Differential Agonist-Induced Displacement of Quinacrine and Ethidium from Their Respective Histrionicotoxin-Sensitive Binding Sites on the *Torpedo* Acetylcholine Receptor†

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ABSTRACT: Fluorescence spectroscopy was used to begin to localize the agonist inhibitory binding site on the nicotinic acetylcholine receptor (AcChR) from *Torpedo californica.* High concentrations of three cholinergic agonists, suberyldicholine (SubCh), acetylcholine (AcCh), and carbamylcholine (CCh), differentially inhibited the binding of two noncompetitive inhibitors (NCIs), quinacrine and ethidium, which bind at distinctly different loci on the desensitized AcChR at zero membrane potential. The agonist-induced inhibition of quinacrine binding occurred at significantly lower (17-fold) concentrations than the inhibition of ethidium binding. Schild plots of SubCh inhibition of ethidium and quinacrine binding showed the competitive nature of the agonist inhibition of the binding of these two NCIs. The quenching constants for short-range quenching of receptor-bound quinacrine and ethidium fluorescence by spin-labeled acetylcholine were about the same as their inhibition constants for agonist-induced displacement of AcChR-bound quinacrine and ethidium. The results demonstrate that agonists can directly bind to both the quinacrine and the ethidium binding sites, albeit at different agonist concentrations. Because the agonist-induced displacement of receptor-bound quinacrine occurs at significantly lower concentrations than the displacement of ethidium, the quinacrine binding site is more likely than the ethidium binding site to form part of the agonist inhibitory binding site.

At micromolar concentrations, acetylcholine (AcCh)[1] and other cholinergic agonists bind at two high-affinity AcCh sites on the muscle-type nicotinic acetylcholine receptor (AcChR). This binding triggers transient openings of a cation channel that allow cations to move down their transmembrane electrochemical gradients. At millimolar concentrations, nicotinic agonists reduce single-channel conductances and/or cause frequent interruptions of open AcChR channels (Adams & Sakmann, 1978; Takeyasu *et al.*, 1983; Pasquale *et al.*, 1983; Ogden & Colquhoun, 1985, 1988; Sine & Steinback, 1985; Forman & Miller, 1988). This inhibitory action is rapid and differs from the slower desensitization process. The fast inhibiting effects of nicotinic agonists resemble the actions of histrionicotoxin (HTX)-sensitive noncompetitive inhibitors (NCIs), suggesting that fast agonist inhibition involves the direct binding of agonists at an HTX-sensitive NCI binding site.

In addition to the similarities between the fast inhibitory effects of agonists and the HTX-sensitive NCIs on single-channel conductances and/or channel interruptions, other evidence also indicates that fast agonist inhibition of the AcChR is mediated via an HTX-sensitive NCI binding site: First, the blocking action of both agonists and HTX-sensitive NCIs is voltage dependent (Takeyasu *et al.*, 1983; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985; Colquhoun & Ogden, 1988; Carter & Oswald, 1993). Second, procaine, an HTX-sensitive NCI, and suberyldicholine (SubCh), an agonist, block AcCh-induced cation fluxes in a mutually exclusive manner (Forman & Miller, 1989). Finally, [^{3}H]-HTX binding is inhibited by high concentrations of (−)-nicotine and decamethonium, two AcChR activators (Eldefrawi *et al.*, 1982).

HTX-sensitive NCIs include ethidium (Herz *et al.*, 1987), quinacrine (Adams, 1981), phencyclidine (PCP) (Eldefrawi *et al.*, 1980), and, of course, HTX (Eldefrai *et al.*, 1977). Although HTX-sensitive NCI binding is associated with a unitary binding stoichiometry, multiple HTX-sensitive NCI binding loci exist (Johnson & Nuss, 1994). A HTX-sensitive NCI binding appears to be highly conformationally dependent, because ligand binding to one HTX-sensitive NCI site precludes simultaneous binding of NCIs at other sites. The most characterized NCI binding locus is about two-thirds of the way (from the extracellular side) into the luminal transmembrane domain. This site is formed in part by the two homologous rings of hydroxyl side chains in the M2 transmembrane segments of each of the five subunits (α_2, β, γ, and δ) that constitute the AcChR (Galzi *et al.*, 1991). An NCI binding site may exist a few amino acid residues above this site (White & Cohen, 1992). An HTX-sensitive NCI binding site may be located at the extracellular entrance to the transmembrane luminal domain of the AChR, because [^{3}H]meproadifen mustard affinity labels a side chain in the extracellular loop between the M2 and M3 transmembrane segments of the α-subunit (αGlu^{262}) (Dreyer *et al.*, 1986;

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[1] Abbreviations: AcChR, muscle-type nicotinic acetylcholine receptor; AcCh, acetylcholine; buffer I, 10 mM sodium phosphate buffer, pH 7.4; CCh, carbamylcholine; HTX, histrionicotoxin; NCI, noncompetitive inhibitor; PCP, phencyclidine; SL-AcCh, spin-labeled acetylcholine [spin-labeled with 4-[*N*-(acetoxyethyl)-*N*,*N*-dimethylammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl]; SubCh, suberyldicholine.

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Pedersen *et al.*, 1990). Quinacrine appears to bind to another locus outside the lumen in the transmembrane region toward the extracellular domain, because [^{3}H]quinacrine azide photoaffinity labels the beginning of the extracellular end of the M1 segment of the α-subunit (Karlin, 1991), and because lipophilic paramagnetic and fluorescent probes readily quench PCP-sensitive quinacrine fluorescence (Valenzuela *et al.*, 1992; Arias *et al.*, 1993a,b). Ethidium appears to bind to still another site that is located in the walls of the extracellular vestibule of the receptor (Johnson & Nuss, 1994).

Although the above-mentioned results indicate that agonists rapidly inhibit cation fluxes by binding at an HTX-sensitive NCI binding site, the particular NCI site involved is, however, unknown. As a first effort to localize more precisely the inhibitory site of action of agonists, we compared the ability of three agonists, AcCh, SubCh, and carbamylcholine (CCh), to inhibit quinacrine and ethidium binding to the *Torpedo* AcChR. Also, to determine whether agonists inhibit quinacrine and ethidium binding by direct displacement or by an indirect allosteric mechanism, the relative accessibility of agonists to receptor-bound quinacrine and ethidium was assessed by measuring the ability of spin-labeled acetylcholine (SL-AcCh) to quench the emissions from receptor-bound quinacrine and ethidium. The results demonstrate that although agonists can directly interact with both the quinacrine and ethidium NCI binding sites on the desensitized AcChR, agonist-induced inhibition of quinacrine binding occurs at significantly lower concentrations than inhibition of ethidium binding.

MATERIALS AND METHODS

Materials. Quinacrine dihydrochloride, suberyldicholine dihydrochloride, acetylcholine hydrochloride, carbamylcholine hydrochloride, and phencyclidine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Ethidium bromide was purchased from Calbiochem (La Jolla, CA). *Torpedo californica* electric rays were obtained from Marinus Inc. (Long Beach, CA).

Isolation of the Membrane-Associated AcChR Receptor. AcChR-associated membranes, designated in the following as AcChR membranes, were prepared from the *Torpedo californica* electric organs following previously described procedures (Johnson & Yguerabide, 1985). The specific activities of the various receptor preparations ranged between 0.9 and 1.5 nmol of SubCh binding sites/mg of total protein, determined as described elsewhere (Johnson & Yguerabide, 1985).

Schild Analysis of SubCh Inhibition of Quinacrine and Ethidium Binding. To assess the nature of agonist inhibition (competitive/noncompetitive), a Schild type of analysis (Schild, 1949) of SubCh inhibition of quinacrine and ethidium binding was performed. Quinacrine and ethidium were titrated into AcChR membranes (0.7 μM in SubCh binding sites) suspended in 10 mM sodium phosphate buffer (pH 7.4) (buffer I), in the presence and absence of PCP (0.5 mM) and fixed concentrations of SubCh (0.17−50.0 mM). SubCh was added to the cuvettes 10 min before the start of each titration. A sufficient amount of CCh (0.77 mM) was also added to each cuvette to prevent quinacrine or ethidium from binding to the high-affinity agonist binding sites and to induce the receptor into a slow-onset desensitized state, which displays a higher affinity toward quinacrine and ethidium. This concentration of CCh was low enough that it did not interfere with NCI binding. The addition of excess PCP, a nonfluorescent HTX-sensitive NCI, to samples defined the specific or PCP-sensitive fluorescence associated with the binding of quinacrine or ethidium to their respective HTX-sensitive NCI sites.

Estimates of the apparent dissociation constants in the absence (K_d) or presence (K_d') of SubCh were made by fitting plots of the specific (PCP-sensitive) changes in quinacrine or ethidium fluorescence versus the free ligand concentration to the equation for a rectangular hyperbola ($Y = B_{max}$[ligand]/(K_d + [ligand])) by using the Marquardt algorithm (Marquardt, 1959). The free-ligand concentration was assumed to equal the total titrant concentration minus the bound titrant concentration. The bound-ligand concentration equaled the fractional specific inhibition of fluorescence multiplied by the concentration of HTX-sensitive binding sites (0.35 μM). These results were plotted using the Schild equation:

$$\log[(K_d'/K_d) - 1)] = \log [B] - \log K_I \qquad (1)$$

where [B] is the concentration of inhibitor and K_I is the inhibition constant, which was determined from the negative antilog of the x-intercept of these plots.

Fluorescence Measurements. Unless stated otherwise, all fluorescence titrations were carried out with 0.5 × 0.5 cm cuvettes held at 15 °C in a Perkin-Elmer Cetus MPF 66 spectrofluorometer. Again, unless stated otherwise, excitation and emission wavelengths were 450 and 502 nm for quinacrine and 520 and 595 nm for ethidium, respectively. To reduce stray-light effects, a Corning 3-71 cutoff filter was placed in the path of the quinacrine emission beam; an Oriel 520-nm narrower band pass and a Perkin-Elmer 610 cutoff filter were placed in the paths of the ethidium excitation and emission beams, respectively.

Chen−Prusoff-Type Analysis of Agonist Inhibition of Quinacrine and Ethidium Binding. Because Schild analysis indicated that SubCh competitively inhibited quinacrine and ethidium binding, the simpler Chen−Prusoff analysis of agonist inhibition was performed to compare the effects of various agonists on both quinacrine and ethidium binding. Suspensions of AcChR membranes (0.7 μM in SubCh binding sites) in buffer I plus CCh (0.77 mM), quinacrine (0.35 μM), or ethidium (2.5 μM) ± PCP (0.5 mM) were prepared 2 h before the start of the SubCh, AcCh, or CCh titrations. The agonist titrations were carried out under the same conditions that were described earlier. To obtain a broad range of final titrant concentrations without excessive dilution, stock solutions were prepared at concentrations that ranged over 0.02−1.8, 0.06−5.9, and 0.04−4.4 M for SubCh, AcCh, and CCh, respectively. The agonists were weighed and dissolved in buffer I by using vigorous vortex mixing, and the final volume was measured to determine molarity. The stock solutions were prepared prior to the start of the titration and were maintained on ice throughout the titration. The apparent inhibition constant (K_I) for each agonist was obtained by using the following expression (Cheng & Prushoff, 1973):

$$K_I = IC_{50}/\{1 + ([NCI]/K_d^{NCI})\} \qquad (2)$$

where IC_{50} is the concentration of agonist that is associated

with a 50% reduction in the PCP-sensitive fluorescence of quinacrine or ethidium. [NCI] and K_d^{NCI} are the concentration and dissociation constant of the added fluorophore in the absence of high concentrations of agonist, respectively.

SL-AcCh Accessibility to Receptor-Bound Quinacrine and Ethidium. To assess the relative accessibility of agonists to specifically bound quinacrine and ethidium, the steady-state quenching constants (K_Q) for SL-AcCh quenching of specific quinacrine and ethidium fluorescence were determined. AcChR membranes (0.7 μM in SubCh binding sites) were suspended in buffer I plus CCh (0.77 mM), quinacrine (0.35 μM), or ethidium (2.5 μM) ± PCP (0.5 mM) and were incubated for 2 h at 15 °C before the start of each titration. Fluorescence values were corrected for the dilution produced by the added titrant. After SL-AcCh was added at each step of the titration, the samples were incubated for 10 min before the quinacrine or ethidium fluorescence was measured. A set of control samples, containing only AcChR membranes in buffer I, was used for the measurement of back-ground and titrant fluorescence (I_{blk}) (Valenzuela *et al.*, 1992).

Because only a fraction (f_B^i) of the added fluorophores was bound to the HTX-sensitive NCI binding sites at each titration step, assessment of the extent of SL-AcCh quenching required the determination of the emission from the receptor-bound fluorophore (I_B). This was accomplished by using the expression,

$$I_B = c[(I_{\text{minus PCP}} - I_{blk}) - (1 - f_B^i)(I_{\text{plus PCP}} - I_{blk})] \quad (3)$$

where $I_{\text{plus PCP}}$ and $I_{\text{minus PCP}}$ are the magnitudes of fluorescence of samples that did or did not contain PCP, respectively (Valenzuela *et al.*, 1992). I_{blk} is the magnitude of fluorescence from samples that contained everything except the fluorophores. The c term is a correction factor for dilution and inner filter effects of titrants. The correction for inner filter effects was calculated from the antilog of the sum of the optical densities of the titrant at the excitation and emission wavelengths divided by 4 (the value 4 was used because 0.5 cm path length cuvettes were utilized). The fraction of added fluorophore that was specifically bound was calculated with the following expression:

$$f_B^i = (0.5\{L_T - K_d' - R_T[L_T^2 + 2L_T(K_d' - R_T) + (K_d' + R_T)^2]^{0.5}\})/L_T \quad (4)$$

where

$$K_d' = K_d(1 + [\text{SL-AcCh}]/K_I^{\text{SL-AcCh}}) \quad (5)$$

and L_T and R_T are the concentrations of total added fluorophore and NCI binding sites, respectively. [SL-AcCh] and $K_I^{\text{SL-AcCh}}$ are the total concentration of added SL-AcCh and its inhibition constant for NCI binding, respectively. Equation 4 corrects for the ability of SL-AcCh to partially displace receptor-bound fluorophore. From the lowest to highest SL-AcCh concentration examined, f changed from 0.27 to 0.24.

K_Q was determined from the slopes of Stern−Volmer plots of specifically bound fluorescence in the absence and presence of SL-AcCh (I_B^0/I_B) versus [SL-AcCh]. The receptor-bound fluorescence intensities were calculated with eq 3.

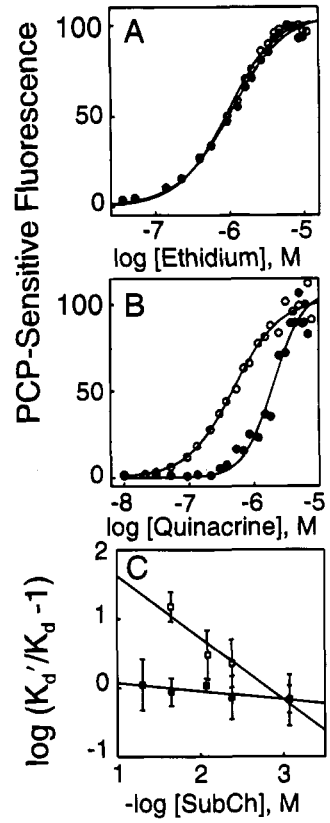

FIGURE 1: Effect of SubCh on PCP-sensitive ethidium and quinacrine binding to the AcChR. Ethidium (A) and quinacrine (B) were titrated into suspensions of AcChR membranes (0.7 μM in SubCh binding sites) and CCh (0.77 mM) ± PCP (0.5 mM) in the absence (○) and presence of 22.5 mM SubCh (●) at 15 °C. Panel C: Schild plots of the inhibition of ethidium (■) and quinacrine (□) binding by SubCh. The calculated K_I's for SubCh inhibition of ethidium and quinacrine binding were 25 ± 38 and 1.5 ± 0.6 mM, respectively. Estimates of the K_d were made by fitting plots of the specific (PCP-sensitive) changes in quinacrine or ethidium fluorescence versus the free-ligand concentration to the equation for a rectangular hyperbola by using the Marquardt algorithm (Marquardt, 1959). Each data point represents the mean ± SD of three determinations.

RESULTS

SubCh Inhibition of PCP-Displaceable Quinacrine and Ethidium Binding. Initial experiments showed that SubCh decreased both PCP-sensitive quinacrine and ethidium fluorescence. To determine whether this effect was because SubCh inhibited their binding, the apparent affinities of quinacrine and ethidium toward their respective PCP-sensitive binding sites were measured in the presence of various concentrations of SubCh. Examples of the results of a set of these titrations in the absence and presence of 22.5 mM SubCh are shown in Figure 1A,B. A 22.5 mM concentration of SubCh shifted the apparent K_D of quinacrine from 0.20 to 3.4 μM (±1.2 μM) without significantly affecting the apparent K_D of ethidium (0.7 μM). To characterize this inhibition further, the results of all of the titrations (not shown) were analyzed with Schild-type plots (Figure 1C). The Schild plots for both quinacrine and ethidium were relatively linear, indicating a competitive type of inhibition. SubCh was much more effective (17-fold) at displacing quinacrine than ethidium from their respective binding sites. The calculated K_I's for SubCh inhibition of quinacrine and ethidium binding were 1.5 ± 0.6 and 25 ± 38 mM, respectively.

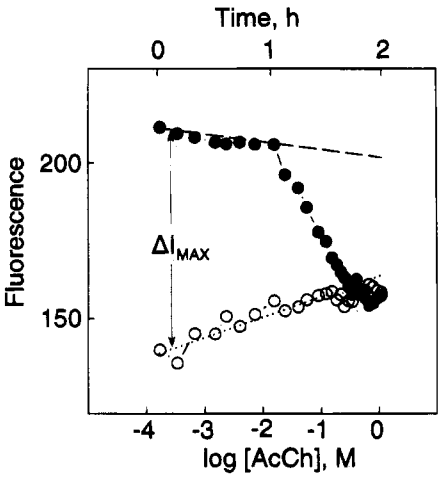

FIGURE 2: AcCh-induced the time-dependent changes in quinacrine fluorescence in the presence of AcChR membranes. AcCh was titrated into a suspension of AcChR membranes (0.7 μM in SubCh binding sites), quinacrine (0.35 μM), and CCh (0.77 mM) in the absence (●) or presence (○) of PCP (0.5 mM) at 15 °C. Time-dependent changes occurred in the AcChR−quinacrine complex without (long dashes) and with PCP (short dashes). See Materials and Methods for experimental details.

Comparison of the Abilities of SubCh, AcCh, and Cch To Inhibit Quinacrine and Ethidium Binding. To more rapidly compare the abilities of various agonists to inhibit quinacrine and ethidium binding, back titrations of agonists were performed into AcChR−membrane suspensions containing quinacrine or ethidium ± PCP. Figure 2 shows an example of such a back titration. Under the experimental conditions, there were time-dependence changes in the fluorescence of both quinacrine and ethidium that were corrected for before the concentration dependence of the agonist inhibition of binding was estimated. Figure 3 shows the time-corrected PCP-sensitive changes in quinacrine and ethidium fluorescence as a function of the concentrations of SubCh, AcCh, and CCh. In all cases, PCP-sensitive quinacrine fluorescence and, therefore, binding were more susceptible to inhibition by all three agonists than PCP-sensitive ethidium fluorescence. Using the IC_{50} values from these plots, the K_I's were calculated (Table 1) with the Cheng−Prusoff expression (eq 2), and they indicate that the agonists examined inhibited quinacrine binding at 17 times lower concentrations on average than they inhibited ethidium binding.

Accessibility of SL-AcCh to Receptor-Bound Quinacrine and Ethidium. To begin to evaluate whether agonists act by direct steric displacement of quinacrine or ethidium binding, we took advantage of the ability of nitroxides to quench emission from fluorophores that are within 5−10 Å. Specifically, the ability of SL-AcCh to quench receptor-bound quinacrine and ethidium fluorescence was examined. Although SL-AcCh is not a nicotinic agonist (Rosen *et al.*, 1976; Goldner & Rosen, 1977), structurally it is very similar to AcCh, and it will inhibit the binding of a fluorescent agonist, dansyl-C_6-choline, to α-cobratoxin-sensitive binding sites with a K_I of about 25 μM (data not shown). Also, SL-AcCh produces 50% inhibition of agonist-induced $^{86}Rb^+$ efflux (10 s) from *Torpedo nobiliana* vesicles at a concentration of 10.6 ± 0.8 mM (data not shown). It was reasoned that if SL-AcCh is accessible to quinacrine and ethidium binding sites at the concentrations that are associated with its displacement of receptor-bound quinacrine and ethidium, then agonist inhibition of quinacrine and ethidium binding

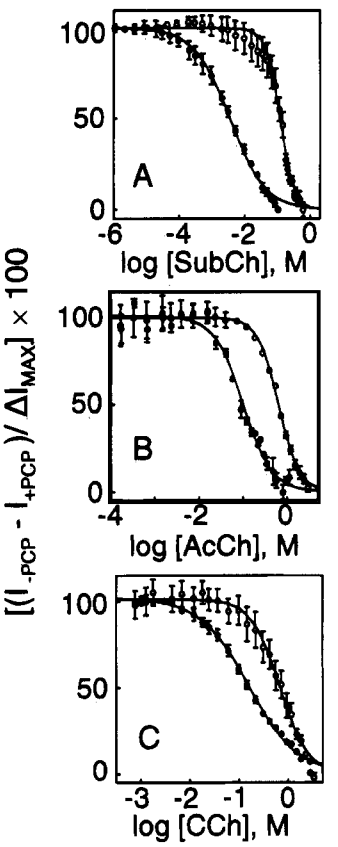

FIGURE 3: SubCh, AcCh, and CCh inhibition of quinacrine and ethidium from the HTX-sensitive NCI binding sites. SubCh (A), AcCh (B), and CCh (C) were titrated into suspensions of AcChR membranes (0.7 μM in SubCh binding sites), CCh (0.77 mM), and quinacrine (0.35 μM) (●) or ethidium (2.5 μM) (○) in the absence and presence of PCP (0.5 mM) at 15 °C. See Materials and Methods for experimental details. Data shown are the mean ± SD of three determinations.

Table 1: Inhibition Constant (K_I) for Agonist Displacement of AcChR-Bound Quinacrine and Ethidium at 15 °C by Chen−Prusoff Analysis

inhibition of the binding of	apparent K_I[a] (mM)		
	SubCh	AcCh	CCh
quinacrine	1.4 ± 0.3	68 ± 29	81 ± 27
ethidium	57 ± 17	278 ± 132	487 ± 139

[a] The apparent inhibition constants for the different agonists were calculated with eq 2. See the legends to Figures 2 and 3 for experimental details. Values represent the mean ± SD of three determinations.

can be explained by a direct steric displacement as opposed to an indirect allosteric mechanism.

To correct for the fact that SL-AcCh displaces both quinacrine and ethidium from their respective binding sites, the K_I's for SL-AcCh inhibition of quinacrine and ethidium binding were determined by measuring the apparent K_D's of quinacrine and ethidium in the presence and absence of various concentrations of SL-AcCh. Figure 4A,B shows examples of these titrations, and Figure 4C shows the Schild plot for each fluorescent NCI. Like SubCh inhibition of quinacrine and ethidium binding (Figure 1C), the Schild plots are relatively linear over the concentrations examined and, therefore, consistent with a competitive inhibition mechanism. The calculated K_I's for the inhibition of quinacrine and ethidium binding were 1.7 and 5.6 mM, respectively (Table 2).

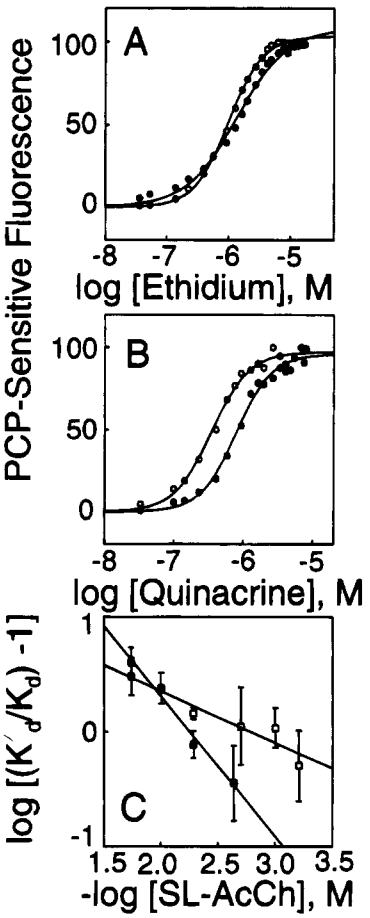

FIGURE 4: Effect of SL-AcCh on PCP-sensitive ethidium and quinacrine binding to the AcChR. Ethidium (A) and quinacrine (B) were titrated into suspensions of AcChR membranes (0.7 μM in SubCh binding sites) and CCh (0.77 mM) ± PCP (0.5 mM) in the absence (○) and presence (●) of SL-AcCh (2 mM) at 15 °C. The difference in fluorescence of samples that contained or did not contain PCP (PCP-sensitive fluorescence) is shown on the ordinate. Panel C: Schild plots of the inhibition of ethidium (■) and quinacrine (□) binding by SubCh. The calculated K_I's for SubCh inhibition of ethidium and quinacrine binding were 5.6 ± 1.2 and 1.7 ± 0.5 mM, respectively. See Materials and Methods for experimental details.

Table 2: Interaction of SL-AcCh with AcChR-Bound Quinacrine and Ethidium: Binding Inhibition Constants (K_I) for Steady-State Stern−Volmer Quenching Constants (K_Q)

fluorescent NCI	K_Q (M^{-1})[a]	$1/K_Q$ (mM)[b]	K_I (mM)[c]
quinacrine	577 ± 20	1.7 ± 0.1	1.7 ± 0.5
ethidium	91 ± 6	11 ± 1	5.6 ± 1.2

[a] Steady-state Stern−Volmer quenching constants obtained from the slopes of Figure 6. These values were corrected for SL-AcCh displacement of AcChR-bound quinacrine and ethidium by using eq 3. [b] The concentration of quencher that produces a 50% reduction in PCP-sensitive fluorescence. [c] The inhibition constants for SL-AcCh displacement of quinacrine or ethidium binding were obtained from the slopes of the Schild plots shown in Figure 4C.

Figure 5 shows the Stern−Volmer plots of the effects of SL-AcCh on receptor-bound quinacrine and ethidium emissions. The quenching constants (K_Q), corrected for the ability of SL-AcCh to displace quinacrine and ethidium from their respective binding sites, were calculated from the slopes of the regression lines of these plots, and they show that the apparent accessibility of SL-AcCh to the PCP-sensitive quinacrine binding site (K_Q = 577 M^{-1}) is 6.3-fold greater

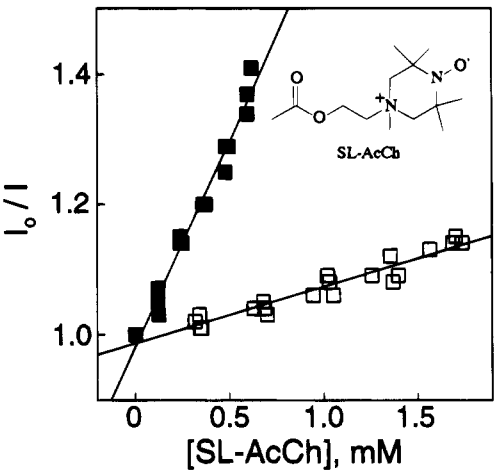

FIGURE 5: Stern−Volmer plots of the effects of SL-AcCh on receptor-bound quinacrine and ethidium fluorescence. The fluorescence from a suspension of AcChR membranes (0.7 μM in SubCh binding sites), CCh (0.77 mM), and quinacrine (0.35 μM) (■) or ethidium (2.5 μM) (□) was measured in the absence and presence of PCP (0.5 mM). Receptor-bound fluorescence from quinacrine and ethidium was calculated with eq 3. All samples were kept at 15 °C. Samples with quinacrine were excited at 450 nm, and the emission was monitored at 502 nm with a Corning 3-71 cutoff filter in the path of the emission beam. Samples with ethidium were excited at 520 nm with a 520-nm narrow band pass Oriel filter in the excitation beam, and the emission was monitored at 595 nm with an internal Perkin-Elmer Cetus 610-nm cutoff filter in the path of the emission beam. Data shown are the results from three (quinacrine) and four (ethidium) determinations.

than that to the ethidium binding site (K_Q = 91 M^{-1}). The concentration of SL-AcCh that produces a 50% reduction in PCP-sensitive quinacrine fluorescence is the same as the K_I for SL-AcCh inhibition of PCP-sensitive quinacrine binding (1.7 mM), while the concentration of SL-AcCh that produces a 50% reduction in PCP-sensitive ethidium fluorescence (11 mM) is slightly (2-fold) higher than its K_I for the inhibition of PCP-sensitive ethidium binding (5.6 mM). This difference between ethidium's K_I and K_Q is of about the same magnitude as the intrinsic difference in the paramagnetic quenching efficiencies of quinacrine and ethidium in various solvents (K_Q^{quin}/K_Q^{eth} ranges between 1.4 and 3.6; Arias *et al.*, 1993b) and suggests that SL-AcCh quenching of quinacrine and ethidium fluorescence occurs at the same concentrations at which SL-AcCh displaces them from their respective NCI binding sites. This implies that SL-AcCh- and agonist-induced displacement of receptor-bound quinacrine and ethidium *probably* occurs by direct binding of agonists to the quinacrine and ethidium binding sites.

DISCUSSION

In this paper, we attempted to contribute to our understanding of the molecular basis of fast agonist inhibition of the AcChR by examining the ability of various agonists to inhibit quinacrine and ethidium binding to their respective NCI binding sites on the desensitized AcChR at zero membrane potential. We found that, at zero membrane potential, quinacrine binding was significantly more sensitive to agonist-induced inhibition than ethidium binding and that the mechanism of agonist-induced inhibition of binding appears to involve direct steric displacement of both NCIs from their respectively binding sites. Importantly, the K_I's for agonist-induced inhibition of quinacrine binding were

comparable to the reported concentrations (K_B) of SubCh, AcCh, and CCh that inhibit 50% of the maximum $^{86}Rb^+$ efflux from *Torpedo nobiliana* vesicles in a 10-s assay with 80−85% of the α-bungarotoxin sites occupied (Forman *et al.*, 1978). For SubCh, AcCh, and CCh, the K_I's equaled 1.4, 68, and 81 nM (Table 1) and the K_B's equaled 3.0, 104, and 244 mM, respectively. The fact that the K_I's for agonist-induced inhibition of quinacrine binding are comparable to the concentrations of agonists that inhibit agonist-induced ion flux through the AcChR *suggests* that fast agonist inhibition involves the binding of agonists to at least a portion of the HTX-sensitive quinacrine binding site.

A potential difficulty with using this correlation between the K_I's and the K_B's to infer that fast agonist inhibition of the AcChR involves agonist binding to the quinacrine binding site is that the K_I's and K_B's provide different information about the interaction of agonists with the receptor. The K_I of agonist-induced inhibition of quinacrine binding provides information about agonist binding to the *slow-onset desensitized state*, while the K_B for agonist inhibition of ion flux provides information on the interaction of agonists with the *open-channel state*. Nicotinic ligands are notoriously sensitive to the conformational state of the receptor; consequently, without additional information it is problematic to infer a relation between agonist binding to the quinacrine binding site and agonist inhibition of ion flux, from a comparison of the K_I's to the K_B's.

However, that said, Grünhagen *et al.* (1977) reported that not only does quinacrine fluorescence rapidly increase upon the mixing of nicotinic agonists with *Torpedo* AcChR membranes but this rapid increase in fluorescence is also inhibited by high concentrations of SubCh. Inspection of the figure depicting the concentration dependence of the SubCh-induced inhibition of this effect [Figure 15 in Grünhagen *et al.* (1977)] indicates an IC_{50} value of about 2.5 mM, which is essentially the same as the K_I (1.4 mM) and K_B (3 mM) values for SubCh inhibition of quinacrine binding (Table 1) and inhibition of agonist-induced $^{86}Rb^+$ efflux (Forman *et al.*, 1987), respectively. Because this rapid increase in fluorescence appears to be associated with quinacrine binding to the open-channel state and occurs at concentrations comparable to those of agonist-induced inhibition of both quinacrine binding and ion flux (Grünhagen *et al.*, 1977), it is probably the case that there is a direct relation between agonist-induced inhibition of quinacrine binding to both the slow-onset desensitized and open-channel states of the AcChR. Therefore, the fact that the two inhibition constants (K_I and K_B) provide information about agonist interaction with different conformational states probably does not pose a significant problem for the proposition that fast agonist inhibition involves the binding of agonists to at least a portion of the HTX-sensitive quinacrine binding site.

A more significant difficulty in using the correlation between the K_I's and the K_B's to infer that fast agonist inhibition of the AcChR is mediated by the HTX-sensitive quinacrine binding site is that there are multiple HTX-sensitive NCI sites on the AcChR, and only two of these NCI sites have been examined in this report. While it seems unlikely that agonist binding to the HTX-sensitive ethidium binding site is involved in agonist inhibition, the possibility that agonist directly interacts with one of the NCI sites in the transmembrane channel luminal binding sites cannot be excluded.

A related difficulty in using the correlation between the constants for agonist-induced inhibition of quinacrine binding and $^{86}Rb^+$ efflux to infer that the quinacrine binding site mediates fast agonist inhibition is that the various HTX-sensitive NCI binding sites are mutually coupled to one another. As discussed in the introduction, ligand binding to one HTX-sensitive binding site precludes the simultaneous binding of a second NCI to the same receptor molecule. Consequently, the agonist-induced displacement of NCIs from one site could be produced either by a direct agonist interaction at the NCI site under study or by an indirect interaction at a distant allosteric site. To begin to address this issue, the ability of a paramagnetic acetylcholine analog (SL-AcCh) to quench the emissions from receptor-bound quinacrine and ethidium was examined. It was found that although SL-AcCh quenched the emissions from receptor-bound quinacrine at significantly lower concentrations than that from receptor-bound ethidium, SL-AcCh-induced quenching occurred at about the same concentrations as displacement of the two fluorescent NCIs (Figure 5 and Table 2), suggesting that the mechanism of inhibition of both quinacrine and ethidium binding involves a direct interaction of agonists at both sites. But clearly, these results do not exclude the possibility of an allosteric interaction. In future studies, more information may be obtained from an analysis of the time course of SL-AcCh quenching of quinacrine and ethidium emissions following rapid mixing.

If the operative mechanism of agonist inhibition involves the quinacrine binding site, then agonists would be expected to be absorbed into the lipid−membrane surface and diffuse (via a membrane approach) to the quinacrine binding site, because the quinacrine site is located at a lipid−protein interface (Valenzuela *et al.*, 1992) near the water−lipid interface (Arias *et al.*, 1993a). A requirement that agonists interact with the lipid membrane might appear to argue against the proposition that agonist inhibition is mediated by the quinacrine binding site, because most agonists are small quaternary amines that have a limited ability to partition into lipid membranes. However, agonist-induced inhibition requires millimolar concentrations, and the membrane partition coefficients of SubCh, AcCh, and CCh are about 4, 0.1, and 0.2, respectively (unpublished results). Consequently, the membrane concentrations of agonists should be quite substantial (≫1 mM) at the agonist concentrations that inhibit 50% of the maximum $^{86}Rb^+$ efflux. Thus, a requirement that agonists take a membrane approach to the inhibitory binding site poses no difficulty for the proposition that the quinacrine binding site mediates, at least in part, agonist-induced inhibition. Indeed, a membrane approach to the inhibitory binding site would be consistent with the very rapid action of agonists to inhibit the AcChR (Forman & Miller, 1989), because the binding kinetics of reactions that involve a membrane approach to the ligand binding site are theoretically several orders of magnitude faster than binding reactions that involve a simple aqueous approach to the binding site (Rhodes *et al.*, 1985).

In conclusion, at zero membrane potential agonists displace AcChR-bound quinacrine at significantly lower concentrations than they displace AcChR-bound ethidium. The mechanism of this action appears to involve direct steric displacement of both quinacrine and ethidium from their

binding sites. The inhibition constants for agonist-induced displacement of quinacrine binding are comparable to the concentrations of agonists that produce a 50% reduction in $^{86}Rb^+$ efflux from *Torpedo* vesicles. Although the results presented in this paper do not conclusively show that fast agonist inhibition of the AcChR is mediated by the quinacrine binding site, they strongly suggest that ethidium binding in the walls of the extracellular domain of the AcChR does not mediate agonist inhibition. Because of the multiplicity of and allosteric interactions between the various HTX-sensitive NCI binding sites on the AcChR, it is a particularly difficult problem to conclusively establish the fast inhibitory site of action of agonists.

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