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The Effect of n-Alkanols on the Lateral Diffusion of Synaptosomal Plasma Membrane Vesicles Isolated from Bovine Cerebral Cortex[1]

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ABSTRACT

Intramolecular excimer formation with the fluorescent probe 1,3-di(1-pyrenyl)propane (Py-3-Py) was used to investigate the effects of methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol and 1-decanol on the lateral diffusion of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex (SPMV). The n-alkanols increased the excimer to monomer fluorescence intensity ratio (I′/I) of Py-3-Py in the SPMV. In a dose-dependent manner, n-alkanols increased lateral diffusion of hydrocarbon region of bulk (inner+outer monolayers) SPMV lipid bilayers, and the potencies of n-alkanols up to 1-nonanol increased with carbon chain length. It appears that the potencies in bilayer fluidization due to the lateral diffusion 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.

Key Words: n-Alkanols, Lateral diffusion, Native membranes, Fluorescent probe technique

INTRODUCTION

It has been generally accepted that n-alkanols are members of membrane perturbing agents that fluidize native and model membranes (Wood and Schroeder, 1988). Changes in membrane fluidity are known to be linked to alterations in physiological processes of the cell membrane like carrier-mediated transport, activities of membrane bound enzymes, receptor binding, phagocytosis, endocytosis, depolarization dependent exocytosis, cytotoxicity, and cell growth (Spector and Yorek, 1985). Membrane fluidity appears to change during development, aging, and drug therapy of cultured cells (Toplak *et al.*, 1990; Yun *et al.*, 1993). Alterations in membrane fluidity may even represent a possible parameter in the evaluation of malignancy (Shinizky, 1984).

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 drugs on the biophysical properties of the membrane lipids. Clearly, no single experimental ap-

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proach 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.

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 rotational diffusion of native and model membranes (Harris and Schroeder, 1981; Lyon *et al.*, 1981; Harris and Bruno, 1985; Perlman and Goldstein, 1984; Harris *et al.*, 1984; Yun and Kang, 1992a,b). However, there is still an important question that remains to be determined: whether n-alkanols produce an effect on the lateral diffusion of native or model membranes. Attempting to answer this question, we studied the effects of n-alkanols on the lateral diffusion of 1,3-di(1-pyrenyl)propane (Py-3-Py) in synaptosomal plasma membrane vesicles isolated from fresh bovine cerebral cortex (SPMV).

MATERIALS AND METHODS

Chemicals

The fluorescent probe Py-3-Py was prepared by the previously reported synthesis (Yun *et al.*, 1990a). n-Alkanols were purchased from Fluka (Buchs, Switzerland). N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid, Ficoll (70,000 M. W.), and bovine serum albumin were obtained 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

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-, 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 (Yun *et al.*, 1990b). Protein was determined by the method of Lowry *et al.* (1959) using BSA as a standard.

Fluorescence measurements

The incorporation of Py-3-Py was carried out by adding aliquots of a stock solution of 5×10^{-5} M in absolute ethanol to intact SPMV (1 mg protein/ml, pH 7.4), 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 hr under gentle stirring. 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 measurements were carried out with a SPF-500C spectrofluorometer (SLM Instruments Inc., Champaign-Urbana, IL, USA) and performed at 37℃. The excitation wavelength was 330 nm. The excimer to monomer fluorescence intensity ratio was calculated from the 480 nm to 379 nm signal ratio (I′/I). 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, served as controls for the fluorometric measurements.

RESULTS

In order to determine the effect of n-alkanols on the lateral diffusion of Py-3-Py in the lipid bilayer structure of SPMV, it is first necessary to demonstrate that these molecules do not interact directly with Py-3-Py 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.

The excimer to monomer fluorescence intensity ratio of Py-3-Py in the intact SPMV was 0.412±

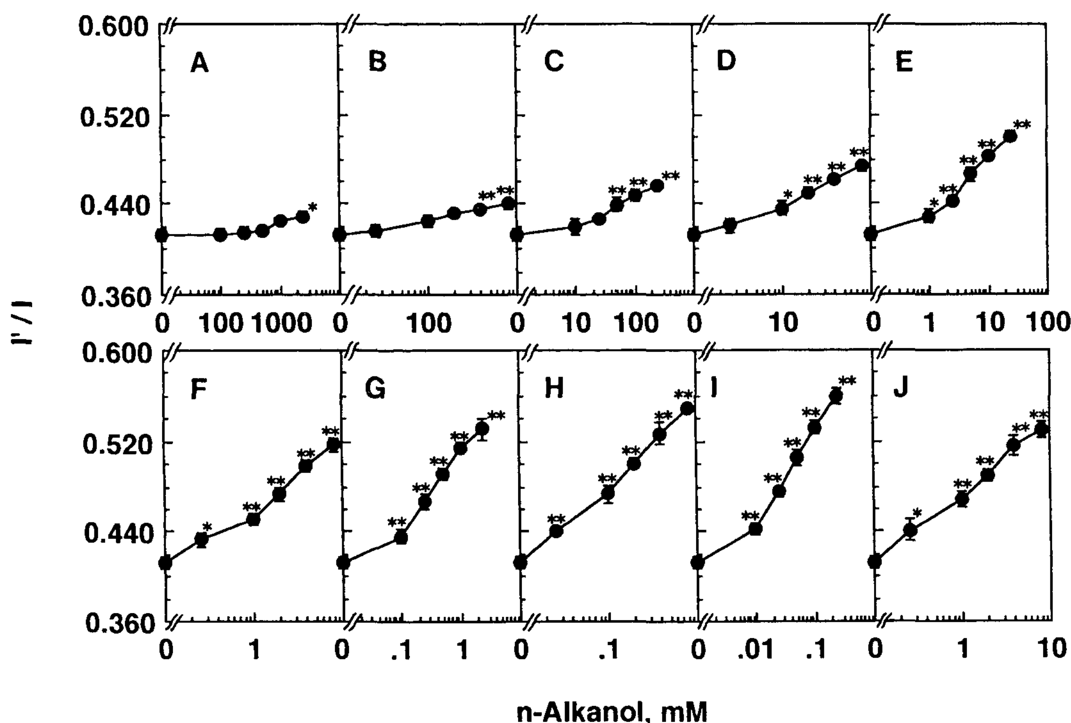

Fig. 1. Effects of n-alkanols on the excimer to monomer fluorescence intensity ratio, I′/I, of 1,3-di(1-pyrenyl)propane incorporated into synaptosomal plasma membrane vesicles isolated from bovine brain. (A) Methanol; (B) ethanol; (C) 1-propanol; (D) 1-butanol; (E) 1-pentanol; (F) 1-hexanol; (G) 1-heptanol; (H) 1-octanol; (I) 1-nonanol; (J) 1-decanol. Fluorescence measurements were performed at 37℃. Each point represents the mean±SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, according to Student's t-test.

0.005 (37℃, pH 7.4). The lowest concentrations which produced a significant increase in the I′/I values of Py-3-Py in the SPMV by methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, and 1-decanol were 2500 (I′/I value: 0.429±0.004), 400 (I′/I value: 0.436±0.003), 50 (I′/I value: 0.439±0.006), 10 (I′/I value: 0.436±0.006), 1.0 (I′/I value: 0.429±0.006), 0.25 (I′/I value: 0.433±0.006), 0.10 (I′/I value: 0.436 ± 0.006), 0.025 (I′/I value: 0.440 ± 0.003), 0.010 (I′/I value: 0.442±0.05), and 0.25 mM (I′/I value: 0.441±0.010), respectively. In a dose-dependent manner, n-alkanols significantly increased the ratio (I′/I) of Py-3-Py 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), where further increase in hydrocarbon length resulted in a decrease in pharmacological activity.

DISCUSSION

Owing to the organization of native and model membrane structures, the resistance to flow at any point in the lipid is quite dependent on the direction of the applied force and on the mode of motion which contributes to the fluidity or microviscosity. Such motions include the following: lat-

eral diffusion of lipid molecules in the plane of the monolayer, with diffusion coefficients in the range of 10^{-9} to 10^{-7} cm sec^{-1} (Devaux *et al.*, 1972; Trauble and Sackman, 1972); rotational diffusion of a lipid molecule around an axis perpendicular to the plane of the bilayer, with reported rotational diffusion frequencies in the range of 10^7 to 10^8 sec^{-1} (Hubbell and McConnell, 1969); in contrast to the relatively rapid motions of the preceding modes, transverse diffusion or "flip-flop" from one monolayer to the other is energetically less favored and relatively slow, particularly in model bilayers (Kornberg and McConnell, 1971); lateral flow of arrays of lipid molecules in bulk under pressure gradients; vertical displacements in and out of the bilayer; rotations of lipid substituents around single bonds, including trans-gauche movements, and vibratory changes in bond length within one molecule (Ladbrooke and Chapman, 1969). A precise definition of "fluidity or microviscosity" in an anisotropic bilayer could be described as a weighted sum of all of these modes of motion.

Unfortunately, no single method is now available to assess all of these motions and to provide such a weighted parameter. Current thinking, as a result, holds that the "fluidity or microviscosity" parameters of bilayers assessed by extant techniques are operational quantities which are technique-dependent and do not necessarily reflect a bulk property of the membrane lipid (Lakowicz *et al.*, 1979).

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. 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. For this purpose, the fluorescence polarization and the excimer fluorescence assess the rotational and lateral diffusion of lipid fluorophores, respectively. 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.

Intramolecular excimer formation of Py-3-Py is sensitive to fluidity and has been used to study fluidity changes and phase transitions of phospholipid vesicles (Zachariasse *et al.*, 1980; Melnick *et al.*, 1981), micelles (Zachariasse, 1978), and biological membranes (Melnick *et al.*, 1981; Zachariasse *et al.*, 1982). This methodology has an advantage over its counterpart based on intermolecular excimer formation, since very small probe concentrations can be employed ($<10^{-6}$M) (Zachariasse *et al.*, 1980, 1982). The use of low probe concentrations is possible as intramolecular excimer formation is a monomolecular process, independent of concentration (Zachariasse *et al.*, 1980, 1982). In this manner, the formation of aggregates in viscous media such as biomembrane is avoided (Zachariasse, 1978). Furthermore, perturbations of the membrane by the probe molecule are minimized.

Intramolecular excimer formation, due to association of an excited and an unexcited aromatic group incorporated in the same molecule, can be described by the excimer to monomer fluorescence quantum yield ratio, ϕ'/ϕ, which is proportional to the fluorescence intensity ratio, I'/I (Zachariasse *et al.*, 1980).

$$\frac{I'}{I} \approx \frac{\phi'}{\phi} = \frac{K_f'}{K_a} = \frac{K_a}{K_d + 1/\tau_o'} \quad \cdots\cdots\cdots\cdots 1$$

Here, K_f', K_f, K_a, and K_d are the rate constants for excimer fluorescence, monomer fluorescence, excimer formation, and excimer dissociation, respectively, and τ_0' is the excimer lifetime in the absence of K_d. At low temperatures, or at sufficiently high local viscosities, $K_d \ll 1\tau_o'$ (Zachariasse *et al.*, 1980), and eq. 1 assumes a simplified form:

$$\frac{I'}{I} \approx \frac{\phi'}{\phi} = \frac{K_f'}{K_a} = K_a\tau_o' \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots 2$$

Therefore, under these conditions, the fluorescence intensity ratio I'/I is primarily determined by the rate constant of excimer formation, K_a, since the radiative rate constants and τ_o' have been found to be essentially independent of temperature (Zachariasse *et al.*, 1980). Since I'/I of Py-3-Py has been shown to increase with lateral

diffusion in highly viscous media (Zachariasse, 1978; Melnick *et al.*, 1981), the technique is suitable to determine the lateral diffusion of the probe environment in media such as biomembranes.

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). Furthermore, 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). And n-alkanols have been shown to decrease rotational relaxation time of DPH in SPMV (Chung *et al.*, 1993). So, n-alkanols increased the range of rotational diffusion of native (Harris and Schroeder, 1981; Lyon *et al.*, 1981; Harris and Bruno, 1985; Perlman and Goldstein, 1984; Harris *et al.*, 1984) and model (Yun and Kang, 1992a,b) membranes and the rate of rotational diffusion of native membranes (Chung *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 membrane, no attention has been given to the effect of n-alkanols on the lateral diffusion of lipid bilayers of native and model membranes. Our data presented herein have shown that n-alkanols increase the ratio of excimer to monomer fluorescence intensity of Py-3-Py in the SPMV lipid bilayers. Therefore, n-alkanols increased the range and rate of lateral diffusion of SPMV lipid bilayers.

n-Alkanols with lipid solubility will enter hydrophobic regions of both proteins and lipids in the core of the membrane (Goldstein, 1984). Although it is too early to explain exactly how the hydrophobic interactions affects functions, it is reasonable to predict that disruption of protein function will result from disorder in any of the hydrophobic regions (Goldstein, 1984). 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 membrane-fluidizing hypothesis of ethanol action in the central nervous system is now being strongly challenged by recent data showing that ethanol specifically and selectively affects the function of the γ-aminobutyric acid-coupled chloride channel (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). So, the function of membrane proteins may be modulated secondarily to changes in membrane fluidity. Conversely, there is also a possibility that n-alkanols may have a direct effect on certain receptors, receptor-gated ion channels, or membrane-bound enzymes, and then on membrane lipids. It may well be that n-alkanols concurrently interact with membrane lipids and membrane-bound proteins. At present, it may be premature to take sides in the controversy about whether membrane lipids or membrane proteins are the site of n-alkanols' action.

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=국문초록=

n-Alkanols가 소의 대뇌피질로부터 분리한 Synaptosomal Plasma Membrane Vesicles의 측방확산운동 범위와 속도에 미치는 영향

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n-Alkanols의 분자적 약리작용기전 탐구에 기초자료를 제공하기 위하여 소의 신선한 대뇌피질로부터 분리한 synaptosomal plasma membrane vesicles (SPMV) 지질 이중층의 측방확산운동에 미치는 n-alkanols의 영향을 형광 probe법으로 검색하였다. n-Alkanols는 SPMV 지질 이중층의 측방확산운동 범위와 속도를 농도 의존적으로 증가시켰고 1-nonanol까지는 탄소수가 두개 증가될 때마다 그 효력은 약 10배 가량 증가되었으나 탄소수 10개인 1-decanol의 효력은 오히려 감소되는 경향을 나타내었다.