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Transbilayer Effects of n-Alkanols on the Fluidity of Phospholipid Model Membranes

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Abstract □ Selective quenching of 1,6-diphenyl-1,3,5-hexatriene (DPH) by trinitrophenyl groups was utilized to examine the transbilayer fluidity asymmetry of model membranes of phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles (SPMV). The polarization (P), anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) of DPH in the inner monolayer were 0.019, 0.014, 0.018, and 0.047, respectively, greater than calculated for the outer monolayer of SPMVPL. Selective quenching of DPH by trinitrophenyl groups was also utilized to examine the effects of n-alkanols on the individual monolayer structure of SPMVPL. n-Alkanols fluidized the hydrocarbon region of bulk SPMVPL and the potencies of n-alkanols up to 1-nonanol increased with carbon chain length. It appears that the potencies in bilayer fluidization increase by 1 order of magnitude as the carbon chain length increases by two carbon atoms. The cut-off phenomenon was reached at 1-decanol, where further increase in hydrocarbon length resulted in a decrease in pharmacological activity. The n-alkanols had greater fluidizing effects on the outer monolayer as compared to the inner monolayer of SPMVPL, even though these selective effects tended to become weaker as the carbon chain length increased. Thus, it has been proven that n-alkanols exhibit selective rather than nonselective fluidizing effects within transbilayer domains of SPMVPL.

Keywords □ n-Alkanols, transbilayer fluidity asymmetry, phospholipid model membranes, fluorescent probe technique.

n-Alkanols are members of the large family of anesthetic drugs whose biological potency correlates with lipid solubility[1]. Many different physicochemical techniques have been used to provide evidence that anesthetic agents and similar drugs have a biophysical action on cell membranes that can be often described as a disordering or fluidizing effect. It has been shown that alkanols at nerve-blocking concentrations expand biological membranes[2] or disturb order-parameters associated with the lipid[3]. In artificial lipid bilayers, both lipid disorder[4] and/or lipid phase transition[5] are affected in a continuous and monotonous fashion throughout the series of saturated aliphatic n-alkanols, up to n=10-12, consistent with the anesthetic potency. However, previous studies that have examined the effects of n-alkanols on native and model membranes have studied changes in the bulk membrane lipids.

Over the past decade, it has been well established that the inner and outer monolayers of the eukaryotic cell plasma membrane have different lipid composition as well as protein composition[6]. This compositional transbilayer asymmetry is expected to confer asymmetry of structure between the monolayers, provided that they are not coupled. Indeed, no, or only a weak, coupling of lipid motion across the bilayer has been experimentally verified[7-9]. The only reported exception is sphingomyelin (SP) containing long-chain fatty acids (*n*-tetracosanoic acid), which illustrated coupling between pure SP monolayers[10].

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 monolayer or the other[11]. This selective effect was dependent on the charge properties of the membrane lipids. Cationic drugs had a greater effect on the negatively charged inner monolayer, whereas anionic drugs acted on the outer monolayer. n-Alkanols are neutral compounds that would not be attracted to one specific monolayer on the basis of charge. However, the more fluid regions in the membrane core are more easily perturbed by ethanol than are the stiffer surface regions[12]. n-Alkanols should have an asymmetric effect if one monolayer differed in fluidity as compared to the other monolayer.

In the present study, selective quenching of 1,6-diphenyl-1,3,5-hexatriene (DPH) by trinitrophenyl groups was utilized to examine the transbilayer fluidity asymmetry of the model membranes of phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles (SPMV) and to examine the effects of n-alkanols on the individual monolayer structure of SPMVPL. The present paper represents the first investigation of transbilayer fluidity asymmetry of SPMVPL and the first proof of transbilayer domain selectiveness of n-alkanols' action.

EXPERIMENTAL METHODS

Chemicals

The fluorescent probe DPH was obtained from Molecular Probes (Junction City, OR, USA). n-Alkanols (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, and 1-decanol) were purchased from Fluka (Buchs, Switzerland). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), Ficoll (70,000 M.W.), Sepharose, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest quality available and water was deionized.

Membrane preparations

Synaptosomal plasma membrane vesicles: The SPMV were isolated from bovine cerebral cortex and characterized by the formerly reported method in our laboratory[13,14]. The purity of SPMV was determined by enzymatic and morphological standards. The specific activities of Na,K-ATPase[13,14], acetylcholinesterase[15], and 5'-nucleotidase[13,14]were about 6-fold, 2.5-fold, 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. Protein was determined by the method of Lowry *et al.*[16] using BSA as a standard.

Lipid extraction: Lipids were extracted from the SPMV as described previously[13]. The individual phospholipid classes were separated by thin layer chromatography[13] and quantitated by measuring the amounts of inorganic phosphate[17] after hydrolysis of the phospholipids at 180℃ in 70% $HClO_4$[18]. Phospholipids were composed of phosphatidylcholine (PC, 43%), phosphatidylethanolamine (PE, 36%), phosphatidylserine (PS, 13%), phosphatidylinositol (PI, 3%), SP (4%), and lysophosphatidylcholine (1%).

Phospholipid model membranes: Large unilamellar liposomes were prepared by the reverse-phase-evaporation technique[19]. The extracted phospholipids in chloroform solution were deposited on the sides of a round-bottom flask by removal of the organic solvent by rotary evaporation. The lipids were then redissolved in diethyl ether which had been redistilled in the presence of $NaHSO_3$ immediately prior to use. Phosphate-buffered saline (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) was added to the solution of phospholipids and the organic/aqueous mixture was placed in an ultrasonic processor (Sonics & Materials, Inc., Danbury, CT, USA), under N_2 at 30℃. It was sonicated for 5 min to form a milky white, homogeneous emulsion. The emulsion was then transferred to a rotary evaporator and the organic solvent was removed under reduced pressure. During evaporation of the solvent, the system foamed. As the process continued, progressively higher vacuum was needed to maintain foaming. As the majority of the solvent was removed, the material first formed a viscous gel and subsequently (within 5-10 min) it became an aqueous suspension. At this time, additional PBS was added, and the preparation foamed and was vented again several times until the foaming ceased. The procedure was finished when no foaming occurred. The preparation was then dialyzed and passed through a Sepharose 4B column.

TNBS labelling reactions

TNBS labelling reactions were performed by the method of Yun and Kang[13] although with several modifications. The SPMVPL were gently resuspended in 0.5 mM TNBS plus 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 pH of reagent was adjusted to 8.5 with NaOH. CO_2 was bubbled through the solution and the treatment was carried out at 4℃ for 20 min. The TNBS labelling reaction was terminated by addition of 2% BSA in PBS (pH 7.4), at 4℃.

Fluorescence measurements

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 of phospholipids as described previously[20]. After incorporation of the probe, the membrane suspension was placed in cuvettes. Control levels of fluorescence were then determined, an aliquot of n-alkanols was added directly to the cuvette, and fluorescence was again determined. The excitation wavelength for DPH was 362 nm and fluorescence emission was read at 424 nm. All fluorescence measurements were obtained with a SPF-500C spectrofluorometer (SLM Aminco Instruments, Inc., Urbana, IL, USA) and performed at 37℃. Before the fluorescence spectra were obtained, all samples were bubbled by dry nitrogen through the solution for at least 30 min in order to eliminate oxygen. Blanks, prepared under identical conditions without 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 exicitation 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 [$r=2P/(3-P)$], limiting anisotropy (r_∞), and order parameter (S). The limiting anisotropy of DPH was determined directly from the anisotropy value using the following relationship[21];

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

The limiting anisotropy reflects restriction to probe motion and can be converted to an order parameter[22], $S=(r_\infty/r_o)^{1/2}$ where r_o, the anisotropy in the absence of motion, is equal to 0.362 for DPH[23].

Determination of individual monolayer structure in SPMVPL: Selective quenching of DPH

This experimental determination of individual monolayer structure in SPMVPL is based on a

Table I. Effects of n-alkanols on transbilayer distribution of 1,6-diphenyl-1,3,5-hexatriene in the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles

n-Alkanol	Concentration, mM	% Quenching of 1,6-diphenyl-1,3,5-hexatriene
None		52.4± 1.1
Methanol	100	52.6± 1.2
Methanol	2500	54.3± 0.6
Ethanol	25	52.1± 1.3
Ethanol	800	55.6± 0.7
1-Propanol	10	53.0± 1.0
1-Propanol	250	54.5± 1.0
1-Butanol	2.5	52.4± 1.1
1-Butanol	80	55.6± 0.8
1-Pentanol	1	51.9± 0.7
1-Pentanol	25	56.0± 1.0
1-Hexanol	0.25	53.9± 1.4
1-Hexanol	8	57.7± 2.1
1-Heptanol	0.1	52.2± 1.1
1-Heptanol	2.5	58.3± 1.8
1-Octanol	0.025	53.0± 1.3
1-Octanol	0.8	58.2± 2.1
1-Nonanol	0.01	53.4± 2.1
1-Nonanol	0.25	56.5± 2.8
1-Decanol	0.25	52.7± 1.7
1-Decanol	8	56.8± 2.6

Phospholipid model membranes were treated± 0.5 mM trinitrobenzenesulfonic acid, pH 8.5, at 4℃ for 20 min. 1,6-Diphenyl-1,3,5-hexatriene was incorporated and the fluorescence was determined in the absence and presence of n-alkanols at the concentrations given at 37℃. Values represent the mean± SEM of 4 determinations.

method previously established for LM plasma membranes[24] and synaptic plasma membranes[25]. 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

$$r = \sum F_j \, r_j$$

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 SPMVPL, 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 SPMVPL incubated with buffer A and buffer A plus TNBS at 4℃ (nonpenetrating conditions), respectively. The values of the fluorophore concentration independent parameter anisotropies, r (anisotropy for both monolayers), r_i (inner monolayer anisotropy), were determined for DPH in SPMVPL incubated with buffer A and buffer A plus TNBS at 4℃ (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

Fluidity asymmetry in SPMVPL : Quenching of DPH fluorescence by trinitrophenyl groups

Several different domains or patches of lipids that differ in their fluidity and lipid composition have been described, e.g., hydrophilic, hydrophobic, lateral, outer, and inner monolayers[12,24-28]. The surface of the membrane is more hydrophilic as compared to the interior which is more hydrophobic. Lateral domains are lipid patches that extend laterally along the horizontal plane of the membrane and are thought to differ in their fluidity and lipid composition. Two other domains are the transbilayer or vertical domains of the membrane (i.e., the outer and inner monolayers).

A wide variety of techniques in many laboratories have provided results consistent with the interpretation that the motional properties (structure) of lipids in the outer and inner monolayers of biological membranes differ[26,29,30]. Hence, in the present study, an impermeable reagent, TNBS, covalently linked to outer monolayer amino groups, was used to quench the fluorescence of DPH, a probe which distributes in both monolayers. Approximately half of the DPH fluorescence was quenched in the trinitrophenylated SPMVPL (Table I). If the TNBS labelling was conducted under penetrating conditions (37℃), nealy 100% of the fluorescence of DPH was quenched. The values of fluorescence parameters of DPH in intact SPMVPL (both monolayers) as compared to those for TNBS-treated SPMVPL (inner monolayer) are listed in Table II. The polarization (P), anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) of DPH in the inner monolayer were 0.019, 0.014, 0.018, and 0.047, respectively, greater than calculated for the outer monolayer of SPMVPL.

Table II. Asymmetry of 1,6-diphenyl-1,3,5-hexatriene motion in the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles

Membrane	Polarization (P)	Anisotropy (r)	Limiting anisotropy (r_∞)	Order parameter (S)
Inner+outer	0.208± 0.001	0.149± 0.001	0.098± 0.001	0.520± 0.004
Inner	0.218± 0.001	0.156± 0.001	0.108± 0.001	0.545± 0.003
Outer	0.199± 0.002**	0.142± 0.002**	0.090± 0.002**	0.498± 0.006**

Phospholipid model membranes were treated± 0.5 mM trinitrobenzenesulfonic acid, pH 8.5, at 4℃ for 20 min. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37℃. Values from untreated membranes represent inner+outer monolayer; Values from 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated membranes represent the inner monolayer; Values for the outer monolayer were calculated as described in Experimental Methods. Values are represented as the mean± SEM of 4 determinations. Double asterisk signifies $P<0.01$ according to Student's t-test.

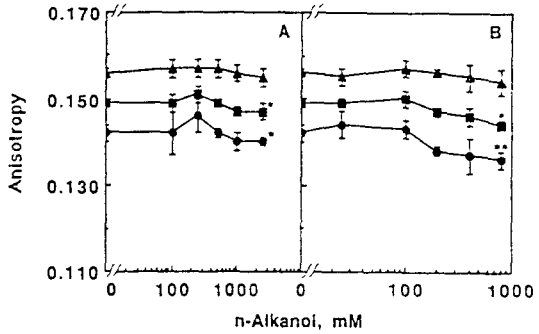

Fig. 1. n-Alkanols alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles.
(A) Methanol; (B) ethanol. Phospholipid model membranes were treated± 0.5 mM trinitrobenzenesulfonic acid, pH 8.5, at 4℃ for 20 min. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37℃. Untreated (inner and outer monolayers, ■); 2,4,6-trinitrobenzenesulfonic acid (TNBS) treated (inner monolayer, ▲); calculated for outer monolayer (●). 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.

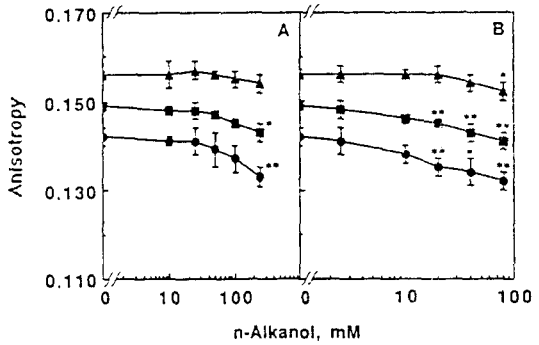

Fig. 2. n-Alkanols alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles.
(A) 1-Propanol; (B) 1-butanol. All conditions were as described in the legend to Fig. 1. Each point represents 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.

Effects of n-alkanols on transbilayer fluidity of SPMVPL

The bulk lipid fluidity change will represent an average of the affected and unaffected portions of the membrane and may underestimate the effect on specific domains. Very little attention has been given to the selective effects of n-alkanols on transbilayer membrane domains. Selective quenching of DPH by trinitrophenyl groups was also utilized to examine the specific effects of n-alkanols on the fluidity of transbilayer domains of SPMVPL. In order to determine the effects of n-alkanols on individual monolayer structure, it is first necessary to demonstrate that these drugs do not interact directly with DPH and thereby quench its fluorescence. As shown in Table I, significant changes in DPH fluorescence intensity distribution between monolayers in the presence of n-alkanols were not detected over the entire concentration range used for n-alkanols. Hence, the possibility of direct quenching of DPH fluorescence by n-alkanols is ruled out.

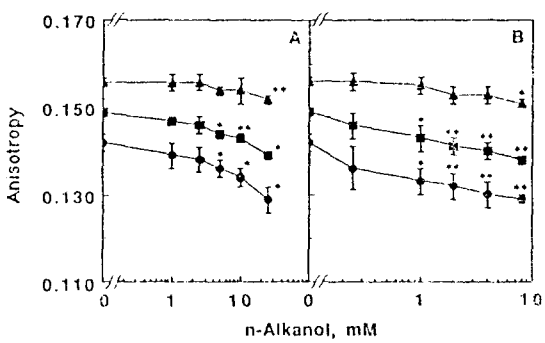

Fig. 3. n-Alkanols alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles.
(A) 1-Pentanol; (B) 1-hexanol. All conditions were as described in the legend to Fig. 1. Each point represents 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.

The effects of increasing concentrations of n-alkanols on the anisotropy of DPH in SPMVPL are shown in Fig. 1-5. All n-alkanols fluidized the bulk lipid (Fig. 1-5, closed squares) and the potencies of n-alkanols up to 1-nonanol increased with the

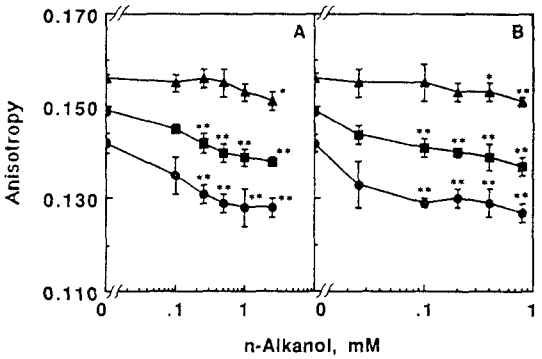

Fig. 4. n-Alkanols alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles.
(A) 1-Heptanol; (B) 1-octanol. All conditions were as described in the legned to Fig. 1. Each point represents 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.

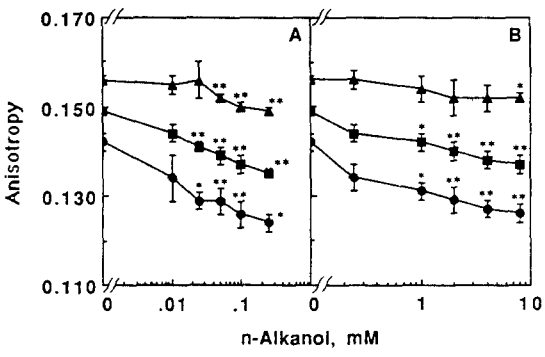

Fig. 5. n-Alkanols alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles.
(A) 1-Nonanol; (B) 1-decanol. All conditions were as described in the legend to Fig. 1. Each point represents 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.

carbon chain length. It appears that the potencies in bilayer fluidization increase by 1 order of magnitude as the carbon chain length increases by two carbon atoms. And the cut-off phenomenon was reached at 1-decanol, where further increases in hydrocarbon length resulted in a decrease in pharmacological activity. The n-alkanols preferentially decreased the anisotropy of DPH in the outer monolayer (Fig. 1-5, closed circles) and there was little effect on the inner monolayer (Fig. 1-5, closed triangles). This indicates that n-alkanols had greater fluidizing effects on the outer monolayer as compared to the inner monolayer, even though these selective effects tended to become weaker as the carbon chain length increased. Thus, it has been proven that n-alkanols exhibit selective rather than nonselective fluidizing effects within transbilayer domains of the SPMVPL.

In biological membranes, the anisotropy (r) reflects mainly the range of motion rather than the rate and membrane perturbants, such as cholesterol and proteins, primarily alter the range of motion rather than rate[31]. Hence, the alterations in the anisotropy (r) are likely to be due to changes in range of motion of the probe. Accordingly, we studied the effects of n-alkanols on the range component of DPH motion in more detail. The range of motion is generally expressed as either the limiting anisotropy (r_∞) or the order parameter (S). The selective effects of n-alkanols on the limiting anisotropy and order parameter of DPH are shown in Table III. The result corroborates the above point.

DISCUSSION

Fluorescence methods have the important advantages of great sensitivity, versatility, and simplicity of instrumentation. Determination of steady-state fluorescence anisotropy of DPH is the easiest and most frequently used method to measure membrane fluidity[32]. DPH has many characteristics of an ideal probe. It has a high extinction coefficient and quantum yield. Its absorption and emission spectra are well separated so that there is little spectral overlap and problems associated with the scattering of excitation light can be minimized. It has a high partition coefficient into lipid assemblies, and it is practically nonfluorescent in aqueous media. DPH absorption and emission dipoles are almost parallel, so that its limiting anisotropy (0.362) is close to the theoretical maximum. The covalently linked trinitrophenyl group displays a broad absorbance with

Table III. Effects of n-alkanols on limiting anisotropy (r_∞) and order parameter (S) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles

n-Alkanol (mM)	Limiting anisotropy (r_∞)			Order parameter (S)		
	Inner+outer	Inner	Outer	Inner+outer	Inner	Outer
Control	0.098± 0.001	0.108± 0.001	0.090± 0.002	0.520± 0.004	0.545± 0.003	0.498± 0.006
Methanol (2500)	0.096± 0.002	0.107± 0.003	0.087± 0.002*	0.514± 0.006	0.543± 0.007	0.491± 0.006*
Ethanol (800)	0.092± 0.001*	0.106± 0.004	0.081± 0.003**	0.504± 0.003**	0.540± 0.009	0.475± 0.007**
1-Propanol (250)	0.090± 0.002*	0.106± 0.002	0.078± 0.003**	0.499± 0.006*	0.540± 0.006	0.465± 0.009**
1-Butanol (20)	0.093± 0.002**	0.108± 0.003	0.081± 0.003**	0.507± 0.005**	0.545± 0.007	0.474± 0.008**
1-Butanol (80)	0.088± 0.002**	0.103± 0.002*	0.076± 0.003**	0.493± 0.006**	0.533± 0.006*	0.461± 0.008**
1-Pentanol (5.0)	0.092± 0.001**	0.106± 0.002	0.081± 0.002*	0.505± 0.003**	0.540± 0.005	0.475± 0.007*
1-Pentanol (25.0)	0.086± 0.001**	0.103± 0.002*	0.072± 0.003**	0.487± 0.003**	0.533± 0.004*	0.450± 0.009**
1-Hexanol (1.0)	0.090± 0.003*	0.106± 0.003	0.076± 0.004*	0.498± 0.009*	0.542± 0.008	0.462± 0.011*
1-Hexanol (8.0)	0.085± 0.002**	0.102± 0.002*	0.072± 0.001**	0.483± 0.005**	0.530± 0.005*	0.449± 0.004**
1-Heptanol (0.25)	0.090± 0.002**	0.108± 0.003	0.075± 0.003**	0.498± 0.006**	0.545± 0.007	0.459± 0.008**
1-Heptanol (2.5)	0.084± 0.002**	0.102± 0.002*	0.071± 0.003**	0.481± 0.005**	0.530± 0.006*	0.445± 0.010**
1-Octanol (0.1)	0.089± 0.003**	0.107± 0.005	0.073± 0.001**	0.494± 0.008**	0.544± 0.013	0.452± 0.004*
1-Octanol (0.4)	0.085± 0.003**	0.103± 0.002*	0.072± 0.004**	0.484± 0.010**	0.534± 0.006*	0.447± 0.011**
1-Nonanol (0.025)	0.089± 0.001**	0.108± 0.005	0.072± 0.002*	0.494± 0.003**	0.546± 0.013	0.451± 0.005**
1-Nonanol (0.050)	0.086± 0.002**	0.103± 0.001**	0.072± 0.004**	0.487± 0.007**	0.533± 0.002**	0.449± 0.011**
1-Decanol (1.0)	0.089± 0.003*	0.105± 0.004	0.075± 0.003*	0.495± 0.008*	0.539± 0.009	0.456± 0.007*
1-Decanol (8.0)	0.083± 0.003**	0.102± 0.001*	0.069± 0.003**	0.479± 0.008**	0.530± 0.002*	0.441± 0.009**

All conditions were as described in the legened to Table II. 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.

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.

Our data explicitly show the transbilayer fluidity asymmetry of SPMVPL (Table II). The outer monolayer was significantly more fluid than the inner monolayer. An explanation for this fluidity gradient may result from asymmetric distribution of phospholipids. It has been well established that neutral or positively charged lipids, such as PC or SP, preferentially reside in the outer monolayer, while anionic phospholipids such as PE, PS, and PI are enriched in the inner monolayer of biological membranes[13,33]. And the inner monolayer of biological membranes contains more unsaturated fatty acids than the outer monolayer[13,34]. Increases in SP content or in the ratio of SP/PC decrease the fluidity[32]. The size and shape of the phospholipid head groups affect fluidity; PE head groups pack into smaller areas and form less fluid bilayers than do PC molecules[35]. The degree of saturation and the length of the fatty acyl side chains of the phospholipids are determinants of fluidity: *cis* double bonds introduce kinks which prevent close packing and thus enhance fluidity; longer chains increase chain-chain interactions and reduce fluidity[36].

As shown in Fig. 1-5 and Table III, all n-alkanols exerted specific fluidizing effects on the outer monolayer as compared to the inner monolayer of SPMVPL. In view of the point that alcohols have greater effects on fluid membranes as compared to more ordered membranes, our results are in agreement with those of previous studies[12,25,26]. However, it still remains to be estabilshed whether changes in fluidity in turn affect the functional activities of the membrane such as transport, signal transduction, drug sensitivity, and tolerance. It has been proposed that there is an optimal fluidity required for membrane function[37]. This hypothesis was based

on the fluidity of the bulk lipid of the membrane. Perhaps optimal fluidity is required not for the bulk membrane but rather for specific membrane domain in relative to each other. In order for normal cell to function, optimal structural asymmetry of the monolayers in terms of fluidity and lipid distribution has been proposed[38-40]. The physiological consequences of altered asymmetry are only now being reported. A reduction in asymmetry of membranes has been found to be associated with various disease e.g., sickle cell disease, acanthocytosis[38,39].

However, n-alkanols' 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[41]. 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[42]. Furthermore, the hypothesis that ethanol is a nonspecific drug that produces its actions *via* perturbation of neuronal membrane lipids is now being challenged by recent data showing that ethanol specifically and selectively affects the function of certain membrane-bound proteins[43,44]. A recent data on the interactions of ethanol and certain receptor- and voltage-gated ion channels concluded that the receptor-gated (γ-aminobutyric acid and N-methyl-D-aspartate) ion channels are more sensitive to acute effects of ethanol than the voltage-gated Ca^{2+} channels[43].

Opinions have been divided as to whether n-alkanols interfered with membrane protein function by direct action 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 n-alkanols readily diffused. Since biological membranes are of highly complex compositions, it has not been feasible to monitor changes in the local lipid environment and to determine its effect on membrane protein function at the same time. Still, a large, diverse collection of physiological agonists produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction[45]. So, the function of membrane proteins may be modulated secondarily to changes in membrane fluidity. In addition, it cannot be ruled out that n-alkanols concurrently interact with neuronal membrane proteins and membrane lipids since the receptor-gated ion channels were found to be tightly associated with membrane lipid through covalent or noncovalent bonds. Thus, it may be premature to take sides in the controversy about whether membrane lipids or membrane proteins are the site of general anesthetic action.

In summary, it is strongly postulated that n-alkanols, in addition to their direct interaction with the ion channels, concurrently interact with membrane lipids, fluidize the membrane, and thus induce conformational changes of the ion channels, which are known to be tightly associated with membrane lipids.

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