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CHLORPROMAZINE INTERACTIONS WITH LIPID BILAYERS

V.M. Ioffe, O.K. Zakharenko, G.P. Gorbenko

V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077

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The effect of cationic drug chlorpromazine (CPZ) on the structural state of model lipid membranes composed of zwitterionic phospholipid phosphatidylcholine (PC) and anionic phospholipid cardiolipin (CL) in molar ratios 8:2 and 3:2 has been investigated using the indicator dye Neutral Red (NR). CPZ incorporation into the PC/CL (8:2) liposomes led to the increase of NR partition coefficients. This effect was interpreted in terms of drug-induced membrane disordering. In contrast, CPZ association with PC/CL (3:2) lipid bilayers suppressed the dye partition which was assumed to be a result of lipid phase transition.

KEY WORDS: lipid bilayer, chlorpromazine, Neutral Red, partition coefficient

Despite the extensive research efforts the exact molecular mechanisms of anaesthesia are far from being fully understood. A wide variety of existing theories diverge along two apparently incompatible lines: one, predicted by Meyer-Overton rule, assumes that membrane lipids are target for anaesthetic compounds and drug potency strongly correlates with their solubility in lipid environment, while the other suggests that the drugs bind directly to the proteins whose altered conformation would subsequently determine the anaesthetic action [1]. However, the above hypotheses could not provide a satisfactory explanation for the exact mechanism of anaesthesia. During the last decades the idea implying the indirect action of drugs on the proteins via perturbations of the lipid bilayer including phase separation, change in order parameter, curvature, lateral pressure, etc. is becoming generally recognized. To develop a unique conception of anaesthesia further investigations of drug-membrane interactions are required. In clarifying the molecular mechanisms of anaesthesia the model lipid and protein-lipid membrane systems appear to be particularly suitable. The present study was undertaken to explore the effect of amphipathic phenothiazine derivative chlorpromazine (CPZ) on the structural state of model lipid membranes composed of zwitterionic phospholipid phosphatidylcholine (PC) and anionic phospholipid cardiolipin (CL) in molar ratios 8:2 and 3:2. Indicator dye Neutral Red (NR) whose acid-base behavior is environmentally-sensitive has been employed to examine chlorpromazine interactions with the model membranes. CPZ is an antipsychotic, antagonistic drug which apart from its traditional medical usage, has also been employed as anticancer agent [2]. Accumulating evidence indicates that chlorpromazine association with membrane is a versatile process. Specifically, anaesthetic influence on microsomal cells was reported to involve membrane protection against loss of fluidity, while in erythrocytes CPZ causes concave membrane bending with formation of stomatocytes [3]. It is assumed that drug actions occur via the perturbations of bilayer integrity and modulation of its physicochemical properties. A vast majority of studies suggest that possible effects of this drug on membrane properties may include the changes in conformation of lipid acyl chains (*trans-gauche* isomerization), membrane curvature, microheterogeneity (phase separation, domain formation), membrane thickness, just to name a few. The magnitude and sign of all these modifications varies with lipid bilayer composition [4]. Deeper understanding of CPZ-lipid interactions is also crucial for the design of liposome-based drug carriers with reduced undesirable side effects since CPZ lipophilicity favors its nonspecific association with biological membranes.

MATERIALS AND METHODS

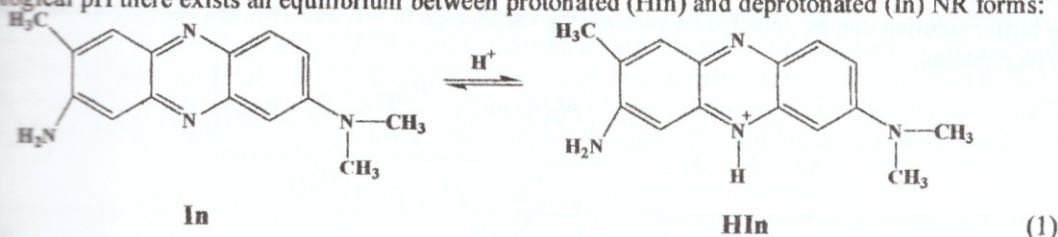
Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Bielek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2, v/v. CPZ was from Sigma (St. Louis, MO, USA). Unilamellar lipid vesicles composed of PC and its mixtures with 20 or 40 mol % CL were prepared by the ethanol injection method, developed by Batzri and Korn [5]. 1 ml of ethanol lipid solution containing appropriate amounts of lipids was injected into 13 ml of 5 mM phosphate buffer (pH 7.4) under continuous stirring. Ethanol was then removed by dialysis. The phospholipid concentration was determined according to the procedure of Bartlett [6]. To incorporate CPZ into the lipid bilayers, liposomal suspensions were incubated with the anaesthetic for 30 min at room temperature to yield a final drug concentration 30 mol %. Absorption measurements with NR were performed using SF-46 spectrophotometer against solvent blanks. NR concentration was determined

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spectrophotometrically using the extinction coefficients of the dye protonated (HIn) and deprotonated (In) forms at 525 nm $\epsilon_{HIn} = 2.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{In} = 2.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

THEORY

At physiological pH there exists an equilibrium between protonated (HIn) and deprotonated (In) NR forms:



The thermodynamic dissociation constant for NR in buffer solution can be written as:

$$K_d = \frac{F_{\text{H}^+} \cdot F_{\text{In}}^0}{F_{\text{HIn}}^0} \quad (2)$$

where F_{H^+} , F_{In}^0 , F_{HIn}^0 are the concentrations of the protons, deprotonated and protonated NR forms ($\text{mol} \times \text{dm}^{-3}$), respectively. Denoting the dye total concentration by D_0 ($4 \times 10^{-5} \text{ M}$ in our experiments) one obtains:

$$D_0 = F_{\text{In}}^0 + F_{\text{HIn}}^0; \quad F_{\text{In}}^0 = \frac{D_0}{1 + \frac{F_{\text{H}^+}}{K_d}}; \quad F_{\text{HIn}}^0 = \frac{D_0 \cdot \frac{F_{\text{H}^+}}{K_d}}{1 + \frac{F_{\text{H}^+}}{K_d}} \quad (3)$$

In liposome suspension the above protolytic equilibrium is shifted due to NR distribution between aqueous (w) and lipid (L) phases. In this case D_0 is given by:

$$D_0 = F_{\text{In}} + F_{\text{HIn}} + B_{\text{In}}^L + B_{\text{HIn}}^L \quad (4)$$

where F_{In} , F_{HIn} are the concentrations of deprotonated and protonated dye forms free in solution, B_{In}^L , B_{HIn}^L are the concentrations of the deprotonated and protonated dye species, respectively, bound to the lipid vesicles. The dye partition coefficients can be defined as:

$$P_{\text{HIn}}^L = \frac{B_{\text{HIn}}^L v_w}{F_{\text{HIn}}^L v_L}; \quad P_{\text{In}}^L = \frac{B_{\text{In}}^L v_w}{F_{\text{In}}^L v_L} \quad (5)$$

where v_w , v_L are the volumes of aqueous and lipid phases. The volume of lipid phase was calculated as:

$$v_L = N_A C_L \sum V_i f_i \quad (6)$$

where C_L is the molar lipid concentration, f_i is mole fraction of the i -th bilayer constituent, V_i is its molecular volume taken as 1.58 nm^3 and 3 nm^3 for PC and CL, respectively [7]. Under the employed experimental conditions ($C_L \leq 1 \text{ mM}$) the v_L value is much less than total volume of the system ($v_t = 1 \text{ dm}^3$), so that

$v_w \approx v_t$.

Taking into account that:

$$F_{\text{HIn}} = \frac{F_{\text{In}} F_{\text{H}^+}}{K_d}; \quad B_{\text{In}}^L = \frac{F_{\text{In}} P_{\text{In}}^L v_L}{v_w}; \quad B_{\text{HIn}}^L = \frac{F_{\text{HIn}} P_{\text{HIn}}^L v_L}{v_w} = \frac{F_{\text{In}} F_{\text{H}^+} P_{\text{HIn}}^L v_L}{K_d v_w} \quad (7)$$

Eq. (4) may be rewritten as:

$$D_0 = F_{\text{In}} \left(1 + \frac{F_{\text{H}^+}}{K_d} + P_{\text{In}}^L v_L + \frac{F_{\text{H}^+} P_{\text{HIn}}^L v_L}{K_d} \right) \quad (8)$$

NR absorbance in buffer (A_0) or lipid phase (A_L) is given by:

$$A_0 = D_0 \cdot G; \quad A_L = (F_{\text{In}} + F_{\text{HIn}})G + \epsilon_{\text{HIn}}^b B_{\text{HIn}}^L + \epsilon_{\text{In}}^b B_{\text{In}}^L \quad (9)$$

where $G = \frac{\varepsilon_{HIn}}{1 + \frac{K_d}{F_{H^+}}} + \frac{\varepsilon_{In}}{1 + \frac{F_{H^+}}{K_d}}$, $\varepsilon_{HIn}^b, \varepsilon_{In}^b$ are the extinction coefficients of lipid-bound protonated and

deprotonated NR species. Assuming that $\varepsilon_{HIn}^b \cong \varepsilon_{HIn}, \varepsilon_{In}^b \cong \varepsilon_{In}$, the dye partitioning of dye into the lipid phase from the buffer solution can be detected by monitoring the change in NR absorbance (ΔA). Combining the Eqs. (3) – (9) one obtains:

$$\Delta A = A_L - A_0 = \frac{D_0 v_L \left[P_{In}^L (G - \varepsilon_{In}) - \frac{P_{HIn}^L F_{H^+}}{K_d} (G - \varepsilon_{HIn}) \right]}{1 + \frac{F_{H^+}}{K_d} + P_{In}^L v_L + \frac{F_{H^+} P_{In}^L v_L}{K_d}} \quad (10)$$

RESULTS AND DISCUSSION

The binding of NR to the lipid vesicles is followed by the increase in its absorbance, with the magnitude of this effect being dependent on membrane surface potential. Addition of chlorpromazine resulted in ambiguous changes in NR spectral properties (Fig. 1). Approximation of the experimental $\Delta A(C_L)$ dependencies by Eq. (10) allowed us to determine the partition coefficients of the protonated (P_{HIn}^L) and deprotonated (P_{In}^L) NR species into the lipid phase in the presence and absence of anaesthetic. The obtained results are summarized in Table 1.

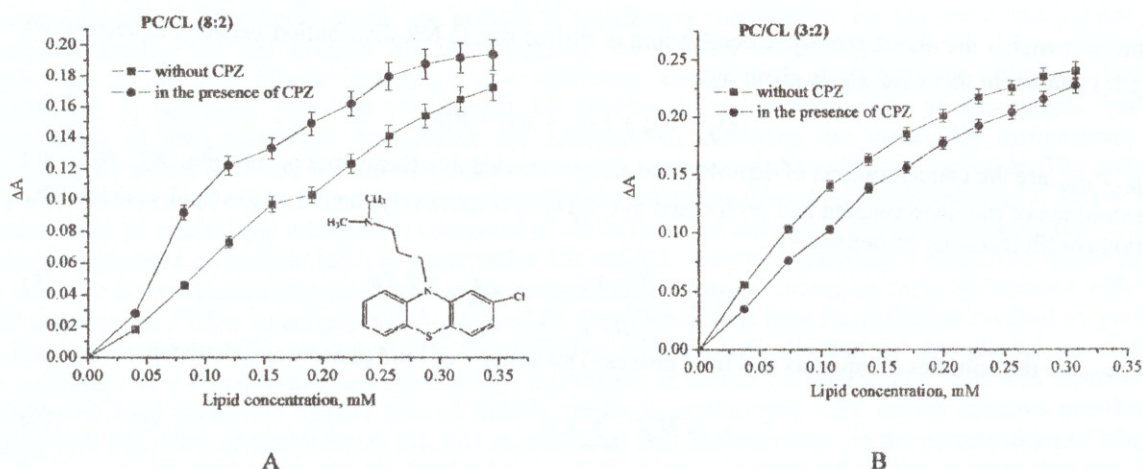


Fig. 1. Changes in NR absorbance upon the dye association with PC:CL (8:2) (A) and (3:2) (B) lipid vesicles in the presence and absence of CPZ. Shown in the inset is CPZ structure.

Higher magnitudes of P_{HIn}^L relative to P_{In}^L can be explained by the fact that electrostatic interactions between positively charged dye protonated form and anionic phospholipids favor the dye partitioning into the lipid phase. Greater values of P_{HIn}^L in the model membranes with CL content 40 mol % are in good harmony with this observation. For energetic reasons positive HIn species would, apparently, localize near the vesicle surface while neutral In species would prefer hydrophobic core of the membrane. Interestingly, enhancement of membrane surface potential also led to the rise in partitioning of NR deprotonated form indicating that increasing negative charge exerts some influence on hydrophobic region of the lipid bilayer.

As seen in Table 1, drug incorporation into PC/CL (8:2) model membranes gives rise to a considerable increase both in P_{HIn}^L and P_{In}^L , while inclusion of anaesthetic into PC/CL (3:2) lipid vesicles results in substantial decrease of the above parameters. Notably, upon formation of drug-lipid complexes change in partition coefficients of deprotonated NR form is much more pronounced for moderately charged membranes (20 mol % CL) than for highly charged ones (40 mol % CL). The above finding suggests that CPZ effect on bilayer hydrophobic core becomes weaker with the increase of membrane surface potential. One of the most probable explanations for the above observation lies in different drug location in the lipid vesicles depending on CL content. Since chlorpromazine has a pK about 9.3 [8], under the employed experimental conditions (pH 7.4) it would exist in the protonated, positively charged form. Therefore, electrostatic CPZ-CL interactions are

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expected to significantly contribute to the anaesthetic complexation with the liposomes. According to the model of CPZ location in the lipid bilayer, proposed by Nerdal *et al.* [9], tricyclic ring system of the drug penetrates the membrane hydrophobic core with the orientation parallel to the acyl chains while positive side group of CPZ resides in proximity to the anionic lipid headgroups due to their electrostatic attraction. This interaction anchors the drug molecule close to membrane surface preventing deep intercalation of the ring system into the hydrophobic interior of the bilayer. It may be expected that this model is adequate for PC/CL membranes, however, at CL content 20 mol % some fraction of CPZ would locate deeper in the acyl chain region and anaesthetic effect on this part of the bilayer is much more pronounced. In turn, when membrane is rich in anionic lipid (40 mol % CL), the number of binding sites for the drug on the bilayer surface significantly increases, CPZ position shifts closer to the lipid headgroups, and anaesthetic influence on membrane nonpolar region is suppressed.

Partition coefficients	Examined system			
	PC/CL (8:2)	PC/CL (8:2) + CPZ	PC/CL (3:2)	PC/CL (3:2) + CPZ
P_{NR}^+	6200±800	17000±650	19000±1774	11000±745
P_{NR}^-	600±100	3600±940	3500±150	1600±80

Of importance also is the fact that CPZ-induced change of NR partitioning in PC/CL (8:2) and PC/CL (3:2) model membranes has different sign. It was reported that the presence of anionic lipids in the membrane is crucial for the drug efficacy. Present results support this idea. Opposite drug effect on dye partition behavior originates, apparently, from different modifications of bilayer properties caused by chlorpromazine. In moderately charged liposomes (20 mol % CL) CPZ addition is likely to bring about the bilayer disordering and increase in membrane fluidity and free volume leading to the enhancement of NR partitioning into the lipid phase. In turn, in highly charged vesicles (40 mol % CL) these consequences of CPZ-lipid association are probably interferes with another possible drug effect – lipid phase transition. It was reported that CPZ is capable of inducing the formation of the lipid hexagonal (H_{II}) phase [10] resulting from the substantial modifications in bilayer curvature, degree of hydration, dynamics of acyl chain and headgroup motions. Due to different sizes of the headgroup and hydrophobic tails CL is known to possess propensity for conversion into H_{II} phase. It may be supposed that drug-promoted lipid phase transition gains importance with CL content, thus in PC/CL (3:2) lipid vesicles CPZ binding results in decrease of NR partitioning.

CONCLUSIONS

Examination of NR partition equilibrium revealed that membrane surface potential is crucial for chlorpromazine action. Specifically, it was found that CPZ effect on bilayer hydrophobic core becomes weaker with increase of CL content. Rise in the partition of protonated and deprotonated dye species in PC/CL (8:2) most likely stems from CPZ-induced membrane destabilization and disordering while decrease in partition coefficients of both NR forms was assumed to be a result of lipid transition into H_{II} phase.

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