Electron Spin Resonance Studies of Acyl Chain Motion in Reconstituted Nicotinic Acetylcholine Receptor Membranes

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ABSTRACT The electron spin resonance spectra of spin-label positional isomers of stearic acid (*n*-SASL) incorporated into nicotinic acetylcholine receptors (nAcChoR) reconstituted into dioleoylphosphatidylcholine (DOPC) were deconvoluted into bilayer- and protein-associated components by subtraction under conditions of slow exchange. The selectivity of *n*-SASL (*n* = 6, 9, 12, and 14) for the lipid-protein interface of the nAcChoR was threefold greater than that of DOPC and independent of the spin label position. The temperature at which exchange became apparent as judged from lineshape broadening of the mobile lipid component spectrum was dependent upon the position of the spin-label moiety; near the bilayer center, exchange broadening occurred at lower temperatures than it did closer to the lipid headgroup. This suggests that the lipid headgroup region of boundary lipids is relatively fixed, whereas its acyl chain whips on and off the protein with increasing frequency near the bilayer center. Motions on the microsecond time scale were examined by microwave power saturation. Each *n*-SASL saturated more readily when incorporated into vesicles containing the nAcChoR than when in pure DOPC liposomes. Therefore, lipid mobility is perturbed by the nAcChoR on the microsecond time scale with an apparent magnitude that is relatively modest, probably due to exchange on this time scale.

INTRODUCTION

The nicotinic acetylcholine receptor (nAcChoR) is a well characterized cation-selective integral membrane protein. It is a member of the superfamily that includes the GABA, glycine, and NMDA receptors (Galzi et al., 1991). It is the only member of this receptor superfamily that can be isolated in sufficient quantities and reconstituted into defined lipid bilayers for biophysical study. Such studies have shown that lipid-protein interactions are important determinates of receptor function (Fong and McNamee, 1987). For example, modifying the membrane lipid composition alters the ion flux activity of the nAcChoR (Fong and McNamee, 1986; Sunshine and McNamee, 1994).

Electron spin resonance (ESR) spectroscopy is a useful method for studying lipid dynamics and can be used to probe lipid-protein interactions (Esmann et al., 1985; Marsh, 1985; Pates et al., 1985; Pates and Marsh, 1987). The spectral lineshapes obtained with conventional ESR spectroscopy are sensitive to motions having correlation times in the range of 10^{-9} to 10^{-7} s. ESR spectra of lipids spin-labeled near the bilayer core and incorporated into membranes containing the acetylcholine receptor reveal the presence of two lipid environments defined by different motional properties. In one environment, the rotational mobility of lipids is relatively high, similar to that in protein-free lipid bilayers (bulk lipids). In the other environment, the mobility of lipids is restricted by the receptor. This motionally restricted environment represents lipids that are in direct contact with the nAcChoR (boundary lipids). Using spectral subtraction, it is possible to determine the fraction of lipids in this region and to estimate their rotational mobility. Such studies of the nAcChoR reconstituted into lipid bilayers at various lipid:receptor ratios indicate that ~50 lipids surround each receptor (Ellena et al., 1983; Abadji et al., 1993). They also conclude that the relative affinity of a lipid for the interface of the nAcChoR varies with headgroup structure. In general, negatively charged lipids such as stearic acid generally exhibit higher affinities than neutral ones.

By measuring the saturation properties of spin-labeled probes, the motional sensitivity of conventional ESR spectroscopy can be extended into the microsecond time domain (Squier and Thomas, 1986; Squier and Thomas, 1989). Therefore, the slow motions of boundary lipid acyl chains can be resolved from bulk lipids even when spectral lineshape overlap precludes spectral subtraction. Such techniques may be useful for studying boundary lipid motions near the lipid headgroup or in the gel phase where spectral subtraction is made difficult by the high degree of anisotropy of the mobile component.

In this study, we have characterized the interactions between the nAcChoR and membrane lipids using a series of fatty acid spin-label positional isomers that sense acyl chain motions at different membrane depths. A population of motionally restricted lipids directly interacting with the nAcChoR was detected at various membrane depths. The spectral lineshapes and saturation characteristics of motionally restricted lipids are distinct from those of more mobile

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Abbreviations used: DOPC, dioleoylphosphatidylcholine; ESR, electron spin resonance; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; nAcChoR, nicotinic acetylcholine receptor; PC, phosphatidylcholine; PA; phosphatidic acid; SA, stearic acid; *n*-SASL, *n*-(4,4-dimethyl-*N*-oxyoxazolidine)-stearic acid, where *n* denotes the position on the acyl chain.

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lipids that are not directly interacting with the nAcChoR. The temperature at which lipid exchange between mobile and restricted environments becomes significant on the conventional ESR time scale was found to vary with spin-label position; spin-labels near the bilayer center exhibited exchange at lower temperatures than those closer to the polar headgroup. These results suggest that the headgroups of motionally restricted lipids are relatively fixed on the conventional ESR time scale, whereas their acyl chains move on and off the protein with increasing frequency near the bilayer center.

MATERIALS AND METHODS

Affi-Gel 102 was purchased from Bio-Rad (Richmond, CA). Diisopropylfluorophosphate was from Sigma Chemical Co. (St. Louis, MO). Buffer A contained 10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% sodium azide, its pH adjusted to 7.4 with concentrated sodium hydroxide. Doubly distilled, deionized water was used to make all buffers. DOPC was purchased from Avanti Polar Lipids (Birmingham, AL). Stearic acid isomers spin-labeled at carbons 5, 6, 9, and 12 were purchased from Molecular Probes (Eugene, OR). Stearic acid spin-labeled at the 14th acyl carbon was a gift from Dr. Anthony Watts (Oxford University, Oxford, U.K.).

Reconstitution of nAcChoR into DOPC

Receptor-rich membranes were obtained from the electric organ of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) and frozen in liquid nitrogen and stored at −80°C until used (Braswell et al., 1984). The nAcChoR was reconstituted into DOPC using a modification of the procedure of Ellena et al. (1983) in which Affi-Gel 201 was derivatized with DL-*N*-acetylhomocysteine thiolactone and used in place of Affi-Gel 401, which is no longer commercially available. The protein content of reconstituted nAcChoR membranes was determined as described by Markwell et al. (1978) using BSA as the standard. The lipid content was determined by assaying for phosphorous (McClare, 1971). The lipid/protein ratio was calculated assuming a receptor molecular weight of 250,000.

Incorporation of spin-labeled lipids into membranes containing the nAcChoR and into DOPC liposomes

Aliquots of ethanolic solutions of spin-labeled lipids were dried to a film under a stream of nitrogen. Reconstituted nAcChoR membranes in buffer A (0.25–0.5 ml; 1–3 mg of protein/sample) were added and equilibrated with the spin-labeled lipid for 15–30 min. The final ratio of DOPC to spin-labeled lipid was always >100:1 to minimize spin-spin interactions. Receptor membranes were washed free of unincorporated label by two cycles of centrifugation (13,600 × *g* for 10 min).

DOPC liposomes were labeled by adding spin-labeled lipid directly to DOPC in chloroform, drying to a film under a stream of nitrogen, and then placing under vacuum for at least 2 h. Liposomes were formed by hydrating the lipid film with buffer and bath-sonicating at a power of 80 W (Heat Systems Ultrasonics, Plainview, NY). The final ratio of DOPC to spin-labeled lipid was always >100:1 to minimize spin-spin interactions.

Spectral subtraction

Samples were transferred into capillary tubes and spectra were obtained on a Bruker ER200 spectrometer interfaced with an IBM 9000 computer. Temperature was controlled to within ±0.5°C using a thermostated insert within the ESR cavity. Microwave frequency was 9.4 GHz, microwave power incident upon the TE_{102} cavity was 10 mW, modulation amplitude was 2 G, modulation frequency was 100 kHz, and sweep width was 120 G. To facilitate spectral deconvolution, spectral acquisition was done at a temperature low enough to minimize exchange of spin-labeled probe between boundary and bulk regions but high enough to allow for visual resolution of bulk and boundary lipid spectral components. Spectral baseline correction and subtraction were performed on an Apple Macintosh computer using a program developed in IGOR (Wave Metrics, Lake Oswego, OR). The mobile component was modeled with a spectrum of the appropriate spin-labeled stearic acid in DOPC liposomes. Except where noted, the DOPC and reconstituted nAcChoR subtraction pairs were obtained at the same temperature. We estimate the error in the determination of the fraction of motionally restricted component to be ~±0.03.

Determination of half-height linewidths, spectral outer hyperfine splittings ($2A_{max}$), and effective rotational correlation times (τ_r)

Lowfield and highfield peak half-height linewidths for motionally restricted components were determined by iterative fitting of these peaks to a Gaussian function as described previously (Raines et al., 1993). The outer hyperfine splittings were calculated from the maxima and minima of the low field and high field Gaussian fits.

Effective rotational correlation times, τ_r, for spin-labels were estimated from their spectral outer hyperfine splitting assuming a Brownian rotational diffusion model for isotropic motions:

$$\tau_r = a\left(1 - \frac{A_{max}}{A^r_{max}}\right)^b \quad (1)$$

where A_{max} is half of the measured outer hyperfine splitting of the motionally restricted component (Freed, 1976). A^r_{max}, half of the hyperfine splitting for the completely immobilized spin-label, was taken as 33.6 G (Gaffney, 1976). The values for a and b are 5.4×10^{-10} and −1.36, respectively, as derived previously by Freed (1976) from spectral simulations for slow isotropic rotational motion assuming a linewidth parameter of 3 G (Freed, 1976).

Effective rotational correlation times for all motionally restricted components were also estimated from their lowfield and highfield peak half-height linewidths. Again, assuming Brownian diffusion:

$$\tau_r = a'\left(\frac{\Delta_i}{\Delta^r_i} - 1\right)^{b'} \quad (2)$$

where Δ_i is the half-height linewidth of the low field ($i = l$) or high field ($i = h$) lines of the motionally restricted component. The values for Δ^r_i, half-height linewidth of the completely immobilized spin-label, have been derived by Freed (1976) using spectral simulations. For a linewidth parameter of 3 G, Δ^r_l is 4.77 G and Δ^r_h is 5.43 G (Freed, 1976). For the lowfield peak, $a' = 1.15 \times 10^{-8}$ s and $b' = -0.943$ and for the highfield peak, $a' = 2.12 \times 10^{-8}$ s and $b' = -0.778$ (Freed, 1976).

Continuous wave saturation studies

Saturation studies were performed on a Varian E-109 Century Series ESR spectrometer equipped with a Medical Advances (Milwaukee, WI) XP-0201 loop gap resonator and a Miteq X-band field effect transistor for signal amplification. The magnetic field sweep of 120 G was controlled by a scan card (University of Denver, Department of Engineering, Denver, CO). The ESR signal (1000 points per 120 G) was digitized by a Real Time Devices (State College, PA) A/D card and routed to a Dell 316SX computer for averaging by the ESR Ware software (Scientific Software Services, Bloomington, IL). Temperature was controlled by flowing chilled nitrogen gas into a Q-band size dewar (Wilmad Glass Co., Buena, NJ) around the resonator. Temperature was measured with an Omega Engineering (Stamford, CT) Model 412B digital temperature indicator fitted

with a 36-gauge copper-constantan thermocouple that was inserted into the resonator. Samples were deoxygenated by flowing nitrogen past the gas-permeable (TPX) sample cell for at least 30 min before study. All measurements were performed at 0 ± 0.5°C.

The microwave field amplitude was calibrated by measuring the broadening of the resonance line of 0.9 mM potassium nitrosodisulfonate solution (Poole, 1983). Conventional in-phase absorption (V_1) spectra of membrane samples were recorded over a range of microwave field amplitudes from 0.015 to at least 0.6 G using a modulation amplitude of 1.0 G. Baseline correction and double integration was performed with the ESR Ware software.

Continuous wave saturation curves were analyzed by nonlinear least-squares fitting to the following equation (Horvath et al., 1993):

$$S_{CW} = S^0_{CW}/[(1 + \sigma^s_{1/2}(P/P^0_{1/2})]^{1/2} \quad (3)$$

where S_{CW} is the double integral of the first derivative ESR spectrum, S^0_{CW} is the double integral of the first derivative ESR spectrum that would be obtained in the absence of saturation (determined from the low power region of each saturation curve), $\sigma^s_{1/2} = 3$, P is the microwave power, and $P^0_{1/2}$ is the microwave power at half-saturation.

RESULTS

Conventional ESR spectroscopy and spectral subtraction

ESR spectra of stearic acid spin-labels incorporated into reconstituted nAcChoR membranes reveal the presence of two motionally distinct environments (Fig. 1): a mobile environment composed of lipid not directly interacting with the nAcChoR and a motionally restricted environment arising from lipid adjacent to the nAcChoR. In contrast, only a single mobile component is observed in ESR spectra of pure DOPC liposomes containing spin-labeled stearic acid.

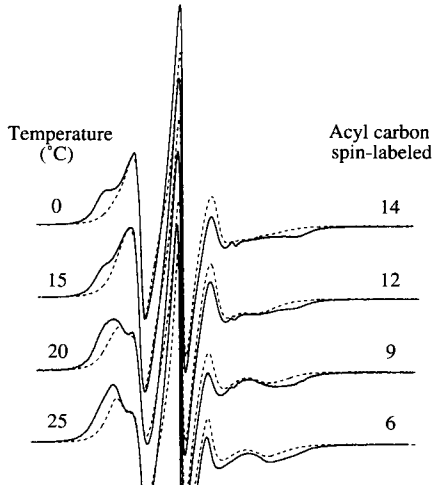

FIGURE 1 ESR spectra of 14-SASL, 12-SASL, 9-SASL, and 6-SASL incorporated into nAcChoR reconstituted into DOPC vesicles (——) and into protein-free DOPC liposomes (· · · · ·). Spectra were obtained at 0°C, 15°C, 20°C, and 25°C for 14-SASL, 12-SASL, 9-SASL, and 6-SASL, respectively. Spectra of nAcChoR reconstituted into DOPC vesicles are normalized to the same central line height. They are aligned to the central peak. Spectra of protein-free DOPC are scaled appropriately for spectral subtraction. The lipid/protein ratio is 245:1.

Provided that the mobilities of the bulk lipids and the protein-associated lipids are sufficiently different, it is possible to resolve these two components by spectral subtraction. This condition is best fulfilled deep in the membrane where bulk lipids exhibit high mobility. Nearer the membrane surface, the motion of the bulk lipids in the bilayer is slower and spectral overlap with motionally restricted lipid increases. In this case, resolution may be enhanced by elevating the sample temperature to decrease the ESR linewidth of spin-labels in the bulk lipid environment. However, at higher temperatures exchange between the two environments may cease to be slow on the conventional ESR time scale. This restriction can be minimized by choosing a spin-labeled lipid that has a relatively high affinity for the lipid-protein interface. For this reason, we chose to use spin-labeled isomers of stearic acid (n-SASL). For each of the spin-labels in Fig. 1 (n = 6, 9, 12, and 14), we selected a temperature that was sufficiently high to permit adequate resolution of the motionally restricted component but low enough to minimize exchange between restricted and mobile environments as judged by broadening of the bulk lipid component. These temperatures were 25, 20, 15, and 0°C for lipids labeled at the 6th, 9th, 12th, and 14th acyl carbons, respectively. For 5-SASL, the two components could not be resolved at any temperature before the onset of fast exchange. Exchange broadening of the mobile component for 16-SASL could be eliminated only by cooling to temperatures below the freezing point of the buffer. Because 16-SASL requires an exceptionally low temperature to adequately reduce exchange and because its position even in simple lipid bilayers is not well defined, we elected not to examine this probe in detail (Ellena et al., 1988; Altenbach et al., 1994).

The spectral lineshapes of 14-SASL and 12-SASL in the bulk environment of reconstituted nAcChoR membranes at 0 and 15°C, respectively, are relatively narrow (Fig. 1). Thus, the highfield and lowfield spectral features of the motionally restricted component can be readily resolved. However, for 9-SASL and 6-SASL, there is considerable overlap of the mobile and motionally restricted spectral components in membrane spectra even at higher temperatures.

The motionally restricted component for 6-SASL, 9-SASL, 12-SASL, and 14-SASL can be derived by subtracting a spectrum of the appropriate spin-labeled stearic acid in pure lipid from the composite reconstituted nAcChoR spectrum (Fig. 2). The resulting lineshape has the lowfield and highfield peaks characteristic of a motionally restricted spin-label (Ellena et al., 1983; Marsh, 1985; Pates et al., 1985; Pates and Marsh, 1987; Raines and Miller, 1993). Frequently, some spectral intensity can be observed as narrow spikes on either side of the central peak. This is commonly observed in motionally restricted lineshapes obtained by spectral subtraction (Ellena et al., 1983; Pates and Marsh, 1987; Raines and Miller, 1993). It results from a

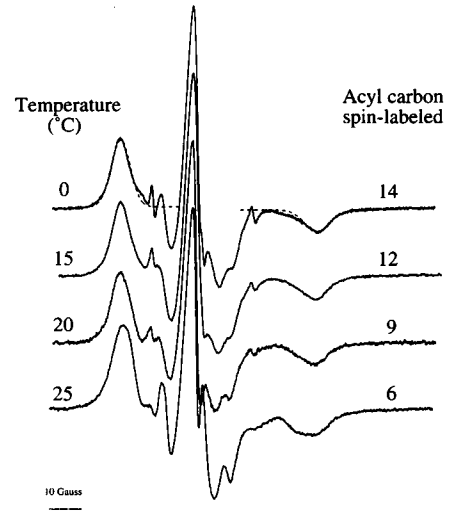

FIGURE 2 The motionally restricted lineshapes obtained by subtraction of the spectral pairs shown in Fig. 1. In the top spectrum, the dotted lines illustrate the Gaussian fits of the high field and low field peaks, which are used to determine half-height linewidths and outer hyperfine splittings.

small quantity of aqueous spin-label (always <2% in this study) and, more significantly, from the difficulty in matching exactly the sharp central line of the spectra. The latter may be the result of exchange broadening of the mobile component. This can be reduced by simulating the bulk lipid component with a lipid bilayer spectrum that has been broadened either by lowering the temperature or by adding cholesterol (Pates and Marsh, 1987; Raines and Miller, 1993). However, we chose experimental temperatures at which exchange broadening was minor so that a reasonable lineshape for motionally restricted spin-labeled stearic acid could be obtained by subtracting the ESR spectrum of pure DOPC vesicles recorded at the same temperature as that used for the nAcChoR membranes.

The results of the above subtractions are presented in Table 1 for a single reconstituted nAcChoR preparation with a lipid/protein ratio of 245:1. The fraction of motionally restricted stearic acid was 0.45, 0.43, 0.38, and 0.45 for 6-SASL, 9-SASL, 12-SASL, and 14-SASL, respectively, at the optimal subtraction temperatures given above. Within the error of the spectral subtraction method, these values show no significant trends so they may be averaged to yield 0.43 ± 0.033 (mean ± SD).

The ESR spectral lineshapes (and, consequently, the estimated rotational correlation times) of the motionally restricted components of 9-SASL, 12-SASL, and 14-SASL incorporated into nAcChoR membranes at 20, 15, and 0°C, respectively, were quite similar. We found that estimates for correlation times calculated from a spectrum's half-height linewidths (23–27 ns) were longer than when determined from its outer hyperfine splitting (14–18 ns). Such differences may arise because acyl chain motion is not completely isotropic (Marsh, 1989).

To examine the effects of temperature on boundary lipid motions, we varied the temperature in nAcChoR membranes and DOPC vesicles labeled with 14-SASL because it is the probe that allows spectral subtractions to be performed over the widest range of temperatures without exposing the nAcChoR to temperatures that might cause degradation of the protein. The rotational correlation times derived from the outer hyperfine splitting decreased steadily when the sample temperature was increased in two to three degree increments from −10 to 5°C. Representative data are given in Table 1. An Arrhenius plot of the temperature dependence of spin-label mobility (log τ_r vs. $1/T$) was linear with an apparent activation energy of 2.8 kcal mol^{-1} deg^{-1}. This compares with 4.3 in rhodopsin (Pates and Marsh., 1987). Extrapolation of the temperature dependence data predicts a doubling of the mobility of 14-SASL with a 20°C increase in temperature. No significant change in mobility with temperature was detected from measurements of the half-height linewidths.

Between −10 and 5°C, we could model the bulk lipid component of 14-SASL in reconstituted receptor membranes with pure DOPC liposomes at the same temperature as that used for the reconstituted membranes indicating that exchange between the bulk and boundary domains was relatively slow on the conventional ESR time scale (Figs. 3 and 4 *A'*). At 10°C, exchange becomes significant as judged by the development of a large spike and marked asymmetry of the highfield and lowfield peaks in the motionally restricted lineshape when using a spectrum of DOPC at 10°C

TABLE 1 The fraction of motionally restricted component, ESR lineshape features, and calculated rotational correlation times for each spin-labeled stearic acid in nAcChoR reconstituted into DOPC (lipid:protein = 245:1)

Spin-label	Temperature (°C)	Fraction motionally restricted	$2A_{max}$ (rotational correlation time*)	Lowfield half-height linewidth (rotational correlation time‡)	Highfield half-height linewidth (rotational correlation time‡)
14-SASL	5	0.43	61.4 G (15 ns)	6.8 (26 ns)	10.1 G (24 ns)
14-SASL	0	0.45	62.1 G (18 ns)	6.7 (27 ns)	9.4 G (27 ns)
14-SASL	−5	0.48	62.4 G (19 ns)	6.8 (26 ns)	9.8 G (25 ns)
14-SASL	−10	0.49	62.8 G (22 ns)	6.8 (26 ns)	9.3 G (28 ns)
12-SASL	15	0.38	61.2 G (14 ns)	6.9 (25 ns)	10.2 G (23 ns)
9-SASL	20	0.43	61.6 G (16 ns)	7.2 (23 ns)	10.0 G (24 ns)
6-SASL	25	0.45	58.8 G (9 ns)	7.7 (18 ns)	14.3 G (14 ns)

* From Eq. 1.
‡ From Eq. 2.

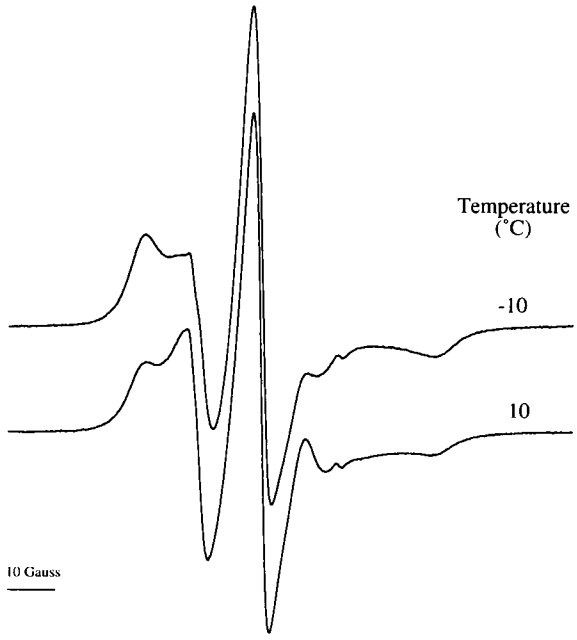

FIGURE 3 ESR spectra of 14-SASL in nAcChoR reconstituted into DOPC (lipid/protein = 167:1) at −10°C (*top*) and 10°C (*bottom*). Spectra are normalized to the same central lineheight.

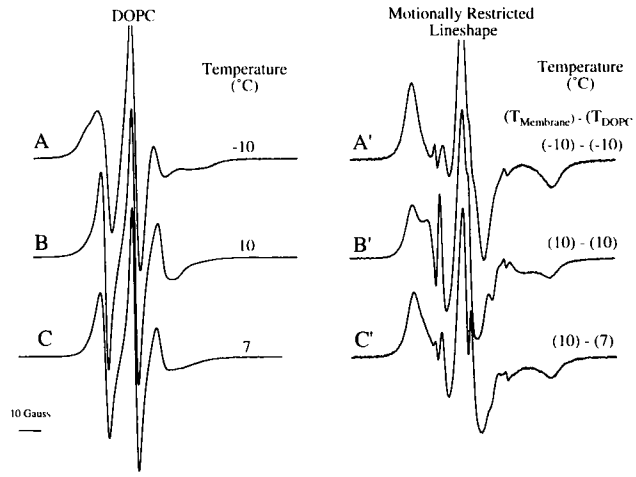

FIGURE 4 ESR spectra of 14-SASL in DOPC liposomes at (*A*) −10°C, (*B*) 10°C, and (*C*) 7°C. (*A'*) The motionally restricted spectral lineshape obtained by spectral subtraction of spectra of 14-SASL in DOPC liposomes (T = −10°C) from nAcChoR reconstituted into DOPC bilayers (T = −10°C; lipid/protein = 167:1); (*B'*) the motionally restricted lineshape obtained by spectral subtraction of DOPC liposomes (T = 10°C) from nAcChoR reconstituted into DOPC (T = 10°C; lipid/protein = 167:1), and (*C'*) the restricted lineshape obtained by spectral subtraction of DOPC (T = 7°C) from nAcChoR reconstituted into DOPC (T = 10°C; lipid/protein = 167:1). The subtraction artifact arising from exchange broadening of the bulk lipid at 10°C can be seen on the highfield portion of the lowfield peak and the lowfield portion of the highfield peak when DOPC at 10°C is used to simulate the bulk (mobile) component (*B'*). This artifact is largely eliminated by using the broader spectrum of DOPC at 7°C (*C'*). The fractions of immobile component obtained by double integration of the ESR spectra are 0.56, 0.58, and 0.57 for *A'*, *B'*, and *C'*, respectively.

to model the bulk lipid spectral component (Fig. 4 *B'*). Using a DOPC spectrum acquired at 7°C to better allow for exchange broadening of the bulk lipid component, a motionally restricted lineshape without much evidence of exchange artifact could be obtained (Fig. 4 *C'*). Thus, for 14-SASL, the onset of exchange on the conventional ESR time scale occurs between 5 and 10°C. Over this temperature range (−10 to 10°C), no difference in the fraction of motionally restricted lipid was detected.

Continuous wave saturation studies

Under appropriate experimental conditions, spectral saturation has been used to detect the motions of boundary lipids (Horvath et al., 1993; Squier and Thomas, 1986). The degree of spectral saturation is a measure of motion (Squier and Thomas, 1989). For motions on the microsecond time scale, spectral saturation decreases with increasing motion.

Fig. 5 shows the low and high power ESR spectra of 5-SASL and 14-SASL incorporated into nAcChoR reconstituted into DOPC vesicles (lipid/protein 85:1) at 0°C. Note that the signal amplitude of each spectrum has been normalized to its corresponding microwave field intensity. A plot of the second integral of the first derivative absorption spectrum as a function of microwave power is shown for 5-SASL and 14-SASL in Fig. 6, *A* and *B*, respectively, incorporated into either reconstituted nAcChoR membranes (lipid/protein = 85:1) or protein-free DOPC liposomes. Fig. 6, *A'* and *B'* show the same data normalized to the second integral that would be obtained in the absence of saturation. These continuous wave saturation curves were analyzed by fitting the data to Eq. 3, which assumes a single component system. We fit the data assuming one component because at a lipid/protein ratio of 85:1, a great majority of stearic acid spin-labels (85% as determined from spectral subtraction of the 14-SASL positional isomer) are at the lipid-protein interface. Second, a two component analysis would assume the absence of exchange, which is unlikely on the time regime of our microwave saturation study. For each spin-label positional isomer, microwave saturation occurred more readily when it was incorporated into vesicles containing the nAcChoR than when incorporated into protein-free liposomes. This was reflected in the values of $P^{o}_{y_2}$ determined from fitting saturation curves to Eq. 3 (Figs. 6 and 7). In DOPC, $P^{o}_{y_2}$ doubles between the 5th and 6th carbon. The 6th, 12th, and 14th carbons all had indistinguishable $P^{o}_{y_2}$, whereas the 9th carbon was similar to the 5th. The chain length dependence in the reconstituted membranes was similar but $P^{o}_{y_2}$ was on average 36% lower (range 15–47%) at each depth than in the pure DOPC vesicles (Fig. 7). Even with a large fraction of the lipids at the lipid-protein interface, the difference in the saturation properties between membranes containing receptor and protein-free lipid is not large. Therefore, we did not study the saturation behavior of reconstituted receptor membranes with higher lipid/protein ratios.

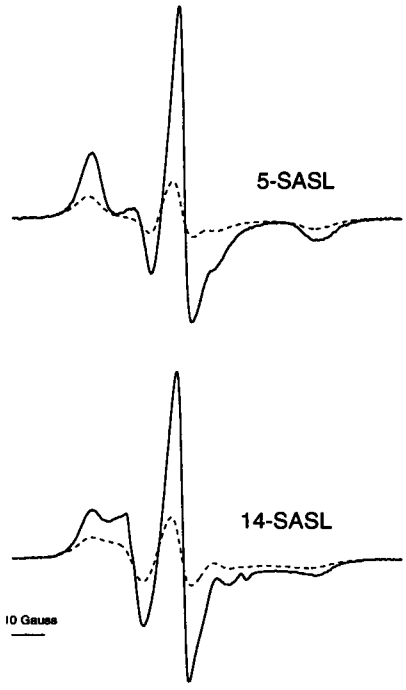

FIGURE 5 Low power (0.20 mW; 0.067 G) and high power (10.0 mW; 0.476 G) spectra of 5-SASL and 14-SASL. High power spectra are shown as dotted lines. Each spectral amplitude is normalized (divided) by its corresponding microwave field amplitude ($\sqrt{\text{microwave power}}$). The lipid/protein ratio is 85:1. T = 0°C.

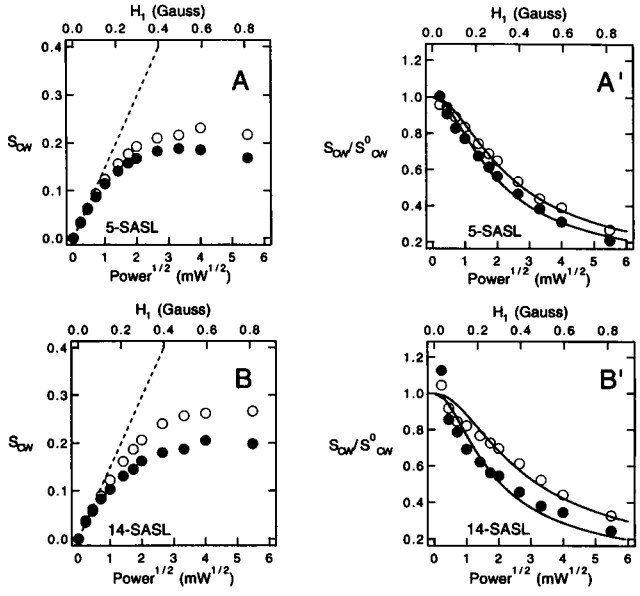

FIGURE 6 Normalized double integral of the first derivative absorption spectra, S_{CW}, as a function of the $\sqrt{\text{microwave power}}$ (top axis is microwave field amplitude in gauss) of 5-SASL (*A*) and 14-SASL (*B*) in reconstituted nAcChoR membranes (●) and DOPC liposomes (○). *A'* and *B'* are the data in *A* and *B*, respectively, replotted as S_{CW}/S^0_{CW}, where S^0_{CW} is the double integral of the first derivative absorption spectra in the absence of saturation. Curves are fits to Eq. 3, and the derived values of $P^0_{1/2}$ are given in Fig. 7. Lipid/protein ratio of reconstituted membranes is 85:1. T = 0°C.

DISCUSSION

ESR spectra of 6-SASL through 14-SASL incorporated into nAcChoR reconstituted into DOPC reveal two components defined by different mobilities. One component has a lineshape that is not significantly different from that of spin-labeled stearic acid in protein-free liposomes. The other component has a lineshape of a motionally restricted spin-label reflecting lipids directly interacting with the nAcChoR. For stearic acid positional isomers, as the spin-label moiety was moved closer to the aqueous interface, the mobile component became progressively broader and it was necessary to increase the sample temperature to resolve the two components. Our ability to deconvolute spectra of lipids spin-labeled as close to the headgroup as the 6th acyl carbon reflects the relatively narrow lineshapes of spin-labeled stearic acid probes in DOPC (relative to some cholesterol-containing native membranes) and to the broad ESR lineshape of motionally restricted stearic acid at the lipid-protein interface of the nAcChoR compared to other membrane proteins (Pates and Marsh, 1987; Squier and Thomas, 1989).

The fraction of stearic acid at the lipid-protein interface exhibited no systematic variation with temperature or depth (Table 1) and, therefore, we conclude that there is no selectivity for different spin-label isomers. Previous studies utilizing stearic acid spin-labels in membranes containing rhodopsin or Ca-ATPase also found stearic acid affinity for the lipid-protein interface to be independent of the position of the nitroxide moiety (Pates and Marsh, 1987; Squier and Thomas, 1989). An estimate of the affinity of each spin-labeled stearic acid for the lipid-protein interface of the nAcChoR relative to DOPC can be made using an equation derived by Brotherus (Brotherus et al., 1981) and assuming ~50 boundary lipids/receptor (Ellena et al., 1983; Abadji et

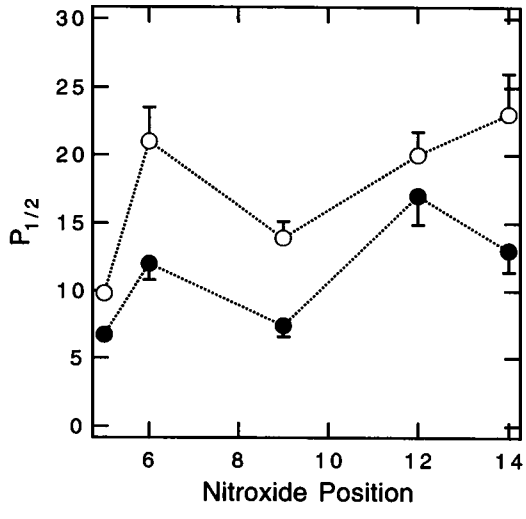

FIGURE 7 Microwave power at half-saturation ($P^0_{1/2}$) for stearic acid spin-label positional isomers for pure DOPC (○) and reconstituted nAcChoR membranes (●). The errors are the SDs derived from the fits. Lipid/protein ratio of reconstituted membranes is 85:1. T = 0°C.

al., 1993). The affinities of 14-SASL, 12-SASL, 9-SASL, and 6-SASL relative to DOPC thus are estimated to be 3.2, 2.4, 2.9, and 3.2, respectively. The average value (±SD) is 2.9 ± 0.38. This finding is consistent with previous reports demonstrating that stearic acid has a relatively high affinity for the nAcChoR and that the doxyl ring does not significantly alter the lipid binding affinity for the nAcChoR (Ellena et al., 1983; Abadji et al., 1993; Raines and Miller, 1993).

At a spin-label's optimal temperature for spectral subtraction, it was generally possible to model adequately the bulk fluid component with a spectrum of pure DOPC recorded at the same temperature as that used to obtain the reconstituted nAcChoR spectrum. Therefore, exchange broadening of the mobile component was not significant on the conventional ESR time scale under these optimal conditions.

In general, the temperature at which exchange becomes significant, as determined from the broadening of the bulk fluid component, decreases with depth; there is little exchange broadening of the bulk component of 12-SASL at 15°C or 9-SASL even at 20°C, whereas it is clearly evident for 14-SASL at 10°C (Fig. 4). Thus, our data suggest that at constant temperature, an "exchange gradient" exists such that the exchange rate of the restricted component is greatest near the bilayer center and less near the headgroup. Squier and Thomas (1989) observed a spectral saturation gradient at constant temperature in sarcoplasmic membranes containing the Ca-ATPase; spectral saturation decreased toward the center of the bilayer. This was attributed to a mobility gradient in which the mobility is greatest near the bilayer center. Alternatively, the decrease in saturation with depth could reflect an increase in the exchange rate near the center of the bilayer (Horvath et al., 1993). Although these possibilities are difficult to distinguish, clearly neither the fraction bound nor the estimated rotational correlation times change significantly with temperature or spin-label position. Therefore, the affinity remains unchanged and the enhanced exchange rate must mean that the on- and off-rates must change in parallel as the chain is descended.

We have shown previously that the relative affinity of 14-SASL for the nAcChoR is largely determined by its headgroup. Titrating the pH through its carboxyl pK_a enhances 14-SASL's affinity for the receptor (Raines and Miller, 1993). The exchange rate of the headgroup is almost certainly slower than that of the acyl chain. This exerts an influence on the acyl chain which diminishes as the spin label is placed further along the chain. The influence of the headgroup is sensed as far as the fourteenth carbon because, in contrast to 14-SASL, 14-PCSL experiences exchange even at 0°C when incorporated into DOPC vesicles containing reconstituted nAcChoRs (Abadji et al., 1993). Consistent with this picture, exchange between restricted and mobile environments has been demonstrated in rhodopsin membranes even when the spin-labeled lipid analog's headgroup was covalently attached to the protein (Davoust and Devaux, 1982). Unfortunately, in this rhodopsin study exchange was examined only at the 16th carbon and, therefore, it is not known whether a similar exchange gradient exists in this membrane. The picture that emerges for the nAcChoR (and perhaps for other protein systems) is that the polar lipid headgroup of boundary lipids is relatively fixed on the conventional ESR time scale, whereas the acyl chain moves like a whip, its frequency of exchange on and off the protein surface being greatest near the center of the bilayer.

We have used continuous wave saturation measurements to further examine lipid-protein interactions in this system. This technique has been shown previously to be useful for examining motionally restricted lipid rotational dynamics because it can selectively detect microsecond motions that are too slow to be resolved by conventional ESR spectroscopy (Squier and Thomas, 1989). It is particularly valuable for detecting changes in the motions of boundary lipids spin-labeled near the polar lipid headgroup or for studying boundary lipids in the gel phase where bulk lipid spectral lineshape overlap hinders resolution of the motionally restricted component (Horvath et al., 1993; Squier and Thomas, 1989). From saturation studies, the mobility of the motionally restricted component can be determined provided that the fraction of motionally restricted component is known independently (Squier and Thomas, 1989). This permits mobility to be assessed at different membrane depths without spectral subtraction. Finally, the microsecond motions detected by saturation studies may be more relevant to nAcChoR function than the faster nanosecond motions reflected in conventional ESR lineshapes because microsecond motions occur on the same time scale as agonist-induced channel opening.

The microwave power at half-saturation was lower for each spin-label incorporated into nAcChoR membranes than when incorporated into pure DOPC liposomes. On average, pure lipid samples required 34% more power to saturate than receptor-containing samples. A previous saturation study by Horvath et al. (1993) found that membranes containing myelin proteolipid protein saturate more readily than protein-free lipid bilayers. This difference in saturation behavior between protein-containing and protein-free lipid bilayers was considerable in the gel phase where lipid exchange between bulk and boundary regions is negligible even on the microsecond time scale; $P^0_{y_2}$ was approximately two- to threefold higher in protein-free lipid bilayers than in membranes containing protein (the fraction of lipid directly interacting with protein was ~0.4). In the liquid crystal phase, however, the difference in between protein-free lipid bilayers and membranes containing protein was smaller, in the range that we report here for nAcChoR reconstituted into DOPC (which at 0°C is in the liquid crystal phase). Therefore, it seems likely that although boundary lipid mobility is restricted on the microsecond as well as the nanosecond time scale, differences in saturation behavior between membranes containing the nAcChoR and pure DOPC liposomes are attenuated by exchange of lipids between mobile and motionally restricted environments on the microsecond time scale. We did not examine the saturation

behavior of membranes in the gel phase because the phase transition temperature of DOPC is approximately −20°C, which cannot be reached with our current temperature control system for the loop gap resonator.

In general, in either protein-free or reconstituted membranes, 5-SASL and 9-SASL are the most readily saturated spin labels. This may reflect a decrease in mobility of 5-SASL due to the proximity of the lipid headgroup and of 9-SASL due to the acyl chain double bond in DOPC. The role of the latter in "anchoring" the center of the acyl chain in homogenous lipids has been discussed in detail by Cevc (1991).

In conclusion, we have examined nAcChoR perturbation of lipid acyl chain motion on both the microsecond and nanosecond time scales. Using spectral subtraction, we have determined that the nAcChoR reduces acyl chain mobility near the bilayer center as well as closer to the lipid headgroup on the nanosecond time scale. Exchange broadening of the bulk lipid spectral lineshape occurs at lower temperatures for stearic acid probes whose spin-label moiety is closer to the bilayer core, consistent with a model in which the polar lipid headgroup interaction is relatively long lived on the nanosecond time scale, and the acyl chain whips on and off the protein surface with increasing frequency as the end of the chain is approached. A more detailed examination of chain motion, for example taking into account the effects of temperature, is prohibited because adequate simulation programs are not available for proteins reconstituted into DOPC (Abadji et al., 1993). Microwave saturation studies indicate that the nAcChoR perturbs mobility of lipid acyl chains on the microsecond time scale, but the observed effects are not large and are probably attenuated by exchange.

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REFERENCES

Abadji, V. C., D. E. Raines, A. Watts, and K. W. Miller. 1993. The effect of general anesthetics on the dynamics of phosphatidylcholine-acetylcholine receptor interactions in reconstituted vesicles. *Biochim. Biophys. Acta.* 1147:143–153.

Altenbach, C., D. A. Greenhalgh, H. G. Khorana, and W. L. Hubbell. 1994. A collisional gradient method to determine the immersion depth of nitroxides in lipid bilayers: application to spin-labeled mutants of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 91:1667–1671.

Braswell, L. M., K. W. Miller, and J. F. Sauter. 1984. Pressure reversal of the action of octanol on postsynaptic membranes from Torpedo. *Br. J. Pharmacol.* 83:305–311.

Cevc, G. 1991. How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of acyl chain unsaturation: an effective chain-length model. *Biochemistry.* 30: 7186–7193.

Davoust, J., and P. F. Devaux. 1982. Simulation of electron spin resonance spectra of spin-labeled fatty acids covalently attached to the boundary of an intrinsic membrane protein: a chemical exchange model. *J. Magn. Reson.* 48:475–494.

Ellena, J. F., S. J. Archer, R. N. Dominey, B. D. Hill, and D. S. Cafiso. 1988. Localizing the nitroxide group of fatty acid and voltage-sensitive spin-labels in phospholipid bilayers. *Biochim. Biophys. Acta.* 940:63–70.

Ellena, J. F., M. A. Blazing, and M. G. McNamee. 1983. Lipid-protein interactions in reconstituted membranes containing acetylcholine receptor. *Biochemistry.* 22:5523–5535.

Esmann, M., A. Watts, and D. Marsh. 1985. Spin-label studies of lipid-protein interactions in (Na^+,K^+)-ATPase membranes from rectal glands of Squalus acanthias. *Biochemistry.* 24:1386–1393.

Fong, T. M., and M. G. McNamee. 1986. Correlation between acetylcholine receptor function and structural properties of membranes. *Biochemistry.* 25:830–840.

Fong, T. M., and M. G. McNamee. 1987. Stabilization of acetylcholine receptor secondary structure by cholesterol and negatively charged phospholipids in membranes. *Biochemistry.* 26:3871–3880.

Freed, J. H. 1976. Theory of slow tumbling ESR spectra for nitroxides. *In* Spin Labeling Theory and Applications. L. J. Berliners, editor. Academic Press, New York. 53–132.

Gaffney, B. J. 1976. Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes. *In* Spin Labeling Theory and Applications. L. J. Berliners, editor. Academic Press, New York. 567–571.

Galzi, J. L., F. Revah, A. Bessis, and J. P. Changeux. 1991. Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain. *Annu. Rev. Pharmacol. Toxicol.* 31:37–72.

Horvath, L. I., P. J. Brophy, and D. Marsh. 1993. Exchange rates at the lipid-protein interface of the myelin proteolipid protein determined by saturation transfer electron spin resonance and continuous wave saturation studies. *Biophys. J.* 64:622–631.

Markwell, M. A. K., S. M. Haas, L. L. Beiber, and N. E. Tolbert. 1978. A Modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87:206–210.

Marsh, D. 1985. ESR spin label studies of lipid-protein interactions. *In* Progress in Protein-Lipid Interactions. A. Watts and J. M. DePonts, editors. Elsevier, Amsterdam, 143–172.

Marsh, D. 1989. Experimental methods in spin-label analysis. *In* Biological Magnetic Resonance. L. J. Berliner and J. Reubens, editors. Plenum Press, New York. 255–303.

McClare, C. W. F. 1971. An accurate and convenient organic phosphorous assay. *Anal. Biochem.* 39:527–530.

Pates, R. D., and D. Marsh. 1987. Lipid mobility and order in bovine rod outer segment disk membranes: a spin label study of lipid-protein interactions. *Biochemistry.* 26:29–39.

Pates, R. D., A. Watts, R. Uhl, and D. Marsh. 1985. Lipid-protein interactions in frog rod outer segment disc membranes: characterization by spin labels. *Biochim. Biophys. Acta.* 814:389–397.

Poole, C. P. 1983. Electron Spin Resonance: A Comprehensive Treatise on Experimental Techniques, 2nd ed. Wiley-Interscience, New York. 780 pp.

Raines, D. E., and K. W. Miller. 1993. The role of charge in lipid selectivity for the nicotinic acetylcholine receptor. *Biophys. J.* 64: 632–641.

Squier, T. C., and D. D. Thomas. 1986. Methodology for increased precision in saturation-transfer electron paramagnetic resonance studies of rotational dynamics. *Biophys. J.* 49:921–935.

Squier, T. C., and D. D. Thomas. 1989. Selective detection of the rotational dynamics of the protein-associated lipid hydrocarbon chains in sarcoplasmic reticulum membranes. *Biophys. J.* 56:735–748.

Sunshine, C., and M. G. McNamee. 1994. Lipid modulation of nicotinic acetylcholine receptor function: the role of membrane lipid composition and fluidity. *Biochim. Biophys. Acta.* 1191:59–64.