

Heparin solubilizes asymmetric acetylcholinesterase from rat neuromuscular junction

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Received 9 February 1983

We are interested in the factors involved in the anchorage of acetylcholinesterase (AChE) to the synaptic basal lamina. Here, we report studies showing that heparin, a sulfated glycosaminoglycan, specifically solubilized AChE from endplate regions of rat diaphragm muscle. Of the several molecular forms of AChE present in that region, heparin only released the asymmetric A₁₂ and A₈ forms of the enzyme. Our results strongly support the involvement of heparin-like macromolecules in the in vivo immobilization of the collagen-tailed forms of AChE to the basal lamina of the neuromuscular junction.

<i>Asymmetric acetylcholinesterase</i>	<i>Heparin</i>	<i>Proteoglycans</i>	<i>Basal lamina</i>
<i>Neuromuscular junction</i>		<i>Rat diaphragm muscle</i>	

1. INTRODUCTION

The acetylcholinesterase (AChE, EC 3.1.1.7) of vertebrate skeletal muscle can be separated by sedimentation analysis into several molecular forms [1]. The different AChE molecules may be classified as globular forms (monomers G₁, dimers G₂, tetramers G₄) and asymmetric forms (containing one, two or three tetramers, plus a collagen-like tail: A₄, A₈ and A₁₂ [2]). In normal adult rat muscle, the A₁₂ form which represents the major asymmetric form, is concentrated at the endplate region [2–4] where it is thought to be anchored to the basal lamina [2,5,6], a thin amorphous sheet of collagen, glycoproteins, and proteoglycans [7,8], which has been implicated in both pre- and post-synaptic differentiation [9,10]. Interactions between the collagen-like tail of A₁₂ AChE and components of the basal lamina might account for the immobilization of the enzyme in this extracellular structure [1,2,6]. Indeed, isolated A₁₂ AChE has

been shown to interact with both fibronectin [11] and glycosaminoglycans (GAGs) [12]. However, there is no evidence that such interactions actually take place in situ.

Here, we report that the GAG heparin solubilizes A₈ and A₁₂ AChE from the rat neuromuscular junction. This result suggests that GAGs are implicated in the in vivo immobilization of asymmetric AChE at the synaptic basal lamina.

2. MATERIALS AND METHODS

The following chemicals were used: acetylthiocholine iodine, *iso*-OMPA, chondroitin sulfate and heparin from bovine lung (Sigma Chemicals); Protamine sulfate (Upjohn); 5,5'-dithio(bis)-dinitrobenzoic acid (Aldrich); Triton X-100 (New England Nuclear). The sucrose used was special Enzyme grade. The specific AChE inhibitor, BW284c51 dibromide was obtained from Burroughs Wellcome Co. The standard enzyme β -galactosidase was obtained from Worthington Biochemicals.

All experiments were made on male Sprague-Dawley rats (200–250 g body wt).

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Abbreviations: AChE, acetylcholinesterase; GAG, glycosaminoglycan

2.1. Rat diaphragm-muscle dissection

Rat diaphragm muscles together with attached ribs were removed, washed in ice-cold saline, rapidly cleaned of most of the connective tissue and separated from the ribs. We divided diaphragms into regions containing endplates (the middle third of the muscle) and those without endplates, by transversely cutting 2–3 mm strips of muscle, under a dissecting microscope [3,6,13].

2.2. Extraction of AChE activity

Endplate and non-endplate regions of rat diaphragm muscle were dissected as above, and homogenized in ice-cold 50 mM Tris-HCl, (pH 8.4) plus 0.5% Triton X-100 or the same Tris-Triton buffer plus heparin, and centrifuged at $20\,000 \times g$ for 20 min. In some experiments 10 mM EDTA, 20 U aprotinin/ml, 20 μ g pepstatin/ml, 1 mM benzamidine, 1 mM *N*-ethylmaleimide and 0.1 mg bacitracin/ml were added to the Tris buffer.

2.3. Linear sucrose density gradients 5–20%

A Hoeffer gradient former was used. In general, a 200 μ l sample was layered on the top of the gradient. Centrifugation was performed in a SW 50.1 rotor at 4°C, at 46 000 rev./min for 8 h in a L5-65 Beckman ultracentrifuge [14,15]. Fractions were collected from the bottom and assayed for AChE activity. Sedimentation coefficients for AChE forms were estimated by comparison with that for β -galactosidase (16.1 S). β -Galactosidase was measured as in [16].

2.4. AChE assay

AChE activity was measured as in [17]. All determinations were done at 37°C in a 1 ml reaction mixture, containing 100 mM sodium phosphate buffer (pH 6.0), 0.75 mM acetylthiocholine iodide, 0.3 mM 5,5'-dithio(bis)-dinitrobenzoic acid and 0.1 mM *iso*-OMPA. BW284c51 dibromide (10 μ M) was used as AChE specific inhibitor.

3. RESULTS

We first compared the total AChE activity released by heparin in both endplate and non-endplate regions of adult rat diaphragm muscle. Heparin was able to increase the solubilization of

AChE activity only from the innervated regions (table 1). As little as 0.05 mg heparin/ml appeared to facilitate extraction of AChE activity. The maximum percentage of solubilization obtained at 1–3 mg heparin/ml is similar to that obtained with high ionic strength solutions (1 M NaCl). When the same experiment was repeated in neonatal rats (postnatal day 13), heparin was able to solubilize AChE activity from both endplate and non-endplate regions (for the latter the values were: Tris-Triton medium $0.4 \pm 0.01 \mu\text{mol AChE} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$; heparin containing medium 0.6 ± 0.01 ; $n = 4$, $P < 0.01$). Because in normal adult rat muscle, the A_{12} form is concentrated in the endplate region [2–4], whereas in neonatal rats this form is distributed over the entire muscle fibre [18,19], the above results suggest a specific solubilization of the asymmetric or collagen-tailed forms of AChE by heparin.

To determine the pattern of molecular forms solubilized by heparin from the endplate regions of adult rat diaphragm muscle, the supernatants were analyzed by sedimentation analysis [2,3,6]. Fig. 1

Table 1

Solubilization of AChE activity from endplate regions of rat diaphragm muscle by heparin (acetylcholinesterase activity, $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g muscle}^{-1}$)

Treatment	Endplate region	Non-endplate region
(a) Buffer		
Tris		
Triton X-100	0.74 ± 0.04	0.38 ± 0.01
(b) Buffer		
Tris		
Triton X-100	1.17 ± 0.03^a	0.39 ± 0.01
Heparin		

^a $P < 0.005$ as compared to control without heparin in the endplate region. Each value is the mean \pm SEM of 4 different experiments run in triplicate

Endplate and non-endplate regions of rat diaphragm skeletal muscle were homogenized in either 50 mM Tris-HCl (pH 8.4), 0.5% Triton X-100, or the same extraction buffer plus 2 mg heparin/ml (from bovine lung, Sigma Chemicals). Homogenates were centrifuged at $20\,000 \times g$ for 20 min. Aliquots of the supernatants were added to assay mixture containing 0.75 mM acetylthiocholine iodide and 0.1 mM *iso*-OMPA [17]. Assays were conducted at 37°C for 30–45 min

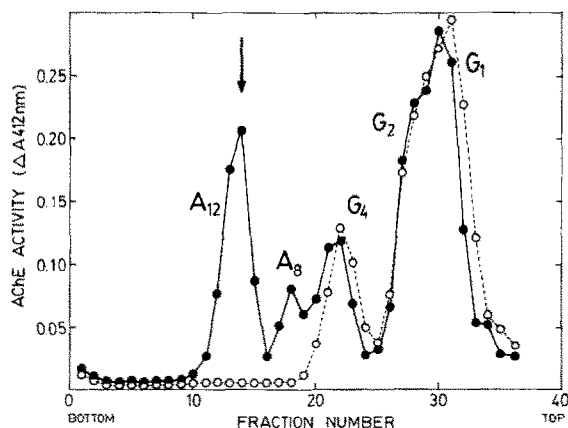


Fig. 1. Sedimentation profiles of AChE in endplate regions of rat diaphragm muscle after solubilization with and without heparin: (○-○-○) extraction in Tris-Triton buffer; (●-●-●) extraction in Tris-Triton-heparin buffer; 200 μ l supernatant obtained as in table 1 were layered over linear 5–20% sucrose gradients containing 50 mM Tris-HCl (pH 8.4), 0.5% Triton X-100 and 1 M NaCl. Gradients were fractionated in ~36 fractions and aliquots assayed as in table 1. (→) Sedimentation position of β -galactosidase (16.1 S) assayed in [16].

shows sucrose gradient profiles of AChE activity solubilized in both buffer-detergent and buffer-detergent-heparin. The additional activity solubilized by heparin corresponds mainly to the A_{12} and A_8 AChE forms (fig. 1). Table 2 shows the relative proportions of the various molecular forms solubilized by this GAG. With minor differences, this profile is similar to that obtained when including 1 M NaCl in the Tris-Triton extraction medium without heparin. The addition of protease inhibitors to the extraction medium did not modify the profile obtained with heparin.

Preliminary experiments on the specificity of the effect of heparin indicated that:

- The presence of protamine in the extraction medium eliminates the capacity of heparin to extract AChE activity;
- Other GAG like chondroitin sulfate at a 10-fold higher concentration than heparin, is not able to solubilize the esterase.

4. DISCUSSION

Our results clearly demonstrate that the GAG heparin solubilizes AChE activity from endplate

Table 2

Molecular forms of AChE in control and heparin extracts of endplate regions of rat diaphragm muscle

	Proportions of molecular forms			
	G_1 - G_2	G_4	A_8	A_{12}
(a) Buffer				
Tris	82	17	-	-
Triton X-100	(81-83)	(16-18)		
(b) Buffer				
Tris				
Triton X-100	62	12	6	19
Heparin	(57-66)	(10-14)	(5-7)	(17-21)

The proportions of molecular forms were estimated from sedimentation profiles obtained from different animals. The values correspond to the mean of 3 animals and the numbers in parentheses to the range of the data. Endplate regions of the left hemidiaphragms were homogenized as in table 1. For the 3 expt, 200 μ l supernatant were run in each gradient. The total AChE activity for the Tris-Triton supernatant was 0.24 ± 0.02 μ mol substrate hydrolyzed $\cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ and for the heparin-containing supernatant, the activity was 0.30 ± 0.02 , mean \pm SEM of 3 expt. Although the G_1 and G_2 forms represent most of the activity in all gradients, they were not well resolved; therefore, only a global percentage is indicated. We could not detect the A_4 form in our analyses, because of the presence of large amounts of globular forms at the corresponding region of the gradient

regions of the rat diaphragm muscle and that most of this activity corresponds to the A_8 and A_{12} forms. There is strong evidence that the A_{12} AChE form is associated to the basal lamina, both in muscle cells in culture [6] and at the adult neuromuscular junction [2]. The collagen-like tail of this form appears to be involved in the attachment of the enzyme, since collagenase is able to release active enzyme from muscle cells [6,20,21]. The collagen-like portion has also been shown to play a role in the interaction of the isolated A_{12} with fibronectin [11] and GAGs [12], two macromolecules present at the muscle basement membrane [7,8]. It has been suggested that the aggregation of asymmetric AChE at low ionic strength, could be related to the immobilization of the collagen-tailed AChE molecules to the basal lamina [1]. In the electric organ of *Electrophorus*, the aggregation depends on a factor related to a GAG of the chondroitin sulfate type [12]. In mam-

mals however, no substantial release of AChE has been obtained after treating mouse myotube cultures [6] and endplate regions of mouse diaphragm [22] with condroitinase ABC and hyaluronidase, respectively.

Heparin, a naturally occurring GAG, has been shown to release lipoprotein lipase from capillary walls [23], to facilitate extraction of fibronectin from lung parenchyma and placental villi [24], and to displace heparan sulfate from the surface of cultured cells [25]. These effects of heparin are probably related to its strongly polyanionic character, its high content of α -L-iduronic acid and other still unclear stereochemical factors [26]. The solubilization of A₁₂ and A₈ AChE by heparin reported here is the first evidence linking this enzyme to GAGs in situ. Our results do not allow conclusions on the nature of such an interaction. Nonetheless, in view of the behavior of heparin in other systems, we suggest that heparin might be competing with heparin-like GAGs for binding sites on the collagen-like tail of the enzyme or other intermediate molecules involved in its anchorage. Because heparin is structurally related to heparan sulfate, and proteoglycans containing this GAG are integral components of basement membranes [27], including the synaptic basal lamina of rat diaphragm muscle [8], heparan sulfate proteoglycans appear as good candidates to be involved in the attachment of the asymmetric AChE to the extracellular matrix. At any rate, our results strongly support the involvement of GAGs in the immobilization of A₈ and A₁₂ AChE forms to the basal lamina of the mammalian neuromuscular junction.

ACKNOWLEDGEMENTS

We thank our colleagues of the Neurophysiology Laboratory and Drs A. González and F. Leighton from our department for their comments on an early draft of the manuscript. We also thank Dr M.I. Behrens for help with the experiments on developing muscles. This work was supported by grants from the Fundación Gildemeister, the Dirección de Investigaciones de la Universidad Católica de Chile (59/82) and from the Fondo Nacional de Ciencias de Chile (645/82).

REFERENCES

- [1] Massoulié, J. and Bon, S. (1982) *Annu. Rev. Neurosci.* 5, 57-106.
- [2] Bon, S., Vigny, M. and Massoulié, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 2546-2550.
- [3] Hall, Z.W. (1973) *J. Neurobiol.* 4, 343-361.
- [4] Vigny, M., Koenig, J. and Rieger, F. (1976) *J. Neurochem.* 27, 1347-1353.
- [5] McMahan, U.J., Sanes, J.R. and Marshall, L.M. (1978) *Nature* 271, 172-174.
- [6] Inestrosa, N.C., Silberstein, L. and Hall, Z.W. (1982) *Cell* 29, 71-79.
- [7] Sanes, J.R. (1982) *J. Cell Biol.* 93, 442-451.
- [8] Daniels, M.P., Vigny, M., Sonderegger, P., Bauer, H.C. and Vogel, Z. (1983) *J. Neurosci.* in press.
- [9] Sanes, J.R., Marshall, L.M. and McMahan, U.J. (1978) *J. Cell Biol.* 78, 176-198.
- [10] Bader, D. (1981) *J. Cell Biol.* 88, 338-345.
- [11] Emmerling, M.R., Johnson, C.D., Mosher, D.F., Lipton, B.H. and Lilien, J.E. (1981) *Biochemistry* 20, 3242-3246.
- [12] Bon, S., Cartaud, J. and Massoulié, J. (1978) *Eur. J. Biochem.* 85, 1-14.
- [13] Inestrosa, N.C. and Fernández, H.L. (1982) *Muscle and Nerve* 5, 33-38.
- [14] Inestrosa, N.C., Méndez, B. and Luco, J.V. (1979) *Nature* 280, 504-506.
- [15] Méndez, B. and Inestrosa, N.C. (1981) *Brain Res. Bull.* 7, 17-24.
- [16] Graven, G.R., Steers, E. Jr. and Anfinsen, C.B. (1965) *J. Biol. Chem.* 240, 2468-2477.
- [17] Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) *Biochem. Pharmacol.* 7, 88-95.
- [18] Sketelj, J. and Brzin, M. (1980) *Neurochem. Res.* 5, 655-660.
- [19] Koenig, J. and Rieger, F. (1981) *Dev. Neurosci.* 4, 249-257.
- [20] Hall, Z.W. and Kelly, R.B. (1971) *Nature New Biol.* 232, 62.
- [21] Inestrosa, N.C., Ramírez, B.U. and Fernández, H.L. (1977) *J. Neurochem.* 28, 941-945.
- [22] Sketelj, J. and Brzin, M. (1979) *Histochemistry* 61, 239-248.
- [23] Olivecrona, T., Bengtsson, G., Marklund, S.E., Lindahl, U. and Höök, M. (1977) *Fed. Proc. FASEB* 36, 60-65.
- [24] Bray, B.A., Mandl, I. and Turino, G.M. (1981) *Science* 214, 793-795.
- [25] Kraemer, P.M. (1977) *Biochem. Biophys. Res. Commun.* 78, 1334-1340.
- [26] Lindahl, U. and Höök, M. (1978) *Annu. Rev. Biochem.* 47, 385-417.
- [27] Farquhar, M.G. (1981) in: *Cell Biology of Extracellular Matrix* (Hay, E.D. ed), pp. 335-378, Plenum, London, New York.