Steric factors limit access to the noncompetitive inhibitor site of the nicotinic acetylcholine receptor

Fluorescence studies

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INTRODUCTION

The nicotinic acetylcholine receptor (AChR) functions as a neurotransmitter-activated cation channel. Previous studies have revealed that noncompetitive inhibitor ligands (NCIs) such as phencyclidine (PCP) and ethidium interact with a single, high affinity site located in the ion channel, that is allosterically regulated by the agonist sites (1, 2). Although analysis of the fluorescent properties of bound ethidium indicate that it is highly immobilized in a hydrophobic pocket, there is also evidence for an important electrostatic component to binding energy (2, 3). Kinetic measurements of [^{3}H]PCP binding to the transient open channel state of the receptor are 10^3–10^4-fold greater than to the closed, desensitized state (4). These findings suggest that the AChR may impose a structural barrier which severely hinders access of NCI ligands to the channel binding site in the desensitized conformational state.

In the present study, we have carried out fluorescence spectroscopic experiments to determine the solute and solvent accessibility of ethidium bound to the NCI site of the AChR. By examining the interactions of bound ethidium with two polar molecules, one charged (iodide) and the other uncharged (D_2O), we are able to discriminate between charge and steric effects influencing accessibility of the bound ligand.

RESULTS AND DISCUSSION

The topography of the NCI binding site was probed by employing iodide (I^-) which we have found to be a highly efficient quencher of ethidium fluorescence in solution. Iodide quenching of ethidium bound to the NCI site was examined under conditions in which fluorescence from the specifically bound ligand accounted for over 93% of the total fluorescence signal. AChR enriched membranes were equilibrated with ethidium (100 nM) and carbamylcholine (100 μM). Under these conditions, the predominant receptor state is the desensitized state which binds ethidium with high affinity (K_d = 200 nM) (2). Dynamic fluorescence quenching can be described by the Stern-Volmer equation:

$$F_0/F = \tau_0/\tau = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q], \qquad (1)$$

where F_0 and F are the fluorescent intensities in the absence and presence of quencher (Q), τ_0 and τ are the corresponding excited state lifetimes, and k_q is the bimolecular quenching rate constant. Fig. 1 shows the Stern-Volmer plots for the steady-state fluorescence quenching of ethidium bound to the NCI site as well as quenching data for ethidium displaced from the site by the addition of 100 μM PCP. Similar results were obtained using either excitation via AChR tryptophan residues (290 nm) or using direct excitation of bound ethidium (520 nm).

To calculate k_q, knowledge of the fluorescence lifetimes are required. It was previously shown that binding of ethidium to the NCI site increases its lifetime from 1.7

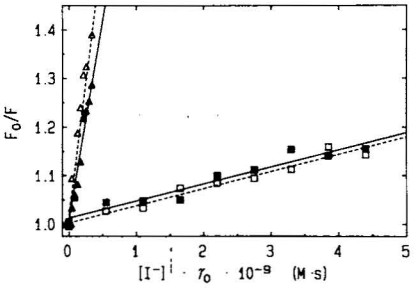

FIGURE 1 Stern-Volmer plots of iodide quenching of the steady-state fluorescence of ethidium bound to the AChR. Quenching of bound ethidium was measured in samples containing AChR membranes (0.5 μM α-toxin sites) suspended in 10 mM Na-phosphate, 1 mM EDTA, 1 mM EGTA, pH 7.4, after incubation with 100 nM ethidium and 100 μM carbamylcholine for 1 hr (□, ■). Alternatively, ethidium was displaced from the NCI site by the addition of 100 μM PCP to above samples (△, ▲). Ionic strength was maintained constant at 0.2M by addition of NaCl. The excitation wavelength was 290 nm (□, △) or 520 nm (■, ▲) and emission was detected at 593 nm. The solid and dashed lines represent the linear least-squares regression fit for each set of data.

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to 21.6 ns (2). The lifetimes of the free and bound species were incorporated into the X-axis in Fig. 1 so that the slope of the lines in the plot are k_q. The steady-state data were also analyzed in terms of a model in which 2 species, ethidium free in solution and ethidium bound to the NCI site, are each undergoing dynamic quenching. Correction for the fractional steady-state fluorescence contributed by the free species yielded k_q values of $2.9 \times 10^7\ M^{-1}s^{-1}$ (290 nm) and $2.4 \times 10^7\ M^{-1}s^{-1}$ (520 nm) for ethidium bound to the NCI site. The bimolecular quenching constant is decreased approximately 100-fold upon binding to the NCI site compared to the value we determined for quenching of ethidium in solution ($k_q = 1.9 \times 10^9\ M^{-1}s^{-1}$). The amount of protection of bound ethidium from the free diffusion of iodide is striking.

Measurements of excited-state lifetimes provides the only direct method to distinguish the effects of quenching on the free and bound ethidium species contributing to the total fluorescence signal in AChR-carbamylcholine-ethidium samples. Because the free and bound species have unique and resolvable lifetimes, the bimolecular quenching constants for each species can be calculated according to Eq. 1. The fluorescence decay of ethidium bound to the NCI site in the presence of 0.2 M NaCl is characterized by two lifetimes of nearly equal amplitudes ($\tau_1 = 1.8$ ns, $\tau_2 = 21.8$ ns). Increasing concentrations of iodide (0 to 200 mM) resulted in a very small reduction in the lifetime of the bound species (21.8 to 21.0 ns), while the lifetime of the free species progressively decreased as expected (1.8 to 1.0 ns). The k_q calculated from the lifetime data for the bound species was found to be $6.15 \times 10^6\ M^{-1}s^{-1}$. Thus, the exceedingly low accessibility of iodide to bound ethidium determined by nanosecond lifetime measurements confirms the results obtained by steady-state methods. Because

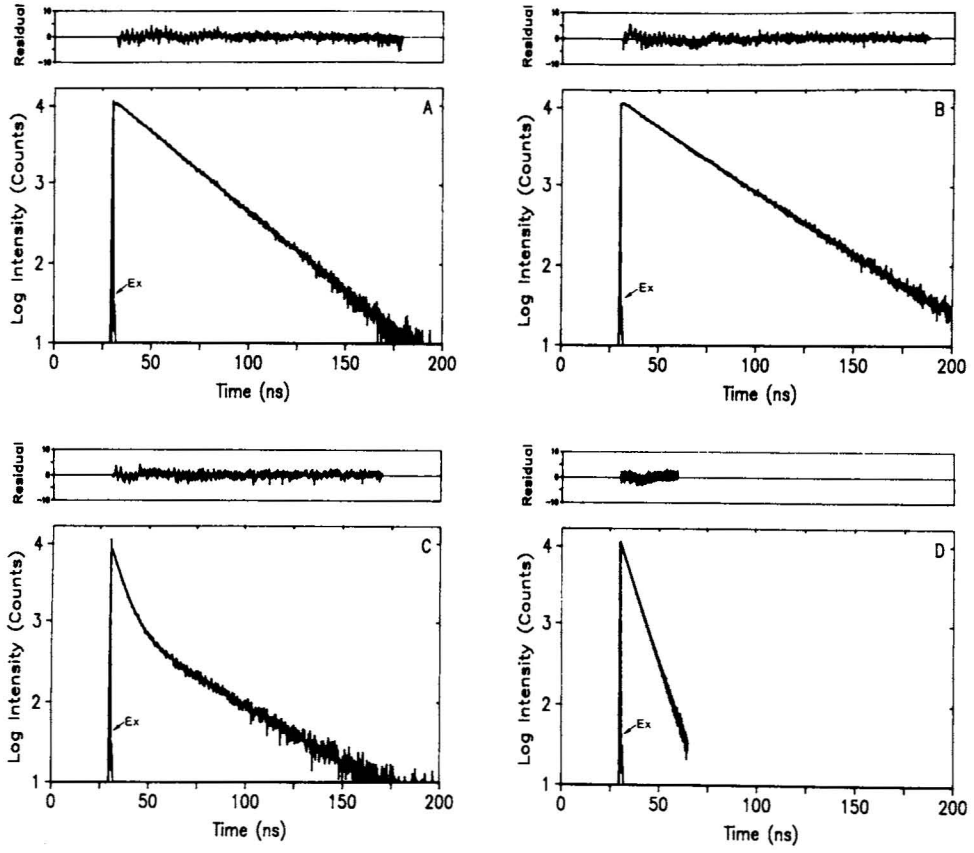

FIGURE 2 Effect of D_2O on the fluorescence decay of ethidium. The nanosecond decay curves of ethidium (0.2 μM) bound to the AChR (0.5 μM α-toxin sites) in the presence of carbamylcholine (200 μM) and (*A*) 0.0 mole fraction D_2O, or (*B*) 1.0 mole fraction D_2O. In (*C*), addition of 100 μM PCP to the sample shown in *B*. (*D*) shows the decay curve for ethidium in 1.0 mole fraction D_2O in the absence of AChR membranes. Each panel (*A–D*) shows the shape of the laser excitation pulse (Ex), the raw data, and a computer calculated decay curve obtained by deconvolution of the experimental decay. Deviations of the experimental data from the fitted single or double exponential decay function are shown in the residual plots above each panel.

quenching efficiency by iodide may be sensitive to electrostatic effects, the large reduction in the collisional rate constant could be ascribed to either charge or steric effects, or some combination thereof.

To resolve the contributions of charge and steric factors, we determined the solvent accessibility of bound ethidium by taking advantage of a large deuterium isotope effect on the lifetime of ethidium. This method involves measurement of the deuterium effect on the lifetime of the excited state both in free solution and bound to the AChR to yield an F factor which may be interpreted as a measure of solvent accessibility of the bound species (5). Because we have shown that the reciprocal of the fluorescence lifetimes (τ^{-1}) for ethidium in solution and bound to the AChR exhibit a linear dependence on the mole fraction of D_2O, the following can be derived to describe the fractional accessibility of the bound ligand (5):

$$F = [\tau^{-1}_{H(AChR)} - \tau^{-1}_{D(AChR)}]/[\tau^{-1}_{(H20)} - \tau^{-1}_{(D20)}], \quad (2)$$

where $\tau_{H(AChR)}$ and $\tau_{D(AChR)}$ are the fluorescence lifetimes of ethidium bound to the NCI site of the AChR site in solutions in which the solvent is H_2O and D_2O, respectively. $\tau_{(H2O)}$ and $\tau_{(D2O)}$ are the lifetimes of ethidium in H_2O and D_2O, respectively.

The fluorescence decay rate for ethidium bound to the AChR in the absence of D_2O obeys a single exponential behavior with a lifetime of 21.6 ns (Fig. 2 *A*). As seen in Fig. 2 *B*, the corresponding decay rate for ethidium bound in the presence of 1.0 mole fraction D_2O exhibits a modest increase to a value of 26.5 ns. In contrast, addition of excess PCP to the 1.0 mole fraction D_2O sample results in a decay rate that is best described by double-exponential decay process (Fig. 2 *C*). The fluorescence decay is characterized by two lifetimes (τ_1 = 5.2, τ_2 = 27.0 ns) in which the shorter lifetime component contributes over 89% of the total amplitude. The lifetime of 5.2 ns is similar to the lifetime measured for free ethidium in 1.0 mole fraction D_2O (Fig. 2 *D*). Substitution of the above lifetimes into Eq. 2 yields F = 0.0021. Thus, solvent accessibility of ethidium bound to the NCI binding site is exceedingly small.

In conclusion, we have shown that ethidium bound to the NCI site of the AChR in the desensitized state is highly inaccessible to charged ions and water molecules. The small F factor for D_2O indicates that the decrease in the bimolecular quenching constant for iodide can be accounted for by the interposition of parts of the AChR macromolecule which present a steric barrier to the collisions between iodide and bound ethidium. Fluorescence energy transfer experiments have mapped the ethidium binding site to a region near the synaptic surface of the transmembrane channel (6). Our current results suggest that the bound NCI ligand is buried within a transmembrane domain of the ion channel which is not accessible to the aqueous phase in the desensitized state. The architecture of the AChR transmembrane channel has been proposed to consist of 5 α-helical M2 sequences, each contributed from one of the five AChR subunits. A significant number of nonpolar and hydrophobic residues in each M2 helix are grouped together in the synaptic portion of the interior of the channel. This cluster of residues could constitute a significant barrier to the entrance of charged ions and polar water molecules in the closed channel state.

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