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THE ASYMMETRIC DISTRIBUTION OF CHLORPROMAZINE AND ITS QUATERNARY ANALOGUE OVER THE ERYTHROCYTE MEMBRANE

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Abstract—The binding of chlorpromazine and its quaternary analogue chlorpromazine methoiodide to open and resealed human erythrocyte ghost membranes was studied. The results were compared with binding to liposomes of phosphatidylcholine or phosphatidylcholine with phosphatidylserine. The results indicate that the quaternary compound is confined to the outside face of the membrane. For both compounds two classes of binding sites are available. The strongest binding sites are mainly located on the inner surface of the membrane. The binding data suggest an asymmetric distribution of chlorpromazine in the membrane.

There is increasing evidence that the erythrocyte membrane is asymmetric with regard to proteins as well as to phospholipids. In the erythrocyte membrane the neutral phospholipids phosphatidylcholine and sphingomyelin appear to be concentrated in the outer half of the lipid bilayer, whereas phosphatidylethanolamine and phosphatidylserine are mainly present in the inner half [1–4].

The asymmetric structure of the membrane may play an important role in the mechanism of action of several membrane-active drugs. Sheetz and Singer [5] have recently proposed that the two halves of an asymmetric membrane can respond differently to a perturbation and thus act as bilayer couples. They attributed the different effects of amphipatic drugs on erythrocyte morphology to a differential distribution of the drugs in the erythrocyte membrane as a consequence of the presence of phosphatidylserine in the inner half of the membrane.

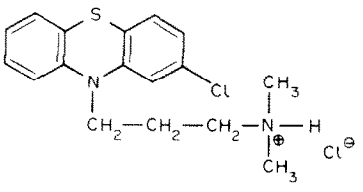
The hypothesis may be applicable in other cases. In the present investigation some quantitative aspects of drug binding in connection with membrane asymmetry will be considered. The binding of a tertiary amine (chlorpromazine HCl) and the corresponding quaternary ammonium compound (chlorpromazine methoiodide) to open and resealed human erythrocyte ghost membranes were compared. The tertiary amine and the quaternary ammonium compound show only small differences in molecular geometry but their ability to diffuse across the membrane is very different. The tertiary amine can be discharged and diffuses easily across the membrane; the quaternary compound cannot be discharged. Apart from experiments with ghost membranes model experiments with liposomes were undertaken. To evaluate the role of phosphatidylserine the binding of chlorpromazine and chlorpromazine methoiodide to liposomes consisting of phosphatidylcholine, with and without phosphatidylserine, were compared.

MATERIALS AND METHODS

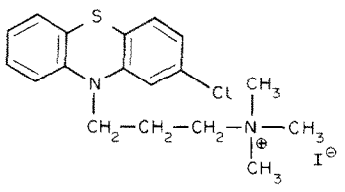
Ghosts. Resealed ghosts were prepared from human erythrocytes according to the method of Schwoch and Passow [6, 7]. Before resealing a concentrated NaCl solution was added to the final hemolysate in order to establish an intracellular concentration of 320 m Osm. Open ghosts were prepared from resealed ghosts by lysis in 20 m Osm phosphate buffer and washing four times with this buffer. The cell suspensions were diluted in order to obtain a stock suspension of 2×10^9 cells ml^{-1}. Experiments with ghosts were carried out in phosphate-buffered saline (PBS).

Reagents. Chlorpromazine-HCl was obtained from SPECIA. Chlorpromazine methoiodide was a gift from Smith, Kline and French Laboratories. Commercial egg phosphatidylcholine was purified by column chromatography; phosphatidylserine was obtained from K and K Laboratories and was used without further purification. Other chemicals were of the highest purity commercially available.

Liposomes. Liposomes were prepared from phosphatidylcholine or a mixture of phosphatidylcholine and phosphatidylserine (4:1 by weight). The lipids



Chlorpromazine – HCl



Chlorpromazine methoiodide

were dissolved in chloroform methanol and evaporated to dryness under nitrogen, forming a lipid film on the wall of the glass vessel. Then PBS was added and the mixture incubated at 40° for 30 min. The suspension was agitated with a Vortex mixer for 2 min and then sonicated (30 min for phosphatidylcholine liposomes, 15 min for phosphatidylcholine + phosphatidylserine liposomes) under a nitrogen atmosphere while cooling with ice. This procedure leads to the formation of unilamellar liposomes [8].

Binding experiments. Binding of drugs to ghosts was determined by adding the drug to a ghost suspension in PBS. Equilibrium was reached rapidly and after 10 min the suspension was centrifuged and in the supernatant the drug concentration was determined by measuring the absorbance at 260 nm. This value was corrected for leakage of material from ghosts, which also gave an absorbance at 260 nm. The value obtained was compared with that of a reference drug solution. Drug binding to liposomes was determined by equilibrium dialysis with a Dianorm apparatus. Liposomes in PBS, with or without drug, were dialyzed against PBS. After 3 hr the drug concentration in the outer compartment was measured. The values obtained were compared with those of reference drug solutions, dialyzed in the same way. This was necessary because there is considerable adsorption of the drug to the dialysis cell. All binding experiments were carried out at 22°.

The bound quantity was expressed as a function of total concentration. The resulting curve was used to construct a Scatchard plot, from which K_{ass} and n were derived [9, 10].

Measurement of protection against hypotonic lysis. The protective action of the drugs with regard to hypotonic lysis of human erythrocytes was measured by adding 0.5 ml of a washed erythrocyte suspension to 4.5 ml of phosphate-buffered saline solution in which the drug was present. The final erythrocyte concentration was 2×10^8 cells ml^{-1}. The tonicity of the mixture was such that about 50 per cent of the erythrocytes were lysed after 10 min at room temperature in the absence of the drug (148 m Osm). After a fixed time—10 min or 2 hr—the mixture was centrifuged and the absorbance of the supernatant was measured at 540 nm and expressed as a percentage of complete hemolysis.

Determination of partition coefficients. Two ml of n-octanol was vigorously shaken with drug containing buffer (2 ml 2×10^{-5} M chlorpromazine methoiodide or 10 ml 10^{-3} M chlorpromazine). The buffer consisted of 280 m Osm NaCl and 40 m Osm phosphate pH 7.4. After centrifugation the absorbance of the drug in the aqueous phase was measured and corrected for the blanco (octanol with buffer without drug). The partition coefficient was calculated as $P = c_o/c_w$, where c_o is the concentration of the drug in the organic phase and c_w is the concentration of the drug in the aqueous phase. The reported value is the mean value of six determinations.

RESULTS

Some preliminary experiments were carried out to compare a few relevant properties of chlorpromazine and chlorpromazine methoiodide, especially with regard to their ability to diffuse across the membrane. The octanol-buffer partition coefficient was determined; this value was 1400 ± 200 for chlorpromazine and 6 ± 2 for chlorpromazine methoiodide. The action of the drugs with respect to hypotonic lysis of intact erythrocytes was determined for different incubation times to find out whether the action of the quaternary compound—as compared to chlorpromazine—is time dependent as a consequence of a slow penetration of the drug through the membrane. As can be seen in Fig. 1, the lytic action of chlorpromazine methoiodide as a function of incubation time is

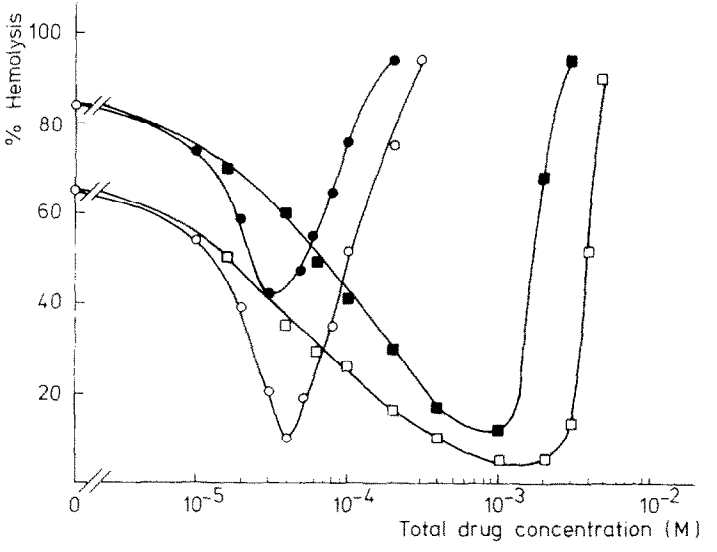


Fig. 1. Effect of chlorpromazine and chlorpromazine methoiodide on the hypotonic lysis of human erythrocytes, for different incubation times with drug before centrifugation. —○— chlorpromazine, after 10 min; —●— chlorpromazine, 2 hr; —□— chlorpromazine methoiodide, 10 min; —■— chlorpromazine methoiodide, 2 hr.

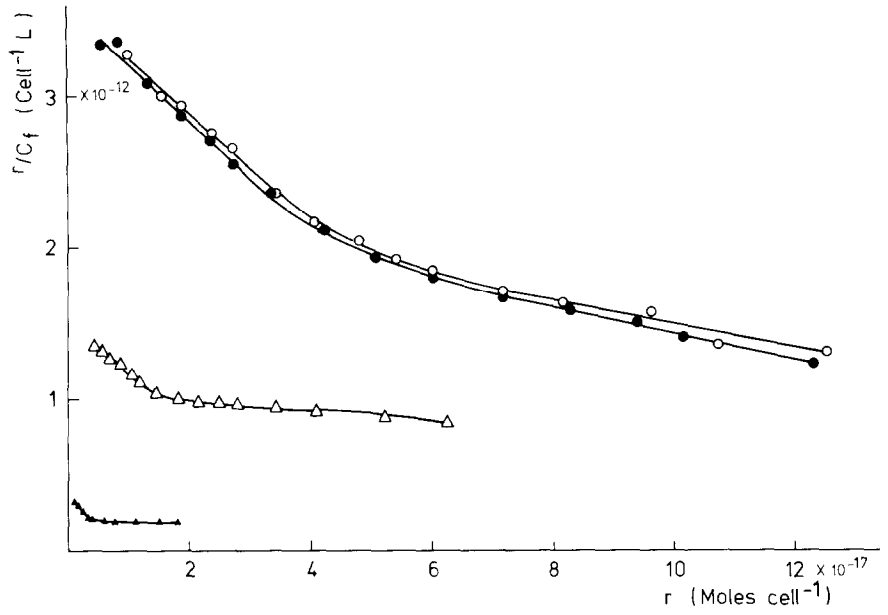


Fig. 2. Scatchard plots for the binding of the drugs to open and resealed ghosts. —○— chlorpromazine, open ghosts; —●— chlorpromazine, resealed ghosts; —△— chlorpromazine methoiodide, open ghosts; —▲— chlorpromazine methoiodide, resealed ghosts. $r = c_b/c_{cells}$, where c_b = concentration of bound drug and c_{cells} = the number of ghosts per liter; c_f = concentration of free drug.

not different from that of chlorpromazine. This was consistent with the finding that for a given drug concentration (2×10^{-5} M, for both drugs) no increase of drug uptake by ghosts can be observed after 2 hr of incubation as compared to 10 min of incubation.

From Fig. 1 it also appears that for the same protective effect a different amount of quaternary drug bound is required as compared to chlorpromazine in the curve for 10 min: the protective action of a 2.5×10^{-5} M chlorpromazine solution corresponds with that of a 6.3×10^{-5} chlorpromazine methoiodide solution. The binding percentage at these concentrations for resealed ghosts—as determined from a plot of total drug concentration versus binding percentage—are 33.5 per cent and 3.7 per cent respectively.

The binding experiments with ghosts gave data which were used to construct Scatchard plots (Figs. 2, 3). These plots are nearly equal for chlorpromazine bound to open and resealed ghosts. The quaternary compound behaves very differently with the two types of ghosts. The binding to ghosts (2×10^8 cells per ml) at 2×10^{-5} M may be used as an illustration: the binding percentage bound vs total is 34 per cent

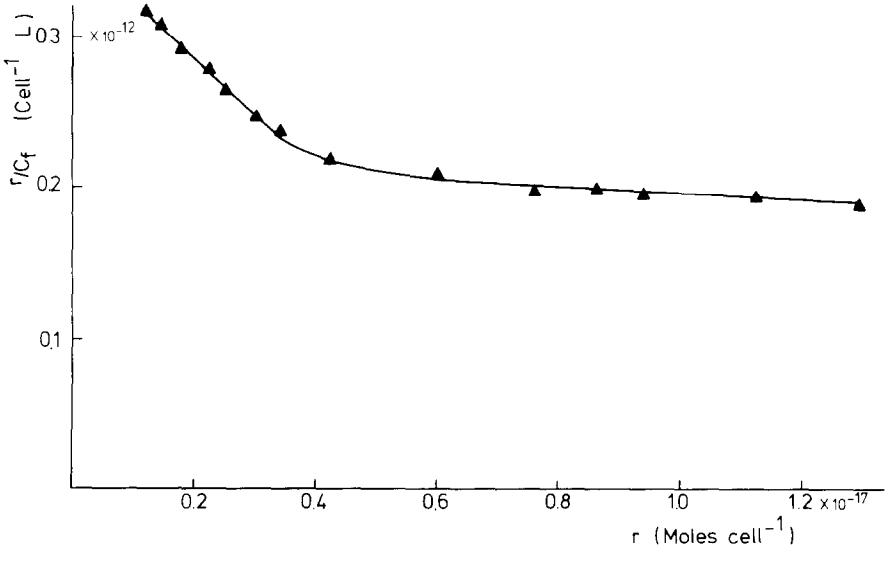


Fig. 3. Scatchard plot for the binding of chlorpromazine methoiodide to resealed ghosts.

Table 1. Association constants (K) and number of binding sites per cell (n) for the binding of chlorpromazine and chlorpromazine methoiodide to open and resealed ghosts*

		$K_1 \times 10^{-4}$ (M^{-1})	$n_1 \times 10^{-7}$	$K_2 \times 10^{-4}$ (M^{-1})	$n_2 \times 10^{-7}$
Chlorpromazine	Open ghosts	4.0 ± 0.5†	3.3 ± 0.8	1.1 ± 0.3	9.6 ± 2.4
	Resealed ghosts	3.8 ± 0.3	3.1 ± 0.7	1.1 ± 0.3	9.3 ± 2.2
Chlorpromazine methoiodide	Open ghosts	3.3 ± 0.5	2.0 ± 0.4	0.35 ± 0.06	11.0 ± 4.2
	Resealed ghosts	3.6 ± 0.4	0.35 ± 0.15	0.12 ± 0.05	7.2 ± 5.6

* For every combination of drugs and ghosts three separate series of binding data were determined. The binding data were converted into Scatchard plots from which the K and the n were determined. The binding parameters, given in the table, are thus the mean of three values.

† The K value for chlorpromazine binding to open ghosts has been determined before. There is considerable difference between the K-values, reported in literature for chlorpromazine binding to ghosts as well as for the values for chlorpromazine binding to albumin [11–16]. It is probable, as suggested by Sharpless [16], that the determination of the K-value is strongly affected by small variations in experimental conditions, e.g. temperature.

for chlorpromazine to both types of ghosts whereas for chlorpromazine methoiodide this is 15 per cent to open ghosts and 4 per cent to resealed ghosts.

The Scatchard plots show two classes of binding sites. The difference between these classes is more pronounced for the quaternary compound than for chlorpromazine. Binding parameters from the Scatchard plots are represented in Table 1. For chlorpromazine the association constants and number of binding sites are about the same for open and resealed ghosts. For the quaternary compound the K_1 is equal to the K_1 of chlorpromazine, for both types of ghosts; n_1 is very low for resealed ghosts and much higher for open ghosts, though still lower than for chlorpromazine. K_2 is lower for chlorpromazine methoiodide than for chlorpromazine. The smallest association constant is in all cases paralleled by a high binding capacity.

It proved to be difficult to derive reliable binding parameters from the Scatchard plots, constructed on the base of binding data of the drugs to liposomes. The first part of the plot showed comparative binding, in all cases. However, as a whole the plots were not suited for deriving binding parameters. In Fig. 4 the binding of the drugs to liposomes has been represented. Assuming impermeability for charged substances the concentration of liposomes for chlorpromazine methoiodide binding was made twice as high as for chlorpromazine, to compare the drug binding to a comparable amount of liposome surface. As can be seen in Fig. 4, binding was significantly lower for the quaternary compound as compared to chlorpromazine, for a comparable amount of liposome surface. As compared to resealed ghosts, there is a considerable binding of drugs to phosphatidylcholine liposomes. The introduction of the negatively charged phosphatidylserine into liposomes causes an increase in drug binding; this applies to both drugs.

DISCUSSION

The biological actions of tertiary amines like chlorpromazine differ from those of quaternary compounds like chlorpromazine methoiodide [17 20]. The hypothesis has been presented that this may be ascribed to the fact that amines in the discharged form can pass the membrane easily. Therefore they can be bound to both faces of the membrane, contrary to the quaternary analogues which are presumed to be bound only to the outer layer of the membrane [21, 22]. However, besides a permanent positive charge chlorpromazine methoiodide also possesses an extensive hydrophobic part in its molecule as is evident from a rather high octanol-buffer partition coefficient. This causes a degree of uncertainty with regard to the statement that charged compounds like chlorpromazine methoiodide cannot pass the membrane. The experiments in this investigation were intended to evaluate this hypothesis and to study the influence of membrane asymmetry on the location of these types of drugs in the membrane.

Erythrocyte ghost membranes, under specific conditions, are able to reseal themselves, forming resealed ghosts with properties which resemble those of intact erythrocytes [6, 7]. Therefore these structures are very suited for this investigation because it might be expected that a difference in drug permeability will be reflected as a difference in drug binding to the membrane. The binding experiments show that the quaternary compound is bound to a much larger extent to open ghosts than to resealed ghosts. With chlorpromazine the difference between open and

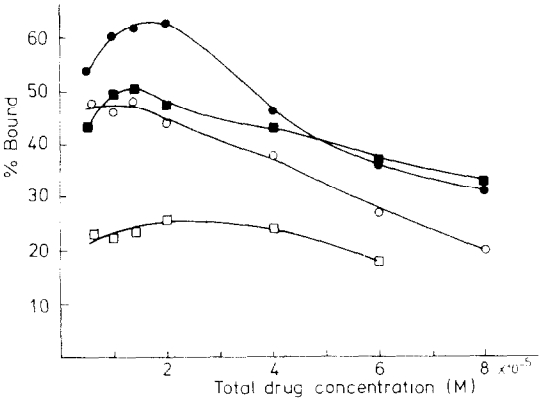


Fig. 4. Binding of the drugs to liposomes of phosphatidylcholine or phosphatidylcholine with phosphatidylserine. Lipid concentration in liposomes: 500 μg lipid/5 ml for chlorpromazine and 1000 μg lipid/5 ml for the quaternary compound. (10^9 human erythrocyte ghosts contain about 500 μg lipid [26, 27]. —○— chlorpromazine, liposomes of phosphatidylcholine; —●— chlorpromazine, liposomes of phosphatidylcholine with phosphatidylserine; —□— chlorpromazine methoiodide, liposomes of phosphatidylcholine; —■— chlorpromazine methoiodide, liposomes of phosphatidylcholine with phosphatidylserine.

resealed ghosts is negligible. Obviously in open ghosts a larger quantity of binding sites is accessible than in resealed ghosts. On this basis we may tentatively conclude that the quaternary compound does not pass the membrane despite the presence of a hydrophobic moiety in the molecule. The results, represented in Fig. 1, are consistent with this view.

Both drugs have two classes of binding sites. The affinity for the strongest binding sites is equal for both drugs in open and resealed ghosts (Table 1). The number of sites for chlorpromazine methoiodide in resealed ghosts is very low. The number of sites for this compound in open ghosts is much larger. Evidently many sites are located on the inner face of the membrane.

With regard to the second class of binding sites there is a considerable difference between the association constants: K_2 being smaller for chlorpromazine methoiodide than for chlorpromazine. This suggests a difference in either the binding site or the nature of the interaction. Studies concerning the interaction of quaternary ammonium compounds and lipid bilayers have shown, that the geometry of the cationic group has an important influence on the way of binding [23]. It may be that in erythrocyte membranes too the geometry of the ammonium group is of crucial importance for the interaction with the binding sites corresponding with K_2. A change of the quaternary compound in the polar headgroup, though of little importance for the geometry of the whole molecule, may result in a different interaction with the binding sites. The same phenomenon may play a role in the interaction of drugs with liposomes. As can be seen in Fig. 4, the binding of chlorpromazine to liposomes is more than that of chlorpromazine methoiodide. Unfortunately it was not possible to find out, whether this was due to a change in K or in number of binding sites. However, it is quite well possible that the difference in binding to liposomes has the same cause as the difference in K_2 for ghost membranes.

Sheetz and Singer [5] have stressed the importance of the negatively charged phospholipid phosphatidylserine in drug action. They suggested that phosphatidylserine in the cytoplasmic half of the membrane could provide a negative field, attracting cationic amphipatic drugs into the cytoplasmic half. The experiments with liposomes indeed show, that the presence of phosphatidylserine enhances drug binding. In the asymmetric erythrocyte membrane this phospholipid may contribute to a preferential binding of chlorpromazine to the inside face. However, the membrane is asymmetric both with regard to the lipid and the protein phase, most protein being located in the inner half of the membrane. In a previous study we have shown that chlorpromazine is bound to the protein as well as to lipid of the membrane [24]. Studying the interaction of spin-labeled anesthetics with the erythrocyte membrane Koblin and Wang [25] similarly reached the conclusion that these drugs were strongly bound to proteins located at the cytoplasmic surface. Therefore it seems likely that the proteins too may contribute to an asymmetric distribution of drugs over the two halves of the membrane.

From the preceding considerations it is obvious that the different effects of chlorpromazine and chlorpromazine methoiodide on intact erythrocytes and perhaps other systems may be due to:

(a) A difference in the total number of molecules bound at a given drug concentration. This is mainly due to a great number of binding sites which are attainable for chlorpromazine but not for the quaternary compound.

(b) An asymmetric distribution of the drug over the both halves of the membrane. Chlorpromazine methoiodide is confined to the outside face of the membrane. The results of the binding experiments for chlorpromazine and chlorpromazine methoiodide suggest that chlorpromazine is preferentially bound to the inside face of the membrane. The greater number of binding sites on the inside face is associated with the presence of phosphatidylserine in that side of the membrane and possibly with the presence of more protein binding sites on the inner half as compared to the outer half of the membrane. The asymmetric distribution is probably the cause of the difference in action between permanently charged compounds on one side, and tertiary amines and organic acids on the other, with regard to erythrocyte morphology [5, 12].

(c) A different binding to one of the both classes of binding sites. This supposition is based on the difference in K_2 for the two drugs, and the difference in binding to liposomes.

From the preceding reasoning it is obvious that a certain quantity chlorpromazine or chlorpromazine methoiodide, bound to the membrane, may result in a different biological effect for the two compounds. The findings, represented in Fig. 1, are in accordance with this condition, because here for the same protective effect less chlorpromazine methoiodide bound is required, as compared to chlorpromazine.

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