Quinacrine Noncompetitive Inhibitor Binding Site Localized on the *Torpedo* Acetylcholine Receptor in the Open State†

David A. Johnson* and Scott Ayres

Division of Biomedical Sciences, University of California, Riverside, California 92521-0121

Received January 18, 1996; Revised Manuscript Received March 20, 1996⊗

ABSTRACT: Open-channel blockers of the nicotinic acetylcholine receptor (nAcChR) are widely thought to act sterically by entering and "plugging" the open channel of the nAcChR. However, quinacrine, a fluorescent open-channel blocker, has been recently shown to bind to the nAcChR at a site near the lipid bilayer while the receptor is in a closed, desensitized state, suggesting that at least one open-channel blocker might act allosterically outside the channel [Valenzuela *et al.* (1992) *J. Biol. Chem. 267*, 8238]. To determine whether or not quinacrine also binds near the lipid bilayer when the receptor is in an open state, a short-range lipophilic quencher (5-doxylstearate, 5-SA) was used to assess the proximity of the nAcChR-bound quinacrine to the lipid bilayer while the receptor was transiently open by an agonist. Initial experiments using a stopped-flow instrument established the conditions required to monitor a portion of the changes in quinacrine fluorescence associated with its binding to the receptor in the open state. 5-SA (80 μM) reduced the amplitude of the rapid agonist-induced change in quinacrine emission to 44% $\pm$ 12% of the control value, indicating that the quinacrine was binding to a site proximal to the membrane-partitioned 5-SA. Control experiments established that 5-SA had no effect on the ability of the receptor to undergo agonist-induced conformational changes, suggesting that little, if any, 5-SA distributed into the channel lumen and perturbed the functional activity of the receptor. Together, the results indicate that quinacrine binds to a site on the open receptor that is in contact with the lipid bilayer and not in the channel lumen.

Nicotinic acetylcholine receptors (nAcChRs)[1] are ligand-gated cation channels that are located on the plasma membranes of a variety of neurons, skeletal muscles, and electroplax. The nAcChR from the electric organs of various species of *Torpedo* is composed of four unique subunits (α, β, γ, and δ in the stoichiometric ratio of 2:1:1:1) (Reynolds & Karlin, 1978; Raftery *et al.*, 1980), each of which has four hydrophobic segments (M1, M2, M3, and M4) that are generally thought to be transmembrane elements (Noda *et al.*, 1982, 1983a,b; Claudio *et al.*, 1983; Blanton & Cohen, 1992, 1994). Functionally, the receptor is capable of existing in at least four conformational states: resting, open, fast-onset desensitized, and slow-onset desensitized [for reviews see Karlin (1991, 1993), and Changeux *et al.* (1992)]. Noncompetitive inhibitors of the nAcChR differ from one another in two respects: (i) the site or sites of their interaction and (ii) the conformation or state dependence of their interaction with the nAcChR (Heidmann *et al.*, 1983; Boyd & Cohen, 1984). One subclass of noncompetitive inhibitors is designated open-channel blockers, which are widely thought to act by entering the open channel and sterically "plugging" the channel lumen (Karlin, 1991; Changeux *et al.*, 1992).

Whether or not *all* open-channel blockers physically enter and plug the lumen is unclear. Studies employing site-directed mutagenesis and affinity labeling strongly suggest that some open-channel blockers act at discrete sites that are located at about the middle of the five putative pore-forming domains (M2s) (Karlin, 1991; Changeux *et al.*, 1992). However, when the nAcChR is in the slow-onset desensitized state, quinacrine, a fluorescent open-channel blocker, binds to a site that is highly accessible to paramagnetic lipid probes (Valenzuela *et al.*, 1992; Arias *et al.*, 1993a,b), which suggests that the quinacrine binding site is in contact with the lipid bilayer and, therefore, is presumably not in the channel lumen.

One difficulty with the experimental support for a non-luminal site of action for quinacrine is that the relative position of receptor-bound quinacrine has only been assessed while the receptor primarily is in the slow-onset desensitized state. Much evidence indicates that conformational changes are associated with the various state changes (Unwin *et al.*, 1988; White & Cohen, 1998; McCarthy & Moore, 1992; Blanton & Cohen, 1994; Akabas & Karlin, 1995). Also, the position of ligand binding sites can radically change following interaction of an antibody with the receptor (Valenzuela *et al.*, 1994b). Consequently, it is conceivable that the quinacrine binding site could move out of the open lumen following the open-to-desensitized state change and,

† This work was supported by National Science Foundation Grant IBN-9515330 to D.A.J.

* To whom correspondence should be addressed at the Division of Biomedical Sciences. Phone: (909) 787-3831. FAX: (909) 787-5504. E-mail: dajohn@mail.ucr.edu.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations and trivial names used: ACh, acetylcholine; nAcChR, muscle-type nicotinic acetylcholine receptor; dansyl-C_6-choline, 6-(5-(dimethylamino)naphthalene-1-sulfonamido)hexanoic acid−β-(*N*-trimethylammonium) ethyl ester; dansyltrimethylammonium, (dimethylamino)naphthalene-5-sulfonamidoethyltrimethylammonium; 5-SA, 5-doxylstearate; MTSEA, methanethiosulfonate ethylammonium; MTSES, methanethiosulfonate ethylsulfonate; α-neurotoxin, *Naja naja siamensis 3* α-neurotoxin; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine; *Torpedo* physiological saline, 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM phosphate buffer, pH 7.0.

S0006-2960(96)00123-7 CCC: $12.00

thus, lead to the false conclusion that quinacrine acts near the lipid bilayer because quinacrine binds near the lipid bilayer after the nAcChR has desensitized.

To address this issue, we measured the ability of a lipid paramagnetic probe to quench the receptor-bound emission from quinacrine as it binds to the transiently open state of the nAcChR. Specifically, we used a stopped-flow instrument to monitor the capacity of paramagnetic 5-SA to quench the rapid, carbamylcholine-induced changes in the emission from quinacrine as it binds to receptor-rich membrane fragments. The magnitude of 5-SA quenching of the rapid, carbamylcholine-induced changes in quinacrine emission was found to be essentially the same as previously observed under conditions that primarily stabilized the receptor in the desensitized state. Consequently, the possible existence of state-dependent changes in the position of the quinacrine binding site does not explain our earlier conclusion that quinacrine binds near a lipid−protein interface and not in the channel lumen.

MATERIALS AND METHODS

Materials. Quinacrine dihydrochloride, suberyldicholine dihydrochloride, carbamylcholine hydrochloride, and phencyclidine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). *Torpedo californica* electric rays were obtained from Marinus Inc. (Long Beach, CA). α-Neurotoxin was isolated following the method of Karlsson *et al*. (1971) from *Naja naja siamensis* venom purchased from the Miami Serpentarium (Salt Lake City, UT). Dansyltrimethylammonium perchlorate was acquired from Pierce Chemical Co. (Rockford, IL). 6-(5-(Dimethylamino)naphthalene-1-sulfonamido)hexanoic acid−β-(*N*-trimethylammonium) ethyl ester (dansyl-C_6-choline) was synthesized as described elsewhere (Valenzuela *et al.*, 1994a).

Isolation of the Membrane-Associated nAcChR. nAcChR-associated membrane fragments were prepared from the *T. californica* electric organs following previously described procedures (Johnson & Yguerabide, 1985). The specific activities of the various receptor preparations ranged between 1.2 and 4.5 nmol of suberyldicholine binding sites/mg of total protein, determined as described elsewhere (Valenzuela *et al.*, 1992).

Stopped-Flow Measurements. The stopped-flow experiments were performed with an Applied Photophysics SX-17MV stopped-flow spectrofluorimeter. An Oriel low-fluorescence 500-nm long-pass filter was placed in front of the photomultiplier tube to select the emission band. The excitation bands were selected with a monochromator in the excitation path. When experimental objectives allowed, fluorophores were excited at 295 nm to enhance the signal-to-noise ratio by tryptophanyl excitation and dipolar energy transfer to the receptor-bound fluorophores. With experiments that involved the use of 5-SA, it was necessary to directly excite the fluorophores (450 nm for quinacrine and 340 nm for dansyl-C_6-choline) to avoid the possibility of indirect quenching from the interaction of the 5-SA with the receptor tryptophan (possibly γ-Trp^{453}) in the transmembrane domain of the receptor (Chattopadhyay & McNamee, 1991). The kinetics of carbamylcholine-induced changes in quinacrine fluorescence or dansyl-C_6-choline binding were the same whether or not the fluorophores were excited directly or indirectly through the receptor tryptophans. Unless stated otherwise, all samples were suspended in 10 mM sodium phosphate buffer, pH 7.4, and the temperature of all samples was held at 15 °C.

RESULTS

Kinetics of Carbamylcholine-Induced Changes in Quinacrine Emission. In an earlier work, Grünhagen *et al.* (1977) reported that the rapid mixing of carbamylcholine or other cholinergic agonists with receptor-rich membrane fragments from *Torpedo marmorata* (suspended in a physiological saline buffer) was associated with both rapid and slow changes in quinacrine emission. The kinetics of the fastest observed rate (k_{obs}) of change of quinacrine fluorescence varied nonlinearly with the carbamylcholine concentration reaching a maximum of about 10 s^{-1} (Grünhagen *et al.*, 1977). Importantly, although not tested with carbamylcholine, acetylcholine (ACh)-induced changes in quinacrine fluorescence did not vary with quinacrine concentration. In a series of experiments using *T. californica*, we confirmed all of the basic results of Grünhagen *et al.* (1977) with the exception that the maximum k_{obs} with the *T. californica* membranes suspended in *Torpedo* physiological buffer was five times faster ($\sim$50 s^{-1}) than that seen with the *T. marmorata* membranes ($\sim$10 s^{-1}) (data not shown). We also found that, like ACh, carbamylcholine-induced changes in quinacrine fluorescence do not vary with quinacrine concentration (data not shown).

Because the kinetics of these rapid changes in carbamylcholine-induced quinacrine fluorescence are independent of the quinacrine concentration, these rapid changes in quinacrine fluorescence are not directly associated with quinacrine binding. Apparently, all the quinacrine binding occurs within the mixing time of the instrument, and only post-quinacrine binding isomerization steps are detectable.

In an attempt to extend the duration of the quinacrine binding process, the above described stopped-flow experiments were repeated using a low ionic strength buffer (10 mM sodium phosphate buffer, pH 7.4). Figure 1A,B shows the changes in quinacrine emission after the rapid mixing of carbamylcholine with nAcChR membrane fragments suspended in this buffer. Both carbamylcholine- and mixing-dependent changes in quinacrine fluorescence are observed (Figure 1A,B). The mixing-dependent effect probably represents a relaxation of quinacrine membrane partitioning as the suspension of nAcChR membrane fragments plus quinacrine is mixed with a buffer solution that contains only quinacrine. This comes about because the suspension of nAcChR membrane fragments plus quinacrine has a lower free quinacrine concentration (due to nonspecific membrane partitioning) than the buffer plus quinacrine solution. Support for this interpretation comes from the following observations: First, under conditions of high ionic strength (250 mM NaCl or *Torpedo* physiological buffer solution), when the membrane surface charge and the surface absorption of organic cations are minimized, a much smaller mixing-dependent effect is observed (data not shown). Second, in the absence of carbamylcholine and when quinacrine is only present in the syringe containing nAcChR membrane fragments, the quinacrine emission decreases, instead of increases, after the mixing as the quinacrine partitions out of the diluted membrane fragments (data not shown).

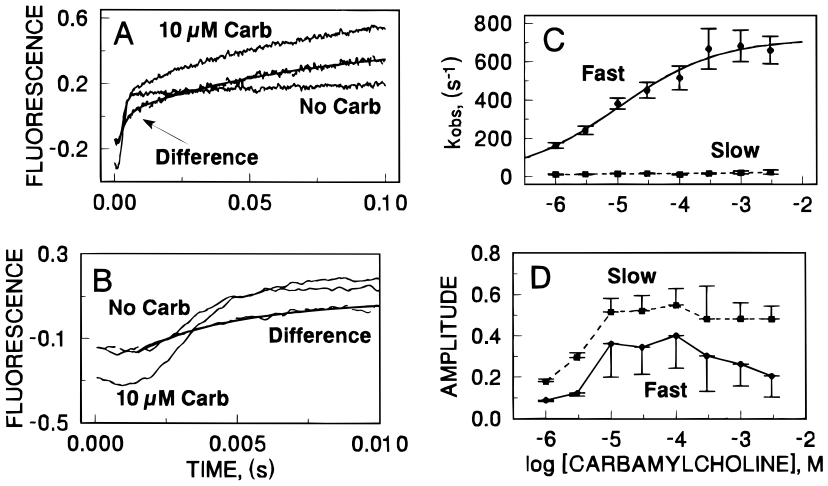

FIGURE 1: Rapid changes in quinacrine emission after the mixing of nAChR membrane fragments with carbamylcholine. In a stopped-flow apparatus (excitation at 295 nm and emission through a 500-nm cutoff filter) an nAChR membrane suspension (0.7 μM in suberyldicholine binding sites) and quinacrine (2 μM) in sodium phosphate buffer were mixed 1:1 with quinacrine (2 μM) and various concentrations of carbamylcholine or α-neurotoxin (2 μM) also in sodium phosphate buffer. (A, B) Time-dependent changes in quinacrine emission following the 1:1 mixing of 20 μM carbamylcholine (upper tracing) with nAChR membrane fragments as described above. The tracings of the effect of removing carbamylcholine or substituting α-neurotoxin (2 μM) for the carbamylcholine are shown as indicated in the figure. The middle tracing (dashed line) is the difference between the upper and the lower tracings, and the smooth line running through this middle tracing was generated with a biexponential equation using the best-fit parameters from a nonlinear regression algorithm provided with the instrumental software. The instrument mixing time is 1.3 ms. Each line tracing represents the average of three mixings. (C) Carbamylcholine concentration-dependent changes in the apparent rate constants (k_{obs}) calculated by first subtracting the "zero carbamylcholine" tracing from each carbamylcholine-induced tracing and then fitting the resultant tracing to a biexponential equation. The filled circles and squares represent the mean (±SD) of the calculated best-fit fast and slow apparent rate constants, respectively. The solid line represents the best fit of the data to an equation for a sigmoid (four-parameter logistic equation) ($EC_{50} = 9.9 \pm 3.4$ μM). The indicated carbamylcholine concentrations represent the values following mixing. (D) Carbamylcholine concentration-dependent changes in the apparent amplitudes from nonlinear regression of the difference tracings to a biexponential equation as described above. The filled circles and the squares represent the mean (±SD) of the calculated best-fit amplitudes of the fast and slow processes, respectively. For panels C and D each point represents the average of three replications using two different nAChR membrane preparations.

To control for the mixing-dependent changes in quinacrine emission, tracings generated with the samples that did not contain carbamylcholine were subtracted from tracings that were generated with comparable samples that contained various concentrations of carbamylcholine. The difference tracings, thus generated, fit well to a biexponential equation and reveal both a fast change in quinacrine fluorescence that is 14-fold faster than was observed with high ionic strength buffers (*vide ante*) and a slow change in quinacrine fluorescence. While the kinetics of the fast phase vary nonlinearly with carbamylcholine concentration, reaching a maximum rate of about 700 s^{-1}, the kinetics of the slower component do not vary with carbamylcholine concentration and averaged (±SD) 18 ± 4 s^{-1} (Figure 1C).

Because quinacrine (at the concentrations used here) only blocks the receptor in the open state (Adams & Feltz, 1980), it would be expected that the kinetics of quinacrine binding to the open receptor would vary linearly with quinacrine concentration and nonlinearly with carbamylcholine concentration. Consequently, the kinetics of the rapid carbamylcholine-induced changes in quinacrine fluorescence were examined as a function of the carbamylcholine and quinacrine concentrations. Figure 1C shows the effects of varying the carbamylcholine concentration on the kinetics of the fast and slow quinacrine response. Although the rate of the slow response is independent of the carbamylcholine concentration, the rate of the fast response varies nonlinearly with carbamylcholine concentration.

Fitting the plot of the observed fast rates versus carbamylcholine concentration to the equation for a sigmoid yields an EC_{50} value of 9.9 ± 3.4 μM. This value is 50− times lower than would be expected (0.5−1.0 mM) of a process that is directly associated with carbamylcholine-induced activation of the nAcChR (Adams, 1980; Forman *et al.*, 1987). Consequently, at first glance, the fast changes in quinacrine fluorescence do not appear to be directly associated with quinacrine binding to the open receptor. However, if the low ionic strength buffer slows the quinacrine binding process sufficiently so that it becomes possible to detect at least a portion of the quinacrine binding, then it is possible to explain the lower than expected EC_{50}. The concentration dependence of the carbamylcholine-induced quinacrine binding reaches an early plateau because quinacrine binding is still relatively rapid and the maximum rate that a stopped-flow instrument can measure is about 700 s^{-1}, well below what is required to accurately measure the carbamylcholine concentration dependence. Consequently, only a fraction of the concentration dependence of carbamylcholine-induced changes in the rate of quinacrine binding can be detected. The capacity of the stopped-flow instrument to monitor quinacrine binding to the open-channel state thus saturates at relatively low agonist concentrations. Consistent with this explanation for the lower than expected EC_{50} is the observed amplitude of the fast response increasing and then decreasing as the carbamylcholine concentration is elevated (Figure 1D). Hence, these data suggest that the fast carbamylcholine-induced response (under low ionic strength conditions) corresponds to at least a portion of the process of quinacrine binding to the open receptor.

With regard to the dependence of the kinetics on the concentration of quinacrine, the fast but not the slow changes in quinacrine fluorescence vary linearly with quinacrine concentration (Figure 2). This indicates again that the fast response is associated with a quinacrine binding-dependent

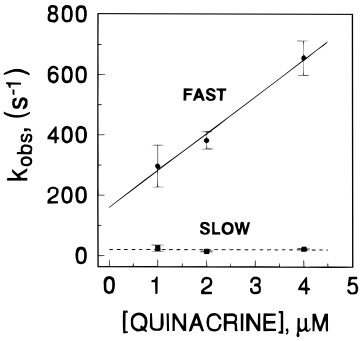

FIGURE 2: Concentration dependence of quinacrine on the observed rate constants. Using the basic procedures described in the legend of Figure 1, the nAcChR membrane fragments (0.7 μM in suberyldicholine binding sites) were rapidly mixed 1:1 with buffer with and without carbamylcholine (20 μM). Various concentrations of quinacrine were present in both syringes. The resultant tracings from mixings that were performed with carbamylcholine were subtracted from tracings of mixings that were performed in the absence of carbamylcholine, and the apparent rate constants (k_{obs}) were the best-fit rate parameters from a nonlinear regression of the difference tracings to a biexponential equation. The filled circles and the filled squares represent the mean (±SD; three determinations) of the calculated best-fit fast and slow apparent rate constants, respectively. The after-mixing concentration of quinacrine binding sites ($^1/_2$ suberyldicholine binding sites) was 0.18 μM.

process and the slow response is not. Furthermore, the apparent bimolecular association rate constant calculated from the slope of the plot of the fast k_{obs} versus the quinacrine concentration is $(1.2 \pm 0.13) \times 10^8$ M^{-1} s^{-1}, a value close to that observed by Adams and Feltz (1980) for the association rate constant for quinacrine inhibition of the frog end-plate (3.8×10^8 M^{-1} s^{-1}).

Taken together, the above results indicate that under low ionic strength conditions the rapid carbamylcholine-induced changes in quinacrine emission correspond to a portion of the process of quinacrine binding to the open receptor. By inference, the quinacrine emission during this fast response phase should be from quinacrine bound to the open, albeit blocked, channel. Therefore, the accessibility of short-range quenchers to quinacrine bound to the open receptor can be assessed by monitoring this fast quinacrine response.

Proximity of the Lipid Bilayer to Quinacrine Bound to the Open Receptor. To assess the proximity of the receptor-bound quinacrine to the lipid bilayer, a comparison was made between the ability of 5-SA and nonparamagnetic stearate to reduce the amplitude of the fast carbamylcholine-induced quinacrine response. Although pretreatment of the nAcChR membrane fragments with stearate (80 μM after-mixing concentration) had no effect on the rapid carbamylcholine-induced changes in quinacrine fluorescence, pretreatment with 5-SA (80 μM after-mixing concentration) reduced the amplitude of the fast response to 44% ± 12% of the control value (Figure 3). This value is similar to what was previously observed for quenching of the emission of quinacrine bound to desensitized receptors (~50% of control value; Arias *et al.*, 1993b), which was greater than the 5-SA quenching of the emission from membrane-partitioned fluorophores. The inset to Figure 3 is a plot of the observed amplitude of the fast carbamylcholine-induced changes in quinacrine emission as a function of 5-SA concentration and shows that the effect of 5-SA is concentration dependent. The observed rate of the fast process was not significantly

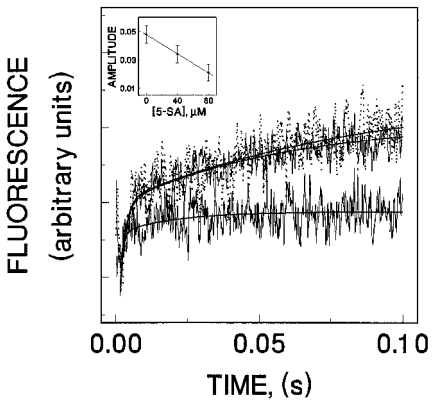

FIGURE 3: 5-SA-induced quenching of the rapid increase in quinacrine emission after the mixing of nAcChR membrane fragments with carbamylcholine. In a stopped-flow apparatus (excitation at 450 nm and emission through a 500-nm long-pass filter) quinacrine (2 μM) and nAChR membrane fragments (0.7 μM in suberyldicholine binding sites) in the presence or absence of 160 μM 5-SA or stearate was mixed 1:1 with quinacrine (2 μM) or quinacrine (2 μM) plus carbamylcholine (20 μM). A parallel set of mixings was preformed in the absence of carbamylcholine to assess the mixing-dependent changes in quinacrine emission. The mixing-dependent tracings were subtracted from the parallel tracings generated with carbamylcholine. The upper solid-line tracing is the control and shows the effect of mixing the nAChR membranes with carbamylcholine in the absence of any exogenous lipid. The upper dotted-line tracing shows the effect of treating the nAChR membrane fragments with stearate. The lower solid-line tracing shows the effect of treating the nAChR membranes with 5-SA. All samples were suspended in 10 mM sodium phosphate buffer, pH 7.4. These tracings are noisier than the tracings illustrated in Figure 1A because the quinacrine was not excited through the receptor tryptophans, which enhances the binding signal.

affected by 80 μM 5-SA (control, 550 ± 180 s^{-1}; 5-SA, 500 ± 310 s^{-1}). These results indicate that when quinacrine is bound to the open receptor, membrane-partitioned 5-SA is very accessible to it.

Proximity of the Lipid Bilayer to Receptor-Bound Dansyl-C_6-Choline. Dansyl-C_6-choline binds to the ACh binding sites that appear to be located on the nAcChR about 35 Å above the extracellular surface of the lipid membrane (Valenzuela *et al.*, 1994a); consequently, membrane-partitioned lipid probes like 5-SA should have no effect on the kinetics of the rapid changes in the dansyl-C_6-choline fluorescence as it binds to the nAcChR. Indeed, 5-SA has been previously shown to have no significant effect on receptor-bound dansyl-C_6-choline fluorescence following prolonged interaction with nAcChR membrane fragments (Valenzuela *et al.*, 1994b).

In an attempt to validate the use of this kinetic approach to assess the proximity of fluorescent receptor-bound ligands to the lipid bilayer, the ability of 5-SA to quench the emission from receptor-bound dansyl-C_6-choline was examined as it binds to the desensitized nAcChR. Specifically, nAcChR membrane fragments in the absence or presence of 5-SA were pretreated with phencyclidine to induce all the receptors into a high-affinity, slow-onset desensitized state (Heidmann & Changeux, 1979b) and then rapidly mixed with dansyl-C_6-choline. A set of mixings was performed with nAcChR membrane fragments that had been pretreated with an excess of α-neurotoxin (2 μM) to show that all the observed changes in fluorescence were specifically associated with the dansyl-C_6-choline binding to the ACh binding sites. Figure 4 illustrates the results of these experiments which indicate,

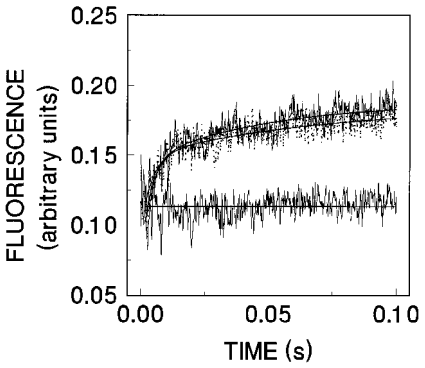

FIGURE 4: Inability of 5-SA to quench the rapid increase in dansyl-C_6-choline emission after mixing with desensitized nAcChR membrane fragments. In a stopped-flow apparatus (excitation at 340 nm and emission through a 500-nm long-pass filter), phencyclidine (200 μM) and nAChR membrane fragments (0.7 μM in suberyldicholine binding sites) in the presence or absence of 5-SA (160 μM) or α-neurotoxin (2 μM) in phosphate buffer was mixed 1:1 with dansyl-C_6-choline (4 μM). In the upper solid-line tracing only the nAChR membranes were mixed with dansyl-C_6-choline, while in the upper dotted-line tracing 5-SA and the nAChR membrane fragments were mixed with dansyl-C_6-choline. In the lower solid-line tracing the nAChR membranes were pretreated with α-neurotoxin. Each tracing represents the averaged results of three mixings, and all samples were suspended in 10 mM sodium phosphate buffer, pH 7.4.

as expected, that 5-SA had no significant effect on the rapid changes in dansyl-C_6-choline fluorescence.

Effect of 5-SA on Carbamylcholine-Induced Receptor Isomerization. The possibility that the accessibility of 5-SA to receptor-bound quinacrine was secondary to a 5-SA-induced structural perturbation of the receptor was evaluated. It was reasoned that if 5-SA induced a significant structural perturbation, then the receptor would be unable to undergo agonist-induced isomerizations. The receptor isomerization pathways are complex. In the absence of any ligands, the *Torpedo* receptor exists largely at equilibrium between two states, low-affinity resting (80%−90%) and high-affinity slow-onset desensitized (10%−20%) (Grünhagen & Changeux, 1976; Weiland *et al.*, 1977). The rapid mixing of an agonist with the receptor is associated with an initial fast binding phase as the agonist molecules bind to the preexisting desensitized receptors. This fast response is followed by a series of slower binding phases as the receptor slowly isomerizes and is ultimately converted into the high-affinity, slow-onset desensitized state (Heidmann & Changeux, 1979a).

To assess the effect of 5-SA on agonist-induced receptor isomerizations, the time courses of the changes in excited-state emission from dansyl-C_6-choline after rapidly mixing with nAcChR membrane fragments were monitored in the absence and presence of 5-SA. With the exception of a small reduction in final magnitude of dansyl-C_6-choline emission that was probably due to an absorptive screening effect (∼6%) produced by 5-SA absorption of the 340 nm excitation beam, 5-SA had little or no effect on the time course of the changes in dansyl-C_6-choline emission following rapid mixing with nAcChR membrane fragments (Figure 5). The fluorescence was recorded and plotted with a log time base to more easily illustrate the complexities of binding. Also, a separate set of mixings were performed with nAcChR membrane fragments that had been pretreated with an excess of α-neurotoxin (2 μM) to show that the observed changes

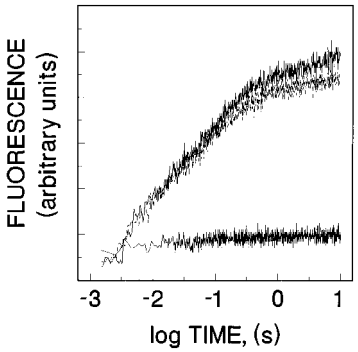

FIGURE 5: Inability of 5-SA to affect agonist-induced nAChR isomerization. In a stopped-flow apparatus (excitation at 340 nm and emission through a 500-nm long-pass filter), dansyl-C_6-choline (4 μM) was mixed 1:1 with nAChR membrane fragments (0.7 μM in suberyldicholine binding sites) in the presence or absence (upper solid line) of 160 μM 5-SA (upper dotted line) or α-neurotoxin (2 μM) (lower solid line) in buffer. All samples were suspended in 10 mM sodium phosphate buffer, pH 7.4.

in fluorescence were specifically associated with the dansyl-C_6-choline binding to the ACh binding sites.

DISCUSSION

The aim of this paper was to further analyze the molecular basis of the noncompetitive inhibitory action of quinacrine on the nAcChR. We previously reported that, when the receptor is primarily in the slow-onset desensitized state, quinacrine binds to a site on the receptor that is very accessible to lipophilic probes and is presumably proximal to the lipid bilayer, a few angstroms below the lipid surface (Valenzuela *et al.*, 1992; Arias *et al.*, 1993a,b). However, because quinacrine is an open-channel blocker and, therefore, acts by preferentially binding to the receptor in the open state, determination of the actual location of the inhibitory site of action of quinacrine requires assessment of the quinacrine binding site on the open receptor. Toward this end, we found a set of conditions that permitted the monitoring of quinacrine emission as it binds to the receptor in the open state and found that 5-SA was just as highly accessible to quinacrine bound to the open receptor as it was to the closed receptor.

The interpretation of these results rests largely on a knowledge of (i) the distance over which significant nitroxide quenching of electronic excited fluorophores occurs and (ii) the distribution of 5-SA in and around the nAcChR. In the absence of spectral overlap between the emission spectrum of the fluorophore and the absorption spectrum of the nitroxide, as it is in the present case, the generally accepted mechanism for excited singlet state quenching by nitroxides involves an electron exchange-induced intersystem crossing to the triplet or internal conversion to the ground state (Green *et al.*, 1973, 1990). For the case of through-space interaction, the distance between the excited fluorophore and the nitroxide quencher in the encounter that leads to quenching has been calculated to be 4−6 Å (Green *et al.*, 1973).

With reference to the distribution of 5-SA in and around the nAcChR, long-chain fatty acids have been shown to distribute not only in the bulk lipid bilayer but also in the phospholipid exchangeable (annular) and nonexchangeable (nonannular) lipid pools which are in contact with the receptor (Jones & McNamee, 1988). There is no evidence that long-chain fatty acids enter the channel lumen at the concentrations (<80 μM) or the temperature (15 °C) used

in present study. Andreasen and McNamee (1980) showed that 5-SA at a concentration of 1 mM (12-fold higher than used in the present study) had no effect on carbamylcholine-induced $^{22}Na^+$ efflux from nAcChR membrane vesicles at 0 °C and only produced a 30% inhibition at 25 °C. Moreover, 5-SA at 80 μM had no significant effect on agonist-induced receptor isomerization (Figure 5). If 5-SA does not interfere with cation movement through the channel, then it seems unlikely that 5-SA would be present in a significant fraction of the channel lumens.

Further insight into the possible distribution of 5-SA in and around the receptor is provided by the studies of nonspecific incorporation of the hydrophobic probe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), into the transmembrane segments of the receptor (Blatton & Cohen, 1992, 1994). [^{125}I]TID nonspecifically incorporates into the M3 and M4 segments of each of the four types of subunits and into the M1 segments of the α and δ subunits. The pattern of [^{125}I]TID incorporation into the apparently α-helical M3 and M4 segments suggests that only one face of the surface of each of these segments is in contact with the lipid bilayer. For the αM1, the labeling pattern is inconsistent with the labeling of either a face of an α-helix or a β-strand. [^{125}I]TID does not incorporate into any of the M2 segments or the βM1 segment. It follows that there probably are zones within the transmembrane domain that exclude lipophilic agents. These exclusion zones appear to include all of the luminal M2 segments, a face of each of the M3 and M4 segments, and the perhaps N-terminal half of the αM1 and the N-terminal third of δM1. Although the delineation of these lipid exclusion zones is clouded by unknown steric factors, the orientation of the transmembrane amino acid residues, and by the differential reactivity of the photoactivated diazirine moiety in the [^{125}I]TID toward the various amino acid side chains, lipophilic agents (and, presumably, 5-SA) probably are not accessible to the entire transmembrane domain. The lack of nonspecific [^{125}I]TID incorporation into any of the luminal M2 segments, again, is consistent with the above conclusion that 5-SA does not distribute within the lumen of the receptor. Of course, given the limited reactivity of diazirines, the absence of labeling does not provide strong proof of inaccessibility of lipophilic agents.

Electrophysiological, biochemical, and molecular biological approaches have yielded results that have been interpreted as supporting the hypothesis that quinacrine acts by binding to the channel lumen. Electrophysiological studies have demonstrated that at low concentrations quinacrine primarily acts by binding to the receptor in the open state (Adams, 1981). In a review article, it was reported that photoactivated [^{3}H]quinacrine azide incorporates into αArg^{209} and αPro^{211}, which are located near the beginning of the N-terminal side of the αM1 segment (Karlin, 1991). The possibility that the N-terminal third of the αM1 segments forms part of the extracellular side of the luminal transmembrane domain of the receptor is supported by the observations that the hydrophobic [^{125}I]TID does not label the N-terminal third of the αM1 segments (Blanton & Cohen, 1992, 1994). Also, following sequential X-to-Cys mutation of the residues comprising the N-terminal half of the αM1 segment, the small, positively charged, sulfhydryl-specific reagent, methanethiosulfonate ethylammonium (MTSEA) has been shown to be accessible to the N-terminal third of the αM1 segment and, therefore, in contact with an aqueous domain (Akabas & Karlin, 1995). It therefore appears that under some circumstances this segment of the αM1 forms part of the channel lumen.

There are several puzzling aspects of the experimental support for a luminal site of quinacrine action. First, the selective binding of a noncompetitive inhibitor to the receptor in the open state carries no information about the location of the inhibitor binding site. An open-channel inhibitor could act either sterically by binding in the lumen or allosterically by binding at a nonluminal site to block the receptor. Second, the accessibility of MTSEA to the αM1 residues is highly dependent on the conformational state of the receptor (Akabas & Karlin, 1995). Under conditions (absence of ACh) in which the channel is in a closed, resting state and toward which quinacrine displays low affinity, MTSEA reacts with six of the fifteen cysteine-substituted αM1 mutants examined to inhibit the ACh-induced response. However, under conditions (presence of ACh) in which the receptor is primarily in either a desensitized or open state and toward which quinacrine displays a high affinity, MTSEA only reacts with one cysteine-substituted mutant (Tyr^{213}-to-Cys^{213}) to block the ACh-induced response. Indeed, when the sulfhydryl-specific reagent reacts with the receptor in the presence of ACh, the cation selectivity of this effect is lost and the negatively charged methanethiosulfonate ethylsulfonate (MTSES) also inhibits the ACh-induced response of the Tyr^{213}-to-Cys^{213} mutant (Akabas & Karlin, 1995). This is inconsistent with the αCys^{213} being located in the cation-selective, channel luminal domain. Furthermore, when MTSEA reacts with the Val^{218}-to-Cys^{218} mutant, the ACh-induced response is potentiated, not blocked (Akabas & Karlin, 1995). This is most perplexing if the αCys^{218} forms part of the lumen and MTSEA is attached to αCys^{218} and protruding into the channel lumen.

With these substituted cysteine-mutant results, it could be argued that in the open state the N-terminal third of the αM1 segment moves out of the transmembrane luminal domain of the receptor. The αM1 segment is much longer (27 residues) than would be required for a single α-helix (20 residues) or β-strand (10 residues) to span the lipid bilayer and, therefore, could undergo a significant rearrangement in which the N-terminal third of the αM1 segment could move away from the channel lumen while the remaining two-thirds could still span the lipid bilayer. Whatever the actual arrangement of the N-terminal third of the αM1 segment is, when ACh is present, little of it appears to be accessible to charged solutes and what is accessible to charged solutes probably does not form the walls of the cation selective domain of the channel lumen.

In conclusion, membrane-partitioned 5-SA was found to be highly accessible to quinacrine as it binds to the receptor in the open state. These results provide critical further support to the hypothesis that quinacrine acts allosterically outside the channel lumen to block ACh-induced ion movements through the nAcChR.

ACKNOWLEDGMENT

We acknowledge the technical assistance of Wendi Wohltmann for the preparation and characterization of the nAcChR membrane fragments.

REFERENCES

Adams, P. R. (1981) *J. Membr. Biol. 58*, 161−174.
Adams, P. R., & Feltz, A. (1980) *J. Physiol. 306*, 261−281.
Akabas, M. H., & Karlin, A. (1995) *Biochemistry 34*, 12496−12500.
Akabas, H. M., Kaufmann, C., Archdeacon, P., & Karlin, A. (1994) *Neuron 13*, 919−927.
Andreasen, T. J., & McNamee, M. G. (1980) *Biochemistry 19*, 4719−4726.
Arias, H. R., Valenzuela, C. F., & Johnson, D. A. (1993a) *J. Biol. Chem. 268*, 6348−6355.
Arias, H. R., Valenzuela, C. F., & Johnson, D. A. (1993b) *Biochemistry 32*, 6237−6242.
Blanton, M. P., & Cohen, J. B. (1992) *Biochemistry 31*, 3738−3750.
Blanton, M. P., & Cohen, J. B. (1994) *Biochemistry 33*, 2859−2872.
Boyd, N. D., & Cohen, J. B. (1984) *Biochemistry 23*, 4023−4033.
Changeux, J.-P., Galzi, J.-L., Devillers-Thiery, A., & Bertrand, D. (1992) *Q. Rev. Biophys. 25*, 395−435.
Chattopadhyay, A., & McNamee, M. G. (1991) *Biochemistry 30*, 7159−7164.
Claudio, T., Ballivet, M., Patrick, P., & Heinemann, S. (1983) *Nature 80*, 1111−1115.
Forman, S. A., Firestone, L. L., & Miller, K. W. (1987) *Biochemistry 26*, 2807−2814.
Green, J. A., & Singer, L. A. (1973) *J. Chem. Physics 58*, 2690−2695.
Green, S. A., Simson, D. J., Zhou, G., & Blough, N. V. (1990) *J. Am. Chem. Soc. 112*, 7337−7346.
Grünhagen, H.-H., & Changeux, J.-P. (1976) *J. Mol. Biol. 106*, 517−535.
Grünhagen, H.-H., Iwatsubo, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 225−242.
Heidmann, T., & Changeux, J.-P. (1979a) *Eur. J. Biochem. 94*, 255−279.
Heidmann, T., & Changeux, J.-P. (1979b) *Eur. J. Biochem. 94*, 281−296.
Heidmann, T., Qswald, R. E., & Changeux, J.-P. (1983) *Biochemistry 22*, 3112−3127.
Johnson, D. A., & Yguerabide, J. (1985) *Biophys. J. 48*, 949−955.
Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364−2374.
Karlin, A. (1991) *Harvey Lect. 85*, 71−107.
Karlin, A. (1993) *Curr. Opin. Neurobiol. 3*, 299−309.
McCarthy, M. P., & Moore, M. A. (1992) *J. Biol. Chem. 267*, 7655−7633.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature 299*, 793−797.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T. Asai, M., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature 301*, 251−255.
Raftery, M. A., Hunkapiller, M. W., Stader, C. D., & Hood, L. E. (1980) *Science 208*, 1454−1457.
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035−2038.
Unwin, N., Toyoshima, C., & Kubalek, E. (1988) *J. Cell Biol. 106*, 1123−1138.
Valenzuela, C. F., Kerr, J. A., & Johnson, D. A. (1992) *J. Biol. Chem. 267*, 8238−8244.
Valenzuela, C. F., Weign, P., Yguerabide, J., & Johnson, D. A. (1994a) *Biophys. J. 66*, 674−682.
Valenzuela, C. F., Dowding, A. J., Arias, H. R., & Johnson, D. A. (1994b) *Biochemistry 33*, 6586−6594.
Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., Taylor, P. (1977) *J. Biol. Chem. 252*, 7648−7656.

BI960123P