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SENSITIVITY OF ERYTHROCYTE ACETYLCHOLINESTERASE TO INHIBITION BY LINOLENOYL SORBITOL

DEPENDENCE ON A TRANSMEMBRANE POTENTIAL

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SUMMARY

Acetylcholinesterase activity of human erythrocytes is known to be inhibited by linolenoyl sorbitol, the inhibition being critically dependent on cell membrane intactness The extent of enzyme inhibition by the added lipid is correlated with the magnitude of Cl^- gradient across the erythrocyte membrane, indicating that enzyme sensitivity is associated with a transmembrane potential If linolenoyl sorbitol is allowed to interact with the erythrocytes while a Cl^- gradient exists, enzyme sensitivity can subsequently be demonstrated not only in the absence of a gradient but even when the cells are lyzed It is concluded that the transmembrane potential determines the accessibility of a membrane component to the added lipid

INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase EC 3 1 1 7) of human erythrocytes is a membrane-bound enzyme [1, 2], which can be assayed in the intact cell [3] Several groups of substances inactivate acetylcholinesterase in intact red cells proteolytic enzymes, antibiotics, sulphydryl and other reagents (for references, note ref 4) Furthermore, the enzyme activity is inhibited by antihemolytic compounds, of which the inhibition by linolenoyl sorbitol, a synthetic lipid, was studied in detail [5] The inhibition of acetylcholinesterase activity by linolenoyl sorbitol is noncompetitive and is critically dependent on cell intactness over a wide temperature range Neither solubilized nor ghost acetylcholinesterase is affected by the acyl sorbitol, while under conditions optimal for ghost resealing, the enzyme resumes the sensitivity to the acyl sorbitol [5] Acetylcholinesterase sensitivity thus appears to be closely related to a certain feature of membrane integrity Efraim Racker (personal communication) suggested that membrane potential is the feature concerned, namely that the added lipid inhibits acetylcholinesterase, provided that a gradient in ion concentration exists across the erythrocyte membrane In the present communication we examine this challenging proposition

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Abbreviation SITS, 4-acetamido-4 -*iso*-thiocyanatostilbene-2,2′-disulfonic acid

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, 5 mM sodium phosphate, pH 7 4, and 10 mM glucose (suspending medium), the top layer and buffy coat were discarded and the erythrocyte suspension was adjusted to hematocrit of 6 % in the same medium

Determination of acetylcholinesterase

Acetylcholinesterase was measured according to Ellman et al [3], with some modifications An aliquot (40 μl) of the 6 % erythrocyte suspension was mixed with 6 ml of the medium, as specified for each experiment The medium also contained 5 mM sodium phosphate buffer, pH 8 0, and, where indicated, the inhibitor (mostly linolenoyl sorbitol) The inhibitor was added in 10 μl ethanol and the control tubes contained the same quantity of ethanol 2,2'-Dinitro-5,5'-dithiobenzoic acid (0 4 mM) was added and the reaction was started by adding 50 μl of 75 mM acetylthiocholine iodide at 37 °C The reaction of a 3 ml aliquot was stopped 2 min after the start (for "zero time") with 20 μl of a solution containing 0 25 mg/ml eserine (physostigmine) and 10 % Triton X-100 The reaction was similarly terminated in the remaining 3 ml aliquot 4 min later The absorbance was recorded at 412 nm in a Bausch and Lomb Spectronic 200 spectrophotometer with the "zero time" reading set as a reference Triton was used to clarify the suspension and eliminate possible changes in reading unrelated to esterase (e g changes in cell volume) When the terminated reaction mixtures were kept in ice, the readings were unaltered for several hours, and thus this procedure is particularly useful for the simultaneous analysis of many reactions Enzyme activity and extent of inhibition recorded by Ellman's original method and the modified procedure agreed within the experimental error (about 5 %) If not indicated otherwise, the results presented are averages of at least 4 experiments, each conducted in triplicate Materials used were as reported earlier [5]

RESULTS AND DISCUSSION

Acetylcholinesterase inhibition and Cl^- gradient

The inhibition of erythrocyte acetylcholinesterase by linolenoyl sorbitol was originally observed when intact cells were assayed in a medium of phosphate buffer [5], in accordance with the conditions described for the assay [3] If the inhibition is indeed conditioned by a membrane potential, it is expected that, on replacing the phosphate medium with isotonic NaCl, the inhibition would be greatly diminished Table I shows that when the cells are assayed in sodium phosphate, sodium citrate or Na_2SO_4, as well as in isotonic sucrose solution, acetylcholinesterase is markedly inhibited by the added lipid However, in media of $NaHCO_3$ and NaCl, the extent of inhibition is indeed very low A rapid dissipation of the Cl^- gradient in a bicarbonate medium is anticipated, since the erythrocyte membrane is known to be highly permeable to HCO_3^- The slight inhibition observed in NaCl was entirely eliminated if the concentration of added lipid was reduced to 2 5 μg/ml, while the inhibition in Na_2SO_4 under these conditions was still about 40 %

TABLE I

INHIBITION OF ERYTHROCYTE ACETYLCHOLINESTERASE ACTIVITY BY LINOLENOYL SORBITOL AS AFFECTED BY THE ASSAY MEDIUM

The figures, averages of triplicates, are results of a single experiment, in which all the media were compared simultaneously Percent inhibition in parenthesis represents the range observed in over 12 other experiments, in which the various media were tested, usually individually All media contained 5 mM sodium phosphate, pH 8 0 Linolenoyl sorbitol added 3 3 μg/ml

Assay medium	Enzyme activity (μmol/ml packed cells per min)	% Inhibition by added lipid
Sodium phosphate, 100 mM	5 2	40 (34–43)
Sodium citrate, 100 mM	4 4	45 (40–54)
Na_2SO_4, 100 mM	4 4	49 (38–55)
Sucrose, 300 mM	3 5	38 (32–46)
$NaHCO_3$, 150 mM	5 1	0 (0–2)
NaCl, 150 mM	4 5	7 (0–12)

The results presented in Table I were not altered if Na^+ was replaced by K^+, thus the anion composition of the medium is a determinant factor

Fig 1 shows that the extent of inhibition of acetylcholinesterase by linolenoyl sorbitol depends linearly on the ratio of SO_4^{2-} to Cl^- concentration in the isotonic medium As already shown, several fatty acids, and particularly stearic acid, also inhibit acetylcholinesterase [5] Fig 1 shows that the inhibition of the enzyme by stearic acid is characterized by a steep dependence on the ratio of SO_4^{2-} to Cl^- When the cells were assayed in isotonic mixtures of sucrose and NaCl (totalling 310

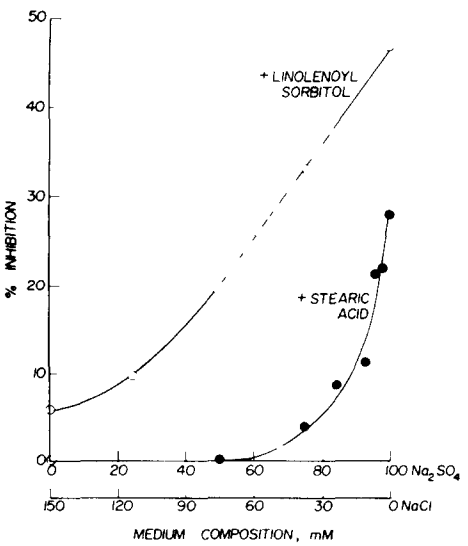

Fig 1 Inhibition of acetylcholinesterase by linolenoyl sorbitol or stearic acid (3 3 μg/ml each) as a function of medium composition The medium was buffered with 5 mM sodium phosphate pH 8 0

mosM), the enzyme inhibition by 3 3 μg/ml linolenoyl sorbitol was gradually decreased (from 45 to 9 %) as the proportion of NaCl increased (from 0 to 155 mM)

Since SO_4^{2-} is able to exchange with Cl^- across the erythrocyte membrane, inhibition of acetylcholinesterase is expected to diminish following an incubation of the erythrocytes in a Na_2SO_4 medium, at a rate compatible with the exchange rate Furthermore, if the transport of SO_4^{2-} is effectively prevented, then incubation of cells in Na_2SO_4 should not much affect the sensitivity of the esterase An experiment designed to test this supposition is presented in Fig 2 4-Acetamido-4′-*iso*-thiocyanto-

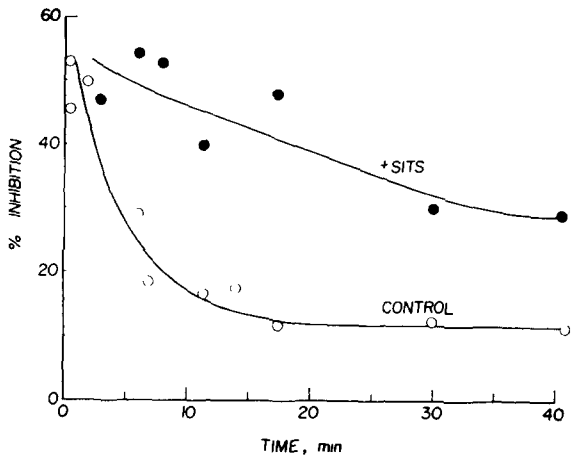

Fig 2 Effect of incubation of erythrocytes in Na_2SO_4 solution on subsequent inhibition of acetylcholinesterase by linolenoyl sorbitol Prior to the incubation, 50 % erythrocyte suspension was treated with 10^{-4} M SITS for 30 min at 0 °C in the dark [6] For "control", SITS was omitted The cells were then washed twice and adjusted to 6 % with the suspending medium An aliquot (0 66 ml) of this suspension was incubated at 37 °C with 100ml 100 mM Na_2SO_4, buffered with 5 mM sodium phosphate, pH 8 0 Samples taken at intervals were augmented with 3 3 μg/ml linolenoyl sorbitol and assayed immediately For 100 % activity, the lipid was omitted Average of 3 experiments

stilbene-2,2′-disulfonic acid (SITS) was chosen to slow down the dissipation of the gradient in Cl^- during incubation in Na_2SO_4, since it is known to be an effective inhibitor of anion transport in human erythrocytes [6] Furthermore, SITS itself at the concentration used caused only slight (< 5 %) inhibition of acetylcholinesterase Fig 2 demonstrates that the inhibition of acetylcholinesterase does indeed decline with time of incubation in Na_2SO_4, while SITS-treated cells show a moderate change Simultaneous influx measurements, using $^{35}SO_4^{2-}$ in the external medium, established that the SITS treatment curtailed about 90 % of SO_4^{2-} transport Incubation in 100 mM sodium phosphate revealed a decline in eventual inhibition, similar to that observed with Na_2SO_4 Anion exchange across the erythrocyte membrane is essentially at standstill at 4 °C [6] As expected, when the incubation in Na_2SO_4 took place at 4 °C for 30 min, hardly any decline in enzyme sensitivity to the acyl sorbitol was apparent

Fig 3 shows that, despite a prolonged incubation of erythrocytes in sodium citrate at 37 °C, the enzyme sensitivity to the added lipid was not altered Since the human erythrocyte membrane is essentially impermeable to citrate, these results are

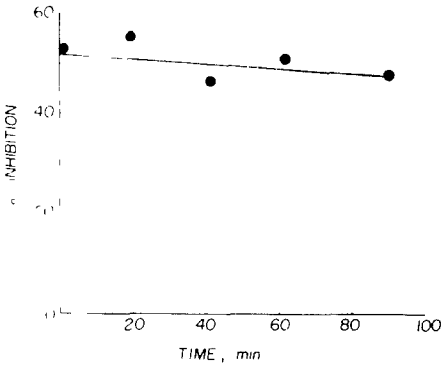

Fig 3 Effect of incubation of erythrocytes in sodium citrate solution on subsequent inhibition of acetylcholinesterase by linolenoyl sorbitol Details are as in Fig 2, except that the treatment with SITS was omitted and the incubation and assay took place in 100 mM sodium citrate, pH 8 0 buffered with 0 5 mM sodium phosphate pH 8 0

in harmony with the proposed relationships between Cl^- gradient and sensitivity of the esterase to the added lipid

Site of action of linolenoyl sorbitol

It is possible that anion composition of the medium affects the extent of binding of the added lipid to the cells and thus determines whether the esterase is inhibited Labeled linolenoyl sorbitol is not available at present It was therefore of value to test another effect of this lipid on the erythrocyte membrane in presence of either NaCl or

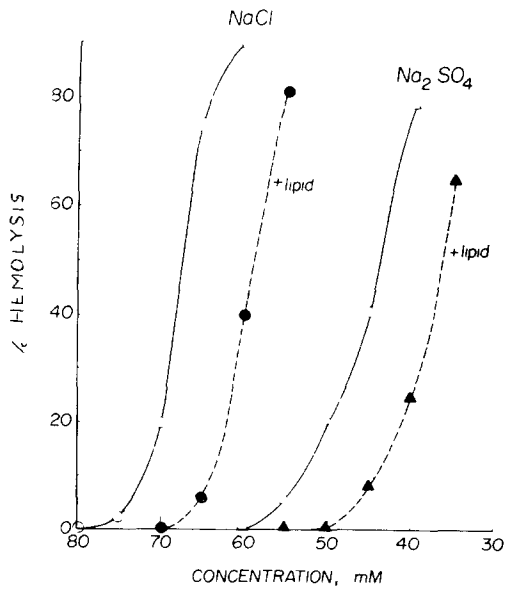

Fig 4 Osmotic fragility of erythrocytes in either NaCl or Na_2SO_4 medium, as affected by 3 3 μg/ml linolenoyl sorbitol The procedure was as described earlier [7]

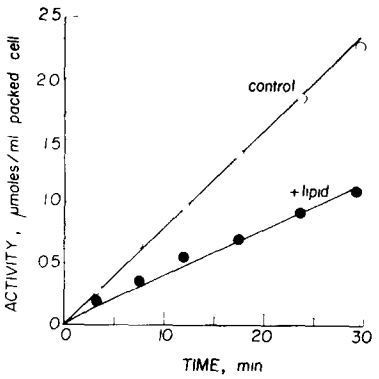

Fig 5 A prolonged assay of erythrocyte acetylcholinesterase as affected by 1 7 μg/ml linolenoyl sorbitol Details are as described in Methods, except that erythrocyte suspension in the assay was reduced to 0 01 % to keep the absorbance values below 1 0 following the prolonged assay

Na_2SO_4 Since linolenoyl sorbitol is known to be antihemolytic [7], probably by expanding the erythrocyte membrane [8], the effect of this lipid on the osmotic fragility of human erythrocytes was studied Fig 4 shows that the shift in hemolysis curve, caused by 3 3 μg/ml lipid, was very similar in the two media

While the partition of the lipid is apparently not affected by the anion of the medium, the site of action of linolenoyl sorbitol might be affected Even when partially inhibited, acetylcholinesterase activity is, curiously, linear with time This is true not only for the time course of the standard assay, but also for a much longer period (Fig 5) Incubation under similar conditions, but without the lipid, would have resulted in a declined sensitivity The linearity of the reaction in presence of linolenoyl sorbitol may indicate that the transmembrane potential does not affect the enzyme directly, but is critical for the accessibility of a membrane component which interacts with the added lipid In this case, it should be possible to demonstrate the enzyme sensitivity to the lipid in absence of an anion gradient, provided that the lipid was allowed to interact with the membrane while the gradient was in effect To test it experimentally, erythrocytes were first incubated for 2 min in a medium of Na_2SO_4 or NaCl (Incubation medium) with or without the lipid Then, following centrifugation, the medium was replaced by either NaCl or Na_2SO_4 solution (Assay medium), containing the lipid, if this had been present originally Table II shows that it is the incubation medium which determines the eventual sensitivity of the enzyme

Is cell intactness really critical for the inhibition [5]? Experiments were conducted essentially as described in Table II, except that, following the first incubation, cells were osmotically lysed in 0 5 ml H_2O and then the components of the reaction mixture were added Again it was clearly verified that once the lipid has interacted with erythrocytes in an Na_2SO_4 medium under conditions of a Cl^- gradient, it inhibits acetylcholinesterase even in the absence of a gradient If, however, the lipid was added after the cells had been lysed, no inhibition took place, as already reported [5], regardless of the medium composition

It is concluded that the sensitivity of erythrocyte acetylcholinesterase to linolenoyl sorbitol is indeed dependent on a transmembrane potential, as proposed by Racker The transmembrane potential, maintained by a Cl^- gradient, apparently

TABLE II

INHIBITION OF ACETYLCHOLINESTERASE BY LINOLENOYL SORBITOL AS AFFECTED BY THE INCUBATION AND ASSAY MEDIA

Erythrocytes (40 μl of the 6 % stock suspension) were incubated for 2 min at 20 C in 6 ml solution of either 100 mM Na_2SO_4 or 150 mM NaCl, buffered with 5 mM sodium phosphate, pH 8 0, with or without 2 5 μg/ml linolenoyl sorbitol (Incubation medium) The suspensions were centrifuged at 2000 *g* for 5 min, the supernatant discarded and the cells suspended in one of the assay media, containing 1 25 μg/ml acyl sorbitol if present during incubation Enzyme activity without the lipid averaged 4 4 μmol/ml packed cells per min

Incubation medium	% Inhibition by added lipid	
	Na_2SO_4 assay medium	NaCl assay medium
Na_2SO_4	51	51
NaCl	43	10

orients membrane components so that certain membrane sites become accessible to the added lipid which, indirectly, affects acetylcholinesterase In view of the relative inhibitory effects of various fatty acids and acyl sorbitols [5], it is unlikely that the interaction of the inhibiting lipid with the hypothetical sites is electrostatic, it is more likely to be hydrophobic

Of the enzymes recognized in the membrane of the human red cells, alterations in activity associated with pathological conditions are found regularly only with acetylcholinesterase [4] Yet, since the functions of this enzyme in the red cell are still obscure, attempts to consider physiological implications of the present study are obviously premature Acetylcholinesterase appears to be a sensitive tool not only to evaluate membrane intactness and to follow the dynamics of the sealing process [5] but also to probe the expression of a transmembrane potential

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