Biochimica et Biophysica Acta, 628 (1980) 286—292

BBA 29195

ACETYLCHOLINESTERASE SOLUBILIZED FROM NORMAL AND DYSTROPHIC MUSCLE BY COLLAGENASE TREATMENT

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(Received May 22nd, 1979)

Key words: Acetylcholinesterase; Butyrylcholinesterase; Collagenase; Muscular dystrophy

Summary

The activity of specific acetylcholinesterase, assayed in the presence of an inhibitor of nonspecific cholinesterase, was significantly lower in the leg muscle of dystrophic mice of Bar Habor strain 129 than in that of normal mice. However, the nonspecific butyrylcholinesterase activity was much higher in dystrophic muscle than in normal muscle.

Collegenase released more acetylcholinesterase activity into the soluble fraction derived from homogenized normal muscle than into that derived from dystrophic muscle. The collagenase-released activity in the normal muscle contained about 95% specific acetylcholinesterase while that from dystrophic muscle contained only 74% specific acetylcholinesterase activity. The acetylcholinesterase activity solubilized by collagenase from control muscle contained the highest activity in 10 S form with decreasing activity of 16 S and 4 S forms, but that from dystrophic muscle contained much less of the 16 S and 10 S forms with more 4 S form, compared to the controls.

Introduction

Acetylcholinesterase (EC 3.1.1.7) is present at motor end-plates and plays an important role in synaptic transmission at the neuromuscular junction by hydrolyzing and thereby terminating the action of the neurotransmitter acetylcholine released from the nerve terminal. Both subcellular fractionation and cytochemical studies indicate that the enzyme is associated with the surface membrane of the excitable cell [1].

Abbreviations: Inhibitor I, 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; Inhibitor II, tetraisopropylpyrophosphoramide.

Previous work from this laboratory showed that there was a striking difference in the pattern of sucrose gradient sedimentation of acetylcholinesterase activities extracted from muscles of normal and dystrophic mice [2]. Three active forms with different sedimentation coefficients (16 S, 10 S and 4 S) of acetylcholinesterase could be solubilized from muscle of normal mice. Decreased activities of the 16 S and 10 S forms with increased activity of 4 S form of acetylcholinesterase were observed in dystrophic muscle as compared with those in the normal muscle. We further showed that much more acetylcholinesterase activity was found in extracts with isotonic sucrose solution from muscles of dystrophic mice than from those of normal animals [3]. The sucrose extract from dystrophic muscle showed, on sucrose gradient centrifugation, one major peak of acetylcholinesterase activity with a sedimentation constant of approx. 4.3 S and this activity was much higher than that from normal muscle.

Since collagenase has been reported to detach acetylcholinesterase activity from muscle end-plates [4,5], we have studied the acetylcholinesterase activities released by collagenase from normal and dystrophic muscles. The present report described the characteristics of acetylcholinesterase released from muscles of dystrophic and control mice. A summary report of these results has been presented recently [6].

Materials and Methods

Animals. In the present study, female, 12—14 week old mice (Bar Harbor strain 129, Jackson Laboratory, Bar Harbor, Maine) of muscular dystrophic (Re *J dy/dy*) and phenotypically normal (Re *J* ?/+) litter mates were used.

Muscle preparations. Leg and thigh muscles were removed immediately after decapitation of the animals, weighed and homogenized in 10 vols. of ice-cold 0.32 M sucrose in a glass homogenizer with a glass pestle. For the preparation of various fractions used in the experiment shown in Table II, muscle homogenate in isotonic sucrose was centrifuged at 15 000 rev./min (27 000 × *g*) for 30 min in a Sorvall RC-5B refrigerated superspeed centrifuge at 0—4° C. The supernatant was designated as sucrose extract and the pellet was suspended in the original volume of collagenase solution (0.18 mg/ml) and incubated at 37° C for 30 min followed by centrifugation at 15 000 rev./min for 30 min at 0—4° C. The resulting supernatant was designated as collagenase extract and the precipitate, after washing with 0.32 M sucrose, was designated as residue which was suspended in NaCl-Triton buffer solution containing 1 M NaCl, 1% Triton X-100, 50 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.2).

Acetylcholinesterase and butyrylcholinesterase assay. Acetylcholinesterase and butyrylcholinesterase activities were assayed by the method of Schrier et al. [7] as modified by Sung [2] using Dowex 50W-X8 (Na^+ form, 100—200 mesh) except that the incubation mixture for butyrylcholinesterase assay contained 50 mM sodium phosphate buffer (pH 7.4) and 5 mM [*butyryl*-1-^{14}C]-butyrylcholine iodide (0.011 μCi/assay). Incubation was carried out for 30 min at 37° C. Unless otherwise stated the acetylcholinesterase activity was always assayed in the presence of $3 \cdot 10^{-5}$ M tetraisopropylpyrophosphoramide, which caused almost complete inhibition of butyrylcholinesterase activity. For the

assay of acetylcholinesterase activity (Table I) muscle homogenate was diluted with NaCl-Triton buffer solution to the appropriate concentration and for the assay of butyrylcholinesterase activity the muscle homogenate was diluted with 0.32 M sucrose solution since NaCl-Triton buffer solution caused a great inhibition of butyrylcholinesterase activity. One unit of acetylcholinesterase or butyrylcholinesterase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate in 30 min at 37°C under the assay conditions. Protein content was determined by method of Lowry et al. [8].

Sucrose density gradient centrifugation. This was carried out essentially as described previously [3] except that after centrifugation, 35-drop fractions were collected from the bottom (fraction 1) of the centrifuge tube and assayed for acetylcholinesterase activity.

Materials. [*Acetyl*-1-^{14}C]Acetylcholine iodide (2.3 Ci/mol) and [*butyryl*-1-^{14}C]butyrylcholine iodide (3.57 Ci/mol) were obtained from New England Nuclear Corporation. Dowex 50W-X8, acetylcholine iodide, butyrylcholine iodide and 1,10-phenanthroline were obtained from Sigma Chemical Co. 1,5-Bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51, Inhibitor I) was a gift from the Wellcome Research Laboratories, U.K. and tetraisopropylpyrophosphoramide (Inhibitor II) was purchased from K & K Laboratories or from Sigma Chemical Co. Chromatographically purified collagenase (code CLSPA, 443 units/mg), β-galactosidase (code BGC) and glucose oxidase (code GOP) were from Worthington Biochemical Corporation and crystalline bovine albumin was from Calbiochem.

Results

In our previous study when acetylcholinesterase activity was assayed without the addition of Inhibitor II, an inhibitor of nonspecific cholinesterase, the acetylcholinesterase activity in the hind-limb muscle of the dystrophic mice was very similar to that of the normal mice [2]. Table I shows that the specific acetylcholinesterase activity, assayed in the presence of $3 \cdot 10^{-5}$ M Inhibitor II, of the muscle from normal mice was significantly higher than that from dystrophic mice either on basis of muscle weight or on protein basis, though the difference on the former basis was greater than on the latter basis. On the other hand, the nonspecific cholinesterase activity measured with butyrylcholine as substrate was much greater in dystrophic muscle than in normal control.

TABLE I

ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE ACTIVITIES IN MUSCLE OF NORMAL AND DYSTROPHIC MICE

Values are expressed as means ± S.D. with numbers of animals in parentheses.

Mice	Acetylcholinesterase		Butyrylcholinesterase	
	units/g muscle	units/mg protein	units/g muscle	units/mg protein
Normal	52.1 ± 2.9 (10)	0.273 ± 0.019 (10)	22.8 ± 3.7 (10)	0.119 ± 0.018 (10)
Dystrophic	33.9 ± 4.0 (6)	0.206 ± 0.022 (6)	51.4 ± 6.6 (6)	0.312 ± 0.041 (6)

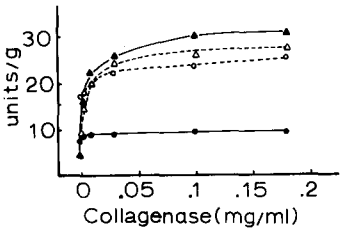

Fig. 1. Effect of increasing amount of collagenase on the release of acetylcholinesterase and butyrylcholinesterase activities from muscle homogenate. 0.5 ml of muscle homogenate (1 : 10) in 0.32 M sucrose was incubated with varying concentrations of collagenase for 30 min at 37° C, followed by centrifugation at 15000 rev./min for 30 min and cholinesterase activity in the supernatant was assayed. Activities of acetylcholinesterase and butyrylcholinesterase rendered soluble by collagenase treatment shown in ordinate are expressed as units of enzyme/g muscle wet weight. ▲———▲, acetylcholinesterase of normal muscle; △- - - - -△, acetylcholinesterase of dystrophic muscle; ●———●, butyrylcholinesterase of normal muscle; ○- - - - -○, butyrylcholinesterase of dystrophic muscle.

When the muscle homogenate was incubated with collagenase (10 μg/ml) for various time intervals, some of the particulate acetylcholinesterase activities were rendered soluble by collagenase treatment. The degree of release of acetylcholinesterase from muscle homogenate into soluble fraction was dependent on the length of time of collagenase treatment. This stimulated release of acetylcholinesterase from muscle homogenate by collagenase was inhibited completely by 1 mM 1,10-phenanthroline, a known inhibitor of collagenase [9]. The release of acetylcholinesterase activity was also dependent on the amount of collagenase present (Fig. 1). From both normal and dystrophic muscle, maximum amount of acetylcholinesterase activity was released from the particulate fraction of muscle with 0.1 to 0.18 mg of collagenase per ml. However, about 80% of maximum release was observed with collagenase at a concentration of 10 μg/ml. Although increasing activity of acetylcholinesterase was released by increasing the amount of collagenase, practically no increase in the release of butyrylcholinesterase was found by collagenase treatment of normal muscle. However, a small increase of butyrylcholinesterase activity was observed with dystrophic muscle (Fig. 1).

When the 27000 × *g* pellet of muscle homogenate in isotonic sucrose was treated with collagenase at a concentration of 0.18 mg/ml, a large amount of

TABLE II

COMPARISON OF ACETYLCHOLINESTERASE ACTIVITY IN ISOTONIC SUCROSE EXTRACT, COLLAGENASE EXTRACT AND THE RESIDUE

Preparation of each fraction from muscle homogenate was described in the text. Data are expressed as means ± S.D. with numbers of separate experiments in parentheses.

Fraction	Acetylcholinesterase activity (units/g muscle)		
	Control	Dystrophic	(% of control)
Isotonic sucrose extract	3.45 ± 0.53 (7)	5.65 ± 0.44 (6)	(163%)
Collagenase extract	31.5 ± 5.1 (7)	21.0 ± 3.5 (7)	(66.6%)
Residue	22.1 ± 1.5 (6)	8.88 ± 1.20 (5)	(40.2%)

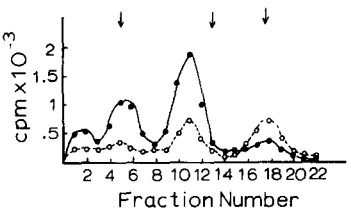

Fig. 2. Sucrose density gradient centrifugation of acetylcholinesterase solubilized by collagenase treatment from normal and dystrophic muscle. Activities of acetylcholinesterase are expressed in the ordinate as cpm/40 µl of each fraction. The arrows represent the position of markers in the gradients which were from left to right, β-galactosidase (16 S), glucose oxidase (8 S) and bovine serum albumin (4.3 S). ●———●, normal; o- - - - -o, dystrophic.

acetylcholinesterase was solubilized. Although small amounts are released by sucrose alone from both types of muscle, much higher activity of acetylcholinesterase was extracted by isotonic sucrose solution from dystrophic muscle than from normal muscle. However, less activity of acetylcholinesterase was solubilized by collagenase treatment from dystrophic muscle than from normal muscle (Table II). The acetylcholinesterase activity in the residue which was not rendered soluble by collagenase treatment was found to be significantly higher in muscle of normal mice than that of dystrophic animals. Collagenase-released cholinesterase contained a higher percentage of specific acetylcholinesterase (Inhibitor II-resistant and BW284C51, Inhibitor I-inhibitable activity) in normal muscle than in dystrophic muscle. Cholinesterase activity extracted with collagenase in the normal muscle was inhibited by 5.4% with $3 \cdot 10^{-5}$ M Inhibitor II and by 92.8% with $5 \cdot 10^{-6}$ M BW284C51, Inhibitor I. In the dystrophic muscle the activity was inhibited by 25.6% with Inhibitor II and by 72.6% with BW284C51, Inhibitor I.

On sucrose density gradient centrifugation, acetylcholinesterase activity solubilized by collagenase treatment from normal muscle contained the highest activity in 10 S form with decreasing activities of 16 S and 4 S forms, but that from dystrophic muscle contained much less 16 S and 10 S forms with more 4 S form compared to the control (Fig. 2).

Discussion

Acetylcholinesterase of skeletal muscle is concentrated at the neuromuscular junction, where it hydrolyzes acetylcholine released from the nerve terminal. Although the precise relationship of acetylcholinesterase to the excitable membrane is not fully understood, acetylcholinesterase was assumed to be associated with the postsynaptic plasma membrane until Hall and Kelly [4] showed that mild protease treatment of intact muscles released active acetylcholinesterase into the medium without apparent damage to the plasma membrane. Recently, direct evidence has been presented to show that junctional acetylcholinesterase is associated with the basement membrane or basal lamina of the synaptic cleft in skeletal muscle [10]. Various lines of evidence suggest that in situ acetylcholinesterase is associated with the collagenous matrix of the basement membrane [11] and collagenase has been reported to detach the enzyme

from muscle end-plates [4,5,12]. Collagenase was also shown to convert purified 18 S and 14 S eel species [13] or 16 S torpedo enzyme [14] to an 11 S form of acetylcholinesterase.

In the present study chromatographically purified collagenase was shown to release acetylcholinesterase from both normal and dystrophic muscle. The collagenase-stimulated release of acetylcholinesterase was possibly due to collagenolytic action, since the release of acetylcholinesterase was inhibited by 1,10-phenanthroline, an inhibitor of collagenase. Though dystrophic muscle was found to contain higher activity of isotonic sucrose soluble acetylcholinesterase activity than normal muscle, the collagenase-released activity was much higher in normal muscle than in the dystrophic (Table II). This might indicate that there is less intrajunctional acetylcholinesterase in the dystrophic muscle than in the normal muscle. The higher activity of 10 S form of acetylcholinesterase than 16 S form of enzyme may be due to the partial conversion of 16 S form to 10 S form by the action of collagenase as was observed by Dudai and Silman in eel acetylcholinesterase [13] and by Lwebuga-Mukasas et al. in the torpedo enzyme [14]. Another possibility is that some of the 16 S form of acetylcholinesterase could be still associated with the residue which was resistant to collagenase action. Our preliminary results showed that about 80% of this residual acetylcholinesterase activity was extracted with NaCl-Triton buffer solution. Therefore, further characterization of this fraction of acetylcholinesterase on sucrose gradient centrifugation will be attempted to see which molecular forms of acetylcholinesterase exist in this fraction.

The 16 S form of acetylcholinesterase activity associated with muscle end-plate has been suggested as an excellent marker of the neuromuscular junction [15—17]. In the present study we have shown that collagenase released more 16 S and 10 S forms of acetylcholinesterase from normal muscle than from dystrophic muscle. This may indicate decreased acetylcholinesterase activity in the junctional area in the dystrophic muscle. The reason why more 4 S form of acetylcholinesterase as well as butyrylcholinesterase activities were released by collagenase from dystrophic muscle than from normal muscle is not clear, since the physiological significance and function(s) of 4 S form of acetylcholinesterase and butyrylcholinesterase in muscle are unknown.

Acknowledgement

This work was supported by grants from the British Columbia Health Care Research Foundation and the Muscular Dystrophy Association of Canada.

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