Association of Spin-Labeled Local Anesthetics at the Hydrophobic Surface of Acetylcholine Receptor in Native Membranes from *Torpedo marmorata*†

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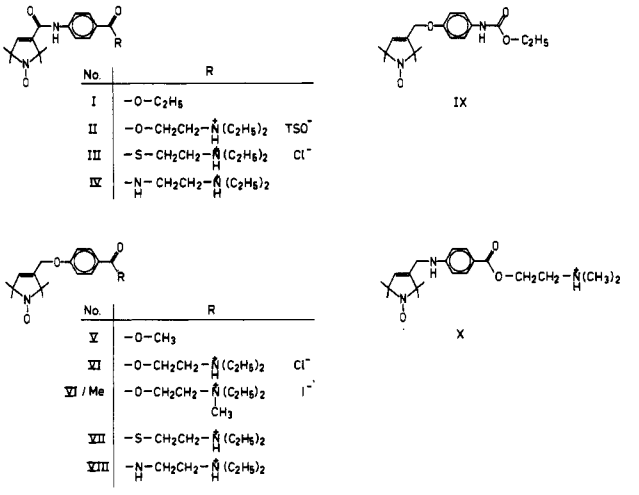
ABSTRACT: The interactions between a series of spin-labeled local anesthetic analogues and the nicotinic acetylcholine receptor (AChR) have been investigated by means of electron spin resonance (ESR) and fluorescence spectroscopy. The paramagnetic local anesthetic analogues quenched the intrinsic tryptophan fluorescence of AChR-rich membranes in an agonist-dependent manner, demonstrating a direct interaction with the AChR. The quenching efficiency was greater for the benzocaine than for the thioprocaine analogue. The protein was found to restrict directly the molecular motion of the spin-labeled analogues, as seen by the appearance of a highly anisotropic component in the ESR spectrum. The relative affinity of the population of local anesthetic probes which interacts directly with the integral protein of the AChR-rich membranes was calculated on the basis of relative association constants, K_r, determined by ESR. By comparison with the relative association constant for spin-labeled phospholipid, K_{ro}, it was possible to differentiate between local anesthetic analogues interacting with high ($K_r/K_{ro} > 2$), intermediate ($K_r/K_{ro} = 1.6–1.9$), and low ($K_r/K_{ro} \leq 1.3$) specificity and to calculate the fraction of protein-associated probe in each case. Differences were observed in the presence of agonist (0.1 mM carbamylcholine) with some, but not all, of the spin-labeled derivatives. The role of the protonatable diethylammonium group in the specificity of the interaction of the procaine and thioprocaine analogues was investigated. Only in the uncharged form, or in the charged form at high ionic strength, was there a preferential association of these two local anesthetic analogues. The specificity of the benzocaine derivative, which lacks the basic side chain, was unaffected by changes in pH or ionic strength.

The nicotinic acetylcholine receptor (AChR)[1] from the electric organ of Torpedinidae is an integral membrane glycoprotein which recognizes and binds acetylcholine, thereby controlling cation permeability of the postsynaptic membrane [for a recent review, see Barrantes (1988)]. The AChR in the native membrane is located in densely packed regions surrounded by a shell of motionally restricted lipids as an interface toward other membrane constituents (Marsh & Barrantes, 1978; Ellena et al., 1983). Of the aminated and quaternary ammonium compounds which interact with AChR as agonists or antagonists, aromatic amines are of particular interest because these molecules can bind either to low-affinity sites at the lipid–protein interface (Lee, 1976) or to discrete high-affinity sites at (or near to) the specific cation recognition site of the acetylcholine ionophore, thereby regulating channel openings (Heidemann et al., 1983). A multiple-site model has been elaborated to explain the positive cooperativity of noncompetitive antagonists such as local anesthetics and histrionicotoxin on the conformational transitions of AChR (Sine & Taylor, 1982).

Spin-label electron spin resonance (ESR) spectroscopy has an inherent time scale optimally suited to resolving the spectra of freely tumbling small molecules in the aqueous phase from those in the fluid bilayer regions of the membrane and of lipids in the latter environment from those motionally restricted at the intramembranous surface or bound to integral membrane proteins [for a review, see Marsh (1985)]. Thus, the ESR spectra of spin-labeled local anesthetics provide a most useful method for studying the molecular details of lipid–local anesthetic–protein interaction in receptor-rich membranes. The synthesis of spin-labeled intracaine[2] has been reported by

Chart I: Molecular Structures of Spin-Labeled Local Anesthetic Analogues



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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Gargiulo et al. (1973), and two classes of association sites have been identified by Earnest et al. (1984); the motionally restricted component observed in the presence of carbamylcholine chloride has been assigned to molecules at the high-affinity allosteric site of the acetylcholine ionophore (Palma et al., 1985). The charge state of the protonatable diethylammonium group has been correlated to the anesthetic potency (Blickenstaff & Wang, 1985).

Here we have examined the interaction of local anesthetics in AChR-rich membrane vesicles from *Torpedo marmorata*, making use of the designed series of spin-labeled local anesthetic analogues shown in Chart I (Hideg et al., 1979). The effects of partitioning between the aqueous and membrane phases and motional restriction at the intramembranous surface of the integral protein in the AChR-rich membranes have been separated, and it is shown that a certain population of the local anesthetics are in contact with the AChR in a manner similar to that of fatty acids and phospholipids. The role of desensitization and amine or quaternary charge has been investigated in an attempt to obtain a coherent picture of the structure and function relationships.

Materials and Methods

Materials. *N*-[^{3}H]Propionyl-α-bungarotoxin (sp act. 107 Ci/mmol) was from Amersham International PLC (Amersham, U.K.); Percoll and Sephadex G-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Unlabeled benzocaine, procaine, and tetracaine were from Sigma (St. Louis, MO). Spin-labeled local anesthetic analogues I–IV (see Chart I) were synthesized as described in Hideg et al. (1979). The other spin-label derivatives, V–X (see Chart I), were synthesized by analogous methods, and their detailed characterization will be reported elsewhere. All other reagents were of the highest purity available.

Purification of AChR-Rich Membrane Vesicles. *T. marmorata* specimens from the Bay of Arcachon, France, were generously provided by Prof. V. Whittaker (Max-Planck-Institut für biophysikalische Chemie) and maintained in aquaria until use. A crude membrane pellet from homogenized electric organs was prepared according to the method of Lindstrom et al. (1980), and membrane vesicles enriched in AChR were obtained by discontinuous sucrose gradient centrifugation as described by Elliot et al. (1980). Sealed and leaky vesicles were further fractionated by the method of Sachs et al. (1982) by exchange of Na^+ within the vesicles for external Cs^+ and by subsequent separation on the basis of density on a Percoll–CsCl gradient. The specific α-bungarotoxin binding activity, assayed by the method of Schmidt and Raftery (1973), was typically 1.1–2.4 nmol of ^{3}H-labeled α-bungarotoxin/mg of protein. Protein determination was performed according to the method of Lowry et al. (1951) with bovine serum albumin as standard. In all ESR and fluorescence quenching experiments the sealed vesicle fraction was used as the native membrane.

ESR Experiments. Sealed membrane vesicles were centrifuged in an Eppendorf centrifuge for 15 min at full speed in order to remove the remaining CsCl–Percoll, and the floating layer was washed in 10 mM sodium phosphate, pH 7.4, or 10 mM Hepes, pH 8.0. The resuspended pellet in 1-mL final volume (0.3–0.6 mg of protein, corresponding to ca. 0.5 μM AChR) was labeled with 20 μg of spin-labeled local anesthetics (concentration range: 46 μM for III to 64 μM for I) previously dissolved in absolute ethanol (2% of the total volume) and allowed to interact at room temperature for 45 min. Then the vesicles were centrifuged at 45 000 rpm in a Beckman 50 Ti rotor for 45 min, and the pellet was transferred into ESR sample capillaries (1-mm i.d.) and concentrated in a bench-top centrifuge. To minimize the aqueous signal, the samples were trimmed to 10-mm height by carefully removing excess vesicle suspension and supernatant. The estimated spin-label/AChR ratio was 15 mol/mol in the membrane, assuming a partition coefficient of 10.

ESR spectra were recorded with a Varian E-12 Century line spectrometer equipped with a nitrogen gas flow temperature regulation system. Temperatures were measured to ± 0.1 °C with a thermocouple placed just above the cavity within the sample capillary. ESR spectra were collected on an IBM personal computer with Labmaster interface (12 bit A/D resolution) using software written by Dr. M. D. King (Max-Planck-Institut für biophysikalische Chemie) and stored as 1-kword data files. Spectral subtractions were performed with a VT-11 interactive graphic display processor (Digital Equipment Co.) using software written by Dr. W. Möller (Max-Planck-Institut für biophysikalische Chemie).

Fluorescence Measurements. Membrane fragments (80–90 nM α-bungarotoxin binding sites, 55–60 μg of protein/mL) were suspended in 1 mL of 10 mM sodium phosphate buffer, pH 8.0, by brief sonication (2 min) and incubated with 1–25 μg of spin-labeled local anesthetics (previously dissolved in 5 μL of ethanol) at room temperature for 70 min. The effect of agonist was measured in parallel experiments. Carbamylcholine chloride (0.1 mM) was allowed to react with AChR-rich membranes for 50 min prior to the addition of local anesthetic (desensitized state) or added simultaneously with the local anesthetic and incubated for 70 min. The vesicle suspensions were placed in 1 × 1 cm quartz cuvettes, and the intrinsic fluorescence of the protein was recorded as the total spectrum above 320 nm (Schott WG-320 filter) with an SLM spectrofluorometer using Xe–Hg arc excitation at 296 nm (200 W). In order to correct for inner-filter effects, the absorption of the procaine thioester (III) and benzocaine (I) spin-labels at the excitation and emission wavelengths was measured in buffer alone. The static absorption factor, I, used to correct the apparent quenching is given by

$$\log I = -A_{ex}(L_{ex}/2)[c]/[c_{ex}] - A_{em}(L_{em}/2)[c]/[c_{em}] \quad (1)$$

where [c] is the concentration of spin-labeled local anesthetic probe used in the fluorescence quenching experiments, $[c_{ex}]$ and $[c_{em}]$ are the concentrations used to determine the absorption at excitation (A_{ex}) and emission (A_{em}) wavelengths, respectively, and L_{ex} and L_{em} are the respective path lengths along the excitation and emission axes.

Results

Assignment of Spectral Components. A typical ESR spectrum of the spin-labeled analogue of procaine thioester (compound III) in native membranes from *T. marmorata* is shown in Figure 1A. The spectrum is dominated by the extremely narrow peaks from the proportion of the spin-labeled molecules remaining in the aqueous phase. The contribution of this aqueous spectral component to the integrated intensity is small; from quantitative double integration, a fraction of 8% was obtained for compound III (Figure 1A), and the fraction varied between 8% and 32% for the other spin-labeled local anesthetic analogues (see f_{aq} values in Table I). By comparison of the ESR spectra obtained in the native membrane and in aqueous dispersions of the extracted lipids

[2] 2-[*N*-Methyl-*N*-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl)amino]-ethyl 4-(hexyloxy)benzoate and 2-[*N*,*N*-dimethyl-*N*-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl)amino]ethyl 4-(hexyloxy)benzoate, which are the nitroxide-labeled tertiary and quaternary analogues of intracaine, respectively (Earnest, 1984; Palma et al., 1985).

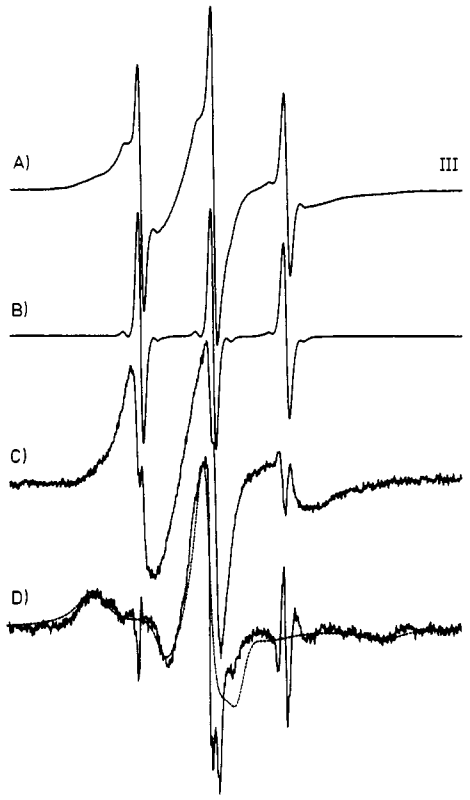

FIGURE 1: Spectral subtractions with a typical ESR spectrum of compound III (spin-labeled analogue of procaine thioester): (A) AChR-rich membranes from *T. marmorata*; (B) aqueous component (downscaled); (C) lipid-associated component; (D) protein-associated component. Spectra C and D were obtained by subtracting the aqueous signal from the spectra of the pH-dependence series (Figure 5) and resolving the two membrane-associated components by intersubtractions between these difference spectra. The lower spectrum is shown together with a simulated slow-motion line shape (dotted line), which was used in all subsequent spectral subtractions. Concentrations are given under Materials and Methods. Total scan width = 100 G; T = 22 °C.

Table I: Lipid/Water Partitioning, f_{aq}, and Relative Association Constant, K_r/K_{ro}, of Spin-Labeled Local Anesthetic Analogues in Acetylcholine Receptor Rich Membranes from *T. marmorata*[a]

compd	f_{aq}	f	K_r/K_{ro}	$\Delta G - \Delta G_o$ (kJ/mol)
III (procaine thioester)	0.08	0.34	2.2	−1.9
I (benzocaine)	0.11	0.35	2.3	−2.0
V	0.12	0.31	1.9	−1.6
VI	0.12	0.27	1.6	−1.1
IX	0.18	0.36	2.4	−2.1
VI$^+$/Me	0.21	0.31	1.9	−1.6
II (procaine)	0.22	0.23	1.3	−0.6
X (tetracaine)	0.23	0.31	1.9	−1.6
VIII	0.30	0.28	1.7	−1.2
IV (procainamide)	0.32	0.29	1.7	−1.4

[a] f_{aq} is the fraction of spin-labeled compound in the aqueous phase. A value of f_o = 0.19, corresponding to spin-labeled phosphatidylcholine, was used in calculating K_r/K_{ro} (cf. eq 2).

(spectrum not shown), the presence of a third spectral component was evident in the outer wings of the membrane spectra. The two membrane-associated spectral components could be resolved by subsequent pairwise intersubtractions with membrane spectra containing different proportions of these two components, as illustrated in Figure 1. The sharp spikes in spectra C and D arise from slight mismatches in the subtraction of the aqueous component and do not contribute appreciably to the total integrated spectral intensity.

The two spectral components, which were observed in both native membranes and dispersions of the extracted lipids, were

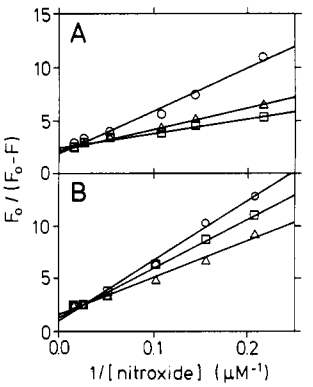

FIGURE 2: Modified Stern–Volmer plots of the quenching of the intrinsic protein fluorescence in AChR-rich membranes (80–90 nM α-bungarotoxin binding sites) by spin-labeled analogues of (A) procaine thioester (III) and (B) benzocaine (I). Fluorescence quenching, after 70-min incubation of spin-labeled drug, with AChR membranes pretreated for 50 min with carbamylcholine chloride (△) or with AChR membranes in the presence (○) or absence (□) of 0.1 mM carbamylcholine.

Table II: Apparent Quenching Constant, K_Q, and Apparent Fraction of Available Fluorophores, f_a, for Quenching by Spin-Labeled Local Anesthetic Analogues in AChR-Rich Membranes[a]

spin-label	carbamylcholine	K_Q (M^{-1})	f_a
III (procaine thioester)	none	1.8×10^5	0.40
	coincubation	5.0×10^4	0.51
	preincubation	1.1×10^5	0.44
I (benzocaine)	none	3.3×10^4	0.67
	coincubation	1.8×10^4	0.99
	preincubation	4.5×10^4	0.65

[a] The apparent parameters were determined from the linear region of the modified Stern–Volmer plot (Figure 2), where f_a = 1/intercept and K_Q = intercept/slope. The slope was determined by linear regression, and the correlation coefficient was at least 0.989. The AChR was in the resting, coincubated, and desensitized states, corresponding to no incubation, coincubation, and preincubation with carbamylcholine, respectively.

assigned to spin-labeled anesthetics partitioning between the aqueous and fluid lipid phases (spectra B and C in Figure 1). The third component was observed only in the native membranes and, therefore, was assigned to anesthetic molecules undergoing restricted motion at the lipid–protein interface (spectrum D in Figure 1). This assignment is consistent with previous ESR results obtained with spin-labeled fatty acids, phospholipids, and steroids, which have also been interpreted in terms of populations in the fluid bilayer and at the interface of intrinsic membrane proteins (Marsh & Barrantes, 1978; Marsh et al., 1981; Ellena et al., 1983). The protein-associated component was analyzed quantitatively by a second spectral subtraction, and as expected, the quality of these ESR difference spectra depended critically on the purity of the difference spectrum from the first subtraction used to remove the aqueous component. The protein-associated component displayed a spectral anisotropy and line shape typical for slow motion ($2A_{max}$ = 63.7 G at 22 °C) and could be simulated with the modified Bloch equations (dotted line in Figure 1D; Horváth et al., 1988).

Local Anesthetic–Protein Interaction from Fluorescence Quenching. Evidence for the contact interaction of spin-labeled local anesthetic analogues with the interface of the AChR was obtained from quenching the intrinsic protein fluorescence by the paramagnetic local anesthetics [cf. Marsh and Barrantes (1978)]. The aromatic chromophores of the AChR represent a heterogeneous population with varying quenching constants, and the apparent fraction of available fluorophores (f_a) and their apparent quenching constant (K_Q) were obtained from

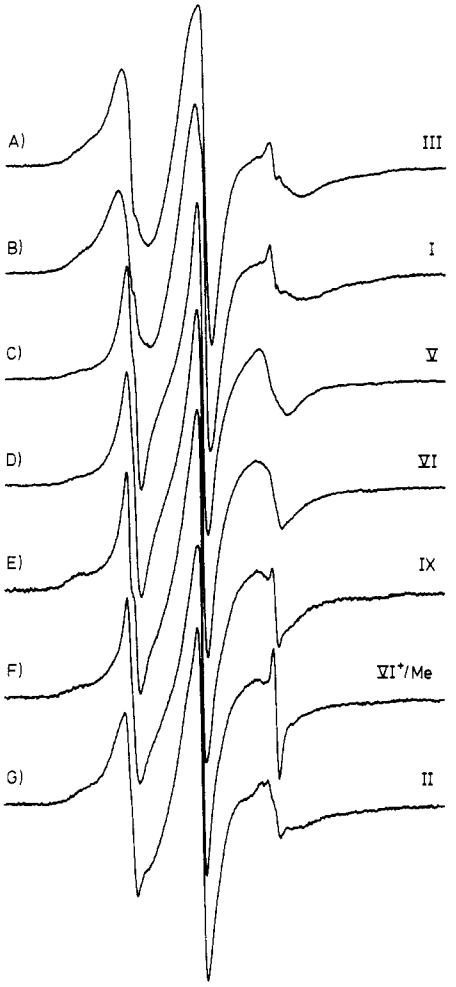

FIGURE 3: Representative ESR spectra of various spin-labeled anesthetics in AChR-rich membrane vesicles from *T. marmorata*, after subtracting the aqueous spectral component. Concentrations used are given under Materials and Methods. All spectra were recorded at 22 °C; scan width = 100 G. Small residual aqueous peaks seen in several of the difference spectra, principally in the high-field region, have a contribution of <0.5% to the total integrated spectral intensity. The molecular structures of compounds I–IX are given in Chart I.

the modified Stern–Volmer plot (Lehrer, 1971). These results are given in Figure 2 and Table II.

Drug Specificity. A series of ESR spectra of the various spin-labeled anesthetics in AChR-rich membranes, which was obtained after subtraction of the various aqueous components, is shown in Figure 3. Small residual aqueous peaks are seen in some of these subtraction end points, mostly in the high-field region, which arise because of difficulties in exactly matching the line widths of the extremely sharp aqueous spectra. However, the contribution of these spurious peaks to the total integrated spectral intensity is less than 0.5% and therefore can be neglected. The fractions of motionally restricted component were quantitated by subtraction of the simulated spectrum of the protein-associated component (dotted line in Figure 1D) from these difference spectra. The quantitative data obtained from the two stages of spectral subtraction are summarized in Table I. The fraction of spin-labeled anesthetic partitioning into the aqueous phase is given by f_{aq}, and the fraction of the membrane-associated spin-labeled anesthetic which is motionally restricted by interaction with the intrinsic membrane protein is given by f. It should be noted that each spectrum recorded with the different spin-labeled anesthetics could be described with the above three components; the combined intensity determined for these components by spectral addition agreed with the total spectral intensity to within 5%.

Table III. Fractions of Protein-Associated Component, f, and Relative Association Constants, K_r/K_{ro}, of Spin-Labeled Local Anesthetic Analogues after Various Treatments of AChR-Rich Membranes[a]

spin-label	treatment	f	K_r/K_{ro}	$\Delta G - \Delta G_o$ (kJ/mol)
III	none	0.35	2.3	−2.0
III	carbamylcholine chloride	0.34	2.2	−1.9
I	none	0.32	2.0	−1.7
I	carbamylcholine chloride	0.31	1.9	−1.6
II	none	0.25	1.4	−0.9
II	carbamylcholine chloride	0.22	1.2	−0.5
III	pH 6	0.34	2.2	−1.9
III	pH 9	0.44	3.4	−3.0
I	pH 6	0.33	2.1	−1.8
I	pH 9	0.35	2.3	−2.0
II	pH 6	0.26	1.5	−1.0
II	pH 9	0.31	1.9	−1.6
III	<0.01 M NaCl	0.34	2.2	−1.9
III	1 M NaCl	0.39	2.7	−2.5
I	<0.01 M NaCl	0.30	1.8	−1.5
I	1 M NaCl	0.31	1.9	−1.6

[a] A value of $f_o = 0.19$, corresponding to spin-labeled phosphatidylcholine, was used in calculating K_r/K_{ro} (cf. eq 2).

The relative contribution of the aqueous component depends on the lipid/water partitioning of the different spin-labeled anesthetics and is seen to increase gradually on going from compounds III, I, VI, and V to compounds IV and VIII (Table I). The second spectral subtraction, performed on the membrane-associated spectra, furnishes information on the relative association constants of the different spin-labeled anesthetics with the protein (Table I). Assuming that the different drugs compete with lipids for sites at the protein interface, one can obtain the relative association constants, K_r, from the fractions of protein-associated spin-label, f_x, compared with the value, f_o, for a nonselective phospholipid [see, e.g., Marsh (1985)]:

$$K_r/K_{ro} = [(1 - f_o)/f_o]/[(1 - f_x)/f_x] \qquad (2)$$

These relative association constants and the differential free energies of association $\Delta G - \Delta G_o = -RT \ln (K_r/K_{ro})$ are given in Table I. A value of $f_o = 0.19$, corresponding to the fraction of motionally restricted spin-labeled phosphatidylcholine [a nonspecific phospholipid; cf. Ellena et al. (1983)] determined in the same membrane preparations, was used as a reference in Table I. On the basis of the relative association constants, the spin-labeled anesthetics can be divided roughly into three groups as follows: high-specificity drugs, such as compounds III, I, and IX ($K_r/K_{ro} > 2$); medium-specificity drugs, such as compounds IV–VI, VI⁺/Me (the quaternized salt of VI), VIII, and X ($K_r/K_{ro} \approx 1.6$–1.9); low-specificity drug, such as compound II ($K_r/K_{ro} = 1.3$).

Allosteric Interaction and Drug Competition. The allosteric interaction between the binding sites for agonists and local anesthetics was studied by comparing the fractions of the protein-associated component for three different spin-labeled local anesthetics, II (spin-labeled procaine analogue), III (spin-labeled procaine thioester analogue), and I (spin-labeled benzocaine analogue), before and after coincubation of the membranes with 0.1 mM carbamylcholine chloride (Figure 4). Of the three spin-labeled molecules tested, the motionally restricted component of compound II decreased, whereas that of compounds III and I remained essentially unchanged. The relative association constants are given in Table III.

The competition of compound III (spin-labeled procaine thioester analogue) with unlabeled procaine, tetracaine, and benzocaine was followed by measuring the fraction of pro-

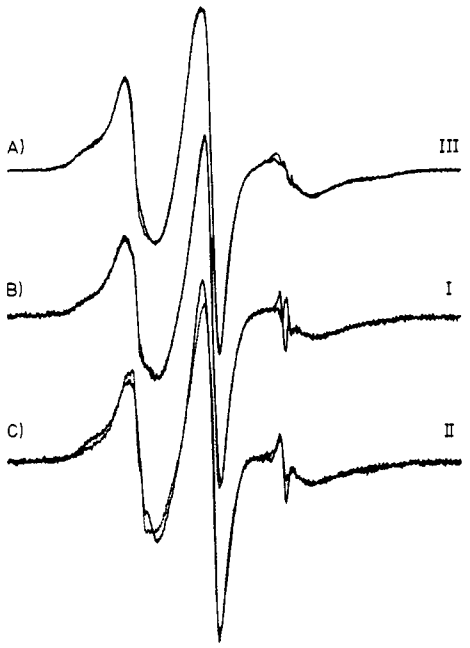

FIGURE 4: Effect of carbamylcholine chloride on the ESR spectra of spin-labeled analogues of procaine and benzocaine in AChR-rich membranes. The overlaid spectra were recorded with (dotted line) and without (full line) the addition of 0.1 mM carbamylcholine chloride. (A) Compound III (spin-labeled analogue of procaine thioester); (B) compound I (spin-labeled analogue of benzocaine); (C) compound II (spin-labeled analogue of procaine). Membrane and spin-label concentrations are given under Materials and Methods. Total scan width = 100 G; T = 22 °C.

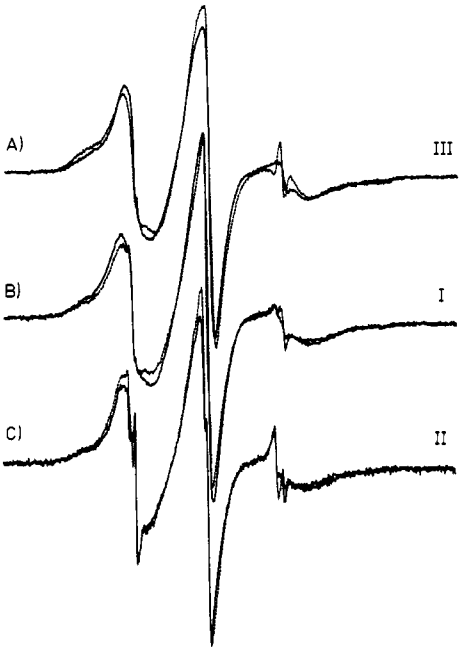

FIGURE 5: Influence of pH on the ESR spectra of spin-labeled local anesthetics in AChR-rich membranes. Each pair was recorded at pH 6 (full line) and at pH 9 (dotted line). (A) Compound III (spin-labeled analogue of procaine thioester); (B) compound I (spin-labeled analogue of benzocaine); (C) compound II (spin-labeled analogue of procaine). Concentrations are given under Materials and Methods. Total scan width = 100 G; T = 22 °C.

tein-associated spin-label component, with drug/spin-label ratios of 1 and 10 mol/mol. No significant displacement of the spin-labeled local anesthetics was observed as estimated from the fraction of the protein-associated component, after addition of the unlabeled drugs at up to 10-fold excess (data not shown).

Dependence on pH and Ionic Strength. The possible role of the protonatable diethylammonium group in local anesthetic–protein interaction was studied by recording ESR spectra at pH 6 and 9, corresponding to the positively charged and uncharged forms, respectively (Figure 5). Of the three compounds chosen for these experiments, the fractions of the protein-associated component significantly increased in the case of compounds II and III (spin-labeled procaine and procaine thioester analogues) in their uncharged forms, whereas compound I (spin-labeled benzocaine analogue) was virtually unaffected, consistent with the lack of a protonatable group in this molecule. The relative association constants at the two pHs are given in Table III. No such trend could be distinguished, however, in a comparison of compound VI and its quaternized salt VI^+/Me (Table I).

In another series of experiments the effect of high monovalent salt concentration on drug–protein interaction was investigated with the charged form of the procaine thioester analogue (III) with the uncharged benzocaine analogue (I) as control (Figure 6 and Table III). An increase in the fraction of protein-associated component was observed for compound III at high ionic strength: the fraction increased from 0.34 to 0.40 with an increase of the salt concentration from 0 to 1 M NaCl, while the ESR spectrum of compound I remained unchanged.

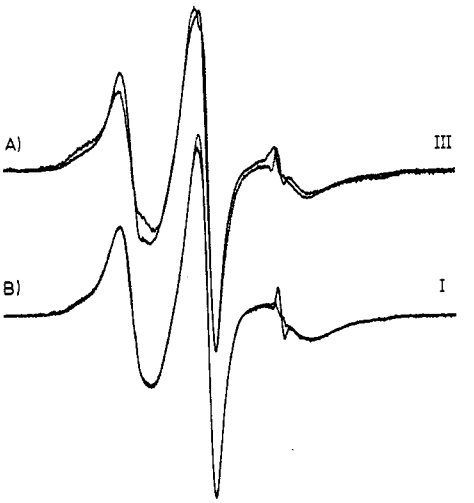

FIGURE 6: ESR spectra of spin-labeled local anesthetic analogues in AChR-rich membranes, as a function of NaCl concentration. Each pair was recorded in <0.01 M NaCl (full line) and in 1 M NaCl (dotted line). (A) Compound III (spin-labeled analogue of procaine thioester); (B) compound I (spin-labeled analogue of benzocaine). Membrane and spin-label concentrations are given under Materials and Methods. Total scan width = 100 G; T = 22 °C.

Discussion

A series of spin-labeled analogues of local anesthetics has been used to investigate the interaction with the AChR protein in its native membrane environment. Moderately low spin-labeled probe to lipid ratios were used (ca 1:25 mol/mol), thus minimizing possible fluidizing effects associated with higher anesthetic concentrations (Boulanger et al., 1981). A common feature in the ESR spectra of all spin-labeled local anesthetic analogues in the AChR-containing membranes was the presence of two components. One component is attributed to probe in the bulk lipid bilayer and the other to the perturbation of the probe mobility in the motionally restricted environment at the interface with the AChR and other integral proteins in the membrane (cf. Figures 1 and 3). Previous work using spin-labeled intracaine and its quaternary analogue[2] also demonstrated motionally restricted spectra in both reconsti-

tuted (Earnest et al., 1984) and AChR-enriched membranes (Palma et al., 1985, 1986). On the basis of the quantitative parameters summarized in Table I, it is possible to compare the apparent degree of association of the different spin-labeled local anesthetics with the integral protein of the AChR membrane.

pH Dependence. The titration behavior of the local anesthetic–protein interaction with pH is of special interest in view of the marked differences, both in steady-state pharmacological effects and in rates of action, between aminated local anesthetics in their uncharged deprotonated forms and the cationic protonated species. The latter are preferentially confined to the aqueous phase; their accessibility to the excitable membrane is lower, and their rate of action is slower than that of the corresponding uncharged form (Hille, 1977). The aminated local anesthetic analogues used here are weak bases; the pK of the spin-labeled intracaine derivative,[2] for instance, has been found to be 7.2–7.4 (Wang et al., 1983). The interfacial contributions to the pK of tetracaine have also been analyzed, and similar conclusions were reached with regard to the pK in neutral micelles (Garcia-Soto & Fernández, 1983). Thus at the physiological pH used in most of the experiments here, the ESR spectra from the spin-labeled local anesthetic analogues will represent the sum of the contributions from the charged and uncharged species [cf. Limbacher et al. (1985)].

The results of the pH titration of the spin-labeled local anesthetic analogues demonstrate that the local anesthetic–protein interaction is strongly dependent on the state of protonation of the probe. A clear pH dependence was observed for compounds II and III (procaine analogue and its thioester derivative). In contrast, no pH dependence was observed for the benzocaine derivative, I, which does not bear a protonatable group (see Figure 5 and Table III), confirming that the pH dependence for compounds II and III arises from titration of the local anesthetic analogue and not of the protein or another membrane constituent. A similar lack of sensitivity to pH has been observed with the permanently charged, quaternary derivative of methylated tetracaine (Limbacher et al., 1985; Earnest et al., 1984).

The finding that the specificity of interaction with the protein is greater in the unprotonated than in the protonated (positively charged) form for the procaine ester (II) and thioester (III) derivatives correlates, in electrostatic terms, with the specificity found of the AChR for negatively charged lipids (Ellena et al., 1983). However, it is not in accord with the results of Earnest et al. (1984), who observed that a greater proportion of the positively charged form of a spin-labeled intracaine derivative[2] is motionally restricted than of the uncharged form. In these latter studies it was also found that the cationic form of the spin-label had a higher potency than the neutral species in inhibiting the ion-flux response of AChR-containing liposomes (Blikenstaff & Wang, 1985). It will be noted that, also in the present studies, a higher proportion of the fully charged, quaternary compound VI$^+$/Me is associated with the protein than of the incompletely protonated analogue, VI, at pH 7.4 (see Table I).

Ionic Strength Dependence. Increasing salt concentration caused a screening of the electrostatic interactions between the protein and the spin-labeled procaine thioester (III) derivative giving rise to an increase in the relative association constant (Figure 6 and Table III). This change in apparent affinity is consistent with the increase observed on titration from pH 6.0 to pH 9.0, i.e., to the deprotonated (uncharged) state (cf. Table III). No salt dependence was observed in the case of the neutral benzocaine derivative, confirming the effects of electrostatic interactions on the affinity in the case of the charged derivatives. It will be noted that the neutral local anesthetic analogues show a considerable degree of affinity for the protein, relative to the zwitterionic phospholipid phosphatidylcholine.

Effects of Agonists. The apparent affinity for the protein of the spin-label derivatives was found not to change very greatly in the presence of carbamylcholine (see Table III). Only for derivative II (procaine analogue) was there a small change in the presence of agonist. In the case of an intracaine spin-label,[2] a high-affinity binding site was observed for ESR in the presence of agonist (Palma et al., 1985, 1986). The apparently small degree of allosteric regulation by cholinergic agonist may well be due to the diluting effect of additional association of the spin-labeled anesthetics with integral proteins other than the AChR.

Fluorescence Quenching Experiments. The amino acid sequence of the AChR from *Torpedo* contains more than 50 Trp and 80 Tyr residues (Noda et al., 1983). The former dominate the fluorescence of the protein and, according to current models [see Barrantes (1988)], are predominantly located in the extramembranous domain of the AChR. In fact, in both the four-chain (Noda et al., 1983; Claudio et al., 1983) and the five-chain (Finer-Moore & Stroud, 1984) receptor models, only one Trp residue is postulated to occur in the bilayer region and hence to be a likely target for paramagnetic quenching by the spin-labeled local anesthetic analogues. Comparison of the quenching efficiency between compounds I and III demonstrates that the fluorophores in native AChR membranes are quenched 1.7-fold more efficiently by the benzocaine than by the procaine thioester spin-labeled analogue.

Receptor activation is a multistep process that may involve a transient intermediate state in which agonist binds at a low-affinity site and doubly liganded AChRs fully open a cation-selective channel. On prolonged exposure to agonist, the average number of open channels decreases, and the receptor affinity increases, reaching the thermodynamically favored desensitized state. The similarity between the quenching values for the native and agonist-incubated states (Table II) indicates that the majority of the AChR channels are unable to reach a transiently activated state in the presence of the local anesthetic analogue. However, the quenching efficiency for compounds I and III differed in the presence of agonist (Figure 2 and Table II), suggesting that the spin-labeled analogues sense two different states of AChR or, alternatively, bind to two sites differing in affinity.

Registry No. I, 71855-57-7; II, 128632-13-3; III, 98647-71-3; IV, 128632-14-4; V, 128632-15-5; VI, 98647-68-8; VI/Me, 128632-20-2; VII, 128632-16-6; VIII, 128632-17-7; IX, 128632-18-8; X, 128632-19-9; acetylcholine, 51-84-3.

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Association of a pH-Sensitive Peptide with Membrane Vesicles: Role of Amino Acid Sequence†

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ABSTRACT: The solution properties and bilayer association of two synthetic 30 amino acid peptides, GALA and LAGA, have been investigated at pH 5 and 7.5. These peptides have the same amino acid composition and differ only in the positioning of glutamic acid and leucine residues which together compose 47% of each peptide. Both peptides undergo a similar coil to helix transition as the pH is lowered from 7.5 to 5.0. However, GALA forms an amphipathic α-helix whereas LAGA does not. As a result, GALA partitions into membranes to a greater extent than LAGA and can initiate leakage of vesicle contents and membrane fusion which LAGA cannot (Subbarao et al., 1987; Parente et al., 1988). Membrane association of the peptides has been studied in detail with large phosphatidylcholine vesicles. Direct binding measurements show a strong association of the peptide GALA to vesicles at pH 5 with an apparent K_a around 10^6. The single tryptophan residue in each peptide can be exploited to probe peptide motion and positioning within lipid bilayers. Anisotropy changes and the quenching of tryptophan fluorescence by brominated lipids in the presence of vesicles also indicate that GALA can interact with uncharged vesicles in a pH-dependent manner. By comparison to the peptide LAGA, the membrane association of GALA is shown to be due to the amphipathic nature of its α-helical conformation at pH 5.

Low molecular weight synthetic peptides have been employed to unravel the role of amino acid sequence in the function of membrane-interactive protein segments. These synthetic peptides are being used as simplified models of lipoproteins, membrane channels, or fusogenic proteins in an attempt to relate peptide conformation, orientation in the lipid bilayer, and supramolecular structure to their function (Spach et al., 1989; Lear et al., 1988).

One of our main objectives was to investigate the role of hydrophobicity and secondary structure, especially the amphipathic α-helix, in defining the interaction of proteins with membranes. We designed a peptide using predictive algorithms governing hydrophobicity (Kyte & Doolittle, 1982;

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