

Direct Evidence for an Adhesive Function in the Noncholinergic Role of Acetylcholinesterase in Neurite Outgrowth

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Acetylcholinesterase (AChE) can promote neurite outgrowth through a mechanism that is independent of its role in hydrolyzing the neurotransmitter acetylcholine. It has been proposed that this neuritogenic capacity of AChE may result from its intrinsic capacity to function in adhesion. In this study we directly tested this hypothesis using neuroblastoma cell lines that have been engineered for altered cell-surface expression of AChE. Using a microtiter-plate adhesion assay and the electrical cell-substrate impedance-sensing (ECIS) method, we demonstrate that the level of cell-substratum adhesion of these cells directly correlates with their level of AChE expression. Furthermore, this adhesion is blocked by either an anti-AChE antibody or a highly specific AChE inhibitor (BW284c51), both of which have also been shown to block neurite outgrowth. In addition, cells that overexpress AChE showed enhanced neurite initiation. By employing cell lines with different levels of AChE expression in two types of cell-substratum adhesion assays, our current studies provide evidence for an adhesive function for AChE. These results, together with the fact that AChE shares sequence homology and structural similarities with several known cell adhesion molecules, support the hypothesis that AChE promotes neurite outgrowth, at least in part, through an adhesive function. *J. Neurosci. Res.* 63:165–175, 2001. © 2001 Wiley-Liss, Inc.

Key words: acetylcholinesterase; cell adhesion; neurite outgrowth; neuroblastoma cells

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7), a protein classically known for its enzymatic role in hydrolyzing acetylcholine, can also promote neurite outgrowth through a mechanism that is independent of its catalytic activity (Dupree and Bigbee, 1994; Layer and Willbold, 1995; Grisaru et al., 1999). A specific role for AChE in neurite outgrowth has been suspected since morphological studies documented a close spatiotemporal correlation between transient AChE expression and presynaptogenic axonal outgrowth in vivo (Robertson, 1987; Weikert et al., 1990; Oudega and Marani, 1990; Layer and Kaulich, 1991; DeCarlos et al., 1995; Brimijoin and Hammond, 1996; Koenigsberger et al., 1998). In vitro studies from our laboratory and others have suggested a

direct role for AChE in outgrowth, in that perturbation of AChE on the surface of cultured neurons using certain AChE-specific pharmacological inhibitors or AChE antibodies reduced neurite outgrowth (Layer et al., 1998; Bigbee et al., 1999).

The neuritogenic capacity of AChE was further supported by studies involving genetic manipulation of endogenous AChE expression. Using neuroblastoma cells that were engineered to either overexpress or underexpress AChE, Koenigsberger et al. (1997) demonstrated that the extent of neurite outgrowth in these cells directly correlated with their levels of AChE expression. In an analogous manner, using adenoviral vectors to manipulate endogenous AChE expression in dorsal root ganglion (DRG) neurons, we have also demonstrated the neuritogenic ability of AChE in primary neurons (Bigbee et al., 2000). Altered neurite outgrowth also results from genetic manipulation of AChE expression in C6 glioma and PC12 neuronal cells (Karpel et al., 1996; Grifman et al., 1998).

Although the mechanism underlying the neuritogenic role for AChE is not fully understood, it has been established that its growth-promoting capacity can occur independently of its catalytic activity. This property, originally proposed by Layer et al. (1993), was best demonstrated by Soreq and colleagues (Sternfeld et al., 1998), who showed that a recombinant form of AChE that is catalytically inactive retains the ability to promote neurite outgrowth. Interestingly, neurite outgrowth from cultured neurons can also be affected by acetylcholine (ACh) or its agonists (Lipton and Kater, 1989; Owen and Bird, 1995; Coronas et al., 2000). Thus, by hydrolyzing ACh, AChE could indirectly affect neurite outgrowth in addition to its direct neuritogenic role, which is independent of ACh or AChE catalytic activity (Layer, 1990). These findings emphasize the multifaceted potential of AChE interactions affecting neurite outgrowth (Grisaru et al., 1999).

Contract grant sponsor: Jeffress Trust; Contract grant number: J-519.

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Received 12 July 2000; Revised 12 October 2000; Accepted 18 October 2000

The direct neuritogenic property of AChE has been attributed to an as yet unidentified structural domain of the protein. Support for an adhesive function for AChE is derived from studies that have identified a family of cell adhesion proteins possessing an AChE-like extracellular domain. These adhesive proteins, which belong to the serine esterase family, include neurotactin (de la Escalera et al., 1990; Hortsch et al., 1990), glutactin (Olson et al., 1990), and gliotactin (Auld et al., 1995) in *Drosophila* and neuroligins in mammals (Ichtchenko et al., 1995). Modeling of these proteins, including AChE has led to the identification of a region of negative electrostatic charge on homologous regions of their surface (Botti et al., 1998; Felder et al., 1998). It has been proposed that this conserved "electrotactin" domain may be of functional importance in cell adhesion (Botti et al., 1998).

Although these findings are consistent with the idea that the neuritogenic property of AChE could be mediated through its intrinsic ability to modulate the adhesive properties of neurons, this possibility has not been tested directly. Thus, in this report, we use two independent cell-substratum adhesion assays to test the adhesive properties of neuroblastoma cell lines that have markedly different levels of AChE expression on their surface (Koenigsberger et al., 1997). These cell lines demonstrate the neuritogenic capacity of AChE by showing a bidirectional correlation between AChE expression and neurite outgrowth and provide an ideal system in which to test the extent to which the neuritogenic ability of AChE is mediated through changes in cell-substratum adhesion.

MATERIALS AND METHODS

Materials

Construction and characterization of the neuroblastoma cell lines employed in these studies have been previously described in detail (Koenigsberger et al., 1997). For the current studies, one high-AChE-expressing sense clone, one low-AChE-expressing antisense clone, the transfection control, and the parental cell line were used. Dulbecco's modified Eagle's medium with glutamine (DMEM), a medium designed for serum-free cell culture (OPTIMEM), heat-inactivated fetal bovine serum (FBS), trypsin, and Geneticin were obtained from Gibco BRL (Grand Island, NY). Polylysine, collagen type 1, collagen type IV, and purified mouse laminin were purchased from Becton-Dickinson (San Jose, CA). All tissue culture plasticware was obtained from Falcon (Oxnard, CA). The slide chambers used for the electrical cell substrate impedance-sensing (ECIS) experiments were purchased directly from the manufacturer, Applied Biophysics Inc. (Troy, NY). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted.

Substrate Preparation

Ninety-six well tissue culture plates were used either uncoated or after coating with polylysine, laminin, collagen type I, or collagen type IV. These representative substrata were chosen because they are commonly used for neuronal cell culture and provide a wide range of potential adhesive interactions.

Laminin and polylysine were diluted to 50 $\mu\text{g/ml}$ in sterile calcium- and magnesium-free phosphate-buffered saline, pH 7.4 (CMF-PBS). Collagen type I and collagen type IV were diluted to the same concentration in sterile 0.1 N acetic acid and sterile 0.05 N hydrochloric acid, respectively. One hundred microliters of each coating agent were added per well, and the plates were incubated overnight at 4°C. Control wells were coated and incubated as described above with a sterile solution of 4% bovine serum albumin (BSA) diluted in CMF-PBS. The plates were then washed three times with 100 μl of CMF-PBS to remove excess coating agents, and 100 μl of OPTIMEM medium were added to each well. Plates were then stored in the cell culture incubator for approximately 1 hr while the cells were prepared for use in the adhesion assays.

Cell-Substratum Adhesion Assays

All neuroblastoma cell lines were maintained in DMEM supplemented with 10% FBS in a cell culture incubator at 37°C and 5% CO₂. All cell lines, with the exception of the wild type, were cultured in the presence of the selection antibiotic Geneticin (500 $\mu\text{g/ml}$). The level of AChE expression in these cell lines was confirmed using the colorimetric assay as previously described (Koenigsberger et al., 1997).

Microtiter Plate Adhesion Assay

Cell adhesion assays were performed according to the method of Hall et al. (1987). Briefly, cells were grown to 80% confluence and detached by treatment with 0.05% trypsin in warm DMEM for 2 min. A single cell suspension was obtained by mechanical dissociation by three sequential passages through a sterile 40- μm -mesh nylon cell strainer. Cell number was determined using an automated Coulter counter and the concentration was adjusted to 4×10^5 cells/ml in OPTIMEM. Next, 40,000 cells in 100 μl were added to each well of the previously prepared plates (for a total volume of 200 μl), and the cells were allowed to adhere for 2 hr.

After this time period, 50 μl of OPTIMEM were forcefully ejected into each well using an eight-channel multiwell pipette, followed by aspiration of the medium under gentle vacuum suction. Any remaining unattached cells were removed by gently rinsing two times with warm PBS. The adherent cells were fixed overnight with 2% glutaraldehyde in PBS and stained for 1 hr with 0.005% crystal violet. Excess dye was removed by washing four times with PBS, and the stained cells were lysed by adding 200 μl of 2% sodium dodecyl sulfate (SDS), a step that releases the bound dye, the amount of which is proportional to the number of adherent cells (Hall et al., 1987). To quantitate the amount of released dye, the absorbance at 490 nm was measured in each well using an automated ELISA plate reader. To confirm the results and to assess cellular morphology and neurite initiation, some of the wells were photographed prior to the addition of SDS.

Electrical Cell Substrate Impedance-Sensing Adhesion Assay

The level of AChE expression was directly correlated to physical parameters of cell adhesion using an ECIS system (Applied Biophysics, Inc., Rochester, NY). The ECIS system utilizes a specially designed multiwell cell culture chamber fitted

with gold electrodes, which are submerged in culture medium. This chamber is inserted into an electrode holder that is contained within a cell culture incubator. The incubator is connected to an amplifier that continuously samples impedance and resistance measurements and transfers the data to a computer. As cells attach and spread on the electrode, the impedance and resistance of the electrode change, because the cells act as insulating particles and restrict current flow. Changes in the impedance reveal dynamic information about both cell attachment rate and strength of adhesion (Kowolenko et al., 1990; Giaever and Keese, 1993).

These experiments were performed in parallel with the microtiter plate adhesion assays and the single cell suspensions were prepared exactly as described above. Eight-well ECIS cell culture chambers were used, and 200 μl of serum-free OPTIMEM were placed in each well of the chamber. The multiwell chamber was then placed in the electrode holder for approximately 1 hr and allowed to equilibrate in the incubator while the cells were being prepared. Two hundred microliters of serum-free OPTIMEM containing 80,000 cells or medium only were then added to each well for a total volume of 400 μl . Resistance measurements were recorded every 2 min for 3 hr after addition of the cells.

Cell-Substratum Adhesion Perturbation

The perturbation studies employed the microtiter plate cell adhesion assay and were performed on uncoated tissue culture plastic dishes using the AChE sense cell line. The assay was performed exactly as outlined above except that, prior to addition to the wells, the cells were preincubated for 30 min at room temperature, with either a 1:400 dilution of an anti-AChE polyclonal antibody (supplied by Dr. T. Rosenberry, Mayo Clinic, Jacksonville, FL) or a 100 μM solution of BW2484c51, a highly specific AChE inhibitor. Both agents were prepared in OPTIMEM. After 2 hr, cells that remained adherent after washing were stained with crystal violet, and cell attachment was quantified as outlined above. As a control, normal rabbit IgG was substituted for the anti-AChE antibody.

Statistical Analysis

Statistical significance was determined using one-way ANOVA and Tukey HSD ad hoc analysis. Results were considered statistically different when their *P* values were <0.05 . Regression analysis for correlating the levels of AChE expression with adhesion was performed using the SigmaStat statistical package.

RESULTS

Correlation Between AChE Expression and Neurite Outgrowth

The neuroblastoma cell lines employed in these experiments were genetically engineered for altered AChE expression by Koenigsberger et al. (1997). When compared to the parental cell line, the AChE-overexpressing cells (AChE sense clone) show a 250% increase in AChE expression whereas the AChE-underexpressing cell line (AChE antisense clone) has only 60% of the AChE levels present in the wild-type cells (Fig. 1A). The transfection

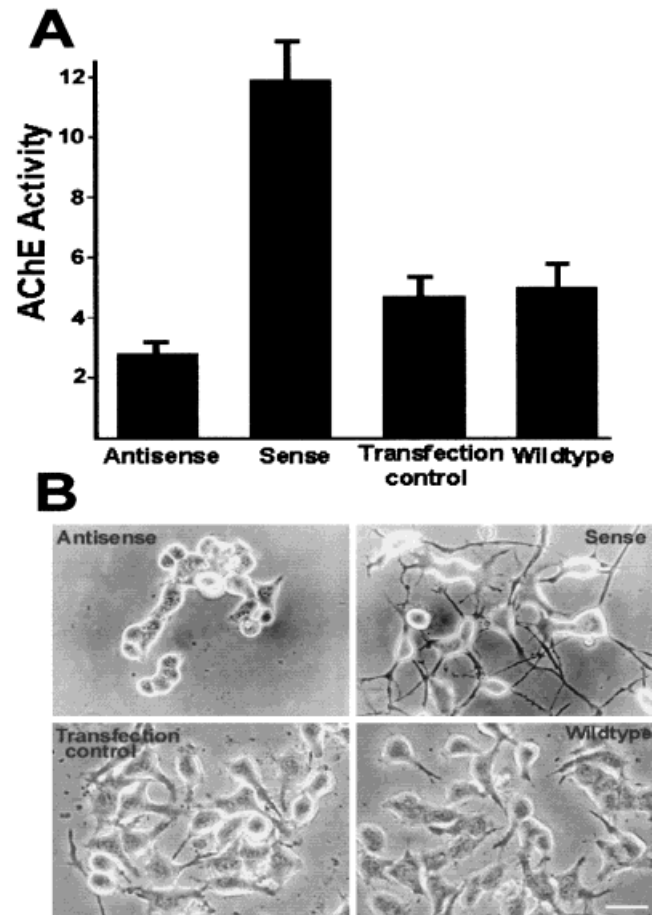


Fig. 1. Characterization of neuroblastoma cell lines genetically engineered for altered AChE expression. Correlation between level of AChE expression and neurite outgrowth in N1E.115 neuroblastoma cells stably transfected with full-length murine AChE cDNA in either the sense or the antisense orientation under the direction of the β -actin promoter (Koenigsberger et al., 1997). As is shown in **A**, stable transfection with β -sense-directing AChE cDNA results in a threefold increase in AChE expression compared to either wild-type or plasmid transfection control. Conversely, transfection with an antisense-directing AChE cDNA results in a 40% reduction in expression. Results are expressed as means \pm SEM for three separate experiments. $P < 0.005$ for sense vs. transfection control; $P < 0.01$ for antisense vs. transfection control. Phase-contrast micrographs (**B**) show the dramatic increase in neurite outgrowth in sense AChE cells compared to either transfection control or wild-type cells. In contrast, antisense AChE cells remain mostly spherical, with occasional short neurites. Scale bar = 60 μm .

control and wild-type cell lines have very similar levels of AChE expression, which is not significantly different. In agreement with the results previously demonstrated by Koenigsberger et al. (1997), the level of AChE directly correlates with the extent of neurite outgrowth (Fig. 1B). AChE-overexpressing cells extended more numerous and longer neurites than either control or wild-type cells. In contrast, underexpressing cells extended significantly fewer and shorter neurites. Insofar as these cells demon-

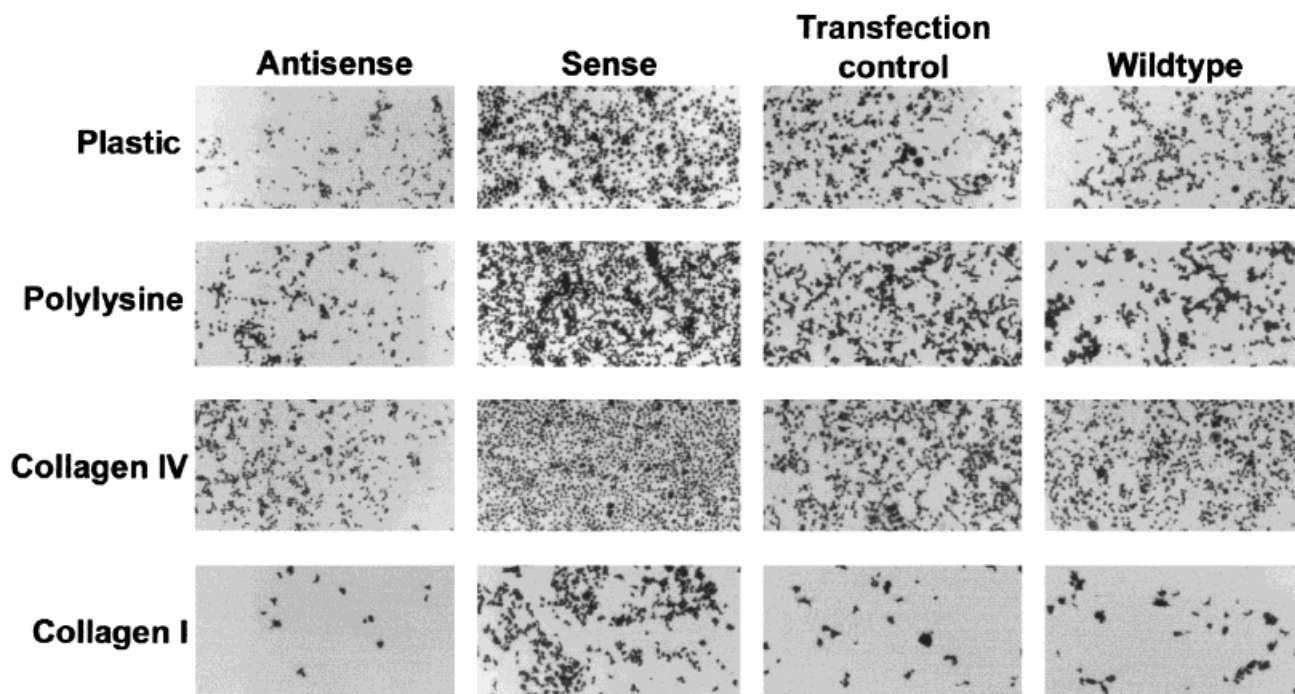


Fig. 2. AChE expression positively correlates with adhesion to plastic, polylysine, collagen type I, and collagen type IV. Low-magnification photomicrographs of crystal violet-stained adherent cells after 2 hr. Note that for each substratum (horizontal rows) the greatest number of adherent cells is seen with the high-AChE-expressing sense cells (sec-

ond vertical column). Conversely, the low-AChE-expressing antisense cells were the least adherent. No significant difference in adhesion was seen between transfection controls and wild-type cells, both of which were intermediate between sense and antisense cells.

strate the direct correlation between AChE expression and neurite outgrowth, they provide a unique tool to investigate the extent to which an adhesive function underlies this correlation.

Correlation of Cell-Substratum Adhesion With AChE Expression

The adhesive properties of these cell lines were first analyzed by testing their capacity to attach to different substrata in a 2 hr microtiter plate cell-substratum adhesion assay. The substrata were selected to provide a range of potential adhesive interactions and are among those commonly used for neuronal cell culture. Figure 2 shows low-magnification photomicrographs of adherent cells stained with crystal violet. When each substratum was examined individually, we consistently observed that the AChE-overexpressing cell line had the greatest number of adherent cells. Conversely, the AChE-underexpressing cell line had the fewest number of adherent cells. The control and wild-type cells had intermediate adhesion levels, which were similar to each other. Insofar as identical numbers of viable cells were introduced at the beginning of the assay, these results imply that variations in the AChE expression directly correlate with the large differences in adhesion to these substrata. Quantification of these results based on the optical density values obtained after extracting the crystal violet dye from the adherent

cells is shown in Figure 3. Compared to the parental control, the high-AChE-expressing cells showed increased binding of 215% for plastic, 130% for polylysine, 222% for collagen type I, and 173% for collagen type IV. In contrast, the low AChE-expressing cells showed 69%, (plastic), 75% (polylysine), 21% (collagen type I), and 66% (type IV collagen) lower binding levels compared to the parental control. For each substratum, the *P* value for high-AChE- vs. low-AChE-expressing cell lines and for high-AChE-expressing cells vs. wild-type and transfection control cells was <0.001 . For low-AChE-expressing cells vs. wild-type and transfection control cells the *P* value was <0.005 . Values for the transfection control cells on all substrata showed no significant difference from the wild-type cell line.

Interestingly, we did not observe a difference in adhesion on the laminin substratum (Fig. 4). In fact, the number of adherent cells on laminin was not different between any of the four cell lines. This result may be due to the fact that laminin, a highly adhesive substratum, provides additional adhesive interactions, such as integrin-mediated adhesion (Smith et al., 1996), which could predominate over the AChE-mediated cell-substratum adhesion. The observation that AChE expression closely correlates with adhesion of neuroblastoma cells to uncoated tissue culture plastic, an interaction in which cell

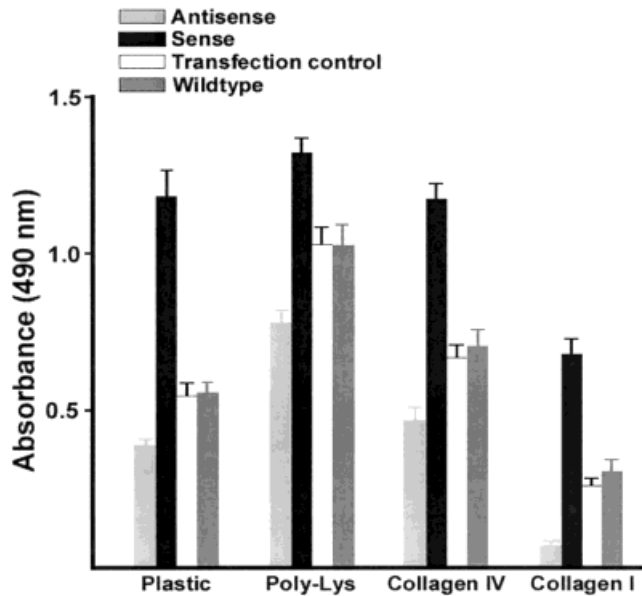


Fig. 3. Quantitation of adhesive properties of neuroblastoma cell lines. Adherent cells were stained with crystal violet; the bound dye was solubilized; and the absorbance, which is proportional to number of adherent cells, was measured in each well. For each substratum tested, the values were highest in wells containing high-AChE-expressing sense cells and lowest in wells containing low-AChE-expressing antisense cells. Results are expressed as means \pm SEM from a minimum of three separate experiments. Statistical significance was determined using one-way ANOVA and Tukey HSD ad hoc analysis. For each substratum, $P < 0.001$ for high- vs. low-AChE-expressing cell lines and for high-AChE-expressing cells vs. wild-type and transfection control cells; $P < 0.005$ for low-AChE-expressing cells vs. wild-type and transfection control cells. Values for the transfection control cells on all substrata were not significantly different from those of the wild-type cell line.

attachment is largely based on electrostatic interaction, may reflect a significant involvement of a charge-based interaction for AChE-mediated adhesion. This finding supports the electrostatic mechanism proposed by Botti et al. (1998) for the serine ester hydrolase family of adhesive proteins.

The correlation between the level of AChE expression and cell-substratum adhesion was examined using regression analysis, and the results are shown in Figure 5. For plastic, polylysine, collagen IV, and collagen I substrata, the level of adhesion, as determined from the amount of solubilized crystal violet dye from adherent cells (values from Figs. 3, 4), shows a linear relationship with increasing AChE expression (values from Fig. 1), with r^2 values ranging from 0.91 to 0.99. These data indicate that a direct correlation exists between the level of AChE expression and adhesion to these substrata. A similar correlation between the level of AChE expression and neurite outgrowth was previously shown for these cell lines (Koenigsberger et al., 1997). The present data suggest that modulation of neuronal adhesion by AChE may underlie

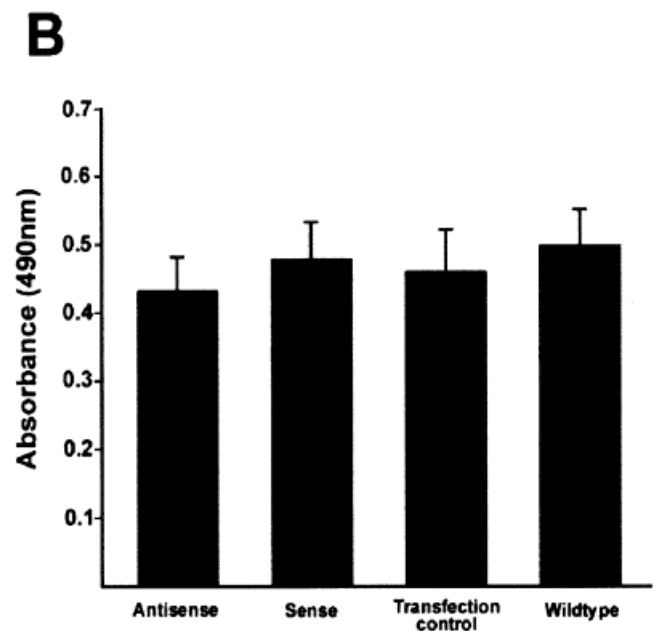
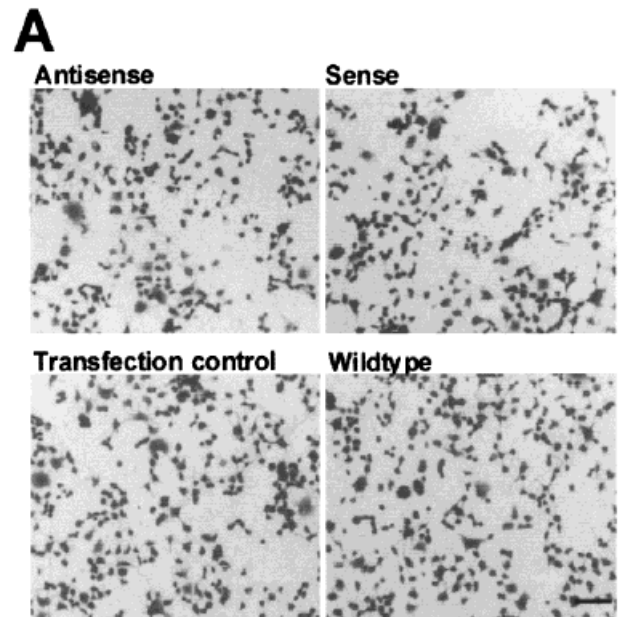


Fig. 4. Cell adhesion on a laminin substratum is independent of AChE expression. When the cell adhesion assays were performed on laminin, results showed that the number of adherent cells was similar among all of the cell lines. **A** shows that similar numbers of cells for each cell line remain adherent after 2 hr. Optical density values for released dye (**B**) also reveal no significant difference in adhesion between the cell lines. Results are expressed as means \pm SEM from a minimum of three separate experiments. Scale bar = 200 μ m.

its ability to promote neurite outgrowth. Interestingly, however, the amount of adhesion to laminin is independent of AChE expression (r^2 value of 0.19), suggesting that other adhesive interactions may predominate on this substratum.

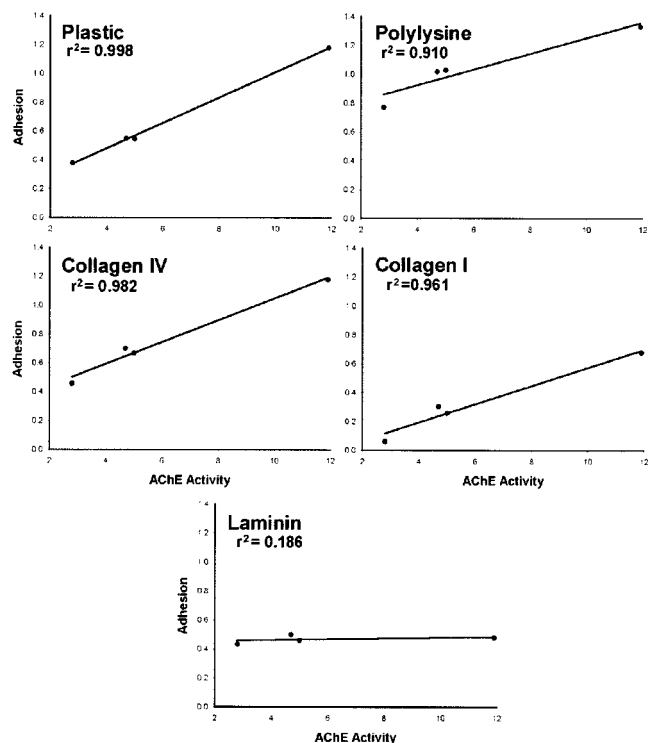


Fig. 5. Regression analysis of AChE expression and cell-substratum adhesion. For each substratum tested, the AChE activity in each cell line (antisense 2.8 OD units/ $\mu\text{g/hr}$, sense 11.9 OD units/ $\mu\text{g/hr}$, wild-type 4.7 OD units/ $\mu\text{g/hr}$, transfection control 5.0 OD units/ $\mu\text{g/hr}$) was plotted against the amount of adhesion for that line as determined by OD measurements of dye solubilized from adherent cells. Whereas the overall level of attachment varied between the different substrata, regression analysis revealed a linear, positive correlation between AChE expression and cell adhesion to plastic, polylysine, collagen IV, and collagen I. In contrast, adhesion to laminin was AChE-independent.

To substantiate the results obtained using the microtiter-plate adhesion assay, we employed a method allowing for continuous monitoring of cell-substratum adhesion beginning at very early time points (ECIS). This approach allowed us to examine the rate of cell attachment as well as the strength of adhesion. In this assay, attachment of cells to an electrode produces a proportional increase in resistance (Kowolenko et al., 1990; Giaever and Keese, 1993). Figure 6 shows one representative tracing from a single ECIS experiment (Fig. 6A) and a composite tracing from seven separate experiments (Fig. 6B). Each cell line is added to previously equilibrated wells at time 0. The initial rise during the first 20 min indicates the *passive* settling of the cells onto the sensing electrode. Thereafter, as cells *actively* attach to the electrode, the resistance increases in proportion to the strength of the attachment. As can be seen in Figure 6, the initial rise in resistance is similar for each cell line, in that equal numbers of cells were added to each well. Thereafter, the largest changes in resistance occurred in wells that contained high-AChE-expressing cells. In contrast, the trace for the low-AChE-expressing

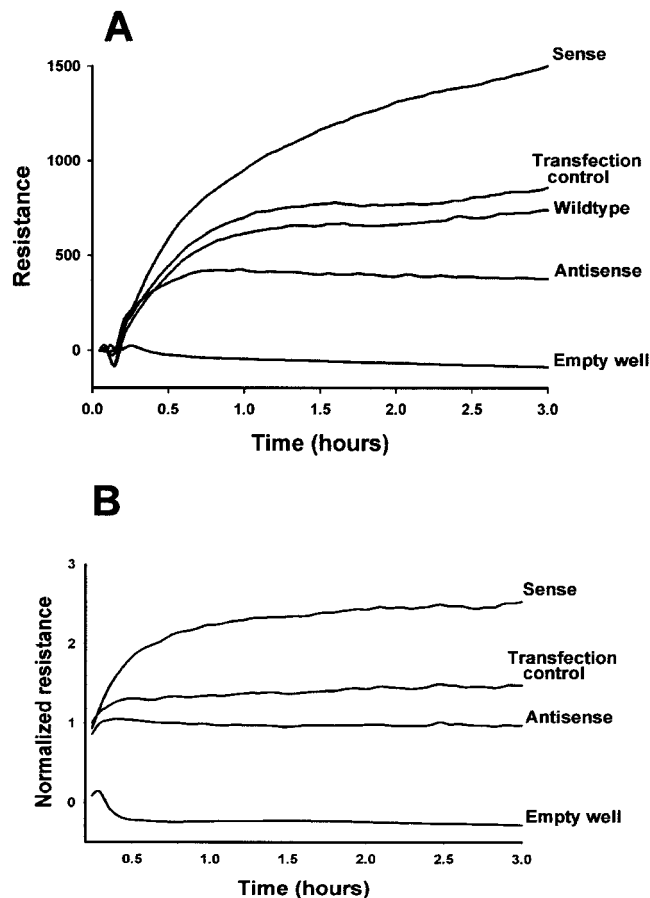


Fig. 6. Cell attachment rate and strength correlate with AChE expression. ECIS recordings of resistance changes in response to cell attachment. The initial resistance increase during the first 20 min after the addition of the cells is due to the passive settling of the cells onto the sensing electrode. As cells actively attach to the electrode, the resistance increases in proportion to the strength of the attachment. A single representative experiment is shown in **A**. Note that the largest resistance changes occur in wells containing high-AChE-expressing sense cells. In contrast, the trace for the low-AChE-expressing antisense cells shows that these cells adhere with the least strength of the four cell lines. The resistance changes for the transfection control and wild-type cells were similar and intermediate between AChE sense and AChE antisense cells. **B**: A summary of multiple experiments ($n \geq 7$) in which resistance values were normalized to the wild-type values within each experiment.

cells shows that they adhere with the least strength. The resistance changes for the transfection controls and wild-type cells were similar and intermediate between the high- and low-AChE-expressing cells.

Agents That Perturb Neurite Outgrowth Also Alter Cell-Substratum Adhesion

We have previously shown that neurite outgrowth can be blocked using either AChE inhibitors or anti-AChE antibodies (Dupree and Bigbee, 1994, 1996; Koenigsberger et al., 1997; Sharma and Bigbee, 1998). To

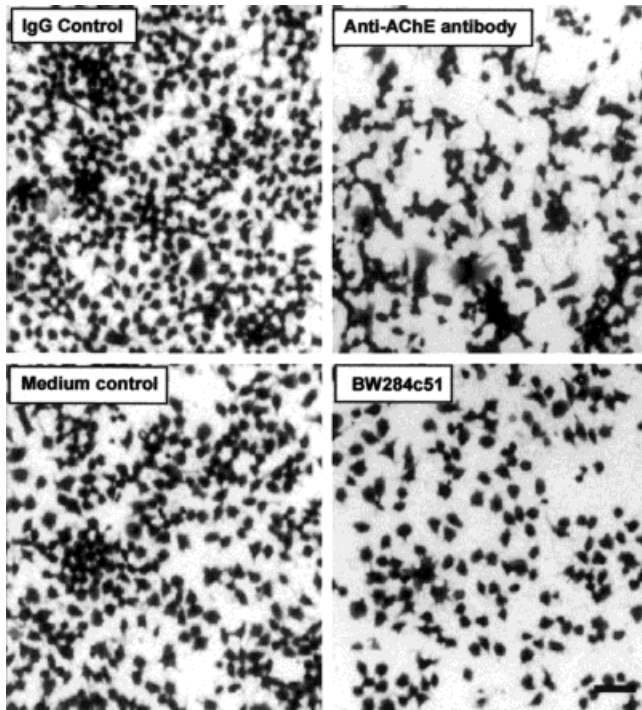


Fig. 7. Perturbation of AChE reduces cell attachment. Photomicrographs of adherent AChE sense cells on plastic after a 30 min preincubation with normal mouse IgG; a polyclonal anti-AChE antiserum (1:400); medium only; or BW284c51, a highly specific AChE inhibitor (100 μ M). BW284c51 and AChE antisera reduced attachment by 30% and 38%, respectively. Scale bar = 100 μ m.

determine the extent to which these same agents could also perturb cell-substratum adhesion, cells overexpressing AChE were incubated either with a 100 μ M solution of BW284c51 or with a 1:400 dilution of an anti-AChE polyclonal antibody prior to addition to the microtiter wells. BW284c51 is a highly specific AChE inhibitor, which has previously been shown to reduce neurite outgrowth (Layer et al., 1993; Dupree and Bigbee, 1994, 1996), and preincubation with BW284c51 reduced cell attachment of the high-AChE-expressing cells by 30% compared to medium alone. The polyclonal AChE antibody labels AChE on the surface of these neuroblastoma cells and has previously been shown to retard neurite outgrowth (Koenigsberger et al., 1997). We show here that preincubation with the AChE antibody reduced adhesion by 38% compared to cells incubated with normal IgG (Fig. 7). These experiments further support the idea that the previously demonstrated inhibition of neurite outgrowth by these agents may involve disruption of AChE-mediated cell-substratum adhesion.

Level of AChE Expression Alters Neurite Initiation

As was described earlier, a direct link has been established between AChE expression and neurite outgrowth in these neuroblastoma cell lines. The results described above indicate that the level of AChE expression

also affects the adhesive properties of these cells. Thus, in the next set of experiments, we examined whether AChE-modulated adhesion might affect the earliest step in neurite outgrowth, namely, neurite initiation. For this, cells were examined at 60 min intervals during a 4 hr period after plating. Upon initial settling, all cells appeared spherical, with smooth outlines. Shortly thereafter, while still maintaining a spherical shape, they extended numerous spikes. Interestingly, comparison of cell lines with different levels of AChE expression revealed dramatic differences in morphology at these early time points (Fig. 8). Whereas the majority of the low-AChE-expressing cells continued to exhibit this spherical morphology, the high-AChE-expressing cells appeared to display a more neuronal phenotype characterized by polygonal cell bodies with long, differentiated neurites. On the other hand, wild-type and transfection control cells displayed a morphology that was intermediate between that of the low- and high-AChE-expressing cell lines.

DISCUSSION

Using neuroblastoma cell lines with markedly different AChE expression levels, the present results show a direct and close correlation between AChE expression and cell-substratum adhesion. We demonstrate that cells with a high level of AChE expression display significantly enhanced cell-substratum adhesion, whereas cells with a low level of AChE expression display decreased adhesion, compared to either the transfection control or the parental cell line. This close and *bidirectional* correlation argues that the level of AChE expression can modulate the adhesive properties of these neuroblastoma cells. Moreover, the enhanced cell-substratum adhesion can be blocked by agents known to perturb the neuritogenic ability of AChE. These results, together with the finding that the level of AChE expression also correlates with neurite initiation, an early event in neurite outgrowth, support the hypothesis that AChE possesses an intrinsic capacity to modulate neuronal adhesion and that, furthermore, this property may underlie the ability of AChE to promote neurite outgrowth.

An adhesive function for AChE in neurite outgrowth was indirectly supported by studies from our laboratory and others demonstrating that treatment of cultured neurons either with AChE inhibitors or with anti-AChE antibodies results in reduced neurite outgrowth and altered neurite fasciculation (Layer et al., 1993; Dupree and Bigbee, 1994; Koenigsberger et al., 1997; Sharma and Bigbee, 1998). Additional evidence in support of an adhesive function for AChE in neurite outgrowth comes from studies demonstrating functional overlap between AChE and other adhesive members of the serine esterase family. Grifman et al. (1998) reported that the expression of neuroligin 1, an AChE-homologous adhesive protein, could partially reverse the reduced neurite outgrowth induced by antisense suppression of AChE in PC12 cells. Functional redundancy in cell adhesion was also demonstrated by expressing a chimeric molecule in which the extracellular domain of neurotactin was replaced with

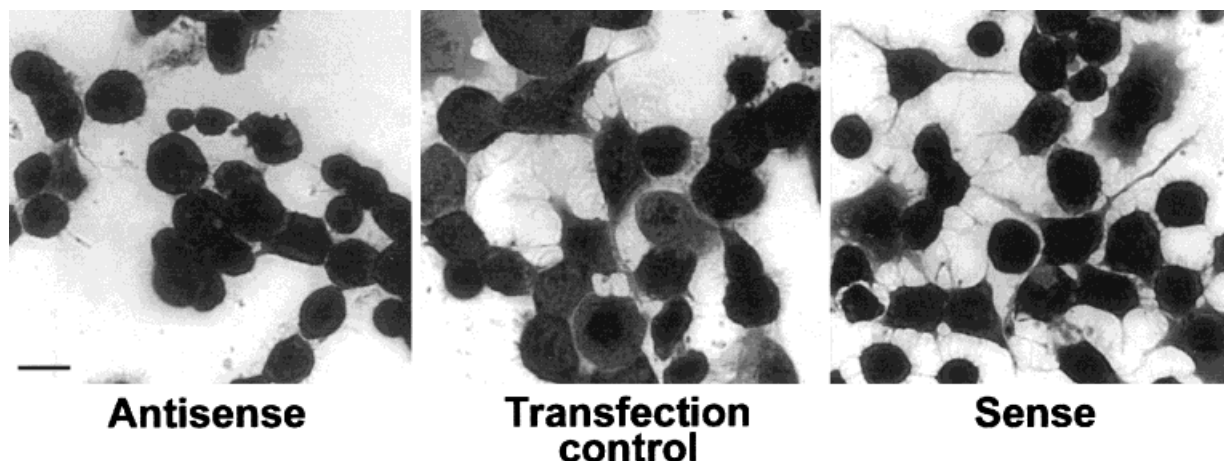


Fig. 8. AChE expression correlates with the rate of neurite initiation. Cell lines were examined every 60 min for 4 hr after plating to assess neurite initiation. Upon initial settling, all cells appeared spherical with smooth outlines and, shortly thereafter, extended numerous filopodia. AChE antisense cells remained mostly spherical, with only occasional

processes observed, whereas high-AChE-expressing sense cells displayed polygonal cell bodies with multiple, differentiated neurites after 4 hr. Wild-type (not shown) and transfection control cells displayed a similar morphology that was intermediate between that of the low- and high-AChE-expressing cell lines. Scale bar = 50 μ m.

Torpedo AChE (Darboux et al., 1996). This study showed that the chimeric molecule remained capable of mediating heterophilic adhesion similar to that provided by native neurotactin. Interestingly, it has been shown, using neurotactin null *Drosophila*, that this AChE-like adhesive protein is required for normal axonal growth and fasciculation in vivo (Speicher et al., 1998). These studies provide indirect evidence for an adhesive function for members of the serine esterase family of adhesive proteins in promoting axonal outgrowth.

Despite this large amount of indirect experimental evidence supporting the involvement of AChE in adhesive interactions (Layer et al., 1995; Bigbee et al., 1999; Grisaru et al., 1999; Muñoz et al., 1999), no studies to date have provided direct evidence to indicate that endogenous AChE expression modulates the adhesive properties of neurons or their processes. By demonstrating a close and direct bidirectional correlation between AChE expression and cell-substratum adhesion in two different cell adhesion assays, the current studies provide strong evidence supporting an adhesive function for AChE.

Requisite for an adhesive function for AChE in vivo is the existence of binding partners present in the environment encountered by growing axons. There is little evidence to suggest that AChE can bind to itself to promote homophilic adhesion (Guenneugues et al., 1996). In fact, none of the adhesive members of the serine esterase family has a homophilic binding mechanism (Barthalay et al., 1990; Darboux et al., 1996; Nguyen and Sudhof, 1997), so it is likely that AChE interactions may also be heterophilic in nature. Our laboratory has been characterizing the neuritogenic ability of AChE in the developing DRG neuronal system, and we have recently detected at least two AChE-binding proteins in embryonic spinal cord using an AChE blot overlay technique (unpublished data).

The existence of AChE-binding partners in the developing CNS is also supported by data from Robertson (1987), who has shown that AChE is transiently expressed by developing thalamocortical axons as they grow into the cerebral cortex. By examining the spatiotemporal binding patterns of AChE-coated microspheres to tissue sections of developing cerebral cortex, Robertson et al. (1998) showed that these microspheres preferentially attach to areas in which these axons grow in vivo. They proposed that the attachment of the microspheres is mediated by AChE binding to as yet unidentified ligand expressed in the developing cortex.

In the current studies, the finding that the level of AChE expression correlates with adhesion to plastic and polylysine substrata indicates the importance of charge-based, electrostatic interactions in AChE-mediated adhesion to these artificial substrata. This importance of charge-based interactions is also consistent with our finding that AChE binding to the spinal cord proteins is highly dependent on ionic strength (unpublished data). Together, these in vitro results predict a charge-based interaction for AChE-mediated adhesion in the more physiological context of developing neuronal systems. Although the direct neuritogenic ability of AChE is now well-established, the adhesive/neuritogenic domain on AChE has not been identified. In this regard, findings that BW284c51 and fasciculins, AChE inhibitors that are likely to interfere with the surface electrostatic domain, can decrease neurite outgrowth and adhesion suggest the involvement of the electrotactin domain (Layer et al., 1993; Dupree et al., 1994, 1996; Bataillá et al., 1998; Marchot et al., 1998; Anderson and Key, 1999; Muñoz et al., 1999; Blasina et al., 2000). Whether the electrotactin domain is responsible for the adhesive/neuritogenic ability of AChE remains to be determined, possibly through the use of site-directed mu-

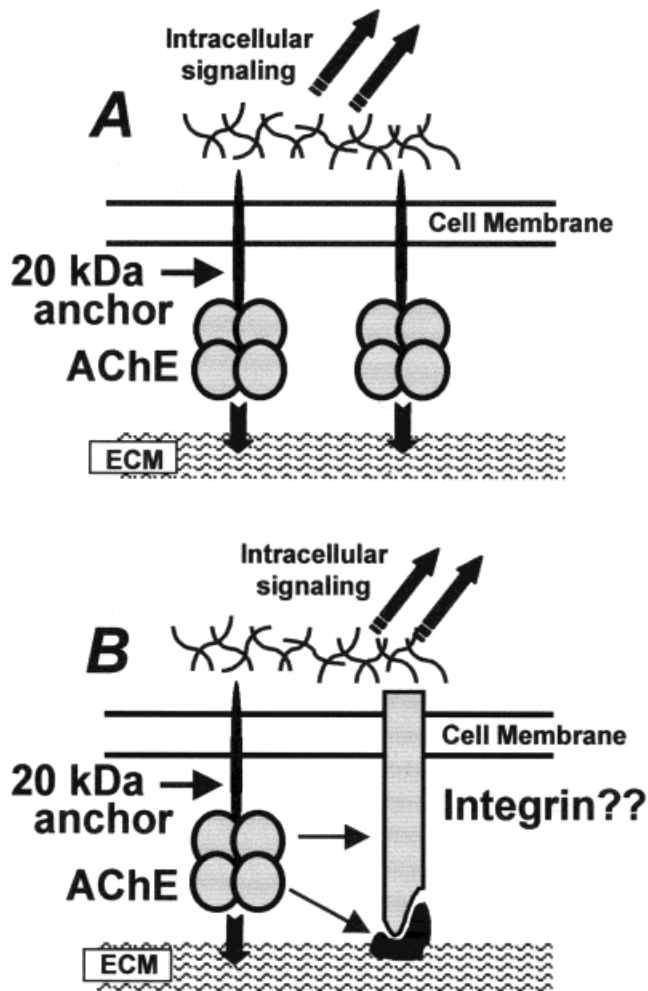


Fig. 9. Potential mechanisms for AChE-mediated cell-substratum adhesion. Tetrameric G4 AChE is anchored in the plasma membrane by a 20 kDa protein, which could potentially signal adhesive events between AChE and the extracellular matrix (ECM; **A**). Through this mechanism, AChE could directly activate intracellular signaling pathways. Alternatively, AChE-mediated adhesion could stabilize or facilitate the binding of other cell adhesion molecules, e.g., integrins, to their ligands, leading to signal pathway activation (**B**). In this “co-receptor” role, AChE could also interact with the receptor or the ligand.

tagenesis to neutralize the electrostatic motif (Botti et al., 1998).

Presently, the mechanism by which AChE-mediated cell-substratum adhesion could lead to enhanced axonal outgrowth is not known. In nervous tissues, the major form of AChE consists of a tetramer of four AChE monomers (G4), which can be anchored to the cell membrane by a 20 kDa hydrophobic protein (Boschetti and Brodbeck, 1996; Fuentes et al., 1988; Navaratnam et al., 2000; Perrier et al., 2000; see Fig. 9). Whereas we and others have previously shown that a soluble form of AChE contributes to the neurotogenic ability of AChE (Small et al., 1995; Srivatsan and Peretz, 1997; Bigbee and Sharma,

1998; Grisaru et al., 1999), the membrane-associated G4 form accounts for the AChE-mediated adhesion in the current studies. This conclusion is based on three observations. 1) The G4 isoform is expressed on the cell surface of these cells, and the intensity of surface immunostaining correlates with the expected modulation of AChE expression (Koenigsberger et al., 1997); 2) the adhesion assays were carried out in serum-free medium, thus eliminating a contaminating source of soluble AChE; and 3) the assays were conducted for short time periods, thus minimizing any significant AChE secretion during the assay.

According to the present hypothesis, AChE modulates neuronal adhesion, and these changes in adhesion may lead to enhanced axonal outgrowth. Presumably these adhesive interactions would activate intracellular signaling pathways, causing enhanced outgrowth. One possibility is that signal activation occurs directly through the 20 kDa anchoring protein. In this scenario, the G4 isoform of AChE can be viewed as a pentameric complex with four AChE monomers responsible for the adhesive interaction and the hydrophobic membrane anchor as a potential signaling subunit. Although multiple candidates for the anchor protein have been described (Boschetti and Brodbeck, 1996; Navaratnam et al., 2000; Perrier et al., 2000), it is not known whether these proteins have the ability to activate intracellular signaling leading to enhanced outgrowth.

A second possibility is that AChE-mediated adhesion could function to facilitate the binding of other classic cell adhesion molecules, e.g., integrins, to their ligands, leading to signal pathway activation. Such a mechanism was recently proposed for transglutaminase (tTG), a cell surface protein that also promotes adhesion independent of its enzymatic activity (Akimov et al., 2000). In this model, tTG serves as a “coreceptor” that can bind to both fibronectin and β 1-integrins, independently of the RGD (Arg-Gly-Asp) tripeptide recognition, and is thought to promote β 1-integrin clustering and intracellular signal activation. In a similar manner, AChE could stabilize the receptor-ligand complex, resulting in signal activation that enhances neurite outgrowth.

Mentioned above are two potential mechanisms by which AChE-mediated adhesion could explain its neurotogenic capacity. However, regardless of the molecular mechanism, the current studies now clearly demonstrate the direct relationship between the level of AChE expression and cell-substratum adhesion. These data further suggest that this intrinsic adhesive property of AChE may also underlie its neurotogenic abilities. Studies are currently underway in our laboratory to examine the molecular mechanisms and signaling pathways mediating the physiological role of AChE in axonal outgrowth during development of the nervous system *in vivo*.

ACKNOWLEDGMENTS

We thank Drs. Gary Bolin and Philippe Lam, Department of Engineering, and Dr. Oliver Bögl, Department of Anatomy, Virginia Commonwealth University, for their assistance with the ECIS experiments; Dr. T.

Rosenberry for supplying the AChE antiserum; and Dr. Carmen Sato-Bigbee for thoughtful discussions and careful review of the manuscript.

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