The role of charge in lipid selectivity for the nicotinic acetylcholine receptor

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ABSTRACT The effect of salt and pH titration on the selectivity of spin-labeled analogues of phosphatidic acid, phosphatidylserine, phosphatidylcholine, and stearic acid for the nicotinic acetylcholine receptor (nAcChoR) reconstituted into dioleoylphosphatidylcholine was examined at 0°C using electron spin resonance spectroscopy. The order of selectivity at pH 7.4 and 0 mM NaCl was phosphatidylserine > stearic acid > phosphatidic acid > phosphatidylcholine. The addition up to 2 M NaCl or titration of pH from 5.0 to >9.0 did not alter the selectivity of the phospholipids for the nAcChoR. For stearic acid, conversely, titration of pH from 5.0 to 9.0 at 0 mM NaCl and titration of NaCl from 0 to 2 M at pH 9.0 both increased selectivity for the nAcChoR. It is concluded that electrostatic interactions do not account for the selectivity of the negatively charged phospholipids, phosphatidylserine, and phosphatidic acid for the nAcChoR. This is consistent with the known orientation of the transmembrane sequences M1 and M4, which predicts a balance in the number of negative and positive charges in the lipid-protein interface and suggests that the two positive charges on each M3 helix are not exposed to the lipid-protein interface.

INTRODUCTION

The nicotinic acetylcholine receptor (nAcChoR)[1] is a ligand-gated, cation-selective ion channel that can be isolated from the electric organ of *Torpedo nobiliana.* It is composed of five transmembrane subunits that are thought to form a central channel. The functional properties of this protein are influenced by its lipid environment. Studies of nAcChoR reconstituted into membranes containing synthetic or native lipids demonstrate that, in addition to cholesterol, a negatively charged phospholipid is necessary to maintain ion-gating activity (1). It has been hypothesized that these lipids act at the lipid-nAcChoR interface to stabilize the secondary structure necessary for formation of the ion channel (2). Ellena et al. (3) have demonstrated using electron spin resonance (ESR) spectroscopy that a spin-labeled analogue of phosphatidic acid (PA) and stearic acid (SA) exhibit selectivity for the nAcChoR relative to phosphatidylcholine (PC). This poses the question of whether this selectivity is related to electrostatic attraction between these lipids and the nAcChoR.

Selectivity of negatively charged lipids for the lipid-protein interface is common in membrane protein systems studied to date. In some, but not all, cases this selectivity has been shown to be due to electrostatic interactions. The electrostatic contribution to lipid selectivity can be assessed by screening the electrostatic interaction with salt or changing it by titrating the pH through the lipid's pK_a. In previous studies, the electrostatic contribution to the selectivity of a particular negatively charged lipid for a protein has been found to vary with the system examined. In the case of the Na^+, K^--ATPase from *Squalus acanthias,* specificity for SA can be ascribed entirely to an electrostatic interaction, whereas that for PA and phosphatidylserine (PS) cannot (4). On the other hand, electrostatic effects only partially account for the negative lipid selectivity of bovine myelin proteolipid apoprotein, since neither protonation nor salt screening completely eliminates the selectivity of SA, PS, or PA for the protein interface (5). Binding of the peripheral protein apocytochrome c to negatively charged phospholipid bilayers is dependent on electrostatic forces (6).

In this study, we investigated the origin of the specificity of negatively charged lipids for the lipid-protein interface of the nAcChoR and determined the rotational mobilities of lipids at that interface using ESR spectroscopy. We concluded that electrostatic forces do not contribute to the selectivity of the negatively charged PS or PA or the zwitterionic phosphatidylcholine for the nAcChoR. In contrast, the selectivity of SA for the lipid-protein interface is partially determined by electrostatic interactions.

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[1] *Abbreviations used in this article:* DOPC, dioleoylphosphatidylcholine; ESR, electron spin resonance; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; nAcChoR, nicotinic acetylcholine receptor; PA; phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; *n*-PASL, *n*-PCSL, and *n*-PSSL, 1-acyl-2-[n-(4,4-dimethyl-*N*-oxyoxazolidine)stearoyl]-*sn*-glycero-3-phosphoric acid, -phosphocholine, and -phosphoserine, respectively; SA, stearic acid; n-SASL, n-(4,4-dimethyl-*N*-oxyoxazolidine)stearic acid, where n denotes the position on the acyl chain.

MATERIALS AND METHODS

Affi-Gel 401 was purchased from Bio-Rad Laboratories (Richmond, CA). Diisopropylfluorophosphate was from Sigma Chemical Co. (St. Louis, MO). Buffer A contained 10 mM 3-(*N*-Morpholino)propanesulfonic acid (MOPS), 100 mM NaCl, 0.1 mM ethylenediaminetetraacetate and 0.02% sodium azide, its pH adjusted to 7.4 with concentrated sodium hydroxide (3). Doubly distilled, deionized water was

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used to make all buffers. Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Phencyclidine was from Research Biochemicals Inc. (Natick, MA). The lipids PC, PA, PS, and SA, spin labeled at the 14th carbon of the acyl chain (14-PCSL, 14-PASL, 14-PSSL, and 14-SASL; 1-acyl-2-[n-2-(4,4-dimethyl-*N*-oxyoxazolidine)stearoyl]-*sn*-glycero-3-phosphacholine, -phosphoric acid, and -phosphoserine, respectively, and n-(4,4-dimethyl-*N*-oxyoxazolidine) stearic acid) were gifts from Dr. Anthony Watts (Oxford University, Oxford, UK).

Reconstitution of nAcChoR into DOPC

The electric organ of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) was dissected and processed as described by Braswell et al. (7) to obtain receptor-rich membranes that were frozen at −80°C until used. The receptor was reconstituted according to the procedure of Ellena et al. (3). All steps were carried out at 0–4°C. In brief, receptor-rich membranes from ~500 g of electric organ were thawed and dissolved in 75 ml buffer A, and the acetylcholinesterase activity was inhibited with 0.3 mM diisopropylfluorophosphate. The mixture was centrifuged (125,100 *g;* 60 min) in a Sorvall type A-641 rotor (DuPont Co., Wilmington, DE), and the supernatant was discarded. The pellet was resuspended in 100 ml buffer A containing 2% (wt/vol) cholate and stirred for 30 min to solubilize the receptor. The mixture was again centrifuged (125,100 *g;* 60 min), and the supernatant was collected.

A glass column was packed with Affi-Gel 401 (Bio-Rad Laboratories) derivatized with bromoacetylcholine bromide and equilibrated with 1% (wt/vol) sodium cholate and 1 mg/ml DOPC in buffer A. The solubilized receptor was applied to the column and washed with two column volumes of 1 mg/ml DOPC and 1% (wt/vol) cholate in buffer A, followed by 1.5 column volumes of 2.5 mg/mL DOPC and 1% (wt/vol) cholate in buffer A, and left to stand overnight. The column was again washed with the high lipid-containing buffer, followed by two washes of 0.15 mg/ml DOPC in 0.5% (wt/vol) cholate in buffer A. The receptor was eluted with the latter buffer and 20 mM carbamylcholine chloride. 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 liters of buffer A. The effects of pH and NaCl on a single lipid:protein ratio are presented to allow for direct comparisons of nAcChoR selectivity among different spin-labeled lipids, but similar results were obtained with separate preparations. The protein and lipid contents were determined by the methods of Lowry et al. (7a) and McClare (7b), respectively. The reconstituted receptor was stored at −80°C until used.

Incorporation of spin-labeled lipids into reconstituted nAcChoR and DOPC liposomes

Aliquots of ethanolic solutions of spin-labeled lipids were dried under a stream of nitrogen. Reconstituted nAcChoR (0.5 ml; 2–3 mg of protein per sample) was added and equilibrated with the spin-labeled lipid for 15–30 min with intermittent vortexing. The final ratio of DOPC to spin-labeled lipid was always >100:1 to minimize spin-spin interactions. Samples were frozen and thawed several times in liquid nitrogen to aid spin-label incorporation and then washed free of unincorporated label by two cycles of centrifugation (13,600 *g;* 10 min). The nAcChoR was resuspended in an excess of the appropriate buffer before ESR spectroscopy. With this procedure, spin-spin interactions resulting from unincorporated spin-label were seen only with some 14-PSSL samples. These were discarded.

DOPC liposomes were labeled by adding the appropriate spin-labeled lipid directly to DOPC in chloroform, drying to a film under a stream of nitrogen, and placing under vacuum for 2 h and hydrating with the appropriate buffer. The final ratio of DOPC to spin-labeled lipid was always >100:1 to minimize spin-spin interactions. All experiments were performed using a 10 mM MOPS buffer with the final buffer pH adjusted with HCl or NaOH.

ESR spectroscopy and spectral deconvolution

Spectra were obtained on a spectrometer (ER200; Bruker Instruments, Billerica, MA) interfaced with a computer (9000; IBM, Danbury, CT). All experiments were conducted at 0 ± 0.5°C using a thermostated insert within the ESR cavity. Temperature stability was ±0.1°C. Microwave frequency was 9.4 GHz, microwave power was 10 mW, modulation amplitude was 2 G, modulation frequency was 100 kHz, sweep width was 100–120 G, and scan rate was 3 min. Typically, 10–20 scans were accumulated and signal averaged for each sample. Spectral baseline correction and subtraction was performed on an Apple Macintosh IIfx (Cupertino, CA) using a program developed in IGOR (Wave Metrics, Lake Oswego, OR). For 14-PSSL, 14-PASL, and 14-SASL, the spectrum of the spin-labeled lipid in DOPC dispersed in buffer at 0°C was used to simulate the fluid component as previously described (3–5). A 2% cholesterol/DOPC dispersion was found to better simulate the exchange broadened fluid component of reconstituted nAcChoR samples labeled with 14-PCSL. The error in the determination of the fraction of motionally restricted component is estimated to be ±0.02.

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. This provided an unbiased estimation of these spectral parameters. In some cases, only the outer half of these peaks were fit because there was a contribution to the inner half that caused an asymmetry. The spectral outer hyperfine splitting was measured using the maxima and minima, respectively, of the lowfield and highfield gaussian fits.

Effective rotational correlation times, τ_r, for the spin labels motionally restricted by the nAcChoR were estimated from their spectral outer hyperfine splitting assuming a Brownian rotational diffusion model:

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

where A_{max} is half of the measured outer hyperfine splitting of the motionally restricted component. A^r_{max}, half of the measured hyperfine splitting measured for the completely immobilized spin label, was taken as 33.6 G (8). The values for a and b were derived by Freed (9) from spectral simulations for slow isotropic rotational motion assuming a linewidth parameter (δ, in his notation) of 3 G and are 5.4×10^{-10} and −1.36, respectively.

Effective rotational correlation times for the motionally restricted components were also estimated from their lowfield and highfield peak half-height linewidths. We again assumed Brownian diffusion:

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

where Δ_i is the half-height linewidth of the lowfield (i = l) or highfield (i = h) linewidths 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 (9) using spectral simulations. Again for δ = 3.0 G, $2\Delta^r_l$ is 4.77 G, and $2\Delta^r_h$ is 5.43 G. 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.

RESULTS

ESR spectroscopy of spin-labeled lipids incorporated into lipid bilayers containing nAcChoR (Fig. 1) reveal

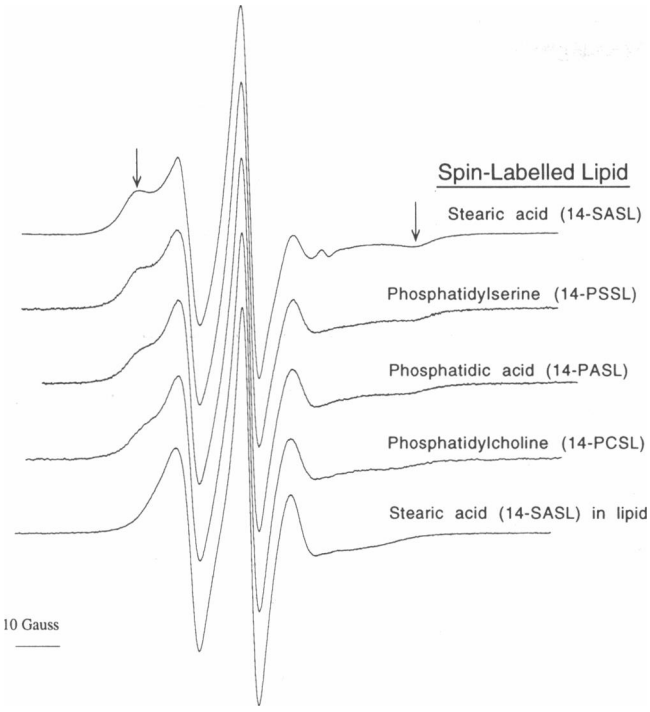

FIGURE 1 ESR spectra of (top to bottom) 14-SASL, 14-PSSL, 14-PASL, and 14-PCSL incorporated into reconstituted nAcChoR having a lipid-protein ratio of 198:1. The bottom spectrum is 14-SASL in DOPC bilayers. Arrows indicate the motionally restricted component. Buffer was 10 mM MOPS, pH = 7.4, T = 0°C. All spectra are normalized to the same central line height.

two spectral components reflecting the existence of two distinct lipid environments: (*a*) a mobile environment in which the rotational mobilities of spin-labeled lipids are similar to those in protein-free lipid bilayers and (*b*) a protein-perturbed environment in which the spin-labeled lipids are motionally restricted. This restricted environment is generally attributed to the annulus of lipids forming the first shell around the protein (boundary lipid). The motionally restricted spectral component (Fig. 2) can be resolved from the two component spectrum of nAcChoR membranes by experimentally simulating the nonperturbed lipid bilayer with protein-free lipid dispersions and digitally subtracting its ESR spectrum from the nAcChoR membrane spectrum. Quantitation of the fraction of spin-labeled lipids in each environment can be achieved by double integration of the intensity of the spectral components.

Spectra of the spin-labeled lipids 14-SASL, 14-PSSL, 14-PASL, and 14-PCSL incorporated into a nAcChoR reconstituted into DOPC at a lipid:protein ratio of 198:1 as well as a spectrum of 14-SASL in DOPC for comparison are shown in Fig. 1. The motionally restricted component is indicated by the arrows. The same buffer solution was used for all samples (10 mM MOPS, pH 7.4, NaCl 0 mM). The spectral lineshape of the motionally restricted component for each spin-labeled lipid derived by spectral subtraction is shown in Fig. 2. The order of selectivity under these conditions as determined by spectral subtraction is 14-PSSL > 14-SASL > 14-PASL > 14-PCSL. A second estimate of the selectivity, obtained by subtracting an immobile component from the composite spectra, was not different (in general, we have found this subtraction strategy to be less satisfactory because the subtraction endpoint is less obvious). The lineshape of the immobile component was obtained from 14-PCSL in DOPC at −35°C.

The effect of pH and NaCl on the ESR spectra of phospholipid spin labels incorporated into reconstituted nAcChoR

The ESR spectra of the spin-labeled lipids in nAcChoR reconstituted into DOPC at extremes of pH are shown in Fig. 3. The motionally restricted component can be best observed in the outer regions of the spectrum where its broad highfield and lowfield peaks can be resolved from

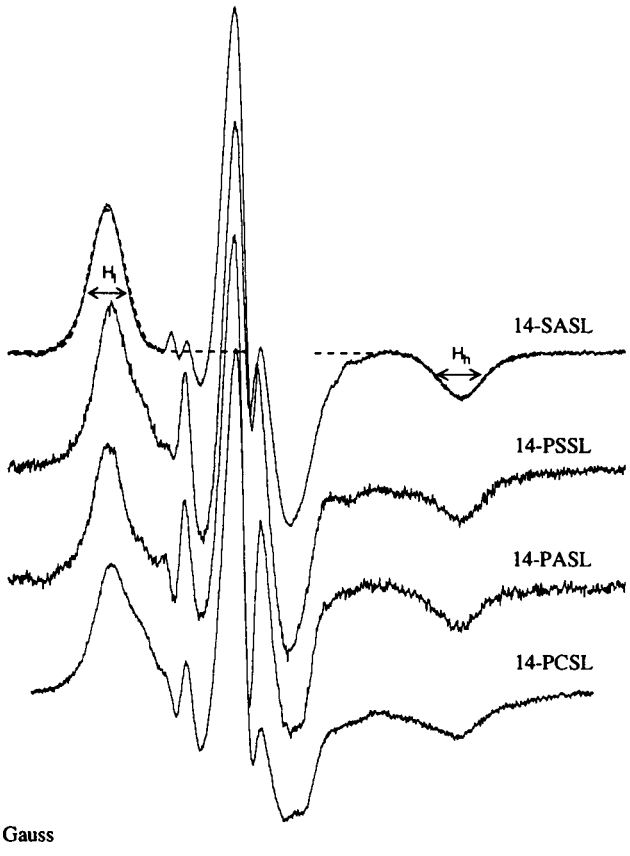

FIGURE 2 The motionally restricted components obtained by spectral subtraction for each of the spin-labeled phospholipids. Dotted lines demonstrate a gaussian fit of the outer portions of the highfield and lowfield peaks. Average values for H_l and H_h, lowfield and highfield half-height linewidths, respectively, are given in Table 1. The lipid-protein ratio was 198:1 for all spin-labeled lipids except 14-PCSL, which was 115:1. Buffer was 10 mM MOPS, pH = 7.4, T = 0°C. All spectra are normalized to the same central line height.

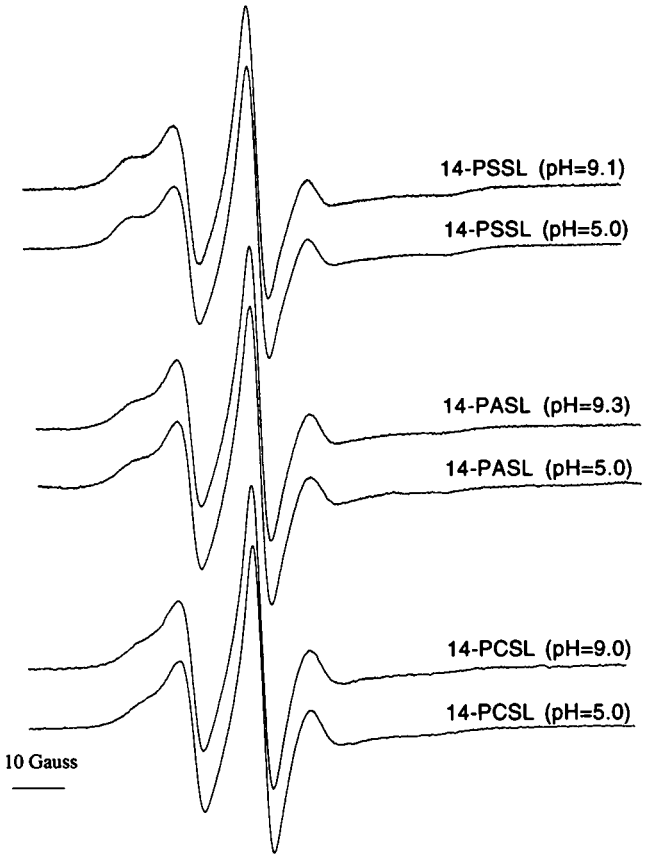

FIGURE 3 ESR spectra for each of the spin-labeled phospholipids at extremes of pH. Deconvolution by spectral subtraction revealed no significant change in the fraction of motionally restricted spectral component. Buffer was 10 mM MOPS, NaCl = 0 mM, T = 0°C. All spectra are normalized to the same central line height.

the sharp lineshape of the fluid component. A single spectrum of the appropriate spin-labeled lipid in DOPC bilayers at 0°C was used for all subtractions from spectra of 14-PSSL and 14-PASL incorporated into nAcChoR since the spectra of these probes in DOPC bilayers were found to be unaffected by titration of pH or NaCl. For similar reasons, a single spectrum of 14-PCSL in DOPC/2% cholesterol bilayers was used for subtractions involving this spin-labeled lipid.

The fraction of motionally restricted component relative to the mobile component was essentially unchanged for the three phospholipids as the buffer pH was increased (Fig. 4). The average fraction of motionally restricted component for 14-PSSL, 14-PASL, and 14-PCSL were (±SD) 0.51 ± 0.007, 0.35 ± 0.013, and 0.32 ± 0.008, respectively.

The outer hyperfine splittings and half-height linewidths of the motionally restricted spectral components were measured, and the rotational mobilities of the negatively charged spin-labeled lipids were calculated from Eq. 1 and 2 and listed in Table 1. In all cases, rotational mobilities were near the limit of what can be determined by conventional ESR spectroscopy. These values could not be determined accurately for the 14-PCSL at this lipid-protein ratio because of the small fraction of motionally restricted lipid and the inability to precisely match the mobile lineshape due to exchange broadening. However, using a sample with a lipid-protein ratio of 115 where this mismatch is less of a problem (because the fluid spectral component is smaller), the rotational mobility was estimated from the 2 A_{max} and the half-height linewidths to be 12 and 19 ns, respectively.

The effect of salt screening of phospholipid negative charge on the fraction of motionally restricted spin label was examined by titrating samples with a 10 mM MOPS buffer at pH 7.4 containing from 0 to 2 M NaCl. No change in either the fraction of motionally restricted spin-labeled lipid or τ_r was observed for 14-PSSL, 14-PASL, or 14-PCSL (Fig. 5 and Table 1).

The Effect of pH and NaCl on the ESR spectra of stearic acid spin label incorporated into reconstituted nAcChoR

The spectral linewidth of 14-SASL in DOPC bilayers increased as the pH was raised. The pK_a of 14-SASL in

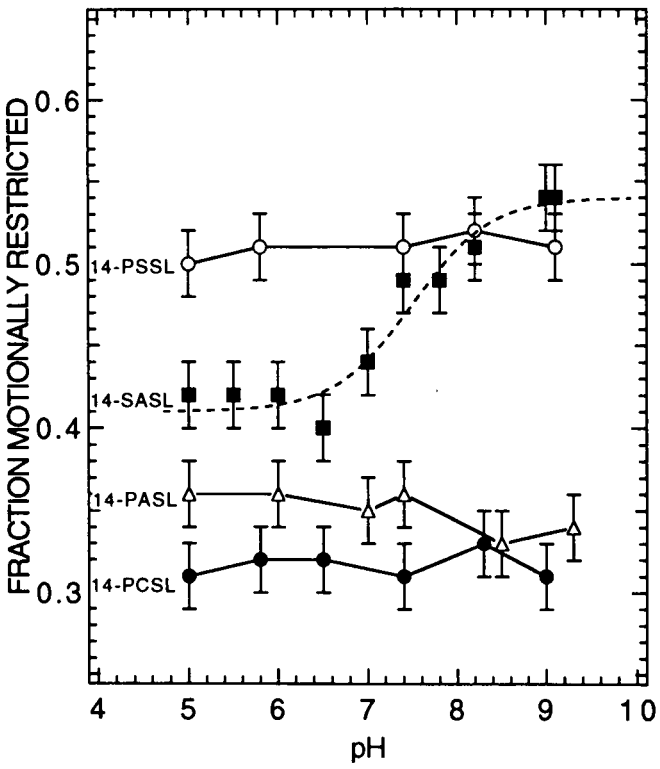

FIGURE 4 Dependence on pH of the fraction of the motionally restricted component for 14-SASL, 14-PSSL, 14-PASL, and 14-PCSL incorporated into reconstituted nAcChoR having a lipid-protein ratio of 198:1. The dotted line is a fit of the 14-SASL data to Eq. 5 with F_{min}, F_{max}, and pK_a equal to 0.42, 0.54, and 7.5, respectively. Buffer was 10 mM MOPS, T = 0°C, NaCl = 0 mM. Error bars indicate our estimate of the subtraction error.

TABLE 1 **ESR lineshape features and calculated rotational correlation times for different motionally restricted spin-labeled lipids in nAcChoR reconstituted into DOPC for pH and NaCl titrations**

Spin-labeled lipid	$2A_{max}$	Lowfield half-height linewidth	Highfield half-height linewidth	τ_r Calculated from $2A_{max}$	τ_r Calculated from lowfield half-height linewidth	τ_r Calculated from highfield half-height linewidth
	G	*G*	*G*	*ns*	*ns*	*ns*
14-PASL*	61.9 ± 0.08	7.1 ± 0.24	8.2 ± 0.63	17 ± 0.4	23 ± 2.3	37 ± 6.9
14-PASL‡	61.8 ± 0.34	6.8 ± 0.16	9.5 ± 0.45	17 ± 1.3	19 ± 0.6	27 ± 2.7
14-PSSL§	61.6 ± 0.12	7.1 ± 0.14	10.2 ± 0.43	16 ± 0.5	23 ± 1.2	24 ± 1.6
14-PSSL‖	61.5 ± 0.20	7.3 ± 0.09	10.2 ± 0.50	16 ± 0.7	21 ± 0.7	23 ± 1.9
14-SASL¶	62.6 ± 0.24	7.3 ± 0.16	9.5 ± 0.26	21 ± 1.4	21 ± 1.2	27 ± 1.4
14-SASL**	62.6 ± 0.16	7.6 ± 0.10	9.7 ± 0.40	21 ± 0.9	19 ± 0.6	26 ± 1.9
14-SASL‡‡	62.9 ± 0.08	7.0 ± 0.09	9.4 ± 0.25	23 ± 0.6	23 ± 0.8	27 ± 1.3
14-PCSL§§	60.5	7.5	11.5	12	19	19

For each titration experiment, lineshape features and calculated rotational correlation times were determined by independently analyzing each motionally restricted lineshape and calculating an average value. The error is the standard deviation. The number of samples can be determined by inspection of Figs. 4 and 5. The lipid-protein ratio was 198:1 (mol:mol), T = 0°C.

* pH = 5.0–9.3, NaCl = 0 mM.

‡ NaCl = 0–2 M, pH = 7.4.

§ pH = 5.0–9.1, NaCl = 0 mM.

‖ NaCl = 0–2 M, pH = 7.4.

¶ pH = 5.0–9.0, NaCl = 0 mM.

** NaCl = 0–2 M, pH = 5.5.

‡‡ NaCl = 0–2 M, pH = 9.0.

§§ ESR lineshape features and calculated rotational correlation times are from a single preparation having a lipid-protein ratio of 115:1, NaCl = 0, pH = 7.4, T = 0°C.

DOPC bilayers can be determined empirically from the change in the half-height linewidth of the lowfield peak as a function of pH (Fig. 6) using the equation:

$$H = H_{min} + (H_{max} - H_{min})/(1 + [H^+]/K_a), \quad (3)$$

where H_{min} and H_{max} are the lowfield half-height linewidths of 14-SASL in the protonated and deprotonated states, respectively. The resulting nonlinear least-squares fit yields a pK_a of 6.8 ($[H^+] = 1.5 \pm 0.28 \times 10^{-7}$).

In contrast, the addition of up to 2 M NaCl did not change the spectral lineshape of 14-SASL in DOPC at a pH of 9.0.

The ESR spectra of the spin-label 14-SASL incorporated into reconstituted nAcChoR at various pHs are shown in Fig. 7, and the motionally restricted components obtained by spectral subtraction are shown in Fig. 8. Since the spectral lineshape of the fluid component broadens with increasing buffer pH, it was critical to use a spectrum of 14-SASL in DOPC at the appropriate pH to simulate this component for spectral subtractions. At high pH and low salt, a small mobile component (always <1% of the integrated intensity) was subtracted from some spectra. This component had the same lineshape as 14-SASL in buffer and was attributed to aqueous spin label. This component did not significantly affect estimation of the fraction of motionally restricted component. The fraction of the motionally restricted component is observed to increase steeply with increasing pH in a manner that is consistent with the titration of a single group (Fig. 4) and can be fitted to:

$$f = f_{min} + (f_{max} - f_{min})/(1 + [H^+]/K_a), \quad (4)$$

where f_{min} and f_{max} are the fractions of motionally restricted 14-SASL in the protonated and deprotonated states, respectively (4). An iterative nonlinear least-squares fit to Eq. 4 gives values of $f_{min} = 0.42$, $f_{max} = 0.54$, and pK_a = 7.5 ($[H^+] = 3.1 \pm 1.21 \times 10^{-8}$). The p$K_a$ is only slightly higher than that measured for the 14-SASL in bilayers of DOPC. It is therefore likely that the increase in the fraction of motionally restricted 14-SASL is due to the titration of the 14-SASL headgroup rather than the titration of a negatively charged amino acid on the nAcChoR.

Since the selectivity of 14-SASL for the nAcChoR is dependent on its protonation state, we examined the effect of salt screening at both low and high pH. As expected, the fraction of motionally restricted 14-SASL was unaffected by titration to 2M NaCl at pH 5.5. However, when the fatty acid was deprotonated by raising the buffer pH to 9.0, there was a small increase in the fraction of motionally restricted 14-SASL (13%) on adding NaCl that reached a plateau by ~200–400 mM NaCl. This result was not specific for NaCl since KCl similarly increased the fraction of motionally restricted 14-SASL (data not shown). This effect is opposite to that expected if selectivity were due simply to the electrostatic attraction of the fatty acid to a positively charged amino acid.

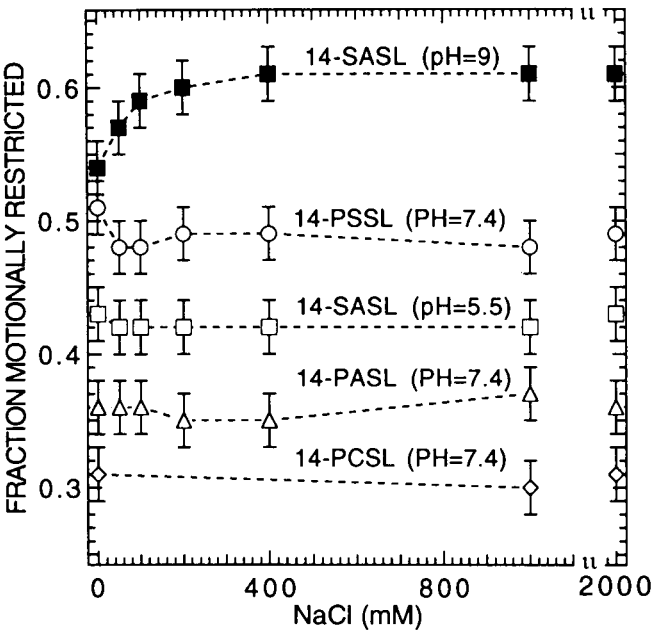

FIGURE 5 Salt dependence of the fraction of the motionally restricted component for 14-SASL, 14-PSSL, 14-PASL, and 14-PCSL incorporated into reconstituted nAcChoR having a lipid-protein ratio of 198:1. Buffer is 10 mM MOPS, T = 0°C. The pH was 7.4 for all spin labels except 14-SASL, which was either 5.5 or 9.0 as indicated. Error bars indicate our estimate of the subtraction error.

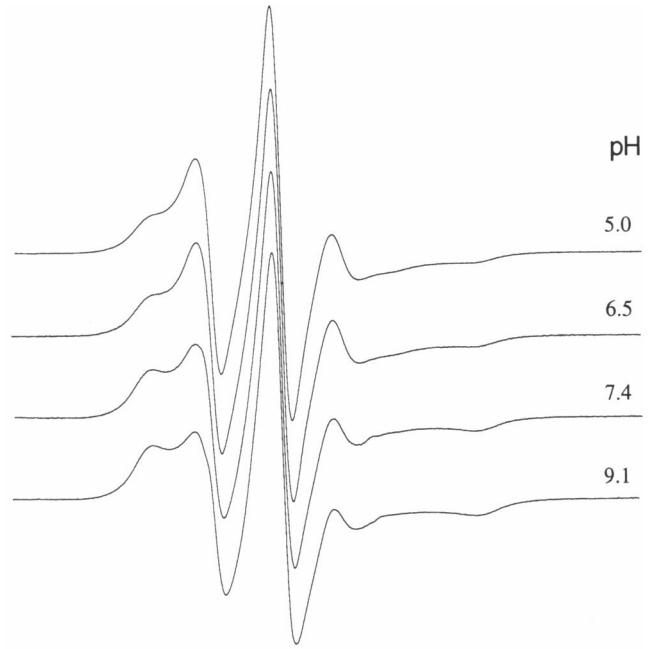

FIGURE 7 Dependence on pH of the ESR spectra of 14-SASL incorporated into reconstituted nAcChoR having a lipid-protein ratio of 198:1. Buffer is 10 mM MOPS, NaCl = 0 mM, T = 0°C. All spectra are normalized to the same central line height.

An intersubtraction of the ESR spectrum in the presence and absence of NaCl yields a restricted component that is independent of any assumption about the fluid component and is very similar to those presented in Fig. 8.

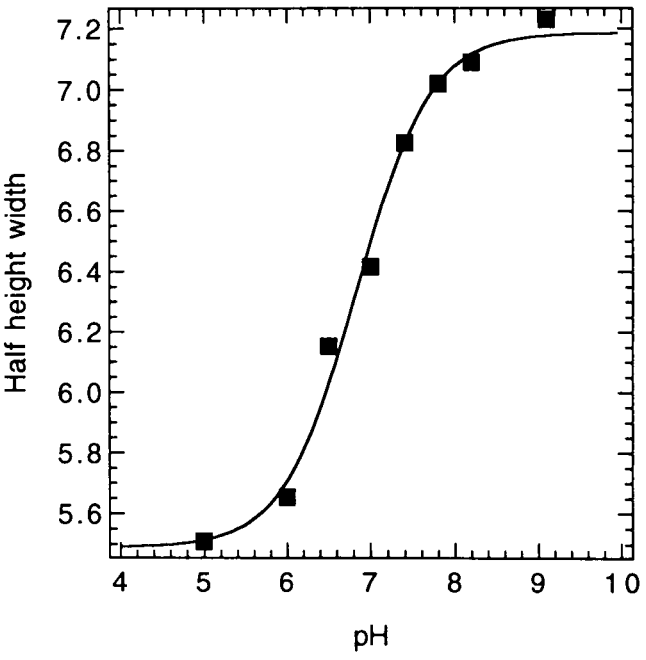

FIGURE 6 Dependence on pH of the lowfield half-height width (in gauss) of 14-SASL in DOPC bilayers. A pK_a of 6.8 is obtained by a fit to Eq. 4. The buffer was 10 mM MOPS, NaCl = 0 mM, T = 0°C.

DISCUSSION

NaCl and pH effects on the affinity of phospholipids for the nAcChoR

Negatively charged lipids are required to preserve the ion-gating function of nAcChoR reconstituted into lipid bilayers, perhaps because negatively charged lipids maintain the nAcChoR secondary structure; studies utilizing Fourier transform infrared spectroscopy indicate that these lipids increase the β-sheet content of the nAcChoR (2). These functionally important lipids also demonstrate selectivity for the nAcChoR. This is most easily studied using ESR spectroscopy since boundary lipids are spectroscopically distinct from those lipids not directly contacting the nAcChoR.

The order of selectivity reported here is 14-PSSL > 14-SASL > 14-PASL > 14-PCSL at pH = 7.4, NaCl = 0 mM. This is identical to that order reported by Ellena et al. (3) for these lipids spin labeled at the 16th carbon of the acyl chain with the exception that 16-PSSL was not found to exhibit any selectivity over 16-PCSL. We cannot explain this difference since it seems unlikely that the selectivity of PSSL is dependent on the position of the spin-label moiety. To ensure the identity of our 14-

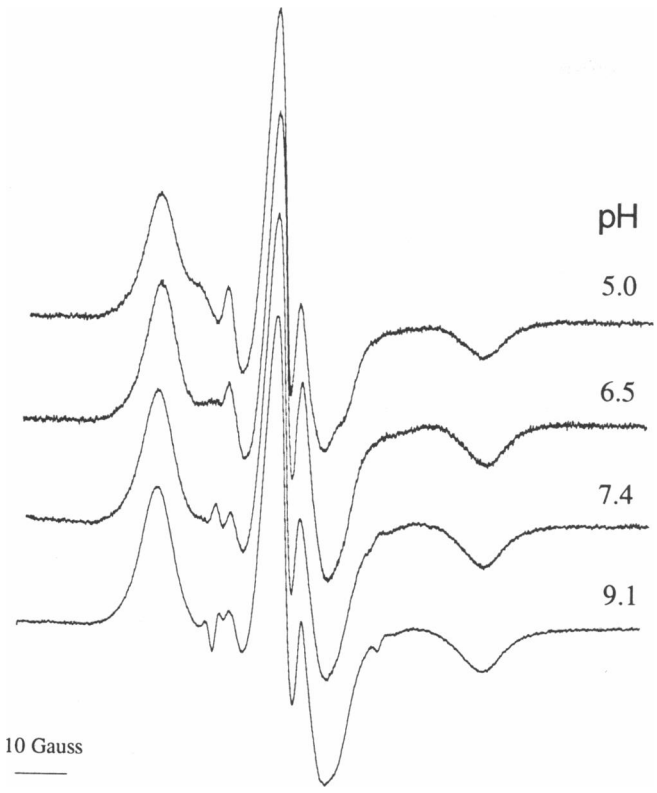

FIGURE 8 Motionally restricted component derived from spectral subtraction of spectra in figure 7. Average values for H_l and H_h are given in Table 1. All spectra are normalized to the same central line height.

PSSL, its structure was checked by nuclear magnetic resonance spectroscopy.

Titrations of pH with 14-PASL extended from 5.0 to 9.3 units and produced no change in the fraction of motionally restricted 14-PASL. This titration is expected to protonate at least one of the charges on the phosphate group of PASL; monolayers of phosphatidic acid have a pK_{a1} and a pK_{a2} of ~4 and 8, respectively (10). Other studies have determined a pK_{a2} of 7.4 for PASL in lipid dispersions composed of DMPC (5). Even if we had not eliminated both negative charges from the PASL, the lack of effect with salt screening argues that electrostatic interactions do not play a significant role in its selectivity for the nAcChoR relative to PC. The spin-labeled lipid 14-PSSL also showed no change in binding with titration of pH or NaCl. The pK_as of its amino, carboxyl, and phosphate groups are 7.5, 4.0, and 3.7, respectively, in monolayers (11). Our pH titration should eliminate at least one charge. And again, although the effective pK_as of two of its titratable groups may be outside the range tested in this study, the lack of any effect by salt at pH 7.4 (or even at pH 10.4; data not shown) supports the conclusion that electrostatic attraction for the nAcChoR does not account for its high affinity for the nAcChoR relative to PC.

For all of the negatively charged lipids, the ESR spectra of the appropriate spin-labeled lipid in pure DOPC bilayers recorded at the same temperature as that of the reconstituted sample could reasonably simulate the fluid component, indicating that exchange between the mobile and protein-perturbed environments is relatively slow on the ESR timescale. It also implies that there is little perturbation of lipids not directly in contact with the nAcChoR. Only for 14-PCSL was exchange significant, requiring the addition of cholesterol to DOPC to broaden its spectrum to reasonably simulate the fluid component. This was empirically found to be more effective than using a lipid sample recorded at a lower temperature. Even so, lineshape mismatch made a determination of the mobility of this lipid at the interface impossible in samples with high lipid-protein ratios.

Origins of selectivity for negatively charged phospholipids

The acetylcholine receptor has been studied extensively, and a review of its structure reveals some insights into the nature of the lipid-protein interface. The acetylcholine receptor has four subunits arranged in a pentameric structure (α_2, β, δ, γ). Based on hydropathy analysis, each subunit is thought to have four transmembrane helices. As a result of recent cross-linking studies with channel blockers (chlorpromazine) and site directed mutagenesis studies, it has been established that the second transmembrane sequences from the NH_2-terminus (the M2 helix) from each subunit forms the channel lining. The 5 M2 helices may be imagined to form a pentameric structure around a central pore with the charged residues at each end of M2 facing toward the lumen. In each subunit the M2 helix is separated by a short loop from the M1 and M3 helices that consequently may pack adjacent to it forming a complete layer around the inner pentameric structure. The M4 subunit is separated by a long cytoplasmic loop and probably lies outside the second ring of helices (see references 12 and 13 for good reviews). Photolabeling experiments with pyrene maleimide and 3-trifluoromethyl-3-(m-[^{125}I]iodophenyl)-diazirine have identified the faces of the M1 and M4 helices that face the bilayer (14, 15).

To estimate how many of the charged amino acids often found at the ends of transmembrane helices faced the bilayer, we arranged the four transmembrane helices of each subunit in a diamond when viewed in cross-section with the M2 helix toward the lumen of the channel and the M4 helix on the opposite corner facing the bilayer. (The centers of the M2 helices formed a pentamer.) Thus, M4 exposes a wide face to the bilayer and M1 and M3 a smaller face. The photolabeling experiments described above enable the approximate orientations of the M1 and M4 helices to be fixed relative to the bilayer. With these assignments, the α, β, and γ M1 subunits have no charged residues in the lipid-protein inter-

face, whereas the δ M1 has one positive and no negative fixed charges facing the bilayer. Similarly, the M4 subunits contribute in all seven positive and eight negative charges to the lipid-protein interface. Thus, the opposing contributions of eight positive and eight negative charges is balanced (note that we have ignored amino acids that have titrateable charges because we saw no evidence for any such contribution in the pH experiments). This observation is consistent with the observed inability within experimental error of pH and salt to titrate the number of charged phospholipids in the lipid-protein interface, because an equal number of repulsive and attractive electrostatic interactions would be titrated. However, the contribution of charge to the lipid-protein interface from the M3 helices has been ignored so far because its orientation is unknown. Each M3 helix has two positive charges, one at each end of the helix arranged one above the other. The limiting case assumptions are to minimize or to maximize the charge in the lipid-protein interface. Thus, the minimum lipid exposed charge from all of the M3 helices would be zero and the maximum plus 10, yielding for the oligomer eight positive and eight negative charges or 18 positive and eight negative charges. Since we observe no net effect of pH or salt, the assignment of M3 with the least net charge facing the bilayer is the most consistent with our data. Recently, Blanton and Cohen (15) have labeled the receptor with azidopyrene. Protease digestion of the receptor and subsequent mapping revealed labeling of fragments containing the M3 hydrophobic segments of both the γ and δ subunits. Future sequencing of the labeled peptide(s) will test our assignment.

Because the electrostatic contributions are balanced, it is difficult to tell if they contribute to lipid selectivity. With the exception of PA, the other lipids have multiple charges and their behavior will not be simple. Most probably the molecular details of the lipid headgroup-protein interaction are important. If this is the case, then the distribution over the lipid-protein surface of a given lipid in a mixture is unlikely to be uniform.

NaCl and pH effects on the affinity of stearic acid for the nAcChoR

Barratt and Laggner (16) have proposed that deprotonation of the fatty acid spin label eliminates its ability to hydrogen bond with the phosphate group of DOPC and allows for an electrostatic attraction between the negatively charged carboxylic acid group on 14-SASL and the positively charged choline nitrogen on DOPC. This results in a change of ~6 Å in stearic acid's vertical position in bilayers composed of PC. Since the spectral lineshape of 14-SASL is dependent on its position in the bilayer, its pK_a can be determined by following changes in spectral lineshape with titration of pH. Our value for the pK_a of 14-SASL in DOPC (6.8) is nearly identical to the values for 14-SASL monolayers (6.9) or for 14-SASL incorporated into bilayers composed of DMPC (6.7) (5, 11).

The association of stearic acid to the nAcChoR was strongly dependent on its protonation state as titration from a pH of 5.0 to 9.1 resulted in a 29% increase in the fraction that is motionally restricted. This finding agrees with the qualitative observation by Ellena et al. (3) that the selectivity of stearic acid is pH dependent. At high pH, the binding of 14-SASL to the nAcChoR is nearly the same as that for 14-PSSL, whereas at low pH its selectivity decreases by roughly twofold to nearly match that exhibited by 14-PASL for the nAcChoR. However, even in the uncharged form, 14-SASL exhibits selectivity for the nAcChoR versus 14-PCSL, indicating that selectivity is not entirely due to charge.

Including our work, three membranes have now been studied in this way. In each case, increasing the pH increased the fraction of n-SASL associated with the protein, but increasing the salt concentration at high pH had disparate effects. In the Na^+, K^--ATPase native membranes from *Squalus acanthia* and the myelin proteolipid apoprotein reconstituted into DMPC, the fraction of 14-SASL at the lipid-protein interface increases with pK_as of 8.0 and 7.7, respectively (4, 5) compared with a value of 7.5 in our study. The Na^+, K^--ATPase presents the simplest case because the pH-induced increase can be titrated completely away with high salt, suggesting the role of electrostatic interactions between the negatively charged stearic acid and positively charged amino acids side chains on the protein (4). On the other hand, in the myelin proteolipid apoprotein reconstituted into DMPC, the interactions are more complex since salt screening only partially reverses the increase in the fraction of motionally restricted 14-SASL seen with deprotonation of the stearic acid (5). Paradoxically, in the nAcChoR, salt screening further increases the association of 14-SASL with the protein.

The simplest explanation for our results invokes the interactions of 14-SASL in the bilayer. Since the selectivity of a lipid for the nAcChoR reflects the difference in free energy between lipid-protein and lipid-lipid interactions, it is necessary to assume that increasing pH weakens lipid-lipid interactions. There is clearly a change in the interaction between 14-SASL and DOPC as reflected in the change in the rotational mobility of 14-SASL in DOPC with titration of pH. Deprotonation of the fatty acid eliminates its ability to hydrogen bond with the phosphate group of DOPC but allows for an electrostatic interaction between the negatively charged carboxylic acid group on 14-SASL and the positively charged choline nitrogen on DOPC. This is bought at the price of pulling some 6 Å of 14-SASL's acyl chain from the bilayer. Subsequent salt screening of the electrostatic interaction between 14-SASL and DOPC might further weaken the lipid-lipid interaction, although we observed no changes in the EPR spectrum. If this explanation is

correct, then a similar pattern should have been observed in the myelin proteolipid apoprotein reconstituted into DMPC system, but it might be difficult to discern in the presence of strong electrostatic lipid-protein interactions.

A second explanation for our results invokes two opposing interactions. As pH is increased, the first interaction, which does not involve charge and may well be the weakening of lipid-lipid interactions that results from eliminating 14-SASL's ability to hydrogen bond discussed above, shifts the equilibrium toward the protein, increasing the fraction of motionally restricted lipid by 0.19 from 0.42 to 0.61. However, at low salt, electrostatic repulsion between the lipid and the protein shifts the equilibrium in the opposite direction, reducing the fraction of motionally restricted lipid by 0.07 to give the net observed change of 0.12 (figure 4). Subsequent salt titration attenuates the second interaction and the fraction of motionally restricted lipid increases by 0.12 to 0.19 (figure 5). This model needs to explain why electrostatic interactions, which yield no net change in 14-PASL and 14-PCSL's interactions with the protein, can do so for 14-SASL. Studies with brominated lipids have lead to the conclusion that some 5–10 additional sites, not accessible to PC, are available for SA (17). The existence of such nonannular sites is thus consistent with this second model, but their location is not known. The channel blocker phencyclidine (100 μM), which interacts with the hydrophobic ring at the mouth of the channel, did not displace 14-SASL under conditions of high pH and salt, ruling out this region as a fatty acid binding site.

Rotational mobility of motionally restricted lipids

Rotational correlation times for boundary lipids have been estimated previously in other lipid-protein systems. For 14-PCSL at the interface of yeast cytochrome oxidase in DMPC, Knowles et al. (18) give a value of ~50 ns. Esmann and Marsh (4) estimate 30–50 ns for stearic acid and phospholipids at the interface of the Na^+, K^--ATPase from *Squalus acanthias* in native membranes. The motionally restricted 14-PSSL component in frog rod outer segment disk membranes has a correlation time of 20 ns (19). Ellena et al. (3) estimate a rotational correlation time of 16 ns for 16-SASL at the lipid-protein interface of the nAcChoR reconstituted into DOPC. Taken together, it appears that the rotational correlation times of protein-perturbed phospholipids and stearic acid in a variety systems are quite similar.

The high quality of the motionally restricted spectral components obtained by subtractions of 14-SASL in nAcChoR should allow for the detection of relatively small changes in rotational mobility with changes in affinity for the nAcChoR. In spite of a nearly 50% increase in the fraction of motionally restricted 14-SASL with increasing pH and NaCl, we could detect no change in the spin label's rotational mobility. Similarly, the rotational mobility was not significantly different for the various spin-labeled lipids despite having dissimilar selectivities for the nAcChoR. This indicates that although the lipid headgroup determines selectivity, it does not determine the mobility of the lipid acyl chains.

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