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Association of a Spin-Labeled Local Anesthetic with the Allosterically Coupled Noncompetitive Inhibitor Site on the Acetylcholine Receptor

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SUMMARY

Radioligand binding and ESR were used to study the association of a spin-labeled local anesthetic, 2-[*N,N*-dimethyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl-4-hexyloxybenzoate iodide (C_6SLMeI), with acetylcholine receptor-enriched membranes from *Torpedo californica*. In the presence of carbamylcholine, we found that C_6SLMeI competitively inhibits [^{3}H]phencyclidine binding with high affinity ($K_D = 8.7 \times 10^{-7}$ M for C_6SLMeI), whereas in the presence of α-toxin or in the absence of agonist, C_6SLMeI binds with lower affinity ($K_D = 2\times10^{-5}$ M). At concentrations lower than 1×10^{-5} M, C_6SLMeI does not bind to the agonist site but enhances [^{3}H]acetylcholine binding. These findings show that C_6SLMeI binds to the allosterically coupled noncompetitive inhibitor site which is regulated by agonist binding. In addition, C_6SLMeI preferentially associates with the desensitized receptor state known to exhibit high affinities for agonists and local anesthetics. ESR measurements of C_6SLMeI bound to receptor membranes in the absence of agonist show moderately immobilized spectra. Addition of carbamylcholine results in the appearance of a strongly immobilized component. Prior exposure to α-toxin blocks the carbamylcholine-induced, strongly immobilized component in the ESR spectrum. Furthermore, in the presence of carbamylcholine, back-titration of bound C_6SLMeI with phencyclidine decreases the highly immobilized component at concentrations consistent with the K_D for phencyclidine. These findings indicate that C_6SLMeI detects conformational changes between the resting and desensitized acetylcholine receptor states that occur at the noncompetitive inhibitor binding site. The strongly mobilized component is not affected by ferricyanide addition, suggesting that the binding site is in a region not readily accessible to anion collision from the aqueous phase.

The AChR functions as an agonist-activated cation channel. The subunit composition and structure of the *Torpedo* AChR have been extensively studied since it can be isolated with high purity in large quantities from the electric organ. The receptor is composed of four homologous subunits (cf. Ref. 1), designated as α, β, γ, and δ, which have molecular weights around 56,000. The subunits form a pentameric complex that is present with a stoichiometry of α_2, β, γ, δ. The amino acid sequence for each subunit has been deduced from the nucleotide sequence of the cloned genes (cf. Ref. 1).

Electron microscopy of receptor-enriched membranes has resolved five subunits which are arranged with pentagonal symmetry around the central axis of the receptor. The subunits together form a cylindrical-shaped structure approximately 80 Å in diameter and 140 Å long which spans the membrane. In addition, a funnel-shaped central hydrophilic cavity of 30 Å maximum diameter is present on the synaptic surface which may represent the ion channel (2). The binding sites for agonists, antagonists, and α-neurotoxins are present on the synaptic surface of the α-subunits (cf. Ref. 1), whereas the location of the binding site(s) for noncompetitive inhibitors has not been established.

Local anesthetics are one class of noncompetitive inhibitors that have been suggested to interact directly with the ion channel on the basis of initiating rapid fluctuations in channel opening (3, 4). These agents also enhance the conversion of the receptor to a state with higher agonist affinity by acting at an allosteric site (5). Conversely, occupation of the agonist/antagonist sites has been found to regulate the affinity of the noncompetitive inhibitor site. Radiolabeled derivatives of phencyclidine (PCP) and histrionicotoxin bind with high affinity to a single class of sites which are coupled to the agonist-antagonist site (6–8). The stoichiometry of the high affinity, allosterically coupled class of sites has been found to be approximately one per receptor monomer for PCP, histrionicotoxin, chlorpromazine, and trimethisoquin (6, 9). Some noncompetitive inhibitors

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; C_6SLMeI, 2-[*N,N*-dimethyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl-4-hexyloxybenzoate iodide; PCP, phencyclidine or 1-[1-phenylcyclohexyl]-piperidine; EDTA, ethylenediaminetetraacetate.

also bind to a second class of lower affinity sites which are present in larger numbers and are not allosterically coupled to the agonist sites (6, 9, 10).

The membrane-bound AChR undergoes conformational changes upon binding cholinergic ligands or local anesthetics and other noncompetitive inhibitors. Using a fluorescent agonist in stopped-flow studies, multiple functional states of the AChR that correspond to activation of the channel and desensitization have been detected (11). Similarly, quinacrine has been utilized as a fluorescence probe of the local anesthetic binding site (12). In addition, equilibrium binding and kinetic studies of ethidium fluorescence have yielded information on AChR conformational states (13). However, the specificity, stoichiometry, and location of the binding site(s) for these probes of the noncompetitive inhibitor binding site have not been clearly established.

Spin-labeled ligands have been utilized to describe binding site topography and to detect changes in binding site conformation. Previous studies of the AChR have employed spin-labeled ligands directed to the agonist/antagonist sites (14–16). In this study we have examined the equilibrium binding of a spin-labeled local anesthetic, C_6SLMeI, to AChR-enriched membranes from *Torpedo*. The structure of C_6SLMeI resembles that of other classical local anesthetics (intracaine, tetracaine) with the addition of the nitroxyl reporter group on the quaternary amine nitrogen (Fig. 1). A previous study has shown C_6SLMeI to be a potent local anesthetic since it exhibited strong frequency and voltage-dependent block of sodium channels in squid giant axon (17). In addition, C_6SLMeI (and C_6SL) inhibits the carbamylcholine-stimulated ion-flux through the AChR in reconstituted vesicles (18). We have employed both radioligand binding and ESR spectroscopy to demonstrate that C_6SLMeI possesses the requisite high-affinity, site specificity, and sensitivity to agonist binding to probe the noncompetitive inhibitor site of the AChR. The site is coupled to the agonist/antagonist site in an allosteric fashion and appears not to be readily accessible to the aqueous phase. Our studies show that a transition from the resting to the desensitized AChR state results in an increase in affinity and concomitant strong immobilization of the bound spin label.

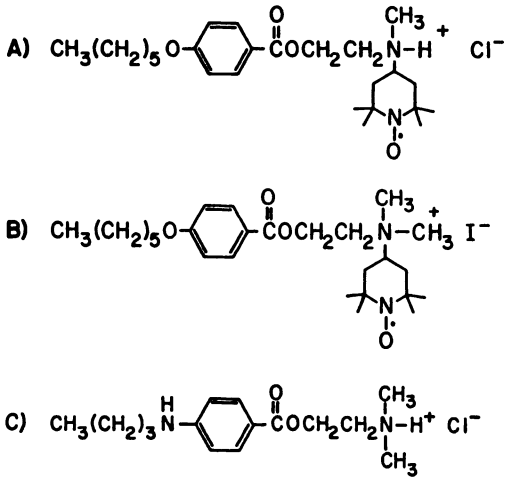


Fig. 1. Chemical structures of the spin-labeled local anesthetics and tetracaine. A, 2-[*N*-Methyl-*N*-(2,2,6,6,-tetramethylpiperidinooxy)ethyl-4-hexyloxybenzoate chloride, abbreviated as C_6SL; B, its methyliodide analog, abbreviated as C_6SLMeI; and c, tetracaine hydrochloride.

Experimental Procedures

Materials. [^{3}H]Phencyclidine (49.9 Ci/mmol) and Na^{125}I were purchased from New England Nuclear. [^{3}H]Ach (3.7 Ci/mmol) was obtained from Amersham. PCP was obtained from the National Institute of Drug Abuse. Cobra α-toxin (*Naja naja siamensis 3*) was generously provided by Dr. David Johnson. Mono-[^{125}I]iodotyrosine 25 α-toxin was prepared and separated from non-iodinated and diiodo species by isoelectric focusing (14). The purity of [^{3}H]PCP and the comigration with unlabeled PCP were checked by thin layer chromotography in *n*-butanol:acetic acid:H_2O (25:4:10) and subsequent autoradiography on Kodak X-Omat film. All other reagents were of the highest purity available. Live *Torpedo californica* were obtained from either Marinus (Westchester, CA) or Santa Cruz, CA.

AChR purification. Receptor-enriched membrane fragments were isolated from the electric organ of *T. californica* by established procedures (19) including base extraction to remove peripheral membrane proteins (20). Specific activities of the receptor preparations were determined by measuring the specific binding of [^{125}I]α-toxin-receptor to DEAE-cellulose filters (21). Specific activities for the receptor ranged from 1.0 to 2.2 nmol of α-toxin-binding sites/mg of protein.

Binding of [^{3}H]PCP. Equilibrium binding of radiolabeled PCP was measured as described by Heidmann *et al.* (6) with the following modifications: AChR-enriched membranes (1.0 μM α-toxin sites) were suspended in 100 mM NaCl, 10 mM $NaPO_4$, pH 7.4. Binding of [^{3}H]PCP was determined under the following conditions: 1) membranes were not treated with cholinergic ligand prior to [^{3}H]PCP addition, 2) a 10-fold stoichiometric excess of α-toxin was incubated with AChR for 1 hr before addition of [^{3}H]PCP, and 3) 200 μM carbamylcholine was incubated with AChR for at least 10 min before the addition of [^{3}H]PCP. Nonspecific binding was determined from bound [^{3}H]PCP in the presence of 1 mM PCP. A 20 μM [^{3}H]PCP stock solution was prepared so that the ratio of radiolabeled to unlabeled PCP was 0.05. Samples were incubated with noncompetitive inhibitor and 1.0 μM [^{3}H]PCP for at least 1 hr at 20° in Beckman polyallomer Airfuge tubes. Bound ligand was separated from free ligand by ultracentrifugation in a Beckman Airfuge for 5 min at 30 psi (160,000 × *g*). Duplicate 10-μl aliquots of the supernatant were removed or, alternatively, aliquots were withdrawn prior to centrifugation to determine total counts. The supernatant was then aspirated, and a 3-mm end of the tube containing the pellet was cut off. The solubilized membrane pellets and aliquots of supernatant were counted in 5 ml of Biofluor (New England Nuclear) using an LKB 1211 Rackbeta.

Binding of [^{3}H]ACh. Binding of [^{3}H]ACh to *Torpedo* membranes was measured in 100 mM NaCl, 10 mM $NaPO_4$, pH 7.4, using the ultracentrifugation sequence described above. To prevent [^{3}H]ACh hydrolysis, acetylcholinesterase was first inactivated by treatment of the concentrated membrane suspension (2.5 μM α-toxin sites) with 1 mM diisopropylfluorophosphate for 1 hr at 25°. Binding studies were subsequently conducted in the presence of 10 μM diisopropylfluorophosphate. AChR membranes (25 nM α-toxin sites) and [^{3}H]ACh (25–50 nM) were incubated in the presence or absence of noncompetitive inhibitors for 1 hr at 25°. Nonspecific binding of [^{3}H]ACh was determined by exposing the AChR with 10-fold excess α-toxin for 1 hr. After ultracentrifugation, free [^{3}H]ACh was removed by aspiration. The membrane pellet containing bound [^{3}H]ACh was then solubilized with of 30 μl of 10% (w/v) Triton X-100 for at least 18 hr and counted.

Data analysis. Competition binding experiments were analyzed by a weighted nonlinear regression computer program, LIGAND, for a single class of binding sites (22). Since the K_D of [^{3}H]PCP is allosterically modulated by occupation of the agonist site, we determined values in our system in the presence of either carbamylcholine or α-toxin. In our analysis, we used values of 0.4 μM for the K_D in the presence of carbamylcholine and 2.0 μM in the presence of α-toxin. Data points are the means of determinations on duplicate samples. Scatchard plots were fit by least squares linear regression analysis. Figures show the results of individual experiments, each of which was performed at least three times with different membrane preparations.

Electron spin resonance. ESR spectra were recorded using a Varian E-3 ESR spectrometer interfaced to a PDP 11/10 computer. Spectra were recorded over a 130-G scan range at a power setting of 10 mW. The modulation amplitude was 1.0 G and 0.1 sec was used as a filter time constant. The sample temperature was maintained at 15 ± 0.1° by a stream of nitrogen regulated at that temperature. The scan time for one spectrum was 6 min. Multiple copies of spectra were accumulated and averaged by the computer on line to the ESR spectrometer.

Samples were prepared for ESR spectroscopy by incubating AChR-enriched membranes (1–2 μM α-toxin sites) in buffer with C_6SLMeI at concentrations ranging from 1 to 10 μM. C_6SLMeI was added from a concentrated aqueous stock solution. Immediately before ESR measurements, samples were centrifuged at 50,000 × *g* for 15 min in conical 1.5-ml polypropylene tubes. Most of the supernatant was removed and the pellet was resuspended in the remaining buffer. The concentration of spin labels in the resuspended pellet therefore remains the same as in the incubation mixture before centrifugation. At low spin label concentrations, most of the labels are bound to the receptor or partitioned into the membrane, and are thus recovered in the pellet. In contrast, at high spin label concentrations, due to saturation of the receptor, a greater proportion and amount of the spin labels are discarded with the supernatant. These considerations make our estimates of spin content at lower spin label concentrations inherently more accurate. The samples in the form of a thick suspension were placed in 100-μl calibrated capillaries to record spectra. Correlation times for nitroxides were estimated as described previously (23).

Results

C_6SLMeI competitively inhibits the binding of [^{3}H]PCP and is allosterically regulated by the agonist site. To determine whether C_6SLMeI binds to a coupled site allosteric to the agonist site on the AChR, competition binding assays with [^{3}H]PCP were performed. [^{3}H]PCP was selected as a marker of the high affinity noncompetitive inhibitor site since its stoichiometry of binding has been precisely determined and its interaction with low affinity sites is minimal (6). In agreement with others, we found that, at concentrations less than 20 μM, [^{3}H]PCP binds to a single class of binding sites on AChR-enriched membranes in the presence of carbamylcholine with a K_D = 0.4 μM and a stoichiometry of 0.82 ± 0.17 per receptor monomer. Since the concentration of [^{3}H]PCP used in the following competition binding experiments was 1.0 μM, only binding to a single high affinity site is measured. As seen in Fig. 2A, increasing concentrations of C_6SLMeI lead to the complete dissociation of bound [^{3}H]PCP. In the absence of cholinergic ligands, the dissociation constant of C_6SLMeI for the receptor was 20 μM and the data were fit well to a single class of binding sites.

The allosteric regulation of C_6SLMeI binding by the agonist site was examined by C_6SLMeI competition with [^{3}H]PCP binding in the presence of α-toxin and carbamylcholine. The affinity of C_6SLMeI for the resting state of the AChR was determined by incubating AChR membranes with a stoichiometric excess of α-toxin prior to the addition of C_6SLMeI and [^{3}H]PCP. Under these conditions, C_6SLMeI exhibits a dissociation constant (K_D = 17.5 μM) which is very similar to that obtained in the absence of cholinergic ligand (Fig. 2A). Since α-toxin binds essentially irreversibly, the possible influence of C_6SLMeI binding to the agonist site on the observed affinity can be excluded. C_6SLMeI competition was also measured in the presence of saturating concentrations of carbamylcholine, which completely converts the receptor to the desensitized

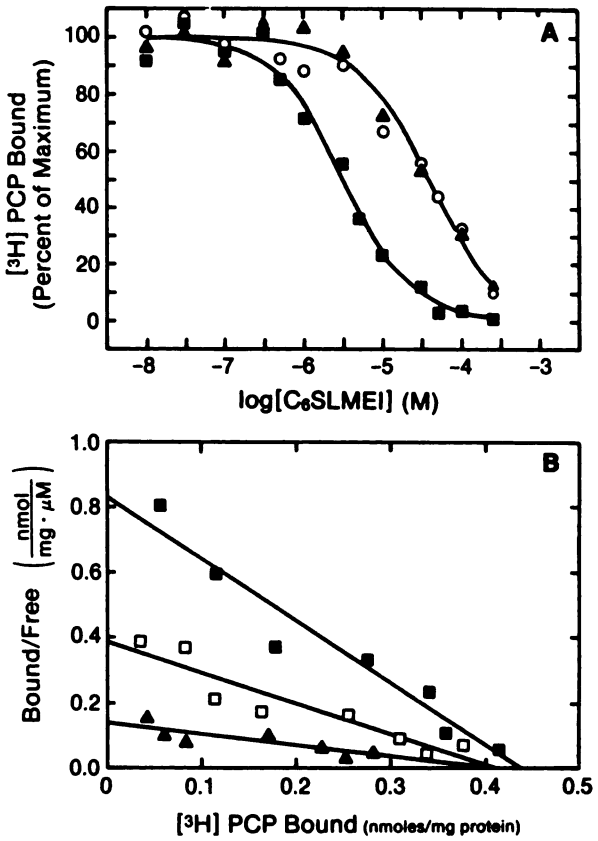


Fig. 2. A, Inhibition of the binding of [^{3}H]PCP by C_6SLMeI and its allosteric regulation by agonist occupation. AChR-enriched membranes (1.0 μM in α-toxin sites) and 1.0 μM [^{3}H]PCP were incubated with the designated ligands (○, control; ■, 0.2 mM carbamylcholine; ▲, 10 μM α-toxin) and C_6SLMeI at the specified concentration. Free and bound [^{3}H]PCP were determined as described in the text. From the K_D values of PCP determined in the absence and presence of carbamylcholine, dissociation constants for C_6SLMeI were determined to be 20.0 μM, no added ligands; 0.87 μM, in the presence of carbamylcholine; and 17.5 μM, in the presence of α-toxin. The *solid lines* through the data were fit by nonlinear least squares analysis assuming a single class of binding sites. B, Scatchard plots of [^{3}H]PCP binding in the presence of C_6SLMeI. The equilibrium binding of [^{3}H]PCP to AChR-enriched membrane (1.0 μM in α-toxin sites) was determined in the presence of 200 μM carbamylcholine and the following C_6SLMeI concentrations: ■, control; □, 0.5 μM; and ▲, 1.0 μM. The *solid lines* are linear regression fits to the data.

state. With agonist present, C_6SLMeI exhibited substantially higher affinity for the receptor (K_D = 0.87 μM). Carbamylcholine also enhanced [^{3}H]PCP binding by increasing its affinity for the receptor. Thus, the data in Fig. 2A show a pattern of allosteric regulation by the agonist site for C_6SLMeI binding typical of amine local anesthetics and other noncompetitive inhibitors of receptor function (6, 9).

C_6SLMeI is a competitive inhibitor of [^{3}H]PCP binding. To characterize further the competition observed between C_6SLMeI and [^{3}H]PCP, Scatchard plots of [^{3}H]PCP binding to the desensitized receptor state in the absence and presence of C_6SLMeI were analyzed. As shown in Fig. 2B, we determined the binding of [^{3}H]PCP to AChR in the presence of 200 μM carbamylcholine and either 0.5 or 1.0 μM C_6SLMeI. The results show a decrease in affinity of [^{3}H]PCP, from a K_D of 0.39 μM in the absence of C_6SLMeI to 0.74 and 1.76 μM in the presence of 0.5 and 1.0 μM C_6SLMeI, respectively. No significant change in the maximal number of [^{3}H]PCP-binding sites was observed

(0.44 versus 0.41 and 0.41 nmol/mg of protein). The *solid lines* are linear regression fits to the data. Thus, Scatchard analysis indicates a competitive interaction between C_6SLMeI and PCP at the allosterically coupled noncompetitive inhibitor site in the desensitized receptor state.

C_6SLMeI and PCP increase [^{3}H]ACh binding. A previous report has shown that several noncompetitive inhibitors, which include tertiary and quaternary amine local anesthetics, may affect binding at the agonist site in two distinct ways (9). First, binding of the anesthetic may give rise to an increase in [^{3}H]ACh binding by regulating the affinity of the receptor at that site. Second, anesthetics may decrease [^{3}H]ACh binding by acting as competitive antagonists. To determine the allosteric effects of C_6SLMeI binding on the agonist site, and the possible direct interaction at the agonist site, equilibrium binding of [^{3}H]ACh was determined in the presence of increasing concentrations of either C_6SLMeI or PCP. A nonsaturating concentration of [^{3}H]ACh was used to achieve fractional occupancy of agonist sites in the absence of noncompetitive ligands.

In the presence of either C_6SLMeI or PCP at concentrations less than 10 μM, there is an increase in [^{3}H]ACh bound at equilibrium due to an increased affinity of the receptor for agonist (Fig. 3). The maximum [^{3}H]ACh bound is similar for both compounds. The half-maximal enhancement of [^{3}H]ACh binding occurs at 0.4 μM for C_6SLMeI and at 1.3 μM for PCP. These values closely correspond to the dissociation constants of C_6SLMeI and PCP directly determined for the allosterically coupled noncompetitive inhibitor site. At higher concentrations, both C_6SLMeI and PCP cause a small decrease in [^{3}H] ACh binding which is probably due to the direct interaction at the agonist binding site. In this respect, C_6SLMeI can act as a competitive antagonist at higher concentrations. These results show that the affinity of C_6SLMeI at the agonist site is low compared to its affinity at the noncompetitive inhibitor site.

C_6SLMeI bound to the resting state of AChR is moderately immobilized. ESR spectra of C_6SLMeI bound to the AChR in its resting state were recorded for different fractional occupancies of spin label at the noncompetitive inhibitor site. AChR membranes (2.0 μM α-toxin sites) were equilibrated at 1–10 molar ratios of C_6SLMeI:AChR in the absence of cholinergic ligand. For comparison, the spectrum of C_6SLMeI in buffer is given in Fig. 4A. The narrow line widths (ΔH_o = 2.6 G), hyperfine splitting ($2a_o$ = 33.5 G), and nearly equal line heights are characteristic of a spin label undergoing rapid,

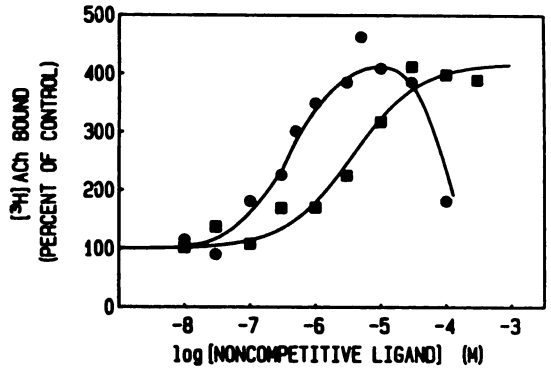


Fig. 3. The influence of C_6SLMeI and PCP on [^{3}H]ACh binding. AChR-enriched membranes were pretreated with 1 mM diisopropylfluorophosphate for 1 hr. The membranes (25 nM α-toxin sites) were incubated with 25 nM [^{3}H]ACh at the specified concentrations of PCP (■) and C_6SLMeI (●). Free and bound [^{3}H]ACh were determined as described in the text.

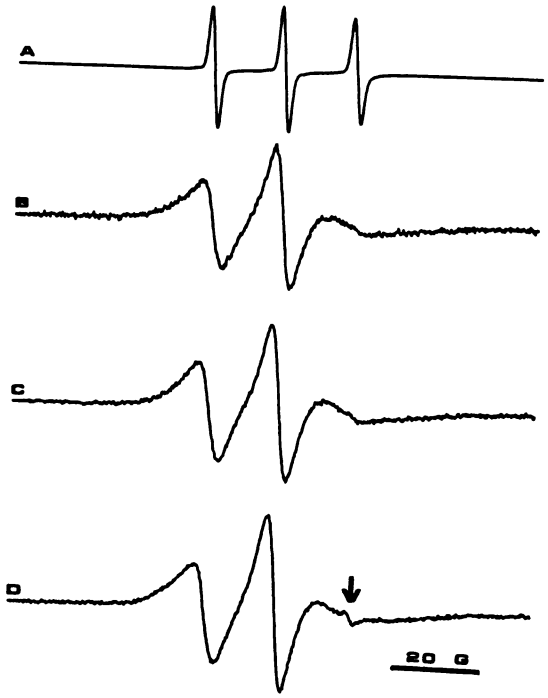


Fig. 4. ESR spectra of C_6SLMeI bound to the AChR-enriched membranes in the absence of cholinergic ligands. A, The spin-labeled local anesthetic C_6SLMeI gives a sharp, three-line spectrum typical of nitroxide free radicals in solution. B–D, AChR-enriched membranes (2.0 μM α-toxin sites) were incubated in the presence of C_6SLMeI for 1.0 hr at molar ratios of C_6SLMeI to AChR of 1:1 (B), 3:1 (C), and 10:1 (D). The spectra were recorded at different gains but the plotted amplitudes were adjusted to equal numbers of spins to facilitate comparison of spectral shapes.

isotropic tumbling in solution. As seen in Fig. 4, B–D, spectra of C_6SLMeI in the presence of AChR membranes exhibit significantly broader line widths of unequal line heights characteristic of moderately immobilized spectra ($\tau_c \simeq 3\times10^{-9}$ sec). The line shapes of the spectra shown are very similar for the 1:1, 3:1, and 10:1 ratios of C_6SLMeI:AChR. A slight difference is seen in the appearance of a small peak at higher C_6SLMeI:AChR ratios (see *arrow* in Fig. 4D) which is due to a small amount of spin-label in the aqueous phase. The moderately immobilized spectra are similar to spectra obtained of C_6SLMeI bound to pure liposomes (24). A saturation binding isotherm using the K_D for C_6SLMeI binding in the absence of agonist (not shown) predicts that at molar ratios of 1:1, 3:1, and 10:1 the local anesthetic site occupancy is 4, 9, and 26%, respectively. Thus, the spectra in Fig. 4, B–D are composed of receptor-specific and nonspecific membrane-bound components.

To resolve the specific ESR receptor component, spectral subtraction of the nonspecific component from the composite spectra was performed. The nonspecific spectral component was defined by two separate methods: 1) in the presence of saturating concentrations of the competing ligand, PCP or 2) in the absence of agonist at low C_6SLMeI concentrations under conditions that yield negligible site occupancy. Minimal differences were observed in difference spectra obtained by either experimental method, indicating that both methods may be used to represent nonspecific binding of C_6SLMeI to AChR membranes. Difference spectra created by subtracting the spectrum in the presence of 200 μM PCP from the spectra in Fig. 4, B–D, are shown in Fig. 5, A–C. For comparison, they are

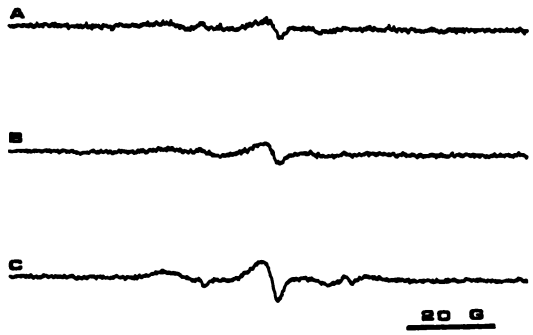


Fig. 5. ESR difference spectra of C_6SLMeI bound to AChR-enriched membranes in the resting state. Difference spectra are obtained by subtracting the spectrum of C_6SLMeI-labeled AChR membranes in the presence of 200 μM PCP (see Fig. 8E) from spectra of AChR membranes in the absence of agonist at C_6SLMeI:AChR ratios of 1:1 (A), 3:1 (B), and 10:1 (C). These difference spectra are all plotted at the same relative scale as the spectra shown in Fig. 4, B–D.

plotted at the same relative scale as the spectra shown in Fig. 4, B–D. At low C_6SLMeI concentrations, the amplitudes of the difference spectra are very small, as predicted by the occupancy levels for the site (Fig. 5, A and B). However, as C_6SLMeI concentration is increased, the difference spectrum becomes distinguishable from the moderately immobilized component (Fig. 5C). This spectral component may represent the low fractional occupation of C_6SLMeI at the noncompetitive inhibitor site seen in the absence of agonist. The immobilization seen for this component is similar to that observed in the presence of agonist.

C_6SLMeI bound to the desensitized state of AChR is strongly immobilized. The binding of agonists to the AChR is coupled to an increase in affinity for C_6SLMeI at the allosteric noncompetitive inhibitor-binding site. High fractional C_6SLMeI occupation at the binding site allows the evaluation of the effects of agonists on the ESR spectra of the AChR-C_6SLMeI complex. Addition of carbamylcholine to AChR membranes (1.5 μM α-toxin sites) in the presence of C_6SLMeI (1–10 μM) resulted in ESR spectra with significantly different line shapes than observed in the absence of carbamylcholine (Fig. 6, A–C). The ESR spectra clearly show an increase in the immobilized spin content as evidenced by the appearance of the broader peaks (Fig. 6A, indicated by *arrows*). However, a population of spin label retains the same moderate mobility seen in the absence of carbamylcholine. Carbamylcholine concentrations between 1 and 1000 μM produced the strongly immobilized ESR component.

The high affinity binding of C_6SLMeI in the presence of carbamylcholine observed by radioligand binding suggested that the strongly immobilized component represented a population of spin-labels bound to the allosterically coupled noncompetitive inhibitor site on the AChR in its high affinity state. Consistent with this expectation, at C_6SLMeI:AChR molar ratios of 1:1, 3:1, and 10:1, the percentage occupancies predicted from the saturation binding isotherm are 53, 78 and 92%, respectively.

At increasing ratios of C_6SLMeI to receptor, the fractional amplitude of the moderately immobilized component increased relative to that of the strongly immobilized component. The nonspecific partitioning of C_6SLMeI into AChR membranes is expected to be a linear function of C_6SLMeI concentration, whereas specific binding sites on the AChR will be saturable.

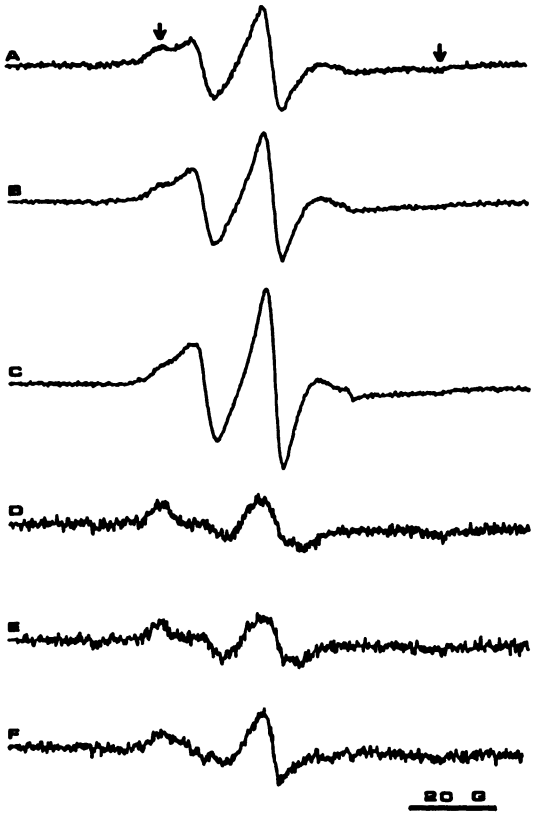


Fig. 6. Effect of carbamylcholine on the ESR spectra of C_6SLMeI bound to AChR-enriched membranes. AChR-enriched membranes (2.0 μM α-toxin sites) were incubated in the presence of 1 mM carbamylcholine at the indicated molar ratios of C_6SLMeI to AChR: A, 1:1; B, 3:1; and C, 10:1. Spectra A–C are normalized to the same number of spins. The *arrows* indicate the appearance of a strongly immobilized component in the ESR spectrum. The difference spectra obtained by subtracting the nonspecific component (i.e., in the absence of carbamylcholine, Fig. 4B) from the composite spectra are shown in D, 1:1; E, 3:1; and F, 10:1. The spectral shapes indicate this population of C_6SLMeI to be highly immobilized. The difference spectra in D–F are plotted to represent the same number of spins, but their original amplitudes represent 48, 30, and 20% of their parent composite spectra A–C, respectively.

Consequently, increasing ratios of C_6SLMeI to AChR will lead to saturation of a specific local anesthetic binding site and a maximal amplitude for that component, as in the case of the strongly immobilized signal. Under the same conditions, the membrane partitioning, represented by the moderately immobilized component, will increase with higher C_6SLMeI:receptor ratios. The relative amplitudes of the two components are consistent with the interpretation that saturation of a specific binding site in the desensitized AChR state gives rise to the strongly immobilized component.

The strongly immobilized component was further defined by resolving the composite ESR spectra. Difference spectra were generated by subtracting the ESR spectrum obtained in the absence of carbamylcholine at 1:1 molar ratios of C_6SLMeI to AChR (i.e., Fig. 4B) from the carbamylcholine-induced composite spectra (Fig. 6, A–C). This subtraction procedure is appropriate since the spectrum in the absence of carbamylcholine primarily represents C_6SLMeI binding nonspecifically to AChR membranes. The composite spectra were resolved as a linear summation of two constant line shapes. The subtraction procedure yielded similar two-component spectra in each case

that consisted of a strongly immobilized and a moderately immobilized spin-signal.

The strongly immobilized spectral components obtained by subtraction from samples at the three C_6SLMeI concentrations are nearly identical in line shape (Fig. 6, D–F). The spectra consist of primarily a single spin population with a central line width of about 8 G and a maximum hyperfine splitting of 64 G (Fig. 6, D–F). Double integration of the strongly immobilized component was performed to determine its contribution to the composite spectra. At molar ratios of C_6SLMeI to AChR of 1:1, 3:1, and 10:1, it represented 48, 30, and 20% of the total spin content of their respective composite spectra. These results are compared with predicted values of 53, 26, and 9.2%, respectively, based on data from radioligand studies. At the lower C_6SLMeI concentrations, the ESR and radioligand methods differed in estimated binding by only 5%. However, at the highest C_6SLMeI concentrations (10:1), the ESR result (20%) is higher than expected; the result can be accounted for by the greater amount of C_6SLMeI discarded in the incubating mixture (see Experimental Procedures). These results further support the assignment of the strongly immobilized component to the high affinity, specifically bound noncompetitive inhibitor site.

α-Toxin blocks formation of the strongly immobilized ESR spectrum. To further test our hypothesis that the strongly immobilized spectrum is due to high affinity binding at the noncompetitive inhibitor site, we determined the effect of α-toxin which blocks formation of the high affinity state by agonist. AChR membranes (1.5 μM α-toxin sites) incubated with C_6SLMeI at a molar ratio of 1:1 in the presence of 0.75 mM carbamylcholine resulted in the spectrum shown in Fig. 7A, which yielded a composite spectrum consisting of a strongly and a moderately immobilized component, as seen previously (Fig. 6, A–C). However, in AChR membranes incubated with α-bungarotoxin at a toxin:receptor ratio of 5:1 for 1 hr under identical carbamylcholine and C_6SLMeI concentrations, no comparable strongly immobilized component was observed (Fig. 7B). The moderately immobilized spectrum observed for the toxin-treated receptor membrane is similar to the spectra obtained for C_6SLMeI in the absence of agonist (Fig. 4B). These spectral data are consistent with the similar low affinity binding observed by radioligand studies for toxin-treated membrane and in the absence of agonist. The difference spectrum shown in Fig. 7C, obtained by spectral subtraction of spectrum 7B from 7A, is similar to the strongly immobilized difference spectrum in Fig. 6D. Thus, the moderately immobilized spectra observed in the toxin-treated membranes also reflects primarily nonspecific binding of C_6SLMeI to AChR membranes.

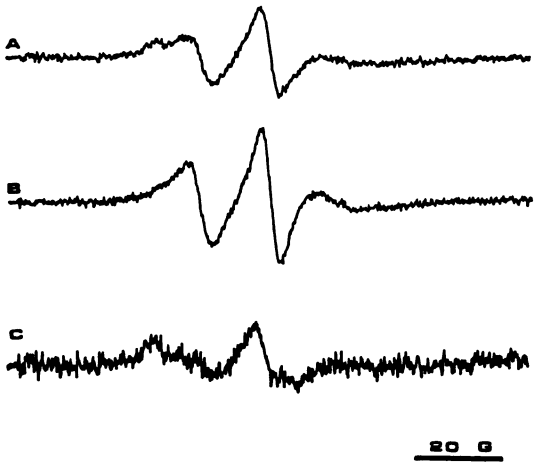


Fig. 7. α-Bungarotoxin blocks formation of the strongly immobilized C_6SLMeI ESR component. AChR-enriched membranes 1.5 μM (α-toxin sites) were incubated with C_6SLMeI at a 1:1 molar ratio with A, 0.75 mM carbamylcholine, or B, pretreatment of AChR membranes with 5:1 molar excess of α-bungarotoxin for 1 hr before addition of C_6SLMeI and carbamylcholine as in A. C, Difference spectrum obtained by subtracting B from A. Spectrum A represents two populations of spin labels at approximately equal numbers; each population is represented by line shapes in spectra B and C, respectively.

PCP competition with strongly immobilized C_6SLMeI. To further demonstrate the selectivity of the C_6SLMeI binding site giving rise to the carbamylcholine-induced, strongly immobilized signal, competition with PCP was examined. AChR membranes (1.47 μM α-toxin sites) at a C_6SLMeI:receptor ratio of 3:1 (2.2 μM C_6SLMeI), in the presence of carbamylcholine (1 mM) showed a typical composite spectrum containing the carbamylcholine-induced component (Fig. 8A). Incubation with increasing concentrations of PCP (1.0, 2.5, 5.0, and 200 μM PCP) decreased the amplitude of the strongly immobilized signal (Fig. 8, B–E). At 200 μM PCP, the strongly immobilized component was completely abolished and the line shape of the spectrum obtained was similar to that obtained in the absence of carbamylcholine (Fig. 4B).

ESR spectral subtraction was conducted to examine the spectral component displaced by increasing PCP concentrations. The difference spectra were generated by subtracting the C_6SLMeI spectrum in the presence of carbamylcholine and 200 μM PCP (defined as nonspecific binding) from the spectra obtained in the presence of carbamylcholine and lower PCP concentrations. The difference spectra obtained for 1.0, 2.5,

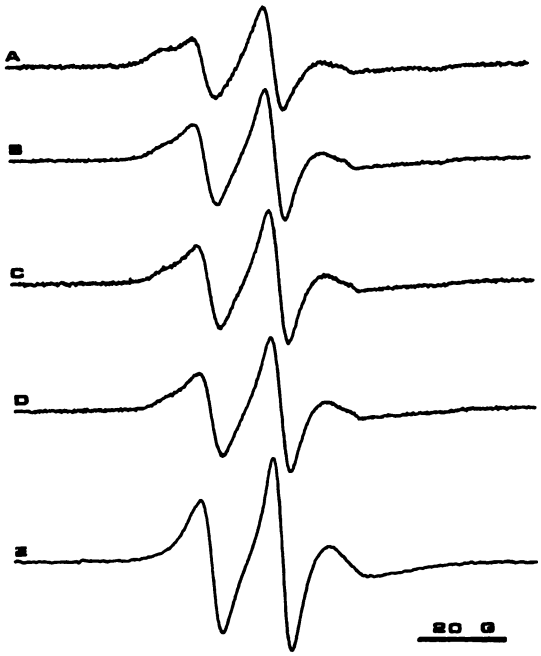


Fig. 8. Dissociation of the strongly immobilized C_6SLMeI ESR component by addition of PCP. AChR-enriched membranes (1.5 μM α-toxin sites) were incubated with 3:1 molar ratio of C_6SLMeI:AChR in the presence of 1 mM carbamylcholine in the presence of the indicated concentrations of PCP: A, control; B, 1.0 μM; C, 2.5 μM; D, 5.0 μM; and E, 200 μM. Note that, at 200 μM PCP, the strongly immobilized component is no longer observable.

and 5.0 μM PCP revealed no significant differences from that of the control (Fig. 9A) in the line shape of the strongly immobilized component (Fig. 9, B–D). The spectra in Fig. 9 are plotted at gains adjusted to represent an equal number of spins in order to facilitate comparison of spectral shape. However, the amplitudes of the difference spectra decreased with increasing PCP concentrations. When the value of the double integral for the difference spectrum (i.e., the strongly immobilized component) obtained in the absence of PCP was defined as 100%, then the fractions of this component present at PCP concentrations of 1.0, 2.5, and 5 μM were 62, 49, and 35%, respectively. Due to the noise level of these spectra, these fractional values should only be considered as estimates. However, the data are consistent with the predicted dissociation of C_6SLMeI due to PCP competition at a single binding site and an approximate IC_{50} for PCP of 2 μM is found. Utilizing the known K_D values for C_6SLMeI and PCP in the presence of carbamylcholine, an IC_{50} of 2.55 μM for PCP displacement of C_6SLMeI was calculated. Thus, the value obtained by ESR is in close agreement with the IC_{50} predicted by [^{3}H]PCP competition binding.

Accessibility of C_6SLMeI bound to AChR to paramagnetic broadening agents. As shown in Fig. 6, the C_6SLMeI spectrum in the presence of carbamylcholine appears to be composed of two components of different mobility. To distinguish spin label populations located at the membrane or protein surface from other labeled sites, the spin exchange broadening between C_6SLMeI bound to AChR membranes and paramagnetic ions in solutions was examined. A high concentration of ferricyanide ions known to broaden completely the spin signal of nitroxides in solution and nitroxides conjugated to small peptides was employed (25).

As seen in Fig. 10, the spectra of C_6SLMeI bound to AChR membranes in the presence of 1 mM carbamylcholine and 95 mM $Na_3Fe(CN)_6^{-3}$ at various C_6SLMeI:AChR ratios show that $Fe(CN)_6^{-3}$ has selectively broadened the more mobile components of the composite spectra (see *arrow* in Fig. 10A) without apparent effect on the strongly immobilized component. This is particularly evident in comparing the decreased relative amplitude of the moderately immobilized component of the low field peak to the strongly immobilized component at higher C_6SLMeI:AChR ratios in Fig. 10, A–C with that of Fig. 6, A–C.

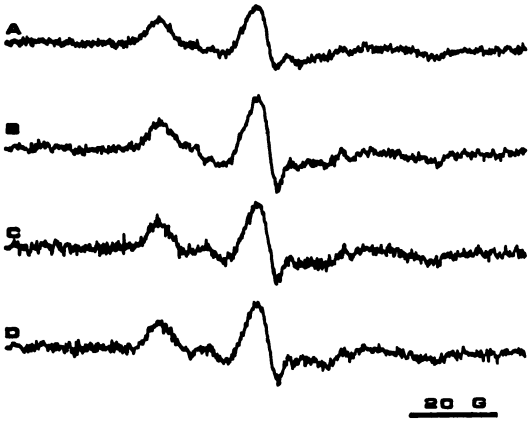


Fig. 9. Difference spectra of the strongly immobilized component displaced by PCP. Difference spectra were obtained by subtracting the spectrum of C_6SLMeI-labeled AChR membranes in the presence of 200 μM PCP (see Fig. 8E) from similarly labeled membranes in the presence of zero (A), 1 μM (B), 2.5 μM (C), and 5 μM (D), PCP, respectively. These spectra are plotted to represent equal numbers of spins (i.e., normalized to the same double integral) to facilitate comparison of the line shape. Their relative magnitudes have ratios of 100, 62, 49, and 35 for spectra A through D, respectively.

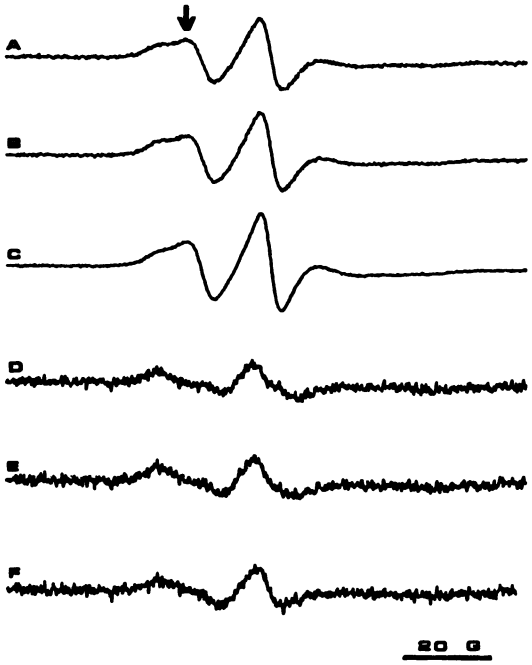


Fig. 10. Differential effects of $Fe(CN)_6^{-3}$ on the strongly and moderately immobilized ESR components of C_6SLMeI bound to the desensitized AChR state. AChR membranes (2 μM α-toxin sites) were incubated with 1 mM carbamylcholine for 30 min at C_6SLMeI. AChR molar ratios were 1:1 (A), 3:1 (B), and 10:1 (C) in the presence of 95 mM $Fe(CN)_6^{-3}$. Note the selective loss in the intensity of the moderately immobilized component (see *arrow*). The difference spectra for the above were obtained by subtracting the AChR membranes in the absence of agonist and in the presence of ferricyanide from the composite spectra in A–C to yield 1:1 (D), 3:1 (E), and 10:1 (F). The spectra are normalized to the same double integral value.

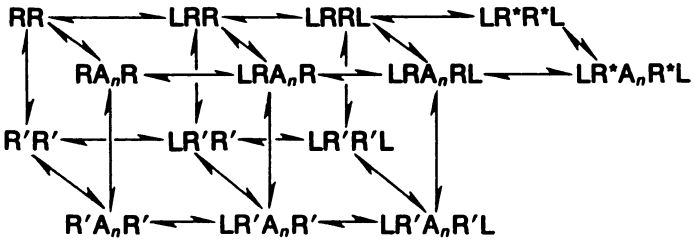
Spectral subtraction of the nonspecific component was carried out by subtracting spectra obtained from C_6SLMeI and ferricyanide-treated AChR membranes in the absence of agonist from the corresponding preparation in the presence of carbamylcholine. The $Fe(CN)_6^{-3}$ difference spectra (Fig. 10, D–F) reveal that the strongly immobilized component with a maximum hyperfine splitting of 64 G has not been significantly broadened. Similar results were obtained using 19 mM Ni-EDTA as the paramagnetic agent (data not shown).

Discussion

This study describes the interaction of a spin-labeled local anesthetic, C_6SLMeI, with native AChR-enriched membranes. AChR-enriched membranes were employed since the direct binding of several local anesthetics and their allosteric interactions with the agonist-binding sites have been extensively studied (6, 9–11, 26, 27). We have utilized both radioligand binding and ESR spectroscopy to characterize the site specificity and affinity of C_6SLMeI. C_6SLMeI was found to displace [^{3}H]PCP completely from its binding site by a competitive mechanism. To determine the selectivity of C_6SLMeI for the allosteric site relative to the agonist site, we also measured the equilibrium binding of [^{3}H]ACh. At concentrations less than 10 μM, C_6SLMeI can be utilized as a specific probe of the noncompetitive inhibitor site. This study has characterized the

binding of C_6SLMeI to AChR membranes by defining a specific binding with respect to allosteric coupling to the agonist site and by competition with PCP. In so doing, we have focused on a class of sites whose stoichiometry has been defined as one per receptor (6). However, it is possible that a second class of C_6SLMeI sites exists which is present in greater number and is not allosterically coupled to the agonist sites since such sites have been described for other quaternary amine local anesthetics (9, 10). We have used C_6SLMeI to examine the environment of the allosterically coupled noncompetitive inhibitor binding site and to monitor changes at this site resulting from receptor changes in state.

Our findings are consistent with a coupled equilibria model which describes the influence of agonists and local anesthetics on receptor state (28). As indicated in Scheme 1, the AChR



exists in at least three distinct states which are: resting (RR), activated or open channel (R^*R^*), and desensitized ($R'R'$). In the absence of local anesthetics (A), the binding of two agonists (L) to the receptor in the resting state will lead to the rapid activation of receptor cation permeability via the LR^*R^*L species. In the continued presence of agonist, the receptor is converted to the desensitized state ($LR'R'L$) in which the channel is refractory to opening. Local anesthetics may inhibit AChR function by interacting directly with the ion channel in the open channel state (LR^*AR^*L) or by stabilizing the desensitized receptor state ($R'R'$) (3, 4, 28). The desensitized receptor state is characterized by having a high affinity for agonists and most local anesthetics (29, 30). Thus, through these coupled equilibria, agonists act to increase the affinity of anesthetics and, conversely, anesthetics can increase the affinity of agonists for the receptor.

To determine whether the affinity of the spin-labeled local anesthetic was sensitive to receptor state, we examined C_6SLMeI equilibrium competition binding with [^{3}H]PCP in the presence of agonists and antagonists that govern the distribution of receptor molecules between resting and desensitized states. In *Torpedo*, 80–90% of the receptors are in the resting state in the absence of agonist (13, 29, 31). The cobra α-toxins bind pseudoirreversibly and stabilize the resting state conformation of the AChR, while equilibration with carbamylcholine converts essentially all receptors to the desensitized state (26). We found that C_6SLMeI binds with a 23-fold higher affinity in the presence of carbamylcholine than in the absence of agonist or in the presence of α-toxin. Hence, C_6SLMeI exhibits a substantial preference for binding to the desensitized or $R'R'$ state. These results are in agreement with studies showing an agonist-induced increase in affinity for other quaternary amine local anesthetics, including [^{14}C]meproadiphen and [^{3}H]trimethisoquin. Similarly, the aromatic amine local anesthetics, dibucaine, dimethisoquin, proadifen, and lidocaine are bound at least 1 order of magnitude more tightly to the desensitized receptor state (8, 32).[1] In contrast, tetracaine, which is structurally related to C_6SLMeI and binds at the same site, exhibits anomalous behavior among the local anesthetics since its affinity for the RR state is greater than for the $R'R'$ state (32, 33). This difference in affinity is not due to the tertiary versus quaternary nitrogen difference in structure since C_6SL, the tertiary amine analog of C_6SLMeI, also binds preferentially to the $R'R'$ state.[2] As previously noted for the structurally related benzylic acid-ester aromatic amine compounds, small changes in structure may markedly influence binding affinity and preference for the AChR state (30).

Many noncompetitive inhibitors have been found to alter receptor conformational equilibria by converting the receptor to the desensitized state (9, 28, 30). It was found that C_6SLMeI increased the amount of [^{3}H]ACh bound with a K_D approximately equal to the K_D determined for the noncompetitive inhibitor site by competition with [^{3}H]PCP. The extent of receptor conversion was equal to that obtained with PCP, the model noncompetitive inhibitor ligand. The conversion of the agonist site to high affinity by local anesthetics has also been directly determined using ESR spectroscopy (27). Thus, C_6SLMeI and other noncompetitive inhibitors may block receptor function by stabilizing the same high affinity, desensitized conformation that is stabilized by agonists (30).

As observed in studies with radiolabeled local anesthetic binding, total C_6SLMeI binding will be composed of specific receptor site interactions as well as nonspecific binding. The nonspecific binding may include partitioning into the lipid membrane and association at the lipid-protein interface. The ESR spectrum of C_6SLMeI bound to desensitized AChR-enriched membranes is clearly a composite spectrum containing a strongly immobilized component and a moderately immobilized component. The nonspecific spectral component was defined by two independent methods: 1) displacement by excess noncompetitive inhibitor ligand, PCP, and 2) maintaining the binding site in a low affinity conformation. Both of these conditions produced single-component spectra that are moderately immobilized and closely resemble spectra for C_6SLMeI in pure lipid liposomes (24). Subtraction of either of these nonspecific spectra from the composite spectra of C_6SLMeI bound to the desensitized AChR produced only strongly immobilized difference spectra. The strongly immobilized spectral line shape observed in this study closely resembles the ESR spectral component of C_6SL bound to affinity-purified AChR in reconstituted lipid vesicles (34). This suggests that the binding site observed in AChR-enriched membranes is preserved in the purified, reconstituted AChR, as previously demonstrated by radioligand binding studies (9). Thus, the strongly immobilized spectra represent C_6SLMeI specifically bound to the high affinity, allosterically coupled, noncompetitive inhibitor site in the desensitized receptor state. This conclusion is supported by several independent criteria: 1) specific effects of carbamylcholine and α-toxin, 2) competition with PCP, and 3) accessibility to paramagnetic quenching agents.

The mobility of C_6SLMeI bound to the local anesthetic site was found to depend on receptor state since the strongly immobilized ESR component required agonist for its appearance. Under these conditions, the high affinity binding of

[1] J. M. Herz, unpublished results.
[2] J. M. Herz, unpublished result.

C_6SLMeI results in complete immobilization of the nitroxide, indicating a highly restricted environment. The hyperfine splitting between the extreme peaks ($2T'_\perp = 64$ G) approaches that of the maximum immobilization measurable (68 ± 1 G) by classical ESR techniques. In contrast, in the absence of agonist or in the presence of α-toxin, specific binding of lower affinity for C_6SLMeI in the resting state showed only a moderately immobilized spectrum. These data may be interpreted as indicating that a conformational change occurs at a single local anesthetic binding site that accompanies a change in receptor state. Our studies clearly show that the mobility of C_6SLMeI reflects the allosteric coupling between the agonist and local anesthetic sites.

Paramagnetic broadening of C_6SLMeI bound to AChR membranes provided an additional means to distinguish the location of the strongly immobilized and moderately immobilized components. Ferricyanide ions were used since they only broaden spins by collisional interactions and have previously been demonstrated to interact only with spin labels at the surfaces of membranes (25). Limbacher *et al.* (24) have shown that C_6SLMeI and C_6SL partitioned into dioleoylphosphatidylcholine liposomes are partially broadened by interactions with $FE(CN)_6^{-3}$. In our studies, the moderately immobilized component is partially broadened by ferricyanide in a similar fashion. However, the strongly immobilized spectral component is not noticeably broadened by $Fe(CN)_6^{-3}$, indicating a low collisional interaction between ferricyanide and C_6SLMeI bound to the high affinity anesthetic site. This finding suggests that the C_6SLMeI binding site is located at a site protected from the ferricyanide anions in the aqueous phase. Although our current data cannot distinguish between possible binding site locations, such as within the AChR channel, at the AChR-lipid interface, or intercalation within transmembrane protein domains, the nitroxide moiety is clearly not accessible to collision by the large ferricyanide anions in the aqueous phase.

Acknowledgments

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