

Lipid–Protein Interactions and Effect of Local Anesthetics in Acetylcholine Receptor-Rich Membranes from *Torpedo marmorata* Electric Organ[†]

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ABSTRACT: The selectivity of lipid–protein interaction for spin-labeled phospholipids and gangliosides in nicotinic acetylcholine receptor-rich membranes from *Torpedo marmorata* has been studied by ESR spectroscopy. The association constants of the spin-labeled lipids (relative to phosphatidylcholine) at pH 8.0 are in the order cardiolipin (5.1) \approx stearic acid (4.9) \approx phosphatidylinositol (4.7) > phosphatidylserine (2.7) > phosphatidylglycerol (1.7) > G_{D1b} \approx G_{M1} \approx G_{M2} \approx G_{M3} \approx phosphatidylcholine (1.0) > phosphatidylethanolamine (0.5). No selectivity for mono- or disialogangliosides is found over that for phosphatidylcholine. Aminated local anesthetics were found to compete with spin-labeled phosphatidylinositol, but to a much lesser extent with spin-labeled stearic acid, for sites on the intramembranous surface of the protein. The relative association constant of phosphatidylinositol was reduced in the presence of the different local anesthetics to the following extents: tetracaine (55%) > procaine (35%) \approx benzocaine (30%). For stearic acid, only tetracaine gave an appreciable reduction (30%) in association constant. These displacements represent an intrinsic difference in affinity of the local anesthetics for the lipid–protein interface because the membrane partition coefficients are in the order benzocaine \gg tetracaine \approx procaine.

The muscle-type nicotinic acetylcholine receptor (nAChR)¹ is a multisubunit integral protein of the postsynaptic membrane that transforms the agonist binding signal into a local membrane depolarization by the opening of a cation-selective channel. The protein is a heterooligomer of approximate molecular mass 268 kDa and is composed of two α subunits

and one each of the β , γ , and δ subunits. The different subunits are arranged in a pentameric structure around the channel, in which all subunits are in contact with the membrane lipid (1). The mechanism by which lipid–protein interactions influence receptor function is not known exactly, but a minimum number of lipids appear to be required for activity (2), and the lipid composition is found to be an important factor for effective reconstitution (3–6). There is an obligate requirement for a negatively charged lipid component and for a neutral lipid component such as cholesterol. In addition, a range of hydrophobic molecules such as fatty acids (7, 8) and local anesthetics (9) are found to block channel activity in a noncompetitive manner by associating at multiple sites at the lipid–protein interface.

nAChR-rich membranes from the electric organ of *Torpedo marmorata* and related species have proved to be a useful system for studying various aspects of the receptor in its native form. Such membrane preparations have an average lipid/protein ratio of 0.69 (w/w) and a molar ratio of phospholipid to cholesterol of ca. 1.7 (10). This lipid composition corresponds to approximately 230 phospholipid molecules and 130 cholesterol molecules per 268 kDa protein molecule. Previous ESR experiments have shown that, at this lipid/protein ratio in the native membranes, it is possible to resolve the population of spin-labeled lipids interacting directly with the intramembranous surface of the integral protein component. This was shown to be the case not only for fatty acids and androstanol which display specificity of interaction with the nAChR protein (11, 12) but also for spin-

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¹ Abbreviations: nAChR, nicotinic acetylcholine receptor; α BTx, α -bungarotoxin; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 14-PCSL, -PESL, -PGSL, -PSSL, and -PISL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphoserine, and -phosphoinositol; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero-3-phospho]-*sn*-glycerol; 14-GM1SL, II³NeuAc-GgOse₄-N-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-GM2SL, II³NeuAc-GgOse₃-N-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-GM3SL, II³NeuAc-Lac-N-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; 14-GD1bSL, II³(NeuAc)₂-GgOse₄-N-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]sphingosine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; DOPC, 1,2-di-oleoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; ESR, electron spin resonance.

labeled phospholipids which show a lower selectivity for the protein (13).

The phospholipids of nAChR-rich membranes from *T. marmorata* are composed principally of 41% phosphatidylcholine, 33% phosphatidylethanolamine, 14% phosphatidylserine, 4% phosphatidylinositol, and 5% sphingomyelin (10). The membranes also contain smaller amounts of other negatively charged phospholipids: cardiolipin (1%) and phosphatidic acid (0.5%). In addition, nAChR-rich membranes also contain sialoglycosphingolipids (gangliosides), the composition of which is 38% G_{M1}, 20% G_{D1a}, 15% G_{D3}, 7% G_{M2}, 5% G_{T1b}, 5% G_{M3}, 4% G_{Q1}, 2% G_{P1}, and 1% G_{D1b} (14). [The lipid-bound sialic acid content of the *Torpedo* electric organ is 27 µg/g wet tissue, roughly 10% of that for elasmobranch brain (15).] It is thus of considerable interest to know how these various different lipids interact with the nAChR protein.

A previous study has determined the specificity pattern for interaction of certain phospholipids with the nAChR reconstituted in a synthetic phosphatidylcholine, DOPC (12), and other work has characterized the interaction of spin-labeled local anesthetic derivatives with the receptor (16–18). In the present work we have studied the phospholipid specificity of the protein in native membranes in greater detail by extending the known pattern of selectivity to more complex phospholipids such as phosphatidylinositol and cardiolipin and to glycosphingolipids, both mono- and disialogangliosides. Cardiolipin is found to possess a rather high specificity for the nAChR protein, and that of inositol lipids may be of particular functional relevance (19, 20). The interaction with glycosphingolipids is especially relevant to organization at the postsynaptic membrane because of recent interest in the formation of spatially differentiated sphingolipid domains (“rafts”) in synaptic plasma membranes (21, 22).

A related problem is the extent to which hydrophobic local anesthetics affect the interaction of the phospholipids with the protein and whether they are able to compete with specific phospholipids for sites at the lipid–protein interface. A variety of fluorescence approaches suggest a heterogeneity in the sites available for different hydrophobic molecules at the lipid–protein interface of the receptor (23–25). Therefore, we also have investigated the competition between local anesthetics, such as tetracaine, procaine, and benzocaine, and phospholipids or fatty acids for sites on the intramembranous surface of the protein.

MATERIALS AND METHODS

Materials. *T. marmorata* specimens from the Bay of Arcachon, France, were provided by Prof. V. P. Whittaker (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany). *Discopyge tschudii* electric rays were caught off the South Atlantic coast near Necochea, Argentina. *N*-(propionyl-³H)propionylated α-bungarotoxin ([³H]αBTx, specific activity 107 Ci/mmol) was obtained from Amersham International, Buchs, U.K. DEAE-cellulose sheets (DE-81) were obtained from Whatman Inc. (Clifton, N. J.). DEAE-Sephadex A-50 (fine) and Percoll (density 1.030 g/mL) were obtained from Pharmacia (Uppsala, Sweden). Benzocaine, procaine hydrochloride, tetracaine hydrochloride, and suberyldicholine dihydrochloride were obtained from Sigma

Chemical Co. (St. Louis, MO). 1-Pyrenedecanoic acid was from Molecular Probes (Eugene, OR) and dansyltrimethylamine from Pierce Chemical Co. (Rockford, IL).

Spin-Label Synthesis. Stearic acid labeled on the C-14 position, 14-SASL, was synthesized according to the method described in ref 26. Spin-labeled phosphatidylcholine, 14-PCSL, was prepared from 14-SASL and lysophosphatidylcholine using the method described in ref 27. The spin-labeled phospholipids 14-PESL, 14-PGSL, and 14-PSSL were prepared from 14-PCSL by a headgroup exchange reaction catalyzed by phospholipase D in the presence of the appropriate alcohol (28). Spin-labeled cardiolipin 14-CLSL was synthesized by a condensation reaction between 14-PGSL and phosphatidic acid derived from egg yolk phosphatidylcholine (Lipid Products, South Nutfield, U.K.), essentially according to the method described in ref 29.

Spin-labeled phosphatidylinositol, 14-PISL, was synthesized from yeast phosphatidylinositol (Lipid Products, South Nutfield, U.K.), as follows, by using a modification for spin-labeled lipids of the method described in ref 30. Yeast phosphatidylinositol (50 mg) was thoroughly dried by repeated evaporation from solution in dry benzene. It was then dissolved in dry CHCl₃ (5 mL) to which acetic anhydride (1 mmol) and (dimethylamino)pyridine (500 µmol) were added. The mixture was stirred at room temperature for 24 h with continuous bubbling of nitrogen gas. The reaction mixture was then washed with CH₃OH/0.1 N HCl (1:1 v/v) solutions and further purified by precipitation from cold (–20 °C) acetone. The pentaacetylphosphatidylinositol so obtained was dispersed in sodium acetate buffer containing 0.1 M CaCl₂, pH 8.0. Porcine pancreatic phospholipase A₂ (Boehringer-Mannheim, FRG.) was added in 1 mg aliquots every 30 min over a period of approximately 4 h, until the desired pentaacetyllysophosphatidylinositol was obtained. The lysolipid was extracted into CH₂Cl₂/CH₃OH (2:1 v/v) and was further purified by chromatography on a silica gel column with CH₂Cl₂/CH₃OH/6 N NH₄OH (65:35:3 v/v/v) as eluent. The pentaacetyllysophosphatidylinositol was acylated with the anhydride of spin-labeled stearic acid, 14-SASL, using 4-pyrrolidinopyridine as catalyst (31). The product was purified by preparative TLC with the solvent system CH₂Cl₂/CH₃OH/6 N NH₄OH (65:35:3 v/v). The 14-pentaacetyl-PISL was deacetylated by hydrazinolysis: 10 µL of hydrazine hydrate was added to the spin-labeled lipid solution in 0.5 mL of CH₃OH/H₂O (3:1 v/v) with stirring. The reaction mixture was incubated at 60 °C for 6 h with stirring and then cooled on ice. The precipitate formed was dissolved in CH₂Cl₂ and washed with CH₃OH/0.1 N NH₄OH (1:1 v/v). The product was further purified by chromatography on a silica gel column with CH₂Cl₂/CH₃OH/6 N NH₄OH (65:35:3 v/v/v) as the eluent. The 14-PISL migrated as a single spot slightly ahead of authentic phosphatidylinositol on TLC with the solvent system CH₂Cl₂/CH₃OH/6 N NH₄OH (65:35:3 v/v/v), as is common for spin-labeled phospholipids, and quenched the fluorescent indicator in the plates indicating the presence of the spin-label group.

Spin-labeled gangliosides were prepared by coupling the *N*-succinimidyl ester of 14-SASL to lysogangliosides obtained from purified brain gangliosides, as described in ref 32. Purification and characterization were carried out as in the same reference (see also ref 33).

Purification of nAChR-Rich Membrane Vesicles. nAChR-containing membranes were prepared from the electric organ of *T. marmorata*, according to the method described in ref 34. Sealed and leaky vesicles were further fractionated by the method described in ref 35, based on the exchange of Na^+ within the vesicles for external Cs^+ and subsequent separation on a Percoll—CsCl density gradient. The specific activity of the nAChR membranes was assayed using the [^3H] αBTx /DE-81 ion-exchange filter paper method (36). Typically, values of 1–1.5 nmol of [^3H] αBTx /mg of protein were obtained. Membranes are enriched in nAChR by up to one-third of the total membrane protein. Electron microscopy shows that the surface of the membranous preparations is substantially covered with receptor (37). Protein was determined according to the method described in ref 38 using bovine serum albumin as standard. Blanks containing Percoll—CsCl were subtracted.

nAChR-containing membranes from *D. tschudii* electric organ were prepared as described in ref 10. Specific activities ranged between 0.7 and 0.9 nmol of suberyldicholine binding sites/mg of protein, assayed by decrease in dansyltrimethylamine (6.6 μM) fluorescence from 0.3 mg protein/mL membrane suspensions containing 0.1 mM phenylcyclidine.

ESR Measurements. Sealed membrane vesicles were centrifuged in an Eppendorf centrifuge for 15 min at full speed in order to remove the remaining Percoll—CsCl. The floating layer was washed in 10 mM Hepes, pH 8.0. The pellet (0.6 mg of protein) was resuspended in 1 mL of buffer, to which was added 0.02 mg of spin-labeled lipid in absolute ethanol (1% of the total volume) for 14-SASL and phospholipids or in ethanol/water (1:1 v/v) for ganglioside spin labels (2% of total volume). Membranes and spin label were incubated together at room temperature for 45 min. The labeled membranes were then centrifuged at 45000 rpm in a Beckman 50 Ti rotor for 45 min. Where necessary, the membranes were washed again in buffer to remove unincorporated spin label, and then the final pellet was transferred into ESR sample capillaries (1 mm i.d.) and concentrated in a benchtop centrifuge. Even if the spin label incorporated completely, the spin label/lipid + cholesterol ratio did not exceed 3 mol %. Previous experiments with *Torpedo* lipid extract gave no evidence for inhomogeneous mixing or phase separation (11, 13). To minimize dielectric loss, samples were trimmed to a height of 10 mm by removing excess supernatant. For the competition experiments with local anesthetics, the resuspended membrane pellet (0.35 mg of protein in 1 mL) was preincubated for 30 min with 0.2 mg of benzocaine, procaine, or tetracaine added as a concentrated solution in ethanol before spin labeling with 14-PISL or 14-SASL. Membranes were then collected by centrifugation, without washing.

ESR spectra were recorded at 9 GHz on a Varian E-12 Century Line spectrometer equipped with a nitrogen gas-flow temperature regulation system. Temperatures were measured with a thermocouple placed just above the cavity adjacent to the sample capillary. ESR spectra were collected on an IBM personal computer with a Labmaster interface (12 bit A/D resolution) using software written by Dr. M. D. King (Max-Planck-Institut für Biophysikalische Chemie). Spectral subtractions were performed as described previously (39) by using extracted nAChR membrane lipids and

sonicated dimyristoylphosphatidylcholine vesicles in the gel phase for reference spectra.

Fluorescence Measurements. Local anesthetic quenching titrations were carried out at 20 °C in an SLM-Aminco 4800 spectrofluorometer with 0.5 \times 0.5 cm cuvettes. Excitation and emission wavelengths of 1-pyrenedecanoic acid (\approx 1.5 mol % with respect to membrane lipid) in nAChR-rich membranes were 337 and 372 nm, respectively. To reduce stray light, a 320 nm cutoff filter was used in the emission beam. Local anesthetics were added from 20 mM stock solutions in dimethyl sulfoxide (procaine and benzocaine) or distilled water (tetracaine) to membrane suspensions at ca. 0.6 mg of protein/mL in 10 mM sodium phosphate buffer, pH 7.4. Fluorescence intensities were corrected for dilution by added titrant. Local anesthetic fluorescence and dimethyl sulfoxide blanks were also subtracted.

Titration with the agonist suberyldicholine were carried out at room temperature in an Aminco SPF-500 fluorometer. Dansyltrimethylamine excitation and emission wavelengths were 280 and 546 nm, respectively. A Corning 7-54 interference filter and a Corning 3-72 cutoff filter were used in excitation and emission beams, respectively, to reduce stray light.

RESULTS

Phospholipid Specificity Pattern. The ESR spectra of C-14 spin-labeled stearic acid (14-SASL) and various spin-labeled phospholipids (14-PCSL, -PESL, -PGSL, -PSSL, -PISL, and -CLSL) incorporated in nAChR-rich membrane vesicles from *T. marmorata* are given in Figure 1. The spectra are all composed of two components, the relative proportions of which vary with the different labeled lipids. One component, which is most prominent in the spectrum of 14-PESL or -PCSL, is very similar to the ESR spectra obtained with aqueous dispersions of lipid extracts from *Torpedo* electric organ (spectra not shown). This component clearly corresponds to fluid lipid bilayer regions in the nAChR-rich membrane. The second component is evident in the outer wings of the membrane spectra, particularly those of 14-CLSL, -SASL and -PISL, for which it is strongest. This latter component arises from those spin-labeled lipids that are undergoing restricted motion at the lipid—protein interfaces in the membrane (cf. refs 11 and 12). The two membrane-associated spectral components could be resolved by spectral subtractions using the extracted lipid spectra for the fluid component, as described previously (11, 40). The different proportions of the two components in the spectra of Figure 1 clearly reflect a specific lipid selectivity pattern for interaction with the protein. [It will be noted that the different spectra in Figure 1 have somewhat different signal/noise ratios. This arises from different specific spin activities of the different labels and different extents of incorporation into the membranes. For reliable quantitation it is essential to work with rather low spin-label concentrations in order to avoid spin—spin broadening of the spectra (cf. refs 12 and 13).]

The values for the fractions, f^L , of the different spin-labeled lipids (L) that are motionally restricted by the protein were determined from the spectral subtractions and are given in Table 1. A clear selectivity between the various lipids is evident, with values for f^L ranging between 0.06 and 0.40

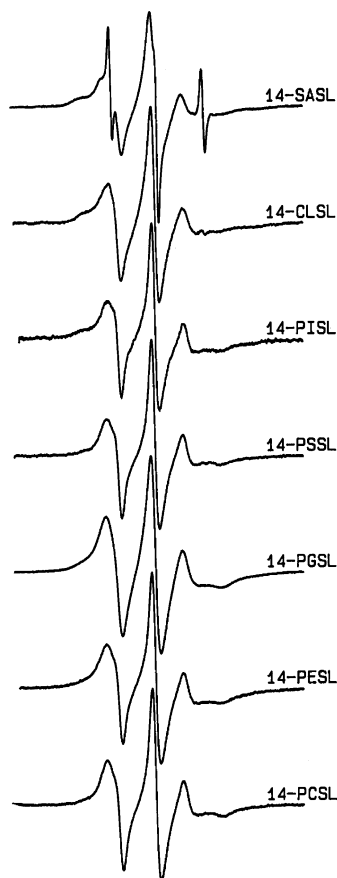


FIGURE 1: ESR spectra of 14-C atom position spin labels in acetylcholine receptor-rich membranes from *T. marmorata*. From top to bottom: stearic acid spin label, 14-SASL; cardiolipin spin label, 14-CLSL; phosphatidylinositol spin label, 14-PISL; phosphatidylserine spin label, 14-PSSL; phosphatidylglycerol spin label, 14-PGSL; phosphatidylethanolamine spin label, 14-PESL; and phosphatidylcholine spin label, 14-PCSL. Buffer: 10 mM Hepes, pH 8.0. $T = 22^\circ\text{C}$; scan width = 100 G. Spectra are normalized to the same maximum positive line height.

Table 1: Fractions of Protein-Associated Component, f^L , Relative Association Constants, K_r^L/K_r^{PC} , and Differential Free Energies of Association, $\Delta G^L - \Delta G^{\text{PC}}$, for Spin-Labeled Lipids, L, in nAChR-Rich Membranes from *T. marmorata* at 22°C

lipid, L	f^L ^a	K_r^L/K_r^{PC}	$\Delta G^L - \Delta G^{\text{PC}}$ (kJ/mol)
14-SASL	0.39	4.9	-3.9
14-CLSL	0.40	5.1	-4.0
14-PISL	0.38	4.7	-3.8
14-PSSL	0.26	2.7	-2.4
14-PGSL	0.18	1.7	-1.3
14-PCSL	0.12	1.0	0.0
14-PESL	0.06	0.5	+1.8

^a Estimated errors in f^L are ca. ± 0.02 .

for 14-PESL and 14-CLSL, respectively. Assuming that specificity arises from differences in lipid affinity, rather than in the stoichiometry of the lipid/protein interaction, the association constant, K_r^L , for spin-labeled lipid L relative to that of spin-labeled PC, K_r^{PC} , can be obtained simply from the values of f^L (see, e.g., ref 40):

$$K_r^L/K_r^{\text{PC}} = [(1 - f^{\text{PC}})/f^{\text{PC}}]/[(1 - f^L)/f^L] \quad (1)$$

where f^{PC} is the fraction of motionally restricted 14-PCSL. The association constant for equilibrium lipid exchange at a

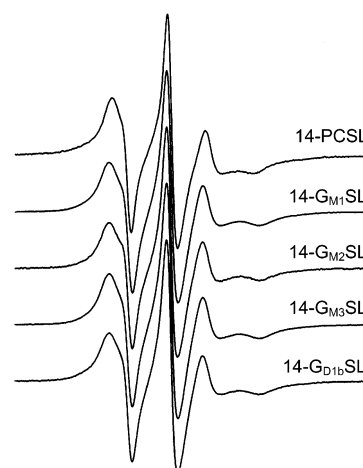


FIGURE 2: ESR spectra of various spin-labeled gangliosides (14-GM1SL, 14-GM2SL, 14-GM3SL, and 14-GD1bSL) in nAChR-rich membranes from *T. marmorata* at 22°C . The spectrum of spin-labeled phosphatidylcholine (14-PCSL) is shown for reference. Buffer: 10 mM Hepes, pH 8.0. Total scan width = 100 G. Spectra are normalized to the same maximum positive line height.

single site, i , is given by

$$K_i = L_{b_i}^* L_f / (L_{b_i} L_f^*) \quad (2)$$

where L_{b_i} and $L_{b_i}^*$ are the moles of unlabeled and labeled lipid that are associated at site i on the intramembraneous surface of the protein and L_f and L_f^* are similarly defined for the free (nonassociated) lipid populations. The average relative association constant, K_r^L , that is measured by ESR at low label concentrations is given by (40)

$$K_r^L = \sum_{i=1}^m n_i K_i / N_b \quad (3)$$

where n_i is the number of association sites of type i and $N_b = \sum n_i$ is the total number of lipid association sites at the intramembraneous surface of the protein.

Values for the relative association constants, K_r^L/K_r^{PC} , normalized to PC, together with the corresponding differential free energies of association $\Delta G^L - \Delta G^{\text{PC}} = -RT \ln[K_r^L/K_r^{\text{PC}}]$, are also listed in Table 1. Because these are average values, they are unable to distinguish between just one site of high affinity and a smaller generalized increase in affinity for all sites or between either and some intermediate between these two extremes. Similarly, the values correspond also to an average between sites on the nAChR and sites on other non-nAChR integral proteins in the membrane.

Ganglioside-Protein Interactions. ESR spectra of C-14 spin-labeled gangliosides, GM₁, GM₂, GM₃, and GD_{1b}, incorporated in nAChR-rich membranes from *T. marmorata* are shown in Figure 2. The spectra from all four gangliosides are very similar and hardly differ from that of the phosphatidylcholine reference, 14-PCSL. This indicates that the ganglioside chains are integrated in the membrane similarly to those of the glycerophospholipids. The similarity extends also to the lipid-protein interactions, as is confirmed by spectral subtractions. These latter demonstrate that the motionally restricted ganglioside component, which has an outer hyperfine splitting, $2A_{\text{max}}$, of approximately 60 G at

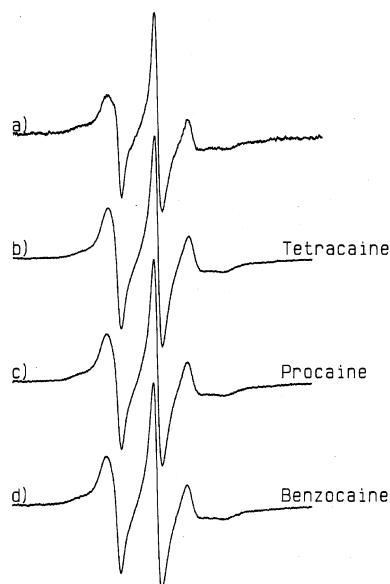


FIGURE 3: ESR spectra of 14-PISL phosphatidylinositol spin label in acetylcholine receptor-rich membranes from *T. marmorata* (1.5 nmol of α -BTx binding sites/mg of protein) in the presence and absence of local anesthetics: (a) membranes alone, (b) 0.2 mg/mL (0.66 mM) tetracaine added, (c) 0.2 mg/mL (0.73 mM) procaine added, and (d) 0.2 mg/mL (1.21 mM) benzocaine added, at a protein concentration of 0.35 mg/mL (0.53 μ M α -BTx sites). Buffer: 10 mM Hepes, pH 8.0. $T = 22^\circ\text{C}$; scan width = 100 G. Spectra are normalized to the same maximum positive line height.

22°C , is very similar to that for 14-PCSL. Also, the fractional population of motionally restricted ganglioside is very similar to that for 14-PCSL. Relative association constants K_r/K_r^{PC} are ≈ 1.1 for 14-GD1bSL and ≈ 1.0 for the spin-labeled monosialogangliosides.

Competition with Local Anesthetics. Phosphatidylinositol is one of the spin-labeled lipids that displays a high specificity for the nAChR (Table 1). The ESR spectra of the phosphatidylinositol spin label (14-PISL) in nAChR-rich membranes that have been preincubated with different local anesthetics are given in Figure 3. From this figure, it is clear that some of the spin-labeled phospholipid is displaced from the protein in the presence of the local anesthetics procaine, tetracaine, and benzocaine. With local anesthetic present, the proportion of the motionally restricted component is reduced relative to that of the fluid component in the spectra of 14-PISL. The values for the fraction of motionally restricted spin-labeled lipid in the presence and absence of the different local anesthetics were obtained by spectral subtraction. These values, together with the parameters governing the lipid–protein selectivity, are given in Table 2 for membranes labeled with 14-PISL and 14-SASL, respectively. The thermodynamic parameters are all referred to 14-PCSL in nAChR-rich membranes in the absence of local anesthetics. The changes are seen to be considerably smaller in the case of 14-SASL than for 14-PISL.

Membrane Partitioning of Local Anesthetics. Figure 4 shows the fluorescence quenching of 1-pyrenedecanoic acid in nAChR-rich membranes on addition of increasing amounts of benzocaine. The quenching of the fluorescence intensity, I , is described by the Stern–Volmer relation:

$$I_0/I = 1 + K_Q c_M \quad (4)$$

Table 2: Effects of Local Anesthetics (0.2 mg/mL) on the Fraction of Protein-Associated Component, f^L , Relative Association Constant, K_r^L/K_r^{PC} , and Change in Free Energy of Association, $\Delta\Delta G = \Delta G^L(\text{LA}) - \Delta G^L(0)$, of Spin-Labeled Phosphatidylinositol, 14-PISL, and Stearic Acid, 14-SASL, in nAChR-Rich Membranes from *T. marmorata* at 22°C

local anesthetic	f^L ^a	K_r^L/K_r^{PC} ^b	$\Delta\Delta G(\text{LA})$ (kJ/mol)
14-PISL	0.38	4.7	0.0
+tetracaine	0.22	2.1	1.9
+procaine	0.29	3.1	1.0
+benzocaine	0.30	3.3	0.9
14-SASL	0.39	4.9	0.0
+tetracaine	0.31	3.4	0.9
+procaine	0.39	4.9	0.0
+benzocaine	0.37	4.5	0.2

^a Estimated errors in f^L are ca. ± 0.02 . ^b Values of K_r^{PC} are for 14-PCSL in the absence of local anesthetics.

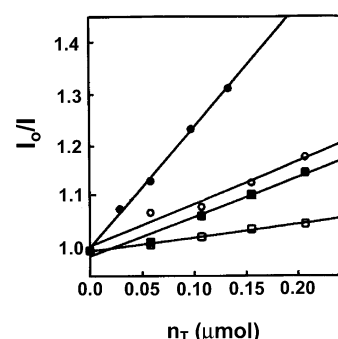


FIGURE 4: Quenching of the fluorescence intensity, I , of 1-pyrenedecanoic acid (7.3 μ M) in nAChR membranes at total lipid concentrations of 0.05 (●), 0.1 (○), 0.2 (■), and 0.4 (□) mg/mL by addition of n_T μ mol of benzocaine. I_0 is the fluorescence intensity in the absence of benzocaine.

where I_0 is the value of I in the absence of quencher, c_M is the membrane concentration of quencher, and K_Q is the quenching constant. The concentration of quencher in the membrane is given by

$$c_M = \frac{n_T}{V_M + V_W/K_P} \quad (5)$$

where n_T is the total number of moles of quencher, V_M and V_W are the volumes of the membrane and aqueous phase, respectively, and K_P is the membrane/water partition coefficient of the quencher. Combination of eqs 4 and 5 allows elimination of K_Q if measurements are made at different values of V_M , as in Figure 4 (see also ref 41). A lipid/protein ratio of 0.62 (w/w) for *D. tschudii* (10) and the lipid partial specific volume are used to determine V_M from the amount of membrane protein. The resulting values for the partition coefficients of benzocaine, procaine, and tetracaine in nAChR-rich membranes from *D. tschudii* are given in Table 3. The partition coefficients are scaled by the partial specific volume, \bar{v}_m , of the membrane lipid (42). For tetracaine, a very similar value of $K_P \bar{v}_m = 2200 \pm 600$ mL/g is also obtained with membranes from *T. marmorata*. To allow for curvature in the Stern–Volmer plots with the partially charged anesthetics, procaine and tetracaine, the method proposed by Lissi et al. (41) was used to deduce the partition coefficients. Evaluations according to this protocol were made consistently at the level of membrane incorporation

Table 3: Partition Coefficients, K_p , of Local Anesthetics (LA) in *D. tschudii* nAChR-Rich Membranes^a

LA	$K_p \bar{v}_m$ (mL/g) ^b	LA/protein (mol/g)
benzocaine	14700 ± 5100	$(2.70 \pm 0.29) \times 10^{-3}$
tetracaine	2500 ± 500	$(0.71 \pm 0.10) \times 10^{-3}$
procaine	2300 ± 1300	$(0.74 \pm 0.34) \times 10^{-3}$

^a Estimated local anesthetic/protein ratios in the membrane, under the conditions applying to Figure 3, are also given. ^b Partition coefficients are normalized by the partial specific volume, \bar{v}_m , of the membrane lipid.

giving a fixed degree of quenching, $I_0/I = 1.1$, for the different total amounts of membrane. This corresponds to an effective anesthetic concentration of ~ 1 M in the membrane.

By using the values of the partition coefficients, it is then possible to determine the membrane concentrations of local anesthetics that correspond to the conditions of the ESR competition experiments (see eq 5). These values calculated for the local anesthetic/protein ratios in the membrane are given also in Table 3. A membrane protein/lipid ratio of 0.69 (w/w) for *T. marmorata* nAChR-rich membranes (10) and a lipid partial specific volume $\bar{v}_m \approx 1$ mL/g (43) are used for this purpose. The corresponding effective molar ratios of local anesthetic to membrane lipid + cholesterol are 0.5 for procaine and tetracaine and 2 for benzocaine.

DISCUSSION

The ESR spectra of the different spin-labeled lipids in nAChR-rich membranes from *T. marmorata* clearly evidence a direct interaction with the membrane protein, as found previously for a more restricted range of lipids (11–13). The various quantitative aspects of the results are discussed below.

Lipid/Protein Stoichiometry. Because phosphatidylcholine is the majority lipid in the membrane, it may be used to make a first estimate of the number of lipid sites associated with the protein. Spin-labeled phosphatidylcholine is associated to a level of approximately 10–15% with the integral membrane protein (Table 1). Neglecting for the moment the selectivity between the different lipids (but see below), the total number of association sites on the protein therefore corresponds to 12% of the total lipids, i.e., 42 ± 7 sites (27 phospholipids and 15 cholesterol) per 268 kDa protein. This estimate assumes that the transmembrane extent of all proteins in the nAChR-rich membranes is comparable to that of the nAChR protein. Nevertheless, the value obtained is close to those determined previously (38–46 lipids/nAChR) by lipid/protein titration with purified nAChR reconstituted in DOPC (12). These values can be compared with the number of lipids which may be accommodated around the intramembranous perimeter of the protein, deduced from structural data. In the nAChR structure determined by Unwin and colleagues (1, 44), the intramembranous cross section of the protein at the level of the lipid chains can be approximated by a pentagon of side ~ 3.5 nm. At the level of the lipid headgroups, the cross section is more circular with a diameter of ~ 7 nm (1). Approximately 41 lipid chains of diameter 0.48 nm can be accommodated around the former structure and 49 chains around the latter. Figure 5 gives a schematic arrangement of the lipid chains around the electron crystallographic cross sections of the receptor from ref 1.

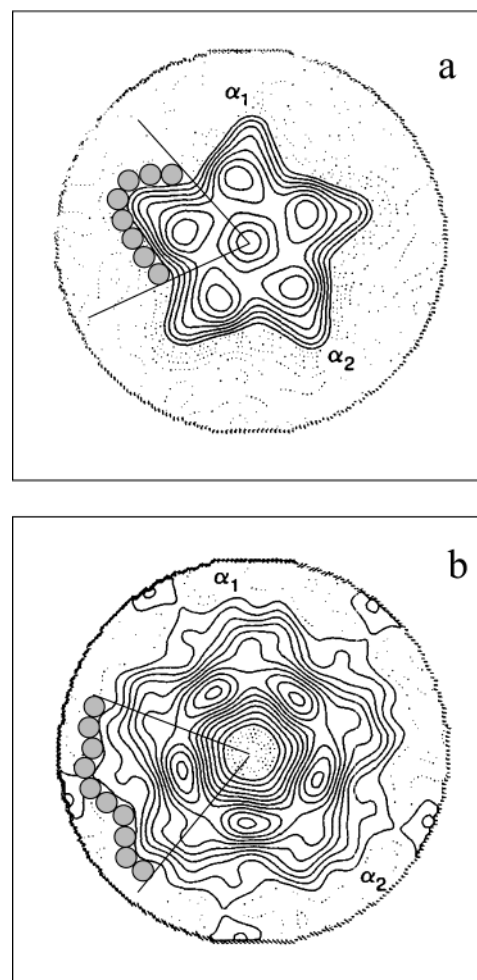


FIGURE 5: Sections normal to the 5-fold axis of the nAChR after 5-fold averaging: (a) at the middle of the synaptic leaflet of the bilayer; (b) at the level of the phospholipid headgroups in the cytoplasmic leaflet (1). Circles represent lipid chains (0.48 nm diameter) adjacent to one subunit of the pentamer. Reprinted with permission from ref 1. Copyright 1993 Elsevier.

These correspond approximately to the narrowest and broadest sections of the transmembrane domain. Therefore, the number of diacyllipids that can be accommodated at the intramembranous perimeter for both sides of the bilayer is estimated to be 40–50. This is in reasonable agreement with the stoichiometry of the motionally restricted lipid component obtained from spin-label ESR measurements. (Note that the cross section of a cholesterol molecule is approximately the same as that of a two-chain lipid.)

Ganglioside–Protein Interactions. The ESR spectra of the spin-labeled gangliosides indicate that they are fully integrated into the native nAChR membrane in a manner similar to that of the endogenous lipids (see Figure 2). Studies on the cellular incorporation of spin-labeled ganglioside analogues also have demonstrated that the spin-labeled derivatives are incorporated in a manner similar to that of the parent unlabeled gangliosides (45, 46).

Although ESR spectra reveal clear evidence for interaction of the gangliosides with the intrinsic protein, the fractions of motionally restricted 14-C gangliosides are practically the same as that for 14-PCSL. Relative to phosphatidylcholine, the majority background lipid of the native membrane, there is no specific enrichment of the gangliosides tested at the

lipid—protein interface of the nAChR. Thus a selective interaction that might target the protein to glycosphingolipid rafts, or serve as the nucleation for raft formation, appears to be lacking in the nAChR. It has been suggested that sphingolipid raft formation may assist, or even precede, cytoskeletal receptor clustering via rapsyn at the postsynaptic membrane (21, 22). In contrast, phosphatidic acid, which displays a specificity for the receptor (12), has been found to nucleate lipid domains around the receptor (47). The lack of association with gangliosides does not necessarily exclude that other sphingolipids may participate in domain formation. In fact, recent studies indicate a moderate affinity of a fluorescent adduct of sphingomyelin for the membrane-bound and purified reconstituted nAChR (48).

Nevertheless, it is significant that there are no energetically unfavorable interactions, relative to phosphatidylcholine, between gangliosides and the acetylcholine receptor. Gangliosides are not excluded from the first shell of lipids surrounding the protein and hence may potentially affect nAChR function via a direct lipid—protein interaction. It is relevant to note that G_{M1} is one of the major gangliosides in nAChR-rich membranes from *T. marmorata*, and G_{M2} , G_{M3} , and G_{D1b} are also found as constituents (14). Furthermore, in the latter referenced work, antibodies to gangliosides of the c-pathway type, such as G_{Q1c} and G_{P1c} , were found to colocalize with the nAChR at the ventral, innervated face of the electrocyte.

Lipid Specificity. On the basis of the data of Table 1, the different lipids can be divided into a high specificity group (14-SASL, -CLSL, and -PISL), an intermediate group (14-PSSL and -PGSL), and a low specificity group (14-PCSL and -PESL). The latter group also includes the gangliosides (14-GD1bSL, -GM1SL, -GM2SL, and -GM3SL). From previous work, it is clear that phosphatidic acid (12) and androstanol (13) also belong to the high specificity group. It also seems likely that cholestane belongs to the low specificity group (49, 50). On the basis of lipid/protein titration of reconstituted nAChR, it was found by Ellena et al. (12) that 14-PSSL displayed a lower selectivity relative to DOPC. The phosphatidylserine selectivity relative to the natural lipid environment of native membranes is apparently somewhat different. This can be attributed, at least in part, to the different background lipid against which the spin-labeled lipid is competing in the two systems.

Previous spin-label ESR studies have found that monosialogangliosides, although negatively charged, display no selectivity relative to phosphatidylcholine for the Na,K-ATPase (33). Also only a modest selectivity of the Na,K-ATPase was found for the disialoganglioside, G_{D1b} . These results are similar to those obtained here for ganglioside interactions with the nAChR. In the electroplax, however, the Na,K-ATPase is confined to the dorsal, noninnervated membrane of the electrocyte, i.e., is segregated from the nAChR-containing innervated membrane domains.

It will be noted that, although the experiments were conducted at low ionic strength, the selectivity between the different lipids that is found here cannot be primarily of direct electrostatic origin, because lipids of the same formal charge display widely different selectivities (see Table 1). Accordingly, the selectivity found for 14-PISL in Table 1 shows no dependence on ionic strength (data not shown). A similar result was obtained also for the selectivity exhibited by the

receptor for stearic acid (51). In addition, a selectivity of certain other negatively charged lipids for the nAChR has been observed at higher ionic strength (12).

Information about the lipid composition of the boundary layer of the nAChR can be obtained if it is assumed that the number, n^L , of lipids of a particular species, L, that is associated with the receptor is approximated by $n^L = n_t^L f^L$, where n_t^L is the total number of lipids of species L per protein and f^L is the fraction of motionally restricted spin label of type L in nAChR-rich membranes. Using the data from Table 1, together with the lipid composition and phospholipid/protein ratio of nAChR-rich membranes, yields the following values for n^L : 11 phosphatidylcholines, 4 phosphatidylethanolamines, 8 phosphatidylserines, 3 phosphatidylinositols, 1 cardiolipin, and 0.3 phosphatidic acid per protein, respectively. (The value for phosphatidic acid is estimated from the data of ref 12.) The errors in these estimates range from 5% to 20% depending on the value of f^L . If it is additionally assumed, as above, that cholesterol also occupies sites at the lipid—protein interface, it can be estimated that there are a total of approximately 44 lipid sites per protein. This again is in agreement with the total number of lipid sites found on the nAChR in reconstituted nAChR/DOPC vesicles (12). It should be noted that this estimate of the average composition of the boundary layer assumes, among other things, that the selectivities registered by spin-labeled lipids at probe concentrations correspond to an average increase in selectivity for all available boundary sites. If a limited number of sites of much higher specificity exists, then the results might be rather different.

Interesting is the high specificity observed here for the *T. marmorata* lipids phosphatidylinositol and cardiolipin. Significant amounts of these two lipids are predicted to be associated with the receptor. Functional studies on reconstituted systems also point to the significance of selectivity between negatively charged lipids, which are necessary for effective reconstitution. The agonist-induced ion flux response was found to be in the ratio 1:0.7:0.4 for phosphatidylcholine/cholesterol vesicles containing 20 mol % of phosphatidic acid, cardiolipin, or phosphatidylserine, respectively (5). For the two latter anionic lipids, these results correlate well with the selectivities given in Table 1.

Of the negatively charged lipids, phosphatidylinositol may be of particular importance. The incorporation of phosphate into phosphatidylinositol was shown to be stimulated by acetylcholine (52). Phosphatidylinositol was found to be more efficient than phosphatidylcholine in restoring activity of the delipidated, purified nAChR (19). In experiments with fluorescent labeled lipids, phosphatidylinositol was also found to behave differently from phosphatidylcholine in nAChR-rich membranes, in a manner which was attributed to specificity in lipid—protein interaction (53). Inositol phospholipids have also been found to be metabolically very active in *Torpedo* electrocytes (54), and their extraction from the membrane results in changes in the phosphorylation state of the nAChR protein (20).

Competition with Local Anesthetics. The results of Table 2 indicate that the aminated local anesthetics are able to compete with phospholipids for sites at the lipid—protein interface. The order of efficiency in competition, under the conditions used, is tetracaine > procaine \approx benzocaine. This pattern correlates neither with the charge on the local

anesthetic, which is expected to be in the order procaine (intrinsic pK 8.9) > tetracaine (intrinsic pK 8.2) > benzocaine (permanently uncharged), nor with the membrane partition coefficients, which are in the order benzocaine \gg tetracaine \approx procaine (see Table 3). The relative association constants for spin-labeled analogues of the local anesthetics in nAChR-rich membranes were previously measured to be in the order benzocaine spin label (2.3) > tetracaine spin label (1.9) > procaine spin label (1.3) (18). However, it is difficult to assess to what extent this pattern of association may be affected by the modification of the molecular structure by the substitution with the spin-label group. The relative association constants given for 14-PISL in Table 2 vary because the background lipid environment with which the spin label is competing is different. As already noted, the local anesthetic content in the membrane is comparable to that of the endogenous lipid (see Table 3, third column). Therefore, it is to be expected that it might modify the thermodynamic standard state (i.e., the background lipid milieu) relative to which the determinations of K_r are referred. An estimate of the relative association constants for the local anesthetics can be obtained from the ratio of K_r without anesthetic to that with anesthetic. This yields $K_r^0/K_r = 2.2$, 1.5, and 1.4 for tetracaine, procaine, and benzocaine, respectively. Although, the relative order differs, these values are comparable in size to the relative association constants for the spin-labeled anesthetics (18). Because the order of these values of K_r^0/K_r differs from that of the partition coefficients (cf. Table 3), it can be inferred that the latter are determined primarily by the hydrophobic environment of the membrane, rather than by protein binding per se. This conclusion is consistent with the magnitude of the observed values of K_r^0/K_r .

The present results on local anesthetic interactions in nAChR-rich membranes can be contrasted with those found for general anesthetics (55). Although a reduction in the motionally restricted lipid population was found in the latter case, this was accompanied in nearly all instances by a disordering of the fluid lipid population. This is not observed for the local anesthetics at the concentrations used here; the spectral characteristics of the fluid lipid component remain unchanged (Figure 3). It is possible that small general anesthetic molecules such as diethyl ether or ethanol may reduce the motionally restricted lipid population by fluidizing some of the boundary lipid chains, i.e., an interstitial rather than a substitutional effect. This would be consistent with the enhanced exchange rate observed at the lipid–protein interface (55).

It is interesting that the local anesthetics are not able to compete so effectively with 14-SASL as with 14-PISL, even though both lipids have rather similar relative association constants in the absence of anesthetics (Table 2). The effect of the local anesthetics therefore does not seem to be simply one of electrostatic competition at the protein surface, as can also be inferred from the partial displacement of 14-PISL by benzocaine. The different responses with 14-SASL as compared to 14-PISL give further support to the proposal that additional sites for fatty acids exist at the intramembranous surface of the receptor, which are different from those for phospholipids (23, 56). Significantly, the general anesthetic hexanol was also found to have relatively little effect on the motionally restricted population of 14-SASL (55). It

has also been found that benzocaine, tetracaine, and procaine are capable of displacing the neutral steroid, spin-labeled cholestane, from its sites at the lipid–protein interface (49). The present results indicate that local anesthetics can occupy phospholipid sites, because 14-PISL is displaced in the competition experiments. Tetracaine, at least, can occupy sites taken up by fatty acid, because 14-SASL was partially displaced. It is possible that there is no very clear distinction between phospholipid and local anesthetic sites at the hydrophobic surface of the protein or at least that some sites overlap. The present results agree with previous ones using spin-labeled local anesthetics (16–18) in demonstrating that sites are available to noncompetitive blockers at the lipid–protein interface.

NOTE ADDED IN PROOF

The most recent electron crystallographic model of the nAChR pore suggests that alcohols and anesthetics might bind in water-filled spaces behind the pore-lining M2 helices (57).

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