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The Effect of Ethanol on Lateral and Rotational Mobility of Plasma Membrane Vesicles Isolated from Cultured Mar 18.5 Hybridoma Cells

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Abstract. Intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to evaluate the effect of ethanol on the rate and range of the lateral mobility and the range of the rotational mobility of bulk bilayer structures of the plasma membrane vesicles (ATCC-PMV) isolated from cultured hybridoma cells (ATCC TIB 216). In a concentration-dependent manner, ethanol increased the excimer to monomer fluorescence intensity ratio (I′/I) of Py-3-Py in the ATCC-PMV and decreased the anisotropy (r), limiting anisotropy (r_∞) and order parameter (S) of DPH in the ATCC-PMV. This indicates that ethanol increased both the lateral and rotational mobility of the probes in the ATCC-PMV. Selective quenching of DPH by trinitrophenyl groups was utilized to examine the range of transbilayer asymmetric rotational diffusion of the ATCC-PMV. The anisotropy (r), limiting anisotropy (r_∞) and order parameter (S) of DPH in the inner monolayer were 0.024, 0.032, and 0.069, respectively, greater than calculated for the outer monolayer of the ATCC-PMV. Selective quenching of DPH by trinitrophenyl groups was also used to examine the transbilayer asymmetric effects of ethanol on the range of the rotational mobility of the ATCC-PMV. Ethanol had a greater increasing effect on the range of the rotational mobility of the outer monolayer as compared to the inner monolayer of the ATCC-PMV. It has been proven that ethanol exhibits a selective rather than nonselective fluidizing effect within the transbilayer domains of the ATCC-PMV.

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Key words: Ethanol — Hybridoma cell plasma membrane vesicles — Transbilayer fluidity asymmetry — Fluorescent probe technique

Introduction

It has been thought that ethanol, as well as the barbiturates and the volatile anesthetics, exerted their depressant effects on the central nervous system (CNS) by dissolving in lipid membranes, thereby perturbing the function of ion channels and other protein embedded therein. The most compelling evidence was the excellent correlation between lipid solubility and anesthetic potency. This hypothesis has been refined by the application of various physical techniques that showed that ethanol caused a local disordering in the lipid matrix, also referred to as membrane fluidization. The enhanced fluidity of neuronal membranes produced by ethanol may alter ion fluxes across the membrane such as chloride, sodium, and calcium (Lee & Becker, 1989). And the enhanced fluidity of neuronal membranes produced by ethanol may alter conformational changes in enzymes such as Na^+,K^+-ATPase, Ca^{2+}-ATPase, 5′-nucleotidase, acetylcholinesterase and adenylate cyclase (Lee & Becker, 1989). In addition, a variety of neurotransmitters such as norepinephrine, dopamine, glutamate and γ-aminobutyric acid (GABA) are affected by the enhanced fluidity of neuronal membranes (Lee & Becker, 1989). However, the membrane-fluidizing hypothesis of 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 & Hoffman, 1991; Sanna et al., 1991). At the present time, the

exact mechanism(s) of action of ethanol in CNS is unclear.

The purpose of this research is two-fold: (i) to provide a basis for studying the mechanism of action of ethanol; and (ii) to provide a basis for studying the characteristics of plasma membrane vesicles of hybridoma cells through the investigation of intrinsic fluidity of the plasma membrane vesicles and sensitivity of the vesicles to the fluidizing effect of ethanol. In the present study, using intramolecular excimerization of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), we examined the effect of ethanol on the bulk bilayer fluidity of the plasma membrane vesicles (ATCC-PMV) isolated from cultured Mar 18.5 (ATCC TIB 216) hybridoma cells. Also, selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the effect of ethanol on the individual monolayer structure of ATCC-PMV.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), L-glutamate, penicillin G, streptomycin and all buffers were purchased from Sigma Chemical (St. Louis, MO). The fluorescent probes DPH and Py-3-Py were obtained from Molecular Probes (Junction City, OR). Ethanol and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Fluka (Buchs, Switzerland). All other reagents were purchased from commercially available sources and were of the highest quality available.

Media and Cell Culture

Mar 18.5 hybridoma cells were purchased from ATCC and used for the membrane analysis. The cells were adopted and routinely maintained in DMEM supplemented with 10% fetal bovine serum and passaged every five days at 5% CO_2, 37°C. This hybridoma formed by fusing spleen cells from SJL/J mice immunized with rat immunoglobulin with the P3X63 Ag8 myeloma cell line secretes an IgG_{2a} monoclonal antibody.

TNBS Labeling Reactions

TNBS labeling reactions were performed by the procedure of Yun and Kang (1990) with a few modifications. We gently resuspended 40×10^6 cells (exponential phase) in 2 mM TNBS + buffer A or buffer A alone. Buffer A was composed of 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose, and 2% BSA. The reagent pH was adjusted to 8.5 with NaOH. To assure complete exposure of all outer monolayers to TNBS, we passed the cells slowly through an Eberbach tissue grinder (three up and down strokes). The treatment was carried out at 4°C for 40 min, unless otherwise specified. The TNBS labeling reaction was terminated by addition of 2% BSA in phosphate-buffered saline (PBS). PBS was composed of 0.14 M NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 7H_2O$ and 2 mM HEPES (pH 7.4). The entire suspension was then sedimented at $1,100 \times g$ for 5 min, and preparation of plasma membrane vesicles was performed as described below.

Membrane Preparation

ATCC-PMV were isolated by the procedure of Yun et al. (1993*a*). All steps were carried out at 0–4°C. Briefly, trinitrophenylated cells were hypotonically lysed by incubation for 20 min with 1 mM $NaHCO_3$. The lysate was centrifuged at $27,000 \times g$ for 15 min. The pellet was resuspended in 46% (w/v) sucrose, adjusted to 44% sucrose, and overlayered with 1 ml of 41% sucrose and 5 ml of 38% sucrose. The gradient was centrifuged for 2.5 hr at $90,000 \times g$ in a SW 41 Ti rotor. The final pellet was resuspended in PBS, divided into small aliquots, quickly frozen in liquid nitrogen and stored at −70°C. The purity was evaluated by morphological and enzymatic standards. Electron microscopic examination showed that the membranes were in vesicular form (Yun et al., 1990*a*). The purity of ATCC-PMV was also confirmed by monitoring the specific activities of marker enzymes (Yun et al., 1990*a*). The specific activities of Na^+,K^+-ATPase and 5'-nucleotidase were about 5.6- and 3.5-fold, respectively, enriched in the plasma membrane fraction compared to crude homogenates. Protein was determined by the method of Lowry et al. (1951) with BSA as a standard.

Fluorescence Measurements

The incorporation of Py-3-Py was carried out by adding aliquots of a stock solution of 5×10^{-5} M in ethanol to ATCC-PMV, so that the final probe concentration was less than 5×10^{-7} M. The mixtures were initially vigorously vortexed for 10 sec at room temperature and then incubated at 4°C for 18 hr under gentle stirring. The fluorescent probe DPH was dissolved in tetrahydrofuran, and a volume of 0.5 μl of tetrahydrofuran per ml of PBS was added directly to the membrane suspension at a concentration of 1 μg/70 μg membrane protein as described previously (Yun et al., 1990*b*). After incorporation of the probes, the membrane suspension was placed in cuvettes. Control levels of fluorescence were then determined, an aliquot of ethanol was added directly to the cuvette, and fluorescence was again determined. The excitation wavelength of Py-3-Py was 330 nm, and the excimer to monomer fluorescence intensity ratio (I'/I) was calculated from the 480 to 379 nm signal ratio. The excitation wavelength for DPH was 362 nm, and fluorescence emission was read at 424 nm. All fluorescence measurements were obtained with an SPF-500C spectrofluorometer (SLM Aminco Instruments, Urbana, IL) and performed at 37°C. Before the fluorescence spectra were obtained, all samples were bubbled by dry nitrogen through the solution for at least 30 min to eliminate oxygen. Blanks, prepared under identical conditions without Py-3-Py or DPH, served as controls for the fluorometric measurements.

The intensity of the components of the fluorescence that were parallel (I) and perpendicular (I) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. The polarization (P) was obtained from intensity measurements using $P = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp})$ where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value 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 [$r = 2P/(3-P)$], the limiting anisotropy (r_∞), and the order parameter (S). The limiting anisotropy of DPH was

determined directly from the anisotropy value using the following relationship (van Blitterwijk, Van Hoeven & van der Meer, 1981):

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

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

Determination of Individual Monolayer Structure in ATCC-PMV: Selective Quenching of DPH

This experimental determination of individual monolayer structure in ATCC-PMV is based on a method previously established for LM plasma membranes (Sweet, Wood & Schroeder, 1987), synaptic plasma membranes (SPM) (Schroeder et al., 1988; Wood, Gorka & Schroeder, 1989) and plasma membrane vesicles of Chinese hamster ovary cells (CHO-K_1-PMV) (Yun et al., 1993*a*). This method does not simply provide a theoretically calculated or average value but is based on the assumption that the system is composed of fluorescing compartments of different accessibility to TNBS. If the fluorescence intensity, F, and anisotropy, r, are measured simultaneously, then

$$r = \Sigma F_j r_j \tag{2}$$

where F_j is the fraction of fluorescence intensity in compartment j. For a binary system composed of the outer and inner monolayers of the ATCC-PMV, this leads to

$$r = \frac{F_i}{F} r_i + \frac{F - F_i}{F} r_o \tag{3}$$

where F and F_i are fluorescence of DPH obtained for ATCC-PMV isolated from cells incubated with buffer A and buffer A plus TNBS at 4°C (nonpenetrating conditions), respectively. The value of the fluorophore concentration-independent parameter anisotropies, r (anisotropy for both monolayers) and r_i (inner monolayer anisotropy), were determined for DPH in ATCC-PMV obtained from cells incubated with buffer A and buffer A plus TNBS at 4°C (nonpenetrating conditions), respectively. The equation was then solved for r_o (outer monolayer anisotropy). Similar calculations were performed by simultaneous measurement of fluorescence intensity and either limiting anisotropy or order parameter.

Results and Discussion

Effects of Ethanol on the Range and Rate of the Lateral Mobility of Bulk Bilayer ATCC-PMV

Py-3-Py, a pyrene derivative which has successfully been used to quantitate lateral mobility within native and model membranes (Zachariasse et al., 1982; Schachter, 1984; Yun et al., 1990*a*, 1993*a,b*), was used to determine the rate and range of the lateral mobility in the ATCC-PMV. Using this probe, one monitors 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 the lateral mobility (Zachariasse et al., 1982; Schachter, 1984; Yun et al., 1990*a*, 1993*a,b*). As probe mobility within membranes increases, emission from the excimer predominates since formation of the intramolecular excimer is dependent upon lateral movement of its two components. Therefore, an increase in the I′/I ratio is an indication of increased lateral mobility of the probe within the membrane. The excimer fluorescence technique of 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 ATCC-PMV by the probe molecule is minimized.

Yun et al. (1993*a,b*) reported that the I′/I values in the CHO-K_1-PMV and the plasma membrane vesicles of mouse myeloma cell line Sp2/0-Ag14 (Sp2/0-PMV) were 0.529 ± 0.016 and 0.608 ± 0.008, respectively (37°C, pH 7.4). In CHO-K_1-PMV and Sp2/0-PMV, ethanol increased the lateral diffusion of Py-3-Py in a concentration-dependent manner, and significant increases in I′/I values were observed at above 50 and 25 mM ethanol, respectively (Yun et al., 1993*a,b*). In this study, the I′/I value in intact ATCC-PMV (ethanol-untreated) was 0.586 ± 0.007 (Fig. 1). Ethanol increased the range and rate of the lateral mobility of the bulk (inner + outer monolayer) ATCC-PMV dose-dependently, and a significant increase in the I′/I value was observed even at 25 mM ethanol (Fig. 1). This is in agreement with the results of our previous studies, indicating that ethanol has a large influence on the lateral mobility of native membranes. However, the important point is the different potency of ethanol between ATCC-PMV (or Sp2/0-PMV) and CHO-K_1-PMV, in terms of minimal ethanol concentration for the significant increase in the I′/I values.

Effects of Ethanol on the Range of the Rotational Mobility of Bulk Bilayer ATCC-PMV

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 & Hoilien, 1987; Yun et al., 1993*a,b*). The results of fluorescence polarization determinations are conveniently expressed as the fluorescence anisotropy (r). The limiting anisotropy (r_∞) reflects the hindrance to 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_∞ value is zero. In native and model membranes, the r_∞ values of the DPH are high and largely determine r. In biological experiments, both the dynamic (rotational relaxation time of fluorophores) and structural (r_∞) or static components may be significant, and it seems reasonable to use "fluidity" to designate both. The structural organization of

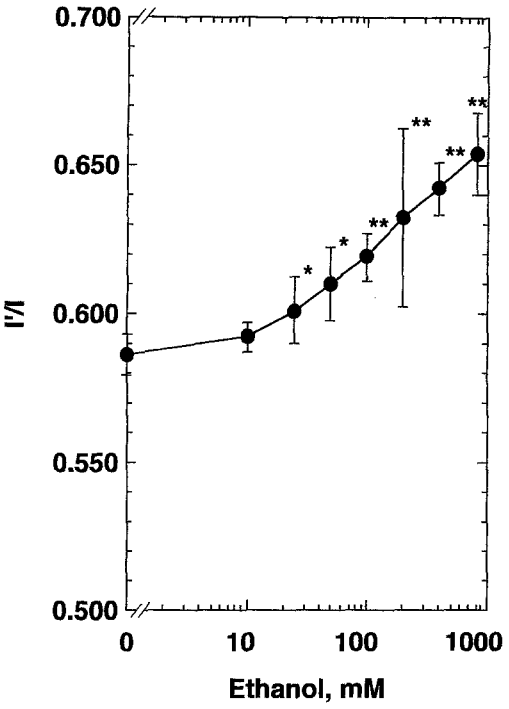

Fig. 1. Effects of ethanol on the excimer to monomer fluorescence intensity ratio, I'/I, of 1,3-di(1-pyrenyl)propane in the plasma membrane vesicles isolated from cultured hybridoma cells (ATCC TIB 216). Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of five determinations. (*) and (**) $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's *t*-test.

Table 1. Asymmetry of 1,6-diphenyl-1,3,5-hexatriene mobility in the plasma membrane vesicles (ATCC-PMV) isolated from cultured hybridoma cells (ATCC TIB 216)

Membrane	Anisotropy (r)	Limiting anisotropy (r_∞)	Order parameter (S)
Inner + outer	0.188 ± 0.003	0.151 ± 0.003	0.645 ± 0.009
Inner	0.198 ± 0.003	0.164 ± 0.004	0.673 ± 0.006
Outer	0.174 ± 0.005*	0.132 ± 0.005*	0.604 ± 0.011*

Cells were treated ±2 mM 2,4,6-trinitrobenzenesulfonic acid, pH 8.5, at 4°C for 40 min, and the plasma membrane vesicles were isolated. 1,6-diphenyl-1,3,5-hexatriene 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 ± SEM of five determinations. *$P < 0.01$ compared to control by Student's *t*-test.

the lipid environment in the bilayers limits the rotational extent or the range of DPH, and the r_∞ can be used to define an order parameter (S).

We reported that the anisotropies (r) of the DPH in the CHO-K_1-PMV (Yun et al., 1993*a*) and the Sp2/0-PMV (Yun et al., 1993*b*) were 0.195 ± 0.002 and 0.183 ± 0.002, respectively. Ethanol increased the rotational diffusion of CHO-K_1-PMV and Sp2/0-PMV in a concentration-dependent manner with a significant decrease in the anisotropy (r), the limiting anisotropy (r_∞) and the order parameter (S) observed even at 25 mM ethanol (Yun et al., 1993*a,b*). In the present study, the anisotropy (r), the limiting anisotropy (r_∞) and the order parameter (S) of DPH in the ATCC-PMV were 0.188 ± 0.003, 0.151 ± 0.003, and 0.645 ± 0.009, respectively (Table 1). Ethanol decreased the anisotropy (r), the limiting anisotropy (r_∞) and the order parameter (S), and a significant decrease in the r, r_∞ and S was observed even at 25 mM ethanol (Fig. 2, filled squares; Table 2). Therefore, even at 25 mM, ethanol significantly decreased the range of the rotational mobility of the bulk ATCC-PMV. This coincides with the studies reported above by Yun et al. (1993*a,b*), and it means that the degree of ethanol-induced decrease in these three parameters, i.e., the r, r_∞ and S, in the ATCC-PMV and the Sp2/0-PMV is more clear compared to that of the CHO-K_1-PMV.

Effects of Ethanol on the Range of the Transbilayer Rotational Mobility of ATCC-PMV

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 DPH. 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 DPH fluorescence was quenched in the trinitrophenylated ATCC-PMV. When the TNBS labeling was conducted under penetrating conditions (37°C), nearly 100% of the fluorescence of the DPH was quenched. The values of fluorescence parameters in intact ATCC-PMV (both monolayers) compared to those for TNBS-treated ATCC-PMV (inner monolayer) are listed in Table 1. The anisotropy (r), limiting anisotropy (r_∞) and order parameter (S) of DPH in the inner monolayer were 0.024, 0.032, and 0.069, respectively, greater than calculated for the outer monolayer of the ATCC-PMV. In this study, ethanol showed a greater fluidizing (range of rotational mobility) effect on the outer monolayer (Fig. 2, filled circles) compared to the inner monolayer (Fig. 2, filled triangles). This is consistent with the results of our previous studies (Yun et al., 1993*a,b*). It suggests that the fluidizing effect (range of rotational mobility) of ethanol is selective rather than nonselective within transbilayer domains of the native membranes.

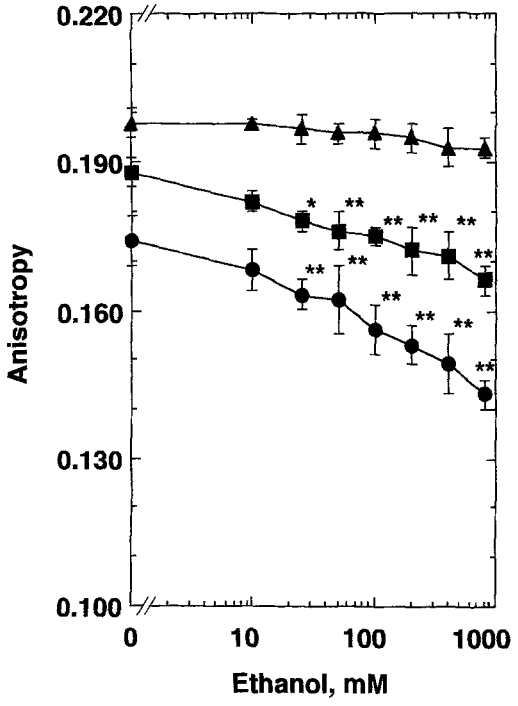

Fig. 2. Ethanol alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the outer monolayer of the plasma membrane vesicles isolated from cultured hybridoma cells (ATCC TIB 216). Cells were treated with ±2 mM trinitrobenzenesulfonic acid, pH 8.5, at 4°C for 40 min and the plasma membrane vesicles were isolated. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated (inner monolayer, ▲); calculated for the outer monolayer (●) as described in Materials and Methods. Each point represents the mean ± SEM of five determinations. (*) and (**) $P < 0.05$ and $P < 0.01$, respectively, compared to control by Student's t-test.

Differences of Intrinsic Fluidity among Native Membranes

The fluidity of intact ATCC-PMV was remarkably higher than that of normal cell plasma membrane vesicles, intact CHO-K_1-PMV. However, its fluidity came out to be slightly lower than that of myeloma cell plasma membrane vesicles, intact Sp2/0-PMV. In the point of view of the preceding results, that is in the aspect of fluidity, ATCC-PMV possesses both the characteristics of the P3X63 Ag8 myeloma cell plasma membranes and the SJL/J mice spleen cell plasma membranes.

Possible Mechanisms of Action of Ethanol

Ethanol and related alkanols have been shown to decrease the temperature of the gel-to-liquid crystalline phase transition of model membranes (Jain & Wu, 1977), expand membranes (Seeman, 1972) and alter the surface charge of membrane lipids (Bangham & Mason, 1979). However, these effects have been obtained with ethanol concentrations in the range of 500–1,500 mM, whereas serum concentrations above 200 mM are usually lethal in humans and laboratory animals (Harris & Schroeder, 1981). Moreover, the accumulated results for the analysis of ethanol effect on the cell membrane fluidity have been focused on the normal cell membranes, not on the cancer and the fused cell membranes. Our data presented herein show that, even at physiologically relevant concentrations, i.e., 25 mM (Majchrowicz & Mendelson, 1971; Harris & Schroeder, 1981), the increased lateral and rotational diffusion induced by ethanol indicates the presence of bulk lipid fluidization, and the increase in bulk bilayer rotational diffusion is mainly due to the increased range of the rotational diffusion in the outer monolayer of the ATCC-PMV.

We demonstrated that ethanol increased the range and rate of the lateral mobility of the CHO-K_1-PMV (Yun et al., 1993*a*), Sp2/0-PMV (Yun et al., 1993*b*), synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex (Chung, Kang & Yun, 1993*a*) and model membranes of total lipid fraction (SPMVTL, Chung, Kang & Yun, 1993*b*) and total phospholipid fraction (SPMVPL, Chung et al., 1993*b*) extracted from SPMV. In addition, ethanol increased the range of the rotational mobility of the CHO-K_1-PMV (Yun et al., 1993*a*), Sp2/0-PMV (Yun et al., 1993*b*), SPMVTL (Yun & Kang, 1992*a*) and SPMVPL (Yun & Kang, 1992*b*) and the rate of the rotational mobility of native (Chung, Cho & Yun, 1993*c*) and model membranes (Chung et al., 1993*d*). Therefore, in a concentration-dependent manner, ethanol significantly increases the range and rate of the lateral and rotational mobility of both native and model membranes. Additionally, the increase in the bulk rotational mobility (primarily the range of the motion) is mainly derived from increases of the motion range in the outer monolayer of native (Yun et al., 1993*a,b*) and model membranes (Yun & Kang, 1992*a,b*). Consequently, native and model membranes that have higher fluidity are much more sensitive to the fluidizing effect of ethanol. Also, this finding can be extended to the transbilayer asymmetric fluidity in native and model membranes.

Several recent data show that ethanol exerts a specific effect on lateral and transbilayer domains in vitro in contrast to a generalized effect on the bulk lipid membrane. Ethanol and butanol increased the diffusion of rhodamine-phosphatidylethanolamine more than the diffusion of 1-acyl-2-(6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminohexanoyl)phosphatidylcholine in *Aplysia* neuronal membranes using fluorescence recovery after photobleaching (Treistman, Moynihan & Wolf, 1987). In synaptic plasma membranes (SPM), it has been reported that ethanol has an ordering and disor-

Table 2. Effects of ethanol on the limiting anisotropy (r_∞) and the order parameter (S) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the plasma membrane vesicles (ATCC-PMV) isolated from cultured hybridoma cells (ATCC TIB 216)

Conc. (mM)	Limiting Anisotropy (r_∞)			Order Parameter (S)		
	Inner + Outer	Inner	Outer	Inner + Outer	Inner	Outer
0	0.151 ± 0.003	0.164 ± 0.004	0.132 ± 0.005	0.645 ± 0.009	0.673 ± 0.006	0.604 ± 0.011
10	0.143 ± 0.004	0.164 ± 0.005	0.124 ± 0.004	0.628 ± 0.005	0.673 ± 0.007	0.585 ± 0.008
25	0.137 ± 0.005*	0.163 ± 0.004	0.117 ± 0.003**	0.616 ± 0.007*	0.670 ± 0.009	0.569 ± 0.007**
50	0.135 ± 0.003**	0.161 ± 0.004	0.116 ± 0.010**	0.610 ± 0.090**	0.668 ± 0.008	0.566 ± 0.018**
100	0.133 ± 0.003**	0.161 ± 0.003	0.108 ± 0.006**	0.607 ± 0.007**	0.668 ± 0.003	0.546 ± 0.012**
200	0.129 ± 0.004**	0.161 ± 0.006	0.104 ± 0.007**	0.597 ± 0.008**	0.665 ± 0.009	0.536 ± 0.013**
400	0.128 ± 0.007**	0.157 ± 0.008	0.099 ± 0.009**	0.595 ± 0.012**	0.659 ± 0.012	0.522 ± 0.016**
800	0.121 ± 0.005**	0.157 ± 0.003	0.091 ± 0.003**	0.579 ± 0.009**	0.659 ± 0.005	0.500 ± 0.008**

All conditions were as described in the legend to Table 1. Values are represented as the mean ± SEM of five determinations. (*) and (**) signify $P < 0.05$ and $P < 0.01$, respectively, compared to control by Student's t-test.

dering effect on the membrane's surface and interior, respectively, as measured by nuclear magnetic resonance (Hitzemann et al., 1986). Using permeant and impermeant fluorescence probes, Chabanel et al. (1985) found that benzyl alcohol had a greater fluidizing effect on the inner monolayer in erythrocytes compared to the outer monolayer. It was reported in that study that the inner monolayer was significantly more fluid than the outer monolayer. The individual monolayers of SPM showed just the opposite asymmetry in fluidity when compared with erythrocyte monolayers, and ethanol fluidized the more fluid outer monolayer of SPM (Schroeder et al., 1988). In the present study, ethanol in vitro preferentially fluidized the more fluid outer monolayer of ATCC-PMV. In view of the point that ethanol has a greater effect on fluid membranes compared to more ordered membranes, our results are in agreement with those of previous studies (Chin & Goldstein, 1981; Chabanel et al., 1985; Schroeder et al., 1988). Hence, the more fluid monolayer in the bilayer organization, whether outer or inner monolayer, is the major target site of the fluidizing effect of ethanol.

Opinions have been divided as to whether ethanol interfered with membrane protein function by directly binding to the proteins, or whether the main modes of action occurred indirectly through a change in the physicochemical properties of the lipid membranes into which the ethanol readily diffused. Because biological membranes are of highly complex composition, it has not been feasible to monitor changes in the local lipid environment and to determine its effect on the membrane protein function at the same time. Ethanol's effects on membranes whether bulk or domains have been studied under the assumption that the membrane is in a bilayer form. In fact, increasing evidence indicates that membrane lipids can adopt a nonbilayer form (Janoff, Boni & Rauch, 1988). In addition, the results of investigations on the effects of higher alkanols and the corresponding alkanes on membrane luciferases indicate that the anesthetic site could be hydrophobic pockets on membrane proteins rather than the lipid part of the membrane (Franks & Lieb, 1987). Ethanol specifically and selectively affects the function of the GABA-coupled chloride channel (Gonzales & Hoffman, 1991; Sanna et al., 1991). Still, a large, diverse collection of physiological agonists produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction (Manevich et al., 1988). So, the function of membrane proteins may be modulated secondarily to changes in membrane fluidity. Conversely, there is also a possibility that ethanol may have a direct effect on certain receptors, receptor-gated ion channels, or membrane-bound enzymes, and then on membrane lipids.

It would be difficult to exclude the possibility that interaction of ethanol with neuronal membrane lipids may exert some influence on the ion channels or receptors which associate tightly with membrane lipids through covalent and noncovalent bonds. That is to say, before, during or even after the interaction of ethanol with the proteins (Gonzales & Hoffman, 1991; Sanna et al., 1991), the fluidization of membrane lipids may provide an ideal microenvironment for optimum anesthetic effects. In conclusion, the present data suggest that ethanol, in addition to its direct interaction with proteins (Gonzales & Hoffman, 1991; Sanna et al., 1991), concurrently interacts with membrane lipids, fluidizes the membrane, and thus induces conformational changes of proteins which are known to be intimately associated with membrane lipids. At present, it may be premature to take sides in the controversy about whether membrane lipids or membrane proteins are the site of ethanol action.

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