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Effects of Barbiturates on the Rotational Relaxation Time of 1,6-Diphenyl-1,3,5-hexatriene in Native and Model Membranes

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Abstract □ Synaptosomal plasma membrane vesicles (SPMV) were isolated from fresh bovine cerebral cortex. The effects of barbiturates on the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) in intact SPMV and model membranes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from SPMV were examined. Barbiturates decreased the rotational relaxation time of DPH in intact SPMV in a dose-dependent manner. In contrast, they did not affect the rotational relaxation time of DPH in SPMVTL and even dose-dependently increased the rotational relaxation time of DPH in SPMVPL.

Keywords □ Barbiturates, native and model membranes, fluidity, fluorescent probe technique.

The physical state of membrane lipids has been shown to influence such membrane enzymes as Na^+, K^+-ATPase, hormone responsive adenylate cyclase and membrane transport processes such as glucose and amino acid uptake[1]. Membrane lipids also play an important role in membrane permeability to sodium, calcium and potassium[1].

In support of the membrane hypothesis, there is an excellent correlation between many diverse *in vitro* actions of barbiturates and their lipid solubilities[2]. There is also evidence that barbiturates penetrate into membrane lipids and alter the fluidity of the membrane[3-8]. However, it seems likely that barbiturates do not have relevant membrane-disordering actions, and the lipid composition of the bilayer strongly affects its response to barbiturates.

Few studies have reported the effects of barbiturates on the rotational relaxation time of native and model membranes. In the present study, to get a better insight into the molecular mechanism of action of barbiturates, the effects of barbiturates on the rotational relaxation time of native and model membrane bilayers were investigated employing 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence technique.

EXPERIMENTAL METHODS

Chemicals

DPH was obtained from Molecular Probes (Junction City, OR, USA). Barbiturates, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepees), Ficoll (70,000 M.W.), Sepharose, 1,4-bis[4-methyl-5-phenyl-2-oxazolyl]benzene (dimethyl-POPOP) 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.

Preparation of synaptosomal plasma membrane vesicles (SPMV)

The SPMV were isolated from bovine cerebral cortex and characterized by the formerly reported method in our laboratory[9,10]. The purity of SPMV was determined by enzymatic and morphological standards. The specific activities of Na^+, K^+-ATPase[9,10], acetylcholinesterase[11] and 5'-nucleotidase[9,10] were about 6-, 2.5- and 3-fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenates. Electron microscopic examination also showed that the membranes were in vesicular form[10]. Protein was determined by the method of Lowry *et al.*[12] using BSA as a standard.

Model membrane preparations

Total lipids and phospholipids were extracted from the SPMV as described previously[9]. Phospholipids were quantitated by measuring the amounts of inorganic phosphate[13] after hydrolysis of the phospholipids at 180℃ in 70% $HClO_4$[14]. The cholesterol to phospholipid molar ratio was 0.59 : 1, and the major phospholipids were phosphatidylcholine (43.6%), phosphatidylethanolamine (35.8%) and phosphatidylserine (13.6%). Phosphatidylinositol (2.9 %), sphingomyelin (4.2%) and lysophosphatidylcholine (1.0%) were relatively minor components while phosphatidylglycerol was absent[9]. Large unilamellar liposomes (0.70 mg of lipids/m*l*) were prepared with total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from SPMV by the reverse-phase-evaporation technique[15].

Fluorescence measurements

The fluorescent probe DPH was dissolved in tetrahydrofuran and a volume of 0.5 μ*l* of tetrahydrofran per m*l* of 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 directly to the membrane suspension at a concentration of 1 μg/70 μg of phospholipids as described previously[15]. After incorporation of the probe, the membrane suspension was placed in cuvettes. Control levels of fluorescence were 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 fluorescence measurements were obtained with a T-format subnanosecond spectrofluorometer (SLM Aminco Instruments, Inc., Urbana, IL, USA) and performed at 37℃. Fluorescence lifetime was measured with an 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-POPOP in absolute ethanol as described previously[16]. This solution, rather than the usual light scattering solution, minimizes wavelength and geometry-dependent time responses of the photomultiplier tubes. The fluorescence lifetime of dimethyl-POPOP in ethanol at 24℃ was 1.45 nsec. Because of this lifetime, the phase angle (θ_R) of dimethyl-POPOP lags behind the exciting light by 3.13° at 6 MHz, 9.13° at 18 MHz and 15.29° at 30 MHz. Phase angles can be corrected for these shifts and are therefore absolute phase angles relative to the phase of the modulated exciation.

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) [$r=2P/(3-P)$]. Alterations in anisotropy were converted to rotational relaxation time, $\bar{P}$, calculated from the Perrin equation[17]: $(1/r-1/3)=(1/r_o-1/3)(1+3\tau/\bar{P})$. In this equation, r_o, the maximal limiting anisotropy of the probe determined under conditions where it can not rotate, is equal to 0.362 for DPH[18], and τ is the fluorescence lifetime of the excited state.

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.

RESULTS

As shown in Table I, the rotational relaxation times ($\bar{P}$) of DPH were shorter in SPMVTL than in intact SPMV, and $\bar{P}$ in SPMVPL was even shorter than those in both SPMV and SPMVTL. Barbiturates did not alter the excited-state lifetimes of DPH in either intact SPMV, SPMVTL or SPMVPL. In a concentration-dependent manner, barbiturates significantly decreased the ratational relaxation times ($\bar{P}$) of DPH in the intact SPMV (Fig. 1). The membrane-disordering potencies followed the order: pentobarbital>hexobarbital>amobarbital>phenobarbital (according to ANOVA test, $p<0.01$). In contrast, the rotational relaxation times ($\bar{P}$) of DPH in SPMVTL were not significantly affected by these barbiturates (Fig. 2). Although the effects were smaller than in intact SPMV, barbiturates even produced a dose-dependent increase in the rotational relaxation time ($\bar{P}$) of DPH in SPMVPL (Fig. 3). The order of membrane-ordering potencies of the barbiturates in SPMVPL was consistent with that of membrane-disordering potencies in SPMV.

DISCUSSION

Numerous techniques have been developed to quantitate the physical state of the membrane. The term "membrane fluidity" has evolved to express this physical state, and a useful functional definition of membrane fluidity is the selective motional freedom of lipid molecules within the bilayer. The fluorescence polarization reflects rotational diffusion of fluorophores. Determination of steady-state fluorescence anisotropy of the probe DPH is the easiest and most frequently used method to measure membrane fluidity. In the present study, the data are expressed as the anisotropy (r) since anisotropies are additive and most theoretical expressions are considerably simpler when expressed in terms of this parameter.

Membrane fluidity is determined by several factors including the fatty acyl chain itself, phospholipids, cholesterol, proteins, temperature and pressure[19]. Proteins and neutral lipids seem to have a significant role in the rotational relaxation time ($\bar{P}$) of membrane lipid bilayer (Table I).

Recently Harris and Groh[20] suggested that ganglioside content and/or composition of biological membranes may be one of the primary factors

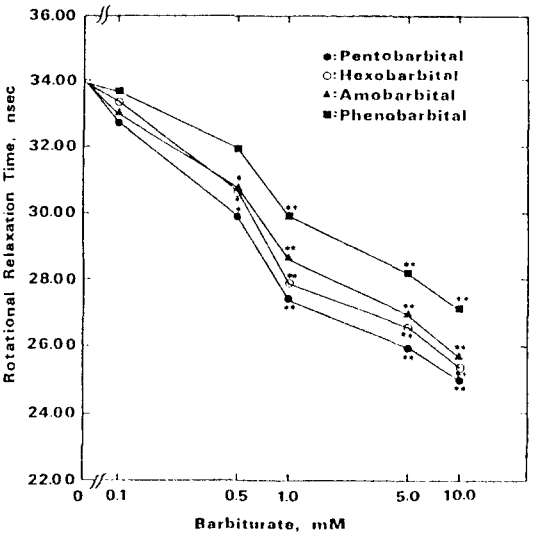

Fig. 1. Effects of barbiturates on the rotational relaxation time ($\bar{P}$) of 1,6-diphenyl-1,3,5-hexatriene in synaptosomal plasma membrane vesicles isolated from bovine brain. Florescence measurements were performed at 37℃. Each point represents the mean of 4 determinations. An asterisk and double asterisk signify $p<0.05$ and $p<0.01$, respectively, compared to control according to Student's t-test.

Table I. The rotational relaxation time ($\bar{P}$) of 1,6-diphenyl-1,3,5-hexatriene in native and model membranes

Membrane	Rotational relaxation time(ns)
SPMV[a]	33.95± 0.43
SPMVTL[b]	27.36± 0.48
SPMVPL[c]	18.50± 0.21

Florescence measurements were performed at 37℃. Values represent the mean± SEM of 4 determinations. [a]Synaptosomal plasma membrane vesicles; [b]model membranes of total lipids extracted from synaptosomal plasma membrane vesicles; [c]model membranes of phospholipids separated from total lipids.

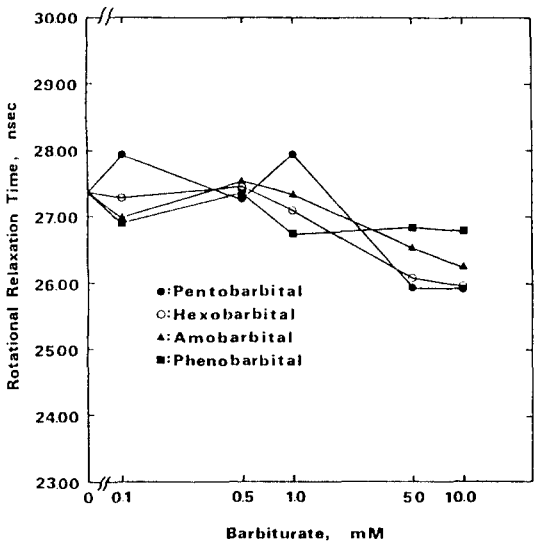

Fig. 2. Effects of barbiturates on the rotational relaxation time ($\overline{P}$) of 1,6-diphenyl-1,3,5-hexatriene in model membranes of total lipids extracted from synaptosomal plasma membrane vesicles. Fluorescence measurements were performed at 37℃. Each point represents the mean of 4 determinations.

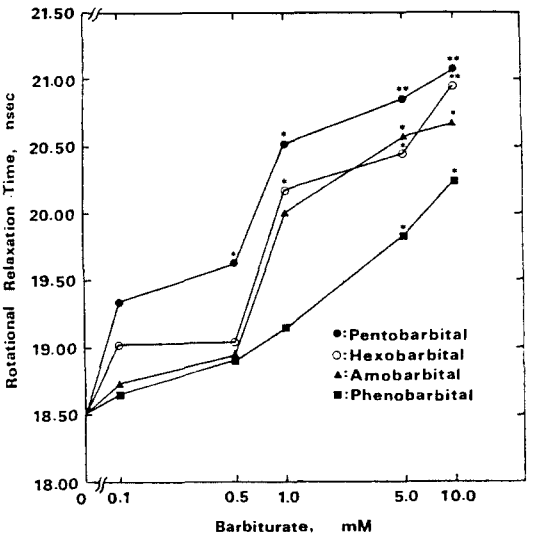

Fig. 3. Effects of barbiturates on the rotational relaxation time ($\overline{P}$) of 1,6-diphenyl-1,3,5-hexatriene in model membranes of phospholipids separated from total lipids. Fluorescence measurements were performed at 37℃. Each point represents the mean of 4 determinations. An asterisk and double asterisk signify $p<0.05$ and $p<0.01$, respectively, compared to control according to Student's t-test.

which determine the sensitivity of the membranes to ethanol and other intoxicant-anesthetic drugs. The results of electron spin resonance (ESR) spectroscopy and differential scanning calorimetry (DSC) experiments indicate that ganglioside oligosaccharide chains undergo mutual cooperative interactions[21]. The ability to interact at both the ceramide bridge and head group levels makes ganglioside prime candidates for lateral segregation either among themselves or in association with the protein and glycoprotein constituents of the membranes. In the present study, gangliosides may be lost during the isolation of phospholipids and neutral lipids. This may partially explain that vesicles composed of phospholipids and neutral lipids extracted from SPMV are relatively insensitive to the effects of barbiturates than intact SPMV.

The results (Fig. 1-3) are in general agreement with the studies of Pang and Miller[7]. Exploiting 5-doxyl stearic acid, they reported that pentobarbital increased the order parameter of phosphatidylcholine-phosphatidic acid liposomes containing no cholesterol, did not affect the order parameter at 14 mol of cholesterol per 100 mol of lipid and decreased the order parameter at 32 mol of cholesterol per 100 mol of lipid. Thus, it appears that the effects of barbiturates on membrane fluidity depend mainly on the initial fluidity of the membrane.

However, the membrane-disordering effects of barbiturates observed in the present study are not consistent with the results of Rosenberg *et al.*[8], who used ESR technique to study the effect of thiopental on rat brain synaptic plasma membranes. Thiopental was excluded in this study because it tended to quench the fluorescence of DPH.

The fluidity change induced by barbiturates may be correlated with an increase in ion permeability. Pang *et al*[22]. 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 membrane perturbations produced by low concentration of barbiturates. There is now substantial support for a γ-aminobutyric acid (GABA)-mediated chloride uptake role in the actions of barbiturates[23]. 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[24] 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-perturbing potencies in the present study is in agreement with Allan and Harris[24], suggesting a close relationship between the membrane-perturbing effects of barbiturates and the chloride fluxes across SPMV.

In summary, barbiturates may 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 GABA with chloride channels. Even though direct evidence for the interaction between membrane perturbation and chloride fluxes should be elucidated, these experiments strongly suggest that barbiturates act, in part, their pharmacological effects by modulating the fluidity of neuronal membranes to a specific level.

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