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ESR studies on the effect of cholesterol on chlorpromazine interaction with saturated and unsaturated liposome membranes

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Abstract

In this study, the effects of chlorpromazine (CPZ) on lipid order and motion in saturated (DMPC, DMPG) and unsaturated (SOPC) liposome membranes were investigated by electron spin resonance (ESR) spin labeling technique. We have shown that above the main phase transition temperature of membrane lipids (T_M), CPZ slightly increases lipid order in membranes without cholesterol, whereas below T_M it has a strong opposite effect. Addition of 30 mol% of cholesterol into DMPC and SOPC membranes changes significantly the CPZ effects both above and below T_M. Additionally, above T_M, the ordering effect of CPZ on pure SOPC membrane is stronger at pH 7.4 than at pH 9.0, whereas below T_M, as well as in the presence of cholesterol, pH does not seem to play a role in CPZ effect on both membranes. Because of the strong influence of membrane composition on CPZ effect on membranes, the use of cholesterol as a marker of CPZ photosensitized reactions has been discussed.

Keywords: Chlorpromazine; Phospholipid membrane; Cholesterol; Spin label; ESR

1. Introduction

Chlorpromazine (CPZ) is a neuroleptic drug of the phenothiazine group, widely used in the treatment of certain psychiatric disorders. Its amphipathic character enables it to partition into the lipid bilayer of cell membranes, and to reach in this way the central nervous system. Interactions of CPZ with biological membranes are linked to the numerous effects in cellular systems, such as a well-known protective effect on red cells osmolysis [1] and redirecting de novo synthesis from neutral to acidic glycerophospholipids by inhibition of PA phosphatase in hepatocytes [2]. Furthermore, it has been shown that neuroleptic drugs affect the function of various receptors including adrenergic, muscarinic, histamine, 5-hydroxytryptamine and dopamine receptor families [3]. Numerous experiments on model membranes have shown that CPZ interacts with membrane phospholipids, especially with negatively charged ones [4–6], and perturbs the membrane structure. CPZ, as a basic molecule with pK = 8.6 [7], remains mostly protonated and positively charged at physiological pH, which makes it locate preferably in the membrane–water interface [8]. Its binding sites (such as negatively charged carboxyl groups) are localized near the surface of the membrane, as shown by the ESR study of Louro et al. [9]. Some data, however, suggest that CPZ may also penetrate into the acyl chain region of phospholipid membranes affecting the acyl chain order [10] and lipid phase transition [4,11]. It has been shown that the partition coefficient (kp) of CPZ into the lipid membrane decreases with the increasing phospholipid alkyl chain length [12], and can be additionally reduced by the presence of cholesterol [12,13].

The understanding of CPZ localization and behavior in membranes is interesting also from the photochemical point of view, since its application combined with sunlight exposure can cause side effects including numerous ocular complications [14] and photoalergy [15,16]. Photochemistry of CPZ is rather complicated and strongly depends on wavelength [17,18]. Although studies by Ljunggren and Moller [19] and Schoonderwoerd et al. [20] indicated that dechloration is responsible for phototoxicity of CPZ, free radicals and singlet oxygen ($^1O_2$) also appear to play an

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important role in CPZ photosensitization. Interestingly, CPZ photogenerates $^1O_2$ in organic solvents, whereas in aqueous solutions does not [21]. The interaction of CPZ with membranes and the extent of its partition into the membrane is then important, since CPZ may play a role of a photosensitizer in the membrane interior by being able to generate $^1O_2$ in its hydrophobic surroundings. Additionally, unsaturated phospholipids and cholesterol in cell membranes are susceptible to reactive oxygen species being generated during photosensitized reactions. Like phospholipids, cholesterol is an important constituent of animal membranes, where it comprises 40–45 mol% of the total lipid, but because it exists as a single molecular species in natural membranes, it presents much fewer oxidation products. In cells under oxidative stress, membranous cholesterol, through the formation of its signature hydroperoxide and diol products serves as a unique marker in situ, allowing discrimination between $^1O_2$ and free radical intermediacy [22–24].

On the other hand, it is well known that cholesterol strongly affects various membrane properties, among which its effects on membrane fluidity, phase transition and penetration of polar and non-polar molecules have been most extensively studied [25–28]. Also, it has been shown that the presence of unsaturated double bonds in the phospholipid alkyl chain strongly affects membrane properties [27,29] and the penetration of different drugs, such as tetracycline [30]. We have been therefore interested in two main problems: (1) how does the membrane composition (especially lipid alkyl chain unsaturation and the presence of cholesterol) influence the CPZ effect on membranes, and (2) how relevant is the use of cholesterol as a marker of CPZ photosensitized reactions in case of altered membrane properties.

To address these issues we have undertaken an investigation of the CPZ effect on lipid order and motion in different liposome membranes. The factors analyzed in the present work were (1) the presence of unsaturated double bonds in lipid alkyl chains; (2) the presence of cholesterol; (3) temperature (as a factor causing the membrane phase transition); (4) pH (affecting CPZ charge); and (5) the surface charge of the membrane.

2. Materials and methods

2.1. Preparation of liposomes

L-α phosphatidylcholine, dimyristoyl (DMPC), L-α phosphatidyl-DL-glycerol, dimyristoyl (DMPG), L-α phosphatidylcholine-β-oleoyl-γ-stearyoyl (SOPC) and cholesterol were purchased from Sigma (Germany), L-α phosphatidylcholine-β-oleoyl-γ-palmitoyl (POPC) from Avanti Polar Lipids (USA), CPZ from Sigma Aldrich (Belgium), and spin labels from Molecular Probes (USA). The membranes used in this work were multilamellar liposomes containing 0 or 30 mol% of cholesterol; 0, 10 or 30 mol% of CPZ and 1 mol% of spin label (in case of ESR measurements), prepared according to Ref. [25]. All compounds were dissolved in chloroform, which was subsequently evaporated under stream of nitrogen to form a lipid film, which was then put under vacuum for at least 14 h. The dried lipids were suspended either in 0.1 M PBS (pH 7.4) or in 0.1 M borate buffer (pH 9.0) and vortexed vigorously. The multilamellar liposome suspension was centrifuged at 7000 rpm, for 15 min at 4 °C and the pellet (about 30 μl) was used as the sample for ESR measurements.

2.2. ESR measurements of lipid bilayer fluidity

The ESR measurements were performed on a Varian E-3 spectrometer at 10 mW of microwave power. Liposome samples were deoxygenated under argon flow, placed in the Pasteur pipettes (Medlab Products, Raszyn, Poland; length 230 mm, $\phi = 1$ mm) and centered in the resonant cavity. The measurements were carried out above and below T_M of the lipid membranes (at 37 and 15 °C in case of the saturated DMPC and DMPG membranes, and at 15 and 2 °C in case of the unsaturated SOPC membrane). SOPC has been chosen for ESR measurements because its T_M is +6 °C, whereas more frequently used unsaturated lipids such as POPC or egg yolk PC (EYPC) have T_M well below 0 °C [31]. Therefore it was possible to do measurements below T_M of SOPC for still not frozen samples. ESR spectra of n-doxylstearic acid spin labels (n-SASL, where $n = 5$ or 16) or cholestane spin label (CSL) incorporated into DMPC, DMPG or SOPC liposomes were analyzed in terms of the order parameter (S), correlation times and maximum splitting parameter (A_{max}).

Above T_M, to monitor the lipid order and motion the order parameter (S) was used. It was calculated using the equations [32]:

$$S = 0.5407(T_{II} - T_{\perp})/a_o) \text{ for } n-\text{SASL} \quad (1)$$

or

$$S = -1.131(T_{II} - T_{\perp})/a_o) \text{ for CSL} \quad (2)$$

where

$$a_o = (T_{II} + 2T_{\perp})/3 \quad (3)$$

and T_{II} and $T_{\perp}$ were measured directly from the ESR spectra.

S parameter as well as T_{II} and $T_{\perp}$ values are in principle static parameters. 16-SASL exhibits however so much motion that a different analysis can also be used [33]. The effective correlation times τ_{2B} and τ_{2C} assuming isotropic rotational diffusion of 16-SASL, were calculated according to the formulas [34]:

$$\tau_{2B} = 6.51 \times 10^{-10}\Delta H_o[(h_o/h_-)^{1/2} - [(h_o/h_+)^{1/2}]s \quad (4)$$

$$\tau_{2C} = 6.51 \times 10^{-10}\Delta H_o[(h_o/h_-)^{1/2} + [(h_o/h_+)^{1/2} - 2]s \quad (5)$$

where: ΔH_o is the peak-to-peak width of the central line in gauss and h_+, h_o and h_- are heights of the low, central and high field peaks, respectively. When τ_{2B} and τ_{2C} are similar, the motion is considered to be isotropic.

Below T_M, a maximum splitting parameter (A_{max}) measured directly from the ESR spectra as the separation between the outer hyperfine lines, was used to monitor the lipid alkyl chain order [32]. A_{max} value decreases as motional freedom increases.

2.3. Spectrophotometric measurements of chlorpromazine partition into liposomes

The studies were performed on DMPC and POPC membranes in the presence and absence of 5 mM cholesterol and at the CPZ concentration of 0.5 mM at pH 7.4. POPC has been chosen instead of SOPC because at 4 °C, which is the temperature required to separating the liposome and buffer phases by centrifugation [25], and at which the equilibrium between these two phases has been achieved, POPC is in the liquid-crystalline state (its T_M is below zero), whereas both DMPC and SOPC are in the gel state (additionally, 4 °C is not a convenient temperature for SOPC, because it is very close to its T_M). Therefore, using of POPC gave us an insight into the CPZ partition into liposome membranes also above T_M. The supernatants of centrifuged multilamellar liposome suspensions were carefully collected and transferred to new eppendorf tubes and their absorbance was measured. All absorption spectra were recorded on an UV–Vis HP 8452 (Hewlett Packard) diode spectrophotometer in the range 200–800 nm with a spectral resolution 2 nm. The supernatants of CPZ containing liposomes were measured versus the supernatants of corresponding pure liposomes, whereas the absorption of CPZ in the buffer was measured versus the buffer alone. The calculation was done by normalization at $\lambda = 308$ nm, which is the third CPZ absorption peak. The absorbance of the CPZ buffer solution (0.5 mM) was set as 100%. The percentage of CPZ which incorporated into or associated with lipid membrane was calculated by comparing the absorbance of the supernatant with the absorbance of the CPZ buffer solution. The measurements at pH 9.0 were impossible due to high scattering effects of the CPZ buffer solution itself.

2.4. Statistical analysis

The bars represent the mean value, and the errors were calculated as standard deviation (SD) of at least four independent measurements. Samples were statistically analyzed by the Student's t-test ($p \leq 0.05$).

3. Results

3.1. Comparison of chlorpromazine effects on lipid order and motion in DMPC and DMPG membranes

The effect of CPZ on lipid order and motion in neutral and negatively charged membranes was investigated by using three spin labels incorporated into DMPC or DMPG liposomes at pH 9.0. A sterol-type CSL (with the nitroxide free radical moiety located very close to the polar head groups) is a probe reflecting the behavior of the whole rigid lipid molecules such as cholesterol, whereas 5-SASL and 16-SASL (with the nitroxide moiety close to the polar head groups and at the membrane center, respectively) are good probes of the segmental motion of phospholipid alkyl chains [25]. The ESR spectra were collected both above and below T_M of DMPC and DMPG membranes (at 37 and 15 °C, respectively).

3.1.1. Measurements above T_M

Table 1 shows the values of S parameter calculated from ESR spectra of CSL, 5-SASL and 16-SASL incorporated into DMPC and DMPG membranes, in the absence and in the presence of 10 mol% of CPZ. A slight increase in S parameter can be seen in the presence of 10 mol% of CPZ, but the differences between pure membranes and those containing CPZ are in most cases very small. The ordering effect of CPZ is very low in all regions of DMPC membrane, unlike in DMPG, where it is stronger for CSL (close to the polar headgroups).

In Table 2 the effect of 10 mol% of CPZ on rotational motion of 16-SASL in DMPC and DMPG membranes is presented. Again, the ordering effect of CPZ (expressed as a decrease in motional freedom of 16-SASL free radical moiety) in both membranes is very low, being slightly stronger for DMPG. In both cases, the addition of 10 mol% of CPZ does not affect the isotropy of the motion.

The stronger effect of CPZ on DMPG liposomes compared to DMPC ones shows that the negative charge at the membrane surface enhances to some extent the interaction of CPZ with the membrane and its penetration through it. This result agrees with most literature data [4,6,35], according to which CPZ interacts stronger with negatively charged membranes. However, the differences are not significant.

3.1.2. Measurements below T_M

Fig. 1 shows the effect of 10 mol% of CPZ on motional freedom of CSL and SASL incorporated into DMPC and DMPG membranes at 15 °C. ΔA_{max} is a difference between the A_{max} value in the presence of 10 mol% CPZ and the control (unperturbed) value of A_{max} in pure liposomes. The figure clearly demonstrates that below T_M

Table 1
Order parameter (S) obtained from ESR spectra of CSL, 5-SASL and 16-SASL incorporated into DMPC and DMPG liposomes at 37 °C, in the presence and in the absence of 10 mol% of CPZ, at pH 9.0

Lipid	Order parameter S of spin labels		
	CSL	5-SAL	16-SASL
DMPC	0.41	0.57	0.08
DMPC + CPZ	0.42	0.58	0.09
DMPG	0.39	0.54	0.08
DMPG + CPZ	0.46	0.55	0.08

Table 2
Correlation times of 16-SASL incorporated into DMPC and DMPG liposomes at 37 °C, in the presence and in the absence of 10 mol% of CPZ, at pH 9.0

Lipid	Correlation time of 16-SASL (ns)	
	τ_{2B}	τ_{2C}
DMPC	0.61	0.59
DMPC + CPZ	0.64	0.65
DMPG	0.67	0.69
DMPG + CPZ	0.76	0.78

the addition of 10 mol% of CPZ strongly increases motional freedom of lipid molecules. The effects are similar in both membranes and much stronger for CSL than for SASL. This can be explained as follows. First, it may suggest that a rigid molecule of CSL is more sensitive to the CPZ effect than lipid alkyl chains of SASL. It was shown before that the effect of some membrane modifiers, such as cholesterol or carotenoids, on steroid-type spin labels (like CSL or ASL) was much larger than their effect on SASL, because CSL is more sensitive to the sum of changes at various depths in the membrane, while SASL reflects the local change [33]. Another explanation for the observed difference between both types of spin labels can also be taken into consideration, namely the localization of their nitroxide groups. If CPZ is present mostly at the membrane–water interface, its strong effect in this region may be best probed by the nitroxide free radical moiety of CSL, which is located very close to the membrane headgroup region.

Because of the strongest effect, CSL has been used in further experiments on the CPZ-membrane interactions. Moreover, CSL has no surface charge, unlike n-SASLs, which are negatively charged at pH used. Therefore the real interaction of CPZ with DMPC or DMPG has been investigated, not its interaction with the spin label carboxyl groups, which was proved to occur [9].

3.2. Effect of chlorpromazine on lipid order and motion in DMPC and SOPC membranes in the presence of cholesterol

It is well known that cholesterol strongly affects membrane fluidity. At the concentration of 30 mol%, cholesterol abolishes the main phase transition rigidifying membranes above T_M and increasing lipid motion below T_M [25,36,37], but lipid unsaturation moderates this effect [25,26,29]. We have been interested in how the presence of cholesterol in both saturated and unsaturated membranes may change the CPZ effect on lipid order and motion. The measurements were carried out above and below T_M of both membranes (at 37 and 15 °C in case of DMPC, and at 15 and 2 °C in case of SOPC). Because of a possible role of the positive charge of CPZ, we studied its interaction with the membranes at two pH: at pH 9.0, in which CPZ is present predominantly in neutral form and at pH 7.4 when it should be protonated and positively charged [35].

3.2.1. Chlorpromazine in DMPC-cholesterol membranes

Fig. 2 shows the effect of 10 and 30 mol% of CPZ on order parameter S (Fig. 2A) and A_{max} parameter (Fig. 2B) of CSL incorporated into DMPC membranes containing 0 and 30 mol% of cholesterol.

ΔS is a difference between the S values for the membranes in the presence of 10 or 30 mol% of CPZ and the control values of S (obtained from the membranes with 0 and 30 mol% cholesterol, respectively). It is clear that for DMPC membranes without cholesterol the ordering effect of CPZ (above T_M) becomes noticeable for the higher CPZ concentration, and at pH 7.4. Interestingly, in the presence

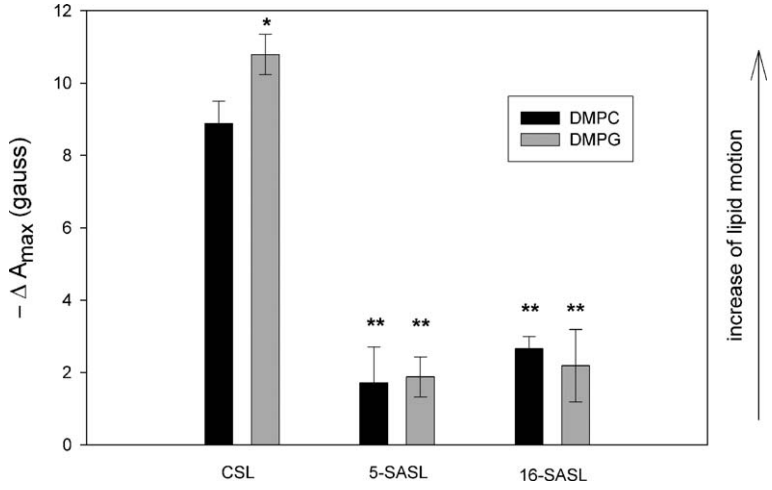

Fig. 1. [$-\Delta A_{max}$] values (in gauss) of CSL, 5-SASL and 16-SASL in DMPC (black bars) and DMPG (gray bars) membranes at 15 °C, pH 9.0. ΔA_{max} was calculated as a difference between the values of A_{max} in the membranes containing 10 mol% CPZ and the control values of A_{max} in pure membranes. The bars indicate mean ± SD of at least four independent measurements; * indicates a statistically significant difference (the Student's t-test, $p \leq 0.05$) relative to DMPC membrane, ** indicates a significant difference relative to CSL.

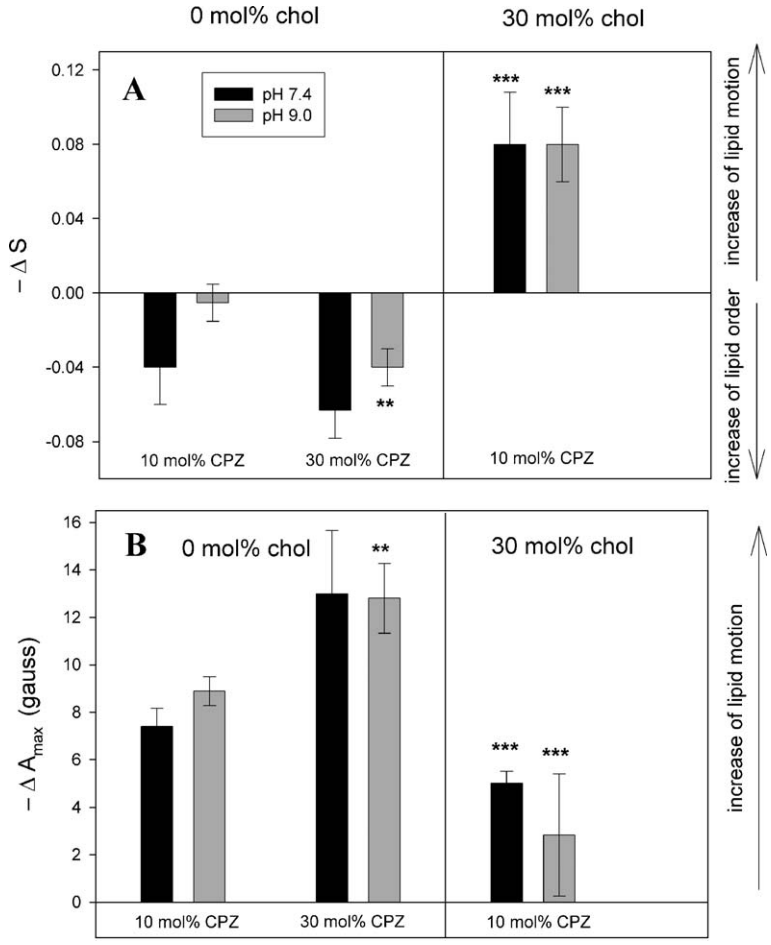

Fig. 2. [$-\Delta S$] values (A) and [$-\Delta A_{max}$] values (in gauss; B) of CSL in DMPC membranes at pH 7.4 (black bars) and pH 9.0 (gray bars). ΔS and ΔA_{max} are the differences between the values of S and A_{max} parameters, respectively, obtained from the membranes in the presence of 10 or 30 mol% CPZ and their control values (obtained from the membranes without cholesterol-left panel, and containing 30 mol% cholesterol-right panel of the graph). S values were obtained at 37 °C and A_{max} values at 15 °C. The bars indicate mean ± SD, and ** indicates a statistically significant difference (the Student's t-test, $p \leq 0.05$) relative to 10 mol% CPZ, *** indicates a significant difference relative to 0 mol% cholesterol.

of 30 mol% of cholesterol, CPZ has the opposite effect on DMPC membrane. Instead of the slight ordering effect, a clear increase in lipid motion has been observed at CPZ concentration of 10 mol%. This effect was observed at both pH with no significant difference.

Below T_M, ΔA_{max} was measured as a difference between the values of A_{max} for the membranes in the presence of 10 or 30 mol% of CPZ and A_{max} control values (obtained from the membranes with 0 and 30 mol% cholesterol, respectively). A strong increase in lipid motion was seen for pure DMPC membranes in the presence of CPZ, at both concentrations and regardless of pH. In the presence of 30 mol% of cholesterol, CPZ also increased lipid motion, but the effect was smaller than in pure membranes.

3.2.2. Chlorpromazine in SOPC-cholesterol membranes

Similar experiments were performed on unsaturated SOPC membranes. Fig. 3 shows the effect of 10 and 30 mol% of CPZ on order parameter S (Fig. 3A) and A_{max} parameter (Fig. 3B) of CSL incorporated into SOPC membranes containing 0 and 30 mol% of cholesterol. Above T_M, the ordering effect of CPZ can be seen in pure SOPC membranes. This effect increases with CPZ concentration and is stronger at pH 7.4 than at pH 9.0. The ordering effect of CPZ observed in pure SOPC membrane has been completely reduced at the presence of 30 mol% cholesterol, but unlike in DMPC-cholesterol membrane, no fluidizing effect has been observed instead.

Below T_M, at both pH, a strong increase in lipid motion has been seen for pure SOPC membranes in the presence of CPZ. In the presence of 30 mol% of cholesterol, the fluidizing effect of 10 mol% of CPZ was smaller than in pure membranes, but still observed.

3.3. Spectrophotometric measurements of the partition of chlorpromazine into saturated and unsaturated membranes containing cholesterol

To better understand the cholesterol effect on CPZ penetration into both membrane types, spectrophotometric

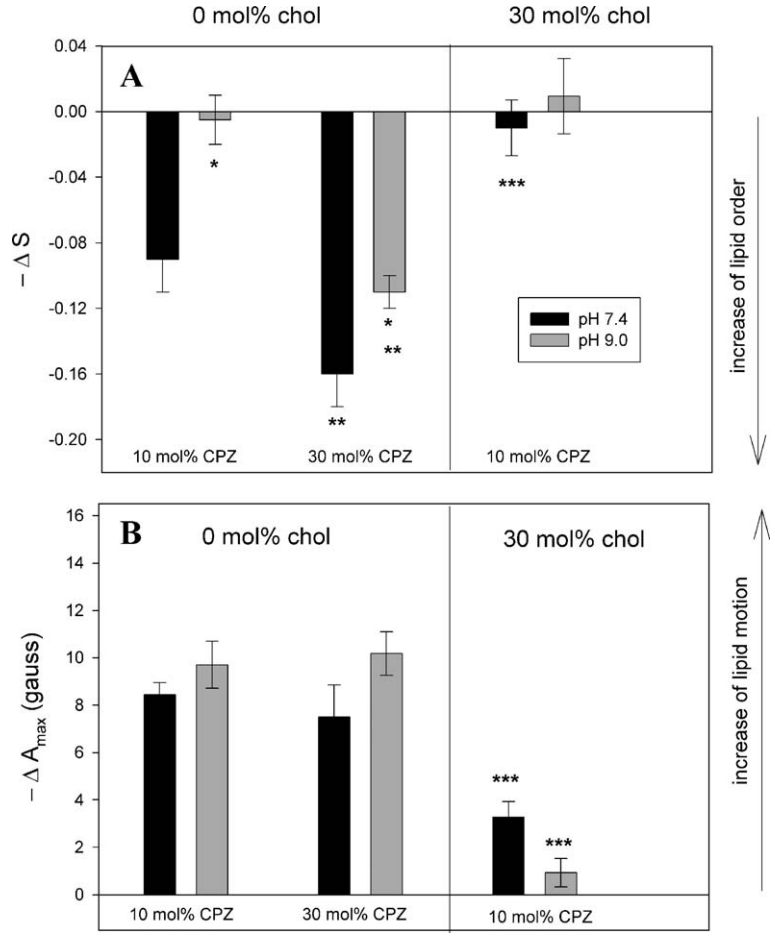

Fig. 3. [$-\Delta S$] values (A) and [$-\Delta A_{max}$] values (in gauss; B) of CSL in SOPC membranes at pH 7.4 (black bars) and pH 9.0 (gray bars). ΔS and ΔA_{max} are the differences between the values of S and A_{max} parameters, respectively, obtained from the membranes in the presence of 10 or 30 mol% CPZ and their control values (obtained from the membranes without cholesterol-left panel, and containing 30 mol% cholesterol-right panel of the graph). S values were obtained at 15 °C and A_{max} values at 2 °C. The bars indicate mean ± SD and * indicates a statistically significant difference (the Student's t-test, $p \leq 0.05$) relative to pH 7.4, ** indicates a significant difference relative to 10 mol% CPZ, *** indicates a significant difference relative to 0 mol% cholesterol.

measurements of CPZ content in liposomes have been performed. The absorption spectra of 0.5 mM CPZ dissolved in the buffer (pH 7.4) were compared with the spectra of CPZ remaining in liposome supernatants after the centrifugation at 4 °C (liposomes made of DMPC, DMPC-cholesterol, POPC, and POPC-cholesterol were employed), and the partition of CPZ into liposomes has been determined. In case of DMPC, the observed partition referred to the gel phase of the membrane, whereas in case of POPC, it concerned the liquid-crystalline phase. The data are presented in Table 3. It can be seen that CPZ quite well incorporates into liposomes, with no significant difference between both membranes. However, a direct comparison between the gel and liquid-crystalline phases cannot be done, since, for experimental reasons (mentioned in Materials and methods), we were not able to determine the CPZ partition into the same membrane below and above T_M. Cholesterol reduces the CPZ content in the membranes of both types, which is in agreement with the data on partition coefficient [12]. It is also clear from the data in Table 3 that cholesterol affects the CPZ content in DMPC membranes more than in POPC. In cholesterol-containing DMPC liposomes the amount of CPZ associated with the membrane is about 30% lower than in pure DMPC liposomes, whereas in POPC liposomes this difference is only about 15%. It is

Table 3
CPZ partition into DMPC and POPC liposomes in the presence and in the absence of 5 mM cholesterol, at pH 7.4; 4 °C. Data at pH 9.0 not available due to high scattering effects of CPZ buffer solution

Lipid	CPZ partition into liposomes (%)
DMPC	78.9 ± 7.03
DMPC + cholesterol	53.7* ± 6.91
POPC	86.8 ± 1.76
POPC + cholesterol	74.0* ± 0.71

The CPZ partition into liposomes has been calculated as follows: $100\% - (A_{308}^{CPZ}\ \text{sup}/A_{308}^{CPZ}\ \text{buf}) * 100\%$, where A_{308}^{CPZ} sup is the absorbance value (at $\lambda = 308$ nm) of CPZ in a given supernatant and A_{308}^{CPZ} buf is the absorbance value of CPZ in the buffer. Errors have been calculated as SD, * indicates a statistically significant difference (the Student's t-test, $p \leq 0.05$) relative to membranes without cholesterol.

possible that cholesterol in DMPC membranes makes CPZ partition into the membrane more difficult, and in unsaturated membranes, in which lipid alkyl chains are not easily miscible with rigid cholesterol molecules [28], CPZ can be better accommodated. Additionally, the difference in the physical states of both membranes is important. The spectrophotometric measurements, however, do not allow telling in what region of the membrane CPZ is located. We assume that during liposome centrifugation all CPZ not associated with the membrane remains in the buffer, and the rest can be either inside the membrane, or at the membrane–water interface, or even bound to the membrane surface.

4. Discussion

4.1. Comparison of the effects of chlorpromazine above and below T_M of the membranes

In the present work we have shown that the effects of CPZ on membranes above and below the main phase transition temperature (T_M) are the opposite, namely ordering and disordering, respectively.

In literature, there is no consistent data on the effect of local anesthetics (LA), tertiary amine amphipathic molecules, on bilayer order at temperatures above T_M. There are some data showing that LA such as benzocaine, lidocaine and tetracaine decrease molecular organization in EYPC membranes [38,39]. Also, CPZ was found to increase mobility of acyl methyl groups of phospholipids in DPPC/PS membranes [4]. On the other hand, some reports indicate that CPZ, cannabinol and pentobarbital increase the order of egg PC-PA membranes [40] and tetracaine increases the organization of micelles [41]. The fluorescence polarization data of Hendrich et al. [42] have shown that another phenothiazine derivative (FPhMS) makes EYPC, DMPC and DPPC bilayers in a liquid-crystalline phase more rigid. Also, molecular dynamics simulation data of Pasenkiewicz-Gierula et al [43] have proved that the order of lipid chains in POPC membranes penetrated by another LA (a carane derivative) was higher than in the pure POPC bilayers. The ordering effect of CPZ observed by us above T_M is stronger in SOPC membrane than in DMPC where it is nearly negligible, especially at lower CPZ concentration. The lack of noticeable effect of 10 mol% CPZ at 37 °C can be explained by fast motion of lipid molecules and high fluidity of the membrane at this temperature. It has been shown before that lipid matrices can accommodate rather high concentrations of LA without aggravating the disordered motion of lipid chains. [44]. SOPC, which has much lower T_M than DMPC [31], was possible to be investigated in liquid-crystalline phase at lower temperature (15 °C), in which lipid chains are less mobile and ordering effects may be better seen.

A decrease in lipid order caused by CPZ or some LA below T_M was observed by most authors [4,12,45,46].

We presume that the opposite effects of CPZ above and below T_M can be explained by different membrane physical state, and, consequently, by the various location of this molecule in membranes of different phases. There are data suggesting that the mechanisms of LA interaction with membranes above and below T_M may be different. The fluorescence quenching measurements of Hutterer et al. [47] showed that the interaction of tetracaine with lipid membranes above T_M is mostly a partitioning process, whereas below T_M a saturable binding one. We think that this can be valid also for CPZ. Above T_M, a membrane can be penetrated by CPZ, whose rigid phenothiazine ring system increases the alkyl chain packing and enhances their extended *trans* conformation in a manner similar to cholesterol or polar carotenoids [33,48]. This suggestion can be supported by molecular dynamics simulation data, according to which the alignment of POPC chain along an LA side chain resembled that between DMPC alkyl chains and cholesterol in DMPC-cholesterol membrane [43]. However, it has to be pointed out that the ordering effect of CPZ is much smaller than the one caused by cholesterol. Below T_M, the membrane penetration by CPZ is much more difficult [12]. CPZ remains mostly at the membrane surface, or at the membrane–water interface, where its polar groups may separate the phospholipid headgroups and decrease the interaction between them. This may lead to increasing mobility of alkyl chains, which enter the *gauche* conformation [4]. The comparison of CPZ effect to that of cholesterol was done by Hendrich et al. [42] also for membranes below T_M. It seems however that the mechanisms of the action of both compounds must be different, because cholesterol, as a much bigger and more hydrophobic molecule, in contrast to CPZ, intercalates into membranes also below T_M.

4.2. Role of cholesterol and unsaturation

Addition of 30 mol% of cholesterol into DMPC and SOPC membranes changes significantly the CPZ effects both above and below T_M. The most striking result is the increase in lipid motion observed above T_M in DMPC-cholesterol membrane in the presence of 10 mol% CPZ (Fig. 2A). Similar results were obtained by Pang and Miller [40], who showed that CPZ ordered egg-PC-PA bilayers with cholesterol content up to 20 mol%, but disordered those with greater than 25 mol%. This can be explained by the well known fact that cholesterol strongly rigidifies the DMPC membrane above T_M [25,37] and changes its phase from liquid-crystalline into less fluid, called liquid-ordered, as illustrated by the DMPC/cholesterol phase diagram [49]. As shown above in the present work, in membranes with ordered lipid chains CPZ may act as a fluidizer, increasing lipid motion (Figs. 1 and 2B). In SOPC-cholesterol membrane no increase in lipid motion was observed in the presence of CPZ, but still the CPZ effect on this membrane remained significantly different from its effect on pure SOPC (Fig. 3A). Cholesterol seems to affect the CPZ-

membrane interaction in both membrane types, but the effect on saturated DMPC membrane is especially pronounced. This result agrees with the known fact that unsaturated membranes are less prone to the rigidifying effect of cholesterol compared to saturated ones [25,26].

According to the DMPC/cholesterol phase diagram [49], below T_M cholesterol affects membrane fluidity in the opposite way—it increases lipid motion, and the membrane is no more in a typical gel-phase, but in a liquid-ordered one. This may be a reason for observed diminishing of the disordering effect of CPZ in both saturated and unsaturated membranes containing cholesterol (Figs. 2B and 3B). The lower disordering effect of CPZ in the presence of cholesterol may also be caused by lower CPZ concentration in a cholesterol-containing membrane. Luxnat and Galla [12] showed that cholesterol reduced CPZ partition coefficient into DMPC membrane quite significantly. Also, our spectrophotometric measurements of CPZ partition into liposomes demonstrated a lower CPZ content in cholesterol-containing membranes compared to the pure ones (Table 3). However, the disordering effect observed in DMPC-cholesterol membrane above T_M does not allow believing that CPZ is not present in the membrane, at least at the region close to the polar head groups. It seems more probable that for observed effects and differences between both cholesterol containing membranes their different phases are responsible.

4.3. Role of pH

Chlorpromazine is a basic molecule with pK = 8.6 (in water) [7] and 8.0 (in the presence of zwitterionic phospholipid bilayers) [35]. Therefore, at pH 9.0 it is predominantly unprotonated and neutral, and in pH 7.4-protonated with a positive charge. Our results obtained for these two pH values show that different forms of CPZ do not influence the membranes equally.

Above T_M, the ordering effect of CPZ on pure SOPC membrane is stronger at pH 7.4 than at pH 9.0 (Fig. 3A). We suppose that for DMPC membrane the dependence should be similar, but because the ordering effect of CPZ in this membrane is in general very small and calculated errors comparably big, therefore we cannot draw a solid conclusion about the pH dependent CPZ effect on DMPC. Anyhow, bearing in mind the limitations of the ESR method at this point, we suggest two possible explanations for observed differences. The most obvious explanation may be that the amount of membrane associated CPZ is pH dependent. Unfortunately, our spectrophotometric measurements of CPZ partition into membranes did not give an answer at this point, because high scattering effects at pH 9.0 made the absorption measurements not reliable. Since CPZ is known to affect the membrane phase transition (causing a slight shift of T_M and decrease in cooperative unit) dependently on its concentration [50], it may be helpful to use DSC as a highly sensitive method to determine the amount of membrane associated CPZ. However, although a stronger CPZ effect at pH 7.4 than at pH 9.0 could suggest more membrane associated CPZ at pH 7.4, we cannot draw the same conclusion from the data obtained below T_M, where we do not see differences in CPZ effects at both pH. Another possible interpretation is that, due to bearing a positive charge at pH 7.4, a CPZ molecule cannot easily penetrate the membrane, and remains mainly at the interface or at the bilayer surface. The charged amine group of CPZ should be located around the phosphate atom of PC and the most perturbing phenothiazine ring system may go deeper into the interface region. Such a localization of CPZ affects mostly this region of the membrane where the CSL free radical moiety is placed. On the contrary, at pH 9.0 CPZ can easier penetrate the membrane because of losing its positive charge. Therefore, it can be expected that in this case CPZ is more evenly distributed within the membrane and affects other regions as well. Also, the molecular dynamics simulation data confirm that carane derivative distribution in the membrane depends on its charge. A neutral form of LA was shown to cross the interfacial region and insert some fragments into the bilayer core, whereas the protonated LA molecules did not cross the membrane–water interface [43]. This interpretation is additionally supported by the data obtained below T_M, where pH does not seem to play a role in CPZ effect on both membranes (Figs. 2B and 3B). As mentioned previously, in the gel-phase membrane CPZ remains mainly at the surface or at the membrane–water interface, and cannot penetrate the membrane neither in neutral nor in a positively charged form.

5. Conclusions

In conclusion, it should be emphasized that CPZ affects lipid order and motion in all investigated membranes and its effects depend first of all on the physical state of the membranes. In saturated membranes, the slight ordering effect observed above T_M becomes a fluidizing one below T_M or in the presence of cholesterol. Cholesterol also reduces the CPZ partition into such membranes. In unsaturated membranes, cholesterol influences the CPZ content in the membrane and its effects to less extent. The pH, which affects the CPZ charge, is also important, but only for pure membranes in the liquid-crystalline phase.

For further photochemical study of CPZ as a sensitizer, liposomes made of unsaturated lipids and containing cholesterol are needed to be employed. However, cholesterol as a molecule altering the state of membranes and the effect of CPZ so strongly, does not seem to be a good probe for such tests as iodometric assay and a HPLC-EC(Hg), where the low temperature of measurements is required. At low temperature, saturated membranes are usually in the gel phase, whereas unsaturated in the liquid-crystalline one. It has to be pointed out that not only the localization and, consequently, the effectiveness of CPZ can be different in both membrane types, but also the addition of cholesterol may influence the

CPZ amount and effects in both membranes in a different way. Therefore, for photochemical studies, we recommend using lower amounts of cholesterol than 30 mol%. It has to be underlined that comparison of the results from different model membranes requires a very careful analysis of experimental conditions, such as temperature, pH and membrane composition, especially cholesterol content.

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