisanti et al., 1989).

Recently, we studied in detail AChE forms found in hepatocytes and adrenal medulla cells (Perelman & Brandan, 1989; Prieto et al., 1989). In rat hepatocytes, 81% of the AChE activity was localized in the hepatocyte surface facing the extracellular media. After PIPLC treatment, almost all the G_2 AChE form present on the surface of the hepatocyte was released. To confirm the presence of a PIPLC-sensitive form of AChE present on rat liver and adrenal gland cell surfaces, a fraction enriched in plasma membrane was incubated with PIPLC. In both cases increasing amounts of PIPLC released about 70% of the total AChE present in the plasma membrane, indicating that the G_2 AChE present in a given cell type is attached via PI anchor to the membrane. The study of the subcellular localization of AChE in rat liver suggested that both G_2 and G_4 forms were found in all organelles, but the G_2 AChE activity was mainly associated with the Golgi apparatus and the plasma membrane (Perelman et al., 1990). Therefore, it was important to determine if the Golgi G_2 form of AChE was linked to the membrane by a similar mechanism. Treatment of permeabilized Golgi vesicles with PIPLC released 30% of AChE. This form was G_2, indicating that the Golgi form is already interacting with the organelle membrane through the glycolipid domain (Perelman et al., 1990). This result is in agreement with the observations made for other PI-linked proteins such as the VSG of *T. brucei*, the murine Thy-1 and N-CAM_{120}, where the transfer of the glycophospholipid can be blocked, during a brief period after protein synthesis, presumably at the endoplasmic reticulum (Low, 1989). However, during biosynthesis of alkaline phosphatase in JEG-3 choriocarcinoma cells, a protein intermediate devoid of the glycolipid anchor was observed (Takami et al., 1988). Therefore, it would be interesting to find out if during the biosynthesis of the G_2 AChE, a protein intermediate without the glycolipid anchor might also be detected.

The Tetrameric G_4 Globular AChE is Associated with Plasma Membranes through a 20-kDa Hydrophobic Peptide

In mammalian brain almost all the AChE activity can be assigned to forms other than the collagen-tailed asymmetric species (Inestrosa & Ruiz, 1985). The bulk of the enzyme comprises tetrameric detergent-soluble G_4 AChE, which has been purified from different sources, including human, chick and bovine brain (Sorensen, Gentinetta & Brodbeck, 1982; Rotundo, 1984*a*; Fuentes & Inestrosa, 1988).

Concerning the hydrophobic domain of the G_4 AChE, involved in the anchorage of the enzyme to the plasma membrane, initially it was thought that, as in the case of the dimeric globular form, a glycophospholipid containing PI would be involved; however, this proved not to be the case. In fact, when homogenates of several brain regions were incu-

bated with PIPLC, no release of AChE was observed (Futerman et al., 1985*b*). This prompted us to purify the bovine brain enzyme and to characterize their subunit composition in order to identify the hydrophobic anchor domain of the tetrameric G_4 AChE.

The subunit structure of the purified bovine brain G_4 AChE under nonreducing conditions in SDS polyacrylamide gels turns out to be complex. There are six components: a major peptide of 65 kD and five minor peptides of 90, 140, 160, 260 and 300 kDa, respectively (Fuentes & Inestrosa, 1988). Each peptide is labeled by (^{3}H) DFP, indicating that all bands include AChE polypeptides (Inestrosa et al., 1987). Following reduction, a single peptide was observed, with relative molecular mass of 68 kDa. To study the association of bovine brain AChE with the neuronal plasma membrane, we used ^{125}I-TID. This reagent has been previously used to label the G_2 anchor domain. TID was reacted with the purified G_4 form of the esterase. SDS-polyacrylamide gel electrophoresis patterns, under reducing conditions, showed that most of the ^{125}I-TID label is in a 20-kDa peptide. The ^{125}I-TID-labeled peptide was not released from the enzyme in the absence of a reducing agent, indicating that the 20-kDa peptide is linked by disulfide bridges to the catalytic subunits of the G_4 AChE.

An obvious question that arises from the previously discussed work on the dimeric G_2 AChE is whether the 20-kDa component of the bovine brain enzyme contains a glycolipid anchor similar to that observed at the C-terminus of the G_2 AChE. Purified G_4 AChE was incubated with PIPLC, and subsequently the different AChE subunits including the 20-kDa band labeled with ^{125}I-TID were separated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, no change in the relative migration of the 20-kDa band was observed, confirming that PIPLC has no effect on the solubilization of the G_4 form in crude brain extracts. Direct chemical analysis to search for compounds known to be present in the glycolipid domain of the dimeric AChE were carried out. Bovine brain AChE contains no inositol, ethanolamine nor glucosamine with free amino groups (Inestrosa et al., 1987).

The existence of a 20-kDa hydrophobic anchor domain for the G_4 AChE, has been confirmed by Gennari, Brunner and Brodbeck (1987) for the human brain AChE. Previous studies have shown that the hydrophobic G_4 AChE form could be converted to a catalytically active hydrophilic enzyme after protease treatment (Gennari & Brodbeck, 1985). We submitted the ^{125}I-TID-labeled AChE to the action of proteinase K. After this treatment 80% of the activity remained; however, almost all the radioactivity associated with the G_4 AChE was released,

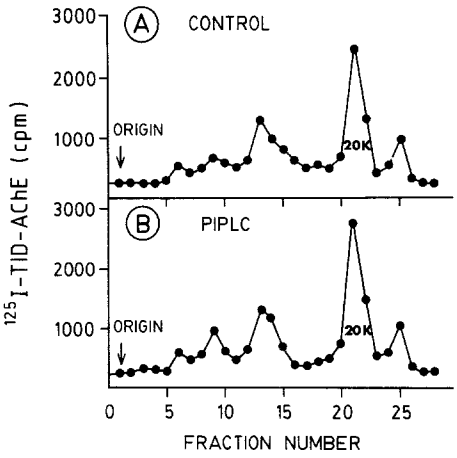

Fig. 2. Resistance of the 20-kDa hydrophobic anchor domain of the G_4 AChE to PIPLC. ^{125}I-TID-labeled AChE from bovine brain was incubated in the presence or absence of 10 μg/ml PIPLC for 60 min; then the enzyme fractions were submitted to SDS-polyacrylamide gel electrophoresis and the resulting gels sliced. Shown are the radioactivity profile of the sliced gel from control (*A*) and PIPLC-treated sample (*B*). *20K* indicates the hydrophobic anchor domain

and instead of the 20-kDa labeled band most of the label migrated close to the front of the SDS-polyacrylamide gel, with an approximate molecular mass of 13 kDa, indicating that proteinase K released most of the hydrophobic domain from the enzyme (Fuentes & Inestrosa, 1988). Under this condition the G_4 hydrophobic form was not able to aggregate in the absence of detergent (Fuentes, Rosenberry & Inestrosa, 1988). To explore whether the 20-kDa component correspond to an hydrophobic polypeptide, the labeled 20-kDa subunit isolated from ^{125}I-TID-AChE was digested with pronase and chromatographed on Sephadex LH-60. The protease-resistant anchor chromatographs at the position of a 7-kDa peptide; however, this fragment also contains fatty acids and thus probably possesses a nonpolypeptide domain (Fig. 3*A*). Recent studies indicate that hydroxylamine, a reagent known to release fatty acids from proteins, was able to remove all the ^{125}I-TID bound to the 20-kDa domain of the G_4 AChE (M.E. Fuentes & N.C. Inestrosa, *unpublished results*).

The 13-kDa fragment previously observed in reducing SDS-polyacrylamide gels after proteinase-K treatment comes from the ^{125}I-TID 20-kD subunit; therefore it is possible to postulate the existence of three different domains in the hydrophobic anchor of the G_4 AChE: (i) a proximal subdomain containing the disulfide linkages involved in the binding of the hydrophobic domain to the 68-kDa catalytic subunit; (ii) an intermediate subunit sensitive to proteinase K; and (iii) a third pronase-resistant 7-kDa subdomain which contains fatty acids and is involved di-

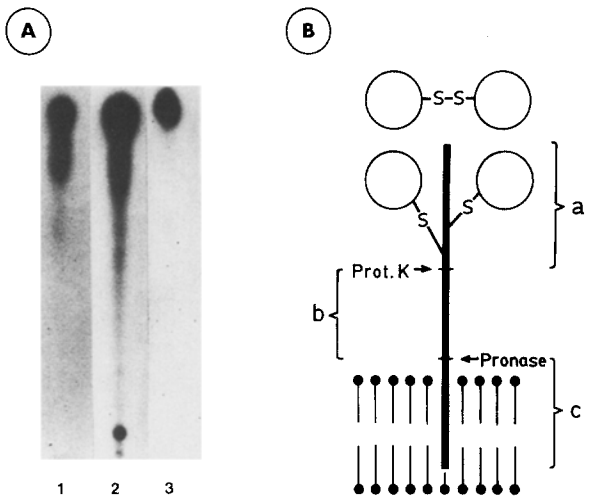

Fig. 3. The hydrophobic anchor subunit of the brain G_4 AChE contains fatty acids. (*A*) Thin-layer chromatography of ^{125}-TID fatty acids released by alkaline hydrolysis from the 20-kDa subunit of the G_4 AChE (*1*) and from the red blood cell G_2 (*2*). Lane *3* corresponds to a standard of ^{125}I-TID-labeled fatty acids. (*B*) Scheme of the G_4 AChE showing the three distinct anchor subdomains: (*a*) the disulfide linkage region, 7 kDa; (*b*) the intermediate domain limited by the proteinase K sensitive site, 6 kDa; and (*c*) the pronase-resistant domain, 7 kDa

rectly in the binding of the G_4 AChE to the neuronal membrane bilayer (Fig. 3*B*).

Coated Vesicles Are Involved in AChE Biosynthesis

The biogenesis of AChE forms has been studied in tissue-cultured nerve and muscle cells, using irreversible AChE inhibitors to block pre-existing molecules and observing the reappearance of newly synthesized, catalytically active enzyme (Rotundo & Fambrough, 1980; Inestrosa, 1984; Inestrosa et al., 1985; Brockman, Usiak & Younkin, 1986) or by density shifting of the AChE molecules in neuroblastoma cells (Lazar et al., 1984). These studies indicate that muscle and neuronal cells synthesize approximately 20% of their total AChE per hour with an important fraction of the enzyme destined for secretion. Only a small subset of molecules are targeted for accumulation on the cell surface (Rotundo & Fambrough, 1980). In 1986, Brockman et al. demonstrated that complex forms of AChE are assembled from active monomeric precursors. The globular dimers and tetramers are assembled in the rough endoplasmic reticulum, while an asymmetric collagen-tailed form of the enzyme is assembled in the Golgi apparatus some 90 min after synthesis of the globular precursor (Rotundo, 1984*b*; Brockman et al., 1986). Recent studies of the distribution of AChE in rat liver also support the notion that dimeric and tetrameric forms are assembled in the rough endoplasmic reticulum (Perelman et al., 1990). Rotundo (1989) has recently shown that the AChE molecule is synthesized as a catalytically inactive precursor which becomes activated and assembled in the rough endoplasmic reticulum. Only a subset of the newly synthesized AChE molecules are activated and assembled into functional enzyme molecules, and the majority of them remain catalytically inactive and are rapidly degraded intracellularly. The manner by which the translocation of AChE molecular forms occurs from their site(s) of assembly to the cell surface has yet to be determined. Some studies have indicated that clathrin-coated vesicles are implicated in the intracellular transport of newly synthesized AChE (Benson et al., 1985; Rotundo et al., 1989). Recent work (Fig. 4) indicates that multiple AChE molecular forms are associated with coated vesicle preparations obtained from rat skeletal muscles (Hodges-Savola et al., 1989). Interestingly enough, coated vesicles isolated from developing muscles exhibit an apparent enrichment in asymmetric AChE, a fact consistent with the increase in this AChE form during postnatal development and synapse formation (Vigny et al., 1976; Inestrosa, Méndez & Luco, 1979; Ziskind-Conhaim, Inestrosa & Hall, 1984).

The Molecular A_{12} and G_2 Forms of AChE Arise from a Single Gene by Alternative *m*RNA Splicing

A fundamental question is the genetic basis of the diversity of cholinesterase species. Cloning of cDNAs encoding the catalytic subunits of AChE from *Torpedo californica* (Schumacher et al., 1986) and *Torpedo marmorata* (Sikorav, Krejci & Massoulié, 1987) and analysis of genomic sequences (Schumacher et al., 1988) indicate that AChE is apparently encoded by a single gene in these species. Sequence analysis of individual *c*DNA clones and amino acid sequencing of the AChE polypeptide chains (Gibney et al., 1988) indicate the presence of at least two *m*RNA transcripts encoding the asymmetric and the dimeric AChE forms. Further studies (Schumacher et al., 1988; Sikorav et al., 1988) demonstrated in *Torpedo* electric organ that the G_2 and A_{12} catalytic subunits arise from a single gene after alternative *m*RNA splicing. The AChE gene contains two exons encoding a leader peptide and the 535 first amino acids of the mature protein which correspond to the catalytic subunit. A third exon

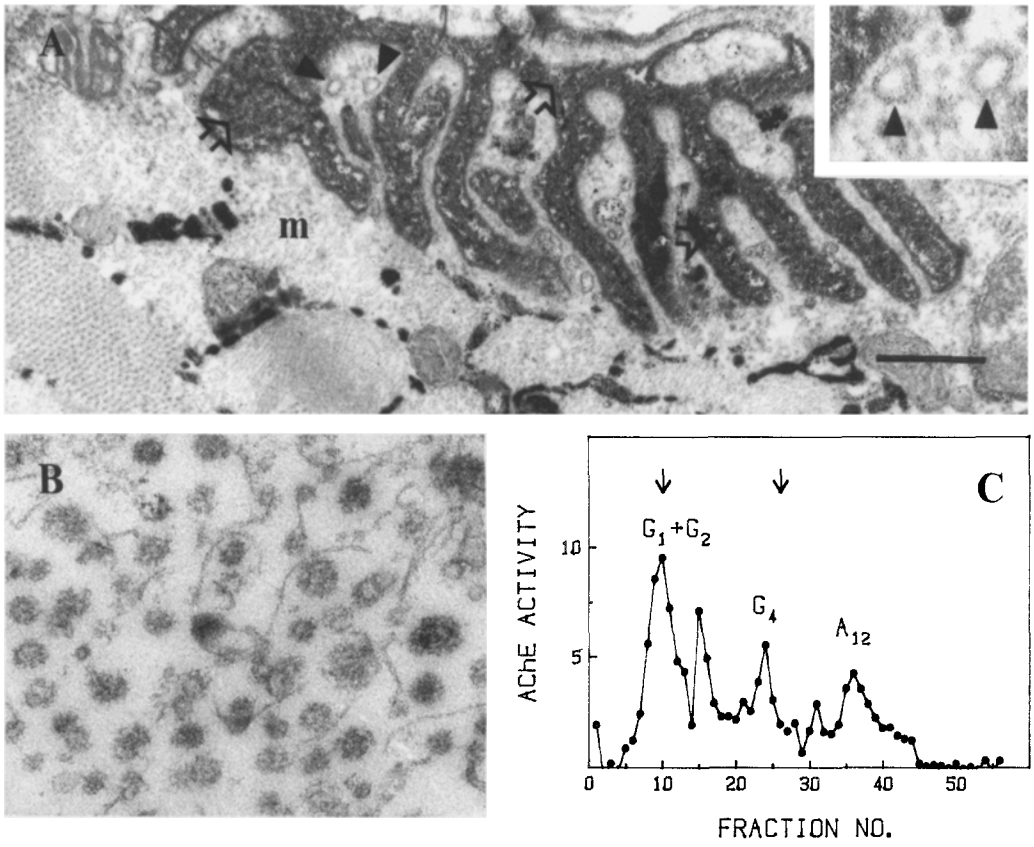

Fig. 4. Coated vesicles and biogenesis of AChE. (*A*) Electron micrograph of a neuromuscular junction from rat (approx. 30 day old) anterior gracilis muscle. *m*, muscle; open arrows, synaptic basal lamina; filled arrows, coated vesicles (CVs); Inset, coated vesicles enlarged × 2.5; scale bar, 0.5 μm. (*B*) Coated vesicle-enriched preparation from developing (18–21 day old) rat skeletal muscles. Muscle microsomes were subjected to equilibrium sedimentation in discontinuous (20–60%) sucrose gradients, and fractions collected from the 50–55% sucrose interface were pelleted and prepared for electron microscopy. Thin sections reveal many CVs, clathrin cages, and some filamentous profiles. Magnification, × 504,000. (*C*) AChE forms specifically associated with rat muscle CV-enriched fractions. Enzymatic activity was extracted in 50 mM Tris-HCl (final pH 8.5), 1 M NaCl, 5 mM EDTA, and 1% Triton X-100. Solubilized AChE was subsequently subjected to velocity sedimentation in linear (5–20%) sucrose gradients and assay to discern individual AChE forms. Bovine serum albumin (4.4S) and catalase (11.3S) were included as sedimentation markers and are indicated by arrows (from left to right). The gradient meniscus is to the left and the major AChE forms are indicated: $G_1 + G_2$, globular monomers and dimers; G_4, globular tetramers; and A_{12}, asymmetric form. It is noteworthy that approximately 80% of the A_{12} AChE form detected in such CV-enriched preparations is retained by the immune complex following CV-specific immunoprecipitation (Hodges-Savola et al., 1989).

(III_H) encodes for the two last amino acids of the mature hydrophobic G_2 form and contains the signal for the linkage of the glycolipid anchor to the catalytic subunit; and, finally, a fourth exon (III_A) encodes for the last 39 residues of the A_{12} form.

Thus, alternate use of exons III_H and III_A within the AChE gene is apparently the molecular basis of the differential tethering of the two AChE forms in synapses. Nothing is known about the gene structure of the tetrameric globular G_4 AChE form. In avian nerves and muscles genetic evidence indicated that all AChE forms arise from a single gene (Rotundo et al., 1988). However, recent studies in rat muscle suggests that the G_4 AChE form might have a different origin than the A_{12} and G_2 AChE (Perelman & Inestrosa, 1989; Perelman et al., 1990).

Summary and Concluding Remarks

Acetylcholinesterase (AChE) shows tissue-specific differences in the distribution of forms and mode of anchoring to the cell surface which can be distinguished by their solubilization characteristics and hydrodynamic properties. Association to the membrane is achieved by post-translational modification of the catalytic subunits. Three such mechanisms are described. The first involves attachment of three

tetramers of subunits to a collagen-like tail, which interacts with heparan sulfate proteoglycans in the basement membrane. The other two modifications involve hydrophobic attachment of globular forms to the plasma membrane. In the G_2 AChE form, the diacylglycerol moiety of a phosphatidylinositol molecule at the C-terminus of each polypeptide anchors the enzyme to the outer layer of the membrane. In the case of the brain G_4 AChE form the modification involves the covalent attachment of a 20-kDa hydrophobic peptide to the plasma membrane. The regulation of alternate RNA splicing, providing selective expression of globular hydrophobic forms on cell plasma membranes or the asymmetric forms in the synaptic basement membrane, should be an interesting starting point for the study of AChE regulation during synapse formation and development of the cholinergic nervous system.

We want to thank Cheryl A. Hodges-Savola and H.L. Fernández for their kind gift and careful preparation of Fig. 4. This review was supported by grants from the Stiftung Volkswagenwerk, FONDECYT (N° 3502/89) and the Fundación Gildemeister to N.C.I.

References

Anglister, L., Rogozinsky, S., Silman, I. 1976. *FEBS Lett.* **60:**129–132

Anglister, L., Silman, I. 1978. *J. Mol. Biol.* **125:**293–311

Benson, R.J.J., Porter-Jordan, K., Buoniconti, R., Fine, R.E. 1985. *J. Cell Biol.* **101:**1930–1940

Bon, S., Cartaud, J., Massoulié, J. 1978. *Eur. J. Biochem.* **85:**1–14

Bon, S., Vigny, M., Massoulié, J. 1979. *Proc. Natl. Acad. Sci. USA* **76:**2546–2550

Brandan, E., Inestrosa, N.C. 1984. *Biochem. J.* **211:**415–422

Brandan, E., Inestrosa, N.C. 1986. *J. Neurosci. Res.* **15:**185–196

Brandan, E., Maldonado, M., Garrido, J., Inestrosa, N.C. 1985. *J. Cell Biol.* **101:**985–992

Brimijoin, S. 1983. *Prog. Neurobiol.* **21:**291–322

Brockman, S.K., Usiak, M.F., Younkin, S.G. 1986. *J. Biol. Chem.* **261:**1201–1207

Brunner, J., Semenza, G. 1981. *Biochemistry* **20:**7174–7182

Cartaud, J., Rieger, F., Bon, S., Massoulié, J. 1975. *Brain Res.* **88:**127–130

Dudai, Y., Herzberg, M., Silman, I. 1973. *Proc. Natl. Acad. Sci. USA* **70:**2473–2476

Eccles, J.C., Katz, B., Kuffler, S.W. 1942. *J. Neurophysiol.* **5:**211–230

Espinoza, B., Tarrab-Hazai, R., Silman, I., Arnon, R. 1988. *Mol. Biochem. Parasitol.* **29:**171–179

Fernández, H.L., Inestrosa, N.C., Stiles, J.R. 1984. *Neurochem. Res.* **9:**1211–1230

Fuentes, M.E., Inestrosa, N.C. 1988. *Mol. Cell. Biochem.* **81:**53–64

Fuentes, M.E., Rosenberry, T.L., Inestrosa, N.C. 1988. *Biochem. J.* **256:**1047–1050

Futerman, A.H., Low, M.G., Ackerman, K.E., Sherman, W.R., Silman, I. 1985*a*. *Biochem. Biophys. Res. Commun.* **129:**312–317

Futerman, A.H., Low, M.G., Michaelson, D.M., Silman, I. 1985*b*. *J. Neurochem.* **45:**1487–1494

Gennari, K., Brodbeck, U. 1985. *J. Neurochem.* **44:**697–704

Gennari, K., Brunner, J., Brodbeck, U. 1987. *J. Neurochem.* **49:**12–28

Gibney, G.G., MacPhee-Quigley, K., Thompson, B., Vadvick, T., Low, M.G., Taylor, S., Taylor, P. 1988. *J. Biol. Chem.* **263:**1140–1145

González, R., Campos, E., Moran, S., Inestrosa, N.C. 1990. *Gen. Pharmacol. (in Press)*

Hall, Z.W. 1973. *J. Neurobiol.* **4:**343–361

Hall, Z.W., Kelly, R.B. 1971. *Nature (New Biol.)* **232:**62–64

Hodges-Savola, C.A., Gregory, E.J., Rummel, S.A., Fernandez, H.L. 1989. *J. Neurosci. Res.* **24:**174–185

Höök, M., Kjellen, L., Johannsson, S., Robinson, J. 1984. *Annu. Rev. Biochem.* **53:**847–869

Inestrosa, N.C. 1984. *Biochem. J.* **217:**377–381

Inestrosa, N.C., Fuentes, M.E., Anglister, L., Futerman, A.H., Silman, I. 1988. *Neurosci. Lett.* **90:**186–190

Inestrosa, N.C., Matthew, W.D., Reiness, C.G., Hall, Z.W., Reichardt, L.F. 1985. *J. Neurochem.* **45:**86–94

Inestrosa, N.C., Méndez, B., Luco, J.V. 1979. *Nature (London)* **280:**504–506

Inestrosa, N.C., Perelman, A. 1989. *Trends Pharmacol. Sci.* **10:**325–329

Inestrosa, N.C., Reiness, C.G., Reichardt, L.F., Hall, Z.W. 1981. *J. Neurosci.* **1:**1260–1267

Inestrosa, N.C., Roberts, W.L., Marshall, T., Rosenberry, T.L. 1987. *J. Biol. Chem.* **262:**4441–4444

Inestrosa, N.C., Ruiz, G. 1985. *Dev. Neurosci.* **7:**120–132

Inestrosa, N.C., Silberstein, L., Hall, Z.W. 1982. *Cell* **29:**71–79

Kuffler, S.W., Martin, A.R., Nicholls, J.G. 1984. From Neuron to Brain. Sinauer, Sunderland, Mass.

Lazar, M., Salmeron, E., Vigny, M., Massoulié, J. 1984. *J. Biol. Chem.* **259:**3703–3713

Lee, S.L., Heinemann, S., Taylor, P. 1982. *J. Biol. Chem.* **257:**12283–12291

Lisanti, M.P., Cordes-Darnell, J., Chan, B.L., Rodríguez-Boulan, E., Saltiel, A.R. 1989. *Biochim. Biophys. Res. Commun.* **164:**824–832

Lisanti, M.P., Sargiacomo, M., Graeve, L., Saltiel, A.R., Rodríguez-Boulan, E. 1988. *Proc. Natl. Acad. Sci. USA* **85:**9557–9561

Low, M.G. 1989. *FASEB J.* **3:**1600–1608

Low, M.G., Saltiel, A.R. 1988. *Science* **239:**268–275

Lwebuga-Mukasa, J.S., Lappi, S., Taylor, P. 1976. *Biochemistry* **15:**1425–1434

Marnay, A., Nachmansohn, D. 1938. *J. Physiol (London)* **92:**37–47

Massoulié, J., Bon, S. 1982. *Annu. Rev. Neurosci.* **5:**57–106

McMahan, U.J., Sanes, J.R., Marshall, L.M. 1978. *Nature (London)* **271:**172–174

Méndez, B., Garrido, J., Maldonado, M., Jaksic, F., Inestrosa, N.C. 1984. *Cell Mol. Neurobiol.* **4:**125–142

Miledi, R., Molenaar, P.C., Polak, R.L. 1984. *J. Physiol. (London)* **349:**663–686

Ott, P. 1985. *Biochim. Biophys. Acta* **822:**375–392

Perelman, A., Abeijon, C., Hirschberg, C., Inestrosa, N.C., Brandan, E. 1990. *J. Biol. Chem.* **254:**214–220

Perelman, A., Brandan, E. 1989. *Eur. J. Biochem.* **182:**203–207

Perelman, A., Inestrosa, N.C. 1989. *Anal. Biochem.* **180:**227–230

Perelman, A., Inestrosa, N.C. 1990. *Gen. Pharmacol. (In Press)*

Prieto, A.L., Fuentes, M.E., Arqueros, L., Inestrosa, N.C. 1989. *J. Neurosci. Res.* **24:**169–173

Quinn, D.M. 1987. *Chem. Rev.* **87:**955–979
Rama-Sastry, B.V., Sadavongvivad, C. 1979. *Pharmacol. Rev.* **30:**65–132
Roberts, W.L., Benedict, H.K., Rosenberry, T.L. 1987. *Proc. Natl. Acad. Sci. USA* **84:**7817–7821
Roberts, W.L., Myher, J.J., Kuksis, A., Low, M.G., Rosenberry, T.L. 1988*a*. *J. Biol. Chem.* **263:**18766–18775
Roberts, W.L., Rosenberry, T.L. 1986. *Biochemistry* **25:**3091–3098
Roberts, W.L., Santikarn, S., Reinhold, V.N., Rosenberry, T.L. 1988*b*. *J. Biol. Chem.* **263:**18776–18784
Rosenberry, T.L. 1979. *Biophys. J.* **26:**263–290
Rosenberry, T.L., Barnett, P., Mays, C. 1980. *Neurochem. Int.* **2:**135–147
Rosenberry, T.L., Richardson, J.M. 1977. *Biochemistry* **16:**3550–3558
Rotundo, R.L. 1984*a*. *J. Biol. Chem.* **259:**13186–13194
Rotundo, R.L. 1984*b*. *Proc. Natl. Acad. Sci. USA* **81:**479–483
Rotundo, R.L. 1989. *J. Biol. Chem.* **263:**19398–19406
Rotundo, R.L., Fambrough, D.M. 1979. *J. Biol. Chem.* **254:**4790–4799
Rotundo, R.L., Fambrough, D.M. 1980. *Cell* **22:**583–594
Rotundo, R.L., Gómez, A.M., Fernández-Valle, C., Randall, W.R. 1988. *Proc. Natl. Acad. Sci. USA* **85:**7805–7809
Rotundo, R.L., Thomas, K., Porter-Jordan, K., Benson, R.J.J., Fernandez-Valle, C., Fine, R.E. 1989. *J. Biol. Chem.* **264:**3146–3152
Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S., Friedmann, T., Taylor, P. 1986. *Nature (London)* **319:**407–409
Schumacher, M., Maulet, Y., Camp, S., Taylor, P. 1988. *J. Biol. Chem.* **263:**18979–18987
Sikorav, J.-L., Duval, N., Anselmet, A., Bon, S., Krejci, E., Legay, C., Osterlund, M., Reimund, B., Massoulié, J. 1988. *EMBO J.* **7:**2983–2993
Sikorav, J.-L., Krejci, E., Massoulié, J. 1987. *EMBO J.* **6:**1865–1873
Silberstein, L., Inestrosa, N.C., Hall, Z.W. 1982. *Nature (London)* **295:**143–145
Silman, I., Futerman, A.H. 1987. *Eur. J. Biochem.* **170:**11–22
Sorensen, K., Gentinetta, R., Brodbeck, U. 1982. *J. Neurochem.* **39:**1050–1060
Stieger, S., Brodbeck, U., Reber, B., Brunner, J. 1984. *FEBS Lett.* **168:**231–234
Takami, N., Ogata, S., Oda, K., Misumi, Y., Ikehara, Y. 1988. *J. Biol. Chem.* **263:**3016–3021
Torres, J.C., Inestrosa, N.C. 1983. *FEBS Lett.* **154:**265–268
Torres, J.C., Inestrosa, N.C. 1985. *Cell. Mol. Neurobiol.* **5:**303–309
Toutant, J.-P., Richards, M.K., Krall, J.A., Rosenberry, T.L. 1990. *Eur. J. Biochem.* **187:**31–38
Toutant, J.-P., Roberts, W.L., Murray, N.R., Rosenberry, T.L. 1989. *Eur. J. Biochem.* **180:**503–508
Vigny, M., Koenig, J., Rieger, F. 1976. *J. Neurochem.* **27:**1347–1353
Vigny, M., Martin, G.R., Grotendorst, G.R. 1983. *J. Biol. Chem.* **258:**8794–8798
Ziskind-Conhaim, L., Inestrosa, N.C., Hall, Z.W. 1984. *Dev. Biol.* **103:**369–377

Received 19 December 1989; revised 23 April 1990