Characterization of Acetylcholinesterase in Caco-2 Cells

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Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was solubilized from cultured Caco-2 cells. It was established that this enzyme activity is acetylcholinesterase by substrate specificity (acetylthiocholine, acetyl-β-methylthiocholine>propionylthiocholine>butyrylthiocholine), substrate inhibition, and specificity of inhibitors (BW284c51>iso-OMPA). The acetylcholinesterase activity increased proportional to the degree of differentiation of the cells. Most of the enzyme was membrane bound, requiring detergent for solubilization, and the active site faced the external fluid. Only one peak of activity, which corresponded to a monomeric form, could be detected on linear sucrose density gradients. The sedimentation of this form of the enzyme was shifted depending on whether Triton X-100 or Brij 96 detergent was used. These results indicate that the epithelial-derived Caco-2 cells produce predominantly an amphiphilic, monomeric form of acetylcholinesterase that is bound to the plasma membrane and whose catalytic center faces the extracellular fluid. [Exp Biol Med Vol. 227(7):480–486, 2002]

Key words: acetylcholinesterase; AChE molecular forms; Caco-2 cells; amphiphilic enzyme

Acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7) is one of the most efficient enzymes known, whose primary function is believed to be termination of the action of the neurotransmitter acetylcholine (ACh). AChE can be differentiated from butyrylcholinesterase (BuChE; acylcholine acylhydrolase, EC 3.1.1.8) on the basis of substrate specificity, affinity for selective inhibitors, and excess substrate inhibition (1, 2).

As a result of alternative splicing and post-translational modifications, there are several molecular forms of AChE that can be distinguished by their quaternary structure. The globular forms (G_1, G_2, and G_4) are monomers, dimers, or tetramers of identical 70-kDa subunits. The asymmetric (A_4, A_8, and A_{12}) forms of the enzyme have a collagen-like tail attached to one, two, or three G_4 units (3). These molecular forms have distinct solubility characteristics and can be differentiated on the basis of their known sedimentation coefficients (4, 5). To date, it is unclear why there are six molecular forms, but it might be possible that each form subserves a different function.

AChE has been found in many locations, including skeletal, cardiac, and smooth muscles and nervous tissues, as well as erythrocytes and plasma (4–6). Specifically, a soluble G_1 form has been identified in skeletal and cardiac muscles and nervous tissue, the membrane-bound G_2 form has been identified in erythrocytes, membrane-bound G_4 has been identified in rat diaphragm and nervous tissue, and freely soluble G_4 was identified in rat plasma. In addition, AChE has been identified in several non-cholinergic sites including human keratinocytes (7) and the intestinal epithelium of several species (8–10). No common pattern of cholinesterase distribution along the intestine was observed among the various species. Soluble G_1 was detected in cat, kitten, and rabbit, whereas membrane-bound G_2 and/or G_4 were also observed in the same species. One major drawback to these studies was the use of tissue samples that could contain mixed cell populations, introducing the possibility for contamination by smooth muscle cells or neural elements. This can be avoided by using an *in vitro* model consisting of only one cell type, i.e., human intestinal epithelial cells.

The Caco-2 cell line was established from a well-differentiated colonic adenocarcinoma of a 72-year-old patient. These cells spontaneously differentiate under defined culture conditions to exhibit structural and functional characteristics of mature enterocytes (11). Therefore, Caco-2 cells represent an unique and ideal *in vitro* model of human enterocytes. As such, they have been widely used to assess membrane permeation properties of drugs across the small intestine (11–16), as well as to characterize intestinal cell differentiation (17, 18). These cells have been used as a model system to study differentiation-associated brush-border hydrolases (19–22). A particular advantage of these

This research was supported in part by a grant from the University of Cincinnati Research Council (to G.M.P.).

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Received November 27, 2001.
Accepted March 13, 2002.

1535-3702/02/2277-0480$15.00

cells is that they can be cultured using a variety of techniques, including monolayers, Transwell inserts, and Ussing diffusion chambers (22).

In this study, we have established that Caco-2 cells contain a monomeric form of AChE, most of which requires detergent for solubilization, suggesting that it is a membrane-bound protein. Furthermore, our results demonstrate that the catalytic site is external to the cell surface. Finally, by using different non-ionic detergents, we have shown that the AChE is primarily amphiphilic in nature.

Materials and Methods

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) at passage 18. As described previously, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, and 100 IU/ml penicillin with 100 μg/ml streptomycin (23). When approximately 80% confluent, cells were detached from the bottom of 75-cm^2 flasks using trypsin/EDTA and they were plated at a density of 5–6 × 10^4 cells/60-mm^2 dish. The culture medium was changed every other day until cells were used for experiments. In this study, cells were used between passages 52 and 62.

Preparation of Caco-2 Cell Homogenates. Cell monolayers were washed three times with ice-cold saline and were scraped into 300 μl of total (150 μl initially plus 150 μl rinse) of low-ionic-strength buffer (50 mM Tris HCl, pH 7.4; 0.2 m*M* EDTA) containing protease inhibitors (2.6 m*M* benzamidine, 1 mg/ml bacitracin, 5 m*M* *N*-ethylmaleimide). All subsequent procedures were conducted at 4°C unless noted. The cell suspension was homogenized using a glass-glass homogenizer and was ultracentrifuged at 50,000*g* for 30 min. The supernatant was withdrawn to a microfuge tube and stored on ice while the pellet was rehomogenized in buffer with detergent (low-ionic-strength buffer with 1 M NaCl and 1% Triton X-100). The detergent buffer supernatant was withdrawn to a tube and stored on ice. Samples were analyzed for protein content, based on the method of Bradford (24), using the Bio-Rad kit (Bio-Rad, Hercules, CA).

Separation of Molecular Forms. Sucrose density gradient centrifugation was performed as previously described (2). Two hundred microliters of supernatant samples was layered on 5% to 20% sucrose gradients prepared in homogenization buffer containing detergent. Gradients were ultracentrifuged at 121,000*g* for 15 hr. Fractions (200 μl) were collected from the bottom of the gradients, and 50-μl samples were assayed for enzyme activity and for marker proteins.

Enzyme Assay. Samples were assayed for total ChE, AChE, and BuChE activities using a microplate modification of the method previously described by Ellman (25). Ten-microliter samples of homogenate supernatants or 50-μl samples of gradient fractions were assayed. Iso-OMPA (tetraisopropylpyrophosphoramide) was used at a final concentration of 45 μ*M* to inhibit BuChE, and 10 μ*M* BW284c51 (1:5-bis[4 allyldimethylammoniumphenyl]-pentan-3-one dibromide) was used to inhibit AChE; both inhibitors were used to obtain blank values. These concentrations have previously been shown to be optimal for differentiating AChE and BuChE activities (2). A 20-min room temperature incubation allowed for complete inhibition. One millimolar acetylthiocholine iodide was used as a substrate. Absorbance was monitored at 412 nm using a UVmax microplate reader (Molecular Devices, Sunnyvale, CA) and SOFTmax software, version 2.01.

Localization of AChE. Plates containing Caco-2 cells 15 days after seeding were washed copiously with Hanks' balanced salt solution (HBSS). Control HBSS or echothiophate (1 μ*M* in HBSS) was added to plates in triplicate. Plates were incubated for 5 min at room temperature and were immediately washed as above. Cells were harvested and homogenized, and the resulting supernatants were assayed for enzyme activity. Preliminary studies had indicated that these conditions were optimal for selective inhibition of external facing enzyme.

Brij 96 Solubilization. The use of Brij 96 as a detergent helps to differentiate between amphiphilic and non-amphiphilic forms of the enzyme. In an effort to extract these forms, 15 days after seeding, three pairs of plates with Caco-2 cells were harvested in 300 μl each of homogenization buffer without detergent. Cells were homogenized as described above, with the following modification: prior to ultracentrifugation, the homogenates were divided in one-half, and detergent, either Triton X-100 or Brij 96, was added to a final concentration of 1%, followed by vigorous mixing. After centrifugation, supernatants were applied to sucrose density gradients prepared with the same detergent as used for solubilization. Molecular forms were separated as described above.

Statistical significance was determined using two-way, repeated measures analysis of variance (ANOVA) or two-tailed Student's *t* test.

Results

Preliminary studies (data not shown) provided evidence that Caco-2 cells contained esterase activity with specificity for choline esters. To determine the nature of this cholinesterase, we conducted studies with specific inhibitors, substrate inhibition, and specific substrates. BW284c51 and iso-OMPA were used as selective inhibitors of AChE and BuChE, respectively. Most of the freely soluble Caco-2 cell cholinesterase activity was inhibited with BW284c51, indicating that it was primarily AChE (Table I). Likewise, detergent-soluble enzyme was primarily inhibited by BW284c51. These results were supported by substrate inhibition studies. As characteristic of AChE, and not BuChE, the Caco-2 cell cholinesterase demonstrated a substrate optimum concentration of about 10 m*M* with substrate inhibition at acetylthiocholine concentrations of 33 and 50 m*M*

Table I. Cholinesterase Activity in Caco-2 Cells

Day[b] (buffer)	Enzyme activity (mean ± SEM)[a]		
	Total ChE	Iso-OMPA treated	BW284c51 treated
3			
(L)[c]	79.7 ± 17.8	109.7 ± 5.9	10.3 ± 3.6
(D)[c]	309.2 ± 62.3	256.2 ± 26.6	33.4 ± 19.9
12			
(L)	96.6 ± 34.2	119.2 ± 28.9	5.9 ± 5.9
(D)	1430.1 ± 405.0	1622.2 ± 389.6	8.8 ± 8.8
21			
(L)	169.5 ± 15.5	144.9 ± 22.5	30.5 ± 14.2
(D)	2831.6 ± 593.8	1953.4 ± 224.1	4.9 ± 3.9

[a] International units/plate; $n = 6$ plates.
[b] Number of days in culture after seeding.
[c] L, detergent free buffer; D, detergent-containing buffer.

(Fig. 1A). Furthermore, the substrate preference studies indicated a predominance of AChE. Acetyl-β-methylthiocholine, which is metabolized by AChE and not BuChE, was hydrolyzed at approximately the same rate as acetylthiocholine. The BuChE substrates propionylthiocholine and butyrylthiocholine were poor substrates for the Caco-2 cholinesterase (Fig. 1B). Based on these results, all subsequent studies were conducted in the presence of 45 μ*M* iso-OMPA to inhibit any residual BuChE.

Caco-2 cells were assayed for AChE activity at different stages of differentiation. Three days after seeding, cells are considered undifferentiated; 12 days after seeding, cells are in mid-differentiation, and by 21 days post-seeding, cells are fully differentiated (23). At all three time points, the majority of the activity was located in detergent-supplemented buffer samples. Enzyme activity increased from Day 3 to Day 21 with activity at Day 21 significantly greater than at Day 3, representing approximately a 7.5-fold increase (Fig. 2; $P < 0.05$). Figure 2 also indicates that this increase is almost exclusively the detergent-soluble AChE. It is not yet known if the freely soluble form is a precursor to the detergent-soluble or if these two forms arise independently.

In order to determine which molecular forms of AChE were present in Caco-2 cells, homogenate supernatants were applied to sucrose density gradients, ultracentrifuged, and fractionated. Figure 3 shows a representative sedimentation profile obtained for cells 18 days after seeding. Similar results were obtained with longer culture times, the only change being an increased amount of overall activity. There was no evidence of asymmetric forms on any of the profiles. Marker proteins (alkaline phosphatase and bovine serum albumin) indicated that there was a single peak of AChE activity sedimenting at about 4S. This was determined to represent the G_1 form of AChE. The apparent peak at fractions 21 and 22 is believed to be anomalous, as it did not occur in other gradients (see, for instance Fig. 5).

Because the majority of the enzyme activity in Caco-2 cells proved to be membrane bound, we investigated where

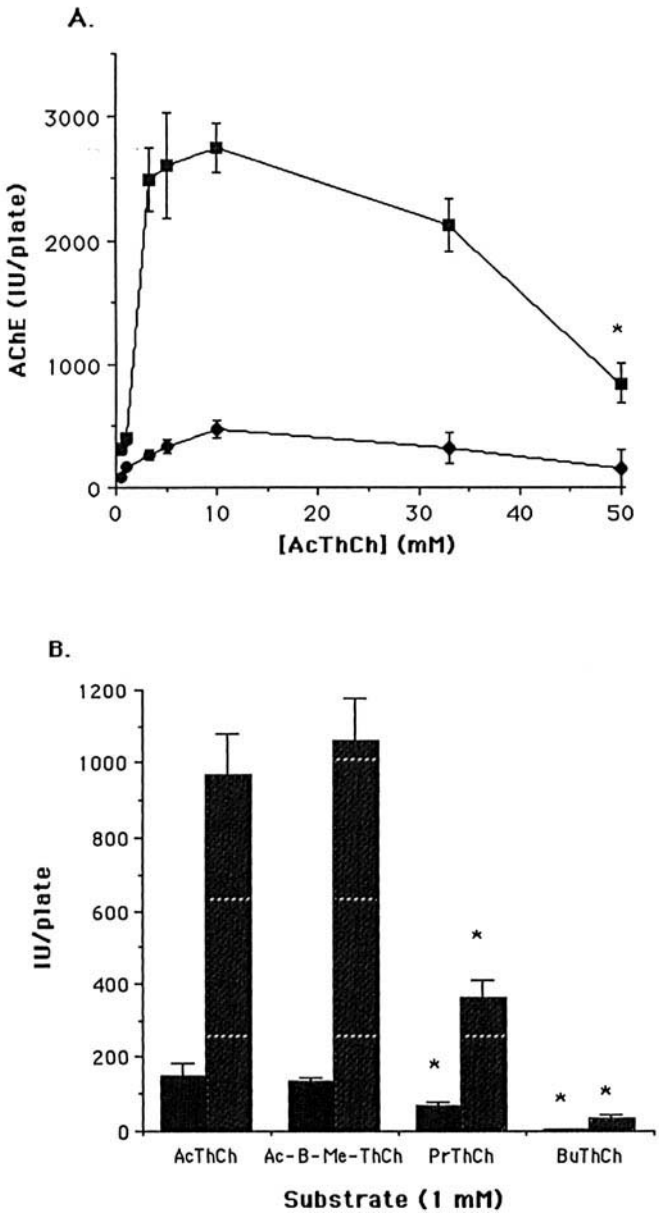

Figure 1. Establishment of characteristics of acetylcholinesterase in Caco-2 cells. (A) Cholinesterase solubilized from three plates was assayed with varying concentrations of acetylthiocholine (0.5–50 m*M*). The enzyme displayed substrate inhibition typical of AChE with the amount of detergent-soluble activity at 50 m*M* different from that at 3.3–33 m*M* (*$P < 0.05$). ●, low-ionic strength soluble; ■, detergent soluble. (B) Cholinesterase solubilized from three plates was assayed with various substrates at a single concentration (AcThCh, acetylthiocholine; Ac-B-Me-ThCh, acetyl-β-methylthiocholine; PrThCh, propionylthiocholine; BuThCh, butyrylthiocholine). No inhibitors were used in this experiment. Filled bars, low-ionic strength soluble; hatched bars, detergent soluble. (*$P < 0.05$ compared with acetylthiocholine).

the enzyme was localized. One micromolar echothiophate, a non-permeant, irreversible inhibitor of AChE, was used to treat cells 15 days after seeding. In cells exposed to echothiophate, 99.3% of detergent-soluble AChE activity was inhibited (Fig. 4, $P < 0.05$) as compared with untreated, suggesting that the enzyme is located on the external leaf of the plasma membrane with the catalytic site exposed to the

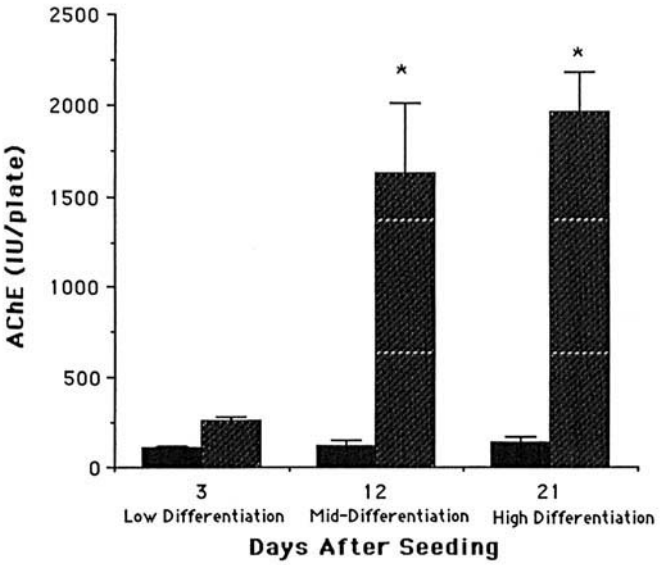

Figure 2. AChE activity in Caco-2 cells relative to different stages of differentiation. Enzyme solubilized from six plates at each time period was assayed (filled bars, low-ionic strength soluble; hatched bars, detergent soluble). Detergent soluble activity at Days 12 and 21 was greater than that at Day 3 (*$P < 0.05$).

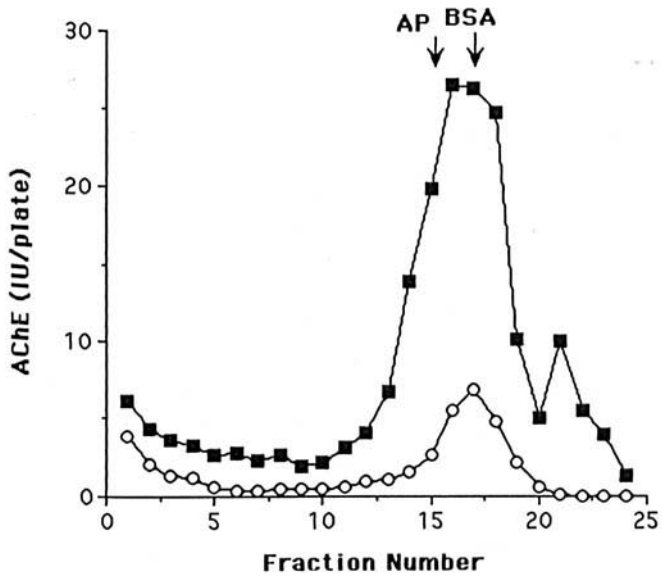

Figure 3. Separation of AChE molecular forms from Caco-2 cells. AChE solubilized from Caco-2 cells 18 days after seeding was separated by sucrose density gradient centrifugation (5%–20% in 50 m*M* Tris HCl buffer containing 1 M NaCl, 0.2 m*M* EDTA, and 1% Triton X-100). Fraction 1 represents 20% sucrose and fraction 24 is 5% sucrose. The peak surrounding fractions 15 through 19 was determined to represent the G_1 form of AChE based on location of marker proteins. Arrows denote marker proteins (A.P., alkaline phosphatase 6.5S; BSA, bovine serum albumin 4.4S). This is a representative profile of enzyme from cells harvested 18 days after seeding. Similar profiles were found with cells at all time periods after seeding. ○, low-ionic strength soluble; ■, detergent soluble.

extracellular fluid. AChE that was soluble in the absence of detergent was not significantly inhibited by the echothiophate.

In an effort to determine if Caco-2 cells possess amphiphilic forms of AChE, cells were homogenized in buffer containing either Triton X-100 or Brij 96. Sedimentation

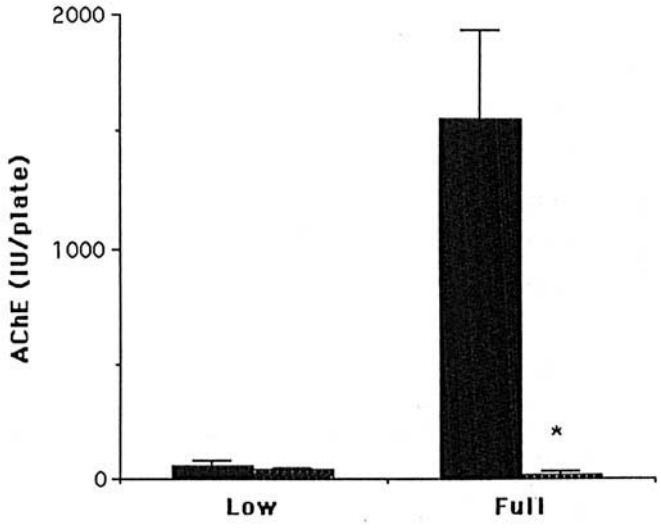

Figure 4. Localization of membrane-bound AChE in Caco-2 cells. Three plates of Caco-2 cells, 15 days after seeding, were incubated for 5 min with 1 μ*M* echothiophate, a non-permeable irreversible inhibitor of AChE, and three plates were untreated under the same conditions. Cell homogenates prepared without (low) and with (full) detergent were assayed for AChE activity. Greater than 99% of the detergent solubilized AChE activity was inhibited (*$P < 0.05$), whereas AChE solubilized without detergent was not significantly affected by the echothiophate (filled bars, untreated controls; hatched bars, echothiophate treated).

profiles were generated from homogenate supernatant samples run on sucrose density gradients containing the same detergent used for solubilization. A shift in the peak of activity, corresponding to the G_1 form of AChE, between the Triton X-100 and Brij 96 sedimentation profiles indicated that Caco-2 cells do contain amphiphilic forms of the monomer (Fig. 5).

Discussion

Caco-2 cells represent a valid *in vitro* model for assessing membrane permeation properties of drugs across the small intestine (11, 12) and as models of small intestinal differentiation (17, 18). Studies of small intestine cells demonstrate that functional differentiation is homogeneous in Caco-2 cells (26). As a model of differentiation, it has been possible to use these cells to study brush-border associated hydrolases (19–21). One such enzyme, not previously characterized in these cells, is AChE.

In the present study, we demonstrated that Caco-2 cells contain AChE as the enzyme assayed exhibited substrate inhibition, a distinct order of substrate affinity as well as differential inhibition. These results are similar to results of others (5) that serve to establish the presence of AChE.

Caco-2 AChE activity was found to increase with differentiation. Activity in detergent-supplemented extracts at Days 12 and 21 was significantly greater than enzyme activity at Day 3. Because the increase in enzyme activity (7.5-fold) was not proportional to the increase in cell number (3-fold; L.R. Plageman, G.M. Pauletti, K.A. Skau, unpublished results), it is suggestive that the enzyme activity increase can be attributed to differentiation as opposed sim-

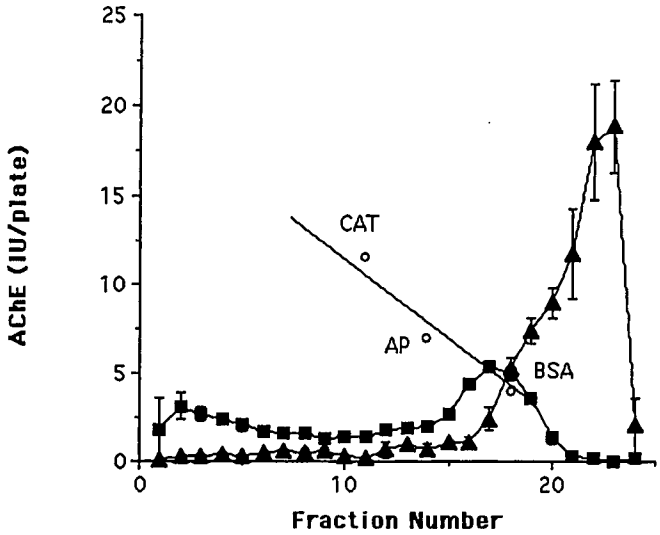

Figure 5. Amphiphilic forms of AChE in Caco-2 cells. Cells from three sets of three plates each were homogenized and the homogenates were subsequently divided in one-half for solubilization with either Triton X-100 (■) or Brij 96 (▲). The solubilized enzyme forms were separated on density gradients prepared with the same detergent as used for solubilization. A shift in the peak of activity with the use of Brij-96 was characteristic of amphiphilic forms. Marker proteins used in each gradient were: BSA, bovine serum albumin 4.4S; A.P., alkaline phosphatase 6.5S; cat., catalase 11.2S.

ply to proliferation. This is consistent with the pattern of induction of other known markers of differentiation. For example, the induction pattern of alkaline phosphatase, a marker protein of brush-border development and of differentiation, increased linearly after confluence, and the activity in differentiated cells (14 days after seeding) was approximately 25 times greater than undifferentiated cells (21).

Sine *et al.* (9) obtained gut mucosal cells by scrapings from various mammalian species, including ox, pig, sheep, cat, rabbit, rat, mouse, and kitten. The intestine was arbitrarily divided into seven sections and scrapings were taken from each section. No common pattern of AChE distribution along the intestine was observed for the various species, and they did not detect any asymmetric forms, a finding with which our results agree. In addition, they found only soluble G_1 in the cat, kitten, and rabbit, whereas they observed membrane-bound G_2 and/or G_4 in the same species. They report "pseudohydrophilic" forms of BuChE in the mouse and rat in which the enzyme was solubilized in detergent-free buffer but whose sedimentation coefficient significantly decreased when analyzed in the presence of Triton X-100. In 1991, Sine *et al.* (10) detected both AChE and BuChE in human intestinal cells isolated by scraping, and they suggested that the enzyme activity was localized near the apex of the villi. In cells scraped from the duodenum, jejunum, ileum, and colon, they found the amount of soluble cholinesterase to decrease distally from the pyloric sphincter. Molecular characterization of soluble fractions indicated AChE existed mainly as the G_1 form in the duodenum and jejunum, whereas G_4-AChE predominated in the ileum and colon. BuChE was essentially present as the G_4 form in all sections of the intestine. The detergent-soluble fraction contained a greater amount of cholinesterase activity, which they attributed to AChE. Again, the G_1 form predominated in the duodenum and jejunum. However, the dimer was predominant in the ileum and colon. Detergent-soluble tetramers were present only in low amounts and only as BuChE. In the current study, Caco-2 cells, which originated as colon carcinoma but differentiate into cells with transport properties indicative of small intestinal cells, exhibited only a monomeric AChE form. The sedimentation profiles were consistent at Days 3, 12, and 21, with the only remarkable change being the overall amount of activity. The major presence of the monomer as well as greater overall activity in the detergent-soluble fraction is consistent with the data reported by Sine *et al.* (9, 10). That we do not detect G_2 or G_4 forms of the enzyme could be attributed to the fact that cultured Caco-2 cells represent a single cell type, whereas scrapings result in heterogeneous populations. It is also possible that the differences could be attributed to the fact that Caco-2 cells are cancer cells, despite a demonstrated maturation similar to normal enterocytes. When Caco-2 AChE solubility characteristics were examined using buffers of different ionic strength and detergent content, the majority of the enzyme was found to be membrane bound (Table I). It is interesting to note that in tissues such as nerve and muscle, it is the G_4 form and not the G_1 that is membrane bound. Thus, membrane-bound G_1 AChE in Caco-2 cells is a unique observation for mammalian cells. There was no evidence that Caco-2 cells secrete AChE (data not shown), which is consistent with the finding that the majority of the activity is membrane bound.

As previously stated, Sine *et al.* (9) reported "pseudohydrophilic" forms of BuChE in mouse and rat in which the enzyme solubilized in detergent-free buffer, but whose sedimentation coefficient significantly decreased when analyzed in the presence of Triton X-100. As reviewed by Massoulie *et al.* (5), amphiphilic forms of AChE, defined as enzyme forms that sediment slower in Brij 96 than in Triton X-100 buffers, have been found in a variety of species and tissues. Caco-2 cells proved to be no exception to this. The sedimentation profile of enzyme solubilized with Brij 96 demonstrated a distinct shift to lower density compared with enzyme solubilized with Triton X-100. Gradients were assayed for marker proteins to calibrate them and demonstrate linearity. Using a linear trendline constructed for the marker proteins, the shifted peak of activity in the Brij 96 solubilized samples exhibited a sedimentation coefficient of 1. The figure clearly shows the presence of amphiphilic forms of the enzyme, but it was difficult to determine whether some or all of the enzyme was amphiphilic. Additional studies are being conducted to clarify this.

To further explore the localization of AChE in these cells, we used the irreversible organophosphate inhibitor, echothiophate. This quaternary agent does not readily penetrate cells and, in our experience, when incubated for short time periods, it will inhibit AChE of living cells if the

catalytic site faces the external milieu. Using these techniques, we found greater than 99% of the membrane-bound activity in Caco-2 cells was externally facing. These data are consistent with those reported by Mizobe *et al.* (27), who found the majority of AChE activity in primary cultures of chromaffin cells to be associated with the outer surface of the cells. Furthermore, because Caco-2 cells grown in culture tend to polarize with the basolateral side adhering to the stratum and the apical side exposed to the medium, it seems likely that the majority of the AChE is on the apical side. However, this cannot be definitively established with cells grown under the current conditions. Studies are underway to more conclusively demonstrate the localization of the enzyme.

The presence of AChE in these cells raises the question of what function the protein subserves. Rimele *et al.* (28) established that intestinal epithelial cells have muscarinic cholinergic receptors that are involved in secretory responses. However, the apparent localization of AChE on the apical side of Caco-2 cells is not consistent with hydrolysis of ACh that would occur on the basolateral side. A nonhydrolytic function of AChE that is gaining increasing support is that of a cell adhesion molecule (29, 30). *In vitro* studies have shown that exogenous AChE promotes neurite growth, that the action is independent of ACh hydrolysis, and that the peripheral allosteric site of AChE may be involved in neurite extension (29). Koenigsberger *et al.* (30) reported that transfecting cultured neurons with a sense AChE cDNA construct led to an increase in AChE expression and an increase in neurite outgrowth, whereas cell lines transfected with an antisense construct demonstrated significant decreases in neurite outgrowth. These effects on neurite outgrowth are generally interpreted to reflect cell adhesion properties. It is possible that AChE may exhibit an adhesive function in Caco-2 cells. However, because our data suggest that the enzyme is located on the apical side of the cell, it remains problematic as to what the protein would adhere.

The complex, polymorphic structure (globular versus asymmetric and hydrophilic versus amphiphilic), as well as the wide tissue distribution of AChE in a variety of species, suggests different functions for the different molecular forms. It is possible that AChE serves to metabolize exogenous esters, but this is unlikely given the enzyme's high degree of substrate specificity; if anything, BuChE would more likely serve such a function. A comprehensive characterization of AChE in Caco-2 cells represents a novel model, an improvement over currently available models, for studying cholinesterases in non-cholinergic sites.

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