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Membrane-Ordering Effects of Barbiturates on Pure Phospholipid Model Membranes

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Abstract □ Intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to investigate the effects of barbiturates on the fluidity of model membranes of phosphatidylcholine (SPMVPC), phosphatidylserine (SPMVPS), and phosphatidylinositol (SPMVPI) fractions of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex. In a dose-dependent manner, barbiturates decreased the excimer to monomer fluorescence intensity ratio (I'/I) of Py-3-Py and increased the anisotropy(r), rotational relaxation time(ρ), limiting anisotropy(r_∞), and order parameter (S) of DPH in SPMVPC, SPMVPS and SPMVPI. This indicates that barbiturates decreased both the lateral and rotational diffusion of the probes in SPMVPC, SPMVPS and SPMVPI. The relative potencies of barbiturates in ordering the membranes were in the order: pentobarbital>hexobarbital>amobarbital>phenobarbital. This order correlates well with the anesthetic potencies of barbiturates and the potencies for enhancement of γ-aminobutyric acid-stimulated chloride uptake. Thus, it is strongly suggested that a close relationship might exist between the membrane-ordering effects of barbiturates and the chloride fluxes across SPMV.

Keywords □ Barbiturates, membrane-ordering effects, fluorescent probe technique.

With increasing frequency, it is being recognized that the effects of drugs on the physical state or fluidity of biological membranes are no less important than the drug-specific receptor interaction in studying the mechanism of action of drugs, especially those having anesthetic and tranquillizing actions. The fluidity of the lipid bilayer component of biological membrances has been shown to influence a number of cellular functions, including carrier-mediated transport, the properties of certain membranes-bound enzymes, binding to the insulin and opiate receptors, phagocytosis, endocytosis, depolarization-dependent exocytosis, immunologic and chemotherapeutic cytotoxicity, prostaglndin production and cell growth[1,2].

In support of the membrane hypothesis, there is an excellent correlation between many diverse *in vitro* actions of barbiturates and their lipid solubilities[3]. There is also evidence that barbiturates penetrate into membrane lipid and alter the fluidity of the membrane. Phenoibarbital and pentobarbital have been shown to form hydrogen bonds with phosphatidylcholine (PC), a major lipid component of brain membrane[4]. Exploiting fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), Harris and Schroeder[5] have shown that barbiturates increased the fluidity of hydrophobic core of synaptic membranes prepared from mouse brain. In contrast, one electron spin resonance (ESR) study indicates that thiopental increases synaptic membrane surface rigidity without affecting membrane core fluidity[6]. Studies with ESR probes indicate phospholipid-cholesterol vesicle fluidization by barbiturates, although pure phospholipids or phospholipids

mixed with small amount of cholesterol appear to be made more rigid by barbiturate[7]. However, barbiturates have been shown to decrease the temperature of the gel-to-liquid crystalline phase transition of PC[8], phosphatidylethanolamine (PE)[8], dipalmitoylphosphatidylcholine (DPPC)[9] and DPPC-cholesterol[9] multilamellar vesicles. Therefore, it seems likely that barbiturates do not have relevant membrane-ordering or membrane-disordering actions and the lipid composition of the bilayer strongly affects its response to barbiturates.

In the present study, exploiting intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py-3-Py) and fluorescence polarization of DPH, we examined the effects of barbiturates on the fluidity of model membranes of PC (SPMVPC), phosphatidylserine (PS) (SPMVPS) and phosphatidylinositol (PI) (SPMVPI) fractions of synaptosomal plasma membrane vesicles (SPMV) isolated from bovine cerebral cortex.

EXPERIMENTAL METHODS

Materials

The fluorescent probe Py-3-Py was prepared by the previously reported synthesis[10]. The other fluorescent probe DPH and barbiturates were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest quality avaliable and water was deionized.

Membrane preparations

The SPMV were isolated from bovine cerebral cortex and characterized by the formerly reported method in our laboratory[11,12]. The specific activities of Na, K-ATPase, acetylcholinesterase and 5′-nucleotidase were about 5.6-fold, 2.5-fold and 3.3-fold respectively, enriched in the plasma membrane fraction as compared to crude homogenate. The phospholipids (PC, PS and PI) were extracted from SPMV as decribed previously[11]. The individual phospholipid classes were quantitated by measuring the amount of inorganic phosphate after hydrolysis of phospholipids at 180℃ in 70% $HClO_4$. The model membranes (SPMVPC, SPMVPS and SPMVPI) were prepared, separated and suspended in phosphate-buffered saline (PBS) (pH 7.4) by the procedure described earlier[10].

Fluorescence measurements

The incorporation of Py-3-Py was carried out by adding aliquots of a stock solution of 5×10^{-5}M in ethanol to model membranes (0.70 mg of phospholipids/m*l*), so that the final probe concentration was less than 5×10^{-7}M. The mixtures were initially vigorously vortexed for 10 sec at room temperature and then incubated at 4℃ for 18 h under gentle stirring. The fluorescent probe DPH was dissolved in tetrahydrofuran and a volume and a volume of 0.5 μ*l* of tetrahydrofuran per m*l* of PBS was added directly to the membrane suspension at a concentration of 1 μg//70 μg phospholipids as described previously[10]. After incorporation of the probes, the membrane suspsension 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 of Py-3-Py was 330 nm and the excimer to monomer fluorescence intensity ratio (I′/I) was calculated from the 480 nm tp 379 nm signal ratio. The excitation wavelength for DPH was 362 nm and fluorescence emissioin was read at 424 nm. All fluorescence measurements were obtained with a T-format SLM-4800 subnanosecond spectrofluorometer (SLM Instruments Inc., Champaign-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 Py-3-Py or DPH, served as controls for the fluorometric measurements.

The intensity of the components of the fluorescence that were parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. The polarization (P) was obtained from intensity measurements using $P=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+GI_{\perp})$ where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal components when the exciting light is polarized in the horizontal direction. The polarization was expressed as the anisotropy [$r=2P/(3-P)$], limiting anisotropy (r_∞), and order para-

meter (S). The limiting anisotropy (r^{∞}) was determined directly from the anisotropy value using the following relationship[13];

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

The limiting anisotropy reflects restriction to probe motion and can be converted to an order parameter, $S = (r_{\infty}/r_o)^{1/2}$ [14] where r_o, the anisotropy in the absence of motion, is equal to 0.362 for DPH[15]. Fluorescence lifetimes (τ) were measured with a SLM-4800 using modulation frequencies of 6, 18 and 30 MHz. Fluorescence lifetimes were measured with excitation polarizer set at 0 and the emission polarizers set at 55℃ in order to correct for instrumentally induced anisotropy (grating correction). Fluorescence lifetimes were measured relative to a reference solution of dimethyl p-bis[2-(5-phenyloxazolyl)] benzene (dimethyl POPOP) in absolute ethanol as described previously[10,16]. The rotational relaxation time (ρ) was calculated from the Perrin equation[17];

$$\left(\frac{1}{r} \cdot \frac{1}{3}\right) = \left(\frac{1}{r_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

Barbiturates were dissolved in a minimum volume of 0.1 N-NaOH, diluted with deionized water and the pH adjusted to 9 to 10. Barbiturates, at the desired concentrations, were added directly to SPMVPC, SPMVPS and SPMVPI resuspended in PBS. The pH of the buffered sample was not changed significantly by addition of barbiturates. Measurements commenced usually within 1 min after addition. No effect of longer incubation times was noted.

RESULTS

Effects of barbiturates on the excimer to monomer fluorescence intensity ratio of Py-3-Py in SPMVPC, SPMVPS and SPMVPI

intramolecular excimer formation of Py-3-Py was used to evaluate the effects of pentobarbital, hexobarbital, amobarbital and phenobarbital on the organization of SPMVPC, SPMVPS and SPMVPI. The advantage of intramolecular over intermolecular excimer probes lies primarily in the possibility that the former offers to use the probe molecule in very small concentrations, 10^{-6} M and lower[18,19]. This minimizes the perturbation of the medium under evaluation and avoids the formation of probe aggregates, a phenomenon that has been encountered with pyrene[20]. At low temperatures, or at sufficiently high local viscosities, the excimer to monomer fluorescence intensity ratio of Py-3-Py, I′/I, is primarily determined by the rate constant of excimer formation, since the radiative rate constants and the excimer lifetime have been found to be essentially independent of temperature[18]. The excimer to monomer fluorescence intensity ratio of Py-3-Py, I/I, increases with the lateral diffusion of the viscous media[1] and has been used to monitor the fluidity changes and phase transitions of phospholipid vesicles[18,21,22], micelles[20] and biological membranes[10,19,21-23]. In a dose-dependent manner, barbiturates decreased the excimer to monomer fluorescence intensity ratio of Py-3-Py in SPMVPC, SPMVPS and SPMVPI (Fig. 1). This indicates that barbiturates decreased the lateral diffusion or the mobility of this hydrophobic probe within SPMVPC, SPMVPS and SPMVPI[1]. In all model membranes, the relative potencies of barbiturates to decrese the ratio were in the order: pentobarbital>hexobarbital>amobarbital>phenobarbital (according to ANOVA test, $p<0.05$).

Effects of barbiturates on the fluorescence polarization of DPH in SPMVPV, SPMVPS and SPMVPI

In order to confirm the results of intramolecular excimer fluorescence technique, the estimation of steady-state fluorescence polarization, using DPH as a lipid probe, was also carried out. The fluorescence polrization mainly reflects the rotational diffusion of lipid fluorophores. DPH has many of the characteristic 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 intoi lipid assemblies and it is practically noinfluorescent in aqueous media. DPH absorption and emission dipoles are almost parallel, so that its maximal limiting anisotropy (0.362) is close to the theoretical maximum. As shown in Fig. 2, the results were in consistent with those obtained by excimer fluorescence technique. Barbiturates decreased the fluorescence anisotropy of SPMVPC, SPMVPS and SPMVPI-bound DPH in a concentration-dependent ma-

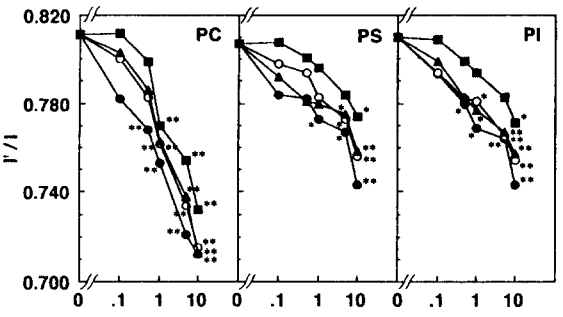

Fig. 1. Effects of barbiturates on the excimer to monomer fluorescence intensity ratio, I/I, of 1,3-di(1-pyrenyl)propane in the phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) model membranes. Fluorescence measurements were performed at 37℃ (pH 7.4). Each point represents the mean of 5 determinations. An asterisk and double asterisk signify $p<0.05$ and $p<0.01$, respectively, compared to control according to Student's t-test. ●, pentobarbital; ○, hexobarbital; ▲, amobarbital; ■, phenobarbital.

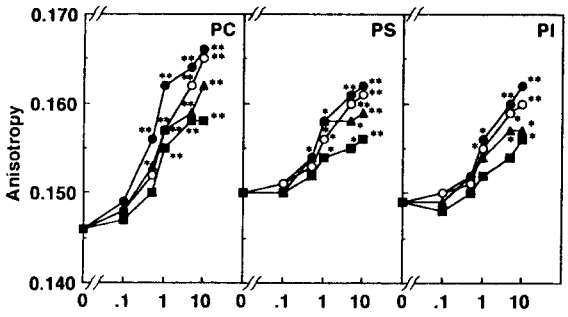

Fig. 2. Effects of barbiturates on the anisotropy (r) 1,6-diphenyl-1,3,5-hexatriene of in the phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) model membranes. Fluorescence measurements were performed at 37℃ (pH 7.4). Each point represents the mean of 4 determinations. An asterisk and double asterisk signify $p<0.05$ and $p<0.01$, respectively, compared to control according to Student's t-test. ●, pentobarbital; ○, hexobarbital; ▲, amobarbital; ■, phenobarbital.

nner. The relative potencies of barbiturates to decrease the rotational diffusion of the model membranes followed the order: pentobarbital>hexobarbital >amobarbital>phenobarbital (according to ANOVA test, $p<0.05$). Thus, this experiment coinfirms that barbiturates order the hydrocarbon region of the pure phospholipid model membranes.

According to a theory of Perrin recently modified by several authors[24], the anisotropy r is made up of two components. The first components (r_∞) is known as the order, static, or range component. The second omponent (r_f) is referred to as either the rate, microviscosity, or dynamic component.

$$r = r_\infty + r_f = r_\infty + \frac{r_o - r_\infty}{1+\tau/\phi}$$

where ϕ is the rotational correlation time, which varies inversely with the speed of rotation and r_∞ is the limiting anisotropy. From the above equation, it is evident that the changes is anisoitropy (r) may reflect the alterations in the rate and/or range of rotational motion of the fluorophore or may be due to the alterations in the lifetime of the excited state (τ). Moreover, if direct quenching of DPH by barbiturates occurred, fluorescence lifetime would decrease. To distinguish among these possibilities, we

Table I. Effects of barbiturates on the lifetime (τ) of 1,6-diphenyl-1,3,5-hexatriene in pure phospholipid model membranes

Barbiturate(mM)	Lifetime(nsec)		
	SPMVPC	SPMVPS	SPMVPI
Control	10.18± 0.12	10.03± 0.18	10.12± 0.14
Pentobarbital(5.0)	9.95± 0.09	9.95± 0.08	9.82± 0.09
Hexobarbital(10.0)	10.10± 0.11	9.85± 0.07	9.86± 0.13
Amobarbital(5.0)	9.95± 0.09	9.94± 0.13	10.10± 0.11
Phenobarbital(10.0)	9.97± 0.09	9.83± 0.08	10.05± 0.17

Lifetime measurements were performed as described in Methods. Values represent the mean± SEM of 4 determinations.

examined the effects of barbiturates on the excited-state lifetime (τ) of DPH. Further, calculation of rotational relaxation tiems ($\rho=3\phi$) from fluorescence anisotropies requires knowledge of the fluorescence lifetime. As shown in Table I, the fluorescence lifetimes of DPH were not changed significantly even by high doses of these barbiturates in the model membranes. Therefore, it is clear that the changes in anisotropy result from variations in the rate and/or range of rotational motion of DPH. In addition, the possibility of direct quenching of DPH

fluorescence by barbiturates is ruled out. By contrast, the rotational relxation times (ρ) of DPH were significantly increased in the model membranes (Fig. 3). 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[25]. Hence, the alterations in the anisotropy (r) or the rotational relaxation time (ρ) are likely to be due to changes in range of motion of the probe. Accordingly, we studied the effects of barbiturates on the range component of DPH motion in more detail. The result corroborates the above point (Table II).

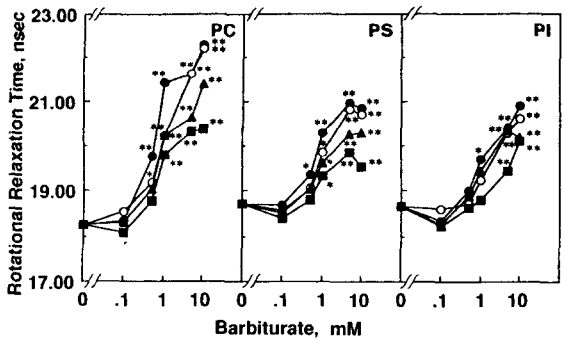

Fig. 3. Effects of barbiturates on the rotational relaxation time (ρ) of 1,6-diphenyl-1,3,5-hexatriene of in the phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) model membranes. Fluorescence measurements were performed at 37 ℃ (pH 7.4). Each point represents the mean of 4 determinations. An asterisk and double asterisk signify $p<0.05$ and $p<0.01$, respectively, compared to control according to Student's t-test. ●, pentobarbital; ○, hexobarbital; ▲, amobarbital; ■, phenobarbital.

DISCUSSION

The methods of quantifying membrane fluidity can be categorized as probe-independent and probe-dependent. In probe-independent techniques, including calorimetry, 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, the non-probe methods exhibit other limitations particularly in relation to studies of biological membranes. 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. So, for studies of biological membranes, probe-dependent spectroscopic methods, which utilize fluorescence, ESR and NMR, have proven particularly effective. However, it bears emphasis that possible errors to the use of exogenous probes must be evaluated in each type of application[1]. Fluorescence methods have the important advantages of great sensitivity, versatility and simplicity of instrumentation. The estimation of excimer fluorescence intensity and fluorescence polarization is particularly simple and dependable for the assessment of membrane fluidity. Given the anisotropic lipid environment of the bilayer membrane, it is useful to characterize the fluidity of a given membrane in terms of several modes of motion. The fluorescence polarization and the excimer fluorescence reflect the ro-

Table II. Effects of barbiturates on limiting anisotropy(r_∞) and order parameter(S) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in pure phospholipid model membranes

Barbiturate(mM)	Limiting anisotropy (r_∞)			Order Parameter (S)		
	PC	PS	PI	PC	PS	PI
Control	0.095± 0.003	0.100± 0.002	0.099± 0.002	0.512± 0.008	0.526± 0.005	0.522± 0.006
Pentobarbital(1.0)	0.116± 0.002**	0.110± 0.004*	0.108± 0.002*	0.565± 0.006**	0.552± 0.009*	0.546± 0.006*
Hexobarbital(1.0)	0.110± 0.003**	0.108± 0.004*	0.105± 0.002	0.550± 0.008**	0.547± 0.011*	0.537± 0.005
Amobarbital(1.0)	0.109± 0.002**	0.109± 0.002*	0.106± 0.002	0.550± 0.004**	0.550± 0.006*	0.540± 0.006
Phenobarbital(1.0)	0.106± 0.001**	0.105± 0.003	0.102± 0.002	0.542± 0.003**	0.538± 0.004	0.531± 0.007

Limiting anisotropy (r_∞) and order parameter (S) of DPH were calculated as described in Methods. Values represent 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.

tational diffusion and lateral diffusion, respectively, of the fluorophores. However, the structural organization of membrane lipids is highly heterogeneous and therefore any absolute approach to lipid fluidity is of a formidable complexity. As with all other biophysical methods, the resolution by this method of the highly heterogeneous fluidity regions in biological membranes is at best only partial.

The decrease in I'/I of Py-3-Py and the increase in anisotropy (r), rotational relaxation time (ρ), limiting anisotropy (r_∞) and order parameter (S) of DPH indicate that barbiturates decreased both the lateral and rotational diffusion in SPMVPC, SPMVPS and SPMVPI. In the model membranes of PE (SPMVPE), the same results were obtained[10]. Exploiting intramolecular excimer fluorescence and fluorescence polarization of DPH, we also demonstrated that barbiturates fluidized intact SPMV, had ordering effects on the liposomes of SPMV phospholipids (SPMVPL) in a dose-dependent manner and had little effects on the liposomes of SPMV total lipids ((SPMVTL)[22]. At 37℃, the I'/I values of Py-3-Py in intact SPMV, SPMVTL, SPMVPL, SPMVPC, SPMVPE, SPMVPS and SPMVPI were 0.489± 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 vlaues 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 order words, barbiturates fluidized the most ordered membranes (intact SPMV), did not affect the membranes of medium fluidity (liposomes of SPMV total lipids) 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[5] and Pang and Miller [7]. However, barbiturates have been demoinstrated to lower the phase transition temperature of pure phospholipid model membranes[8,9]. 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 fluidity change induced by barbiturates may be correlated with an increase in ion permeability. Pang *et al.*[26] have shown that relatively small changes in membrane fludity resulted in substantial changes in ion permeability, suggesting the existence of a mechanism for amplifying the weak membrane perturbations produced by low concentrations of barbiturates. There is now substantial support for a GABA-mediated chloride uptake role in the actions of barbiiturates[27]. It is generally assumed that barbiturates enhance and mimic GABA-ergic inhibition *via* opening or prolonging the lifetime of chloride channels, primarily acting through allosteric modulatory sites on the receptor-ionophore complex. Allan and Harris[28] 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[28], suggesting a close relationship between the membrane-perturbing effects of barbiturates and the chloride fluxes acrose SPMV.

In summary, barbiturates appear to modulate the fluidity 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 the 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.

ACKNOWLEDGEMENTS

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