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A study of human erythrocyte acetylcholinesterase inhibition by chlorpromazine

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Membrane-bound acetylcholinesterase (AChE) from the human erythrocyte is inhibited by chloropromazine (CPZ) in a concentration range within which this amphiphilic drug has been demonstrated to interact with erythrocyte membranes, causing a large spectrum of physical and structural effects; membrane solubilization with 0.5% Triton X-100 results in a complete loss of CPZ inhibitory potency. Although these observations might suggest a role for membrane lipid environment in mediating human erythrocyte AChE inhibition, we observed that CPZ retains its full inhibitory effect on the fraction of enzyme (5–6% of total) that is solubilized from erythrocytes upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*; furthermore, Triton X-100 is able to reverse the CPZ effect also in the case of PI-PLC-solubilized enzyme. These results demonstrate unequivocally that CPZ inhibits human erythrocyte AChE through direct molecular interaction. The inhibition kinetics displayed by CPZ on human erythrocyte AChE are dependent on drug concentration: evidence is provided that this phenomenon may be related to formation of CPZ micellar aggregates.

INTRODUCTION

The activity of membrane-bound acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) (Ott, 1985) from both mammalian erythrocytes and nervous tissue is sensitive to a number of amphiphilic molecules of pharmacological interest, which also affect membrane structural and physical properties (Sidek *et al.*, 1984; Deliconstantinos & Tsakiris, 1985; Mazzanti *et al.*, 1986; Spinedi *et al.*, 1989): this circumstance could be suggestive of a role of the membrane lipid environment in mediating amphiphile effects. Previous investigations carried out with the human erythrocyte enzyme, both in the native state and after detergent solubilization, suggested that human erythrocyte AChE activity is not sensitive to changes of membrane physical state (Spinedi *et al.*, 1989); it is evident, on the other hand, that unequivocal assessment of the lack of any role for the lipid environment in modulating enzyme activity and, in particular, in mediating amphiphile effects may only be achieved by using a detergent- and lipid-free system.

AChE solubilization from a variety of membranes can be obtained by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment (Futerman *et al.*, 1985). Owing to covalent modification of the inositol ring (Roberts *et al.*, 1988), human erythrocyte AChE is largely resistant to PI-PLC hydrolysis; a small fraction of the enzyme, however, can be solubilized by this treatment (Futerman *et al.*, 1985). A parallel study was carried out on this fraction as well as on the native enzyme to investigate the mechanism by which the cationic amphiphile chlorpromazine (CPZ) affects enzyme activity.

EXPERIMENTAL

Erythrocyte membranes were prepared as described by Steck (1974). AChE activity was determined as described by Ellman *et al.* (1961) at 37 °C in 25 mM-Tris/HCl buffer/100 mM-NaCl (pH 7.4), with 0.5 mM-acetylthiocholine as substrate. For AChE solubilization, erythrocyte membranes (5 mg of protein/ml) were incubated for 1 h at 37 °C in the above-mentioned buffer in the presence of 4.0 units of PI-PLC from *Bacillus thuringiensis* (obtained through the courtesy of Dr. M. G. Low, Columbia University)/ml. After phospholipase treatment, membranes were centrifuged at 25000 *g* for 1 h; only a small fraction of the total enzymic activity is released by this treatment (5–6% under our conditions). In order to avoid possible contamination by uncleaved enzyme bound to membrane vesicles, the supernatant after PI-PLC digestion was routinely filtered through an Ultrogel AcA22 (LKB) column (60000–1000000 Da fractionation range), and experiments were carried out on the activity eluted within the column volume.

Stock solutions (20 mM) of CPZ hydrochloride (Sigma) were freshly prepared; either native or PI-PLC-solubilized AChE (routinely 100 m-units/ml) were preincubated for 10 min at 37 °C with CPZ before carrying out activity measurements. Absorption spectra of CPZ were recorded by a Perkin–Elmer Lambda 9 spectrophotometer; fluorescence measurements were performed with a Perkin–Elmer LS-5 luminescence spectrometer. Measurements were carried out at 37 °C, and fluorescence values were corrected for sample absorption as described by Lakowicz (1983).

RESULTS AND DISCUSSION

The effects of CPZ on the activity of human erythrocyte AChE in its native state, as well as after solubilization with 0.5% Triton X-100, are shown in Fig. 1. Native enzymic activity was inhibited in a micromolar concentration range within which CPZ has been reported to be non-lytic for intact erythrocytes and to enhance the motional freedom of membrane-inserted e.s.r. probes (Minetti & Di Stasi, 1987); enzyme solubilization with 0.5% Triton X-100 and enzyme-activity measurement in the presence of the same detergent concentration resulted in an almost complete loss of CPZ inhibitory potency.

In order to assess whether the membrane lipid environment had any role in mediating the inhibitory effect of CPZ, we

Abbreviations used: AChE, acetylcholinesterase; CPZ, chlorpromazine; PI-PLC, phosphatidylinositol-specific phospholipase C; c.m.c., critical micellar concentration.

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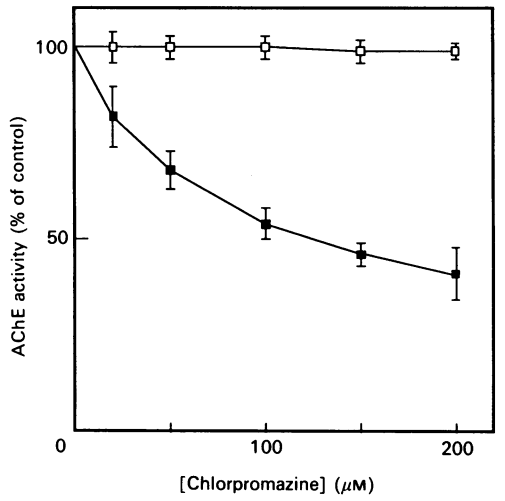

Fig. 1. Effect of CPZ on the activity of human erythrocyte AChE in the native state (■) and after solubilization with 0.5% Triton X-100 (□)

CPZ concentration causing 50% inhibition of native enzymic activity was estimated as about 120 μM. In the absence of CPZ (control), no change in human erythrocyte AChE specific activity was observed after membrane solubilization with 0.5% Triton X-100 in comparison with the native enzyme. Results are means ± S.D. of five independent experiments.

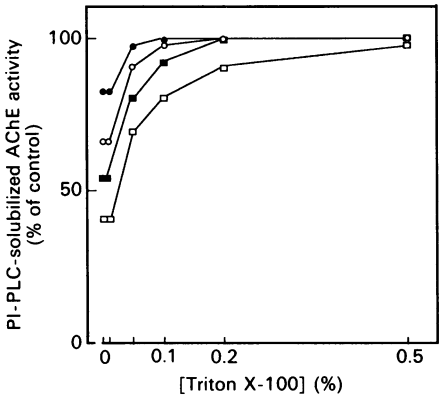

Fig. 2. Effect of CPZ on the activity of PI-PLC-solubilized human erythrocyte AChE in the absence or presence of Triton X-100 at different concentrations

Results are means of five independent experiments; the S.D. never exceeded 10% of mean values. [CPZ]: ●, 20 μM; ○, 50 μM; ■, 100 μM; □, 200 μM.

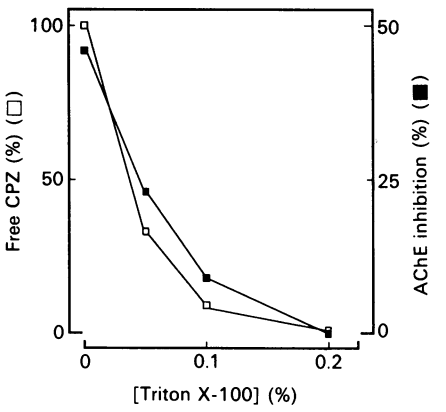

Fig. 3 Correlation between loss of CPZ inhibitory potency and amphiphile partition into Triton X-100 micelles

CPZ (100 μM) was mixed with the indicated concentrations of Triton X-100, and the fractions of amphiphile inserting into detergent micelles and remaining in the aqueous phase were determined by Sephadex G-15 gel filtration, as described in the text. Results are from a representative experiment repeated three times.

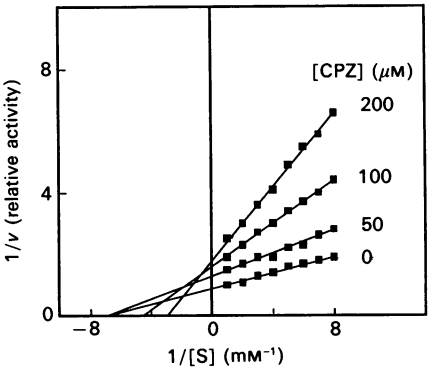

Fig. 4. Inhibition kinetics of CPZ at various concentrations on the activity of PI-PLC-solubilized human erythrocyte AChE

Each point in the Lineweaver–Burk plots represents the average value of duplicate determinations from a representative experiment repeated three times. Similar results were obtained with the enzyme in the native state. In the absence of CPZ a K_m value of 0.14 mM was calculated, which remained unchanged at 50 μM-CPZ and rose to 0.22 and 0.32 mM in the presence of 100 μM- and 200 μM-CPZ respectively. $V_{max.}$ at 50 μM, 100 μM- and 200 μM-CPZ was 69, 57 and 50% respectively of the value in the absence of CPZ.

investigated the action of this amphiphilic drug on the fraction of human erythrocyte AChE which is released upon membrane treatment with PI-PLC from *Bacillus thuringiensis*, testing, in addition, whether even in this case Triton X-100 was able to reverse the inhibitory effect of CPZ. Fig. 2 shows that, in the absence of detergent, CPZ at concentrations of 20, 50, 100 and 200 μM, diminished PI-PLC-solubilized human erythrocyte AChE activity to about 81, 69, 53 and 40% respectively; these values, as compared with those obtained for the native enzyme (Fig. 1), indicate that the effectiveness of CPZ in inhibiting human erythrocyte AChE activity does not depend on whether the enzyme is in its native state or solubilized by PI-PLC treatment. Fig. 2 also shows that Triton X-100, at concentrations below 0.02%, i.e. below its critical micellar concentration (c.m.c.) (Wiedmer *et al.*, 1979), had no effect in decreasing inhibition by CPZ of PI-PLC-solubilized human erythrocyte AChE; as detergent concentration was raised above the c.m.c., however, a dramatic decrease of CPZ inhibitory potency was observed, which, as with the native enzyme, was almost complete at 0.5% Triton X-100 for all the drug concentrations tested.

The possibility was investigated that the loss of CPZ inhibitory potency was due to amphiphile partition into detergent micelles, and hence to a decrease of effective drug concentration in the aqueous phase. In preliminary experiments we observed that CPZ is strongly retained when filtered through a Sephadex G-15 (Pharmacia) column, but can be easily eluted when forming mixed micelles with Triton X-100. Samples (1 ml) containing 100 μM-CPZ as well as increasing concentrations of Triton X-100 were applied on to a 0.4 ml Sephadex G-15 column, previously packed by centrifugation at 1600 *g* for 5 min to minimize the void volume, and filtered by centrifugation at 1600 *g* for 5 min. The eluate was collected into centrifuge tubes, and the A_{307} was

measured to determine the fraction of CPZ eluted with Triton X-100, and hence the drug fraction present in free form (i.e. the fraction bound to Sephadex G-15). Fig. 3 shows that the amount of CPZ remaining free after addition of increasing concentrations of Triton X-100 closely correlates with the expected degrees of enzyme inhibition, as derived from data reported in Fig. 1.

Taken together, these results demonstrate that the inhibitory effect of CPZ on native human erythrocyte AChE is not modified after membrane solubilization by PI-PLC, thus unequivocally confirming that AChE inhibition by amphiphilic drugs is not a membrane-mediated phenomenon. The use of PI-PLC-solubilized human erythrocyte AChE also allowed an unequivocal demonstration that the loss of inhibitory potency displayed by CPZ in the presence of Triton X-100 is due to its partition into detergent micelles.

Some aspects of CPZ interaction with PI-PLC-solubilized human erythrocyte AChE were investigated. Fig. 4 shows the inhibition kinetics of CPZ, at various concentrations, on PI-PLC-solubilized human erythrocyte AChE. At low CPZ concentrations, i.e. up to 50 μM, human erythrocyte AChE was non-competitively inhibited, whereas at higher drug concentrations the inhibition kinetics turned to a mixed type. A study on the native enzyme gave similar results (not shown). Interestingly, results by Luxnat & Galla (1986), supported by data by Zachowski & Durand (1988), pointed to a value of 30–50 μM for CPZ c.m.c. in a buffer system similar to that employed in our study; we therefore investigated whether, in our system also, formation of CPZ micelles occurred within the above-mentioned drug-concentration range. Fluorescence of CPZ at various concentrations was recorded at 450 nm after excitation at 307 nm and corrected for sample absorption; relative quantum yields were then calculated as the ratios of absorption-corrected fluorescence to absorption, for each individual drug concentration. It was observed that CPZ quantum yield was fairly constant up to about 30 μM-CPZ, but abruptly decreased at higher drug concentrations, a phenomenon possibly due to fluorescence quenching by dye aggregation (results not shown). To explain the concentration-dependent inhibition kinetics of CPZ on human erythrocyte AChE activity, we propose that CPZ, at concentrations below its c.m.c., binds to an allosteric site, producing non-competitive inhibition, whereas when present as micellar aggregates, thus exposing polar groups, the drug interacts with another site, possibly the active site, producing the observed competitive-inhibition component.

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