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Biphasic nature of the binding of cationic amphipaths with artificial and biological membranes

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We have studied the interaction with liposomes and red cell membrane of various cationic amphipaths, chlorpromazine, methochlorpromazine, imipramine and propranolol. At low concentrations the interaction is a partition of the molecule between the lipid hydrophobic phase and the aqueous medium. The extent of the partition is dependent on the membrane composition or physical properties, on the incubation conditions (pH, ions) and on the amphipath used. After a given amount of amphipath has entered in the membrane, a new type of interaction appears which leads to an apparent saturable association. This association, which probably involves the anionic groups of the membrane components, might result from structural or/and electrical membrane perturbations induced by the presence of drug molecules between the phospholipids. Thus the interaction of a molecule of cationic amphipath with a membrane varies according to the amount of drug present.

Interaction of amphipathic molecules with membranes have been extensively studied over the past decades. It has been widely accepted that amphipaths partition into the membrane bilayer by interaction between the lipid molecules [1–4]. Few years ago, Conrad and Singer [5,6] questioned this concept. Using hygroscopic desorption, they found that amphipaths have a different interaction with biological membranes than with artificial membranes. Responsible for that would be the presence of proteins and/or cholesterol in the former ones which would reduce drastically the partition coefficients. Nevertheless, conflicting results obtained with the same system were later published [7–9]. Luxnat and Galla [9] showed that chlorpromazine concentration used in the experiments may be critical for the interaction mechanism involved between the drug and the lipidic structure; amphipaths behaving as detergents may solubilize the membrane [9,10]. Consequently the loss of some membrane material will lower the apparent association of drug to the membrane.

The goal of the present study is to extend these observations to a set of cationic amphipaths and to membranes of various composition, i.e. phospholipids with or without cholesterol and/or proteins. We have shown that even for amphipath concentration lower than the critical micellar concentration, i.e. at a concentration where membrane solubilization does not happen, the association to membranes departs from simple partitionning which is detected only at very low concentration (e.g. 1 μM chlorpromazine). Thus at intermediate concentration (2 to 200 μM) the interaction cannot be anymore considered as a partition but looks like a saturable phenomenon. The question remains whether this apparent saturable as-

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sociation reflects a binding to some sites or is the consequence of electrical properties of the membrane. However, this association occurs presumably after some changes in the physical chemistry of the membrane induced by the first molecules of amphipath which have intercalated in the bilayer.

L-α-Phosphatidylcholine from soybean (Sigma), a mixture of several phospholipids, will be referred to as soybean lipids. Pure phosphatidylcholine was prepared from soybean lipids by high performance liquid chromatography on silica column. Methochlorpromazine and its tritiated analog were synthesized as described [11]. The radioactive analogs of the other amphipaths were of the highest specific activity available and obtained from New England Nuclear or Amersham. M^{13} coat protein was prepared according to Knippers and Hoffman-Berling [12]. Freshly drawn human blood was used as a source of erythrocytes. Unsealed red cell ghosts were prepared by hypotonic lysis (15 mOsM). Large unilamellar liposomes were prepared by the method of Szoka and Papahadjopoulos [13]; one volume of buffer supplemented with 5% inulin ($d = 1.019$) was sonicated with three volumes of lipid solution (20 mg/ml in diethyl ether or chloroform) and evaporated slowly. The fraction of liposome used was non-sedimentable after centrifugation at $3000 \times g$ for 10 min, but pelleted after centrifugation at $20000 \times g$ for 20 min. Liposomes could pass through a polycarbonate filter (pore diameter: 0.2 μm). Binding experiments were run as follows: Tubes containing a fixed amount of membranes (usually 100 μM of lipids) and various concentrations of drug (0.1 to 600 μM) in a total volume of 1 ml (130 mM NaCl, 0.1 mM EDTA, 20 mM Tris-HCl buffer, pH 7.4) were incubated at 37 °C (unless otherwise specified) for 30 min. At that time two 100-μl aliquots were taken in order to measure the total drug concentration and the remaining 800 μl centrifuged 30 min at $27000 \times g$. Then two 100-μl aliquots were sampled from the supernatant in order to determine the free drug concentration.

The amount of chlorpromazine associated with the pelleted membranes as a function of drug concentration is displayed in Fig. 1. Whether vesicles are made from phosphatidylcholine or from soybean lipids, chlorpromazine seems to associate in a saturable manner to these membranes. The same type of curve is obtained with red cell ghosts. However, data obtained with the lower chlorpromazine concentrations can be plotted at an expanded scale (Fig. 2). The conclusions are then drastically different: up to approx. 1 μM chlorpromazine the amount of drug associated with the membranes is proportional to the amount

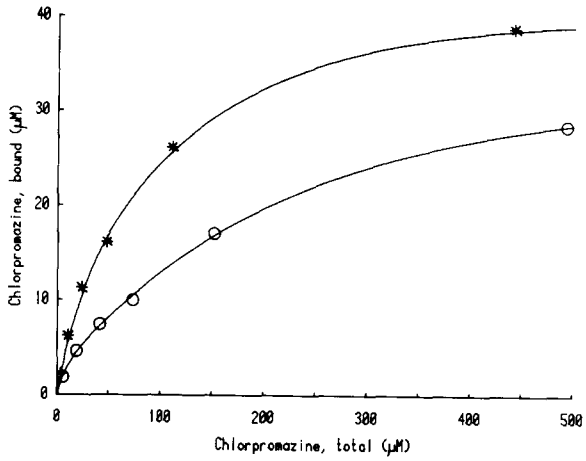

Fig. 1. Plot of the concentration of chlorpromazine bound to phosphatidylcholine (○) or soybean lipid (*) vesicles vs. total concentration in the incubation (100 nmol of lipid in 1 ml; 37 °C, pH 7.4).

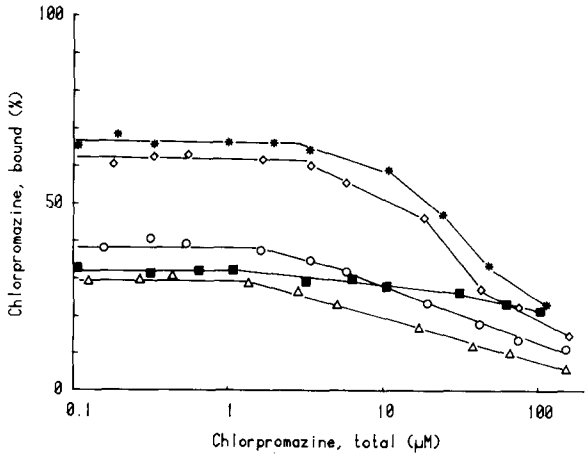

Fig. 2. Percentage of the incubated chlorpromazine associated with the membrane as a function of the concentration (logarithmic scale) present in the incubation (conditions as in Fig. 1). ○, Phosphatidylcholine vesicles; *, soybean lipid vesicles; △, phosphatidylcholine-M^{13}coat protein vesicles; ◇, soybean lipid-cholesterol vesicles; ■, red cell ghosts.

TABLE I

PARTITION COEFFICIENTS OF CHLORPROMAZINE INTO DIFFERENT MEMBRANES (37 °C, pH 7.4)

The coefficients were obtained using low chlorpromazine concentrations ('plateau region' of Fig. 2) and lipid amounts from 100 to 1600 nmol per incubation. Data are the mean of at least three independent sets of experiments.

Membrane composition	K_p
Soybean lipids	23200 ± 1900
Soybean lipids + cholesterol (4 : 1, mol/mol)	17000 ± 1400
Phosphatidylcholine	8500 ± 800
Phosphatidylcholine + M^{13}-coat protein (80 : 1, mol/mol)	5400 ± 300
Red cell ghosts	6300 ± 700

present in the medium. Above this threshold, the proportionnality is lost. From the plateau region of the curves displayed in Fig. 2, we can infer partition coefficients:

$$K_p = (CPZ_m/CPZ_{aq}) \cdot (V_{aq}/V_m)$$

where CPZ is chlorpromazine (in moles) in membrane (m) or aqueous buffer (aq), V_{aq} is the volume of the aqueous buffer and V_m is the volume of membrane calculated assuming a density of 1 g/ml for lipids. As expected, the coefficients depend on the membrane composition (Table I). This partition ends when relatively few molecules of chlorpromazine are inserted between the lipid molecules; 1 : 200 for ghosts or 1 : 50 for soybean lipid vesicles. For high concentrations, the association becomes apparently saturable. One explanation of such a behavior results from changes in electrical properties of the membrane (see the discussion below). But if this saturable association is considered to reflect binding of chlorpromazine molecules to sites, data may be linearized as Langmuir isothermal adsorption curves, suitable for interactions with surfaces, using the following equation [14]:

$$A_f = (N \cdot PL \cdot A_f/A_b) - K_d$$

where A_f and A_b are the concentrations of free and bound amphipath; PL is the concentration of phospholipid; N is the asymptote of A_b/PL and K_d is the dissociation constant. Data can be fitted by two straight lines (not shown), arguing for two isothermal adsorption processes [15] whose parameters are listed in Table II. Here again, although membrane chemical composition affects the calculated values, the behavior of chlorpromazine is grossly similar in any system tested.

As in pure homogeneous lipid systems temperature affects the physical properties of a membrane (liquid crystal-gel phase transition), we assayed chlorpromazine association to dimyristoylphosphatidylcholine liposomes at 10 and 37 °C, i.e. below and above the transition temperature. As already reported [9], the partition coefficient is lower in the gel phase than in the liquid crystal (K_p values 1400 and 8000, respectively). A striking difference with the other membranes tested is the chlorpromazine concentration where the partition coefficient ceases being constant; in accordance with Luxnat and Galla [9] K_p remains constant up to 30 μM chlorpromazine added before decreasing for higher drug concentrations.

TABLE II

BINDING CHARACTERISTICS OF CHLORPROMAZINE TO VARIOUS MEMBRANES (37 °C, pH 7.4)

First and second bindings refer to the two curves obtained in the isotherms. N represents the number of moles of chlorpromazine bound per mole of phospholipid at saturation. Values are given ± S.E.

Membrane	First binding		Second binding	
	N	K_d(μM)	N	K_d(μM)
Phosphatidylcholine	0.108 ± 0.007	17.3 ± 1.8	0.378 ± 0.028	158.3 ± 21.3
Phosphatidylcholine + M^{13}-coat protein	0.052 ± 0.004	12.0 ± 1.1	0.211 ± 0.028	139.5 ± 46.8
Soybean lipids	0.167 ± 0.046	8.3 ± 2.6	0.430 ± 0.045	47.1 ± 8.2
Soybean lipids + cholesterol	0.177 ± 0.012	9.6 ± 0.8	0.536 ± 0.044	116.5 ± 27.1
Red cell ghosts	0.192 ± 0.021	28.4 ± 3.3	1.245 ± 0.046	236.5 ± 15.9

Moreover, the temperature also plays a role on the saturable binding as the dissociation constant is three times higher at 10 °C than at 37 °C (90.2 ± 6.8 μM and 29.4 ± 6.6 μM, respectively). Calcium is antagonist to chlorpromazine binding to membranes if negatively charged phospholipids are present; addition of 10 mM Ca^{2+} in the incubation has no effect as far as phosphatidylcholine vesicles are used, neither the partition nor the subsequent apparent saturable binding being noticeably modified. With red cell ghosts, calcium ions affect both the partition coefficient (33% decreased) and the dissociation constant of the first binding which doubles (from 28.4 to 49.7 μM). Depending on the pH of the medium, chlorpromazine may be more or less protonated on the nitrogen atom. As membranes, especially biological, protein-containing ones, also bear dissociable groups which might be involved in the apparent saturable association, the effect of pH was studied solely on the partition. For phosphatidylcholine vesicles at pH 3.0 (formic acid-sodium formate buffered saline), 4.0 (acetic acid-sodium acetate buffered saline), 7.4 (Tris-buffered saline) and 8.5 (phosphate-buffered saline), K_p is respectively 3900, 7200, 8500 and 12000. With red cell ghosts, the pH scale was reduced and the partition coefficient also increases with the pH, from 5100 (pH 6.0, acetic acid-sodium acetate buffered saline) to 6300 (pH 7.4) and 7000 (pH 8.5).

Methochlorpromazine, imipramine and propranolol behave like chlorpromazine. Partition coefficients at low drug concentration (Table III) are of the same order of magnitude than those deduced for chlorpromazine. Nonetheless, the differences between these compounds are at the concentration where the amount bound ceases to be proportional to the amount added in the incubation. For instance, with imipramine, the partition ends when approximately one molecule of drug is present for 20 molecules of phospholipid in the membrane, which is more than for chlorpromazine (approx. 1 for 100).

The data reported above show that the association of cationic amphipaths such as chlorpromazine to membranes represents a complex phenomenon: the first stage of the interaction involves a partition of the compound between the buffer and the phospholipid bilayer; then, the association departs from a simple equilibrium of respective solubilities in a hydrophilic buffer and a hydrophobic lipid bilayer.

TABLE III

PARTITION COEFFICIENTS OF SOME CATIONIC AMPHIPATHS INTO VARIOUS MEMBRANES (37 °C, pH 7.4)

Values are obtained as in Table I.

Amphipath	Membrane composition	K_p
Imipramine	Soybean lipids	32900 ± 2300
	Phosphatidylcholine	11900 ± 900
	Red cell ghosts	6200 ± 400
Methochlor-promazine	Soybean lipids	9800 ± 700
	Phosphatidylcholine	2100 ± 400
Propranolol	Red cell ghosts	3100 ± 400

The existence of partition at low amphipath concentration is characterized by a percentage of association which does not vary with the amount of compound; this type of results was previously obtained with artificial membranes [9] and red cell membranes [7] and the partition coefficients determined here are in agreement with those published. Several parameters such as the nature of the phospholipids affect the partition. Indeed a higher interaction is found with soybean total lipids than with soybean phosphatidylcholine. The presence of cholesterol decreases the partition as well as a gel phase in the membrane [9]. The presence of proteins in the lipid bilayer also reduces the partition coefficient as shown with ghosts and also with proteoliposomes containing the coat protein of phage M^{13}. However, the presence of cholesterol and proteins does not abolish the partition phenomenon since the partition coefficient remains well above one. The electrical properties of the membrane bilayer [16] may help to understand some of these results. For instance, the presence of a negative net charge on phosphatidylserine (in soybean lipid vesicles) produces a double layer potential which enhances the concentration of the drug at the membrane/solution interface. Thus more amphipath will be available for partition. However, the fact that the molecules tested bind to phosphatidylcholine liposomes, whose surface is uncharged, shows that the inter-

action drug-membrane is not purely electrostatic in nature and that hydrophobic forces must contribute to the binding energy [16,17]. Apart from the membrane content, the buffer composition also plays a role on the equilibrium. For phosphatidylcholine vesicles or red cell ghosts, increasing the pH of the aqueous phase led to higher partition coefficient; this coefficient estimated with red cell ghosts changes by about one-third between pH 8.5 and pH 6.0. As chlorpromazine pK is 9.2, the proportion of uncharged species varies 260 times in this pH interval; consequently both uncharged and protonated forms of chlorpromazine are involved in the partition. The involvement of the two forms of an amphipath in membrane binding was already demonstrated with other cationic amphipaths [18] or with anionic fatty acid analogues [19]. The compound should then intercalate its hydrophobic moiety between the phospholipid molecules and point its hydrophilic moiety towards the membrane surface.

If, with increasing amphipath concentration, results are still expressed as partition coefficient, one can see that this parameter value decreases as already quoted [7,9,20]. It must be stressed that, with the exception of membranes made from dimyristoylphosphatidylcholine, this phenomenon takes place at chlorpromazine concentrations lower than the micellar concentration (50 μM) where some membrane solubilization occurs [9]. Thus the departure from partition cannot be explained solely by a detergent-like activity of chlorpromazine on the membrane. In fact, if the incubation contains 60 μM chlorpromazine for instance, it is possible to pellet all the drug provided enough membranes are present, showing that no significant solubilization has occurred. Omitting the conditions where partition takes place, binding data could be interpreted as if two distinct populations of sites exist in the membrane: for chlorpromazine, the first population exhibits between 10 and 20 sites for 100 lipids with a dissociation constant varying between 10 and 30 μM; the second population possesses more sites (up to 1 site per phospholipid) of lower affinity (K_d around 100 μM). A saturable interaction of chlorpromazine with red cell membranes [3], even on two sites [4], was already described for a drug concentration of several micromolar. The occupancy of these sites may be related to well known action of such molecule on cell behavior: the first population would be related to membrane stabilization as detected by hemolysis protection which takes place below 20 μM chlorpromazine [3] and the second population would represent the sites related to cell lysis by membrane solubilization. However, one must keep in mind that this second binding site population might be artefactual and due to micellization of the amphipath: once micellation occurs, there is an almost insignificant increase in activity of the amphipath with increasing concentration of the aqueous phase leading to flattening of the binding curve.

The question of the mechanism underlying this apparent saturable association remains unanswered. One explanation considers the electrical properties of the membrane. The charged form of the amphipath adsorbed to the bilayer would produce, in the case of phosphatidylcholine membranes, an electrical double layer which would repulse the other drug molecules. If the membrane contains negatively charged lipids, the original double layer potential would be reduced by the cationic amphipath molecules in the membrane, thus being less attractive to other molecules. The apparent saturation would be due to this change in the surface potential, having for consequence that the concentration of chlorpromazine in the aqueous phase adjacent to the membrane does not increase proportionnaly to the bulk aqueous concentration [21]. Consequently, even with the partition phenomenon unaltered, the ratio drug bound/drug present in the bulk phase will diminish, leading to an apparent saturation. The decreased association to the latter type of membrane noticed in presence of Ca^{2+} is the consequence of binding of this ion to the surface negative charges which reduces the surface potential [16]. Nevertheless, some direct binding of cationic amphipath to anionic membrane residues could occur. In the case of liposomes, these negative groups belong to the phospholipid molecule. If phosphatidylserine is present, the carboxyl residue can be one of these groups, but in the case of phosphatidylcholine, the only anionic group is the phosphate which was shown to be engaged into strong electrostatic interactions (binding) with cationic amphipaths [22,23]. In a biological membrane, many other

anionic groups, namely some amino-acid and sialic acid residues, are available [22,24,25]. As these groups are always present, it seems reasonable to think that the emergence of the saturable binding results from some membrane alterations. The limit of the strict proportional binding corresponds to a number of incorporated molecules which varies with the amphipath (five times more imipramine molecules than chlorpromazine ones), and a mere change of surface potential cannot be solely accounted for this binding modification. In fact, the cationic amphipaths affect the packing of the phospholipid molecules in a membrane [26] and can lead to membrane alterations, such as hemolysis protection [3], or morphological changes [27]. One might hypothetize that the disturbance due to the intercalation of few amphipath molecules between the lipids affects sufficiently the membrane to change the binding characteristics. This change is not more pronounced in presence of cholesterol and proteins and thus artificial membranes are fairly good representatives of biological membranes in these studies.

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