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Effects of Ethanol on the Fluidity of Plasma Membrane Vesicles Isolated from Cultured Mouse Myeloma Cell Line Sp2/0-Ag14[1]

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ABSTRACT

Intramolecular excimerization 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 examine the effects of ethanol on the rate and range of lateral diffusion of bulk bilayer structures of plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14 (Sp2/0-PMV). In a concentration-dependent manner, ethanol increased the excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py in the Sp2/0-PMV and decreased the anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) of DPH in the Sp2/0-PMV. This indicates that ethanol increased both the lateral and rotational diffusion of the probes in the Sp2/0-PMV. Selective quenching of DPH by trinitrophenyl groups was utilized to examine the transbilayer asymmetric rotational diffusion 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 utilized to examine the transbilayer asymmetric effects of ethanol on the range of rotational diffusion of the Sp2/0-PMV. Ethanol had a greater fluidizing effect on 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 transbilayer domains of the Sp2/0-PMV.

Key Words: Ethanol, Myeloma plasma membrane vesicles, Transbilayer fluidity asymmetry, Fluorescent probe technique

INTRODUCTION

For many years it has been thought that ethanol and related alkanols exerted their depressant effects on the central nervous system (CNS) by dissolving in membrane lipid bilayers, thereby perturbing the function of ion channels and other proteins embedded therein (Wood and Schroeder, 1988; Yun and Kang, 1992a,b; Yun *et al.*, 1993). The most compelling evidence was the excellent correlation between lipid solubility and anesthetic potency. This hypotheis has been refined by the application of various physical techniques

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that showed that anesthetics caused a local disordering in the lipid matrix, also referred to as membrane fluidization.

Previous studies on ethanol-induced changes in membrane fluidity have measured changes in rotational diffusion of bulk lipid bilayers. However, membrane lipid motions include the following: lateral diffusion of lipid molecules in the plane of the hemileaflet; rotational diffusion of a lipid molecule around an axis perpendicular to the plane of the bilayer; in contrast to the relatively rapid motions of the preceding modes, transverse diffusion or "flip-flop" from one hemileaflet to the other is energetically less favored and relatively slow, particularly in model bilayers (Kim *et al.*, 1992). There is increasing evidence that the membrane consists of domains or patches of lipids that differ in their fluidity and lipid composition (Chin and Goldstein, 1981; Chabanel *et al.*, 1985; Schroeder *et al.*, 1988; Yun and Kang, 1990). Recent data indicate that ethanol exerts a specific effect in contrast to a nonspecific effect on the bulk membrane lipid bilayers (Chin and Goldstein, 1981; Chabanel *et al.*, 1985; Wood *et al.*, 1989; Yun and Kang, 1992a,b; Yun *et al.*, 1993).

Nowadays there is an increasing tendency of cancer patients' drinking. Although it seems that ethanol reduces the severe pain of cancer patients, it is not yet quite clear that ethanol increases or decreases the growth rate of cancer cells. And there is few reports whether ethanol inceases or decreases the fluidity of cancer cell lipid bilayers.

In this paper, exploiting 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), we investigated the effects of ethanol on the bulk bilayer fluidity of the plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14 (Sp2/0-PMV). Also, selective quenching of DPH fluorescence by trinitrophenyl groups was utilized to examine the effects of ethanol on the individual monolayer structure of the Sp2/0-PMV.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium, fetal calf 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 Co. (St. Louis, MO, USA). The fluorescent probe DPH was obtained from Molecular Probes (Junction City, OR, USA) and the other probe Py-3-Py was prepared by the previously reported synthesis (Yun *et al.*, 1990a). Ethanol and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Fluka (Buchs, Switzerland). All other reagents were of the highest quality available and water was deionized.

Media and cell culture

Mouse myeloma cell line Sp1/0-Ag14 was kindly provided by Dr. Chang-Mo Kang (Pusan National University, Korea). Sp2/0-Ag14 was derived from P3-X63Ag8×BALB/c. These cell lines were permanently growing in tissue culture medium as described previously (Horibata and Harris, 1970).

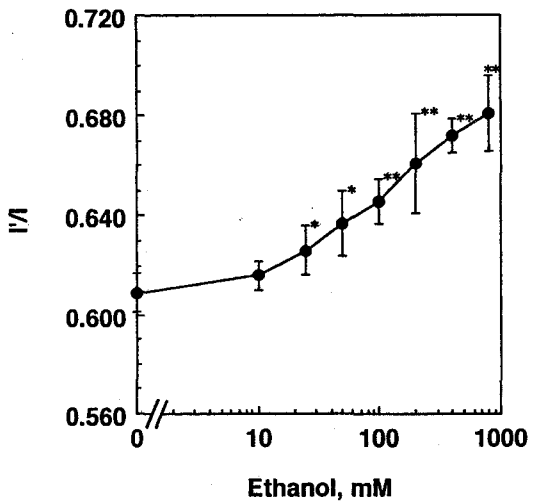

Fig. 1. Effect of ethanol on the excimer to monomer fluorescence intensity ratio, I′/I, of 1,3-di(1-pyrenyl)propane in the plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14. Fluorescence measurements were performed at 37℃ (pH 7.4). Each point represents the mean ± SEM of 5 determinations. An asterisk and double asterisk signify $P < 0.05$ and $P < 0.01$, respectively, compared to control according to Student's t-test.

Table 1. Asymmetry of 1,6-diphenyl-1,3,5-hexatriene motion in the plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14

Membrane	Polarization (P)	Anisotropy (r)	Limiting anisotropy (r_∞)	Order parameter (S)
inner+outer	0.251±0.003	0.183±0.002	0.145±0.002	0.635±0.005
inner	0.266±0.002	0.195±0.002	0.161±0.003	0.668±0.007
outer	0.240±0.004**	0.173±0.004**	0.132±0.005**	0.605±0.011**

Cells were treated ±2 mM trinitrobenzenesulfonic acid, pH 8.5, at 4℃ for 30 min, and the plasma membrane vesicles were isolated. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37℃ (pH 7.4). Values from untreated membranes represent inner + outer monolayer; Values from 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated membranes represent the inner monolayer; Valuese for the outer monolayer were calculated as described in Materials and Methods. Values are represented as the mean ± SEM of 4 determinations. Double asterisk signifies $P<0.01$ compared to control by Student's t-test.

Cells were grown in Dulbecco's modified Eagle 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℃ in a moist atmosphere containing 5% CO_2 with subculturing every 4 days. All cultures were grown in disposable plastic Petri dishes (Falcon Plastics, LA, Calif., 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, Flo.) with 0.1% trypan blue exclusion.

Preparation of plasma membrane vesicles

The Sp2/0-PMV were isolated from cultured mouse myeloma cell line Sp2/0-Ag14 and characterized by the formerly reported method in our laboratory (Yun and Kang, 1990; Yun *et al.*, 1990b). The purity of Sp2/0-PMV was determined by enzymatic and morphological standards. The specific activities of Na,K-ATPase (Yun and Kang, 1990; Yun *et al.*, 1990b) and 5′-nucleotidase (Yun and Kang, 1990; Yun *et al.*, 1990b) were about 5- and 3-fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenates. Electron microscopic examination also showed that the membranes were in vesicular form (Yun *et al.*, 1990b). Protein was determined by the method of Lowry *et al.* (1959) using BSA as a standard.

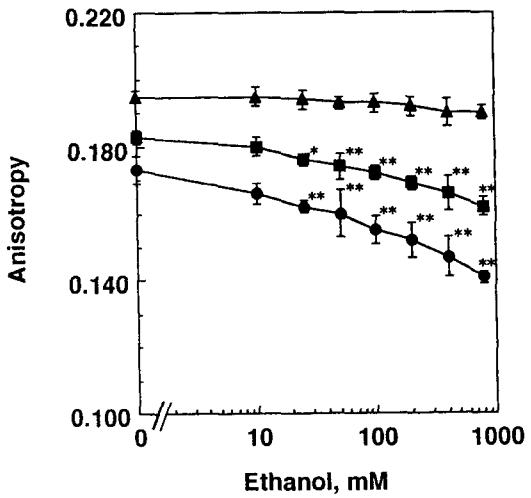

Fig. 2. Ethanol alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14. Cells were treated ± 2 mM trinitrobenzenesulfonic acid, pH 8.5, at 4℃ for 30 min and the plasma membrane vesicles were isolated. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37℃ (pH 7.4). Untreated (inner and outer monolayers, ■); 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated (inner monolayer, ▲); calculated for outer monolayer (●) as described in Materials and Methods. Each point represents the mean ± SEM of 4 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

Table 2. Effects of ethanol on the limiting anisotropy (r_∞) and order parameter (S) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14

Conc. (mM)	Limiting anisotropy (r_∞)			Order parameter (S)		
	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**	0.576±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±SEM of 4 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

TNBS labelling reactions

TNBS labelling reactions were performed by the procedure of Yun and Kang (1990) with a few modifications. The cultured cells were gently resuspended 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, the cells were passed slowly through an Eberbach tissue grinder (3 up and down strokes). Unless otherwise specified, the treatment was carried out at 4℃ for 30 min. The TNBS labelling reaction was terminated by addition of 2% BSA in PBS (8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l $Na_2HPO_4 \cdot 7H_2O$, 0.48 g /l Hepes, pH 7.4). The entire suspension was then sedimented at 1,100×g for 5 min and preparation of plasma membrane vesicles was performed as described above.

Fluorescence measurements

The fluorescence measurements were performed as described previously (Yun *et al.*, 1990a; Yun and Kang, 1992a,b; Yun *et al.*, 1993; Chung *et al.*, 1993a)

RESULTS AND DISCUSSION

Studies were undertaken to evaluate the effects of ethanol on the lateral mobility or diffusibility of the Sp2/0-PMV. To accomplish this, the fluorescent probe Py-3-Py was used. Py-3-Py, a pyrene derivative, which has been successfully used to quantitate lateral mobility within native and model membranes (Schachter, 1984; Zachariasse, 1980; Zachariasse *et al.*, 1982; Yun *et al.*, 1990a), was used to determine the rate and range of lateral mobility in the Sp2/0-PMV. Using this probe one monitors emission of both the monomer (I) and excimer (I′) components in such a way that a ratio can be derived and used as a measure of lateral mobility (Schachter, 1984; Zachariasse, 1980; Zachariasse *et al.*, 1982; Yun *et al.*, 1990a). 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 Py-3-Py excimer fluorescence technique has an advantage over its counterpart based on intermolecular excimerization since very small probe concentrations can be employed ($<10^{-7}$ M), and the perturbation of the Sp2/0-PMV by the probe molecule is mini-

mized.

We reported that the I′/I value in intact plasma membrane vesicles isolated from Chinese hamster ovary K_1-cells (CHO-K_1-PMV) was 0.529±0.016 (37℃, pH 7.4). Ethanol increased the lateral diffusion of the bulk CHO-K_1-PMV in a concentration-dependent manner, and a significant increase in I′/I value was observed at 50 mM ethanol (Yun *et al.*, 1993). Our study extended from the above experiments demonstrated a very interesting phenomenon in the Sp2/0-PMV. Ethanol increased the range and rate of lateral mobility of the bulk Sp2/0-PMV dose-dependently, and a significant increase in I′/I value was observed even at 25 mM ethanol (the control value: 0.608±0.008). This result is in agreement with that of our previous study, indicating ethanol has a large influence on the lateral mobility of native membranes. However, the important point is the different potency of ethanol between Sp2/0-PMV and CHO-K_1-PMV, in terms of minimal ethanol concentration for the significant increase in the I′/I values.

To evaluate the effects of ethanol on the rotational mobility of the Sp2/0-PMV, the estimation of steady-state fluorescence polarization, using DPH as a lipid probe, was carried out. DPH is a rod-shaped molecule that orients with high affinity in hydrophobic regions (core) of the bilayer structures. The fluorescence polarization mainly reflects the rotational mobility of lipid fluorophores (Schachter, 1984; Molitoris and Hoilien, 1987; Yun and Kang, 1992a,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 DPH are high and largely determine r. In biological membranes, 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 r_∞ can be used to define an order parameter (S). Yun *et al.* (1993) reported that the limiting anisotropy (r_∞) and order parameter (S) of DPH in the CHO-K_1-PMV were 0.160±0.004 and 0.666±0.007, respectively.

Ethanol increased the rotational diffusion of the membrane in a concentration-dependent manner with a significant decrease in the anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) observed even at 25 mM ethanol. In the present study, the anisotropy (r), limiting anisotropy (r_∞), and 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. Ethanol decreased the anisotropy (r), limiting anisotropy (r_∞), and order parameter (S), and a significant decrease in the r, r_∞ and S was observed even at 25 mM ethanol (Fig. 2, closed squares). Therefore, even at 25 mM, ethanol significantly decreased the range of rotational mobility of the bulk Sp2/0-PMV. This is in agreement with the study previously reported by Yun *et al.* (1993), and it means that the degree of ethanol-induced decrease in these three parameters, i.e., r, r_∞ and S, in the Sp2/0-PMV is more clear compared to that of CHO-K_1-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 is quenched in the trinitrophenylated Sp2/0-PMV. If the TNBS labelling is conducted under penetrating conditions (37℃), nearly 100% of the fluorescence of the DPH is quenched. The values of fluorescence parameters in intact Sp2/0-PMV (both monolayers) as 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 the 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. Ethanol showed a greater fluidizing (range of rotational mobility) effect on the outer monolayer (Fig. 2, closed circles) as compared to the inner monolayer (Fig. 2, closed triangles). It suggests that the fluidizing effect (range of rotational mobility) of ethanol is selective rather than nonselective within transbilayer domains of the Sp2/0-PMV.

Ethanol and related alkanols have been shown

to decrease the temperature of the gel-to-liquid crystalline phase transition of model membranes (Jain and Wu, 1977), expand membranes (Seeman, 1972), and alter the surface charge of membrane lipids (Bangham and Mason, 1979). However, these effects have been obtained with ethanol concentrations in the range of 500-1500 mM, which are usually higher than the lethal serum concentrations in humans and laboratory animals (Harris and Schroeder, 1981). Moreover, the accumulated results for the analysis of ethanol effect on the cell membrane fluidity has been focused on the normal cell membranes, not on the cancer cell membranes. Our data presented herein shows that, even at physiologically relevant concentrations (Harris and Schroeder, 1981; Majchrowicz and Mendelson, 1971), 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 rotational diffusion in the outer monolayer of the Sp2/0-PMV.

Our previous studies reported that ethanol increased the range and rate of lateral mobility of the CHO-K_1-PMV (Yun *et al.*, 1993) and synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex (Chung *et al.*, 1993a). In addition, ethanol increased the range of rotational mobility of the CHO-K_1-PMV (Yun *et al.*, 1993) and the rate of rotational mobility of the SPMV (Chung *et al.*, 1993b). Therefore, in a concentration-dependent manner, ethanol significantly increases the range/rate of the lateral/rotational mobility in both native and model membranes. Additionally, the increase in the bulk rotational mobility (primarily the range of the motion) is mainly derived from increases in the motion range in the outer monolayer of native and model membranes. Consequently, the native 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. The more fluid monolayer in the bilayer organization, whether outer or inner monolayer, is the major target site of the fluidizing effect of ethanol.

The physical state of membrane lipids has been shown to influence such membrane enzymes as Na,K-ATPase (Chong *et al.*, 1985), hormone-responsive adenylate cyclase (Dipple and Houslay, 1978), and membrane transport processes such as glucose and amino acid uptake (Balcar *et al.*, 1980; Carriere and Le Grimellec, 1986). Membrane lipids also play an important role in membrane permeability to sodium, calcium, chloride, and potassim (Green *et al.*, 1980). Changes in membrane fluidity are known to be linked to alterations in physiological processes of the cell membrane like carrier-mediated transport, activity of membrane bound enzymes, receptor binding, phagocytosis, endocytosis, depolarization dependent exocytosis, cytotoxicity, and cell growth (Spector and Yorek, 1985). Possibly, the effects of ethanol on the membrane may increase the growth rate of myeloma cells, since the increased membrane fluidity (by ethanol) is directly related to an increase in the membrane permeability and to the cell growth. If someone observed the suppressive effect of ethanol on the growth rate of cancer cells *in vivo*, it might be resulted from indirect effects of ethanol through central nervous system rather than direct effect of ethanol.

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=국문초록=

Ethanol이 배양된 Mouse Myeloma Cell Line Sp2/0-Ag14로부터 분리한 형질막의 유동성에 미치는 영향

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Ethanol이 암세포 증식 속도에 미치는 직접적인 영향 검색의 일환으로 배양된 mouse myeloma cell line Sp2/0-Ag14로부터 분리한 형질막 (Sp2/0-PMV) 유동성에 미치는 ethanol의 영향을 형광분석법으로 측정하였다. 그 결과 ethanol은 Sp2/0-PMV 지질 이중층 측방확산운동의 범위와 속도를 증가시켰고 회전확산운동 범위도 증가시켰다. 특히 ethanol은 Sp2/0-PMV 지질 이중층 중 내부단층 (inner monolayer)에 비하여 비교적 선택적으로 외부단층 (outer monolayer)의 회전확산운동 범위를 증가시킨다는 것을 확인하였다.