Quinacrine and Ethidium Bind to Different Loci on the *Torpedo* Acetylcholine Receptor†

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ABSTRACT: Fluorescence spectroscopy was used to determine whether quinacrine and ethidium, two high-affinity noncompetitive inhibitors of the *Torpedo* acetylcholine receptor (AcChR), bind to the same loci. The ability of three nitroxide spin-labels, 5-doxylstearate (5-SAL), spin-labeled androstane (ASL), and TEMPO, to quench receptor-bound quinacrine and ethidium fluorescence was measured. When bound to a phencyclidine-displaceable site on the AcChR, quinacrine was 16.9 and 19 times more efficiently quenched than ethidium by the highly lipophilic 5-SAL and ASL, respectively. TEMPO, which has a limited ability to partition into *Torpedo* plasma membranes (<1%), was only twice as efficient at quenching receptor-bound quinacrine than ethidium fluorescence. The relative sensitivity of quinacrine and ethidium fluorescence to paramagnetic quenching was examined in three solvents, 1-butanol, sodium phosphate buffer, and acetonitrile, with TEMPO as a quencher. The results from the different solvents demonstrate that quinacrine fluorescence is intrinsically 1.4–3.6 times more sensitive than ethidium fluorescence to quenching by nitroxide spin-labels. Examination of the effect of high concentrations of 5-SAL on ethidium and quinacrine dissociation constants showed that quinacrine but not ethidium binding was competitively inhibited. Together, these results indicate that although quinacrine and ethidium bind in a mutually exclusive manner, the two inhibitors interact at different loci on the AcChR. Whereas the ethidium binding site is at a distance from membrane lipids, probably in or near the lumen, the quinacrine binding site appears to be at a lipid–protein interface in the transmembrane domain and at a distance from the lumen.

Noncompetitive inhibitors (NCIs)[1] of the muscle-type acetylcholine receptor (AcChR) block acetylcholine-induced cation fluxes through the central lumen of the receptor without inhibiting acetylcholine binding. Chemically, NCIs are a heterogeneous class of compounds that includes quinacrine, ethidium, triphenylmethylphosphonium, phencyclidine (PCP), chlorpromazine, histrionicotoxin (HTX), local anesthetics, and general anesthetics (Forman & Miller, 1989; Taylor et al., 1991). In vitro observations have led to the subclassification of NCI binding sites into low- and high-affinity sites (Heidmann et al., 1983). The stoichiometry of the low-affinity NCI sites is 10–30 sites/receptor (Heidmann et al., 1983). The high-affinity NCIs bind with a stoichiometry of 1 site/receptor (Eldefrawi et al., 1978; Strnad & Cohen, 1983; Heidmann et al., 1983; Herz et al., 1987; Arias et al., 1993).

To develop a molecular understanding of NCI action, much effort has been directed at the localization of NCI binding sites. Affinity and photoaffinity labeling (Oberthür et al., 1986; Dreyer et al., 1986; Hucho et al., 1986; Giraudat et al., 1986, 1987, 1989; Revah et al., 1990; White & Cohen, 1992), site-directed mutagenesis (Imoto et al., 1986, 1988; Leonard et al., 1988; Charnet et al., 1990), and fluorescence spectroscopic (Herz et al., 1989, 1991; Herz & Atherton, 1992; Valenzuela et al., 1992a; Arias et al., 1993) approaches have been utilized. Much evidence points to the existence of multiple hydrophobic domains on the AcChR that can bind high-affinity NCIs. Significantly, the binding of these inhibitors is mutually exclusive. This mutually exclusive behavior suggests that the association of a single ligand is sufficient to prevent the interaction of additional ligands to other sites (Taylor et al., 1991).

Although much evidence supports the existence of one or more high-affinity NCI binding sites within the lumen of the receptor (Taylor et al., 1991; Changeux et al., 1992), there is only limited support for the existence of nonluminal sites of high-affinity NCI binding. Assuming that the first transmembrane domain (M1) of the α subunit does not form part of the lumen, the ability of photoactivated [^{3}H]quinacrine azide to react with residues in the α-subunit M1 transmembrane domain suggests that the quinacrine binding site is not in the lumen (DiPaola et al., 1990; Karlin, 1991). However, it is unknown whether or not the M1 domain forms part of the lumen. A second line of evidence suggesting nonluminal sites of NCI binding centers around the observation that PCP-sensitive quinacrine fluorescence is readily quenched by fluorescent and spin-labeled lipophilic probes (Valenzuela et al., 1992a; Arias et al., 1993). These quenching experiments assume that the lipid probes are inaccessible to the lumen of the AcChR. However, little is known about the distribution of the lipid probes in the AcChR-associated membranes, and therefore, the issue of the existence of nonluminal NCI binding sites is problematic.

Because ethidium, another fluorescent NCI, has been suggested to interact with the lumen of the AcChR (Herz et al., 1989, 1991; Herz & Atherton, 1992), we reasoned that a comparison of the accessibility of lipophilic spin-labels to

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[1] The abbreviations and trivial names used are as follows: AcChR, nicotinic acetylcholine receptor; NCI, noncompetitive inhibitor; CCh, carbamylcholine hydrochloride; PCP, phencyclidine; 5-SAL, 5-doxylstearate; ASL (spin-labeled androstane), 3-doxyl-17β-hydroxy-5α-androstane; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TLC, thin-layer chromatography; Me_2SO, dimethyl sulfoxide; buffer I, 10 mM sodium phosphate buffer, pH 7.4; SL-PC (spin-labeled phosphatidylcholine), 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphatidylcholine; 7-AS, 7-(9-anthroyloxy)stearic acid.

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receptor-bound quinacrine and ethidium could reveal whether quinacrine, in fact, interacts with a nonluminal site on the AcChR. Consequently, we compared the ability of 5-doxylstearate (5-SAL), spin-labeled androstane (ASL), and TEMPO to quench PCP-sensitive quinacrine and ethidium fluorescence in the presence of membrane-associated AcChR and carbamylcholine (CCh). To further assess the differences between these binding sites, the ability of high concentrations of 5-SAL to competitively inhibit PCP-displaceable quinacrine and ethidium binding was examined.

MATERIALS AND METHODS

Materials. Quinacrine dihydrochloride, CCh, PCP, 5-SAL, ASL, and suberyldicholine were obtained from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (Me_2SO) (HPLC grade), 1-butanol, and TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) were from Aldrich Chemical Co. (Milwaukee, WI). Ethidium bromide was purchased from Calbiochem (La Jolla, CA). *Torpedo californica* electric rays were purchased from Marinus Inc. (Long Beach, CA).

The purity of quinacrine and ethidium was analyzed with thin-layer chromatography (TLC) by using a mixture of chloroform/methanol/acetic acid/water (25:12:4:2 by volume) as the mobile phase through a silica gel stationary phase (Sil Gel G, Fisher Scientific). The purity of the spin-labeled lipids was also assessed by means of TLC with silica gel plates (Sil Gel GF, Fisher) impregnated with a fluorescent indicator; a mixture of cyclohexane/ethyl ether/acetic acid (30:20:0.5 by volume) was used as the mobile phase. The presence of free radicals was revealed by the ability of the nitroxide moiety to quench the fluorescence of the indicator in the silica gel. Only single spots of fluorescent quenching were detected for each of the spin-labeled lipids used.

Isolation of the Membrane-Associated Receptor. AcChR-enriched membranes were obtained from a *T. californica* electric organ by using differential sucrose gradient centrifugation (Johnson & Yguerabide, 1985). The specific activities of these preparations ranged between 1.4 and 3.6 nmol of suberyldicholine binding sites/mg of total protein, determined as described elsewhere (Valenzuela et al., 1992a,b).

Quinacrine and Ethidium Fluorescence Quenching with ASL, 5-SAL, and TEMPO. Quinacrine (0.46–0.53 μM) or ethidium bromide (2.5 μM) was suspended in 10 mM sodium phosphate buffer, pH 7.4 (buffer I), with membrane-associated AcChR (0.7 μM in suberyldicholine binding sites) and 1 mM CCh ± PCP (500 μM). The CCh was added to prevent quinacrine or ethidium from binding to agonist binding sites and to induce the receptor into the slow-onset desensitized state, which displays a higher affinity for quinacrine and ethidium. All samples were incubated for 1 h at 15 °C before fluorescence measurements were made. Fluorescence titrations were carried out in 0.5- × 0.5-cm cuvettes held at 15 °C in a Perkin-Elmer Cetus MPF 66 spectrofluorometer. 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 narrow band and a Perkin-Elmer 610 filter were placed in the path of ethidium excitation and emission beams, respectively. Fluorescence values were corrected for dilution resulting from added titrant and for the intrinsic fluorescence of the sample. Titrants, previously dissolved in Me_2SO, which had no measurable effect on quinacrine or ethidium emission, were added to the cuvettes with a Hamilton syringe. After each addition of titrant (0.5–1.0 μL) the samples were incubated for 5 min before the quinacrine or ethidium fluorescence was measured. Initial control experiments established that the titrants equilibrated with the membrane within 5 min. One set of control samples, containing only AcChR suspensions in buffer I, was used for the measurement of background and titrant fluorescence (I_{blk}). A second set of control samples was used to monitor any time-dependent changes in the instrumental output or in the quinacrine fluorescence. In the case of ethidium, no measurable time-dependent fluorescence changes were observed.

Because only a fraction (f) of the added fluorophores were bound to the high-affinity NCI binding sites, assessment of the extent of quenching for the different spin-labeled probes required subtraction of the receptor-bound (I_B) from the free fluorophore fluorescence (Valenzuela et al., 1992a). This was accomplished by using eq 1,

$$I_B = c_t c_d (I_{-PCP} - I_{blk}) - c_d(1-f)(I_{+PCP} - I_{blk}) \quad (1)$$

where I_{+PCP} and I_{-PCP} are the magnitudes of fluorescence of samples that did or did not contain PCP, respectively. The c terms are correction factors for time-dependent changes in the receptor-bound quinacrine fluorescence (c_t) and for dilution and inner filter effects of titrants (c_d). No time-dependent corrections were necessary for the samples containing PCP. 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 quinacrine or ethidium that was free in solution ($1-f$) was determined from the plot of the concentration vs PCP-dependent quinacrine or ethidium fluorescence (Valenzuela et al., 1992a,b).

The addition of excess PCP to samples helped to define the specific or PCP-sensitive fluorescence associated with the binding of quinacrine or ethidium to the high-affinity NCI sites. PCP is a nonfluorescent high-affinity NCI that displaces both AcChR-bound quinacrine and ethidium and, thus, allows detection of the NCI site-bound quinacrine and ethidium fluorescence. The receptor-bound fluorescence intensities were analyzed with the steady-state Stern–Volmer equation

$$I_0/I = 1 + K_Q[Q] \quad (2)$$

where I_0 and I are the calculated bound intensities measured plus/minus various concentrations of quencher ([Q]) and K_Q is the Stern–Volmer quenching constant derived from steady-state data. The bound fluorescence intensities were calculated with eq 1.

Relative Sensitivity of Quinacrine and Ethidium to Nitroxide Quenching. The relative sensitivities to paramagnetic quenching were assessed by comparison of the bimolecular quenching rate constants, k_q, of quinacrine and ethidium in three solvents (1-butanol, buffer I, and acetonitrile) with TEMPO as a nitroxide quencher. The k_q were determined by measuring the fluorescence lifetimes of the two fluorophores in the absence (τ_0) and presence (τ) of various concentrations of TEMPO. The results were then analyzed with the lifetime Stern–Volmer expression

$$\tau_0/\tau = 1 + K_Q'[Q] = 1 + k_q\tau_0[Q] \quad (3)$$

where K_Q' is the lifetime Stern–Volmer quenching constant.

Fluorescence lifetimes were determined by the single-photon counting technique with an EEY Scientific nanosecond spectrofluorometer (La Jolla, CA), equipped with a high-pressure hydrogen arc lamp. The excitation wavelengths for

most samples were selected with a Corning 7-54 interference filter. For acetonitrile, the quinacrine excitation wavelength was selected with an Oriel 400-nm broad-band interference filter. The emission from quinacrine and ethidium was selected with Corning 3-71 and 2-73 cutoff filters, respectively. Stock solutions of TEMPO (4.75 M) were freshly prepared with 1-butanol. Appropriate volumes of pure 1-butanol were added to control samples so that corrections could be made for any butanol-induced effects. The instrumental arrangement and principles of data treatment are discussed elsewhere (Yguerabide, 1972).

Fractional Partitioning of TEMPO into AcChR-Enriched Membranes. TEMPO (4.3 mM) in the presence and absence of membrane-associated AcChR (0.7 μM in suberyldicholine binding sites) was suspended in 1.5 mL of buffer I. The samples were then centrifuged (200000g) for 1 h at 15 °C. The amount of TEMPO in the supernatant was determined by absorption spectroscopy (absorbance 423 nm).

Effect of Spin-Labels on Quinacrine and Ethidium Binding. To confirm that the paramagnetic probes quenched quinacrine and ethidium fluorescence without affecting their association properties, the effect of 5-SAL, ASL, and TEMPO on the dissociation constant (K_d) of quinacrine and ethidium binding was measured. Direct titrations of quinacrine and ethidium into suspensions of AcChR (0.7 μM in suberyldicholine binding sites) and CCh (1 mM) ± PCP (0.5 mM) and fixed concentrations of 5-SAL (73 μM), ASL (109 μM), and TEMPO (4.3 mM) were used to determine the K_d. The spin-labels were added to the cuvettes 10 min before the start of the titrations. Estimates of the K_d were made by fitting plots of the specific (PCP-sensitive) changes in quinacrine or ethidium fluorescence vs the free ligand concentration to the equation for a rectangular hyperbola by using the Marquardt algorithm (Marquardt, 1959). The free ligand concentration was assumed to be the total titrant concentration minus the bound titrant concentration.

RESULTS

Relative Sensitivity of Quinacrine and Ethidium to Paramagnetic Quenching. Because fluorophores differ in their intrinsic sensitivities to paramagnetic quenchers (Eftink, 1992), the relative sensitivity of quinacrine and ethidium fluorescence to quenching by TEMPO in three solvents (buffer I, 1-butanol, and acetonitrile) was assessed. To evaluate the relative quenching independent of nondiffusional factors, quenching was measured as a reduction of the fluorescence lifetime. Stern–Volmer plots of these results are shown in Figure 1. Table I presents the slopes, K_Q, of these plots and the calculated bimolecular quenching rate constants, k_q. While both quinacrine fluorescence and ethidium fluorescence were quenched by TEMPO, quinacrine was between 1.4 and 3.6 times more sensitive than ethidium to quenching by TEMPO when assessed in terms of the k_q. The relative sensitivities to paramagnetic quenching were not well correlated with solvent polarity, following the order buffer I > 1-butanol > acetonitrile. The insolubility of the quaternary ethidium in highly nonpolar systems limited the range of solvent polarities that could be examined; consequently, a more thorough analysis of the polarity dependence of quenching could not be performed.

Comparison of the steady-state results (data not shown) with the lifetime results, not surprisingly, showed that the steady-state results were produced by a mixture of dynamic and static quenching, with predominance of the static quenching process. This static process probably originates, at least in part, by the high concentration of quencher and by the fact that the quenching radius extends beyond the van der Waals radii (Green et al., 1990; Matko et al., 1992). At the high concentrations of quencher required for paramagnetic quenching the probability of a fraction of quencher molecules being adjacent to the fluorophore increases, with the subsequent and immediate deactivation of the fluorophore at the moment of excitation.

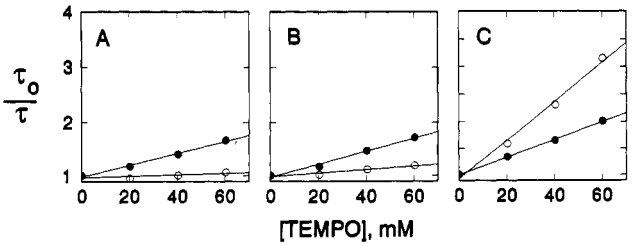

FIGURE 1: Stern–Volmer plots of the effect of TEMPO on the fluorescence lifetime of quinacrine and ethidium in buffer I, 1-butanol, and acetonitrile: (A) 10 mM sodium phosphate, pH 7.4, (B) 1-butanol, and (C) acetonitrile. Quinacrine (●) and ethidium (○) were dissolved in each solvent at 2.5 μM final concentration and titrated with 4.75 M TEMPO (dissolved in butanol) at 15 °C. With samples in buffer and butanol, excitation and emission wavelengths were selected with a Corning 7-54 interference filter and Corning 3-71 (for quinacrine) or 2-73 (for ethidium) cutoff filters, respectively. With acetonitrile, an Oriel 400-nm broad-band interference filter and a Corning 3-71 cutoff filter were used to select the excitation and emission wavelengths, respectively. Shown is the mean of three determinations.

Table I: Relative Sensitivity of Quinacrine and Ethidium to Quenching by TEMPO in Various Solvents

solvent	NCI fluorophore	K_Q' [a] (M^{-1})	$\langle\tau\rangle$ [b] (ns)	k_q [c] $\times 10^{-9}$ (M^{-1} s^{-1})	k^Q_q/k^E_q [d]
buffer I	quinacrine	11	3.8	2.9	3.6
	ethidium	1.3	1.6	0.81	
1-butanol	quinacrine	8.0	6.0	1.3	2.2
	ethidium	3.3	5.5	0.60	
acetonitrile	quinacrine	17	3.4	5.0	1.4
	ethidium	36	10.0	3.6	

[a] Lifetime Stern–Volmer quenching constant derived from the data illustrated in Figure 1. [b] Geometric average fluorescence lifetime (mean of at least five determinations). [c] Bimolecular quenching rate constant, obtained from eq 3. [d] Ratio of quinacrine (Q) and ethidium (E) bimolecular quenching constants.

Membrane Partitioning of TEMPO into Torpedo Membranes. Although it is well-established that ASL partitions completely into artificial and biological membranes (Marsh & Barrantes, 1978), and that 96% of 5-SAL partitions into the *Torpedo* membrane at the concentrations used in the quenching studies described below (Arias et al., 1993), it was unknown how much TEMPO partitions into *Torpedo* membranes. Consequently, the measured fractional partitioning of TEMPO into the AcChR-associated membranes was determined by measuring the visible absorbance of the supernanant following centrifugation to be slightly less than 1%.

Quenching of AcChR-Bound Quinacrine and Ethidium Fluorescence by Spin Labels. Because quinacrine and ethidium binding are both displaceable by excess PCP, it was possible to calculate (eq 1) the receptor-bound quinacrine and ethidium fluorescence in the absence and presence of the various quenchers. Steady-state Stern–Volmer plots of these results are shown in Figure 2 and the quenching constants summarized in Table II. No effort was made to correct for differences in the fluorescence lifetimes of the receptor-bound fluorophores, because the reduced translational freedom of

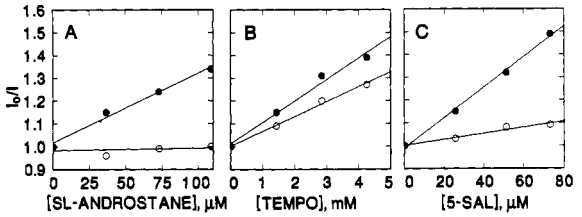

FIGURE 2: Stern–Volmer plots of spin-label quenching of receptor-bound quinacrine and ethidium fluorescence: effect of ASL (A), TEMPO (B), and 5-SAL (C) on AcChR-bound quinacrine (●) and ethidium (○) fluorescence. The fluorescence from a suspension of AcChR-associated membranes (0.7 μM in suberyldicholine binding sites with an estimated total lipid content of 270 μM), CCh (1.0 mM), and quinacrine (0.46–0.53 μM) or ethidium (2.5 μM) was measured in the absence and the presence of PCP (0.5 mM). Fluorescence from receptor-bound NCIs was calculated with eq 1. All samples were kept at 15 °C. Quinacrine-containing samples 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; ethidium-containing samples 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 filter in the path of the emission beam. Shown is the mean of three determinations.

Table II: Paramagnetic Quenching of AcChR-Bound Quinacrine and Ethidium Fluorescence by Spin-Labels

spin-labeled probe	AcChR-bound NCI	K_Q[a] ($M^{-1} \times 10^3$)	K^Q_Q/K^E_Q[b]
ASL	quinacrine	3.1 ± 0.3	19.0
	ethidium	0.16 ± 0.61	
5-SAL	quinacrine	14.1 ± 2.8	16.9
	ethidium	0.83 ± 0.30	
TEMPO	quinacrine	0.14 ± 0.004	2.0
	ethidium	0.069 ± 0.003	

[a] Apparent steady-state Stern–Volmer quenching constant, obtained from the slopes of Figure 2. [b] Ratio of quinacrine (Q) and ethidium (E) steady-state Stern–Volmer quenching constants.

the membrane-partitioned spin-labels produces quenching that is independent of the fluorescent lifetime of fluorophores with $\tau < 50$ ns (London & Feigenson, 1981).

The results show that receptor-bound quinacrine was 16.9 and 19 times more efficiently quenched than ethidium by the highly lipophilic 5-SAL and ASL, respectively (Table II). TEMPO, which has a limited ability to partitioning into *Torpedo* membranes (<1%), was only twice as efficient at quenching receptor-bound quinacrine than ethidium fluorescence (Table II). Whereas it is impossible to know a priori what the intrinsic nitroxide quenching efficiencies of receptor-bound quinacrine and ethidium are, relative estimates of the intrinsic quenching efficiencies can be made. If TEMPO is assumed to be equally accessible to both receptor-bound quinacrine and ethidium, then the relative nitroxide-quenching efficiency of quinacrine would be twice as great as ethidium, and receptor-bound quinacrine would be 8–10 times more accessible to lipids partitioned into the membrane than ethidium. Taking a more conservative estimate of their relative quenching efficiencies obtained in the buffer I (relative quenching efficiency 3.6, Table I), receptor-bound quinacrine would be at least about 5 times more accessible to quenching by the lipid membrane probes 5-SAL and ASL than ethidium. Whichever approach is used to estimate the relative intrinsic nitroxide-quenching efficiencies of receptor-bound quinacrine and ethidium, the results indicate a substantial difference in lipid accessibility to the two receptor-bound high-affinity NCIs.

Effect of Spin-Labels on Quinacrine and Ethidium Binding. To determine if the paramagnetic quenchers used in the quenching experiments influence the binding of quinacrine and ethidium to the high-affinity NCI sites on the AcChR, we measured the apparent dissociation constant for both ligands in the absence and in the presence of the different spin-labels. TEMPO, ASL, and 5-SAL at the highest concentrations used in the quenching experiments (4.3 mM, 109 μM, and 73 μM, respectively) produced no significant inhibition of either quinacrine or ethidium binding to the high-affinity (PCP-sensitive) NCI site(s) (Table III).

Table III: Effect of Spin-Labels on the Apparent Dissociation Constants (K_d) of PCP-Sensitive Quinacrine and Ethidium Binding to the AcChR[a]

spin-labels	quinacrine K_d (μM)	ethidium K_d (μM)
control	0.29 ± 0.19	1.6 ± 0.3
5-SAL	0.38 ± 0.15	1.8 ± 0.1
ASL	0.21 ± 0.16	1.3 ± 0.2
TEMPO	0.31 ± 0.19	1.6 ± 0.2

[a] K_d were calculated assuming a stoichiometry of 1 NCI/receptor functional unit. The spin-labels SAL, ASL, and TEMPO were incubated with the AcChR for 10 min on ice at the concentrations of 73 μM, 109 μM, and 4.3 mM, respectively. These concentrations were the highest used in the quenching experiments shown in Figure 2. Shown are the mean and standard deviation of three determinations.

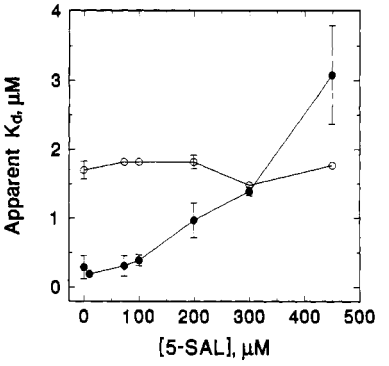

FIGURE 3: Effect of 5-SAL on the apparent K_d of quinacrine and ethidium toward the AcChR. The apparent K_d of quinacrine (●) and ethidium (○) toward PCP-displaceable binding sites on the AcChR was determined by titrating quinacrine or ethidium into suspensions of AcChR (0.7 μM in suberyldicholine binding sites) and CCh (1 mM) ± PCP (0.5 mM) and various concentrations of 5-SAL. Estimates of the K_d were made by fitting plots of the specific (PCP-sensitive) changes in quinacrine or ethidium fluorescence vs the free ligand concentration to the equation for a rectangular hyperbola by using the Marquardt algorithm (Marquardt, 1959).

To gain more information on possible differences or similarities between PCP-displaceable quinacrine and ethidium binding, the ability of high concentrations of 5-SAL to influence quinacrine or ethidium binding was assessed. High concentrations of 5-SAL were previously shown to competitively inhibit PCP-displaceable quinacrine binding (Arias et al., 1993). If ethidium binds to the same loci as the quinacrine, then 5-SAL would be expected to also inhibit ethidium binding. Plots of the apparent K_d of quinacrine and ethidium as a function of added 5-SAL concentration (10–450 μM) are shown in Figure 3. As we previously observed, 5-SAL produced a concentration-dependent increase in the apparent K_d of quinacrine. Schild analysis of the quinacrine results (Schild, 1949) yielded an inhibition constant of 170 μM in agreement with our previous estimate (160 μM; Arias et al., 1993). The apparent K_d of ethidium toward PCP-displaceable sites was unaffected by any concentration of 5-SAL examined. The differential effect of 5-SAL on quinacrine and ethidium binding to the PCP-displaceable binding sites further indicates that these two high-affinity NCIs bind to different loci.

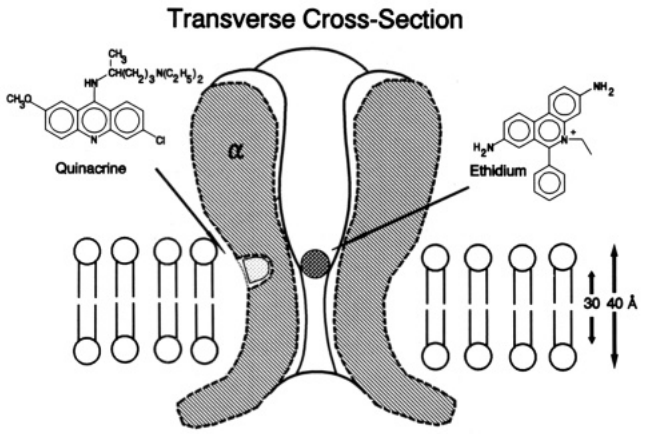

FIGURE 4: Representation of the apparent locations of the high-affinity NCI binding sites for ethidium and quinacrine on the membrane-associated AcChR.

DISCUSSION

Theoretically, noncompetitive inhibitors of the AcChR can act either *directly* by "plugging" the lumen (sterically) or *indirectly* at a nonluminal site to block agonist-induced cation fluxes. Although it is unclear what the molecular basis of the low-affinity NCI action is, the high-affinity NCIs have been frequently suggested to act by a steric mechanism (Adams, 1981; Changeux et al., 1992; Lester, 1992). The unitary binding stoichiometry (Eldefrawi et al., 1978; Strnad & Cohen, 1983; Heidmann et al., 1983; Herz et al., 1987; Arias et al., 1993), the ability of reactive NCI derivatives to label the putative luminal M2 transmembrane segments of the receptor (Oberthür et al., 1986; Dreyer et al., 1986; Hucho et al., 1986; Giraudat et al., 1986, 1987, 1989; Revah et al., 1990; White & Cohen, 1992), the effects of site-directed mutagenesis of the M2 transmembrane segments on NCI action (Imoto et al., 1986, 1988; Leonard et al., 1988; Charnet et al., 1990), and the voltage dependence of their blocking action (Adams, 1981) point to a steric mechanism of action for the high-affinity NCIs.

In this paper, we examined the binding sites of two high-affinity NCIs, ethidium and quinacrine, that interact in a mutually exclusive manner with the AcChR. Like other high-affinity NCIs, ethidium binds to a single PCP- or HTX-displaceable site on the AcChR (Herz et al., 1987). Receptor-bound ethidium exists in a hydrophobic environment as evidenced by a >10-fold increase in its fluorescence lifetime and a >40-nm red shift in its excitation spectrum relative to its spectrum in water (Herz et al., 1987; Valenzuela et al., 1992b). Curiously, despite the restricted accessibility of iodide and deuterium oxide to receptor-bound ethidium (Herz & Atherton, 1992), cations competitively inhibit PCP-displaceable ethidium binding (Herz et al., 1991). The existence of significant Förster energy transfer between AcChR-bound fluorescent agonists and ethidium indicates that the ethidium binding site is on or near the extracellular side of the receptor (Herz et al., 1989). Taken together, research on the PCP-displaceable ethidium binding is consistent with a locus in a hydrophobic pocket in or near the lumen, toward the extracellular side of the AcChR.

Quinacrine, like ethidium, appears to bind to a single PCP- or HTX-displaceable site on the AcChR that is relatively inaccessible to iodide (Arias et al., 1993). Because of the solvent insensitivity of the excitation and emission spectra of quinacrine, the polarity of the quinacrine binding site is unclear, but the 2.3-fold increase in the emission of quinacrine upon binding to the AcChR (Valenzuela et al., 1992b) is consistent with a hydrophobic microenvironment, at least around its acridine moiety. The ability of a photoreactive derivative, [^{3}H]quinacrine azide, to label the residues (αArg^{209} and αPro^{211}) near the interface between the putative extracellular and M1 transmembrane domains of the α subunits also suggests that the quinacrine binding site is toward the extracellular side of the transmembrane segment of the AcChR (Karlin, 1990).

Despite the similarities between receptor-bound quinacrine and ethidium, these two NCI binding sites are shown in this paper to differ in the accessibility of two lipophilic agents, i.e., 5-SAL and ASL (Figure 2), and in the differential ability of high concentrations of 5-SAL to competitively inhibit their binding (Figure 3). These differences strongly indicate that quinacrine and ethidium bind to different loci. The possibility that both quinacrine and ethidium might share some but not all of their points of interaction with the AcChR cannot be completely excluded. However, the quenching ability of nitroxide radicals extends somewhat beyond their van der Waals contact distances (Green et al., 1990). Indeed, some intramolecular fluorescence quenching has been observed by a nitroxide radical separated from a fluorophore by a relatively rigid ~20-Å polyproline spacer (Matko et al., 1992). Consequently, the significant quenching radii of nitroxide free radicals greatly limit the possible arrangements for two partially overlapping binding sites in which paramagnetic lipids would differentially quench the fluorescence of ligands binding to one of the sites. Thus, the possibility that quinacrine and ethidium share some of their points of AcChR interaction seems unlikely.

Jones and McNamee (1988) demonstrated that lipids differentially interact with the receptor. Fatty acids but not cholesterol competitively displace phosphatidylcholine from annular lipid sites in agreement with their higher affinity for the lipid–protein interface (Ellena et al., 1983). Moreover, dibromocholesterol partially quenches the receptor intrinsic fluorescence and produces additional quenching in pure dibromophosphatidylcholine membranes. These observations are consistent with cholesterol and fatty acids binding to nonannular lipid binding sites, not displaceable by phosphatidylcholine. Consequently, the ability of *n*-doxylstearate (Arias et al., 1993) and ASL (Valenzuela et al., 1992a) to quench quinacrine fluorescence opens the possibility that the quinacrine binding site is localized in a nonannular lipid domain. To address this issue, an attempt was made to examine the accessibility of spin-labeled phosphatidylcholine (SL-PC) to receptor-bound quinacrine and ethidium, but SL-PC (200 μM) was found to be a relatively weak lipophilic quenching agent. Even when a fluorescent fatty acid, 7-(9-anthroyloxy)stearic acid (7-AS), was allowed to partition into *Torpedo* membranes, SL-PC (200 μM) only produced 8.4% quenching of membrane-partitioned 7-AS fluorescence (data not shown). Given the fact that 7-AS fluorescence is about 1.7 times more sensitive than quinacrine fluorescence to TEMPO quenching in organic environments (1-butanol and acetonitrile; data not shown), SL-PC (200 μM) should be expected to produce no more than about 5% quenching of receptor-bound quinacrine fluorescence. A 5% change in fluorescence would be too small to obtain conclusive results on the proximity of receptor-bound quinacrine to the annular lipid domain, particularly when the addition of SL-PC increases the sample turbidity.

Although it is unclear whether or not the quinacrine binding site is near the annular lipid domain, the limited accessibility of 5-SAL and ASL to receptor-bound ethidium suggests that

the quinacrine binding site is probably not located in the lumen of the AcChR. If ethidium binds in or near the transmembrane luminal region of the AcChR, as suggested by the evidence cited above, and given the inaccessibility of spin-labeled lipids to AcChR-bound ethidium, then it is reasonable to conclude that 5-SAL and ASL are not accessible to the lumen of the receptor. The great accessibility of 5-SAL and ASL to AcChR-bound quinacrine suggests that its binding site is at a distance from the lumen, and therefore, quinacrine appears to act *indirectly* to inhibit agonist-induced cation fluxes.

Temperature may also distinguish ligand binding to either the quinacrine or the ethidium sites. PCP-displaceable quinacrine binding is more sensitive to temperature changes than ethidium binding. The affinity of quinacrine decreased ~4-fold when the temperature was increased from 15 ($K_d \simeq$ 0.2 μM) to 23 °C ($K_d \simeq$ 0.8 μM) (data not shown). In contrast, ethidium's K_d changed little over a similar change in temperature ($K_d \simeq$ 1.6 μM at 15 °C and $\simeq$ 1.5 μM at 23 °C) (data not shown). This and other factors need to be systematically explored to gain a fuller understanding of the differences between these binding sites.

One final comment, the value of the K_d for PCP-sensitive ethidium binding to the AcChR reported in this paper (1.6 μM) is more than 4 times greater than that previously reported by Herz *et al.* (1987) (0.25–0.36 μM). In unpublished experiments, we have compared the affinity of ethidium from different suppliers and examined the effect of temperature, buffer ionic strength, and NaOH treatment of the membranes. We have yet to find any explanation for this difference other than that there appears to be some uncontrolled factors in the preparation of the *Torpedo* membranes which produce significant preparation-to-preparation differences that range in our hands from 0.4 to 1.9 μM.

In conclusion, quinacrine and ethidium have been shown to bind to different PCP-displaceable loci on the desensitized *Torpedo* AcChR. Although receptor-bound ethidium is highly inaccessible to charged ions and water molecules, small slightly polar molecules can get within a few angstrŏms of receptor-bound ethidium. Moreover, because of the differential accessibility of 5-SAL and ASL to receptor-bound quinacrine and ethidium, the binding site for quinacrine is probably not in the lumen of the AcChR, and therefore, quinacrine appears to act in an indirect manner to noncompetitively block agonist-induced cation fluxes.

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