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Effects of n-Alkanols on the Rotational Relaxation Time of 1,6-Diphenyl-1,3,5-hexatriene in the Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Cerebral Cortex

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The effects of n-alkanols on the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) in synaptosomal plasma membrane vesicles isolated from fresh bovine cerebral cortex were investigated. n-Alkanols decreased the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene in the native membranes and the potencies of n-alkanols up to 1-nonanol increased 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 an increase in the rotational relaxation time of DPH in the native membranes.

Key words: n-Alkanols, Membrane-disordering effects, Fluorescent probe technique

INTRODUCTION

n-Alkanols are neutral compounds and are members of the large family of anesthetics whose biological potency correlates with lipid solubility (Seeman, 1972). The exact mechanisms by which ethanol and similar drugs cause depression of the central nervous system (CNS) and the subsequent behavioral manifestations of intoxicaton remain undefined. In recent years, a great deal of research has focused on the effects of n-alkanols on the physical properties of native and model membranes (Goldstein, 1984; Lyon *et al.*, 1981; Yun and Kang ,1992a, b; Yun *et al.*, 1993).

Most of published data about the effects of n-alkanols on the biophysical characteristics of native and model membranes have been obtained from the analysis of the influence on the phase transition temperature of model membranes (Jain and Wu, 1977) or on the restriction of rotational diffusion of native and model membranes (Goldstein, 1984; Lyon *et al.*, 1981; Yun and Kang, 1992a, b; Yun *et al.*, 1993). However, there are still important questions that remain to be determined: (i) whether n-alkanols produce an effect on the rotational relaxation time of native or model membranes. Attempting to answer these questions, the effects of n-alkanols on the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) in synaptosomal plasma membrane vesicles isolated from fresh bovine cerebral cortex (SPMV) were investigated and report the results.

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MATERIALS AND METHODS

Chemicals

The fluorecent probe DPH was obtained from Molecular Probes (Junction City, OR, USA). n-Alkanols were purchased from Fluka (Buchs, Switzerland). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), Ficoll (70,000 M.W.), 1,4-bis[-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

2-year-old Korean cattle of either sex were killed at Daeyoung slaughterhouse (#192 Kupo-dong, Buk-ku, Pusan, Korea). The whole brain was rapidly excised, placed in ice-cold 0.32 M sucrose plus 3 mM Hepes (pH 7.5), and then immediately carried to our laboratory. All steps, subsequent to decaptitation and removal of the cortex, were carried out at 0-4℃. The SPMV were isolated from bovine cerebral cortex and characterized by the formerly reported method in our labora-

tory (Yun *et al.*, 1990; Yun and Kang, 1990). The purity of SPMV was determined by enzymatic and morphological standards (Yun *et al.*, 1990; Yun and Kang, 1990). Each membrane suspension was either used immediately or frozen in liquid nitrogen and stored at −70°C for no longer than a month. Protein was determined by the method of Lowry *et al.* (1959) using BSA as a standard.

Fluorescence measurements

The fluorescent probe DPH was dissolved in tetrahydrofuran and a volume of 0.5 μl of tetrahydrofuran per ml of phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 2 mM Hepes, pH 7.4) was added directly to the membrane suspension at a concentration of 1 μg/100 μg of membrane protein. The suspension was incubated in the dark at 37°C for 30 min with frequent vortexing. After incorporation of the probe, the membrane suspension (protein 1 mg/ml, 4 ml) was placed in cuvettes. Control levels of fluorescence were 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 T-format subnanosecond spectrofluorometer (SLM Aminco Instruments, Inc., Urbana, IL, USA) and performed at 37°C (pH 7.4). 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°C 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 (Lakowicz *et al.*, 1980). 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°C 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 excitation.

The anisotropy was determined as described earlier (Yun and Kang, 1992a, b). Alterations in anisotropy were converted to rotational relaxation time, $\bar{P}$, calculated from the Perrin equation (Shinitzky and Inbar, 1976): $(1/r - 1/3) = (1/r_o - 1/3)(1 + 3\tau/\bar{P})$. 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 (Lokowicz *et al.*, 1979), 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

The specific activities of Na^+, K^+-ATPase, acetylcholinesterase and 5'-nucleotidase were about 6-, 2.5- and 3-fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenates (Yun *et al.*, 1990; Yun and Kang, 1990). Electron microscopic examination also showed that the membranes were in vesicular form (Yun *et al.*, 1990).

Numerous methods including the fluorescent probe technique have been employed to determine the biophysical properties of native and model membranes or to study the influence of n-alkanols and other drugs on the biophysical properties of the membrane lipids. Clearly, no single experimental approach can encompass the richness and complexity inherent in problems of membrane structure and dynamics. A number of quite different physical and chemical techniques must be applied and their results correlated.

In order to determine the effect of n-alkanols on the rotational relaxation time of DPH in the lipid bilayer structure of SPMV, it is first necessary to demonstrate that these molecules do not interact directly with DPH and thereby quench its fluorescence. Quenching of absorbance-corrected fluorescence intensity by n-alkanols is not observed over the entire concentration range used for n-alkanols (Table 1).

Table 1. Effects of n-alkanols on the fluorescence lifetime (τ) of 1,6-diphenyl-1,3,5-hexatriene in synaptosomal plasma membranes vesicles isolated from bovine cerebral cortex

n-Alkanols	Concentration, mM	Lifetime
None		10.20± 0.17
Methanol	1000	10.26± 0.13
Ethanol	100	10.23± 0.07
1-Propanol	25	10.15± 0.10
1-Butanol	40	10.18± 0.12
1-Pentanol	2.5	10.18± 0.07
1-Hexanol	0.25	10.19± 0.11
1-Heptanol	0.1	10.16± 0.24
1-Octanol	0.025	10.23± 0.10
1-Nonanol	0.010	10.25± 0.07
1-Decanol	0.25	10.24± 0.018

1,6-Diphenyl-1,3,5-hexatriene was incorporated, and fluorescence lifetime was determined in the absence and presence of n-alkanols at the concentrations given at 37°C (pH 7.4). Values represent the mean SEM of 4 determinations.

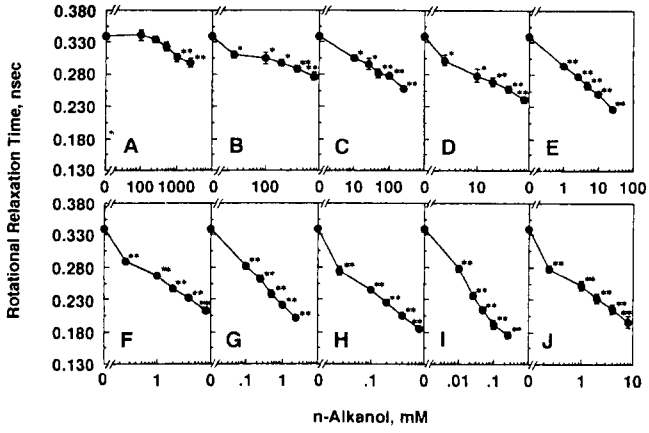

Fig. 1. Effects of n-alkanols on the rotational relaxation time ($\bar{P}$) of 1,6-diphenyl-1,3,5-hexatriene in synaptosomal plasma membrane vesicles isolated from bovine brain. (A) Methanol; (B) ethanol; (C) 1-propanol; (D) 1-butanol; (E) 1-pentanol; (F) 1-hexanol; (G) 1-heptanol; (H) 1-octanol; (I) 1-nonanol; (J) 1-decanol. Fluorescence measurements were performed at 37℃ (pH 7.4). Each point represents the mean of 4 determinations. An asterisk and double asterisk signify $\bar{P}<0.05$ and $\bar{P}<0.01$, respectively, according to Student's t-test.

The rotational relaxation time ($\bar{P}$, nanosecond) of DPH in the intact SPMV was 33.95± 0.43. In a concentration-dependent manner, n-alkanols significantly decreased the rotational relaxation time ($\bar{P}$) of DPH in the intact SPMV and the potencies of n-alkanols up to 1-nonanol increased by 1 order of magnitude as the carbon chain length increases by two carbon atoms (Fig. 1). The cut-off phenomenon was reached at 1-decanol (Fig. 1-J), where further increase in hydrocarbon length resulted in an increase in the rotational relaxation time of DPH.

DISCUSSION

Fluorescence methods offer the biologist the important advantages of great sensitivity, versatility, and simplicity of instrumentation. Hence, many laboratories are now equipped to perform the estimation of steady-state fluorescence polarization and excimer fluorescence intensity, two methods which are particularly simple and dependable for the assessment of lipid fluidity. 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.

Ethanol and related alkanols have been shown to decrease the temperature of the gel-to-liquid crystalline phase transition of pure phospholipid model membranes (Jain and Wu, 1977), expand model membranes (Seeman, 1972), and alter the surface charge of membrane lipids (Bangham and Mason, 1979). These effects have been obtained with ethanol concentrations in the range of 500-1500 mM, whereas serum concentrations above 200 mM are usually lethal in humans and laboratory animals (Harris and Schroeder, 1981). n-Alkanols have been shown to decrease anisotropy of DPH in mouse brain membranes (Harris and Schroeder, 1981; Lyon *et al.*, 1981; Harris and Bruno, 1985; Perlman and Goldstein, 1984; Harris *et al.*, 1984) model membranes (Yun and Kang 1992a, b), and ovarian cell plasma membranes (Yun *et al.*, 1993). Although evidence is accumulating to suggest that the effect of n-alkanols at the cellular level is the result of biophysical changes in membranes, no attention has been given to the effect of n-alkanols on the rotational relaxation time of DPH in the native membranes. Our data presented herein have shown that, even at physiologically relevant concentrations (Harris and Schroeder, 1981; Majchrowicz and Mendelson, 1971), n-alkanols decrease rotational relaxation time of DPH in the SPMV, indicating the bulk lipid (inner + outer monolayer) fluidization of the native membranes. In biological membranes, the anisotropy reflects mainly the range of rotational diffusion rather than the rate and membrane perturbants , such as cholesterol and proteins, primarily alter the range of the motion rather than rate (Kinosita *et al.*, 1981). Kinosita *et al.* (1977) suggested that the rotational diffusion of DPH is limited to a cone formed by the surrounding membrane structures, and the range of the motion of the fluorophore depend on the resistance to the motion offered by the microenvironment. The alterations in the anisotropy are likely to be due to changes in range of rotational diffusion of the probe. The rotational relaxation time of DPH in biological membranes, however, is referred to as either the rate (the speed of rotational diffusion), microviscosity or dynamic component.

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 (Franks and Lieb, 1987). 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 (Gonzales and Hoffman, 1991; Sanna *et al.*, 1991). Still, a large, diverse collection of physiological agonists produces the alterations in membrane fluidity as well as their specific ligand-receptor interaction (Manevich *et al.*, 1988). A recent data on the interactions of ethanol and certain receptor- and voltage-gated ion channels concluded that the recep-

tor-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 (Gonzales and Hoffman, 1991).

Opinions have been divided as to whether n-alkanols interfered with membrane protein function by directly binding 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. Because biological membranes are of highly complex composition, it has not been feasible to monitor changes in the local lipid environment and to determine its effect on the membrane protein function at the same time. It would be difficult to exclude the possibility that the interaction of n-alkanols with neuronal membrane lipids may exert some influences on the ion channels or receptors which associate tigntly with membrane lipids through covalent and noncovalent bonds (Catterall, 1987; Lipowsky, 1991). That is to say, before or during, even after the interaction of n-alkanols with the proteins, the fluidization of membrane lipids may provide an ideal microenvironment for optimum anesthetic effects. In conclusion, the present data suggest that n-alkanols, in addition to its direct interaction with proteins (Franks and Lieb, 1987; Gonzales and Hoffman, 1991; Sanna *et al.*, 1991), concurrently interacts with membrane lipids, fluidizes the membrane (Goldstein, 1984; Lyon *et al.*, 1981; Yun and Kang, 1992a, b; Yun *et al.*, 1993), and thus induces conformational changes of proteins which are known to be tightly associated with membrane lipids (Catterall, 1987; Lipowsky, 1991).

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