THE EFFECT OF Ca^{2+} ON INTERACTION OF ACETYLCHOLINESTERASE WITH SUBCELLULAR FRACTIONS OF ELECTRIC ORGAN TISSUE FROM THE ELECTRIC EEL*

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1. Introduction

Acetylcholinesterase (EC 3.1.1.7) (AChE) is believed to be a membrane-bound enzyme [1–3] although its relationship to the membrane has not been fully clarified [4, 5]. Massoulié et al. [6] showed that tryptic treatment of electric organ tissue from the electric eel led to release of the enzyme from the innervated surface of the electroplax membrane, and that this release appeared to be correlated with a change in the molecular properties of the enzyme. In extracts of fresh electric organ tissue, the major molecular species of AChE have sedimentation coefficients of 14 S and 18 S, and aggregate at low ionic strength [7]. A minor 8 S component is also observed which does not aggregate. After tryptic digestion or autolysis of electric organ tissue, all the AChE is converted to an 11 S form which does not aggregate at low ionic strength [7, 8]. Burger et al. [9] have shown that the release of AChE from bovine erythrocyte membranes is almost completely prevented in the presence of divalent cations such as Ca^{2+} and Mg^{2+}. In the following we will show that the aggregating forms of AChE derived from fresh electric organ tissue (14 S and 18 S forms) differ from the non-aggregating forms (8 S and 11 S) with respect to their behavior in the presence of Ca^{2+} ions. It will be suggested that this difference may be related to the mode of attachment of AChE to the electroplax membrane.

* This paper is dedicated to the memory of Professor Arieh Berger.

2. Materials and methods

Fresh electric organ tissue from the electric eel, *Electrophorus electricus*, was used in all the experiments to be described. Homogenization of the tissue in the various media employed was performed using a Sorvall Omnimixer at speed 6, for 2 min at 4°. Crude membrane fractions (MF) of electric organ tissue were prepared as follows: Fresh tissue was homogenized in the Omnimixer in 4 vol of 0.3 M sucrose. The pellet obtained by centrifuging the homogenate at 30,000 *g* for 30 min was resuspended in 5 vol of 0.3 M sucrose using a glass–Teflon homogenizer. This suspension was centrifuged at 2,000 *g* for 20 min, and the supernatant obtained was taken as the crude membrane fraction (MF). A high-speed supernatant fraction (SF) was prepared by centrifuging the 0.3 M sucrose homogenate of fresh tissue at 200,000 *g* for 180 min. Purified 14 + 18 S AChE was prepared as described previously [10]. Pure 11 S AChE was prepared as described previously [8].

AChE activity was routinely measured by the pH-stat method [11], and units of activity were as defined previously [8]. Sucrose gradient centrifugation in 5–20% sucrose was performed as previously described [8] and the fractions were assayed by the method of Ellman et al. [12].

3. Results

The presence of Ca^{2+} ions in the medium employed for homogenization of electric organ tissue causes a large decrease in the AChE activity found in the

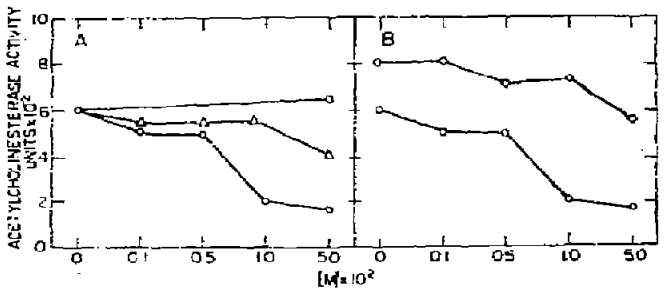

Fig. 1. AChE activity in supernatants from electric organ tissue homogenates as a function of Ca^{2+} concentration. Homogenization was performed as described under Materials and methods, in 5 vol of 0.1 M NaCl–0.05 M Tris, pH 7.4, or 1.0 M NaCl–0.05 M Tris, pH 7.4. After homogenization, 5 ml aliquots were adjusted to the appropriate molarity of Ca^{2+} or Mg^{2+}, an equivalent molarity of NaCl being added to a control sample. The samples were stirred overnight at 4°, and centrifuged at 10,000 *g* for 10 min prior to determination of AChE activity in the supernatants. A) Effects of Ca^{2+} and Mg^{2+} at low ionic strength (0.1 M NaCl). (○—○—○), Ca^{2+}; (△—△—△), Mg^{2+}; (□—□—□), Na^{+}. B) Effect of Ca^{2+} at high and low ionic strength. (●—●—●), 0.1 M NaCl; (○—○—○), 1 M NaCl. The abscissa gives the molarity of the ion added.

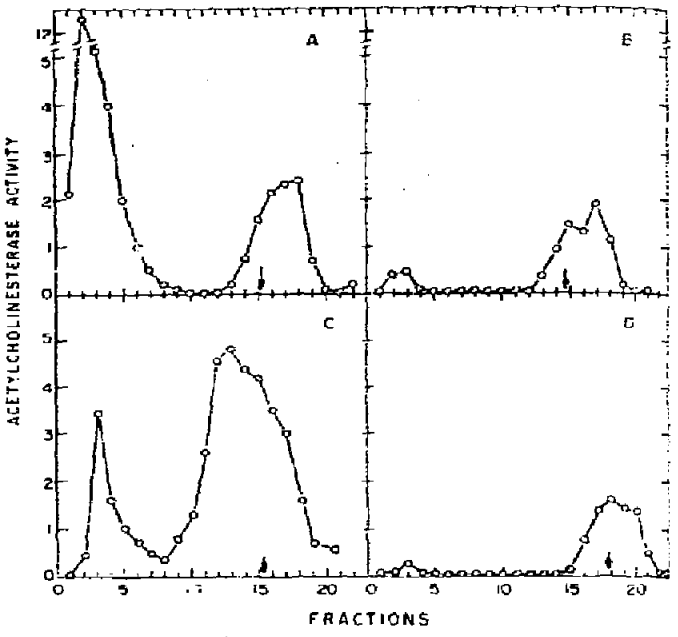

Fig. 2. Sucrose gradient centrifugation of supernatants from electric organ homogenates. Homogenization was performed as under Materials and methods in 5 vol of 2.5 mM EDTA, pH 7.5 (control) or 10 mM $CaCl_2$, pH 7.5 (Ca^{2+}). The homogenates were centrifuged at 27,000 *g* for 20 min. Aliquots from the supernatants were centrifuged on sucrose gradients prepared as described previously [8], containing either 0.05 M NaCl–0.05 M Tris, pH 7.4 (A and B) or 1.0 M NaCl–0.05 M Tris, pH 7.4 (C and D). A) Control supernatant. B) Ca^{2+} supernatant. C) Control supernatant. D) Ca^{2+} supernatant. The arrows indicates the position of the catalase marker taken as 11.4 S [8].

Table 1

Recovery of AChE activity from electric organ homogenates subsequent to addition of Ca^{2+}.

	AChE activity (units)			
	Supernatant	Pellet I	Pellet II	Total
Control	600	120	186	906
Ca^{2+}	165	345	303	813

Aliquots of 5 ml obtained as in fig. 1 at low ionic strength (0.1 M NaCl), by incubation of homogenates overnight with or without 50 mM Ca^{2+}, were centrifuged at 10,000 *g* for 10 min. The supernatant was assayed for AChE activity and the pellet (pellet I) suspended in 3 ml of 0.1 M NaCl–0.01 M EDTA–1% Triton X-100–0.05 M Tris, pH 7.4. After 16 hr at 4° the suspension was centrifuged at 10,000 *g* for 10 min, and the AChE activity determined in the supernatant (defined as activity of pellet I). The residual pellet (pellet II) was suspended in 3 ml 0.1 M NaCl–0.05 M Tris, pH 7.4 containing 0.6 mg trypsin. After 4 hr at room temp. the samples were centrifuged at 10,000 *g* for 10 min, and AChE activity was determined in the supernatant (defined as activity of pellet II).

supernatant after centrifugation at 10,000 *g* for 10 min. The effect is most pronounced if the Ca^{2+} concentration is varied prior to homogenization, but similar results are obtained if the Ca^{2+} is added to the medium after homogenization, as shown in fig. 1A, or even if the homogenates are dialyzed against solutions containing the appropriate concentration of Ca^{2+}. Mg^{2+} ions have a much smaller effect (fig. 1A). If the homogenization medium contains 1 M NaCl (fig. 1B), the effect of Ca^{2+} is largely abolished. Treatment of the homogenate with trypsin also abolishes the effect of Ca^{2+}.

The AChE activity in the pellet obtained in the presence of Ca^{2+} can be recovered in soluble form by treatment of the pellet with EDTA or by tryptic digestion (table 1).

Table 2
Effects of Ca^{2+} on interaction of purified AChE with subcellular fractions of electric organ tissue.

	AChE activity (units)		
	Supernatant	Pellet	Total
MF	6.0	8.3	14.3
MF + 10 mM Ca^{2+}	4.8	8.7	13.5
MF + 14 and 18 S AChE	102.5	26.7	129.2
MF + 14 and 18 S AChE + 10 mM Ca^{2+}	54.4	55.2	109.6
MF + 11 S AChE	27.6	8.0	35.6
MF + 11 S AChE + 10 mM Ca^{2+}	24.9	9.9	34.8
14 and 18 S AChE	90.0	1.5	91.5
14 and 18 S AChE + 10 mM Ca^{2+}	80.0	2.0	82.0
11 S AChE	29.0	0.0	29.0
11 S AChE + 10 mM Ca^{2+}	26.5	0.8	27.3
SF + 14 and 18 S AChE	102.0	1.4	103.4
SF + 14 and 18 AChE + 10 mM Ca^{2+}	14.0	57.0	71.0
SF + 11 S AChE	25.0	0.4	25.4
SF + 11 S AChE + 10 mM Ca^{2+}	22.5	5.0	27.5

The incubation mixtures in a final volume of 0.4–0.8 ml 0.025 M NaCl–0.025 M Tris, pH 7.4, were stirred overnight at 4° and then centrifuged for 10 min in an Eppendorf 3200 microcentrifuge at 4°. AChE activities of the supernatants were then determined. The pellets were suspended in 0.2–0.3 ml of 0.05 M NaCl–0.01 M EDTA–1% Triton X-100–0.05 M Tris, pH 7.4, stirred overnight at 4°, and centrifuged as above. AChE activity in the supernatant thus obtained was taken as the activity in the pellets. The pure molecular forms of AChE, the crude membrane fraction (MF) and the high-speed supernatant (SF) were prepared as described under Materials and methods. Where MF was employed the aliquot added contained 680 μg protein, and where SF was added, 380 μg protein.

Sucrose gradient centrifugation reveals that the molecular forms of AChE in the homogenate which are affected by the Ca^{2+} concentration are the aggregating 14 S and 18 S forms, whereas the non-aggregating forms are not influenced (fig. 2). Thus it can be seen that when homogenization is performed in the presence of 10 mM Ca^{2+}, only the non-aggregating forms of AChE, of low sedimentation coefficient, appear in the supernatant (fig. 2B and 2D). If homogenization is performed in the presence of 2.5 mM EDTA, much more AChE appears in the supernatant, most of it as the heavier molecular forms of the enzyme (fig. 2C) which aggregate at low ionic strength (fig. 2A).

When purified 14 + 18 S AChE is incubated with the crude membrane fraction (MF), part of the enzyme appears in the pellet on centrifugation at 10,000 *g* for 10 min. When Ca^{2+} is added to the incubation medium, the amount of enzyme in the pellet is much larger. This effect is not seen with 11 S AChE (table 2). When the same experiments are performed in the presence of 1 M NaCl, Ca^{2+} has no effect.

In control experiments it was found that 14 + 18 S AChE alone did not sediment at 10,000 *g* for 10 min in the presence of Ca^{2+} (table 2), nor did Ca^{2+} directly affect enzymatic activity. However, sedimentation could be achieved by adding to a solution of the enzyme an aliquot of the high-speed supernatant (SF) of electric organ tissue (table 2). In this case, too, the presence of Ca^{2+} is essential, and again the effect on the 11 S enzyme is small.

4. Discussion

Our results show clearly that the addition of Ca^{2+} ions to an electric organ homogenate causes a marked redistribution of the AChE in the subcellular fractions. The results also reveal a great difference between the behavior of the major molecular forms of AChE present in fresh electric organ tissue (14 S and 18 S), and the behavior of the non-aggregating forms present in small amounts in fresh tissue (8 S) and obtained by proteolysis or autolysis (11 S). The 14 and 18 S forms are sensitive to the presence of Ca^{2+}, whereas the 8 and 11 S forms are not.

Since the concentration of inorganic phosphate in electric organ tissue is rather high [13], the addition of Ca^{2+} may lead to formation of calcium phosphate precipitates which are known to adsorb proteins [14]. Indeed, in control experiments it was found that at low ionic strength calcium phosphate precipitates 14 S and 18 S AChE, whereas the 11 S form is adsorbed to a lesser degree. In 1 M NaCl none of the molecular forms are adsorbed to calcium phosphate.

However, it does not seem that these observations can explain our results, for the following reasons: i) The pattern obtained on incubation of AChE with crude washed membrane fractions (MF) containing low concentrations of inorganic phosphate (table 2), was similar to that observed in the homogenates (fig. 1 and table 1). ii) When the supernatant (SF), containing a high concentration of inorganic phosphate, is treated with trypsin prior to the addition of AChE, its ability to cause sedimentation of 14 + 18 S AChE in the presence of Ca^{2+} is greatly reduced. It is, therefore, reasonable to assume that one or more proteins in the supernatant are involved in the Ca^{2+}-induced sedimentation.

Our observation that high-speed supernatants from electric organ homogenates also form sediments in the presence of Ca^{2+}, which adsorb 14 + 18 S enzyme but not 11 S enzyme, leads us to treat our data on enzyme–membrane interactions with caution. Rapidly aggregating sediments formed by various subcellular fractions in the presence of Ca^{2+} have been observed in other cases (see, for example, [15, 16]). The ability of the 14 S and 18 S forms of AChE to adsorb to such sediments, while distinguishing them from the 8 S and 11 S forms, may not be directly related to their mode of association with the membrane. However, as in the studies of Burger et al. [9] on the AChE of bovine erythrocyte stroma, our data may indeed indicate a requirement of Ca^{2+} for attachment of AChE to the electroplax membrane. This attachment would then involve a site on the AChE molecule which is destroyed during proteolytic treatment of the electroplax membrane or of the purified enzyme.

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