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Effects of Chlorpromazine · HCl on the Structural Parameters of Bovine Brain Membranes

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Fluorescence probes located in different membrane regions were used to evaluate the effects of chlorpromazine · HCl on structural parameters (transbilayer lateral mobility, annular lipid fluidity, protein distribution, and lipid bilayer thickness) of synaptosomal plasma membrane vesicles (SPMVs) isolated from bovine cerebral cortex. The experimental procedure was based on the selective quenching of 1,3-di(1-pyrenyl)propane (Py-3-Py) by trinitrophenyl groups, radiationless energy transfer from the tryptophan of membrane proteins to Py-3-Py, and energy transfer from Py-3-Py monomers to 1-anilinonaphthalene-8-sulfonic acid (ANS). In this study, chlorpromazine · HCl decreased the lateral mobility of Py-3-Py in a concentration dependent-manner, showed a greater ordering effect on the inner monolayer than on the outer monolayer, decreased annular lipid fluidity in a dose dependent-manner, and contracted the membrane lipid bilayer. Furthermore, the drug was found to have a clustering effect on membrane proteins.

Keywords: Annular lipid fluidity, Chlorpromazine · HCl, Membrane protein clustering, Membrane thickness, Transbilayer lateral mobility

Introduction

Antipsychotics originally helped in the discovery of dopaminergic receptors, and cloned versions of the seven known dopaminergic receptors are now helping with the identification 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). This overactivity may stem from either an excessive release of dopamine or an overactive response mediated by the dopaminergic receptors. The dopamine theory essentially is dependent 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 post-synaptic neurons, whereas D_2-like receptors are found on both post- and presynaptic neurons (Sunahara *et al*., 1993). Moreover, chlorpromazine is a postsynaptic dopaminergic receptor blocking agent.

Changes in membrane fluidity are known to be linked to alterations in the physiological processes of cell membranes, for example, with carrier-mediated transport, the activities of membrane bound enzymes, receptor binding, phagocytosis, endocytosis, depolarization dependent exocytosis, cytotoxicity, and cell growth (Spector and Yorek, 1985). Moreover, membrane fluidity appears to change during development, aging, and cultured cell drug therapy (Toplak *et al.*, 1990), and alterations in membrane fluidity may even offer parameters for the evaluation of malignancy (Shinitzky, 1984).

The ability to regulate cell volume is one of the elementary requirements of cell survival, and volume regulatory mechanisms are an integral part of the intracellular cascade responsible for transmitting hormonal signals (Lang *et al.*, 1993, 1998). It is important to note that cell volume is linked to ion channels and carriers at the cell membrane and to cellular metabolic activity (Lang *et al.*, 1993, 1998).

Researches on the mechanism of the pharmacological action of chlorpromazine · HCl have been relatively limited to chlorpromazine's blocking of dopaminergic receptors due to its binding to dopaminergic receptors in competition with dopamine. Since receptors are embedded in the membrane, several authors have advised that the possibility cannot be excluded that change in lipid fluidity may modify the binding of chlorpromazine · HCl to the receptors. It was reported that

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chlorpromazine has a reducing effect on the bulk (inner + outer monolayers) rotational mobility of rat liver plasma membranes (Keefe *et al*., 1980), platelet membranes (Zubenko and Cohen, 1984; Zubenko and Cohen 1985a, 1985b, 1985c), and rat intestinal brush border membrane vesicles (Iseki *et al.*, 1988). On the other hand, chlorpromazine has been shown to increase the bulk rotational mobility of synaptic membranes (Breton *et al.*, 1977), pigeon erythrocyte membranes (Saless *et al*., 1982a, 1982b), and human erythrocyte membranes (Noji *et al*., 1982; Widmer *et al*., 1987; Lejoyeux *et al.*, 1993). Moreover, chlorpromazine · HCl has reduces the rotational mobility of bulk bilayer structures and reduces the mobility of the inner monolayer more so than the outer monolayer of synaptosomal plasma membrane vesicles (SPMVs) from the bovine cerebral cortex (Ahn *et al*., 2000).

As indicated above, with only a few exceptions (Ahn *et al*., 2000), most analyses of the effects of chlorpromazine on cell membrane fluidity have focused on bulk bilayer rotational mobility, not on the transbilayer rotational mobility. Furthermore, little attention has been given to the effect of chlorpromazine on either the bulk or the transbilayer lateral mobility of biological membranes.

It was the aim of this study to provide a basis for the study of the mode of action of chlorpromazine · HCl. Thus, we carried out a comprehensive study of the action of chlorpromazine · HCl on SPMVs. The scope of this research was as follows. First, to exploit the intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py-3-Py), we examined the effect of chlorpromazine · HCl on the rate and range of the lateral mobility of the bulk SPMV lipid bilayer. Second, by employing a fluorescence quenching method, which we developed specifically for this study to measure the rate and range of asymmetrical lateral mobility between the inner and outer monolayers of the lipid bilayer, we evaluated the effect of chlorpromazine·HCl on the transbilayer lateral mobility of the SPMV lipid bilayer. Third, we examined the effects of chlorpromazine · HCl on both annular lipid fluidity and protein distribution in the SPMVs. Fourth, by employing a fluorescence quenching method, we examined the effect of chlorpromazine · HCl on SPMV lipid bilayer thickness.

Materials and Methods

Materials The fluorescent probes, Py-3-Py and 1-anilinonaphthalene -8-sulfonic acid (ANS) were obtained from Molecular probes (Eugene, USA). Chlorpromazine · HCl, and the other reagents were obtained from Sigma (St. Louis, USA) and were of analytical grade.

Preparation of SPMVs SPMVs were isolated from bovine cerebral cortex by a previously reported method (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 transmission electron microscopic examination of the prepared of SPMVs showed a high level of purity (Yun *et al.*, 1990). The vesicles, which were separated according to size, demonstrated a homogeneous distribution and no longer showed the presence of intracellular organelles or leakage. The protein concentration of SPMVs was determined using the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Fluorescence measurements Fluorescence measurements were performed using a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (Model; ISS K2-003). Cuvette temperature was maintained at 37.0 ± 0.1ºC with a circulating water bath (pH 7.4). Bandpass slits were 10 nm on excitation and 5 nm on emission. Blanks, prepared under identical conditions without fluorescent probes, served as controls for the fluorometric measurements.

The incorporation of Py-3-Py was carried out by adding aliquots of a stock solution of 5×10^{-5} M in absolute ethanol to the SPMVs, so that the final probe concentration was less than 5×10^{-7} M (Yun *et al.*, 1994; Kang *et al.*, 1996). The mixtures were initially vigorously vortexed for 10 s at room temperature and then incubated at 4ºC for 18 h with gentle stirring (Yun *et al.*, 1994; Kang *et al.*, 1996).

After incorporation of the Py-3-Py or ANS, the membrane suspension was placed in cuvettes. Control levels of fluorescence were then determined. A concentrated solution of chlorpromazine · HCl was prepared in 10 mM Tris-HCl (pH 7.4) and added to the labeled membrane suspension to give the desired agent concentration.

Excitation wavelengths were 280 nm for tryptophan, 330 nm for Py-3-Py, and 380 nm for ANS. Emission wavelengths were 335 nm for tryptophan, 379 nm for Py-3-Py monomer and 480 nm for Py-3-Py excimer. A GG-455 cut-off filter was used for Py-3-Py excimer emission. The excimer to monomer fluorescence intensity ratio, I'/I, was calculated from the 480 nm to 379 nm signal ratio.

The pH of the buffered sample was not changed significantly by the addition of chlorpromazine · HCl. Measurements commenced usually within 1 min of addition; no effect of incubation time was noticed.

TNBS labeling reactions To determine the fluorescence parameters of probe molecules in each membrane monolayer, 2,4,6-trinitrobenzenesulfonic acid (TNBS) labeling reactions were performed using modifications of published procedures (Yun and Kang, 1990; Yun *et al.*, 1994; Kang *et al.*, 1996). Breifly, SPMV was gently resuspended in 50 ml of 4 mM TNBS plus buffer A or buffer A alone; buffer A was composed of 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose and 1% BSA. Reagent pH was adjusted to 8.5 with NaOH. To assure complete exposure of all SPMV outer monolayers to TNBS, the SPMV pellet was passed slowly through an Eberbach tissue grinder (3 up and down strokes). Unless otherwise specified, the treatment was carried out at 4ºC for 90 min. The TNBS labeling reaction was terminated by adding an equal volume of 1% BSA in phosphate-buffered saline (PBS: 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; pH adjusted to 7.4 with Tris base).

Effect of chlorpromazine · HCl on individual monolayer structures in SPMVs: Selective quenching of Py-3-Py To determine individual monolayer structures in SPMVs, we devised a new method involving the selective quenching of Py-3-Py fluorescence by trinitrophenyl groups. This method is based on the

assumption that the system is composed of fluorescing compartments that are differentially accessible to TNBS. The excimer to monomer fluorescence intensity ratios, *I′/I*, of the Py-3-Py in bulk (inner plus outer), inner and outer monolayers were calculated using the following equations:

$$(I'/I)_t = I'_t / I_t \quad (1)$$
$$(I'/I)_i = I'_i / I_i \quad (2)$$
$$(I'/I)_o = (I'_t - I'_i) / (I_t - I_i) \quad (3)$$

where $(I'/I)_t$, $(I'/I)_i$ and $(I'/I)_o$ are the excimer to monomer fluorescence intensity ratio of Py-3-Py (I'/I) in bulk, inner, and outer monolayers, respectively. The values of I'_t (excimer fluorescence intensity for inner plus outer monolayers) and I'_i (excimer fluorescence intensity for inner monolayer) were determined for Py-3-Py in SPMVs obtained from SPMVs incubated with buffer A and buffer A plus TNBS at 4°C (pH 8.5) (nonpenetrating conditions), respectively.

Determination of annular lipid fluidity in SPMVs Incorporated Py-3-Py in SPMVs was excited by radiationless energy transfer (RET) from tryptophan (excitation at 286 nm) and the excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py was calculated from the 480 nm to 379 nm signal ratio. Taking into account that the Förster radius (the RET-limiting distance) for the tryptophan-Py-3-Py donor-acceptor pair is 3 nm (Dobretsov *et al.*, 1982), only Py-3-Py located in annular lipids (close to proteins) was excited; the fluidity of annular lipids was considered proportional to I'/I (Schachter, 1984; Almeida *et al*., 1986; Yun *et al*., 1993; Kang and Yun, 1994; Yun *et al*., 1994; Kang *et al*., 1996). The efficiency of RET from tryptophan to Py-3-Py was calculated using the equation:

$$\text{RET} = (I_d - I_{da})/I_d \quad (4)$$

where I_d and I_{da} represent the fluorescence intensities of donor (in this case, endogenous tryptophan) in the absence and presence of acceptor (Py-3-Py), respectively.

Determination of protein clustering in the SPMV lipid bilayer The fluorescence intensity of endogenous tryptophan in SPMVs was determined. Following this measurement, the probe Py-3-Py was incorporated at a concentration of 10^6 M (1 ml of 10^3 M in ethanol), and after 10 min, tryptophan emission fluorescence intensity was remeasured. The efficiency of RET from tryptophan to Py-3-Py was calculated using:

$$\text{RET} = (I_d - I_{da}) / I_d \quad (4)$$

where I_d and I_{da} represent the fluorescence intensities of donor (in this case, endogenous tryptophan) in the absence and presence of acceptor (in this case, Py-3-Py), respectively. The wavelengths of excitation and emission of tryptophan were 286 and 335 nm, respectively.

Determination of the thickness of the SPMV lipid bilayer We determined the effect of chlorpromazine · HCl on the thickness of the SPMV lipid bilayer using energy transfer between the surface fluorescent probe ANS and the hydrophobic fluorescent probe Py-3-Py. Py-3-Py was excited at 379 nm, and SPMV lateral mobility was considered proportional to the ratio I'/I obtained at an excitation wavelength of 480 nm. Next, 30 μM ANS (60 μl of 10^{-3}M solution in water) was added. After 1 min, Py-3-Py monomer fluorescence was remeasured. ANS is located on the membrane surface and the above concentration was chosen because it produced 50% quenching of Py-3-Py monomer fluorescence in SPMVs. The efficiency of Py-3-Py quenching by ANS was calculated using equation 4, considering Py-3-Py monomer as the donor and ANS as the acceptor. Membrane thickness was considered to be proportional to the distance (D) between the donor and the acceptor and can be calculated as follows:

$$D = [(\pi\Phi/6)(\langle E\rangle^{-1} 1)]^{1/3} \quad (5)$$

where F is the number of acceptors per volume and <E> is the average efficiency of RET (Eisinger and Flores, 1982). To calculate D, we used a modified equation:

$$D = [(F'/F_{max})(\langle E\rangle^{-1} - 1)]^{1/3} \quad (6)$$

where F' is the fluorescence intensity of the acceptor, the concentration utilized in RET experiments, and F_{max} is the maximal fluorescence of acceptor in the membrane (both values were obtained by ANS binding studies in the absence of Py-3-Py). The ratio F'/F_{max} provides a measure of the binding of ANS to the membrane surface and provides a correction for the effects of chlorpromazine · HCl on this binding.

To calculate F' and F_{max}, ANS (2 μl of 10^{-2} M solution) was added repeatedly to the sample at 15 sec intervals and measurements were made as soon as the probe was added. In each case, at least 14 concentrations of the probe were used. Dissociation constants and F_{max} values for the ANS-membrane complex were calculated from double-reciprocal plots by linear regression analysis. Correlation coefficients were > 0.99.

Results and Discussion

In order to determine the effects of chlorpromazine · HCl on the bulk and asymmetric lateral mobility of SPMV monolayers, annular lipid fluidity in SPMVs, clustering of proteins in SPMVs, and thickness of the SPMV lipid bilayer, it was first necessary to demonstrate that this drug does not interact directly with Py-3-Py and thereby quench its fluorescence. No quenching of absorbance-corrected fluorescence intensity by chlorpromazine · HCl was observed at any tested concentration. Furthermore, if direct quenching of Py-3-Py by chlorpromazine · HCl occurred, fluorescence lifetime would decrease. However, the lifetime of Py-3-Py in SPMV was 15.3 ± 0.02, 15.4 ± 0.01, 15.1 ± 0.02, 15.4 ± 0.05 and 15.3 ± 0.01 ns at 0.1, 0.5, 1, 5 and 10 mM of chlorpromazine · HCl, respectively. Hence, the possibility of direct quenching of probe fluorescence by the drug was ruled out.

Changes in membrane structure including fluidity, cause changes in membrane properties including functions. In the present study, we found that neuronal membrane fluidity and structure are changed by chlorpromazine · HCl. Therefore, it can be presumed that the properties and functions of neuronal membranes are changed by the antipsychotic agent.

Effect of chlorpromazine · HCl on the rate and range of the lateral mobility of the bulk SPMV lipid bilayer Py-3-Py, a pyrene derivative, which has been successfully used to quantitate lateral mobility within native and model membranes (Zachariasse *et al*., 1982; Schachter, 1984; Yun *et al*., 1993, 1994; Kang *et al*., 1996), was used to determine the rate and range of lateral mobility in SPMVs. Using the probe, one monitors the emission of both the monomer (*I*) and the excimer (*I'*) components in such a way that a ratio can be derived and used as a measure of lateral mobility (Zachariasse *et al*., 1982; Schachter, 1984; Yun *et al*., 1993, 1994; Kang *et al*., 1996). As probe mobility within membranes increase, emission from the excimer predominates, since the formation of the intramolecular excimer is dependent upon the lateral movement of its two components. Therefore, an increase in the *I'/I* ratio is an indication of increased lateral probe mobility within a membrane. The excimer fluorescence technique utilizing Py-3-Py has an advantage over its counterpart based on intermolecular excimerization since very small probe concentrations can be used ($<10^{-7}$ M), and the perturbation of the SPMV by the probe molecule is thus minimized.

In this study, the *I'/I* value in intact SPMVs (chlorpromazine · HCl untreated) was 0.412 ± 0.005. In SPMVs, chlorpromazine · HCl reduced the rate and range of the lateral mobility of Py-3-Py in a concentration dependent-manner, and significant decreases in *I'/I* values were observed at above 70×10^{-6} M of chlorpromazine · HCl (Fig. 1). The reduction in the *I'/I* values of Py-3-Py found in the bulk SPMV lipid bilayer after adding 70×10^{-3} M chlorpromazine · HCl was 0.058. The *I'/I* values of Py-3-Py in the bilayer were 0.412 ± 0.005 (n = 5), 0.356 ± 0.006 (n = 5) at 37 and 25ºC (pH 7.4), respectively. Based on the aforementioned results at these two different temperatures, the observed effect by 70×10^{-3} M chlorpromazine · HCl (different value 0.058) was the same as that produced by a temperature fall of approximately 12.4ºC.

Psychiatric patients receiving treatment with neuroleptics typically develop plasma levels of drug in the 10^{-9}-10^{-6} M range (Cohen, 1983; Curry, 1983). Rats receiving comparable doses of neuroleptics typically develop 10-100 μM concentrations of drug in brain tissue (Bickel *et al*., 1983). In this study, SPMVs were exposed to concentrations of chlorpromazine spanning this range. Our results suggest that the administration of chlorpromazine · HCl to psychiatric patients may be associated with alterations in the biophysical properties of cell membranes in the brain, and possibly in other tissues.

In support of this hypothesis Zubenko and Cohen (1985a) have found that, independent of psychiatric diagnosis, platelet membranes prepared from patients treated with phenothiazines exhibit an increase in structural order, as reflected by an increase in the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). The effects of chlorpromazine on the molecular dynamics of native and model lipid bilayers are complex and appear to depend on; the membrane system, the concentration of chlorpromazine relative to other membrane components, temperature, and the membrane region examined (Zimmer, 1984). Moreover, interpretations of membrane "structural order" or "fluidity" based upon data from fluorescence spectroscopy, electron spin resonance spectroscopy, nuclear magnetic resonance spectroscopy, and differential calorimetry, while internally consistent are not always in agreement when the same system is examined by multiple methods (Perlman and Goldstein, 1984; Zimmer, 1984). In part, these discrepancies may be related to the use of different reporter molecules and the different time windows over which measurements are made.

Effect of chlorpromazine · HCl on the rate and range of transbilayer lateral mobility of the SPMV lipid bilayer The covalently linked trinitrophenyl group displays a broad absorbance with a maximum near 420 nm. This absorption peak has a large overlap with the fluorescence emission of Py-3-Py. Moreover, this spectral overlap of donor emission and acceptor absorbance is responsible in part for the high transfer (quenching) efficiency of the probe. Approximately half of the Py-3-Py fluorescence was quenched in trinitrophenylated SPMVs. When the TNBS labeling was conducted under penetrating conditions (37ºC), nearly 100% of the fluorescence of the Py-3-Py was quenched. The *I´/I* of Py-3-Py in the outer monolayer was 0.066, greater than calculated for the inner monolayer of SPMVs. This means that the rate and range of lateral mobility of the outer monolayer is greater than that of the inner monolayer.

The effects of increasing concentrations of chlorpromazine · HCl on the *I´/I* values in the individual monolayers of SPMVs are shown in Fig. 1. Chlorpromazine · HCl had a greater ordering effect on the rate and range of lateral mobility of the inner monolayer (Fig. 1, filled circles) than on the outer monolayer (Fig. 1, filled triangles). Since changes observed in the *I´/I* values of Py-3-Py derive primarily from changes in the inner monolayer, we studied the selective effects of the drug on the components of the rate and range of probe mobility. To the best of our knowledge, the results presented herein demonstrate for the first time that the Sheetz-Singer hypothesis (Sheetz and Singer, 1974; see below) is valid for membranes.

Plasma membranes consist of two monolayers that are asymmetric in terms of; lipid distribution, electrical charge, fluidity, and protein distribution and function, and they do not appear to be coupled. In the case of membrane fluidity, it is worth noting that effects vary considerably according to the lipid composition of membranes. Membrane cholesterol is one of the major lipids of the plasma membranes and is asymmetrically distributed in the outer and inner monolayers (Kier *et al*., 1986; Wood *et al*., 1990; Schroeder *et al*., 1991a, 1991b). 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 is less fluid

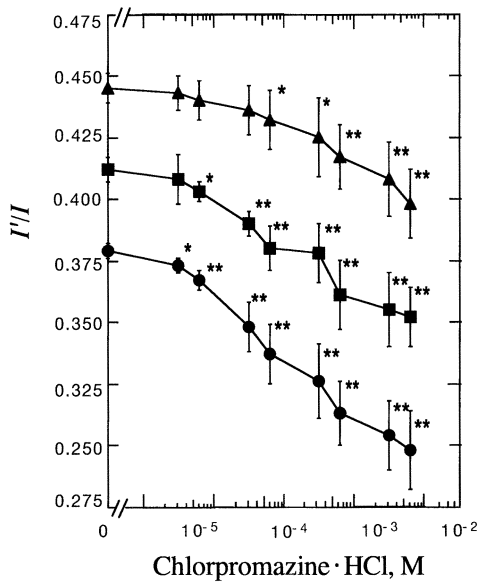

Fig. 1. Effects of chlorpromazine · HCl on excimer to monomer fluorescence intensity ratio (*I'/I*) of Py-3-Py in SPMVs. The excitation wavelength of Py-3-Py was 330 nm and *I'/I* values were calculated from the 480 nm to 379 nm signal ratio. SPMVs were treated ± 4 mM TNBS, pH 8.5, at 4°C for 90 min. Py-3-Py was incorporated into SPMVs and fluorescence measurements were performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS treated (inner monolayer, ●); calculated for outer monolayer (▲) by eq. 3 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, versus the control by the Student's *t*-test.

(Seigneuret *et al*., 1984; Chabanel *et al*., 1985), whereas other studies have found the opposite (Cogan and Schachter, 1981; Schachter *et al*., 1983). The finding that the inner monolayer of synaptic plasma membrane isolated from rat brain (SPM) is less fluid than the outer monolayer is consistent with data showing that the SPM inner monolayer contains approximately 7-times as much cholesterol as the outer monolayer (Wood *et al*., 1990). Thus, a possible explanation for the range of asymmetric lateral mobilities observed between the outer and inner monolayers of SPMVs in this study is that the amount of cholesterol may have differed 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); however, cholesterol does not seem to bear sole responsibility for such differences. This is because 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 SPMVs were 0.452 ± 0.008 and 0.500 ± 0.013, respectively (*I'/I* values from bispyrenyl propane; Min, 1997). The differences in asymmetrical lateral diffusion between inner and outer monolayers of the model membrane lipid bilayer made with phospholipids that were separated from SPMVs were also 0.540 ± 0.013 and 0.572 ± 0.016, respectively (*I'/I* values from bispyrenyl propane; Lee, 1999). Thus, we presume that the asymmetrical mobility between inner and outer monolayers of the model membranes formed with total phospholipids free of cholesterol and protein can be attributed to the types of phospholipids (which are likely to be distributed asymmetrically between the 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 to maintain lipid bilayer stability.

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 from the drive to create 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 used (shape, charge, location etc.) (Stubbs and Williams, 1992). It also depends on the assumption that the hydrophobic region of cell membranes is structurally and dynamically homogeneous; an assumption that is now under serious challenge. Thus, while it may be true to state that the bulk or average spectroscopic properties of cell membranes may not be useful for building a hypothesis for the molecular mechanism(s) of the pharmacological action(s) of drug(s), local properties pertaining to domains may be very relevant.

Investigations of the binding sites of drugs at a cellular level provide important basic materials for research on the pharmacological actions of drugs. This is because the binding site of a drug at the cellular level coincide with its site action, though this is not necessarily always the case.

Effect of chlorpromazine · HCl on the annular lipid fluidity of SPMVs The *I'/I* values of Py-3-Py were used to measure the annular lipid fluidity of SPMVs (intact membranes), and was found to be 0.156 ± 0.003 (37°C, pH 7.4). *In vitro* the addition of chlorpromazine · HCl decreased the annular lipid fluidity of SPMVs in a dose-dependent manner. Significant decreases in *I'/I* values by chlorpromazine · HCl were observed at all concentration levels (70×10^{-6}, 35×10^{-5}, 70×10^{-5}, 35×10^{-4}, 70×10^{-4}, 35×10^{-3} and 70×10^{-4} M) examined in the present study, except at 35×10^{-6} M (Fig. 2).

The clear mechanism of action of chlorpromazine · HCl with respect to the reduced annular lipid fluidity of SPMVs is unknown. However, a mechanism by which chlorpromazine · HCl decreases the annular lipid fluidity of the SPMV lipid bilayer can be proposed, as follows.

Annular lipids are known to either surround proteins in a circle and to be tightly associated with receptors through covalent and noncovalent bonds, or to closely encircle proteins almost brushing past them. First, it is assumed that

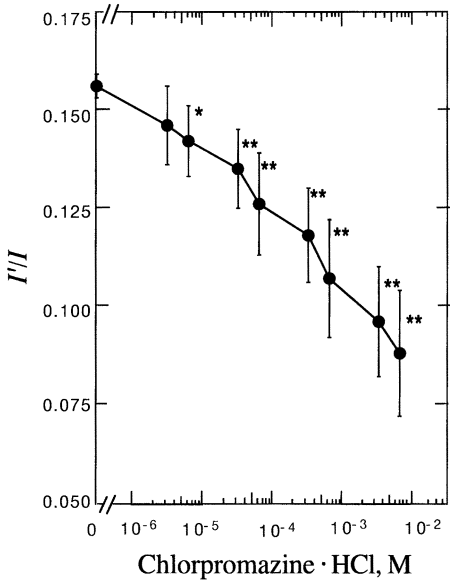

Fig. 2. Effects of chlorpromazine · HCl on the annular lipid fluidity of SPMVs. Py-3-Py was excited through RET from tryptophan (excitation wavelength, 286 nm) and the excimer to monomer fluorescence intensity ratio (*I'/I*) was calculated from the 480 to 379 nm signal ratio. Fluorescence measurements were performed at 37°C (pH 7.4).

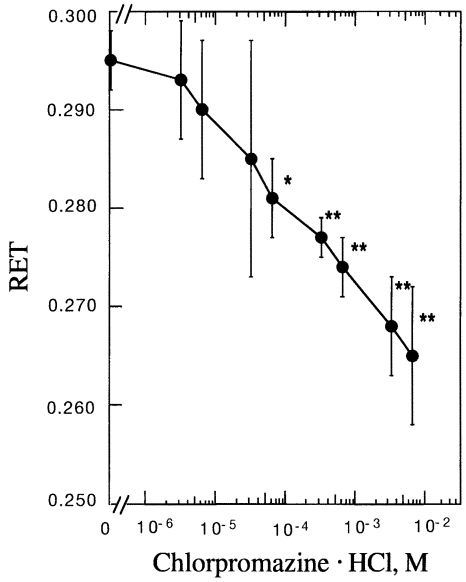

Fig. 3. Effects of chlorpromazine · HCl on protein clustering in SPMVs. The efficiency of RET from tryptophan to Py-3-Py was taken as a measure of protein clustering and calculated using eq. 4. Fluorescence measurements were performed at 37°C (pH 7.4).

chlorpromazine · HCl influences the stereostructure or dynamics of proteins. In addition, it is also assumed that the drug is combined with lipids, especially with annular lipids, and decreases their mobilities thereby indirectly affecting the dynamics of proteins. Second, because biological membranes have highly complex compositions, it has not been feasible to monitor changes in the local lipid environment and to determine the drugs effect on membrane protein functions simultaneously. The aforementioned effects are not solely due to the influence of chlorpromazine · HCl on lipids, but they are magnified by the interaction between lipids and dopaminergic receptor.

Effect of chlorpromazine · HCl on the SPMV protein clustering Protein distribution was evaluated by RET from tryptophan to Py-3-Py in SPMVs. The RET value of intact SPMVs (chlorpromazine · HCl-untreated) was 0.295 ± 0.003 (37°C, pH 7.4). Chlorpromazine · HCl *in vitro* significantly reduced the RET from tryptophan to Py-3-Py in SPMVs in a dose-dependent manner (Fig. 3) (37°C, pH 7.4), a significant decrease in the RET value by chlorpromazine · HCl was observed at even 70×10^{-5} M (Fig. 3).

It is assumed that protein clustering is caused (in part) by decreased annular lipid fluidity. Thus, it is difficult to exclude the possibility that interactions with annular lipids may exert some influence on the receptors or proteins that are closely associated with annular lipids through covalent and noncovalent bonds (Butterworth and Strichartz, 1990; Yun *et al*., 1993; Kang *et al*., 1996).

Effect of chlorpromazine · HCl on the thickness (D) of the SPMV lipid bilayer Cell volume is an integral element within the cellular machinery and regulates cellular performance. The role of cell volume in cell functions involves erythrocyte volume and shape, epithelial transport, the regulation of metabolism, receptor recycling, hormone and transmitter release, excitability and contraction, migration, pathogen host interactions, cell proliferation, cell death, and others (Lang *et al.*, 1998).

It is known that increases or decreases in molecular movement eventually increase or decrease molecular volume. However, it is not known to what extent molecular movement increases or decreases molecular volume. In the present study, we determined to what extent membrane thickness (D) is increased (or decreased) by chlorpromazine · HCl, by using the fluorescence quenching technique.

Membrane thickness (D) was measured by energy transfer from Py-3-Py to ANS in SPMVs. F'/F_{max} and E values are listed in Table 1. Intact membrane thickness (D) in arbitrary units was 1.044 ± 0.008 (37°C, pH 7.4), and chlorpromazine · HCl increased thickness (D) in a dose-dependent manner (Fig. 4) (37°C, pH 7.4). Moreover, a significant increase in thickness (D) by the drug was observed even at 35×10^{-5} M (Fig. 4).

It is a natural consequence that the thickness (D) of the SPMV lipid bilayer is increased by chlorpromazine · HCl, which means that the membrane has contracted. For, as having discussed above, the drug decreases the bulk lateral mobility of the SPMV lipid bilayer and a significant reduction in the bulk lateral mobility by the drug was observed at 70×10^{-6} M. Nevertheless, the thickness (D) of the membrane lipid

Table 1. F'/F_{max} and E values

Chlorpromazine · HCl Conc. (M)	F'/F_{max}	E
0	0.724 ± 0.016	0.389 ± 0.003
35×10^{-6}	0.734 ± 0.015	0.386 ± 0.002
70×10^{-6}	0.739 ± 0.010	0.383 ± 0.004
35×10^{-5}	0.748 ± 0.007	0.378 ± 0.003
70×10^{-5}	0.754 ± 0.012	0.368 ± 0.002
35×10^{-4}	0.758 ± 0.018	0.363 ± 0.004
70×10^{-4}	0.782 ± 0.013	0.357 ± 0.002
35×10^{-3}	0.787 ± 0.009	0.350 ± 0.002
70×10^{-3}	0.794 ± 0.007	0.346 ± 0.004

F' is the fluorescence intensity of 5 μM 1-anilinonaphthalene-8-sulfonic acid (ANS) and F_{max} is the maximal fluorescence of ANS in synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. Both values were obtained by ANS binding studies in the absence of 1,3-di(1-pyrenyl)propane (Py-3-Py). Values are presented as the means ± SEM of 5 determinations.

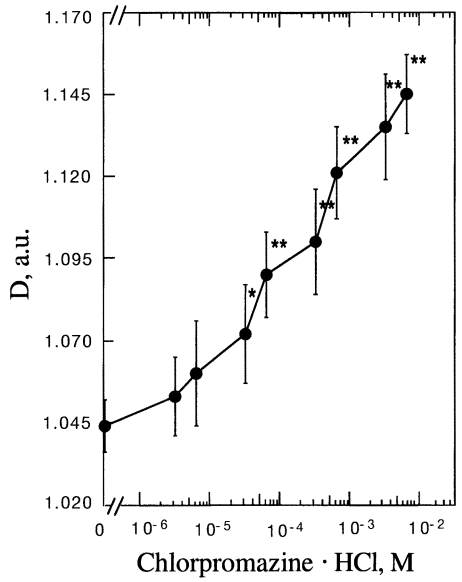

Fig. 4. Effects of chlorpromazine · HCl on lipid bilayer thickness in SPMVs. D (a measure of bilayer thickness) values were calculated using eq. 6 and expressed in arbitrary units (a.u.). Fluorescence measurements were performed at 37°C (pH 7.4).

bilayer was not changed by the same concentration (70×10^{-6} M) of chlorpromazine · HCl. It was only when its concentration reached 35×10^{-5} M that the thickness (D) of the membrane lipid bilayer significantly increased. This means that the thickness (D) of the lipid bilayer can be changed and affected only by a considerable change in lipid mobility. Or, if not, then maybe the measuring fluorescence probe technique employed in the present research lacks the sensitivity to catch the infinitesimal change of membrane thickness (D).

The volume of matter is proportional to molecular movement. It is judged that the reason for the increased thickness of the SPMV lipid bilayer by chlorpromazine · HCl is due to the lateral and rotational mobility (Ahn *et al*., 2000) of the SPMV lipid bilayer, and annular lipid fluidity was found to be reduced by chlorpromazine · HCl.

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