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Chondroitinases release acetylcholinesterase from chick skeletal muscle

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Bacterial chondroitinases (both ABC and AC types) release asymmetric and globular forms of AChE from chick skeletal muscle samples. Heparinases, however, including heparitinase I, fail to do so under different incubation conditions. These results do not support the direct implication of the heparin/heparan sulfate family of GAGs in the interaction of the different AChE molecular forms with the muscle ECM. GAGs of the chondroitin/dermatan sulfate group could however be involved, either directly or indirectly, in the attachment of the AChE collagen-like tail to the muscle basal lamina.

Skeletal muscle; Asymmetric acetylcholinesterase; Collagen-like tail; Basal lamina proteoglycan; Heparinase; Chondroitinase

1. INTRODUCTION

Experimental evidence has accumulated during recent years which supports the involvement of the glycosaminoglycan moiety of proteoglycans in the anchorage of the asymmetric, collagen-tailed forms (A-forms) of acetylcholinesterase (AChE; EC 3.1.1.7) to the basal lamina, at the motor endplate, in vertebrate skeletal muscle [1–8]. Among glycosaminoglycans (GAGs), heparan sulfate has been singled out as the most likely candidate for this role in view of the proven affinity of the tailed AChE species for heparin [3–6,8], and of the reported ability of heparinases (but not chondroitinases) to dissociate aggregative AChE-GAG complexes [2], and to release asymmetric AChE from electric organ samples [5]. Our recent work has shown, however, that the affinity of heparin for the asymmetric AChE tail is not a selective phenomenon, being shared by a number of polyanions including sulfated GAGs, dextran sulfates, some acidic polyaminoacids and even polyvinylsulfates [9]. Since this finding puts additional weight on digestion experiments as a means to identify the molecule(s) responsible for the AChE-ECM interactions, we have carried out a detailed analysis of the effect of some GAG-lyases on chick skeletal muscle AChE.

Abbreviations: AChE, acetylcholinesterase; A-forms, asymmetric molecular forms; ECM, extracellular matrix; GAGs, glycosaminoglycans; GAG-lyases, glycosaminoglycan-lyases; G-forms, globular molecular forms; TT buffer, 10 mM Tris-HCl, pH 7/0.5% Triton X-100; TTSE buffer, TT plus 1 M NaCl/5/mM EDTA

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2. MATERIALS AND METHODS

2.1. *Enzymes*

Flavobacterium heparinum heparinases I, II and III (heparitinase I), and chondroitinase AC, and *Proteus vulgaris* chondroitinase ABC were purchased from Sigma. Enzymes from other sources (e.g. Miles heparitinase) were also occasionally used, with identical results. Since all these enzymes exhibit markedly different temperature and pH optima (which are in turn substrate-dependent) [10,11] special care was exercised to use them within their high activity range. Heparinases were initially tested in the following temperature ranges: I, 30–37°C; II, 30–42°C; and III, 37–42°C [11], using chondroitinases always at 37°C [10]. As for pH, an interval of 7.0–7.4 was considered for all enzymes, except that chondroitinase ABC was checked between 7.4 and 8.0. No significant differences in activity were found within these ranges, perhaps due to the complexity of the substrate and to the high enzyme concentrations. Accordingly, and to facilitate experiments with enzyme mixtures, we adopted 37°C/pH 7.4 as the standard incubation conditions for all enzymes.

2.2. *Procedure*

Muscle samples from 10-day chick hindlimbs were first homogenized (4°C) in 10 mM Tris-HCl pH 7.0/0.5% Triton X-100 (TT buffer), containing 0.1 mg/ml bacitracin, to remove the bulk of the globular AChE forms. After centrifugation (140 000 × *g*) the pellet was resuspended in 1 ml of 10 mM Tris-HCl pH 7.4/0.1% Triton X-100/100 mM NaCl/5 mM calcium acetate, containing an antiprotease cocktail (0.1 mg/ml bacitracin, 0.3 mg/ml benzamidine, 0.1 mg/ml leupeptin, 0.1 mg/ml trypsin inhibitor) and 5 U of the corresponding GAG-lyase. Two control samples were always carried along with the experimental samples: the first was a background control, homogenized in the same buffer plus antiproteases, but containing no enzymes, to assess the spontaneous release of AChE during the incubation. The second was a measure of the A-forms available for extraction and consisted of the same sample as above but homogenized in TT buffer supplemented with 1 M NaCl and 5 mM EDTA (TTSE buffer) plus antiproteases. Incubations were carried out at 37°C for 30 min.

Two independent procedures were used to properly evaluate the AChE activity released by GAG-lyases, especially A-forms which would not be soluble, even after release, due to the low salt concentration in the incubation medium. In some experiments, the digested samples and the controls were quickly filtered through coarse glass fiber filters to remove most of the solid tissue (but not the released

Table I

Release of molecular forms of AChE from chick hindlimb muscle samples by different GAG-lyases

	Background control (no enzymes added)	Heparinases			Chondroitinases		TTSE control (maximal release of A-forms)
		I	II	III	ABC	AC	
A-forms	3.2 ± 0.8	2.9 ± 0.6	3.1 ± 0.5	2.8 ± 0.8	5.9 ± 0.8*	5.9 ± 0.8*	19.2 ± 1.6
G-forms	4.0 ± 0.5	3.7 ± 0.4	4.0 ± 0.5	3.2 ± 0.6	6.6 ± 0.5*	6.8 ± 0.8*	4.2 ± 0.5

All enzymes were used at a concentration of 5 U/ml, as explained in the text. Values (mean ± SD) in the Table are expressed as % of the total AChE activity in the initial muscle sample (i.e. before the TT extraction: see section 2). The results for A-forms are based on 8 experiments (4 of each type described in section 2), whereas, in the case of G-forms, only the results of 4 experiments (like the one depicted in Fig. 1) have been taken into account.

*Differences with background controls are significant with $P<0.001$.

asymmetric AChE aggregates, if any), and the filtrate layered on top of a 5-20% sucrose gradient made up in TTSE buffer containing the usual sedimentation marker enzymes [8]. The rationale for this procedure was to effect the total solubilization of the released AChE, while minimizing the exposure to high salt of the A-forms remaining attached to the particulate material (i.e. not released by the GAG-lyases). The differences between gradients containing samples and background controls would then be due to the specific action of the enzymes. The second protocol was a modification of the one used by Inestrosa and co-workers [5]; the digested samples and the controls were layered on top of 1.5 ml of 45% sucrose, and centrifuged for 10 min at 10 000 × g. Both the supernatant and the heavy sucrose layer were immunoprecipitated with a monoclonal antibody specific for chick asymmetric AChE to measure released A-forms. This antibody, prepared by immunization of mice with partially-purified chick muscle asymmetric AChE, and further selection of the specific clone, has been found to react with collagen-tailed AChE species, but not with G-forms, either buffer- or detergent-soluble, in both ELISA and immunoprecipitation tests (Pérez-Tur et al., unpublished). Four independent experiments, including all enzymes, were carried out using each procedure. The procedure using gradient separation gave an estimate of both the asymmetric and globular AChE forms released by each enzyme. The method involving the sucrose cushion and immunoprecipitation was only used to measure A-forms.

3. RESULTS AND DISCUSSION

We have analyzed the release of the molecular forms of AChE by incubation of chick skeletal muscle samples in the presence of specific GAG-lyases: heparinases I, II and III (heparitinase I) and chondroitinase ABC and AC. We have checked three different heparinases taking into account the structural heterogeneity of the heparin/heparan sulfate family of GAGs: it is, for instance, a proven fact that heparan sulfates do often contain long heparin-like stretches [12]. Besides, in our hands, heparin shows consistently more affinity for tailed AChE forms than heparan sulfate itself [8,9]. Actually, these enzymes show mixed specificities: 'heparinase' (heparinases I and II) acts equally well upon heparin and heparitin sulfates C and D, whereas heparitinase I (heparinase III) degrades only heparitin sulfates A and B [13]. In any case, Table I shows quite clearly that neither form of heparinase was ble to extract A-forms or G-forms above the background level. Chondroitinases (both ABC and AC, although the quantitative results with each type are no directly comparable [10]), however, released small but consistently significant amounts of A- and G-forms. The enzyme concentrations used were much higher than usual [2,5], and we found that increasing them, or lengthening the incubation times only resulted in higher backgrounds. Anyway, chondroitinases released about one third of the A-forms that could be extracted with high salt (TTSE, maximal release control) from an identical sample incubated (without enzymes) under the same conditions. High backgrounds, such as ours, were also reported by Brandan et al. [5]. The most critical aspect of these digestion experiments concerns the procedures used to isolate the AChE released by the enzymatic digestion by GAG-lyases. Our first procedure (see section 2) relies on a limited exposure of the digested material to high salt which allows a separation in sucrose gradients to the released AChE forms, so that both A- and G-forms can be accurately quantified, while preventing the massive solubilization of more A-forms in the presence of high salt (Fig. 1). When first using the second protocol, taken from Brandan et al. [5], we found that some 25% of A-forms entered the sucrose cushion (see section 2). To estimate all A-forms, we then had to immunoprecipitate the supernatant and the heavy sucrose (not a suitable sample for gradients) with an antibody specific for A-forms. It is reassuring that both methods gave results (controls and A-forms) which were statistically similar, so that all the experiments have been analyzed together to obtain the mean values given in Table I.

Our data suggest that GAGs of the chondroitin/dermatan sulfate family could be involved in the anchorage of asymmetric (and also, perhaps, globular) AChE to the muscle basal lamina. The low yield of A-forms in our experiments could be tentatively explained by the existence, in the muscle basal lamina, of a type of chondroitin sulfate only partially sensitive to the bacterial chondroitinases used. Alternatively, the anchorage mechanisms could be heterogeneous, with chondroitin sulfates being the critical link in just a subpopulation of AChE molecules. To explore, within the latter hypothesis, the possibility of a joint involvement of both heparan and chondroitin sulfates in the attachment of extracellular muscle AChE we also measured the release of A-forms by a mixture of all heparinases

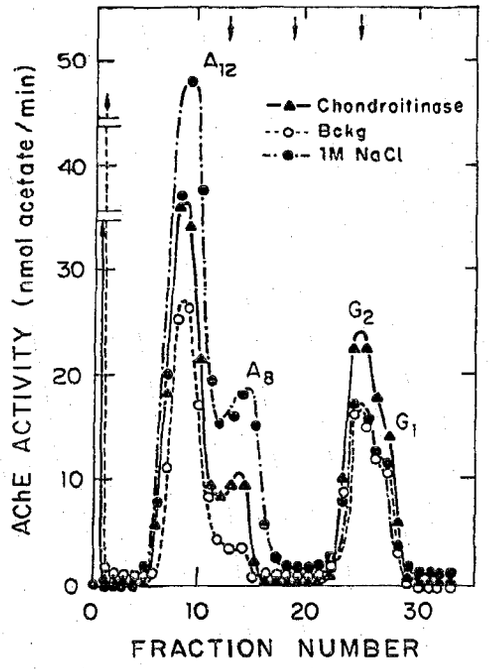

Fig. 1. Release of A- and G-forms of AChE by chondroitinase ABC from chick hindlimb muscle. Ten-day chick muscle samples, pre-extracted with TT buffer, were incubated with 5 U/ml of chondroitinase, as described in section 2, along with control samples (background - Bckg - control, containing no enzyme, and maximal release control - valid only for A-forms -, containing high salt and EDTA). The digested samples were usually freed of the coarse particulate material by filtration, and then layered on top of a 5-20% sucrose gradient, made up in TTSE buffer, together with sedimentation markers [8]. These gradients were run, fractionated and assayed for AChE (nmol [^{14}C]acetate released per min and fraction) and marker enzyme activities as described [8]. The arrows point the position of these marker enzymes, namely, from left to right, β-galactosidase (16S), catalase (11.3S), and alkaline phosphatase (6.1S).

plus chondroitinase ABC, with the result of 6.3% A-forms released by the mixture vs 6.4% by chondroitinase alone. Thus, the alleged role of (heparin-like) heparan sulfate GAGs [2,5] is not supported by our results.

The release of G-forms by chondroitinases above control level is a potentially interesting finding. On the one hand, it would seem to confirm previous reports by Jedrejczyk et al. [14], and Nicolet et al. [15,16] describing the presence in muscle ECM, including the basal lamina, of typical G-forms, even perhaps involved in synaptic function. After all, as we have already shown, some G-forms do interact with GAGs at physiological ionic strength [8]. However, they could also derive from the degradation of A-forms during the incubation process.

The lack of specificity of the heparin-AChE tail affinity, shared as a matter of fact by many polyanions [9], and the results reported in this paper are at variance with the findings and hypotheses of Inestrosa and coworkers [5,14] and make it necessary to re-evaluate the role of the different polyanions possibly present in the muscle basal lamina, including chondroitin sulfate-like GAGs, which incidentically were the first polyanions ever suggested to be involved in the well-known aggregation of A-forms at low ionic strength [1].

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