Penetration of Barbiturates into Model Membranes of Phospholipids Extracted from Brain Membranes

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=국문초록=

인지질들로서 제제한 인공세포막에서의 Barbiturates 침투 정도

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소의 신선한 대뇌피질로부터 synaptosomal plasm membrane vesicles(SPMV)를 분리하고 이 SPMV로부터 추출한 모든 인지질들로서 제제한 인공세포막(SPMVPL)에서의 barbiturates 침투정도를 형광 probe법으로 검색하였다.

Barbiturates는 SPMVPL 외부 단층(outer monolayer)의 표면에 주로 분포되되 지질용해도와 마취능에 비례하여 소수성 부위에 분포되는 양이 증가되는 현상을 보여주었다. Thiopental sodium, pentobarbital, hexobarbital, amobarbital 및 phenobarbital은 SPMPL 외부 단층의 표면(친수성 부위)에 분포되는 것이 소수성 부위에 분포되는 것에 비하여 각각 7.4, 28.6, 30.2, 54.3 및 65.5배가 된다는 것을 확인하였다.

Key Words: Barbiturates, Penetration, Model membranes, Modified Stern-Volmer equation, Fluorescence probe technique

INTRODUCTION

For many years it has been thought that barbiturates, as well as the primary alcohols and the volatile anesthetic agents, exerted their depressant effects on the central nervous system by dissolving in lipid membranes, thereby perturbing the function of ion channels and other protein embedded therein¹⁻⁷⁾. However, the membrane-fluidizing hypothesis of barbiturates

action in the central nervous system is now being strongly challenged by recent data showing that barbiturates specifically and selectively affects the function of the γ -aminobutyric acid-coupled chloride channel⁸⁾.

At the present time, the exact mechanism(s) of action for barbiturates in the central nervous system is unclear. The present study on synthetic lipid systems is included to the extent that it contributes to an understanding of the behavior of naturally occurring phospholipids in

native membranes. The investigation of the primary site of action of barbiturates can provide a basis for studying the mechanism of action of these drugs. In the present study, we examined the penetration site of barbiturates into model membranes of phospholipids (SPMVPL) extracted from synaptosomal plasma membrane vesicles (SPMV), employing two fluorescent probes N-octadecyl naphthyl-2-amine-6-sulfonic acid (ONS) and 12-(9-anthroyl) stearic acid (AS). The analysis of preferential quenching of these probes by barbiturates revealed the relative accessibility of these drugs into the hydrocarbon region of the outer monolayers of SPMVPL lipid bilayer structures.

MATERIALS AND METHODS

1) Materials

The fluorescent probe, 12-(9-anthroyl) stearic acid (AS), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other fluorescent probe, N-octadecyl naphthyl-2-amine-6-sulfonic acid (ONS) was synthesized by DR. Yun (Department of Dental Pharmacology and Biophysics, College of Dentistry, Pusan National University) and was kindly donated to us. Barbiturates were obtained from Sinjun Pharmaceutical Co. (Seoul, Korea). All other reagents were of the highest quality available. Water was deionized.

2) Membrane preparations

The SPMV were isolated from bovine cerebral cortex and characterized by the formerly reported method as described earlier⁹⁾. Lipids were extracted from the SPMV by the procedures of Yun and Kang¹⁰⁾. The individual phospholipid classes were separated by thin layer chromatography and quantitated by measuring the amounts of inorganic phosphate after hydrolysis of the phospholipids at 180°C in 70%

HClO₂¹⁰⁾. Phospholipids were composed of phosphatidylcholine (PC, 43%), phosphatidylethanolamine (PE, 36%), phosphatidylserine (PS, 13%), sphingomyelin (SM, 4%), phosphatidylinositol (PI, 3%), and lysophosphatidylcholine (LPC, 1%). In the present study, gangliosides might have been lost during the isolation of the phospholipids and neutral lipids. Large unilamellar liposomes (SPMVPL; 0.7 mg of phospholipids/ml pH 7.4) were prepared by the method described earlier¹¹⁾.

3) Fluorescence measurements

These measurements were identical to procedures of our previous study¹²⁾.

RESULTS AND DISCUSSION

Fluorescence quenching in homogeneous solution has been described in terms of the Stern-Volmer equation:

$$F_0/F = 1 + k_t t_a [Q] = 1 + K [Q] \dots \dots \dots \text{equation 1}$$

In this equation F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, k_t is the bimolecular quenching constant, t_a is the lifetime of the fluorophore in the absence of quencher, $[Q]$ is the concentration of quencher, and $K = k_t t_a$ is the Stern-Volmer quenching constant. A plot of F_0/F versus $[Q]$ yields an intercept of one on the y axis and a slope equal to K . In the present study, the Stern-Volmer equation was modified for uneven distribution of the quencher in the SPMVPL. For chloroform-soluble barbiturates, the concentration in the aqueous phase is excessively smaller than that in the lipid bilayer at pH 7.4. Consequently, $[Q]_L \cong P[Q]_T$ and equation 1 can be modified:

$$F_0/F = 1 + fKP[Q]_T \dots \dots \dots \text{equation 2}$$

In this equation $[Q]_i$ and $[Q]_o$ are the concentrations of the quencher in the outer monolayers of the SPMVPL lipid bilayer structures and the total concentration of the quencher in the system, respectively, P is the partition coefficient, and f is a regional correction factor for uneven distribution of the quencher between the surface area and the interior area in the outer monolayers of the SPMVPL lipid bilayer structures. Studies have shown that the most probable position of the naphthalene sulfonate moiety of the ONS molecule is at the surface of membrane's outer monolayer, and the anthroyl moiety of the AS molecule is most likely located

in the hydrocarbon region of the outer monolayers of the membrane lipid bilayer structures¹³⁾. Hence, ONS or AS quenching in the SPMVPL gives the following:

$$F_o/F = 1 + f_o K_{ONS} P [Q]_T \quad \text{.....equation 3}$$

$$F_o/F = 1 + f_i K_{AS} P [Q]_T \quad \text{.....equation 4}$$

In these equations K_{AS} and K_{KS} are the Stern-Volmer constants of the fluorescence quenching of ONS and AS by the quencher, respectively. f_i/f_o is the ratio of the regional correction factors in the surface area and the interior area of the outer monolayers of the SPMVPL, and becomes the concentration gradient of the quencher be-

Fig. 1. Stern-Volmer plot of quenching of ONS fluorescence in SPMVPL by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

Fig. 2. Stern-Volmer plot of quenching of AS fluorescence in SPMVPL by thiopental sodium, pentobarbital, hexobarbital, amobarbital and phenobarbital. Lines were fitted by a least-squares analysis.

Table 1. Stern-Volmer Constant of Quenching of ONS and AS Fluorescence by Barbiturates in SPMVPL Liposomes(S_s and S_i values).

Barbiturates	Stern-Volmer constant(l/M) ^a	
	ONS(S_s)	AS(S_i)
Thiopental sodium	199.8	85.0
Pentobarbital	186.2	15.1
Hexobarbital	185.4	13.5
Amobarbital	150.4	7.8
Phenobarbital	120.3	6.3

^a; Values are taken from Fig. 1 and 2.

Table 2. Stern-Volmer Constant of Quenching of ONS and AS Fluorescence by Barbiturates in Dimethylsulfoxide and Tetrahydrofuran Mixture(1:1).

Barbiturates	Stern-Volmer constant(l/M) ^a	
	K_{ONS}	K_{AS}
Thiopental sodium	9.4	29.6
Pentobarbital	7.5	17.4
Hexobarbital	7.3	16.1
Amobarbital	4.9	13.8
Phenobarbital	3.5	12.0

Data were adapted from our previous study(reference number, 12)

tween these two areas.

The plots of F_0/F vs. $[Q]_T$ of equations 3 are shown in Fig. 1. And the plots of F_0/F vs. $[Q]_T$ of equations 4 are shown Fig. 2. From this line, F_s/F_i can be obtained:

$$f_s/f_i = \frac{S_s K_{AS}}{S_i K_{ONS}} \dots \dots \dots \text{equation 5}$$

In this equation S_s and S_i are the slopes of the plots of equations 3 and 4, respectively, and listed in Table 1. For water-insoluble barbiturates, $[Q]_L \cong [Q]_T$, and equation 5 could be easily reached.

Our previous study, Stern-Volmer plots were drawn for quenching of ONS and AS fluores-

Table 3. Ratio of the Concentration of Barbiturates in the Surface area to the Concentration in the Hydrocarbon Interior of the Lipid Bilayer of SPMVPL Liposomes(f_s/f_i).

Barbiturates	Ratio ^a
Thiopental sodium	7.4
Pentobarbital	28.6
Hexobarbital	30.2
Amobarbital	54.3
Phenobarbital	65.5

^a; The ratio values were obtained from equation (5) where the values of K_{ONS} , K_{AS} , S_s and S_i and those as shown in tables 1 and 2.

cence by barbiturates in 1:1 mixture of DMSO and THF⁽¹²⁾. The slopes of these plots yield the K_{ONS} and K_{AS} values, and the values are listed in Table 2. Assuming K_{ONS} and K_{AS} values in bulk solution are not much different from the values in the SPMVPL, these values can be substituted into equation 5. The values of f_s/f_i calculated by this method are listed in Table 3.

In accordance to the analysis of preferential quenching of ONS and AS fluorescence by barbiturates the penetration of the drugs into hydrocarbon interior increased directly proportional to their lipid solubility and anesthetic efficacy. We reported that the major distribution area of barbiturates in the SPMV and SPMVTL was the surface of the outer monolayers of the membranes^(12,14). Our study extended from the above experiments demonstrated an interesting phenomenon in the SPMV, SPMVTL and SPMVPL. This result is in agreement with those of our studies, indicating barbiturates have a larger distribution on the surface area of the outer monolayers of the SPMV, SPMVTL and SPMVPL than on the hydrocarbon region of the membranes' monolayers. Furthermore, the relative penetrabilities of individual barbiturates into the hydrocarbon region were iden-

tical to our previous studies^{12,14}). However, the important point is the different penetrability of barbiturates among SPMV, SPMVTL and SPMVPL in terms of the amount of penetration of the drugs into the hydrocarbon region of the outer monolayers of the membranes. According to previous studies^{6,7}), the relative fluidities of SPMV, SPMVTL and SPMVPL in relation to range and rate of lateral and rotational mobility were in the order of SPMVPL, SPMVTL, SPMV. There is an excellent correlation between intrinsic membrane fluidity and penetrability of the barbiturates into the hydrocarbon region of the outer monolayers of native and model membranes. The results of this study strongly suggest that penetrabilities of the barbiturates into hydrocarbon region of outer monolayers decrease inversely proportionate to intrinsic fluidity of native and model membranes. Comparing the penetrability of barbiturates in SPMV, SPMVTL and SPMVPL, SPMV was the most permeable. Then was SPMVTL, SPMVPL being the least. The reasons of the different penetrability of the barbiturates can be analyzed as follows: (i) the penetrability of barbiturates into the SPMV may be amplified by the presence of proteins(the lipid-protein interactions) which are found to be tightly associated with lipids through covalent or noncovalent bonds, (ii) gangliosides may have a significant role in the penetrability of the barbiturates, and (iii) the differences between SPMVTL and SPMVPL in the penetrabilities of barbiturates cannot be fully explained. However, such phenomenon can be explained by following mechanisms. Cholesterol is one of the major components in biological membranes, yet the role of cholesterol in membranes is not clear enough. In native and model membranes, there is evidence that cholesterol is inhomogeneously distributed and creates regions of differing fluid-

ity¹⁵). Although native and model membranes are usually in the liquid-crystalline state, it seems that they have some gel-phase regions which are interposed sparsely¹⁶). Thus, the penetrability of barbiturates may be amplified by the cholesterol(cholesterol-gel phase interactions) which are concentrated in the gel-phase regions of SPMV and SPMVTL.

To the best of our knowledge, the results presented herein are the first to demonstrate the penetration site of barbiturates into SPMVPL.

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REFERENCES

- 1) Harris RA, Groh GI. *Membrane disordering effects of anesthetics are enhanced by gangliosides. Anesthesiology* 1985; 62: 115-119.
- 2) Harris RA, Schroeder F. *Interaction of barbiturates with brain membranes. Soc Neurosci Abstr* 1980; 6: 722.
- 3) Lee AG. *Interactions between phospholipids and barbiturates. Biochem Biophys Acta* 1976; 455: 102-108.
- 4) Novak RF, Swift TJ. *Barbiturate interaction with phosphatidylcholine. Proc Natl Acad Sci U.S.A.* 1972; 69: 640-642.
- 5) Pang KY, Chang TL, Miller KW. *On the coupling between anesthetic induced membrane fluidization and cation permeability in lipid vesicles. Mol Pharmacol* 1979; 15: 729-738.
- 6) Yun I, Kim HI, Hwang TH, et al. *Effects of barbiturates on the fluidity of phosphatidylethanolamine model membranes. Korean J Pharmacol* 1990; 26: 209-217.
- 7) Kang JS. *Effects of barbiturates on the fluidity of native and model membranes. Ph.D. Thesis(Pusan National University)* 1990; 34-42.
- 8) Rall TW. *Goodman and Gilman's the pharmacological basis of therapeutics. 8th ed, Tokyo: Per-*

- gamon Press. 1991; 358-360.*
- 9) Yun I, Kim YS, Yu SH, et al. *Comparison of several procedures for the preparation of synaptosomal plasma membrane vesicles.* Arch Pharm Res 1990; 13: 325-329.
 - 10) Yun I, Kang JS. *The general lipid composition and aminophospholipid asymmetry of synaptosomal plasma membrane vesicles isolated from bovine cerebral cortex.* Mol Cells 1990; 1: 50-20.
 - 11) Yun I, Kang JS. *Effects of barbiturates on transbilayer fluidity domains of phospholipid model membrane monolayers.* Korean J Pharmacol 1992; 28: 103-114.
 - 12) Kim IS, Baik SY, Baik SW, et al. *The penetration site of barbiturates into brain membranes.* J Korean Soci Anesth 1993; 26: in press.
 - 13) Yun I, Jung IK, Kim IS, et al. *The penetration of local anesthetics into the synaptosomal plasma membrane vesicles isolated from bovine brain.* Asia Pacific J Pharmacol 1990; 5: 19-26.
 - 14) Kim IS, Kim HK, Chung KS. *The penetration of barbiturates into model membranes of cholesterol plus phospholipids extracted from brain membranes.* J Korean Soci Anesth 1993; 26: in press.
 - 15) Harris RA, Schroeder F. *Ethanol and the physical properties of brain membranes.* Mol Pharmacol 1981; 20: 128-137.
 - 16) Yun I, Kang JS. *Transbilayer effects of N-alkanols on the fluidity of phospholipid model membranes.* Arch Pharm Res 1992; 15: 152-161.
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