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A Study of the Effect of General Anesthetics on Lipid–Protein Interactions in Acetylcholine Receptor Enriched Membranes from *Torpedo nobiliana* Using Nitroxide Spin-Labels†

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Received June 14, 1989; Revised Manuscript Received September 29, 1989

ABSTRACT: Stearic acid, phosphatidylcholine, and phosphatidylglycerol nitroxide spin-labels were used to probe the effect of 1-hexanol, urethane, diethyl ether, and ethanol on lipid–protein interactions in nicotinic acetylcholine receptor (nAcChoR) rich membranes from *Torpedo nobiliana*. For stearic acid spin-labeled at the C-14 position of the *sn*-1 acyl chain, 1-hexanol induced little change (over a wide concentration range, 0–16.7 mM) in either the ESR line shape or the proportion of motionally restricted spectral component from labels probing the protein interface. The main effect of 1-hexanol was limited to an increase in the mobility of stearic acid spin-labels probing the non-protein-associated environment. In contrast, for C-14 phosphatidylcholine spin-label, 1-hexanol decreased the fraction of spin-labels motionally restricted at the protein interface from 0.33 without 1-hexanol to 0.20 with 16.7 mM 1-hexanol, with no change in the line shape of the spectral component of these labels. The ESR spectral line shape of the fluid component due to phosphatidylcholine labels in sites away from the protein interface displayed a gradual decrease in spectral anisotropy on addition of increasing amounts of 1-hexanol. At a concentration of 1-hexanol that desensitizes half the receptors, the fraction of motionally restricted phosphatidylcholine spin-label is reduced by approximately 15%. The effect of 1-hexanol on phosphatidylglycerol spin-labels was intermediate between these two cases. Similar effects were measured with other general anesthetics, including urethane, diethyl ether, and ethanol. Thus, action at the lipid–protein interface by general anesthetics provides a plausible alternative molecular mechanism for desensitization of the nAcChoR, and possibly general anesthesia, but does not preclude some synergistic lipid disordering by anesthetics of the surrounding bilayer.

Despite much investigation, there is no consensus on the molecular mechanism of anesthesia. The wide variety of chemical substances that may produce anesthesia points to nonspecific sites able to accommodate molecules of quite different molecular architectures. It is established that the anesthetic potency of a chemical species correlates with its octanol–water partition coefficient (Franks & Lieb, 1978) and that general anesthesia can be reversed by high pressure (Lever et al., 1971). Theories diverge along two apparently incompatible lines, one suggesting that lipids of neuronal membranes are the prime site of anesthetic action and the other proposing that anesthetic action arises from direct anesthetic–protein interactions (Franks & Lieb, 1982; Miller, 1985). An alternative that has attracted little experimental attention, except in model systems (Galla & Trudell, 1981), is that the size of action is at the lipid–protein interface of excitable channels in neuronal membranes (Trudell et al., 1973; Lee, 1976; Heidmann et al., 1983; Elliot & Haydon, 1989). However, in a system containing both lipids and proteins, it is far more difficult to deduce whether the conformation of a protein is affected either by a direct anesthetic–protein interaction at the bilayer–protein interface or by motional or conformational modification of the lipids themselves which exist at the lipid–protein interface.

† D.M.F. is the recipient of a Medical Research Council research studentship. S.R.W.L. is supported in part by the Brazilian agency CAPES. L.I.H. thanks the Science and Engineering Research Council for a traveling fellowship (GR/E 89070). The research was supported in part by a grant to K.W.M. (NIAAA 07040) and by grants to A.W. (SERC GR/D 69846; from the E.P.A. Cephalosporin Fund).

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0006-2960/90/0429-2664$02.50/0

Although the central sites of general anesthetic action remain ill-defined, general anesthetics are known to act on postsynaptic ion channels including that of the nicotinic acetylcholine receptor. In particular, there is a strong correlation between the ability to cause anesthesia and the ability to desensitize the nAcChoR[1] (Miller et al., 1986; Gage & Hamill, 1981), which is an exceptionally well-characterized integral membrane protein (Changeux et al., 1984; Hucho, 1986), and it has been suggested that alcohols may act at the lipid–protein interface of this receptor (Heidmann et al., 1983); diethyl ether, also a general anesthetic, has been shown to affect the lipid–protein interactions of an integral membrane protein, the Ca^{2+}-ATPase of sarcoplasmic reticulum (Bigelow & Thomas, 1987).

Electron spin resonance methods using nitroxide-labeled lipids have been used to probe lipid–protein interactions in a number of protein-containing membranes (Marsh & Watts, 1982, 1988) including nAcChoR-rich membranes from *Torpedo* electroplax, as used in this study (Marsh & Barrantes, 1978; Marsh et al., 1981; Rousselet et al., 1979). Integral membrane proteins restrict the motion of lipids at their hydrophobic boundary with the lipid bilayer. This restriction can be quantified by diffusible spin-labeled lipid probes, which have shown the presence of a proportion of the membrane lipids with slow motion on the nitroxide hyperfine anisotropy averaging time scale ($\tau_R > 3$ ns) (Marsh & Barrantes, 1978; Marsh et al., 1981; Ellena et al., 1983). In general, the exchange rate (τ_{ex}^{-1}) for lipid labels between the two motionally distinct environments is slow ($<10^7$ s^{-1}) on the ESR anisotropy averaging time scale (Marsh & Watts, 1988). For the nAcChoR in enriched membranes, fatty acids, and androstane, spin-labels have been shown to partition preferentially to the lipid–receptor interface compared to phospholipid spin-labels (Marsh & Watts, 1982; Jones & McNamee, 1988); this has also been shown for the nAcChoR in reconstituted systems (Ellena et al., 1983).

Materials and Methods

Preparation of Membranes. Nicotinic acetylcholine receptor (nAcChoR) rich membranes were obtained from the electric organ of *Torpedo nobiliana* by differential and sucrose density gradient centrifugation according to procedures adapted from Cohen et al. (1972). The buffer used was Ca^{2+}-free *Torpedo* Ringer (TR), pH 7.4. Membranes, at a total protein concentration of 10 mg·mL^{-1} and specific activity of 1.2 μmol of nAcChoR·(g of protein)$^{-1}$, were stored at −70 °C in 0.3-mL aliquots.

Lipids were extracted from nAcChoR-rich membranes into $CHCl_3$–MeOH (2:1 v/v) by a Folch–Lees procedure, and insoluble debris was removed by filtering through a glass wool plug in a Pasteur pipet followed by bench centrifugation. The organic phase was back-washed by shaking with an equal volume of buffer, the lipids being retained in the organic phase. The extraction was repeated twice more, and the lipids were dried and stored at −20 °C. Typically, 5 mg of total lipid extract was obtained from membranes containing 10 mg of protein.

Spin-Label Preparation and Spin-Labeling of Membranes. The stearic acid, phosphatidylcholine, and phosphatidylglycerol spin-labels (14-SASL, 14-PCSL, and 14-PGSL, respectively) were synthesized as described (Marsh & Watts, 1982). Vesicles, either those obtained from *Torpedo* electroplax as described above or dispersions of extracted lipid, were labeled as described (Watts et al., 1979; Marsh et al., 1982) at a label to endogenous lipid ratio of 1:100 (mol/mol) under the assumption that the protein/lipid ratio in nAcChoR-rich membranes is approximately 1:1 (w/w) (Leibel et al., 1987). Unless otherwise stated, all labeling operations were carried out at 4 °C. Upon thawing, all vesicle suspensions were washed twice in TR (5000*g*, 20 min). For 14-SASL, a dried film of label was gently shaken with a membrane suspension for some minutes at room temperature, left to stand at this temperature for 15 min, and then stored at 4 °C overnight to allow full partitioning of spin-label. The membranes were pelleted by centrifugation (5000*g*, 20 min) and then resuspended in TR buffer. For labeling with 14-PCSL or 14-PGSL, a solution of spin-label in ethanol (6 mg·mL^{-1}) was injected into a dilute buffered membrane suspension at a buffer/ethanol ratio of greater than 1000:1 (v/v) as previously described (Watts et al., 1979; Marsh et al., 1982). In all cases, the vesicles were pelleted and washed three times to remove unincorporated spin-label and ethanol (40000*g*, 10 min); the vesicles were then resuspended in 1 mL of TR buffer.

Addition of Anesthetics. Anesthetics, from concentrated anesthetic solutions in TR buffer, were added to a set of 200-μL samples made from a larger spin-labeled stock solution (10 mg/mL in protein) to give the desired final concentration; the capped samples were vigorously mixed and incubated (1 h, R. T.; then 8 h, 4 °C). Immediately prior to each ESR measurement, 100-μL aliquots were transferred to 100-μL capillaries in which the membranes were repelleted (8000*g*, 10 min). The capillaries were then flame-sealed. Following the recording of an ESR spectrum of certain samples, the concentrations of 1-hexanol and diethyl ether in the supernatant in the capillary above the pelleted vesicles were determined by gas chromatography. Concentrations quoted are those in the buffer.

Recording of ESR Spectra and Spectral Manipulation. ESR measurements were carried out at 0 and 7 °C on a Bruker ESP300 spectrometer and with ESP1600 software for data accumulation. A modulation amplitude of 0.07 mT and microwave power of 20 mW were used. The spectral width of all spectra was 10 mT (100 G). The digitized spectra were converted to ASCII format and transferred to a Research Machines (Oxford) NIMBUS AX computer on which software for performing spectral simulations and subtractions was implemented.

In several cases, a proportion of underlying spin-exchange broadened spectrum was observed from membranes labeled with 14-PCSL or 14-PGSL, indicating that some unincorporated spin-label aggregates were present in the dispersions (Marsh & Watts, 1982). The ESR spectra of pure phospholipid spin-label aggregates in buffer were subtracted away from the ESR spectra of 14-PCSL- and 14-PGSL-labeled membranes until the high- and low-field base lines were collinear; the resulting spectra were then renormalized. This contribution was estimated to be less than 8% of the total spectral intensity for 14-PCSL and 14-PGSL.

Calculation of Order Parameter. The effective order parameter S_{eff} was calculated according to Gaffney (1976) from

$$S_{eff} = \{(A_{\parallel} - A_{\perp})/[A_{zz} - (A_{xx} + A_{yy})/2]\}a_0'/a_0$$

where $2A_{\parallel}$ and $2A_{min}$ are obtained from the outer hyperfine splitting and inner hyperfine splitting, respectively, with $A_{\perp} = A_{min} + 1.4\{1 - (A_{\parallel} - A_{min})/[A_{zz} - (A_{xx} + A_{yy})/2]\}$. Label

[1] Abbreviations: ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; nAcChoR, nicotinic acetylcholine receptor; 14-SASL, 14-(4,4-dimethyl-*N*-oxyoxazolidine)stearic acid; 14-PCSL and 14-PGSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxyoxazolidine)stearoyl]-*sn*-glycero-3-phosphocholine and -phosphoglycerol, respectively; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; L/P ratio, lipid to protein mole ratio; TR, *Torpedo* Ringer's buffer.

polarity was normalized from a_0'/a_0, which was calculated with $a_0' = (1/3)(A_{zz} + A_{xx} + A_{yy})$ and $a_0 = (1/3)(A_{\parallel} + 2A_{\perp})$. Values for A_{zz}, A_{xx}, and A_{yy} were taken from Griffiths and Jost (1976).

Simulation of ESR Spectra. Spectral simulations of two-component spectra from spin-labeled membranes were achieved with exchange-coupled Bloch equations as described elsewhere for similar systems (Horváth et al., 1988). First, the component line shapes were derived by spectral subtractions; a spectrum from a dispersion of extracted *Torpedo* electroplax lipid provided the line shape for the fluid component corresponding to the bulk lipid of the nAcChoR-rich membranes. The motionally restricted line shape was obtained by either subtraction of pure lipid reference spectra or intersubtraction between two-component spectra of different proportions (Marsh & Watts, 1982). These component spectra were then simulated by setting the **g** and **A** tensor values and the linewidths with the modified Bloch equations (Davoust & Devaux, 1982; Horváth et al., 1988). Second, the fraction of the motionally restricted component (f_b) and the off-rate (τ_b^{-1}) of labels were varied in the two-component simulation until the simulated spectrum most closely matched the experimental spectrum. Best fitting was aided by least-squares minimization of residuals between simulated and experimental spectra. The estimated error in the fraction of restricted label is of the order of ± 0.01 and that for the exchange rate is $\pm 1.5.10^6$ s^{-1}.

Results

Two-Component Spectra of Spin-Labeled Lipids in Torpedo Electroplax Membranes. Spin-labeled lipids display two-component spectra in electroplax membranes due to the rather different motional properties of lipids in the bulk fluid membrane (f) and at the solvation shell sites (b) at the protein interface. Spectral subtractions, as performed previously (Marsh & Barrantes, 1978; Ellena et al., 1983), or intersubtractions in the case of urethane (see below) can be used to obtain the line shapes of the two spectral components. All experiments were conducted at 0–7 °C since this temperature range gave the best resolution of the two components and is close to the physiological temperature for *Torpedo*. The spectra recorded in dispersions of extracted lipids for all the labels studied matched closely the fluid, narrower line shape from labeled electroplax membranes. To obtain the best subtraction end points of the broad component, fluid lipid spectra were recorded at up to 5 °C lower than corresponding membrane spectra, as found in earlier work (see superimposed spectra in Figure 1d) (Marsh & Watts, 1988). This subtraction strategy was used for all labels and anesthetics and has been shown to emulate the line-broadening effects of chemical exchange to give consistent values for the proportions of the two spectral components (Horváth et al., 1988).

The line shape of the motionally restricted component (spectrum c in Figure 1) may also be obtained by intersubtracting two spectra (Knowles et al., 1981), one with and one without anesthetic (spectra a and b, respectively, in Figure 1), when the line shape of either component does not significantly change upon addition of anesthetic. This was only possible in the case for urethane, which did not change the order of lipid spin-labels (and thus spectral shape) for the bulk lipid or motionally restricted spectral component (see below). Quantitation of the two spectral components obtained conventionally by subtracting the appropriate spectrum recorded in dispersions of extracted lipids (spectra not shown) gave very similar values (within 5%) of f_b, the fraction of motionally restricted labels at the protein interface (spectra not shown), to those obtained by intersubtraction methods. From the

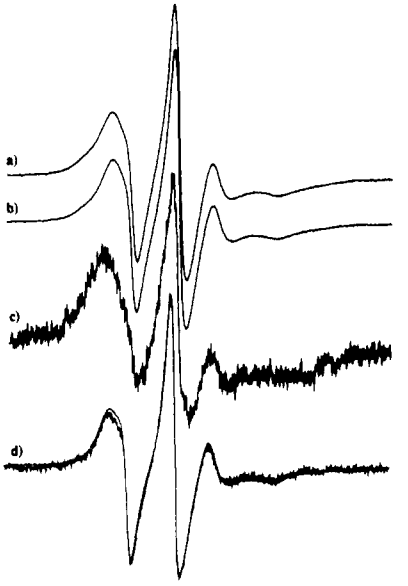

FIGURE 1: Intersubtraction end points obtained from the ESR spectra of 14-PCSL in nAcChoR-enriched membranes (a) and in a sample with 0.33 M urethane (b) both at 0 °C. The slow motion spectrum (c) typical for motionally restricted lipids at the nAcChoR interface was obtained by subtracting 88% of the intensity of spectrum a from spectrum b. The motionally averaged fluid spectrum (d), typical for bulk lipids, was obtained by subtracting 84% of the intensity of spectrum b from spectrum a; this fluid end-point spectrum is superimposed on the ESR spectrum of 14-PCSL at −2 °C in dispersions of the total lipid extract (dashed line) from nAcChoR-enriched membranes. Total scan width, 10 mT.

slow-motion spectra of motionally restricted spin-labeled lipids derived by either subtraction method, effective correlation times (τ_r) for the acyl chain at the protein interface can be estimated from the incipient collapse of spectral anisotropy due to motional averaging (Freed, 1976); with the assumption that the acyl chains undergo Brownian motion, $\tau_R \sim a(1 - S)^b$, where $a = 5.4 \times 10^{-10}$ s, $b = -1.36$, and $S = A_{max}/A_{zz}^R$, where A_{max} and A_{zz}^R are the outer spectral hyperfine splittings corresponding to the motionally restricted component and its rigid limit, respectively. The effective correlation times, τ_r, for 14-SASL, 14-PGSL, and 14-PCSL were ~30, ~7, and ~7 ns, respectively, and were not significantly altered by anesthetics at any concentration measured.

Effect of 1-Hexanol on ESR Spectra of 14-PCSL, 14-PGSL, and 14-SASL in Torpedo Electroplax Membranes. The effect of up to 16.7 mM 1-hexanol on the ESR spectra of 14-PCSL, 14-PGSL, and 14-SASL in suspensions of nAcChoR-rich membranes was studied. Representative experimental spectra (full lines) recorded with these spin-labels are given in pairs (Figure 2) which corresponds to controls in the absence of anesthetic (spectra a, c, and e) and samples treated with 16.7 mM 1-hexanol (spectra b, d, and f). An apparent decrease in the spectral anisotropy of the fluid component reflects an increase in the mobility of spin-labels away from the protein interface on addition of 1-hexanol, and therefore, lipid reference spectra recorded at higher temperatures were used in these subtractions in order to allow for the decreasing molecular order.

In the case of 14-SASL, spectral subtractions and simulations show that 1-hexanol causes a small change (from $f_b = 0.44$ to $f_b = 0.40$) in the proportions of the fluid and restricted labels (spectra e and f in Figure 2), whereas in the case of 14-PCSL a much greater change in the proportions of the two components (spectra a and b in Figure 2) was obtained (from $f_b = 0.33$ to $f_b = 0.20$) over the concentration range for 1-hexanol used, 0–16.7 mM. For 14-PGSL a moderate change

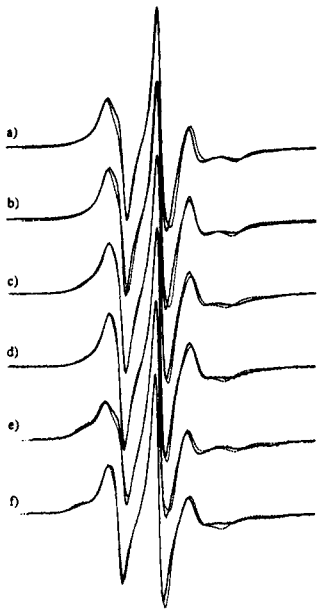

FIGURE 2: Representative ESR spectrum of various spin-labeled lipids in nAcChoR-rich membranes given in pairs corresponding to controls (a, c, and e) and samples treated with 16.7 mM 1-hexanol (b, d, and f) at 7 °C for the three spin-labels examined: (a and b) 14-PCSL; (c and d) 14-PGSL; (e and f) 14-SASL. The experimental spectra (full lines) are overlaid with the best-fitting simulated (dashed lines) spectra, which have been computed from the exchange-coupled, modified Bloch equations (see Materials and Methods). Total scan width, 10 mT.

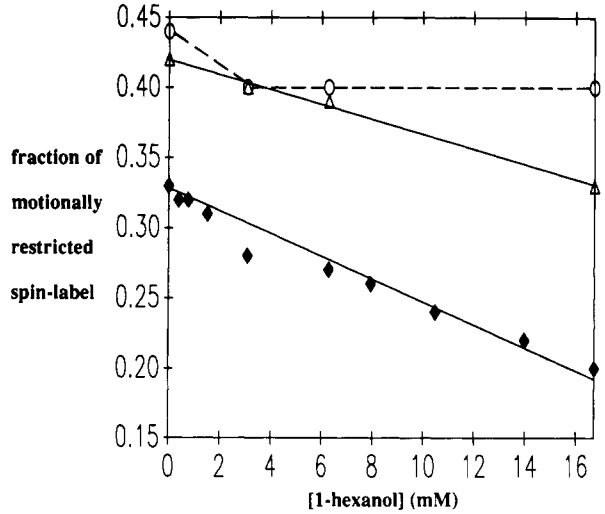

FIGURE 3: Fraction of motionally restricted, spin-labeled lipid determined at various concentrations of added 1-hexanol, as derived from computer simulations of experimental spectra from 14-SASL (○), 14-PCSL (♦), and 14-PGSL (△) probing nAcChoR-enriched membranes at 7 °C.

in the proportion of motionally restricted labels was obtained (spectra c and d in Figure 2) with added 1-hexanol which was intermediate (from f_b = 0.42 to f_b = 0.33) between that of 14-SASL and 14-PCSL. The results obtained by spectral subtractions and simulations are summarized in Figure 3 where a close-to-linear dependence of f_b for all spin-labeled lipids used is observed in the range of 0–16.7 mM 1-hexanol (see Discussion).

Effect of 1-Hexanol on Lipid Exchange Dynamics at the Protein Interface. The changes in ESR line shape caused by exchange of lipids between fluid and motionally restricted membrane sites (Davoust & Devaux, 1982) were simulated and compared with the experimental spectra. Sample best-fitting simulated spectra are displayed in Figure 2 (dashed lines), superimposed on their respective experimental spectra. The off-rates (τ_b^{-1}) of the different spin-labels from the motionally restricted environment to the bulk fluid phase increased upon addition of 1-hexanol, which is consistent with the changing proportions of spin-labeled lipids in the two motionally distinct environments (Horváth et al., 1988) as shown in Table I.

Table I: Relative Fractional Changes in Motionally Restricted Spin-Labels ($\Delta f_b/f_b$) at the Lipid–Protein Interface of nAcChoR-Rich Membranes and Off-Rates (τ^{-1}) for Spin-Labeled Lipids from the Protein Interface for Acetylcholine Binding to nAcChoR in Receptor-Rich Membranes from *T. nobiliana*[a]

spin-label	$\Delta f_b/f_b$[b]	$\Delta f_b/f_b$[c]	$(\Delta f_b/f_b)_c$ (M^{-1})[c,d]	τ_b^{-1} $(10^6\ s^{-1})$[e]
(a) 14-SASL				
control	0			8
hexanol	0	−0.02	−1.40	8
(b) 14-PGSL				
control	0			12
hexanol	−0.09	−0.21	−12.80	15
(c) 14-PCSL				
control	0			18
hexanol	−0.15	−0.35	−23.60	27
urethane	−0.09	−0.23	−0.69	25
diethyl ether	−0.14	−0.21	−2.11	26
ethanol	−0.03	−0.08	−0.09	22

[a] Determined by spectral subtractions and simulations, in the presence of 1-hexanol, urethane, diethyl ether, and ethanol at approximately half-desensitizing ($\sim EC_{50}$) and at greater than desensitizing ($>EC_{100}$) concentrations. All data correspond to measurements at 0 or 7 °C (see text). [b] $\Delta f_b/f_b$, relative fractional change in the proportion of motionally restricted spin-labels (f_b) determined as described under Results at an anesthetic concentration (Miller et al., 1987) sufficient to cause ~50% desensitization of the nAcChoR: 6 mM 1-hexanol, 165 mM urethane, 48 mM diethyl ether, and 450 mM ethanol. Value for f_b without added lipophile is 0.44 for 14-SASL, 0.42 for 14-PGSL, and 0.33 for 14-PCSL (from Figure 3). [c] As for footnote *b* but at anesthetic concentrations (Miller et al., 1987) sufficient to cause >100% desensitization of the nAcChoR: 16.7 mM hexanol, 330 mM urethane, 99 mM diethyl ether, and 850 mM ethanol. [d] $(\Delta f_b/f_b)_c$, $\Delta f_b/f_b$ (as defined in footnote *c*) extrapolated to a concentration of 1 M for each lipophile. [e] τ_b^{-1}, off-rate constant for spin-labels at the protein interface as deduced from computer simulation of experimental spectra (see text) at $>EC_{100}$ concentrations for the lipophiles tested.

Comparison of Effects of 1-Hexanol with Urethane, Diethyl Ether, and Ethanol. The effect of 1-hexanol was compared with those of urethane, diethyl ether, and ethanol, which are also known to desensitive the nAcChoR, in order to compare their actions in receptor-rich membranes. For these experiments 14-PCSL was chosen because it was this spin-label which had the least specificity toward the protein interface and hence was the most readily displaced from the interface by lipophilic molecules (Figures 2 and 3). Unlike with 1-hexanol, no major line-shape changes were observed in the fluid component on addition of these other general anesthetics (spectra not shown, see later). The results of spectral subtractions obtained for greater than full ($>EC_{100}$) desensitizing and approximately half desensitizing ($\sim EC_{50}$) concentrations of the general anesthetics (Firestone et al., 1986b; Miller et al., 1987) indicate significant reductions in the fraction of motionally restricted lipid by the nAcChoR at the protein–lipid interface. The changes in the fractional proportions ($\Delta f_b/f_b$) are summarized in Table I together with the off-rates (τ_b^{-1}) for lipid spin-labels from the nAcChoR interface.

Two-site chemical exchange simulations provide a more detailed insight into the dynamic aspects of lipid and anesthetic competition at the interface of nAcChoR molecule. All these molecules, like 1-hexanol (see above), are in free exchange between the protein interface and the bulk membrane (Table

I), and on addition of anesthetics in concentrations used for desensitization of the nAcChoR, the off-rates (τ_b^{-1}) gradually increase in agreement with the decreasing values of f_b for 14-PCSL and 14-PGSL at the protein interface.

Effect of 1-Hexanol, Urethane, Diethyl Ether, and Ethanol on Order of Lipids in Torpedo Electroplax Membranes. The effective order parameter (S_{eff}) was derived for the fluid spectral component of the experimental spectra for each label in membranes obtained after subtraction of the broad motionally restricted component (for example, Figure 1d). In all cases, the order parameter changed approximately linearly with 1-hexanol concentration from 0 to 16.7 mM. The rate of change of order parameter with 1-hexanol concentration was obtained by a linear least-squares fit. In membranes, the values of −30, −60, and −7 ($\Delta S_{eff}/S_{eff}$, M^{-1}) for 14-PCSL, 14-SASL, and 14-PGSL, respectively, were measured. The value for 14-SASL is similar to that determined previously for 12-SASL (Firestone et al., 1986a). For urethane, little relative change was detected in lipid order by 14-PCSL (which allowed intersubtraction methods to be used as described above), and the other lipophiles produced intermediate order changes.

Discussion

The general anesthetic 1-hexanol was shown to modify the interactions of three spin-labeled lipids, 14-SASL, 14-PCSL and 14-PGSL, with the nAcChoR in *Torpedo* electroplax membranes. Both spectral simulation and subtraction methods suggest that, for each of the labels studied, the motionally restricted and bulk spectral components were affected in different ways by the alcohol. For each probe the fraction (f_b) of spin-labels that were motionally restricted by their interaction with protein decreased with increasing 1-hexanol concentration. However, there was an order of magnitude difference in the change in f_b over the concentration range studied between the probes which were most (14-PCSL) and least (14-SASL) sensitive to displacement from the nAcChoR interface with the bilayer. For the 14-PCSL, which could be readily displaced from the protein interface, a significant reduction in f_b was observed at low aqueous concentrations of 1-hexanol between 0.8 and 1.5 mM, and the population of interacting sites was halved by an extrapolated aqueous concentration of about 21 mM 1-hexanol. The actual concentration of 1-hexanol in the bilayer was not determined but may be estimated by its partition coefficient in red cell ghosts, which is 13 [for a review of partition coefficients of anesthetics, see Firestone et al. (1986a)]. The decrease in f_b was largely linear with hexanol concentration, but a slight curvilinearity was apparent, suggesting the rate of change in $\Delta f_b/f_b$ with concentration is greater at lower concentrations that at higher concentrations. The average change, over the whole concentration range, in this parameter is 24 per molar 1-hexanol. The 14-PGSL showed a similar sensitivity of 13 per molar 1-hexanol whereas the 14-SASL was much less sensitive with a value of 1–2 per molar 1-hexanol. Because of its low sensitivity to hexanol, 14-SASL was not studied further, but the nonlinearity of the decrease in f_b with increasing hexanol concentration suggests some heterogeneity in SASL binding sites at the nAcChoR interface, as previously suggested (Jones & McNamee, 1988), perhaps involving interstitial sites.

The action of 1-hexanol on the bulk, mobile component of the spectra was also dissimilar for all three spin-labels. Order parameters decreased approximately linearly with increasing 1-hexanol concentration in the order 14-SASL > 14-PCSL > 14-PGSL in the membrane possibly suggesting that 14-SASL is a more sensitive probe of bulk phospholipid bilayer perturbations than the structurally more analogous phospholipid spin-labels.

Within the limits of the methods, both simulations and subtractions reveal no change in the motional properties or dynamics of interfacial lipid in any situation studied. Bigelow and Thomas (1987) investigated the action of diethyl ether, also a general anesthetic, on the dynamics of spin-labeled lipids and $Ca^{2+}Mg^{2+}$-ATPase of sarcoplasmic reticulum (SR). Ether, in up to 10-fold higher concentration (1 M) than we use here (100 mM), increased the mobility of spin-labeled lipids at the lipid–protein interface to a greater extent than the mobility of labels in the bulk lipids away from the protein. However, in the SR system, no reduction in the fraction of motionally restricted labels at the protein interface was observed. Since nitroxides with slow motion ($\tau_c \sim 10^{-7}$ s) are relatively insensitive to small motional changes (Freed, 1976) by conventional ESR detection, it may be that any motional changes that do occur are not detected. In addition, the aggregation states of the nAcChoR and $Ca^{2+}Mg^{2+}$-ATPase are probably different and may, in the case of the receptor, be altered by lipophilic molecules, thereby altering the lipid/protein stoichiometries; increased aggregation decreases f_b (Watts, 1987).

Previous studies have been interpreted to show a correlation between the lipid disordering effects of general anesthetics and their ability to stabilize the desensitized state of the acetylcholine receptor (Miller et al., 1986). Although similar changes in lipid order can be induced by removing some cholesterol from the membrane, these are not accompanied by enhanced desensitization (Leibel et al., 1987) suggesting that bulk lipid disordering may not in itself be sufficient to enhance receptor desensitization. This may, therefore, imply some role for the lipid–protein interface in functional modulation of the receptor, in addition to bulk lipid disordering, as suggested by two observations.

Firstly, enhancement of desensitization by 1-hexanol has been studied quantitatively at 4 °C, and an increase in the fraction of receptors that preexist in the desensitized state in the absence of agonist is first observed on going from 1 to 4 mM 1-hexanol (Miller et al., 1987). This enhancement has a steep concentration dependence with half-desensitization occurring at 6 mM and complete desensitization occurring at ~10 mM. Therefore, desensitization and reduction in the fraction (f_b) of motionally restricted 14-PCSL (Figure 3) are both initially observed over the same concentration range of 1-hexanol, and in addition, f_b for 14-PCSL is reduced by approximately 15% at a concentration of 1-hexanol (16.7 mM) which causes full desensitization. Comparable changes occur with 14-PGSL but not with 14-SASL. Because of its intermediate behavior, the anionic phospholipid phosphatidylglycerol could occupy both desensitization sites and cationic sites which themselves are not involved in maintaining the resting receptor states. It has been previously speculated that anionic phospholipids are necessary to support elements of the secondary structure of the receptor which are related to ion channel function (Fong & McNamee, 1987).

Secondly, we examined three other general anesthetics to determine if the reduction of motionally restricted 14-PCSL was constant at concentrations which caused similar degrees of receptor desensitization. There is some difficulty in this approach since the concentrations that cause half-desensitization vary considerably depending on the assay procedure used and the conditions of the experiments, leading to an uncertainty by about a factor of 2 in the half-desensitizing concentration. For example for ethanol, estimates vary from

320 mM at 20 °C (Firestone et al., 1986b) to 700 mM at 4 °C (Miller et al., 1987). However, in Table I, the results of analyzing representative spectra determined at concentrations which cause approximately half-desensitization ($\sim EC_{50}$) or complete desensitization ($\sim EC_{100}$) are given. Although the parameter $(\Delta f_b/f_b)_c$ (Table I) for the four anesthetics varies about 300-fold (and increases with the lipophilicity of the reagent), when compared at the concentration of each anesthetic that causes an equal degree of desensitization, the variability is reduced to no greater than 5-fold, which may be limited considerably by experimental uncertainties. Thus, it appears that general anesthetic induced desensitization may be accompanied by significant and consistent changes at the lipid–protein interface. However, changes in the aggregation state of the nAcChoR upon addition of lipophiles might also produce spectral changes similar to those presented here and is a possibility that awaits further experiment.

Although there is no evidence defining the central sites of general anesthetic action, there are nicotinic acetylcholine receptors in the central nervous system. In addition, the *Torpedo* nAcChoR is one of a superfamily of receptors that includes the GABA and glycine receptors. Thus, our results may have more general implications. The constraints imposed by nitroxide ESR spectral deconvolution required that our experiments were performed at low temperatures where there is little available general anesthetic data. Consequently, the hypothesis that general anesthesia per se arises from changes in the lipid–protein interactions of excitable membranes cannot be immediately tested. However, for ethanol, the concentration required for EC_{50} for general anesthesia at 10 °C is 340 mM (Miller et al., 1987), close to the concentration and temperature used here, suggesting that significant changes may occur at the lipid–protein interface during anesthesia. Synergistically induced lipid disordering may also be a prerequisite of receptor desensitization by general anesthetics, although spin-label studies reported here showed no such effect was seen with urethane which did, however, displace labels from the lipid–protein interface.

Acknowledgments

We thank J. Rowntree (Department of Biochemistry, Oxford University) for improving the computer program used for spectral manipulations other than simulation, P. Fisher for spin-label synthesis, and B. Bugge for performing GC analysis.

Registry No. 1-Hexanol, 111-27-3; urethane, 51-79-6; diethyl ether, 60-29-7; ethanol, 64-17-5.

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