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Effects of normal alcohols and isoflurane on lipid headgroup dynamics in nicotinic acetylcholine receptor-rich lipid vesicles

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Abstract

The trend of evidence suggests that general anesthetics act directly on proteins in the neural membrane. However, the fact that the functions of nicotinic acetylcholine receptor (sodium permeability, desensitization rate) are modulated by the composition of the membrane in which it is reconstituted has been thought to be a result of the variation of interactions between acetylcholine receptor and membrane. In this study, protein–lipid interaction at the level of the lipid headgroup was investigated using electron paramagnetic resonance (EPR) and headgroup spin label. Lipid headgroup mobility was evaluated with rotational correlation time from the EPR spectrum. Protein–lipid interaction at headgroup depth was demonstrated from the motionally restricted component of the spectrum. Rotational correlation time increased to 13 ns from 7 ns due to protein–lipid interaction. The effect of anesthetic (ethanol, 1-hexanol, and isoflurane) on protein–lipid interaction was investigated, and the correlation time was 13 ns. It is concluded that the anesthetics used in this study did not alter protein–lipid interaction at the level of the lipid headgroup, so far as observed by rotational correlation time, without excluding the possibility that anesthetics that perturb protein–lipid interactions modulate receptor functions via this mechanism.

Keywords: Nicotinic acetylcholine receptor; Reconstitution; Lipid–protein interaction; Electron paramagnetic resonance; Headgroup spin label; Anesthetic

1. Introduction

Both proteins and lipids have been proposed as important sites of action for general anesthetics in the central nervous system (CNS). The superfamily of ligand-gated ion channels, including the γ-aminobutyric acid A ($GABA_A$), nicotinic acetylcholine (nACh), and glutamate receptors are key excitable proteins in the CNS [1], and they have common amino acid sequences in transmural domains [2]. At surgical concentrations, volatile anesthetics potentiate inhibitory synaptic transmission by effects on the $GABA_A$ receptor [3], while inhibiting neuronal nACh receptor-mediated excitatory transmission [4,5]. Of all members of this superfamily, muscle type nACh receptor from *Torpedo* electroplaque (following isolation, purification, and reconstitution) is the best characterized, and it can be obtained in sufficient quantity for biophysical studies.

Function of the nACh receptor is dependent on its membrane lipid environment [6]. Reconstitution studies support the importance of both bulk and boundary lipid properties; both affinity-state transi-

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tions (desensitization) and permeability responses to agonists have been demonstrated to depend on the lipid species employed [7,8]. From EPR experiments with reconstituted nACh receptor, it is known that presence of the protein affects lipid rotational correlation time, and that the relative affinity of spin-labeled lipid for the nACh receptor depends on the specific lipid headgroup identity [9]. Relations between ion gating function and boundary lipid dynamics have been established [10].

One hypothesis for the molecular mechanism of volatile anesthetics and alcohols at nACh receptors is that the protein–lipid interface is perturbed by weak binding of numerous anesthetic or alcohol molecules to low-affinity noncompetitive blocking sites [11]. Attempts to address this hypothesis include EPR experiments demonstrating that general anesthetic-induced desensitization of *Torpedo* nACh receptor correlates with bulk lipid disordering [12]. However, using 14-PCSL, a spin probe labeled at the acyl region of phosphatidylcholine, it was shown that even supraphysiological concentrations of ethanol, diethyl ether, and urethane failed to change either the number of boundary lipid molecules, or their rotational correlation times in nACh receptor-rich membranes, although very high concentrations of 1-hexanol modestly reduced the number of boundary lipids [13]. In studies with reconstituted nACh receptors, ethanol, 1-hexanol, pentobarbital, and isoflurane changed neither boundary lipid number nor rotational correlation times [14,15]. However, these experiments limited their focus to lipid dynamics at the acyl chain level of the membrane. To date, no investigation of lipid headgroup dynamics has been undertaken in the presence of anesthetics.

Therefore, to examine the hypothesis that general anesthetics affect nACh receptor function by modulating protein–lipid interactions at the level of lipid headgroups, we chose a spin-label with a nitroxyl moiety incorporated into the phosphatidylcholine headgroup ('HGSL,' Fig. 1). HGSL is able to reflect molecular dynamics at the headgroup level of lipid bilayers [16]. It was found that ethanol and isoflurane did not change the rotational correlation time at the headgroup region of the boundary lipid, while1-hexanol enhanced the exchange between the bulk and boundary lipid.

2. Materials and methods

2.1. Chemicals

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine (HGSL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Bromoacetylcholine bromide was obtained from Research Biochemical International (Natick, MA, USA). All other chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. AffiGel-10 was obtained from Bio-Rad Laboratories (Hercules, CA, USA). 'Buffer A' (below) consisted of 100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% NaN_3, adjusted to pH 7.4 with 10 N NaOH. Isoflurane was obtained from Ohmeda PPD Inc. (Liberty Corner, NJ, USA).

2.2. Methods

2.2.1. Preparation of nACh affinity gel

AffiGel-10 was derivatized for acetylcholine affinity following the method of Chak and Karlin [17]. Half-life of the affinity site was 5 days at 4°C in buffer with 0.02% NaN_3. The gel was prepared fresh for each purification.

2.2.2. Preparation of nACh rich membrane

Frozen electroplaque tissue from freshly dissected *Torpedo nobiliana* (BioFish Associates, Georgetown, MA, USA) was thawed and purified by a modification of Chak and Karlin's procedure [17]. The entire procedure was carried out at 4°C. In brief, 1000 g of *T. nobiliana* electroplaque tissue and 6 ml of 0.3 mM PMSF were added to 1200 ml of phosphate buffer (1 mM EDTA, 10 mM sodium phosphate, pH 7.4) and homogenized in a Osterizer processor (Oster Corp., Milwaukee, MN, USA) for 60 s at the 'liquefy' setting. The homogenate then was filtered through four layers of cheesecloth and then centrifuged at $30\,000 \times g$ for 90 min. The pellets were rehomogenized in 700 ml of the phosphate buffer in the Osterizer for 20 s. Homogenates were again filtered and centrifuged as above, and 90 ml of clear, colorless, soft pellet was obtained.

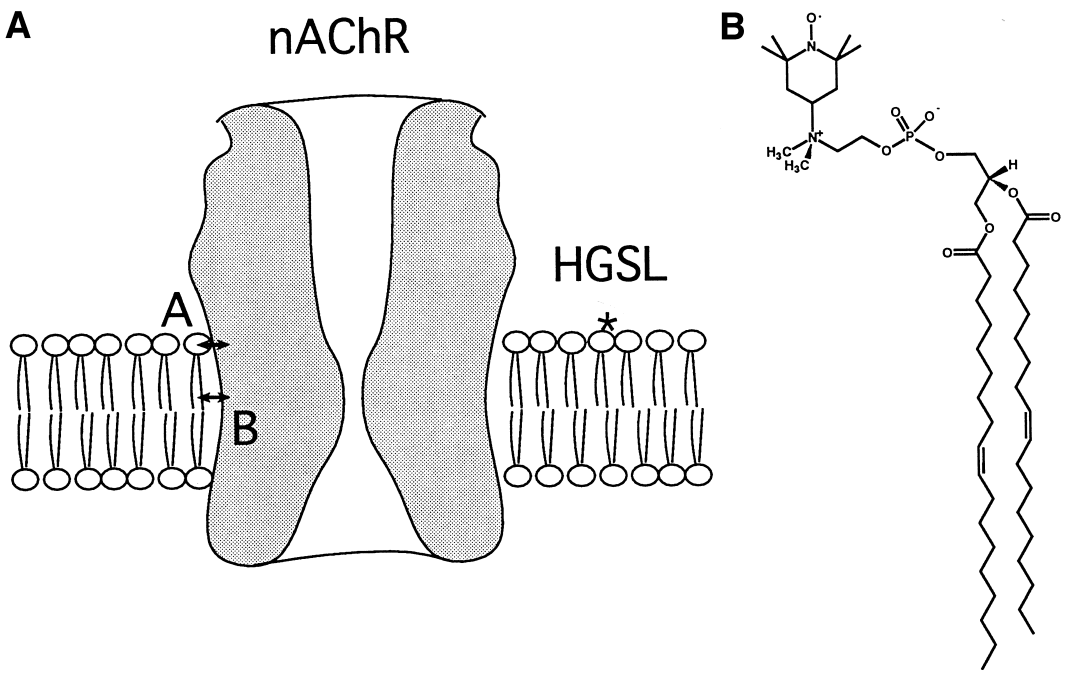

Fig. 1. Structure of headgroup spin-label (HGSL): 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine.

2.2.3. Purification and reconstitution of nACh receptor

The pellets from 1000 g of *Torpedo* were resuspended in 600 ml of 'Buffer A' containing 1% (w/v) cholate and stirred for 2 h to solubilize the receptor. The mixture was centrifuged at 256 000×g for 30 min and the supernatant was collected. Extracts from 500 g of *Torpedo* tissue were gently stirred with 50 ml of bromoacetylcholine-derivatized Affi-Gel-10 for 2 h, to adsorb the receptor. The receptor-bound gel was packed in a 5-cm (internal diameter) column. The column then was washed with 200 ml of 'Buffer A' containing 1% cholate, at the rate of 100 ml/h, after which it was washed at the same rate with 200 ml of 'Buffer A' containing 0.043 mM DOPC and 0.5% cholate. The receptor was eluted with 'Buffer A' containing 20 mM carbamylcholine chloride, 0.043 mM DOPC, and 0.5% cholate. Protein-rich fractions were collected by measuring the absorbance at 280 nm, and then dialyzed against six changes of 6 l of 'Buffer A' for 12 h. The reconstituted protein thus obtained was concentrated by Centriprep-50 (Amicon Inc., Beverly, MA, USA) to a few milliliters. To adjust vesicle size, the concentrate was put through six cycles of freeze and thaw, with the freeze phase carried out at the temperature of liquid nitrogen, and thaw at room temperature. The reconstituted protein was stored at −70°C; lipid to protein ratio (L:P) of the reconstituted receptor was adjusted by changing the DOPC concentration in the column eluate.

2.2.4. Protein and lipid analysis of reconstituted nACh

Protein amount was determined by the BCA method [18] using BSA standard. Lipid amount was determined by inorganic phosphor analysis [19]. The L:P ratio was calculated assuming a receptor molecular weight of 250 kDa. Polyacrylamide gel electrophoresis was performed following the method of Karlin [20]. Reconstituted protein (50 μg) was dialyzed against 250 ml of 73 mM SDS, 2 mM EDTA, 10 mM Tris-acetate (pH 8.0) for 2–4 h at room temperature. Protein was precipitated by acetone and dried in a vacuum. The sample was dissolved in *Lamielli* sample buffer and held at 50°C for 0.5–2 h. *N*-ethylmaleimide was then added to a concentration of 30 mM, which was held at 50°C for 15 min. 7.5% of gel containing 37 mM SDS, 2 mM EDTA, 0.1 M Tris-acetate (pH 8.0) was used for separation. The gel was then stained with Coomassie Blue; purity was evaluated by gel densitometry.

2.2.5. Preparation of DOPC vesicles

15 mg of DOPC was dissolved in 40 ml of 0.5% cholate in 'Buffer A'. The DOPC solution was dialyzed six times with 6 l of 'Buffer A' for 12 h. The vesicle obtained was concentrated with Centriprep-50 (Amicon, MA, USA) and passed through seven cycles of freeze-and-thaw (frozen at the temperature of liquid nitrogen and thawed at room temperature). Vesicles were stored at −70°C.

2.2.6. Incorporation of HGSL into reconstituted nACh receptor and DOPC vesicles

1.5×10^{-8} mol of HGSL in chloroform was dried down in a stream of nitrogen gas (grade 4.8), and added to 8×10^{-7} mol lipid-only vesicles or reconstituted nACh receptor-rich vesicles. The sample was vortexed for 30 min at room temperature. The spin-labeled vesicle was sealed in a 3-ml UltraLok (Nalge, Rochester, NY, USA) centrifuge tube with 'Buffer A,' containing a defined concentration of each anesthetic. The vesicles were centrifuged at $200\,000\times g$ for 45 min at 4°C. The obtained pellet was resuspended in 30 μl of 'Buffer A', both with and without anesthetics, and transferred to an EPR capillary tube. Air in the free space of the capillary was replaced with nitrogen gas. The capillary was heat-sealed in flame. The EPR samples were frozen in liquid nitrogen and thawed at room temperature seven successive times, then stored at −20°C.

2.2.7. Preparation and analysis of 'Buffer A' with isoflurane

'Buffer A' was saturated by isoflurane at 4°C following a previously published method [21]. Isoflurane buffer was diluted to one half the concentration of the saturated stock solution and used for the pelleting procedure described above. Following centrifugation, the supernatant was analyzed using a gas chromatograph (Perkin-Elmer Model 8500).

2.2.8. EPR measurements

Spectra were obtained with a Brucker ER300 EPR spectrometer equipped with the EPS 1600 data acquisition and processing system (Billerica, MA, USA). Acquisitions were performed using a TE102 cavity equipped with a variable temperature unit. Spectra were obtained and recorded 100–196 times from each reconstituted and pure DOPC vesicle preparation, then averaged and digitally stored. Spectral acquisition parameters were: microwave frequency 9.5 GHz and power 1.58 mW; modulation frequency 100 kHz and modulation amplitude 0.2 mT; and sweep width 12 mT. Microwave power was chosen to avoid signal saturation. Temperature was observed by thermocouple at the bottom of the cavity, and maintained at 278 K by passage of a stream of dry nitrogen.

2.2.9. Spectral digitization and integration

The magnetic field axis was digitized to 1024 points, and the spectral amplitude was digitized to 32 bits. Data processing was performed with Brucker EPS 1600 software from digitally stored spectra. Reconstituted nACh receptors and the corresponding difference spectra were double integrated by numerical integration to quantify the boundary lipid fraction. First, a first-derivative spectrum was integrated and a tentative absorption spectrum was obtained. Then the spectrum was linearly baseline-corrected to obtain the zero-average of baselines in the lower and higher fields of the absorption spectrum. Iteratively, the baseline was corrected so that the first integral baseline values averaged zero. After these baseline corrections, the absorption spectrum was again integrated to obtain absorption intensity.

2.2.10. Spectral subtraction and boundary fraction (f_b)

Spectra from protein-free HGSL-labeled DOPC vesicles at 278 K were digitally aligned and subtracted from reconstituted nACh receptor spectra at 278 K, to yield 'difference' spectra of the motionally restricted lipid component. The subtraction coefficient for endpoint was determined following the criteria by Jost and Griffith [22]. First, the gross endpoint was determined by cancelling out the second lower-field peak of the experimental spectrum. After the tentative subtraction, the difference spectrum was integrated to detect over-subtraction (over-subtracted spectrum would result in a decreasing baseline). Iteratively, a subtraction coefficient was carefully determined so as not to produce over-subtraction. Poor signal:noise ratio spectra were Fourier-transformed and zero-filled, then reverse-transformed to improve apparent signal to noise ratio (Figs. 3C, 5C and 6). Zero-filling points were

carefully selected so as to avoid distortion of the original spectral line shape. The f_b was calculated as f_b = (absorption intensity of the difference spectra)/(absorption intensity of nACh reconstituted vesicles spectra). The error in determination of the fractional components is estimated to be ±0.05 [22].

2.2.11. Rotational correlation times (τ_c)

For DOPC vesicles, and for vesicles containing nACh receptors, the rotational correlation time, τ_c, was calculated with the assumption that the anisotropy of hyperfine interactions are not completely averaged out, so that:

$$\tau_c = 6.5\times10^{-10}\ (\Delta H_0)((h_0/h_{-1})^{1/2}-1) \quad (1)$$

where ΔH_0 is the line width of the central line (in Gauss), and h_0 and h_{-1} are the amplitude of the center and high-field EPR lines [23].

For difference spectra, τ_c was calculated from the hyperfine splitting 2 A_{max} (distance between low-field peak to high-field dip) assuming a Brownian rotational diffusion model for isotropic motions as follows:

$$\tau_c = a\ (1-A_{max}/A^r_{max})^b \quad (2)$$

where A^r_{max} is half of the hyperfine splitting of immobilized spin-label, measured from HGSL–DOPC as 34.9 G at 233 K.[2] The values for '*a*' and '*b*' are 5.4×10^{-10} s and −1.36, respectively, based on the simulation of Freed [24].

3. Results

3.1. Characterization of reconstituted nACh receptor

Reconstituted DOPC vesicles containing purified acetylcholine receptor were prepared from the electroplaques of *T. nobiliana*. Typically, 20 mg of nACh receptor (BCA method) was obtained from 1000 g of tissue. Higher DOPC concentration in washing and elute solutions led to decreased yield of nACh receptor. The L:P ratios of the final reconstituted vesicles were adjusted by equilibration of the receptor with controlled concentrations of DOPC in washing and eluant solutions. The solutions contained 0.143 and 0.0456 mM DOPC reconstituted to L:Ps of 218 (mol:mol) and 124, respectively. The L:P ratio was confirmed from the protein and lipid analyses. Protein composition of the reconstituted nACh receptor was analyzed by SDS polyacrylamide gel electrophoresis. It showed only the four bands corresponding to the α,β,γ, and δ subunits. Integration of densitometer traces of four bands indicated that 90% of protein on the gel was nACh receptor; the subunit ratio of α:β:γ:δ was 2:1:1:1.

3.2. Spectra of head spin-labeled DOPC vesicles

DOPC vesicles made by ultrasonication [14,15] did not yield a regular, reproducible EPR baseline; signals were dependent on sonication batch. We therefore used dialysis to prepare the DOPC vesicle for HGSL–DOPC spin-label. The baselines of the HGSL–DOPC spectra were wavy initially, even though the DOPC ratio was designed to be less than 2% of DOPC molecule number to avoid spin–spin interactions. However, following a seven-cycle freeze-and-thaw process, the spectral baseline became linear, and independent of both spin-label batch and vesicle batch. EPR spectra of DOPC labeled with HGSL at 5°C are shown in Fig. 2. A single component signal from the bulk lipid environment was observed. The effective rotational correlation time was determined to be 7 ns from line width measurement.

3.3. Spectra of headgroup spin-labeled reconstituted nACh receptors

The nACh receptor was reconstituted in DOPC vesicles and labeled with HGSL, a phosphatidylcholine spin-label. Typical EPR spectra at 5°C are shown in Fig. 2. As in previous reports using an acyl chain spin-label, the spectra from nAChR reconstituted in DOPC showed two motionally distinct components. The portion of motionally restricted component increased with decreasing L:P ratio in the reconstituted vesicles. Therefore, this motionally restricted component is assigned to HGSL associated

[2] Apparent hyperfine coupling constant, A_{max}, from HGSL–DOPC in the temperature range from 233 to 293 K were measured and evaluated. A_{max} decreased rapidly above 250 K, showed slight temperature dependence below it. Thus, 34.9 G at 233 K was taken as rigid limit of molecular motion of headgroup spin label in DOPC vesicles.

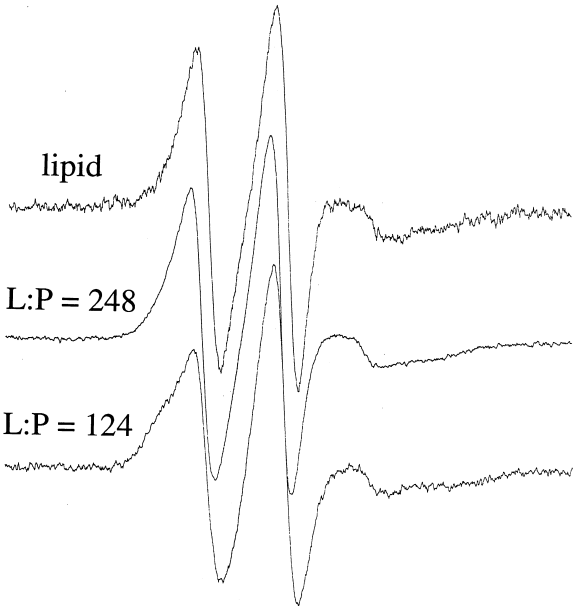

Fig. 2. EPR spectra of HGSL in DOPC vesicle (lipid), and reconstituted nAChR–DOPC (L:P = 124) at 278 K. Spectral change is shown as a function of L:P ratio. Spectra were normalized to the same central peak height.

with the nACh receptor (boundary lipid). The other environment is more mobile, and characteristic of lipids not associated with the receptor (bulk lipid). The mobile component showed an effective correlation time of 7 ns (Table 1), corresponding to that of pure DOPC vesicle bilayers.

3.4. Deconvolution of spectra from reconstituted nACh receptors

The line-shape of pure DOPC vesicles was matched to the mobile component of nAChR in DOPC at 5°C for subtractions. Fig. 3 shows the spectrum of nAChR reconstituted into DOPC with 124 lipids per receptor (A), the spectrum of pure DOPC vesicles (B) at 5°C, and the difference spectrum (C), which represents the motionally restricted component. A small, low-field, second peak in the difference spectrum originated from residual mismatching during subtraction; however, this did not contribute significantly to integrated signal intensity.

The difference spectrum (Fig. 3C) showed hyperfine splitting, $2A_{max}$, as 63.2 G. The spectral lineshape resembled that of pure DOPC vesicles at low temperature. The rotational correlation time of the motionally restricted component was estimated to be 13 ns; that of the mobile component was 7 ns, coinciding with that obtained from pure DOPC vesicles (Table 1).

Double integration of spectrum A and C indicated that the f_b was 0.31. The number of boundary lipids adjacent to nACh receptor was estimated to be 43 by the method of Brotherus [25], assuming that the affinity of HGSL for nACh receptors was not significantly different from 1.0.

Table 1
Effect of anesthetics on calculated correlation times (τ_c) for headgroup spin-labeled nACh receptor reconstituted into DOPC at 278 K

[DOPC]:[nAChR] (mol:mol)	Anesthetics	[ethanol] (mM)	τ_c (ns)	f_b	τ_c^b (ns)
124		0	7	0.31	13
138	ethanol	190	8	0.40	13
138	ethanol	380	8	0.34	12
147	isoflurane	8.2	7	0.30	13
147	1-hexanol	8	6	0.38	9
147	1-hexanol	16	6	0.36	–
lipid		0	5	–	–
lipid	ethanol	95	7	–	–
lipid	ethanol	190	6	–	–
lipid	ethanol	285	6	–	–
lipid	ethanol	380	6	–	–
lipid	isoflurane	8.2	7	–	–
lipid	1-hexanol	8	6	–	–
lipid	1-hexanol	16	5	–	–

f_b and correlation times (τ_c^b) of motionally restricted labels at lipid–protein interface at 278 K.

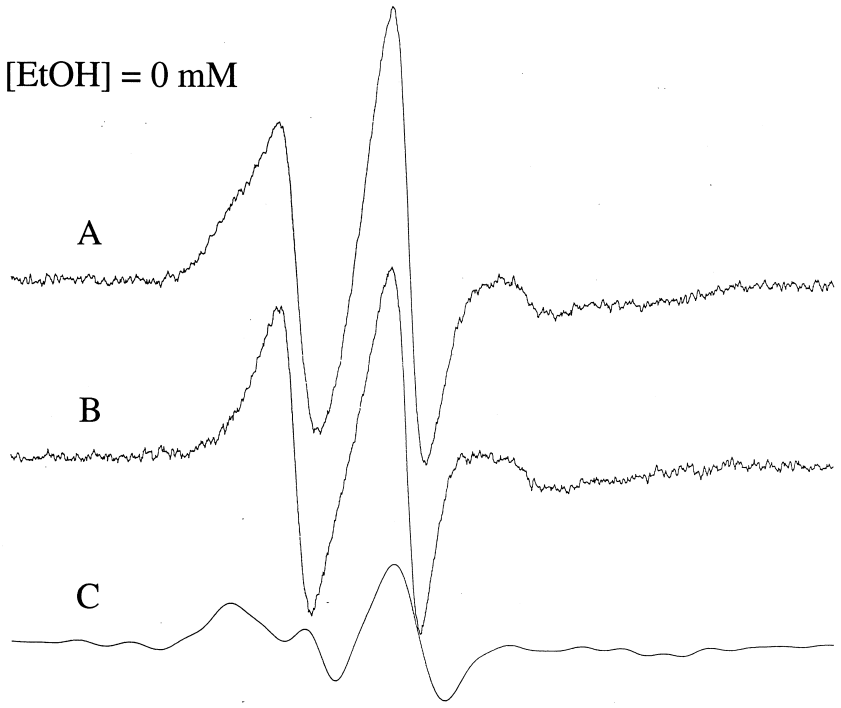

Fig. 3. EPR spectra of HGSL in nAChR reconstituted in DOPC (L:P = 124) (A); DOPC vesicle (B); and their difference spectrum (C), the motionally restricted, or protein-associated component at 278 K. The relative proportion of spectral heights was preserved. Spectrum C was smoothed after subtraction by the zero-filling method.

3.5. *Effect of ethanol, 1-hexanol and isoflurane on DOPC vesicles*

EPR spectra of HGSL in pure DOPC vesicles in the presence of supraphysiological concentrations of ethanol or isoflurane showed no significant difference in line-shape. The rotational correlation time of HGSL in DOPC proved to be 6–7 ns in the presence of ethanol (95–380 mM) and 7 ns in the presence of isoflurane (8.2 mM) (Table 1).

In the presence of 8–16 mM of 1-hexanol, EPR spectra of HGSL in pure DOPC vesicles were narrowed at 5°C, as previously reported [14]. Hexanol increased DOPC mobility and enhanced the exchange rate between spin-labeled lipids; the rotational correlation times of HGSL in pure DOPC in the presence of 8 and 16 mM of 1-hexanol at 5°C, were 6 and 5 ns, respectively.

3.6. *Effect of ethanol on reconstituted nAChRs*

Fig. 4 shows the EPR spectra of HGSL in reconstituted nACh receptor-rich vesicles in the presence

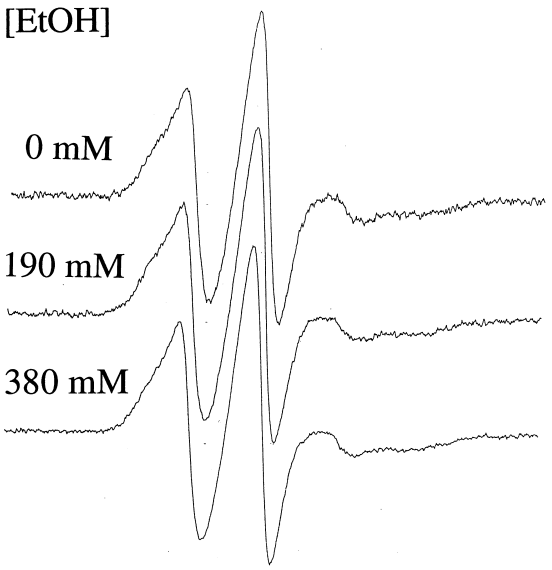

Fig. 4. Effect of increasing concentrations of ethanol on nAChR reconstituted in DOPC, spin-labeled with HGSL at 278 K (L:P = 138). Spectra were normalized to the same central peak height.

of ethanol at 5°C. The spectra from nAChR–DOPC vesicles showed two motionally distinct lipid environments over the range 0–380 mM, with minimal effects on spectral line-shapes.

Over the same range of ethanol, subtractions produced undistorted difference spectra, meaning that the separation of the two spin systems was complete, and any exchange effects were minimal. In the presence of 380 mM of ethanol, Fig. 5 shows the spectrum of nAChR reconstituted into DOPC with 138 lipids per receptor (A); and the spectrum of pure DOPC vesicles (B) at 5°C. The mobile component of the spectra showed the same effective correlation time of 8 ns.

Fig. 6 shows the difference spectrum (C), which represents the motionally restricted components at 190 and 380 mM of ethanol. The rotational correlation times of the motionally restricted components were estimated to be 13 and 12 ns, respectively, while that of the mobile component was estimated to be 8 ns. At concentrations of 190 and 380 mM, the restricted component showed no change in hyperfine splitting (63.2 and 63.7 G, respectively), or in the fraction of the protein-associated spectral component (0.40 and 0.34, respectively), within experimental error (Table 1).

3.7. *Effect of isoflurane on reconstituted nAChR vesicles*

The spectra from reconstituted nAChR–DOPC vesicles in the presence of isoflurane at 5°C showed two motionally distinct lipid environments; a concentration of 8.2 mM isoflurane caused minimal effect on spectral line-shapes. The restricted component showed no change in hyperfine splitting (63.0 G), rotational correlation time (13 ns), or the fraction of protein-associated component (0.30), within experimental error (Table 1).

3.8. *Effect of 1-hexanol on reconstituted nAChR vesicles*

Fig. 7 shows the EPR spectra of HGSL in reconstituted nACh receptors, in the presence of 8 mM and 16 mM of 1-hexanol at 5°C. Spectral line-shape changed; hexanol decreased the spectral 'shoulder' of the motionally restricted component.

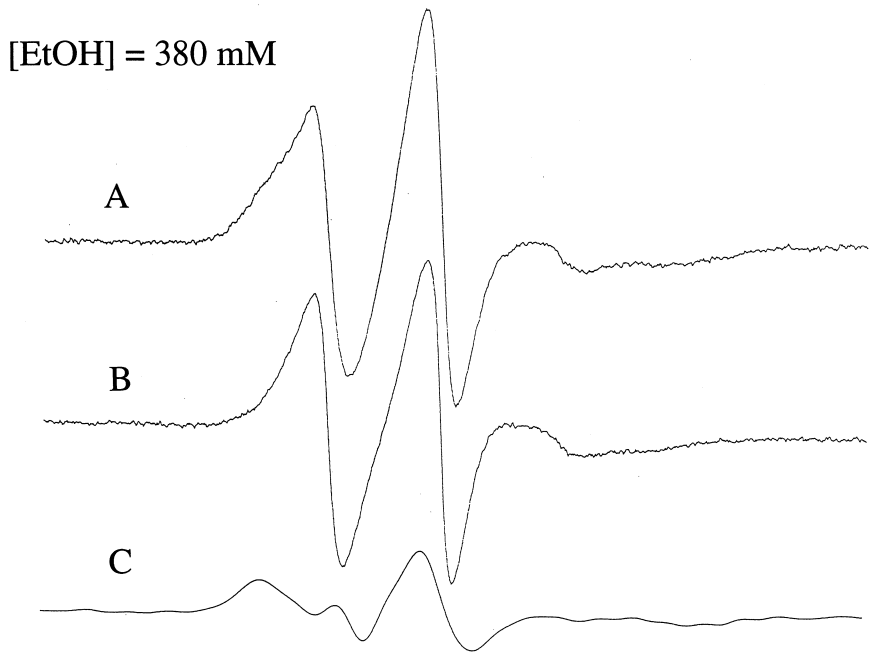

Fig. 5. EPR spectra of HGSL in nAChR reconstituted in DOPC (L:P = 138) with 380 mM of ethanol (A); DOPC vesicle (B) with 380 mM of ethanol; and their difference spectrum (C), the motionally restricted, or protein-associated component with 380 mM of ethanol at 278 K. The relative proportion of spectral heights was preserved. Spectrum C was smoothed after subtraction by the zero-filling method.

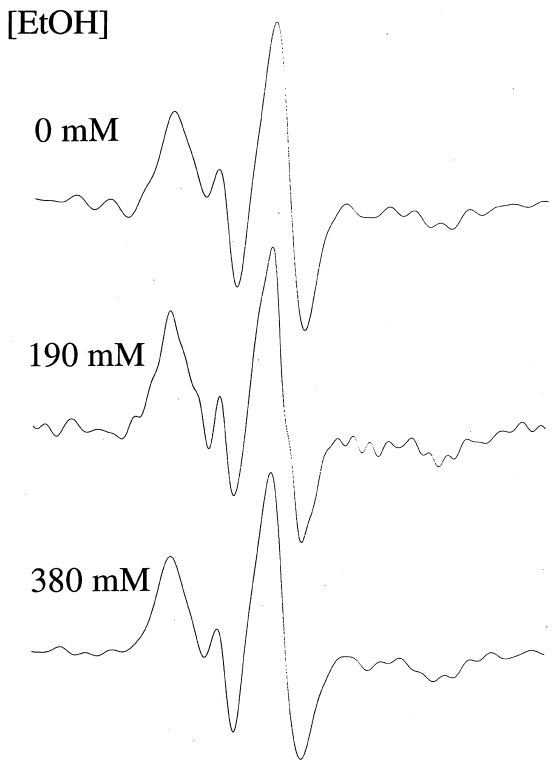

Fig. 6. Spectra of the protein-associated component of HGSL in DOPC exposed to increasing concentrations of ethanol at 278 K (L:P = 138). Spectra obtained were by subtraction of DOPC vesicle from reconstituted nACh receptor. Spectra were normalized to the same central peak height. Spectra were smoothed after subtraction by the zero-filling method.

Spectral subtraction of DOPC vesicles labeled with HGSL from those from nAChR–DOPC produced a clear, protein-associated, motionally restricted component (Fig. 8). The rotational correlation time of this motionally restricted component was estimated to be 9 ns; that of mobile component, 6 ns. The restricted component at the concentrations of 8 mM of 1-hexanol showed hyperfine splitting of 60.8 G, and the fraction of protein-associated component was 0.38 (Table 1).

4. Discussion

HGSL has relatively low affinity for the nACh receptor, similar to that of phosphatidylcholine [9]. Nonetheless, with HGSL, as well as with 14-PCSL, small perturbations of protein–lipid interactions can be detected with high sensitivity [14]. It is thought that there is no significant difference in receptor affinity between HGSL and DOPC, as DOPC has the phosphatidylcholine structure. Thus, molecular dynamics of bulk and boundary DOPC can be probed by HGSL, and anesthetic effects on HGSL dynamics can be faithfully reflected.

Differing from the more typically used spin-labels containing nitroxyl moieties in the acyl region of phospholipid, HGSL nonetheless partitions to the interfacial region of the lipid bilayer. From molecular dynamics simulations of lipid bilayers [26], water molecules exist in abundance at the lipid headgroup region. Recently, interactions between lipid bilayers and anesthetics, investigated by T_2 relaxation time of anesthetics themselves, show that anesthetics are preferentially distributed in this amphiphilic layer of the bilayer, at which site, anesthetics are also readily accessible to the bulk water phase [27]. Thus the use of HGSL allows direct observation of the molecular dynamics of this interfacial zone.

In DOPC, nACh receptors are limited to transitions between resting and desensitized states [7,14]. Thus lipid headgroup contacts seems to limit the functional states of the receptor. HGSL thus provides a method to investigate whether or not anesthetic effects occur at this crucial protein–lipid interface.

Two component spectra were observed in DOPC

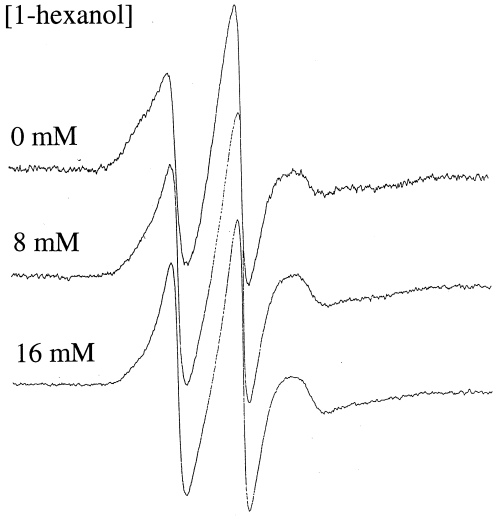

Fig. 7. Effect of increasing concentrations of 1-hexanol on nAChR reconstituted in DOPC spin-labeled with HGSL at 278 K (L:P = 138). Spectra were normalized to the same central peak height.

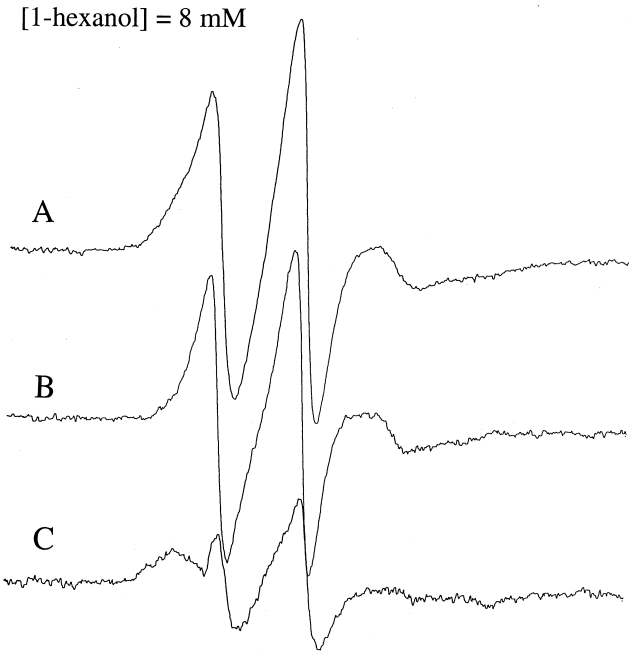

Fig. 8. EPR spectra of HGSL in nAChR reconstituted in DOPC (L:P = 138) with 8 mM of 1-hexanol (A); DOPC vesicle (B) with 8 mM of 1-hexanol; and their difference spectrum (C), the motionally restricted, or protein-associated component with 8 mM of 1-hexanol at 278 K. The relative proportion of spectral heights was preserved. Spectrum C was not smoothed in any part.

vesicles of reconstituted nACh receptor containing trace HGSL. As the ratio of protein to lipid increases, the motionally restricted portion increases; these two components can be attributed to HGSL in bulk DOPC, and in boundary DOPC adjacent to the membrane-bound nACh receptor [9,14]. The number of nAChR boundary lipids has been reported to be 46; 56 ± 2 using 14-PCSL [9,14], and been calculated to be 43 based on the nACh receptor's dimensions [9]. These values are not significantly different from ours (43), particularly when considering the approximations inherent in spectral subtraction, and integration. As for lipid dynamics, rotational mobility of protein-associated 14-PCSL (9.4–11 ns) has been reported to be 40% slower than that of bulk DOPC in bilayers [14]. In the present experiment, rotational mobility of HGSL in boundary lipid (13 ns) was 55% slower than that of bulk DOPC.

With HGSL, spectral separation of the two components is somewhat less well defined than that with 14-PCSL. The exchange rate calculated from hyperfine splittings of the two spectral components obtained with HGSL is less than 7.4×10^7 s^{-1} at 5°C. This would correspond to a rate some 30% higher than that obtained with 14-PCSL at 0°C (5.6×10^7 s [14]). While this may accurately reflect faster diffusion in the place of the bilayer at the headgroup level, uncertainty with spectral subtraction could also account for this difference.

We found that the rotational correlation time derived from the hyperfine splitting of the protein-associated component was 13 ns at 5°C. Adbaji et al. reported this rotational correlation time to be 10 ns at 0°C at the 14-acyl position [14]. Thus the rotational correlation time of the headgroup is comparable to that measured at the 14-acyl position. This implies that motionally restricting interactions at the headgroup are roughly equivalent to those experienced in the acyl region. Similarly, the number of boundary lipids at the headgroup is not different from that at the acyl region. This finding is consistent with Raines and Miller's conclusion that electrostatic interactions do not account for the selectivity of the negatively charged phospholipid [10].

Our results indicate that ethanol and isoflurane do not alter the rotational dynamics of lipid at the headgroup level. Isoflurane (2 mM) is reported to be distributed to the amphiphilic interface of the lipid bilayer [27]. 1-hexanol increases rotational mobility in this same zone and increases lipid disorder in a concentration-dependent manner. But since the ED_{50} of 1-hexanol is 0.7 mM [28], the concentration used in this and previous reports corresponds to supraphysiological concentrations; rotational dynamics of the headgroup only begin to increase at these toxicological concentrations. Overall, headgroup dynamics are insensitive to therapeutic levels of anesthetics.

An anesthetic effect on boundary lipid was observed from 14-PCSL in DOPC-reconstituted nACh receptor [14,15]. It was concluded that 1-hexanol (16 mM), isoflurane (7 mM) or ethanol (1.6 M) altered neither the rotational dynamics nor the fractions of boundary lipid. Similarly, with HGSL, rotational mobility, and the number of boundary lipids, were not altered by ethanol and isoflurane. In this study, 8 mM 1-hexanol slightly increased boundary lipid mobility, but we can safely conclude that therapeutic levels of anesthetics do not change rotational dynamics of boundary lipid.

Using nACh receptor-rich native membranes,

Fraser et al. [13] concluded that boundary lipid dynamics (probed by 14-SASL and 14-PGSL) were unaltered by 6 mM of 1-hexanol. However, with 14-PCSL, hexanol decreased the number of boundary lipids in a concentration-dependent manner, and accelerated exchange between bulk and boundary lipids. Similarly, with 14-PCSL, urethane, diethyl ether, and ethanol decreased the f_b. In our study, HGSL was chosen, since it has the same phosphatidylcholine structure with DOPC which presumably has the same affinity to nACh receptors.

Under the conditions of this study, DOPC-reconstituted nACh receptors could transition from the resting to the desensitized state. The anesthetics we used are able to desensitize receptors. Therefore, our observations were made on a receptor whose state should correspond to desensitized. Hidemann et al. hypothesized that about 60 molecules of noncompetitive blocker occupied the interface of the receptor, and induced desensitization [11]. Yet, although we used anesthetics in high enough concentrations to desensitize receptors and disorder bulk DOPC's, they did not alter the rotational dynamics of boundary lipids or the lipid number binding to the receptor.

Even in the context of our findings, it is still possible that lipid membranes contribute to anesthetic actions at receptors. For example, an investigation of X-ray structure and function of membrane protein with the volatile anesthetic analogue, bacteriorhodopsin with diiodomethane, demonstrated that diiodomethane binds to the interfacial sites between protein and lipid and reduces the longevity of M-intermediate in flash photolysis [29]. As a further example, sodium channels were reconstituted into membranes in which lipid composition was systemically altered by the addition of cholesterol. Suppression of sodium channels by pentobarbital was inhibited by cholesterol, and lipid composition modulated the sodium channel function [30]. Results in this study were limited to discussion of EPR measurement of the rotational correlation time of the lipid molecule. It cannot be generalized nor concluded that anesthetics did not change protein–lipid interactions. A recent estimation of the exchange rate between isoflurane binding sites on the nACh receptor and on the membrane [31] demonstrated that the membrane has an important role in isoflurane's interaction with nACh receptor.

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References

[1] N.P. Franks, W.R. Lieb, Molecular and cellular mechanisms of general anesthesia, Nature 367 (1994) 607–614.
[2] H. Betz, Homology and analogy in transmembrane channel design: lessons from synaptic membrane proteins, Biochemistry 29 (1990) 3591–3599.
[3] M. Wakamori, Y. Ikemoto, N. Akaike, Effects of two volatile anesthetics and a volatile convulsant on the excitatory and inhibitory amino acid responses in dissociated CNS neurons of the rat, J. Neurophysiol. 66 (1991) 2014–2021.
[4] P. Flood, J. Ramirez-Latorre, L. Role, $\alpha_4\beta_2$ neuronal nicotinic acetylcholine receptors in the central nervous system are inhibited by isoflurane and propofol, but α_7-type nicotinic acetylcholine receptors are unaffected, Anesthesiology 86 (1997) 859–865.
[5] J.M. Violet, D.L. Downnie, R.C. Nakisa, W.R. Lieb, N.P. Franks, Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anesthetics, Anesthesiology 86 (1997) 866–874.
[6] F.J. Barrantes, Structural–functional correlates of the nicotinic acetylcholine receptor and its lipid microenvironment, FASEB J. 7 (1993) 1460–1467.
[7] T.M. Fong, M.G. McNamee, Correlation between acetylcholine receptor function and structural properties of membranes, Biochemistry 25 (1986) 830–840.
[8] C. Sunshine, M.G. McNamee, Lipid modulation of nicotinic acetylcholine receptor function: the role of neutral and negatively charged lipids, Biochim. Biophys. Acta 1108 (1992) 240–246.
[9] J.F. Ellena, M.A. Blasing, M.G. McNamee, Lipid–protein interactions in reconstituted membranes containing acetylcholine receptor, Biochemistry 22 (1983) 5523–5535.
[10] D.E. Raines, K.W. Miller, The role of charge in lipid selectivity for the nicotinic acetylcholine receptor, Biophys. J. 64 (1993) 632–641.
[11] T. Heidmann, R.E. Oswald, J.-P. Changeux, Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from *Torpedo marmorata*, Biochemistry 22 (1983) 3112–3127.
[12] L.L. Firestone, J.K. Alifimoff, K.W. Miller, Does general anesthetic-induced desensitization of the *Torpedo* acetylcho-

line receptor correlate with lipid disordering?, Mol. Pharm. 46 (1994) 508–515.
[13] D.M. Fraser, S.R.W. Louiro, L.I. Horvath, K.W. Miller, A. Watts, A study for the effect of general anesthetics on lipid–protein interactions in acetylcholine receptor enriched membrane from *Torpedo nobiliana* using nitroxide spin-labels, Biochemistry 29 (1990) 2664–2669.
[14] V.C. Abadji, D.E. Raines, A. Watts, K.W. Miller, The effect of general anesthetics on the dynamics of phosphatidylcholne-acetylcholine receptor interactions in reconstituted vesicles, Biochim. Biophys. Acta 1147 (1993) 143–153.
[15] V.C. Abadji, D.E. Raines, L.A. Dalton, K.W. Miller, Lipid–protein interactions and protein dynamics in vesicles containing the nicotinic acetylcholine receptor: a study with ethanol, Biochim. Biophys. Acta 1194 (1994) 25–34.
[16] R.D. Kornberg, H.M. McConnell, Inside–outside transitions of phospholipids in vesicle membrane, Biochemistry 10 (1971) 1111–1120.
[17] A. Chak, A. Karlin, Purification and reconstitution of nicotinic acetylcholine receptor, Methods Enzymol. 207 (1992) 546–555.
[18] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[19] C.W. McClare, An accurate and convenient organic phosphorus assay, Anal. Biochem. 39 (1971) 527–530.
[20] A. Karlin, M.G. McNamee, C.L. Weill, R. Valderrama, Method of isolation and characterization of the acetylcholine receptor, in: Method in Receptor Research, 1976, pp. 1–35.
[21] T. Seto, T. Mashimo, I. Yoshiya, K. Kaneshiro, Y. Taniguchi, Solubility and preparation of volatile anesthetic solution, J. Anesth. 12 (1998) 41–42.
[22] P.C. Jost, O.H. Griffith, The spin-labeling technique, Methods Enzymol. 49 (1978) 369–418.
[23] A. Keith, G. Bulfield, W. Snipes, Spin-labeled neurospora mitochondria, Biophys. J. 10 (1970) 618–629.
[24] J.H. Freed, Theory of slow tumbling ESR spectra for nitroxides, in: L.J. Berliner (Ed.), Spin-Labeling, Theory and Applications, Academic Press, New York, 1976, pp. 53–132.
[25] J.R. Brotherus, O.H. Griffith, M.O. Brotherus, P.C. Jost, J.R. Silvius, L.E. Hokin, Lipid–protein multiple binding equilibria in membranes, Biochemistry 20 (1981) 5261–5267.
[26] O. Berger, O. Edholm, F. Jahnig, Molecular dynamics simulations of a fluid bilayer of dipalmitoylphosphatidylcholine at full hydration constant pressure, and constant temperature, Biophys. J. 72 (1997) 2002–2013.
[27] P. Tang, B. Yan, Y. Xu, Different distribution of fluorinated anesthetics and nonanesthetics in model membrane: a ^{19}F NMR study, Biophys. J. 72 (1997) 1676–1682.
[28] L.L. Firestone, J.C. Miller, K.W. Miller, Tables of physical and pharmacological properties of anesthetics, in: S.H. Roth, K.W. Miller (Eds.), Molecular and Cellular Mechanisms of Anesthetics, Plenum, New York, 1986, pp. 455–470.
[29] T. Nakagawa, T. Hamanaka, S. Nishimura, T. Uruga, Y. Kito, The specific binding site of the volatile anesthetic diiodomethane to purple membrane by X-ray diffraction, J. Mol. Biol. 238 (1994) 297–301.
[30] B. Rehberg, B.W. Urban, D.S. Duch, The membrane lipid cholesterol modulates anesthetic actions on a human brain ion channel, Anesthesiology 82 (1995) 749–758.
[31] Y. Xu, T. Seto, P. Tang, L. Firestone, NMR study of volatile anesthetic binding to nicotinic acetylcholine receptors, Biophys. J. 78 (2000) 746–751.