Neural 16S acetylcholinesterase is solubilized by heparin

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The effect of heparin, a sulphated glycosaminoglycan, on the solubilization of rat sciatic-nerve acetylcholinesterase (acetylcholine acetylhydrolase; AChE; EC 3.1.1.7) was studied. It was found that heparin solubilized esterase activity from ligated nerves. Sedimentation analysis revealed this activity to be mainly the 16S form. Chondroitin sulphate did not solubilize AChE activity, and protamine eliminated the solubilizing effect. Our results suggest the involvement of sulphated glycosaminoglycans in the intra-axonal localization and transport of 16S AChE.

AChE is synthesized in the neuronal cell body and transported to the axon by axoplasmic flow (Kasa, 1968; Ranish & Ochs, 1972). In rat sciatic nerves only a small proportion of the total AChE activity is transported by fast axonal flow, and most of this enzyme corresponds to the 16S molecular form (DiGiambardino & Couraud, 1978). So far, however, the intra-axonal localization of 16S AChE during transport remains unknown.

On the other hand, more than a decade ago, the fast axonal flow of sulphated glycosaminoglycans was demonstrated in the goldfish visual system, and a possible function for these highly charged anionic molecules, as carriers of specific cations to the synapses, was proposed (Elam *et al.*, 1970). Samson (1971) suggested that anionic polyelectrodes of microtubule-associated filaments (Tani & Ametani, 1970; Hinkley, 1973) might play a role in the mechanism of axoplasmic transport.

The main objective of the present study was to determine the effect of sulphated mucopolysaccharides on the solubility of sciatic-nerve AChE, as a way to explore the possible involvement of glycosaminoglycans in the intra-axonal localization of nerve 16S AChE. We report in the present paper that heparin solubilizes 16S AChE conveyed by fast axonal transport in rat sciatic nerve.

aseptic conditions both sciatic nerves were exposed proximally from their entrance into the sciatic notch and distally to the muscles of the hind leg. One sciatic nerve (experimental) was tightly tied with fine silk thread (gauge 00). The contralateral sciatic nerve (control) was left intact. Exposed regions were washed with 0.9% NaCl (saline), the skin incisions were closed and the animal was allowed to recover.

Preparation of sciatic-nerve extracts

At different times post-operation (12–36 h) the animals were stunned by a blow on the head. Both experimental and control sciatic nerves were exposed, quickly removed and 3 mm nerve segments were taken either proximal to the ligature or from the corresponding contralateral (control) sciatic nerve. The samples were immediately weighed (0.003–0.005 g wet wt.), finely minced with scissors and homogenized (1:50, w/v) in 0.05 M-Tris/HCl ice-cold buffer, pH 8.4, containing 0.15 mM-EDTA and 0.5% Triton X-100, or the same Tris-Triton buffer plus heparin (2 mg/ml). The homogenates were centrifuged at 10000g for 20 min at 4°C in a Sorvall centrifuge. Pellets were discarded and the supernatants were assayed for total AChE activity (Ellman *et al.*, 1961) and analysed on sucrose gradients.

Sucrose-gradient sedimentation analysis

Portions of supernatants (0.1–2 ml) containing 0.05–0.1 mg of protein were layered on to linear sucrose gradients (5 ml; 5–20% sucrose, in the homogenization Tris-Triton buffer plus 1 M-NaCl) generated by a Hoefer gradient former. Sedimentation ultracentrifugation was performed in a

Methods

Animals and surgical procedures

Male Sprague-Dawley rats (200–250 g) were anaesthetized with sodium pentobarbital (36 mg/kg body wt.), administered intraperitoneally. Under

Abbreviation used: AChE, acetylcholinesterase.

Beckman L2-65B ultracentrifuge, in an SW 50.1 rotor as described previously (Hall, 1973; Inestrosa *et al.*, 1981). Samples were centrifuged at 254 300 *g* for 8 h at 4°C and approx. 30 or 40 fractions were collected from the bottom of each gradient. A portion (0.1 ml) of each fraction was assayed for AChE activity (Ellman *et al.*, 1961).

Materials

Chondroitin sulphate and heparin were obtained from Sigma Chemical Co; protamine sulphate was from Upjohn Co.; β -galactosidase was from Worthington Biochemicals; and BW284c51 was from Burroughs Wellcome Co.

Results

In order to explore the type of components and interactions involved in the intra-axonal compartmentalization and transport of 16S AChE, we studied the solubilization of neural AChE by heparin. In control non-ligated nerves, heparin (2 mg/ml) did not solubilize more activity than that solubilized by Tris-Triton buffer alone. In contrast, in extracts from ligated nerves the addition of heparin solubilized 10% more AChE activity than the Tris-Triton buffer alone. Since it has been shown that 16S AChE form accumulates in ligated sciatic nerves (Fernández *et al.*, 1980) our results suggest that heparin might solubilize this particular form of the enzyme. To determine whether this was in fact the case, 24 h-ligated nerves were homogenized in Tris-Triton buffer and centrifuged [to eliminate some of the detergent-soluble globular forms (Inestrosa *et al.*, 1981)]. The pellet was re-extracted with the Tris-Triton buffer and then it was re-homogenized in either Tris-Triton or Tris-Triton-heparin buffer. After centrifugation these latter supernatants were subjected to sucrose-gradient sedimentation analysis. As can be seen in Fig. 1(b), it is clear that the additional activity solubilized by heparin corresponds mainly to the 16S AChE form. At this stage we wanted to find out how selective this effect of heparin might be. As can be seen in Table 1, chondroitin sulphate, another sulphated glycosaminoglycan, at a 10-fold higher concentration did not solubilize any additional activity from ligated sciatic nerves. On the other hand, protamine, a polycationic agent that has been shown to eliminate specifically both the anticoagulant activity of heparin and its inhibitory effect on the uptake of plasma lipids by endothelial cells (Ehrlich & Stivala, 1973) neutralized the solubilizing activity of this mucopolysaccharide (Table 1). Both results suggest that some specific domain of heparin is responsible for the detachment of the 16S form from its anchoring site in the nerve.

Sedimentation analysis of accumulated enzyme

Fig. 1. Sedimentation analysis of AChE activity solubilized by heparin

Five segments (3 mm) from 24 h-ligated nerves were homogenized in 50 mM-Tris/HCl (pH 8.4)/0.5% Triton X-100/0.15 mM-EDTA in a glass/glass homogenizer, and centrifuged at 10 000 *g* for 20 min. The pellet was re-extracted again in the Tris-Triton buffer, re-homogenized in the same buffer and then diluted in either the same buffer (a) or the same buffer plus 2 mg of heparin/ml (b). Centrifugation was repeated and portions of the supernatants were loaded on 5–20% sucrose gradients. Gradients were prepared in the extraction buffer containing 1 M-NaCl. They were run in an SW50.1 Beckman rotor at 254 300 *g* for 8 h. Forty fractions were recovered and assayed as detailed in the Methods section. The arrow indicates the position of sedimentation of β -galactosidase (16.1 S). A second extraction with heparin did not solubilize more 16S AChE activity, suggesting that at this concentration heparin is quite efficient as extracting agent. In (b) it is apparent that the 16S form represents around 30% of the total AChE activity; this value is obtained only after partial removal of the globular forms. The approximate sedimentation coefficients of the forms are (from left to right) 16S, 10S and 4S.

carried out at several time intervals after nerve ligation (Fig. 2) clearly showed that the 16S AChE activity solubilized by heparin increased much more rapidly than the 10S AChE activity, indicating that the appearance of the asymmetric AChE correlated well with ligation time. Our results reveal, then, that the heparin-extraction procedure can be used to

Fig. 2. *Sedimentation analysis of AChE activity solubilized by heparin at different periods of ligature* Segments (5 mm) from two nerves were homogenized after 0, 12 and 24 h ligature as described in Fig. 1 and the first pellet was re-extracted in Tris-Triton buffer. The second pellet was re-homogenized in Tris-Triton-heparin, centrifuged and the supernatant loaded on 5–20% sucrose gradients. Gradients were run in an SW50.1 Beckman rotor at 254 300g for 6.5 h. Thirty fractions were collected and assayed as described in the legend to Fig. 1. The arrow indicates the position of sedimentation of β -galactosidase (16.1S). The approximate sedimentation coefficients of the forms are 16S and 10S.

solubilize selectively the 16S AChE form not only from long-term ligated nerves, but also from early-ligated ones. This procedure could be useful in axonal transport kinetic studies.

Discussion

Our results show that heparin is able to solubilize 16s AChE from ligated sciatic nerves. Since solubilization has been, in this and other systems (Singer & Nicholson, 1972), a valuable tool to explore the nature of the interactions involved in the specific association of a macromolecule to a sub-

Table 1. *Specificity of the effect of heparin*

A pool of three nerves (24h-ligated) was homogenized in 50 mM-Tris/HCl (pH 8.4)/1 mM-EDTA without detergent, in a glass/glass homogenizer, and centrifuged at 10000g for 20 min to remove most of the soluble globular forms. The pellet was re-homogenized in the same buffer and four portions were taken and diluted in the extraction buffer as shown in the Table. The centrifugation was repeated and the AChE activity was determined in the supernatant. Values are means $\pm$ S.E.M. of three experiments. Percentage extraction was calculated taking Tris-extracted activity as 100%.

Extraction buffer	AChE activity solubilized (nmol/ min per g of nerve)	Extraction (%)
Tris/HCl (50 mM), pH 8.4	14.6 ± 1.09	100
Tris/HCl (50 mM), pH 8.4; heparin (0.5 mg/ml)	$23.4 \pm 1.70^*$	160
Tris/HCl (50 mM), pH 8.4; heparin (0.5 mg/ml); protamine (2 mg/ml)	13.4 ± 1.4	93
Tris/HCl (50 mM), pH 8.4; chondroitin sulphate (5 mg/ml)	15.3 ± 1.3	104

* $P < 0.001$.

cellular structure, our finding should help us to understand how 16S AChE is localized in the axon, and what type of structure is involved in its transport.

We suggest that heparin solubilized the enzyme by competing with other mucopolysaccharides for binding sites probably located in the collagen-like tail of the 16S form. In fact, heparin has been shown to have a number of biological effects that depend on its ability to bind to specific domains of proteins (Lindahl & Höök, 1978). Among these it displaces lipoprotein lipase from capillary walls and liver phospholipase from their anchoring site; both lipases are associated with glycosaminoglycans (Waite & Sisson, 1973; Olivecrona *et al.*, 1977). Heparin is also able to bind collagen and displace other glycosaminoglycans bound to this protein (Ruoslathi & Engvall, 1980). Moreover, it has been shown that heparin displaces heparan sulphate from the surface of cultured cells (Kraemer, 1977). Furthermore, we have recently shown that heparin also solubilizes 16S AChE from rat neuromuscular junction (Torres & Inestrosa, 1983).

On the other hand, the specific histochemical staining of intra-axonal structures with Alcian Blue (Hinkley, 1973) and Ruthenium Red (Tani & Ametani, 1970) suggested that sulphated mucopolysaccharides are in fact present in axons. Also, it has been shown in several preparations that glycosaminoglycans are transported by fast axonal transport (Elam *et al.*, 1970; Edstrom & Mattson,

1972; Elam & Peterson, 1976). These sulphated mucopolysaccharides are mostly of the heparan sulphate type (Elam *et al.*, 1970; Elam & Peterson, 1976). So far, the intra-axonal localization of these polyanions is partially known, and there exists evidence for their association with axonal plasma membrane (Elam, 1982), secretory vesicles (Kiang *et al.*, 1982; Stadler & Dowe, 1982) and microtubule-associated filaments (Hinkley, 1973). In any case, no matter what is the actual structure involved in the transport of 16S AChE, our results strongly suggest the participation of an enzyme-glycosaminoglycan interaction. Also, they provide a new methodological tool to study both the enzyme and its mechanism of axonal transport.

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