Regulated and constitutive secretion of distinct molecular forms of acetylcholinesterase from PC12 cells

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SUMMARY

PC12 cells secrete the enzyme acetylcholinesterase (AChE) while at rest, and increase the overall rate of this secretion 2-fold upon depolarization. This behavior is different from the release of other markers by the constitutive or regulated secretory pathways in PC12 cells. Both the resting and stimulated release of AChE are unchanged after treatment with a membrane-impermeable esterase inhibitor, demonstrating that it represents true secretion and not shedding from the cell surface. The stimulated release of AChE is Ca\(^{2+}\)-dependent, while the unstimulated release is not. Analysis of the molecular forms of AChE secreted by PC12 cells indicates that the release of AChE actually involves two concurrent but independent secretory processes, and that the G\(_4\) form of the enzyme is secreted constitutively, while both the G\(_2\) and G\(_4\) forms are secreted in a regulated manner, presumably from regulated secretory vesicles. Compared with other regulated secretory proteins, a much smaller fraction of cellular AChE is secreted, and the intracellular localization of this enzyme differs from that of other regulated secretory proteins. The demonstration that a cell line that exhibits regulated secretion of acetylcholine (ACh) is also capable of regulated secretion of AChE provides additional evidence for the existence of multiple regulated secretory pathways within a single cell. Moreover, there appears to be a selective packaging of different molecular forms of AChE into the regulated versus the constitutive secretory pathway. Both the specificity of sorting of AChE and the regulation of its secretion suggest that AChE may play a more dynamic role in synaptic function than has been recognized previously.

Key words: acetylcholinesterase, PC12, secretion, constitutive, regulated, sorting, vesicles, acetylcholine, cholinergic

INTRODUCTION

At cholinergic synapses, acetylcholinesterase (AChE) plays an important role in the termination of synaptic transmission by hydrolyzing ACh in the synaptic cleft after this transmitter is released from the pre-synaptic nerve terminal. AChE is a prominent and highly specific marker for cholinergic synapses in both the central and peripheral nervous systems, including at the neuromuscular junction, where specific synaptic forms are localized to the extracellular matrix in the immediate vicinity of the synaptic cleft (Hall, 1973; Schumacher et al., 1988). The origin and dynamics of this synaptic AChE has been a matter of substantial interest (for reviews, see Brimijoin, 1983; Appleyard, 1992), and although much recent work has focused on the expression of AChE by (post-synaptic) muscle cells (Bursztajn et al., 1991; Rossi and Rotundo, 1992), there is some suggestion that pre-synaptic neurons also synthesize and secrete this enzyme (Chubb and Smith, 1975). Details about what portion of the extracellular AChE is contributed by the pre-synaptic and post-synaptic cells are still unclear, as are the processes responsible for externalization.

AChE is expressed in several different molecular forms, with different structural features but identical catalytic sites. Symmetric AChE molecules are made of from 1 to 4 globular subunits of the enzyme (G\(_1\), G\(_2\), and G\(_4\)), while asymmetric forms (A\(_4\), A\(_8\) and A\(_{12}\)) possess, in addition to varying numbers of catalytic subunits, a collagen tail, which serves to anchor these forms in the extracellular matrix (Brimijoin, 1983). Some molecules of symmetric AChE are also bound to membranes by a variety of covalent linkages. While both membrane-bound and extracellular matrix forms of AChE appear on the surface of cells, the 16S extracellular matrix form is highly localized to the synapse (Abramson et al., 1989). All these forms of AChE are produced from a single gene as a result of alternative mRNA splicing (Sikorav et al., 1987; Schumacher et al., 1988; Rachinsky et al., 1990; Maulet et al., 1990; Li et al., 1991), but the specific structural differences between the various polymeric forms of soluble AChE remain undefined. PC12 cells have been shown to synthesize three identifiable molecular forms of AChE, which are generally characterized by their different sedimentation coefficients (Rieger et al., 1980; Inestrosa et al., 1981); 6S and 10S AChE are symmetric (globular) forms while 16S is an asymmetric form induced by nerve growth factor (NGF) and found associated with the extracellular matrix (Inestrosa et al., 1982).

At cholinergic synapses like the neuromuscular junction,
the available evidence suggests that the post-synaptic muscle cell is likely to be a major source of the synaptic AChE: contributions by the pre-synaptic cholinergic nerve cell are less certain. It is clear, for example, that cultured muscle cells can synthesize AChE, including the typical synaptic forms, in the absence of nerve cells (Silberstein et al., 1982; Inestrosa et al., 1983). While denervation (Fadic and Inestrosa, 1989) or blockade of axonal transport (Fernández and Inestrosa, 1976) results in a decrease in the AChE content of the end-plate, it is not certain whether this is due to a direct contribution of AChE from the nerve or to a trophic effect of the nerve on synthesis and secretion of AChE from the muscle cell.

In vivo and in vitro experiments have demonstrated effects of activity on the levels of AChE that appear in the extracellular fluid. Perfusion of adrenal glands with depolarizing solutions led to the increased appearance of AChE in the perfusate (Chubb and Smith, 1975). Although such experiments do not indicate whether the origin of the AChE is pre- or post-synaptic, they do demonstrate that the levels of this enzyme are dynamic, and can be altered by nerve activity. Experiments on substantia nigra (Llinás and Greenfield, 1987), isolated adrenal cells (Mizobe et al., 1984), and non-innervated myotubes (Bursztajn et al., 1991) have demonstrated that all these cells, which are post-synaptic in the sense that they receive cholinergic input, can secrete AChE in an activity-dependent fashion. At least in the case of cultured myotubes, the activity- and Ca<sup>2+</sup>-dependent release occurs over a period of days, and is likely to result from increases in gene expression rather than a regulation of secretion itself. In isolated cell culture systems, secretion of AChE has also been observed from neuroblastoma cells (Steiger et al., 1989), transfected kidney cells (Velan et al., 1991), and transfected CHO cells (Legay et al., 1993).

In contrast, there is little evidence for the ability of a pre-synaptic cholinergic neuron to secrete AChE into the synaptic cleft. Injection of anti-AChE antibodies produces the selective removal of pre-synaptic neurons in sympathetic ganglia (Brimijoin and Lennon, 1990) and in the brain (Bean et al., 1991), but the exact mechanism of this destruction is unknown, and does not necessarily require that the pre-synaptic neurons are the source of the AChE that is the target of the antibodies. If AChE were to be secreted by pre-synaptic neurons, it is not clear through which of the secretory pathways it would be released from the cells. The constitutive pathway is known to secrete extracellular matrix components such as laminin (Schweitzer and Kelly, 1985) and heparin sulfate proteoglycan (Toozie and Huttnner, 1990), but the evidence, cited above, that the secretion of AChE is modulated by neural activity suggests that it is secreted at least in part by a regulated pathway.

PC12 cells (Greene and Tischler, 1976) express a variety of neuronal properties in culture, including the ability to synthesize and secrete acetylcholine (ACh) as well as catecholamines such as norepinephrine (NE) (Greene and Rein, 1977; Schubert and Klier, 1977). In addition to secreting neurotransmitters in a highly regulated fashion, PC12 cells secrete proteins by at least two distinct pathways. Gene transfection experiments have clearly defined the existence of both a regulated and a constitutive secretory pathway, and have demonstrated the selective sorting of native and transgenic proteins within these pathways (Schweitzer and Kelly, 1985; Lowe et al., 1988); the behavior of all the regulated secretory proteins so far examined has paralleled the secretion of norepinephrine (NE), suggesting that they are packaged into the large dense-core secretory vesicles containing catecholamines. However, evidence from studying the distribution of vesicle-specific monoclonal markers (Schweitzer and Paddock, 1990; Lowe et al., 1988) and vesamicol, a marker for cholinergic vesicles (Blumberg and Schweitzer, 1992), suggests that additional pathways for vesicular secretion exist in these cells. The data presented here indicate that the cholinergic PC12 cell line secretes AChE by both a constitutive and a highly regulated pathway, and that this secretion involves specific sorting of the different molecular forms of AChE.

**MATERIALS AND METHODS**

**Materials**

All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Heps-buffered saline (HBS) contained 150 mM NaCl and 10 mM HEPES, pH 7.4. Calcium- and magnesium-containing HBS (CM-HBS) consisted of HBS with an additional 2.0 mM CaCl<sub>2</sub> and 0.8 mM MgCl<sub>2</sub>. Na incubation medium was made by mixing 1 part HBS and 2 parts DMEM (Dulbecco's Modified Eagle's Medium, high glucose (Gibco, Grand Island, NY)); bovine serum albumin (Sigma-Aldrich, recombinant and lyophilized) was then added to a final concentration of 1%. K incubation medium was identical except for the substitution of 150 mM KCl + 25 mM HEPES for the HBS. The final concentration of K<sup>+</sup> in the K incubation medium was therefore approximately 55 mM. Na (0 Ca) and K (0 Ca) media were identical to Na medium and K medium, except that 2 parts DMEM was added to 1 part Na or K solution lacking Ca and containing 30 mM Mg-EGTA. The final concentration of Ca was therefore 0.67 mM, and of Mg-EGTA was 10 mM. AChE extraction buffer contained 1 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 20 mM EDTA, 5 mM EGTA, and 20 units/ml aprotinin as described by Inestrosa et al. (1981). NGF was purified from mouse salivary glands as described by Mobley et al. (1976).

**Cell culture**

PC12 cells, subclone A, were cultured in DMEM, with the addition of 25 mM HEPES, 5% supplemented calf serum (HyClone Laboratories, Logan, UT), 5% horse serum (HyClone), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco) at 37°C and 10% CO<sub>2</sub>. The experiments comparing AChE and human growth hormone (hGH) release were carried out with PC12 cells stably transfected with a metallothioneine promoter-hGH construct (PC12/pMET-hGH; Schweitzer and Paddock, 1990), grown under identical conditions except for the inclusion of 0.25 mg/ml genetin (Gibco) in the culture medium.

**Release experiments**

Cells were grown in 10 cm tissue culture plates (Falcon) to no more than 30% confluence. Prior to the experiment, the cells were incubated overnight with 1-5 µCi [<sup>3</sup>H]NE (Amersham). For some experiments, cholinesterase inhibitors (phospholine or paroxon) were added to the medium and incubated for 10 minutes just prior to rinsing the cells. Remaining inhibitor, extracellular esterase (both from the cells and from the sera) and [<sup>3</sup>H]NE were removed by gently rinsing the dishes with Na incubation medium, at 37°C.
5 times for 2 minutes each. Between manipulations, the cell dishes were placed on top of a metal plate in contact with a water bath at 37°C. During the release experiments themselves, the medium in each dish was changed at 2 or 4 minute intervals by tilting the dish, removing the medium, and then adding 2 ml of fresh incubation medium (at 37°C) to the side of the dish. The dishes were immediately swirled and replaced on the metal plate at 37°C. The collected media were transferred to test tubes in an ice bath, and at the end of the experiment all samples were centrifuged at 3000 g for 5 minutes to remove any cells that might have washed off the dishes during the manipulations. There was no visible cell loss from the dishes, nor was there any visible pellet in any of the sample tubes after centrifugation. Aliquots of the supernatants were then assayed for [3H]NE by scintillation counting, for AChE activity, or for hGH immunoreactivity.

At the end of the incubation experiments, the remaining AChE and NE was extracted from the cells by adding 2 ml of extraction buffer (Inestrosa et al., 1981).

**AChE assays**

For measurements of AChE activities in cell extracts and media, the colorimetric enzyme assay described by Ellman et al. (1961) was modified for use in 96-well microplates by scaling down the volumes while keeping the reagent concentrations the same. The absorbance change at 405 nm was monitored with a Molecular Dynamics UVMax microplate spectrophotometer. For each sample, a blank containing identical reagents plus 100 µg/ml eserine was performed and the blank values were subtracted from those of the samples. Under the conditions of the assay, the rate of absorbance change was linear with time and amount of sample added.

For analyzing AChE activities in velocity gradient fractions (see below), a radiochemical assay using [3H]ACh was performed as described by Johnson and Russell (1975). As described above, eserine blanks were carried out in parallel for each fraction and this blank value was subtracted from the experimental activity.

**hGH assay**

A sandwich monoclonal radioimmunoassay (Hybritech) was used to assay supernatants and extracts for hGH.

**AChE histochemistry**

PC12 cells, pre-treated with 2 ng/ml NGF for 3-5 days to induce neurite outgrowth were plated onto poly-D-lysine-coated glass coverslips and grown in the presence of NGF for an additional 2 days. The cells were then rinsed with CM-HBS and fixed with 4% formaldehyde (made fresh from parafomaldehyde) in HBS. AChE was visualized by a modification of the bright-field procedure described by Hedreen et al. (1985). After fixation and rinsing, cells were incubated for 3 hours at room temperature in a solution of 0.5 mg/ml acetylthiocholine iodide, 0.1 mM K ferricyanide, 3 mM CuSO4, 65 mM Na acetate pH 6.0, and 4 mM Na citrate. After 2 rinses of 2 minutes each in 0.1 M Na acetate, pH 6.0, the cells were then incubated with 0.1% ammonium sulfide for 20 seconds, rinsed 5 times with 0.1 M Na nitrate for 2 minutes each, and then rinsed twice with 0.1 M Na acetate. For negative controls, cells were incubated with 0.2 mM diisopropylfluorophosphate (DFP) for 20 minutes prior to fixation.

**Sedimentation analysis**

Different molecular forms of AChE were separated by velocity sedimentation essentially as described by Inestrosa et al. (1981) using a 5-20% sucrose gradient in 1 M NaCl, 0.5% Triton X-100, 20 mM EDTA, 5 mM EGTA, and 10 mM Tris-HCl, pH 7.4. Two units of β-galactosidase were added to the media samples or cell extracts as a sedimentation marker (16S), and the samples were layered on top of gradients and centrifuged in an SW41 Ti rotor at 200,000 g for 17 hours at 4°C. The gradients were collected from the bottom by puncturing the tubes with a hypodermic needle. β-galactosidase was measured colorimetrically by monitoring the conversion of O-nitrophenyl-β-galactoside as described (Bahl et al., 1980).

**RESULTS**

**Regulation of AChE release**

Depolarization increases the rate of secretion of the neurotransmitters ACh and NE, as well as a variety of other regulated secretory molecules, from PC12 cells. This effect is illustrated in Fig. 1A, in which the secretion of NE was stimulated by directly depolarizing the PC12 cells with elevated extracellular K+. As reported previously for carbachol-treated cells (Schweitzer and Kelly, 1985), this stimulated release was rapid, phasic, and many times the resting level of release. Moreover, an examination of the medium bathing these cells for total AChE enzymatic activity revealed that readily detectable amounts of AChE were

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**Fig. 1.** Release of NE and AChE from PC12 cells. Duplicate dishes of PC12 cells were loaded overnight with [3H]NE and then rinsed with Na medium. The culture media were then removed and fresh media added at 4 minute intervals. At time 0, K medium was added to the dishes, and the medium collected 4 minutes later. The media were analyzed for [3H]NE (A) or AChE activity (B). Amounts are expressed as % total cellular content, calculated by adding up all material released plus the amount recovered in the extracts at the end of the experiment. All values are means ± range of duplicate dishes.
released from the cells at rest, and that the rate of this release was increased by depolarization (Fig. 1B). Assay of the bathing media by themselves, containing no serum and 0.1% recrystallized BSA, showed no detectable AChE activity. Moreover, control experiments, in which known amounts of purified AChE were assayed in Na medium or K medium demonstrated that the substitution of K for Na did not alter the measured activity of the enzyme (data not shown). It therefore appears that PC12 cells secrete AChE into the extracellular space, and that at least a portion of this secretion occurs via a regulated pathway.

Despite the clear increase in the rate of release of AChE with depolarization, the secretion of AChE differed in several respects to that of NE. In contrast to NE, overall AChE release was stimulated only about 2-fold by depolarization with 50 mM KCl (vs > 25-fold for NE). Another striking difference between the secretion of NE and AChE was the fraction of material within the cells that was secreted. Nearly half of the total cellular content of NE was secreted within 4 minutes of stimulation; similar amounts have been observed for the stimulated release of transgenic human growth hormone (hGH) (Schweitzer and Kelly, 1985) and insulin (E. S. Schweitzer, unpublished results) after stimulation of PC12 cells with the cholinergic agonist, carbachol. In contrast, only about 1% of the total cellular AChE was secreted during a 4 minute stimulation (Fig. 1B).

It was important to prove that the release of AChE in the medium was due to secretion, rather than the shedding of cell-surface AChE molecules. Such shedding is known to occur for other proteins including cell-surface viral glycoproteins, and would not reflect the actual kinetics of exocytosis. This was of particular concern since some forms of AChE are known to be associated with extracellular matrix and are released by collagenase or heparitinase treatment (Hall and Kelly, 1971; Brandan et al., 1985). In theory, both the stimulated as well as the basal release of AChE could be explained by shedding of cell surface AChE if such degradative enzymes were contained in regulated secretory vesicles. The availability of an irreversible membrane-impermeable inhibitor of AChE, phospholine (Inestrosa et al., 1981) permitted a critical test of this possibility. After treatment of intact cells with phospholine, any AChE present on the outside of the cells should be irreversibly inactivated by this treatment, while the AChE protected in intracellular compartments should be unaffected.

As shown in Fig. 2A, NE secretion was unaffected by prior treatment of the cells with 1 µM phospholine. Fig. 2B shows that the phospholine treatment also failed to reduce either the basal (filled circles) or stimulated release (filled triangles) of AChE, demonstrating that all of the AChE that appeared in the medium was being released from some compartment that protected the AChE from the membrane-impermeable inhibitor; this compartment presumably corresponded to secretory vesicles located in the cytoplasm of the PC12 cells. In contrast, pre-treatment with the irreversible membrane-permeable inhibitor, paroxon, resulted in a complete disappearance of AChE activity from the medium, either before or after depolarization; this treatment also abolished all activity in the cell extracts (data not shown). Separate experiments, in which phospholine was added to the medium after collection, demonstrated that 1 µM phospholine was effective in eliminating all detectable AChE activity when the enzyme was not protected by membranes (data not shown). The absence of effect of phospholine on the release of AChE in the medium demonstrated that the source of the AChE was intracellular.

It was also important to eliminate the possibility that the differences between the extent of stimulation of NE and AChE release were the result of comparing a small, rapidly diffusible molecule like NE with a protein such as AChE. To address this question, release experiments were carried out using PC12 cells engineered to express hGH (Schweitzer and Kelly, 1985; Schweitzer and Paddock, 1990). The secretion of hGH from these cells parallels that of NE, and shows a large stimulation upon the addition of carbachol to the medium. By a variety of criteria, the transgenic hGH appears to be packaged into the same catecholaminergic vesicles that contain NE (Schweitzer and
Kelly, 1985; Lowe et al., 1988; Schweitzer and Paddock, 1990). As shown in Fig. 3A and B, direct depolarization by elevated K had identical effects on stimulating the secretion of NE and hGH, as would be expected if they were being released from the same vesicles. As with untransfected PC12 cells, the secretion of AChE from the PC12/pMTP:hGH cells showed only a two-fold stimulation (Fig. 3C). This experiment clearly indicated that the unusual pattern of AChE secretion could not be attributed to artifacts of diffusion or trapping that could result from comparing the release of a secreted protein with a small neurotransmitter such as NE.

Despite the fact that the stimulation media were placed on ice immediately after collection, and centrifuged to remove any detached cells or debris, it was of some concern that cell detachment or lysis was responsible for the release of AChE from intracellular sites. One of the characteristic features of regulated secretion from neurons is the dependence on extracellular Ca\(^{2+}\). Such a dependence would not be expected if the AChE was being released by cell lysis or other non-specific means. For this reason, it was of considerable interest to examine the effect of removing Ca\(^{2+}\) on the secretion of AChE from PC12 cells. Fig. 4 shows the results of such an experiment, in which extracellular Ca\(^{2+}\) was removed prior to and during depolarization by elevated K. Such treatment completely abolished the stimulated release of NE from these same cells (data not shown). This experiment demonstrated that removal of extracellular Ca\(^{2+}\) had no significant effect on the basal release of AChE, but that it blocked completely the two-fold increase that normally resulted from depolarization. This result is consistent with the known properties of the regulated and constitutive pathways, and provides additional evidence that the stimulated release reported here represents true neurosecretion.

Although the level of stimulation of AChE release was modest, Figs 1-4 demonstrate that the 2-fold increase in the rate of release was consistently seen in experiments under different conditions, using different collection times, and with different strains of PC12 cells (wild type and transfected). The secretion of AChE is therefore clearly regu-
lated, though to a lesser extent than are other previously described components of neurosecretory vesicles.

**Localization of intracellular AChE**

The quantitative difference between AChE secretion and NE secretion suggested that the AChE was being released, at least in part, from different vesicle(s) than the regulated secretory vesicles containing NE. Several possible models could explain this behavior. (1) AChE could be packaged into a single population of vesicles, distinct from the constitutive vesicles or the regulated vesicles responsible for NE secretion; this model requires that the AChE-containing vesicles exhibit an interaction with the plasma membrane that is intermediate between these other two recognized vesicle populations. (2) This secretory pattern could result from AChE being packaged indiscriminately into both the constitutive and regulated catecholaminergic vesicles as a result of the absence of any sorting. (3) AChE could be actively sorted into both regulated and constitutive vesicles, producing a segregation of distinct fractions of the total AChE into each of these different types vesicles. In such a model, the portion of the AChE targeted to regulated vesicles could be packaged into the catecholaminergic vesicles or into some other, uncharacterized population of regulated secretory vesicles.

In an attempt to clarify further the type of compartment(s) from which AChE was being released, the intracellular compartmentalization of AChE was examined using both subcellular fractionation and immunohistochemical localization. A variety of fractionation procedures, including density equilibrium gradients using sucrose, metrizamide, and/or Ficoll proved inconclusive. Multiple peaks of AChE were apparent from such gradients, and their location varied with the different density medium used (data not shown). While much of the AChE activity purified away from the NE, it was impossible to determine which peak

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**Fig. 5.** Histochemical localization of AChE in PC12 cells. Cells were treated with NGF to induce neurites, fixed and stained for AChE. The presence of AChE enzyme activity is indicated by the deposition of brown reaction product (A and B). Cells shown in (C) and (D) were incubated with 0.2 mM DFP prior to carrying out the immunohistochemical reaction for AChE. (A and C) Phase-contrast images of the cells shown in bright field in (B) and (D). Bar, 20 µm.
of AChE was the source of the secreted enzyme, since this constituted only a tiny fraction of the total cellular AChE. Similarly, fractionation of PC12 membranes on a sizing column using Sephacryl S-1000 showed about 1/3 of the AChE activity eluting in the excluded volume along with the NE-containing vesicles; the remainder eluted in multiple peaks, some of which probably corresponded to the rough endoplasmic reticulum, Golgi membranes, and coated vesicles that have been reported to be major sites of localization of AChE in chick myotubes (Rotundo et al., 1989).

In order to examine the spatial distribution of AChE within PC12 cells, NGF-differentiated cells were fixed and processed for AChE histochemistry. Fig. 5A (phase-contrast image) shows several PC12 cells with well-developed neurites and nerve terminals. Consistent with the localization of AChE in multiple intracellular sites, AChE histochemistry indicated that AChE activity (brown reaction product) was present in vesicular compartments scattered throughout the cell, with dense concentrations in the perinuclear area of the cell body (Fig. 5B, bright-field image). Only a small number of AChE-positive puncta appeared in the neurite terminals, where the catecholaminergic vesicles are concentrated. The specificity of this staining was demonstrated by the virtual absence of reaction product in cells that were pre-treated with the irreversible AChE inhibitor, DFP (Fig. 5C,D). Although it is clear that the majority of AChE in the cells was localized to a portion of the cell that does not contain large numbers of regulated secretory vesicles, it was not possible to define more fully the localization of the small percentage of AChE that is destined to be secreted in either a constitutive or a regulated manner.

**Molecular forms of AChE**

In order to define more fully the nature of AChE release, cell extracts and bathing media were examined for the presence of different molecular forms of AChE. In the absence of antibodies that can discriminate between the various forms, cell extracts and bathing media were analyzed by sedimentation gradients. As shown in Fig. 6, and as reported previously (Rieger et al., 1980; Inestrosa et al., 1981), velocity sedimentation of PC12 cell extracts revealed three distinct forms of AChE. The 6S and 10S species (peaks at fractions 15 and 9) are globular forms (G2 and G4, respectively), while the (minor) 16S form (sedimenting slightly faster than β-galactosidase) is a collagen-tailed asymmetric form of the enzyme known to be associated with the extracellular matrix and induced by NFG. The majority of AChE in total cell extracts was in the smallest, 6S form. Consistent with the observation that stimulation released only a small percentage of the total AChE within the cells, there was no significant difference between the AChE content of stimulated versus non-stimulated cells.

A different picture emerged when bathing media were analyzed for the various molecular forms of AChE. Fig. 7 shows that the resting secretion of AChE from non-stimulated cells (filled circles) appeared to be exclusively the 10S form of the enzyme (peak at fraction 12). Since the majority of AChE inside the cells was the 6S form, some selective targeting was occurring that segregated the 10S form of AChE into (or excluded the 6S form from) the vesicles responsible for this unstimulated release. Moreover, the molecular profile of the AChE released from stimulated cells (filled triangles) was distinct not only from the total AChE in the cells, but also from the profile observed for unstimulated secretion, showing peaks at both 10S and 6S (peak at fraction 17). Since the medium collected during a 5 minute period represented the sum of the stimulated...
release plus any ongoing release, the AChE attributable to the stimulated release alone was obtained by subtracting the values for the non-stimulated medium from the total for the stimulated medium (open triangles).

By integrating the AChE activities recovered in fractions 11-13 and 16-18, it was possible to compare the relative amounts of 10S and 6S AChE that were released constitutively and by stimulation. Whereas cells under resting conditions released 10S and 6S AChE in a ratio of at least 13:1, the corresponding ratio for AChE release caused by depolarization was 2.4:1. Thus not only was the mix of secreted forms of AChE distinct from the total AChE within the cells, but the mix of molecular forms released from the regulated pathway differed from that released by non-stimulated cells. This result complements the data presented in Fig. 4, which showed that the resting and stimulated release of AChE differed in their dependence on extracellular Ca\(^{2+}\); taken together, these results support the conclusion that the release of AChE that occurs from non-stimulated cells represents secretion from a separate, constitutive pathway rather than basal activity of the regulated secretory pathway. Thus AChE is sorted into both regulated and constitutive pathways, and this sorting appears to be selective with respect to the molecular forms of AChE. By comparing the amounts of 6S AChE released under resting and stimulated conditions, it appears that there is at least a 10-fold increase in release upon depolarization, indicating that a subset of the intracellular AChE is sorted into a secretory pathway that is capable of a high degree of regulation.

**DISCUSSION**

The experiments presented here demonstrate the regulated secretion of AChE from a cholinergic cell line. The overall profile of this secretion, which is stimulated approximately 2-fold by depolarization, appears to be quite different from that of other proteins examined, which are either highly regulated or completely constitutive. However, careful examination of the molecular forms of the secreted AChE suggests that this pattern is actually the result of at least two concurrent, but independent, secretory pathways. Only 10S AChE is secreted constitutively, and this secretion is carried out independently of extracellular Ca\(^{2+}\). In contrast, depolarization causes an increase in the rate of secretion of both 10S and 6S AChE; this increase in secretion absolutely requires extracellular Ca\(^{2+}\). The simplest explanation for this result is that only the 10S enzyme is sorted into constitutive secretory vesicles while both 10S and 6S forms are sorted into regulated vesicles in a ratio of about 2.4:1; this ratio is quite different from the overall ratio found in cell extracts. The packaging of AChE into both constitutive and regulated secretory pathways therefore appears to result from specific sorting events, rather than being the result of limitations or inefficiencies in the sorting machinery.

The ability of PC12 cells to segregate two closely related molecular forms of AChE suggests that the sorting machinery is recognizing subtle structural elements that differ between the 6S (G\(_2\)) form and 10S (G\(_4\)) form of the enzyme. While the symmetrical and asymmetrical forms of AChE differ as a result of alternative splicing of mRNA from a single gene, the differences between the various globular forms of the enzyme are obscure. Fuentes and Inestrosa (1988) have suggested that the G\(_2\) and G\(_4\) forms differ with respect to glycosylation, but this study compared enzymes from different species. An understanding of the structural differences between the forms of AChE within a single cell could provide clues about the recognition signals responsible for sorting in the secretory pathways.

PC12 cells are known to package a variety of regulated secretory proteins selectively into the catecholaminergic vesicles, including dopamine-β-hydroxylase, secretogranins, as well as hGH and insulin in transgenic cells expressing these foreign proteins. It is not possible to determine from the present data whether the regulated portion of the AChE is packaged into these same vesicles or into another population of regulated secretory vesicles. The histochromological localization of AChE suggests that little AChE is present in the nerve terminals where the catecholaminergic vesicles are concentrated; in addition, the detailed kinetics of AChE release are slower than those for NE and hGH. While these observations argue against AChE being packaged into the catecholaminergic secretory vesicles, additional experiments are necessary to determine whether the regulated secretion of AChE is occurring from a novel population of regulated secretory vesicles distinct from the catecholaminergic vesicles. The recent availability of antibody probes directed against the chromaffin granule amine transporter (Liu et al., 1992, and unpublished data) should open the way to a more rigorous examination of the question of intracellular localization.

Regardless of which vesicles AChE is secreted from, the present data support the conclusion that multiple regulated secretory pathways exist in PC12 cells. This conclusion was previously based on our demonstration that it is possible to separate vesicles that bind vesamicol (a marker for cholinergic vesicles, see Parsons et al., 1993) from NE-containing vesicles in PC12 cells (Blumberg and Schweitzer, 1992). The ability of PC12 cells to secrete both ACh and the enzyme responsible for its degradation in a regulated fashion also supports the existence of at least two distinct populations of regulated secretory vesicles, since the presence of these two entities in the same compartment would presumably lead to the rapid destruction of all ACh. Although it is possible that ACh and AChE could coexist inside the same vesicle if the AChE were in some inactive state, there is no evidence at present for such azymogen-like form of AChE. Moreover, cholinergic vesicles are known to contain little or no soluble protein (Wagner et al., 1978). It therefore appears that the simplest model that explains the data is that AChE is secreted from a population of vesicles that differs from cholinergic vesicles, and perhaps from catecholaminergic vesicles as well. Recent evidence that muscle cells can secrete ACh in a regulated fashion (Dan and Poo, 1992) implies the existence of multiple pathways for the externalization of ACh and AChE in these cells as well, and suggests that such multiple regulated secretory pathways may be a more common phenomenon than has been previously recognized.

The ability of PC12 cells to secrete AChE in a regulated fashion also suggests that pre-synaptic neurons may con-
tribute, in a controlled fashion, to the AChE in cholinergic synaptic clefts. The extent to which this occurs in vivo, and its significance for synaptic function remains to be determined. Engel et al. (1980) have described a clinical syndrome of muscle weakness associated with small nerve terminals and a deficiency of AChE at the neuromuscular junction. Whether the primary defect resulting in this syndrome is pre- or post-synaptic is not known, but the present experiments raise the possibility that the pre-synaptic nerve cell may contribute directly to the AChE at a synapse. While the major function of acetylcholinesterase appears to be the hydrolysis of ACh released from pre-synaptic terminals, there are suggestions that this enzyme may play other roles in the functioning of the nervous system (Ismael et al., 1986; Greenfield, 1984; Greenfield, 1992). The regulated and specific secretion of AChE from PC12 cells implies a more dynamic role for AChE that has been previously recognized, and suggests that pre-synaptic cholinergic neurons may contribute AChE to the synaptic cleft in an activity-dependent manner.

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