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Relationship between Drug- and Phospholipase-Induced Shape Changes of Human Erythrocytes

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Invagination or crenation of human erythrocytes induced by pretreating the cells with an invaginator or crenator was reversed by treatment with phospholipase A_2 (from bee venom) or phospholipase C (from *Clostridium perfringens*), respectively, under non-hemolytic conditions. In this study, flurbiprofen and lysophosphatidylcholine were used as crenators, and chlorpromazine and cepharanthine were used as invaginators. Crenation or invagination induced by pretreating the erythrocytes with phospholipase A_2 or phospholipase C, which attacks mainly phosphatidylcholine in the outer layer of the membrane lipid bilayer, was also reversed by treatment with an invaginator or crenator, respectively.

We also found that pretreatment of the erythrocytes with any of the drugs tested does not significantly affect the susceptibility of the cells to the phospholipases, and also that pretreatment of the erythrocytes with any of the phospholipases used does not affect the binding of the drug to the cells.

Thus, the findings suggest that the shape change of human erythrocytes induced by amphiphilic drugs occurs through a mechanism similar to that by which the shape change due to partial hydrolysis of the outer layer phospholipids by phospholipase action occurs, possibly through a change in the lipid bilayer balance of the membrane.

Keywords——amphiphilic drug; erythrocyte membrane; erythrocyte shape change; membrane lipid bilayer; membrane phospholipid; phospholipase

Many amphiphilic drugs have been shown to induce shape changes in human erythrocytes. Generally, anionic compounds produce crenation of the cells while cationic compounds cause invagination.[1,2] Quarternary ammonium compounds generally induce crenation first and then invagination, and are sometimes called crenators in experiments with relatively short time course. Sheetz and Singer[3] proposed that such shape change may be due to an asymmetric expansion of the membrane lipid bilayer caused by a drug which selectively enters one of the two leaflets of the lipid bilayer. Cationic drugs intercalate mainly into the inner half of the bilayer because of their interaction with the acidic phosphatidylserine (PS) localized in this region. This expands the inner half relative to the outer half of the bilayer, thus inducing invagination. Because of their charge repulsion with respect to PS, anionic drugs have the opposite effect, causing the cell to crenate. This hypothesis, however, has not yet been experimentally confirmed.

We reported previously[4,5] that a similar type of shape change of human erythrocytes can also be produced by modifying only the outer half of the membrane lipid bilayer under non-hemolytic conditions with exogenously added phospholipase (PLase). PLase A_2 induced crenation, while PLase C or D induced invagination, in parallel with hydrolysis of phosphatidylcholine (PC) of the erythrocyte membrane. We proposed that these shape changes induced by PLase treatments may also be due to an asymmetric expansion or shrinkage of the outer leaflet of the lipid bilayer relative to the inner one.

In the present work, we examined the relationship between these two apparently similar

kinds of shape changes of human erythrocytes, the drug- and phospholipase-induced ones, in order to elucidate the mechanism of the drug-induced shape change.

Materials and Methods

Erythrocytes——Human erythrocytes from freshly drawn ACD (acid citrate dextrose) blood, kindly supplied by the Kyoto Prefectural Red Cross Blood Center, were washed three times with isotonic Tris-buffered saline (TBS) containing 0.87% NaCl and 50 mM Tris buffer, pH 7.4, and resuspended in TBS. Ghosts were prepared from the washed erythrocytes by hypotonic hemolysis as reported previously.[6]

Chemicals——The following drugs were kindly supplied by the companies in parenthesis; flurbiprofen, 2-(2-fluoro-4-biphenyl)propionic acid, (Kakenyaku Kako Co., Ltd., Tokyo); cepharanthine, a biscoclaurine alkaloid, (Kaken Pharmaceutical Co., Ltd.); chlorpromazine and palmitoyl-lysophosphatidylcholine (lysoPC) were purchased from Sigma Co., Ltd. Structural formulas of these chemicals are shown in Fig. 1.

Enzymes——The following PLases were employed; commercial preparations of PLase A_2 from bee venom (Sigma Co., 1320 IU/mg protein) and PLase C from *Clostridium perfringens* (P. L. Biochemicals Inc. 1 IU/mg

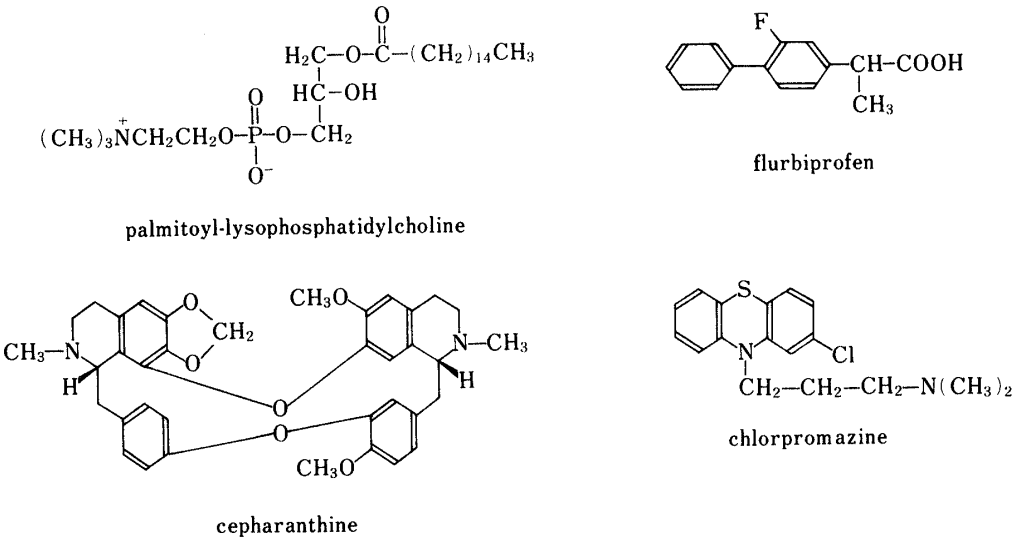
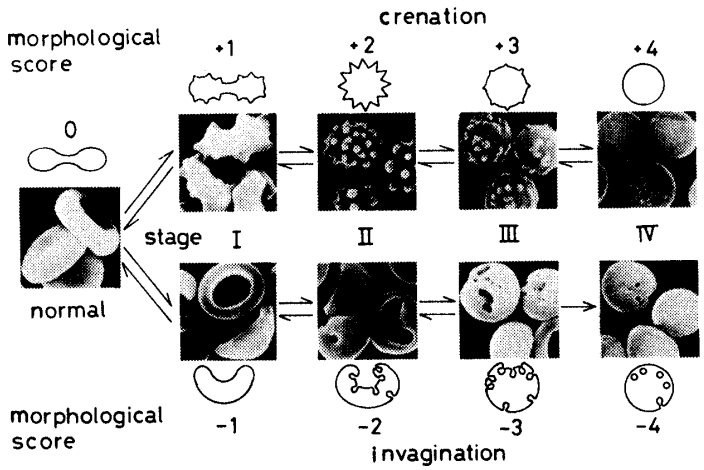


Fig. 1. Structural Formulas of the Drugs Tested



$$\text{morphological index} = \sum\left(\text{score} \times \frac{\text{number of transformed cells}}{\text{total number of cells}}\right)$$

Fig. 2. Illustration of Two Types of Shape Changes of Human Erythrocytes Induced by Drugs or PLase Treatments and Definition of the Morphological Index

protein) which had been heated for 10 min at 95 °C to inactivate possibly contaminating protease. No protease activity was detected in any of these PLase preparation against denatured hemoglobin as a substrate.

Drug Treatment of Erythrocytes——A solution of a drug was added to a suspension of intact or PLase-treated erythrocytes to make a final hematocrit of 10%. The mixture was incubated at 37 °C for 10 min.

PLase Treatment of Erythrocyte——One ml (40 unit/ml) of a solution of PLase A_2 or PLase C (200 μg/ml) in TBS containing 20 mM $CaCl_2$ was added to 10 ml of a 10% suspension of intact or drug-treated erythrocytes. The mixture was incubated at 37 °C for an appropriate time.

Morphological Observation of the Erythrocytes——Each sample (0.5 ml) was mixed with 2 ml of 0.9% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4. An aliquot of each sample was washed, air-dried and coated with carbon and gold. The preparation was then observed under a JEOL JSM-35 scanning electron microscope. The extent of the induced shape change was expressed in terms of the morphological index,[2] as defined in Fig. 2.

Quantification of the Phospholipids of Erythrocyte Membrane——Erythrocyte lipids were extracted and phospholipid classes were separated by two-dimensional thin layer chromatography on silica gel plates, and the lipid phosphorus was determined as described before.[4]

Quantification of the Amount of Drug Incorporated into Erythrocytes——Erythrocytes were treated with a drug and centrifuged at $900 \times g$ for 10 min. Ghost suspension equivalent to a 10% suspension of the original washed erythrocytes was also treated with each drug under the same conditions and centrifuged at $20000 \times g$ for 30 min. The amount of drug incorporated into erythrocytes and ghosts was determined by measuring the decrease in ultraviolet (UV) absorption of the supernatant of the incubation mixture, as described already.[6] The wavelengths used were 254, 282 and 247 nm for chlorpromazine, cepharanthine and flurbiprofen, respectively.

Results

We first examined whether or not pretreatment of intact human erythrocytes with a drug alters the susceptibility of the treated cells to the action of PLase and also whether or not pretreatment with PLase alters the extent of drug incorporation. Table I indicates that, when acting on intact erythrocytes, PLase A_2 hydrolyzes only the membrane PC to yield lysoPC, and PLase C acts mainly on PC and slightly on sphingomyelin (SM), as already demonstrated in our previous papers.[4,5] PLase A_2 attacks erythrocytes pretreated with 0.1 mM chlorpromazine solution in a similar manner except that it hydrolyzes, in addition to PC, a small portion of phosphatidylethanolamine (PE) to yield the corresponding lyso-compound. There

Table I. Quantification of the Membrane Phospholipids and Their Hydrolysis Products Remaining in the Membrane after PLase A_2 or C Action on Intact or Drug-Treated Erythrocytes

Treatment of erythrocytes		Phospholipid content (μmol/10^{10} cells)					
1st	2nd	PC	LysoPC	PE	LysoPE	PS	SM
None	None	1.03±0.03	0.13±0.01	0.99±0.04	0.02±0.01	0.33±0.04	1.07±0.09
None	PLase A_2	0.53±0.07	0.57±0.08	1.03±0.03	0.03±0.02	0.34±0.04	1.10±0.04
0.1 mM Chlorpromazine	PLase A_2	0.59±0.04[a]	0.49±0.04[a]	0.88±0.02[b]	0.14±0.01[b]	0.34±0.02[a]	1.07±0.02[a]
None	PLase C	0.61±0.09	0.16±0.01	0.85±0.05	0.06±0.01	0.46±0.06	0.74±0.10
1.0 mM Flurbiprofen	PLase C	0.65±0.08[a]	0.12±0.02[a]	0.94±0.06[a]	0.05±0.02[a]	0.46±0.05[a]	0.79±0.09[a]

Erythrocytes were treated with PBS or 0.1 mM chlorpromazine for 10 min, then treated with PLase A_2 for 15 min. Other erythrocytes were treated with PBS or 1.0 mM flurbiprofen for 10 min, then treated with PLase C for 15 min. Phospholipids of each sample were extracted, and separated by TLC.

Values are shown as means ± S.D. for $n=3$.

a) No significant difference ($p>0.1$) in phospholipid contents after each PLase action between the intact and drug-treated erythrocytes.

b) Significant difference ($p<0.001$) in the phospholipid contents after each PLase action between the intact and drug-treated erythrocytes.

PLase, phospholipase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

TABLE II. Incorporation of Added Drug into Intact or PLase-Treated Erythrocytes

Drug	The concentration (mM) of drug	The amount (μmol/10^{10} cells) of drug incorporated into the		
		Intact cells	PLase A_2-treated cells	PLase C-treated cells
Chlorpromazine	0.05	0.392±0.029	0.399±0.024	—
	0.075	0.611±0.025	0.650±0.032	—
	0.10	0.769±0.015	0.784±0.021	—
Flurbiprofen	0.25	0.845±0.009	—	0.837±0.013
	0.50	1.626±0.042	—	1.582±0.022
	1.00	2.887±0.295	—	2.793±0.511

Intact or PLase A_2-pretreated erythrocytes were mixed with 0.05—0.10 mM chlorpromazine. Other intact or PLase C-pretreated erythrocytes were mixed with 0.25—1.00 mM flurbiprofen. The amounts of the drug incorporated into the cell were determined by measuring the decrease in UV absorption of the supernatant of the incubation mixture.

Values are shown as means±S.D. for $n=4$.

No significant difference ($p>0.1$) in the amount of the bound drug between the intact and PLase-treated erythrocytes.

was practically no difference between the phospholipid hydrolysis patterns of PLase C acting on intact and flurbiprofen-treated erythrocytes. Similar results were obtained when erythrocytes were pretreated with 0.1 mM cepharanthine or with 0.015 mM lysoPC (data not shown).

As shown in Table II, when intact and PLase A_2-treated erythrocytes were treated with 0.05—0.1 mM of chlorpromazine solution, and also when intact and PLase C-treated erythrocytes were further treated with 0.25—1.0 mM flurbiprofen solution, no significant difference was noted in the amount of either drug incorporated between the two runs. Similar results were also obtained by using 0.05—0.1 mM cepharanthine and 0.005—0.015 mM lysoPC (data not shown).

Figure 3 shows that the altered cell shape of erythrocytes treated with a crenator or an invaginator was reversed by treatment with PLase C or PLase A_2, respectively. Crenation equivalent to a morphological index of +1.9 induced by 1.0 mM flurbiprofen or of +2.1 induced by 20 μM lysoPC was completely reversed to the normal biconcave disc shape (index, 0) after a 20 min incubation with PLase C. Upon more prolonged incubation (30 min), invagination with an index of −1.1 to −1.0 occurred. Treatment of intact cells with this enzyme yielded invaginated cells with an index of −1.8 or −2.3 after 20 or 30 min incubation, respectively. In contrast, invagination equivalent to an index of −1.3 induced by 75 μM chlorpromazine or of −1.5 induced by 75 μM cepharanthine was reversed to almost the normal shape after an incubation with PLase A_2 for 10 min. Upon more prolonged incubation (30 min), crenated cells showed an index of +1.2 to +1.5. Incubation of the intact cells with the enzyme for 10 or 30 min yielded crenation equivalent to an index of +1.3 or +2.5, respectively. The erythrocytes treated with each crenator or invaginator were incubated with 10 mM $CaCl_2$ for 30 min at 37 °C, as a control for the PLase retreatment. The degrees of crenation or invagination were similar to those observed before the incubation.

As demonstrated in Figs. 4 and 5, crenation brought about by PLase A_2 action or invagination induced by PLase C action was reversed by subsequent treatment with an invaginator or a crenator, respectively. The PLase A_2/invaginator (chlorpromazine or cepharanthine) antagonism in Fig. 4 appears to be almost the inverse of the invaginator/PLase A_2 antagonism in Fig. 3. A similar result was also obtained in the case of PLase C/crenator

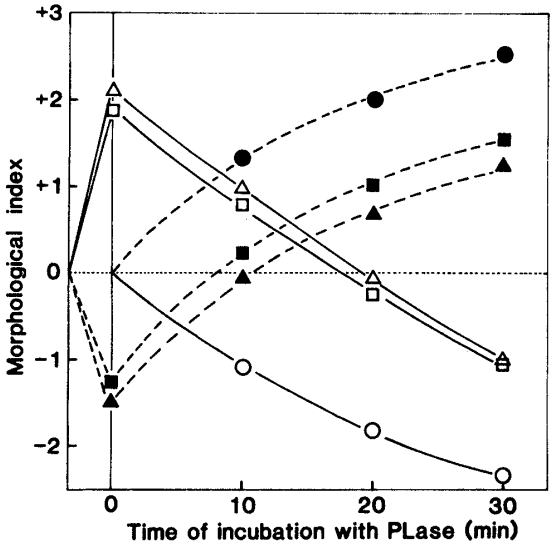


Fig. 3. Shape Change of Human Erythrocytes Observed When Cells Treated with an Invaginator or Crenator were then Further Treated with PLase A_2 or C

Erythrocytes treated with 1.0 mM flurbiprofen (□) or 20 μM palmitoyl-lysoPC (△) were then further treated with PLase C (——) in the presence of the residual drug. Cells treated only with PLase C (○). Erythrocytes treated with 75 μM chlorpromazine (■) or 75 μM cepharanthine (▲) and then treated with PLase A_2 (------) in the presence of the residual drug. Cells treated only with PLase A_2 (●).

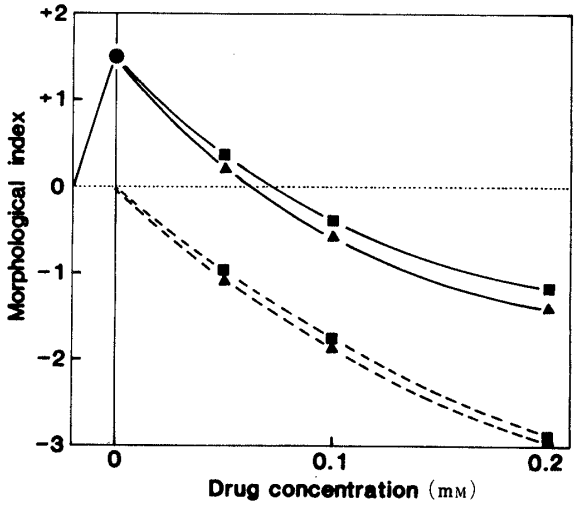


Fig. 4. Shape Change of PLase-Treated Erythrocytes upon Further Treatment with Cepharanthine or Chlorpromazine at Various Concentrations

Erythrocytes were treated with PLase A_2 for 15 min, then washed with PBS (●) and further treated with cepharanthine (—▲—) or chlorpromazine (—■—). Cells treated only with cepharanthine (---▲---) or chlorpromazine (---■---).

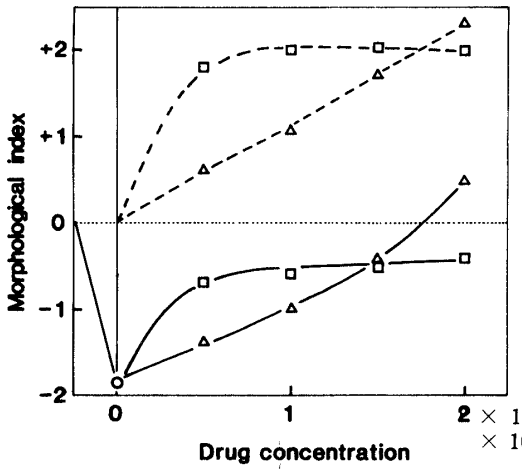


Fig. 5. Shape Change of PLase C-Treated Erythrocytes upon Further Treatment with LysoPC or Flurbiprofen at Various Concentrations

Erythrocytes were treated with PLase C for 15 min, then washed with PBS (○) and further treated with palmitoyl-lysoPC (—△—) or flurbiprofen (—□—). Cells treated only with palmitoyl-lysoPC (---△---) or flurbiprofen (---□---).

antagonism when lysoPC was used as a crenator. However, flurbiprofen, another crenator, was unable to reverse even at a high concentration (Fig. 5).

Some representative electron micrographs of erythrocytes treated as in Figs. 4 and 5 are shown in Fig. 6. Intact erythrocytes (A) became crenated after PLase A_2 action (B) and a further treatment of this preparation with 50 μM chlorpromazine reversed the shape change to very slightly crenated morphology (C). Upon removal of the bound chlorpromazine from the erythrocytes of (C) by washing with PBS, the PLase A_2-treated erythrocytes regained their original shape (D). Intact erythrocytes became invaginated after PLase C treatment (E) and the invaginated shape was then reversed by further treatment with 15 μM lysoPC to provide a slightly cupped shape (F). Upon removal of the bound lysoPC from the erythrocytes of (F)

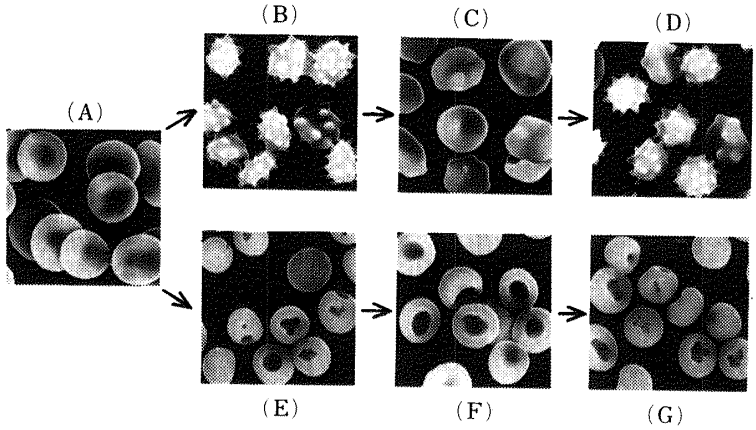


Fig. 6. Shape Change of PLase A_2- or C-Treated Erythrocytes upon Further Treatment with Chlorpromazine or LysoPC as Seen in Scanning Electron Micrographs of the Treated Cells

Erythrocytes treated for 15 min with PLase A_2 (B) were further treated with 50 μM chlorpromazine (C), and then washed PBS (D). Erythrocytes treated for 15 min with PLase C (E) were further treated with 15 μM palmitoyl-lysoPC (F), and then washed with PBS containing 1% bovine serum albumin (G). Erythrocytes without treatment (the control) were also incubated under the same conditions (A).

TABLE III. Drug Incorporation into Intact Erythrocytes or Ghosts

Drug	Concentration (mM) of drug added	Amount (μmol/10^{10} cells) of drug added	Amount (μmol/10^{10} cells) of drug incorporated into the	
			Intact cells	Ghosts
Chlorpromazine	0.05	0.50	0.392±0.029 (78)[a]	0.390±0.019 (78)
	0.10	1.00	0.769±0.015 (77)	0.728±0.015 (73)
Cepharanthine	0.05	0.50	0.285±0.031 (57)	0.279±0.014 (56)
	0.10	1.00	0.525±0.026 (53)	0.493±0.037 (49)
Lysophosphatidylcholine	0.005	0.05	0.044±0.002 (88)	0.042±0.002 (84)
	0.01	0.10	0.088±0.001 (88)	0.086±0.002 (86)
Flurbiprofen	0.5	5.0	1.626±0.042 (33)	0.081±0.011 (2)
	1.0	10.0	2.887±0.295 (29)	0.152±0.019 (2)

Intact erythrocytes or ghosts were incubated with each drug for 10 min. The amounts of the drug incorporated into the whole cells or ghosts were determined by measuring the decrease in UV absorption of the supernatant of the incubation mixture.

Values are shown as means±S.D. for $n=4$.

a) The figure in parenthesis is the % of the total amount added.

by washing with PBS containing bovine serum albumin, the PLase C-treated erythrocytes regained their original shape (G).

Certain additive effects of PLase and drug treatments on the cell shape were observed. For example, when the PLase A_2-treated erythrocytes with a morphological index of +1.0 were further treated with 10 μM lysoPC, the index of the cells became +2.2. When the PLase C-treated cells with an index of −1.0 were further treated with 50 μM chlorpromazine, their index became −2.2. Similar additive effects were also observed when the crenator-treated cells were further treated with PLase A_2, when the invaginator-treated cells were further treated with PLase C, and when the intact cells were treated with two crenators or two invaginators successively (data not shown).

Binding of crenator and invaginator drugs to the erythrocyte membrane was also

investigated. Table III shows the amounts taken up by whole erythrocytes and ghosts (the membrane fraction) when the same amount of each drug was added to the suspensions. About 77—78% of chlorpromazine, 53—57% of cepharanthine and 88% of palmitoyl-lysoPC added were incorporated into the whole cells while only 29—33% of flurbiprofen was incorporated. Furthermore, about 73—78, 49—56, 84—86 and only 2% of chlorpromazine, cepharanthine, palmitoyl-lysoPC and flurbiprofen, respectively, were taken up by the ghosts. These results suggest that chlorpromazine, cepharanthine and lysoPC are predominantly taken up by the cells into the membrane fraction, whereas only a small portion of flurbiprofen taken up by the cells may be bound to the membrane.

Discussion

As the present results clearly demonstrate, the drug-induced crenation of whole human erythrocytes was reversed by incubating the drug-treated cells with phospholipase C under non-hemolytic conditions which partially hydrolyzes the PC and SM molecules located in the outer leaflet of the membrane lipid bilayer. The drug-induced invagination was similarly reversed by incubation with PLase A_2 which partially hydrolyzes only the PC molecules in the outer leaflet. In contrast, the crenation induced by incubating the whole cells with PLase A_2 was reversed by a subsequent treatment of the cells having the modified lipid bilayer with an invaginator, and the invagination induced by incubation with PLase C was reversed by subsequent treatment with a crenator. In addition, pretreatment of the erythrocytes with any of the drugs tested did not significantly affect the susceptibility of the cells to subsequent phospholipase action, and also pretreatment of the cells with any of the phospholipases used did not affect the binding of the drugs to the cells (Tables I and II). For unknown reasons, a small portion of the membrane PE was hydrolyzed by PLase A_2 when the erythrocytes had been treated with chlorpromazine, but this was regarded as negligible for the following reasons; there was no significant difference in the amount of the total lysophospholipid produced (lysoPC + lysoPE) as shown in Table I, and another invaginator, cepharanthine, did not affect the susceptibility of the membrane PE to PLase A_2 (data not shown). Thus, we conclude that a kind of "functional antagonism" exists between the drug-induced and phospholipase-induced shape changes of human erythrocytes, namely, between drug-induced crenation and PLase C-induced invagination and also between drug-induced invagination and PLase A_2-induced crenation.

As we reported previously, a similar antagonistic effect on the cell shape was also observed between a crenator and invaginator[2] and between PLase A_2 and PLase C treatments.[4]

We have also shown[4,5,7] that treatment of intact human erythrocytes with PLase A_2 from bee venom under non-hemolytic conditions produced a pH-dependent shape change of crenation type (echinocyte formation), probably because partial hydrolysis of the outer leaflet phospholipids (mainly PC) of the membrane lipid bilayer yielded additional head groups (not phosphorylcholine of lysoPC, but COOH of fatty acid) exposed to the outer medium which are dissociable in a certain pH range and thus cause increased charge repulsion among the lipid molecules, which might lead to asymmetric expansion of this bilayer half. In contrast, treatment of erythrocytes with PLase C from *Clostridium perfringens* induced shape change of invagination type (stomatocyte formation), which was not influenced by the medium pH, probably because removal of polar head groups (phosphorylcholine) of the outer leaflet phospholipids by this enzyme resulted in asymmetric shrinkage of the bilayer half.[4,5,7]

Considering the results of our present and previous studies as well as the Sheetz–Singer "bilayer couple hypothesis" already mentioned,[3] the crenator/PLase C antagonism may indicate that the probable asymmetric shrinkage of the outer half of the lipid bilayer produced

by this enzyme can be alleviated by asymmetric expansion of the same bilayer half due to crenator action. Similarly, the invaginator/PLase A_2 antagonism may imply that the probable expansion of the outer half of the bilayer produced by PLase A_2 can be alleviated by expansion of the inner half of the bilayer due to invaginator action. Thus, our results strongly suggest that the drug-induced shape changes of human erythrocytes are also due to a change in the lipid bilayer balance of the membrane, namely, due to asymmetric expansion of only one half of the bilayer, although the precise site(s) of drug binding on the membrane is not established yet. We consider that, even though drugs may be bound to the membrane lipid bilayer, to the membrane protein(s) or to both components, they may directly or indirectly lead to expansion of one of the lipid bilayer halves; the crenators induce an expansion of the outer half while the invaginators induce an expansion of the inner half.

As regards crenators, however, the situation is not necessarily so simple, because we found some differences between the two crenator compounds employed in this study, with respect to binding behavior to and action on the erythrocyte membrane. Namely, whereas lysoPC can completely reverse the invaginating action of PLase C, flurbiprofen can reverse it only partially (Fig. 5). Furthermore, whereas lysoPC was found to have high affinity to the membrane, like the invaginators (such as chlorpromazine), flurbiprofen has only weak affinity (only about 6—7% of flurbiprofen taken up by the whole erythrocytes is considered to be localized in the membrane) and is predominantly localized in the cytosol (Table III). We have already reported that some anionic crenators having a phenothiazine nucleus also have only weak affinity to the erythrocyte membrane, in contrast with the strong affinity of cationic phenothiazines such as chlorpromazine, and are mostly found in the cytosol fraction of erythrocytes.[6] It is possible, therefore, that such anionic crenators may have a mode of action different from that of the quarternary ammonium crenators of lipid character, such as lysoPC. This point remains to be investigated.

In addition to the drug-induced shape changes and those due to manipulation of the membrane lipid bilayer, some other phenomena of the erythrocyte shape change are well known, including those induced by changes in the intracellular level of ATP,[8] in which participation of any change in the membrane lipid seems to be unlikely. It is probable that cell shape in general is regulated directly by the cytoskeletal structures, and therefore the erythrocyte shape is probably regulated by the spectrin–actin–ankyrin network.[9,10] We speculate that changes in the membrane lipid bilayer may act as a trigger for induction of the shape change, in the sense that such changes may be recognized by mediating membrane protein(s) and finally transmitted to the cytoskeletal system, leading to modification of the conformation.

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