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Biochimica et Biophysica Acta 1281 (1996) 157–163

BBA Biochimica et Biophysica Acta

Effects of ethanol on lateral and rotational mobility of plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14

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Received 25 July 1995; revised 16 November 1995; accepted 14 December 1995

Abstract

Intramolecular excimerization of Py-3-Py and fluorescence polarization of DPH were used to evaluate effects of ethanol on the rate and range of the lateral mobility and the range of the rotational mobility of bulk bilayer structures of the Sp2/0-PMV. In a concentration-dependent manner, ethanol increased the rate and range of the lateral mobility and the range of the rotational mobility of bulk bilayer structures of Sp2/0-PMV. Selective quenching of DPH by trinitrophenyl groups was utilized to examine the range of transbilayer asymmetric rotational mobility of the Sp2/0-PMV. The anisotropy (r), limiting anisotropy (r_∞) and order parameter (S) of DPH in the inner monolayer were 0.022, 0.029 and 0.063, respectively, greater than calculated for the outer monolayer of the Sp2/0-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 Sp2/0-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 Sp2/0-PMV. It has been proven that ethanol exhibits a selective rather than nonselective fluidizing effect within the transbilayer domains of the Sp2/0-PMV.

Keywords: Ethanol; Asymmetric fluidity; Lateral mobility; Rotational mobility; Native membrane; Model membrane; (Mouse); (Cell line Sp2/0-Ag14)

1. Introduction

The molecular mechanism of action of ethanol in the CNS has long been a subject of great interest. It has been thought that ethanol, as well as the barbiturates and volatile anesthetics, exerted their depressant effects on the CNS by dissolving in lipid membranes, thereby perturbing the function of ion channels and other proteins 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. A number of theories propose perturbations of bulk physical properties of the lipids of cell membranes as the primary event leading to inebriation or anesthesia.

Previous studies [1–16] on effects of ethanol on membrane bulk lipids had examined the average or total change in the membrane lipid environment. We [17,18] and other investigators [19–24] take the position that membrane lipids are an important site of action of ethanol but ethanol has a very specific effect on different lipid areas or domains in the membrane. With few exceptions [17,18,23–29], little attention has been given to the effect of ethanol on lateral mobility of biological membranes. Furthermore,

Abbreviations: ATCC-PMV, plasma membrane vesicles isolated from cultured Mar 18.5 hybridoma cells; BSA, bovine serum albumin; CHO-K_1-PMV, plasma membrane vesicles isolated from cultured Chinese hamster ovary K_1 cells; CNS, central nervous system; DPH, 1,6-diphenyl-1,3,5-hexatriene; GABA, γ-aminobutyric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; Py-3-Py, 1,3-di(1-pyrenyl)propane; Sp2/0-PMV, plasma membrane vesicles isolated from cultured myeloma cell line Sp2/0-Ag14; SPM, synaptic plasma membranes; SPMV, synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex; SPMVPL, liposomes of SPMV total phospholipids; SPMVTL, liposomes of SPMV total lipids; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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SSDI 0005-2736(95)00301-0

most of 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 cell membranes.

The aim 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 mouse myeloma cell line Sp2/0-Ag14 through the investigation of intrinsic fluidity of the vesicles and sensitivity of the vesicles to the fluidizing effect of ethanol. In the present study, using intramolecular excimerization of Py-3-Py and fluorescence polarization of DPH, we examined the effect of ethanol on the bulk bilayer fluidity of the Sp2/0-PMV. Also, selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the effect of ethanol on the individual monolayer structure of the Sp2/0-PMV.

2. Materials and methods

Dulbecco's modified Eagle's medium, fetal calf serum, BSA, Hepes, L-glutamate, penicillin G, streptomycin and all buffers were purchased from Sigma (St. Louis, MO, USA). The fluorescent probe DPH and Py-3-Py were obtained from Molecular Probes (Junction City, OR, USA). Ethanol and TNBS were obtained from Fluka (Buchs, Switzerland). All other reagents were purchased from commercially available sources and were of the highest quality available. Water was deionized.

2.1. Media and cell culture

Mouse myeloma cell line Sp2/0-Ag14 was kindly provided by Dr. Chang-Mo Kang (Pusan National University, South Korea). Sp2/0-Ag14 was derived from P3X63Ag8 × BALB/c. These cell lines were permanently growing in tissue culture medium as described previously [30]. Briefly, cells were grown in Dulbecco's modified Eagle's medium which was supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 μg streptomycin per ml, and 2 mM L-glutamine. Cultures were maintained at 37°C in a moist atmosphere containing 5% CO_2 with subculturing every 4 days. All cultures were grown in disposable plastic Petri dishes (Falcon Plastics, Los Angeles, CA, USA, although several other manufacturers' products were equally suitable), usually of the bacteriological type. The viable cell numbers were counted on a Coulter Counter Model F (Coulter Electronics, Hialeath, FL, USA) with 0.1% Trypan blue exclusion.

2.2. TNBS-labeling reactions

TNBS-labeling reactions were performed according to procedures described by us [17,18,31] and by other investigators [20–22,32] with a few modifications. We gently resuspended $40 \cdot 10^6$ 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 PBS. PBS was composed of double distilled water, 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 $1100 \times g$ for 5 min and preparation of plasma membrane vesicles was performed as described below.

2.3. Membrane preparation

Sp2/0-PMV were isolated as previously described [17,18,31]. 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 h 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 of Sp2/0-PMV was evaluated by morphological and enzymatic standards, according to Yun et al. [33]. Electron microscopic examination showed that the membranes were in vesicular form. The specific activities of Na^+,K^+-ATPase and 5'-nucleotidase were about 5- and 3-fold, respectively, enriched in the plasma membrane fraction compared to crude homogenates. Protein was determined by the procedure of Lowry et al. [34] with BSA as a standard.

2.4. Fluorescence measurements

The incorporation of Py-3-Py was carried out by adding aliquots of a stock solution of $5 \cdot 10^{-5}$ M in ethanol to the Sp2/0-PMV, so that the final probe concentration was less than $5 \cdot 10^{-7}$ M. The mixtures were initially vigorously vortexed for 10 s at room temperature and then incubated at 4°C for 18 h 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 10 mg/50 μg membrane protein as described previously [17,18,35]. 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 nm 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, USA) and performed at 37°C. Before the fluorescence spectra were obtained, all samples were bubbled by dry nitrogen through the solution for at least 5 min in order to eliminate oxygen. Blanks, prepared under 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 ($I_{\parallel}$) and perpendicular ($I_{\perp}$) 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 [36]:

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

The limiting anisotropy reflects restriction to probe motion and can be converted to an order parameter, $S = (r_\infty/r_o)^{1/2}$ [37] where r_o, the anisotropy in the absence of motion, is equal to 0.362 for DPH [38].

2.5. Determination of individual monolayer structure in Sp2 / 0-PMV: selective quenching of DPH

This experimental determination of individual monolayer structure in Sp2/0-PMV is based on a method previously established for CHO-K_1-PMV [17], ATCC-PMV [18], SPM [20–22], SPMV [31] and LM fibroblast plasma membranes [32]. 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

$$r = \Sigma F_j r_j$$

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 Sp2/0-PMV, this leads to

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

where F and F_i are fluorescence of DPH obtained for Sp2/0-PMV isolated from cells incubated with buffer A and buffer A plus TNBS at 4°C (pH 8.5) (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 Sp2/0-PMV obtained from cells incubated with buffer A and buffer A plus TNBS at 4°C (pH 8.5) (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.

3. Results

3.1. Effects of ethanol on the range and rate of the lateral mobility of bulk bilayer Sp2 / 0-PMV

Intramolecular excimer formation of Py-3-Py is sensitive to fluidity and has been used to study fluidity changes [17,18,25–29,39–43] and phase transitions of phospholipid vesicles [29,40,42], micelles [39] and biological membranes [29,41,42]. Py-3-Py, a pyrene derivative which has successfully been used to quantitate the lateral mobility within native [17,18,25,27–29] and model [26,29,39,40,43] membranes, was used to determine the rate and range of the lateral mobility in the Sp2/0-PMV. 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 excimer (I') to monomer (I) intensity ratio (I'/I) is an indication of increased lateral mobility of the probe within the membrane. This methodology has an advantage over its counterpart based on intermolecular excimer formation, since very small probe concentrations can be used ($< 10^{-7}$ M), and the perturbation of the Sp2/0-PMV by the probe molecule is minimized.

The I'/I value of intact Sp2/0-PMV (ethanol-untreated) was 0.608 ± 0.008 (at 37°C, pH 7.4) (Fig. 1). Ethanol increased the range and rate of the lateral mobility of the bulk bilayer (inner + outer monolayer) Sp2/0-PMV dose-dependently and a significant increase in the I'/I value was observed even at 25 mM ethanol (Fig. 1).

3.2. Effects of ethanol on the range of the rotational mobility of bulk bilayer Sp2 / 0-PMV

The fluorescence polarization mainly reflects the rotational mobility of lipid fluorophores [17,18,43,44]. The results of fluorescence polarization determinations are conveniently expressed as the fluorescence anisotropy (r). Limiting anisotropy (r_∞) is primarily determined by the

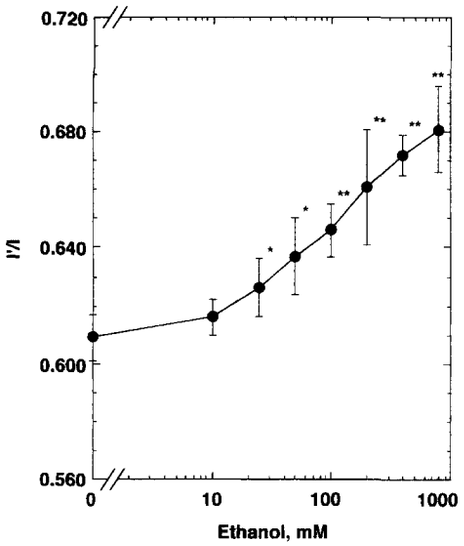

Fig. 1. Effects of ethanol on the excimer to monomer fluorescence intensity ratio, I'/I, of Py-3-Py in the Sp2/0-PMV. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ±S.E. of five determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's t-test.

degree of limitation to rotation range and may vary independently of rotational rate. The limiting anisotropy 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 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).

The anisotropy (r), the limiting anisotropy (r_∞) and the order parameter (S) of DPH in the Sp2/0-PMV were 0.183 ± 0.002, 0.145 ± 0.002 and 0.635 ± 0.005, respectively (at 37°C, pH 7.4) (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 Sp2/0-PMV. This coincides with the study reported by Yun et al. [17,18] and others [20].

Table 1
Asymmetry of mobility of DPH in the Sp2/0-PMV

Membrane	Anisotropy (r)	Limiting anisotropy (r_∞)	Order parameter (S)
Inner + outer	0.183 ± 0.002	0.145 ± 0.002	0.635 ± 0.005
Inner	0.195 ± 0.002	0.161 ± 0.003	0.668 ± 0.007
Outer	0.173 ± 0.004 **	0.132 ± 0.005 **	0.605 ± 0.011 **

Sp2/0-Ag14 cells were not treated or treated with 2 mM TNBS, pH 8.5, at 4°C for 40 min, and the Sp2/0-PMV were isolated. DPH was incorporated, and fluorescence measurements were performed at 37°C (pH 7.4). Values from untreated membranes represent inner + outer monolayer; values from TNBS-treated membranes represent the inner monolayer; values for the outer monolayer were calculated as described in Section 2. Values are represented as the mean ±S.E. of five determinations. Double asterisk signifies $P < 0.01$ according to Student's t-test.

3.3. Effects of ethanol on the range of the transbilayer rotational mobility of Sp2/0-PMV

The structures of the intact Sp2/0-PMV (whole membrane, inner + outer monolayer), the outer monolayer (extracellular monolayer) and the inner monolayer (cytoplasmic monolayer) were examined with DPH as a fluorescent reporter molecule and trinitrophenyl groups as a quenching agent. Trinitrophenylation of the intact Sp2/0-Ag14 cells at 4°C (nonpenetrating conditions) and subsequent isolation of plasma membranes result in covalent attachment of trinitrophenyl quenching agents to the outer monolayer. The covalently linked trinitrophenyl group displays a broad absorbance with a maximum near 420 nm. This absorption peak has a large overlap with the fluores-

Table 2
Effects of ethanol on the limiting anisotropy (r_∞) and the order parameter (S) of DPH in the Sp2/0-PMV

Concn.	Limiting anisotropy (r_∞)			Order parameter (S)		
(mM)	inner + outer	inner	outer	inner + outer	inner	outer
0	0.145 ± 0.002	0.161 ± 0.003	0.132 ± 0.005	0.635 ± 0.005	0.668 ± 0.007	0.605 ± 0.011
10	0.141 ± 0.004	0.160 ± 0.004	0.122 ± 0.004	0.625 ± 0.009	0.666 ± 0.008	0.583 ± 0.009
25	0.135 ± 0.003 *	0.159 ± 0.004	0.116 ± 0.003 **	0.611 ± 0.008 *	0.664 ± 0.009	0.566 ± 0.006 **
50	0.132 ± 0.005 **	0.157 ± 0.002	0.113 ± 0.010 **	0.605 ± 0.011 **	0.659 ± 0.005	0.558 ± 0.019 **
100	0.128 ± 0.003 **	0.156 ± 0.004	0.106 ± 0.006 **	0.597 ± 0.006 **	0.656 ± 0.008	0.540 ± 0.013 **
200	0.124 ± 0.003 **	0.156 ± 0.004	0.102 ± 0.007 **	0.586 ± 0.007 **	0.655 ± 0.009	0.529 ± 0.014 **
400	0.120 ± 0.007 **	0.153 ± 0.006	0.096 ± 0.009 **	0.576 ± 0.013 **	0.649 ± 0.012	0.516 ± 0.018 **
800	0.116 ± 0.004 **	0.152 ± 0.003	0.089 ± 0.003 **	0.568 ± 0.009 **	0.647 ± 0.007	0.498 ± 0.008 **

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

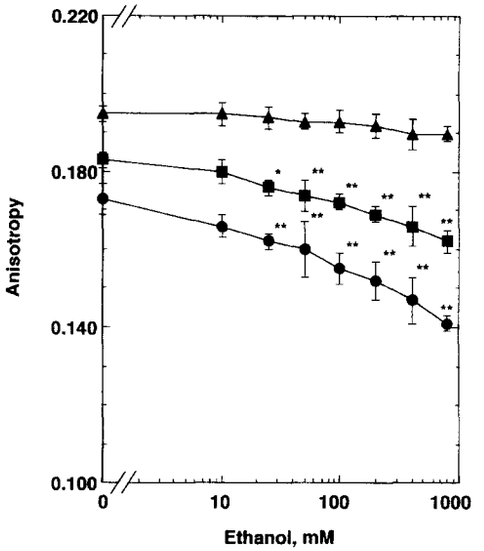

Fig. 2. Ethanol alters the anisotropy (r) of DPH in the outer monolayer of the Sp2/0-PMV. Sp2/0-Ag14 cells were treated ±2 mM TNBS, pH 8.5, at 4°C for 40 min, and the plasma membrane vesicles were isolated. DPH was incorporated, and fluorescence measurements were performed at 37°C (pH 7.4). Untreated (inner and outer monolayers, ■); TNBS-treated (inner monolayer, ▲); calculated for outer monolayer (●) as described in Section 2. Each point represents the mean ±S.E. of five determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control by Student's t-test.

cence 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 Sp2/0-PMV. When the TNBS labeling was conducted under penetrating conditions (37°C), greater than 90% of the fluorescence of the DPH was quenched. The values of fluorescence parameters in intact Sp2/0-PMV (both monolayers) compared to those for TNBS-treated Sp2/0-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 0.022, 0.029 and 0.063, respectively, significantly greater than calculated for the outer monolayer of the Sp2/0-PMV, as demonstrated in Table 1. The significance of the magnitude of difference in anisotropy of DPH between monolayers, namely, 0.022, can be illustrated by comparison to the effect of temperature on this parameter. The anisotropy of DPH in the Sp2/0-PMV is 0.227 ± 0.001 ($n = 5$) and 0.183 ± 0.002 ($n = 5$) at 25 and 37°C, respectively. Thus, the difference in anisotropy between the inner and outer monolayer is as large as that produced by approximately a 6 C° change in temperature on bulk membrane anisotropy. These data indicate that the range of the rotational mobility of DPH in the Sp2/0-PMV is considerably more restricted in the inner than the outer monolayer. As shown in the preceding section, the effect of increasing concentrations of ethanol on the anisotropy of DPH in the Sp2/0-PMV is shown in Fig. 2. The anisotropy of the untreated membrane shows a gradual decrease (fluidization) with increasing ethanol concentration (closed squares). A similar pattern (but a more gradual decrease) is observed for the TNBS-treated membrane (inner monolayer) (Fig. 2, closed triangles). The anisotropy of DPH calculated for outer monolayer is significantly decreased at 25 mM ethanol (Fig. 2, closed circles). However, a statistically significant decrease of anisotropy of DPH in the inner monolayer is not observed even at 800 mM, 32-fold higher concentration than needed for the decrease in the outer monolayer. This is consistent with the result of previous studies by Yun et al. [17,18] and others [20]. It suggests that the fluidizing effect (range of rotational mobility) of ethanol is selective rather than nonselective within transbilayer domains of the native membranes.

3.4. Difference of intrinsic fluidity between native membranes

The fluidity of intact Sp2/0-PMV was remarkably higher than that of normal cell plasma membrane vesicles (intact CHO-K_1-PMV) [17] and SPMV [25]. It appears that the bulk bilayer fluidity of cancer cell membrane is greater than that of normal cell membrane. However, its fluidity came out slightly higher than that of hybridoma cell plasma membrane vesicles, intact ATCC-PMV [18]. In the point of view of the preceding results, that is in the aspect of fluidity, ATCC-PMV (hybridoma formed by fusing spleen cell from SJL/J mice immunized with rat immunoglobulin with the P3X63Ag8 myeloma cell line secretes an IgG_{2a} monoclonal antibody) possesses both the characteristics of the P3X63Ag8 myeloma cell plasma membranes and SJL/J mice spleen cell plasma membranes.

4. Discussion

We [17,18,25] had previously reported that the I'/I values in the CHO-K_1-PMV, ATCC-PMV and SPMV were 0.529 ± 0.016, 0.586 ± 0.007 and 0.412 ± 0.005, respectively. Ethanol increased the range and rate of the lateral mobility of Py-3-Py in the Sp2/0-PMV (in this study), CHO-K_1-PMV [17], ATCC-PMV [18] and SPMV [25]. Avdulov et al. [27,28] have also reported that ethanol (100 mM) increased bulk fluidity of rat brain SPM lipid bilayer. However, the important point is the different potency of ethanol among Sp2/0-PMV, CHO-K_1-PMV, ATCC-PMV, SPMV and rat brain SPM, in terms of minimal ethanol concentration for the significant increase in the I'/I values: 25 mM, 50 mM, 25 mM, 400 mM and 100 mM in Sp2/0-PMV, CHO-K_1-PMV, ATCC-PMV, SPMV and rat brain SPM, respectively. It would be of interest to understand this phenomenon but the differences in the sensitivity to ethanol cannot be fully explained. The

results of several studies [17,18,25,27,28] strongly suggest that the sensitivity of lateral mobility of natural membrane lipid bilayer to ethanol is proportional to intrinsic fluidity of membranes. Thus, it seems likely that there might be a correlation between intrinsic membrane fluidity and sensitivity to ethanol's fluidizing effect.

In the present study, the Sp2/0-PMV outer monolayer is more fluid than the inner monolayer. This is in agreement with the results of previous studies [17,18,20–22,45–47] but inconsistent with two studies [48,49]. Plasma membranes consist of two monolayers that are asymmetric in lipid distribution, electrical charge, fluidity, protein distribution and function, and do not appear to be coupled [50]. It had been widely known that different lipids could affect the physical properties of the membrane. Membrane cholesterol is one of the major lipids of plasma membranes and is asymmetrically distributed in the outer and inner monolayers of membranes [21,45,51,52]. Interest in cholesterol derives from the fact that cholesterol has a regidifying effect on membrane above the phase transition temperature of the membrane lipid [50]. In erythrocytes, differences in fluidity between the two monolayers have not been consistently observed. Some studies have reported that the outer monolayer was less fluid [48,49], whereas other studies have found that the outer monolayer was more fluid compared with the inner monolayer [46,47]. The finding that the SPM inner monolayer was less fluid than the outer monolayer was consistent with data showing that the SPM inner monolayer contains approximately 7-times as much cholesterol compared with the outer monolayer [21]. Thus, a possible explanation for the asymmetric rotational mobility range between outer and inner monolayers of Sp2/0-PMV in this study is that the amount of cholesterol may differ in the outer and inner monolayers.

Previous studies showed that ethanol increased the range and rate of the lateral mobility of the CHO-K_1-PMV [17], ATCC-PMV [18], SPMV [25], SPMVTL [26], SPMVPL [26], SPM [27,28] and sarcoplasmic reticulum membranes [29]. In addition, ethanol increased the range of the rotational mobility of CHO-K_1-PMV [17], ATCC-PMV [18], SPM [20,21], SPMVTL [53] and SPMVPL [54], and the rate of the rotational mobility of native [55] and model [56] membranes. 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 [17,18,20,21] and model [53,54] membranes. Consequently, native and model membranes that have higher fluidity are much more sensitive to fluidizing effect of ethanol. Also, this finding can be extended to the transbilayer asymmetric fluidity in native and model membranes.

Using permeant and impermeant fluorescent probes, Chabanel et al. [48] found that benzylalcohol had a greater fluidizing effect on the inner monolayer in erythrocytes compared to the outer monolayer. It was reported in that study [48] that the inner monolayer was significantly more fluid than the outer monolayer. The individual monolayers of CHO-K_1-PMV [17], ATCC-PMV [18] and SPM [20,21] showed just the opposite asymmetry in fluidity when compared with erythrocyte monolayers and ethanol fluidized the more fluid outer monolayer of CHO-K_1-PMV [17], ATCC-PMV [18] and SPM [20,21]. In the present investigation, ethanol in vitro preferentially fluidized the more fluid outer monolayer of Sp2/0-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 [5,17,18, 20,21,48]. 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. The results of investigations on the effects of higher alkanols and the corresponding alkanes on membrane luciferases and several proteins indicate that the anesthetic site could be hydrophobic pockets on membrane proteins rather than the lipid part of the membrane [57–60]. Using firefly luciferase as a model, Franks and Lieb [58] have shown that it obeys the Meyer–Overton rule and that it displays the 'cut off' effect. From this they argued that the primary anesthetic site could be a hydrophobic pocket on a protein and even independent of membrane lipids. However, to date, no specific protein hydrophobic site critical for anesthesia or ethanol intoxication has been identified. Thus, it is supposed that the following possibility cannot be excluded completely. 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. In conclusion, the present data suggest that ethanol, in addition to its direct interaction with proteins [57–60], 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.

Acknowledgements

This paper was supported in part by a research grant from the Korea Science and Engineering Foundation (KOSEF 88-1013-01), the Non-directed Research Fund (1991) of the Korea Research Foundation and PNU ORAL BIOTECH research grant (1993).

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