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Lipid–protein interactions and protein dynamics in vesicles containing the nicotinic acetylcholine receptor: a study with ethanol

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Abstract

Electron paramagnetic resonance (EPR) spectroscopy was used to study the action of ethanol on the protein side chain motions of the nicotinic acetylcholine receptor (nAcChoR) in alkaline extracted membranes from *Torpedo nobiliana*. EPR spectra of the nAcChoR derivatized with maleimide spin label contain both strongly and weakly immobilized components. The rotational correlation time of the strongly immobilized component decreases by a factor of 2–3-fold with the addition of 1.6 M ethanol, while that of the weakly immobilized component is not significantly altered. EPR spectroscopy was also used to probe the lipid environment immediately surrounding the nAcChoR with stearic acid and phosphatidylcholine spin labeled at the fourteenth acyl carbons (14-SASL and 14-PCSL, respectively), and the steroid spin label androstanol (ASL). EPR spectra of these probes reveal a component corresponding to lipids that are motionally restricted by the receptor (annular lipids) in addition to a more fluid component arising from bulk lipid. Using spectral subtraction, the order of selectivity of these spin labels for the nAcChoR was determined to be ASL ≥ 14-SASL > 14-PCSL. The estimated rotational correlation times of the high affinity 14-SASL and ASL probes ranged from approx. 20 to 35 ns. The correlation times of the lower affinity 14-PCSL were generally shorter than those for 14-SASL and ASL and ranged from about 10 to 25 ns. The addition of up to 0.9 M ethanol altered neither the affinity nor the mobility of the motionally restricted EPR component. This suggests that ethanol's actions on the nAcChoR are not mediated via changes at the lipid/protein interface near the center of the bilayer.

Key words: Ethanol; Lipid–protein interaction; Reconstitution; Acetylcholine receptor; EPR

1. Introduction

Ethanol is one of the most widely used psychoactive substances [1]. Although there is a general consensus that it acts on the central nervous system, there is nevertheless very little known about its molecular mechanism or its specific site(s) of action [2].

Early theories have centered on the idea that ethanol acts by partitioning into neuronal membranes, thus altering the dynamics of cell membranes by perturbing the bulk lipid bilayer [3]. More recent lines of evidence suggest that ethanol acts specifically on various ligand-gated channels within the CNS [4]. Electrophysiological and neurochemical studies have shown that acute exposure to ethanol enhances the response to GABA and serotonin at $GABA_A$ and $5\text{-}HT_3$ receptors, respec-

Abbreviations: AcCho, acetylcholine; nAcChoR, nicotinic acetylcholine receptor; AER, alkaline extracted nAcChoR-rich membranes; BSA, bovine serum albumin; DOPC, dioleoylphosphatidylcholine; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ASL, androstanol spin label; MSL, 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy spin label; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxyoxazolidine)stearoyl]-*sn*-glycero-3-phosphocholine; 14-SASL, 14-(4,4-dimethyl-*N*-oxyoxazolidine)stearic acid; SDS, sodium dodecyl sulfate; ST-EPR, saturation transfer EPR; τ_r, correlation time for rotation.

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tively [5–7]. Conversely, it has been shown that ethanol attenuates the excitatory action of glutamate at both the NMDA and kainate receptors and inhibits function of voltage-sensititve Ca^{2+} channels [8,9]. The effects of ethanol have also been demonstrated on the ligand-gated cationic nAcChoR ion channel: at the neuromuscular junction, ethanol enhances nAcChoR-mediated cation flux and promotes desensitization [10–13].

The molecular mechanism of action of ethanol on different biomembrane systems has been addressed in several EPR studies. Goldstein and Chin [14] examined the membrane effects of ethanol at concentrations ranging from 0.02 to 0.35 M by spin labeling erythrocyte, synaptosomal plasma and mitochondrial membranes from mice with 5-doxyl stearic acid (5-SASL). A decrease in order parameter, reflecting an increase in membrane 'fluidity', was observed in each case. Subsequent studies [15,16] addressed the effect of ethanol as a function of temperature, depth of spin label probe and membrane cholesterol content. The addition of 0.35 M ethanol in mouse synaptosomal and brain membranes resulted in a disordering effect which increased linearly with temperature between 10 and 28°C. Surprisingly, however, the disordering effect of ethanol was larger in the interior of the membrane as monitored by 12- and 16-SASL rather than near the surface of the membrane. Membrane fluidity was also examined as a function of temperature. It was shown that at higher temperatures the effects of ethanol and temperature were similar but not identical. Miller et al. [12] examined alcohol effects on the order parameter of acetylcholine receptor-rich membranes labeled with 12-SASL and found that the ethanol concentration required to desensitize half of the receptors changes the membrane order parameter by 4.4%. However, these studies did not examine the effect of ethanol on protein motions or on the lipid–protein interactions which are important determinants of protein function.

In this study, we compare the action of ethanol on protein side chain motion and lipid–protein interactions of the nAcChoR. This was accomplished by covalently labeling the receptor with a maleimide spin probe. We chose to label the receptor in alkaline extracted native membranes in which the nAcChoR is the major protein and retains the ability to undergo state transitions. Using nAcChoR reconstituted into DOPC, we also tested the hypothesis that ethanol alters interactions between the nAcChoR and spin labeled representatives of three classes of lipids: phosphatidylcholines, fatty acids, and steroids. A reconstituted system was used since it allows the lipid : protein ratio to be controlled, giving rise to EPR spectra where the two spectral components – annular and bulk – can be well resolved. A more accurate assessment of ethanol's effect on the quantity, shape and rotational mobility of each component can thus be obtained.

2. Materials and methods

2.1. Chemicals

Affi-Gel 401 was obtained from Bio-Rad (Richmond, CA). Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidylcholine spin label (14-PCSL) and 14-doxyl stearic acid (14-SASL) were generous gifts from Professor Tony Watts. Androstanol and MSL were obtained from Aldrich (Milwaukee, WI). Anhydrous ethanol ($\geq$ 99.9% purity) was from Pharmco (Dayton, NJ). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Buffer A consists of 10 mM Mops (pH 7.4), 100 mM NaCl, 0.1 mM EDTA and 0.02% sodium azide [17].

2.2. Methods

2.2.1. Preparation of nAcChoR-rich membranes

Postsynaptic membranes from freshly dissected electroplaque tissue of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared using differential and sucrose density gradient centrifugation as described in [18]. A typical membrane preparation contained 5–10 mg protein/ml (as assessed by the method of Lowry et al. [19]). Membranes were kept frozen at −80°C until used.

2.2.2. Alkaline extracted nAcChoR-rich membranes

Alkaline extraction of the above AcChoR-enriched membranes was carried out by diluting 8 mg protein to 0.5 mg/ml in 10% sucrose and adjusting the pH to 11.0 by dropwise addition of 0.2 M NaOH. After stirring on ice for 1 h, the alkaline suspension was diluted 5-fold with distilled water and the membranes were pelleted by centrifugation at 41 000 rpm in a DuPont A-641 rotor for 1 h at 4°C. The pellet, AER, was resuspended in 16 ml (0.5 mg/ml) buffer B consisting of 250 mM NaCl, 5 mM KCl, 10 mM Mops, 0.1 mM EDTA. 0.02% azide (pH 7.4) [20].

2.2.3. Derivatization of AER membranes

Cysteines accessible to aqueous alkylation reagents were blocked with *N*-ethylmaleimide prior to spin labeling a subclass of sensitive cysteines with MSL as follows: 1.8 ml of 120 mM *N*-ethylmaleimide in acetone was added to yield a final concentration of 12 mM [21] and allowed to react for 12 h at 4°C. Excess reagent was removed by two cycles of 5-fold dilution and centrifugation at 41 000 rpm for 1 h. Cystines were reduced by treatment of the pellet with 0.2 mM DTT for 4 h and excess reagent was removed by dilution and centrifugation as above. MSL in acetone was added to the resuspended pellet (buffer B) to a final concentration of 1 mM. After 24 h, the excess reagent was

removed from MSL-AER by five dilution/centrifugation cycles.

2.2.4. Preparation and characterization of DOPC reconstituted membranes containing the nAcChoR

The receptor was reconstituted into DOPC liposomes according to the procedure of Ellena et al. [17]. Acetylcholine receptor-rich membranes were thawed on ice and dissolved in Buffer A containing 2% cholate to solubilize the nAcChoR. The mixture was centrifuged and the collected supernatant was applied to an Affi-Gel 401 affinity column derivatized with bromoacetylcholine bromide [22]. The receptor was eluted from the column with carbamylcholine chloride and fractions were assayed for protein content by measuring the absorbance at 280 nm. Protein-rich fractions were pooled and dialyzed against 2 l of Buffer A for 48 h. The reconstituted receptor was stored at −80°C.

The protein content of the reconstituted vesicles was determined using BSA as standard [19]. The phospholipid content was measured by the method of McClare [23].

Polyacrylamide gel electrophoresis under denaturing conditions was performed to verify the identity and purity of the receptor [24].

2.2.5. Spin labeling of liposomes

An ethanolic stock solution of spin label was added to the desired lipid in chloroform at a lipid to spin-label molar ratio of 100. The lipids were dried under a stream of nitrogen and then placed under 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.

2.2.6. Preparation of spin labeled vesicles

Reconstituted vesicles were spin labeled with 14-PCSL by adding an ethanolic stock solution of the label to a membrane suspension containing 1–3 mg of nAcChoR and stirring overnight at 4°C. The molar ratio of 14-PCSL : lipid was less than 1 : 100. The final ratio of ethanol to buffer was always less than 1 : 1000 (v/v). The receptor was pelleted by centrifugation and washed three times with Buffer A to remove unincorporated spin label. The final pellet was transferred into a capillary tube for spectroscopic studies. In the case of androstanol and stearic acid spin labels, aliquots of the label were deposited as a thin film from a chloroform solution and vacuum dried. The membrane suspension was added to the label and processed as above for 14-PCSL.

Ethanol, in the desired concentration, was added to the capillary tube as described by Fraser et al. [25]. Final ethanolic concentrations were determined by a Hewlett-Packard 5890 Gas Chromatograph equipped with a J & W DB-5 column and an integrator (Hewlett Packard 3393A). The free aqueous concentration is reported throughout this study.

2.2.7. Conventional EPR spectroscopy and analysis

Spectroscopic measurements were performed with a Bruker ER200 EPR spectrometer interfaced with an IBM 9000 computer and a variable temperature unit. Temperature was measured to ±0.5°C by placing a thermocouple just above the cavity. Spectra were obtained using the following acquisition parameters: microwave frequency, 9.5 GHz; microwave power, 10 mW; modulation amplitude, 0.2 mT; modulation frequency, 100 kHz, and spectral width of 12 mT. Spectral subtraction and deconvolution were performed as described in Abadji et al. [26]. We estimate that the error in the calculation of the fraction of motionally restricted component is ±0.02 [27].

Effective rotational correlation times, τ_r, for the spin labels motionally restricted by the nicotinic receptor were calculated by two different methods:

(1) From their outer hyperfine splittings assuming a Brownian diffusion model for isotropic motions [28]. The relationship between A'_Z, half the 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 and the rotational correlation time is given by

$$\tau_r = a(1 - A'_Z/A^r_Z)^b \quad (1)$$

where $A_Z = 3.36$ mT and a and b are $5.4 \cdot 10^{-10}$ s and -1.36, respectively, based on simulations of Freed [29].

(2) Their low- and high-field half-height widths, still employing the Brownian diffusion model, according to the relationship

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

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. For the low-field peak $a' = 1.15 \cdot 10^{-8}$ s, $b' = -0.943$ and $\Delta^r_l = 0.24$ mT and for the high-field peak $a' = 2.12 \cdot 10^{-8}$ s, $b' = -0.778$ and $\Delta^r_h = 0.27$ mT [29].

2.2.8. Saturation transfer EPR spectroscopy

ST-EPR spectra were recorded on a Varian E109 EPR spectrometer equipped with a Medical Advances XP-0201 loop-gap resonator and the signal was amplified by a Miteq X-band field effect transistor installed in the E-102 bridge as described by Hubbell et al. [30].

Data were digitized using an A/D card from Real Time Devices (State College, PA) and interface hardware and software from Scientific Software Services (Bloomington, IL). Temperature was regulated (to ± 0.1°C) using the Varian E-257 assembly with the loop-gap resonator positioned inside a 'Q-band' size glass dewar. Temperature was confirmed with an Omega Engineering (Stamford, CT) Model 412B digital temperature indicator.

The effective microwave field intensity ($B_{1\text{eff}}$) in the resonator was calibrated by measuring the line broadening of a reference sample consisting of 0.9 mM potassium nitrosodisulfonate in 20 mM potassium carbonate [31]. The modulation field amplitude was calibrated at each frequency (100 kHz for conventional V_1 EPR and 50 kHz for V_2' ST-EPR) by over-modulated broadening of the same reference sample [32]. Conventional EPR spectra were recorded with a subsaturating microwave power of 50 μW, equivalent to an effective microwave field of 3.32 μT, incident upon the resonator and with a modulation amplitude of 0.1 mT. V_1 EPR spectra were also recorded at higher power levels, corresponding to $B_{1\text{eff}}$ of 25 μT, to measure the fractional loss of integrated signal intensity [33,34].

Several complementary methods were used for measurement of correlation times of strongly and weakly immobilized nitroxides in composite spectra. Correlation times for the strongly immobilized subset of labels were determined from the integrated signal intensity 'sigma' parameter calibration curves [33]. The integrated intensity of the subsaturated EPR spectrum, normalized for receiver gain and effective microwave field amplitude, was used as the reference for comparison of normalized intensity of the partially saturated V_1 spectrum. Sigma is numerically evaluated as the ratio of $\{[\int\int V_1(B_{1\text{eff}} = 3\ \mu\text{T})/3]/[\int\int V_1(B_{1\text{eff}} = 25\ \mu\text{T})/25]\} - 1.0$. An alternative method for enhancing sensitivity to the slow-motion component is analysis of V_2' ST-EPR spectra. ST-EPR spectra were recorded at 3 mW incident power ($B_{1\text{eff}}$ of 25 μT) and with applied modulation amplitude of 0.5 mT at 50 kHz with phase quadrature signal detection at 100 kHz [35]. ST-EPR amplitude ratio parameters were measured as specified by Squier and Thomas [33] with a one-milliTesla interval between the field positions L'' and L and H'' and H (Fig. 1B). Correlation times were calculated by comparison with the calibration plots of Squier and Thomas [33] and from the polynomial fits of ratio parameter data to correlation time described by Horvath and Marsh [36]. The reproducibility of the amplitude ratio parameter measurement is about $\pm 10\%$ [33], which propagates to an uncertainty in correlation times of a factor of two in the optimal range of sensitivity (near 10 μs) for a single-component spectrum. Since the mole fraction of weakly immobilized nitroxides has been shown to influence the calculated result for the strongly immobilized labels [33], the fraction of weakly immobilized nitroxides was estimated from the ratio of amplitudes of the low-field weakly immobilized signal to the strongly immobilized signal in the conventional EPR spectrum. This ratio, along with the intermediate motion correlation time calculated from the empirical relationship of Likhtenshtein [37], was used to estimate the composition of labeled membranes by comparison with calculations given by Squier and Thomas [33].

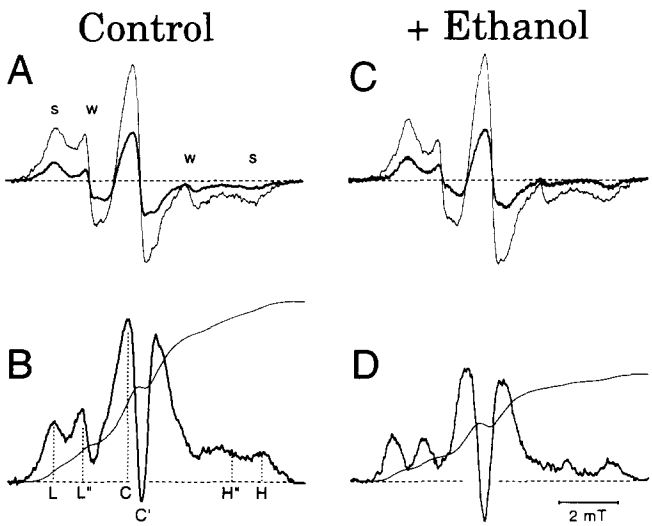

Fig. 1. EPR and ST-EPR spectra of maleimide spin labeled Ac-ChoR-enriched membranes without (A, B) and with 1.6 M ethanol (C, D). EPR spectra (A, C), recorded at the same receiver gain and modulation amplitude (0.1 mT) are scaled so that their low power second integrals are equivalent. The heavy line corresponds to the conventional V_1 spectrum at low incident power ($B_{1\text{eff}} = 3$ μT) and the thin line corresponds to the partially saturated V_1 spectrum at $B_{1\text{eff}} = 25$ μT. The V_2' saturation transfer EPR spectra (B, D), recorded at high power ($B_{1\text{eff}} = 25$ μT) and modulation amplitude 0.5 mT, are normalized according to the relative spin concentrations in each sample, i.e., the double integral of the low power V_1 spectra A and C. The first integral of V_2', a measure of correlation time, is superimposed on each ST-EPR spectrum. The sweep width is 10 mT for all spectra.

3. Results

3.1. Covalently labeled receptors

The maleimide spin label derivatized several sites on the electroplax membrane which resulted in both strong and weak immobilization of the label. The major fraction of labels (approx. 95%) was associated with strongly immobilized sites, as is shown in the V_1 EPR spectra (Fig. 1A and C). This level of contamination in the labeling was consistent with the polyacrylamide gel analysis which showed there to be no peripheral 43 K protein and only a small amount of low molecular weight (< 30 K) species ($\sim$ 10–15% of dye intensity).

Maleimide labels strongly immobilized by nearby protein side chains are manifest as broad resonances in the low- and high-field wings of the EPR spectrum, while the weakly immobilized nitroxides that possess flexibility independent from the protein matrix have narrower resonance lines positioned inward toward the central region (lower hyperfine splitting) due to motional averaging. The hyperfine splitting of the strongly immobilized component, 6.76 mT, is near the rigid-lattice limit of sensitivity of V_1 EPR spectra. The hyperfine splitting of the weakly immobilized component, 3.42 mT, and the differential narrowing of low- and high-field hyperfine components ($m_I = +1$ and -1, respectively) indicates motional averaging in the intermediate motion regime, wherein the rotational correlation time can be determined from line amplitudes and widths [37].

Since the strongly immobilized labels are characterized by correlation times longer than the V_1 EPR rigid lattice limit, the application of saturation transfer methods is merited. However, the well-known method for extraction of correlation times for reorientation of the nitroxide about one radian by analysis of ST-EPR spectra in terms of spectral amplitude ratios [38] could not be applied as a singularly reliable method due to the overlap of signals from the weakly immobilized component in the L'', C, C' and H'' regions of the ST-EPR spectra (Fig. 1B and D). The application of several complementary spectral analysis strategies to compare MSL-AE-AcChoR in buffer A (control) and buffer A supplemented with 1.6 M ethanol (Table 1) was necessary. The L'' and H'' positions reside in spectral regions subject to overlap of strongly and weakly immobilized labels, but the high-field region is less affected by the overlap problem than the low-field reigion, so τ for wobble is calculated from H''/H and reported in Table 1. Although the apparent τ for libration calculated from the C'/C ratio is not a quantitative measure of protein motion due to contributions to the V_2' lineshape from the weakly immobilized components, it is nonetheless useful for comparison of the effect of ethanol upon the labeled electroplax preparation. The decrease in $\tau(C'/C)$ from 3 to 1 μs upon ethanol addition to buffer A, does not appear to be an artifact of spectral overlap since the relative fraction of weakly immobilized component does not change with ethanol.

Two supplementary methods of analysis that discriminate between the contributions from strongly and weakly immobilized nitroxides, namely, measurement of the loss of integrated intensity from the V_1 EPR spectrum upon saturation, and measurement of the gain in intensity in the V_2' ST-EPR spectrum, were used. The relative losses of saturation from the V_1 in-phase signal as a function of microwave field intensity, expressed as the sigma parameter (Table 1) were determined for the control and ethanol-containing membrane preparations. The correlation time derived for the control (200 μs) was twice that of the sample in ethanol/buffer. The relative increase in integrated intensity of the V_2' signal mirrored the loss of intensity of the V_1 signal. The relative intensity of the V_2' signal in the presence of ethanol was 80% that of the control, consistent with a 2-fold decrease in correlation time.

Table 1
Correlation times of strongly and weakly immobilized labels on MSL-derivatized alkaline-extracted AcChoR (units given for each method)

Method of spectral analysis	MSL-derivitized alkaline-extracted AcChoR	
	Control	1.6 M ETOH
V_1 EPR WI line height ratio [a]	3.93	3.69
τ fast motion component	0.25 ns	0.21 ns
V_1 EPR SI sigma [b]	0.96	0.83
τ slow motion	200 μs	100 μs
V_1' ST-EPR integral [c]	100%	80%
V_2' ST-EPR H''/H [d]	0.95	0.59
τ slow motion wobble	100 μs	20 μs
V_2' ST-EPR C'/C [e]	−0.13	−0.35
τ slow motion libration	3 μs	1 μs

[a] Correlation time of weakly immobilized (WI) subset of labels was calculated from linewidths and amplitudes of the $m_I \pm 1$ lines using empirical formulae of Likhtenshtein [37]. In this case, we have used the relationship: $\tau = \{[(h_{(+1)}/h_{(-1)})^{1/2} - 1][\Delta H_{(+1)}]\}/1.2E + 10$ where $h_{(+1)}$ and $h_{(-1)}$ are the amplitudes of the low- and high-field weakly immobilized lines and $\Delta H_{(+1)}$ is the width (in Gauss) of the low-field line.

[b] The sigma parameter is a measure of fractional integrated intensity lost from the partially saturated V_1EPR signal relative to the intensity at subsaturated $B_{1\text{eff}}$. Sigma is differentially sensitive to the strongly immobilized (SI) component. It is defined as $\{[\int\int V_1(B_{1\text{eff}} = 3\ \mu\text{T})/3]/[\int\int V_1(B_{1\text{eff}} = 25\ \mu\text{T})/25]\} - 1.0$. The calibration curve for σ vs. τ (Squier and Thomas [33]) was used to obtain the correlation time. $\sigma = 1.2$ is the rigid limit value for this parameter, where $\tau > 5$ ms.

[c] The integral of the V_2' signal, shown in Figs. 1B, D is selectively sensitive to the strongly immobilized component(s) of the mixture of nitroxides on MSL-AE-AcChoR, because the lineshape of weakly immobilized components sums to a value approaching zero. The values of $\int V_2'$ normalized by the spin concentration ($\int\int V_1$ at 3 μT) are below the MSL-hemoglobin calibration curve reported by Squier and Thomas [33], so the relative values are reported for MSL-AE-AcChoR. The difference between our values of $\int V_2'/\int\int V_1$ and those of Squier and Thomas may derive from our use of the loop-gap resonator contrasted with their use of a cavity resonator which operates at higher power.

[d] The positions for determination of V_2' spectral amplitudes H and H'' were at the high-field turning point and 1 mT downfield, as specified by Squier and Thomas [33]. H''/H was chosen for estimation of apparent correlation time of the strongly immobilized component(s) because the overlap with resonances attributable to weakly immobilized nitroxides was less in this region of the spectrum than in the low-field region.

[e] The apparent correlation time derived from the C'/C amplitude ratio provides a qualitative description of differences in nitroxide motion of MSL-AE-AcChoR in the two media, buffer A and A+EtOH. The central region of the V_2' spectrum contains overlapping resonances from both strongly and weakly immobilized labels.

Calculation of correlation times for the weakly immobilized component was straightforward since the high-field and low-field lines were adequately separated in field position from the broader strongly immobilized resonance lines in the wings. Correlation times obtained from the ratio of amplitudes of the high-field and low-field lines according to Likhtenshtein [37] were comparable for the control (0.25 ns) and ethanol-containing (0.21 ns) preparations.

3.2. Reconstituted vesicles

3.2.1. Effect of ethanol on the lipid / protein interface

The nAcChoR was reconstituted in DOPC vesicles and labeled with three different kinds of spin labels: 14-SASL, a fatty acid spin label; androstanol, a spin labeled cholesterol analog; and 14-PCSL, a phospholipid spin label. EPR spectra were recorded at 0°C in the absence or presence of ethanol. The spectra of all three spin labels incorporated in vesicles containing the nAcChoR revealed two motionally distinct lipid environments. One environment corresponded to motionally restricted lipids directly adjacent to the receptor (annular region). The other environment was more mobile and corresponded to lipids more distant from the receptor (bulk region). In order to assess the effect of ethanol on the individual lipid components, spectra were deconvoluted by subtracting the spectrum of spin labeled DOPC liposomes from that of the reconstituted receptor labeled with the same spin label [17]. The resulting motionally restricted components were quantitated by double integration as previously described [26]. The mobility of the derived components was assessed by measuring the separation and the line widths of the low- and high-field peaks (Table 2) and calculating the rotational correlation time using Eqs. (1) and (2).

Spectra of reconstituted vesicles (nAcChoR : DOPC 1 : 198) spin labeled with 14-SASL are shown in Fig. 2 in the absence (A) and presence (D) of 0.9 M ethanol.

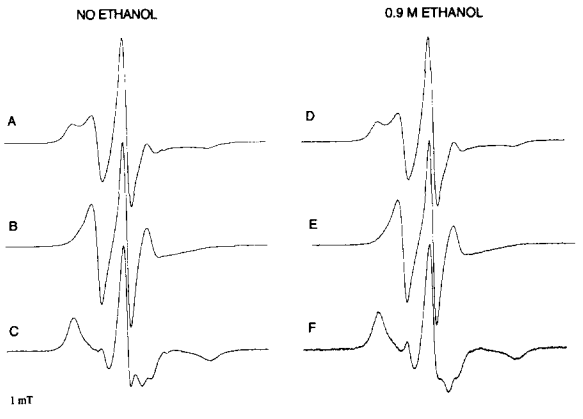

Fig. 2. EPR spectra of 14-SASL in reconstituted vesicles DOPC/nAcChoR 198:1 (A and D), DOPC vesicles (B and E) and the resulting motionally restricted components (C and F) in the absence and presenece of 0.9 M ethanol. The protein associated components were obtained by subtracting spectra B from A and E from D. Spectra were recorded at 0°C and normalized to the same central line height. In the deconvoluted spectra C and F the narrow lines on either side of the central peak arise from difficulties in exactly matching the central peak linewidth in the parent spectra. These artifacts contribute a negligible amount to the total intensity [27].

From each composite spectrum, the spectrum of the corresponding DOPC liposome, recorded in the absence and presence of 0.9 M ethanol was subtracted. Double integration revealed the fraction of the motionally restricted components obtained in each case to be 0.54 and 0.57, respectively. The outer hyperfine splitting of 6.22 mT (no ethanol) and 6.26 mT (0.9 M ethanol), is typical of a spin label in a motionally highly restricted environment (Table 1). Thus, ethanol did not significantly change either the relative fraction of protein associated component or its outer hyperfine splitting.

Similar trends were observed when spectra of the reconstituted vesicles spin labeled with the steroid analog, androstanol, were recorded in the absence and presence of 0.9 M ethanol. Fig. 3 shows the spectra of

Table 2
Effect of ethanol on EPR lineshape features and calculated rotational correlation times for various motionally restricted spin-labeled lipids in nAcChoR reconstituted in DOPC

Spin-label (Lipid/Protein) (mole : mole)	[Ethanol] (M)	f_b	$2A_{max}$ (mT)	Low-field half-height linewidth (mT)	High-field half-height linewidth (mT)	τ_r calculated from $2A_{max}$ (ns)	τ_r calculated from low-field half-height (ns)	τ_r calculated from high-field half-height (ns)
14-PCSL	0	0.50	6.05	0.75	1.15	12	19	19
(115 : 1)	0.4	0.48	5.99	0.82	1.55	11	16	16
	0.9	0.45	6.11	0.72	1.16	14	22	22
	1.6	0.45	6.09	0.76	1.00	14	19	24
14-SASL	0	0.54	6.22	0.69	0.94	18	25	27
(198 : 1)	0.9	0.57	6.26	0.69	0.92	21	27	28
ASL	0	0.58	6.33	0.67	0.85	26	27	33
(198 : 1)	0.9	0.58	6.33	0.68	0.85	26	26	33

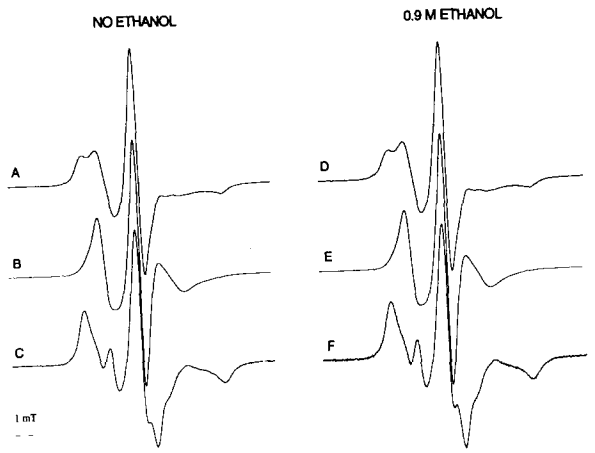

Fig. 3. EPR spectra of ASL in reconstituted vesicles DOPC/nAcChoR 198:1 (A and D), DOPC vesicles (B and E) and the resulting motionally restricted components (C and F) in the absence and presence of 0.9 M ethanol. The protein associated components were obtained by subtracting spectra B from A and E from D. Spectra were recorded at 0°C and normalized to the same central line height.

composite (A), fluid model (B) and deconvoluted motionally restricted (C) components. Addition of ethanol to the sample had no effect on the fraction of the highly immobilized protein perturbed component, since it remains 0.58. There was virtually no change in the outer hyperfine splitting of the protein associated spectral component or any detectable effect in the low-field or high-field half-height linewidths (Table 2).

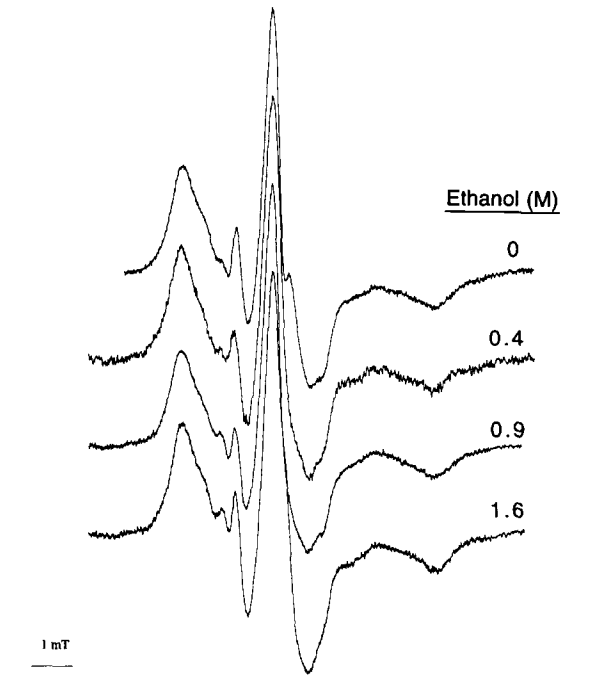

Fig. 4. Effect of increasing concentrations of ethanol on the motionally restricted component derived by subtraction of the spectrum of DOPC liposomes labeled with 14-PCSL from reconstituted vesicles containing DOPC/nAcChoR 115:1 spin labeled with 14-PCSL. Spectra were recorded 0°C and normalized to the same central line height.

The phosphatidylcholine spin label, 14-PCSL, has been shown to have a lower affinity for the nAcChoR than 14-SASL and ASL and to be exchanging on the EPR timescale [17,27]. Fig. 4 shows the deconvoluted protein associated spectral components obtained by subtracting DOPC liposomes containing 2% cholesterol and spin labeled with 14-PCSL from reconstituted vesicles containing the nAcChoR at ethanol concentrations ranging from 0 to 1.6 M. We have found that DOPC containing 2% cholesterol better emulates the mobile component in DOPC/nAcChoR membranes [26]. While a slight trend might be inferred from the data presented in Table 1 for the fraction of motionally restricted 14-PCSL, it is fairly small and within the error of the subtraction technique. In fact, no change in the fraction of motionally restricted 14-PCSL is expected since this spin label has the same headgroup as DOPC [26]. No systematic change in the maximum hyperfine splitting, the high and low-field peak lineshape and the derived rotational correlation times was observed for 14-PCSL (Table 2).

4. Discussion

4.1. Protein dynamics

We have labeled the acetylcholine receptor in the electroplax membrane with MSL at a subclass of sites consisting of disulfide bridges amenable to reduction with mild reagents. Since our objective was to compare ethanol-induced changes in the receptor structure compared to those in the lipid bilayer, it was more important to ensure that all labels were on the nAcChoR than to selectively label a single site on the nAcChoR. Therefore, we elected to strip the 43 kDa peripheral membrane proteins by alkaline extraction prior to labeling. Our labeling protocol followed the concept of procedure II of Rousselet et al. [20], i.e., blocking of accessible cysteines with *N*-ethylmaleimide (NEM) and mild reduction of disulfide bridges, followed by derivatization with MSL which then labels the alpha subunit and 43 kDa peripheral proteins almost exclusively. About half the alpha subunit labels in their study were at the agonist site (cysteine residues 192 and 193). We made a number of improvements. First, we stripped the peripheral proteins by alkali extraction before, rather than after, labeling. Second, the labeling procedure itself was modified based on the thorough study of Mosckovitz and Gershoni [21], which emphasizes the particular sensitivity to reduction of the cysteine residues 192 and 193 on the alpha subunits. Therefore, we modified Rousselet et al.'s [20] procedure in order to improve the specificity of labeling,

namely we used a 12-fold higher concentration of NEM for better protection of free —SH and one-fifth of their concentration of reducing agent to open the disulfides more selectively. Third, and of equal importance, was the addition of EDTA to all buffers to minimize proteolysis. With our derivatization protocol, we achieved EPR results similar to those reported by Rousselet et al. [20], i.e., the labels resided in sites of strong and weak immobilization on the receptor. The correlation time of the strongly immobilized subset of labels in our preparation (100 μs) was comparable to that reported by Rousselet et al. (100–120 μs).

That our preparation of spin-labeled AER contains labels at more than one site enables us to compare ethanol-induced changes on different parts of the nAcChoR, specifically the ligand binding site of the receptor with other uncharacterized sites. In the derivatized receptor, it is likely that most receptors exist in an inactive conformation because the Cys192-Cys193 disulfide domain is critical for maintaining capability to bind ligands [39].

In the presence of 1.6 M ethanol, the correlation time of the strongly immobilized fraction of labels decreases 2–3-fold. The magnitude of this decrease is consistent with ethanol facilitating side-chain motion on the electroplax proteins, but inconsistent with major alterations in protein structure or quaternary organization. Since an appreciable fraction of MSL is inferred to reside in or near the ligand binding site on the alpha subunit of nAcChoR, a small increase in protein tertiary structural flexibility may reflect a relaxation of the contour of the acetylcholine binding pocket. Confirmation of this tentative conclusion will require much more detailed work, but the consequences of such a small alteration could be manifest as (1) a change in rate of binding of acetylcholine to the receptor or (2) a change in occupancy of ligand binding sites which might alter the distribution of receptor conformational states. Ethanol has been shown to enhance the occupancy of acetylcholine sites [40], with a plateau being achieved in the range of 1.0 to 1.2 M ethanol.

The weakly immobilized nitroxides are essentially unaffected by the presence of ethanol. The calculated correlation times of 0.25 and 0.21 ns for control and ethanol-containing preparations are within the range of error typical for this method. Nor does ethanol induce a major interconversion of labels between stongly and weakly immobilized sites, since the fraction of EPR signal intensity associated with the strongly immobilized labels is similar in both control (95%) and ethanol-containing (96%) preparations (Fig. 1A and C).

The decrease in correlation time of strongly immobilized labels on the electroplax membrane calculated by various methods for the ethanol-containing samples (Table 1) is consistent for all methods. The 2–3-fold increase in the overall nitroxide label mobility reflects a small perturbation of local protein structure near the labeled sites in the presence of ethanol.

In our system, complexities arising from the inevitable overlap of weakly and strongly immobilized components prevent more detailed interpretation. For example, the strongly immobilized components might consist of an ensemble of nitroxides characterized by slightly different slow reorientational motion rates. Squier and Thomas [33] have examined the calculated correlation times (using the methods listed in Table 2) for two-component systems over a range of mole fractions of fast and slow motion components and they have concluded that the mole fraction of the fast motion component influences the calculated result for the slow motion component. In our work, there is no change in the mole fraction of the fast component. This strongly suggests that the decrease in correlation times in the presence of ethanol, calculated by several complementary methods, are genuine.

4.2. Lipid–protein interactions

Previous studies of the effect of ethanol on membranes treated the bilayer as a homogeneous environment. They failed to distinguish between the differences in the fraction and mobility of the two lipid environments that are evident in our EPR spectra, i.e. the bulk phospholipid bilayer and the annular lipid. In order to assess the effect of ethanol on the two distinct lipid environments, we spin labeled vesicles containing the nAcChoR reconstituted at high protein/lipid ratio.

4.2.1. Comparison of the spin labels

Spectral subtraction revealed that the order of selectivity of the spin labels used in this study was ASL $\geq$ 14-SASL > 14-PCSL, consistent with previous reports [17,27]. The affinities (relative to DOPC) of each label can be calculated from the following equation [41]:

$$y = \frac{x}{NK_{AV}} - \frac{1}{K_{AV}}$$

where y = fraction mobile/fraction motionally restricted, x = lipid/protein molar ratio, N = number of annular lipids, and K_{av} = the affinity of the spin label relative to 14-PCSL for the nAcChoR. Assuming that there are approximatey 56 annular lipids/receptor, the relative affinities of ASL, 14-SASL, and 14-PCSL for the nAcChoR are 3.5, 3.0 and 1.1, respectively. For the range of values for N reported in the literature, these affinities vary by $\pm$0.2 [17,26]. Thus the affinity of 14-PCSL in DOPC is not significantly different from 1.0.

The outer hyperfine splittings of the motionally restricted components of all three spin labels were also

measured. In the case of 14-SASL and ASL the outer hyperfine splittings were larger than that of 14-PCSL (6.24 and 6.33 mT, respectively, versus 6.06 mT) perhaps reflecting the higher affinty of 14-SASL and ASL for the receptor. The $2A_{max}$ values obtained in this study are similar to those reported elsewhere for the nAcChoR reconstituted into DOPC: Ellena et al. [17] measured a $2A_{max}$ of 6.16 mT G for 16-SASL in reconstituted vesicles containing the nAcChoR; Raines and Miller [27] report a value of 6.28 mT for 14-SASL and 6.05 mT for 14-PCSL.

The exchange rate of lipid between protein-perturbed and mobile environments seems to be slow on the EPR timescale in the case of 14-SASL and ASL, since we could successfully simulate the mobile component in the composite spectra of the nAcChoR reconstituted in DOPC with pure DOPC vesicles at the same temperature. However, in the case of 14-PCSL, 2% cholesterol must be added to the DOPC vesicles to allow reasonable simulation of the exchange broadened bulk component [26,27].

Rotational correlation times for the protein-associated components were estimated for each spin label using the values of the outer hyperfine splitting and the low-field and high-field half-height linewidths for our calculations. The motionally restricted components had rotational correlation times in the range of 10–30 ns, an order of magnitude slower than that of pure lipid bilayers. In general, the two high affinity spin labels, ASL and 14-SASL, had larger outer hyperfine splittings, smaller low-field and high-field linewidths and hence slower estimated rotational correlation times than 14-PCSL (Table 2).

4.2.2. Comparison of the spin labels in the presence of ethanol

A priori, ethanol might be expected to change the affinity of ASL or SASL relative to PCSL either allosterically by altering the conformation of the receptor, or sterically by directly competing with lipids for the 50 annular sites on the receptor. Alternatively, ethanol could exert effects on the headgroup region of the lipid bilayer thus altering selectivity [42]. However, we observed that the addition of ethanol had no effect on the order of selectivity for the three spin probes used, implying that either the lipid/protein interface is inaccessible to ethanol or that the alcohol is unable to displace any of these lipids.

The effect of ethanol on the outer hyperfine splitting of the motionally restricted component was assessed for each spin probe. There was some variation in the $2A_{max}$ and half-height linewidths of 14-PCSL as the ethanol concentration was varied from 0 to 1.6 M. However, a trend with ethanol concentration was not observed. In the cases of 14-SASL and ASL these parameters remained virtually unaltered with ethanol. There is a larger variation between the $2A_{max}$ values obtained for the different spin probes than for a given spin probe in the presence versus the absence of ethanol.

4.3. Conclusion

Ethanol increases agonist-induced ion flux and desensitization in the nAcChoR over the concentration range studied here [13]. However, ethanol changes neither the fraction of motionally restricted lipid nor its rotational correlation time as measured with the spin label probes 14-SASL, 14-PCSL, and ASL indicating that the lipid/protein interface, at least deep in the bilayer, is quite insensitive to the presence of ethanol. Conversely, ethanol decreases the rotational correlation time of the strongly immobilized component in covalently labeled receptor as measured by a variety of EPR methods. These results are consistent with the hypothesis that ethanol-induced alterations in nAcChoR function result either from a direct interaction between ethanol and the receptor or a disruption of lipid–protein interactions in the lipid headgroup, but not in the acyl chain region of the lipid/protein interface.

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