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Effects of Barbiturates on Transbilayer Fluidity Domains of Phospholipid Model Membrane Monolayers[1]

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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 domains of the model membranes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles. At 37°C, all anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) values of DPH in the SPMVTL were larger than those in SPMVPL. The anisotropy, limiting anisotropy, and order parameter of DPH in the inner monolayer were 0.025, 0.033, and 0.070, respectively, greater than calculated for the outer monolayer of SPMVTL. In SPMVPL, the anisotropy, limiting anisotropy, and order parameter of DPH in the inner monolayer were 0.014, 0.018, and 0.047, respectively, greater than calculated for the outer monolayer. Selective quenching of DPH by trinitrophenyl groups was also utilized to examine the effects of barbiturates on the transbilayer fluidity domains of SPMVTL and SPMVPL. Barbiturates did not affect the anisotropy of DPH in the transbilayer domains of SPMVTL. In contrast, barbiturates increased the fluorescence anisotropy, limiting anisotropy, and order parameter of DPH in the SPMVPL in a dose-dependent manner. Barbiturates showed a greater ordering effect on the outer monolayer as compared to the inner monolayer of SPMVPL. Hence, it has been demonstrated for the first time that the Sheetz-Singer hypothesis (1974) may be valid for phospholipid model membranes.

Key Words: Barbiturates, Model membranes, Transbilayer fluidity domains, Fluorescence probe technique

INTRODUCTION

With increasing frequency, it is being recognized that the effects of drugs on the fluidity of biological membranes are as important as the drug-specific receptor interaction in studying the mechanism of action of drugs, especially those having anesthetic and tranquillizing actions. In support of the membrane hypothesis, there is an excellent correlation between many diverse *in vitro* actions of barbiturates and their lipid solubilities (Hansch and Anderson, 1967). There is also evidence that barbiturates penetrate into membrane lipid and alter the fluidity of the membrane (Harris and Schroeder, 1982; Lawrence and Gill, 1975; Lee, 1976; Novak and Swift, 1972; Pang and Miller, 1978; Rosenberg *et al.*, 1977). However, it seems likely that barbiturates do not have relevant membrane-perturbing actions and the lipid composition of the bilayer strongly affects its response to barbiturates.

It has been well established that phospholipids are asymmetrically distributed in biological membranes (Op den Kamp, 1979; Yun and Kang, 1990). Neutral or positively charged lipids, such as

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phosphatidylcholine (PC) or sphingomyelin (SP), preferentially reside in the outer monolayer, while anionic phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are enriched in the inner monolayer.

About two decades ago, Sheetz and Singer (1974) proposed that the asymmetry in net charge at the surface of the two monolayers of biological membranes could establish an asymmetric transbilayer distribution of charged amphipaths (e.g., charged anesthetics) intercalating into the monolayers. It is expected that the anionic barbiturates might have preferential fluidizing action on the positively charged outer monolayer. This selective effect of barbiturates has been demonstrated only in rat liver plasma membranes (Houslay *et al.*, 1981; Dipple *et al.*, 1982) and in LM fibroblast plasma membranes (Kier *et al.*, 1986; Sweet and Schroeder, 1986).

In the present study, to get a better insight into the molecular mechanism of action of barbiturates, the effects of barbiturates on the transbilayer fluidity domains of model membrane bilayers of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles (SPMV) were investigated employing selective quenching of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence by trinitrophenyl groups.

MATERIALS AND METHODS

Materials

The fluorescent probe DPH was obtained from Molecular Probes (Junction City, OR, USA). Barbiturates, 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 purchased 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 (Yun and Kang, 1990; Yun *et al.*, 1990b). The purity of SPMV was determined by enzymatic and morphological standards. The specific activities of Na,K-ATPase (Yun and Kang, 1990; Yun *et al.*, 1990b), acetylcholinesterase (Ellman *et al.*, 1961), and 5′-nucleotidase (Yun and Kang, 1990; Yun *et al.*, 1990b) 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.* (1951) using BSA as a standard.

Model membranes. Total lipids and phospholipids were extracted from the SPMV as described previously (Yun and Kang, 1990). Phospholipids were quantitated by measuring the amounts of inorganic phosphate (Bartlett, 1959) after hydrolysis of the phospholipids at 180°C in 70% $HClO_4$ (Madeira and Antunes-Madeira, 1976). Large unilamellar liposomes were prepared by the reverse-phase-evaporation technique (Szoka and Papahadjopoulos, 1978). The extracted lipids 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 lipids and the organic/aqueous mixture was placed in an ultrasonic processor (Sonics & Materials, Inc., Danbury, CT, USA), under N_2 at 30°C. 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 (1990) although with several modifications. The model membranes 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 reagent pH was adjusted to 8.5 with NaOH. CO_2 was bubbled through the solution and the treatment was carried out at 4°C for 20 min. The TNBS labelling reaction was terminated by addition of 2% BSA in PBS (pH 7.4), at 4°C.

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 (Yun *et al.*, 1990a). After incorporation of the probe, the membrane suspension was placed in cuvettes. Control levels of fluorescence were then determined, an aliquot of barbiturates 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 florescence measurements were obtained with a SPF-500C spectrofluorometer (SLM Aminco Instruments, Inc., 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 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 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 [$r = 2P/(3-P)$], limiting anisotropy (r_∞), and order parameter (S). The limiting anisotropy of DPH was determined directly from the anisotropy value using the following relationship (van Blitterswijk *et al.*, 1981).

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

The limiting anisotropy reflects restriction to probe motion and can be converted to order parameter, $S = (r_\infty/r_0)$ (Kawato *et al.*, 1978) where r_0, the anisotropy in the absence of motion, is equal to 0.362 for DPH (Lakowicz *et al.*, 1979).

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

This experimental determination of individual monolayer structure in SPMVTL and SPMVPL is based on a method previously established for LM plasma membranes (Sweet and Schroeder, 1986) and synaptic plasma membranes (Schroeder *et al.*, 1988). 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 model membranes, this leads to

$$r = \frac{F_i}{F} r_i + \frac{F - F_i}{F} r_0$$

where F and F_i are fluorescence of DPH obtained for the model membranes incubated with buffer A and buffer A plus TNBS at 4°C (nonpenetrating conditions), respectively. The values of the fluorophore concentration independent parameter anisotropies, r (anisotropy for both monolayers) and r_i (inner monolayer anisotropy), were determined for DPH in the model membranes incubated with buffer A and buffer A plus TNBS at 4°C (nonpenetrating conditions), respectively. The equation was then solved for r_0 (outer monolayer anisotropy). Similar calculations were performed by simultaneous measurement of the fluorescence

intensity and either limiting anisotropy (r_∞) or order parameter (S).

RESULTS

Transbilayer fluidity domains of SPMVTL and SPMVPL: Quenching of DPH fluorescence by trinitrophenyl groups

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. As shown in Table 1, approximately half of the DPH fluorescence was quenched in the trinitrophenylated SPMVTL and SPMVPL. If the TNBS labelling was conducted under penetrating conditions (37°C), nearly 100 % of the fluorescence of DPH was quenched. The values of fluorescence parameters in intact SPMVTL and SPMVPL (both monolayers) as compared to those for TNBS-treated SPMVTL and SPMVPL (inner monolayer) are listed in Table 2. At 37°C, all anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) values of DPH in the SPMVTL were larger than those in the SPMVPL. The anisotropy, limiting anisotropy, and order parameter of DPH in the inner monolayer were 0.025, 0.033, and 0.070, respectively, greater than calculated for the outer monolayer of SPMVTL. In SPMVPL, the anisotropy, limiting anisotropy, and order parameter of DPH in the inner monolayer were 0.014, 0.018, and 0.047, respectively, greater than calculated for the outer monolayer.

Effects of barbiturates on transbilayer fluidity domains of model membranes: SPMVTL versus SPMVPL

The above data distinctly show that both SPMVTL and SPMVPL consist of transbilayer domains or monolayers that differ in fluidity. Very little attention has been given to the selective effects of barbiturates on transbilayer membrane domains. Hence, selective quenching of DPH by trinitrophenyl groups was also utilized to examine the specific effect of barbiturates on the transbilayer fluidity domains of SPMVTL and SPMVPL. In order to determine the effects of barbiturates 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 1, significant changes in DPH fluorescence intensity

Table 1. Effects of barbiturates on transbilayer distribution of 1,6-diphenyl-1,3,5-hexatriene in the model membranes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles

Barbiturate	Concentration, mM	% Quenching of 1,6-diphenyl-1,3,5-hexatriene	
		SPMVTL	SPMVPL
None		52.5±1.5	52.4±1.1
Pentobarbital	0.1	52.7±1.5	53.6±1.5
Pentobarbital	1.0	54.8±1.0	53.6±1.6
Pentobarbital	10.0	55.6±2.1	56.1±1.0
Hexobarbital	0.1	54.4±1.9	53.1±1.9
Hexobarbital	1.0	53.7±1.6	54.1±1.6
Hexobarbital	10.0	53.4±1.9	55.4±1.8
Amobarbital	0.1	53.7±1.5	54.8±1.2
Amobarbital	1.0	54.4±0.8	53.7±1.5
Amobarbital	10.0	55.4±1.0	54.9±1.3
Phenobarbital	0.1	54.1±1.3	53.6±1.3
Phenobarbital	1.0	54.8±1.5	54.9±1.0
Phenobarbital	10.0	55.4±1.3	54.1±1.7

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

Table 2. Asymmetry of 1,6-diphenyl-1,3,5-hexatriene motion in the model membranes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles

Membrane	SPMVTL			SPMVPL		
	Anisotropy (r)	Limiting Anisotropy (r_∞)	Order Parameter (S)	Anisotropy (r)	Limiting Anisotropy (r_∞)	Order Parameter (S)
inner+outer	0.183±0.002	0.144±0.003	0.630±0.006	0.149±0.001	0.098±0.001	0.520±0.004
inner	0.196±0.003	0.161±0.004	0.667±0.008	0.156±0.001	0.108±0.001	0.545±0.003
outer	0.171±0.002**	0.128±0.002**	0.597±0.005**	0.142±0.002**	0.090±0.002**	0.498±0.006**

Model membranes were treated±0.5 mM trinitrobenzenesulfonic acid, pH 8.5, at 4°C for 20 min. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37°C. 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 Materials and Methods. Values are represented as the mean± SEM of 4 determinations. Double asterisk signifies $P<0.01$ according to Student's t-test.

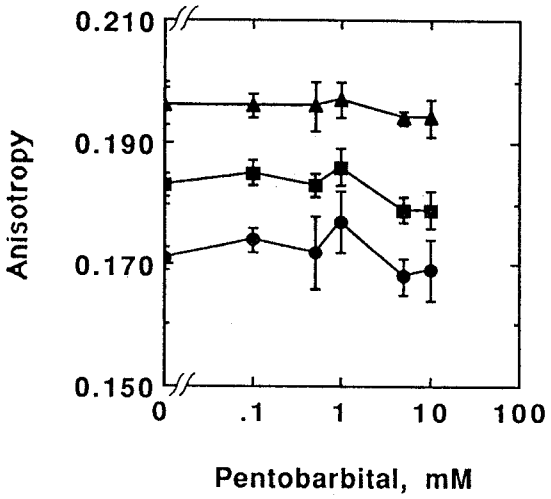

Fig. 1. Pentobarbital does not alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the model membranes of total lipids extracted from synaptosomal plasma membrane vesicles (SPMVTL). Model membranes were treated±0.5 mM trinitrobenzenesulfonic acid, pH 8.5, at 4°C for 20 min. 1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence measurements were performed at 37°C. 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.

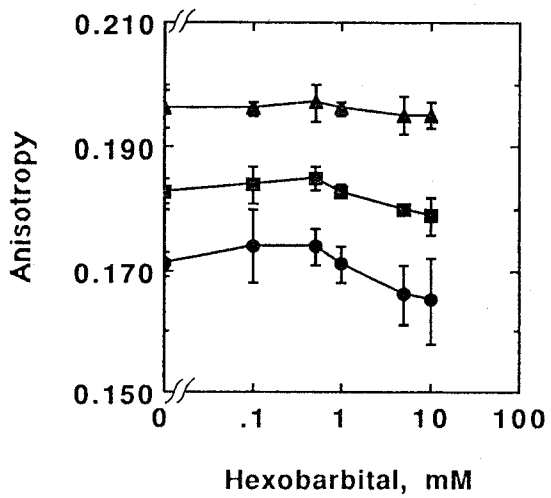

Fig. 2. Hexobarbital does not alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the model membranes of total lipids extracted from synaptosomal plasma membrane vesicles (SPMVTL). All conditions were as described in the legend to Fig. 1.

distribution between monolayers in the presence of barbiturates were not detected over the entire concentration range used for barbiturates. Hence, the possibility of direct quenching of DPH fluorescence by barbiturates is ruled out.

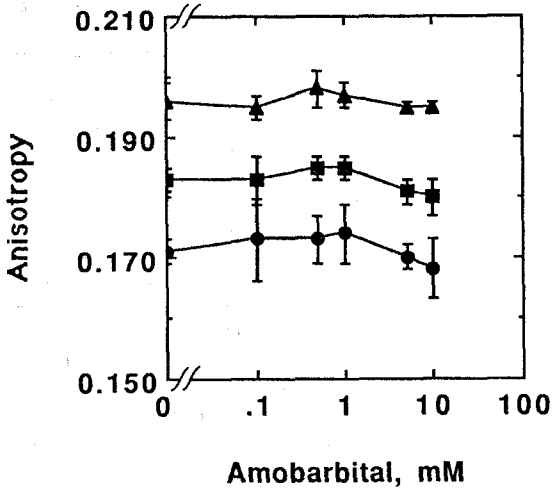

Fig. 3. Amobarbital does not alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the model membranes of total lipids extracted from synaptosomal plasma membrane vesicles (SPMVTL). All conditions were as described in the legend to Fig. 1.

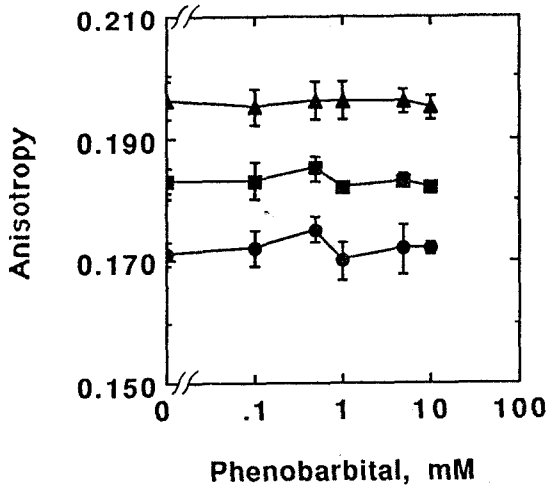

Fig. 4. Phenobarbital does not alter the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the model membranes of total lipids extracted from synaptosomal plasma membrane vesicles (SPMVTL). All conditions were as described in the legend to Fig. 1.

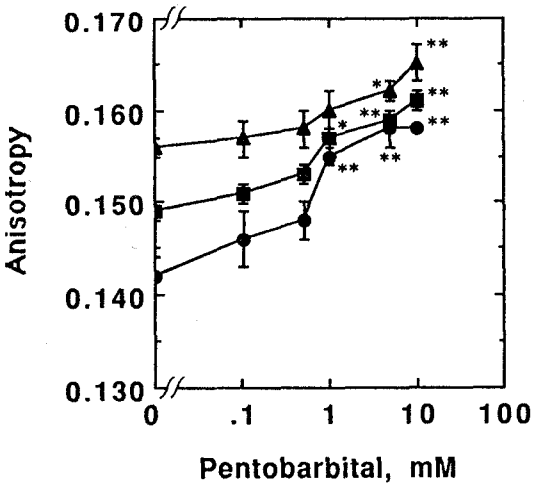

Fig. 5. Pentobarbital alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles (SPMVPL). All conditions were as described in the legend to Fig. 1. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

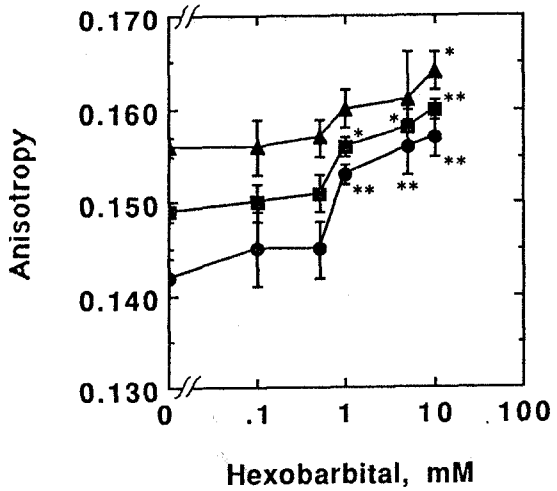

Fig. 6. Hexobarbital alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles (SPMVPL). All conditions were as described in the legend to Fig. 1. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

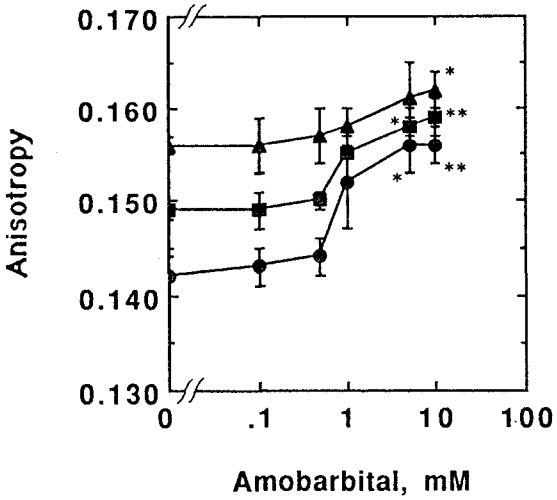

Fig. 7. Amobarbital alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles (SPMVPL). All conditions were as described in the legend to Fig. 1. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control by Student's t-test.

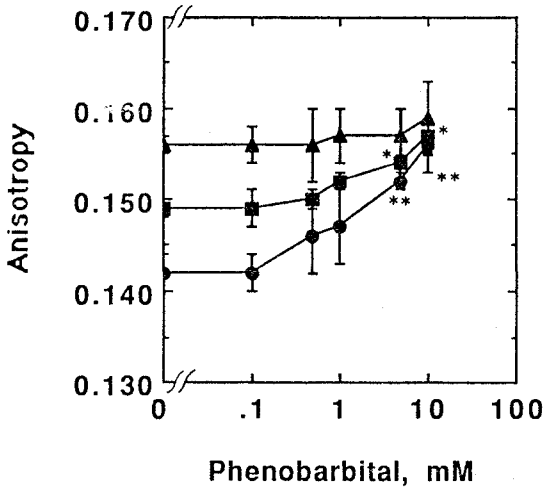

Fig. 8. Phenobarbital alters the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in outer monolayer of the model membranes of phospholipids extracted from synaptosomal plasma membrane vesicles. All conditions were as described in the legend to Fig. 1. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$. respectively, compared to control by Student's t-test.

Table 3. Effects of barbiturates 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 (SPMVPL)

Barbiturates (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
Pentobarbital (1.0)	0.109±0.001*	0.113±0.003	0.107±0.001**	0.550±0.003*	0.558±0.007	0.542±0.003**
Pentobarbital (5.0)	0.112±0.002**	0.116±0.002*	0.110±0.002**	0.557±0.004**	0.566±0.004*	0.550±0.006**
Hexobarbital (1.0)	0.108±0.002*	0.113±0.003	0.104±0.001**	0.546±0.004*	0.558±0.007	0.535±0.003**
Hexobarbital (5.0)	0.111±0.003*	0.115±0.007	0.108±0.004**	0.553±0.008*	0.563±0.017	0.546±0.010**
Amobarbital (5.0)	0.111±0.003*	0.114±0.005	0.108±0.004*	0.553±0.007*	0.561±0.013	0.548±0.011*
Amobarbital (10.0)	0.111±0.003*	0.116±0.003*	0.108±0.003**	0.555±0.006**	0.565±0.007*	0.546±0.007**
Phenobarbital (5.0)	0.106±0.002*	0.110±0.004	0.102±0.000**	0.540±0.004*	0.550±0.009	0.531±0.001**
Phenobarbital (10.0)	0.110±0.003*	0.112±0.006	0.108±0.004**	0.550±0.008*	0.554±0.015	0.548±0.010**

All conditions were as described in the legend to Table 2. 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.

Fig. 1-4 show that barbiturates did not affect the anisotropy of DPH in the transbilayer fluidity domains of SPMVTL. In contrast, barbiturates increased the anisotropy (r) of DPH in the bulk SPMVPL (Fig. 5-8, closed squares) in a dose-dependent manner. This indicates that barbiturates

decreased the rotational diffusion of DPH in SPMVPL. The bulk membrane-ordering potencies were in the order: pentobarbital > hexobarbital > amobarbital > phenobarbital. In the model membranes of PC (SPMVPC) (Kang *et al.*, 1992), PE (SPMVPE) (Yun *et al.*, 1990a), PS (SPMVPS) (Kang *et al.*, 1992), and PI (SPMVPI) (Kang *et al.*, 1992) fractions extracted from SPMV, the same results were obtained. Barbiturates showed a greater ordering effect on the outer monolayer (Fig. 5-8, closed circles) as compared to the inner monolayer (Fig. 5-8, closed triangles) of SPMVPL. Since changes observed in the anisotropy derive primarily from changes in limiting anisotropy, we studied the selective effects of barbiturates on the range component of DPH motion. The selective effects of barbiturates on the limiting anisotropy and order parameter of DPH are shown in Table 3. Barbiturates preferentially increased the limiting anisotropy and order parameter of DPH calculated for the outer monolayer.

DISCUSSION

The methods of quantifying membrane fluidity can be categorized as probe-dependent and probe-independent. In probe-independent techniques, including differential scanning calorimetry (DSC), X-ray diffraction, electron diffraction, light scattering, and certain applications of nuclear magnetic resonance (NMR), possible perturbation of the bilayer by a foreign molecule is avoided. However, they are relatively insensitive and require larger samples; the signal-to-noise ratio is low and the resulting signals are complex and difficult to interpret; the data obtained may provide only a limited and indirect assessment of fluidity. Thus, for studies of biological membranes, probe-dependent spectroscopic methods, which utilize fluorescence, electron spin resonance, and NMR, have proven particularly effective. 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 (Shinitzky and Barenholz, 1978). The covalently linked trinitrophenyl group displays a broad absorbance with a maximum near 420 nm. This absorption peak has a large overlap with 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.

The increase in anisotropy (r), limiting anisotropy (r_∞), and order parameter (S) of DPH indicates that barbiturates decreased the rotational diffusion of DPH in SPMVPL. In contrast, barbiturates did not affect the rotational diffusion of DPH in SPMVTL lipid bilayers. Exploiting intramolecular excimer fluorescence of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of DPH, we also demonstrated that barbiturates fluidized intact SPMV (unpublished data), and had ordering effects on SPMVPC (Kang *et al.*, 1992), SPMVPE (Yun *et al.*, 1990), SPMVPS (Kang *et al.*, 1992), and SPMVPI (Kang *et al.*, 1992).

At 37°C, the excimer to monomer fluorescence intensity ratios of Py-3-Py in intact SPMV, SPMVTL, SPMVPL, SPMVPC, SPMVPE, SPMVPS, and SPMVPI were 0.493 ± 0.008, 0.606 ± 0.007, 0.790 ± 0.010, 0.811 ± 0.009, 0.804 ± 0.009, 0.807 ± 0.008, and 0.810 ± 0.009, respectively. And the anisotropy values of DPH in intact SPMV, SPMVTL, SPMVPL, SPMVPC, SPMVPE, SPMVPS, and SPMVPI were 0.202 ± 0.001, 0.183 ± 0.002, 0.149 ± 0.001, 0.146 ± 0.002, 0.152 ± 0.001, 0.150 ± 0.001, and 0.149 ± 0.002, respectively. In other words, barbiturates fluidized the most ordered membranes (intact SPMV), did not affect the membranes of medium fluidity (SPMVTL), and ordered the most fluid membranes (SPMVPL, SPMVPC, SPMVPE, SPMVPS, and SPMVPI). This indicates that barbiturates function to modulate the fluidity of neuronal membranes to a certain level. Our results are in general agreement with the studies of Harris and Schroeder (1982) and Pang and Miller (1978). However, barbiturates have been demonstrated to lower the phase transition temperature of pure phospholipid model membranes (Lee, 1976; Yu *et al.*, 1990). This is not consistent with the results of the present study. The differences in the results cannot be fully explained, but may be probably due to the differences in detection methods. Moreover, it seems likely that the phase transition temperature might not be directly related to membrane fluidity.

The experiments with shape changes in red blood cells after treatment with charged amphipaths suggested that the asymmetry of composition produced asymmetric interaction with the

lipids of two monolayers. Electrostatic interactions, with cationic amphipaths preferentially interacting with the anionic inner monolayer, was the proposed mechanism (Sheetz and Singer, 1974). The Sheetz-Singer hypothesis has been tested only indirectly. First, the activities of enzymes of known transbilayer orientation (Houslay and Palmer, 1978; Houslay *et al.*, 1980; Dipple *et al.*, 1982), or fluorescent or spin-labelled hydrophobic compounds (Salesse *et al.*, 1982) were used as probes of lipid order. Arrhenius plots produce an inflection in slope of the parameter temperature curve, at the presumptive phase change. Combining this with the correlation between fluidity and phase transition temperature of pure lipids, the specificity of amphipath-membrane interaction could be determined. However, the correlation between fluidity and phase transition temperature is weak, particularly across lipid classes. Further, the extent of coupling between lipid order and enzyme activity is not well quantified, and many soluble enzymes, without a lipid structure bounding them, exhibit Arrhenius discontinuities. Also, the change in lipid order cannot be quantitated. Direct evidence using differential polarized phase fluorometry on LM fibroblast plasma membranes was recently obtained (Kier *et al.*, 1986; Sweet and Schroeder, 1986).

As shown in Fig. 5-8, barbiturates preferentially increased the anisotropy, limiting anisotropy, and order parameter of DPH in the outer monolayer of SPMVPL. To our knowledge, the results reported herein demonstrated for the first time that the Sheetz-Singer hypothesis (1974) may be valid for phospholipid model membranes.

Cholesterol seems to have a significant role in the membrane actions of barbiturates. Early experiments recognized that cholesterol acts to buffer the fluidity of model phospholipids (Oldfield and Chapman, 1972). Above the thermal transition temperature of lipid, cholesterol increases ordering; conversely, cholesterol incorporation below the lipid melting point serves to fluidize the mixture. Although the stoichiometry of phospholipid-cholesterol interactions is still controversial, recent studies on either model or native membranes show that cholesterol does not ideally mix with phospholipids. Cholesterol-rich and cholesterol-poor domains were detected in binary mixtures of cholesterol and phospholipid using electron microscopy (Copeland and McConnell, 1980), electron diffraction (Hui and Parsons, 1975), fluorescent cholesterol analogs (Smutzer and Yeagle, 1985), and DSC (Estep *et al.*, 1978). Moreover, DSC experiments on ternary mixtures indicate that cholesterol has varying affinities for phospholipids. van Dijck (1979) reported that phospholipids show a decrease in affinity for cholesterol in the following order: SP$\gg$PS, phosphatidylglycerol$>$PC$\gg$PE. The nonuniform distribution of cholesterol may partly explain that the differences in transbilayer fluidity asymmetry in SPMVTL were larger than those in SPMVPL (Table 2).

The fluidity change induced by barbiturates may be correlated with a change in ion permeability. Pang *et al.* (1979) have shown that relatively small changes in membrane fluidity resulted in substantial changes in ion permeability, suggesting the existence of a mechanism for amplifying the weak perturbations produced by low concentrations of barbiturates. There is now substantial support for a γ-aminobutyric acid (GABA)-mediated chloride uptake role in the actions of barbiturates (Nicoll, 1980; Yu and Ho, 1990). It is generally assumed that barbiturates enhance and mimic GABAergic inhibition via opening or prolonging the lifetime of chloride channels, primarily acting through allosteric modulatory sites on the receptor-ionophore complex. Allan and Harris (1986) reported that the anesthetic potencies of the barbiturates were significantly correlated with the potencies for enhancement of GABA-stimulated ^{36}Cl uptake. The order of membrane-ordering potencies in the present study is in good agreement with Allan and Harris (1986), suggesting a close relationship between the membrane-perturbing effects of barbiturates and chloride fluxes across SPMV.

In summary, barbiturates appear to modulate the fluidity of of neuronal membranes to a certain level, which in turn facilitates chloride fluxes. There is also a possibility that the specific fluidity induced by barbiturates might help the interaction of them with chloride channels. Even though direct evidence for interaction between membrane perturbation and chloride fluxes should be elucidated, the results of the present study, together with our previous reports, strongly suggest that barbiturates act, in part, their pharmacological effects by modulating the fluidity of neuronal membranes to a specific level, preferentially acting on the positively charged outer monolayer.

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=국문초록=

Barbiturates가 소의 대뇌피질 Synaptosomal Plasma Membrane Vesicles로 부터 추출 제제한 총지질 및 총인지질 인공세포막에 형성된 비대칭적 유동성에 미치는 비대칭적 영향

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한국산 2년생 소의 신선한 대뇌피질로 부터 synaptosomal plasma membrane vesicles (SPMV)를 분리한 후 이 SPMV로 부터 추출한 총지질 (SPMVTL) 및 총인지질 (SPMVPL)로서 인공세포막을 제제하였다. SPMVTL 및 SPMVPL의 outer monolayer에 trinitrophenyl groups로 공유결합시킴으로써 형광 probe 1,6-diphenyl-1,3,5-hexatriene (DPH)의 outer monolayer 형광을 선택적으로 소광케한 후 SPMVTL 및 SPMVPL의 inner와 outer monolayer 사이의 비대칭적 유동성 존재 유무를 확인하였던 바 inner monolayer에 비하여 outer monolayer가 유동성이 크다는 것을 확인하였으며 SPMVTL에 비하여 SPMVPL의 유동성이 높았다. 뿐만 아니라 barbiturates는 SPMVTL의 유동성에 대하여는 유의성이 있을 만큼의 증감 효과를 나타내지 않았고 SPMVPL의 유동성은 감소시키되 주로 outer monolayer의 유동성을 감소시킨다는 것을 확인하였으며 barbiturates 종류에 따른 SPMVPL의 총유동성 감소의 크기는 pentobarbital > hexobarbital > amobarbital > phenobarbital의 순이었다.