Decidium

A NOVEL FLUORESCENT PROBE OF THE AGONIST/ANTAGONIST AND NONCOMPETITIVE INHIBITOR SITES ON THE NICOTINIC ACETYLCHOLINE RECEPTOR*

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We have examined the interaction of the nicotinic acetylcholine receptor with decidium diiodide, a bisquaternary analogue of ethidium containing 10 methylene groups between the endocyclic and trimethylamino quaternary nitrogens. Decidium inhibits mono-[^125]I]iodo-α-toxin binding, inhibits agonist-elicited \( 25^\circ \text{Na}^+ \) influx in intact cells, augments agonist competition with mono-[^125]I]iodo-α-toxin binding, and enhances \(^3\text{H}\)phenacyclidine (PCP) binding to a noncompetitive inhibitor site. These effects occur over similar concentration ranges (half-maximum effects between 0.1 and 0.4 \( \mu \text{M} \)). Thus, decidium binds to the agonist site and converts the receptor to a desensitized state exhibiting increased affinity for agonist and heterotropic inhibitors. These properties are similar to mepiphalic antagonists characterized in classical pharmacology. At higher concentrations decidium associates directly with the noncompetitive inhibitor site identified by \(^3\text{H}\)phenacyclidine binding. Dissociation constants of decidium at this site in the resting and desensitized states are determined to be 29 and 1.2 \( \mu \text{M} \), respectively. Analysis of fluorescence excitation and emission maxima reveal that binding to both the agonist and noncompetitive inhibitor sites is associated with 2-fold enhancement of fluorescence. The excitation maximum for decidium bound at the agonist site appears at 490 nm while that for decidium bound at the noncompetitive inhibitor site appears at 530 compared to 480 nm in buffer. These results suggest that decidium experiences a more hydrophobic environment upon binding to the nicotinic acetylcholine receptor sites, particularly to the noncompetitive inhibitor site. Fluorescence energy transfer between \( \text{N}^\prime\)-fluorescein isothiocyanate-lysine-23 α-toxin (FITC-toxin), and decidium is not detected when each is bound to one of the two agonist sites on the receptor. This allows a minimal distance to be estimated between fluorophores. In contrast, energy transfer is observed between decidium nonspecifically associated with the membrane or with nonspecific sites and the FITC-toxin at the agonist sites.

The nicotinic acetylcholine receptor (AcChR)\(^*\) represents a prototypic transmembrane ligand-regulated ion channel. Coupling of receptor occupation with ion permeability arises from an interplay between interconvertible functional states, modulated by the history of exposure to agonist or a variety of heterotropic ligands, such as phencyclidine (PCP) and local anesthetics. The emerging description of ligand-response coupling reveals a dynamic receptor entity possessing discrete but allosterically interactive sites and functional domains on the receptor oligomer.

Fluorescent ligands containing 5-dimethylaminonaphthalene-1-sulfyl (Dansyl) (Heidmann and Changeux, 1979), pyrenebutyl (Tan and Barrantes, 1980), or 7-nitrobenzo-2-oxa-1,3-diazole (Prinz and Maelicke, 1980a, 1980b; Bolger et al., 1981) moieties have been employed to characterize effector sites and mechanisms underlying functional transitions of the nicotinic receptor. These ligands emit at wavelengths shorter than 550 nm. A ligand which emits at wavelengths greater than 600 nm would be useful for assessing intersite distances through excitation energy transfer. To this end, we have characterized the interaction of decidium diiodide with the AcChR. Decidium is a fluorescent phenylphenanthridium analogue of ethidium which contains 10 methylene groups between the endocyclic quaternary nitrogen and the exocyclic trimethylamino quaternary nitrogen. Hence, it contains an interquaternary distance identical to decamethonium and its congeners which have been widely studied in nicotinic receptor function.

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The abbreviations used are: AcChR, nicotinic acetylcholine receptor; FITC-toxin, \( \text{N}^\prime\)-fluorescein isothiocyanate-lysine-23 cobra α-toxin; α-toxin, \text{Naja naja siamensis} 3 α-toxin; \(^{125}\text{I}\)-labeled α-toxin, α-mono[^125]I]iodo-α-toxin; PCP, phencyclidine; \( K_p \), protection constant.

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played to determine the distance between the two AcChR agonist-binding sites using N'-fluorescein isothiocyanate-labeled 23 cobra α-toxin (FITC-toxin) bound to one and decidium to the other.

To characterize the properties of decidium, we utilized both the BCh1-1 clonal muscle cell line and AcChR-enriched membrane fragments from Torpedo californica. BCh1-1 cells grow in monolayer cultures and elaborate uniformly distributed surface AcChRs permitting simultaneous measurement of receptor occupation and functional response in the same population of intact cells. Torpedo electric organs provide highly enriched source of receptors for fluorescence measurements.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and fetal calf serum were obtained from Gibco. Radionuclides, [125I] and [3H]Na+, and [125I]PCP were obtained from Du Pont-New England Nuclear. PCP was obtained from the National Institute of Drug Abuse. d-Tubocurarine chloride was a gift from Lilly. Dinaphthylecsemethionin 1 was a gift from Dr. H. P. Ring (University College, London, England). Proadifen was obtained from Smith Kline and French. Mebroadifen was prepared by methyleinating proadifen with iodomethane. Lidocaine and carbachol were from Sigma. The synthesis and chemical characterization of decidium is described in Berman et al. (1987). Freshly prepared solutions of decidium in buffer were clarified by filtration and the decidium concentration determined from the absorbance at 480 nm (εm = 6000 mM−1 cm−1). Concentrated stock solutions were dissolved in acetonitrile, protected from light, and stored at −10°C.

FITC-toxin was prepared as described elsewhere (Johnson and Taylor, 1982) and purified by column isoelectric focusing (Weiland et al., 1976). Cobra α-toxin (siamensis 3) was isolated following the method of Karlsson et al. (1971) from Naja naja siamensis venom (Miami Serpentarium, Salt Lake City, UT). Mono[125I]iodo-α-toxin (siamensis 3) (125I-labeled α-toxin) was prepared and separated from noniodinated and diiodo species by column isoelectric focusing. All other reagents were at least reagent grade. Except where noted, all binding and spectroscopic analyses were performed with samples suspended in 100 mM NaCl, 10 mM sodium phosphate buffer at pH 7.4.

Receptor Isolation—Receptor-rich membrane fragments were isolated from T. californica electric organ following published procedures (Johnson and Yguerabide, 1985; Reed et al., 1975). The specific binding activities of the receptor preparations were measured by adsorption of 125I-labeled α-toxin–receptor complexes onto DEAE-cellulose filters (Johnson and Yguerabide, 1985) and ranged between 1 and 2 nmol of α-toxin-binding sites/mg of protein.

Assays of Receptor Occupation and Agonist-stimulated Permeability Response—Kinetic assays to measure ligand competition with the initial rate of 125I-labeled α-toxin binding to AcChR in Torpedo membrane were performed using DE52 filter discs to adsorb selectively the receptor-toxin complex as described previously (Weiland et al., 1976). To reduce nonspecific adsorption of 125I-labeled α-toxin, the filter discs were acid-washed as follows. Filters were placed in a 43°C HCl/methanol solution (1:2, v/v) and allowed to cool to room temperature over a 45-min period. The filters were washed three times in distilled water with an overnight soak and then dried before use.

Assays of ligand competition with initial rates of 125I-labeled α-toxin binding and agonist stimulated 2Na+ permeability in intact BCh1-1 cells were performed as described previously (Sine and Taylor, 1986; Brown and Taylor, 1985).

FH/PAC Binding—Equilibrium binding of radiolabeled PAC was measured by a modification of the centrifugation assay of Heidmann et al. (1983) as described by Palma et al. (1986). Competition binding experiments were analyzed by the LIGAND nonlinear curve-fitting program, as developed by Munson and Rodbard (1980). PAC affinity toward the desensitized and resting states were determined to be 0.4 and 2 μM, respectively. Data points are the mean of duplicate determinations, and 17% will have two FITC-toxin molecules bound (cf Table I in Damle and Karlin, 1978). With 15% of the α-toxin-binding sites occupied by FITC-toxin, 82% of the FITC-toxin-occupied receptors will have only one toxin molecule bound, and 17% will have two FITC-toxin molecules bound (cf. Table I in Damle and Karlin, 1978). After the FITC-toxin was incubated

Fluorescence Titrations of Decidium Association with the Receptor—Titrations were carried out in 1-cm cuvettes using a water-jacketted four-sample thermostated compartment. All fluorescence values were corrected for dilution resulting from added tetrath, incident light scatter, and innerfilter effects. Except where noted, excitation and emission wavelengths were 290 and 620 nm, respectively. In the case of direct titrations with decidium, fluorescence contributions of the free ligand were subtracted from total fluorescence, and the specific fluorescence enhancement versus ligand concentration was plotted directly.

For measurements of competition with other ligands, decidium and receptor concentrations were 2.5 and 0.25 μM, respectively. Data are plotted to the formulation

$$\log \frac{f_b - f} {f_a} = -\frac{K_a}{f_a} (C) - \frac{K_b}{f_b} [D]$$

where, f, f0, and f1 are the observed decidium fluorescence, fluorescence when sites on the receptor are fully occupied, and the fluorescence when decidium is fully dissociated from the receptor. [D] and [C] represent the estimated free decidium and competing ligand concentrations, and K and K represent their respective dissociation constants (Taylor and Lappi, 1975). Equation 1 assumes that [D] ≫ [K] and that [D] is in excess of its binding sites.

Fluorescence Lifetime Analysis—Fluorescence lifetimes were determined by the single-photon-counting technique using an ESY Scientific nanosecond fluorescence spectrophotometer (La Jolla, CA) equipped with a high pressure hydrogen arc lamp. Excitation and emission bands of the FITC-toxin were selectively monitored with 480 nm broad and 560 nm narrow band interference filters, respectively. The filters used for measurement of the FITC-toxin lifetime were selected to eliminate interference from decidium emission. In addition to the chromatic filters, Polaroid HN'B dichroic film polarizers (Norwood, MA) were placed in the excitation and observation paths. The emission polarizer was rotated at an angle of 55° relative to the vertically oriented excitation polarizer to eliminate anisotopic contributions to the observed light. Decay curve was corrected by subtracting the decay curves for samples containing all the components except the fluorophore. The instrumental arrangement and principles of its measurement have been discussed in detail (Yguerabide, 1972; Johnson and Yguerabide, 1985).

Energy Transfer—The efficiency of dipolar resonance energy transfer between a discrete donor and acceptor pair is related to the distance (R) separating the pair by Equation 2 (Forster, 1965).

$$R = R_0(1/E - 1)^{1/6}$$

R0, the distance at which transfer efficiency equals 50%, is 9.765 × 10^-10 (A/F0)^-1/6. The overlap integral, I, between excited state donor and acceptor dipole is the integrated area of overlap between the donor emission spectrum, I(λ), and the acceptor absorption spectrum, ε(λ) (Equation 3).

$$J = \int I(\lambda)ε(\lambda)d\lambda$$

Q(λ) denotes the donor quantum yield in the absence of acceptor and λ, represents the refractive index of the medium between donor and acceptor, ε(λ), the orientation factor, accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles. λ is the wavelength in cm.

When donor and acceptor are at separate sites on a macromolecule, the efficiency of energy transfer (E0) can be measured by

$$E_0 = 1 - \tau_0/\tau_d$$
with the AcChR, the remaining unoccupied sites were occupied by a near saturating concentration of decidium, 10 μM. PCP (100 μM) was present to prevent decidium binding to the noncompetitive inhibitor site.

**RESULTS**

**Decidium Inhibition of Initial Rate of \(^{125}\text{I}\)-Labeled α-Toxin Binding and Agonist-stimulated \(^{22}\text{Na}^+\) Permeability**—Decidium inhibition of the initial rate of \(^{125}\text{I}\)-labeled α-toxin binding to both Torpedo AcChR-enriched membrane fragments and intact BC3H-1 cells was examined after instantaneous and prolonged (30 min) exposure to decidium. Decidium inhibits α-toxin binding to AcChR in Torpedo membranes (Fig. 1A) and in intact BC3H-1 cells (Fig. 1B). In each case the binding profiles exhibit Hill coefficients \((n_H)\) less than unity. Decidium also inhibits \(^{22}\text{Na}^+\) influx stimulated by carbamylcholine in BC3H-1 cells (Fig. 1C) at slightly lower concentrations but with a somewhat steeper profile (Table 1). As seen in Torpedo (Neubig and Cohen, 1979; Weilard and Taylor, 1979) and in BC3H-1 cells (Sine and Taylor, 1981), the Hill slope of less than unity for occupation suggests nonequivalence of decidium \(K_R\) for the two agonist sites on the receptor. The dependence on lower antagonist concentrations for blockade of the functional response is consistent with the concept that occupation of only one of the two sites is adequate to achieve functional antagonism, as documented for the classical antagonists (Sine and Taylor, 1981). In control experiments, decidium alone did not increase \(^{22}\text{Na}^+\) permeability over basal values, indicating that it has no partial agonist activity (data not shown).

The decidium protection constants decreased 2–4-fold when decidium was added 30 min prior to instead of simultaneously with radiolabeled α-toxin addition (Fig. 1, Table 1). This observation suggests that decidium possesses a limited capacity to convert AcChR to a state which displays increased affinity for decidium. Such a conversion in AcChR state might occur by decidium acting as a metaphilic antagonist (Rang and Ritter, 1970a, 1970b) or by acting as an allosteric site as a noncompetitive, heterotropic inhibitor. In both cases, a slow conversion to a state possessing a higher affinity for decidium is predicted.

**Decidium Inhibition of Agonist Activation of AcChR Permeability Response**—To examine the mechanism of decidium inhibition, the concentration dependence for carbamylcholine activation of \(^{22}\text{Na}^+\) permeability was measured in BC3H-1 cells in the presence of decidium. For comparison, a metaphilic antagonist, dinaphthyldecamethonium (Rang and Ritter, 1970a, 1970b), and a classical antagonist, \(d\)-tubocurarine were also examined. Increasing decidium concentrations progressively shift the carbamylcholine concentration-response curve to higher agonist concentrations as well as reduce the maximal response obtained at saturation (Fig. 2A). This behavior indicates both competitive and noncompetitive components of decidium inhibition are present. Like decidium, dinaphthyldecamethonium displays apparent noncompetitive inhibition, consistent with conversion of AcChR to a desensitized state incapable of channel opening (Fig. 2B). \(d\)-Tubo-

![Diagram](image-url)

**FIG. 1.** Decidium inhibition of initial rates of cobra \(^{125}\text{I}\)-labeled α-toxin and carbamylcholine-stimulated \(^{22}\text{Na}^+\) influx in Torpedo membranes and BC3H-1 cells. Initial rates of \(^{125}\text{I}\)-labeled α-toxin binding to AcChR in Torpedo membrane fragments (A) or monolayer BC3H-1 cultures (B) were measured in the presence of the indicated decidium concentrations. Data were normalized to the rate of α-toxin binding in the absence of decidium. C, initial rates of carbamylcholine-stimulated \(^{22}\text{Na}^+\) influx were measured in BC3H-1 cells in the presence of 60 μM carbamylcholine plus the indicated decidium concentrations. Experimental determinations were made in duplicate. Filled circles (●), equilibrium exposure (30 min) to buffer containing the indicated decidium concentrations followed by initial rate measurements of either \(^{125}\text{I}\)-labeled α-toxin or carbamylcholine-stimulated \(^{22}\text{Na}^+\) influx in the presence of the indicated decidium concentrations. Open circles (○), after equilibration in buffer alone for 30 min, the instantaneous addition of decidium and α-toxin (panels A and B) or 60 μM carbamylcholine (panel C) starts the respective initial rate measurements. The solid lines represent nonlinear best fits to the Hill equation.

**TABLE 1**

| AcChR source and exposure duration | \(K_p\) (m) \((^{22}\text{Na}^+\) influx) | \(n_H\) | \(K_p\) (m) \((^{125}\text{I}\)-labeled α-toxin) | \(n_H\) |
|----------------------------------|-----------------------------------|--------|-----------------------------------|--------|
| Intact BC3H-1 cells 30 min       | 1.2 ± 0.5 × 10^{-7}               | 0.92 ± 0.18 (4) | 3.6 ± 0.8 × 10^{-7} | 0.69 ± 0.01 (3) |
| Instantaneous                   | 3.5 ± 1.0 × 10^{-7}               | 0.66 ± 0.07 (4) | 5.0 ± 0.7 × 10^{-7} | 0.66 ± 0.07 (3) |
| Torpedo membranes 30 min        | 1.9 ± 0.2 × 10^{-7}               | 0.73 ± 0.03 (3) | 3.3 ± 0.5 × 10^{-7} | 0.77 ± 0.01 (3) |
| Instantaneous                   |                                   |        |                                   |        |
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Decidium modulation of \( [^3H] \)PCP binding to the Torpedo membranes. Assays solutions contained membranes (1.0 mM in \( \alpha \)-toxin sites), \( [^3H] \)PCP (1.0 mM), plus specified effector ligand concentrations. •, in the presence of carbamylcholine (200 mM); △, in the presence of \( \alpha \)-toxin (10 mM); ○, in the absence of both \( \alpha \)-toxin and carbamylcholine.

When the agonist sites are occupied with saturating concentrations of carbamylcholine or \( \alpha \)-toxin increasing concentrations of decidium displace \( [^3H] \)PCP, consistent with direct action of decidium at the noncompetitive inhibitor site in its high and low affinity states, respectively. As expected, carbamylcholine alone enhances the fraction of bound \( [^3H] \)PCP. Using previously determined values for the \( K_d \) of PCP toward the desensitized and resting receptor states, the \( K_d \) of decidium toward the site in these two states were calculated to be 1.2 and 29 μM, respectively.

In the absence of either carbamylcholine or \( \alpha \)-toxin, decidium exhibits biphasic effects on \( [^3H] \)PCP binding (Fig. 4). Low concentrations of decidium increase \( [^3H] \)PCP binding to the level observed in the presence of carbamylcholine, suggesting that decidium interaction at the agonist site converts the receptor to a state exhibiting increased \( [^3H] \)PCP binding. At higher concentrations decidium inhibits \( [^3H] \)PCP binding, indicating decidium directly competes with the PCP-binding site.

Decidium and meproadifen enhancement of carbamylcholine competition with the initial rate of \( [^{125}I] \)-labeled \( \alpha \)-toxin binding to Torpedo membranes and BC3H-1 cells. Decidium and meproadifen were added in the specified concentrations 30 min before initiating the toxin-binding reaction. Carbamylcholine was added either 10 s or 30 min before addition of \( \alpha \)-toxin. Initial rates of toxin binding were measured over 40 s and expressed as a percent of the rate observed in the absence of added effector. ○, rate of binding measured with 10 s of prior exposure to agonist; •, rate of binding with 30 min of prior exposure to agonist; △, rate of binding with exposure only to decidium or meproadifen. A, decidium, Torpedo membranes. \( ED_{50} \) for 10 s, 30 min, and no exposure to carbamylcholine were 0.10, 0.60, and 0.16 μM, respectively. The test carbamylcholine concentration was 1 μM. B, decidium, BC3H-1 cells. \( ED_{50} \) for 10 s, 30 min, and no exposure to carbamylcholine were 0.29, 0.74, and 0.47 μM, respectively. The test carbamylcholine concentration was 30 μM. C, meproadifen, Torpedo membranes. \( ED_{50} \) for 10 s, 30 min, and no exposure to carbamylcholine were 0.79, 0.63, and 1.0 μM, respectively. The test carbamylcholine concentration was 1 μM. Asterisk indicates overlapping data points.

Decidium Modulation of \( [^3H] \)PCP Binding—\( [^3H] \)PCP has been shown previously to bind selectively to a site on the receptor which is noncompetitive with and allosterically coupled to agonist binding (Albuquerque et al., 1980; Heidmann et al., 1983). Prolonged occupancy by agonists and certain antagonists converts the receptor to a desensitized state exhibiting increased agonist affinity as well as increased affinity for noncompetitive inhibitors such as PCP. Occupancy of the agonist site by \( \alpha \)-toxin prevents the conversion of the receptor to the desensitized state. Conversely, occupancy of the noncompetitive inhibitor site by PCP converts the receptor to a state exhibiting increased affinity for certain agonists (Palma et al., 1986). We, therefore, examined the influence of decidium on \( [^3H] \)PCP binding to the Torpedo AcChR in the absence and presence of excess carbamylcholine or \( \alpha \)-toxin (Fig. 4). A subsaturating concentration of \( [^3H] \)PCP was used in order to monitor both increases or decreases in the capacity of \( [^3H] \)PCP to bind to the AcChR.

FIG. 2. Antagonist inhibition of concentration-dependent carbamylcholine elicited Na\(^+\) permeability in BC3H-1 cells. \(^{22}\)Na\(^+\) permeability elicited by the specified concentrations of carbamylcholine plus antagonist was measured over a 20-s interval in sets of culture dishes. Results are expressed as percent of the maximum rate in the absence of antagonist. A, decidium: ■, no added decidium; ●, 10\(^{-7}\) M; △, 3 × 10\(^{-7}\) M; ○, 10\(^{-5}\) M; □, 3 × 10\(^{-5}\) M. B, dinaphthyldecamethonium: ■, no added dinaphthyldecamethonium; ●, 6 × 10\(^{-7}\) M; △, 3 × 10\(^{-6}\) M; ○, 10\(^{-4}\) M. C, d-tubocurarine: ■, no added d-tubocurarine; ●, 3 × 10\(^{-5}\) M; △, 10\(^{-4}\) M; ○, 6 × 10\(^{-4}\) M. D-tubocurarine, by contrast, shifts the curve to higher agonist concentrations without altering the maximal permeability response (Fig. 2C).

Decidium Modulation of Agonist Competition with \( \alpha \)-Toxin Binding—The previous data do not distinguish whether decidium converts AcChR to a state of increased agonist affinity and decreased functional responsiveness by association at the agonist site or at heterotropic, allosteric site(s). If decidium acted solely at the agonist site, then decidium enhancement of the apparent agonist affinity should occur over the same concentration range as decidium competition with \( [^{125}I] \)-labeled \( \alpha \)-toxin binding. Alternatively, if decidium converts the AcChR to a state of increased agonist affinity by an allosteric mechanism as has been demonstrated for the noncompetitive inhibitors (Sine and Taylor, 1980; Boyd and Cohen, 1980; Quast et al., 1978; Weiland et al., 1978), we should observe a separation between the concentration dependences for direct decidium competition and allosteric enhancement of agonist competition with \( \alpha \)-toxin binding. To address this question, we examined the concentration dependence for decidium augmentation of the competition of agonist with the initial rate of \( [^{125}I] \)-labeled \( \alpha \)-toxin binding, and these results were compared to the concentration dependence for decidium competition of the initial rate of \( [^{125}I] \)-labeled \( \alpha \)-toxin binding. Data on Torpedo membranes (Fig. 3A) and BC3H-1 cells (Fig. 3B) show identical decidium concentration at the agonist site or at heterotropic, allosteric site(s). If decidium converts AcChR to a state of increased agonist affinity as well as increased affinity for certain agonists (Palma et al., 1986). We, therefore, examined the influence of decidium on \( [^3H] \)PCP binding to the Torpedo AcChR in the
Spectroscopic Characterization of Decidium-AcChR Complexes—Excitation and emission fluorescence spectra of decidium complexed with the agonist and high affinity noncompetitive inhibitor sites were obtained either in the presence of excess PCP, to block the noncompetitive inhibitor site, or excess carbamylcholine, to block the agonist site. Contributions to the spectra arising from free ligand and light scatter were subtracted to yield the difference spectra. The corrected excitation maximum of decidium complexed with the agonist site appears at 490 nm, while the excitation maximum of decidium at the noncompetitive inhibitor site appears at 530 nm (Fig. 5A). The fluorescence emission maximum of decidium complexed to the agonist or the high affinity noncompetitive inhibitor sites appear between 605 and 610 nm (Fig. 5B). The apparent quantum yield of decidium increases about 2-fold upon binding to either the agonist or the noncompetitive inhibitor sites.

Fluorescence Titrations—Equilibrium binding parameters for decidium association at the agonist sites were determined by fluorescence titrations (Fig. 6). Excitation at 290 nm was used to maximize the signal from the bound fluorophore. The spectral overlap between receptor tryptophanyl emission and decidium absorbance in the 340-nm band results in substantial enhancement of the bound decidium signal relative to 480 nm of excitation. All samples contained 100 μM PCP to prevent decidium binding to the noncompetitive inhibitor site. Fig. 6B shows the specific fluorescence from decidium bound to the agonist sites, where nonspecific binding was defined using excess α-toxin. In four replicate titrations we obtained composite values of \(K_d = 0.57 \pm 0.34 \text{ μM} \) and \(n_H = 0.89 \pm 0.19\). The observation of a Hill coefficient less than unity suggested nonequivalence of decidium dissociation constants at the agonist sites. We also carried out displacement titrations of decidium by the agonist carbamylcholine, which displays a unitary Hill coefficient for binding (Weiland et al., 1977) (Fig. 7). At saturation, carbamylcholine reduces decidium fluorescence to a similar extent as excess α-toxin. Hill analysis of the data (Fig. 7B) reveal \(n_H = 0.85\). The observation of Hill coefficients less than unity for displacement of decidium further suggests some nonequivalence for the interaction of decidium at the agonist sites, in accord with the pharmacological studies. Analogous results were obtained by competition with the partial agonist decamethonium (data not shown).

Energy Transfer between FITC-toxin and Decidium—To examine the distance between the two agonist sites on the individual receptor molecules, we assessed dipolar fluorescence energy transfer between FITC-toxin and decidium bound to the agonist sites on the receptor. Doubly liganded receptors were prepared to achieve the fractional occupation described under “Experimental Procedures.” Saturating concentrations of PCP were added to prevent decidium binding to the noncompetitive inhibitor site. The spectral overlap integral between FITC emission and decidium excitation, \(J\), was calculated to be \(2.49 \times 10^{-14} \text{ cm}^2 \cdot \text{M}^{-1} \) (cf. Fig. 4 and data not shown). Assuming a refractive index of 1.4 and an orientation factor \((α^2)\) of 0.67, \(R_0\) was calculated to be 30Å.

Various factors render quantitation of steady-state energy transfer between FITC-toxin and decidium bound to the AcChR difficult. These factors include the significant overlap of decidium and FITC emission spectra and the innerfilter effects of decidium at the FITC absorption maxima. Since fluorescence lifetimes are unaffected by innerfilter effects and since an appropriate combination of interference filters could be chosen which reduces the contribution of decidium fluorescence to less than 5% of the FITC fluorescence signal, we have chosen to determine energy transfer as the extent of reduction of donor FITC fluorescence lifetime (Equation 4) in the presence of the acceptor decidium. The results of studies on membrane-associated and detergent-solubilized receptor preparations are presented in Fig. 8 and Table II. In the absence of added decidium and with 15% occupancy by FITC-toxin, the exponential decay of FITC-toxin fluorescence was satisfactorily described by a single fluorescence lifetime \((τ = 3.9 \text{ ns})\). When decidium was added to saturate the remaining agonist sites, a 16% decrease was observed in the fluorescence lifetime of FITC-toxin bound to the membrane-associated AcChR (Fig. 8A). This quenching was not altered by the further addition of native α-toxin (Fig. 8B) and was abolished by solubilizing the receptor with cholate (Fig. 8, C and D). Taken together, the reduction in FITC-toxin fluorescence

**Fig. 5.** A, normalized and corrected difference excitation spectrum of decidium bound to the enriched AcChR membranes. Dashed line represents the difference spectrum for decidium bound to the agonist sites obtained from samples (AcChR, 0.25 μM in α-toxin sites; decidium, 1.0 μM; PCP, 100 μM) in the absence and presence of carbamylcholine (500 μM). Solid line represents difference spectrum for decidium bound to the noncompetitive inhibitor site obtained from samples (AcChR, 1.0 μM in α-toxin sites; decidium, 2.0 μM; carbamylcholine, 500 μM) in the absence and presence of PCP (100 μM). Dotted line represents the corrected emission spectrum of decidium in buffer. B, normalized uncorrected difference emission spectrum of decidium bound to the membrane-enriched AcChR. Dashed line represents the difference spectrum for decidium bound to the agonist sites obtained from samples (AcChR, 0.25 μM in toxin sites; decidium, 1.0 μM; PCP, 100 μM) in the absence and presence of carbamylcholine (500 μM). Solid line represents difference spectrum for decidium bound to the noncompetitive inhibitor site obtained from samples (AcChR, 1.0 μM in toxin sites; decidium, 2.0 μM; carbamylcholine, 500 μM) in the absence and presence of PCP (100 μM). Dotted line represents the uncorrected excitation spectrum of decidium in buffer.
least three types of states: resting (R), activated (R*), and desensitized (R') (Scheme 1). Additional transient desensitized states have been shown to exist but after equilibrium exposure a single desensitized state is presumed to predominate.

**DISCUSSION**

The dynamics of the nicotinic AcChR function have been extensively studied from several perspectives (Karlin, 1980; Adams, 1981; Taylor et al., 1983; Changeux et al., 1984). Within the timescale in which our studies are conducted, the AcChR can be thought to exist in equilibrium between at least three types of states: resting (R), activated (R*), and desensitized (R') (Scheme I). Additional transient desensitized states have been shown to exist but after equilibrium exposure a single desensitized state is presumed to predominate.

Changes in the population distribution of receptor molecules between these states govern the functional and binding properties of the receptor. In the absence of agonist (L) or nonselective binding with the receptor molecule but reflects energy transfer between FITC-toxin bound to the agonist site and decidium partitioned nonspecifically into the lipid bilayer. If we assume a detection limit of 5% transfer between agonist sites, then Equation 2 predicts a minimum separation between fluorophores at the agonist sites of 1.63 × R0, or 49 Å. Hence, the greater than 49 Å separation between the agonist sites is too distant to produce the reduction in lifetime of FITC-labeled toxin in the presence of decidium. The minimal but measurable excitation transfer efficiency indicates that at high concentrations decidium can partition into the membrane.

**FIG. 6.** A, titration of membrane-enriched AcChR with decidium. Incremental quantities of decidium were added to 2.0 ml of membrane suspension of AcChR (0.2 μM in α-toxin sites) and 100 μM PCP. Fluorescence was measured at 620 nm with excitation at 290 and corrections made for dilution and inner filter effects. □—□, membranes in the absence of α-toxin; Δ—Δ, membranes in the presence of 2 μM α-toxin; ○—○, buffer only. B, plot of specific enhancement of fluorescence as percentage of receptor bound versus the concentration of free ligand. The inset represents a Hill plot of this data.

**FIG. 7.** Displacement titration of the decidium-AcChR complex by carbamylcholine. Panel A, decidium (2.5 μM) and PCP (100 μM) were equilibrated with receptor-enriched membranes (0.25 μM in α-toxin sites). Incremental quantities of carbamylcholine were then added. Data were corrected for nonspecific fluorescence determined in a companion titration in the presence of excess α-toxin. Panel B, plot of the data shown in panel A according to Equation 1. f, fB, and fC represent the observed fluorescence, fluorescence with decidium alone, and fluorescence in presence of excess competing ligand. [D] and [C] are the estimated free concentrations of decidium and competing ligand, respectively.

Agents which perturb receptor functional responses may do so by competitive or noncompetitive mechanisms. Classical antagonists inhibit agonist-stimulated cation fluxes by direct competition at the agonist-binding sites (Neubig and Cohen, 1979), exhibit little or no preference for the R'R' relative to RR states and do not effect transitions to the active state (Sine and Taylor, 1981). A large and heterogeneous class of inhibitors noncompetitively inhibit channel opening by agonists. Most, but not all, of these noncompetitive inhibitors enhance the conversion of receptor to a desensitized state. Typical of such agents are the local anesthetics, general anesthetics, PCP, and certain toxins such as histrionotoxin. Certain antagonists competitively inhibit agonist activation and in addition convert the receptor to a desensitized state.
Fig. 8. Decidium quenching the fluorescence lifetime of FITC-toxin bound to the AcChR. Nanosecond decay curves of FITC-toxin (0.6 μM) were recorded in the presence of AcChR (4 μM in α-toxin complex), decidium (10 μM), and PCP (100 μM). The timing calibration was 0.43 ns/channel. Dashed lines represent the lamp pulse. Deviations of the experimental data points from a single exponential theoretical function (solid lines) are shown in the upper curves. AcChR is membrane-associated in panels A and B and solubilized with 4% cholate in panels C and D. In panels B and D native α-toxin (10 μM) was incubated with the AcChR 30 min after the addition of FITC-toxin to inhibit decidium binding to the agonist sites. Decidium was added to the sample cuvettes just before initiating the 1-h data accumulation period. The data accumulation interval was limited to 1 h in order to minimize the displacement of FITC-toxin from the agonist sites by decidium. The apparent decay curves generated from samples that did not contain FITC-toxin were subtracted from the AcChR samples to correct for light scatter.

TABLE II

|                     | FITC-toxin + AcChR | FITC-toxin + decidium + AcChR | FITC-toxin + α-toxin + decidium + AcChR |
|---------------------|--------------------|-------------------------------|----------------------------------------|
| Lifetimes in ns ± S.D. | 3.9 ± 0.1 (2)     | 3.8 ± 0.1 (2)                 | 3.8 ± 0.1 (3)                          |

Classical pharmacologic studies distinguish these agents as metabolic antagonists (Rang and Ritter, 1970a, 1970b). Metaphilic antagonists exhibit higher affinity for the desensitized state of the receptor and therefore show a greater capacity to block the permeability response after exposure to carbacholino. The actions of decidium are consistent with it exhibiting metaphilic properties. Decidium exhibits mixed competitive and noncompetitive inhibition of agonist-stimulated $^{22}$Na$^+$ influx (Fig. 2). Equilibrium exposure of the receptor to decidium converts the receptor to a state exhibiting increased decidium binding affinity (Fig. 1 and Table I). Decidium binding to the agonist site enhances $[^3H]$PCP binding to an allosterically coupled noncompetitive site (Fig. 4). Decidium by itself does not stimulate $^{22}$Na$^+$ influx in BC3H-1 cells, so it is not a partial agonist. Hence, these results indicate that upon occupation of the agonist sites decidium converts the AcChR to a functionally blocked state toward which agonists and noncompetitive inhibitors display a higher affinity. In addition to its functional antagonism and ability to convert the receptor to a high affinity state by occupying the agonist sites, decidium at higher concentrations binds directly to the PCP-binding site (Fig. 4).

Fluorescence characterization of decidium bound to the two classes of binding sites indicates significant shifts in both the excitation and emission spectra, which are consistent with an environment of greater hydrophobicity upon binding to the AcChR. A red-shift in the excitation spectrum of far greater magnitude is associated with decidium binding to the noncompetitive site (~50 nm) than to the agonist sites (~10 nm). This suggests that the noncompetitive site for PCP is more hydrophobic than the agonist-binding sites, i.e. in a domain less susceptible to solvent proton transfer from the excited singlet state (Olmstead and Kearns, 1977).

In a previous study of the interaction of ethidium with the AcChR, we observed that this phenylphenanthridium ligand binds selectively to the noncompetitive inhibitor site for PCP, particularly when the receptor is in a desensitized state ($K_d$ ~ 0.4 μM) (Herz et al., 1987). Like decidium, ethidium displays a large red-shift in the excitation (~47 nm) and a blue-shift in the emission (~35 nm) maxima upon binding to the noncompetitive inhibitor site for PCP. These results also show that a portion of the binding domain of the noncompetitive site is particularly hydrophobic. Furthermore, addition of a second quaternary group separated by 10 methylene groups dramatically changes the relative binding selectivity of these phenylphenanthridium congeners for the two respective sites.

Fluorescence titrations confirm that functional antagonism by low concentrations of decidium occurs via occupation of the agonist site and that decidium binds nonequivalently to the two agonist sites on the receptor oligomer, although the degree of nonequivalence is small relative to antagonists such as pancuronium or dimethyl-d-tubocurarine. Studies on reversible (Weiland et al., 1976; Sine and Taylor, 1981; Neubig and Cohen, 1979) and site-directed irreversible antagonists (Damele and Karlin, 1978) also show nonequivalence of the sites. Despite the identity in the sequence of the α subunits (Noda et al., 1982) differential post-translational glycosylation has been reported (Ratnam et al., 1986). Thus, either the
differential glycosylation or the lack of identity of subunits neighboring the α-subunit could be responsible for the nonequivalence in binding sites.

To measure distances between the two agonist sites on the receptor, we estimated the efficiency of excitation energy transfer between a slowly reversible α-toxin and a highly reversible ligand, decidium. We observed no energy transfer between the FITC-toxin and the decidium when each is bound to one of the two agonist sites on doubly liganded receptors. Previous studies using a hybrid receptor-fluorescent-α-toxin complex, where FITC-toxin is on one of the subunits and tetramethylrhodamine-toxin occupies the other subunit, exhibit intersubunit and interreceptor transfer in the membrane-associated receptor and intersubunit transfer in the solubilized receptor (Johnson et al., 1984). Intersubunit excitation transfer measurements afforded a distance of 67 Å (range 55–84 Å) between the fluorophores conjugated to lysine 23 of the α-toxin, consistent with location of these lysine 23-conjugated fluorophores at the outer perimeter of the 85–90 Å diameter receptor molecule. However, interpretation of these data was limited by uncertainty in the location of the fluorophore relative to the orientation of bound α-toxin molecule. Although the α-carbon of lysine 23 is no more than 19 Å away from any other α-carbon in the α-toxin, the α-toxin molecule is an asymmetric, peptide (M_r = 7820) with dimensions of 20 × 30 × 40 Å (Walkinshaw et al., 1980). The absence of energy transfer between lysine 23-FITC-toxin and decidium in the present study confirms our previous estimate of the intersite transfer between lysine 23 on the two α-toxin molecules. With a critical transfer distance of 30 Å for fluorescein to decadium, 5% transfer of energy will occur at 49 Å. This value is significantly less than the 67 Å measured between fluorophores for the two fluorescent α-toxins. If the agonist sites where decidium binds are located on the extracellular surface near the pseudo-axis of symmetry, then energy transfer should have been observed.

The absence of detectable energy transfer confirms our previous suggestion that the agonist sites reside at a distance from rather than near the pseudo-axis of symmetry of the receptor. Our findings are consistent with previous suggestions that the central loop of the α-toxin, which contains lysine 23, overrides the agonist site. Thus, energy transfer studies with decidium have allowed us to place further constraints on the location of the agonist sites when ligands are associated with the receptor.

While no site-specific energy transfer was detected between the decidium and FITC-toxin specifically bound to the agonist sites, minimal dipolar transfer efficiency (16%) can be detected between FITC-toxin and decidium associated non-specifically with the lipid or the receptor molecule. Decidium has sufficient hydrophobicity to partition into the lipid bilayer. Since we also found no energy transfer with the solubilized receptor, the membrane itself is the likely site of non-specific decidium association. Due to uncertainty in the donor-acceptor stochiometry, precise estimates of distance between the agonist site and the lipid bilayer cannot be obtained. Nevertheless, our results suggest proximity of the agonist-binding sites to the lipid bilayer, which is consistent with the recent finding the Cy5-192 and Cy5-193 is proximal to both the acetylcholine-binding site and the beginning of the proposed M1 membrane spanning hydrophobic region (Ile-210) of the α-subunit (Kao et al., 1984).

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