Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug-Erythrocyte Interactions

(membrane asymmetry/cell morphology/amphipathic drugs/anesthetics)

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ABSTRACT We propose that membranes whose proteins and polar lipids are distributed asymetrically in the two halves of the membrane bilayer can act as bilayer couples, i.e., the two halves can respond differently to a perturbation. This hypothesis is applied to the interactions of amphipathic drugs with human erythrocytes. It is proposed that anionic drugs intercalate mainly into the lipid in the exterior half of the bilayer, expand that layer relative to the cytoplasmic half, and thereby induce the cell to creenate, while permeable cationic drugs do the opposite and cause the cell to form cup-shapes. This differential distribution of the drugs is attributed to interactions with the phosphatidylserine that is concentrated in the cytoplasmic half of the membrane. Impermeable amphipathic drugs intercalate only into the exterior half of the bilayer, and therefore are creenators of the intact cell. Several predictions of this hypothesis have been confirmed experimentally with erythrocytes and erythrocyte ghosts. The bilayer couple hypothesis may contribute to the explanation of many membrane-mediated phenomena in cell biology.

It is generally agreed that the fluid mosaic model of membrane structure (1-4) provides a satisfactory general description of the molecular organization of the proteins and lipids of most functional membranes. In this model, the integral proteins are amphipathic molecules with their hydrophobic ends embedded in the matrix of the membrane, and their hydrophilic ends protruding from it. The matrix of the membrane is a bilayer of phospholipids, at least some part of which is fluid under physiological conditions. There is increasing evidence that both the proteins and the phospholipids of membranes are asymmetrically distributed in the two halves of the bilayer. This evidence is most substantial for the erythrocyte membrane with respect to its proteins (5, 6) and phospholipids (7, 8). Phosphatidylincholine and sphingomyelin appear to be concentrated in the exterior half of the bilayer, and phosphatidylethanolamine and phosphatidylserine in the cytoplasmic half, as was suggested by Bretscher (9). While the first three of these phospholipids have zwitterionic head groups, phosphatidylserine bears a net negative charge at neutral pH. It seems likely that all cell membranes are characterized by asymmetrical distributions of their integral proteins and polar lipids, but as the evidence on this point is still only fragmentary, in what follows we will largely confine ourselves to the erythrocyte membrane.

The nature of these asymmetrical distributions, and in particular, whether they are equilibrium or nonequilibrium distributions, are matters that we have briefly considered elsewhere (2-4, 10). Whatever the nature or origins of these asymmetrical distributions, however, their existence in the erythrocyte membrane leads us to formulate the bilayer couple hypothesis. In its most general form, the hypothesis is that the two halves of the closed membrane bilayer may respond differently to various perturbations while remaining coupled to one another. For example, one half of the bilayer, either through effects on its protein or its lipid components, may expand rapidly in the plane of the membrane relative to the other half of the bilayer, while the two layers remain throughput in van der Waals contact with one another. This would lead to various functional consequences, including shape changes of the intact cell. We call this the bilayer couple hypothesis because of the rough analogy to the response of a bimetallic couple to changes in temperature.

The bilayer couple hypothesis is briefly presented elsewhere (10). In this paper, we use the hypothesis to provide a new and consistent molecular explanation for the nonspecific noncovalent interaction of a wide range of amphipathic small molecules with the intact human erythrocyte. In order to present this material, we first give a brief summary of the salient features of the vast literature dealing with drug-erythrocyte and related interactions.

An excellent recent review of the binding properties of amphipathic molecules (many of which are anesthetics) to intact erythrocytes, erythrocyte ghosts, and other membranes is that of Seeman (11). For our present purposes, the important characteristics of such binding are as follows:

1) The binding is usually biphasic. In the range of low concentrations of the drug, the Scatchard plot (12), and the intact erythrocyte is protected against hypotonic lysis. At higher concentrations, however, the drug increasingly promotes lysis.

2) These drugs can vary widely in their hydrophobic portions and can be anions, cations, or neutral molecules at pH 7. A significant feature is that an increase in ionic strength increases the binding of anionic amphipaths to erythrocyte ghosts, and decreases the binding of cationic ones (13). Therefore, the binding of both kinds is influenced by a negative field associated with the membrane.

Many of these same compounds induce profound shape changes in the intact human erythrocyte. Two different kinds of changes are observed, either by light microscopy, or in greater detail by scanning electron microscopy. In one change,
It is presumed that the amphipathic compounds intercalate their hydrophobic ends primarily into the nonpolar interior of the lipid portions of the mosaic membrane, while their polar or ionic ends are exposed at the membrane--water interface. To a first approximation, the integral proteins of the erythrocyte membrane are not involved in the binding of the compounds, nor in the cell shape changes that result.

(ii) Cup-formers distribute preferentially into the lipid portions of the cytoplasmic half of the bilayer (Fig. 1), causing that half to expand relative to the exterior half, and thereby inducing the observed invagination. Crenators, on the other hand, distribute preferentially into the lipid of the exterior half of the bilayer, expanding that half relative to the cytoplasmic half and inducing crenation.

(iii) The asymmetry of these distributions is determined by the sign of the charge of the amphipathic compound at neutral pH, and by the rate at which it diffuses across the membrane of the intact cell. We suggest that the cationic amphipathic compounds distribute preferentially into the cytoplasmic half of the bilayer because of the negative field generated by the anionic phosphatidylserine concentrated in that layer, provided that these compounds can diffuse across the membrane at an appreciable rate. It is assumed that they diffuse across as the neutral (discharged) species, and reacquire a proton and a positive charge while bound at the other membrane surface. Anionic amphipathic compounds distribute preferentially into the lipid in the exterior half of the bilayer because they are repelled by the negative field generated by the phosphatidylserine. Furthermore, it is proposed that all amphipathic compounds (whatever their electric charge) that bind to the membrane but cannot diffuse across it at a significant rate are crenators, since they must performe concentrate in the lipid portion of the exterior half of the bilayer.

This molecular explanation of the binding characteristics of drugs to intact erythrocytes and of the erythrocyte shape changes they induce leads to many predictions that are capable of being tested experimentally. One set of predictions concerns a comparison of the erythrocyte binding characteristics of two compounds that are structurally closely similar, except that one is a tertiary and the other a quaternary amine. The tertiary amine (which can be discharged and diffuses fairly rapidly across the membrane) should be a cup-former of the intact erythrocyte, whereas its quaternary analogue (which cannot be discharged and should, therefore, diffuse only slowly across the membrane) should be a crenator. On the other hand, if similar shape changes could be observed with permeable erythrocyte ghosts, the quaternary amine should now have direct access to the cytoplasmic half of the membrane, and both analogues should cause the ghost to cup. Furthermore, the quaternary amine should be less strongly bound than its tertiary analogue to the intact erythrocyte because the confinement of the former to the exterior layer would not permit it to interact as strongly with the phosphatidylserine. On the other hand, with erythrocyte ghosts, the two compounds should be about equally well bound. Another set of predictions concerns the altered shapes of the erythrocytes induced by crenators and cup-formers, which should be accounted for quantitatively as an expansion of one-half of the bilayer relative to the other. Such experiments and analyses are reported in this paper.

FIG. 1. Schematic representation of the proposed binding of amphipathic compounds that are crenators or cup-formers to the phospholipid regions of the erythrocyte membrane. Crenators intercalate preferentially into the exterior half of the bilayer, causing it to expand relative to the cytoplasmic half, thereby producing the observed crenation. Cup-formers do the opposite.

The cells are converted from the normal biconcave-disk shape to a highly crenated form (see Fig. 4); in the other, to an invaginated or cupped form. Deuticke (14) noted that most of the compounds that crenate the erythrocyte (crenators) are anionic amphipathic compounds, such as free fatty acids, 2,4-dinitrophenol, and barbiturates. However, some crenators are not anionic, as for example, lysolecithin. The compounds that convert the erythrocyte into cup shapes (cup-formers), on the other hand, are almost exclusively cationic amphipathic compounds, such as the phenothiazine tranquilizers and local anesthetics. At sufficiently large concentrations, both crenators and cup-formers cause the erythrocytes to become spherical and lyse.

Various investigators have suggested several mechanisms to account for one or another of these effects of amphipathic compounds on erythrocytes, but none has been sufficiently comprehensive or satisfactory (11). On the basis of the bilayer couple hypothesis, we propose a coherent qualitative explanation for these phenomena consisting of the following elements. In this treatment, we confine ourselves to the low concentration range in which a compound affords the erythrocyte some protection against hypotonic hemolysis.

(i) It is presumed that the amphipathic compounds intercalate their hydrophobic ends primarily into the nonpolar interior of the lipid portions of the mosaic membrane, while their polar or ionic ends are exposed at the membrane--water interface. To a first approximation, the integral proteins of the erythrocyte membrane are not involved in the binding of the compounds, nor in the cell shape changes that result.

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MATERIALS AND METHODS

Reagents. The structures of the amphipathic amines used in this study are shown in Fig. 2. Chlorpromazine·HCl was obtained from Smith, Kline, and French Laboratories, Philadelphia, Pa., and lidocaine from Astra Pharmaceutical Co., Worcester, Mass. Methochlorpromazine iodide and QX-222 were the generous gifts of Dr. Glenn Ulloty of Smith, Kline, and French, and Dr. Bertil Takman of Astra Pharmaceutical, respectively. HK-25 and HK-27 were synthesized by Dr. Hansreudi Kiefer. The purity of these compounds was assessed by thin-layer chromatography. The alcohols used—benzyl (Aldrich Chemical Co., puriss. grade), heptyl and decyl (Matheson, Coleman, and Bell Co., reagent grade), and octyl (Eastman reagent grade)—were all first extracted with 1 M NaOH to remove any acidic contaminants. Fresh human blood was obtained in citrate–phosphate–dextrose and was used within 5 days.

Hemolysis Protection Experiments. Washed erythrocytes at a hematocrit of about 3% were incubated with isotonic [146 mM
NaCl, 10 mM Tris·HCl (pH 7.4) solutions of a drug for 5 min at room temperature, and then diluted into a hypotonic solution of the same concentration of the drug. The tonicity of this solution (72 mM NaCl) was chosen so that in the absence of the drug, about 30% of the erythrocytes lysed after 10 min at room temperature. The drug and control samples were then centrifuged, and the absorbances of the supernatants were measured at 543 nm.

**Scanning Electron Microscopy.** Erythrocytes at about 3% hematocrit were suspended in isotonic drug solutions for 10 min at room temperature. Four parts of this suspension were then added to one part of 5% glutaraldehyde in isotonic NaHPO₄ (pH 7.4) and the samples were cooled to 0°C. After 1 hr, the cells were washed thoroughly with distilled water. They were then treated for 20 min at room temperature with 1% osmium tetroxide in phosphate buffer (pH 7.4). The cells were then washed with distilled water, dehydrated in acetone, and triple-point dried with CO₂ in a Bomar SPC-900 apparatus. After rotary shadowing with Au-Pd, the cells were examined in a Cambridge S4 scanning electron microscope.

When erythrocytes to which the drugs were added were treated in this manner and observed in the scanning electron microscope, they were found to have bizarre shapes, perhaps because of some reaction of the drugs with the glutaraldehyde fixative. The erythrocytes treated with isotonic solutions of the drugs were therefore observed in the unfixed condition by phase optics in a light microscope. Samples were placed between siliconized glass slides and coverslips to avoid the glass effect.

In one set of experiments, erythrocytes were first treated with the enzyme neuraminidase to remove sialic acid residues from the cell surface. A reaction mixture of 10⁶ cells and 50 units of the enzyme (Neuraminidase: *Vibrio cholerae*, Calbiochem, San Diego, Calif.) in a buffer containing 146 mM NaCl, 10 mM cacodylate, and 2 mM CaCl₂ (pH 6.5) was incubated for 30 min at 37°C. The treated cells were no longer agglutinable by influenza virus.

**Experiments with Erythrocyte Ghosts.** Erythrocytes were lysed in 20 mosM phosphate buffer (pH 7.4) at 0°C and centrifuged for 7 min at 6000 × g. Fifty microliters of packed membranes was added to 800 µl of a solution of a drug in the lysis buffer at 0°C, and after 1 min, 200 µl of 5% glutaraldehyde in the same buffer was added to fix the membranes. The ghosts were then prepared for scanning electron microscopy as described above for the intact cells.

**RESULTS**

The effects of three pairs of tertiary amine-quinary amine analogues on the hypotonic hemolysis of human erythrocytes are summarized in Fig. 3. Lidocaine and HK-27 show unusual behavior in that they do not protect the erythrocyte against hemolysis at any concentration; instead, they promote lysis. The data for chlorpromazine and methochlorpromazine are similar to those obtained earlier (15). For our purposes, these results show that the tertiary amine of each pair has its effect at a lower concentration than its

**FIG. 2.** The structural formulas of three pairs of analogues used in this study. HK-25 and HK-27 were Br salts, methochlorpromazine was the I salt, and the others were Cl salts.

**FIG. 3.** The effect of the compounds in Fig. 2 on the hypotonic lysis of erythrocytes. Cp and M-Cp are chlorpromazine and methochlorpromazine, respectively.
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quaternary analogue, which implies that the tertiary amine binds to the membrane of the intact erythrocyte more strongly than does the quaternary.

The effects of these compounds on the shape of the intact erythrocyte were determined in the lower range of concentrations defined by the hemolysis results in Fig. 3. For each pair, the tertiary amine caused the erythrocytes to become cup-shaped, while the quaternary analogue caused them to become crenated. The results for the pair chlorpromazine-methochlorpromazine on intact cells are shown in Fig. 4.

Erythrocyte ghosts prepared as indicated in Materials and Methods show a somewhat mottled surface morphology (Fig. 5A). There is no difficulty, however, in recognizing the crenation of the ghosts induced by 10 mM 2,4-dinitrophenol (Fig. 5B) or the cupping induced by 0.1 mM chlorpromazine (Fig. 5C). Methochlorpromazine (0.1 mM) also cups the ghosts (Fig. 5D); at this low concentration it has hardly any effect on the shape of the intact erythrocyte (Fig. 4).

In order to analyze the shape changes induced by drugs in intact erythrocytes, consider the results for crenators first. As the concentration of a crenator was increased up to the concentration affording maximum hemolysis protection, the character of the crenation of the erythrocyte changed progressively. Although a considerable heterogeneity of crenated shapes was observed (Fig. 4) at any one concentration of added crenator, it is clear that the number of projections increased and their average size decreased as the crenator concentration increased. From the numbers and geometry of these projections the areas of the outer and inner surfaces of the entire cell could be approximated. These calculations will be presented in detail elsewhere. At concentrations of the crenator methochlorpromazine of 0.2 mM, 0.6 mM, and 2.0 mM (Fig. 4, top), the overall ratios of the areas of the outer and inner surfaces of the intact erythrocyte (Fig. 4).

With increasing concentrations of the cup-former chlorpromazine, a more complex sequence of shape changes occurs (Fig. 4, bottom). Small invaginations increasingly appear, sometimes within larger invaginations. These small invaginations, because of their small radii of curvature, must contribute to the values significantly less than 1.00. It is probable, therefore, that there is a monotonic decrease of with increasing chlorpromazine concentration, which

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**Fig. 4.** Scanning electron micrographs of erythrocytes treated with (top row, left to right) 0.2 mM, 0.6 mM, and 2.0 mM methochlorpromazine; and (bottom row, left to right) 6.0 \( \mu M \), 20 \( \mu M \), and 60 \( \mu M \) chlorpromazine.

**Fig. 5.** Scanning electron micrographs of erythrocyte ghosts. (A) Control; (B) with 10 mM 2,4-dinitrophenol; (C) with 0.1 mM chlorpromazine; and (D) with 0.1 mM methochlorpromazine.
is parallel but opposite to the monotonic increase observed with the crenator methochlorpromazine.

With concentrations of benzyl (25 mM), heptyl (5 mM), octyl (1 mM), or decyl (0.5 mM) alcohol that afforded erythrocytes greater than 50% protection against hemolysis, no change in the biconcave cell shape was observed.

Neuraminidase treatment of intact erythrocytes did not change detectably the extent of cup-formation induced by 20 μM chlorpromazine.

**DISCUSSION**

We propose that membranes forming a closed surface, whose proteins and polar lipids are asymmetrically distributed in the two halves of the membrane bilayer, can act as bilayer couples. That is, the two halves can respond differently to a perturbation while remaining coupled to one another. We have applied this hypothesis to suggest a detailed molecular mechanism for the interactions of various drugs with erythrocytes. This mechanism can explain qualitatively the manifold characteristics of drug-erythrocyte interactions, including the shape changes induced in the intact cell. In addition, several experimental predictions of the proposed mechanism have been confirmed. That methochlorpromazine is a crenator of the intact erythrocyte but induces permeable erythrocyte ghosts to form cup-shapes, is particularly striking evidence for the propositions that (i) differential expansion of the exterior half of the bilayer leads to crenation, and of the cytoplasmic half to cup-formation; and (ii) that a negative field on the cytoplasmic surface of the membrane attracts the cationic cup-formers to that surface. The erythrocyte shape changes induced by crenators can be explained by the expansion of the exterior surface area of the membrane relative to the cytoplasmic surface area. It is particularly impressive that less than a 1% increase in this area ratio can account for such a profound change in the morphology of the cell (Fig. 4, top left).

While these new experimental findings, therefore, provide strong additional support for the bilayer-couple mechanism of drug-erythrocyte interactions, they do not prove that each of the many individual elements in the proposal is correct. It would be desirable, for example, to obtain direct evidence about the proposed distributions of different drugs in the two halves of the erythrocyte bilayer by appropriate binding and exchange experiments with right-side-out and inside-out ghosts (5).

That the hydrophobic alcohols, at concentrations that protect erythrocytes against hypotonic hemolysis, do not alter the shape of the cell further demonstrates the importance of electrostatic factors in the shape changes induced by crenators and cup-formers. The sialic acid residues of the membrane glycoproteins do not appear to be involved, however, since the drug-induced shape changes are unaffected by their removal. Also, the fact that ghosts and intact erythrocytes undergo very similar drug-induced shape changes indicates that the resting potential of the intact cell [about 10 mV negative inside (16)] is not of primary importance to these changes. These results are all consistent with, but do not prove, the suggestion that it is the phosphatidylserine in the cytoplasmic half of the membrane bilayer that provides the relevant negative field that attracts cationic amphipaths into, and repels anionic amphipaths out of, the cytoplasmic half. If the enzyme phosphatidylserine decarboxylase (17) could act on erythrocyte ghosts, this might provide a direct test of the role of phosphatidylserine in drug-binding.

The possible generality of the proposed bilayer couple mechanism of drug-membrane interactions to other than erythrocyte membranes cannot be dwelt upon here. Whether, for example, an asymmetrical distribution of anionic phospholipids exists in other membranes is not known. It is worth noting, however, the striking correspondence of certain results that have been obtained with nerve membranes to those described for erythrocytes. Narahashi and his colleagues (18, 19) have provided strong evidence that it is the positively charged form of local anesthetics, acting on the cytoplasmic side of the nerve membrane, that is important in blocking the action potential. In particular, they have shown that quaternary lidocaine derivatives are much more effective blocking agents when applied on the inside than on the outside of the axon.

To explain the interactions of amphipathic drugs with membranes, differential expansion of the lipid portions of the two surfaces of the membrane bilayer has been proposed. It is possible that other perturbations, by acting on the protein components of the membrane, may also lead to a differential expansion of the two surfaces. We suggest that pinocytosis, for example follows upon the "capping" of B lymphocyte membranes by bivalent antibodies directed to receptor immunoglobulin molecules in the membrane surface, is such a phenomenon. Experiments bearing on this suggestion, involving the inhibition by erythrocyte crenators of antibody-induced lymphocyte pinocytosis, will be published elsewhere (M. Sheetz, R. G. Painter, and S. J. Singer, in preparation).

Finally, the proposed behavior of molecularly asymmetric membranes as bilayer couples may be significant to many phenomena in cell biology that involve shape changes in a cell or organelle, including cell locomotion and ruffling, microvillus formation, cell-cell and cell-virus fusion, secretion, phagocytosis, and mitochondrial energization.

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