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Transbilayer effects of ethanol on fluidity of brain membrane leaflets

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Previous work on membrane effects of ethanol focused on fluidization of the bulk membrane lipid bilayer. That work was extended in the present study to an examination of ethanol's effect on lipid domains. Two independent methods were developed to examine the effects of ethanol on the inner and outer leaflets of synaptic plasma membranes (SPM). First, differential polarized phase and modulation fluorometry and selective quenching of diphenyl-1,3,5-hexatriene (DPH) were used to examine individual leaflets. Both limiting anisotropy and rotational relaxation time of DPH in SPM indicated that the outer leaflet was more fluid than the inner leaflet. Second, plasma membrane sidedness selective fluorescent DPH derivatives, cationic 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) and anionic 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (PRO-DPH), confirmed this transmembrane fluidity difference. TMA-DPH and PRO-DPH preferentially localized in the inner and outer leaflets of SPM, respectively. Ethanol in vitro had a greater fluidizing effect in the outer leaflet as compared to the inner leaflet. Thus, ethanol exhibits a specific rather than nonspecific fluidizing action within transbilayer SPM domains. This preferential fluidization of the SPM outer leaflet may have a role in ethanol affecting transmembrane signaling in the nervous system.

Introduction

Ethanol is a member of the large family of anesthetic drugs whose biological potency correlates with lipid solubility. Ethanol, at physiologically relevant concentrations, fluidizes biological membranes as indicated by electron spin resonance (ESR) order parameter [1] and fluorescence polarization [2]. Previous studies that have examined the effects of ethanol on membranes have studied changes in the bulk membrane. There is, however, increasing evidence for domain structure

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Abbreviations: SPM, synaptic plasma membrane; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene; PRO-DPH, 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid; TNBS, trinitrobenzenesulfonic acid; PBS, phosphate-buffered saline; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; BSA, bovine serum albumin.

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in membranes that may be significant to membrane function [3]. Two such domains are the outer and inner leaflets of the membrane bilayer that are asymmetric in fluidity and lipid composition [4–12].

Not only is asymmetry present with respect to fluidity and lipid distribution, but certain drugs that differ in their charge properties have been found to differentially affect one leaflet or the other [10–17]. This selective effect was dependent on the charge properties of the membrane lipids. Cationic drugs had a greater effect on the negatively charged cytofacial leaflet, whereas anionic drugs acted on the exofacial leaflet.

Ethanol is a neutral compound that would not be attracted to one specific leaflet on the basis of charge. However, ethanol should have an asymmetric effect if one leaflet differed in fluidity as compared to the other leaflet. Such a proposed effect is based on ethanol having a greater effect on more fluid as compared to less fluid membranes [1,18], and led to the following hypothesis. We predicted that the fluidity of the two leaflets of SPM would differ and that the more fluid leaflet would be more susceptible to the fluidizing actions of ethanol [50]. This hypothesis was tested in mouse brain SPM using selective quenching of DPH fluorescence that allows examination of fluidity of the outer and inner leaflets [15,17]. The results were confirmed with sidedness selective probes that localize in one leaflet or the other in the absence of quenching agents. The present report represents, to our knowledge, the first in-depth examination of transbilayer structural domains in SPM and the first demonstration of transbilayer domain selectiveness of ethanol action. A preliminary account of this work was presented as an abstract [50].

Methods

Chemicals

The fluorescent probes DPH, TMA-DPH and PRO-DPH were obtained from Molecular Probes, Junction City. Hepes, Ficoll (70000 M_w), TNBS, picryl sulfonic acid, BSA and Tris-HCl were obtained from Sigma, St. Louis.

Membrane isolation and TNBS labeling

Male C57BL/6NNIA (6 mo old) mice were obtained from the National Institute on Aging Colony maintained by Charles River Laboratories, Wilmington, MA. Animals were decapitated, brains dissected, and synaptosomes isolated based on methods described earlier [6]. The brain was homogenized in 10 vol. of 0.32 M sucrose/2 mM Hepes (pH 7.5) (buffer A). All subsequent procedures were performed at 4°C or on ice. The homogenate was sedimented at $1200 \times g$ for 5 min with a SS34 rotor on a Sorvall RC2B centrifuge (E.I. duPont de Nemours & Co., Newtown, CT). The pellet was discarded and the supernatant was further sedimented at $17000 \times g$ for 12 min. The pellet was retained, resuspended in buffer A and again sedimented at $17000 \times g$ for 12 min. The pellet was then gently resuspended in buffer A and layered over 7.5 and 12.0% (w/v) Ficoll in buffer A, such that the volume ratio of the three layers was 1:1:1. Each gradient tube (Beckman Ultra Clear, 1 × 3.5 inch) was layered with resuspended crude synaptosomal pellets generated from 3–5 mice. Gradients were sedimented in a SW28 rotor at $75000 \times g$ for 60 min using an L8-80 ultracentrifuge (Beckman Instruments). The 7.5–12.0% interface was removed, suspended and the buffer A synaptosomes were sedimented at $18120 \times g$ for 20 min with the Sorvall SS34 rotor. The supernatant was discarded and the pellet was gently resuspended in TNBS + buffer B or buffer B alone. Buffer B was composed of 50 ml double-distilled water/0.5 g BSA/0.0877 g NaCl/0.5041 g $NaHCO_3$/0.0991 g glucose (pH 8.3). After TNBS was added, CO_2 was bubbled through the solution and the tube was capped and placed on ice for 45 min. The trinitrophenylation reaction was terminated by addition of 1% BSA in PBS (pH 7.3). PBS was composed of double-distilled water/8 g NaCl/0.2 g KCl/0.92 g Na_2HPO_4 (anhydrous)/0.20 g KH_2PO_4 (pH 7.3). The entire suspension was then underlayered with 7.5% Ficoll in buffer A and 12.0% Ficoll in buffer A. The gradient was centrifuged at $75000 \times g$ for 60 min. The 7.5–12.0% interface was then removed and diluted with 1% BSA/PBS and sedimented at $40000 \times g$ (SS34 rotor) for 20 min. The pellet was then vigorously resuspended in 5 mM Tris-HCl (pH 8.5), homogenized with three up and down strokes as above and allowed to lyse for 30 min at 4°C. After lysis was completed, the membranes

were sedimented at 40000 × g for 20 min. The pellet was resuspended in cold double-distilled water, underlayered with 0.95 M sucrose/50 mM Tris (pH 7.4) and sedimented at 40000 × g for 20 min. The 0.95 M sucrose/water interface was removed and sedimented at 40000 × g for 20 min; and the final SPM pellet was resuspended in 1 ml 50 mM Tris (pH 7.3). Purification of Na^+/K^+-ATPase, a marker enzyme of SPM was 4- to 5-fold greater in SPM as compared to whole brain homogenate in each condition [6].

Fluorescence spectroscopy and differential polarized phase fluorescence

Absorbance, absorption-corrected fluorescence, relative fluorescence efficiency (0.1 μg per 100 μg membrane protein) and corrected fluorescence emission of DPH in SPM were determined concurrently with a computer-centered spectrofluorometer as described [19–22]. Excitation and emission wavelengths were 355 and 430 nm. Fluorescence lifetimes were measured by phase and modulation with a SLM 4800 (SLM Instrument, Champaign, IL) instrument updated by ISS, Urbana, IL, to become a multifrequency phase and modulation fluorometer (1–250 MHz), described elsewhere [23]. A He/Cd laser (Model 4240NB, Liconix, Sunnyvale, CA), whose emission intensity at 325 nm was modulated sinusoidally with a Pockels cell, was the light source. Ethanol, at the concentrations indicated, was added directly to the sample cuvette which was continuously stirred for 5 min. Longer incubation times with ethanol did not result in further alterations in fluorescence parameters. All fluorescence measurements were made at 24°C.

Determination of SPM individual leaflet structure: selective quenching of DPH

This experimental determination of individual leaflet structure in SPM is based on a method previously established for cultured cell plasma membranes [10–12,17,26,27]. We extended this method to determination of SPM individual leaflet fluidity. The appropriateness of this extension derives from Weber's law of anisotropy additivity [28,29], which requires intensity weighing for the addition of fractional anisotropies and from results reporting a lipid compositional transbilayer asymmetry in mouse and rat brain SPM [6,8]. Since lipids are the primary determinant of membrane structure, their asymmetric transbilayer distribution is expected to confer asymmetry of structure between the leaflets. A necessary and experimentally verified corrollary of this prediction is that the leaflets are not coupled (i.e., fluidizing or rigidifying one leaflet does not result in a structural change in the other membrane leaflet) [4,10–12,30–33].

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 [28] to TNBS. If the fluorescence intensity, F, and anisotropy, r, are measured simultaneously, then

$$r = \Sigma F_i r_i \tag{1}$$

where F_i is the fraction of fluorescence intensity in compartment i. For a binary system composed of the outer and inner leaflet of the SPM this leads to

$$r = \frac{F_c}{F} r_c + \frac{F - F_c}{F} r_e \tag{2}$$

where F and F_c are fluorescence of DPH obtained for SPM isolated from synaptosomes incubated with buffer B and buffer B + TNBS at 4°C (nonpenetrating conditions), respectively. The values of the fluorophore concentration independent parameter anisotropy, r (anisotropy for both leaflets) and r_c (inner leaflet anisotropy), were determined for DPH in SPM obtained from synaptosomes incubated with buffer B and buffer B + TNBS at 4°C (nonpenetrating conditions), respectively. The equation is then solved for r_e (outer leaflet anisotropy). Similar calculations were performed by simultaneous measurement of fluorescence intensity and either limiting anisotropy, rotational relaxation time or lifetime. It should be noted that each fraction F_i in Eqn. 1 is individually determined for each preparation and ranges between 45 to 55%. Lastly, the method depends on essentially complete quenching of DPM fluorescence in the outer leaflet without significant quenching of DPH in the inner leaflet. The validity of this approach in SPM was tested/confirmed and is reported in Results.

Determination of SPM individual leaflet structure: leaflet selective derivatives of DPH

This method is based on the prediction that cationic TMA-DPH and anionic PRO-DPH probes would preferentially localize in the SPM inner and outer leaflets, respectively. Because the negatively charged phospholipids of SPM are localized in the inner leaflet [6,8], electrostatic attraction should preferentially localize the positively charged TMA-DPH in the inner leaflet, while electrostatic repulsion should preferentially localize the negatively charged PRO-DPH in the SPM outer leaflet. Fluorescence parameters measured for the two probe molecules should then report on static and dynamic properties of the lipids in the SPM inner and outer leaflets. The validity of this approach in SPM was also tested/confirmed and is presented in Results.

ESR spectroscopy

SPM that had been treated with TNBS + buffer B or buffer B alone were incubated with 5-doxylstearic acid spin label for 1 h at 37°C. All other procedures for determination of ESR order parameter were exactly as described earlier [12,34,35].

Results

Trinitrophenylation and transbilayer location of trinitrophenyl groups in SPM

The first method for examining the SPM individual leaflet structure utilized selective quenching of DPH in the outer leaflet. This was accomplished by treating SPM with TNBS under nonpenetrating conditions. The TNBS-labeling ratio must be carefully monitored to assure that the reagent did not penetrate into the inner leaflet. For this purpose, five control procedures were used to determine effects of TNBS on the membrane.

First, fluorescence quenching was used to estimate the transbilayer distribution of DPH. Over the concentration range 2–8 mM TNBS, approximately one-half of the DPH fluorescence/mg membrane protein was quenched in the SPM trinitrophenylated at 4°C. This quenching was maximal, since addition of 0–54 μM trinitrophenylglycine, a water-soluble, nonpenetrating quenching agent [21,26,36], quenched outer leaflet fluorescence to the same degree as did covalent attachment of trinitrophenyl groups to the outer leaflet by TNBS. Incubation with TNBS for longer time periods, up to 80 min, at 4°C did not further decrease the DPH fluorescence (Fig. 1).

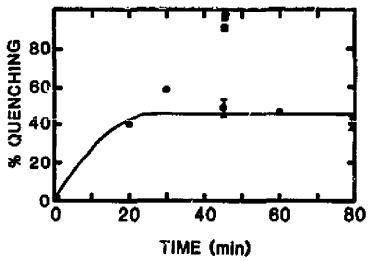

Fig. 1. Optimal time for TNBS reaction with synaptosomes. Synaptosomes were treated with 2 mM TNBS in buffer B for 0–80 min at 4°C (●———●), 37°C (▲) or 4°C lysed (■) as described in Methods. Values with error bars indicate the mean ± S.E. ($n = 5$).

If the TNBS reaction was performed at 37°C or at 4°C with lysed SPM (penetrating conditions) greater than 90% of the fluorescence of DPH was quenched with 2 mM TNBS (Fig. 1). Most important, with 2 mM TNBS all dynamic and static fluorescence parameters of DPH (limiting anisotropy, order parameter, rotational relaxation time and lifetime) achieved plateau values after 20 min incubation time at 4°C (Fig. 2). These results indicate that treatment with 2 mM TNBS for 45 min resulted in maximal quenching of the outer leaflet DPH fluorescence. This result is consistent with but not definitive proof that all of the outer leaflet and little of the inner leaflet DPH fluorescence is quenched by externally located trinitrophenyl groups. Further proof is provided by the following four control procedures.

The Forster distance for energy transfer of the DPH/trinitrophenyl donor/acceptor pair in the SPM was used as a control to examine whether quenching of DPH in the outer leaflet affected DPH in the inner leaflet. The distance between a donor and an acceptor for 50% of the transfer efficiency, R_o, was calculated using Forster's equations [37–39] to be 16.6 Å for the DPH/trinitrophenyl donor/acceptor pair. For probe molecules located at the surface of a bilayer membrane,

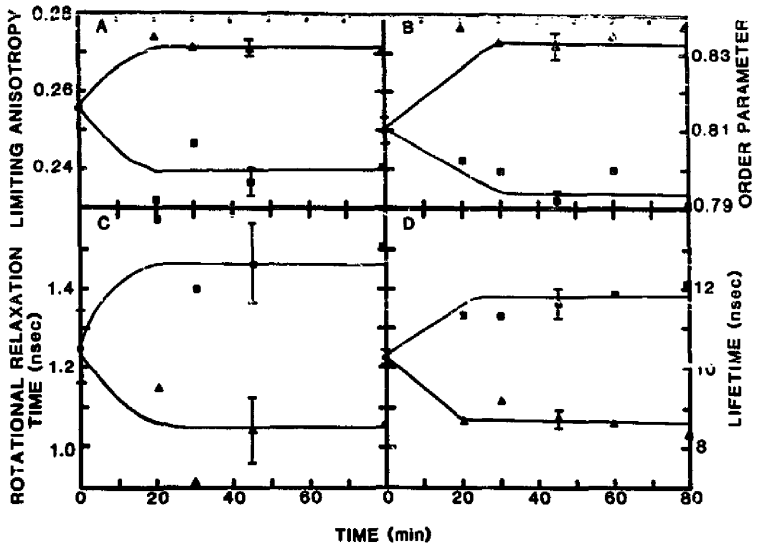

Fig. 2. Trinitrophenylation alters static and dynamic properties of DPH in SPM. The effect of incubation time at 4°C with 2 mM TNBS in buffer B on DPH dynamic and static properties in SPM inner (▲——▲) and outer (■——■) leaflets was determined as described in Methods. Values with error bars indicate the mean ± S.E. ($n = 5$).

there is little energy transfer from one leaflet to acceptors in the opposite one if the R_0 value is less than 40 Å [40]. Since biological membrane thickness is usually between 50 and 80 Å, the likelihood of transbilayer energy transfer in SPM may be calculated [12]. If the center of the bilayer is 25 Å from the surface, for example, the transfer efficiency from DPH in the bilayer center to a surface-located trinitrophenyl group is expected to be only 3.6%. Thus, it would be even less likely that the trinitrophenyl groups quench DPH molecules in the opposite SPM leaflet.

Intrasynaptosomal mitochondria were isolated from the synaptosomes labeled with TNBS as a third control to determine penetration of TNBS into the cell. If significant penetration of TNBS into the synaptosome occurred during incubation, intracellular mitochondrial phosphatidylethanolamine would become extensively labeled. Under nonpenetrating conditions (4°C), only trace

TABLE I

LIFETIME ANALYSIS OF DPH FLUORESCENCE IN SPM

Synaptosomes were isolated and treated in buffer B with or without 2 mM TNBS under nonpenetrating conditions (4°C for 45 min) as described in Methods. SPM were then isolated, DPH was incorporated (1:100 mole ratio to lipid), and lifetime analysis was performed also as described therein. Values represent the mean ± S.E. (in = 4–8). Buffer-treated SPM refers to values for the inner and outer leaflets. Buffer + TNBS refers to values for the inner leaflet.

SPM Leaflet	EtOH (mM)	Single lifetime analysis		Two lifetime analysis		
		τ (ns)	χ^2	τ_1 (ns)	τ_2 (ns)	χ^2
Buffer	0	10.21 ± 0.14	4 ± 2	10.21 ± 0.29	2.75 ± 0.93	25 ± 6
	200	10.02 ± 0.17	10 ± 2	10.37 ± 0.17	3.10 ± 0.78	65 ± 5
Buffer	0	8.76 ± 0.19	306 ± 70	10.81 ± 0.36	3.88 ± 0.38	6 ± 2
+ TNBS	200	8.71 ± 0.19	122 ± 14	10.52 ± 0.24	3.71 ± 0.27	4 ± 2

amounts of mitochondrial phosphatidylethanolamine were labeled.

As a fourth control, transbilayer quenching as well as penetration of TNBS to the inner leaflet was further examined by resolution of lifetime components. Forster nonradiative energy transfer substantially reduces the fluorescence lifetime [41,42]. When synaptosomes were treated with buffer alone (absence of TNBS), the fluorescence lifetime of DPH in SPM was best expressed as a single component at 10.2 ± 0.01 ns, $\chi^2 = 4$ (Table I). Attempts at fitting the data to two components did not substantially decrease values of χ^2. Such results demonstrate that inclusion of an additional component is not justified from the data. When SPM were treated under penetrating (37°C) conditions with TNBS, a single lifetime component is again obtained but with a much shorter lifetime at 3.9 ns. If as little as 10% of the DPH in the inner leaflet were quenched by TNBS that had leaked into the synaptosome, the fluorescence lifetime in the inner leaflet would be significantly reduced. Under nonpenetrating (4°C) labeling conditions with buffer + TNBS, two lifetime components, 10.8 ± 0.4 and 3.9 ± 0.4 ns ($\chi^2 = 6$), representing the lifetimes of unquenched DPH in the inner leaflet and quenched DPH in the outer leaflet, respectively, were obtained (Table I). Thus, the lifetime of DPH in the inner leaflet was not reduced. The mole fractions of DPH associated with each lifetime are 43 and 57% for the inner and outer leaflet, respectively. This distribution is in good agreement with the fluorescence intensity quenching data presented above.

Finally, the relatively mild TNBS-labeling procedure used did not significantly alter the function or structure of SPM. The activity of Na^+/K^+-ATPase was not significantly altered by the TNBS reaction under nonpenetrating conditions, 4°C. TNBS treatment at 4°C did not affect the structure of the SPM as determined by 5-doxylstearic acid. The 5-doxylstearic acid was incorporated into SPM from synaptosomes treated with buffer B alone and into SPM from synaptosomes treated at 4°C with buffer B + TNBS. The order parameters determined by ESR were 0.625 ± 0.001 and 0.627 ± 0.001 in the untreated and TNBS-labeled SPM, respectively.

Dynamic and static properties of DPH in SPM

The static (limiting anisotropy) and dynamic (rotational relaxation time) parameters of DPH in the bulk membrane and in the inner and outer leaflets were resolved by differential polarized phase fluorometry. Limiting anisotropy and order parameter of DPH in the bulk membrane indicate that at 24°C there is considerable restriction to motion of DPH in SPM (Table II). Rotational relaxation time indicated rapid motion of DPH near 1.1 ns.

Table II shows that the limiting anisotropy and order parameter of DPH in the inner leaflet were significantly higher than calculated for the outer leaflet ($P < 0.01$). The rotational relaxation time

TABLE II

STRUCTURAL PROPERTIES OF DPH IN SPM

Synaptosomes were treated with buffer B ± TNBS under nonpenetrating conditions (4°C, 2 mM, 45 min) as described in Methods. The reaction was terminated, SPMs were isolated, DPH was incorporated (1:100 probe/lipid ratio), and fluorescence parameters were determined at 24°C also as described in Metnods. Values from synaptosomes treated with buffer A alone represent inner + outer leaflets; values from synaptosomes treated with buffer A + 2 mM TNBS for 45 min at 4°C represent the inner leaflet; values for the outer leaflet were calculated as described in Methods. Individual values represent the mean ± S.E. ($n = 8$–9). * and ** represent $P < 0.05$ and $P < 0.01$, respectively, compared to inner leaflet by Student's t-test.

SPM leaflet	Polarization	Limiting anisotropy	Rotational relaxation time (ns)	Order parameter
Inner + outer	0.3561 ± 0.0018	0.2556 ± 0.0028	1.118 ± 0.063	0.8095 ± 0.0031
Inner	0.3741 ± 0.0031	0.2715 ± 0.0015	1.240 ± 0.005	0.8323 ± 0.0040
Outer	0.3408 ± 0.0028 **	0.2366 ± 0.0013 **	1.023 ± 0.003 *	0.7924 ± 0.0065 **

TABLE III

DYNAMIC PROPERTIES OF CHARGED DPH DERIVATIVES, DPH-PROPIONIC ACID AND TRIMETHYLAMMONIO-DPH IN SPM

Synaptic plasma membranes were isolated from synaptosomes as described in Methods. Instead of DPH, PRO-DPH or TMA-DPH was incorporated and all fluorescence parameters were determined as described in Methods. Values represent the mean ± S.E. ($n = 3$–4). * and ** signify $P < 0.05$ and $p < 0.025$, respectively, according to Student's t-test.

Parameter	PRO-DPH	TMA-DPH
Polarization	0.357 ± 0.002	0.365 ± 0.002 *
Limiting anisotropy	0.254 ± 0.002	0.263 ± 0.001 **
Rotational relaxation time (ns)	1.510 ± 0.084	1.783 ± 0.039 *
Lifetime τ_1 (ns)	7.09 ± 0.57	7.70 ± 0.33
Lifetime τ_2 (ns)	1.91 ± 0.26	2.38 ± 0.13
Fraction F_1	0.72 ± 0.09	0.80 ± 0.06

of DPH was also significantly longer in the inner leaflet than calculated for the outer leaflet ($P < 0.05$).

Dynamic and static properties of TMA-DPH and PRO-DPH in SPM

Under nonpenetrating conditions, TNBS quenches about 50, 21 and 68% of DPH, TMA-DPH and PRO-DPH fluorescence intensity, respectively in SPM. When SPMs were labeled with TNBS under penetrating conditions (37°C), greater than 95% of the fluorescence of all three probe molecules was quenched. These results are consistent with nearly equal transbilayer distribution of DPH (also obtained from lifetime heterogeneity analysis) and preferential distribution of TMA-DPH and PRO-DPH in SPM inner and outer leaflets, respectively. Polarization and limiting anisotropy of TMA-DPH in SPM are significantly higher ($P < 0.05$) than those of PRO-DPH (Table III). The rotational relaxation time of TMA-DPH was also longer than that of PRO-DPH.

Effect of ethanol on SPM individual leaflet structure

In order to determine the effect of ethanol on individual leaflet structure, it is first necessary to demonstrate that ethanol itself does not directly affect the fluorescent probes. If direct quenching of DPH, TMA-DPH or PRO-DPH by ethanol occurred, fluorescence lifetime would decrease. Fluorescence lifetimes of DPH (Table I) and the sidedness selective probes were not changed by ethanol in the SPM. Second, redistribution of DPH in the presence of ethanol would result in a change in the fraction of fluorescence quenched by TNBS. Significant changes in DPH fluorescence intensity distribution between leaflets in the presence of ethanol were not detected. The percent quenching of DPH in SPM from synaptosomes treated under nonpenetrating conditions with TNBS was 50 ± 4% and 48 ± 4% at 0 and 650 mM ethanol, respectively. Ethanol did interact with TMA-DPH and PRO-DPH. The percent of TMA-DPH and PRO-DPH fluorescence quenched at 400 mM ethanol was 53% and 50% as compared to 21% and 68% in the absence of ethanol.

The effect of increasing concentrations of ethanol on fluorescence polarization of DPH in SPM individual leaflets is shown in Fig. 3. At sublethal concentrations (25–100 mM), ethanol significantly decreased the fluorescence polarization of DPH in the outer leaflet. There was little, if any, effect on the inner leaflet. Whether this

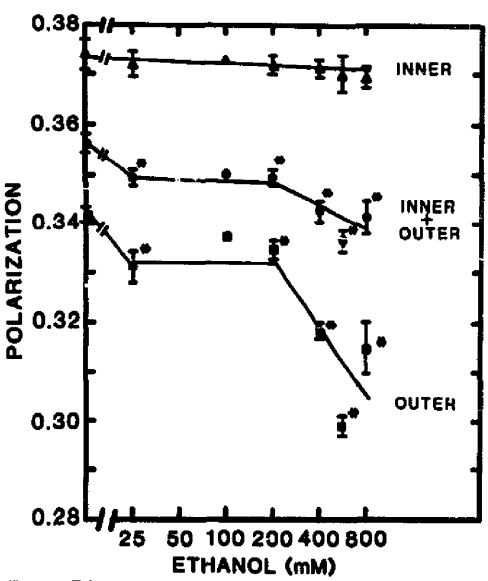

Fig. 3. Ethanol alters fluorescence polarization of DPH in outer leaflet of SPM. All conditions were as described in the legend to Table II except that fluorescence polarization was determined in response to increasing ethanol concentration. Values represent the mean ± S.E. ($n = 5$). An asterisk refers to $P < 0.05$ as compared to no ethanol by Student's t-test.

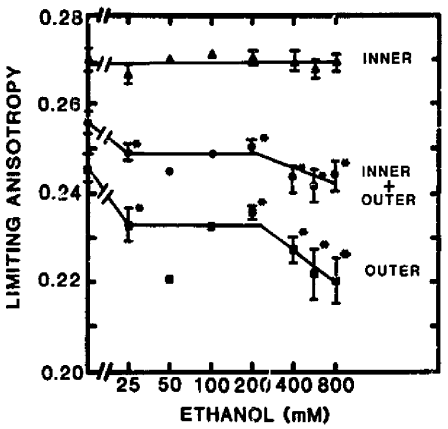

Fig. 4. Ethanol decreased DPH-limiting anisotropy in SPM outer leaflet. All conditions were as described in legend to Fig. 3 except that limiting anisotropy was determined.

difference in individual leaflet sensitivity to ethanol was due to resistance of membrane lipids to probe motion (order) or rate of probe motion (dynamics) was resolved by differential polarized phase fluorometry. The effect of increasing ethanol on limiting anisotropy of DPH in SPM is shown in Fig. 4. The limiting anisotropy of DPH in the bulk SPM shows a gradual decrease (fluidization) with increasing ethanol concentration (closed circles). No effect of ethanol was observed in the inner leaflet (closed trianges), even at 800 mM ethanol. In contrast, the limiting anisotropy of DPH calculated for the outer leaflet was significantly decreased at 25 mM ethanol (closed squares). Ethanol did not have a significant effect on the rotational relaxation time of DPH in the whole membrane, the inner leaflet or the outer leaflet.

Discussion

The present experiments were designed to test two hypotheses: 1) that fluidity of the outer and inner leaflets of SPM would differ; and 2) that ethanol would have a greater effect on the more fluid leaflet. The results of the experiments supported those hypotheses. The outer and inner leaflets of SPM differed in terms of both static and dynamic properties using DPH. The outer leaflet was more fluid than the inner leaflet. This result was confirmed by use of two leaflet-selective probe molecules, TMA-DPH and PRO-DPH. The transbilayer difference in SPM fluidity observed here is similar to that found for LM fibroblast plasma membranes [10–12] and B-16 melanoma plasma membrane [27]. Other studies have found that the outer leaflet of erythrocyte membranes was more rigid than the inner leaflet [4,5,30]. An explanation for differences in fluidity of SPM, LM plasma membrane and B-16 melanoma plasma membrane leaflets as compared to those of erythrocytes may result from asymmetric transbilayer distribution of cholesterol. LM and erythrocyte plasma membrane bilayers differ in transbilayer distribution of cholesterol [20,21]. Cholesterol was enriched in the inner leaflet of LM cell plasma membranes and in the outer leaflet of red blood cell membranes (see Refs. 9 and 17 for reviews). Cholesterol distribution in SPM may be similar to that of LM fibroblast and B-16 melanoma plasma membranes, since all three of these membranes are derived from nucleated cells while red blood cells are not nucleated. This difference in cholesterol between the two leaflets is consistent with the greater fluidity of the outer leaflet of SPM reported here.

Ethanol had a specific effect on the outer leaflet of SPM rather than on the inner leaflet. This asymmetric effect of ethanol was related to differences in membrane fluidity of the two leaflets. In the absence of ethanol, the outer leaflet was more fluid and was found to be disordered more by ethanol than was the inner leaflet. These results are consistent with findings that show that ethanol has a greater effect on fluid membranes as compared to more ordered membranes [1]. It was recently shown that benzyl alcohol had a greater effect on the more fluid inner leaflet of erythrocyte membranes than the outer leaflet [5]. The inner leaflet of the red blood cell membrane was more fluid than the outer leaflet [4,5,30].

Most studies that have examined effects of drugs on membrane leaflets have explained the asymmetric effects as resulting from the charge properties of the individual drugs [10–17]. The present results with ethanol and the study on benzyl alcohol [5] demonstrate that differences in fluidity of the leaflets can also determine where these alcohols and probably other neutral lipophilic drugs may act. The possibility that ethanol

may preferentially affect specific regions or lipid domains of the membrane has been considered [43–46]. Previously, these regions have been conceptualized as representing lateral patches, hydrophilic and hydrophobic domains or protein boundary lipid within the bulk membrane environment. To this end, it has been shown in *Aplysia* neuron membrane studies that the alcohols ethanol and butanol differentially altered the diffusion coefficient of fluorescent-labeled phosphatidylethanolamine and fluorescent-labeled phosphatidylcholine in the bulk membrane using fluorescence photobleaching recovery [45]. Alcohols differing in chain length have also been found to have qualitative differences on potassium currents in *Aplysia* neurons [46]. The present work demonstrates that ethanol also has a differential effect on vertical (transbilayer) domains.

Fluidity of SPM leaflets differ between each other both in the presence and absence of ethanol. The functional consequences of this gradient in fluidity between leaflets of membranes is not well understood. In the erythrocyte, changes in lipid distribution between the two leaflets may affect clotting and it has been suggested that externalization of negatively charged phospholipids may act as antigenic sites for antiphospholipid antibodies [47]. What effect chronic ethanol consumption may have on asymmetry has not been determined. Ethanol consumption may alter the mechanism that maintains asymmetry of fluidity and lipid distribution and in turn affect membrane function [9]. Normal function may require an optimal asymmetry between the two leaflets. A reduction in asymmetry of membranes has been found to be associated with various disease, e.g., sickle cell disease, acanthocytosis [48,49].

The results presented here show for the first time a difference in structure between the two leaflets of SPM. In addition, ethanol rather than being a nonspecific membrane fluidizing drug, instead displays great specificity for one but not both leaflets of the SPM membrane.

Acknowledgements

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