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SHORT COMMUNICATIONS

Highly cooperative inhibition of acetylcholinesterase by pentachlorophenol in human erythrocytes

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Abstract—Pentachlorophenol (PCP) inhibited acetylcholinesterase (AchE) activity in human erythrocyte membranes with high cooperativity. The Hill coefficient for the inhibition was 4–5 in "untreated" membranes. Differences in the temperature (13, 25 and 37°) or treatment with 1% Triton X-100 did not clearly affect the cooperativity which, however, increased after the erythrocyte membranes were treated with 2-mercaptoethanol and iodoacetatic acid, suggesting that higher cooperativity in the inhibition of AchE by PCP may reflect conformational changes of AchE. Thus, PCP may be useful for the study of AchE in human erythrocytes.

Erythrocytes, especially human erythrocytes, have high acetylcholinesterase (AchE*) activity (acetylcholine hydrolase, EC 3.1.1.7). Although its physiological function is unknown, AchE in erythrocytes has proved to be a valuable model system because it is easily available and it shows great similarities to a number of neutral enzymes [1]. In addition, the enzyme appears to be a useful probe to study erythrocyte membranes [1, 2]. In the case of rat erythrocytes, AchE is inhibited cooperatively by fluoride and it could be used to examine the enzyme and its environment in the membrane [2]. In human erythrocytes, however, such cooperative inhibition was not observed [1, 3]. To our knowledge, no chemicals have been found that can inhibit AchE with cooperativity in human erythrocytes. Here, we present results showing that pentachlorophenol (PCP) inhibits human erythrocyte AchE with high cooperativity.

Materials and Methods

Chemicals. PCP was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), [^{3}H]Acetylcholine from Amersham (Tokyo, Japan), acetylthiocholine iodide from Wako Pure Chemicals (Osaka, Japan) and scintillator (Scintisol AM-1) from Dojin (Kumamoto, Japan). All other chemicals were reagent grade.

Erythrocyte membrane. Blood was obtained from healthy males and erythrocyte membrane (white ghost) was prepared as described [3] and kept at −80°. The protein concentration of the membrane suspension was determined by the method of Lowry *et al.* [4].

Treatment of the membrane. Reduction and alkylation of the membrane (treatment with 2-mercaptoethanol and iodoacetic acid) were done as described by Ott *et al.* [5] except that 10 mM Tris–HCl pH 7.4 was used to wash the treated membrane. When treated with Triton X-100, the membrane suspension was diluted (30–40 times) with 0.1 M potassium phosphate buffer pH 7.4 and Triton X-100 was added up to 1%, and the protein concentration was made 0.1 mg/mL. After left on ice overnight and centrifuged for 2 hr at 4° and 100,000 *g*, the supernatant was used. When comparing effects of these treatments, sham treatments (sedimentation and washing of the membrane after incubation without 2-mercaptoethanol and iodoacetate) were carried out at the same time.

Enzyme assay. AchE activity was measured using radiometric [6] or colorimetric [7] methods with slight modification. In all experiments, PCP was first dissolved in ethanol and added to the reaction mixture containing the enzyme and incubated for 30 min before the substrate was added.

For the radiometric measurement, the pre-incubation mixture contained 0.165 mL of 0.1 M potassium phosphate buffer pH 7.4, 0.005 mL of ethanol with various amount of PCP and 0.025 mL of the enzyme source. After this mixture was incubated with gentle shaking at 25°, 0.005 mL of [^{3}H]acetylcholine (100 mM) (or 0.003–0.02 mL of the substrate diluted five times) was added, and incubated for a further 15 min. The reaction was terminated by adding 0.1 mL of the "stopping mixture" which contained 1.5 M chloroacetic acid, 0.75 M NaOH and 3 M NaCl. After 6 mL of scintillation mixture (Scintisol AM-1 containing 10% isoamyl alcohol) was added, the radioactivity was determined in a Beckman LSC-7000 counter.

When using the colorimetric method, the pre-incubation mixture contained 2.9 mL of 0.1 M potassium phosphate buffer pH 7.4, 0.1 mL of the enzyme source and 0.05 mL of ethanol with or without PCP. After the preincubation, 0.03 mL of 25 mM dithiobis-nitrobenzoic acid (in 1% sodium citrate) and 0.05 mL of 40 mM acetylthiocholine were added, and the changes of the absorbance at 412 nm were recorded using a Hitachi 557 or a Beckman DU-7HS spectrophotometer. The temperature of the cuvette holder was controlled with a circulation water bath (Lauda RM3 or RM6).

All radiometric measurements were done in duplicate and each experiment repeated at least twice.

Hill plot and statistical analysis. Hill plot analysis was done as described previously [3]. When necessary, using the REG procedure in SAS/STAT (release 6.03, SAS Institute, Cary, NC), the slope was calculated (regression analysis) and statistical differences of the slopes examined (analysis of covariance).

Results

AchE activity was inhibited by PCP and the inhibition was apparently non-competitive (Fig. 1). When the concentration of PCP was varied (at 25°), a sigmoidal dose–responsive curve was obtained and the Hill coefficient for the inhibition, calculated with statistical software, was 4.7 ± 0.5 (mean ± SD, three experiments) (Fig. 2).

In all the following experiments, AchE activities were measured colorimetrically using acetylthiocholine as substrate. On varying the temperature over 7–42° (without ethanol and PCP), a "break" was observed at 19° on the

* Abbreviations: AchE, acetylcholinesterase; PCP, pentachlorophenol.

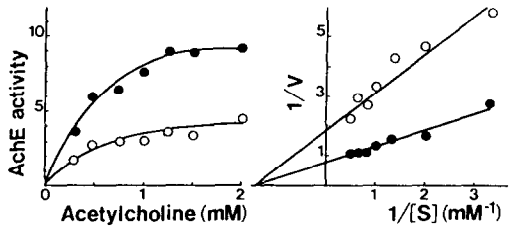

Fig. 1. AchE activity in human erythrocyte membrane in the presence of 0.75 mM PCP (○) and the absence of PCP (●). The concentration of [^{3}H]acetylcholine was varied and the enzyme activity was measured radiometrically. The activity is expressed in an arbitrary unit (cpm/5 μg membrane protein/15 min at 25°). Lineweaver–Burk plot is shown on the right. The K_m for AchE and K_i for PCP obtained from this graph are 0.71 and 0.56 mM, respectively.

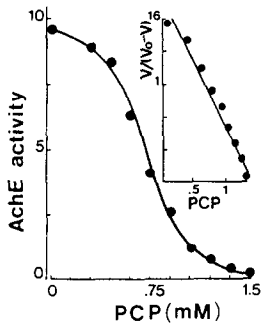

Fig. 2. Effects of PCP on AchE activity in human erythrocyte membrane. The enzyme activity was measured and expressed in the same way as in Fig. 1 except that 2.5 mM acetylcholine was used. Hill plot of the same data is shown in the inset. V and V_0 indicate the enzyme activity with and without PCP, respectively. The Hill coefficient obtained from this graph (and by calculation on SAS statistical software) was 4.1.

Arrhenius plot (data not shown). The effects of the temperature, therefore, were examined at 13, 25 and 37°. The dose–response curve was sigmoidal at any temperature examined and the Hill coefficient did not clearly change (data not shown).

In the experiment comparing the membranes treated in three ways, treatment with 2-mercaptoethanol and iodoacetic acid significantly increased the cooperativity for the inhibition of AchE activity by PCP (Fig. 3), while the increase by Triton X-100 was not statistically significant.

Discussion

PCP has been known as a potent uncoupler of oxidative phosphorylation in mitochondria [8]. It has also been shown that PCP decreases fluidity of plasma membrane [9]. Its effects, however, on enzymes in erythrocyte membrane have not been reported. In our previous study [10], we noted the PCP can hemolyse human erythrocytes and that the erythrocyte membrane has multiple binding sites for PCP with different affinities. Hence, it seemed

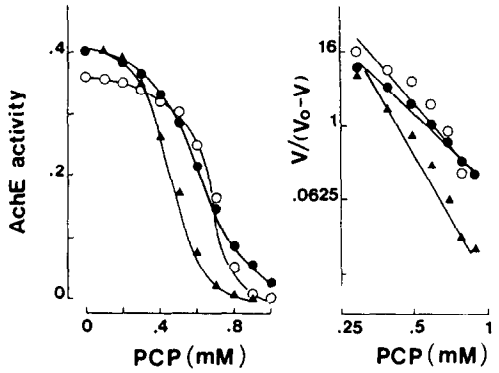

Fig. 3. Effects of PCP on AchE activity in human erythrocyte membrane treated with: sham treatment (●), 1% Triton X-100 (○), and 2-mercaptoethanol and iodoacetate (▲). The enzyme activity was measured colorimetrically (at 37°) and expressed in an arbitrary unit (increase of the absorbance at 412 nm for 5 min). The activity after sham treatment was 0.90 μmol acetylthiocholine hydrolysed/min/mg protein assuming the complete recovery of the protein. Hill plot of the same data is shown on the right. The Hill coefficients (calculated on SAS statistical software) were: 3.2 (●), 4.5 (○) and 6.6 (▲). The difference between the sham treatment and that with 2-mercaptoethanol and iodoacetic acid was statistically significant ($P < 0.001$) (analysis of covariance), while the difference between the sham treatment and that with 1% Triton X-100 was not ($0.1 < P < 0.2$).

that PCP may affect not only the lipid bilayer but also the membrane-bound enzyme(s) in erythrocytes.

Indeed, as shown in the present experiments, PCP inhibited erythrocyte AchE activity non-competitively. Furthermore, the dose–response curve was sigmoidal either when the natural or synthetic substrate was used. Thus, it is obvious that PCP has the potential to inhibit AchE in human erythrocytes cooperatively (and allosterically).

The Hill coefficient for the inhibition of AchE by PCP was not clearly altered by the temperature, whether it was above or below the "break" in the Arrhenius plot, or by treatment of the erythrocyte membrane with 1% Triton X-100. This indicates that conformational changes of the enzyme caused by the different temperatures (whether it was a direct effect on the enzyme or through alterations of the fluidity in the hydrophobic environment) or by solubilization of the membrane do not markedly affect the cooperativity of the inhibition by PCP. However, reduction and alkylation of the membrane increased the cooperativity. The mechanism of this increase is not clear. AchE in erythrocyte membrane is a dimer whose identical subunits are linked by disulfide bridges and anchored in the membrane by a glycoinositol phospholipid at the C-terminus of each peptide [11]. Hence, a treatment that disrupts the disulfide bridges may allow greater changes of the conformation of the enzyme, and this might be the basis of the increased cooperativity of the inhibition. To clarify these, further experiments are necessary using purified AchE.

Thus, while its mechanism has not been fully elucidated, PCP exerts cooperative inhibition of human erythrocyte AchE, and the cooperativity is even higher than that of rat erythrocytes by fluoride in which the Hill coefficient for the inhibition was approximately 2 [2]. Therefore, PCP may be used to study properties of AchE (and possibly its

environment) in human erythrocytes. Moreover, since AchE in synaptosomes from rat brain was also inhibited by PCP with a high cooperativity (Igisu, unpublished), usefulness of PCP for the study of AchE may not be limited to human erythrocytes.

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