Protective Effects of Neutral Amino Acids against Amphipathic Drug-Induced Hemolysis

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Some neutral amino acids were compared for their anti-hemolytic effects with sugars which are well-known colloid-osmotic protectants. The kinetic studies in isotonic suspensions of erythrocytes indicated that the hemolysis induced by the amphipathic drug chlorpromazine (CPZ) or flufenamic acid (FA) was retarded by addition of sugars, and the degree of the anti-hemolytic effect increased with increases in molecular size. Phenylalanine (Phe), the largest among the amino acids tested, showed the greatest inhibitory effect on CPZ-induced hemolysis, but not on FA-induced hemolysis. This demonstrated that the anti-hemolytic effects of amino acids were not the result of colloid-osmotic protection. Hemolytic actions of amino acids were also examined to determine their interaction with the erythrocyte membrane, and the mechanism of their inhibitory effects against amphipathic drug-induced hemolysis was discussed.

Key words chlorpromazine; flufenamic acid; hemolysis; neutral amino acid; colloid-osmotic protectant; sugar

It is known that amphipathic drugs such as chlorpromazine (CPZ) elicit detergent-like hemolysis.¹⁾ Lieber *et al.*²⁾ reported that CPZ induced formation of holes with a diameter of approximately 14 Å in cell membranes causing colloid-osmotic hemolysis. The hole size was estimated from the rate constants for uptake of probes such as mannitol and sucrose to which the cells were initially impermeable. In addition, nonelectrolytes of varying size such as raffinose, dextran and polyethylene glycol are well known protectants against colloid-osmotic hemolysis because they counterbalance the osmotic pressure of the intracellular macromolecules (hemoglobin, *etc.*)³⁾

We have studied the membrane-stabilizing action of neutral amino acids by evaluating their anti-hemolytic activities against CPZ-induced hemolysis, and reported that the addition of amino acids reduced the membrane affinity of CPZ and inhibited hemolysis.⁴⁾ Phenylalanine (Phe), the largest of the amino acids we tested, showed the greatest anti-hemolytic action,^{4a)} suggesting that this hydrophobic amino acid might interact with erythrocyte membranes strongly and reduce the membrane affinity of CPZ. However, it was suspected that the remarkable anti-hemolytic action of Phe may be due to a colloid-osmotic protective effect. In this study, we compared the anti-hemolytic effects of amino acids with those of sugars to clarify the mechanism of the membrane protecting effects of amino acids. An anionic amphipath, flufenamic acid (FA), was also used to induce colloid-osmotic hemolysis.

MATERIALS AND METHODS

Materials CPZ·HCl was purchased from Wako Pure Chemical Industries, Ltd. FA, L-glycine, L-alanine, L-valine, L-leucine, L-Phe, mannitol, sucrose and raffinose were purchased from Nacalai Tesque Inc. These chemicals and other reagents used in this study were all of analytical grade.

Preparation of Erythrocyte Suspension Fresh blood

from healthy rabbits was drawn by heart puncture into heparinized tubes and red blood cells were separated by centrifugation at 2000 rpm for 3 min. The cells were then washed three times with saline and then once with 10 mM phosphate buffered saline (PBS) (pH = 7.0). Two-milliliter aliquots of the cells were then suspended in PBS to a final volume of 50 ml (4% erythrocyte suspension).

Hemolysis Measurement The kinetic parameters (lag time and half-life for hemolysis) were determined as described previously.⁴⁾ Preincubated (37 °C, 10 min) 0.66 mM CPZ solutions (pH = 7.0) with or without 44.4 mM anti-hemolytic agent (amino acid or sugar) and osmotically equivalent to PBS (4.5 ml) were mixed with aliquots (0.5 ml) of the cell suspensions. The mixtures were incubated for an appropriate time at 37 °C with shaking and then centrifuged at 3000 rpm for 1 min to separate unhemolyzed cells. The absorbance of the supernatant was measured at 543 nm (A_{sample}) and the percent hemolysis ($H\%$) of each sample was calculated using the following equation:

$$H\% = A_{\text{sample}}/A_{100} \times 100$$

where A_{100} is the absorbance of 100% hemolyzed cells, *i.e.* cells incubated in distilled water. When hemolysis was evoked by FA, the solution containing 1.11 mM FA was used in place of CPZ. The calculated final concentrations of CPZ, FA and anti-hemolytic agents in this experiment were 0.6, 1.0 and 40 mM, respectively.

Hemolytic activities of amino acids were examined as described above except that the test solutions were prepared with 10 mM TES (*N*-tris(hydroxymethyl)methyl-2-aminoethansulfonic acid) buffer. Preincubated 111 mM amino acid solutions (18 ml) were mixed with aliquots (2 ml) of the cell suspensions. The mixtures were incubated for 60 min at 37 °C with shaking and then centrifuged at 3000 rpm for 1 min to separate unhemolyzed cells. The supernatants were used for hemolytic measurement and phospholipid determination.

Phospholipid Determination The lipid of the super-

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natant was extracted according to the Bligh-Dyer method.⁵ The supernatant (12 ml) and chloroform-methanol (1:2) mixture (45 ml) were mixed in a 100 ml volume separating funnel and well shaken. Fifteen ml of both distilled water and chloroform were added to the mixture and well shaken. The chloroform solution separated and filtrated with exsiccated sodium sulfate was dried to a lipid film in a round bottom flask under reduced pressure in a rotary evaporator. The lipid was resolved into a chloroform-methanol (1:2) mixture in a test tube and dried to a lipid film. The phospholipid was determined according to the method of Bartlett.⁶ The lipid film was resolved in 0.4 ml of 70% perchloric acid and the solution was heated at above 100 °C for more than 1 h until this became clear and uncolored. Aliquots of distilled water (4.2 ml), 5% molibden ammonium solution (0.2 ml) and amidol test solution (0.2 ml) were added to the lipid solution and the mixture was heated at 90 °C for 7 min. A quarter hour after the heating, the absorbance of the solution was measured at 820 nm. The lipid concentration of sample was calculated by using a linear equation between concentration and absorbance (820 nm) obtained with L- α -phosphatidylcholine (Sigma Chemical Co., 99% from egg yolk).

RESULTS

Effects on CPZ-Induced Hemolysis Rabbit erythrocytes treated with 0.6 mM CPZ in the presence or absence

Fig. 1. Typical Time Course of Hemolysis Induced by CPZ in the Presence or Absence of Sugars

Concentrations: rabbit red blood cells, 0.4%; CPZ, 0.6 mM; saccharides, 40 mM. ●, without sugar; ○, with mannitol; ■, with sucrose; □, with raffinose.

Fig. 2. Typical Time Course of Hemolysis Induced by CPZ in the Presence or Absence of Amino Acids

Concentrations: rabbit red blood cells, 0.4%; CPZ, 0.6 mM; amino acids, 40 mM. ●, without amino acid; ○, with Gly; ■, with Ala; □, with Val; ▲, with Leu; △, with Phe.

of 40 mM amino acids or sugars at pH 7.0 were hemolyzed to different degrees. Typical curves of hemolysis are shown in Figs. 1 and 2. As shown in Fig. 1, all sugars tested remarkably retarded both the time at which the hemolysis occurred (lag time) and the rate of CPZ-induced hemolysis. In contrast, some of the amino acids prolonged the lag time but none influenced the hemolytic rate (Fig. 2). From the hemolytic curves shown in these two figures, the lag time and the half-life for hemolysis in the presence or absence of each anti-hemolytic agent were calculated as hemolytic behavior following apparent first order kinetics.⁴ The results are plotted as the molecular weights of agents in Fig. 3 and are summarized in Table 1. The degree of retardation in kinetic parameters for CPZ-induced hemolysis by each agent was expressed as the ratio of the value obtained in the test solution containing the agent to the value in the control solution (without agent). The degree of retardation in lag time was decreased as the molecular size of the agent decreased (raffinose > sucrose > mannitol > Phe > Leu > Val). Ala and Gly did not noticeably retard the lag time. The half-life for hemolysis was not changed significantly by any amino acid tested, in contrast to the remarkable increases by the sugars with

Fig. 3. Plots of the Kinetic Parameters for CPZ-Induced Hemolysis vs. the Molecular Weights of Anti-hemolytic Agents

The kinetic parameters, lag time (●) and half-life (○), were estimated from each hemolytic time-course shown in Fig. 1 or 2.

Table 1. Effects of Anti-hemolytic Agents on Parameters for CPZ-Induced Hemolysis

Agent	Lag time	<Ratio>	Half-life	<Ratio>
None	4.84 ± 1.38	<1.0>	3.67 ± 1.79	<1.0>
Gly	5.95 ± 1.38	<1.2>	4.28 ± 0.26	<1.2>
Ala	6.42 ± 2.82	<1.6>	3.50 ± 0.48	<1.0>
Val	8.07 ± 2.07 ^a	<1.7>	3.89 ± 0.93	<1.1>
Leu	8.09 ± 2.02 ^a	<1.7>	4.12 ± 0.67	<1.1>
Phe	9.64 ± 3.21 ^a	<2.0>	5.76 ± 2.40	<1.6>
Mannitol	10.84 ± 4.19 ^a	<2.2>	7.72 ± 0.09 ^a	<2.1>
Sucrose	40.23 ± 5.59 ^c	<8.3>	22.16 ± 6.48 ^b	<6.0>
Raffinose	89.25 ± 5.62 ^c	<18.4>	103.09 ± 59.67 ^a	<28.1>

Results are shown as means of three experiments ± standard deviation. Concentration: rabbit red blood cells, 0.4%; CPZ, 0.6 mM; anti-hemolytic agent, 40 mM. Significantly different from the values for control (without agent): a) $p < 0.05$; b) $p < 0.005$; c) $p < 0.001$.

increments in the effect with increasing molecular weight.

Effects on FA-Induced Hemolysis Figure 4 and Table 2 show the ability of amino acids to inhibit the colloid-osmotic hemolysis induced by the other amphipathic drug, FA. Degree of retardation of both lag time

Fig. 4. Plots of the Kinetic Parameters for FA-Induced Hemolysis vs. the Molecular Weights of Anti-hemolytic Agents

The kinetic parameters, lag time (●) and half-life (○), were estimated from each hemolytic time-course.

Table 2. Effects of Anti-hemolytic Agents on Parameters for FA-Induced Hemolysis

Agent	Lag time	<Ratio>	Half-life	<Ratio>
None	33.41 ± 5.67	<1.0>	14.70 ± 3.63	<1.0>
Gly	59.32 ± 11.09 ^{a)}	<1.8>	37.49 ± 19.95	<2.6>
Ala	66.67 ± 9.45 ^{b)}	<2.0>	41.82 ± 20.71	<2.8>
Val	66.10 ± 9.35 ^{b)}	<2.0>	28.50 ± 10.33	<1.9>
Leu	59.45 ± 12.77 ^{a)}	<1.8>	24.96 ± 4.16 ^{a)}	<1.7>
Phe	46.36 ± 10.47	<1.4>	17.30 ± 9.05	<1.2>
Mannitol	58.00 ± 1.59	<1.7>	16.59 ± 4.99	<1.1>
Sucrose	68.05 ± 11.73 ^{a)}	<2.0>	36.20 ± 15.04	<2.5>
Raffinose	98.75 ± 6.37 ^{b)}	<3.0>	63.86 ± 23.91	<4.3>

Results are shown as means of three experiments ± standard deviation. Concentration: rabbit red blood cells, 0.4%; FA, 1.0 mM; anti-hemolytic agent, 40 mM. Significantly different from the values for control (without agent): a) $p < 0.05$; b) $p < 0.005$.

Fig. 5. Hemolysis and Leakage of Membrane Lipids Induced by Amino Acids of Various Molecular Weights

Concentrations: rabbit red blood cells, 0.4%; amino acids, 100 mM. H% (●) and phospholipid concentration in the supernatant (○) were determined 60 min after mixing of amino acid with erythrocytes.

and half-life for FA-induced hemolysis increased with increasing molecular size of the sugars added (mannitol < sucrose < raffinose); however, no such relationship to the molecular size of amino acids was observed. This demonstrates that those amino acids could not act as colloid-osmotic protectants.

Hemolytic Action of Amino Acids The hemolytic activities of amino acids were examined, and the addition of 100 mM was found to induce hemolysis (Fig. 5). The degrees of hemolysis observed with Phe or Leu were greater than those observed with Gly or Ala, so that apparently the larger amino acids could more strongly interact with erythrocyte membranes than the smaller ones. Phe also caused phospholipid leakage, suggesting its very strong interaction with the membrane.

DISCUSSION

The sugars tested in this experiment delayed amphipathic drug-induced hemolysis with increasing molecular size (Figs. 3, 4), and evidently counterbalanced the osmotic pressure of hemoglobin across the porous membranes, while no such molecular size dependence of anti-hemolytic effects of amino acids was found (Fig. 4). Thus, the mechanism of the anti-hemolytic actions of amino acids could not be explained by colloid-osmotic protective effects.

Fujii *et al.*⁷⁾ and other researchers⁸⁾ have reported that the shape of erythrocytes changed in the presence of CPZ from discocytes to stomatocytes. Such a shape change was proposed to be due to incorporation of CPZ, a cationic amphipathic drug, into the inner erythrocyte membrane leaflet which is rich in acidic phospholipids.⁹⁾ Further binding of CPZ to the inner leaflet likely causes the shape change from stomatocytes to spherocytes accompanied by endovesiculation of lipids and formation of holes in the membranes. Lieber *et al.*²⁾ reported that CPZ-laden erythrocyte membranes showed increases in thickness of more than two-fold in comparison to controls, and possibly formation of a CPZ-rich zone at the center of the bilayers. Also, we reported previously that the innermost portion of liposome membranes where 16-doxyl stearate was located was extremely perturbed by CPZ, suggesting the entrance of CPZ molecules into the center of the bilayers.¹⁰⁾ Based on these findings, it is probable that CPZ binds to the outer leaflet of erythrocyte membranes, is transmitted through the center of the bilayer and accumulates in the innerleaflet. The binding of CPZ could induce membrane fluidization¹¹⁾ and form holes through which hemoglobin could leak out of the cells.²⁾ FA, an anionic amphipathic drug, has been reported to induce a shape change to echinocyte as a result of its binding to the outer leaflet of erythrocyte membranes.⁷⁾ Its further binding could induce exovesiculation of lipids and form holes in membranes. Thus, the hemolytic potency was considered to be dependent on the membrane binding characteristics of lytic substances.

With respect to the amino acids tested in the present study, the larger ones were more hydrophobic. The 1-octanol-water partition coefficients (log *P*) for Phe, Leu, Val, Ala and Gly were reported to be -1.44, -1.72,

−2.29, −2.77 and −3.00, respectively, and there is a strong correlation between partition coefficients and hydrophobicity of amino acids.¹²⁾ Therefore, it is likely that the larger amino acids incorporate to erythrocyte membranes; indeed, Phe, Lue and Val induced severe hemolysis (Fig. 5), suggesting their strong incorporation to those membranes. Such incorporation of amino acids might shut out amphipathic drugs which would otherwise be incorporated into membranes and induce hemolysis. Among the amino acids we tested, Phe showed the greatest interaction with cell membranes and induced lipid leakage. The degree of hemolytic action of Phe (Fig. 5) was sufficiently weaker than CPZ which caused >90% hemolysis within 10 min after its treatment (Fig. 1), but it was not as weak as FA which took about 60 min to cause >90% hemolysis (data not shown). Taking this finding into consideration, the convex relationship of inhibitory effects of amino acids on FA-induced hemolysis depending on their molecular size (Fig. 4) might be explained as due to the hemolytic activity of Phe being superior to their abilities to prevent entrance of FA to the cell membranes.

CONCLUSION

The present kinetic study of amphipathic drug-induced hemolysis demonstrated that the anti-hemolytic effects of amino acids were not the result of colloid-osmotic protective effects such as those which have been well documented in sugars. Investigation showed the strong interaction of hydrophobic amino acids with erythrocyte

membranes. It is likely that the protective effects of amino acids against amphipathic drug-induced hemolysis result from their binding to the membrane thus blocking access of the drugs.

REFERENCES

- 1) Seeman P., Weinstein J., *Biochem. Pharmacol.*, **15**, 1737—1752 (1966); *idem, ibid.*, **15**, 1753—1766 (1966).
- 2) Lieber M. R., Lange Y., Weinstein R. S., Stech T. L., *J. Biol. Chem.*, **259**, 9225—9234 (1984).
- 3) Thelen P., Deuticke B., *Biochim. Biophys. Acta*, **944**, 285—296 (1988); Katsu T., Ninomiya C., Kuroko M., Kobayashi H., Hirota T., Fujita Y., *ibid.*, **939**, 57—63 (1988).
- 4) a) Morimoto Y., Kotani K., Watanabe M., Fukumori Y., Fukuda T., Takeuchi Y., *Yakuzaigaku*, **49**, 215—220 (1989); b) Takeuchi Y., Yamaoka Y., Morimoto Y., Kaneko I., Fukumori Y., Fukuda T., *J. Pharm. Sci.*, **78**, 3—7 (1989).
- 5) Bligh E. G., Dyer W. J., *Can. J. Biochem. Physiol.*, **37**, 911—917 (1959).
- 6) Bartlett G. R., *J. Biol. Chem.*, **234**, 466—468 (1959).
- 7) Fujii T., Sato T., Tamura A., Wakatsuki M., Kanaho Y., *Biochem. Pharmacol.*, **28**, 613—620 (1979).
- 8) Deuticke B., *Biochim. Biophys. Acta*, **163**, 494—500 (1968); Ogiso T., Imai S., Hozumi R., Kurobe M., Kato Y., *Chem. Pharm. Bull.*, **24**, 479—486 (1976).
- 9) Elferink J. G. R., *Biochem. Pharmacol.*, **26**, 2411—2416 (1977).
- 10) Morimoto Y., Hosokawa M., Sayo H., Takeuchi Y., *Chem. Pharm. Bull.*, **42**, 123—129 (1994).
- 11) Suda T., Shimizu D., Maeda N., Shiga T., *Biochem. Pharmacol.*, **30**, 2057—2064 (1981); Noji S., Takahashi T., Kon H., *ibid.*, **31**, 3173—3180 (1982).
- 12) Van de Waterbeemd H., Karajannis H., El Tayar N., *Amino Acids*, **7**, 129—145 (1994); Klein R. A., Moore M. J., Smith M. W., *Biochim. Biophys. Acta*, **233**, 420—433 (1971).