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Short communication

# Transbilayer Effects of Chlorpromazine · HCl on Rotational Mobility of Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Brain

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**Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to evaluate the effects of chlorpromazine · HCl on the range of the rotational mobility of bulk bilayer structure of the synaptosomal plasma membrane vesicles (SPMV) isolated from a bovine brain. In a dose-dependent manner, chlorpromazine · HCl increased the anisotropy ($r$), limiting anisotropy ($r_\infty$) and order parameter ($S$) of DPH in the membranes. Cationic 1-[4-(trimethylammonio)-phenyl]-6-phenylhexa-1,3,5-hexatriene (TMA-DPH) and anionic 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid (PRO -DPH) were utilized to examine the range of transbilayer asymmetric rotational mobility of the neuronal membranes. The anisotropy ($r$) of TMA-DPH in the inner monolayer was 0.034 greater than the value of PRO-DPH in the outer monolayer of the membranes. Both cationic TMA-DPH and anionic PRO-DPH were also used to examine the transbilayer asymmetric effects of chlorpromazine · HCl on the range of rotational mobility of the membranes. Chlorpromazine · HCl have a decreasing effects on the rotational mobility of the bulk bilayer structures and have a greater decreasing effect on the mobility of the inner monolayer as compared to the outer monolayer of the membranes. It has been proven that chlorpromazine · HCl exhibit a selective rather than nonselective fluidizing effect within the transbilayer domains of the SPMV.**

**Keywords:** Chlorpromazine · HCl, Neuronal membranes, Transbilayer fluidity, Fluorescent probe technique

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## Introduction

Dopaminergic receptors are the primary targets in the treatment of schizophrenia, Parkinson's disease, and Huntington's disease. Antipsychotics originally helped to discover dopamine receptors, the seven cloned dopaminergic receptors that are now facilitating the discovery of selective antipsychotic and antiparkinson drugs. The dopamine hypothesis of schizophrenia proposes that brain dopamine systems are overactive (Seeman, 1992; Seeman and Van Tol, 1993; Seeman *et al.*, 1993). The overactivity may stem from either an excess release of dopamine or an overactive response that is mediated by the dopaminergic receptors. The hypothesis mainly relies on the fact that neuroleptics block dopaminergic receptors in direct relation to their clinical antipsychotic potencies (Seeman, 1992; Seeman and Van Tol, 1993). $D_1$-like receptors are found on the post-synaptic neuron, while $D_2$-like receptors are found on both the post- and presynaptic neurons (Sunahara *et al.*, 1993). Chlorpromazine is one of postsynaptic dopaminergic receptor blocking agents. In the absence of exogenous dopamine, the binding of [$^3$H] raclopride to $D_2$ receptors was monophasically inhibited by the $D_1$ receptor antagonist SCH23390 at concentrations above $10^{-4}$ M. However, in the presence of 100 mM exogenous dopamine, the binding of [$^3$H] raclopride to $D_2$ receptors was increased by $10^{-8}$ M to $10^{-7}$ M SCH23390 (Ellenbroek, 1993; Seeman *et al.*, 1994; Cristina *et al.*, 1998).

Research on mechanism of the pharmacological action of chlorpromazine · HCl has been limited to chlorpromazine's blocking of dopaminergic receptors due to its binding to dopaminergic receptors in competition with dopamine. Because receptors coexist with membrane lipids, we cannot entirely exclude the possibility that changes of lipid fluidity may be accompanied before or after receptor blocking agents bind to receptors. If chlorpromazine · HCl changes mobility of

a neuronal membrane lipid bilayer, it will evenly act on both inner and outer monolayers. Sheetz and Singer (1974) proposed that the asymmetry net charge at the surface of the two monolayers of the biological membranes could establish an asymmetric transbilayer distribution of charged amphipaths intercalating in the two monolayers. Will the positively charged chlorpromazine in the water solution change mainly the mobility of the negatively charged inner monolayer of neuronal membrane lipid bilayer?

Exploiting fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), we examined the effects of chlorpromazine · HCl on the rotational mobility of a bulk lipid bilayer of synaptosomal plasma membrane vesicles (SPMV). Using sidedness selective fluorescent DPH derivatives, cationic 1-[4-(trimethylammonio)-phenyl]-6-phenylhexa-1,3,5-hexatriene (TMA-DPH) and anionic 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid (PRO-DPH), we evaluated the effects of chlorpromazine · HCl on the transbilayer differential rotational mobility of SPMV.

## Materials and Methods

**Materials** The fluorescent probes DPH, PRO-DPH and TMA-DPH were obtained from Molecular probes (Eugene, USA). Chlorpromazine · HCl, and other reagents were obtained from Sigma (St. Louis, USA) and were of analytical grade.

**Preparation of SPMV** The SPMV was isolated from a whole bovine brain by the formerly reported method in our laboratory (Yun and Kang, 1990; Yun *et al.*, 1990). The specific activities of Na,K-ATPase, acetylcholinesterase and 5'-nucleotidase in the plasma membrane fraction were approximately 4-, 2.5- and 3-times higher than those in crude homogenates. The electron microscopic examination of the prepared SPMV showed very high purity. The vesicles, which were separated according to size, demonstrated homogeneous distribution and no longer showed the presence of intracellular organelles or leakage. The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

**Fluorescence measurements** The fluorescent probe DPH was dissolved in tetrahydrofuran and a volume of 0.5 ml of tetrahydrofuran per ml of phosphate-buffered saline (PBS) was added directly to the membrane suspension at a concentration of 0.1 μg/100 μg membrane protein. PBS was composed of 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $KH_2PO_4$, 1.15 g/l $Na_2HPO_4 \cdot 7H_2O$, 0.48 g/l Hepes (37°C, pH 7.4). The suspension was incubated in the dark at 37°C for 30 min with frequent vortexing. The excitation wavelength for DPH in SPMV was 362 nm, and the fluorescence emission was monitored at 424 nm.

The polarization ($P$) was obtained from intensity measurements using $P = (I_{//} - GI_{\perp})/(I_{//} + GI_{\perp})$ where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This polarization ($P$) value is the ratio of the fluorescence intensities of the vertical to horizontal components when the excited light is polarized in the horizontal direction. This condition yields an equivalent fluorescence intensity ($I$) entering the monochromator irrespective of the orientation of the observation polarizer. The polarization was expressed as the anisotropy [$r = 2P/(3-P)$], the limiting anisotropy ($r_\infty$) and the order parameter ($S$). The limiting anisotropy ($r_\infty$) of membrane-bound DPH, PRO-DPH and TMA-DPH was determined directly from the anisotropy value using the following relationship (van Blitterwijk *et al.*, 1981)

$$r_\infty = (4/3)r - 0.10 \qquad 0.13 < r < 0.28 \qquad (1)$$

The limiting anisotropy ($r_\infty$) reflects restriction to probe motion and can be converted to an order parameter, $S = (r_\infty / r_o)^{1/2}$ (Kawato *et al.*, 1978) where $r_o$, the anisotropy in the absence of motion, is equal to 0.362 for DPH (Lakowicz *et al.*, 1979).

Chlorpromazine · HCl, at the concentrations indicated, were added directly to membranes resuspended in PBS. The pH of the buffered sample was not changed significantly by the addition of chlorpromazine · HCl. Measurements commenced usually within 1 min after addition. There was no effect from longer incubation time.

All fluorescence measurements were obtained with an Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003, IL, USA) and performed at 37°C (pH 7.4). Before the fluorescence spectra were obtained, all samples were bubbled by dry nitrogen through the solution for at least 5 min in order to eliminate oxygen. Blanks, prepared under the identical conditions without fluorescent probes, served as a control for the fluorometric measurements.

**Determination of individual monolayer structure in SPMV: preferential distribution of charged DPH derivatives** In order to evaluate transmembrane asymmetry of rotational mobility, plasma membrane sidedness selective fluorescent DPH derivatives, cationic TMA-DPH and anionic PRO-DPH were utilized. Since the negatively charged phospholipids are located preferentially in the inner monolayer of SPMV, TMA-DPH and PRO-DPH are expected to be located preferentially in the inner monolayer and in the outer monolayer of SPMV, respectively. The excitation wavelength for TMA-DPH and PRO-DPH was 362 nm, and the fluorescence emission was read at 424 nm.

## Results and Discussion

In order to determine the effects of the chlorpromazine · HCl on the bulk and differential rotational mobility of monolayers of SPMV, it is first necessary to demonstrate that this drug does not interact directly with DPH, TMA-DPH and PRO-DPH and thereby quench its fluorescence. Quenching of absorbance-corrected fluorescence intensity by the chlorpromazine · HCl is not observed at all of the concentration levels where chlorpromazine · HCl was tested. Furthermore, if direct quenching of DPH, TMA-DPH and PRO-DPH by chlorpromazine · HCl occurred, fluorescence lifetime would decrease. However, the fluorescene lifetime of DPH is not changed by chlorpromazine · HCl in the SPMV. For example, the lifetime of DPH in the SPMV was 9.7±0.02,

9.6±0.2, 9.7±0.3, 9.5±0.1 and 9.6±0.2 ns at 0.1, 0.5, 1.5 and 10 mM chlorpromazine · HCl, respectively. Similar results were with TMA-DPH and PRO-DPH. Hence, the possibility of the direct quenching of fluorescence of the probes by the drug is ruled out.

Our data presented herein have shown that, even at physiologically relevant concentrations (from $10^{-4}$ to $10^{-3}$ M, Ellenbroek, 1993; Cristina *et al.*, 1998), chlorpromazine decreases the rotational diffusion of DPH, TMA-DPH and PRO-DPH in the SPMV, indicating that chlorpromazine has a decreasing effect on the rotational mobility of the bulk lipid bilayer and inner and outer monolayers.

**Effects of chlorpromazine · HCl on the range of rotational mobility of bulk SPMV lipid bilayer** DPH is a rod-shaped molecule that orients with high affinity in hydrophobic regions (core) of the bilayer structures. The fluorescence polarization mainly reflects the rotational mobility of lipid fluorophores (Schachter, 1984; Molitoris and Hoilien 1987; Yun *et al.*, 1993a,b). The results of fluorescence polarization determination are conveniently expressed as the fluorescence anisotropy ($r$). The limiting anisotropy ($r_\infty$) reflects the hindrance to a full 90° rotation of a fluorophore in a particular microenvironment. For example, the rod-like hydrocarbon DPH is free to rotate a full 90° in certain organic solvents, and the $r_\infty$ value is zero. In native and model membranes, the $r_\infty$ values of the DPH are high and largely determine $r$. In biological experiments, both dynamic (rotational relaxation time of fluorophores) and structural ($r_\infty$), or static components may be significant. It seems reasonable to use "fluidity" to designate both. The structural organization of the lipid environment in the bilayers limits the rotational extent or the range of DPH. The $r_\infty$ value can be used to define order parameter ($S$).

The bulk anisotropy ($r$), bulk limiting anisotropy ($r_\infty$) and bulk order parameter ($S$) of intact SPMV (chlorpromazine · HCl-untreated) were 0.202±0.001, 0.169±0.002 and 0.683±0.003 (Table 1). The effect of increasing concentrations of chlorpromazine · HCl on the anisotropy ($r$), limiting anisotropy ($r_\infty$) and order parameter ($S$) of DPH in bulk SPMV lipid bilayer are shown in Figs. 1-3. In the SPMV, chlorpromazine · HCl increases the anisotropy ($r$), limiting anisotropy ($r_\infty$) and order parameter ($S$) of DPH (decreasing effect of chlorpromazine on rotational mobility) dose-dependently. The significant increases in the anisotropy ($r$) by chlorpromazine · HCl were observed even at $70 \times 10^{-6}$, $35 \times 10^{-5}$, $70 \times 10^{-5}$, $35 \times 10^{-4}$, $70 \times 10^{-4}$, $35 \times 10^{-3}$ and $70 \times 10^{-3}$ M (Fig.1). The variation in the anisotropy ($r$) of DPH found in the bulk SPMV lipid bilayer before and after adding $70 \times 10^{-3}$ M chlorpromazine · HCl was 0.030. The anisotropy ($r$) values of DPH in the bilayer are 0.202±0.001 (n = 5), 0.257±0.002 (n = 5) at 37 and 25°C (pH 7.4), respectively. Based on the aforementioned results at the two different temperatures (25 and 37°C), the observed effect by $70 \times 10^{-3}$ M chlorpromazine · HCl (different value 0.030) was the same as produced by the temperature fall of approximate 17.5°C.

**Effects of chlorpromazinePHCl on the range of transbilayer rotational mobility of SPMV lipid bilayer** The anisotropy ($r$) value of TMA-DPH in the inner monolayer was 0.034 greater than the value of PRO-DPH in the outer monolayer of the SPMV. This means that the range of rotational mobility of the outer monolayer is greater than that of the inner monolayer. The membrane consists of domains or patches of lipids that differ in their fluidity and lipid composition. Several different domains have been described, e.g., hydrophilic, hydrophobic, lateral, outer and inner monolayers (Chin and Goldstein, 1981; Seigneuret *et al.*, 1984; Chabanel *et al.*, 1985; Hitzemann *et al.*, 1986; Treistman and Wilson, 1987). The surface of the membrane is more hydrophilic as compared to the interior which is more hydrophobic. Lateral domains are lipid patches that extend laterally along the horizontal plane of the membrane and are thought to differ in their fluidity and lipid composition (Treistman and Wilson, 1987). Two other domains to be considered are the transbilayer or vertical domains of the membrane (i.e., the outer and inner monolayers). Our data explicitly show that the SPMV consists of vertical domains or monolayers that differ in rotational mobility. Hence, the bulk lipid fluidity change obtained will represent an average of the affected and unaffected portions of the membrane core and may underestimate the effect on specific domains. Very little attention has been given to the selective effects of chlorpromazine · HCl on vertical domains.

The effects of increasing concentrations of chlorpromazine · HCl on the anisotropy ($r$), limiting anisotropy ($r_\infty$) and order parameter ($S$) of TMA-DPH and PRO-DPH in the SPMV individual monolayers are shown in Figs. 1-3, respectively. Chlorpromazine · HCl shows a greater decreasing effect on the range of rotational mobility of the inner monolayer (Figs.

**Table 1.** Structural parameters of 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylammonio)-phenyl] -6-phenylhexa-1,3,5-hexatriene (TMA-DPH) and anionic 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid (PRO-DPH) in synaptosomal plasma membrane vesicles isolated from bovine brain (SPMV)

| Parameters | DPH | TMA-DPH | PRO-DPH |
|---|---|---|---|
| Anisotropy ($r$) | 0.202±0.001 | 0.220±0.002 | 0.186±0.001 |
| Limiting anisotropy ($r_\infty$) | 0.169±0.002 | 0.193±0.003 | 0.148±0.002 |
| Order parameter ($S$) | 0.683±0.003 | 0.730±0.005 | 0.639±0.004 |

Fluorescence measurements were performed at 37°C (pH 7.4). Values are represented as the mean±SEM of 5 determinations.

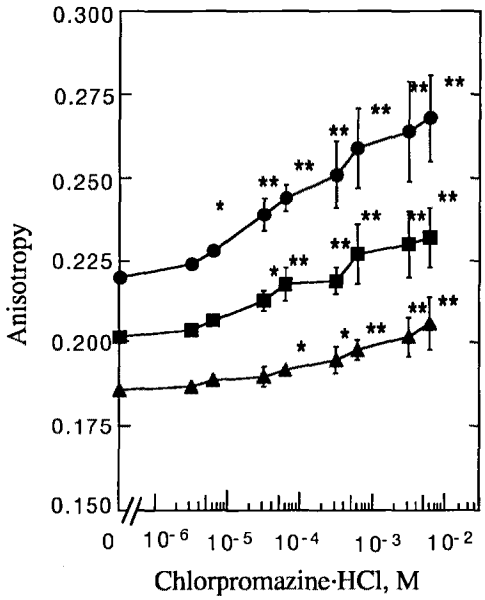


**Fig. 1.** Effects of chlorpromzine · HCl on the anisotropy ($r$) values of DPH, TMA-DPH and PRO-DPH in the SPMV isolated from bovine brain. The excitation and emission wavelengths of the probes were 362 nm and 424 nm, respectively. The probes were incorporated into SPMV and fluorescence measurements were performed at 37°C (pH 7.4). Inner plus outer monolayer (DPH, ■); inner monolayer (TMA-DPH, ●); outer monolayer (PRO-DPH, ▲) as described in Materials and Methods. Each point represents the mean±SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's $t$-test.

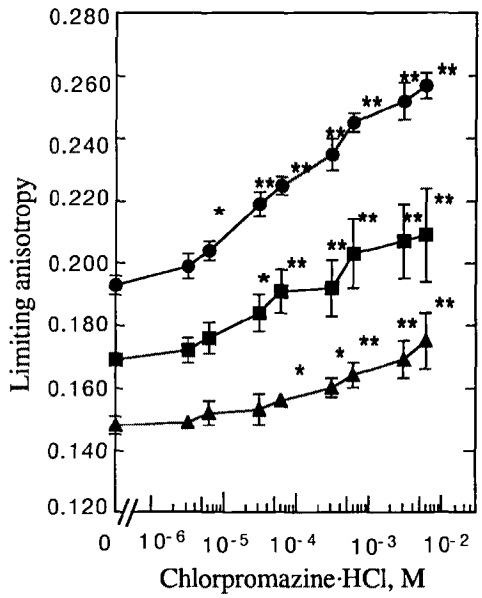


**Fig. 2.** Effects of chlorpromzine · HCl on the limiting anisotropy ($r_\infty$) values of DPH, TMA-DPH and PRO-DPH in the SPMV isolated from bovine brain. The excitation and emission wavelengths of the probes were 362 nm and 424 nm, respectively. The probes were incorporated into SPMV and fluorescence measurements were performed at 37°C (pH 7.4). Inner plus outer monolayer (DPH, ■); inner monolayer (TMA-DPH, ●); outer monolayer (PRO-DPH, ▲) as described in Materials and Methods. Each point represents the mean±SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's $t$-test.

1-3, filled circles) compared with the outer monolayer (Figs. 1-3, filled triangles). Since changes observed in the anisotropy ($r$), limiting anisotropy ($r_\infty$) and order parameter ($S$) of TMA-DPH and PRO-DPH derive primarily from changes in the inner monolayer, we studied the selective effects of the drug on the component of the range of mobility of the probes. To the best of our knowledge, the results presented herein are the first to demonstrate that the Sheetz-Singer hypothesis (1974) is valid in neuronal membranes.

Plasma membranes consist of two monolayers that are asymmetric in lipid distribution, electrical charge, fluidity, protein distribution and function, and do not appear to be coupled. It had been widely known that different lipids could affect the physical properties of the membrane. Membrane cholesterol is one of the major lipids of plasma membranes and is asymmetrically distributed in the outer and inner monolayers of membranes (Kier *et al.*, 1986; Wood *et al.*, 1990; Schroeder *et al.*, 1991a,b). Interest in cholesterol derives from the fact that cholesterol has a rigidifying effect on the membrane above the phase transition temperature of the membrane lipid. In erythrocytes, differences in fluidity between the two monolayers have not been consistently observed. Some studies have reported that the outer monolayer was less fluid (Seigneuret *et al.*, 1984; Chabanel *et al.*, 1985), whereas, other studies have found that the outer monolayer was more fluid than the inner monolayer (Cogan and Schachter, 1981; Schachter *et al.*, 1983). The finding that inner monolayer of the synaptic plasma membrane isolated from rat brain (SPM) was less fluid than the outer monolayer was consistent with data showing that the SPM inner monolayer contains approximately 7-times as much cholesterol compared with the outer monolayer (Wood *et al.*, 1990). Thus, a possible explanation for the range of asymmetric rotational mobility between outer and inner monolayers of SPMV in this study is that the amount of cholesterol may differ in the outer and inner monolayers. These differences have been ascribed to cholesterol (which is asymmetrically distributed between the inner and outer monolayers of the neuronal membrane), but cholesterol does not seem to be solely responsible for such differences. This is because the differences in asymmetrical lateral diffusion between the inner and outer monolayers of the model membrane lipid bilayer (prepared from total lipids that were isolated from SPMV) were 0.452±0.008 and 0.500± 0.013, respectively ($I'/I$ values from bispyrenyl propane, Min, 1997). The differences in asymmetrical lateral diffusions between

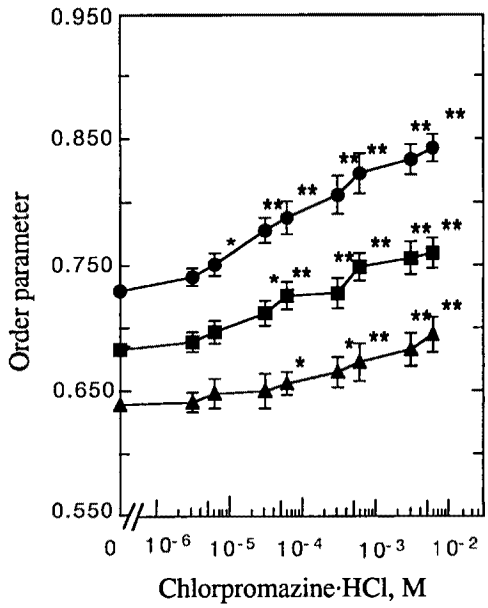


**Fig. 3.** Effects of chlorpromzine · HCl on the order parameter (*S*) of DPH, TMA-DPH and PRO-DPH in the SPMV isolated from bovine brain. The excitation and emission wavelengths of the probes were 362 nm and 424 nm, respectively. The probes were incorporated into SPMV and fluorescence measurements were performed at 37°C (pH 7.4). Inner plus outer monolayer (DPH, ■); inner monolayer (TMA-DPH, ●); outer monolayer (PRO-DPH, ▲) as described in Materials and Methods. Each point represents the mean±SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's *t*-test.

inner and outer monolayers of the model membrane lipid bilayer (made with phospholipids that were separated from SPMV) were also 0.540±0.013 and 0.572±0.016, respectively (*I'/I* values from bispyrenyl propane, Lee, 1999). It is presumed that the asymmetrical mobility between inner and outer monolayers of the model membranes (formed with total phospholipids where cholesterol and protein are not present) can be attributed to the types of phospholipids, which are likely distributed asymmetrically between inner and outer monolayers, and to the composition of each phospholipids unsaturated or saturated fatty acids. We can also presume that the law of physics may dictate asymmetrical movements for the stability of lipid bilayer.

The term "membrane fluidity" is often misused. It arose from a combination of spectroscopic studies; the realization that a membrane can be regarded as a two-dimensional fluid, and the drive to obtain a simple single physical parameter that would describe the property. The difficulty with the membrane fluidity concept is that any physical parameter chosen will be a property of the spectroscopic method employed, specifically its particular time window and the properties of the probe (shape, charge, location *etc*) (Stubbs and Williams, 1992). The membrane fluidity concept also depends on the assumption that the hydrophobic region of cell membranes is structurally and dynamically homogeneous, an assumption is now being seriously challenged. Thus, while it may be true. That bulk or average spectroscopic properties of cell membranes may not be useful in building a hypothesis for the molecular mechanism(s) of pharmacological action(s) of drug(s), local properties pertaining to domains or in the immediate environment of a membrane protein maybe very relevant.

The investigation of the binding site(s) of drug(s) at a cellular level provides important basic materials for the research of pharmacological actions of drug(s). This is because the binding site(s) of drug(s) at a cellular level coincides with the site(s) of drug action, even though not necessarily all the time. From present results, the reactions between the membrane lipids and the drugs is assumed to be as follows. The unusually hydrophobic ion channel macromolecule has an anomalously high detergent-binding capacity, due in part to more than a dozen long-chain fatty acids associated with each channel molecule (Butterworth and Strichartz, 1990). Bound to the protein by covalent or noncovalent bonds, these acyl chains may anchor and orient the channel in the membrane, stabilizing the channels three-dimensional structure. Long-chain fatty acids also may participate in binding of lipophilic drug(s) such as chlorpromazine · HCl. The drugs' binding site(s) may exist in the channel's pore at the membrane-protein interface, or within the protein subunits of the channel. The clear mechanism of action of the drugs in the ordering effects on the lipid bilayer of the neuronal membrane is unknown. However, the mechanism through which chlorpromazine · HCl decreases the rotational mobility of the SPMV lipid bilayer can be assumed as follows.

The phospholipid molecules in the bilayer of the SPMV, the chlorpromazine · HCl effectively establish formation of hydrogen bonds with the carbonyl moiety. Present results show that the chlorpromazine · HCl may interact with carbonyl moiety of phospholipids in the bilayer. The interaction of the chlorpromazine · HCl with the bilayer's hydrocarbon region will generate rearrangements of the intermolecular hydrogen-bonded network among phospholipid molecules and/or protein molecules that are associated with the liberation of hydrated water molecules on the monolayer of the membranes. The interaction will also change the orientation of the P-N dipole of phospholipid molecules. The decreasing effects on the rotational mobility of the neuronal membrane lipid bilayer by chlorpromazine · HCl provide proper environment for the binding of chlorpromazine-dopaminergic receptor, and due to the decreased rotational mobility of the neuronal membrane lipid bilayer resulting from the binding of chlorpromazine-dopaminergic receptor, influx of inorganic ions are inhibited or blocked, thereby leading to preventing of depolarization of postjunctional membrane.

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