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Axonal Transport and Subcellular Distribution of Molecular Forms of Acetylcholinesterase in Rabbit Sciatic Nerve

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SUMMARY

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Rabbit sciatic nerves were incubated *in vitro* with local cooling of their proximal or distal regions to produce local accumulations and depletions of acetylcholinesterase (AChE) undergoing rapid axonal transport. Some nerves were also exposed to echothiophate, an anticholinesterase that is partially selective for external or extracellular enzyme. Samples from various regions of the treated nerves were analyzed by differential ultracentrifugation. Particulate AChE activity was found to be transported at a higher average velocity than soluble activity. Somewhat surprisingly, the latter fraction was more sensitive to echothiophate than was the former. On sucrose density gradients, three forms of AChE were resolved, with sedimentation coefficients of 4S, 10S, and 16S. All three forms were subject both to orthograde and to retrograde axonal transport. The 10S form, which predominated in control samples, was also responsible for most of the accumulation of AChE activity at the borders of cooled regions, but the percentage changes were much larger for the 16S form. Although the results indicated large differences among the average transport velocities of the molecular forms of AChE, these differences are probably explained by differences in the proportion of moving enzyme rather than in the true velocity. The echothiophate-sensitivity of the forms of AChE followed the order, 4S > 10S > 16S, but the differences among forms were not statistically significant, suggesting a similar distribution of these forms between internal and external compartments.

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is a glycoprotein that is subject to rapid transport in both directions along the axons of peripheral nerves (1-3). A large proportion of this enzyme, however, is essentially stationary and seems to be contained within an external compartment that may include the axolemmae of cholinergic nerves and the basement membranes of neuronal capillaries (4-6). Thus, AChE has various locations and fates in peripheral nerves.

The discovery that AChE occurs in different molecular forms distinguishable by their sedimentation velocities (7-9) raises the possibility that each form might have a special location and fate. This possibility is strengthened by a recent report that 16S AChE, previously believed unique to the neuromuscular junction (7), not only is present in rat sciatic nerves but also is transported down them faster than any other form of the enzyme (10).

We undertook the present study in order to compare the locations and fates of the molecular forms of AChE. The apparent subcellular distribution of these forms and

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their transport in proximal as well as distal directions were examined in the rabbit sciatic nerve. In addition, echothiophate, a quaternary organophosphate anticholinesterase that is largely excluded from axons (6), was used as a tool for analyzing the distribution of the forms of AChE between internal and external compartments in this nerve. The results of this study revealed some differences among the molecular forms of AChE but showed no characteristic that was exclusive to any one form.

METHODS

A. *Individual Procedures*

1. *Preparation of nerves.* Adult New Zealand white rabbits (3–5 kg, of both sexes) were killed by injection of sodium pentobarbital (250 mg) via an ear vein. The sciatic nerves were removed and transferred in 0.9% NaCl solution at room temperature to a dissection microscope, under which the surrounding fatty tissue was dissected away without disturbance to the epineurium. Except when the experiment called for fresh tissue, pieces of nerve approximately 7 cm long (weighing about 30 mg/cm) were ligated at both ends with silk thread (4-0 gauge) and were then transferred to incubation chambers for local cooling or exposure to echothiophate.

2. *Local cooling.* Local cooling was used to interrupt axonal transport so that one could examine the characteristics of the AChE that accumulated at the border of the cooled region. Isolated sciatic nerves were incubated in barrier-free chambers constructed to allow exposure of discrete regions to solutions differing in temperature (11). In these chambers, the proximal or the distal 1-cm of the nerve was kept at 5° while the remaining part was kept at 37° by means of separate currents of bicarbonate-buffered physiological saline solution containing glucose and continuously aerated with 95% O_2-5% CO_2 (11).

3. *Exposure to echothiophate.* Sciatic nerves were immersed for 10 min at 37° in beakers containing echothiophate, 0.1 mM, dissolved in the aerated saline solution described above. To rinse away free echothiophate and to allow inhibition to develop fully, the nerves were subsequently incubated in the absence of drug at 37° for a total of 50 min. *Procedure a* was used to rinse nerves exposed to echothiophate without prior treatment; it simply involved immersion in a large volume of physiological saline solution at 37°. *Procedure b* was used for nerves that had been locally cooled. In this procedure, nerves were rinsed in saline solution only for 2 min. Afterwards, in order to prevent AChE from redistributing within axons by rapid transport, 6-mm samples were cut from selected regions of the nerves. These samples were then incubated individually at 37° for 48 min in glass homogenizers containing 2 ml of 0.5 M Tris HCl, pH 7.4 and 0.2% (w/v) bovine serum albumin. In all cases nerve samples were cooled to 4° and homogenized when one hour had elapsed from the onset of drug exposure.

4. *Apparent subcellular distribution.* Differential ultracentrifugation was used to separate "soluble" from "particulate" AChE. Preliminary experiments were conducted to determine the effect of the ionic strength and pH of the homogenization buffer on the apparent subcellular distribution of this enzyme. It was found that between 25% and 32% of the AChE activity was solubilized by homogenization in Tris HCl buffers ranging in ionic strength from 0.01 to 0.05 M and in pH from 6.8 to 8.6. Since a hypotonic buffer was desired in order to lyse the nerve cells and since the exact composition was not crucial to the apparent subcellular distribution of AChE, it was arbitrarily decided to homogenize samples routinely in 0.05 M Tris-HCl, pH 7.4, containing 0.2% (w/v) bovine serum albumin. Homogenates, 2 ml each, prepared from 6-mm pieces of nerve, were centrifuged at 100,000 × g_{av} for 1 hr at 4° (Beckman L2-65B ultracentrifuge; 50 Ti rotor). Aliquots of the supernatant fractions (referred to as "soluble" AChE) were saved for assay, the rest of the supernatant fractions were aspirated and discarded, the tubes were wiped, and the pellets were rehomogenized in the Tris-HCl buffer with Triton X-100 added to a concentration of 0.1% (v/v). Aliquots of the rehomogenized pellets (referred to as "particulate" AChE)

were also saved for assay.

5. *Molecular forms of AChE.* Sucrose density gradients were used to analyze the molecular forms of AChE in experiments modelled after those of Hall (7) and Vigny, Koenig and Rieger (8). Pieces (6 mm) of sciatic nerve were homogenized in glass homogenizers each containing 1 ml of 0.05 M Tris HCl, pH 7.4; 1 M NaCl; and 1.0 (v/v) Triton X-100. The homogenates were centrifuged at 15,000 × *g* for 15 min at 4°. Supernatant fractions were then collected and the pellets were rehomogenized in the original volume of fresh buffer. Supernatant fractions prepared from these extracts by another centrifugation at 15,000 × *g* were combined with the first ones and then centrifuged one last time at 15,000 × *g* for 10 min in order to clarify them. Portions (0.1 ml) of these preparations were then layered onto linear gradients of 5–20% sucrose made up in 12 × 50 mm centrifuge tubes by means of a Beckman Density Gradient Former. Tris-HCl, Triton X-100, and NaCl were present throughout the gradients in the same concentrations as in the homogenization buffer. The gradients were centrifuged at 41,000 rpm (120,000 × g_{av}) in an SW65 rotor in an L2-65B ultracentrifuge for 16 hr at 4°. Afterwards, 0.2 ml fractions were collected from the bottoms of the tubes for assay of AChE, and portions of these fractions were examined with a refractometer to verify the concentration of sucrose. To calibrate the gradients, identical replicates were centrifuged with (1) β-galactosidase (16S), (2) catalase (11.2S), and (3) bovine serum albumin (4.4S) applied as samples; the positions of these markers in the gradients were established by (1) assay for galactosidase activity (12) and (2) catalase activity (13) and by (3) determinations of the total concentration of protein (14).

In some experiments, apparently soluble and apparently particulate AChE were separated before samples were placed on the gradients. In these cases, tissues were initially homogenized and subjected to differential ultracentrifugation as described under "apparent subcellular distribution." The high-speed pellets were then extracted twice by homogenization in 167 μl of buffer per cm, containing 1% Triton X-100 and 1 M NaCl, and the extracts were centrifuged at 15,000 × *g* for 15 min, as above. Samples (0.1 ml) of the high-speed supernatants (referred to as "soluble fractions") and of the combined extracts from the high-speed pellets (referred to as "particulate fractions") were finally layered on gradients of sucrose, prepared and handled as already described.

6. *Acetylcholinesterase assay.* AChE activity was measured by the radiometric assay of Potter (15) with minor modifications (3), using 50 μl samples of tissue-extracts and using acetylcholine iodide labeled with carbon-14 (New England Nuclear Corp.) as a substrate. Each assay tube contained 25 nCi of radioactively labeled substrate and enough nonradioactive acetylcholine chloride to bring the final concentration of substrate to 1 mM in a final volume of 100 μl. Buffer blanks were used routinely since they did not differ from blanks produced by addition of physostigmine to the reaction mixture in a final concentration of 10 μM. Ethopropazine was added to all samples in a concentration of 100 μM, in order to prevent butyrylcholinesterase (EC 3.1.1.8) from contributing to measured cholinesterase activity.

B. Experimental Design

1. Solubility, transport, and accessibility to AChE. Nerves were subjected to distal cooling for 3 hr. Half of these were then exposed to echothiophate and rinsed (Procedure A3.b), while the control half were treated similarly except that echothiophate was omitted from the media. After 1 hr, samples taken from the middle of the warmed and cooled regions and from the boundary between regions were homogenized, subjected to differential ultracentrifugation, and assayed.

2. Solubility of molecular forms of AChE. Samples taken from the middle of fresh nerves were homogenized and subjected to differential ultracentrifugation. Soluble and particulate fractions were then separately subjected to density-gradient ultracentrifugation and assayed.

3. Orthograde transport of molecular forms of AChE. Nerves were subjected to distal cooling for 6 hr, after which samples

were taken from the middle of the cooled and warmed regions and from the boundary between regions. These samples were then homogenized and subjected to density-gradient ultracentrifugation and assayed.

4. Retrograde transport of molecular forms of AChE. Nerves were subjected to proximal cooling for 6 hr and samples were taken from the middle of the cooled and warmed regions and from the boundary between regions. These samples were then homogenized and subjected to density-gradient ultracentrifugation and assayed.

5. Accessibility of molecular forms of AChE. Fresh nerves were exposed to echothiophate and rinsed (Procedure A3a). Control nerves were treated similarly, but without drug. After 1 hr, samples were taken from the distal third of the nerves, homogenized, subjected to density-gradient ultracentrifugation and assayed.

C. Drugs

Echothiophate (phospholine iodide, O,O′-diethyl S-ethyltrimethylammonium phosphorothiolate iodide) was obtained from Ayerst Laboratories through a local pharmacy. Although this opthalmic preparation contained potassium acetate, the highest concentration of the salt in the solutions applied to nerve was 1 mM. Ethopropazine (10-2-diethylaminopropyl phenothiazine) HCl was kindly suppled by Dr. J.D. Stein, Warner-Lambert Research Institute, Morris Plains, New Jersey.

RESULTS

Apparent subcellular distribution of AChE. Experiments were first designed to determine the solubility of AChE in different neural fractions, especially those that were: 1) rapidly transported; 2) stationary; 3) accessible to echothiophate; and 4) relatively inaccessible to echothiophate. Nerves were subjected to local cooling of their distal portions for 3 hr and were then exposed to echothiophate as described in the METHODS, B.1. A control group was treated similarly but without drug. Samples were then taken from various regions of the nerves, homogenized, and subjected to differential ultracentrifugation (Table 1). As expected, the concentrations of AChE activity were highest at the boundary of the cooled region, where rapidly transported enzyme is known to accumulate (3), and were lowest in the middle of the warmed region, which probably loses most of its transported enzyme. The differences in concentration of AChE activity were pro-

TABLE 1

Effect of distal cooling and treatment with echothiophate on the apparent subcellular distribution of AChE

Nerves were distally cooled for 3 hr and then exposed to echothiophate or physiological saline solution (see METHODS, B.1). "Warmed" and "cooled" segments were taken from the midpoints of the proximal and distal regions, respectively, while other segments were taken from the boundary between regions. The percentage of soluble activity was calculated for each individual segment by dividing the activity in the supernatant fraction by the sum of activities in that fraction and in the pellet. Means and standard errors of the means are represented, and *n* indicates the number of nerves per group.

Treatment	Nerve region	*n*	AChE activity		
			Soluble	Particulate	(% soluble)
			(nmol/mm·hr)		
Local cooling only	Warmed	8	18 ± 2	47 ± 2	27 ± 1
	Boundary	7	26 ± 2[a]	99 ± 5[b]	20 ± 1
	Cooled	8	20 ± 2	53 ± 5	27 ± 1
Local cooling plus echothiophate	Warmed	6	1 ± 0.2	6 ± 1	18 ± 3
	Boundary	6	7 ± 0.6[c]	32 ± 2[c]	18 ± 1
	Cooled	6	2 ± 0.2	12 ± 1	16 ± 1

Statistical significance is indicated: [a]($p < 0.05$ vs. warmed and cooled regions of normal nerves); [b]($p < 0.01$ vs. warmed and cooled regions of normal nerves); and [c]($p < 0.01$ vs. warmed and cooled regions of nerves treated with echothiophate.

portionately more marked in the drug-treated nerves, although the total content of enzyme activity was much less than in the control nerves.

The enzyme activity remaining in the supernatant fractions after ultracentrifugation was called "soluble" and that which was recovered in the pellets was called "particulate." Thus defined, both soluble and particulate AChE were subject to transport, as indicated by an accumulation of enzyme activity at the boundary of the cooled region (Table 1). However, in control nerves there was only about a 30% increase in soluble enzyme activity at this boundary, compared with a nearly 100% increase in particulate AChE activity. Consequently, the soluble AChE activity was a significantly lower percentage of the total in samples taken from the boundary region than in samples from other parts of the nerves (Table 1). Treatment with echothiophate inhibited about 80% of the AChE activity overall, but this inhibition was not uniform, being greater in the soluble fraction than in the particulate fraction. When data from all regions of echothiophate-treated nerves were averaged, soluble AChE activity was found to constitute 17 ± 1% of the total, while in control nerves it was 25 ± 1% of the total (the difference is significant, $p < 0.001$). Inexplicably, treatment with echothiophate abolished the difference between the rate of accumulation of soluble activity and the rate of accumulation of particulate activity at the boundary of the cooled region.

Molecular forms of AChE. The subcellular fractions of fresh nerves were recentrifuged on linear gradients of 5–20% sucrose under conditions suitable for resolving molecular forms of AChE with differing sedimentation velocities (METHODS, B.2). Both in the soluble and in the particulate fractions, two main forms of the enzyme were found with sedimentation coefficients of approximately 4S and 10S (Fig. 1). The 4S form was a minor component of each fraction, but it did constitute a much greater percentage of the soluble activity than of the particulate activity.

The dynamics of the various forms of AChE were examined next in extracts placed on gradients without prior separation of soluble and particulate fractions. At the boundary of a distal nerve-region that was cooled to 5° for 6 hr *in vitro* (METHODS, B.3), there was a substantial accumulation of 10S AChE and a smaller accumulation of 4S AChE (Fig. 2). In addition, a peak of enzyme activity with a sedimentation coefficient of approximately 16S appeared in samples taken from this region, although very little 16S enzyme was detected in the controls (Fig. 2). When a second group of nerves was subjected to cooling of their proximal regions for 6 hr (METHODS, B.4), there was a similar but smaller accumulation of the three forms of AChE (not shown for reasons of clarity). Exposing a third group of nerves to echothiophate (METHODS, B.5) led to substantial inhibition of all forms of the enzyme (Fig. 2).

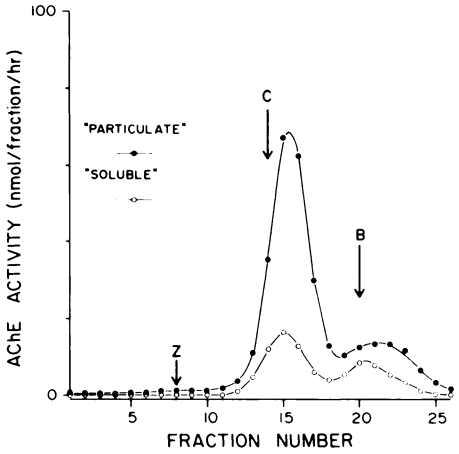

FIG. 1. *Subcellular distribution of molecular forms of AChE*

Soluble and particulate fractions of fresh rabbit sciatic nerve were placed on linear 5–20% sucrose density gradients and centrifuged as described in the METHODS (B.2). The arrows indicate the positions of the following markers after centrifugation: B (bovine serum albumin, 4.4S); C (catalase, 11.2S), and Z (beta-galactosidase, 16S).

Quantitative comparisons are best made by reference to Table 2, in which the total activity in each fraction of the experimental samples is expressed as a percentage of the activity in the corresponding fraction of the controls. The main features of the results may be summarized as follows: 1) The accumulation of all three forms of AChE at

the boundaries of cooled regions in the proximal and the distal parts of the nerves was statistically significant. 2) There was also a statistically significant depletion of all three forms from a warmed region in the distal part of proximally cooled nerves. 3) Although some of the differences among forms were not statistically significant, the rates of accumulation and depletion followed a consistent order: 16S > 10S > 4S. 4) The order of sensitivity of the various forms to echothiophate was reversed from that of the rates of accumulation and depletion: 4S > 10S > 16S, although in this case, none of the differences among forms was statistically significant.

The most striking difference among the forms of AChE was in the accumulation and depletion of 16S enzyme as compared to 10S and 4S enzyme. Although the largest absolute increases and decreases were in the activity associated with the predominant 10S form, the percentage changes were by far the largest in the 16S form. As judged from the relative rates of accumulation of enzyme activity in distally cooled nerves, the average transport velocity of 16S AChE was about 3 times as great as that of the two other forms (Table 2). Likewise as judged from the depletion of enzyme activity from warm nerve-regions, the fraction of 16S AChE subject to removal by axonal transport was also about three times as great as that of the other forms (Table 2).

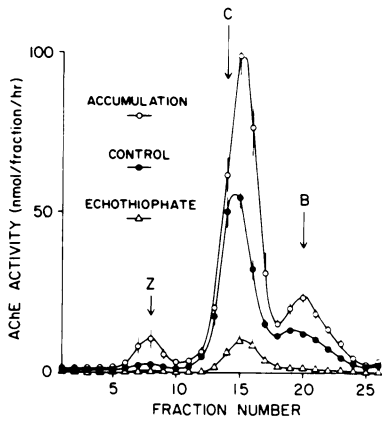

FIG. 2. *Axonal transport and echothiophate-sensitivity of molecular forms of AChE*

"Accumulation" indicates activity in segments taken from the temperature-boundary in four nerves subjected to distal cooling for 6 hr (see METHODS, B.3). Four fresh nerves were exposed to echothiophate as described in the METHODS (B.5). The control samples consisted of segments: a) from the middle of the distal region of the cooled nerves and b) from nerves that were handled exactly like those treated with echothiophate, except for the exposure to drug. Since the control samples were all similar in the content of AChE activity and its sedimentation properties, the data were combined. Gradients were centrifuged and calibrated as described in the METHODS. Mean values and standard errors are shown.

DISCUSSION

The present results are consistent with previous reports that mammalian peripheral nervous tissue contains multiple forms of AChE with sedimentation coefficients of 4S, 10S, and 16S (7-9). The minor 6.5S form recently demonstrated in rat sciatic nerve by DiGiamberardino and Couraud (10) was not resolved on our gradients, perhaps because of the relatively small number of fractions collected. Our results agree with those of DiGiamberardino and Couraud (10) in that the largest percentage accumulations of enzyme activity occurred in the 16S fraction, while the largest absolute accumulations occurred in the 10S fraction. On the other hand, we differ in finding a highly significant accumulation of 4S AChE as well as of the higher molecular weight forms.

The disproportionately rapid accumulation of 16S AChE at the boundary of a cooled distal region might be taken to reflect an unusually rapid orthograde axonal transport of this form of the enzyme. Our results do indicate large differences among the average transport velocities of the different forms of AChE. However, the differences in rates of accumulation were paralleled by differences in the amount of enzyme activity that was depleted from the middle of a warmed region of nerve. This depletion or "clearance" is probably related to the amount of material in motion (16, 17). Therefore, it is likely that differences among the average transport velocities of 4S, 10S and 16S AChE reflect differences among the distributions of the forms between moving and stationary phases in the

TABLE 2

Effect of local cooling and treatment with echothiophate on the molecular forms of AChE

Local cooling of either the proximal or the distal parts of nerves was carried out as described in the METHODS (B. 3&4). Control samples consisted of segments from the middle of the cooled regions. Echothiophate treatment was as described in the METHODS (B.5). Control samples consisted of segments from contralateral nerves treated similarly but without drug. Samples were applied to sucrose density gradients and centrifuged. The gradients were calibrated as described in the legend to Fig. 1. The combined activity in fractions 19–21 was considered to represent 4S AChE, in fractions 13–17 to represent 10S AChE, and in fractions 7–9 to represent 16S AChE. Means and standard errors of the means are shown, and *n* indicates the number of nerves per group. All values are significantly different from control.

Treatment	Nerve region	*n*	AChE activity		
			4S	10S	16S
			(% of control)		
Local cooling	Boundary (distal cooling)	4	163 ± 10	176 ± 11	297 ± 19*
	Boundary (proximal cooling)	6	128 ± 6	134 ± 4	147 ± 14
	Warmed	6	89 ± 4	83 ± 3	67 ± 2*
Echothiophate	Distal	4	11 ± 4	16 ± 3	22 ± 7

Asterisks indicate values for 16S enzyme that differ significantly from corresponding values for 4S and 10S enzyme ($p < 0.005$).

axons. The present evidence is entirely consistent with the view that the true velocity of the moving fraction is the same for each form of the enzyme. It is not necessary to suppose that 16S AChE is transported unusually rapidly or that 4S AChE is carried by "slow axonal transport."

Accumulation of enzyme activity at the borders of a cooled proximal region is an index of retrograde transport (18). Since it is unlikely that AChE is synthesized in axons (3), this retrograde transport probably involves the recirculation of enzyme originally formed in cell bodies and delivered to distal axonal regions by rapid transport. The proportion of enzyme recirculated may be gauged from the ratio of enzyme activity accumulating in proximally cooled nerves to that accumulating in distally cooled nerves. It is interesting that this ratio was twice as large for 4S and 10S AChE (about 0.4) as for 16S AChE (about 0.2, see Table 2). This could mean that the 16S form, on arrival at the nerve terminals, is more likely to be fixed, degraded, or released than are the lower molecular weight forms.

Some insight into the localization of AChE can be obtained from an analysis of the effects of echothiophate. This quaternary organophosphate anticholinesterase is slow to penetrate nerve cells (19) and can inhibit most of the AChE in external sites while sparing much of the intracellular enzyme. For example, under conditions like those used in the present experiments, exposing rabbit sciatic nerves to 100 μM echothiophate was found to inhibit about 85% of the total AChE, while inhibiting only about 30% of the rapidly transported, presumably intracellular enzyme (6). A similar effect can be seen in both soluble and particulate fractions of Table 1, where the ratio of AChE activity at the boundary of a cooled region to activity in other parts of the nerve is enhanced by echothiophate, despite substantial inhibition overall.

The most surprising effect of echothiophate was the greater inhibition of soluble than of particulate AChE (Table 1). It is difficult to reconcile this result with the idea that soluble AChE derives mainly from the axoplasm. Perhaps the majority of this fraction comes from external sites, from which it is readily solubilized either because it is sheared off the cell membrane surface during homogenization or because it has actually been secreted from the neuron (20).

Because the soluble fraction of rabbit sciatic nerve is particularly susceptible to echothiophate and is disproportionately rich in 4S AChE, one might ask whether 4S AChE is unusually well represented in the

external compartment. Unfortunately, the results of the density gradient experiments do not provide an unequivocal answer. Although the order of susceptibility to echothiophate was 4S > 10S > 16S, there were no statistically significant differences among these molecular forms. Therefore, even though minor differences may exist among the localizations of the forms of AChE, each form probably occurs in external sites as well as inside axons, along which they are all subject to rapid transport in both proximal and distal directions.

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