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The temperature-dependence of human erythrocyte acetylcholinesterase activity is not affected by membrane cholesterol enrichment

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The temperature-dependence of both the lipid order parameter (S_{DPH}) and acetylcholinesterase (AChE) activity from native and cholesterol-enriched human erythrocyte membranes was investigated. Cholesterol enrichment abolishes an inflection observed around 30 °C in the temperature-dependence of native membrane lipid order parameter, whereas the Arrhenius plot of the enzymic activity is substantially unaffected. These results support the view that the breaks in the Arrhenius plot of the enzyme activity are not related to sudden changes of bulk membrane physical state, but arise from a direct effect of temperature on enzyme conformation.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7; AChE) occurs in several molecular forms in vertebrate tissues, a major distinction being made between asymmetric (collagen-tailed) and globular forms (Massoulié & Bon, 1982). It appears that, whereas asymmetric forms are anchored to extracellular structures, such as the basal lamina in the neuromuscular synaptic cleft, via a collagen tail (Taylor *et al.*, 1987), globular forms include detergent-soluble species inserted into the membrane lipid bilayer by a hydrophobic domain (Ott, 1985). Such a membrane-binding domain, for detergent-soluble AChE from a number of sources, is not provided by a hydrophobic amino acid sequence, but is given by a glycolipid moiety covalently bound to an otherwise soluble protein structure (Futerman *et al.*, 1985; Roberts & Rosenberry, 1986; Haas *et al.*, 1986; Low *et al.*, 1987; Inestrosa *et al.*, 1987). Modulation of detergent-soluble AChE by membrane lipid environment has been widely investigated: Beauregard & Roufogalis (1979*a,b*) reported that the enzyme from bovine erythrocytes is tightly associated with cardiolipin and that this phospholipid might regulate its activity; Farias and associates demonstrated that diets of different lipid composition can influence the activity of rat erythrocyte AChE with respect to the temperature-dependence (Bloj *et al.*, 1974) and to the co-operative behaviour in case of fluoride inhibition (Bloj *et al.*, 1973, 1976). Effects of a number of anaesthetics and drugs on enzyme activity from rat (Mazzanti *et al.*, 1986) and dog (Delicostantinos & Tsakiris, 1985) synaptosomes have been observed and related to modification of membrane fluidity. As far as human erythrocyte AChE is concerned, the enzyme is inserted into the lipid bilayer by a non-peptide hydrophobic domain (Rosenberry & Scoggin, 1984; Dutta-Choudhury & Rosenberry, 1984; Roberts & Rosenberry, 1985, 1986; Haas *et al.*, 1986). The purified enzyme requires a hydrophobic environment for expression of activity; such an environment, however, can be provided not only by lipids and detergents, but also by interaction between the hydrophobic tails, which occurs after enzyme purification and detergent removal (Wiedmer *et al.*, 1979). Although human erythrocyte AChE cannot be regarded as a lipid-dependent enzyme in a strict sense, investigations with the purified enzyme have demonstrated that its temperature-dependence is modulated by the composition and physical state of the lipid system in which it is reconstituted (Frenkel *et al.*, 1980). Since the Arrhenius plot of the native enzyme has been reported to be non-linear (Aberlin & Litman, 1979), the question arises as to whether also the temperature-dependence of AChE activity in native membranes is modulated by the physical state of its lipid environment. In the present study, we investigated whether cholesterol enrichment of human erythrocyte membranes brings about any modification in the Arrhenius plot of AChE activity as compared with enzyme activity in control membranes, monitoring, at the same time, the effects produced by cholesterol enrichment on the physical state of membrane lipids, by means of steady-state DPH (1,6-diphenylhexa-1,3,5-triene) fluorescence polarization.

MATERIALS AND METHODS

Cholesterol enrichment of erythrocytes and plasma-membrane preparation

Fresh blood from healthy donors of both sexes was used within 2 h. Erythrocytes were separated from plasma by centrifugation at 900 ***g*** for 20 min, and the buffy coat was removed by aspiration; the cells were then washed twice with 10 vol. of 310 mOsM-Tris/HCl, pH 7.4, by re-centrifugation as described above.

For cholesterol enrichment of erythrocytes (Incerpi *et al.*, 1983), 20 μl of a stock solution (250 mg/ml) of cholesterol hemisuccinate in tetrahydrofuran was added,

Abbreviations used: AChE, acetylcholinesterase; DPH, 1,6-diphenylhexa-1,3,5-triene.

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with vigorous mixing, to 5 ml of 310 mOsM-Tris/HCl buffer (pH 7.6) containing 3.5% (w/v) polyvinylpyrrolidone and 2% (w/v) bovine serum albumin. The dispersion was then sonicated for 6 min at 100 W and added to 1 ml of packed cells in 45 ml of the Tris buffer. After incubation with frequent mixing at 37 °C for 30 min, the cells were washed twice with 310 mOsM-Tris/HCl, pH 7.6.

Erythrocyte membranes were prepared as described by Steck (1974), by lysis in 5 mM-sodium phosphate buffer, pH 8.0, in order to avoid inside-out ghost resealing. By this procedure no increase in AChE activity, assayed at 37 °C, was observed after treatment of membranes with 1% Triton X-100, by comparison with untreated membranes.

Assay of AChE activity

AChE activity was determined as described by Ellman *et al.* (1961). The assay mixture (3 ml) contained 0.125 mM-5,5′-dithionitrobenzoic acid and 5–10 μg of membrane protein in 100 mM-sodium phosphate buffer, pH 7.4. $K_{app.}$ and $V_{max.}$ were determined from Lineweaver–Burk plots by using acetylthiocholine concentrations of 0.06–0.5 mM. K_i was calculated from the equation $v_0 = V_{max.}/(1+[S_0]/K_i)$, by using inhibitory substrate concentrations of 1.5–5.0 mM. After temperature equilibration for 15 min in a thermostatically controlled bath, the reaction was started by addition of the appropriate amount of substrate, and the A_{412} was recorded at 0, 10 and 20 min, to ensure linearity with time. Blanks were also run at each selected temperature for each substrate concentration tested.

Lipid extraction and determination

Lipids were extracted by the method of Bligh & Dyer (1959). Phospholipid P was determined as described by Marinetti (1962). Cholesterol was measured by the cholesterol oxidase method with a high-performance 'Monotest' kit (Boehringer, Mannhein, Germany), after hydrolysis of the cholesterol hemisuccinate incorporated into erythrocyte membranes by 0.9% NaOH.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Biochemical characterization of control and cholesterol-enriched erythrocyte membranes

The cholesterol/phospholipid molar ratio and AChE units/mg of membrane protein (at 36 °C) were respectively 0.95 and 1.28 in control membranes and 1.40 and 0.91 in cholesterol-enriched membranes. The observed loss of enzymic activity (about 30%) was largely due to enzyme released from erythrocytes during incubation for cholesterol enrichment and washings.

Although we did not investigate the form in which AChE was released, owing to the possibility of microvesiculation, the phospholipid pattern of cholesterol-enriched erythrocyte membranes was analysed as reported previously (Spinedi *et al.*, 1987). No significant compositional differences were observed with respect to the phospholipid pattern of control membranes.

Fluorescence polarization

For fluorescence labelling, a 2 mM stock solution of DPH (Fluka, Buchs, Switzerland) in tetrahydrofuran was diluted 1:1000 immediately before use with 100 mM-sodium phosphate buffer, pH 7.4, and then mixed by vigorous agitation with a suspension of erythrocyte membranes to a final protein concentration of about 50 μg/ml; the mixture was then kept at room temperature for 30 min. Fluorescence-polarization measurements were carried out on a Perkin–Elmer LS-5 luminescence spectrometer. Excitation was set at 355 nm and emission was detected at 450 nm by using a 5 nm band-pass on both light-paths. The lipid order parameter S_{DPH} was calculated as described by Van Blitterswijk *et al.* (1981) from the equation:
and

$$S^2_{DPH} = r_\infty/r_0$$

where

$$r_0 = 0.362,$$
$$r_\infty = 4r_s/3-0.10$$

and

$$r_s = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$$

$I_\parallel$ and $I_\perp$ being the fluorescence intensities recorded with the analysing polarizer oriented, respectively, parallel and perpendicular to the plane of the excitation beam (Shinitzky & Barenholz, 1978). Temperature control was achieved by a water-bath-operated circulation around the jacketted cuvette; the temperature of the sample in the cuvette was routinely checked by a precision thermocouple thermometer.

RESULTS AND DISCUSSION

The temperature-dependence of the lipid order parameter S_{DPH}, in the range 10–42 °C, is reported in Fig. 1 for control and cholesterol-enriched membranes. Control membranes display values of S which are shifted by 5–10 °C towards lower temperatures as compared with cholesterol-enriched membranes; in addition, the plot for native membranes displays an inflection around 30 °C which is not observed for the cholesterol-enriched preparation. Caution should be used in relating discontinuities in the temperature-dependence of spectroscopic parameters with the occurrence of lipid-phase transitions in biological membranes (Devaux & Signeuret, 1985); in this connection, on the other hand, breaks around 30 °C in the temperature-dependence of spectroscopic parameters have been reported, by use of various e.s.r. probes, in studies with human erythrocyte ghosts and related to the occurrence of a lipid-phase transition (Tanaka & Ohnishi, 1976; Ogiso *et al.*, 1981). If this is the case for the inflection observed by us in native membranes, then it appears that such a phase transition is either abolished by membrane cholesterol enrichment or is shifted to higher temperatures, as previously observed for other membrane systems (Whetton *et al.*, 1983).

The temperature-dependence of AChE activity was then investigated in the same temperature range. Artifacts arising in obtaining enzyme Arrhenius plots have been extensively discussed (Dixon & Webb, 1964; Silvius *et al.*, 1978): the most trivial deal with changes which may occur with temperature, concerning either intrinsic kinetic parameters (enzyme $K_{app.}$) or extrinsic factors (pH modification of the assay buffer); with AChE, substrate inhibition should also be considered (Ellman *et al.*, 1961). In our conditions, the pH of the phosphate buffer used for enzyme assay was fairly constant throughout the temperature range studied. The effect of

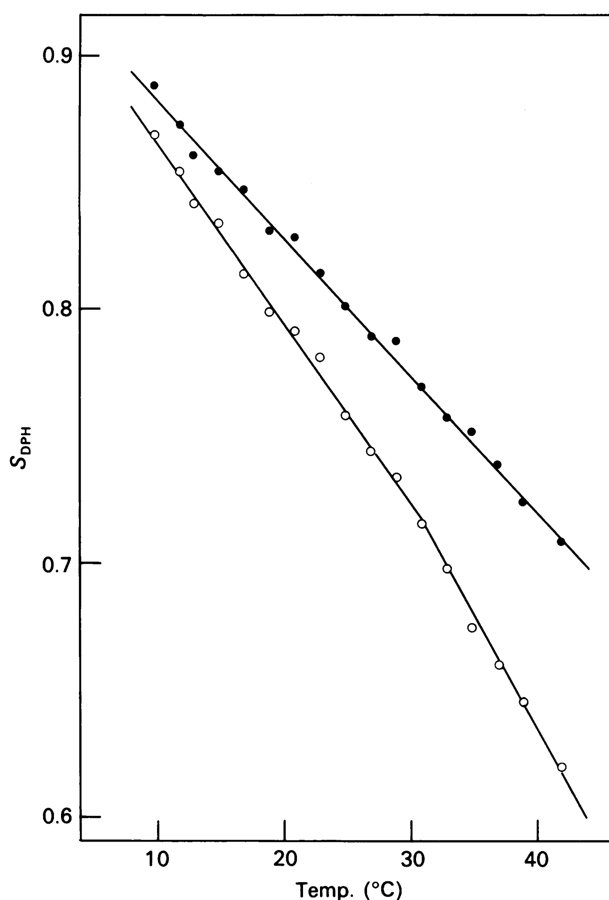

Fig. 1. Temperature-dependence of the lipid order parameter S_{DPH} in native (○) and cholesterol-enriched (●) erythrocyte membranes

Each point is the average of three independent determinations.

Table 1. $K_{app.}$ values at different temperatures for AChE in native and cholesterol-enriched erythrocyte membranes and for the enzyme solubilized with 1% Triton X-100

Enzyme activity was assayed and $K_{app.}$ calculated as described in the text. Results are means of three determinations.

	$K_{app.}$ (mM)			
Temp. (°C)...	10	20	30	40
Control membranes	0.078	0.075	0.072	0.077
Cholesterol-enriched membranes	0.076	0.079	0.077	0.073
Triton-solubilized enzyme	0.080	0.082	0.078	0.078

cholesterol enrichment on enzyme $K_{app.}$ was investigated at different temperatures and compared with the $K_{app.}$ of the enzyme in control membranes (Table 1). Cholesterol enrichment does not affect the enzyme $K_{app.}$, which holds constant throughout the temperature range investigated, as does the enzyme $K_{app.}$ in control membranes.

Concerning substrate inhibition, Table 2 shows that K_i, for the enzyme in both native and cholesterol-enriched membranes, increases as a function of the assay temperature, from about 6 mM at 10 °C to an average of 24 mM at 35 °C; at 40 °C the substrate inhibition appears to be drastically decreased.

Changing the pH of the enzyme assay from 7.0 to 8.0 changed the break point of Arrhenius plots of the AChE activity from 20 °C to 30 °C in rat erythrocyte membranes (Bloj *et al.*, 1974). In the human system too, the change in pH as well as the change in substrate concentration strongly modify the Arrhenius plots of AChE activity (results not shown).

To avoid the possibility that enzyme measurement might suffer from either substrate inhibition or non-saturating conditions, we extrapolated at each temperature the enzyme $V_{max.}$, obtained from double-reciprocal plots in the range of substrate concentration 0.06–0.5 mM. The Arrhenius plots thus obtained do not differ absolutely from those obtained with a final substrate concentration of 0.5 mM; in addition, our experiments were carried out at the physiological pH of 7.4.

The Arrhenius plots of AChE activity from control and cholesterol-enriched human erythrocyte membranes are shown in Figs. 2(*a*) and 2(*b*) respectively. Both plots show that: (i) the increase in enzyme activity with temperature abruptly stops around 36 °C; (ii) data in the range 10–36 °C are best fitted, by minimizing χ^2, with two lines rather than with one line, with intersection around 23 °C for control membranes and around 25 °C for cholesterol-enriched membranes; (iii) activation energy (kJ/mol) below and above the break point is 26.9 and 12.5 for control membranes and 25.4 and 12.9 for cholesterol-enriched membranes respectively. These results suggest that modifications of the physical state of the lipid environment by cholesterol enrichment *in vitro* scarcely affect the temperature-dependence of the activity of this membrane enzyme.

The inflection observed at 30 °C in the temperature-dependence of the lipid order parameter of native membranes can hardly be related to the discontinuities observed in the Arrhenius plot of the enzyme activity; in addition, although membrane cholesterol enrichment abolishes such an inflection, it does not abolish the breaks observed in the Arrhenius plot of the enzyme activity, leaving activation energies substantially unchanged. Although the possibility should be considered that the enzyme operates in a lipid environment having physical properties which are not affected by cholesterol enrichment, results reported herein support the view of Barton *et al.* (1985), who suggested that breaks in the Arrhenius plot of AChE activity from rat erythrocytes can hardly be expected to arise from changes in the physical state of the lipid environment, on the basis of the similar pattern displayed by the Arrhenius plots of the activity of the enzyme in the native state and solubilized by proteinase and phosphatidylinositol-specific phospholipase C. Those authors, on the other hand, reported a break at 14 °C over the temperature range 0–35 °C, at a concentration of 3.0 mM-acetylcholine. In our case, the Arrhenius plot of the enzyme activity after membrane solubilization with 1% Triton X-100 (Fig. 2*c*) displays a break around 24 °C, with activation energies of 27.5 kJ/mol and 15.9 kJ/mol respectively below and above the transition point; above 36 °C, the activity of the soluble enzyme increases with temperature, with an apparent activation energy of 6.5 kJ/mol, where-

Table 2. K_i values for substrate inhibition measured at different temperatures for AChE in native and cholesterol-enriched erythrocyte membranes and for the enzyme solubilized with 1% Triton X-100

Enzyme activity was assayed and K_i calculated as described in the text. Results are means of three determinations.

	K_i (mM)						
Temp. (°C)...	10	15	20	25	30	35	40
Control membranes	5.6	8.1	10.5	12.7	18.5	21.7	97.3
Cholesterol-enriched membranes	5.8	7.7	10.3	12.3	18.6	26.6	89.1
Triton-solubilized enzyme	5.7	7.6	10.3	12.9	16.2	19.9	40.7

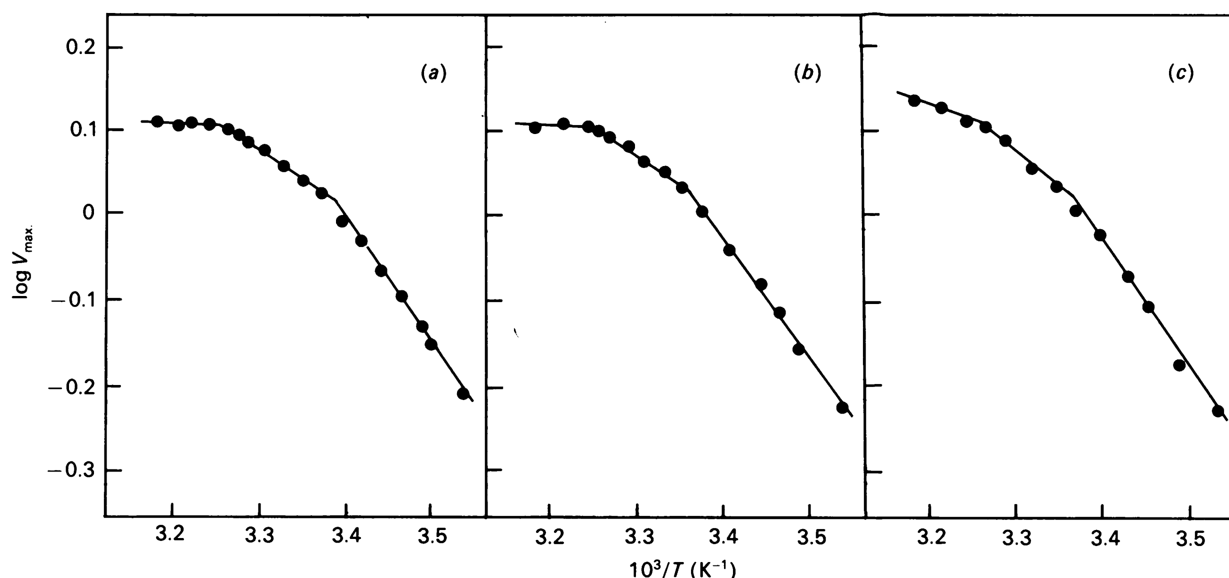

Fig. 2. Temperature-dependence of AChE activity in native (*a*) and cholesterol-enriched (*b*) erythrocyte membranes and after enzyme solubilization with 1% Triton X-100 (*c*)

Each point is the average of three independent determinations. Data are normalized for 1.28 units of enzyme activity at 36 °C.

as, as mentioned above, the increase in the native enzyme activity with temperature abruptly stops. Also, for the solubilized enzyme, as for the enzyme in the native state, the $K_{app.}$ holds constant and K_i for substrate inhibition increases with temperature, as reported respectively in Tables 1 and 2. As reported above, cholesterol-enriched membranes displayed a lower enzyme specific activity, compared with control membranes, which was largely accounted for by enzyme release from erythrocytes during incubation for cholesterol enrichment and washings. To determine whether cholesterol had any effect on the absolute enzyme activity, the specific activity of AChE in cholesterol-enriched membranes was measured, at 36 °C, before and after solubilization with 1 % Triton X-100: no difference was observed, indicating that cholesterol enrichment does not affect the absolute enzyme activity. We also carried out an Arrhenius plot of AChE activity from cholesterol-enriched membranes solubilized with 1 % Triton X-100: break-point positions as well as activation energies were the same as observed in the Arrhenius plot of AChE activity from native membranes solubilized with 1 % Triton X-100 (results not shown).

Data from the group of Van Deenen on the human reconstituted enzyme (Frenkel *et al.*, 1980) and from the group of Farias on the native rat erythrocyte enzyme (Bloj *et al.*, 1974, 1976, 1979) indicate that AChE activity is sensitive to the lipid environment in which it is embedded. In particular, for AChE from rat erythrocytes, it was pointed out that the cholesterol effect on Arrhenius plots depends on the membrane fatty acid composition, which in turn depends on the dietary fatty acid composition (Bloj *et al.*, 1979). The modification of the lipid environment through feeding conditions leads to some particular regulatory pattern for several membrane-associated enzymes (Farias & Trucco, 1977).

In the present experiments with humans, no control on dietary fatty acid effect was carried out. At present, although discrepant data about the sensitivity of detergent-soluble AchE to its lipid environment cannot easily be reconciled, it can be hypothesized that temperature directly affects the enzyme activity by inducing different conformational states, which, moreover, can be modulated by the composition of the lipid environment: such a modulation could involve direct enzyme–lipid molecular interaction or, in addition, could be related to the

physical state of a restricted lipid domain. In this connection, the question arises as to the role played by the non-peptidic membrane binding domains, which have been demonstrated to be covalently linked to the enzyme (Futerman *et al.*, 1985; Roberts & Rosenberry, 1986; Haas *et al.*, 1986; Inestrosa *et al.*, 1987): whether they simply anchor the enzyme to the membrane or are also involved in targeting the enzyme in a particular membrane domain is yet to be established.

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