Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–569.
Shapiro, H. M. (1981) *Cytometry 1*, 301–312.
Vassilev, P., Kanazirska, M., & Ti Tien, H. (1985) *Biochem. Biophys. Res. Commun. 126*, 559–565.
Waggoner, A. S. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 47–68.
Wehland, J., Willingham, M. C., Gallo, M. G., & Pastan, I. (1982) *Cell (Cambridge, Mass.) 28*, 831–841.
Whitfield, C., Abraham, I., Ascherman, D., & Gottesman, M. M. (1986) *Mol. Cell. Biol. 6*, 1422–1429.
Wilson, H. A., Seligman, B. E., & Chused, T. M. (1985) *J. Cell. Physiol. 125*, 61–71.

Binding of Local Anesthetics to Reconstituted Acetylcholine Receptors: Effect of Protein Surface Potential†

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Received February 11, 1986; Revised Manuscript Received May 16, 1986

ABSTRACT: Nicotinic acetylcholine receptor isolated from *Torpedo californica* electric organ is reconstituted into lipid bilayers of zwitterionic dioleoylphosphatidylcholine. These membranes are labeled with a spin-labeled quaternary amine local anesthetic (C6SLMeI), which has been shown previously to be a noncompetitive blocker of acetylcholine receptor–ion channel function in the micromolar concentration range. The electron spin resonance spectral component corresponding to protein-immobilized anesthetic spin-label can be resolved from the composite data spectrum by using spectral subtraction of lipid components. This protein-immobilized component is shown to represent C6SLMeI bound to a finite number of sites on the receptor. We demonstrate that C6SLMeI binds to the receptor as a function of the surface potential on the protein and suggest that the acetylcholine receptor reconstituted into zwitterionic phospholipid, which has no surface potential of its own, provides an excellent model system with which to study effects of protein surface charge. We hypothesize that the primary pathway of interaction of C6SLMeI with the acetylcholine receptor is via the aqueous medium.

Amphiphilic tertiary amine local anesthetics such as procaine, lidocaine, and tetracaine are known to interact hydrophobically with membrane lipids (Skou, 1954; Lee, 1977). On the other hand, there is much evidence that local anesthetics interact directly with a variety of membrane proteins. Yeh demonstrated a difference in sodium channel blocking potency between two isomeric forms of a local anesthetic in squid giant axon (Yeh, 1980). Hille (1977) demonstrated that there are both hydrophilic and hydrophobic pathways of interaction of local anesthetics with the sodium channel, suggesting that lateral diffusion to the protein from the lipid phase may not be required for pharmacological effect. The interactions of local anesthetics with sodium channels and acetylcholine receptors (AChR)[1] are similar in that the positively charged form is pharmacologically active at micromolar concentrations (Aracava et al., 1984; Blickenstaff and Wang, 1985) and are different in that the location of the pharmacologically relevant binding site(s) is (are) intracellular for the sodium channel and extracellular for the AChR (Aracava et al., 1984; Aracava & Albuquerque, 1984). Several electrophysiological studies on the acetylcholine receptor suggest a direct blockade of the ion channel by local anesthetics (Neher and Steinbach, 1978; Oswald et al., 1983; Ikeda et al., 1984), although there have been reports that some local anesthetics may block acetylcholine receptor–ion channel activity via the lipid bilayer (Koblin and Lester, 1979; Ribera et al., 1985).

Electrophysiological studies of local anesthetic binding are limited because only channel-associated activity is monitored. Biochemical approaches to determining binding sites on acetylcholine receptor membranes are difficult because of the noncovalent interactions of the local anesthetic with multiple sites on the receptor membrane. An accurate study of local anesthetic–membrane interactions in vivo must include careful characterization of the lipid domain, regardless of the location of the pharmacological local anesthetic binding site. The use of a spin-labeled local anesthetic and electron spin resonance spectroscopy (ESR) allows us to observe more directly the drug's interactions with membrane components.

Local anesthetic binding to membrane components is examined in a reconstituted membrane containing acetylcholine receptor (RAChR membrane). The defined composition of the RAChR membrane allows unambiguous identification of membrane components with which spin-labeled local anesthetic populations are associated. The use of the zwitterionic phospholipid dioleoylphosphatidylcholine permits examination

† This work was supported in part by NIH Grant GM28087 and a Faculty Research Grant from the University of California, Santa Cruz.
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[1] Abbreviations: AChR, acetylcholine receptor; carb, carbamylcholine; C6SL and C6SLMeI, tertiary and quaternary amine local anesthetic spin-labels, respectively (see Figure 1); C6MeI, quaternary amine local anesthetic without spin-label; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ESR, electron spin resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NCB, noncompetitive blocker; PCP, phencyclidine; PMSF, phenylmethanesulfonyl fluoride; RAChR, reconstituted acetylcholine receptor; SDS, sodium dodecyl sulfate; TC, *d*-tubocurarine; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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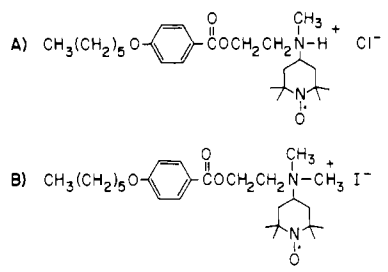


FIGURE 1: Chemical structures of spin-labeled local anesthetics: (A) tertiary amine derivative (2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxy)]ethyl *p*-hexyloxybenzoate), abbreviated C6SL; (B) methylated quaternary analogue, abbreviated C6SLMeI.

of effects of surface potential on the AChR without the interference of a surface potential on the lipid bilayer.

The spin-labeled local anesthetics used in this study are shown in Figure 1. These compounds, synthesized in our laboratory (Gargiolo et al., 1973), have been shown to be potent local anesthetics: they exhibit a frequency-dependent block of the sodium channel in squid giant axon (Wang et al., 1982), and preliminary studies indicate that they are effective inhibitors of AChR flux response in both native and reconstituted membranes (Blickenstaff & Wang, 1985). The tertiary amine, abbreviated C6SL, has a pK value of 7.2–7.4 (Wang et al., 1983).

The nitroxide reporter group of the spin-labeled local anesthetic is attached to the polar amine. This allows the ESR spectra to reflect anesthetic motion at the membrane–water interface. This is an important distinction between the anesthetic spin-labels used in this study and the acyl-chain-labeled spin-labels commonly used to examine lipid phenomena in membranes. The latter type of spin-labels typically give rise to ESR spectra with greater mobility than do the spin-labels used in this study (see Ellena et al., 1983; Brotherus et al., 1981; Griffith & Jost, 1976). By examination of the ESR spectra recorded from reconstituted acetylcholine receptor membranes labeled with tertiary and quaternary anesthetic spin-labels, we can quantify distinct populations of spin-labeled local anesthetics existing within the membrane. We can observe changes in these spin-label populations under various experimental conditions that are relevant to local anesthetic action and to acetylcholine receptor function.

Materials and Methods

Isolation and Reconstitution of the Acetylcholine Receptor. The methods of isolating and reconstituting the acetylcholine receptor are adaptations of those described in Ellena et al. (1983). All procedures were carried out at 4 °C unless noted.

(*A*) *Preparation of Receptor-Rich Membranes.* Electroplax tissue was dissected from live *Torpedo californica*. The tissue was cut into one-inch cubes, washed with dialysis buffer (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, 0.02% sodium azide, pH 7.4), and quick-frozen in liquid nitrogen. Six hundred grams of slightly thawed electroplax tissue was further chopped with a knife, then homogenized in a Waring blender (3 × 30 s) with 500 mL of homogenization buffer (10 mM $NaPO_4$, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 10 mM iodoacetamide, 0.1 mM PMSF, pH 7.5). The homogenate was centrifuged in a Sorvall GSA rotor at 5000 rpm for 10 min, and the supernatant was filtered through four layers of cheesecloth. The pellets were combined with 200 mL of homogenization buffer and homogenized with a high-speed Virtis homogenizer at a setting of 70% for 3 × 30 s. This homogenate was centrifuged at 5000 rpm for 10 min. The supernatant was filtered through four layers of cheesecloth, and the combined supernatants were centrifuged in the Sorvall GSA rotor at 12 500 rpm for 4.5 h. The membrane pellet was suspended to a volume of approximately 40 mL with dialysis buffer. The protein concentration of the membrane suspension was 15–20 mg/mL as measured by a Lowry protein assay (Lowry et al., 1951). The crude membranes were divided into 4.5-mL cryotubes and stored in liquid nitrogen.

(*B*) *Reconstitution of Purified Acetylcholine Receptors into Synthetic Lipid Bilayers.* Crude membranes derived from 600 g of electroplax tissue were thawed and diluted to a total volume of 250 mL with dialysis buffer, and 250 mL of 2% w/v sodium cholate in dialysis buffer was added for a final cholate concentration of 1% (w/v). This suspension was stirred at 4 °C for 40 min and then centrifuged in a Beckman type 50.2Ti rotor at 35 000 rpm for 40 min. The supernatant containing solubilized protein was filtered through four layers of cheesecloth and applied to an affinity column prepared from bromoacetylcholine bromide and a reactive sulfhydryl gel (AffiGel-401, BioRad). The column was prepared according to the procedures of Ellena et al. (1983). Before application of the supernatant, the column was washed with 150 mL of dialysis buffer containing 1% w/v cholate and 1 mg/mL dioleoylphosphatidylcholine (DOPC). The supernatant was applied to the column at a flow rate of 1 mL/min. The column was washed with 150 mL of dialysis buffer containing 1% w/v cholate and 1 mg/mL DOPC and then equilibrated overnight with dialysis buffer containing 1% w/v cholate and 2.5 mg/mL DOPC to allow complete exchange of protein-bound native lipids for DOPC. After two more 50-mL washes with the 2.5 mg/mL DOPC solution, the column was washed with 150 mL of dialysis buffer containing 0.1% w/v cholate and 0.1–0.4 mg/mL DOPC, depending on the desired final lipid-to-protein ratio. (All column solutions were prepared by drying DOPC in chloroform to a thin film in a round-bottomed flask with a rotary evaporator for 30 min, adding 2% w/v cholate in dialysis buffer, stirring until the film of DOPC was gone, and then adding dialysis buffer to a final cholate concentration of 1 or 0.5% w/v.) Bound acetylcholine receptor was eluted from the column by washing with dialysis buffer (using 80 mM NaCl instead of 100 mM NaCl) containing 0.5% w/v cholate, 0.1–0.4 mg/mL DOPC, and 10 mM carbamylcholine. Protein content of the eluted fractions was monitored by measuring the absorbance at 280 nm. A final adjustment of the desired lipid-to-protein ratio was made by pooling the appropriate eluate fractions. The pooled fractions were dialyzed for 48 h against 4 × 4 L dialysis buffer and then stored in liquid nitrogen. The total yield of purified receptor from 600 g of electroplax tissue was 30–60 mg at a protein concentration of 0.3–0.8 mg/mL. The column was washed with and stored in an aqueous solution of 0.5 μM physostygmine sulfate, and 0.02% sodium azide. Purity of the receptor was assayed by binding of ^{125}I-α-bungarotoxin (Andreasen & McNamee, 1977).

Preparation of Liposomes. Liposomes of DOPC were prepared by pooling the protein-free fractions of column eluate and dialyzing against 4 × 4 L dialysis buffer. This method produced small liposomes which were designed to accurately model the lipid domain of the reconstituted AChR membrane.

Preparation of Membrane Samples for ESR Spectroscopy. A suspension of reconstituted acetylcholine receptor (AChR) containing 1.5–3.0 mg of AChR (or an equivalent aliquot of DOPC liposomes) was annealed by six freeze–thaw cycles. Ionic strength and pH were adjusted before freeze–thawing to ensure symmetrical distribution of charge. An aliquot of aqueous spin-label, either C6SL or C6SLMeI, was added to

give the desired anesthetic-to-protein or anesthetic-to-lipid ratio. After a 15-min incubation on ice, the samples were centrifuged in a Sorvall SS34 rotor at 18000 rpm for 30 min. The pellet was drawn up into a 100-μL thin-walled microcapillary tube.

Competitive or noncompetitive blockers, agonists, or covalent protein modifiers were added to the membrane suspension by adding a 2-fold solution concentration of test substance in dialysis buffer to an equal volume of membrane suspension and incubating on ice for 1 h. The membranes were then treated with spin-label as above.

ESR Procedures. ESR spectra were recorded with a Varian E-3 spectrometer interfaced to a PDP 11/10 computer. The scan range was 130 g at 100-kHz modulation frequency, 10-mW power, 1-G modulation amplitude, and 0.1–s filter time constant. Six thousand data points across the 130-G scan range were sampled 300 times at each point, digitized, averaged, and stored. Shifting of x-axis registration between spectra could be done on the 6000-point file; all other spectral manipulations were done on 1000-point files, which were averaged from the 6000-point data files. The scan time for one spectrum was 6 min; multiple copies of noisy spectra were recorded and summed unless a measurement of the signal-to-noise ratio was desired. Data analysis was done by using BASIC and FORTRAN software and plotted on a Hewlett-Packard x–y plotter. The temperature of the sample was regulated at 15 °C.

To compare mobility of different ESR spectra, the spectra were normalized to a constant area under the absorbance curve. A spectrum of high mobility (i.e., having a narrow line width), therefore, will be seen as having a greater low-field peak amplitude than does a spectrum of lower mobility.

To quantify the contribution of a spectral component to the composite spectrum, the component spectrum is subtracted from the composite spectrum until a negative inflection or "glitch", attributed to over-subtraction, appears in the difference spectrum. The relative contribution of the subtrahend and the difference to the minuend spectrum can then be determined by double-integration of the spectra.

Electron Microscopy. Parlodian-coated, carbon-shadowed copper grids were floated on 15 μL of membrane suspension at 0.02 mg/mL AChR. Grids were washed with 3 drops of dialysis buffer, stained with 1% uranyl acetate, and examined by using a Phillips EM400 electron microscope at 100 kV.

Other Procedures. Phospholipid content of the reconstituted vesicles was measured by the Ames method (1966). The final lipid-to-protein molar ratio of the reconstituted membranes was determined by division of the total phosphate molar concentration minus 7 mol of phosphate per mol of receptor by the molar concentration of receptor. SDS polyacrylamide gels were prepared by a modification of the Laemmli method (1970), using urea in the gel. Influx of $^{86}Rb^+$ was measured by the method of Walker et al. (1981) but without d-tubocurarine to quench the reaction.

Results

Receptor Purity and Functional Capabilities. The reconstituted AChR membranes used in these studies were of DOPC-to-AChR molar ratios of 100–400. The lipid-to-protein molar ratio is a parameter used often in these studies: for convenience we refer to this molar ratio as "ϕ". An electron micrograph of a RAChR membrane of $\phi = 200$ is shown in Figure 2. Receptor purity was verified by using an ^{125}I-α-bungarotoxin equilibrium binding assay; specific activities ranged from 7.5–8.5 nmol of toxin sites per mg of protein, consistent with the known properties of pure AChR (Damle & Karlin, 1978). SDS gel electrophoresis of the reconstituted vesicles demonstrated the presence of four major polypeptide subunits corresponding to the α, β, γ, and δ subunits of AChR. Assaying receptor channel function in pure DOPC membranes was difficult. AChR requires acidic phospholipids and cholesterol for maximum flux assay response, and measurement of radioactive cation flux through the ion channel was limited by the internal volume of the vesicles and by the lipid-to-protein ratio of the membrane (Ochoa et al., 1983). Recent evidence suggests that the difficulty in measuring ion flux in DOPC–AChR membranes is an artifact of the method of reconstitution and does not mean that the AChR is inactive (Earnest et al., 1986). To ensure that the DOPC–AChR membranes used in the electron spin resonance studies contained functional AChR, these membranes were reextracted with a cholate–asolectin solution and then dialyzed to form vesicles of asolectin, DOPC, and AChR at a total lipid-to-protein ratio of 10000. Receptor function was then measured as carbamylcholine-stimulated uptake of $^{86}Rb^+$. These "re-reconstituted" vesicles showed a 4-fold increase in uptake of cations in the presence of carb, comparable to that of receptors which had been initially reconstituted into asolectin vesicles. Manipulations of pH and lipid composition of the RAChR membranes for these ESR studies did not irreversibly inhibit the receptor-mediated ion flux.

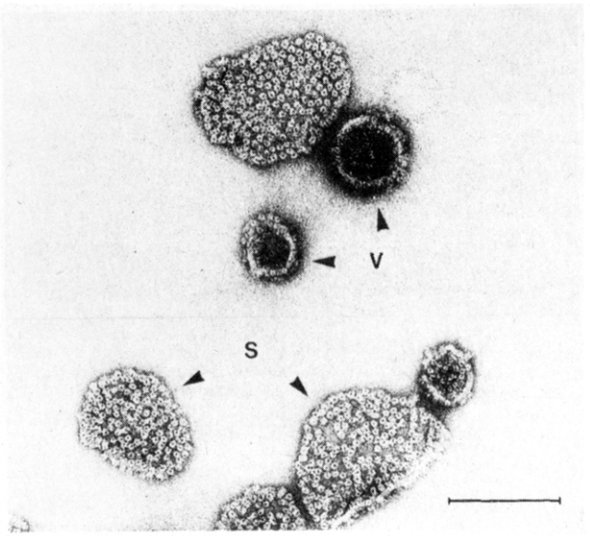


FIGURE 2: Electron micrograph of AChR reconstituted into DOPC ($\phi = 200$). Small doughnut-shaped objects (about 10 nm in diameter) represent top views of individual receptor molecules. In this sample, membranes were present as sheets (S) and as vesicles (V). Samples were stained with 1% uranyl acetate and visualized in a Philips EM-400 electron microscope. Bar = 100 nm.

Resolution of the Composite Spectra into Spectral Components. An ESR spectrum, obtained from C6SL incubated with reconstituted AChR membranes at pH 6.0, is shown in Figure 3A. The spectrum taken from the same membranes at pH 9.5 is also shown (Figure 3B). Charged C6SL at pH 6.0 results in a broad ESR spectrum, indicating immobilization of the nitroxide group of the C6SL. Neutral C6SL at pH 9.5 results in a narrow ESR spectrum, indicating a more fluid, unrestrictive environment at the nitroxide reporter group. In its positively charged form, therefore, the local anesthetic spin-label is motionally restricted by electrostatic interactions with components of the membrane. A comparison of the spectral shape of C6SL in the AChR membrane at pH 6.0 with C6SL in corresponding protein-free liposomes at pH 6.0 [spectrum not shown; see Earnest et al. (1984)] suggests that the motionally restricted component visible in the low-field

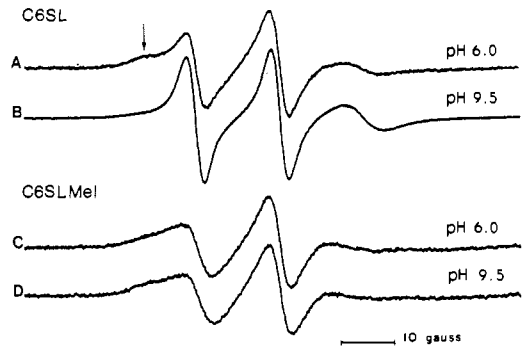


FIGURE 3: Effects of pH on spectral shape. (Top) ESR spectrum of C6SL in reconstituted DOPC–AChR membranes (ϕ = 275) at pH 6.0 (A) and pH 9.5 (B). Arrow points to protein-immobilized component. (Bottom) ESR spectrum of C6SLMeI in reconstituted DOPC–AChR membranes (ϕ = 225) at pH 6.0 (C) and pH 9.5 (D).

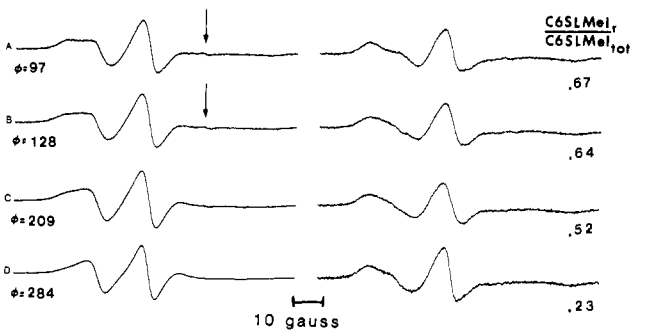


FIGURE 4: Resolution of several RAChR membrane samples labeled with C6SLMeI (left-hand column) into their corresponding protein components (right-hand column). ϕ = lipid-to-protein mole ratio; C6SLMeI$_r$/C6SLMeI$_{tot}$ = fraction of spin-label bound to AChR. Arrows point to solution spectrum components.

peak of the AChR membrane spectrum (see arrow, Figure 3A) is due to interaction of charged C6SL with the AChR, and the pH experiments above indicate that this interaction is electrostatic. It is demonstrated below that this motionally restricted population of spin-labels represents local anesthetics interacting with the AChR at a finite number of binding sites. This spectral component will therefore be referred to as the "immobile" or "protein-bound" component.

The permanently charged spin-labeled local anesthetic, C6SLMeI, gives a braod spectrum (Figure 3C) similar to the pH 6 C6SL spectrum, but the shape of the C6SLMeI spectrum is much less dependent on pH (for a discussion of why the high-pH C6SLMeI spectrum is slightly broader than the low-pH C6SLMeI spectrum, see section on salt effects below). It has been demonstrated that the positively charged spin-labeled local anesthetic is responsible for blocking carbamylcholine-stimulated cation flux through the ion channel: the approximate ED50 values for the spin-labeled local anesthetics are 1×10^{-5} M for C6SLMeI, 2.5×10^{-5} for C6SL at pH 6.5, and 1.5×10^{-4} M for C6SL at pH 8.5 (Blickenstaff, G., personal communication). Because the population of charged and uncharged C6SL are dependent upon local surface charge density, it is simpler to study the binding of the quaternary C6SLMeI to the acetylcholine receptor.

It has been shown that ESR spectra of C6SLMeI in pure DOPC membranes can be resolved into two distinct spectral populations of differing line widths (Limbacher et al., 1985). These two lipid populations are therefore represented by single-component spectra that can be subtracted sequentially from the composite spectra of C6SLMeI in AChR reconstituted into DOPC bilayers (Wang et al., 1983; Earnest et al., 1984). The difference spectrum resulting from subtracting the two lipid components from the composite membrane spectrum represents the population of C6SLMeI interacting with the AChR. When the lipid components were subtracted from the composite data spectra, the protein components from membranes of varying lipid-to-protein ratios were resolved, and their relative contribution to the composite spectrum was quantified by double-integration of the components.

The resolution of several reconstituted acetylcholine receptor (RAChR) membranes of different lipid-to-protein ratios into their corresponding protein components is shown in Figure 4. All spectra in this figure have been normalized to a constant double-integral value after resolution so that spectral shapes may be compared. As the protein concentration within the RAChR membrane increases, the low-field immobile component of the spectrum increases relative to the sharper peak attributed to the lipid component. The resolved protein components all have a similar shape, and their contribution to the corresponding unresolved spectra increases with increasing concentration of AChR.

There was another component present in several of the unresolved spectra that has been attributed to a solution spectrum component. This component has a narrow line width and slightly broader hyperfine splitting, consistent with rapidly tumbling spin-label in the aqueous medium (see arrows in Figure 4). The spectrum with the largest solution spectral component was calculated to contain only 4.6% of the entire spins in this component, so the contribution of the solution spectral component was usually ignored.

Competition for Binding Sites. Reconstituted AChR membranes treated with both dibucaine and C6SLMeI gave a narrower ESR spectrum than RAChR membranes treated with C6SLMeI alone. The spectra were resolved by subtraction of the lipid components from the composite spectra (the spectral line shapes of the lipid components were unaffected by the addition of dibucaine at these concentrations), and the contribution of the resulting protein-bound component to the composite spectrum was determined. The decrease in the protein-bound component at 2 μM dibucaine was accompanied by a corresponding increase in the less immobilized spectral components, which include both the lipid components and the solution component. These results suggest competition between dibucaine and C6SLMeI for binding site(s) on the AChR (Table I). At a molar ratio of dibucaine to C6SLMeI of 10, C6SLMeI bound to the receptor was 90.6% of the control.

A problem is encountered in experiments designed to test competition of the spin-labeled local anesthetics with other charged amphiphiles. The tertiary amine C6SL is sensitive to the membrane surface pH, so the intercalation of other charged amphiphiles into the membrane increases the surface pH and increases the population of uncharged C6SL, according to the equilibrium model proposed by Limbacher et al. (1984). This is illustrated in Figure 5A, in which an ESR spectrum of RAChR membranes with C6SL at a lipid-to-anesthetic mole ratio of 100 is compared to an ESR spectrum of the same membranes with the same mole ratio of C6SL but with the addition of 50-fold excess of the non-spin-labeled analogue of C6SL (Figure 5B). The narrowing of the spectrum is due to the increase in the proportion of uncharged C6SL which causes an increase in the population of free C6SL relative to the lipid-bound or protein-bound C6SL.

A different problem is encountered with the quaternary amine C6SLMeI. Figure 5 (parts c and d) compares an ESR spectrum of RAChR membranes with C6SLMeI at a lipid-to-anesthetic mole ratio of 164 (Figure 5C) with the same membrane which had been first treated with a 50-fold molar

Table I: Effects of Cholinergic Ligands and Noncompetitive Inhibitors Other than C6SLMeI on the Size of the Protein-Associated Component of C6SLMeI (C6)

	[C6] (μM)	[ligand] (μM)	C6[a]/AChR	ligand[a]/AChR	ligand[a]/C6	$C6_r'$[b]/C6 (control)
control	0.2		2			1.0
+non-spin-labeled C6SLMeI	0.2	0.2	2	2.0	1.0	0.71
+dibucaine	0.2	2.0	2	20.0	10.0	0.88
+PCP	0.2	10.0	2	8.0	4.0	0.79
+carb	0.2	1000.0	2	850.0	425.0	0.95
+TC	0.2	100.0	2	85.0	42.5	0.98
+α-Bgtx[c]	0.2	(excess)	2	(excess)	(excess)	0.80

[a] These values are given in molar ratios. Ligand concentrations were chosen on the basis of published values of potencies and/or partition coefficients. [b] Ratio of the AChR-bound portion of total anesthetic spin-label (C6SLMeI) in a given experiment ($C6_r'$) relative to the control experiment in the absence of other ligands ($C6_r$(control)). The AChR-bound portions are determined by resolving ESR spectra (see text). [c] α-Bungarotoxin. Moles of α-Bgtx were estimated to be around 50 per mole of AChR; given the extremely low K_d for α-Bgtx, this value is considered to be in excess.

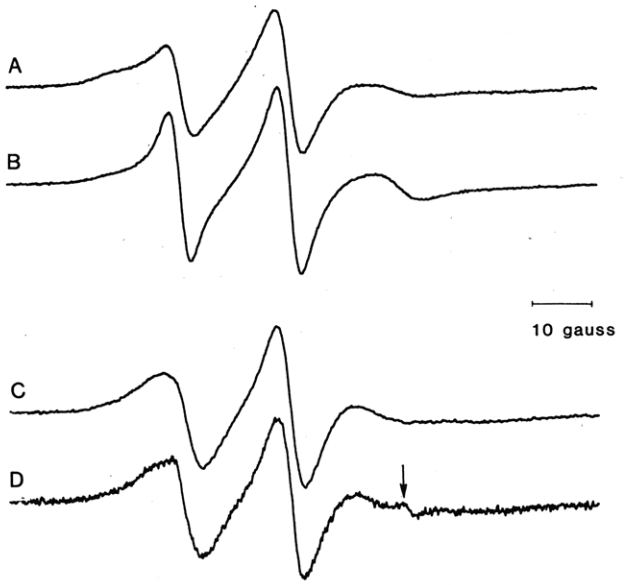


FIGURE 5: Effects of adding additional charges to the membrane surface: (A) C6SL-treated membranes, pH 6.5; (B) C6SL-treated membranes with addition of 100 mol of non-spin-labeled C6 per mol of C6SL, pH 6.5 (ϕ = 140 for (A) and (B)); (C) C6SLMeI-treated membrane, pH 6.5; (D) C6SLMeI-treated membrane, prior treatment with 50 mol of dibucaine per mol of C6SLMeI, pH 6.5 (ϕ = 284 for (C) and (D)). Arrow points to solution-spectrum component.

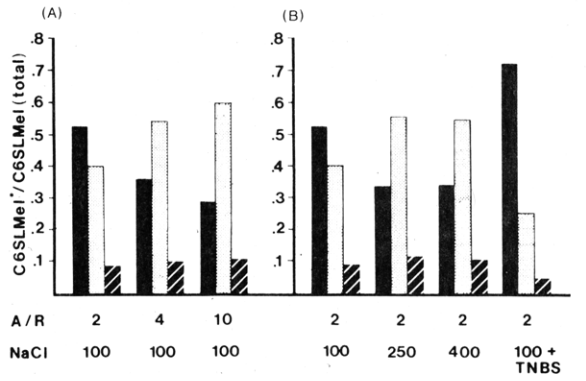


FIGURE 6: (A) Effects of increasing anesthetic concentration. (B) Effects of increasing salt concentration and of TNBS modification. (Solid bars) protein-immobilized component; (dotted bars) lipid-immobilized component; (striped bar) mobile lipid component. A/R = anesthetic-to-receptor mole ratio; NaCl concentrations given in mM; TNBS (5 mM) increases receptor surface potential (see Table II).

excess of dibucaine, a tertiary amine local anesthetic (Figure 5D). The sample was kept at pH 6.5 so the dibucaine was charged. It can be seen that the signal-to-noise ratio is decreased with the addition of high concentrations of dibucaine, and the solution spectral component increases (see arrow). Excess dibucaine in the RAChR membrane lowers the effective partition coefficient of C6SLMeI, perhaps by charge screening on the membrane surface (McLaughlin, 1975).

The above experiments demonstrate two important points: (1) binding of the positively charged species of C6SL or of other tertiary amine noncompetitive blockers cannot be accurately measured due to the effect of surface pH on the concentration of the charged species and (2) the concentration of the permanently charged C6SLMeI or of other quaternary amine noncompetitive blockers cannot exceed the value that significantly effects the partition coefficient of the putative competing amphiphile. In addition, demonstrating saturable binding by increasing the concentration of C6SLMeI is not possible due to spin–spin broadening; such broadening can change the spectral shape in an uninterpretable fashion (Sauerheber et al., 1977).

With these constraints in mind, saturation of the receptor-associated binding sites was demonstrated by treating the RAChR membrane with the non-spin-labeled analogue of C6SLMeI before treatment with C6SLMeI. In the experiments shown in Figure 6, the final concentration of spin-labeled anesthetic was maintained at 2 mol per mol of AChR, and the molar ratios of non-spin-labeled anesthetic were increased from 0 to 2–8 mol per mol of AChR. Pretreatment with equimolar and 4-fold excess non-spin-labeled C6MeI decreased C6SLMeI binding by 29% and 42%, respectively. As these concentration changes are well below the salt concentrations which are shown below to affect the AChR-bound local anesthetics, the decrease in the AChR-bound C6SLMeI in the presence of non-spin-labeled local anesthetic is clearly due to the specific displacement of C6SLMeI from saturable binding site(s). These results are compared to the effects of other noncompetitive blockers in Table I.

Effects of Agonists and Competitive Antagonists on Binding of C6SLMeI to the AChR. The addition of 1 mM carbamylcholine (850 mol of carb per mol of AChR) ratio) or 0.1 mM *d*-tubocurarine (85 mol of TC per mol of AChR) did not affect the shape of the C6SLMeI-treated RAChR membrane. The addition of excess α-bungarotoxin caused a 20.6% decrease in the proportion of C6SLMeI bound to the receptor. These results are summarized in Table I. The inability of carb or *d*-tubocurarine to affect the interactions of C6SLMeI with the AChR demonstrates that (1) C6SLMeI does not bind to the agonist binding site at these concentrations and (2) binding of C6SLMeI to the AChR is not enhanced by agonists. This is examined in greater detail in the Discussion. The basis for the effect of α-bungarotoxin on C6SLMeI binding is unclear, but may be due to steric hindrance (see Discussion).

Effects of Salt on the Protein-Immobilized Spectral Components. Increasing the salt concentration of the dialysis buffer from 100 to 250–400 mM NaCl decreased the protein-bound population of C6SLMeI from 51% to 33%–34% of the total (Figure 6). The ability of increasing ionic strength to decrease

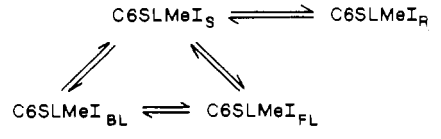
the highly immobilized component of the spin-labeled local anesthetic, without affecting the spectral shapes of the lipid components, suggests an effect of protein surface charge on C6SLMeI–AChR interactions. Figure 3 shows that the ESR spectrum of C6SLMeI at pH 9.5 is slightly less mobile than that at pH 6.0 (Figure 3C,D). The decrease in mobility at high pH suggests that the increase in surface charge density on the receptor surface results in an increase in the protein-bound component. Control experiments using DOPC liposomes in increasing ionic strengths or at various pH values have demonstrated that salt has no effect on the ESR spectrum of C6SLMeI within the bilayer (Limbacher et al., 1985).

Effects of Chemical Modification by TNBS. The surface electronegativity of the AChR can be increased by using trinitrobenzenesulfonic acid (TNBS), which neutralizes reactive amino groups (Poensgen & Passow, 1971). TNBS modification has been useful in several studies of the influence of surface charge density on ion channel function (e.g., Cahalan & Pappone, 1981). TNBS at 5 mM caused a 31.4% *increase* in the population of anesthetic bound to the receptor, consistent with the hypothesis that C6SLMeI–AChR binding is influenced by the surface charge on the receptor. It is emphasized that the effects on C6SLMeI immobilization of salt, pH, and TNBS must be effects on the surface of the AChR, as there is no surface charge on the DOPC bilayer and there are no amino groups on DOPC which can react with TNBS. Furthermore, the C6SLMeI concentrations used here (at ϕ = 200 there is one C6SLMeI per 200 lipids) are too low for there to be a bilayer surface charge induced by the local anesthetic itself.

Discussion

Spin-Label Equilibria. It has been demonstrated that C6SLMeI in RAChR membranes exists in at least three populations of distinct spectral shape, (1) C6SLMeI immobilized by the receptor, (2) C6SLMeI immobilized by the lipid, and (3) motionally unrestricted C6SLMeI within the lipid bilayer. The contributions of these three different components to the composite spectrum are plotted vs. the lipid-to-protein ratio of the membrane sample (Figure 7). The receptor-immobilized component increases with increasing protein concentration within the membrane and is accompanied by a decrease in the lipid-immobilized component. The mobile component increases slightly with increasing lipid concentration, but stays below 10% of the total spin-label population. The distribution of C6SLMeI between the two lipid populations is consistent with the multiequilibrium model of Limbacher et al. (1985) which predicts that for completely charged C6SL in DOPC liposomes, 8.7% of the spin-labels should be mobile within the lipid bilayer and 91.3% should be immobilized by the phospholipids.

The effects of salt and pH on the protein-associated C6SLMeI demonstrate that C6SLMeI–AChR interaction is mediated by surface charge. It was postulated that the pathway of interaction of C6SLMeI with the AChR was via the aqueous solution, as in the multiple equilibria



where C6SLMeI$_S$ is C6SLMeI in solution; C6SLMeI$_{BL}$ is C6SLMeI immobilized by lipid; C6SLMeI$_{FL}$ is C6SLMeI within the lipid, but motionally unrestricted; and C6SLMeI$_R$ is C6SLMeI immobilized by the receptor (AChR).

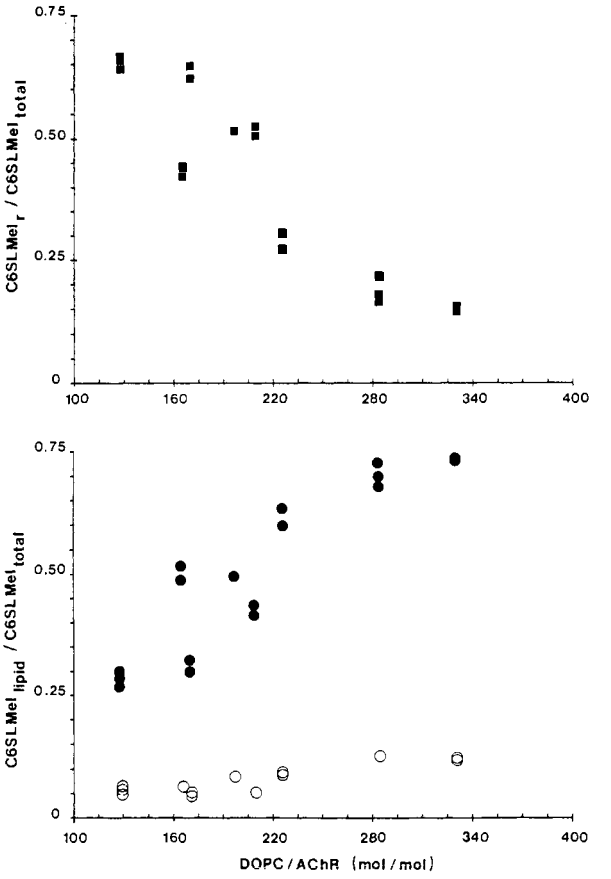


FIGURE 7: Contributions of the protein-immobilized (filled squares), lipid-immobilized (filled circles), and mobile (open circles) components to the composite spectrum of C6SLMeI in RAChR membranes are plotted against the lipid-to-protein ratio (ϕ) of the membrane.

In this model, the interaction of the anesthetic with the lipid is governed by its membrane–buffer partition coefficient and is independent of the interaction of the anesthetic with the AChR, which is characterized by its dissociation constant. The dissociation constant can be calculated for the equilibrium

$$LA_s + AChR_f \rightleftharpoons LA\text{–}AChR$$

so

$$K_d = \frac{(LA_s/\text{liter})(AChR_f/\text{liter})}{(LA\text{–}AChR/\text{liter})}$$

where LA_s is moles of local anesthetic in solution, $AChR_f$ is moles of free AChR, and LA–AChR is moles of local anesthetic–protein complex. K_d can be solved for by quantifying these three species, using the ratios of immobilized C6SLMeI to the total C6SLMeI (as derived from the ESR data) and the partition coefficient of C6SLMeI into the lipid. The complete derivation and computation of K_d is given in the Appendix. For the membrane sample referred to in the Appendix, K_d = 0.91 μM. This value is quite comparable to the K_d of 0.87 μM calculated for C6SLMeI binding to AChR in AChR-enriched native membranes, as measured by competition with radioactive phencyclidine (Palma et al., 1986).

The observation that binding of C6SLMeI to the AChR was saturable at a stoichiometry well below the number of annular lipids surrounding the receptor (see next section) precludes the possibility of applying the type of analysis used by Brotherus et al. (1981). Their analysis depends on the assumption that the labeled amphiphile is in equilibrium with other phospholipids for binding sites within the annular lipids; data presented here suggest that C6SLMeI immobilization by the AChR is not analogous to the phospholipid immobi-

Table II: Comparison of AChR-Bound Portions of Total Anesthetic Spin-Label with Calculated Concentrations of Anesthetic at the AChR–Water Interface[a]

sample	ψ_0[b] (mV)	σ_0[b] ($e^-/Å^2$)	charge[b] per AChR (e^-/AChR)	calcd[c] [LA]'/[LA]	$(C6_r)'$[d]/$(C6_r)$
115 mM NaCl, pH 7.5	−43	1/455	−55	1.0	1.0
250 mM NaCl, pH 7.5	−34	1/444	−56	0.70	0.65
400 mM NaCl, pH 7.5	−27	1/437	−57	0.52	0.68
115 mM NaCl, pH 7.5, + 5 mM TNBS[e]	−71	1/216	−116	3.1	1.3
115 mM NaCl, pH 9.5	−47	1/405	−62	1.0	1.0
115 mM NaCl, pH 6.0	−26	1/800	−31	0.43	0.67

[a] Abbreviations: ψ_0 = surface potential; σ_0 = surface charge density; [LA]'/[LA] = ratio of surface concentration of anesthetic in a particular experiment to that of a standard, defined in the table as 1.0; TNBS = 2,4,6-trinitrobenzenesulfonic acid, an amino-blocking reagent. [b] Calculations of ψ_0, σ_0 (from the Grahame equation), and charge per AChR use a surface area per receptor of 25 000 Å^2. Conditions for these calculations, which differ slightly from those of our data, are the addition of 2.5 mM KCl and 2 mM $CaCl_2$ (Love, 1986). [c] Calculations for surface concentration ratios of local anesthetic were made by using the Boltzmann distribution for the dependence of surface concentration of a soluble cation on bulk concentration ($[LA]_s$), ψ_0, and T: $[LA]'/[LA] = ([LA]_s'/[LA]_s)(\exp(-(e\psi_0'/kT))/(\exp(-(e\psi_0/kT))$. The bulk concentrations are assumed equal, so $([LA]_s'/[LA]_s) = 1$. For $T = 15$ °C, $e/kT = 0.0403$/mV. [d] Ratio of the AChR-bound portion of total anesthetic spin-label in a given experiment ($C6_r'$) to that of a standard experiment ($C6_r$). The standard for each set of membrane preparations is defined by a ratio of 1.0 in the table. The AChR-bound portions are determined by resolving ESR spectra (see text). [e] TNBS reacts primarily with amino groups. Its effect on ψ_0 was calculated by assuming all lysines in the 25 000-Å^2 region were neutralized.

lization seen by Ellena et al. (1983).

Competition for Binding Sites. If the spin-labeled local anesthetic binds to the acetylcholine receptor at the same site(s) as other drugs that have been demonstrated to have local anesthetic action on the AChR (these compounds are known collectively as noncompetitive blockers or NCBs), then sufficient quantities of these ligands should displace the spin-labeled anesthetic from the receptor. If binding sites on the AChR are depleted by competitive ligands, more of the C6SLMeI will reside in the lipid components and the composite ESR spectrum will become narrower. As shown in Table I, both dibucaine and PCP compete with C6SLMeI for binding sites on the receptor. Dibucaine and PCP are both high-affinity NCBs and are able to displace C6SLMeI at physiological concentrations (0.2 μM C6SLMeI, 2 μM dibucaine, 10 μM PCP). It should be mentioned that another interpretation of spectral narrowing in the presence of other NCBs is that the other NCBs affect C6SLMeI binding allosterically rather than directly. While it is not possible to prove that the NCBs tested in this study are not allosterically inhibiting the binding of C6SLMeI, most if not all amphiphilic noncompetive blockers of acetylcholine receptor function are thought to bind to the receptor at the same site(s) (Heidmann and Changeux, 1984; Karpen et al., 1982; Albuquerque et al., 1980).

Pretreatment with the non-spin-labeled analogue of C6SLMeI (C6MeI) blocks the binding of C6SLMeI to sites on the receptors, demonstrating that these sites are saturable. If the binding properties of the non-spin-labeled compound were identical with those of the C6SLMeI, the addition of equimolar C6MeI would reduce the protein-bound C6SLMeI component by 50%. Equimolar C6MeI decreased the protein-bound C6SLMeI by 29%, and a 4-fold excess of C6MeI decreased the protein-bound C6SLMeI by only 42%. Although it is clear that these two compounds have a common binding site, the reduced potency of the non-spin-labeled compound is consistent with the observations that spin-labeled local anesthetics were more potent at blocking sodium channel response in squid giant axons than were the non-spin-labeled analogues (H. Wang, personal communication). Table I demonstrates that non-spin-labeled C6MeI is more effective at displacing C6SLMeI than are PCP or dibucaine.

Effects of Surface Charge on Binding of C6SLMeI. The effects of salt and pH on C6SLMeI binding to the AChR support the involvement of receptor surface charge. An increase in anionic surface charge density increases the local concentration of the cationic C6SLMeI and favors interaction with sites on the receptor. Without specific knowledge of the receptor topology it is impossible to predict where these binding sites are. Binding sites at the lipid–protein interface could be affected by surface charge density, but the observation that the binding of C6SLMeI to the receptor is saturable at less than 4 mol of anesthetic per mol of receptor is not consistent with the immobilized C6SLMeI component reflecting spin-labels *non-specifically* bound at the lipid–protein interface. Furthermore, the pharmacological potency of a series of spin-labeled local anesthetics is inversely proportional to the chain length of the compound (Blickenstaff et al., 1985). This suggests that the pathway of interaction of these local anesthetics is via the aqueous medium rather than via the lipid bilayer.

A prediction of the AChR surface potential has been made that allows us to calculate the effect this surface potential should have on binding of C6SLMeI to the AChR (Love, 1986). Love calculated the surface charge density of the hydrophilic extracellular portion of the AChR from the experimentally derived and the predicted transmembrane distribution of amino acids proposed in Finer-Moore and Stroud (1984). The Grahame equation was used to calculate the surface potential in varying ionic environments, assuming a uniformly distributed planar surface charge. The ions in solution are assumed to exert a charge screening effect and do not penetrate the planar modeled surface. Love predicts that the extracellular portion of the receptor bears a net charge of 55 e^- per molecule at pH 7.4, 115 mM NaCl, 2.5 mM KCl, and 2 mM $CaCl_2$ (Love, 1986). According to the Gouy–Chapman theory, this highly anionic region will induce a local surface concentration of soluble cations (such as C6SLMeI) that is higher than the bulk concentration. These cations would include the positively charged agonist in vivo and the positively charged quaternary amine anesthetics in the reconstituted system considered here. Table II gives predicted values for surface potentials and surface charge densities in three ionic strengths and two pH values which closely match the conditions used in these experiments. The effect that the protein surface potential is expected to have on the surface concentration of C6SLMeI has been calculated by using the Boltzmann distribution for the dependence of surface concentration of a soluble cation on its bulk concentration, ψ_0, and T (see Table II). The actual changes in the amount of protein-bound C6SLMeI from the ESR data are also given, and there is a close match with the predicted values of surface concentration

changes. These data support the hypothesis that a surface potential generated by the acetylcholine receptor favors the binding of charged molecules such as C6SLMeI. The ability to predict the concentration of charged amphiphiles at the receptor surface without interference from a bilayer surface potential makes this reconstituted membrane system an important experimental tool for further research on protein surface charge effects.

The effect of TNBS is also consistent with C6SLMeI binding favored by the negative surface potential of the AChR. The reaction of TNBS with protein amino groups decreases the fixed positive surface charge, i.e., increases the receptor electronegativity (Koblin & Wang, 1976). C6SLMeI-labeled RAChR membranes treated with 5 mM TNBS showed an increase in the immobile component attributed to C6SLMeI–protein interaction. The effect on the surface potential of removing the positive charges on the lysines in the region modeled by Love were calculated (Table II). On the basis of this value of ψ_0 the surface concentration of C6SLMeI was predicted to be 3.1 times that of the reference state, i.e., the sample without TNBS treatment. That the data showed only a 1.3 times increase indicates that while receptor surface potential has clearly affected the binding of C6SLMeI, the extent of covalent modification was possibly not as complete as that modeled by Love.

Effects of Agonists and Noncompetitive Inhibitors. A wide range of compounds, including local anesthetics, PCP, histrionicotoxin, and chlorpromazine, are known to block the agonist-stimulated cation flux through the ion channel of the acetylcholine receptor. These compounds exert their effects by binding to the receptor at sites other than the agonist binding site and are therefore called "noncompetitive blockers". Binding sites of differing affinities have been proposed for several of these compounds, and it has been observed that the equilibrium affinity of the high-affinity binding site is regulated by agonists and some antagonists (Sine & Taylor, 1982; Heidmann et al., 1983; Covarrubias et al., 1984). In most cases, binding of the inhibitor to its high-affinity binding site is enhanced by agonists. Oswald et al. (1983) found the rate of PCP–receptor association to be 3 orders of magnitude higher when PCP and agonist were mixed with the membrane simultaneously as opposed to membranes preincubated with agonist. The prolonged presence of agonist is known to cause a conformational change of the receptor leading to desensitization of the receptor.

There is not yet a clear picture of the relationship between compounds which bind at the agonist-binding site and binding of noncompetitive blockers (NCB) at other sites, nor is it known what conformation state the AChR is in during NCB binding. In this study the addition of 1 mM carbamylcholine (an agonist) or 0.1 mM *d*-tubocurarine (a high-affinity competitive antagonist) had no effect on the spectral shape of C6SLMeI-treated RAChR membranes. These experiments indicate that C6SLMeI does not bind to the agonist binding site at these concentrations. On the other hand, α-bungarotoxin significantly reduced the protein-bound component. Although the toxin-binding region is thought to include the agonist binding site, the large size of the toxin may physically block C6SLMeI from binding to sites other than the agonist binding site. In support of this hypothesis, hydrogen–tritium exchange experiments have shown that α-bungarotoxin restricts solvent accessibility while agonists, TC, and noncompetitive antagonists have little effect (McCarthy et al, 1986).

That agonists increase the affinity of AChR for local anesthetics has been observed with C6SLMeI in AChR membranes, but not with the reconstituted membranes used in this study. Other work in our lab (Palma et al., 1986) has shown that 1 mM carbamylcholine increases the relative size of the protein-associated C6SLMeI component in AChR-rich native membranes. The ESR spectrum of C6SLMeI plus agonist in native membranes is very similar to the ESR spectrum of C6SLMeI in reconstituted AChR membranes with or without agonist. The effect of agonist on the binding of C6SLMeI to native AChR may be to provide access to binding sites that are already available to C6SLMeI in DOPC-reconstituted AChR in the absence of carbamylcholine. A comparison of the dissociation constants for these samples supports this theory: for native membranes, in the absence of agonist, C6SLMeI binds with lower affinity (K_d = 20 μM) than in the presence of agonist (K_d = 0.87 μM); for AChR–DOPC membranes, with or without agonist, C6SLMeI binds with high affinity (K_d = 0.91 μM).

It has been proposed by several researchers that the voltage-dependent ability of agonists to enhance the binding of local anesthetics to the AChR is evidence that local anesthetics bind within the ion channel, thus the term "open channel blockers" has been applied to many of these compounds which are quite similar to C6SLMeI. If C6SLMeI binds to AChR in DOPC bilayers at the same site(s) as it binds to AChR in native membranes in the presence of agonist, then this implies that AChR in DOPC bilayers exists in either the open channel or the desensitized state. Toxin rate-binding experiments suggest that AChR in DOPC is present in the desensitized state and the state of low affinity for toxin (Fong & McNamee, 1986), but do not exclude the possibility that open channels are present (T. M. Fong, personal communication). AChR function is completely reversible when re-reconstituted into a bilayer which is more easily assayed for ion flux capability. Rather than modeling the complete functional properties of the native membranes, the DOPC–AChR membranes used in this study may prove to be uniquely suited to the examination of independent states of the receptor.

Conclusions

The method employed here of resolving the ESR spectrum of a spin-labeled local anesthetic into several identifiable components has proven to be a unique and informative way of monitoring molecular interactions within the plane of the membrane. Four populations of quaternary amine local anesthetic interacting with reconstituted acetylcholine receptor membranes have been isolated: two distinct populations are present in the lipid bilayer, one population is present in the aqueous solution, and one population is bound to the acetylcholine receptor through a combination of electrostatic and hydrophobic interactions.

The effect of protein surface charge on binding of local anesthetics is important in that previous analyses of AChR surface charge effects had to consider the charge densities on both the protein and the lipid. It has been established that the membrane surface charge greatly affects local anesthetic binding (McLaughlin, 1975; Limbacher et al., 1985). It has also been proposed that a postsynaptic surface potential modulates AChR channels, on the basis of the observation that increasing the divalent ion concentration decreases the potency of charged agonists and antagonists (Schmidt & Raftery, 1974; Chang & Neumann, 1976; Spivak & Taylor, 1980; Van der Kloot & Cohen, 1979) and that the miniature end-plate current is slowed by increasing external ionic strength (Van der Kloot & Cohen, 1979) or by decreasing pH (Mallart & Molgo, 1978). In these studies it was difficult to distinguish between ionic effects on the bilayer and effects on the AChR.

The system used here of AChR reconstituted into DOPC allows visualization of surface charge effects that are attributed solely to the acetylcholine receptor: there are no other contaminating membrane proteins, and most importantly there is no surface potential on the bilayer surface. This type of membrane may provide an excellent model system with which to study protein surface charge effects, and quantitative measurements can be made which will confirm or refute structural predictions of surface charge densities.

ACKNOWLEDGMENTS

We thank Dr. Robert L. Love (Department of Chemistry, University of Pennsylvania, Pittsburgh, PA) for performing the calculations of AChR surface potentials and for generously allowing us to use and cite his manuscript prior to publication. We are grateful to Prof. Robert M. Stroud and the Department of Biochemistry and Biophysics at UCSF for help with electron micrographs and other research support.

APPENDIX

Definition and Computation of Dissociation Constants from ESR Data. For AChR reconstituted into DOPC bilayers and treated with C6SLMeI, the mass action law is

$$K_d = \frac{(LA_s/\text{liter})(AChR_f/\text{liter})}{(LA\text{–}AChR/\text{liter})} \quad (1)$$

for the equilibrium $LA_s + AChR_f \rightleftharpoons LA\text{–}AChR$, where LA_s is moles of local anesthetic (C6SLMeI) in solution, $AChR_f$ is moles of free AChR (within the bilayer but with no C6SLMeI bound), and LA–AChR is moles of C6SLMeI–AChR complex (assumes one LA per AChR).

This equilibrium is for the original 8.5-mL sample when partitioning of the C6SLMeI into the bilayer and binding of C6SLMeI to the AChR came to equilibrium, as well as for the pellet collected for ESR. We assume that all the lipid and AChR is pelleted and that the equilibrium is unaffected by pelleting the sample. Canceling two of the volume terms and dividing both numerator and denominator of eq 1 by the total C6SLMeI in the pellet give

$$K_d = \frac{(LA_s/\text{liter})(AChR_{pf}/LA_{ptot})}{(LA\text{–}AChR_p/LA_{ptot})} \quad (2)$$

where $AChR_{pf}$ is moles of free AChR (within the pelleted bilayer but with no C6SLMeI bound), LA_{ptot} is the total moles of C6SLMeI in pellet, and $LA\text{–}AChR_p$ is moles of C6SLMeI–AChR complex in pellet.

$AChR_{pf}$ can be expressed as the total AChR in the pellet minus the AChR which is bound to C6SLMeI in the pellet, so

$$\frac{(AChR_{pf})}{(LA_{ptot})} = \frac{(AChR_{ptot})}{(LA_{ptot})} - \frac{(LA\text{–}AChR_p)}{(LA_{ptot})} \quad (3)$$

The ratio of AChR-bound spin-labeled local anesthetic to the total spin-labeled local anesthetic in the bilayer is available from the resolved ESR data and can be substituted for $(LA\text{–}AChR_p)/(LA_{ptot})$ above. This ratio, which is derived from the ESR data, is referred to as "$C6_r/C6_{tot}$". Therefore, substituting this expression and eq 3 back into eq 2, we have

$$K_d = \frac{(LA_s/\text{liter})((AChR_{ptot}/LA_{ptot}) - (C6_r/C6_{tot}))}{C6_r/C6_{tot}} \quad (4)$$

The value of $AChR_{ptot}$ is assumed to be the same as the original moles of AChR in the sample, so the only remaining unknowns are LA_s and LA_{ptot}, which can be calculated by using the partition coefficient of C6SLMeI into DOPC liposomes, adjusted for the presence of AChR. The partition coefficient of C6SLMeI into DOPC liposomes is defined as P_{DOPC} (Colley et al., 1971)

$$P_{DOPC} = \frac{(LA_l/\text{weight of lipid})}{(LA_s/\text{weight of solution})}$$

where LA_l is moles of C6SLMeI within the lipid bilayer and LA_s is moles of C6SLMeI in solution.

The C6SLMeI in solution will partition into both lipid and protein, and we know from the ESR data for a sample of $\phi = 128$, 66.3% of the spin-label will associate with the AChR and 33.7% will associate with the lipid. The protein-associated C6SLMeI is therefore 1.97 times the lipid-associated C6SLMeI, and the total (1.97 + 1) represents the factor by which the P_{DOPC} must be multiplied to get the true value for partitioning into AChR–DOPC membrane. The value of 2500 for P_{DOPC} is estimated from the partitioning of the tertiary amine C6SL (Figure 1) at pH 5.5, where the spin-label is totally charged, into unilamellar DOPC liposomes (Limbacher et al., 1985).

$$P_{AChR\text{-}DOPC} = 2.97 \times P_{DOPC} = 2.97 \times 2500 = 7400$$

This value can then be used to calculate the moles of C6SLMeI in the aqueous phase and in the membrane pellet:

$$P_{AChR\text{-}DOPC} = \frac{((LA_{tot} - LA_s)/\text{weight of DOPC})}{(LA_s/\text{weight of solution})}$$

For the sample in which $LA_{tot} = 1.6 \times 10^{-8}$ mol, weight of DOPC = 8.02×10^{-4} g, weight of solution = 8.5 g, and $P_{AChR\text{-}DOPC} = 7400$, $LA_s = 9.41 \times 10^{-9}$ mol. Subtracting this value from LA_{tot} gives $LA_{ptot} = 6.59 \times 10^{-9}$ mol, the amount of C6SLMeI in the total pellet.

By substituting these values and that for $C6_r/C6_{tot}$ given above, we can now calculate K_d from eq 4. For the membrane sample referred to previously ($\phi = 128$, 2 C6SLMeI added per AChR, 1.02×10^{-6} mol of DOPC, and 8.0×10^{-9} mol of AChR, 8.5-mL-total volume), $K_d = 0.91$ μM.

This method of calculating dissociation constants should be generally applicable to any experiment in which a lipophilic substance binds to a membrane protein from the aqueous phase, as long as the ratio of bound-to-unbound species is known and partition coefficients are calculated.

It is important to appreciate the sources of error in the resolution of the ESR spectra. There are errors in spectral resolution which are multiplied as three or four resolutions are done in sequence and the ratios between the components are calculated. Each resolution was performed at least twice, and average values for spectral proportions were used. ESR data taken from the same membrane preparation and treated in the same fashion give consistent resolutions. The assignation of a lipid-to-protein ratio based on biochemical assays was another source of error. The steep slope of the curve representing spin-label bound to protein vs. lipid-to-protein ratio demonstrates that any inaccuracy in the x-axis value leads to a great deal of scatter in the data. Values for contributions of protein components of less than 20% were less accurate than large values due to the noise in the spectrum. Finally, it was observed that even though *liposomes* containing spin-label at lipid to spin-label mole ratios of 100 or even 50 showed no signs of spin–spin interaction, spin–spin interaction was seen in several *membrane* samples in which the lipid to spin-label ratio was maintained at 100. This indicates that the high affinity of the spin-labels for the receptor caused the labels to be concentrated out of the bulk-phase lipid and increased the likelihood of interaction near the protein. Although ESR

spectra which could be seen to clearly contain spin–spin interaction were not included with the data presented here, small amounts of spin–spin interaction are difficult to detect. The effect of a slight degree of spin–spin interaction would be to artificially increase the size of the immobile component by broadening parts of the spectrum (Sauerheber et al., 1977).

Registry No. C6SLMeI, 82473-54-9.

REFERENCES

Albuquerque, E. X., Tsai, M.-C., Aronstam, R. S., Witkop, B., Eldefrawi, A. T., & Eldefrawi, M. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1224–1228.

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

Andreasen, T. J., & McNamee, M. G. (1977) *Biochem. Biophys. Res. Commun. 79*, 958–965.

Aracava, Y., & Albuquerque, E. X. (1984) *FEBS Lett. 174*, 267–274.

Aracava, Y., Ikeda, S. R., Daly, J. W., Brookes, N., & Albuquerque, E. X. (1984) *Mol. Pharmacol. 26*, 304–313.

Blickenstaff, G. D., & Wang, H. H. (1985) *Biophys. J. 47*, 261a.

Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., & Hokin, L. E. (1981) *Biochemistry 20*, 5261–5267.

Cahalan, M. D., & Pappone, P. A. (1981) *J. Physiol. (London) 321*, 127–139.

Chang, H. W., & Neumann, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3364–3368.

Colley, C. M., Metcalfe, S. M., Turner, B., Burger, A. S. V., & Metcalfe, J. C. (1971) *Biochim. Biophys. Acta 233*, 720–729.

Covarrubias, M., Prinz, H., & Maelicke, A. (1984) *FEBS Lett. 169*, 229–233.

Damle, V. N., & Karlin, A. (1978) *Biochemistry 17*, 2039.

Earnest, J. P., Wang, H. H., & McNamee, M. G. (1984) *Biochem. Biophys. Res. Commun. 123*, 862–868.

Earnest, J. P., Fong, T.-M., Stroud, R. M., & McNamee, M. G. (1986) *Biophys. J. 49*, 522a.

Ellena, J. F., Blazing, M. A., & McNamee, M. G. (1983) *Biochemistry 22*, 5523.

Finer-Moore, J., & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155–159.

Fong, T. M., & McNamee, M. G. (1986) *Biochemistry 25*, 830–840.

Gargiolo, R., Giotta, G., & Wang, H. H. (1973) *J. Med. Chem. 16*, 707–708.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 454–523, Academic, New York.

Heidmann, T., & Changeux, J.-P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1897–1901.

Heidmann, T., Oswald, R. E., & Changeux, J.-P. (1983) *Biochemistry 22*, 3112–3127.

Hille, B. (1977) *J. Gen. Physiol. 69*, 497–515.

Ikeda, S. R., Aronstam, R. S., Daly, J. W., Aracava, Y., & Albuquerque, E. X. (1984) *Mol. Pharmacol. 26*, 293–303.

Karpen, J. W., Aoshima, H., Abood, G. L., & Hess, G. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2509–2513.

Koblin, D., & Wang, H. H. (1976) *Biochem. Pharmacol. 25*, 1405–1413.

Koblin, D. D., & Lester, H. A. (1979) *Mol. Pharmacol. 15*, 559.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, A. G. (1977) *Mol. Pharmacol. 13*, 474–487.

Limbacher, H. P., Jr., Blickenstaff, G. D., Bowen, J. H., & Wang, H. H. (1985) *Biochim. Biophys. Acta 812*, 268–276.

Love, R. A. (1986) *Biophys. J.* (submitted for publication).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mallart, A., & Molgo, J. (1978) *J. Physiol. (London) 276*, 343–352.

McCarthy, M., Earnest, J. P., Young, E. F., Choe, S., & Stroud, R. M. (1986) *Annu. Rev. Neurosci. 9*, 383–413.

McLaughlin, S. (1975) *Prog. Anesthesiol. 1*, 193–200.

Neher, E., & Steinbach, J. H. (1978) *J. Physiol. (London) 277*, 153–176.

Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) *Biochim. Biophys. Acta 721*, 151–162.

Oswald, R. E., Heidmann, T., Changeux, J.-P. (1983) *Biochemistry 22*, 3128–3136.

Palma, A., Herz, J., Wang, H. H., & Taylor, P. (1986) *Mol. Pharmacol.* (in press).

Poensgen, P., & Passow, H. (1971) *J. Membr. Biol. 6*, 210.

Ribera, A., Trautmann, A., Pinset, C., & Changeux, J.-P. (1985) *Biophys. J. 47*, 40a.

Sauerheber, R. D., Gordon, L. M., Crosland, R. D., & Kuwahara, M. D. (1977) *J. Membr. Biol. 31*, 131–169.

Schmidt, J., & Raftery, M. A. (1974) *J. Neurochem. 23*, 617–623.

Sine, S. M., & Taylor, P. (1982) *J. Biol. Chem. 257*, 8106–8114.

Skou, J. C. (1954) *Acta Pharmacol. Toxicol. 10*, 325–337.

Spivak, C. E., & Taylor, P. (1980) *Mol. Pharmacol. 18*, 413–420.

Van der Kloot, W., & Cohen, I. (1979) *Science (Washington, D.C.) 203*, 1351–1353.

Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191–2199.

Wang, H. H., Yeh, J. Z., & Narahashi, T. (1982) *J. Membr. Biol. 66*, 227–233.

Wang, H. H., Earnest, J., & Limbacher, H. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5297–5301.

Yeh, J. Z. (1980) *Prog. Anesthesiol. 2*, 35–43.