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Co-solubilization of asymmetric acetylcholinesterase and dermatan sulfate proteoglycan from the extracellular matrix of rat skeletal muscles

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We have previously communicated that heparin released asymmetric acetylcholinesterase (AChE) from cholinergic synapses. Here we report studies showing that heparin, besides releasing asymmetric AChE from the skeletal muscle extracellular matrix (ECM), specifically solubilizes a dermatan sulfate proteoglycan (DSPG) which accounts for more than 95% of the ^{35}S-released material. The co-solubilization of AChE and the proteoglycan opens up the possibility that both macromolecules could be involved in the formation of the soluble AChE complex observed after incubation of muscle homogenate with heparin. Our results suggest a possible association between asymmetric AChE and DSPG at the muscle ECM, moreover this work is the first report of the existence of DSPG at the skeletal muscle cell surface.

Acetylcholinesterase; Heparin; Dermatan sulfate proteoglycan; (Muscle extracellular matrix)

1. INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7) is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine. The enzyme exercises this role at synaptic junctions where it is thought to be bound via extracellular HSPGs to muscle, electric organ and neuronal cells [1–3]. The AChE at the synaptic ECM is displaced by the action of exogenously applied heparin through a mechanism involving competition for enzyme binding between the polyanion and the endogenous protein-linked GAG [4–6]. We have recently found that the ECM of rat skeletal muscles contained a HSPG of 5×10^5 Da which is apparently concentrated at the neuromuscular junction [7]. The aim of this paper is to examine whether endogenous HSPGs could be displaced from the muscle ECM on addition of heparin; this is important because heparin removed AChE from the ECM by a mechanism that involves the formation of soluble complex between the GAG (HSPG?) and the asymmetric AChE [2,4,6].

We report here that heparin solubilizes asymmetric AChE and DSPG, but not HSPG, from the rat neuromuscular junction. This result is important for two reasons, (i) it is the first evidence linking AChE to DSPG, and (ii) it provides direct evidence for the presence of DSPG in the ECM of rat skeletal muscle.

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Abbreviations: AChE, acetylcholinesterase; HSPG, heparan sulfate proteoglycan; ECM, extracellular matrix; GAG, glycosaminoglycan; DSPG, dermatan sulfate proteoglycan

2. MATERIALS AND METHODS

The following chemicals were used: chondroitin sulfate from shark and whale cartilage, heparin

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from bovine lung and chondroitinase ABC and AC (Sigma); guanidine-HCl (Bethesda Research Labs); $Na_2{}^{35}SO_4$ (100 Ci/mmol) was obtained from New England Nuclear. Other reagents, where not specified, were obtained from commercial sources.

In all experiments male Sprague-Dawley rats (200 g body wt) were used. The rats were injected i.p. with a total of 10 mCi of [^{35}S]sulfate in saline. Four injections were carried out during the first 12 h.

2.1. *Preparation of ECM-like material*

A detergent-insoluble fraction enriched in basal lamina components was prepared by modifications of previous procedures [8,9]. Rat skeletal muscles were homogenized in 10 mM Tris-HCl buffer, pH 7.4, containing 20 U/ml aprotinin, 5 mM benzamidine-HCl, 100 mM 6-aminohexanoic acid, 0.5% (v/v) Triton X-100. The homogenate was centrifuged at 15000 × *g* for 15 min. The precipitate was resuspended in the same homogenization medium and centrifuged again. This procedure was repeated three times, and the final insoluble precipitate ECM-like material was used for the experiments described in this paper.

2.2. *Characterization of proteoglycans*

The ^{35}S-material solubilized by heparin from the muscle ECM, was diluted with 8 M urea, 0.2 M sodium chloride and 0.05 M sodium acetate, pH 6.0. The sample was then applied to a DEAE-Sephacel column pre-equilibrated in the same 8 M urea buffer with the addition of 0.5% (v/v) Triton X-100. After sample application, the column was washed with 5–10 ml of the same buffer, and then eluted with a continuous NaCl gradient in the same solvent using a total volume of 40 ml [10]. A bed volume of 3 ml was used for the DEAE-Sephacel column. Analytical Sepharose CL-4B chromatography was carried out in columns of 0.7 × 120 cm prepared in 1% SDS, 0.1 M NaCl and 50 mM Tris-HCl buffer, pH 8.0. Samples were applied to columns together with previously fractionated Blue Dextran 2000 (Pharmacia) and phenol red, respectively to mark the void and total volumes.

2.3. *Enzymatic and chemical treatments*

Chondroitinase digestion of ^{35}S-labelled proteoglycans was done in 50 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl. Digestion with 0.1 U/ml was carried out for 4 h at 37°C [11]. Degradation of heparan sulfate was done by nitrous acid treatment using reaction 'A' of Lindahl et al. [12]. GAG chains free from the protein were obtained from the proteoglycans by a mild alkali treatment (24 h at 37°C in 100 mM NaOH and 1 M $NaBH_4$) [13].

2.4. *AChE assay and sucrose gradients*

AChE activity was measured as in [14]. Velocity sedimentation in 5–20% sucrose gradients was carried out as described [15].

3. RESULTS

If it is assumed that asymmetric AChE is bound to polysaccharide 'receptors', it may be postulated that added heparin would displace completely the

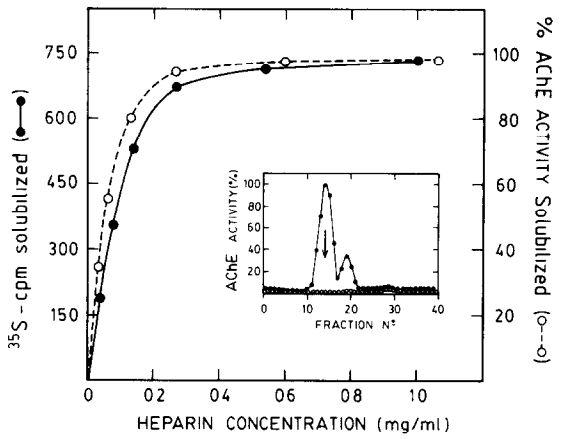

Fig.1. Solubilization of ^{35}S-labelled polysaccharide and asymmetric AChE from ECM-like material of rat skeletal muscles. A detergent-insoluble ECM fraction was incubated in 50 mM Tris-HCl, pH 8.4. Heparin was added to the different samples to obtain the final concentration shown in the figure. After homogenization, the samples were centrifuged at 15000 × *g* during 15 min and the AChE activity (o---o) was determined in the supernatant. In parallel experiments the ^{35}S-radioactivity released by heparin was also evaluated (●——●). In each case a point represents the average of three different experiments. The inset shows the velocity sedimentation of the AChE molecular forms solubilized by heparin (●——●) after separation in 5–20% sucrose gradients [1,15]. The arrow indicates the sedimentation position of *β*-galactosidase (16.1 S).

particular surface-bound proteoglycan. As shown in fig.1, the addition of heparin to skeletal muscle ECM [2,8] displaced around 35% of the total endogenous ^{35}S-labelled polysaccharide and most of the asymmetric AChE (see inset in fig.1). Maximal amounts of ^{35}S-labelled polysaccharide and AChE were released by heparin at 0.2–0.3 mg/ml. All the class I and part of the class II forms of AChE were solubilized by heparin (not shown).

[^{35}S]Sulfate-labelled polysaccharide released by heparin (1 mg/ml) was analyzed by DEAE-Sephacel chromatography (fig.2A). This method has proved to be very effective in separating heparan sulfate from dermatan sulfate proteoglycans because of the large charge density difference in the two classes of GAGs [10]. Most of the bound material to the DEAE-Sephacel column eluted at a salt concentration of 0.5 M NaCl. In order to know how many kinds of proteoglycans of similar charge and different size were released by heparin, the above DEAE peak was pooled and analyzed by Sepharose CL-4B chromatography in 4 M guanidine-HCl. The elution profile is shown in fig.2B (●——●). The ^{35}S-labelled polysaccharide eluted at K_{av} = 0.6 which corresponds to a single species of M_r = 0.5–0.8 × 10^5 [16]. Incubation of this material with alkaline borohydride released polysaccharide chain from the core protein (fig.2B, ○---○) indicating that this ^{35}S-labelled material represents a proteoglycan.

In order to determine the nature of this proteoglycan, the ^{35}S-labelled material solubilized by heparin from the muscle ECM was concentrated and submitted to different mucopolysaccharides and chemical treatments. When the ^{35}S-labelled proteoglycan was treated with nitrous acid (degrades heparan sulfate and heparin) and chromatographed on Sepharose CL-4B (fig.3A), almost all the material eluted at a similar position to that of the control (fig.2B, ●——●). This result indicated that a HSPG was not released by heparin. When the ^{35}S-labelled material was treated with chondroitinase ABC (degrades chondroitin and dermatan sulfate) more than 95% of the ^{35}S-labelled proteoglycan was hydrolyzed and the fragments eluted at the total volume of the column (fig.3B). Finally when the ^{35}S-labelled material solubilized by heparin was treated with chondroitinase AC II (degrades only chondroitin sulfate [11]) no change in the elution position of

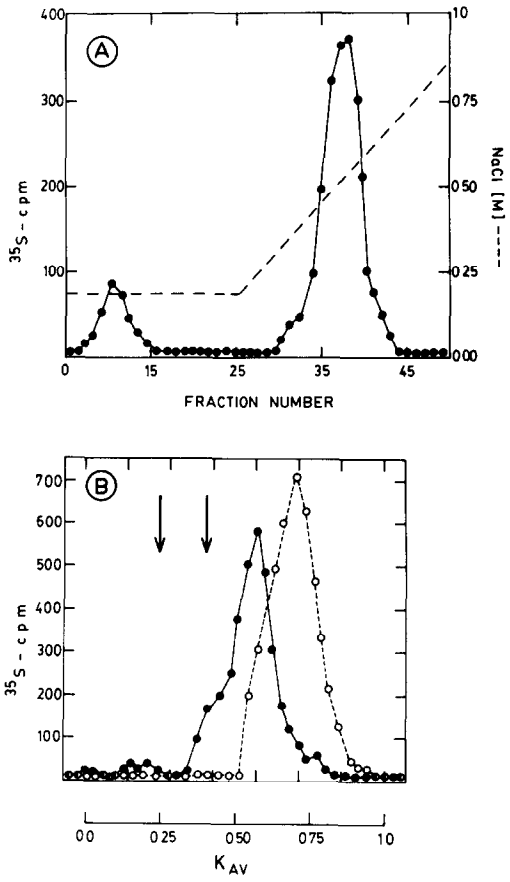

Fig.2. Characterization of the ^{35}S-labelled polysaccharide released by heparin as a proteoglycan. (A) DEAE-Sephacel chromatography: ^{35}S-labelled material solubilized by heparin from a muscle ECM fraction, was diluted in 8 M urea, 0.2 M NaCl and was then applied to a DEAE-Sephacel column previously equilibrated in the same solution. The material bound to the column was eluted in a linear gradient of 0.2–0.9 M NaCl. (B) Sepharose CL-4B: The ^{35}S-labelled material bound to the DEAE-Sephacel column, was concentrated and applied to a Sepharose CL-4B (●——●). The proteoglycan nature of the ^{35}S-labelled material released by heparin was demonstrated after treatment of the material bound to the DEAE-Sephacel column with sodium borohydride (○---○). Under these conditions the free GAG chains eluted from the Sepharose CL-4B after the untreated material (K_{av} 0.72 against K_{av} 0.54). The recovery of radioactivity from both columns was better than 90%. The two arrows at K_{av} 0.25 and 0.41 corresponded to the elution of the two major HSPGs present in the muscle ECM [7].

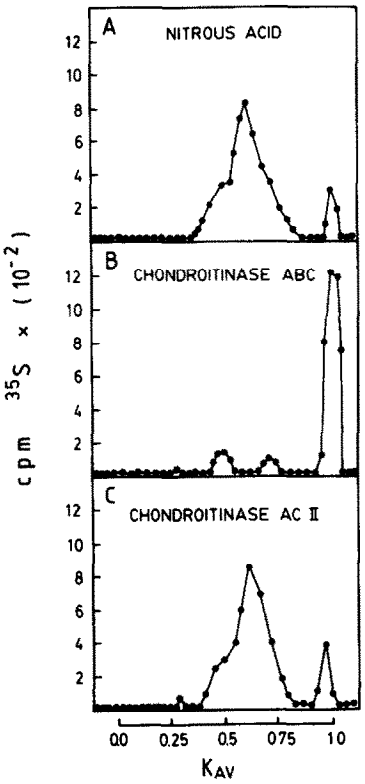

Fig.3. The ^{35}S-labelled proteoglycan released by heparin is a DSPG. The ^{35}S-labelled proteoglycan solubilized by heparin from the skeletal muscle ECM was treated with (A) nitrous acid, which degrades heparan sulfate and heparin; (B) chondroitinase ABC, which degrades chondroitin and dermatan sulfate; and (C) chondroitinase AC II, which only degrades chondroitin sulfate. The products of these reactions were filtrated in Sepharose CL-4B (pre-equilibrated in 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 1% SDS). The recovery in the three columns was around 87 and 95%.

the ^{35}S-labelled proteoglycan was observed in the Sepharose CL-4B (fig.3C). This result indicates that heparin releases from the muscle ECM a DSPG previously unknown.

4. DISCUSSION

Our results clearly demonstrate that heparin co-solubilizes both the asymmetric AChE and a DSPG from the skeletal muscle ECM. This result was unexpected, in fact; following the rationale that heparin released AChE by the removal of the polysaccharide receptor involved in its anchorage, we anticipated the solubilization of HSPG. However, this was not the case and almost all the ^{35}S-labelled proteoglycan solubilized corresponds to DSPG, a proteoglycan not previously observed in the basement membranes of skeletal muscles. The significance of the co-liberation of the asymmetric AChE and a dermatan sulfate type of proteoglycan is uncertain and of course does not necessarily prove that DSPG is bound to AChE in situ, in fact its release might well fortuitously coincide with that of AChE. Alternatively, our results could be related to a possible interaction between both macromolecules in the muscle ECM.

In studies on the binding of *Discopyge* asymmetric AChE to bovine endothelial cells, we have previously shown that treatment with chondroitinase ABC (degrades both chondroitin and dermatan sulfate) determines some decrease in the binding of the asymmetric AChE to such cells [17], this result is interesting because chondroitin sulfate was not able to dissociate previously bound AChE, suggesting that besides HSPG, dermatan sulfate but not chondroitin sulfate proteoglycans could also be involved in the association of AChE to endothelial cells. In the case of C_2 mouse myotube cultures however, treatment with chondroitinase ABC did not release asymmetric AChE, indicating that neither chondroitin sulfate proteoglycan nor DSPGs are involved in the anchorage of the esterase to mouse myotubes [18]. The same was true for experiments carried out with purified cholinergic basal lamina from *Discopyge* electric organ [2].

DSPGs which differ greatly in size and structure are present in the basement membranes, plasma membranes, and ECM of a variety of different normal and neoplastic tissues [19]. DSPGs of relatively large size are components of the basement membranes of teratocarcinoma cells [20,21], however the most widely distributed DSPGs are the small, interstitial proteoglycans present in the ECM of fibrous connective tissues [22].

The DSPG solubilized by heparin from the muscle ECM is a small proteoglycan of 50000–80000 Da, a size consistent with that of a potential aggregating factor for the solubilized asymmetric AChE. In this context it is interesting to mention that different laboratories [4,6] have reported that heparin released asymmetric AChE, not as in-

dependent macromolecules, but as soluble complex of 24–30 S. Since a wide variety of polyanions, including heparin [23], form complexes with asymmetric AChE, it is possible that the soluble AChE-polysaccharide complex could be formed by the association of asymmetric AChE and the small DSPGs identified in the present communication. However further work is necessary to establish whether dermatan sulfate presents a higher affinity for the enzyme than heparin.

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