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SHORT COMMUNICATIONS

Effect of cholesterol supplementation on acetylcholinesterase activity from sheep platelet plasma membrane

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Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7, AChE) is found in a variety of neuronal and a few non-neuronal cells such as erythrocytes, leucocytes and platelets of different animal species [1, 2]. The sheep platelet plasma membrane-bound AChE (AChEm) (exists as a dimeric globular form (G2) [3] and can be released by either *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PIPLC) or deoxycholate-activated platelet PIPLC [3]. However, the real contribution of this attachment and/or its lipidic environment to the catalytic activity of platelet AChE has not been fully elucidated. Investigations with human erythrocyte AChE have demonstrated that its temperature-dependence is modulated by the composition and physical state of the lipid system in which it is reconstituted [4]. However, Spinedi *et al.* [5] support the view that the breaks in the Arrhenius plots of human erythrocyte AChE are not related to sudden changes in the bulk membrane physical state but rather arise from a direct effect of temperature on enzyme conformation. We have reported recently that sheep platelet membrane-bound AChEs show a clear break in the Arrenius plot at around 17°, which disappears when activities are assayed in the presence of Triton X-100 [6]. In order to shed more light on the effects of the environment on AChE from platelet plasma membrane, we modified the lipid composition of sheep platelet plasma membranes by increasing the cholesterol content, using a polyvinylpyrrolidone (PVP)/cholesterol dispersion [7]. Cholesterol has been shown to be involved in the modulation of the activities of certain membrane-associated proteins at moderate to high cholesterol levels [8] and in the arrangement of aminophospholipids [7].

Materials and Methods

Materials. Acetylthiocholine chloride, bovine serum albumin (BSA) (essentially fatty acid- and globulin-free), cholesterol, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), eserine (physostigmine), ethopropazine, polyvinylpyrrolidone (PVP) and Tris (hydroxymethyl) aminomethane were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). D-Sucrose was from Aldrich-Europe (Beerve, Belgium). The remaining products were from Probus (Barcelona, Spain). All organic solvents were glass-distilled before use.

Preparation and cholesterol enrichment of platelet plasma membrane. Platelets were isolated from the fresh blood of adult sheep (*Ovis aries* L. var. *domestica*) collected in a local slaughterhouse, as described earlier [6]. The cholesterol enrichment of platelet plasma membrane under *in vitro* conditions was performed as indicated by Sánchez-Yagüe *et al.* [7]. Whole sheep platelets [9×10^8 cells/mL of buffer solution A (5 mM Na_2HPO_4, 0.154 M NaCl, pH 7.4, containing 0.2% glucose)] were incubated in a PVP/cholesterol dispersion [cholesterol stock solution (5 mg/mL ethanol) diluted 1:20 in solution A containing 0.05% BSA and 3.5% PVP (w/v)] at room temperature with gentle shaking for 2–2½ hr. Cells were washed twice with buffer solution A. In the control mixture, sheep platelets were incubated in solution A containing PVP and ethanol, without cholesterol, under similar conditions to those described above. Platelet lysis after treatment was measured by the determination of lactate dehydrogenase [9] in the supernatant and always remained below 7%.

The isolation of plasma membranes from control or cholesterol-enriched sheep platelets was carried out under identical conditions to those described by Sánchez-Yagüe *et al.* [6]. The platelet homogenate fractionated in a discontinuous sucrose gradient yielded one soluble fraction (band A) and three interface bands (B, C and D). The B band (located in the interface of the 0.6/1.0 M sucrose solutions) was diluted with solution B (5 mM Tris–HCl, pH 7.4–7.5) at a sucrose concentration of between 0.2 and 0.3 M and centrifuged at 105,000 g_{av} for 1 hr at 4°, thus yielding the plasma membranes. This fraction was finally resuspended in solution B for enzymatic analysis.

AChE activity was determined by measuring the hydrolysis of 1 mM acetylthiocholine chloride (final concentration) at 25° under conditions indicated in a previous paper [6]. One unit of AChE is defined as the amount of enzyme that forms 1 μmol of thiocholine per min, which yields 1 μmol of nitromercaptobenzoate (molar absorptivity of 13,600 M^{-1} cm^{-1} at 412 nm) under the assay conditions. The protein content of the different fractions was determined by the method of Lowry *et al.* [10], using BSA as standard.

Lipid analysis. Total lipid extraction from platelet plasma membranes, determination of their lipid phosphorus and evaluation of the total cholesterol content of the lipid extracts were carried out as reported by Sánchez-Yagüe and Llanillo [11].

AChE thermal stability. Samples were heated for varying periods of time (5–90 min) at different temperatures (35°, 45° and 55°) in a thermostatically regulated bath with constant shaking. Control samples were kept at 4°. All samples were then assayed for remaining enzyme activity, as described above.

AChE kinetic parameters. Michaelis constants (K_m) and maximal velocities (V_{max}) were calculated from Lineweaver–Burk plots [12] using acetylthiocholine concentrations of 0.05 to 2.8 mM at temperatures of 12°, 25° and 33°. Data were fitted to the plots by non-linear regression analysis.

AChE temperature-dependence assay. The temperature-dependence of AChE activity was measured over a temperature range of 12 to 37°. Samples were assayed in triplicate at 1° intervals, keeping the samples at a constant temperature by a thermostatted cell holder coupled to a circulating water bath. Blanks without enzyme were run at each temperature to correct for spontaneous hydrolysis of acetylthiocholine. Lines were fitted to the data points in Arrhenius plots by regression analysis using the least squares method. X^2 statistical analyses were used to choose the two-line fits.

Results and Discussion

The modulation of solubilized membrane-bound AChE by a lipid environment has been widely investigated [4, 5, 13]. As far as AChEm is concerned, the enzyme is inserted into the outer half of the lipid bilayer by a non-peptide hydrophobic domain, thus being included within the so-called glycosyl-phosphatidylinositol (GPI)-anchored proteins [2]. Discussion of the influence of the lipid environment of these GPI-anchored proteins, especially AChE, is still an open question and although the influence of these surroundings could be less marked than for proteins that penetrate more deeply into the lipid bilayer, the data reported in the literature point to different results [4, 5, 13, 14]. In the present study, we have investigated whether modifications of the lipid environment by *in vitro* cholesterol enrichment of sheep platelet plasma membranes affects the kinetic, physicochemical and temperature-dependence characteristics of AChEm. Treatment of cells with lipid dispersion in PVP was found to be more efficient for cholesterol enrichment or depletion than treatment by established methods with liposomes or with lipid-modified sera [15]. Cell viability was practically unaffected and remained above 85% after all treatments [15]. In our case, more than 90% of intact cells were obtained after treatment, because cell lysis always remained below 7% and the cholesterol/phospholipid molar ratio was increased more than twice. On the other hand, the presence of ethanol in the incubation medium does not affect AChE activity significantly [16].

AChE thermal stability has been shown to be different in cholesterol-enriched platelet plasma membranes with respect to the controls, showing a less stable general behaviour in the treated samples at all the temperatures assayed (Fig. 1). At 35°, AChE was very stable throughout the preincubation time in the control plasma membranes. Nevertheless, AChE from plasma membranes with a cholesterol/phospholipid molar ratio of 1.05 ± 0.05 lost 20% of its original activity after preincubation at 35° for 50 min. Cholesterol enrichment also favoured an almost total inactivation of AChEm after preincubation at 55° for 10 min.

AChEm followed typical Michaelis–Menten kinetics for the hydrolysis of the acetylthiocholine substrate, regardless of the degree of cholesterol enrichment of the plasma membrane. In order to avoid serious errors in the interpretation of the Arrhenius plots [17], the K_m and V_{max} values for acetylthiocholine at different temperatures (12, 25 and 33°) were determined in all samples (Table 1). The values obtained both in the presence and absence of Triton X-100 were essentially identical in all cases for a given temperature (data not shown). The K_m values at different temperatures were also essentially coincident in control samples (0.122 ± 0.004 SD mM), resulting in a value that was increased significantly (0.155 ± 0.004 SD mM) in the cholesterol-enriched samples ($P < 0.001$). The absence of a significant effect of temperature on the K_m values of the system studied is in agreement with observations reported previously [4, 14]. Cholesterol supplementation was also associated with a decrease in the efficacy of the enzyme against the hydrolysis of the acetylthiocholine substrate, as suggested by the V_{max}/K_m ratio.

The temperature-dependence of AChEm activity was

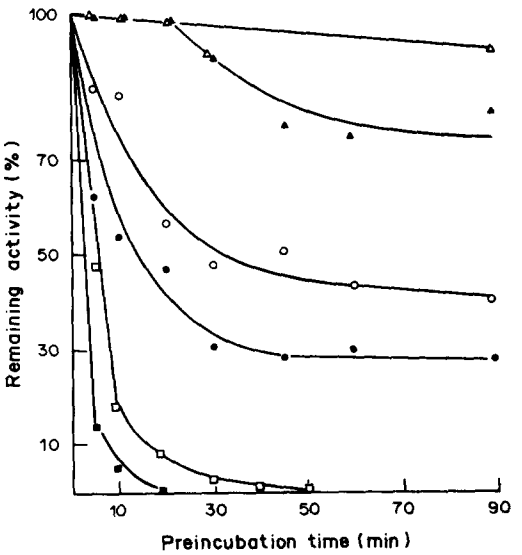

Fig. 1. Thermal stability of AChE from sheep platelet plasma membranes with cholesterol/phospholipid molar ratios of 0.66 ± 0.05 (control samples, open symbols) and 1.05 ± 0.05 (closed symbols), after heating at 35° (△, ▲), 45° (○, ●) and 55° (□, ■) for different times. The remaining activity is calculated at 1 mM acetylthiocholine substrate (final concentration) as indicated in Materials and Methods.

Table 1. Kinetic parameters for AChE located in sheep platelet plasma membranes with a cholesterol/phospholipid molar ratio of 0.66 (native membranes incubated in PVP/ethanol solution considered as control) or 1.05 ± 0.05 (native membranes enriched in cholesterol as indicated in Materials and Methods)

Cholesterol/phospholipid molar ratio	K_m (mM)*			V_{max}† (mUnits/mg)	V_{max}/K_m‡ (mUnits/mg/mM)
	12°	25°	33°		
0.66	0.127	0.121	0.119	17.5 ± 1.8	143.4
1.05	0.159§	0.154§	0.151§	16.3 ± 1.9	105.8

The K_m and V_{max} values were determined from Lineweaver–Burk plots using acetylthiocholine chloride as substrate.

* Data represent the average values of duplicate determinations from two different experiments.

† Values represent the mean ± SD of duplicate data for V_{max} obtained at each temperature: 12°, 25° and 33°.

‡ The ratio V_{max}/K_m was found using the mean of K_m and V_{max}.

§ Significantly different from control; $P < 0.001$ (Student's t-test, two-tailed).

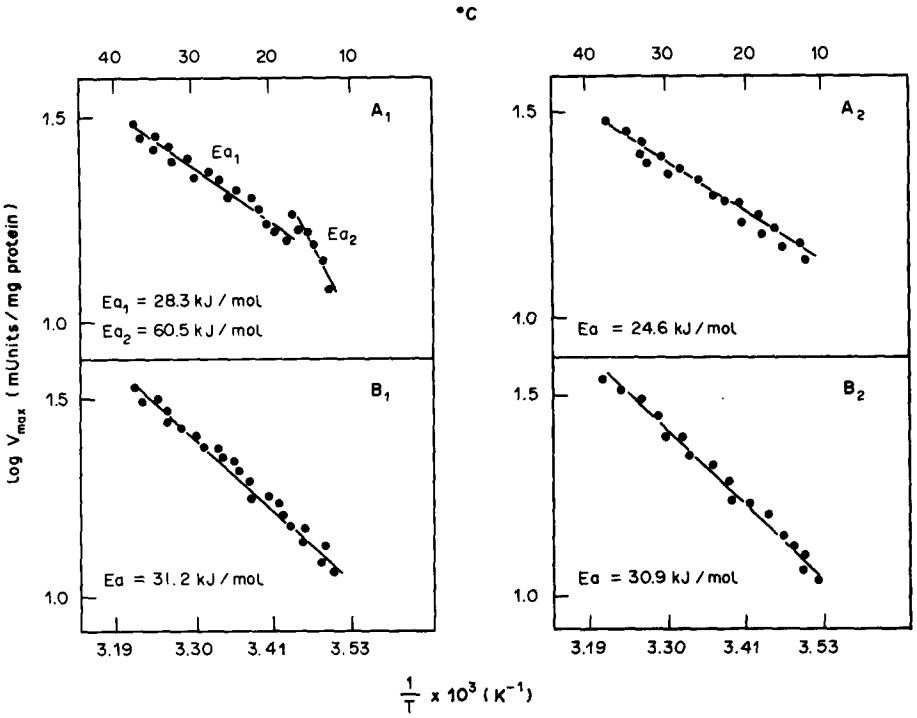

Fig. 2. Temperature-dependence of AChE measured over the 12–37° range at 1 mM acetylthiocholine final concentration in native sheep platelet plasma membranes (control samples: A, cholesterol/phospholipid molar ratio: 0.66) and cholesterol-enriched platelet plasma membranes (B, cholesterol/phospholipid molar ratio: 1.40), in the absence (A_1, B_1) or presence (A_2, B_2) of detergent (0.02% Triton X-100, final concentration). For experimental details see section on Materials and Methods. Each point represents the average of duplicate values from two different experiments. The straight lines were fitted by the least square method.

visualized as a plot of log V_{max} versus 1/T which for homogenously reacting systems has been called the Arrhenius plot. These plots of AChEm activity from control and cholesterol-enriched sheep platelet plasma membranes (Fig. 2) show that: (1) data in the range of 12 to 37° are fitted with two lines; in control plasma membranes, when AChE was assayed in the absence of Triton X-100 (Fig. 2A_1), with the intersection around 17° and with the finding of apparent activation energies of 28.3 kJ/mol above the break point and 60.5 kJ/mol below the discontinuity point; (2) the break point was abolished by cholesterol supplementation of the plasma membranes (a cholesterol/phospholipid molar ratio of 1.4) (Fig. 2B), or in controls treated with Triton X-100 (Fig. 2A_2), showing a constant apparent activation energy in the 12–37° range of about 30 kJ/mol. Thus, these discontinuities in the Arrhenius plots of native AChEm could be considered to reflect a phase transition in the lipid environment of the activity and, therefore, to indicate enzyme modulation by lipids [18], since the possibility of discontinuities arising from a variation in the K_m is discarded in the present study. As our results suggest, cholesterol supplementation affects the temperature-dependence of sheep platelets AChEm. This could be due to the fact that the enzymatic protein is located in a new lipid environment depleted in cholesterol, as has been suggested previously for other enzymes [19]. A modulation of AChE by a lipid environment has also been described for other sources [4, 14]. By contrast, Barton *et al.* [13] have claimed that both membrane bound and enzymically solubilized rat erthrocyte AChE show a discontinuity in the Arrhenius plot which seems to rule out the possibility that the discontinuity may be due to a membrane lipid transition, and also supports the view of a conformational change in the enzyme protein. A similar kind of behaviour was reported by Spinedi *et al.* [5] for human erythrocyte AChE. Within this framework, the possibility could be entertained that molecular species of GPI-anchored AChE from various sources may display differing sensitivities to the physical state of the environment in which they are embedded.

The changes in the surface charge of the plasma membrane may lead to certain alterations in the catalytic properties of AChE. The progressive inclusion of free cholesterol into the sheep platelet plasma membrane causes a gradual movement of phosphatidylserine from the inner to the outer side of the lipid bilayer [7] which increases the negative charge density of the membrane [20]. Since acetylthiocholine is positively charged and the enzyme has an anionic site for binding the substrate, an increase in the density of negative charges on the membrane surface would raise the apparent K_m of AChEm for its substrate, as shown in Table 1.

In summary, these results demonstrate that the AChE activity from sheep platelet plasma membrane is affected by variations in the lipid bilayer produced by cholesterol enrichment. The enzyme shows a considerable decrease in its thermal stability and an increase in the K_m against acetylthiocholine substrate, with a "linearization" in the Arrhenius plots that shows a constant activation energy between 12 and 37°.

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A second site of action of soman on acetylcholinesterase

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Acetylcholinesterase (AChE, acetylcholine acetylhydrolase, EC 3.1.1.7) is involved in terminating the actions of acetylcholine at cholinergic synapses [1]. AChE is also located in a number of noncholinergic tissues and has been shown to possess a peptidase activity in addition to its ability to hydrolyse acetylcholine [2–5]. In addition to the esteratic and tryptic sites, AChE possesses peripheral sites which modify the esteratic activity. The modulation of AChE activity is carried out at these sites by a broad range of compounds [6–10]. AChE is inhibited by a number of irreversible or slowly reversible inhibitors. Many of these compounds are used as therapeutic agents [11] or insecticides [12]. One group of such compounds, referred to as nerve agents, has been produced for use as chemical weapons [13].

The mechanism by which these compounds inhibit AChE