The Effect of Dimyristoylphosphatidylethanol on the Lateral and Rotational Mobilities of Liposome Lipid Bilayers

Hye-Ock Jang, Min-Hoi Huh, Seung-Woo Lee, Young-Ho Lee, Jong-Hwa Lee, Jun-Bong Seo, Kyo-Il Koo, Seong-Deok Jin, Je-Hyung Jeong, Jang-Seop Lim, Moon-Kyung Bae, and Il Yun

College of Dentistry and Research Institute for Oral Biotechnology, Pusan National University, Busan 602-739, Korea

The aim of this study was to provide the basis to further examine the mode of action of ethanol. Fluorescent probes reported to have different membrane mobilities were used to evaluate the effect of dimyristoylphosphatidylethanol (DMPEt) on the lateral and rotational mobilities of liposome lipid bilayers. An experimental procedure, based on the selective quenching of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1,3-di(1-pyrenyl)propane (Py-3-Py) by trinitrophenyl groups, was used. DMPEt increased the bulk lateral and rotational mobilities, and had a greater fluidizing effect on the outer than the inner monolayer. These effects of DMPEt on liposomes may be responsible for some, but not all, of the general anesthetic actions of ethanol.

Key words: Dimyristoylphosphsatidylethanol, Liposomes, Transbilayer lateral and rotational mobility, Fluorescence quenching technique

INTRODUCTION

The use of ethanol goes back to the dawn of civilization, and its ingestion is known to exert diverse physiological effects on many body organs, including the central nervous system (CNS). The molecular mechanism of action of ethanol in the CNS has long been a subject of great interest. There are several proposals for the molecular mechanism of action of ethanol, and include both lipid and protein theories. However, the precise location of molecular action has continued to be a subject of controversy to the present day. The current consensus is that ethanol has a site(s) of action located within the cell membrane, presumably at the synapse. Debate has continued as to whether the site is located exclusively in the lipid bilayer, at a hydrophobic site on a protein or at the membrane protein-lipid interface.

A number of theories propose perturbations of the bulk physical properties of the lipids of cell membranes as the primary event leading to inebriation or anesthesia. An important version attributed the anesthetic and intoxicating properties of alcohols to their lipid-fluidizing action in membranes (Armbrecht *et al.*, 1983). The enhanced fluidity of neuronal membranes produced by ethanol may alter many cell surface processes, including membrane-bound enzymes, receptors, neurotransmitters, and ion channel activities (Bangham and Mason, 1979; Chin and Goldstein, 1977a). Ethanol and related alkanols have been shown to decrease the temperature of the gel-to-liquid crystalline phase transition of model membranes (Chin and Goldstein, 1977b), expand membranes (Chin and Goldstein, 1981) and alter the surface charge of membrane lipids (Chin and Goldstein, 1984).

However, the hypothesized membrane-fluidizing action of ethanol in the CNS is now being strongly challenged by recent data showing that ethanol specifically and selectively affects the function of the GABA-coupled chloride channel (Gonzales and Hoffmann, 1981; Sanna *et al.*, 1991) and membrane specific protein (Franks and Lieb, 1985, 1987, 1993, 1994).

A pathway of ethanol metabolism with an unusual phospholipid, phosphatidylethanol (PET), as the product has been reported (Alling *et al.*, 1984). The conformation and properties of this phospholipid could be of considerable interest since it is at the polar region level that membranes interact with the external environment. Omodeo-Salê *et al.* (1989) investigated the thermotropic behavior of PET in vesicular dispersions of phosphatidylcholine

Correspondence to: Il Yun, Department of Dental Pharmacology and Biophysics, College of Dentistry and Research Institute for Oral Biotechnology, Pusan National University, Busan 602-739, Korea
Tel: 82-51-240-7813, Fax: 82-51-254-0576
E-mail: iyun@pusan.ac.kr

using high-sensitivity differential scanning calorimetry. They showed that PET can markedly influence the physicochemical properties of membranes where it is occasionally synthesized. PET decreased the anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in native membrane lipid bilayers (isolated from the crude mitochondrial fraction of the brain in Sprague-Dawley rats) and in 1,2-dimyristoyl-phosphatidylcholine (1,2-DMPC) model membrane lipid bilayers (Omodeo-Salê *et al.*, 1991). However, analysis of the effects of PET on the fluidity of native and model membranes have focused on the average change in their lipid environment, not on any asymmetric effect on the inner and outer monolayers. Furthermore, the change in the lateral mobilities of native and model membranes in response to PET has not been determined.

Furthermore, it has already been reported that while DMPEt increased the lateral and rotational mobilities of the neuronal lipid bilayer, it had a greater impact on increasing the mobility of the outer monolayer compared to that of the inner (Jang *et al.*, 2004a). There is a good possibility that the effects of ethanol on the CNS result not only from a direct action on neuronal membranes, but from the action of DMPEt also (Jang *et al.*, 2004a). However, since neuronal membranes are protein containing lipid bilayers, we can not entirely exclude the possibility that the effects of DMPEt on proteins may increase or decrease depending on the lipid-protein interactions. Therefore, the effects of DMPEt on the fluidity of lipid bilayers were specifically examined in order to study the effects of DMPEt on lipids only, with the results reported herein.

MATERIALS AND METHODS

Materials

The DPH and 1,3-di(1-pyrenyl)propane (Py-3-Py) fluorescent probes were obtained from Molecular Probes (Eugene, OR, U.S.A.). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Fluka (Buchs, Switzerland). 1,2-DMPC, phospholipase D, and other reagents were obtained from Sigma (St. Louis, MO, U.S.A.) and were of analytical grade. Phospholipase D was partially purified from Savoy cabbage, using acetone precipitation, according to the method of Davidson & Long (1958). After acetone precipitation the enzyme was suspended in water and lyophilized. The powder obtained was stored at -20°C.

Preparation of synaptosomal plasma membrane vesicles (SPMV)

The SPMV were prepared according to the procedure reported in earlier studies (Yun and Kang, 1990; Yun *et al.*, 1990). The specific activities of Na,K-ATPase, acetylcholinesterase and 5'-nucleotidase in the plasma mem-

brane fraction were approximately 4-, 2.5-, and 3-times higher than those in the 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 BSA as a standard.

Liposome preparation

Total lipids were extracted from the SPMV, as previously described (Yun and Kang, 1990). The cholesterol content of the extracted total lipids was determined using the Liebermann-Buchard reaction (Huang *et al.*, 1961). Phospholipids were quantified by measuring the amounts of inorganic phosphate (Bartlett, 1959) after hydrolysis of the phospholipids in 70% $HClO_4$ at 180°C (Madeira and Antunes-Madeira, 1976). The SPMV had a high lipid to protein ratio (0.942 mg total lipids/1 mg protein) and a low cholesterol to phospholipid molar ratio (0.593 ± 0.011: cholesterol 0.208 ± 0.010, phospholipids 0.702 ± 0.025). For our calculations, the average molecular weight of the phospholipids was assumed to be 775; that of cholesterol is 387. The phospholipids were composed (mol%) of phosphatidylcholine (41.55 ± 0.91), phosphatidylethanolamine (36.83 ± 0.48), phosphatidylserine (13.60 ± 0.26), sphingomyelin (4.15 ± 0.16), phosphatidylinositol (2.90 ± 0.09), and lysophosphatidylcholine (0.97 ± 0.03).

Stock solutions of the total lipids or phospholipids extracted from the SPMV, SPMVTL, and SPMVPL, respectively, were prepared in chloroform. The concentration of the lipid stock solutions was 0.2 mg/mL. Giant unilamellar vesicles (GUVs: SPMVTL or SPMVPL), with a mean diameter of 45 μm, were prepared by the method developed by Angelova & Dimitrov (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1987; Angelova *et al.*, 1992). To grow the GUVs, a previously described special temperature-controlled chamber was used (Bagatolli and Gratton, 1999, 2000; Yun *et al.*, 2002). The experiments were carried out in the same chamber following vesicle formation, using an inverted microscope (Axiovert35: Zeiss, Thornwood, NY). The following steps were used to prepare the GUVs: 1) ~3 μL of the lipid stock solution was spread on each Pt wire under a stream on N_2. To remove the organic solvent residues the chamber was placed in a liophilizer for ~2 h. 2) For the addition of aqueous solvent into the chamber (Millipore water 17.5 MΩ/cm), the bottom part of the chamber was sealed with a coverslip. Millipore water, previously heated to the desired temperature (80°C for SPMVTL, 60°C for SPMVPL), was then to sufficiently cover the Pt wires. Immediately after this step, the Pt wires were connected to a function generator (Hewlett-

Packard, Santa Clara, CA), and a low-frequency AC field (sinusoidal wave function with a frequency of 10 Hz and an amplitude of 3 V) applied for 90 min. After vesicle formation, the AC field was turned off.

Synthesis of 1,2-dimyristoylphosphatidylethanol

DMPEt was synthesized by the procedure of Omodeo-Salè et al. (1989), which involved the transphosphatidylation of 1,2-DMPC/ethanol by phospholipase D. The yield was 70-80%, with the DMPEt produced purified by ion-exchange chromatography and characterized by fast atom bombardment-mass spectrometry (FAB-MS).

Labeling of 2,4,6-trinitrobenzene sulfonic acid (TNBS)

To determine the fluorescence parameters of the probe molecules in each of the membrane monolayers, TNBS labeling reactions were performed as previously described (Kang et al., 1996; Yun and Kang, 1990; Yun et al., 1993; 1994, Jang et al., 2004a,b; Bae et al., 2004), but with a few modifications. The SPMVTL (or SPMVPL) was gently resuspended in 50 mL of 4 mM TNBS in buffer A for 80 min (in the case of asymmetric lateral mobility) or 50 mL of 2 mM TNBS in buffer A for 40 min (in the case of asymmetric rotational mobility) or in buffer A alone. Buffer A consisted of 30 mM NaCl, 120 mM NaHCO$_3$, 11 mM glucose, and 1% BSA. The reagent pH was adjusted to 8.5 with NaOH. To assure complete exposure of all SPMVTL (or SPMVPL) outer monolayers to TNBS, the pellet was passed slowly through an Eberbach tissue grinder (3 up and down strokes). Unless otherwise specified, treatment was carried out at 4°C. The TNBS labeling reaction was terminated by the addition of 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$_2$PO$_4$, 1.15 g/L Na$_2$HPO$_4$·7H$_2$O, 0.48 g/L Hepes, pH 7.4).

Fluorescence measurements

All fluorescence measurements were made with a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (Model; ISS K2-003). The cuvette temperature was maintained at 37.0 ± 0.1°C in a circulating water bath (pH 7.4). The bandpass slits were 10 and 5 nm on excitation and emission, respectively. Blanks, prepared under identical conditions, but without the fluorescent probes, served as controls.

Py-3-Py was incorporated by the addition of aliquots of 5×10^{-5} M stock solution in absolute ethanol to the SPMVTL (or SPMVPL), such that the final probe concentration was less than 5×10^{-7} M (Kang et al., 1996; Yun et al., 1994; Jang et al., 2004a,b; Bae et al., 2004). Mixtures were initially vigorously vortexed for 10 s at room temperature, and then incubated at 4°C for 18 h, with gentle

stirring (Kang et al., 1996; Yun et al., 1994; Jang et al., 2004a,b; Bae et al., 2004).

DPH was dissolved in tetrahydrofuran, and 0.5 µL tetrahydrofuran per mL of PBS was added directly to the liposome suspension, to a final concentration of 0.01 µg/50 µg membrane phospholipids (fluorescent probe DPH 2: membrane phospholipids 10,000), as described previously (Kang et al., 1996; Yun et al., 1993, 1994; Jang et al., 2004a,b; Bae et al., 2004). After probe incorporation, the membrane suspension was placed in cuvettes, and the control fluorescence determined. Concentrated solutions of DMPEt were prepared in 10 mM Tris-HCl (pH 7.4) and added to the labeled membrane suspension (or untreated both SPMVTL and SPMVPL suspension) to give the desired concentration of DMPEt (in this case, for 30 min incubation).

Excitation wavelength for Py-3-Py was 330 nm and the emission wavelengths for the Py-3-Py monomer and excimer were 379 and 480 nm, respectively. For the Py-3-Py excimer emission, a GG-455 cut-off filter was used. The excimer to monomer fluorescence intensity ratio, I'/I, was calculated from the ratio of the 480 to 379 nm signals. The excitation and emission wavelengths for DPH were 362 and 424 nm, respectively.

The effect of DMPEt on the lateral mobility of the individual monolayers of both SPMVTL and SPMVPL: selective quenching of Py-3-Py

This method was based on the assumption that the system is composed of fluorescing compartments that are differentially accessed by TNBS. The excimer to monomer fluorescence intensity ratios, I'/I, of Py-3-Py in the bulk (inner plus outer), and in the inner and outer monolayers were calculated by the following equations:

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

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

The effect of DMPEt on the rotational mobility of bulk SPMVTL and SPMVPL

The intensities of the components of fluorescence parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light were determined by measuring the light emitted through polarizers oriented

vertically and horizontally. The polarization (P) was obtained from the intensity measurements, using $P = (I_{\|} - GI_{\perp}) / (I_{\|} + GI_{\perp})$, where G is a grating correction factor for the transmission efficiency of the monochromator for vertically and horizontally polarized light. This is given by the ratio of the fluorescence intensities of the vertical to horizontal components when the exciting light is polarized in the horizontal direction. The polarization was expressed as the anisotropy as the follow:

$$r = 2P/(3-P) \qquad (4)$$

The effect of DMPEt on the rotational mobility of individual monolayers of both SPMVTL and SPM-VPL: selective quenching of DPH

The experimental determination of the separate mono-layer structures of both the SPMVTL and SPMVPL were based on a method previously established for the plasma membrane vesicles of Chinese hamster ovary cells (CHO-K₁-PMV, Yun et al., 1993), plasma membranes of Mar 18.5 hybridoma cells (ATCC-PMV, Yun et al., 1994), and of the myeloma cell line Sp2/0-Ag14 (Sp2/0-PMV, Kang et al., 1996) and SPMV (Yun and Kang, 1990; Jang et al., 2004a,b; Bae et al., 2004). This does not simply provide a theoretically calculated or average value: instead it is based on the assumption that the system is composed of fluorescing compartments of different accessibility to TNBS. If the fluorescence intensity, F, and the anisotropy, r, are measured simultaneously, then:

$$r = \Sigma F_j r_j \qquad (5)$$

where F_j is the fraction of the fluorescence intensity in compartment j. For a binary system, composed of the outer and inner monolayers of the SPMVTL (or SPMVPL), this leads to:

$$r = \frac{F_i}{F} r_i + \frac{F - F_i}{F} r_o \qquad (6)$$

where F and F_i are the DPH fluorescence obtained for both SPMVTL and SPMVPL incubated at 4°C with buffer A and buffer A plus 2 mM TNBS (pH 8.5, non-penetrating conditions), respectively. The values of the fluorophore concentration-independent parameter anisotropies, r (anisotropy for both monolayers) and r_i (inner monolayer anisotropy), were also determined for DPH in both SPMVTL and SPMVPL incubated at 4°C with buffer A and buffer A plus TNBS, respectively. The equation was then solved for r_o (outer monolayer anisotropy).

RESULTS AND DISCUSSION

The characteristics of the lipid samples, such as size, lamellarity, radius of curvature, and shape, are strongly dependent on the method used to form the vesicles (Lasic, 1988). As a consequence of the preparation method, the parameters that characterize the lipid phase equilibrium in lipid mixtures are affected by the lipid sample characteristics. Because the size (a mean diameter of 45 μm) of the GUVs is in the same order as that of the cells, the vesicles are becoming objects of intense scrutiny in diverse areas that focus on membrane
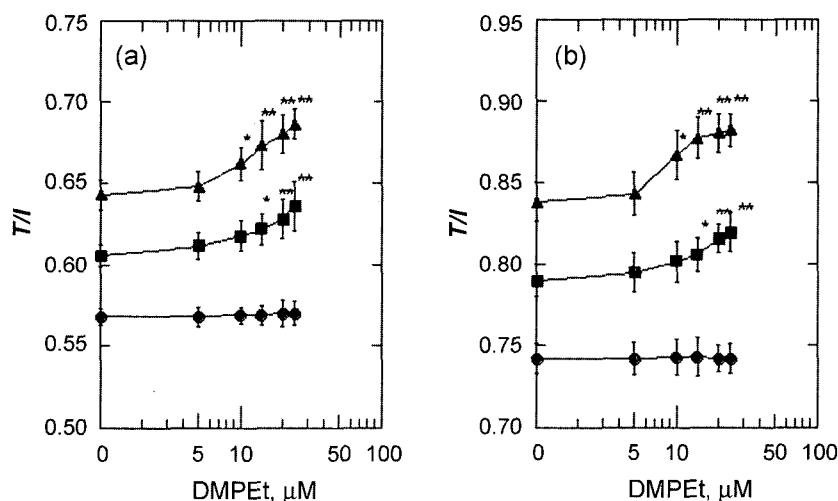

Fig. 1. Effects of DMPEt on the excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py in liposomes [SPMVTL (a) and SPMVPL (b)]. The excitation wavelength of Py-3-Py was 330 nm. The I'/I values were calculated from the ratios of the 480 to 379 nm signals. The SPMVTL and SPMVPL were treated 4 mM TNBS, pH 8.5, at 4°C for 80 min. Py-3-Py was incorporated into both SPMVTL and SPMVPL, and fluorescence measurements performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS treated (inner monolayer, ●); calculated for the outer monolayer (▲) using eq. 3, as described in Materials and Methods. Each point represents the mean ± SEM of 5 determinations. The asterisk and double asterisks signify $P < 0.05$ and $P < 0.01$, respectively, compared to the control, using the Student's t-test.

behavior (Menger and Keiper, 1998).

The effect of DMPEt on the rate and range of lateral mobility in bulk bilayers of SPMVTL and SPMVPL

We used Py-3-Py, a pyrene derivative that has been used to quantify the lateral mobility within native and model membranes (Bae et al., 2004; Jang et al., 2004a,b; Kang et al., 1996; Schachter, 1984; Yun et al., 1993, 1994; Zachariasse et al., 1982), to determine the rate and range of lateral mobility in the SPMVTL and SPMVPL. With this probe, the emission of both the monomer (I) and the excimer (I') components are monitored in such a way that a ratio can be derived and used as a measure of the lateral mobility (Bae et al., 2004; Jang et al., 2004a,b; Kang et al., 1996; Schachter, 1984; Yun et al., 1993, 1994; Zachariasse et al., 1982). The probe mobility increases the 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 indicates increased lateral mobility of the probe within the membranes. The excimer fluorescence technique using Py-3-Py has advantages over its counterpart based on intermolecular excimerization, in that very low probe concentrations can be used ($<10^{-7}$ M) and perturbation of the liposomes by the probe molecule is minimized.

The I'/I values in intact SPMVTL and SPMVPL (DMPEt-untreated) were 0.606 ± 0.007 and 0.790 ± 0.010 (at 37 °C, pH 7.4), respectively. Incubation with DMPEt increased the range and rate of the lateral mobility in the bulk (inner

+ outer monolayer) SPMVTL and SPMVPL at concentrations as low as 15.0 μM ($n = 5$, $P < 0.05$), as demonstrated in Figs. 1 and 3.

The I'/I values of Py-3-Py in the bulk SPMVTL and SPMVPL, incubated with 25.0 μM DMPEt, were $0.636 \pm 0.015^{**}$ ($n = 5$, $P < 0.01$) and $0.820 \pm 0.012^{**}$ ($n = 5$, $P < 0.01$), respectively, and the changes in I'/I values before and after the addition of DMPEt were 0.030 and 0.030, respectively. The I'/I values of Py-3-Py in the SPMVTL bilayer were 0.606 ± 0.007 ($n = 5$) and 0.524 ± 0.005 ($n = 5$) at 37 and 25°C (pH 7.4), respectively. Hence the effect of 25.0 μM DMPEt was equivalent to that produced by a temperature increase of approximate 4.4°C (in the case of SPMVTL). The I'/I values of Py-3-Py in the SPMVPL bilayer were 0.790 ± 0.010 ($n = 5$) and 0.715 ± 0.007 ($n = 5$) at 37 and 25°C (pH 7.4), respectively. Hence the effect of 25.0 μM DMPEt was equivalent to that produced by a temperature increase of approximate 4.8°C (in the case of SPMVPL). We reported that the effect of 25.0 μM DMPEt on the neuronal membranes (SPMV) was as large as that produced by a temperature rise of approximate 6°C (Jang et al., 2004a). Thus, in terms of the increase in mobility of the neuronal and liposome lipid bilayers by DMPEt, the magnitude of effects of DMPEt was found to be greater in the neuronal membranes (SPMV) than in the liposome (both SPMVTL and SPMVPL). The sensitivities to the increasing effect of the lateral mobility of the bulk bilayer due to DMPEt differed depending on the native and liposomes membranes, in the descending order: SPMV, SPMVPL, and SPMVTL.
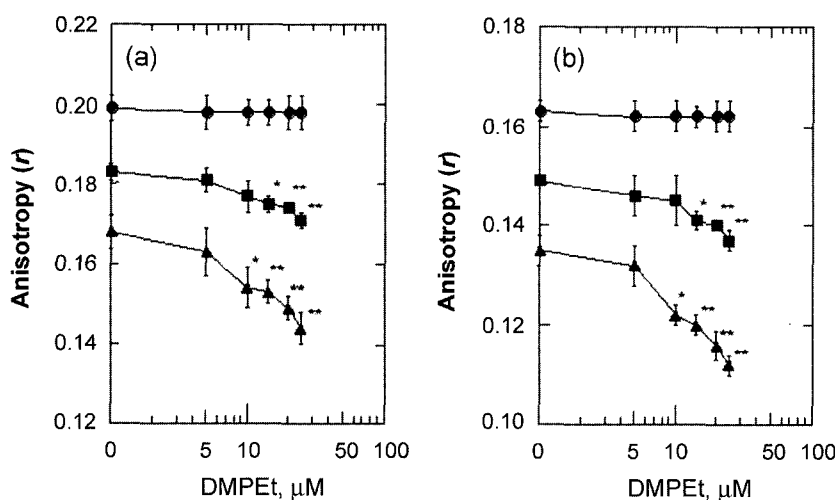

Fig. 2. Effects of DMPEt on the anisotropy (r) of DPH in liposomes [SPMVTL (a) and SPMVPL (b)]. The excitation and fluorescence emission wavelengths for DPH were 362 and 424 nm, respectively. The SPMVTL and SPMVPL were treated 2 mM TNBS, pH 8.5, at 4°C for 40 min. DPH was incorporated into both SPMVTL and SPMVPL, and fluorescence measurements performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS treated (inner monolayer, ●); calculated for the outer monolayer (▲) using eq. 6, as described in Materials and Methods. Each point represents the mean ± SEM of 5 determinations. The asterisk and double asterisks signify $P < 0.05$ and $P < 0.01$, respectively, compared to the control, using the Student's t-test.

The effect of DMPEt on the range and rate of transbilayer asymmetric lateral mobility of liposome monolayers

The covalently linked trinitrophenyl group has a broad absorbance range, with a maximum near 420 nm. This peak has a large overlap with the fluorescence emission of Py-3-Py. This overlap is responsible, in part, for the high transfer (quenching) efficiency of the probe. Approximately half the Py-3-Py fluorescence was quenched in the trinitrophenylated SPMVTL and SPMVPL. When TNBS labeling was conducted under penetrating conditions (37°C), nearly all of the fluorescence of the Py-3-Py was quenched. Values for the excimer to monomer fluorescence intensity ratio (*I'/I*) of Py-3-Py in intact SPMVTL and SPMVPL (both monolayers) and in TNBS-treated SPMVTL and SPMVPL (inner monolayer) are listed in Table I. The *I'/I* of Py-3-Py in the outer monolayer was 0.075, which was greater than that calculated for the inner monolayer (in the case of SPMVTL). The *I'/I* of Py-3-Py in the outer monolayer was 0.096, which was greater than that calculated for the inner monolayer (in the case of SPMVPL). This means that the rate and range of the lateral mobility of the outer monolayer was greater than that of the inner monolayer.

The effect of increasing concentrations of DMPEt on the *I'/I* values in the individual SPMVTL (or SPMVPL) monolayers is shown in Fig. 1 (a and b). DMPEt significantly increased the rate and range of lateral mobility of the outer monolayer of the SPMVTL and SPMVPL, 0.662 ± 0.010, $P < 0.05$, n = 5 [Fig. 1(a)] and 0.867 ± 0.015, $P < 0.05$, n = 5 [Fig. 1(b)], respectively. DMPEt caused a greater increase in the fluidity of the outer [Fig. 1 (a and b), filled triangles] than the inner monolayer [Fig. 1 (a and b), filled circles]. Since the changes in *I'/I* values are derived primarily from the effect on the outer monolayer, we studied the selective effects of DMPEt on the rate and range of mobility of the probe. To the best of our knowledge, the results presented here are the first to demonstrate that the Sheetz-Singer hypothesis (1974) is valid in liposomes.

Although many researchers have reported that the fluidity in inner and outer monolayers of native and model membranes differ, all previous studies on asymmetric bilayer fluidity have examined the rotational range, but not the rate and range of lateral mobility. In this study, using the selective quenching of Py-3-Py and DPH fluorescence due to trinitrophenyl groups, we examined the transbilayer asymmetric lateral and rotational mobilities.

The TNBS labeling reaction must be carefully monitored in order to ensure the reagent does not penetrate into the inner monolayer, and thereby label both sides of the liposomes. When the TNBS treatment is performed under penetrating conditions (37°C), 60-80% of the phosphatidylethanolamine in liposomes is trinitrophenylated (Yun and Kang, 1990). Approximately half the Py-3-Py fluorescence was quenched in both the trinitrophenylated SPMVTL and SPMVPL.

It is important to note that the term "membrane fluidity" is often misused. It arose from a combination of spectroscopic studies, the realization that membranes can be regarded as two-dimensional fluids, and the desire to obtain a simple single physical parameter that would describe their properties. The difficulty with the membrane fluidity concept is that any physical parameter chosen will be a function 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 that is now under serious challenge. Thus, while it may be true to say that the bulk or average spectroscopic properties of cell membranes may not be useful in building a hypothesis for the pharmacological action(s) of drug(s), local properties pertaining to domains or the immediate environment of a membrane protein may be very relevant.

As already pointed out, the membrane bilayer mobility is an important factor controlling the membrane microviscosity or fluidity. The membrane bilayer mobility includes both the lateral and rotational mobilities, as well as the "flip-flop", with the lateral mobility well known as the most important of these factors. We are pleased to have been able to develop and describe a fluorescence quenching technique that can measure the membrane transbilayer

Table I. Structural parameters of intact liposomes (SPMVTL and SPMVPL)

SPMVTL	Anisotropy (*r*) [a]	*I'/I* [b]	SPMVPL	Anisotropy (*r*) [a]	*I'/I* [b]
Inner + Outer	0.183 ± 0.002	0.606 ± 0.007	Inner + Outer	0.149 ± 0.001	0.790 ± 0.010
Inner	0.199 ± 0.003	0.568 ± 0.005	Inner	0.163 ± 0.002	0.742 ± 0.009
Outer	0.168 ± 0.004	0.643 ± 0.009	Outer	0.135 ± 0.003	0.838 ± 0.012

[a]Liposomes were treated $\pm$ 2 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS), pH 8.5, at 4°C for 40 min (anisotropy), [b]liposomes were treated $\pm$ 4 mM TNBS, pH 8.5, at 4°C for 80 min (*I'/I*). 1,6-Diphenyl-1,3,5-hexatriene (or 1,3-di(1-pyrenyl)propane) was incorporated, and fluorescence measurements were performed at 37°C (pH 7.4). Values from TNBS-treated membranes represent the inner monolayer; values for the outer monolayer were calculated as described in Materials and Methods. Values are represented as the mean $\pm$ SEM of five determinations.

The effect of DMPEt on the range of rotational mobility of bulk bilayer liposomes

The anisotropies (r) of DPH in the intact SPMVTL and SPMVPL were 0.183 ± 0.02 and 0.149 ± 0.001, respectively (37°C, pH 7.4) (Table I). DMPEt decreased the anisotropies of the liposome lipid bilayers at 15.0 μM [Fig. 2(a and b)]. The differences in the anisotropies of the bulk SPMVTL and SPMVPL lipid bilayers before and after the addition of 25.0 μM DMPEt were 0.012 and 0.012, respectively. This can be evaluated by comparison with the effect of temperature on this parameter. The anisotropies of DPH in the bilayer of the SPMVTL were 0.183 ± 0.002 (n = 5) and 0.242 ± 0.003 (n = 5) at 37 and 25°C (pH 7.4), respectively. The effect of 25.0 μM DMPEt was; thus, the same as that produced by a temperature increase of approximate 2.4°C. The anisotropies of DPH in the bilayer of the SPMVPL were 0.149 ± 0.001 (n = 5) and 0.201 ± 0.002 (n = 5) at 37 and 25°C (pH 7.4), respectively. The effect of 25.0 μM DMPEt was; thus, the same as that produced by a temperature increase of approximate 2.8°C.

The effect of DMPEt on the range of transbilayer asymmetric rotational mobility of liposome monolayers

The structures of the intact SPMVTL (or SPMVPL) (inner plus outer monolayers), and the outer (extracellular) and inner (intracellular) monolayers, were evaluated using DPH as a fluorescent reporter, with trinitrophenyl groups as quenching agents. Trinitrophenylation of the intact SPMVTL (or SPMVPL) at 4°C (non-penetrating conditions) resulted in the covalent attachment of trinitrophenyl quenching agents to the outer monolayers. Approximately half the DPH fluorescence was quenched in the treated SPMVTL (or SPMVPL) outer monolayer. When TNBS labeling was conducted under penetrating conditions (37°C), more than 98% of the DPH fluorescence was quenched. The values of the fluorescence parameters in intact (both monolayers) and TNBS-treated liposomes (inner monolayer) are listed in Table I. The anisotropy of DPH in the inner monolayer of the liposomes was 0.031, which was significantly greater than that calculated for the outer monolayer, as demonstrated in Table I.

Fig. 2 (a and b) shows that the anisotropy of DPH in the TNBS untreated membrane (inner plus outer monolayers) decreased gradually (fluidization) with increasing DMPEt concentration [Fig. 2 (a and b), filled squares]. There was a similar, but more gradual, decrease in the calculated anisotropy of the outer monolayer [Fig. 2 (a and b), filled triangles]. However, there was no statistically significant decrease in the anisotropy (the rotational mobility range)

of the inner monolayer at any of the DMPEt concentrations used. These results suggest that the fluidizing effect (range of rotational mobility) of DMPEt is selective.

Thus, DMPEt affects the lateral and rotational mobilities of liposomes, mainly *via* an effect on their outer monolayer. This is the first demonstration that DMPEt has a differential effect on the transbilayer asymmetric lateral and rotational mobilities of the inner and outer monolayers of liposomes. The sensitivities to the increasing effect of the rotational mobility on the hydrocarbon interior by the DMPEt differed depending on the native and model membranes, in the descending order: SPMV (Jang *et al.*, 2004a), SPMVPL, and SPMVTL. From the results of this study, DMPEt undoubtedly increases the rotational mobility of the hydrocarbon interior of the membrane. These effects are not solely due to the influence of the DMPEt on lipids, but are magnified by the interaction between lipids, proteins and water. Water plays a fundamental role in the cell membrane structure, in that it drives the formation of the lipid bilayer, with a polar surface facing the aqueous environment and a hydrophobic interior containing the fatty acyl chains and transmembrane proteins. In general, the structures and dynamics of proteins are also, to a large extent, governed by interactions with water (Teeter, 1991). Water penetrates into lipid bilayers at least as far as the glycerol backbone, but deeper between fatty acyl chain packing defects. Water at the protein-lipid interface is an additional factor influencing the lipid bilayer structure. The introduction of small peptides, consisting of three amino acids, can cause a shift of water deeper into the bilayer, indicating increased hydration (Jacobs and White, 1989). Altered hydration may have marked effects on membrane protein/lipid functioning, possibly due to the formation of hydrogen bonds between the interchain water and protein amino acid side chains or lipid acyl chains that face into the hydrophobic interior of the membrane. It is possible that the proteins organize the lipid in such a way as to make them more susceptible to DMPEt.

Ethanol increased the lateral and the rotational mobilities of plasma membrane vesicles (CHOK1-PMV) of cultured Chinese hamster ovary K1 cells (Yun *et al.*, 1993), the plasma membrane vesicles (ATCC-PMV) of cultured hybridoma cells (ATCC T1B 216, Yun *et al.*, 1994), plasma membrane vesicles of the cultured mouse myeloma cell line Sp2/0-Ag14 (Kang *et al.*, 1996) and SPMV (Bae *et al.*, 2005). Ethanol had a greater effect on increasing the range of rotational mobility of the outer compared to the inner monolayer of CHOK1-PMV (Yun *et al.*, 1993), ATCC-PMV (Yun *et al.*, 1994), Sp2/0-Ag14 (Kang *et al.*, 1996) and SPMV (Bae *et al.*, 2005). Furthermore, as mentioned in the introduction, a pathway for ethanol metabolism, where the product was an unusual phospholipid, PET

(Alling *et al.*, 1984), has been reported. Judging from the results of the present study, as well as those from other studies (Alling *et al.*, 1984; Omodeo-Salê *et al.*, 1989, 1991; Jang *et al.*, 2004a), there is a good possibility that the effects of ethanol on the CNS result not only from a direct action on neuronal membranes, but also from the action of DMPEt.

REFERENCES

Alling, C., Gustavsson, L., Månsson, J.-E., Benthin, G., and Anggård, E., Phosphatidylethanol formation in rat organs after ethanol treatment. *Biochim. Biophys. Acta,* 793, 119-122 (1984).

Angelova, M. L. and Dimitrov, D. S., Liposome electroformation. *Faraday Discuss. Chem. Soc.,* 81, 303-311 (1986).

Angelova, M. L., Soléau, S., Meléard, P. H., Faucon, J. F., and Bothorel, P., Preparation of giant vesicles by external AC fields, kinetics and application. *Prog. Colloid. Polym. Sci.,* 89, 127-131 (1992).

Armbrecht, H. J., Wood, W. G., Wise, R. W., Walsh, J. B., Thomas, B. N., and Strong, R., Ethanol-induced disordering of membranes from different age groups of C57BL/6NNIA mice. *J. Pharmacol. Exp. Ther.,* 226, 387-391 (1983).

Bae, M. K., Huh, M. H., Lee, S. W., Kang, H. G., Pyun, J. H., Kwak, M. H., Jang, H. O., and Yun, I., Effects of dopamine·HCl on structural parameters of bovine brain membranes. *Arch. Pharm. Res.,* 27, 653-661 (2004).

Bae, M. K., Jeong, D. K., Park, N. S., Lee, C. H., Cho, B. H., Jang, H. O., and Yun, I., Effects of ethanol on the physical properties of Neuronal Membranes. *Mol. Cells,* 19, 356-364 (2005).

Bagatolli, L. A. and Gratton, E., Two photon fluorescence microscopy observation of shape change at the phase transition in phospholipid giant unilamellar vesicles. *Biophys. J.,* 77, 2090-2101 (1999).

Bagatolli, L. A. and Gratton, E., Two photon fluorescence microscopy of coexisiting lipid domains in giant unilamellar vesicles of binary phospholipid mixtures. *Biophys. J.,* 78, 290-305 (2000).

Bangham, A. D. and Mason, W., The effect of some general anesthetics on the surface potential of lipid monolayers. *Br. J. Pharmacol.,* 66, 259-265 (1979).

Bartlett, G. R., Phosphorous assay in column chromatography. *J. Biol. Chem.,* 234, 466-468 (1959).

Chin, J. H. and Goldstein, D. B., Effects of low concentrations of ethanol on the fluidity of spin-labeled erythrocyte and brain membranes. *Mol. Pharmacol.,* 13, 435-441 (1977a).

Chin, J. H. and Goldstein, D. B., Drug tolerance in biomembranes: a spin label study of the effects of ethanol. *Science,* 196, 684-685 (1977b).

Chin, J. H. and Goldstein, D. B., Membrane-disordering action of ethanol: variation with membrane cholesterol content and

depth of the spin label probe. *Mol. Pharmacol.,* 19, 425-431 (1981).

Chin, J. H. and Goldstein, D. B., Cholesterol blocks the disordering effects of ethanol in biomembranes. *Lipids,* 19, 929-935 (1984).

Davidson, F. M. and Long, C., The structure of the naturally occurring phosphoglycerides. 4. Action of cabbage-leaf phospholipase D on ovolecithin and related substances. *Biochem. J.,* 69, 458-466 (1958).

Dimitrov, D. S. and Angelova, M. L., Lipid swelling and liposome formation on solid surfaces in external electric fields. *Prog. Colloid. Polym. Sci.,* 73, 48-56 (1987).

Franks, N. P. and Lieb, W. R., Mapping of general anesthetic target sites provides a molecular basis for cutoff effects. *Nature,* 316, 349-351 (1985).

Franks, N. P. and Lieb, W. R., Neuron membranes: anesthetics on the mind. *Nature,* 328, 113-114 (1987).

Franks, N. P. and Lieb, W. R., Do general anesthetics act by competitive binding to specific receptors? *Nature,* 310, 599-601 (1993).

Franks, N. P. and Lieb, W. R., Molecular and cellular mechanisms of general anesthesia. *Nature,* 367, 607-614 (1994).

Gonzales, R. A. and Hoffman, P. L., Receptor-gated ion channels may be selective CNS targets for ethanol. *Trends Pharmacol. Sci.,* 12, 1-3 (1991).

Huang, N.-N., Florine-Casteel, K., Feigenson, G. W., and Spink, C., Effect of fluorophore linkage position of *n*-(9-anthroyloxy) fatty acids on probe distribution between coexisting gel and fluid phospholipid phases. *Biochim. Biophys. Acta,* 939, 124-130 (1988).

Jacobs, R. E. and White, S. H., The nature of the hydrophobic binding of small peptides at the bilayer interface. Implications for the insertion of transbilayer helices. *Biochemistry,* 28, 3421-3437 (1989).

Jang, H. O., Jeong, D. K., Ahn, S. H., Yoon, C. D., Jeong, S. C., Jin, S. D., and Yun, I. Effects of chlorpromazine·HCl on the structural parameters of bovine brain membranes. *J. Biochem. Mol. Biol.,* 37, 603-611 (2004b).

Jang, H. O., Shin, H. G., and Yun, I., Effects of dimyristoyl-phosphatidylethanol on the structural parameters of neuronal membrane. *Mol. Cells,* 17, 485-491 (2004a).

Kang, J.-S., Choi, Ch.-M., and Yun, I., Effects of ethanol on lateral and rotational mobility of plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14. *Biochim. Biophys. Acta,* 1281, 157-163 (1996).

Lasic, D. D., The mechanism of vesicle formation. *Biochem. J.,* 256, 1-11 (1988).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* 193, 265-275 (1951).

Madeira, V. M. C. and Antunes-Madeira, M. C., Lipid composition of biomembranes: a complete analysis of sarcoplasmic reticulum phospholipids. *Cienc. Biol., (Coimbra)* 2, 265-291

(1976).

Menger, F. M. and Keiper, J. S., Chemistry and physics of giant vesicles as biomembrane models. *Curr. Opin. Chem. Biol.*, 2, 726-732 (1998).

Omodeo-Salê, M. F., Cestaro, B., Mascherpa, A., Monti, D., and Masserini, M., Enzymatic synthesis and thermotropic behavior of phosphatidylethanol. *Chem. Phys. Lipids*, 50, 135-142 (1989).

Omodeo-Salê, M. F., Lindi, C., Palestini, P., and Masserini, M., Role of phosphatidylethanol in membranes. Effects on membrane fluidity, tolerance to ethanol, and activity of membrane-bound enzymes. *Biochemistry*, 30, 2477-2482 (1991).

Sanna, E., Concas, A., Serra, M., Santoro, G., and Biggio, G., *Ex vivo* binding of t-[35S)butylbicyclophosphorothionate: a biochemical tool to study the pharmacology of ethanol at the gamma-aminobutyric acid-coupled chloride channel. *J. Pharmacol. Exp. Ther.*, 256, 922-928 (1991).

Schachter, D., Fluidity and function of hepatocyte plasma membranes. *Hepatology*, 4, 140-151 (1984).

Sheetz, M. P. and Singer, S. J., Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4457-4461 (1974).

Stubbs, C. D. and Williams, B. W., Fluorescence in membranes; in Fluorescence Spectroscopy in Biochemistry, In Lakowicz, J. R. (Ed). Vol. III, Plenum Press, New York, pp 231-263, (1992).

Teeter, M. M., Water-protein interaction: the theory and experiment. *Annu. Rev. Biophys. Biophys. Chem.*, 20, 577-600 (1991).

Yun, I., Cho, E. S., Jang, H. O., Kim, U. K., Choi, C. H., Chung, I. K., Kim, I. S., and Wood, W. G, Amphiphilic effects of local anesthetics on rotational mobility in neuronal and model membranes. *Biochim. Biophys. Acta*, 1564, 123-132 (2002).

Yun, I. and Kang, J.-S., The general lipid composition and aminophospholipid asymmetry of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex. *Mol. Cells*, 1, 15-20 (1990).

Yun, I., Kim, Y.-S., Yu, S.-H., Chung, I.-K., Kim, I.-S., Baik, S.-W., Cho, G.-J., Chung, Y.-Z., Kim, S.-H., and Kang, J.-S., Comparision of several procedures for the preparation of synaptosomal plasma membrane vesicles. *Arch. Pharm. Res.*, 13, 325-329 (1990).

Yun, I., Lee, S.-H., and Kang, J.-S., Effects of ethanol on lateral and rotational mobility of plasma membrane vesicles isolated from cultured Mar 18.5 hybridoma cells. *J. Membr. Biol.*, 138, 221-227 (1994).

Yun, I., Yang, M.-S., Kim, I.-S., and Kang, J.-S., Bulk *vs.* transbilayer effects of ethanol on the fluidity of the plasma membrane vesicles of cultured Chinese hamster ovary cells. *Asia Pacific J. Pharmacol.*, 8, 9-16 (1993).

Zachariasse, K. A., Vaz, W. L. C., Stomayer, C., and Kuhnle, W., Investigation of human erythrocyte ghost membranes with intramolecular excimer probes. *Biochim. Biophys. Acta*, 688, 323-332 (1982).