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Disturbing Effect of Cationic Amphiphilic Drugs on Phospholipid Asymmetry of the Membrane Lipid Bilayer of Human Erythrocytes

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After treatment of human erythrocytes at 37°C with a cationic amphiphilic drug (one of various tertiary amine phenothiazines, primaquine or *n*-decylamine), up to 45% of the membrane phosphatidylethanolamine (PE) was degraded by added phospholipase A_2 from bee venom (untreated erythrocytes, 1—2%) and about 35% of the PE could be labelled by trinitrobenzenesulfonate (untreated erythrocytes, about 14%). Pancreatic phospholipase A_2, which is virtually unable to hydrolyze the phospholipids of intact erythrocytes, degraded significant amounts of phosphatidylcholine and PE in the erythrocyte membrane pretreated with the above drugs.

These results suggest that cationic drugs in general may alter the protein-lipid interaction of the membrane and thus release the inner layer phospholipid PE from possible locational restriction under the influence of certain proteins.

Keywords——cationic amphiphilic drug; erythrocyte membrane; lipid bilayer perturbation; phospholipase A_2; phosphatidylethanolamine translocation; membrane phospholipid asymmetry

Introduction

It has been reported that amphiphilic drugs have a variety of effects on human erythrocytes, such as shape change,[1,2] change in osmotic fragility[3,4] and increase in K^+ efflux.[5]

Recently, it has been shown that the asymmetric arrangement of phospholipid in the erythrocyte membrane is disturbed by various drug treatments. Local anesthetics such as dibucaine and tetracaine cause an increase in the hydrolysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in chicken erythrocytes by phospholipase A_2 or C,[6] and an increase in the amount of PE labelled with trinitrobenzenesulfonate (TNBS) in human erythrocytes,[7] probably as a result of movement of PE from the inner to the outer layer of the membrane lipid bilayer. The asymmetric distribution of aminophospholipids in human erythrocyte membrane was also disturbed by treating the membrane protein-SH with an SH-oxidizing reagent such as tetrathionate or diamide.[8] On the basis of these results, it is suggested that many externally added drugs may have a direct or indirect disturbing effect on membrane lipid arrangement.

In the present study, we investigated the possibility that cationic amphiphilic drugs in general may have a similar disturbing effect on lipid-protein or lipid-lipid interaction in human erythrocyte membrane. For this purpose, we employed chlorpromazine, perazine, prochlorperazine, primaquine and *n*-decylamine, as representative cationic amphiphilic drugs which can produce invagination type shape change of the erythrocyte membrane, like dibucaine *etc.*[2] The extent of perturbation of the membrane lipid bilayer induced by treatment with each of the above drugs was measured by determining the topological distribution of the membrane aminophospholipid, PE, using phospholipase A_2 and TNBS as reagents to attack only phospholipid located in the outer layer of the lipid bilayer.

Materials and Methods

Erythrocytes——Human erythrocytes from freshly drawn ACD blood, kindly supplied by the Kyoto Prefectural Red Cross Blood Center, were washed three times with isotonic phosphate-buffered saline (PBS,

140.5 mM NaCl and 10 mM phosphate buffer, pH 7.4), and resuspended in PBS.

Chemicals——The drugs used were kindly supplied by the following companies; flurbiprofen (Kakenyaku Kako Co., Tokyo); chlorpromazine hydrochloride, perazine dimaleate, prochlorperazine dimaleate (Yoshitomi Pharmaceutical Co., Osaka). All others were the purest available commercial products. Each chemical was dissolved in PBS to give an appropriate concentration to produce shape change of stomatocyte~spherostomatocyte stages of the erythrocyte membrane.[2]

Drug Treatment of Erythrocytes——A drug solution was added to the erythrocyte suspension to make a final hematocrit of 10%. The mixture was incubated at 37°C for 10 min and then the erythrocytes were washed twice with isotonic saline (0.9% NaCl) or isotonic Tris-buffered saline (TBS, 0.8% NaCl, 0.25 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 50 mM Tris buffer, pH 7.4).

Phospholipase Treatment of Erythrocytes——One volume of a solution of phospholipase A_2 from bee venom (Sigma Co.) or porcine pancreas (Boehringer Mannheim GmbH) dissolved in TBS in which the $CaCl_2$ concentration had been raised to 5 mM, was added to one volume of a 10% suspension of intact or drug-treated erythrocytes. The mixture, pH 7.4, was incubated at 37°C and the reaction was stopped by adding two volumes of an isotonic saline solution containing 10 mM EDTA. The methods of lipid analysis were the same as described previously.[9]

Trinitrobenzenesulfonate (TNBS)-labelling of Erythrocytes——TNBS-labelling was carried out by the method of Gordesky *et al.*;[10] drug-treated or intact erythrocytes were incubated at 37°C for 60 min with 2.0 mM TNBS and then the erythrocytes were washed three times with isotonic saline. Lipids were extracted from these erythrocytes and subjected to unidimensional thin layer chromatography with $CHCl_3$–CH_3OH–NH_4OH–H_2O (70: 30: 4: 1, v/v). The trinitrophenyl PE and PE spots were scraped from the plates and phosphorus was determined as described before.[9]

Determination of ATP and GSH Contents of Erythrocytes——Intracellular ATP and GSH contents of drug-treated erythrocytes were determined by the methods of Adam[11] and Kay,[12] respectively.

Polyacrylamide Gel Electrophoresis of Erythrocytes——Ghosts from intact or drug-treated erythrocytes, prepared by the method of Dodge *et al.*,[13] were solubilized with 1% sodium dodecyl sulfate at 100°C for 3 min and then subjected to electrophoresis by the method of Laemmli.[14]

Results

Table I shows the effect of pretreatment of human erythrocytes with some phenothiazine neuroleptics on the hydrolysis of the membrane phospholipids by bee venom phospholipase A_2. When intact erythrocytes were treated with this enzyme, hydrolysis of PC, the major phospholipid of the outer layer of the membrane lipid bilayer, and corresponding production of lysoPC, occurred.[9] Neither phospatidylserine, localized exclusively in the inner layer, nor PE was split significantly. We employed mild conditions of enzymatic action, so in spite of the presence of about 20% of PE in the outer layer of the lipid bilayer,[10,15] essentially no PE was hydrolyzed by bee venom phospholipase A_2. However, in the case of erythrocytes which had been treated with a phenothiazine neuroleptic and then washed with saline, PE in the membrane was hydrolyzed by the phospholipase A_2 to an extent that depended on the drug concentration. After pretreatment with a high concentration of a drug, such as 0.3 mM chlorpromazine, 0.3 mM perazine or 0.2 mM prochlorperazine, 35 to 45% of PE of the membrane was hydrolyzed without causing hemolysis. The amount of cleaved PC in intact erythrocytes was not altered significantly by such drug treatment, and phosphatidylserine was not degraded at all in any case.

A similar result was also obtained when the erythrocytes were treated with other cationic drugs, such as *n*-decylamine and primaquine (Table II). The extents of PE degradation in the erythrocytes treated with 0.6 mM decylamine and 5.0 mM primaquine were 35.5 and 38.7%, respectively.

Even when the phospholipase A_2 treatment was carried out immediately after drug addition to the erythrocyte suspension (without washing), the amounts of PE hydrolyzed were almost the same as shown in Tables I and II (data not shown).

In further studies, erythrocytes pretreated with *n*-decylamine or chlorpromazine were exposed to pancreatic phospholipase A_2, which is unable to degrade the phospholipids of intact erythrocytes.[15] Although the action of this enzyme on the erythrocytes treated with 0.1 mM chlorpromazine was essentially the same as that on intact erythrocytes, it degraded

TABLE I. Influence of Phenothiazine Neuroleptics Pretreatment on the Hydrolysis of the Membrane Phospholipids of Human Erythrocytes by Bee Venom Phospholipase A_2[a]

Treatment of erythrocytes		Membrane phospholipid class composition (%)						Phospholipid hydrolysis (%)[b]	
1st	2nd	PC	LysoPC	PE	LysoPE	PS[c]	Others	PC	PE
None	None	27.3 ±0.9	2.1 ±0.3	26.0 ±1.0	0.7 ±0.3	11.0 ±1.2	32.9 ±2.6	-	-
None	Phospho-	15.4	13.2	25.8	1.5	11.6	32.5	44.9	1.6
	lipase A_2	±1.8	±2.3	±0.9	±0.7	±1.1	±1.2	±4.0	±1.0
Chlorpromazine	Phospho-	16.7	12.3	26.2	1.5	11.3	32.0	39.4	2.7
0.1 mM	lipase A_2	±1.0	±1.0	±0.4	±0.3	±0.4	±0.6	±3.4	±0.6
0.2 mM	Phospho-	12.8	14.7	17.8	10.7	9.4	34.6	53.4	32.2[d]
	lipase A_2	±1.9	±0.6	±3.8	±2.8	±0.6	±2.2	±6.5	±8.9
0.3 mM	Phospho-	12.9	14.8	14.8	14.1	9.3	34.1	52.9	43.6[d]
	lipase A_2	±1.6	±1.2	±3.3	±3.5	±0.8	±1.2	±5.6	±9.5
Perazine	Phospho-	14.1	14.3	21.5	7.3	10.8	32.0	41.5	17.4[d]
0.15 mM	lipase A_2	±3.7	±0.8	±1.1	±2.1	±0.9	±1.7	±6.3	±0.3
0.3 mM	Phospho-	15.4	11.8	16.2	10.7	12.0	33.9	42.3	38.1[d]
	lipase A_2	±0.9	±0.8	±2.3	±0.7	±0.6	±1.6	±1.7	±6.1
Prochlorperazine	Phospho-	15.1	14.8	23.3	4.1	10.8	31.9	43.4	10.3[d]
0.05 mM	lipase A_2	±0.9	±0.6	±0.6	±1.1	±1.8	±2.9	±2.0	±2.4
0.2 mM	Phospho-	15.3	14.8	16.1	11.2	11.0	31.6	42.5	44.4[d]
	lipase A_2	±1.7	±1.4	±1.5	±2.9	±1.2	±2.5	±4.6	±2.7

a) Drug-treated erythrocytes were incubated with 10 μg of phospholipase A_2 from bee venom per 10^{10} cells in the presence of 5 mM $CaCl_2$ at 37 °C for 30 min.
b) Hydrolysis (percent) of PC and PE of membrane by phospholipase A_2 treatment was calculated from the decreases of PC and PE, respectively. Values are shown as means ± S.D. for n=4.
c) PS; phosphatidylserine.
d) Significantly (p<0.001) increased from the value without drug pretreatment (t-test).

TABLE II. Influence of Pretreatment with *n*-Decylamine or Primaquine on the Hydrolysis of the Membrane Phospholipids of Human Erythrocytes by Bee Venom Phospholipase A_2[a]

Pretreatment of erythrocytes		Phospholipids hydrolysis (%)[b]	
		PC	PE
None		45.5±3.9	0.5±0.6
n-Decylamine	0.4 mM	37.4±2.1	21.9±2.4[c]
	0.6 mM	40.2±1.4	35.5±6.9[c]
Primaquine	2.0 mM	41.7±6.6	8.1±0.2[c]
	5.0 mM	43.6±3.2	38.7±0.5[c]

a) Drug-treated cells were incubated with 20 μg of bee venom phospholipase A_2 per 10^{10} cells in the presence of 5 mM $CaCl_2$ at 37 °C for 10 min.
b) Hydrolysis (percent) of PC and PE of the membrane by phospholipase A_2 treatment was calculated from the decreases of PC and PE, respectively. Values are shown as means ± S.D. for n=4.
c) Significantly (p<0.001) increased from the value without drug pretreatment (t-test).

10—15% of PC and PE of erythrocytes pretreated with 0.6 mM decylamine or 0.3 mM chlorpromazine (Table III).

In order to confirm the above results, we estimated the amount of PE in the outer layer of the membrane lipid bilayer by a different method, using a chemical probe, TNBS. Although TNBS has been used as impermeable amino-reactive reagent for biological membranes,[10] most recently it was reported by Haest *et al.*[16] that this reagent was able to penetrate into the human erythrocyte membrane under conditions of depletion of intracellular GSH, which can react with TNBS. Under our experimental conditions, however, the amounts of intracellular

TABLE III. Influence of Pretreatment with *n*-Decylamine or Chlorpromazine on the Hydrolysis of the Membrane Phospholipids of Human Erythrocytes by Pancreatic Phospholipase A_2[a]

Pretreatment of erythrocytes		Phospholipids hydrolysis (%)[b]	
		PC	PE
None		0.4±0.1	0.4±0.1
n-Decylamine	0.4 mM	6.5±2.5[c]	7.0±1.3[d]
	0.6 mM	12.7±2.2[d]	10.7±3.1[d]
Chlorpromazine	0.1 mM	1.1±0.2[d]	0.7±0.2[c]
	0.3 mM	17.1±1.1[d]	15.9±2.0[d]

a) Drug-treated erythrocytes were incubated with 500 μg of pancreatic phospholipase A_2 per 10^{10} cells in the presence of 5 mM $CaCl_2$ at 37 °C for 60 min.
b) Hydrolysis (percent) of PC and PE of membrane by phospholipase A_2 treatment was calculated from the decreases of PC and PE, respectively. Values are shown as means ± S.D. for n=4.
c) Significantly ($0.001<p<0.01$) increased from the value without drug pretreatment (t-test).
d) Significantly ($p<0.001$) increased from the value without drug pretreatment (t-test).

GSH of intact and TNBS-treated erythrocytes at 37°C for 60 min were found to be 2.06 and 1.63 μmol per 10^{10} cells, respectively. Thus, for the following reasons, we used TNBS as a reagent considered to react only with PE located on the outer layer of the lipid bilayer; 1) the decrease in the amount of GSH as a reasult of TNBS treatment observed in our study (*ca.* 21%) is not as great as that reported by Haest *et al.* (*ca.* 60%), and 2) even if the TNBS can penetrate slightly into the erythrocyte membrane, it should react with GSH remained in the intracellular fluid before reacting with PE locating in the inner layer, because the rate of reaction of TNBS with GSH is higher than that of reaction with PE.[16] Trinitrophenyl PE as percentage of the total PE is shown in Table IV. The percentage in chlorpromazine- or primaquine-treated erythrocytes was about 35% of the total PE, in contrast to 14% in intact erythrocytes. No phosphatidylserine in the intact or drug-treated erythrocytes was labelled with TNBS.

TABLE IV. Influence of Chlorpromazine or Primaquine on Trinitrophenylation of PE in the Human Erythrocyte Membrane[a]

Pretreatment of erythrocytes		% PE trinitrophenylated[b]
None		14.0±2.0
Chlorpromazine	0.3 mM	36.5±3.0[c]
Primaquine	5.0 mM	34.0±4.6[d]

a) Intact or drug-treated erythrocytes were incubated with 2 mM TNBS at 37 °C for 60 min.
b) Trinitrophenylated-PE as a percentage of total PE of the membrane was calculated from the amount of PE labelled. Values are shown ± S.D. for n=3.
c) Significantly ($p<0.001$) increased from the value without drug pretreatment (t-test).
d) Significantly ($0.001<p<0.01$) increased from the value without drug pretreatment (t-test).

The intracellular ATP contents of the intact erythrocytes and the erythrocytes treated with 0.3 mM chlorpromazine for 30 min at 37°C were 1.40 and 1.32 μmol per 10^{10} cells, respectively. Thus, no significant ATP depletion seems to occur in human erythrocytes treated with such a drug.

Furthermore, it was confirmed that no detectable polymerization of the membrane proteins occurred after treatment of the erythrocytes with 0.3 mM chlorpromazine, as demonstrated by an unaltered electrophoretic pattern on SDS-polyacrylamide gel (data not shown).

Discussion

In this paper, it is demonstrated that some amphiphilic drug caused severe perturbation of the membrane lipid bilayer of human erythrocytes, resulting in rearrangement of the topological distribution of the major aminophospholipid, PE, between the bilayer halves, as well as acquired susceptibility of the membrane phospholipids to pancreatic phospholipase A_2, which cannot attack them in intact erythrocytes.

The existence of an asymmetric distribution of phospholipid classes in the human erythrocyte membrane has already been proved by specific determinations of the phospholipid located in the outer layer of the membrane lipid bilayer by means of treatment of the intact erythrocytes with phospholipases[15] or with membrane-impermeable reagents that react with PE molecules[10] added to the suspending medium. It is now evident that about 20 and 75% of the total PE and PC, respectively, are present in the outer layer, while practically all the phosphatidylserine (PS) is localized in the inner layer. In the present study, it was found that when intact human erythrocytes were treated with one of the amphiphilic drugs tested, about 35—45% of the total PE became accessible to added bee venom phospholipase A_2 (untreated erythrocytes, 1—2%) and about 35% of the total PE became accessible to TNBS labelling (untreated erythrocytes, about 14%).

Because our treatments did not cause any hemolysis of the treated cells, the enzyme and the reagent are considered not to have gained access to the inner layer. The TNBS reagent, which was once reported to be able to permeate partially through plasma membrane under certain conditions,[16] was confirmed to be practically unable to permeate through the membrane of intact human erythrocytes under the experimental conditions employed in this study, as described earlier. Therefore, we conclude that the drug treatment caused an increase in the amount of PE located in the outer half of the lipid bilayer, probably due to migration of PE molecules from the inner to the outer half.

Our observations suggest that such a migration of PE is not accompanied by any detectable concomitant movement of PC in the reverse direction, because the maximal cleavage of the membrane PC by the bee venom phospholipase A_2 was not altered after the drug treatment.

It is noteworthy that such a perturbation in the membrane lipid bilayer appears to be an irreversible one, since the effect of the drug persisted after washing of the treated erythrocytes with saline to remove the drug bound to the membrane. Furthermore, the perturbation seems to be similar to, although not identical with, that already reported in erythrocytes treated with SH-oxidizing agents, such as diamide and tetrathionate.[8] The difference is that, whereas the SH-oxidizing agents caused increased cleavage of both PE and PS, the drugs used in this study caused increased cleavage of PE only. Since PS, as well as PE and PC, is susceptible to the action of bee venom phospholipase A_2 employed in our study,[8] this result may suggest that PS, in contrast with PE, did not migrate from the inner to the outer leaflet of the lipid bilayer upon drug treatment. Although the reason for such a difference in the behavior of these two phospholipids in the membrane is not clear at present, we can speculate that the PS-protein interaction in the membrane might be stronger than the PE-protein interaction and consequently the rather mild perturbation in the lipid bilayer caused by the drugs used here may not be enough to induce the migration of PS, whereas stronger perturbation caused by an SH-oxidizing agent might do so. The effect of inducing migration of only PE molecules in the drug-treated erythrocytes is very similar to the effect of some local anesthetics, demonstrated in TNBS labelling experiments by Bradford and Marinetti.[7]

The occurrence of such perturbation in the membrane lipid bilayer is also supported by the data described in Table III, which indicated that the drug-treated erythrocytes become susceptible to the action of pancreatic phospholipase A_2, which is unable to attack membrane lipid of intact erythrocytes.

All the cationic amphiphilic drugs tested in this study induce membrane shape change of an invagination type (stomatocyte type).[1,2] Chlorpromazine, for example, produces stomatocytes at 0.1 mM and sphero-stomatocytes at 0.3 mM concentration. However, the lipid-perturbing effect of the drugs does not seem to be necessarily correlated with their shape-transforming effect, since invaginated erythrocytes produced by the action of phospholipase C did not show any such perturbation, as judged by the unaltered susceptibility of the membrane PE to the action of bee venom phospholipase A_2 (data to be published).

In our experiments, neither significant decrease in ATP content nor cross-linking of membrane proteins in the drug-treated erythrocytes was observed, so it appears that the perturbation of the erythrocyte membrane observed in this experiment may be induced by a mechanism different from dephosphorylation of membrane proteins resulting from the depletion of intracellular ATP[6] and from polymerization of membrane proteins such as spectrin.[8]

Thus, considering the reported finding that tertiary amine local anesthetics can interact with charged groups of membrane protein located at the inner surface of the membrane,[17] we speculate that cationic amphiphilic compounds in general may have a disturbing effect on the asymmetric distribution of phospholipids in the membrane through an interaction with certain proteins with affinity for PE at the inner surface of the membrane.

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