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Expression of Dimeric and Tetrameric Acetylcholinesterase Isoforms on the Surface of Cultured Bovine Adrenal Chromaffin Cells

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Abstract Acetylcholinesterase is a highly polymorphic enzyme, which can be anchored to the cell surface through several different mechanisms. Dimeric (G2) acetylcholinesterase isoforms are attached by a glycosyl-phosphatidyl-inositol (GPI) linkage, whereas tetrameric (G4) forms are linked through a 20 kilodalton hydrophobic subunit. Although cells of haemopoietic origin contain large amounts of G2 GPI-linked acetylcholinesterase, most tissues express only trace amounts of this isoform. We examined the expression of acetylcholinesterase isoforms in cultured bovine adrenal medullary chromaffin cells. Two major isoforms (G2 and G4) were identified on the cell surface. The G2 isoform, which accounted for approximately half the cell-surface enzyme activity, was linked to the membrane through a GPI anchor. After treatment with diisopropylfluorophosphate to completely inhibit cellular acetylcholinesterase, the G4 isoform was found to be resynthesised and transported to the cell surface more rapidly than the G2 isoform. As the addition of GPI anchors is known to be a very rapid step, this finding suggested that the G2 and G4 isoforms might be transported to the cell surface by two different mechanisms. This conclusion was supported by results from subcellular fractionation experiments. The ratio of G4/G2 membrane-bound acetylcholinesterase varied between different subcellular fractions. The membrane-bound G2 isoform was greatly enriched in a high-speed "microsomal" fraction. G4 acetylcholinesterase is known to be actively secreted by chromaffin cells in culture. Although the G4 isoform was present on the cell surface, most of the secreted enzyme was derived from an intracellular pool. Thus, it is unlikely that the cell-surface G4 isoform contributes significantly to the pool of secreted enzyme. Instead, the expression of two different membrane-bound isoforms may provide a means by which chromaffin cells can target the enzyme to different locations on the cell surface.

Key words: protein transport, chromaffin cell, organophosphorus, GPI-linked, phosphatidyl-inositol-specific phospholipase C

Acetylcholinesterase (AChE, EC 3.1.1.7) is the enzyme responsible for inactivating the neurotransmitter acetylcholine at cholinergic synapses [Silver, 1974]. Many isoforms of AChE have been identified in cholinergic and noncholinergic tissues of vertebrate and invertebrate organisms [Massoulié and Bon, 1982]. The catalytic subunit of AChE has a molecular mass of approximately 70 kilodaltons (kD). Dimeric globular (G2) forms of AChE are attached to membranes through a glycosyl-phosphatidyl-inositol (GPI) anchor [Silman and Futerman, 1987], whereas tetrameric globular (G4) forms are attached through a 20 kD noncatalytic subunit [Inestrosa et al., 1987]. Asymmetric (A) isoforms containing up to 12 subunits are anchored to basement membranes through a collagen-like "tail" component [Anglister and Silman, 1978], which is the product of a separate gene [Krejci et al., 1991]. High proportions of GPI-linked AChE have been identified in *Torpedo* electric organ [Futerman et al., 1985], *Xenopus* skeletal muscle [Inestrosa et al., 1988], sheep and rabbit platelets [Majumdar and Balasubramanian, 1985; Shukla, 1985], and mammalian erythrocytes [Low and Finean, 1977]. Minor amounts have also been localised to the motor end plates of mouse muscle [Garcia et al., 1988].

The expression of G2 GPI-linked AChE is controlled by a specific mRNA splicing event which generates a unique C-terminal amino acid sequence that is subsequently trimmed by a specific protease [Ferguson and Williams, 1988;

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Sikorav et al., 1988]. In contrast, the production of other membrane-bound AChEs such as the G4 isoform is probably regulated by the expression of the specific membrane-anchoring subunits [Krejci et al., 1991]. The mechanism by which the membrane-bound G4 and G2 isoforms are subsequently targeted to the cell surface is not well understood.

In this study, we examined the expression and cell-surface targeting of AChE isoforms using isolated adrenal medullary chromaffin cells in culture. Chromaffin cells are of neural crest origin and are known to express both soluble and membrane-bound forms of AChE [Mizobe and Iwamoto, 1983; Mizobe and Livett, 1983; Mizobe et al., 1984; Prieto et al., 1989; Bon et al., 1990]. AChE is actively secreted by chromaffin cells [Mizobe and Livett, 1980]. Stimulation of chromaffin cells with nicotinic agonists in vitro causes increased secretion of AChE and catecholamines [Mizobe and Livett, 1983; Small et al., 1993]. Increased AChE secretion is also observed in isolated perfused bovine adrenal glands following electrical field stimulation of splanchnic nerve terminals [Small et al., 1993].

As GPI-linked AChE has been reported in homogenates of adrenal medulla and in fractions enriched in plasma membrane or chromaffin granules [Bon et al., 1990; Prieto et al., 1989], we examined whether G2 GPI-linked AChE was expressed on the surface of chromaffin cells in culture. We show that adrenal medullary chromaffin cells express high levels of GPI-linked G2 AChE, and we provide evidence that the transport of the G2 isoform to the cell surface differs from that of the membrane-bound G4 isoform.

EXPERIMENTAL PROCEDURES

Materials

Acetylthiocholine iodide, dithionitrobenzoic acid, diisopropylfluorophosphate (DFP), BW284c51 (BW), Fast Red TR, β-galactosidase (from *E. coli*), catalase (from bovine liver), Naphthol AS-MX phosphate, and alkaline phosphatase (from *E. coli*) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylinositol-specific phospholipase C (PIPLC) purified from *B. cereus* was purchased from Boehringer-Mannheim (Mannheim, Germany).

Isolation and Culture of Chromaffin Cells

Bovine adrenal glands were obtained fresh from a local abattoir and digested with collagenase to isolate chromaffin cells as previously described [Livett et al., 1987]. Cells were cultured on collagen-coated plates at densities of either 1.6×10^6 cells/well (in 6 well plates) or 0.5×10^6 cells/well (in 24 well plates), and used 3 days after plating [Livett et al., 1987]. For experiments requiring serum-free medium, cells were cultured in a medium containing equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco Laboratories, Grand Island, NY).

Incubation With AChE Inhibitors

To inactivate total cell AChE, chromaffin cells were incubated with 10^{-4} M DFP in Hank's Balanced Salt Solution (HBSS) containing $CaCl_2 \cdot 2H_2O$ (185.5 mg/l), $MgSO_4 \cdot 7H_2O$ (200 mg/l), KCl (400 mg/l), KH_2PO_4 (60 mg/l), NaCl (8.0 g/l), Na_2HPO_4 (4.75 mg/l), D-glucose (1.0 g/l), and $NaHCO_3$ (350 mg/l), pH 7.4, at ambient temperature for 15 min with gentle shaking. To determine the proportion of AChE present on the surface of isolated chromaffin cells in culture, cells were incubated with the nonpermeant AChE inhibitor BW (1 mg/ml) to inhibit cell-surface AChE reversibly. BW has a low lipid solubility and does not cross cell membranes. To selectively inactivate intracellular AChE, chromaffin cells were incubated with 1 mg/ml of BW in HBSS for 15 min at ambient temperature; then DFP was added to a final concentration of 10^{-4} M, and the cells were incubated at ambient temperature for a further 15 min. Following this treatment, inhibitors were removed by washing the cells three times with HBSS. The cells were then extracted with a high-salt buffer containing detergent and the extracts assayed for AChE.

High-Salt Detergent Extraction of AChE

Chromaffin cell monolayers were scraped from the surface of culture dishes into 50 mM Tris-HCl + 0.5 M NaCl, pH 7.4, containing 0.5% (v/v) Triton X-100 (extraction buffer) at a ratio of 0.2 ml buffer per 10^6 cells. Cell suspensions were chilled on ice and then sonicated (20 bursts at 50% intermittency, setting 3 using a Branson sonifier) to disrupt cells. Extracts were centrifuged (12,000*g* at 4°C for 30 min) in a Beckman microfuge E and the supernatant fraction assayed for AChE activity or analysed by sucrose density gradient centrifugation.

AChE Assay

AChE was routinely assayed by the method of Ellman et al. [1961]. One unit of activity was defined as the number of micromoles of acetylthiocholine hydrolysed per minute at 30°C. For the assay of cell-surface AChE activity, the method of Rotundo [1983] was employed, except that acetylthiocholine (ASCh) was used as the substrate rather than radiolabelled acetylcholine. The product of the reaction, thiocholine (SCh), was detected and quantified using the method of Ellman et al. [1961]. Briefly, chromaffin cells were incubated on ice in the presence of HBSS containing 10 mM HEPES buffer, pH 7.4, containing 10 mM ASCh. After an appropriate time on ice, the medium was removed and the level of SCh in the medium determined. Control experiments showed that when assays were performed on ice, there was negligible contribution to the assayed AChE by secreted AChE.

Incubation With PIPLC

Cells were incubated in phosphate-buffered saline (PBS) with 1 U/ml of phosphatidylinositol-specific phospholipase C (PIPLC) at 37°C for 1 h. Control incubations contained PBS alone. The incubation medium was collected and centrifuged at 12,000*g* at 4°C for 15 min using a Beckman microfuge E, or a JA-20 rotor in a Beckman J2-21M/E refrigerated centrifuge.

Density Gradient Analysis of AChE Isoforms

Prior to sucrose gradient analysis, samples were centrifuged at 40,000*g* at 4°C for 30 min. Samples (up to 200 μl) were loaded onto 12 ml linear gradients of 5–20% (w/v) sucrose in extraction buffer. Gradients were centrifuged at 150,000*g* for 20 h at 4°C in a Beckman SW40 rotor. Approximately 40 fractions of 0.3 ml were collected from the bottom of each tube.

Subcellular Fractionation

Chromaffin cells were fractionated by a modification of a previously described procedure [Bon et al., 1990]. All procedures were carried out on ice or at 4°C. Cells were scraped into 0.3 M sucrose buffered with 5 mM Tris-HCl, pH 7.2 (buffered sucrose), and homogenized on ice with a hand glass homogenizer. The homogenate was centrifuged in a Beckman L2-21M/E centrifuge with JA-20 rotor at 1,000*g* (3,000 rpm) for 15 min and the pellet retained (low-speed pellet). The supernatant from the 1,000*g* spin was centrifuged at 20,000*g* (13,000 rpm, JA20 rotor, 30 min) and the pellet retained (medium-speed pellet). The supernatant from the 20,000*g* spin was then centrifuged in a Beckman L8-8M ultracentrifuge using a Ti 40.3 rotor at 100,000*g* (38,500 rpm) for 1 h and the pellet retained (high-speed pellet). A chromaffin granule-enriched fraction was prepared from the medium-speed pellet. The pellet was resuspended in 1.0 ml of buffered sucrose and then washed by a second centrifugation at 20,000*g* (13,000 rpm, JA20 rotor, 30 min). The washed pellet was resuspended in 1.0 ml of buffered sucrose and layered onto 5.0 ml of 1.6 M sucrose containing 5 mM Tris-HCl buffer, pH 7.2, in a 13 × 64 mm Ultra-Clear centrifuge tube (Beckman). The sucrose gradient was then centrifuged at 127,000*g* (40,000 rpm, Ti 40.3 rotor) for 1 h. The pellet enriched in chromaffin granules was retained (chromaffin granule fraction). The material remaining in the upper sucrose layer was collected, diluted 1:5 with 5 mM Tris-HCl, pH 7.2, and centrifuged for 30 min at 20,000*g* to yield a pellet (nongranule fraction). To remove contaminating soluble AChE, each membrane fraction was resuspended in 5 mM Tris-HCl, pH 7.2, hypotonically lysed by sonication (20 bursts in a Branson sonifier at 50% intermittency, setting 3), subjected to a freeze-thaw cycle, and then centrifuged at 127,000*g* (Ti 40.3 rotor) for 1 h and the supernatant fractions discarded. The pellets were resuspended in extraction buffer, sonicated again, and then centrifuged at 100,000*g* for 1 h. The supernatant fractions, containing the solubilized membrane-associated AChE isoforms, were analysed by sucrose density gradient centrifugation.

Other Assays

β-Galactosidase and catalase were assayed according to the methods of Sambrook et al. [1989] and Cohen et al. [1970], respectively. Alkaline phosphatase was assayed by incubating 30 μl aliquots with 300 μl of a freshly prepared 1:1 mixture of 1 mM Naphthol ASMX phosphate in H_2O and 20 mM Fast Red TR in 50 mM sodium borate containing 5 mM $MgCl_2$, pH 9.8, at ambient temperature until colour developed (approximately 30 min).

RESULTS

Cell-Surface AChE

In the absence of BW, DFP inactivated greater than 99% of the total cellular AChE (Fig. 1).

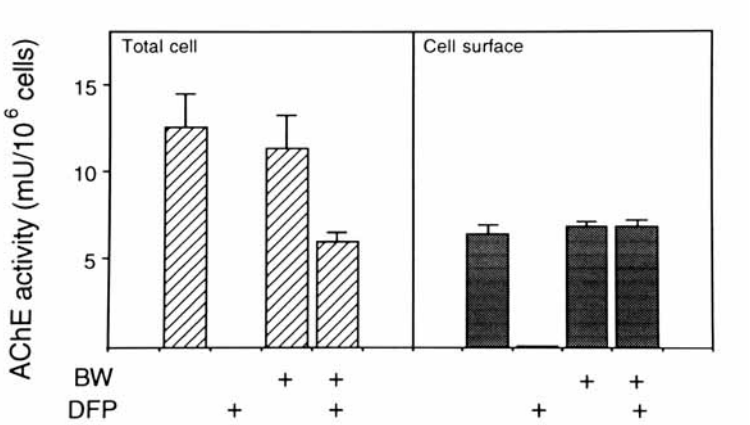

Fig. 1. Effect of AChE inhibitors on the levels of total cell AChE and cell-surface AChE. Bovine chromaffin cells were cultured for 3 days after plating and then incubated in the presence or absence of 1 mg/ml BW at ambient temperature for 15 min. Diisopropylfluorophosphate (DFP) was added in some incubations to a final concentration of 10^{-4} M, and the cells were incubated for a further 15 min. Following this, the cells were washed three times with HBSS. To assay for total cell AChE, the cells were homogenised in 50 mM Tris-HCl + 0.5 M NaCl, pH 7.4, containing 0.5% (v/v) Triton X-100, and a supernatant fraction from the homogenate was assayed by the method of Ellman et al. [1961]. Cell-surface AChE was assayed on intact cells by a modification of the method of Rotundo [1983]. Values are means of four determinations ± SD.

However, cells pretreated with BW prior to incubation with DFP retained 50% of the activity of the untreated cells. This indicated that approximately 50% of the total cell AChE was localised on the cell surface. To confirm this result, cell-surface AChE was assayed directly by incubating intact monolayer cultures of chromaffin cells with the substrate ASCh. The assay was performed on ice to prevent the active secretion of AChE by the cells. BW completely protected cell-surface AChE from inhibition by DFP, as cells incubated with BW prior to inhibition with DFP retained all of the cell-surface AChE activity (Fig. 1). The amount of activity assayed when the incubations were performed on ice was approximately 25% of that determined at 30°C (data not shown), a value similar to the value of 30% calculated previously by Rotundo [1983] for myocyte cell-surface AChE. Therefore, the amount of activity measured on ice was multiplied fourfold to give the amount that would have been measured at 30°C. Using this method, the amount of cell-surface AChE activity was approximately 50% of the total cellular activity (Fig. 1), a value consistent with the value obtained from the assay of the cell homogenates following BW and DFP treatment.

Analysis of AChE Isoforms

To identify the isoforms of AChE on the surface of chromaffin cells, high-salt detergent extracts of the cells were analysed by sucrose density gradient centrifugation. Cells that were not incubated with AChE inhibitors contained three major forms of AChE, corresponding to tetrameric (G4), dimeric (G2), and monomeric (G1) species (Fig. 2). The sucrose density gradient profile was similar to that reported previously [Livett et al., 1983], although the peaks corresponding to the G2 and G1 forms were not well resolved in the previous study. In cultures treated with BW and DFP (to inactivate intracellular AChE), two peaks of activity were observed, corresponding to tetrameric (G4) and dimeric (G2) isoforms. Similar results were obtained from three separate experiments. This result showed that G4 and G2 forms are present on the cell surface, whereas the G1 form is exclusively intracellular.

To examine the possibility that G2 AChE was GPI-linked to the plasma membrane, chromaffin cells were incubated with BW and DFP to inactivate intracellular AChE and then incubated with PIPLC. The cells incubated with PIPLC retained 50% of their cell-surface AChE activity (data not shown), consistent with the previous observation from the sucrose density gradient centrifugation, which indicated that approximately 50% of the cell-surface AChE was in the G2 form. Analysis of the solubilised AChE by sucrose gradient centrifugation showed that only the G2 form was released by PIPLC digestion (Fig. 2).

Substrate Hydrolysis by G2 and G4 AChE

We also compared the abilities of cell-surface G2 and G4 AChE to hydrolyse the substrate

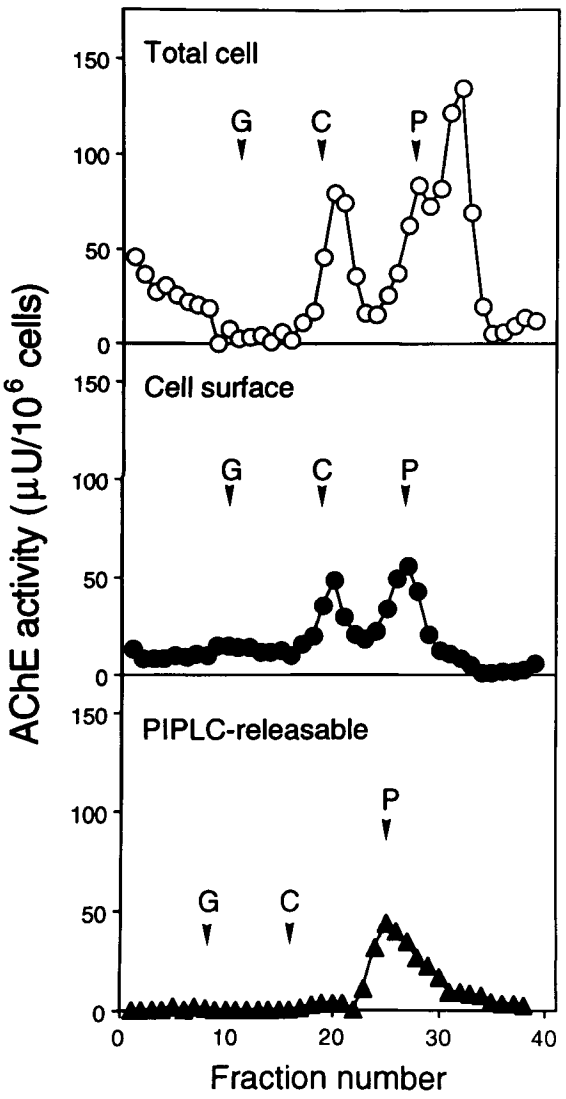

Fig. 2. Sucrose density gradient analysis of AChE isoforms in chromaffin cells. Cells were scraped into 50 mM Tris-HCl + 0.5 M NaCl, pH 7.4, containing 0.5% Triton X-100 and sonicated, the extract centrifuged at 40,000*g* at 4°C for 30 min, and the pellet discarded. Samples (200 μl) were loaded onto 12 ml linear gradients of 5–20% sucrose in the extraction buffer. Tubes were centrifuged in a Beckman SW40 rotor at 150,000*g* for 20 h at 4°C. Fractions of approximately 300 μl were collected from the bottom of the tube and assayed for AChE activity. **Total cell:** Activity profile of total cell homogenate AChE. **Cell surface:** Activity profile of cell-surface AChE. To inactivate intracellular AChE, chromaffin cells were incubated with 1 mg/ml BW for 15 min at room temperature; then DFP was added to a concentration of 10^{-4} M, and cells were incubated for a further 15 min. Inhibitors were removed with three washes of HBSS. **PIPLC-releaseable:** AChE released from chromaffin cells by PIPLC (filled triangles). Cells were incubated with BW and DFP to inactivate intracellular AChE and then incubated in the presence of 1 U/ml PIPLC in PBS for 1 h at 37°C. The positions of the peak fractions of marker enzymes are shown by arrowheads: G, β-galactosidase (16S); C, catalase (11.4S); and P, alkaline phosphatase (6.1S).

ASCh. Chromaffin cells were incubated with BW and DFP to inactivate the intracellular AChE. Following this treatment, the G2 cell-surface AChE was solubilised by incubating the cells with PIPLC. The remaining activity associated with the G4 cell-surface isoform was then solubilised with extraction buffer containing NaCl and Triton X-100. The two preparations of solubilized cell-surface AChE were assayed in the presence of various concentrations of ASCh. The data were analysed by a double reciprocal plot of 1/velocity vs. 1/ASCh concentration (Fig. 3, insert) and the Km values for ASCh calculated from a direct fit of the enzyme kinetic data (Fig. 3) to the Michaelis-Menten equation using an iterative computer-assisted least-squares algorithm. The values for Km obtained were 950 μM for the G2 isoform and 460 μM for the G4 isoform.

Resynthesis of Cell-Surface and Secreted Forms of AChE After DFP Treatment

To examine the rate at which G2 and G4 AChE are transported to the cell surface, chromaffin cells were treated with DFP to inhibit all

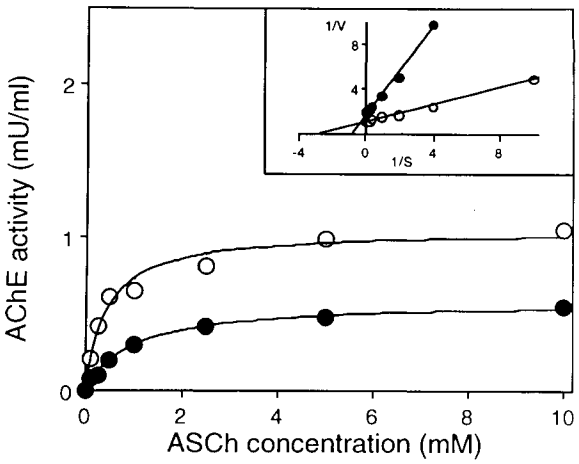

Fig. 3. Activity of G4 (open circles) and G2 (filled circles) AChE as a function of the concentration of substrate (ASCh). Chromaffin cells were treated with BW and DFP to inactivate intracellular AChE, and then cells were incubated with PIPLC to solubilise cell-surface G2 AChE. The remaining G4 AChE was extracted by homogenisation in 50 mM Tris-HCl + 0.5 M NaCl, pH 7.4, containing 0.5% Triton X-100. Extracts of G2 and G4 AChE were centrifuged (40,000*g* at 4°C, 30 min) prior to AChE assay in the presence of various concentrations (0–10 mM) of ASCh. Inset shows a Lineweaver-Burke double reciprocal plot of the data: V, AChE activity in mU/ml; S, concentration of ASCh (mM).

of the cellular AChE. The cells were then incubated in serum-free medium for several hours to allow the recovery of cell-surface AChE activity. A time course study revealed that G2 and G4 AChE were transported to the cell surface at different rates. G4 (PIPLC-resistant) AChE was detected within 1 h of removal of DFP (Fig. 4). However, G2 (PIPLC-sensitive) AChE was not detected until 4 h after removal of DFP. Between 2 and 8 h after removal of DFP from the medium, the ratio of G2 to G4 AChE increased from 0 to 0.65.

As chromaffin cells are known to secrete soluble G4 AChE, we examined the possibility that cell-surface G4 AChE might contribute to the pool of secreted enzyme. Cells were treated with DFP in the presence or absence of BW. Following removal of the inhibitors, the time course of reappearance of AChE in the incubation medium was examined. In cultures not exposed to inhibitors, AChE was detected in conditioned medium within 30 min (Fig. 5). However, in cultures treated with DFP, there was a delay of at least 4 h before the appearance of AChE in the medium. Preincubation with BW before the addition of DFP had no effect on the amount of AChE secretion. This result indicated that most of the AChE detected in the incubation medium was derived from an intracellular pool.

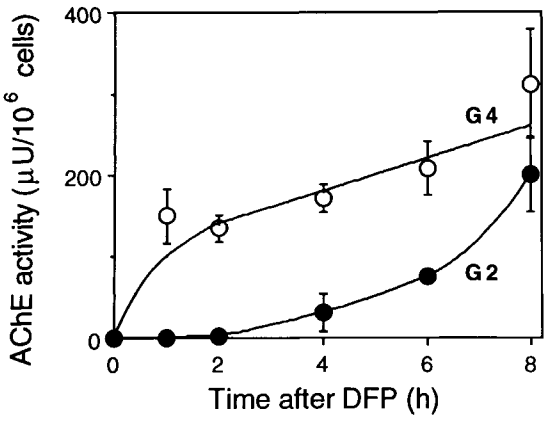

Fig. 4. Time course of de novo synthesis of cell-surface G4 (open circles) and G2 (filled circles) AChE. Three days after plating, cells were incubated with 10^{-4} M DFP in HBSS for 15 min to inactivate both cell-surface and intracellular forms of AChE. The cells were washed three times with HBSS and then incubated with serum-free medium at 37°C. At various times after removal of DFP, the amount of G2 and G4 AChE was assayed. The amount of G2 AChE was defined as the amount released by incubation with PIPLC. The amount of G4 AChE was defined as the amount of cell-surface AChE that was not released by incubation with PIPLC. Values are means of three determinations ± SD.

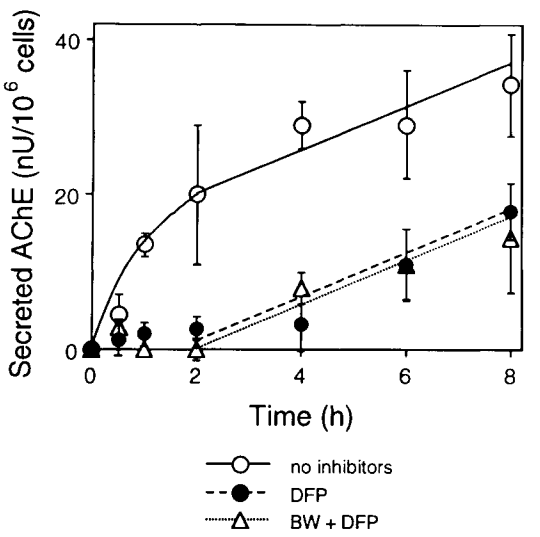

Fig. 5. Time course of AChE secretion from chromaffin cells after pretreatment with DFP in the presence or absence of BW. Chromaffin cells were preincubated at ambient temperature with HBSS only for 30 min (open circles), HBSS for 15 min followed by 10^{-4} M DFP for 15 min (filled circles), or 1 mg/ml BW for 15 min, with DFP added to a final concentration of 10^{-4} M for a further 15 min (open triangles). After washing with HBSS, the cells were incubated in serum-free medium. At various times, the medium was assayed for AChE. Similar results were obtained in two separate experiments. Points show the mean of four determinations ± SD.

Subcellular Fractionation of AChE Isoforms

The different rates of expression of cell-surface G2 and G4 AChE suggested that each isoform may be translocated to the plasma membrane by a different vesicular pathway or mechanism. To investigate this possibility, we examined the distribution of membrane-bound G2 and G4 AChE in subcellular fractions prepared by differential centrifugation through sucrose. Using this approach, four membrane fractions were obtained: a heavy membrane fraction obtained from a low-speed pellet (LS), a light "microsomal" fraction obtained from a high-speed pellet (HS), a chromaffin granule-enriched fraction (CGM), and a nonchromaffin granule membrane fraction (NGM) of intermediate density, which was recovered in the course of preparing the chromaffin granule fraction. The isoforms of membrane-bound AChE in each fraction were analysed on sucrose density gradients (Fig. 6). The results were consistent with the possibility that the G2 and G4 AChEs are associ-

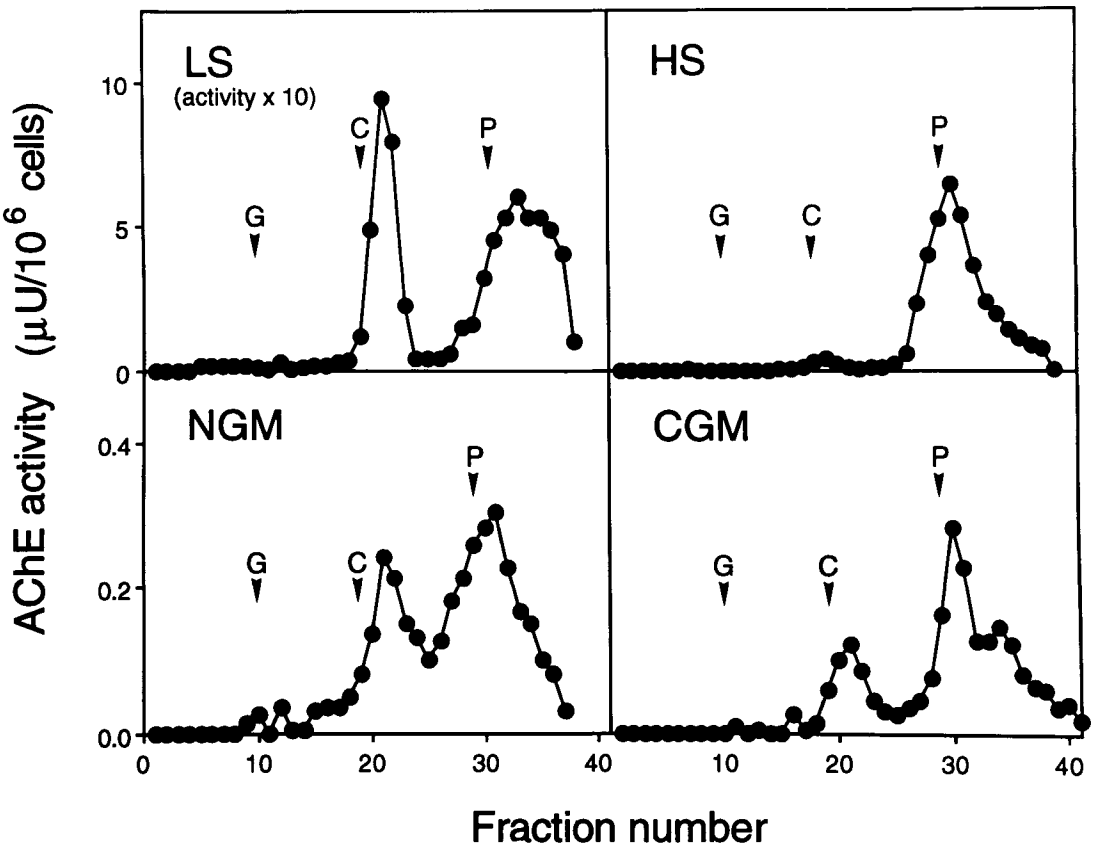

Fig. 6. Isoforms of membrane-bound AChE in subcellular fractions. A membrane preparation was made from four different subcellular fractions, a low-speed (1,000*g*) pellet (LS), a high-speed (100,000*g*) pellet (HS), and a chromaffin granule-enriched pellet (CGM) and a nongranule fraction (NGM) which were prepared from a medium-speed (20,000*g*) pellet. The membrane-bound AChE from these fractions was solubilised in a high-salt detergent-containing buffer and analysed on 12 ml 5–20% sucrose gradients in extraction buffer. Tubes were centrifuged in a Beckman SW40 rotor at 150,000*g* for 20 h at 4°C. Fractions were collected from the bottom of the tube and assayed for AChE activity. Marker enzymes are shown by arrowheads: G, β-galactosidase (16S); C, catalase (11.4S); and P, alkaline phosphatase (6.1S).

ated with different vesicle populations. Marked differences in the AChE activity profiles from the different fractionation procedures were obtained. The low-speed membrane fraction contained the greatest proportion of G4 AChE, whereas the high-speed fraction (HS) contained the greatest proportion of G2 AChE. Intermediate amounts of each isoform were detected in the chromaffin granule-enriched membrane fraction (CGM) and in the nonchromaffin granule fraction (NGM).

DISCUSSION

This study demonstrates that two major forms of AChE (G4 and G2) are expressed on the surface of bovine adrenal medullary chromaffin cells in culture. The G4 AChE was not released when cells were incubated with PIPLC. This form of AChE was similar in its molecular weight to the major membrane-bound form found in bovine brain, which is anchored to membranes through a 20 kD noncatalytic subunit [Inestrosa et al., 1987]. The G2 isoform was released from the membrane when cells were incubated with PIPLC, indicating that it was associated with the membrane through a GPI moiety. The G2 isoform was similar to the form of AChE that is associated with erythrocyte membranes [Futerman et al., 1985; Low and Finean, 1977; Toutant et al., 1989].

Time course studies following DFP treatment demonstrated that the G4 isoform is resynthesised rapidly. Within 1 h of removal of inhibitor, more than 10% of the original G4 cell-surface isoform was resynthesised. However, the G2 isoform was not detected until 4 h after removal of the DFP. Studies on GPI-linked proteins

suggest that the step of adding a glycolipid anchor to proteins is very rapid [Ferguson and Williams, 1988]. The 4 h delay before resynthesised G2 AChE was detected on the cell surface may therefore have been due to a slower transport rate to the cell surface. A slower rate of transport would be consistent either with the possibility that G4 and G2 AChE are processed through two different intracellular pathways or that each isoform is translocated through the same pathway but at a different rate. We cannot completely eliminate the possibility that DFP treatment may have had a nonspecific effect on the processing of one AChE isoform. However, the results of an analysis of the membrane-bound isoforms of AChE in different subcellular fractions were also consistent with the conclusion that the G4 and G2 AChE are normally processed separately. The ratio of G4/G2 AChE varied in different subcellular fractions, suggesting that each AChE isoform may be associated with a different vesicle population.

The finding that most of the membrane-bound AChE activity was associated with a high-speed "microsomal" fraction is also consistent with previous findings. Studies by Lewis and Shute [1969], Somogyi et al. [1975], and Carmichael [1984] have shown that in chromaffin cells AChE is predominantly localized to the rough endoplasmic reticulum. Our own studies using Brefeldin A, an inhibitor of the translocation of proteins from the endoplasmic reticulum to the Golgi apparatus [Fujiwara et al., 1988], indicate that soluble AChE can reside within the endoplasmic reticulum for several hours prior to secretion [Small et al., 1993]. Thus certain forms of AChE may need to be extensively posttranslationally processed in the endoplasmic reticulum prior to their transport to the cell surface for secretion or incorporation into the plasma membrane.

The major secreted AChE is known to be a G4 isoform [Hodgson and Chubb, 1983]. However, our experiments clearly indicate that the G4 cell-surface isoform is not the precursor of most of the secreted AChE. Instead, the secreted AChE is derived from an intracellular pool. The evidence for this is as follows. First, inhibition of intracellular AChE isoforms under conditions in which the cell-surface AChE was protected from inhibition led to complete inhibition of AChE secretion. Second, the time course of resynthesis of the membrane-bound G4 isoform after DFP inhibition was more rapid than the time course of appearance of soluble AChE in the incubation medium. Previous studies [Mizobe and Livett, 1983; Mizobe et al., 1984; Small et al. 1993] have demonstrated that secreted AChE is unlikely to be derived from chromaffin granules, as the release of AChE following nicotinic stimulation does not parallel the release of catecholamines. Somogyi et al. [1975] proposed that AChE can be released from cells directly from an endoplasmic reticulum compartment. However, as AChE release from chromaffin cells is blocked by Brefeldin A, AChE is more likely to be secreted from a population of post-Golgi vesicles distinct from the chromaffin granules.

The reason for the presence of two different forms of AChE on the surface of chromaffin cells is unclear. It is unlikely that the two AChE isoforms have significantly different abilities to hydrolyse acetylcholine, as the Km values for hydrolysis of ASCh were similar for both isoforms. The Km values calculated for bovine chromaffin cell AChE were within the range of values (10^{-4}–10^{-3} M) reported for the hydrolysis of ASCh by AChE purified from other mammalian tissues [Ellman et al., 1961; Silver, 1974]. It has been proposed that a population of GPI-linked proteins may represent a pool of readily releasable protein [Cross, 1986; Low et al., 1986; Silman and Futerman, 1987]. However, as G2 AChE is not released by adrenal chromaffin cells [Chubb and Smith, 1975], this possibility also seems unlikely.

The presence of two different membrane-anchoring mechanisms may provide a means by which chromaffin cells can target AChE to two different sites on the cell surface. There is increasing evidence to suggest that AChE regulates more than one physiological process [Small, 1990]. It is unlikely that all of the AChE synthesised by chromaffin cells is needed to terminate the cholinergic response, as almost all of the AChE activity in chromaffin cells must be inhibited before a significant increase in responsiveness to acetylcholine is seen [Mizobe and Livett, 1982]. Thus some of the AChE on chromaffin cells may have functions which are unrelated to the termination of cholinergic neurotransmission.

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REFERENCES

Anglister L, Silman I (1978): Molecular structure of elongated forms of electric eel acetylcholinesterase. J Mol Biol 125:293–311.

Bon S, Bader MF, Aunis D, Massoulié J, Henry JP (1990): Subcellular distribution of acetylcholinesterase forms in chromaffin cells. Eur J Biochem 190:221–232.

Carmichael SW (1984): Acetylcholinesterase activity of adrenal chromaffin vesicles. Acta Histochem 75:183–188.

Chubb IW, Smith AD (1975): Isoenzymes of soluble and membrane-bound acetylcholinesterase in bovine splanchic nerve. Proc R Soc Lond [Biol] 191:263–269.

Cohen G, Dembiec D, Marcus J (1970): Measurement of catalase activity in tissue extracts. Anal Biochem 34:30–38.

Cross GMA (1986): Eukaryotic protein modification and membrane attachment via phosphatidylinositol. Cell 48: 179–181.

Ellman GL, Courney KD, Andres V, Featherstone RM (1961): A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95.

Ferguson MAJ, Williams AF (1988): Cell-surface anchoring of proteins via glycosylphosphatidylinositol structures. Annu Rev Biochem 57:285–320.

Fujiwara T, Oda K, Yokota S, Takatsuki A, Ikehara Y (1988): Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J Biol Chem 263:18545–18552.

Futerman AH, Low MG, Michaelson DM, Silman I (1985): Solubilization of membrane-bound acetylcholinesterase by a phosphatidylinositol-specific phospholipase C. J Neurochem 45:1487–1494.

Garcia L, Verdiere-Sahuqu M, Dreyfuss PA, Nicolet M, Rieger F (1988): A dimeric form of acetylcholinesterase anchored through a glycolipid in mouse skeletal muscle. Neurochem Int 13:327–332.

Hodgson AJ, Chubb IW (1983): Isolation of the secretory form of soluble acetylcholinesterase by using affinity chromatography on edrophonium-Sepharose. J Neurochem 41:654–662.

Inestrosa NC, Roberts WL, Marshall TL, Rosenberry TL (1987): Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterases in other tissues. J Biol Chem 262:4441–4444.

Inestrosa NC, Fuenes ME, Anglister L, Futerman AH, Silman I (1988): A membrane-associated dimer of acetylcholinesterase from *Xenopus* skeletal muscle is solubilized by phosphatidylinositol-specific phospholipase C. Neurosci Lett 90:186–190.

Krejci E, Coussen F, Duval N, Chatel JM, Legay C, Puype M, Vanderkerckhove J, Cartaud J, Bon S, Massoulié J (1991): Primary structure of a collagenic tail peptide of *Torpedo* acetylcholinesterase: Co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. EMBO J 10:1285–1293.

Lewis PR, Shute CCD (1969): An electron-microscopic study of cholinesterase distribution in the rat adrenal medulla. J Microsc 89:181–193.

Livett BG, Boksa P, Dean DM, Mizobe F, Lindenbaum MH (1983): Use of isolated chromaffin cells to study basic release mechanisms. J Auton Nerv Syst 7:59–86.

Livett BG, Mitchelhill KI, Dean DM (1987): Adrenal chromaffin cells—their isolation and culture. In Poisner A, Trifaro J (eds): "The Secretory Process." Amsterdam: Elsevier North Holland, pp 171–175.

Low MG, Finean JB (1977): Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidyl-inositol-specific phospholipase C. FEBS Lett 82:143–146.

Low MG, Ferguson MAJ, Futerman AH, Silman I (1986): Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. Trends Biochem Sci 11:212–215.

Majumdar R, Balasubramanian AS (1985): The solubilisation of platelet membrane-bound acetylcholinesterase and aryl acylamidase by exogenous or endogenous phosphatidylinositol specific phospholipase C. Biochem Pharmacol 34:4109–4115.

Massoulié J, Bon S (1982): The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. Annu Rev Neurosci 5:57–106.

Mizobe F, Iwamoto I (1983): Multiple localisation of cellular acetylcholinesterase in cultured adrenal chromaffin cells. Biomed Res 4:543–548.

Mizobe F, Livett BG (1980): Production and release of acetylcholinesterase by a primary cell culture of bovine adrenal medullary chromaffin cells. J Neurochem 35:1469–1472.

Mizobe F, Livett BG (1982): Biphasic effect of eserine and other acetylcholinesterase inhibitors on the nicotinic response to acetylcholine in cultured adrenal chromaffin cells. J Neurochem 39:379–385.

Mizobe F, Livett BG (1983): Nicotine stimulates secretion of both catecholamine and acetylcholinesterase from cultured adrenal chromaffin cells. J Neurosci 3:871–876.

Mizobe F, Iwamoto M, Livett BG (1984): Parallel but separate release of catecholamines and acetylcholinesterase from stimulated adrenal chromaffin cells in culture. J Neurochem 42:1433–1438.

Prieto AL, Fuentes ME, Arqueros L, Inestrosa NC (1989): Phosphatidylinositol-specific phospholipase C solubilised G2 acetylcholinesterase from plasma membranes of chromaffin cells. J Neurosci Res 24:169–173.

Rotundo RL (1983): Acetylcholinesterase biosynthesis and transport in tissue culture. Meth Enzymol 96:353–357.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning: A Laboratory Manual," Vol 3. New York: Cold Spring Harbour Laboratory Press.

Shukla SD (1985): Action of phosphatidylinositol specific phospholipase C on platelets: Non-lytic release of acetylcholinesterase, effect on thrombin and PAF induced aggregation. Life Sci 38:751–755.

Sikorav JL, Duval N, Anselmet A, Bon S, Krejci E, Legay C, Osterlund M, Reimund B, Massoulié J (1988): Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ: Primary structure of the precursor of the glycolipid-anchored dimeric form. EMBO J 7:2983–2993.

Silman I, Futerman AH (1987): Modes of attachment of acetylcholinesterase to the surface membrane. Eur J Biochem 170:11–22.

Silver A (1974): "The Biology of Cholinesterases." Amsterdam: North Holland Publishing.

Small DH (1990): Non-cholinergic actions of acetylcholinesterases: Proteases regulating cell growth and development? Trends Biochem Sci 15:213–216.

Small DH, Michaelson S, Marley PD, Friedhuber A, Hocking A, Livett BG (1993): Regulation of acetylcholinesterase secretion from perfused bovine adrenal gland and isolated bovine chromaffin cells. J Auton Nerv Syst 42:131–142.

Somogyi P, Chubb IW, Smith AD (1975): A possible structural basis for the extracellular release of acetylcholinesterase. Proc R Soc Lond [Biol] 191:271–283.

Toutant JP, Roberts WL, Murray NR, Rosenberry TL (1989): Conversion of human erythrocyte acetylcholinesterase from an amphiphilic to a hydrophilic form by phosphatidylinositol-specific phospholipase C and serum phospholipase D. Eur J Biochem 180:503–508.