Biochimica et Biophysica Acta, 339 (1974) 359–366

BBA 76588

ACETYLCHOLINE ESTERASE AS A PROBE FOR ERYTHROCYTE-MEMBRANE INTACTNESS

BENY ALONI and AVINOAM LIVNE

Research and Development Authority and Department of Biology, University of the Negev, Beer Sheva (Israel)

(Received October 3rd, 1973)

SUMMARY

Erythrocyte acetylcholine esterase can be assayed in intact cells and was tested as a probe for membrane changes. Acetylcholine esterase activity correlated with the erythrocyte relative volume. Antihemolytic acyl sorbitols, fatty acids and phenothiazines inhibit to varying extents the activity of acetylcholine esterase. The inhibition of acetylcholine esterase by linolenoyl sorbitol was further characterized and found to be non-competitive and critically dependent on cell intactness over a wide temperature range. Neither solubilized nor ghost acetylcholine esterase was affected by the acyl sorbitol while under conditions optimal for ghost resealing, the enzyme resumed the sensitivity to the acyl sorbitol. Acetylcholine esterase sensitivity thus appears to be a promising tool to follow the dynamics of membrane integrity.

INTRODUCTION

Erythrocyte ghosts are commonly used for studies of membrane properties. However, for understanding cell function, membrane properties of the intact cell should ultimately be investigated. To study intact cell membrane it is desirable to utilize a membraneous enzyme which can be assayed in the intact cell and which signals changes in the membrane. Acetylcholine esterase (acetylchloline hydrolase EC 3.1.1.7) appears to fulfill at least part of these requirements, since it is a membraneous enzyme [1, 2] and its activity can be assayed in the intact cell [3]. It was, therefore, of interest to evaluate changes in acetylcholine esterase activity as affected by various membrane alterations.

Since acetylcholine esterase can be solubilized by high concentration of NaCl [4], it is apparently tied to the membrane electrostatically. On this ground acetylcholine esterase is expected to be insensitive to modification in the membrane lipids. However, recent investigations [5, 6] show that changes in fatty acid composition in rat erythrocyte membrane, following feeding with fat-deficient or fat-sufficient diets, caused alterations in the allosteric properties of acetylcholine esterase. If this enzyme is indeed bound to the membrane by a certain hydrophobic interaction, added lipids might affect its activity. The present study tests this supposition, using com-

pounds which are known to stabilize red cells against osmotic fragility [7–9]. The sensitivity of acetylcholine esterase to these compounds is shown to be critically dependent on cell intactness.

MATERIALS AND METHODS

Erythrocyte suspension

Freshly drawn human blood was obtained in heparin. The cells were washed three times with a solution containing 155 mM NaCl, 2 mM sodium phosphate, pH 7.4, and 10 mM glucose, the top layer and buffy coat were discarded and the erythrocyte suspension was adjusted to hematocrit of 6% in the same medium.

Determination of acetylcholine esterase

Acetylcholine esterase was measured according to Ellman et al. [3]. An aliquot (20 μl) of the 6% erythrocyte suspension was mixed with 3 ml of 155 mM sodium phosphate buffer, pH 8.0, containing (when specified) one of the antihemolytic compounds. The mixtures were incubated for 10 min at 25 °C and 0.4 mM 2,2′-dinitro-5,5′dithiobenzoic acid was added. The reaction was started by adding 20 μl of 75 mM acetylthiocholine iodide and was followed by a colorimeter (Klett–Summerson, Filter 42) with readings taken at 1 min and 7 min after the start. Hydrolysis of 1 nmole of substrate yielded 9.25 Klett units. Unless specified otherwise, the reaction took place at 37 °C.

Solubilization of acetylcholine esterase

Erythrocyte acetylcholine esterase was solubilized from the membrane according to Mitchell and Hanahan [4]. Hemoglobin-free membranes [10] were suspended in 10 vol. of 1.4 M NaCl, incubated for 15 h and then centrifuged at 25 000 $\times g$ for 30 min. The supernatant was dialyzed against distilled water for 17 h, adjusted to pH 8.0 with sodium phosphate buffer (1 M) and diluted to 0.15 mg protein per ml with 0.1 M sodium phosphate, pH 8.0. The resultant solution was transparent. All steps were conducted at 4 °C.

Resealing of ghosts

The procedure of Redman [11] for lysis of cells and resealing was followed. Washed red cells (60%) were lysed in 10 vol. of 10 mM Tris–HCl, pH 7.2, containing 1 mM $MgCl_2$. For resealing, 3 M KCl/NaCl (3 : 1, v/v) was added to a final concentration of 0.17 M. The mixture was incubated at 37 °C (or, for control, at 4 °C) for 40 min, centrifuged at 20 000 $\times g$ for 20 min and the ghosts suspended in 0.155 M NaCl, pH 7.4. For the experiment shown in Fig. 5, the time of incubation varied as specified and the final centrifugation was omitted.

Materials

Acyl sorbitols were synthesized by transesterification at the Organic Chemistry Laboratory of this Institute by Mrs R. Rahav and Dr S. Bittner. The synthetic esters were highly pure, with a sorbitol: fatty acid ratio of 1 : 1. The phenothiazines were kindly supplied by Taro Ltd, Haifa. Other chemicals were obtained from Sigma Chemical Co., St. Louis. The fatty acids were 98–99% pure.

The experiments were repeated at least four times, with the exception of the experiment given in Table III (twice only), with essentially identical results. Average values are presented.

RESULTS

Acetylcholine esterase and cell volume

Acetylcholine esterase is located on the outer surface of the membrane [1, 2], thus it is possible that its activity is affected by alteration of cell shape. Red-cell shape can be controlled by the tonicity of the medium; however, increasing NaCl concentration increases the activity of red-cell acetylcholine esterase [12], while sucrose does not affect its activity. Therefore, to modify the relative cell volume, sucrose tonicity was changed, while NaCl was kept constant at an optimal concentration for enzyme activity (40 mM). Red-cell relative volume was determined as described [7, 8]. Fig. 1 shows that acetylcholine esterase activity is correlated with red-cell relative volume. Shrinkage of the red cell under hypertonic conditions reduces acetylcholine esterase activity while expansion of the red cell to about 1.4 of its original volume apparently reveals all the masked enzyme activity.

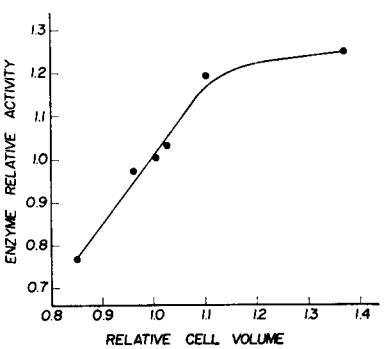


Fig. 1. Erythrocyte relative volume and acetylcholine esterase activity. In addition to NaCl (40 mM), the reaction mixtures contained the following sucrose concentrations for increasing cell volume: 310, 280, 230, 200, 160 and 100 mM. Acetylcholine esterase activity in isotonic medium (310 mosM): 6.6 μmoles/ml packed cells per min.

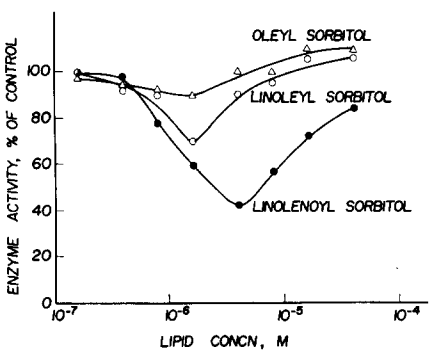


Fig. 2. Inhibition of erythrocyte acetylcholine esterase activity by acyl sorbitols. Erythrocyte cells suspension (0.04 %) was incubated with an acyl sorbitol as specified in 155 mM sodium phosphate buffer, pH 8.0, for 10 min at 25 °C and the enzyme activity was determined as described in Materials and Methods.

Inhibition of acetylcholine esterase by antihemolytic compounds

Lipids which protect red cells against hypotonic hemolysis [7–9] inhibit the activity of acetylcholine esterase to varying extents. Fig. 2 shows that the extent of inhibition of acetylcholine esterase increases with the degree of unsaturation of acyl sorbitols. The inhibition is clearly biphasic, with decreasing effectiveness at superoptimal concentrations of acyl sorbitols. No hemolysis was detected during the experiment, even at the higher lipid concentration. Hemolysis was evident, however, following an additional period of incubation (1 h) with the lipids at over $5 \cdot 10^{-6}$ M.

TABLE I

INHIBITION OF ERYTHROCYTE ACETYLCHOLINE ESTERASE BY 18-CARBON FATTY ACIDS

Fatty acids were added from ethanol stock solutions to 0.04 % erythrocyte suspension (about $1.3 \cdot 10^6$ cells per ml) in 155 mM sodium phosphate buffer, pH 8.0. Equal quantity of ethanol (up to 10 μl in 3 ml of suspension) was also added to the control. The mixtures were incubated for 10 min at 25 °C and the enzyme activity determined as described in Materials and Methods. Activity of control cells amounted to 5.75 μmole/ml packed cells/min.

Fatty acid	Concentration for 50 % inhibition (M)
Linolenic	$4.3 \cdot 10^{-6}$
Linoleic	$2.3 \cdot 10^{-6}$
Oleic	$1.5 \cdot 10^{-6}$
Stearic	$1.0 \cdot 10^{-6}$

Unlike the acyl sorbitols, unesterified fatty acids show a declining inhibitory effect with an increasing number of double bonds (Table I). Phenothiazine tranquilizers also inhibit acetylcholine esterase activity, with an effectiveness correlated with the clinical potency, as well as with their stabilization capability in erythrocytes [9]. Table II shows that on molar basis chlorpromazine is several fold more effective than promethazine in arresting acetylcholine esterase activity. Chlorpromazine differs from promethazine in one of the phenothiazine benzene rings (a Cl substitution). The inhibitory potency of the phenothiazines in our tests correlates with octanol–water partition coefficients of the tranquilizers [17].

TABLE II

INHIBITION OF ERYTHROCYTE ACETYLCHOLINE ESTERASE BY CHLORPROMAZINE AND PROMETHAZINE

The phenothiazines were added from aqueous stock solutions. Other details as in Table I. Activity of control cells: 6.28 μmole/ml packed cells/min.

Concentration (M)	Inhibition of enzyme activity %	
	Chlorpromazine	Promethazine
$1 \cdot 10^{-5}$	0	5
$2 \cdot 10^{-5}$	10	6
$5 \cdot 10^{-5}$	40	12
$1 \cdot 10^{-4}$	49	19

Characterization of acetylcholine esterase inhibition

To characterize the inhibition of erythrocyte acetylcholine esterase activity, the effect of linolenoyl sorbitol was studied to a greater extent. Acyl sorbitol does not modify the erythrocyte volume in isotonic medium, as already reported [7]. Fig. 3 presents a Lineweaver–Burk plot of acetylcholine esterase activity and its inhibition by the added acyl sorbitol. The inhibition is clearly non-competitive. Enzyme inhibi-

TABLE III

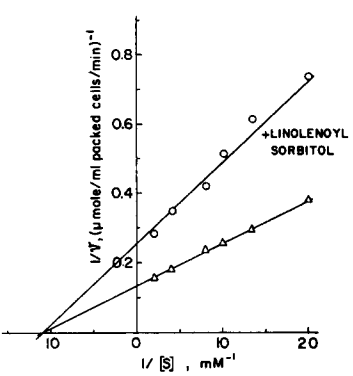
INSENSITIVITY OF SOLUBILIZED ERYTHROCYTE ACETYLCHOLINE ESTERASE TO LINOLENOYL SORBITOL

An aliquot of solubilized acetylcholine esterase (6.3 μg protein/ml) was incubated in 150 mM sodium phosphate buffer, pH 8.0, with linolenoyl sorbitol as specified for 10 min at 25 °C and the enzyme activity was determined as described in Materials and Methods. Protein concentration in reaction mixture: 6.3 μg/ml.

Linolenoyl sorbitol concentration (M)	Enzyme activity (μmoles/mg protein per h)
0	8.3
$4 \cdot 10^{-7}$	8.3
$4 \cdot 10^{-6}$	8.3
$8 \cdot 10^{-6}$	8.3
$4 \cdot 10^{-5}$	8.8
$8 \cdot 10^{-5}$	7.7
$4 \cdot 10^{-4}$	7.7

tion may result from the solubilization of the enzyme by the added lipid. However, this possibility is excluded, since the lipid did not release any acetylcholine esterase activity from cells to the medium. Furthermore, acetylcholine esterase solubilized from red-cell membranes by high ionic strength [4], was not affected by the acyl sorbitol over a wide concentration range (Table III). This insensitivity might be related to the presence of lipids in the preparation of the solubilized enzyme [4].

The inhibition of acetylcholine esterase by linolenoyl sorbitol is critically dependent on the intactness of the erythrocytes: following hemolysis the inhibition is no longer apparent (Table IV). This dependence is observed over a temperature of 2–40 °C, as can be seen in Fig. 4. It is noteworthy that the plots of $1/T$ vs log acetylcholine esterase activity coincide for untreated intact cells and for either lipid-treated



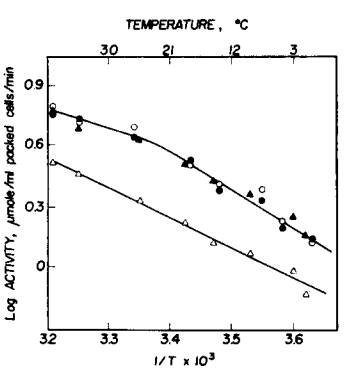


Fig. 3. Kinetics of erythrocyte acetylcholine esterase activity as affected by $4 \cdot 10^{-6}$ M linolenoyl sorbitol. For details, see Materials and Methods.

Fig. 4. Temperature dependence (Arrhenius plot) of acetylcholine esterase activity in presence (open symbols) or absence (closed symbols) of $4 \cdot 10^{-6}$ M linolenoyl sorbitol. Triangles, intact cells; circles, lyzed cells. The activity for lyzed cells is related to the equivalent intact cells. For details, see Materials and Methods.

TABLE IV

SENSITIVITY OF ACETYLCHOLINE ESTERASE TO LINOLENOYL SORBITOL AS DEPENDENT ON ERYTHROCYTE INTACTNESS

An aliquot (50 μl) of 6 % cell suspension was incubated either with $5 \cdot 10^{-4}$ M linolenoyl sorbitol or without the lipid (control) for 10 min at 25 °C. The cells were lyzed by adding 150 μl H_2O and then 0.155 M sodium phosphate buffer, pH 8.0, was added to a total volume of 3 ml for determination of enzyme activity. The activity is related to the equivalent intact cells. For "intact cells", the aliquots were brought to 3 ml with the same buffer without hemolysis. Final lipid concentration: $8.5 \cdot 10^{-6}$ M.

	Treatment	Enzyme activity (μmoles/ml packed cells per min)
Intact cells	Control	6.2
	+linolenoyl sorbitol	2.6
Lyzed cells	Control	6.7
	+linolenoyl sorbitol	6.5

of untreated broken cells. The plot is characterized by two slopes, with corresponding apparent energy of activation of 8.2 and 4.5 kcal/mole. The transition is at about 24 °C. The slope for treated intact cells yields an apparent energy of activation of 6.6 kcal/mole, with no clear change in the slope over the entire temperature range.

If the inhibition of acetylcholine esterase by added lipid is indeed a characteristic of cell intactness, it is anticipated that the enzyme of resealed ghosts should also reveal this property, in contrast to unsealed ghosts. Table V shows that under conditions required for resealing (40 min, 37 °C, ref. 11) the inhibition is reestablished, although to a smaller extent. Ghosts kept for control at 4 °C during the resealing incubation ("unsealed ghosts") are not affected by linolenoyl sorbitol with respect to acetylcholine esterase activity. This virtue may enable the detection of the

TABLE V

RECOVERY OF SENSITIVITY OF ACETYLCHOLINE ESTERASE TO LINOLENOYL SORBITOL BY ERYTHROCYTE-GHOST SEALING

The procedure of Redman [11] for lysis of cells and resealing was followed as described in Materials and Methods. Intact cells (0.04 % suspension), or ghosts obtained from these cells, were incubated with linolenoyl sorbitol in 155 mM sodium phosphate buffer, pH 8.0, for 10 min at 25 °C and the enzyme activity was determined. The activity of the ghosts relates to the equivalent intact cells.

	Linolenoyl sorbitol concentration (M)	Enzyme activity (μmoles/ml packed cells per min)	Percent inhibition
Intact cells	0	6.6	56
	$4 \cdot 10^{-6}$	2.9	
Unsealed ghosts (incubated at 4 °C)	0	9.8	0
	$4 \cdot 10^{-6}$	10.5	
Sealed ghosts (incubated at 37 °C)	0	8.2	33
	$4 \cdot 10^{-6}$	5.6	

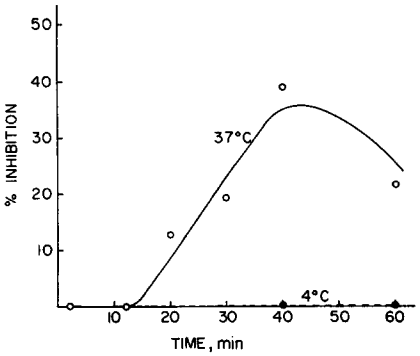


Fig. 5. Time course of recovery of sensitivity of acetylcholine esterase to linolenoyl sorbitol in erythrocyte ghosts incubated at 37 °C. Ghosts incubated at 4 °C showed no inhibition. The procedure of Redman [11] for lysis of cells and resealing was followed as described in Materials and Methods. The ghosts were sampled during the course of incubation and were analyzed for enzyme sensitivity to linolenoyl sorbitol according to our standard procedure.

membrane sealing process. Fig. 5 demonstrates the time dependence for the revealing of enzyme inhibition, apparently signalling the process of ghost resealing, or a certain organization of the sealed ghost membrane. The decreasing portion of Fig. 5 was repeatedly observed, but its significance is not clear.

DISCUSSION

Singer and Nicolson [13] distinguish between two categories of membrane-bound proteins, termed peripheral and integral proteins. According to their criteria, acetylcholine esterase may be considered as a "peripheral protein", since it dissociates from the membrane by high ionic strength [4] and in the dissociated state the enzyme preparation is soluble in neutral aqueous buffer [12]. However, such a definition is equivocal. Acetylcholine esterase is dissociable by high ionic strength from the erythrocyte ghosts but not from the intact erythrocytes. The enzyme must be at least partly embedded in the lipid matrix even in isolated membrane, since its allosteric properties are markedly affected by the fatty acid composition of the membrane [5, 6]. Thus, the distinction between peripheral and integral membrane proteins must be reevaluated, particularly with respect to intact erythrocytes.

Several observations presented in this study support a conclusion that the inhibition of acetylcholine esterase activity by the added lipid is an indirect effect due to a modification of the membrane. The solubilized enzyme is not affected by linolenoyl sorbitol (Table III). Erythrocyte acetylcholine esterase is inhibited non-competitively (Fig. 3), indicating that the substrate–enzyme affinity is not altered by the added lipid. Furthermore, different groups of antihemolytic compounds are inhibitory and this diversity is compatible with an indirect effect. It is noteworthy, however, that a certain specificity is apparent within each group of antihemolytic compounds (Fig. 2, Tables I and II). The biphasic inhibitory effect observed in Fig. 2 may signal some transitory changes in the membrane in the presence of relatively high concentration of added acyl sorbitol. These changes are possibly due to alterations of packing of membrane components [14], which eventually lead to hemolysis.

An outstanding feature of the inhibition of acetylcholine esterase by antihemolytic agents, as shown in this study, is its clear dependence on cell intactness. Upon lysis the inhibition is obviated, but it reappears when sealing of the erythrocyte ghosts is favorable (Table V). This feature further illustrates the marked differences in properties between intact membranes in situ and isolated membranes, as amply documented in the literature (reviewed in ref. 14). Acetylcholine esterase appears to be a sensitive tool to evaluate the intactness of the membrane and to follow the dynamics of the sealing process. In terms of the fluid mosaic model of membrane structure [13], erythrocyte rupture may be considered as a cooperative phenomenon being transmitted by some structural coupling through the entire membrane (*cis* effect). The structural coupling in this case could be associated with the membrane tension [15, 16]. The change of acetylcholine esterase activity by a change in relative cell volume (Fig. 1) may also be a manifestation of the dependence of acetylcholine esterase properties on membrane conformation.

In conclusion, we would like to stress the concept of the "integral structure" typical for the intact cell membrane. Much as macromolecules are distinguished by features which are not present in the individual monomers, the membrane of the intact cell is endowed with some specific characteristics, which may not be predicted from those of the fragmented membrane.

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs Judith Tal is gratefully acknowledged. We thank our colleagues, Mrs Rachel Rahav and Dr Shmuel Bittner for the synthesis of the acyl sorbitols. The study was supported in part by a grant from the Council for Research and Development, Israel.

REFERENCES

1 Hertz, F., Kaplan, E. and Stevenson, J. H. (1963) Nature 200, 901–902
2 Branley, T. A., Coleman, R. and Finean, J. B. (1971) Biochim. Biophys. Acta 241, 752–769
3 Ellman, G. L., Courtney, D. K., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
4 Mitchell, C. D. and Hanahan, D. J. (1966) Biochemistry 5, 51–57
5 Moreno, R. D., Bloj, B., Farias, R. N. and Trucco, R. E. (1972) Biochim. Biophys. Acta 282, 157–165
6 Bloj, B., Moreno, R. D., Farias, R. N. and Trucco, R. E. (1973) Biochim. Biophys. Acta 311, 67–79
7 Livne, A., Kuiper, P. J. C. and Meyerstein, N. (1972) Biochim. Biophys. Acta 255, 744–750
8 Raz, A. and Livne, A. (1973) Biochim. Biophys. Acta 311, 222–229
9 Seeman, P. and Weinstein, J. (1966) Biochem. Pharmacol. 15, 1737–1752
10 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
11 Redman, C. M. (1972) Biochim. Biophys. Acta 282, 123–134
12 Heller, M. and Hanahan, D. J. (1972) Biochim. Biophys. Acta 255, 251–272
13 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720-731
14 Gitler, C. (1972) Annu. Rev. Biophys. Bioeng. 1, 51–92
15 Lopez, L., Duck, I. M. and Hunt, W. A. (1968) Biophys. J. 8, 1228–1235
16 Adams, K. H. (1973) Biophys. J. 13, 209–217
17 Krieglstein, J., Meiler, W. and Staab, J. (1972) Biochem. Pharmacol. 21, 985–997