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PHENOTHIAZINES INHIBIT ACETYLCHOLINESTERASE BY CONCENTRATION-DEPENDENT-TYPE KINETICS

A STUDY WITH TRIFLUOPERAZINE AND PERPHENAZINE

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Abstract—The properties of perphenazine (PPZ) and trifluoperazine (TFP) as fluorescent dyes were exploited to calculate their critical micellar concentrations. The relative fluorescence quantum yield of the two amphiphiles was dependent on their concentration, abruptly decreasing above 30–40 μM PPZ and 20–30 μM TFP. Evidence is presented that this phenomenon is driven by the formation of non-fluorescent drug aggregates. The type of inhibition kinetics displayed by PPZ and TFP on human erythrocyte acetylcholinesterase (AChE) was also dependent on drug concentration, turning from non-competitive to a "mixed" inhibition type at concentrations at which PPZ and TFP were demonstrated to undergo micelle formation. Results support the notion that phenothiazines may interact with AChE both as monomers and micellar aggregates, producing different inhibitory effects.

Phenothiazines are cationic amphiphilic drugs with neuroleptic properties exerting a wide range of effects on cell metabolism [1–6] as well as on membrane structural and physical characteristics [7–11]. Owing to their amphiphilic nature, these molecules are expected to undergo aggregation at a certain critical micellar concentration (c.m.c.‡), when present in aqueous solution. Chlorpromazine (CPZ) has been reported to undergo micellar aggregation at a concentration of 30–50 μM [12]. We have demonstrated recently that CPZ inhibits human erythrocyte acetylcholinesterase (EC 3.1.1.7; AChE) activity by direct molecular interaction and we have observed, incidentally, that the type of inhibition kinetics displayed by this drug is concentration-dependent, turning from non-competitive to "mixed" type at an amphiphile concentration of around 50 μM [13]. This observation raised the possibility that CPZ may interact with the enzyme as both free monomers and micellar aggregates, resulting in different inhibitory effects: thus, we were prompted to investigate further the phenomenon, extending our observation to other phenothiazines. Unfortunately, determination of c.m.c. for phenothiazines other than CPZ has received scarce attention, albeit the biochemical properties of these drugs have been tested over a broad concentration range. In the present study we attempted to calculate the c.m.c. for perphenazine (PPZ) and trifluoperazine (TFP) exploiting the properties of these two drugs as fluorescent dyes. We then investigated the effect of these two drugs on the activity of human erythrocyte AChE in order to clarify whether the aggregation state of phenothiazines is indeed critical in determining the type of interaction with the enzyme.

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‡ Abbreviations: PPZ, perphenazine; TFP, trifluoperazine; CPZ, chlorpromazine; AChE, acetylcholinesterase; c.m.c., critical micellar concentration.

MATERIALS AND METHODS

Perphenazine hydrochloride and trifluoperazine were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Absorption spectra of PPZ and TFP, dissolved in 25 mM Tris–HCl buffer/100 mM NaCl (pH 7.4), were recorded by a Perkin–Elmer Lambda 9 spectrophotometer. Fluorescence measurements were performed by a Perkin–Elmer LS-5 luminescence spectrometer, using a bandpass width of 5 nm for excitation and 10 nm for emission. Fluorescence values were corrected for sample absorption as described [14].

Red cell membranes were prepared as described previously [15]. AChE activity was determined at 37° according to Ellman *et al.* [16]. The assay mixture (3 mL) contained 0.125 mM 5,5′-dithionitrobenzoic acid and 10 μg membrane protein in 25 mM Tris–HCl buffer/100 mM NaCl (pH 7.4); for routine assay 0.5 mM acetylthiocholine was used, whereas enzyme K_m and V_{max} were determined from Lineweaver–Burk plots using substrate concentrations from 0.5 to 0.1 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 absorbance at 412 nm recorded. Blanks were also run for each substrate concentration tested.

RESULTS AND DISCUSSION

The absorption spectra of PPZ and TFP are

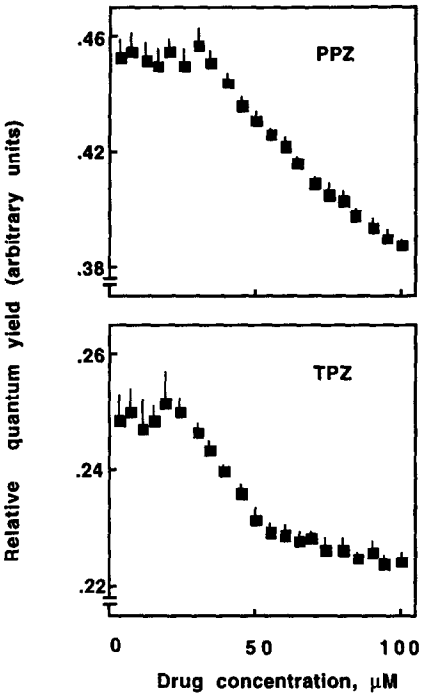

Fig. 1. Concentration dependence of PPZ and TFP relative quantum yield. Fluorescence of PPZ (upper panel) and TFP (lower panel) at various concentrations was recorded at 37° and corrected for sample absorption as described in the text. Relative quantum yield was then calculated for each drug concentration as the ratio of absorption-corrected fluorescence to absorption. Each point is the mean ± SD of data from four different experiments.

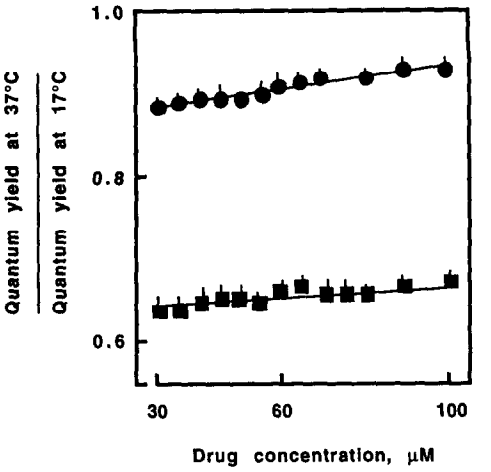

Fig. 2. Temperature dependence of PPZ and TFP fluorescence at various drug concentrations. Fluorescence of PPZ (■) and TFP (●) at various concentrations was recorded at 37° and 17° and corrected for sample absorption as described in the text. Each point is the mean ± SD of data from four different experiments.

characterized by a major band of absorption peaking at 254 nm and a minor and broad band peaking at 307 nm. At wavelengths higher than 400 nm absorption is negligible for drug concentrations below 100 μM. PPZ and TFP also behave as fluorescent dyes, with an emission maximum at 455 nm. In the drug concentration ranges employed we observed that the main features of the spectra remained unchanged (not shown); however, while absorption at 307 nm increased linearly with drug concentration at least up to 100 μM PPZ and TFP (not shown), the absorbance-corrected fluorescence, measured after excitation at 307 nm and emission at 455 nm, did not. In Fig. 1, in fact, it can be observed that the relative fluorescence quantum yield of the two amphiphiles (calculated as the ratio of fluorescence to absorption) is dependent on their concentration. In the case of PPZ, quantum yield held fairly constant up to a drug concentration of about 30–40 μM, linearly decreasing at higher amphiphile concentrations. For TFP, quantum yield decrease was already evident at concentrations higher than 20–30 μM and was particularly pronounced in a narrow drug concentration range, i.e. up to 50 μM. The phenomenon can not be explained in terms of energy transfer between drug molecules, since, as observed above, the fluorescence emission band of phenothiazines overlaps a wavelength range in which light absorption by drugs is negligible.

The most likely explanation for the observed concentration-dependent decrease in fluorescence quantum yield is that phenothiazines quench their own fluorescence. This phenomenon could occur either through formation of non-fluorescent aggregates or by a process involving molecular collision. To discriminate between these two possibilities, concentration-dependent changes in relative quantum yields were monitored at 17°: if quenching is due to a collisional process it should be attenuated at lower temperatures [17]. Figure 2 shows that after a temperature shift from 37° to 17° the phenomenon was not attenuated, it was in fact somewhat more pronounced. This circumstance supports the notion that the concentration-dependent decrease in fluorescence quenching may be due to amphiphile aggregation.

It was of interest to correlate the physical aspects of drug behaviour with the drug effects on human erythrocyte AChE activity. In a set of experiments which are not reported we observed that TFP and PPZ inhibit AChE activity within a micromolar concentration range: drug concentration causing 50% enzyme inhibition, as assessed in the presence of 0.5 mM substrate, was around 60 μM for TFP and 100 μM for PPZ. The inhibition kinetics displayed by the two drugs at various concentrations were investigated; the results are reported in Fig. 3. It can be seen that 20 μM TFP and 40 μM PPZ brought about non-competitive inhibition kinetics; in fact, over a 20% decrease in enzyme V_{max} was observed, but no change in enzyme K_m took place. At higher drug concentrations, namely above the concentrations indicated by fluorescence studies as those at which the two drugs start to undergo aggregation, a dramatic increase in enzyme K_m was

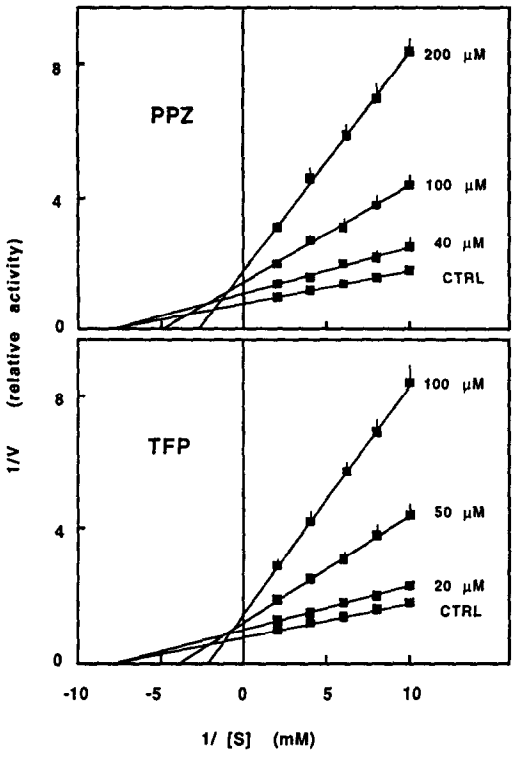

Fig. 3. Inhibition kinetics of PPZ and TFP at various concentrations on the activity of human erythrocyte AChE. AChE activity was mesured at 37° in the presence of various concentrations of PPZ (upper panel) or TFP (lower panel) as described in the text. Each point in the Lineweaver–Burk plots is the mean ± SD of data from three different experiments; bars are not shown when they fall within the symbol. K_m was 0.13 mM in controls as well as in the presence of 40 μM PPZ and 20 μM TFP. In the presence of 100 μM PPZ, 200 μM PPZ, 50 μM TFP and 100 μM TFP, K_m increased to 0.25, 0.45, 0.20 and 0.37 mM, respectively. V_{max} at 40, 100 and 200 μM PPZ was 74%, 55% and 45% of control, respectively; V_{max} at 20, 50 and 100 μM TFP was 78%, 63% and 52% of control, respectively.

observed, yielding "mixed" inhibition kinetics, with intersections among slopes progressively approaching the *y* axis. Various amphiphilic drugs have been reported by others [18] and by us [19] to affect AChE activity through "mixed" inhibition kinetics. Although this mode of action has been ascribed to inhibitor binding to a peripheral modulatory site which is not highly discriminatory with respect to ligand size and shape, the dependence of enzyme inhibition kinetics on drug concentration was not investigated. Data herein reported provide circumstantial evidence that, at least for phenothiazines, the phenomenon may be more complex and may involve enzyme interaction with amphiphiles both as monomers and aggregates. We have suggested recently that phenothiazines in the monomeric state may bind to an allosteric site producing non-competitive inhibition, whereas when present as micellar aggregates, thus exposing the polar groups, they may interact with another site, possibly the active site, leading to the appearance of a competitive inhibition component [13]. Indeed, the latter hypothesis no longer holds since the recent elucidation of the three-dimensional structure of AChE from *Torpedo californica* [20]; in fact, it appears that the enzyme active site is located at the bottom of a deep and narrow hydrophobic "gorge" through which an aggregate with polar characteristics could hardly have access. An alternative explanation for the phenomenon could be that amphiphile aggregates block the opening of the "gorge" thus impairing substrate access to the active site. Further studies are required in order to verify this hypothesis.

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