A study of the mechanism by which some amphiphilic drugs affect human erythrocyte acetylcholinesterase activity

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The effects of the local anaesthetics procaine, tetracaine and lidocaine and of the antidepressant imipramine on human erythrocyte acetylcholinesterase were investigated. All four amphiphilic drugs inhibited enzymic activity, the IC_{50} (the concentration causing 50% inhibition) being about 0.40 mM for procaine, 0.05 mM for tetracaine, 0.70 mM for imipramine and 7.0 mM for lidocaine. Procaine and tetracaine inhibited acetylcholinesterase activity competitively at concentrations at which they did not perturb the physical state of the membrane lipid environment, as assessed by steady-state fluorescence polarization, whereas lidocaine and imipramine displayed mixed inhibition kinetics at concentrations at which they induced an enhancement of membrane fluidity. The question was addressed as to whether membrane integrity is a prerequisite for imipramine and lidocaine action. Membrane solubilization by 1% Triton X-100 and a decrease, by dilution, in the detergent concentration to 0.05% [which is above the Triton X-100 critical micelle concentration (c.m.c.)] did not substantially affect the inhibitory potency of the two amphiphilic drugs at their IC_{50} ; in the presence of increasing detergent concentrations the inhibitory potency of imipramine was gradually decreased, but not abolished, whereas the inhibitory effect of lidocaine was only slightly diminished, even at 1% Triton X-100. It is suggested that neither competitive nor mixed inhibition kinetics arise from conformational changes of the protein driven by a modification of the physical state of the lipid environment, but from a direct interaction of the amphiphilic drugs with acetylcholinesterase. In particular, the partial loss of the inhibitory potency of imipramine and lidocaine that is observed upon increasing Triton X-100 concentration well above its c.m.c. could be explained in terms of amphiphile partition in detergent micelles and, in turn, of a decreased effective concentration of the two inhibitors in the aqueous phase.

INTRODUCTION

Acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7; AChE) occurs in detergent-soluble forms (DSACHe) in the central nervous system as well as in erythrocytes of mammals (Massoulié & Bon, 1982). The function of the erythrocyte enzyme is unknown; however, it has been extensively investigated, being regarded as a valid model system for substituting the brain enzyme in 'in vitro' studies (Ott, 1985). Although DSACHe mainly occurs in mammalian brain in a tetrameric (G4) form, whereas dimeric (G2) forms are mainly found in erythrocytes, recent data suggest that, in both systems, the membrane-binding domain is not provided by a hydrophobic amino acid sequence but by a glycolipid moiety covalently bound to an otherwise-soluble proteic structure (Futerman *et al.*, 1985; Roberts & Rosenberry, 1986; Haas *et al.*, 1986; Low *et al.*, 1987; Inestrosa *et al.*, 1987).

In many instances lipid modulation of DSACHe has been suggested on the basis of changes occurring in the temperature-dependence of the enzymic activity after modification of the composition, as well as of the physical properties, of the lipid environment in which the enzyme is embedded (Bloj *et al.*, 1973, 1974, 1976; Frenkel *et al.*, 1980; Foot *et al.*, 1983). In this connection, however, Barton *et al.* (1985) suggested that breaks in the Arrhenius plot of AChE activity from rat erythrocytes can hardly be expected to arise from changes of the physical state of

the lipid environment; in line with this observation we have recently observed that cholesterol enrichment, although affecting the temperature-dependence of the lipid order parameter of human erythrocyte membranes, as measured by 1,6-diphenylhexa-1,3,5-triene (DPH) steady-state fluorescence polarization, does not affect the temperature-dependence of AChE activity (Spinedi *et al.*, 1988).

Effects of a number of amphiphilic drugs and anaesthetics on enzyme activity from dog synaptosomes (Deliconstantinos & Tsakiris, 1985) and rat brain membrane preparations (Mazzanti *et al.*, 1986) have been reported and related to modification of membrane fluidity. Mazzanti *et al.* (1986), on the other hand, suggested that drugs with membrane-perturbing properties might affect AChE activity independently from the effect on the physical state of the lipid environment.

Within this framework we studied the effects of a number of amphiphilic drugs on the activity of human erythrocyte AChE, investigating in particular whether their action is due to their membrane-fluidity-perturbing properties or is brought about by a direct molecular interaction with the enzyme.

MATERIALS AND METHODS

Erythrocyte membrane preparation

Fresh blood from healthy donors of both sexes was

Abbreviations used: IC_{50} , concentration causing 50% inhibition; c.m.c., critical micelle concentration; (DS)AChE, (detergent-soluble) acetylcholinesterase; DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenylhexa-1,3,5-triene.

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used within 2 h. Erythrocytes were separated from plasma by centrifugation at 900 *g* for 20 min, and the buffy coat removed by aspiration; the cells were then washed twice with 10 vol. of 155 mM-sodium phosphate buffer, pH 7.4, by re-centrifugation as described above. 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 of AChE activity, assayed at 37 °C, was observed after treatment of membranes with 1% Triton X-100 (Sigma) in comparison with untreated membranes.

Membrane solubilization

Erythrocyte membranes (100 µg/ml) were solubilized with 1% Triton X-100. After centrifugation at 100 000 *g* for 1 h, AChE activity was quantitatively recovered in the supernatant. Aliquots of the supernatant were diluted 1:20 with 25 mM-Tris/HCl buffer/120 mM-NaCl, pH 7.4, alone or plus Triton X-100, to achieve a final concentration of 5 µg of protein/ml in the presence of Triton X-100 (final concns. 0.05–1%).

Drug treatment

Procaine hydrochloride was obtained from Fluka (Buchs, Switzerland); tetracaine hydrochloride, imipramine hydrochloride and lidocaine were from Sigma.

Stock solutions of 100 mM-procaine, 30 mM-tetracaine, 100 mM-imipramine and 300 mM-lidocaine in 25 mM-Tris/HCl buffer/120 mM-NaCl, pH 7.4, were freshly prepared. Amphiphilic drugs were preincubated for 10 min at 37 °C with native or solubilized erythrocyte membranes before carrying out enzyme-activity and fluorescence measurements. As far as native membranes are concerned, enzymic activity, fluorescence quenching and fluorescence-polarization measurements were also carried out at the same membrane protein final concentration (50 µg/ml) in order to obtain directly comparable data without taking into account the coefficients of drug partition between the membrane lipid environment and the aqueous phase.

Assay of AChE activity

AChE activity was determined at 37 °C as described by Ellman *et al.* (1961). The assay mixture (3 ml) contained 0.5 mM-acetylthiocholine, 0.125 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and membrane protein (40 µg/ml) in 25 mM-Tris/HCl buffer/120 mM-NaCl, pH 7.4. K_m and V_{max} were determined by Lineweaver-Burk plots, using acetylthiocholine concentrations from 0.06 to 0.5 mM.

Protein determination

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

Fluorescence spectroscopy

For fluorescence labelling, 1 mM stock solutions of 1,6-diphenylhexa-1,3,5-triene (DPH; Fluka), in tetrahydrofuran or 1-(4-trimethylammoniumphenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH; Molecular Probes, Junction City, OR, U.S.A.) in *NN*-dimethylformamide were diluted 1:1000 immediately before use with 25 mM-Tris/HCl buffer/120 mM-NaCl, pH 7.4, and then mixed by vigorous agitation with a suspension of erythro-

cyte membranes in the same buffer to a final protein concentration of about 50 µg/ml; the mixture was then left at room temperature for 120 min. Absorption-corrected fluorescence of DPH and TMA-DPH as well as 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 455 nm. The degree of fluorescence polarization (*P*) was calculated according to the equation:

$$P = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities recorded with the analysing polarizer oriented parallel with, and perpendicular to, respectively the plane of the excitation beam (Shinitzky & Barenholz, 1978). Corrections for light-scattering from the sample, as well as for a grating correction factor were introduced (Chen & Bowman, 1965). Temperature control was achieved by a water-bath-operated circulation around the jacketed cuvette.

RESULTS

Fig. 1 shows the effect of the local anaesthetics procaine, lidocaine and tetracaine, as well as of the antidepressant imipramine, on human erythrocyte AChE activity. All the amphiphilic compounds tested inhibited enzyme activity, the IC_{50} being about 0.40 mM for procaine, 0.05 mM for tetracaine, 0.70 mM for imipramine and 7.0 mM for lidocaine; in all cases enzyme inhibition was fully reversible, as assessed after amphiphile removal by membrane washing (results not shown).

The type of inhibition produced by the various amphiphilic compounds was assessed by Lineweaver-Burk plots. As shown in Fig. 2, procaine and tetracaine inhibited AChE activity competitively, whereas the action of imipramine and lidocaine was consistent with mixed inhibition kinetics.

The question was addressed as to whether the tested amphiphilic drugs affected the physical properties of the membrane lipid environment, as assessed by steady-state

Fig. 1. Human erythrocyte AChE inhibition by amphiphilic drugs

Data are presented in form of Hill plots; v and v_0 are the reaction rates in the presence or absence of inhibitor respectively. Hill coefficients were 0.98 for procaine (■), 0.96 for tetracaine (●), 0.98 for imipramine (○) and 0.96 for lidocaine (□). Each point is the mean of three independent determinations.

Fig. 2. Kinetics of AChE inhibition by amphiphilic drugs

Results were plotted by the Lineweaver-Burk method as described in the Materials and methods section in the absence (■) or in the presence (●) of the indicated inhibitor at its IC_{50} . The K_m was 0.10 mM for the enzyme in control membranes and 0.72 and 0.69 mM in the presence of tetracaine and procaine respectively. For imipramine, the K_m was 0.35 mM and V_{max} was 71 % of that in control membranes; for lidocaine the above-mentioned values were 0.24 mM and 61 % respectively. Each point represents the average value of duplicate determinations from a typical experiment.

fluorescence polarization, over the same concentration ranges at which they inhibited AChE activity.

Since procaine, tetracaine, lidocaine and imipramine quenched DPH fluorescence in ethanol solution at millimolar concentrations (results not shown), we investigated, as a preliminary approach, whether fluorescence quenching by the above-mentioned amphiphilic compounds also occurred after DPH incorporation into erythrocyte membranes.

Fluorescence quenching was also investigated by using the trimethylammonium derivative of DPH (TMA-DPH); TMA-DPH probes the lipid environment next to the hydrophobic/polar interface, whereas DPH is believed to locate in the inner core of the lipid bilayer (Donner & Stoltz, 1985).

Fig. 3 shows that imipramine and lidocaine did not substantially affect DPH fluorescence, at least up to concentrations at which 80 % inhibition of AChE activity is achieved; TMA-DPH fluorescence was quenched by imipramine over the drug concentration range investigated, whereas quenching by lidocaine was significant at drug concentrations above 5 mM ($P < 0.05$ at least, as from paired-data analysis of fluorescence intensities). As far as competitive inhibitors are concerned, procaine had no effect on either DPH or TMA-DPH fluorescence over the concentration range tested, whereas tetracaine significantly affected TMA-DPH fluorescence only at concentrations above 0.125 mM. Indeed, in a set of experiments which we do not report for sake of brevity,

procaine significantly quenched TMA-DPH fluorescence, and tetracaine quenched both TMA-DPH and DPH fluorescence, at concentrations well above those relevant for AChE inhibition.

In Fig. 4 we report the effects displayed by the amphiphilic drugs under investigation on the physical state of erythrocyte membranes, as assessed by steady-state DPH and TMA-DPH fluorescence polarization, over the same amphiphile concentration ranges at which inhibition of AChE activity was studied. Fluorescence-polarization measurements using TMA-DPH were carried out only in those experimental conditions in which fluorescence quenching did not occur. It can be seen that, at concentrations relevant for AChE inhibition, neither procaine nor tetracaine affects the physical state of the lipid environment, whereas an enhancement of membrane fluidity is induced by both imipramine and lidocaine.

In order to assess whether membrane integrity is a prerequisite for the inhibitory action of imipramine and lidocaine, AChE activity was measured in presence of the above-mentioned amphiphilic drugs at their IC_{50} after membrane solubilization with 1 % Triton X-100 and dilution with buffer plus detergent or buffer alone to reach final Triton concentrations ranging from 0.05 % to 1 %. The concentrations tested are all above the Triton X-100 critical micellar concentration (c.m.c.), which is at about 0.02 % (Wiedmer *et al.*, 1979). Fig. 5 shows that: (i) membrane disruption does not substantially abolish

Fig. 3. Effects of amphiphilic drugs on the absorbance-corrected fluorescence of DPH and TMA-DPH in human erythrocyte membranes

Data were plotted according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{app}[Q]$$

F_0 and F being DPH (○) and TMA-DPH (▲) absorbance-corrected fluorescence intensities respectively in the absence of quencher (Q) and at various quencher concentrations ([Q]) and K_{app} being the apparent quenching constant. Each value is the average value of three independent determinations.

Fig. 4. Effect of amphiphilic drugs on steady-state fluorescence polarization of TMA-DPH (▲) and DPH (●) in erythrocyte membranes

Data are average values of three independent determinations.

Fig. 5. Effect of membrane solubilization and Triton X-100 concentration on AChE inhibition by imipramine and lidocaine

Membranes were solubilized and Triton X-100 final concentrations achieved as described in the text. The final protein concentration was 10 μ g of protein/ml; 100% enzymic activity was taken as the activity, in the absence of inhibitors, of the enzyme solubilized with 1% Triton. ●, Imipramine; ■, lidocaine. Each point is the average value of three independent determinations.

the inhibitory effect of the amphiphilic drugs, in comparison with native membranes, when AChE activity is measured at a Triton X-100 final concentration of 0.05%; (ii) only a partial recovery of the enzymic activity is observed when the detergent final concentration is increased well above its c.m.c.; (iii) the degree of enzymic activity recovery, at the same final concentration of detergent, is different for the two inhibitors, being higher in the case of imipramine in comparison with lidocaine.

DISCUSSION

The present results indicate that lidocaine and imipramine, which inhibit human erythrocyte AChE activity by mixed inhibition kinetics, are effective at the same concentrations at which they are able to perturb the physical state of the lipid environment. This observation, although suggestive of a role of the membrane lipid environment in mediating inhibitory effects, does not, however, constitute an unequivocal proof of the existence of a direct relationship between membrane-fluidity modification and enzyme inhibition. In this connection, Mazzanti *et al.* (1986), investigating the effect of the general anaesthetic ketamine on rat synaptosomal AChE, demonstrated that synaptic membranes from anaesthetized animals displayed both an increased fluidity and a decreased enzymic activity (consistent with mixed inhibition kinetics) with respect to membranes prepared from control animals. Those authors suggested that changes in AChE activity could arise from the effect of

ketamine on membrane fluidity; however, the possibility was also considered that the two effects could arise independently. Our results, relating to the effect of lidocaine and imipramine on AChE activity after membrane solubilization with Triton X-100, suggest that the latter possibility could be true for the above-mentioned inhibitors. The evidence that the inhibitory effect of lidocaine and imipramine is fully retained after membrane solubilization, at Triton X-100 concentrations immediately above its c.m.c., calls for a direct interaction of the two inhibitors with the enzyme; on the other hand, the partial loss of inhibitory potency of imipramine and lidocaine which is observed as the Triton X-100 concentration is increased well above its c.m.c. could be explained in terms of a partial amphiphile partition in detergent micelles and, in turn, of a reduced effective concentration of the two inhibitors in the aqueous phase.

As far as the competitive inhibitors procaine and tetracaine are concerned, the data presented indicate that they affect AChE activity at concentrations at which they do not affect membrane fluidity, ruling out the possibility that competitive inhibition may arise from a conformational change of the protein driven by a modification of the physical properties of the lipid environment.

It is interesting to compare our results with those of Sidek *et al.* (1984) who, studying the rat brain synaptosomal enzyme, reported that the inhibitory potency of amphiphiles displaying mixed inhibition kinetics (lidocaine, dibucaine and chlorpromazine) is linearly related to their octanol/water partition coefficient, whereas anaesthetics displaying competitive inhibition kinetics (procaine and tetracaine) are stronger inhibitors than expected on the basis of their partition coefficients as compared with those of the other three compounds. Those authors, who did not take into account the possibility that the drug-inhibitory effect could be mediated by perturbation of the physical state of the lipid environment, concluded that the ester anaesthetics procaine and tetracaine bind to the enzyme active site, whereas lidocaine, dibucaine and chlorpromazine bind to a peripheral 'modulatory' site which is not highly discriminatory with respect to ligand size and shape. Indeed, the present data point to a direct interaction of the investigated amphiphilic drugs with human erythrocyte AChE not only when their effect is consistent with competitive, but also with mixed, inhibition kinetics. Although the possibility must be entertained that molecular species of AChE from various sources may display a different sensitivity to the physical state of the lipid environment in which they are embedded, the existence

of a direct relationship between membrane perturbation and acetylcholinesterase inhibition (or activation) by amphiphilic drugs should be cautiously considered.

This research was partly supported by grants of the Italian Ministry of Education (40% Funds) to P.L.

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Received 21 November 1988/20 February 1989; accepted 28 February 1989