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Noncompetitive Inhibitors Reach Their Binding Site in the Acetylcholine Receptor by Two Different Paths

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SUMMARY

Electron spin resonance was used to contrast the accessibility of tertiary and quaternary local anesthetics to their high affinity binding site in the desensitized acetylcholine receptor (AChR). The time dependence of agonist addition on the association of spin-labeled local anesthetics with the nicotinic AChR-enriched membranes from *Torpedo californica* was studied. Preincubation of AChR-enriched membranes with agonist for more than a few minutes before the addition of C6SLMeI, a quaternary amine local anesthetic, resulted in substantial reduction in the initial association of the label with the receptor. The time-dependent reduction in the initial association of the label with the receptor is modeled by an exponential function having a rate constant of approximately 0.2 min^{-1}. In contrast, agonist preincubation did not produce a comparable decrease in the association of C6SL, a tertiary amine analog, with the AChR. These findings show that whereas the affinity of either anesthetic for the AChR is dependent on the presence of agonist, for C6SLMeI the timing of agonist addition is an important factor in determining the rate of anesthetic association with the receptor. Our results are concerned with the desensitized receptor at an early phase, when the average open-channel time limits the anesthetic binding to the receptor.

We interpret our results by a model in which the cationic local anesthetic reaches its high affinity binding site in the receptor by an aqueous path that is accessible only when the channel is open. On the other hand, anesthetic in its uncharged form is not restricted only to the aqueous path of access. An additional path, probably through the lipid bilayer, allows uncharged forms of anesthetics to reach the high affinity binding site in the AChR even when the aqueous path is closed. During the "open state" of the receptor both cationic and uncharged anesthetics have access to the high affinity site through the aqueous path. However, after this open state, the channel opens only intermittently. The rapidly decreasing open time results in the time-dependent reduction in the binding of cationic anesthetics. This model is consistent with the open channel hypothesis of anesthetic binding to the AChR immediately after agonist stimulation; however, our model also includes an additional hydrophobic path of access for uncharged and reversibly charged anesthetics.

The AChR is a chemically gated postsynaptic ion channel. Each channel is an asymmetric pentameric protein having a subunit stoichiometry of $\alpha_2\beta\gamma\delta$. The amino acid sequence for each subunit is known from the nucleotide sequence of the cloned gene (cf. Refs. 1–3). All subunits are transmembrane integral proteins that are believed to form the wall of the membrane channel. Morphological studies by electron microscopy have shown that the subunits form a protein complex having a 80 Å diameter, 140 Å length, and a central pore of about 8 Å (1). It is also established that binding of agonist molecules to α subunits (4, 5) results in opening of the channel to allow transmembrane cation transport. Mechanisms of the gating process are not known although it is no doubt mediated by a protein conformational change. Under experimental conditions, persistent presence of agonist has been shown to result in receptor desensitization in which agonist binding affinity is potentiated with concurrent decrease in ion conductance (6, 7). The desensitized receptor also exhibits an increased binding affinity for NCB such as local anesthetics (8–11).

Local anesthetics and a number of other related compounds are known to bind to the AChR at a site distinct from the agonist site, and they inhibit ion conductance; the compounds are known collectively as NCB (12, 13). Electrophysiological evidence from single-channel studies (14, 15) suggests that NCB block channel function during the open state of the channel. The open channel block provides a model that is consistent with the belief that local anesthetics enter the channel mouth to the NCB site located in the ion channel. Results from photoaffinity labeling experiments also support the hypothesis that the NCB site is located in the pore of the channel (16, 17) although there have been some discrepancies in labeling patterns (1). In addition to the voltage-dependent anesthetic effects and the action of anesthetics on the open channel state of the receptor, (18, 19), Koblin and Lester (20) also reported

ABBREVIATIONS: AChR, acetylcholine receptor; NCB, noncompetitive blocker; C6SL, 2-[*N*-methyl-*N*-(2,2,6,6,-tetramethylpiperidinooxy)]ethyl-4-hexyloxybenzoate; C6SLMeI, the quaternary methyl iodide analog of C6SL; PCP, phencyclidene; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*,′*N*′-tetraacetic acid.

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that the effects of the more hydrophobic local anesthetics are voltage-dependent and have potencies that parallel their hydrophobicity. Similar observations of these dual anesthetic actions have also been reported (17, 21, 22). These reports demonstrate that the open channel model is not a sufficient model of local anesthetic action.

In this report, we examined the binding of two spin-labeled local anesthetics to native AChR membranes. We will restrict our attention primarily to the high affinity binding of the anesthetics. By correlating the differences in anesthetic properties with differences in binding of the anesthetic to AChR, we can infer functional properties of the AChR. The spin-labeled local anesthetics C6SL and C6SLMeI (Fig. 1) are potent local anesthetics in blocking sodium currents in squid axons (23, 24) and in blocking rubidium flux across reconstituted and native AChR membranes (25). The spin-labeled local anesthetics have been shown to bind AChR at high affinity in native and reconstituted AChR membranes, with K_d values in the micromolar range (11, 26). The compound C6SLMeI has been shown to compete with PCP for the NCB site and to induce receptor desensitization (11). These two local anesthetics are homologous except that C6SLMeI is permanently charged whereas C6SL has a pH-dependent charge. Both anesthetics are amphiphiles with nitroxide reporter group attached to the polar head. These compounds are ideally suited for detecting binding of anesthetics (26) and are not probes of liquid mobility. C6SL in its uncharged state is highly hydrophobic and can penetrate membranes effectively.

Methods

Preparation of receptor-rich native AChR-membranes. Electroplax tissue was dissected from the live *Torpedo californica*. The tissue was cut into 1-inch cubes, washed with dialysis buffer (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% sodium azide, pH 7.4), and quick frozen in liquid nitrogen. A total of 600 g of a slightly thawed electroplax tissue was further chopped with a knife, then homogenized in a Waring blender (3 × 30 sec) with 500 ml of homogenization buffer (10 mM $NaPO_4$, 5 mM EGTA, 0.02% sodium azide, 10 mM iodoacetamide, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5). The homogenate was centrifuged in a Sorvall GSA rotor at 5000 rpm for 10 min, and the supernatant was filtered through four layers of cheesecloth. The pellets were combined with 200 ml of homogenization buffer and homogenized with a high-speed Virtis homogenizer at a setting of 70% for 3 × 30 sec. This homogenate was centrifuged at 5000 rpm for 10 min. The supernatants were filtered through four layers of cheesecloth and the combined supernatants were centrifuged in the Sorvall GSA rotor at 12,500 rpm for 4.5 hr.

The crude membrane pellet was then resuspended to a volume of approximately 40 ml with dialysis buffer and brought to 32% sucrose (w/w) and 400 mM NaCl with 60% sucrose and 4 M NaCl. Approximately 12 ml of the preparation were layered onto 20 ml of a 43–35% continuous sucrose gradient. This was centrifuged in a Ti 50.2 rotor at 35,000 rpm for 100 min at 4°. The diffuse receptor band at 37% sucrose was then removed using a Hamilton syringe. The sucrose was removed by diluting with several volumes of dialysis buffer, pH 7.4, centrifuging, and then resuspending the pellet in approximately 35 ml of dialysis buffer, pH 7.4. The protein concentration of the enriched membrane suspension was 2–10 mg/ml as measured by the method of Lowry *et al.* (27). Specific activities of receptor preparations were determined by measuring the specific binding of ^{125}I-α-bungarotoxin to receptors on DEAE-cellulose filters (28). Specific activities for the receptor is about 1 nmol of α-toxin binding sites/mg of protein.

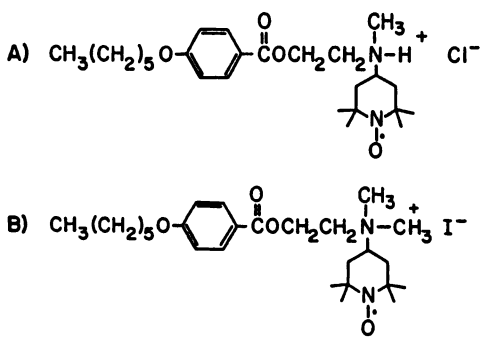

Fig. 1. Chemical structure of the spin-labeled local anesthetics C6SL (A) and its methyliodide analog C6SLMeI (B).

Preparation of membrane samples of ESR spectroscopy. A suspension of enriched AChR membranes containing 2 mg of protein was incubated in a conical 1.5-ml polypropylene tube at room temperature. Aqueous solutions of either spin-labeled C6SL or C6SLMeI (Fig. 1) with or without aqueous solutions of PCP, agonist (carbamylcholine or acetylcholine), or antagonists were incubated together in various combinations and at different addition times. When appropriate, 1 mM carbamylcholine was used at the optimal concentration at which receptors are maximally stimulated (25, 29). Upon addition of the final solution, which in most cases were the spin-labeled anesthetics, samples were centrifuged at 20,000 rpm for 20 min. Most of the supernatant was removed and the pellet was resuspended in the remaining buffer. The samples in the form of thick suspensions were placed in 100-μl calibrated capillaries to record ESR spectra. The elapsed time between addition of the final solution and completion of the recording was under 30 min.

ESR procedures. ESR spectra were recorded with a Varian E-3 spectrometer interfaced to a PDP 11/10 computer. The scan range was 130 gauss, with 100-KHz modulation frequency, 32-mW power, 1-gauss modulation amplitude, and 0.1-sec filter time constant. Six thousand data points across the 130 gauss scan range were sampled 300 times at each point, digitized, averaged, and stored. The scan time for one spectrum was 6 min; when necessary, multiple copies of noisy spectra were recorded and averaged. Data analysis was done using BASIC and FORTRAN software and plotted on a Hewlett/Packard x-y plotter. The temperature of the sample was regulated at 15° with a flow of nitrogen gas.

In order to compare mobility differences between individual ESR spectra, the spectra were normalized to a constant area under the absorbance curve and hence a constant number of spin labels per spectrum. A population of spin labels that are freely mobile will exhibit a spectrum characterized by a narrow linewidth and a greater low field peak amplitude, whereas a spectrum of spin labels with reduced mobility will exhibit a broader linewidth and decreased amplitude.

To resolve the specific receptor-bound component, spectral subtraction of the nonspecific component from the composite spectra was performed. The nonspecific component was defined at the spectra produced in the presence of either spin-labeled anesthetic but in the absence of cholinergic ligands. The difference spectra represent the strongly immobilized receptor component. The relative contribution of the difference spectra to the composite spectra can then be determined by double integration of the spectra. Detailed procedures used in spectral subtraction were previously published (30).

Results

Spin-labeled local anesthetic bind the AChR membranes. The spin-labeled local anesthetics C6SL and C6SLMeI in the presence of biological membranes gave reproducible results as we have previously reported (11). In free solution these labels yield a characteristically sharp three-line spectrum. In the presence of lipid bilayers, these labels yield broader spectra representing moderate immobilization of anesthetic spin labels intercalated into the bilayer. In native and

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reconstituted AChR membranes, these anesthetic labels exhibit composite spectra, which can be resolved into their individual components. These component spectra represent various populations of spin-labeled anesthetics in the membrane preparation as described previously (11, 26, 30). Resting AChR membranes, in the presence of C6SLMeI, give rise to a composite spectrum consisting of a moderately immobilized lipid-intercalated component and a negligibly small solution component (Fig. 2B). Addition of agonist (1 mM carbamylcholine) to such preparations results in the appearance of a highly immobilized spectral component (Fig. 2A), which was previously identified as the binding of anesthetic labels to AChR at the NCB site (11). This result may be interpreted in several ways as follows: 1) in the presence of agonists the desensitized receptor has an increasing binding affinity for anesthetic; 2) the addition of agonists made the NCB site accessible to anesthetics; or 3) the agonist stimulates both the accessibility and the increased binding affinity of the NCB site for local anesthetics.

C6SLMeI binding is time dependent. In the course of these experiments we discovered that the sequence and timing of the addition of C6SLMeI and agonists to the AChR preparation are significant factors in the binding of C6SLMeI to the NCB site. In the control experiment the addition of carbamylcholine after or at the same time as C6SLMeI gave the highly immobilized component (Fig. 3A). However, addition of C6SLMeI after carbamylcholine resulted in decreased C6SLMeI binding to the NCB site in time-dependent manner. The amounts of anesthetics bound to the receptor is dependent on the agonist preincubation time.

Addition of C6SLMeI to the AChR preparation 1 min after adding carbamylcholine gave ESR spectra having a highly immobilized component (Fig. 3B), which was not significantly different from the control (Fig. 3A). However, increasing agonist preincubation times significantly reduced the highly immobilized ESR component (Fig. 3, C–E). Comparison between these "late" carbamylcholine spectra (Fig. 3E) with the zero-carbamylcholine spectrum (Fig. 3F) showed a great similarity. The reduction in the binding of C6SLMeI to the AChR as inferred by the decrease in the highly immobilized ESR component (Fig. 4) is represented by a double exponential ($y = 85e^{-k_1 t} + 15e^{-k_2 t}$) with k_1 and k_2 being 0.19 and 0.03 min^{-1}, respectively. In this expression, y equals the per cent anesthetic bound relative to the control, and t is the duration of agonist preincubation. In addition, it was also shown that even after extensive agonist preincubation time (30 min), C6SLMeI association returned to maximum with increased anesthetic incubation time over 60 min. These findings suggest that the rate of anesthetic association to the NCB site is significantly faster during and immediately after addition of agonist, whereas the anesthetic binding rate is slowed after longer periods of agonist stimulation (i.e., longer than a few minutes). These results suggest that binding of C6SLMeI to the NCB site is not simply due to increased binding affinity of the desensitized receptor; at least in this case it can also be ascribed to the accessibility of the NCB site (i.e., increased kinetics in reaching equilibrium) during and immediately after stimulation of the receptor by agonist. The double exponential suggests the presence of two populations of AChR, with the majority (85%) of receptors having a faster response to agonist stimulation. The smaller population (15%) may represent inactive receptors as previously noted (8). We have also represented the decrease in C6SLMeI binding by a single-exponential decay with a rate constant of 0.19 min^{-1}. This model fits the initial reduction in C6SLMeI binding (Fig. 4), suggesting an exponential decrease in accessibility of C6SLMeI to the NCB site. Although the two exponential model gave the better fit, we attach no great significance to the presence of the smaller population in our interpretation. We interpret the decrease in C6SLMeI binding in the majority of the receptors as resulting from a rapid drop in the average open time of the receptor channels that are

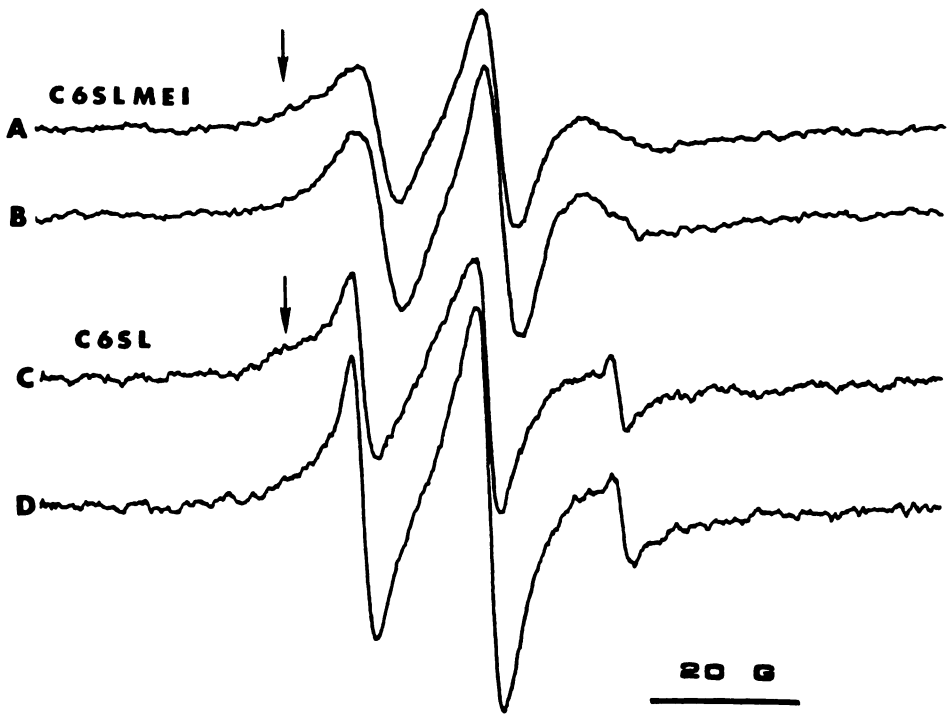

Fig. 2. Effect of carbamylcholine on the ESR spectra of C6SLMeI or C6SL bound to AChR-enriched membranes at pH 7.5. Note the highly immobilized component as indicated by *arrows*. AChR-enriched membranes (2.0 μM α-toxin sites) were incubated in the presence of C6SLMeI at a molar ratio of 3:1 C6SLMeI:AChR (A and B), C6SL at a molar ratio of 5:1 C6SL:AChR (C and D), in the absence of cholinergic ligands (B and D), and in the presence of 1 mM carbamylcholine (A and C).

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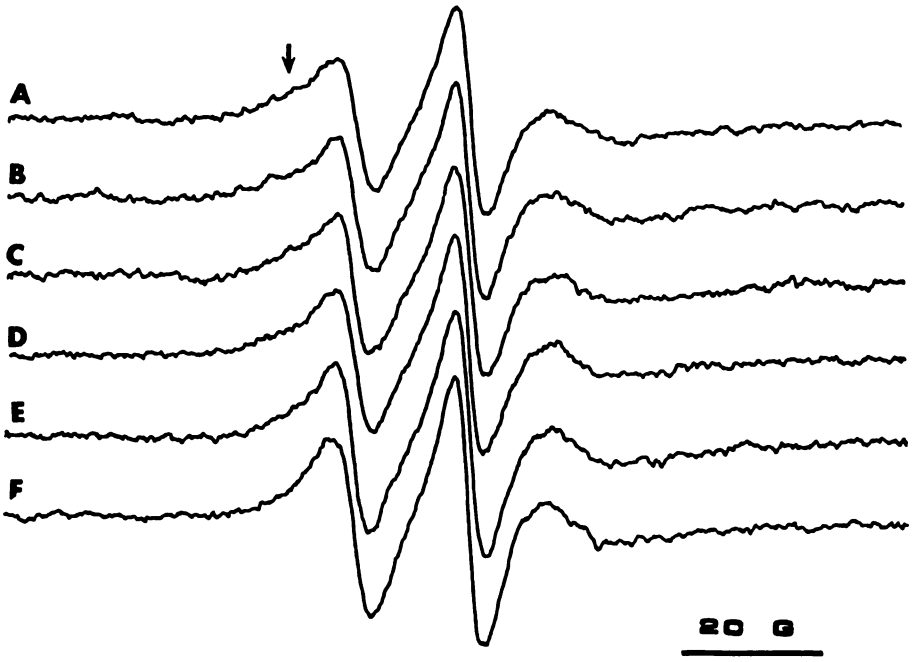

Fig. 3. Effect of agonist preincubation time on the strongly immobilized C6SLMeI ESR component. Note the decrease of the highly immobilized component (*arrow*) from the spectra A to F. AChR-enriched membranes (1.5 μM α-toxin sites) were incubated with a molar ratio of 3:1 C6SLMeI:AChR in the presence of 1 mM carbamylcholine. A, The spin-labeled local anesthetic C6SLMeI and 1 mM carbamylcholine were added simultaneously. (B–E), AChR-enriched membranes were preincubated with 1 mM carbamylcholine for 2 min (B), 5 min (C), 16.5 min (D), or 26 min (E) before the addition of C6SLMeI. F, AChR-enriched membranes were incubated with C6SLMeI in the absence of carbamylcholine.

intermittently open. Our method does not measure changes in kinetics during the transient open state (i.e., changes during the first minute), but we examine changes in the desensitized receptor.

C6SLMeI is a permanently charged, quaternary amine local anesthetic, which suggests an aqueous path of access. During the open state and when the average open time is long, the anesthetic is rapidly accessible to the AChR, as predicted by the open channel model of anesthetic action. As the average time becomes shorter, the access becomes limited; eventually, the binding of C6SLMeI to the late desensitized AChR requires prolonged anesthetic incubation of over 60 min.

Binding of C6SL to the AChR is pH dependent. To further characterize the binding of anesthetic to AChR we used C6SL, a tertiary amine analog of C6SLMeI. C6SL has a p*K* of 7.3 and is thus positively charged at low pH. AChR in the presence of C6SL and carbamylcholine showed a highly immobilized component in the ESR spectrum for samples at low pH, but not for samples at high pH (see Fig. 5A; a similar result for reconstituted membranes was previously described by us in Ref. 30). At pH 6, C6SL is more than 90% charged and the spectrum showed a highly immobilized component that resembled that obtained in the presence of C6SLMeI. However, at pH 10, with almost all C6SL in the uncharged state, the highly immobilized component is not significantly present. These pH-dependent effects can be explained in two ways, 1) the changes in pH altered the AChR and its ability to bind anesthetics, or 2) the binding of anesthetics to AChR requires that the anesthetic be positively charged. Control experiments using the permanently charged C6SLMeI at pH 6 and 10 showed no comparable difference in their ESR spectra (compare Fig. 5, A and B). This result supports the latter interpretation and demonstrates that the highly immobilized component in the ESR spectrum represents binding of positively charged local anesthetics to the NCB site in the AChR. We have used two extreme pH values to demonstrate that the immobilization of the spin-labeled anesthetics is charge dependent. However, although the binding is not greatly altered by the pH as shown by the above control experiment, we do not mean to imply that the receptor is insensitive to pH. We have previously found that the agonist-induced ^{86}Rb flux shows a maximum at pH 7.5–8, and about 57% of the maximum flux remains at pH 6.5 (see Ref. 25). All our experiments are carried out at pH 7.4 unless otherwise specified.

Binding of C6SL to the AChR is not time dependent. In the following experiments we used both pH 6.4 and 7.4 buffer solutions as the incubating medium; the results were not significantly different at either pH. Addition of agonists to AChR in the presence of C6SL resulted in the presence of the highly immobilized component in the ESR spectrum. However, unlike results obtained from C6SLMeI studies, C6SL binding is not time dependent; an immobilized component in the ESR spectrum is present regardless of agonist preincubation time (Fig. 6, A–C). Addition of C6SL at 0, 1, 5, 15, 20, and 30 min after agonist was added to the AChR did not decrease the amplitude of the bound component (Fig. 4). In the control experiment, AChR preparation in the presence of C6SL but without agonists gave an ESR spectrum showing no significant amount of the immobilized component (Fig. 6D).

C6SL showed no decrease in binding when the agonist was added to the AChR before the anesthetic. The binding is only dependent on the presence of agonists and the availability of charged anesthetics at the binding site. The results show that the tertiary amine C6SL achieves equilibrium binding to the NCB site rapidly in the desensitized receptor in which the channel openings are infrequent. We interpret the results to mean that, in addition to open channels, another path of access to the NCB site is available to C6SL. We believe that both C6SL and C6SLMeI follow the rapid changes in binding kinetics demonstrated previously for the initial transient opening of the receptor (8, 10) although our method does not measure kinetics on such short time scales. However, on the time scale of minutes our result does demonstrate the dramatic difference between quaternary and tertiary amine anesthetics in their

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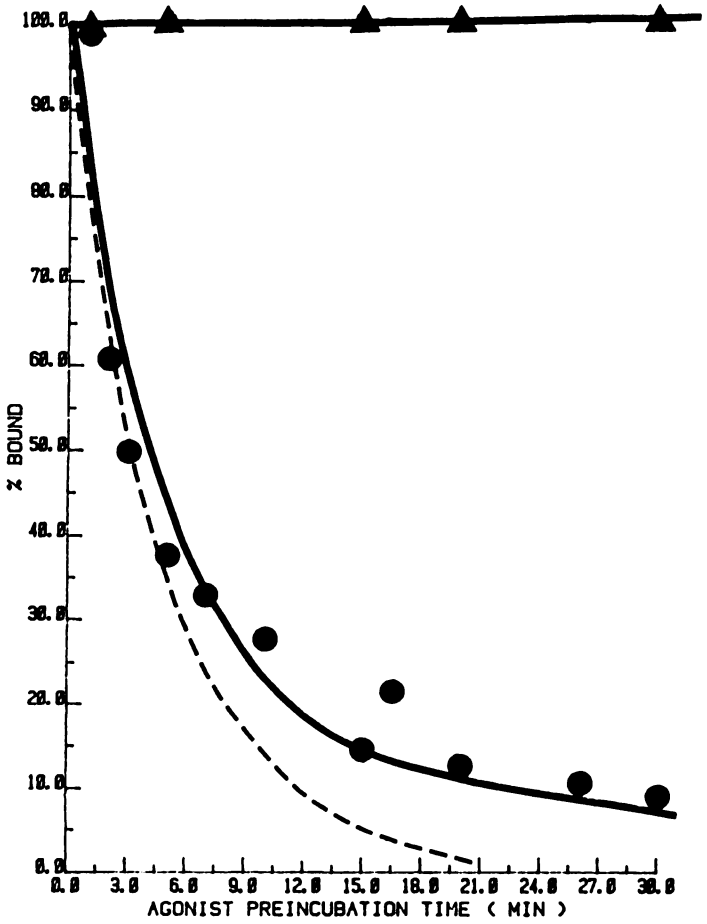

Fig. 4. Effect of agonist preincubation on the strongly immobilized C6SL ESR component (▲) and C6SLMeI component (●). The *solid line* represents the double exponential fit ($y = 85e^{-k_1t} + 15e^{-k_2t}$), in which k_1 = 0.19 min^{-1} and k_2 = 0.03 min^{-1}; the *dashed line* is a single exponential fit. The nonspecific spectral component defined as the C6SLMeI or C6SL spectra produced in the absence of agonist (i.e., such as Fig. 3F or 6D) was subtracted from spectra shown in Fig. 3, A–E for C6SLMeI and shown in Fig. 6, A–C for C6SL. The difference spectrum represents the spectral component of the highly immobilized, AChR-bound anesthetic population. The double integral value for the difference spectra obtained from spectra produced under different agonist preincubation times are represented as the fraction of the 100% (control) value. The 100% value is defined by the difference spectrum obtained from experiments in which agonist and anesthetics were added simultaneously (i.e., Fig. 3A or Fig. 6A). For C6SLMeI agonist preincubation times of 1, 2 (3B), 3, 5 (3C), 7, 10, 15, 16.5 (3D), 20, 26 (3E), and 30 min, the fraction of anesthetic bound to the control value was 99, 61, 50, 38, 33, 29, 14.7, 22, 12.7, 10.8, and 9.1% respectively. For C6SL preincubation times of 1, 5, 15, 20, and 30 min resulted in 100% anesthetic bound in each case.

rates of binding to the AChR in the desensitized receptor. The primary difference in the physical property of C6SL and C6SLMeI is that C6SLMeI is permanently charged and C6SL (pK < 8) is partially uncharged at neutral pH. The presence of a population of uncharged anesthetics would thus permit C6SL to reach its binding site through a hydrophobic path.

C6SL and PCP compete for the NCB site. The differences in the time-dependent binding between C6SL and the quaternary amine C6SLMeI could be due to binding of these anesthetics at different sites. We therefore would like to establish the identity of these binding sites. C6SLMeI has been shown to compete for the PCP binding site in the AChR (11). Thus we examined the effect of PCP on C6SL binding. AChR incubated in the presence of C6SL and agonists showed the bound component as described earlier (Fig. 6). With addition of PCP, the bound component was decreased in a dose-dependent fashion (Fig. 7B and *inset*). We further examined the effect of C6SL on PCP binding by competitive radioligand assays. The inhibition of [^{3}H]PCP binding by C6SL gave a K_i of 3.2 μM for C6SL binding to AChR.[1] The Hill plot had a slope of −1.26 and gave a K_i of 3.5 μM. These results are consistent with the interpretation that C6SL and PCP compete for the same site, as was demonstrated previously for C6SLMeI (11).

Discussion and Conclusion

The spin-labeled C6SL and C6SLMeI are both potent local anesthetics as established by voltage-clamped studies of squid giant axons (24). In enriched AChR membranes, binding of C6SLMeI was shown to increase the binding affinity of AChR for agonists (11). Both C6SL and C6SLMeI also block rubidium flux in reconstituted and in native AChR vesicles (25). The concentration of spin-labeled local anesthetics required to block rubidium flux by 80% was the same concentration required to demonstrate the appearance of the highly immobilized component in the ESR spectrum of reconstituted AChR membranes (25). The highly immobilized ESR component was also shown to arise from a PCP-titratable population of bound C6SLMeI in the AChR (11). These are sufficient conditions to define the spin-labeled local anesthetics used in this study as NCB of AChR; furthermore, the highly immobilized component observed in the ESR spectra may be attributed to anesthetic binding at the NCB site.

The location of NCB binding site is not unequivocally established; however, electrophysiological evidence (14, 15, 17, 20–22, 31, 32) strongly suggest a location in the ion channels. Results from studies using photoaffinity labels (16, 17) are also consistent with a NCB binding site on the inside wall of the channel. Studies using the spin label C6SLMeI in native AChR membranes and the paramagnetic ion ferricyanide suggested a local anesthetic binding site that is protected from the bulk aqueous phase (11). Although a location for the NCB site other than somewhere inside the channel has not been ruled definitely out, the weight of the evidence and the simplicity of explanation strongly favor a NCB binding site associated with the ion channel.

Presence of the NCB site inside the ion channel would require charged anesthetics such as C6SLMeI to enter through the channel mouth. The time dependency that we have observed for C6SLMeI suggests that this local anesthetic is readily accessible to the binding site during the initial few minutes after agonist stimulation, after which access follows an increasingly longer time course. There are two ways to model the process. 1) The C6SLMeI binds only to open channels and establishes an equilibrium with NCB sites during the open phase of activated channels. However, the duration of the open state is less than 100 msec, much shorter than the time that C6SLMeI is accessible to the binding site. Furthermore, a required assumption in this model is that the NCB site is in its high affinity state during the open state phase so that equilibrium binding can be established (see Diagram 1). 2) The C6SLMeI binds to channels rapidly in their early desensitization phase (D_1) and open state. This model assumes that the NCB site is accessible during D_1 period via intermittently open

[1] Methods were published in Ref. 11; detailed data are to be published elsewhere.

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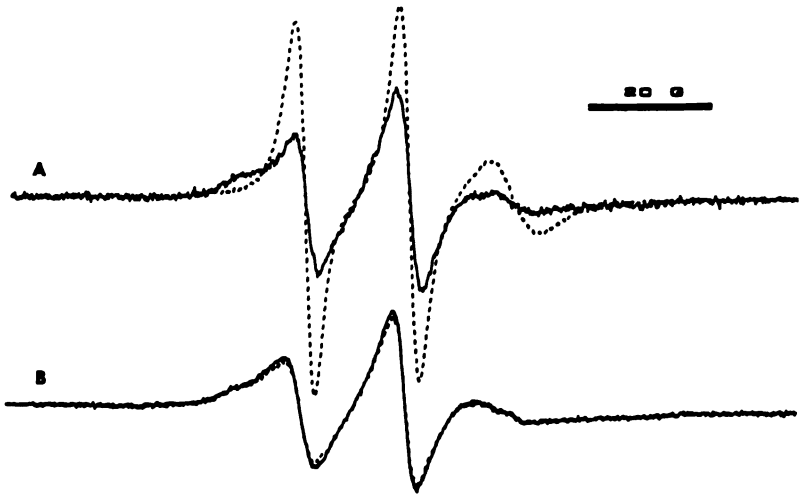

Fig. 5. Effect of pH on spectral shape. AChR-enriched membranes (ranged between 1 and 2 nmnol/mg of protein) were incubated at the indicated pH with spin labels at an AChR to label ratio of 1:3. Supernatant was discharged after centrifugation. A, ESR spectrum of C6SL at pH 6.0 (*solid line*) and pH 10 (*dotted line*). B, ESR spectrum of C6SLMeI at pH 6.0 (*solid line*) and pH 10 (*dotted line*).

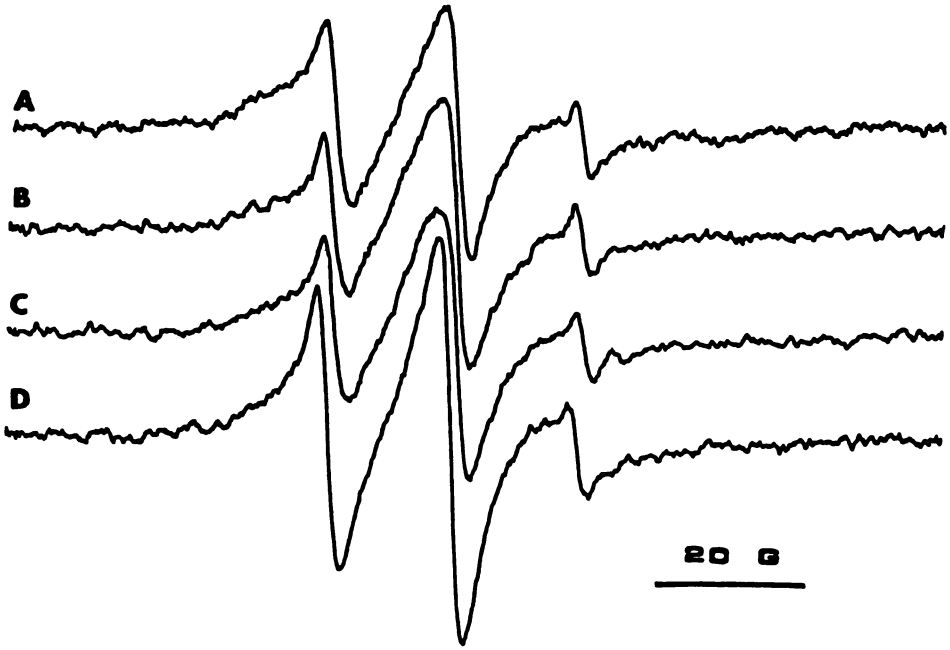

Fig. 6. Effect of agonist preincubation time on the strongly immobilized C6SL spectral component. AChR-enriched membranes (1.5 μM α-toxin sites) were incubated with C6SL at a molar ratio of 5:1 C6SL:AChR, in the presence of 1 mM acetylcholine (1 mM carbamylcholine gave identical results). A–C, AChR-enriched membranes were incubated with 1 mM acetylcholine at times of 0 (A), 1 (B), or 15 min (C) before the addition of C6SL. (D), AChR-enriched membranes were incubated with C6SL in the absence of cholinergic ligands.

channels. As the average open time of the channel is decreased during D_1, accessibility to the NCB site becomes limiting. Consequently, binding of charged local anesthetic is decreased. As the channel reaches the late desensitized phase (D_2) the accessibility to the NCB site is substantially reduced (see Diagram 2). The second model, which we favor, is supported by the time dependence of C6SLMeI binding. In this model the channel in the D_1 state opens intermittently and binds anesthetics at high affinity, but the average open time decreases rapidly during this period. The channel in the D_2 state opens at a greatly reduced frequency and would thus bind charge anesthetics at a very slow rate. The two mechanisms are summarized by Diagrams 1 and 2 in which *A* is the anesthetic, *R* is the resting state, *O* is the open activated state, and D_1 and D_2 are the early and late desensitized states.

$$\begin{array}{ccccc} R & \rightleftharpoons & O & \rightleftharpoons & D_1 \rightleftharpoons D_2 \\ & & \downarrow\uparrow & & \\ & & OA & & \end{array} \qquad (1)$$

$$\begin{array}{ccccc} R & \rightleftharpoons & O & \rightleftharpoons & D_1 \rightleftharpoons D_2 \\ & & \downarrow\uparrow & & \downarrow\uparrow \\ & & OA & \rightarrow & D_1A \rightarrow D_2A \end{array} \qquad (2)$$

Comparison of our findings with results from other techniques should be taken with caution. We define binding by the immobilization of spin-labeled anesthetics, which provides a different criterion of binding than do other methods. Furthermore, our procedures are not intended to measure receptor changes on the rapid time scale; we are primarily concerned with the difference in binding of C6SL and C6SLMeI to the AChR in its high affinity state. Another caution in evaluating experiments using other tertiary amine anesthetics is the need to establish the pK_a of the anesthetic. A tertiary amino anesthetic with a high pK_a may have very few uncharged species at neutral pH; consequently, such an anesthetic may behave more like C6SLMeI. We predict that tertiary amine NCB with pK_a near neutrality would behave like C6SL.

The binding site for local anesthetics undoubtedly has a negatively charged component. C6SL with a pK below 8 is reversibly charged depending on the pH of the medium. At pH 10 most of the C6SL are uncharged and, at the concentrations used in our experiments, did not exhibit significant binding. In comparison, at pH 6, the binding of C6SL to the AChR is clearly present. It is therefore possible to conclude that the positive charge of the local anesthetics is required in order to

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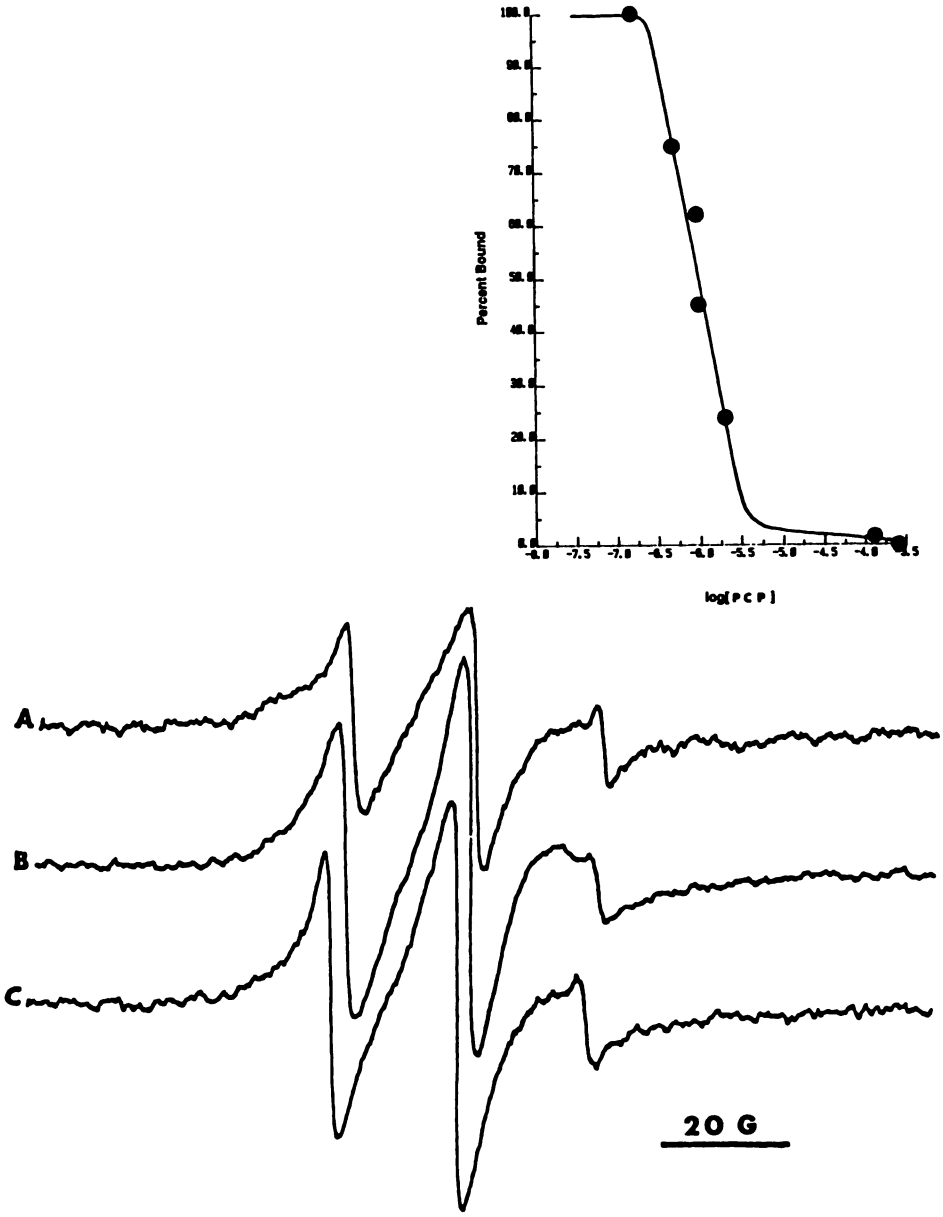

Fig. 7. Dissociation of the strongly immobilized C6SL ESR component by addition of PCP. AChR-enriched membranes were incubated with C6SL and AChR at a molar ratio of 5:1 in the presence of 1 mM carbamylcholine. After 15 min of incubation, PCP was added at concentrations: (A) control, (B) 250 μM, (C) no agonist. Note that at 250 μM PCP the strongly immobilized component is no longer observable. Additional concentrations of PCP were also used to examine the inhibition of the strongly immobilized C6SL binding to AChR (see *inset*). The per cent bound is determined from the strongly immobilized component in the ESR spectra in a similar way as described in Fig. 4. The 100% value is defined by the difference spectrum obtained from experiments in which agonist and anesthetics were added simultaneously in the absence of PCP (i.e., Fig. 7A). At 250 μM PCP, there is no significant binding of C6SL to the AChR (i.e., Fig. 7B).

bind at the NCB site under concentration conditions used in our experiments. The effective pH in the ion channel and the effective pK of C6SL determine the available cationic species of C6SL for binding and are the primary reason for the somewhat higher K_d value measured for C6SL when contrasted with that of the permanently charged C6SLMeI. On the other hand, the relatively low effective pK of C6SL, which gives rise to a large fraction of uncharged species at physiological pH, may account for the high accessibility of C6SL to the NCB site. Other tertiary amines that are predominantly charged at physiological pH may exhibit lower accessibility.

Both C6SLMeI and C6SL compete with PCP for its binding site. Because PCP is believed to bind to a single class of sites on the AChR (8–11), our results suggest that C6SL and C6SLMeI both bind to the same site to exhibit the highly immobilized ESR component. Because the suggestion is that both C6SL and C6SLMeI interact with the same site on the AChR, the differences in the ability of the respective anesthetics to interact with the AChR suggest a difference in accessibility rather than in affinity. Unlike C6SLMeI, which has access to the NCB site for a limited time period immediately after agonist stimulation, C6SL is not comparably restricted in its access to the AChR. It is clear that C6SL is able to gain access readily to the receptor in the desensitized state. In other words the tertiary amine local anesthetic, which can exist in both charged and uncharged states, is readily accessible to the high affinity NCB site when the channel is "closed" or in the D_2 state. C6SL did not show the same binding to the AChR in the resting state (in the absence of agonists). This result is most likely due to the low binding affinity at the NCB site in the resting state of the receptor. We did not investigate the binding at high concentrations of C6SL and thus have no results regarding the affinity of the resting site.

The binding of C6SLMeI is in accordance with the model in which the high affinity NCB site is reached through the open channel or by means of a restricted path available during the early desensitized state. C6SL, on the other hand, has no comparable restrictions on its ability to reach the high affinity NCB site. Because the only significant difference between C6SL and C6SLMeI is that C6SL exists as an equilibrium of charged and uncharged species, we base our explanation on these anesthetic properties. Two models are proposed to explain

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the experimental results. 1) A charged gate in the channel mouth presumably restricts the entry of the permanently charged quaternary amine local anesthetic to the NCB site in the desensitized receptor. Conversely the charged gate poses no barrier to the access of the uncharged form of the tertiary amine local anesthetic. After entering the channel the anesthetic can then reestablish its charged state, depending on local solvent polarity and pH, allowing it to bind. 2) The uncharged form of C6SL can penetrate hydrophobic regions where charged anesthetics are excluded. Conceivably, uncharged C6SL is able to diffuse into the lipid bilayer and gain access to the ion channel through a corridor in the annular lipid surrounding the receptor (Fig. 8). Depending on the environment in the channel, it can then reestablish itself in the charged state and bind to the NCB site. The existence of this hydrophobic path is consistent with results from patch-clamp studies (31, 32) in which tertiary amine local anesthetics are effective, but only with delayed onset when applied intracellularly or outside the patch electrode. In contrast, permanently charged anesthetics are only effective when applied to the extracellular side of the receptor channel (31, 32). Our latter model, which provides an aqueous path (ion channel) for charged and uncharged local anesthetics, is consistent with results reported by Oswald *et al.* (33). They observed rapid kinetics for PCP binding under open channel conditions and slower, temperature-dependent kinetics under closed channel conditions for the tertiary amine PCP. Furthermore, they observe little change in equilibrium parameters between simultaneous addition and prior addition of agonist; this result is predictable for tertiary amine compounds from our model. It is also interesting to note that they observed a pH-dependent change in the equilibrium binding of PCP, which may be due to a change in PCP ionization. The observation by Koblin and Lester (20) suggests that small charged anesthetics bind to the NCB site by an open channel path, whereas large hydrophobic anesthetics are able to reach the NCB site by an additional membrane path that is voltage-independent.

Our results suggest a primary time window in the range of minutes ($k = 0.19$ min^{-1}) during which NCB site is accessible to charged anesthetics. However, the open channel state is generally in the range of milliseconds (29, 34, 35). This would necessarily imply that intermittent channel openings must take place through the early desensitized phase (or a transition state) of the receptor if charged anesthetics are to enter through the channel mouth. Channel openings during this period are reported; estimates of the time required for ion flux to each nonsignificant levels in the *Torpedo* ranged from seconds to minutes (29, 34). In the *Electrophorus* the time required is much slower, in the range of hours (34). However, our results provide only information on the accessibility of local anesthetics to the NCB site in the AChR, which may or may not correspond to the activation of ion conductance. We do conclude that charged anesthetics reach the NCB site by an agonist-dependent path. In contrast, uncharged anesthetics are able to reach the NCB site by an additional agonist-independent path that may be hydrophobic in nature. Regardless of the path of access, high affinity binding of local anesthetics to the NCB site is agonist- and charge-dependent.

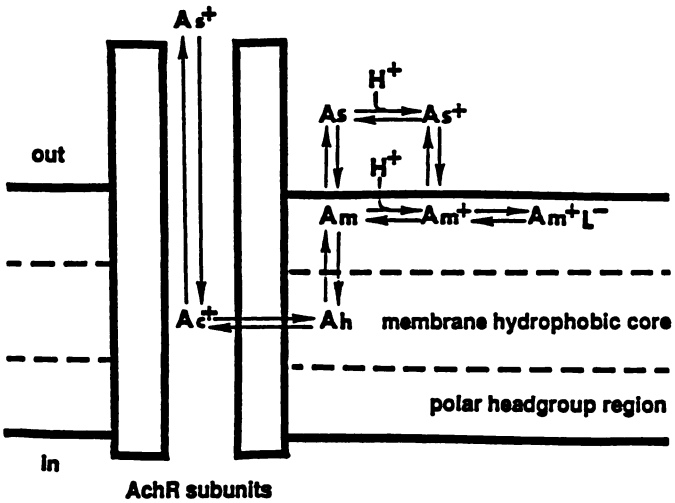

Fig. 8. Model to illustrate membrane lipid pathway of anesthetic access to the AChR. *As* and *As*$^+$ represent uncharged and charged anesthetics in solution; *Am*, *Am*$^+$, and *Am*$^+L^-$ represent membrane-intercalated anesthetics that are uncharged, charged, and lipid-associated, respectively. *Ah* represents uncharged anesthetics penetrating into the hydrophobic interior of the bilayer. *Ac*$^+$ represents anesthetics in the ion channel. Free protons in the AChR channel are not shown. The equilibrium between As$^+$ and Ac$^+$ is dependent on the open channel as illustrated. When the receptor is in the open state, uncharged As can also enter the channel rapidly by diffusion (not shown) through the channel mouth. When the channel is closed, by the uncharged or reversibly charged anesthetics are in equilibrium with the channel lumen through a hydrophobic membrane path.

References

1. McCarthy, M. P., J. P. Earnest, E. F. Young, S.-H. Choe, and R. M. Stroud. The molecular neurobiology of the acetylcholine receptors. *Ann. Rev. Neurosci.* **9**:383–413 (1986).
2. Changeux, J. P., A. Devillers-Thiery, and P. Chemouilli. Acetylcholine receptor: an allosteric protein. *Science (Wash. DC)* **225**:1335–1345 (1984).
3. Conti-Tronconi, B. M., and M. A. Raftery. The nicotinic cholinergic receptor: correlation of molecular structure with functional properties. *Annu. Rev. Biochem.* **51**:491–530 (1982).
4. Spivak, C. E. and E. X. Albuquerque. Dynamic properties of the nicotinic acetylcholine receptor ionic channel complex: activation and blockade, in *Progress in Cholinergic Biology*, (I. Hanin and A. M. Goldberg, eds.), Vol. 2. Raven Press, New York, 323–325 (1982).
5. Weiland, G., and P. Taylor. Ligand specificity of state transitions in the cholinergic receptor: behavior of agonists and antagonists. *Mol. Pharmacol.* **15**:197–212 (1979).
6. Sakmann, B., J. Petlack, and E. Neher. Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature (Lond.)* **286**:71–73 (1980).
7. Weiland, G., R. Georgia, V. T. Wee, C. F. Chignell, and P. Taylor. Ligand interactions with cholinergic receptor-enriched membranes from *Torpedo:* influence of agonist exposure on receptor properties. *Mol. Pharmacol.* **12**:1091–1105 (1976).
8. Heidmann, T., R. E. Oswald, and J.-P. Changeux. Multiple sites of action for noncompetitive blockers on acetylcholine receptor-rich membrane fragments from *Torpedo marmorata. Biochemistry* **22**:3112–3127 (1983).
9. Elliot, J., and M. Raftery. Binding of perhydrohistrionicotoxin to intact and detergent-solubilized membranes enriched in nicotinic acetylcholine receptor. *Biochemistry* **18**:1868–1877 (1979).
10. Aronstam, R. S., A. T. Eldefrawi, I. N. Pessah, J. W. Daly, E. X. Albuquerque, and M. E. Eldefrawi. Regulation of [^{3}H]perhydrohistrionicotoxin binding to *Torpedo ocellata* electroplax by effectors of the acetylcholine receptor. *J. Biol. Chem.* **225**:2863–2850 (1981).
11. Palma, A., J. M. Herz, H. H. Wang, and P. Taylor. Association of a spin-labeled local anesthetic with the allosterically coupled noncompetitive inhibitor site on the acetylcholine receptor. *Mol. Pharmacol.* **30**:243–251 (1986).
12. Peper, K., R. J. Bradley, and F. Dreyer. The acetylcholine receptor at the neuromuscular junction. *Physiol. Rev.* **62**:1271–1340 (1982).
13. Krodel, E. K., R. A. Beckman, and J. B. Cohen. Identification of a local anesthetic binding site in nicotinic post-synaptic membranes isolated from *Torpedo marmorata* electric tissue. *Mol. Pharmacol.* **15**:294–312 (1979).
14. Ruff, R. L. A quantitative analysis of local anesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol. (Lond.)* **264**:89–124 (1977).
15. Neher, E., and J. H. Steinbach. Local anesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol. (Lond.)* **277**:153–176 (1978).

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16. Heidmann, T., and J.-P. Changeux. Time-resolved photolabeling by the noncompetitive blocker chlorpromazine of the acetylcholine receptor in its transiently open and closed ion channel conformation. *Proc. Natl. Acad. Sci. USA* **81**:1897–1901 (1984).
17. Changeux, J.-P., C. Pinset, and A. Ribera. Effects of chlorpromazine and PCP on mouse C_2 acetylcholine receptor kinetics. *J. Physiol. (Lond.)* **378**:497–513 (1986).
18. Albuquerque, E. X., M.-C. Tsai, R. S. Aronstam, B. Witkop, A. T. Eldefrawi, and M. B. Eldefrawi. Phencyclidine interactions with the ionic channel of the acetylcholine receptor and electrogenic membrane. *Proc. Natl. Acad. Sci. USA* **77**:1224–1228 (1980).
19. Adams, P. R., and B. Sarmann. Decamethionium both opens and blocks endplate channels. *Proc. Natl. Acad. Sci. USA* **75**:2994–2998 (1978).
20. Koblin, D. R., and H. Lester. Voltage-dependent and voltage-independent blockade of acetylcholine-receptors by local anesthetics. *Mol. Pharmacol.* **15**:559–580 (1978).
21. Farley, M. J., J. Z. Yeh, S. Watanabe, and T. Narahashi. Endplate channel block by guanidine derivatives. *J. Gen. Physiol.* **77**:273–293 (1981).
22. Adams, P. R. Voltage jump analysis of procaine action at frog end-plate. *J. Physiol. (Lond.)* **268**:291–318.
23. Gargiolo, R., G. Giotta, and H. H. Wang. Spin-labeled analogs of local anesthetics. *J. Med. Chem.* **16**:707–708 (1973).
24. Wang, H. H., J. Z. Yeh, and T. Narahashi. Interaction of spin-labeled local anesthetics with the sodium channel of squid axon membranes. *J. Membr. Biol.* **66**:227–233 (1982).
25. Blickenstaff, G. The effect of local anesthetics on rubidium flux across reconstituted acetylcholine receptor membranes, Ph.D. dissertation, University of California, Santa Cruz (1986).
26. Limbacher, H. P., G. D. Blickenstaff, J. H. Bowen, and H. H. Wang. Multiequilibrium binding of a spin-labeled local anesthetic in phosphatidylcholine bilayers. *Biophys. Biochim. Acta* **812**:268–276 (1985).
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1981).
28. Schmidt, M., and M. A. Raftery. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* **52**:345–355 (1973).
29. Walker, J. W., K. Takeyasu, and M. G. McNamee. Activation and inactivation kinetics of *Torpedo californica* acetylcholine receptor in reconstituted membranes. *Biochemistry* **21**:5384–5389 (1982).
30. Earnest, J. P., H. P. Limbacher, M. G. McNamee, and H. H. Wang. Binding of local anesthetics to reconstituted acetylcholine receptors: the effect of protein surface charge. *Biochemistry* **25**:5809–5818 (1986).
31. Aracava, Y., S. R. Ikeda, J. W. Daly, N. Brookes, and E. X. Albuquerque. Interactions of bupivacaine with ionic channels of the nicotinic receptor: analysis of single-channel currents. *Mol. Pharmacol.* **26**:304–313 (1984).
32. Ikeda, S. R., R. S. Aronstam, J. W. Daly, Y. Aracava, and E. X. Albuquerque. Interaction of bupivacaine with ionic channels of the nicotinic receptor: electrophysiological and biochemical studies. *Mol. Pharmacol.* **26**:293–363 (1984).
33. Oswald, R. E., M. J. Bamberger, and J. T. McLaughlin. Mechanisms of phencyclidine binding to the acetylcholine receptor from *Torpedo* electroplaque. *Mol. Pharmacol.* **25**:360–368 (1984).
34. Aoshima, H. A second, slower inactivation process in acetylcholine receptor-rich membrane vesicles prepared from *Electrophorus electricus*. *Arch. Biochem. Biophys.* **235**:312–318 (1984).
35. Hess, G. P., D. J. Cash, and H. Aoshima. Acetylcholine receptor-controlled ion translocation: chemical kinetic investigations of the mechanism. *Annu. Rev. Biophys. Bioeng.* **12**:443–473 (1983).

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