Biochimica et Biophysica Acta, 1147 (1993) 143–153

BBAMEM 75917

The effect of general anesthetics on the dynamics of phosphatidylcholine-acetylcholine receptor interactions in reconstituted vesicles

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(Received 26 August 1992)

Key words: General anesthetic; Anesthetic; Lipid–protein interaction; Acetylcholine receptor; Reconstitution; Spin label

The interaction of general anesthetics at the lipid/protein interface of the nicotinic acetylcholine receptor reconstituted in dioleoylphosphatidylcholine bilayers at various lipid/protein ratios has been studied using the electron spin resonance spectra of phosphatidylcholine spin-labeled at the fourteenth acyl carbon (14-PCSL). In addition to the bilayer spectrum, the spin label reported a more motionally restricted environment whose contribution increased with increasing protein/lipid ratio. Exchange between thses two environments occurred at a rate of approx. $6 \cdot 10^7$ s^{-1}. The motionally restricted, protein-associated 14-PCSL had a rotational correlation time of about 10–20 ns, an order of magnitude slower than when in the bilayer. Addition of 1-hexanol (up to 16 mM) to the reconstituted receptor perturbed the acyl chains of the bulk lipid phase, but the motional properties of the lipid acyl chains at the protein/lipid interface near the membrane center were not significantly perturbed on the EPR motional time-scale. Similarly, anesthetics that were less effective at perturbing the bilayer, such as pentobarbital (up to 2 mM) and isoflurane (7 mM), did not perturb the lipid/protein interface on the conventional EPR motional time scale.

Introduction

General anesthetics are thought to exert their actions on the central nervous system by perturbing excitable membranes. While anesthetic potency correlates with lipid solubility and the ability to perturb the anisotropy of lipid bilayers, decades of work has failed to produce a consensus on the molecular nature of the site of action in excitable membranes [1]. More recently researchers have turned to the lipid/protein interface in order to probe how lipid perturbations might influence a protein's function.

Among the proteins likely to be of relevance to general anesthesia are the ligand-gated channels of the four transmembrane domain superfamily [2]. One member of this family is available in the quantities required for biophysical studies. This is the muscle subtype of the nicotinic acetylcholine receptor, much studied by physiologists [3], which occurs in such large quantities in the electric tissue of *Torpedo* that it has become the best characterized of all ligand-gated ion channels [4]. Heidmann et al. have suggested that anesthetics bind with low affinity at a number of sites located at the lipid/protein interface resulting in a stabilization of the desensitized state [5].

Fraser et al. approached this hypothesis by monitoring the action of general anesthetics on lipid–protein interactions with various spin-labeled lipids incorporated into acetylcholine receptor-rich membranes from *Torpedo nobiliana*. [6]. A doxylstearic acid spin labeled on the fourteenth carbon (14-SASL) reported disordering in the bilayer but little change at the lipid/protein interface, a result confirmed by a preliminary report of Firestone and Ferguson using 12-SASL [7]. However,

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Abbreviations: AcCho, acetylcholine; nAcChoR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; α-BTX, alpha-bungarotoxin; Carb, carbamylcholine chloride; DFP, diisopropylfluorophosphate; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; PCP, 1-(1-phenylcyclohexyl)piperidine; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxyoxazolidine)stearoyl]-*sn*-glycero-3-phosphocholine; *n*-SASL, *n*-(4,4-dimethyl-*N*-oxyoxazolidine)stearic acid.

with a spin-labeled phosphatidylcholine (14-PCSL), Fraser et al. found that, in addition to decreasing bilayer anisotropy, general anesthetics appeared to reduce the affinity of labels for the lipid/protein interface [6]. The 14-PCSL has the weakest affinity for the lipid/protein interface of all the spin labels studied. While this makes it more sensitive to perturbants, it also increases the difficulties associated with the spectral subtraction used to resolve the lipid and the protein-associated components.

To overcome this problem, we have affinity-purified the nAcChoR, reconstituted it into dioleoylphosphatidylcholine (DOPC) at various lipid-to-protein ratios and labeled it with 14-PCSL. This reconstituted system focuses attention on the interaction between a single protein and a single lipid. By using a spin label with the same headgroup as DOPC, changes in the affinity of the lipid for the protein will appear solely as changes in the label's dynamics at the lipid/protein interface and its exchange rate with the surrounding bilayer. A further simplification is that DOPC maintains the nAcChoR in its desensitized conformation which is also the conformation stabilized by the addition of general anesthetics to nAcChoRs in the resting state [8]. Thus, potential complications of effects arising from conformational changes of the protein are eliminated. Therefore, our study is focused on the question of whether perturbants can change the dynamics of phosphatidylcholine–nAcChoR interactions. An additional advantage, of an experimental nature, of our design is that the fraction of protein-associated label can be controlled allowing one to compare different methods of spectral subtraction, a procedure that is particularly difficult in the presence of general anesthetics.

Materials and Methods

Materials

Carbamylcholine chloride (Carb), sodium dodecyl sulfate (SDS), buffer reagents and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Affi-Gel 401 was obtained from Bio-Rad (Richmond, CA).

Buffer A contained 10 mM 3-(*N*-morpholino)propanesulfonic acid, 100 mM NaCl, 0.1 mM EDTA and 0.02% sodium azide and the pH was adjusted to 7.4 with concentrated sodium hydroxide. Doubly distilled, deionized water was used to make all buffers.

Lipids. Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Birmingham, AL). The phosphatidylcholine spin label (14-PCSL) was synthesized as described by Marsh and Watts [9].

Anesthetics. 1-Hexanol was from Aldrich (Milwaukee, WI), pentobarbital from Sigma (St. Louis, MO) and isoflurane (CF_3-CHCl-O-CHF_2) was from Anaquest (Anaheim, CA). 1-(1-Phenylcyclohexyl)piperidine hydrochloride (PCP) was from Research Biochemicals (Natick, MA).

Methods

Reconstitution of nAcChoR into DOPC. The electric organ of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) was dissected and processed according to the procedure of Braswell et al. (1984) and the high speed pellets frozen at −80°C until used [10]. The receptor was reconstituted as described by Ellena et al. [11]. All liquid chromatographic steps were carried out at 4°C, unless otherwise noted. The high speed pellets prepared from approx. 500 g of tissue were thawed and dissolved in 75 ml Buffer A and the acetylcholinesterase activity was inhibited with 0.3 mM DFP. The mixture was centrifuged (125 100 × *g*; 60 min) in a Sorvall A-641 rotor and the supernatant discarded. The pellet was resuspended in 100 ml Buffer A containing 2% (w/v) cholate and stirred for 30 min to solubilize the receptor. The mixture was again centrifuged (125 100 × *g*; 60 min) and the supernatant collected.

A 2.0 × 70 cm glass column was packed with Affi-Gel 401 derivatized with bromoacetylcholine bromide and equilibrated with 1% (w/v) sodium cholate and 1 mg/ml DOPC in Buffer A. The solubilized receptor was applied to the column and washed with 2 column volumes of 1 mg/ml DOPC and 1% (w/v) cholate in Buffer A, followed by 1.5 column volumes of 2.5 mg/ml DOPC and 1% (w/v) cholate in Buffer A and left to stand overnight. The column was again washed with the high lipid-containing buffer, followed by 2 washes of 0.1–0.4 mg/ml DOPC (depending on the desired final lipid to protein ratio) in 0.5% (w/v) cholate in Buffer A. The receptor was eluted with Buffer A containing the concentration of DOPC used above, 0.5% cholate and 20 mM Carb. Fractions were assayed for protein content by measuring the absorbance at 280 nm and the protein-rich fractions were pooled and dialyzed against six changes of 2 liter of Buffer A. Finally, the reconstituted receptor was centrifuged, resuspended in Buffer A and stored at −80°C.

Characterization of reconstituted membranes. The protein content of the reconstituted vesicles was determined by the procedure of Lowry et al. using BSA as standard [12]. A molecular weight of 250 000 was assumed in calculating molar ratios. The phospholipid content was measured as described by McClare [13].

Polyacrylamide gel electrophoresis under denaturing conditions was performed to verify the identity and purity of the receptor [14]. The state of the receptor function was ascertained by a fluorescence assay [15,16]. Fluorescence measurements were made on a SLM Aminco SLM 48000S fluorimeter interfaced to an IBM PC2 computer. Titrations were performed in a 3 ml cuvette where 2 ml of *Torpedo* Ringer buffer (250 mM

NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaP_i, and 0.02% NaN_3, pH 7.0) was added along with 1 μM ethidium bromide. The change in relative fluorescence intensity and the shift in the emission wavelength were monitored upon the successive addition of ~ 0.3 μM (final concentrations) of nAcChoR and 50 μM carbachol, respectively.

Preparation of spin-labeled reconstituted membranes. An ethanolic stock solution of 14-PCSL was added to reconstituted vesicles containing 2–4 mg of protein in 70 ml of Buffer A and stirred overnight at 4°C. The ratio of 14-PCSL/lipid was always $< 1:100$ (mole/mole) and the ratio of ethanol to buffer was $< 1:1000$ (v/v). After incubation, the receptor was pelleted by centrifugation ($125\,100 \times g$; 60 min) and washed three times with Buffer A to remove any unincorporated spin label. The final pellet was transferred into a capillary tube for spectroscopic studies. While establishing conditions for these experiments we noticed that if the ratio of spin label to lipid was higher than 1:100 problems with spin–spin interactions were encountered. For example, if the spectra obtained from the same DOPC/nAcChoR recombinant labeled at 1:200 and 1:50 were subtracted, a broad spectrum that could easily be mistaken for a protein-associated component was obtained. If the 1:50 sample was subtracted from one exposed to 16 mM hexanol, a similar broad spectrum resulted, presumably because hexanol-induced lateral membrane expansion changed the spin–spin interactions.

Anesthetic, in the desired concentration, was added to the capillary tube and mixed with the membranes using a stilette from a 22-gauge spinal needle (Becton Dickenson, Rutherford, NJ). After centrifugation, the supernatant was discarded and the pellet resuspended in anesthetic-containing buffer. This cycle was repeated three times to ensure that dissolution of anesthetic into the membranes did not lower the concentration of anesthetic in the buffer. After the third centrifugation, the sample was immediately placed on ice and the capillary flame-sealed. A Hewlett-Packard 5890 Gas Chromatograph equipped with a J&W DB-5 column and an integrator (Hewlett Packard 3393A) was employed to determine anesthetic concentrations. With isoflurane, which has a much higher vapor pressure than hexanol, it was found necessary to sample the final supernatant above the EPR sample, but for hexanol negligible loss was found to occur on handling and only the stock solutions were routinely chromatographed.

Spin labeling of liposomes. An ethanolic stock solution of 14-PCSL was added to the desired lipid in chloroform at a lipid to spin label molar ratio of one hundred to one. The lipids were dried under a stream of nitrogen and then placed under high vacuum for 4 h. Buffer A was added to make a final lipid concentration of approx. 20 mg/ml. The mixture was vortexed and transferred into a capillary tube for EPR spectroscopy.

EPR spectroscopy. Electron paramagnetic resonance spectra were obtained with a Bruker ER200 interfaced with an IBM 9000 computer and a variable temperature unit. Temperature fluctuations were within ± 0.1 C° as measured by placing a thermocouple just above the cavity. Spectral acquisition parameters were: microwave frequency, 9.5 GHz; microwave power, 10 mW; modulation amplitude, 0.2 mT; modulation frequency, 100 kHz, and scan width of 10–12 mT. Baseline correction, which was much more secure when the wider scan was used, normalization and spectral subtraction were performed on a Macintosh IIfx using a program developed in this laboratory on Igor (Wave Metrics, Lake Oswego, OR). Peak positions and half-height widths were estimated using the same program by fitting appropriate sections of the spectra to Gaussian curves. If these line widths were not symmetrical, only the outer half of the peak was fitted and the peak widths estimated by doubling the half width [17].

Effective order parameters, S, were calculated with correction for solvent polarity and $A_\perp$ [18,19]. Effective rotational correlation times, τ_r, for the spin labels motionally restricted by the protein were estimated from their outer hyperfine splittings and their low- and high-field half-height widths with a Brownian diffusion model for isotropic motions [20]. The relationship between A'_z, half the outer hyperfine splitting of the motionally restricted component (equivalent to the experimental A_{max}), and A^r_z, half the outer hyperfine splitting of the completely immobilized label at the rigid limit in a similar membrane system obtained from a spectrum of 14-PCSL in DOPC at -100°C, and the rotational correlation time is given by

$$\tau_r = a(1 - A'_z/A^r_z)^b \qquad (1)$$

where values for a and b used in previous studies of the nAcChoR and based on simulations of Freed are $5.4 \cdot 10^{-10}$ s and -1.36, respectively, and $A^r_z = 33.6$ G [11,21]. Further estimates of the rotational correlation times were made from the relationship

$$\tau_r = a'(\Delta_i/\Delta^r_i - 1)^{b'} \qquad (2)$$

where Δ_i is the half line width at half height of the low ($i = l$) or high ($i = h$) field peak of the motionally restricted component and Δ^r_i is the corresponding half line width at half height of the completely immobilized label in a similar membrane system. The parameters a' and b' have been given by the workers cited above. For the low-field peak $a' = 1.15 \cdot 10^{-8}$ s, $b' = -0.943$ and $\Delta^r_l = 2.4$ G and for the high-field peak $a' = 2.12 \cdot 10^{-8}$ s, $b' = -0.778$ and $\Delta^r_h = 2.7$ G. Exchange rates were estimated as described by Davoust and Devaux [22].

Results

Characterization of the nAcChoR

The nAcChoR from *Torpedo nobiliana* was reconstituted in DOPC vesicles at various lipid-to-protein molar ratios. Polyacrylamide gel electrophoresis revealed only the four bands characteristic of the nAcChoR. The conformational state of the reconstituted nAcChoR was determined by fluorescence spectroscopy as previously described [16]. Ethidium has a higher affinity for the desensitized than for the resting state of the nAcChoR (K_d of 0.4 μM and ~ 1 mM, respectively [15]). Addition of native nAcChoR membranes or of nAcChoR reconstituted into DOPC to aqueous ethidium solutions caused an enhancement of fluorescence and a shift of the maxima to lower wavelength. Subsequent addition of carbachol, which desensitized the receptor, caused a further enhancement and shift in native membranes, but in DOPC/nAcChoR membranes this effect is absent or attenuated, consistent with reports that in DOPC membranes the nAcChoR is always in the desensitized state [23]. Finally, addition of PCP, which occupies the same site as ethidium, reversed the membrane-induced enhancement of fluorescence in both native and reconstituted receptors and established that the observed effects were specific.

Spectra of spin labeled phosphatidylcholine in DOPC / nAcChoR membranes

Typical spectra of vesicles spin-labeled with 14-PCSL at 0°C are shown in Fig. 1. In agreement with previous reports for both native and reconstituted membranes containing the nAcChoR, the spectra exhibit two distinct components, corresponding to environments with different motional characteristics of the spin label [6,11,21,24]. The proportion of motionally restricted spin-label component increased with increasing protein concentration in the reconstituted complexes, as can be seen from the increase in spectral amplitude in the lowest and highest field peaks. The intensity of the corresponding high-field dip increased in parallel and is shown with an expanded vertical scale for the lowest lipid/protein ratio in the figure. Thus, this motionally restricted component can be assigned to 14-PCSL associated with the nAcChoR, whereas the narrower spectral component represents the label in the bulk phospholipid bilayer.

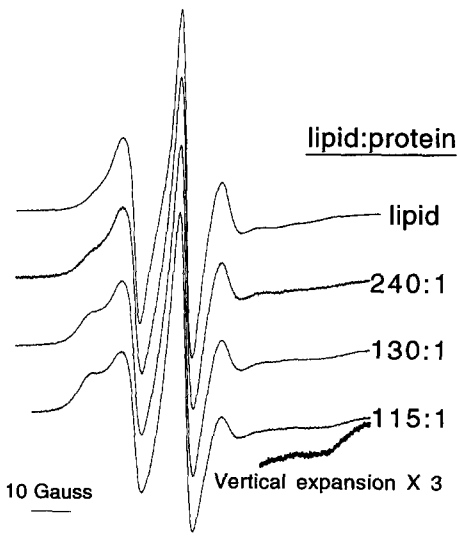

Fig. 1. EPR spectra of 14-PCSL in DOPC bilayers (top spectrum) and in reconstituted vesicles containing the nAcChoR (bottom three spectra) recorded at 0°C. The change in spectral line shape is shown as a function of lipid/protein ratio.

Deconvolution of spectra from DOPC / nAcChoR membranes

This was achieved both by the conventional subtraction method and by pairwise subtraction of reconstitutions having different lipid/protein ratios [11]. We found, as have others, that subtraction of spectra recorded from DOPC vesicles labeled with 14-PCSL from those from DOPC/nAcChoR membranes did not produce satisfactory protein-associated, motionally-restricted component spectra, even when the temperature of the DOPC bilayers was lowered as much as 5–10°C below that of the reconstituted membrane to allow for exchange effects [11]. This was because the line widths of the DOPC bilayer's spectra were too narrow to match the mobile component in the DOPC/nAcChoR membranes. We found that DOPC liposomes containing 2% cholesterol provided a much better empirical model of the bilayer component for spectral subtraction.

Fig. 2 shows: (A) the spectrum of nAcChoR reconstituted into DOPC with 115 lipids per receptor; (B) a typical EPR spectrum of DOPC/2% cholesterol liposomes at 0°C; and (C) the lineshape of the motionally restricted component that results when spectrum B is subtracted from spectrum A. The estimate of the restricted component in Fig. 2C suffered much less from residual mismatching of the central line shapes (as revealed by narrow spikes either side of the central peak) than did any subtraction achieved with DOPC alone (not shown), but even so residual mismatching of the central line remained. These, however, did not contribute significantly to the integrated intensity and could be ignored to a first approximation. Double integration showed the restricted component to be 55% of the composite spectrum and its maximum hyperfine splitting, $2A_{max}$, was 60.5 G, indicative of a spin label in a motionally restricted environment. We found, like others, that the subtraction technique was difficult to apply quantitatively if the fraction of protein-associated label fell below ~ 0.3; hence the $2A_{max}$

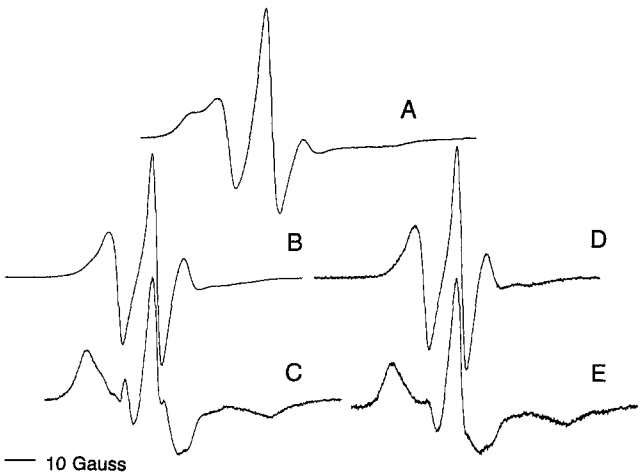

Fig. 2. Comparison of subtraction methods using spectra from nAcChoR reconstituted into DOPC with 115 lipids per receptor and labeled with 14-PCSL. The experimental spectra obtained at 0°C (A) were analyzed either by: (1) subtraction of a mobile spectrum (B) obtained from DOPC/ 2% cholesterol liposomes recorded at −2°C to yield an estimate of the immobile, protein associated, spectrum (C); or (2) by pairwise subtraction of spectra of reconstituted vesicles with lipid/protein ratio of 115:1 and 240:1 to yield estimates of the mobile (D) and restricted (E) components. The fraction of restricted component was estimated to be 0.55 and 0.56 and the $2A_{max}$ was 60.5 and 59.6 G by method 1 and 2, respectively.

values in Table I for the 240:1 DOPC/nAcChoR are less reliable [11].

By recording the spectra of 14-PCSL in several membranes reconstituted with different DOPC to nAcChoR ratios, we were also able to resolve the spectral components by pairwise subtraction after suitably scaling their intensities. Figs. 2D and E show the result of pairwise subtraction of spectra with lipid/protein ratios of 115:1 and 240:1, both recorded at 0°C. The motionally restricted component obtained by this method has identical outer hyperfine splitting and comparable line shape to that obtained by subtraction (compare Figs. 2C and E), but does not suffer from line mismatching artifacts. The mobile component, D, differed only subtly from that of DOPC/2% Chol (spectrum B). The fraction of lipids perturbed by the receptor was 0.56 and $2A_{max}$ was 59.6 G in satisfactory agreement with the estimates obtained above by the subtraction method.

Effect of general anesthetics on DOPC / nAcChoR membranes

EPR spectra of 14-PCSL in reconstituted complexes of DOPC/nAcChoR were recorded in the presence of 1-hexanol, isoflurane and pentobarbital. Typical results obtained at 0°C are shown in Fig. 3 for a membrane preparation with a lipid/protein ratio of 115:1 (mole/mole). Hexanol, over the range 3–16 mM, narrowed the lines of the more mobile component and apparently decreased the amplitude of the less mobile component. Pentobarbital (up to 2 mM) and isoflurane (7 mM) caused minimal effects on the spectral line shapes of these membranes. In order to assess whether general anesthetics altered the shape or the fraction of restricted component (f_b), it was necessary to examine how far the conventional subtraction techniques could be applied in a situation where the mobile phase is being perturbed by hexanol.

Spectral subtraction in the presence of general anesthetics

The presence of membrane perturbing agents complicates spectral subtraction because each component may change line shape due both to changes in lipid mobility and to enhancement of the exchange rate of

TABLE I

Effects of 1-hexanol on the lipid–protein interactions of nicotinic acetylcholine receptors reconstituted in DOPC membranes

[DOPC]/ [nAcChoR] (mole/mole)	[Hexanol] (mM)	Fraction of protein-associated DOPC		$2A_{max}$ (gauss) [a]	τ_r [b] (ns)	Line width (gauss) [a]	
		subtraction	pairwise subtraction			low-field	high-field
115	0	0.55	0.56	59.6	9.4	7.6	14.0
	3	0.56	0.59	59.0	11	7.5	11.8
	7	0.55	0.59	59.9	11	7.3	10.9
	16	0.58	0.56	59.8	10	7.0	12.6
130	0	0.42	0.41	60.0	11	7.6	13.6
	3	0.38	0.38	59.4	9.6	7.2	10.5
	7	0.36	0.38	59.4	10	7.8	8.8
	16	0.43	0.44	59.3	9.9	8.5	8.9
240	0	0.25		61.6			
	3	0.23		61.7			
	7	0.22		62.8			
	16	0.22		62.6			

[a] Calculated from pairwise subtraction for 115:1 and 130:1 and from subtraction for 240:1.
[b] Calculated from Eqn. 1 using data from pairwise subtractions and parameters given in Materials and Methods.

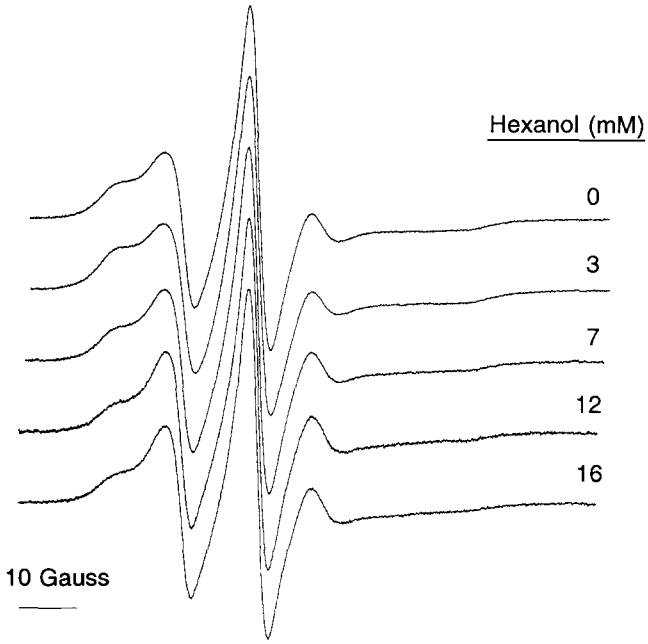

Fig. 3. Effect of increasing concentrations of 1-hexanol on nAcChoR reconstituted into DOPC with 115 lipids per receptor and labeled with 14-PCSL. Temperature 0°C.

the spin-labeled lipid between bilayer and protein-associated states. Consequently, we compared the pairwise technique of subtracting the spectra from membranes with different DOPC/nAcChoR ratios to the more generally applicable technique of subtracting a model lipid spectrum (see Figs. 4C–F). Pairwise subtraction of DOPC/nAcChoR membranes of mole ratio 115:1 and 240:1, both in the presence of 16 mM hexanol (C and D), is compared with subtraction of a suitable model lipid spectrum (DOPC/2 mole percent Chol treated with 16 mM hexanol recorded at −2°C) from the 115:1 DOPC/nAcChoR membrane also in 16 mM hexanol (E and F).

Both the model lipid spectrum and that obtained by pairwise subtraction are considerably narrowed by the hexanol (compare Figs. 4D and F with B). The protein-associated components obtained by both methods of subtraction were comparable in the high- and low-field region (compare Figs. 4C and E), but the difficulty of matching the narrow central lines of the spectrum from DOPC/AcChoR membranes with those from the model lipid is accentuated in the presence of hexanol (compare Figs. 4E and 2C) presumably because the lipid component is narrower in the presence of hexanol. The outer hyperfine splittings obtained by the two subtraction techniques did not differ systematically. In Table I the fraction of protein-associated 14-PCSL estimated by the two subtraction techniques is compared. Average variation is within five percent and does not exceed seven percent. Thus, while pairwise subtraction is the method of choice, subtraction techniques provide fair estimates of the outer extrema and the fraction of protein-associated component provided the presence of anesthetic is allowed for in the mobile bilayer component.

An alternative subtraction strategy was to increase the temperature at which the spectrum of 14-PCSL was recorded in DOPC/2% Chol bilayers to mimic the addition of hexanol. For example, a spectrum of this

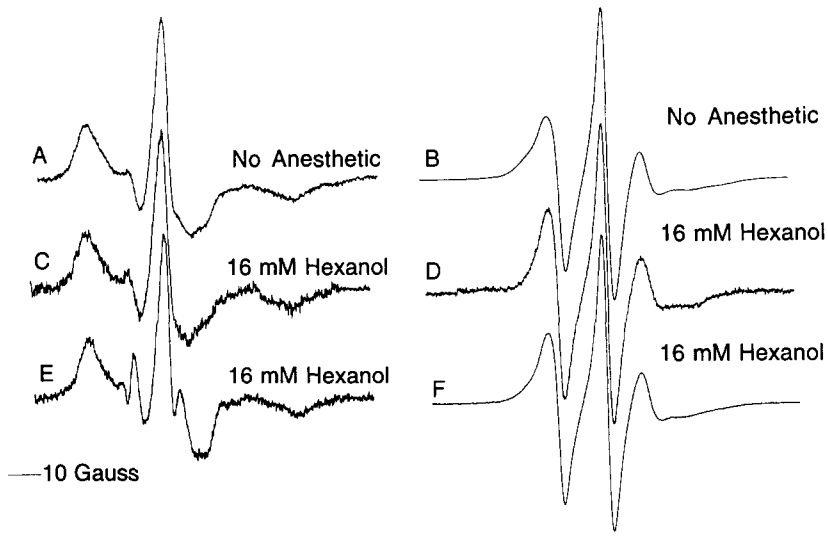

Fig. 4. EPR spectra of 14-PCSL in nAcChoR reconstituted in DOPC resolved into the protein-associated component (A, C and E) and bilayer-associated components (B and D). Spectra A–D were obtained by pairwise subtraction of reconstituted vesicles with lipid/protein ratios of 115:1 and 240:1. Spectrum E was obtained by subtracting spectrum F, acquired from DOPC/2% cholesterol liposomes exposed to 16 mM 1-hexanol, from the experimental spectrum with 115 DOPC per receptor (Fig. 2). All spectra were recorded at 0°C, except (F) which was recorded at −2°C.

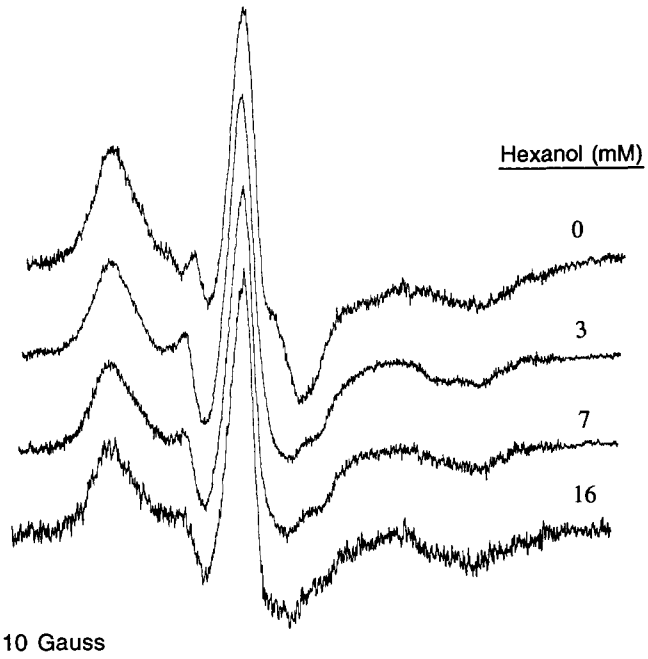

Fig. 5. Spectra of the protein-associated component of 14-PCSL in DOPC bilayers exposed to increasing concentrations of 1-hexanol at 0°C. Spectra were obtained by pairwise subtraction of reconstituted membranes with a lipid to receptor ratio of 115 and 240. Other parameters are given in Table I.

bilayer at 7°C was very similar to one exposed to 16 mM hexanol at 0°C.

Effect of general anesthetics on the two environments in DOPC / nAcChoR membranes

The effect of hexanol on nAcChoR reconstituted into DOPC was determined at each of the three lipid-to-protein ratios examined. All three reconstitutions were analyzed by conventional subtraction and, in addition, pairwise subtraction was performed between the membranes with lipid-to-protein mole ratios of 115 : 1 and 240 : 1 (Fig. 5) and of 130 : 1 and 240 : 1. The data are summarized in Table I. The fraction of motionally restricted spectral component, f_b, did not vary in any systematic way with hexanol concentration, indicating that this agent did not change f_b within the accuracy of the method, that is by more than five percent. Nor did the protein-associated component's maximum hyperfine splitting, $2A_{max}$, or the width at half height of the low or high-field peaks change significantly with hexanol concentration (Table I). However, control spectra showed a shoulder on the right of the center ($m = 0$) peak (Fig. 5) which may be evidence of unaveraged g-value anisotropy due to slow motions of the lipid probe. Although this shoulder was small, it did not appear to be an artifact of the subtraction because it could not be removed by acceptable shifts of the spectra along the field axis before subtraction. Furthermore, its presence was independent of lipid/protein ratio and the subtraction technique. This anisotropy was abolished at the lowest concentrations of hexanol studied (3 mM).

On the other hand, the mobile component was significantly and systematically disordered by hexanol (compare Figs. 4B with 4D and F). This effect was hard to quantify because the order parameter of this component was low in the control ($S \sim 0.3$) and the subtracted spectra were relatively noisy. In DOPC/2% Chol bilayers, where the signal was much larger, the order parameter decreased systematically with increasing hexanol concentration, with a change of nearly ten percent at 16 mM.

Samples of the membranes reconstituted at a DOPC/nAcChoR mole ratio of 115 : 1 were exposed to pentobarbital (up to 2 mM) and isoflurane (7 mM). The spectra obtained were resolved by subtraction of spectra of 14-PCSL in DOPC/2% Chol, which themselves were essentially unperturbed by these general anesthetics. The restricted components obtained showed no change in the maximum hyperfine splitting, the high- and low-field peaks' line shape or the fraction of the protein-associated component. Once again, there was evidence of unaveraged g-value anisotropy which was abolished by isoflurane (7 mM) and largely attenuated when the pentobarbital concentration was raised from 1 to 2 mM.

Interaction of phosphatidylcholine with the nAcChoR

The number of lipids surrounding the receptor, N_{av}, and their affinity for the lipid/protein interface relative to the bulk lipid bilayer, K_{av}, may be estimated using the analysis of Brotherus et al. [25]. All the data using both subtraction methods in Table I were used in this analysis since hexanol had no significant effect on f_b. An additional determination, made at a lipid to protein ratio of 198 ($f_b = 0.31$), was included to avoid weighting the values at 240 unduly (Fig. 6). From the dependence of the ratio of the fraction of lipid in the bilayer to that associated with the protein upon the molar lipid-to-protein ratio, we derived an average equilibrium constant, K_{av}, of 0.8 ± 0.23 and a N_{av} of 63 ± 11. If the value of K_{av} was constrained to the theoretical value of 1.0, a value of N_{av} of 56 ± 2 was obtained.

For the restricted component spectra of 14-PCSL resolved from the DOPC/nAcChoR membranes by pairwise subtraction, the maximum hyperfine splittings ($2A_{max}$) and width at half heights were found to be the same for all complexes studied, independent of the presence or absence of anesthetics or of temperature variation in the range 0–7°C. For over ten independent determinations of membranes with lipid to protein ratios of 115 : 1 and 130 : 1 the maximum hyperfine splitting was found to be 59.5 ± 0.36 G, the width at

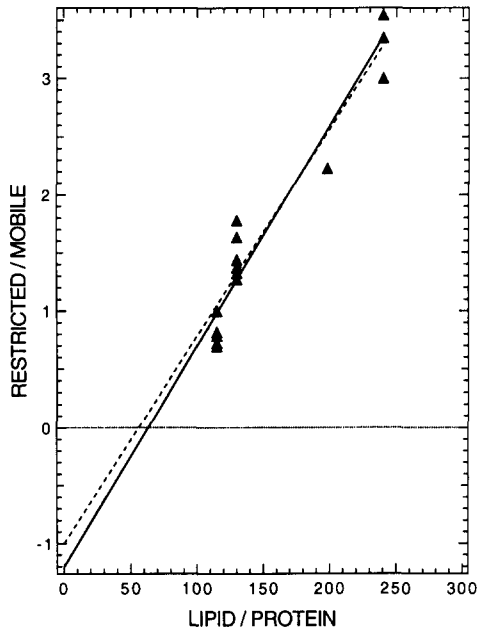

Fig. 6. A Brotherus plot [34] of the fraction of protein-associated 14-PCSL as a function of the mole ratio of DOPC/nAcChoR. The slope of the least squares line (solid) is 0.019 ± 0.0012 and the intercept -1.2 ± 0.19 which yields an estimate of 63 ± 11 protein associated lipids having an average binding constant of 0.8 ± 0.23 (see text). The dotted line is a fit of the data with the intercept constrained to -1 and yields an estimate of 56 ± 2 protein-associated lipids.

half height to be 7.7 ± 0.50 G for the low-field peak and 11 ± 1.9 G for the high-field peak.

The rotational correlational time, τ_r, of 14-PCSL at the lipid/protein interface can be estimated from our data and Eqn. 1 which yields an estimate of 10 ns. Another estimate of τ_r can be obtained from the high- and low-field linewidths and Eqn. 2; this yielded values in the region of 20 ns. The disparity in the two methods is common and may arise from the failure of the isotropic motion assumption made in Freed's treatment or from exchange broadening of the linewidths. Estimates of τ_r from A_{max} for the bilayer component were around 1 ns, and the exchange rate between the bulk bilayer and the lipid/protein interface estimated from the outer hyperfine splittings of the two components was $5.6 \cdot 10^7$ s^{-1}. It should be emphasized that these methods only provide a semiquantitative estimate of the parameters because of the nature of the assumptions.

Neither $2A_{max}$ nor the linewidths of the protein-associated component were altered in any systematic way by addition of general anesthetic, implying that the rotational correlation time was unaffected within experimental error. On the other hand, the sharper lines of the bilayer-associated label revealed a small but significant decrease in $2A_{max}$. For example, on addition of 16 mM hexanol, $2A_{max}$ decreased from 38.8 to 37.0 G. Thus, if the $2A_{max}$ of the protein-associated component is assumed to be constant, the exchange rate increases very slightly on addition of hexanol, reaching a value of $5.8 \cdot 10^7$ s^{-1} at 16 mM.

Discussion

Phosphatidylcholine at the lipid / protein interface

EPR spectra of nAcChoR-rich membranes reveal two components, indicative of two distinct lipid environments: a motionally restricted component which arises from the lipid in contact with the intramembraneous portion of the receptor and a mobile component attributed to the bulk lipid bilayer [11,21]. In a typical native nAcChoR-rich membrane preparation the ratio of the mobile to motionally restricted component varies with spin label and for 14-PCSL is only about 10:1 because of the high lipid-to-protein ratio. Indeed in the earliest study with membranes from *Torpedo marmorata* no protein associated signal was reported for phosphatidylcholine [26].

Although a subsequent study with higher specific activity membranes succeeded, it was not until the application of reconstitution techniques that significant information was obtained [11,24]. These workers concentrated their efforts on spin labels with higher affinity for the lipid/protein interface than phosphatidylcholine, such as stearic acid. However, for acetylcholine receptors reconstituted into DOPC at molar lipid to protein ratios of 100:1 to 250:1 they deduced from the spectra of 16-PCSL that 45–55 lipids were in the lipid/protein interface and that the average affinity for the interface was one. These values are not significantly different from ours (K_{av} 0.8 ± 0.23 and a N_{av} of 65 ± 11) especially considering the difficulty of spectral subtraction when the interfacial lipid represents a small fraction of the total.

Our value of 10 ns for the rotational correlation time of protein-associated 14-PCSL, derived from the maximum hyperfine splitting, may be compared to that deduced by Fraser et al. of 7 ns for 14-PCSL in native membranes at 0°C [6]. The latter workers also obtained a value of 30 ns for 14-SASL (0°C), comparable to values of 12 (34°C) and 45 (-4°C) ns for 12-SASL and 16-SASL, respectively, reported by Marsh and Barrantes in native membranes and of 15 (0°C) ns for 16-SASL reported by Ellena et al. in DOPC/nAcChoR membranes [11,21]. The overall picture that emerges from our data is that the rotational correlation time of protein-associated 14-PCSL is about a factor of two faster than the more tightly bound n-SASL and about an order of magnitude slower than that in a lipid bilayer.

In the intersubtracted spectra of the control at 0°C (Figs. 2E, 4A and 5) a feature suggestive of unaveraged g-value anisotropy appears on the high-field side of the central peak. Such a feature might arise from slight

misalignment of the spectra during pairwise subtraction, but attempts to remove it by shifting the component spectra slightly resulted in unacceptable sharp components between the low and central field peaks. Since g-value anisotropy is averaged only at correlation times lower than 2 ns, its observation is quite consistent with our estimate of a rotational correlation time of 10–20 ns estimated from A_{max} and linewidths using analysis for motions averaging on an intermediate timescale for hyperfine interactions [20].

The exchange rate calculated from the difference in the maximum hyperfine splitting of the two components is $5.6 \cdot 10^7\ s^{-1}$ equivalent to an off rate of about $10^8\ s^{-1}$, a value very close to the rotational correlation time. Such an exchange rate is intermediate on the EPR time-scale, and suggests that broadening of the pure component lines by two site chemical exchange might be occurring [22]. In fact, the spectrum of the bilayer-associated label, obtained by pairwise subtraction, does have wider linewidths than that of DOPC at a comparable temperature, consistent with the bilayer- and protein-associated 14-PCSL exchanging on the EPR time scale. We attempted to investigate this further by simulations of the two component spectra by exchange-coupled Bloch equations [21]. However, it was not possible to simulate satisfactorily the narrow lines of labels in pure DOPC bilayers at 0°C.

Effects of general anesthetics on the lipid / receptor interface

In all cases examined to date phospholipids spin-labeled on the acyl chain have had the same affinity as the parent lipid for the lipid/protein interface [27]. Consistent with this, the association constant, K_{av}, derived in our work for 14-PCSL is not significantly different from unity. Therefore, it is unlikely that general anesthetics would have a differential effect on the affinity of DOPC and 14-PCSL, and indeed none is seen.

Our results for hexanol, isoflurane and pentobarbital (Results, Table I and Figs. 4 and 5) show that quite high concentrations of general anesthetics failed to significantly perturb the rotational dynamics or exchange rate of phospholipids at the lipid/protein interface on the conventional EPR time-scale. This was true even at concentrations (for example 16 mM hexanol) adequate to cause a 10 percent decrease in the order parameter of an already quite disordered bilayer.

Overall, at the lipid/protein interface of the acetylcholine receptor, general anesthetics have few effects on the conventional EPR time-scale. However, hexanol may have some qualitative effects which are not easily expressed by the quantitative parameters above. There appears to be an abolition of g-value anisotropy by addition of as little as 3 mM hexanol, suggesting that some preferential averaging of slower motions has occurred. This could occur if the motion of the protein, which is slower than that of the lipids, is altered by the anesthetics and this leads to averaging of g-value anisotropy. Such a hypothesis might be testable using saturation transfer EPR techniques which are more sensitive to slow molecular motions than are conventional EPR techniques.

Several studies have examined the effect of anesthetics on lipid–protein interactions in native *Torpedo* membranes. Firestone and Ferguson labeled *Torpedo* membranes with 12-SASL and showed that general anesthetics such as diethyl ether, halothane and octanol all failed to induce changes in the motionally perturbed component [7]. Similar results were also reported by Fraser et al. [6] when native *Torpedo* membranes were labeled with 14-SASL. However, when 14-PCSL, which has a much lower affinity for the lipid/protein interface, was used as a label both 1-hexanol and diethyl ether induced a change in the fraction of the motionally restricted component and caused the exchange rate to increase. The decrease of the fraction of protein-associated component would not be detected in our experiments because only a single lipid is present (see discussion above), but we would have detected exchange rate effects had they occurred. The effects observed by Fraser et al. in native membranes might have resulted from a decrease in the affinity of phosphatidylcholine for the receptor relative to the other lipids, to a conformational change in the receptor or to interactions with proteins other than the acetylcholine receptor. On the other hand, the difficulties of spectral subtraction when the fraction of protein-associated component is low should not be underestimated.

Implications for mechanisms of action of general anesthetics on acetylcholine receptors

The agents studied all share at least two actions on the acetylcholine receptor. They inhibit agonist-induced ion flow through the channel on a millisecond timescale and they cause desensitization on the minute timescale. Only the latter effect is relevant for the present studies because in DOPC the receptor is trapped in the desensitized state. Heidemann et al. have proposed that induction of desensitization by such nonspecific agents occurs through occupation of some 30 sites in the lipid/protein interface [5]. Occupation of these sites by such small molecules is unlikely to 'displace' lipids, and indeed in our experimental design such effects are precluded, but would it modify the interaction between phospholipids and the receptor? Our results suggest that neither the rotational correlation time nor the exchange rate of phospholipids at the lipid/receptor interface are changed at concentrations of hexanol, isoflurane and pentobarbital sufficient to desensitize all the receptors in native membranes. On

the other hand, g-value anisotropy may be lost at concentrations of hexanol and isoflurane which desensitize, while pentobarbital, which causes only modest desensitization at the highest concentration studied, partially removes it [28]. It is unclear whether this reflects occupation of the sites proposed for anesthetics in the lipid/protein interface [5]. A recent study suggests that such sites for local anesthetics are accessible only to steroids, a possibility we have not tested [29].

Comparison to other proteins

Bigelow and Thomas have made a detailed study of diethyl ether, an agent that can activate the Ca-ATPase in sarcoplasmic reticulum membranes [30]. They used 14-PESL, the ethanolamine analog of 14-PCSL, and subtracted its spectra in lipids extracted from sarcoplasmic reticulum membranes from its spectra in intact sarcoplasmic reticulum membranes. Ether was added to both membranes as appropriate and mobilized the lipid chains at the lipid/protein interface more effectively than those in the bulk bilayer, while the number of lipids surrounding the protein remained unaltered. The protein-associated component's rotational correlation time calculated from its maximum hyperfine splitting decreased from 11.5 ns to 5.5 ns upon addition of ~500 mM diethyl ether (equivalent to a change in $2A_{max}$ from 59 to 54 G). The rotational correlation time calculated from the low field linewidth decreased over 2-fold from 41 ns to 17 ns. Therefore, they suggested a preferential partitioning site for diethyl ether at the lipid/protein interface. Similar studies by Cobb et al. on the anion exchange protein in intact human erythrocytes revealed that the rotational mobility of band 3 itself increased at lower concentrations (approximately physiologic anesthetic concentrations) of diethyl ether than those which caused disordering of the bulk bilayer lipids [31]. They suggested that diethyl ether may produce larger effects at or near the band 3/lipid interface than in the bulk bilayer.

A more recent study on the action of n-alkanols on sarcoplasmic reticulum membranes labeled with 14-PCSL came to different conclusions [32]. The alcohols reduced the fraction of 14-PCSL at the lipid/protein interface without affecting their mobility. While all these agents can activate the enzyme, the authors suggest the discrepancy with diethyl ether's action correlated with different abilities of alcohols and ethers to influence the nonspecific leak of cations across the membrane [30]. It is unfortunate that these two studies differ in the general anesthetics studied and the spin labels used, so that further work will be required before the observed differences can be resolved.

General anesthetics affect the function of all the proteins studied above, simultaneously altering dynamics at their lipid/protein interfaces over the same concentration range. Whether these two effects are mechanistically linked remains uncertain. However, at concentrations relevant to general anesthesia (25 mM for diethyl ether, 0.7 mM for hexanol, 0.29 mM for isoflurane and 0.16 mM for pentobarbital), none of the agents would have much effect on either the fraction or the dynamics of lipid associated with the lipid/protein interface in so far as this could be detected on the EPR timescale and with a reporter group deep in the bilayer [33]. Slower motions associated with the protein may be more sensitive, however [31].

Acknowledgements

This research was supported in part by grants from the National Institute on Alcohol Abuse and Alcoholism (AA-07040) and from the National Institute of General Medical Sciences (GM15904) to K.W.M. and from the E.P.A Cephalosporin Fund (SERC GR/D 69846) to A. Watts. D.E.R. was supported by Basic Science Research Training for Anesthetists (GM07592-14). We thank Adam Hill for help with computer programming.

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