A Spin Probe Study of the Effects of Chlorpromazine and its Derivatives on Lipid-Protein Interactions in Synaptosomal Membranes

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Abstract. The electron spin resonance spectra of 16-doxyl stearic acid (16-SA) incorporated into synaptosomes mostly showed a fluid lipid component and a minor motionally-restricted component (MRC) of the molar fraction of 10–20%, measured at 0 °C. At 10 mmol/l concentration, thioridazine (TRZ), chlorpromazine (CPZ), chlorprothixene (CPT), perphenazine (PFZ) and levopromazine (LPZ) raised the MRC molar fraction in the synaptosomes to 100, 92, 65, 41 and 39%, respectively (as detected by the spin probe at 0 °C). At 4% concentration, TRZ, CPZ, CPT, PFZ, and LPZ the respective MRC percentages were 100, 75, 41, 24 and 17%. In synaptosomal membranes, A_{MRC} splitting values of MRC, induced by TRZ and CPZ, were similar to those of the probe in human serum albumin. MRC induced by CPZ and TRZ was constant (±15%) within the temperature range from 0 to 30 °C. At drug/lipid ratios $\geq 2:1$, TRZ and CPZ formed rigid complexes with total lipids isolated from the rat brain. The complexes melted upon increasing the temperature of the samples over 10–20 °C. The drugs decreased the lipid concentrations in synaptosomes in the order of potency TRZ > CPZ > CPT > PFZ ≥ LPZ; this was similar to their effect on MRC increase. The drugs tested increased the membrane dynamics/disordering, and their potency fairly correlated with their MRC increasing effects. It is supposed that the drug-induced 16-SA probe MRC increase in synaptosomes was a result of mainly decreased lipid/protein ratio in the synaptosomal membranes, which in turn probably is connected with perturbation of lipid-protein interactions and/or membrane proteins. The perturbation of lipid-protein interactions and/or membrane proteins may be connected with the drug perturbation effect on the bulk lipid membrane part.

Key words: Tranquilizers — Lipid-protein interaction — Synaptosomes — EPR spectroscopy

Introduction

Chlorpromazine and its derivatives influence various biological processes in which drug-membrane interactions have been supposed to play an important role (Hruban et al 1978, Fujii et al 1979, Salhab et al 1979, Tsao et al 1982, Ondriaš et al 1989a) The mode of action of these agents has not been understood as yet To understand the membrane effects at molecular level, their interactions with proteins, lipids and the lipid-protein interface should be considered

EPR spectroscopy of spin probes has been found useful in the study of interactions of lipids with membrane proteins (Marsh 1985) EPR spectra of spin probes in biological membranes revealed two separate components one corresponding to the fluid bilayer and the other one, with a higher degree of motional restriction, being attributed to the lipids interacting directly with the intramembrane surface of membrane proteins (Jost et al 1973, Marsh 1985) Changes of the second component of EPR spectra reflect changes in lipid-protein interface and/or membrane proteins Yamaguchi et al (1985) reported chlorpromazine to induce motionally restricted signal of spin probes in erythrocyte ghosts Recently thioridazine was found to strongly influence the second EPR component in synaptosomes (Ondriaš et al 1992) In the present work, the effects of five tranquilizers on lipid-protein interaction and/or membrane proteins were compared in synaptosomal membranes

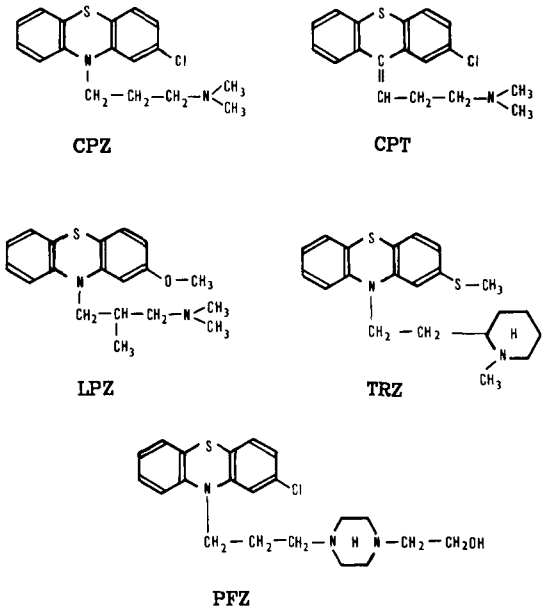

Figure 1. Structural formulas of chlorpromazine (CPZ), chlorprothixene (CPT), levopromazine (LPZ), thioridazine (TRZ), and perphenazine (PFZ)

Materials and Methods

Chemicals Thioridazine (TRZ), chlorpromazine (CPZ), chlorprothixene (CPT), perphenazine (PFZ) and levopromazine (LPZ) (Fig 1) were provided by the Research Centre of Mental Health in Pezinok Stearic acid spin labelled by doxyl group at 16 position (16-SA) was from Sigma Dodecylsulfate Na salt (SDS) was from Serva All other chemicals were from commercial sources and of analytical grade

Samples Total lipids (TL) were extracted from rat brains according to the method of Folch et al (1957) Dried TL were hydrated with a buffer containing (in mmol/l) NaCl 145, KCl 5, $MgCl_2$ 1 4, $CaCl_2$ 1, HEPES HCl 20, pH 7 4 The lipid/buffer weight ratio in the samples was 0 1 To prepare liposomes, the samples were sonicated in a bath and subjected to repeated freeze-thaw-vortex cycles The liposomes thus prepared (55 μl) were added to appropriate amounts of the powdered drugs containing 20 μg of the spin probe, and sonicated and vortexed for several minutes The lipid drug molar ratio in the samples was calculated assuming a lipid molecular weight of 775

Six mg of human serum albumin (HSA) and the spin probe were vortexed in 100 μl of the buffer for 1 minute and then incubated for 60 minutes at 37 °C The drug spin probe samples were prepared by hydrating 20 μg of dry spin probe and 5 mg of dry drug with 100 μl buffer and vortexing for 1 min

Isolated synaptosomes (Krueger et al 1979) (3 mg protein) were incubated with or without the respective drug at 37 °C for 60 minutes in 50 μl buffer (procedure A) or 5 ml buffer (procedure B) The samples containing 50 μl of buffer were mixed with dry spin probe at 15 μg probe/3 mg protein and vortexed for 3–5 minutes Since procedure A avoided sample centrifugation the EPR measured samples contained all membrane components The samples prepared by procedure B containing 5 ml of buffer, were centrifuged at $10,000 \times g$ for 10 minutes, and the pelletted synaptosomes (50 μl) were mixed with the dry spin probe at 15 μg spin probe/3 mg protein, and vortexed for 3–5 minutes The samples were further incubated for 30 minutes at 37 °C, transferred into capillaries (1 mm i d), and EPR spectra were recorded using a BRUKER ER 200 D SRC spectrometer

The amount of lipid phosphorus was determined in the supernatant of pelletted synaptosomes according to the slightly modified method of Mrsny et al (1986), with the samples digested in perchloric acid at 180 °C for 3 hours The error of lipid phosphorus determination was ±20%

The inner splitting, A_{min}, (arrows in Fig 8) and outer splitting, A_{max}, (arrows in Figs 4 and 6) parameters were evaluated from the ESR spectra of the probe in synaptosomes or TL-drug aggregates An increase of parameter A_{min} and a decrease of parameter A_{max} indicate a higher disorder or dynamics of the hydrophobic part of membranes (Gaffney 1976) A_{max} splitting of immobilized ESR spectra was expressed in terms of A_{MRC} (arrows in Fig 4)

To separate fluid and MRC components from the complex spectra (see e g Fig 2-D), MRC was subtracted using the spectra of HSA in buffer, or the fluid lipid component was subtracted using the spectra of isolated total lipids from the rat brain in the presence of a drug with similar A_{min} values (Jost et al 1973, Marsh 1985, Ondriaš et al 1989b) To find the absolute MRC value, the double integral of the MRC spectrum was compared with the double integral of the original complex spectrum (Jost et al 1973, Marsh 1985, Ondriaš et al 1989b) The standard error of the MRC determinations was ±10% The concentrations of the drugs reported in this paper correspond to those in the buffer phase

immediately after drug addition rather than to the final concentrations in the aqueous phase after equilibration with membranes.

Results

Fig. 2. shows 16-SA probe EPR spectra in control synaptosomes and in those treated with the drugs, measured at 0 °C. The samples were prepared by procedure B. The spectra of control synaptosomes (Fig. 2-A) mostly exhibited a fluid component which corresponded to the single component spectra of the probe in rat brain TL (shown in Fig. 6) and a minor portion of the MRC (10–20%).

In the presence of 10 mmol/l of the drugs in the synaptosomal samples (Figs. 2-

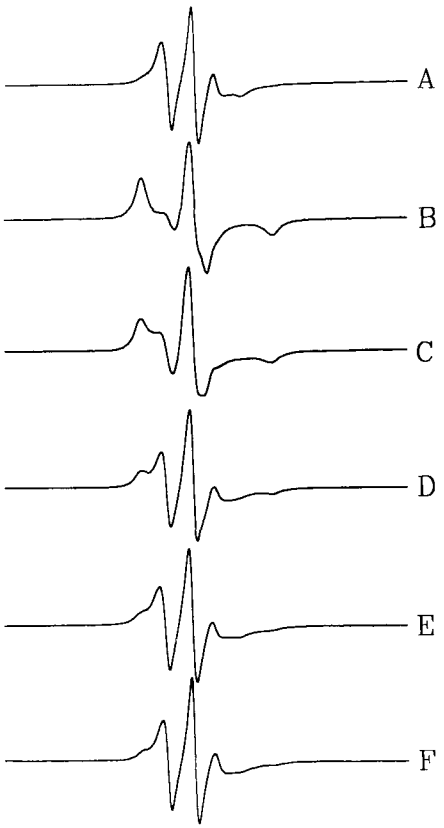

Figure 2. EPR spectra of spin probe 16-SA in control synaptosomes and in synaptosomes treated with 10 mmol/l concentration of the drugs at 0 °C. A, control synaptosomes; B, TRZ; C, CPZ; D, CPT; E, PFZ; and F, LPZ. Spectral width 20 mT. Samples prepared by procedure B (see text).

Table 1. Effects of the drugs tested on MRC (at 0 °C) and A_{min} (at 37 °C) parameters of 16-SA spin probe in synaptosomes, and on lipid concentration (relative units) in supernatant above pelleted synaptosomes. (n.d., not determined)

Drugs	%MRC 10 mmol/l	%MRC 4%	%MRC 1 mmol/l	A_{min} [mT] 1 mmol/l	lipid c. 10 mmol/l
No drug	15	15	15	1.160	1
TRZ	100	100	36	1.250	8.2
CPZ	92	75	20	1.235	7.9
CPT	65	41	17	1.211	2.7
PFZ	41	24	20	n.d.	1.6
LPZ	39	17	15	1.189	0.9
SDS	n.d.	<20	n.d.	1.431	n.d.

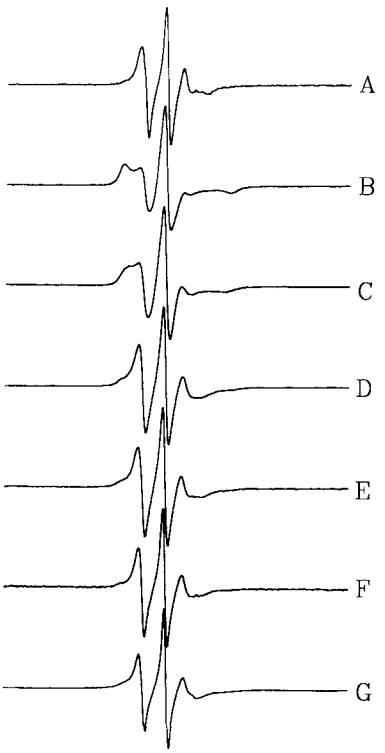

Figure 3. EPR spectra of spin probe in control synaptosomes and in synaptosomes treated with 4% concentration of the drugs at 0 °C. A, control synaptosomes; B, TRZ; C, CPZ; D, CPT; E, PFZ; F, LPZ; and G, SDS. Spectral width 20 mT. Samples prepared by procedure A (see text).

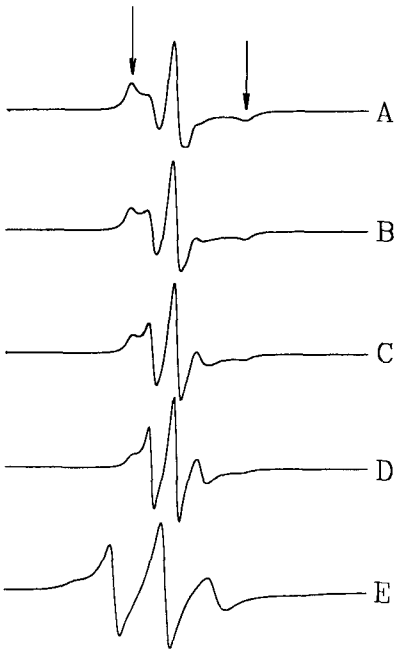

Figure 4. Temperature dependence of EPR spectra of spin probe in synaptosomes treated with 10 mmol/l CPZ. Spectra A, B, C, D and E were measured at 0, 10, 20, 30 and 37 °C, respectively. Arrows indicate MRC. Samples prepared by procedure B. Spectral width was 20 mT, with the exception of spectrum E where spectral width was 10 mT.

B to F) the ESR spectra changed: in addition to alterations in the fluid component (A_{min} splitting increased) the MRC proportion increased. A higher degree of motional restriction of the probe and/or higher membrane ordering was characteristic for MRC. At 4%, the drug tested increased MRC when the samples were prepared by procedure A (Fig. 3). However, SDS did not increase MRC. As seen in Figs. 2 and 3, the drugs had different potencies in increasing MRC, the potency order being TRZ > CPZ > CPT > PFZ > LPZ.

The drugs tested decreased the concentration of lipids in synaptosomal membranes in the same potency order as with MRC. The relative values of lipid phosphorus (average values from two measurements) for TL in the supernatant of control synaptosomes and those treated with the drugs are shown in Table 1.

The values of MRC in the presence of 10 mmol/l CPZ and TRZ, the only drugs tested, were similar within 0 to 30 °C (see CPZ, Fig. 4). The MRC values for the spin probe in synaptosomes treated with CPZ at 1, 10, 20 and 30 °C were 92, 84, 78, and 72%, respectively. The temperature dependence of A_{MRC} values of the probe in CPZ-treated synaptosomes was similar to that of A_{MRC} values of the probe in HSA, but significantly higher in comparison to the values for the probe in control synaptosomes (Fig. 5).

Raising the molar fraction of CPZ in TL-CPZ aggregates in the range of the TRZ/TL molar ratio from 4:1 to 1:0 immobilized the probe motion and increased

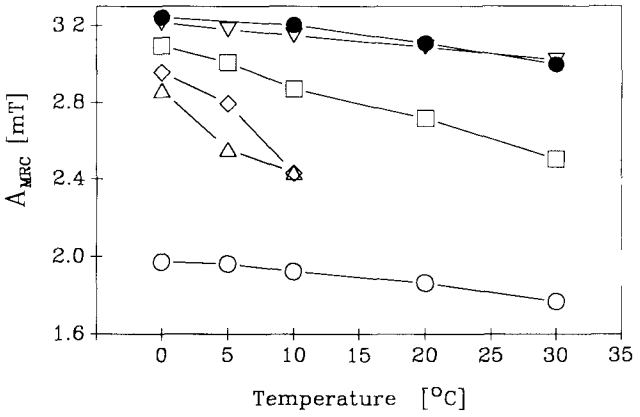

Figure 5. Temperature dependence of A_{MRC} values of spin probe in the HSA (inverse triangles), control synaptosomes (open circles), synaptosomes treated with 10 mmol/l CPZ (filled circles); at CPZ:TL molar ratios of 4:1 (triangles), 8:1 (diamonds) and 1:0 (squares).

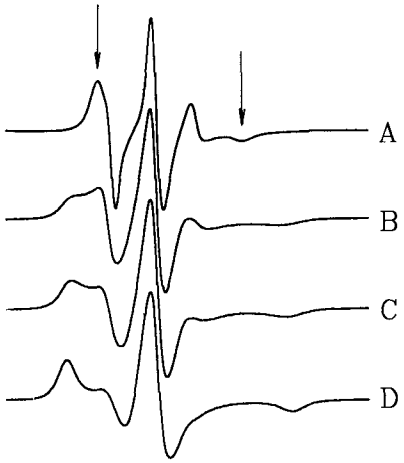

Figure 6. EPR spectra of spin probe at 0 °C in aggregates of CPZ:TL at molar ratios of 0:1 (A), 4:1 (B), 8:1 (C) and 1:0 (D). Arrows indicate outer splitting ($2A_{max}$) determination. Spectral width 10 mT.

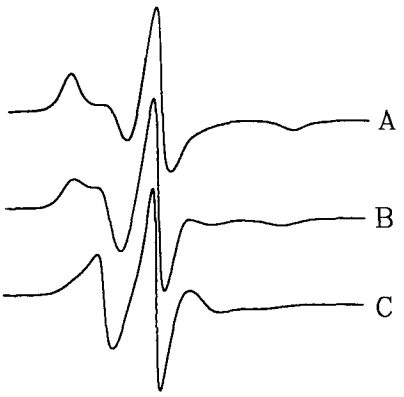

Figure 7. Temperature dependence of spin probe in CPZ aggregates at 0 (A), 10 (B) and 2 °C (C). Spectral width 10 mT.

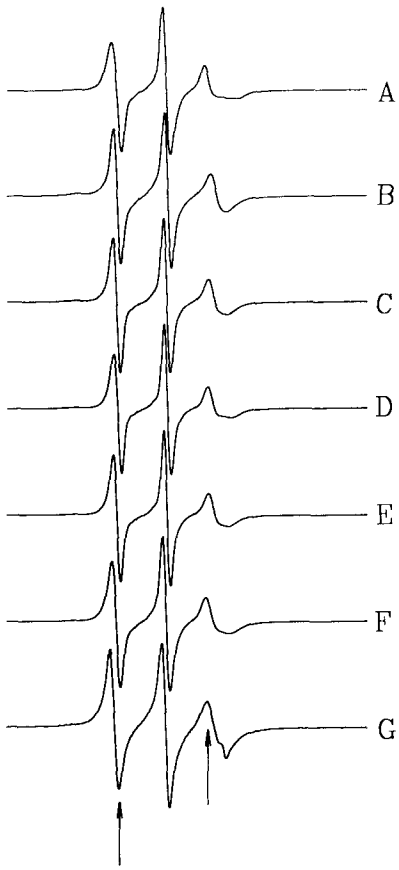

Figure 8. EPR spectra of spin probe in control synaptosomes and in synaptosomes treated with 1 mmol/l concentration of the drugs at 37 °C A, control synaptosomes, B, TRZ, C, CPZ, D, CPT, E, PFZ, F, LPZ, and G, SDS Arrows indicate inner splitting ($2A_{min}$) determination Spectral width 20 mT Samples prepared by procedure B

the ordering (measured at 0 °C) of the probe in the aggregates (Fig 6) The spin probe bound easily to CPZ aggregates in the buffer, showing a very high immobilized motion and/or high ordering of the complexes at 0–10 °C (Fig 7), MRC disappeared at 20 °C, indicating that CPZ aggregates dissolved between 10 and 20 °C The temperature dependence of the CPZ induced A_{MRC} values for the probe interacting with TL or in CPZ aggregates differed from that for A_{MRC} in CPZ-treated synaptosomes (Fig 5)

At 1 mmol/l, the drugs tested had different potencies in increasing parameter A_{min} of the probe in synaptosomes (Fig 8) they increased the dynamics/disordering of the bulk lipid part of synaptosomes (Table 1) Since at 4% or 10 mmol/l drug concentrations the MRC contribution to parameter A_{min} was significantly high, A_{min} values were not evaluated at these drug concentrations The potencies of the drugs to induce MRC and to increase membrane dynamics/disordering correlated fairly well (Fig 9) However, the high A_{min} value induced

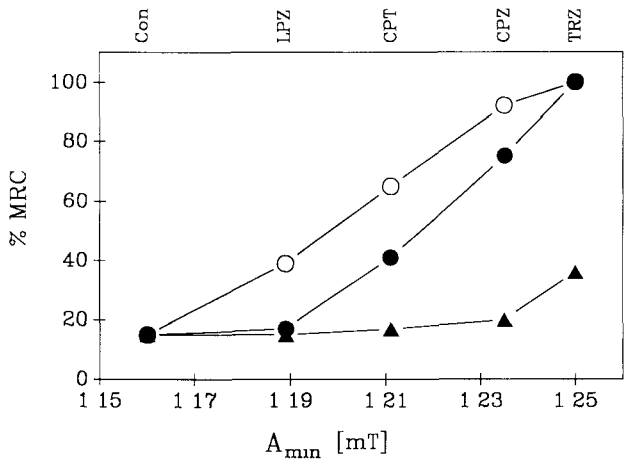

Figure 9. Correlation between the effect of 1 mmol/l concentration of the drugs at 37 °C on A_{min} values in synaptosomes and the effect of the drugs at 0 °C to induce MRC in synaptosomes at 1 mmol/l (triangles), 4% (filled circles) and 10 mmol/l (open circles) drug concentrations.

by SDS (Table 1) did not correlate with the nonsignificant MRC increase.

Discussion

MRC of the EPR spectra indicates immobilized motion of the spin probes and/or a large increase of the membrane order. In biological membranes MRC has been attributed to the spin probes in lipids either interacting directly with the intramembranous surface of the integral proteins (Jost et al. 1973; Marsh 1985; Horvath et al. 1988) and/or trapped between aggregated proteins (Fabre et al. 1979; Watts et al. 1981). Drugs may perturb membrane proteins and so increase the number of binding sites for the spin probes.

The highest capacity to induce MRC was found for TRZ and CPZ causing immobilization of the spin probe in synaptosomal membranes. Our study confirmed that the probe immobilization resulted from the involvement of membrane proteins interacting with the probe. CPZ and TRZ formed aggregates in the buffer. The spin probe and TL bound firmly to the aggregates yielding EPR spectra similar to MRC seen in synaptosomes treated with the drugs tested. However, upon increasing the temperature of the samples, the A_{MRC} values for probes in CPZ or CPZ-TL aggregates felt more rapidly than those for probes in CPZ-treated synaptosomes. Since SDS did not induce MRC in the synaptosomes, the detergent-like properties of the drugs studied could not be responsible for the MRC increase.

The studied drugs exhibited distinct differences as to their ability to induce MRC, to disorder the lipid part of synaptosomal membranes, and to decrease the lipid concentration in synaptosomes. The order of potencies of the studied drugs to induce MRC in synaptosomes correlated with their potency to decrease the lipid concentration in synaptosomes, and correlated with their capacity to increase the dynamics/disorder of the lipid part of synaptosomal membranes. Thus, the increase of MRC induced by the drugs is supposed to have resulted mainly from the decreased lipid concentration in synaptosomes, where perturbation of lipid-protein interactions and membrane proteins can be presumed, and from their perturbation effect on the lipid membrane part.

All the drugs studied have a similar heterocyclic skeleton, substituted in position 2 by -Cl in CPZ, CPT and PFZ, by $-OCH_3$ in LPZ, and by $-SCH_3$ group in TRZ. Moreover, CPT differs from the other drugs in its tertiary amino group ($>N-CH_2-$) in position 10 being replaced by $>C=CH_2$-group. The aliphatic substitutes on the nitrogen in position 10 are different in each of the five drugs, which decisively modifies their properties. Thus, CPZ has a dimethyl amino group in position 14, whereas PFZ has piperazine with a polar 2-hydroxyethyl moiety. TRZ followed by CPZ are the most potent drugs to form MRC, whereas LPZ has the lowest potency. The effect on MRC is also evident on replacing the amino group ($>N-CH_2-$) by $>C=CH_2$ group in position 10. CPZ was more potent than CPT. The effect of 2-substitution on the heterocyclic skeleton could not be clearly established. Although CPZ and LPZ have similar alkyl chains, they differ in position 2 (-CL in CPZ, $-OCH_3$ in LPZ), CPZ being very potent and LPZ showing very low potency in forming MRC. This implies that secondary and even higher structure orders may be responsible for the observed effect in the investigated systems.

It was suggested that hydrogen-bonds spanned between membrane components can play an important role in membrane behavior (Kamaya et al. 1980; Brockerhoff 1982; 1986; Boggs et al. 1986; Cevc 1987). Some drugs were found to interrupt the hydrogen bonds (Kamaya et al. 1980; Brockerhoff 1982; Hanpft and Mohr 1985; Veiro and Hunt 1985) and thus to destroy the specific structural arrangement of the membrane components. Rearrangement of the hydrogen-bonds may play a role in the effect of the studied drugs on lipid-protein interactions and/or membrane proteins observed in this study.

General anesthetics at pharmacological concentrations were found to decrease MRC of spin labeled lipid probe in nicotinic acetylcholine receptor-rich membranes (Fraser et al. 1990). In our previous studies an increase of MRC of a 16-SA probe was induced by local anesthetics and β-adrenoceptor blocking drugs (Ondriaš and Staško 1986; Ondriaš et al. 1989b), but the effects were relatively small in comparison to the effect of tranquilizers observed in the present study.

Chlorpromazine and its derivatives influence various biological membrane processes (Hruban et al. 1978; Salhab et al. 1979; Fujii et al. 1979; Tsao et al. 1982).

They were found to interact strongly with biological or lipid membranes. CPZ has a high partition coefficient in lipid membranes (Luxnat and Galla 1986) and was found to form 1:1 molar complexes with a negatively charged lipid (Schwendener 1988), and to induce immobilization of spin probe in erythrocyte ghosts (Yamaguchi et al. 1985). Maximal uptake of CPZ in erythrocyte membrane exceeded 2×10^9 molecules/red cell (Lieber et al. 1984). These findings indicate that in some *in vitro* experiments the membrane concentrations of the drugs can be high enough to perturb lipid-protein interactions and/or membrane proteins. However, the concentrations of the drugs used in our study were much higher than those encountered *in vivo*. Further studies are necessary to demonstrate whether the studied drugs perturb lipid-protein interactions and/or membrane proteins at pharmacological concentrations. We suppose that the strong perturbation effect of the tranquilizers on lipid-protein interactions and/or membrane proteins found in our study may be responsible for some of their biological effects.

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