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Mechanism of Binding of a Benzomorphan Opiate to the Acetylcholine Receptor from *Torpedo* Electroplaque

ROBERT E. OSWALD, LEE MICHEL, and JUDITH BIGELOW

New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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SUMMARY

The mechanism of binding of the benzomorphan opiate, (−)-*N*-allylnormetazocine [(−)-ANMC], to *Torpedo* acetylcholine receptor (AcChR)-rich membranes was investigated. Using a centrifugation assay, two equilibrium binding affinities were observed with K_D values of 0.4 and 2 μM. The K_D and the apparent B_{max} of the higher affinity component were decreased by cholinergic agonists and antagonists but not by α-bungarotoxin alone. The high affinity binding site (K_D = 0.4 μM) was found to be distinct from the binding site for tetracaine, a noncompetitive blocker. The apparent association rate constant was essentially independent of receptor concentration both in the presence and absence of the cholinergic agonist, carbamoylcholine. When carbamoylcholine was equilibrated with the AcChR prior to (−)-[^{3}H]ANMC addition, the association rate constant was 2- to 3-fold greater than in the absence of cholinergic effectors. When carbamoylcholine and (−)-[^{3}H]ANMC were added simultaneously to AcChR-rich membranes, association was too rapid to resolve manually and binding measured at 5 sec was greater than the equilibrium level both in the presence and absence of carbamoylcholine. Binding decreased as a function of time, reaching its equilibrium level with a time constant of approximately 1 min. This effect appeared to be agonist specific since it was not observed when the antagonist, *d*-tubocurarine, replaced carbamoylcholine. In the absence of cholinergic ligands, dissociation of (−)-ANMC was biphasic ($t_{½}$ values of less than 5 sec and approximately 2.5 min) and, in the presence of cholinergic ligands, was monophasic ($t_{½}$ of 40 sec). The simultaneous addition of carbamoylcholine and (−)-[^{3}H]ANMC to the membranes initially results in a biphasic dissociation ($t_{½}$ of 5 and 30 sec) which becomes monophasic with increasing times of incubation. A mechanism is proposed involving an isomerization of the receptor-ligand complex which agrees quantitatively and qualitatively with the data.

The nicotinic AcChR from *Torpedo* electric organ mediates ion flux across the postsynaptic electrocyte membrane in response to the binding of AcCh. It is a multisubunit complex consisting of four different types of glycosylated subunits with protein molecular weights (1–3) of 50, 116 (α), 53, 681 (β), 56, 279 (γ), and 57, 565 (δ), existing in a stoichiometry of $\alpha_2\beta\gamma\delta$ (4–6). Reconstitution experiments (reviewed in Ref. 7) and insertion of specific mRNA into oocytes (8) have demonstrated that these four types of subunits are necessary and sufficient for binding AcCh and mediating ion flux.

Various pharmacological agents interact with the AcChR of skeletal muscle and electroplaque. These include: (a) the agonists (e.g., AcCh, carbamoylcholine, nicotine), which open the ion channels; (b) the competitive antagonists (e.g., *d*-tubocurarine, gallamine), which probably bind to the agonist site and prevent the binding of AcCh; (c) the snake α-neurotoxins (e.g., α-Bgt), which block the effect of AcCh by binding to a site that may overlap the AcCh site; (d) the noncompetitive blockers (e.g., histrionicotoxin, PCP, local anesthetics), which bind to sites distinct from the AcCh site but which block the effect of AcCh; and (e) the benzomorphan opiates (e.g., *N*-allylnormetazocine, pentazocine), which seem to bind to a distinct site but which have unknown effects on the AcChR (9).

The noncompetitive blockers have been studied extensively by direct ligand binding (10–14) and affinity labeling (15–19) studies and by measuring the effects of noncompetitive blockers on agonist binding (12), ion flux (20, 21) and electrophysiological responses (22–24). The general conclusion from these studies is that noncompetitive blockers inhibit ion flux through the channel without interfering with the binding of AcCh and bind to one high affinity site as well as a variable number of low affinity sites that are capable of inducing cooperative effects on the conformational transitions of the AcChR (12, 21). In most cases, the affinity of the noncompetitive blocker is increased by the presence of cholinergic agonists and antagonists. An exception to this is the local anesthetic, tetracaine, which binds with much higher affinity in the absence (rather than in the presence) of agonist (25, 26).

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ABBREVIATIONS: AcCh, acetylcholine; AcChR, acetylcholine receptor; (−)-ANMC, (−)-*N*-allylnormetazocine; α-Bgt, α-bungarotoxin; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetracetic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PCP, phencyclidine.

The interaction of benzomorphans with the AcChR was first observed as an inhibition of the binding of [^{3}H]perhydrohistrionicotoxin and [^{3}H]PCP (27, 28). A stereoselective, high affinity binding site for an optically pure benzomorphan, (−)-[^{3}H]ANMC, was subsequently reported (9). (−)-[^{3}H]ANMC was found to bind to the AcChR with higher affinity in the absence than in the presence of cholinergic ligands. This property was distinct since most noncompetitive blockers bind with higher affinity in the presence of cholinergic effectors than in their absence. An additional property which makes (−)-[^{3}H]ANMC a useful probe for the study of allosteric binding sites on the AcChR is that it can be crosslinked quantitatively to the AcChR with UV irradiation (60–70% yield). Using this technique, the binding site for (−)-[^{3}H]ANMC has been localized to the δ subunit of the AcChR (9). The studies described here characterize further the interaction of (−)-[^{3}H]ANMC with the *Torpedo nobiliana* electroplaque AcChR. The binding mechanism was found to be complex, and a model is proposed that accounts for the data currently available.

Materials and Methods

Preparation of membranes. AcChR-rich membranes were prepared from frozen electroplaque from *Torpedo nobiliana* as described previously (29). The membranes were stored until use in liquid nitrogen at 5–10 μM (expressed in α-[^{125}I]Bgt binding sites). Preparations ranged from 1 to 2 nmol of α-[^{125}I]Bgt sites/mg of protein.

Binding assays. The binding of (−)-[^{3}H]ANMC and [^{3}H]PCP was measured either with a filtration assay (30) or a centrifugation assay (12, 14). (−)-[^{3}H]ANMC was diluted with nonradioactive (−)-[^{3}H]ANMC in some cases to conserve radioligand. Nonspecific binding, measured in the presence of a 200-fold excess of nonradioactive (−)-ANMC or PCP, ranged between 5 and 20% of the total binding for the filtration assay and between 10 and 40% of the total binding for the centrifugation assay (depending upon the concentrations of (−)-[^{3}H]ANMC and AcChR). Titration of constant concentrations of (−)-[^{3}H]ANMC (100 nM) and AcChR (400 nM) with increasing concentrations of nonradioactive (−)-ANMC demonstrated that an excess of nonradioactive ligand, between 200- and 5000-fold, displaced all specifically bound radioactivity without artifactually displacing a "nonspecific" component. The buffer used in all experiments was 50 mM MOPS-NaOH (pH 7.5)/1 mM EGTA. MOPS-NaOH was chosen because of its insensitivity to changes in temperature ($\Delta pK_a/°C = -0.006$). EGTA was included because free calcium ions activate a calcium-dependent protease in these membrane fragments (29) and are capable of modifying the conformation of the AcChR (31).

The concentration of α-[^{125}I]Bgt sites was measured with the DE81 filter disc assay (32) using 1% (w/v) Triton X-100 in 20 mM Tris-HCl (pH 7.5) as the buffer. Alternatively, the intial rate of α-[^{125}I]Bgt binding to AcChR-rich membranes was measured by filtration through Millipore EGWP filters (presoaked in 1 mg/ml of bovine serum albumin and 1 μM nonradioactive α-Bgt) (33).

Photoaffinity labeling. (−)[^{3}H]ANMC was cross-linked to the AcChR using UV irradiation as described previously (9, 18), except that the time of irradiation was 15 sec. Immediately after irradiation, sodium dodecyl sulfate was added to the sample to a final concentration of 2%, and 40 μl were loaded on sodium dodecyl sulfate/10% acrylamide/0.26% bis(acrylamide) gels (29). Gels were prepared for fluorography using Autofluor (National Diagnostics), dried, and exposed to Kodak XAR-5 film (preflashed to an absorbance of 0.2 nm) at −80° for 4–7 days.

Data analysis. Equilibrium dissociation constants were calculated from nonlinear least squares fitting routines using a Marquardt-Levenberg algorithm modified from Bevington (34). As described previously (35), the data were fit to both a one-site and a two-site model and the improvement of the fit was analyzed by an F-ratio test. In filtration assays, the concentration of free radioligand was calculated as described previously (35). Pseudo-first order association rate constants (k_{obs}) and dissociation constants (k_{-1}) were determined in an analogous manner.

Microscopic rate constants were determined by numerical integration of the appropriate differential equations using the Runge-Kutta fourth order technique. All calculations were performed using FORTRAN-77 programs developed in the laboratory for use on a MicroVAX I computer.

Materials. ANMC, metazocine, pentazocine, and (−)-[^{3}H]ANMC (49.5 Ci/mmol) were supplied by the National Institute on Drug Abuse. The other opiate derivatives were gifts of A. E. Jacobson (National Institutes of Health) and Prof. A. Gero (Hanemann University). PCP hydrochloride was obtained from the United States Pharmacopeial Convention, Inc. [^{3}H]PCP (48 Ci/mmol) and α-[^{125}I]Bgt (70–140 Ci/mmol) were obtained from New England Nuclear Corp. The radiochemical purity of [^{3}H]PCP and (−)-[^{3}H]ANMC was checked by thin layer chromatography on silica gel [1-butanol/acetic acid/water (25:4:10)]. Frozen *T. nobiliana* electroplaque was purchased from Biofish Associates.

Results

Stoichiometry and the effects of carbamoylcholine. As described previously (9), (−)-[^{3}H]ANMC binds to two sites in AcChR-rich membranes: a high affinity site (K_D of 0.4 μM) and a low affinity site (K_D of 2 μM). The results (9) suggested that the two affinities observed constituted two distinct sites on the AcChR, existing in one copy per AcChR monomer ($\alpha_2\beta\gamma\delta$). Using the filtration assay (see Materials and Methods), only one component ($K_D \sim 0.5$ μM) is observed (Fig. 1C), presumably due to a rapid dissociation from the low affinity site. The B_{max} in the absence of carbamoylcholine is approximately 70% of the total concentration of AcChR monomer (one-half the number of α-[^{125}I]Bgt sites). This may be an underestimate of the total number of sites due to the dissociation of ligand during filtration and the nature of the binding mechanism (see Discussion).

The apparent number of binding sites is decreased dramatically by equilibration in the presence of carbamoylcholine. As shown in Fig. 1A, equilibration of AcChR-rich membranes with carbamoylcholine prior to the addition of 50 nM (−)-[^{3}H]ANMC (prior addition) results in a relatively slow rate of binding which equilibrates at a level lower than that in the absence of carbamoylcholine. In contrast, if carbamoylcholine and (−)-[^{3}H]ANMC are added simultaneously (Fig. 1A, *simultaneous addition*), the association rate cannot be resolved with a manual binding assay. Also, the initial level of binding at the earliest time point (5 sec) is greater than the equilibrium level observed both in the presence and absence of carbamoylcholine. The measured binding then relaxes to the equilibrium level with a $t_{1/2}$ of 45–60 sec. The competitive antagonist, *d*-tubocurarine, differs in that the initial stimulation of binding is not observed and prior addition produces the same results as simultaneous addition (Fig. 1B).

Photoaffinity labeling of the δ-subunit with (−)-[^{3}H]ANMC (Fig. 1D) had the same characteristics of reversible binding. If irradiation began simultaneously with the addition of carbamoylcholine and (−)-[^{3}H]ANMC to the membranes (Fig. 1D, lane *C*), the labeling was greater than if the irradiation was initiated following equilibration (Fig. 1D, lane *E*). An intermediate level of labeling was observed in the absence of carbamoylcholine (Fig. 1D, lanes *B* and *D*).

By measuring binding at various concentrations of (−)-[^{3}H]

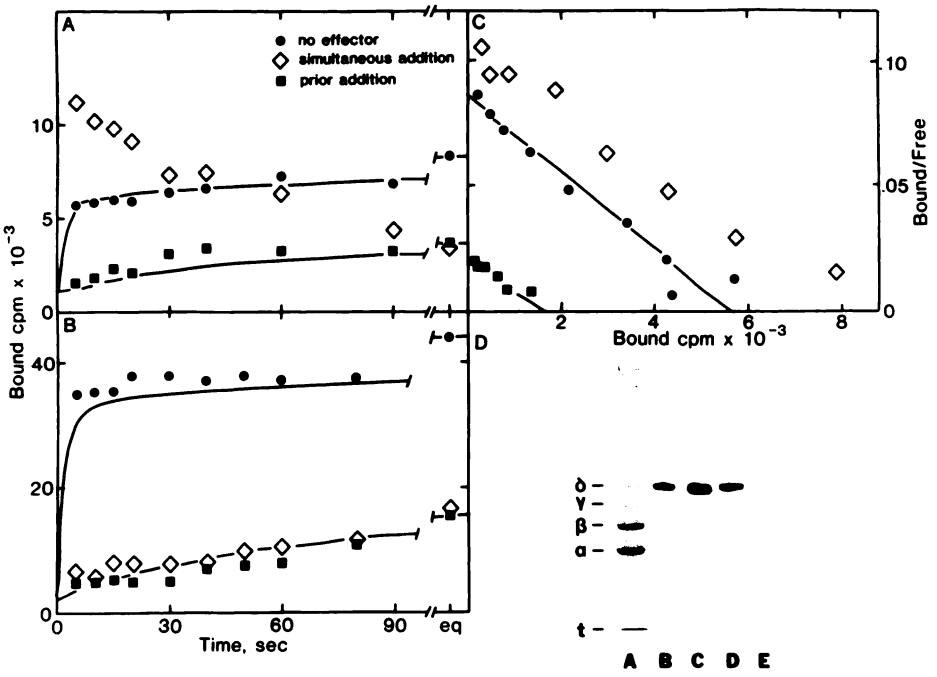


Fig. 1. Kinetics of (−)-[^{3}H]ANMC (50 μM) binding to AcChR-rich membranes (0.6 μM in α-[^{125}I]Bgt binding sites) in the presence (■, ◇) or absence (●) of 0.2 mM carbamoylcholine (A) or *d*-tubocurarine (B). A filtration assay was used to measure binding (see Materials and Methods). Carbamoylcholine or *d*-tubocurarine was added either simultaneously with (−)-[^{3}H]ANMC (◇) or 15 min before the addition of (−)-[^{3}H]ANMC (■). C, Equilibrium binding of (−)-[^{3}H]ANMC to AcChR-rich membranes measured with a filtration assay in the presence (■, ◇) or absence (●) of 0.2 mM carbamoylcholine. AcChR concentration was 0.4 μM, expressed in α-[^{125}I]Bgt binding sites. When carbamoylcholine was used, it was included in the (−)-[^{3}H]ANMC solution and was added simultaneously with (−)-[^{3}H]ANMC to the AcChR-rich membranes. An aliquot was then assayed at 10 sec (◇) and another at 45 min (■). The incubation time in the absence of carbamoylcholine was 45 min. In all cases (A–C), the *solid lines* were drawn by computer modeling using the rate constants given in the text. The conversion factor from radioactivity to concentration was 4 × 10^5 cpm/μM in A, 2.4 × 10^6 cpm/μM in B, and 4.2 × 10^4 cpm/μM in C. D, A 10% sodium dodecyl sulfate gel demonstrating photoaffinity labeling of AcChR-rich membranes (0.5 μM, expressed in α-[^{125}I]Bgt binding sites) by UV irradiation in the presence of (−)-[^{3}H]ANMC (0.1 μM). Shown are: lane *A*, a Coomassie blue stain of the protein; lane *B*, a fluorogram of labeling in which irradiation was begun simultaneously with the addition of (−)-[^{3}H]ANMC; lane *C*, a fluorogram of labeling in which irradiation was begun simultaneously with the addition of (−)-[^{3}H]ANMC and 0.2 mM carbamoylcholine; lane *D*, a fluorogram of labeling in the absence of carbamoylcholine (irradiation begun following equilibration of reactants); and lane *E*, a fluorogram of labeling in the presence of carbamoylcholine (irradiation begun following equilibration of reactants).

ANMC 10 sec after the simultaneous addition of carbamoylcholine and (−)-[^{3}H]ANMC, a "Scatchard" plot was generated (Fig. 1C). The B_{max} of this plot is approximately 90% of the total number of AcChR present. Extrapolating back to time zero, assuming a relaxation half-time of 60 sec, the B_{max} is within 5% of the total number of AcChRs, suggesting one binding site per AcChR monomer, which is in agreement with the results obtained previously with the centrifugation assay (9). The effect of carbamoylcholine on (−)-[^{3}H]ANMC binding was previously reported to be relatively insensitive to α-Bgt (9). As shown in Fig. 2, α-Bgt (2 μM—90% of the sites are occupied with α-Bgt) is capable of decreasing the IC_{50} for carbamoylcholine ("prior addition") but not totally inhibiting the effect. The apparent differences in the results reported previously (9) and those of Fig. 2 seem to be due to dissociation of the ligand during filtration (Fig. 2).

As shown in Fig. 3A, decamethonium and *d*-tubocurarine are also capable of inhibiting the binding of (−)-[^{3}H]ANMC. In all three cases, the Hill coefficient for the inhibition of (−)-[^{3}H]ANMC binding was approximately 0.5, indicating a complex mechanism of inhibition. Decamethonium, *d*-tubocurarine, and carbamoylcholine, under identical conditions, increased the binding of [^{3}H]PCP (a decrease in K_D with no effect on B_{max})[1] with EC_{50} values approximately 10-fold lower than those for the inhibition of (−)-[^{3}H]ANMC binding (Fig. 3B). Only decamethonium significantly decreased the binding of [^{3}H]PCP at higher concentrations. In this case, the IC_{50} was approximately the same as that for the inhibition of (−)-[^{3}H]ANMC binding (7 μM), but the Hill coefficient was approximately 1.0, indicating a simple competitive inhibition. A slight inhibition of [^{3}H]PCP binding is observed at high concentrations of *d*-tubocararine, as is consistent with the results of others (36).

Relationship between the site for benzomorphans and the site for noncompetitive blockers. The inhibition by PCP of (−)-[^{3}H]ANMC binding to its high affinity site has been shown to be noncompetitive (9), suggesting that the two compounds bind to distinct sites on the AcChR. Two additional compounds have been studied in detail, tetracaine and (−)-pentazocine. The local anesthetic, tetracaine, was chosen because previous studies (25, 26) have demonstrated that, like (−)-ANMC, tetracaine binds with higher affinity in the absence than in the presence of carbamoylcholine, raising the possibility that it may bind to the same site as the benzomorphans.

[1] R. E. Oswald, unpublished results.

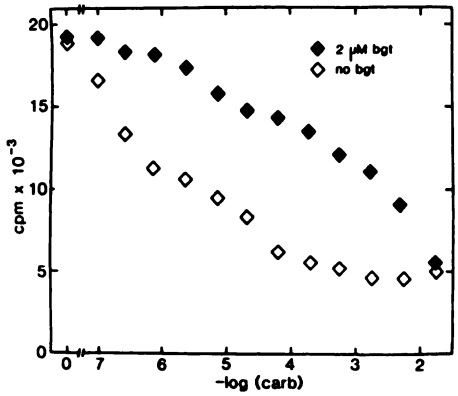


Fig. 2. Effect of α-Bgt (2 μM) on the concentration dependence of carbamoylcholine on the binding of (−)-[^{3}H]ANMC (50 nM) to AcChR-rich membranes (0.6 μM in α-[^{125}I]Bgt sites). α-Bgt was added 30 min before the addition of carbamoylcholine. (−)-[^{3}H]ANMC was added 15 min after the addition of carbamoylcholine. The conversion of radioactivity to concentration was 3.3×10^6 cpm/μM. An aliquot of the carbamoylcholine-α-Bgt-membrane or carbamoylcholine-membrane mixture was removed prior to (−)-[^{3}H]ANMC and dialyzed overnight in dialysis membranes with a 14,000 molecular weight cutoff. The membranes were then analyzed for α-[^{125}I]Bgt binding. When present, nonradioactive α-Bgt occupied approximately 90% of the binding sites for α-[^{125}I]Bgt.

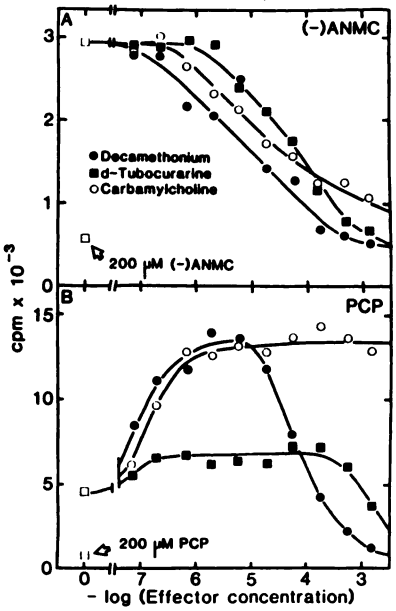


Fig. 3. The effect of carbamoylcholine, decamethonium, and *d*-tubocurarine on the equilibrium binding of (A) (−)-[^{3}H]ANMC (50 nM) and (B) [^{3}H]PCP (50 nM) on AcChR-rich membranes (0.3 μM in α-[^{125}I]Bgt binding sites). A filtration assay was used and the cholinergic effectors were added to the membrane solution 15 min before the addition of the radioactive ligand. Incubation times with the radioactive ligand were 45 min. The conversion of radioactivity to concentration was 3.3×10^5 cpm/μM for (−)-[^{3}H]ANMC and 1.3×10^6 cpm/μM for [^{3}H]PCP.

(−)-Pentazocine was chosen as the control to demonstrate a competitive inhibition of (−)-[^{3}H]ANMC binding by a related, but structurally distinct, benzomorphan opiate. As shown in Fig. 4A, tetracaine inhibits the binding of (−)-[^{3}H]ANMC to its low affinity site. The IC_{50} for this inhibition is 24.4 ± 4.5 μM. Whether tetracaine affects the high affinity site is not clear from these data, except at very high concentrations (e.g., 200 μM); however, the experiments using the filtration assay suggest

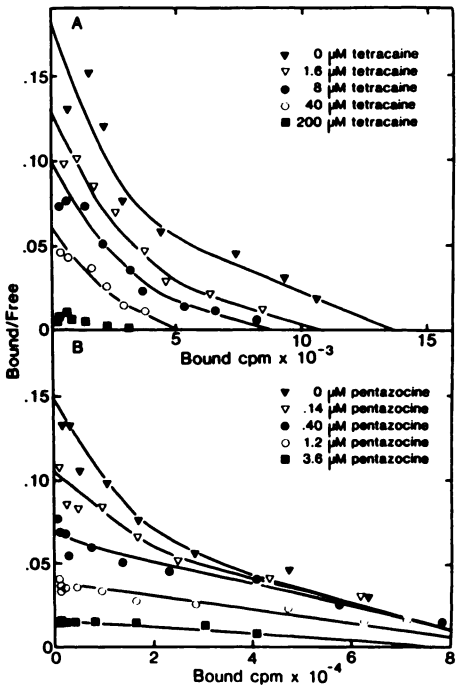


Fig. 4. The effect of tetracaine (A) and (−)-pentazocine (B) on the equilibrium binding of (−)-[^{3}H]ANMC to AcChR-rich membranes (0.4 μM in α-[^{125}I]Bgt sites). A centrifugation assay was used and the incubation times were 45 min. The conversion of radioactivity to concentration was 3.3×10^4 cpm/μM in (A) and 2.0×10^5 in B.

little or no effect on the high affinity site.[2] In contrast, (−)-pentazocine (Fig. 4B) decreases the apparent K_D for (−)-[^{3}H]ANMC binding to its high affinity site, with no change in the B_{max}. The IC_{50} for this effect is 0.97 ± 0.13 μM. These results suggest that tetracaine, like PCP (9), binds to a site distinct from the high affinity site for benzomorphans and that (−)-pentazocine binds to the high affinity benzomorphan site.

Association kinetics of (−)-[^{3}H]ANMC binding. As shown in Fig. 1, the association rate of (−)-[^{3}H]ANMC binding seems to vary with the conformational state of the AcChR. Simultaneous addition of (−)-[^{3}H]ANMC and agonist (carbamoylcholine) leads to an association rate too rapid to measure by manual binding techniques (Fig. 1A), whereas simultaneous addition of (−)-[^{3}H]ANMC and antagonist (*d*-tubocurarine) leads to a rate identical to that observed with prior addition (Fig. 1B). For this reason, association rates were studied in detail only in the absence of carbamoylcholine or following prior addition of carbamoylcholine. In the absence of carbamoylcholine, association was biphasic with a rapid component that was difficult to resolve (see Fig. 1A) and a slower component which could be measured. In the presence of carbamoylcholine, a rapid component of association was not consistently detected. In all cases, binding was measured under pseudo-first order conditions, i.e., the binding measured at equilibrium was less than 10% of the total receptor concentration (37). Because of the difficulty in obtaining accurate results at high ligand and low receptor concentrations, detailed investigations were limited to varying the receptor concentration. As shown in Fig. 5, the pseudo-first order rate constant was 2- to 3-fold greater in the presence (0.005 sec^{-1}) than in the absence of carbamoyl-

[2] R. Oswald and L. Michel, unpublished data.

choline (0.016 sec^{-1}), and the rate constant had little or no dependence on the concentration of AcChR. The findings that the association is biphasic and that the slower rate is essentially independent of AcChR concentration suggest that the mechanism of binding is more complex than a simple biomolecular interaction. (A bimolecular mechanism would predict a monophasic association and a linear increase in the apparent association rate constant as a function of receptor concentration.)

Dissociation kinetics of (−)-[^{3}H]ANMC binding. The dissociation of (−)-[^{3}H]ANMC-AcChR complexes, like the association, is complex and presumably dependent upon the conformational state of the AcChR. In the absence of carbamoylcholine, the characteristics of the dissociation are dependent upon the time of association. As shown in Fig. 6A, if dissociation is initiated by the addition of a 200-fold excess of nonradioactive (−)-ANMC 30 sec after the addition of (−)-[^{3}H]ANMC to AcChR-rich membranes, dissociation is biphasic and dominated by a component with a $t_{½}$ of less than 5 sec. With increasing time after the addition of (−)-[^{3}H]ANMC, the slowly dissociating component becomes a greater percentage of the total binding. At equilibrium, the dissociation is biphasic, with approximately 50% of the complex dissociating with a $t_{½}$ less than 5 sec and 50% dissociating with a $t_{½}$ of approximately 2.5 min.

In the presence of carbamoylcholine under "prior addition" conditions or following equilibration under "simultaneous addition" conditions, the dissociation is monophasic with a $t_{½}$ of dissociation of approximately 40 sec (Fig. 6B). When dissociation is initiated at short intervals following the simultaneous addition of (−)-[^{3}H]ANMC and carbamoylcholine, the dissociation is initially more rapid and is biphasic. The faster rate has a $t_{½}$ of dissociation of less than 5 sec and the slower rate $t_{½}$ is approximately 30 sec.

Structure-affinity relationships. A number of benzomorphan analogs were tested for their ability to inhibit the binding of (−)-[^{3}H]ANMC, [^{3}H]PCP, and α-[^{125}I]Bgt. The compounds shown in Table 1 are organized in six groups. Within the first five groups, all but one substitutent is held constant, with the varying substitutents arranged in the order of decreasing hydrophobicity (38). The sixth group consists of other opiate derivatives. Several general conclusions can be drawn from these comparisons.

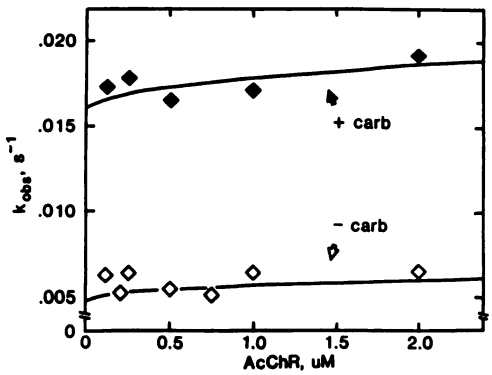


Fig. 5. Plot of k_{obs} (pseudo-first order rate constant measured as an exponential approach to equilibrium) as a function of the concentration of AcChR (expressed as AcChR monomers—two α-[^{125}I]Bgt binding sites per monomer) in the presence and absence of 0.2 mM carbamoylcholine (added 15 min before the addition of (−)-[^{3}H]ANMC. (−)-[^{3}H]ANMC concentration was initially 50 nM in all cases. The *solid lines* were calculated from the kinetic constants given in the text.

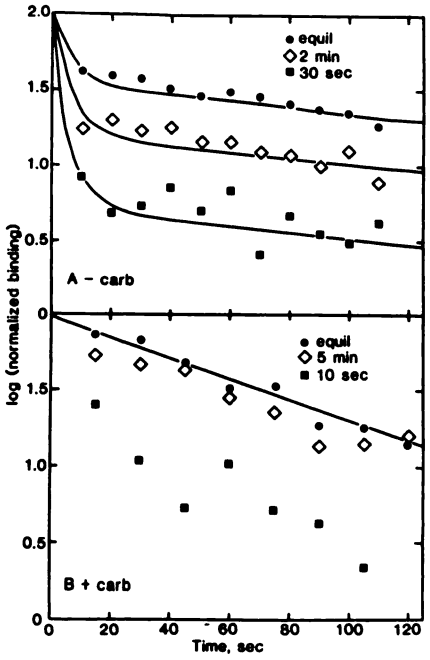


Fig. 6. A, Dissociation of (−)-[^{3}H]ANMC-AcChR complexes in the absence of carbamoylcholine. Dissociation was initiated by the addition of 200-fold excess of nonradioactive (−)-ANMC at the indicated intervals following the addition of (−)-[^{3}H]ANMC. AcChR concentration was 0.4 μM (expressed in α-[^{125}I]Bgt binding sites) and (−)-[^{3}H]ANMC concentration was 100 nM. The *solid lines* were calculated from the kinetic constants given in the text. B, Dissociation of (−)-[^{3}H]ANMC complexes at various times following the simultaneous addition of 0.2 mM carbamoylcholine and 100 nM (−)-[^{3}H]ANMC. Dissociation was initiated with a 200-fold excess of nonradioactive (−)-ANMC. AcChR concentration was 0.4 μM (expressed in α-[^{125}I]Bgt binding sites). The *solid line* for the equilibrium condition was calculated from the kinetic constants given in the text.

1) Hydrophobicity is a major determinant for the inhibition of [^{3}H]PCP binding in the presence of carbamoylcholine. This is particularly evident in positions 0 and 1. For (−)-isomers varying in position 0, the K_I decreases from 34 μM for $-CH_3$ to 1.7 μM for $-CH_2CH_2Ø$. The hydrophobicity is interrelated with steric factors; however, steric hindrance would be expected to follow the opposite rank order. R_1 is on the aromatic ring so that electronic, as well as hydrophobic, parameters could have an effect. Comparisons between fluorine, hydroxyl, and hydrogen are interesting in this regard. The major difference is in the substitution of a hydroxyl group which decreases affinity relative to fluorine and hydrogen. This is consistent with a hydrophobic, rather than electronic, effect.

2) Hydrophobicity seems to be a minor determinant for the inhibition of (−)-[^{3}H]ANMC and α-[^{125}I]Bgt binding. In these cases, the major differences between substituents are dictated by the stereochemistry, with (−)-isomers being more potent in the case of (−)-[^{3}H]ANMC binding and (+)-isomers being more potent in the case of α-[^{125}I]Bgt binding.

3) Modification of the benzomorphan structure significantly changes affinity. In the cases of thebaine, oxymorphone, and a morphinan, the inclusion of an additional ring and ether linkage led to a dramatic decrease in affinity.

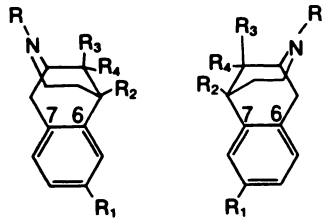
Discussion

The characteristics of the stereoselective binding of the benzomorphan opiate, (−)-[^{3}H]ANMC, to AcChR-rich membranes

TABLE 1

Structure-affinity relationships for the inhibition of [^{3}H]PCP, (−)-[^{3}H]ANMC, and α-[^{125}I]Bgt binding by opiate analogs

The AcChR concentration was 0.4 μM for [^{3}H]PCP and (−)-[^{3}H]ANMC experiments and 10 nM for α-[^{125}I]Bgt experiments (measured in sites for α-[^{125}I]Bgt). Filtration assays were used in all cases. Concentrations of radioligands were: [^{3}H]PCP, 100 nM; (−)-[^{3}H]ANMC, 100 nM; and α-[^{125}I]Bgt, 5 nM. In the case of α-[^{125}I]Bgt, the inhibition of the initial rate of binding was measured; and, in the cases of [^{3}H]PCP and (−)-[^{3}H]ANMC, the inhibition was measured after equilibration for 45 min. The values given in the table are K_i values calculated according to the method of Cheng and Prusoff (44) and are expressed in μM. When included, carbamoylcholine was present at a concentration of 0.2 mM and added 15 min before the addition of radioligand.



R_0	R_1	R_2	R_3	R_4	PCP (+CC)	PCP (−CC)	ANMC	Bgt
Position 0 Comparisons								
(−) $CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	6.6	4.6	0.72	405
CH_2–◁	OH	CH_3	CH_3	H	6.9	1.9	2.6	225
$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	7.0	2.0	2.0	117
$CH_2CH_2Ø$	OH	CH_3	CH_3	H	1.7	3.1	1.5	
CH_3	OH	CH_3	CH_3	H	34.4	4.8	3.2	381
(+) $CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	16.9	13.4	1.8	201
CH_2–◁	OH	CH_3	CH_3	H	9.5	26.7	9.2	218
$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	18.3	15.0	9.4	96
CH_3	OH	CH_3	CH_3	H	31.7	29.2	24.5	76
(*R*) $CH_2CH{=}CH_2$	OH	CH_3	CH_3	H	11.9	9.0	1.3	303
CH_2–◁	OH	CH_3	CH_3	H	3.9	2.2	1.3	266
$CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	12.7	8.5	5.7	107
$CH_2CH_2Ø$	OH	CH_3	CH_3	H	3.2	1.3	7.5	9.4
CH_3	OH	CH_3	CH_3	H	33.3	42.9	2.5	258
H	OH	CH_3	CH_3	H	35.9	171	0.39	103
Position 1 Comparisons								
(*R*) CH_3	OH	CH_3	CH_3	OH	75.9	34.3	22.9	1230
CH_3	H	CH_3	CH_3	OH	10.4	14.0	6.1	761
CH_3	OCH_3	CH_3	CH_3	OH	9.5	12.6	47.9	129
(*R*) CH_3	OH	CH_3	CH_3	H	33.2	17.0	13.8	229
CH_3	H	CH_3	CH_3	H	3.7	11.4	5.1	125
CH_3	F	CH_3	CH_3	H	2.2	4.4	4.7	60
Position 2 Comparisons								
(−) CH_3	OH	CH_3	H	H	91	155	1.7	50
CH_3	OH	C_2H_5	H	H	39	208	0.65	38
(+) CH_3	OH	CH_3	H	H	158	375	24	91
CH_3	OH	C_2H_5	H	H	34	227	10.6	70
(*R*) $CH_2CH{=}C(CH_3)_2$	OH	CH_3	CH_3	H	12.7	8.5	5.7	107
$CH_2CH{=}C(CH_3)_2$	OH	C_2H_5	CH_3	H	1.7	3.3	1.1	43
Position 3 Comparisons								
(−) CH_3	OH	CH_3	H	H	91	155	1.7	50
CH_3	OH	CH_3	CH_3	H	34	4.8	3.2	381
(+) CH_3	OH	CH_3	H	H	158	375	24	91
CH_3	OH	CH_3	CH_3	H	32	29	25	76
Position 4 Comparisons								
(*R*) CH_3	OH	CH_3	CH_3	H	33.2	17.0	13.8	229
CH_3	OH	CH_3	CH_3	OH	75.9	34.3	22.9	1230
Other Opiate Derivatives								
Thebaine			(−)		63	436	56	61
			(+)		251	>1000	89	104
Oxymorphone			(−)		93	388	143	75
			(+)		>1000	>1000	615	112
5-*m*-Hydroxyphenyl-2-methylmorphan			(−)		42	13	31	12
			(+)		44	9	61	27

from *T. nobiliana* have been described. A centrifugation assay is capable of detecting two binding sites, whereas a filtration assay and photoaffinity labeling (see Ref. 9) detect only the higher affinity component. It is the higher affinity component (K_D ~0.4 μM) that was studied in detail. The following observations were made.

1) The high affinity binding site seems to exist in one copy per AcChR monomer ($\alpha_2\beta\gamma\delta$) although, due to the complex binding mechanism, not all of the sites are measured with the filtration assay. The low value obtained for the B_{max} in the absence of carbamoylcholine seems to be the result of a rapid dissociation of approximately 30% of the bound ligand, which would lead to an underestimate of the binding in the filtration but not in the centrifugation assay. The simultaneous addition of carbamoylcholine and (−)-[^{3}H]ANMC leads to a transient detection of a larger number of binding sites.

2) Carbamoylcholine at equilibrium decreases the affinity and apparent number of binding sites for (−)-[^{3}H]ANMC. The EC_{50} for this effect is increased by α-Bgt, but the effect cannot be totally inhibited by α-Bgt. Carbamoylcholine does not seem to be interacting with the same site as α-Bgt because the effect can be observed under conditions where more than 90% of the α-[^{125}I]Bgt binding sites are occupied by nonradioactive α-Bgt.

3) The site for PCP and tetracaine (noncompetitive blockers) seems to be distinct from the high affinity site for benzomorphan opiates in that the binding of (−)-[^{3}H]ANMC is inhibited in a noncompetitive manner by PCP (9) or only at very high concentrations by tetracaine (Fig. 4A) and in a competitive manner by (−)-pentazocine (Fig. 4B) and nonradioactive ANMC (data not shown). The results obtained with tetracaine are interesting in light of the fact that tetracaine binding is regulated by agonists in a manner similar to that of (−)-[^{3}H]ANMC, i.e., carbamoylcholine at equilibrium decreases the binding affinity (25, 26).

4) The kinetics of association of (−)-[^{3}H]ANMC in the absence of carbamoylcholine or following equilibration with carbamoylcholine are biphasic and the slower rate has little or no dependence on the concentration of AcChR. The rate of association of the slower component is 2- to 3-fold higher in the presence than in the absence of carbamoylcholine.

5) The kinetics of dissociation of (−)-[^{3}H]ANMC seem to be dependent upon the conformational state of the AcChR. In the absence of effector, dissociation is biphasic, with one component having a $t_{1/2}$ of less than 5 sec and the other component having a $t_{1/2}$ of approximately 2.5 min. Immediately (i.e., 5 sec) following the simultaneous addition of (−)-[^{3}H]ANMC and carbamoylcholine, the dissociation rate is biphasic with one component having a $t_{1/2}$ of approximately 5 sec and the second with a $t_{1/2}$ of approximately 30 sec. After equilibration, dissociation is monophasic with $t_{1/2}$ of 40 sec.

6) The structure-affinity relationships indicate that the substituents added to the benzomorphan backbone have only minor effects on the binding activity as compared with changes in the benzomorphan backbone itself. In terms of the interaction of benzomorphans with the PCP site, the hydrophobicity of the substituents added to the benzomorphan backbone seems to be a major determinant of the binding affinity. This is consistent with the finding that the driving force for the binding of PCP is an increase in entropy (39).

The results suggest that the binding mechanism is complex. One of the major problems in defining a precise mechanism is that the contribution of the low affinity site to the kinetic

measurements is unknown. For example, association under conditions of simultaneous addition of carbamoylcholine and (−)-[^{3}H]ANMC (Fig. 1A) could be interpreted either as an increase in the number of observable high affinity binding sites or as a recruitment of low affinity sites which are observable due to a decrease in the dissociation rate of the low affinity site. The pseudo-Scatchard plot in Fig. 1C showing one binding component and the quantitation of the number of sites support the first alternative. The finding that the labeling of the δ-chain by UV-irradiation is increased by the simultaneous addition of carbamoylcholine also supports the first alternative. The second problem with defining the binding mechanism is that some of the steps involved cannot be resolved temporally (for example, the rapid component of association and dissocation in the absence of carbamoylcholine).

Assuming that the binding observed is exclusively to the high affinity binding site in the absence of carbamoylcholine or following equilibration with respect to carbamoylcholine, a tentative mechanism can be proposed that fits the data. The biphasic dissociation (Fig. 6A) observed in the absence of carbamoylcholine, the apparent independence of the slow association rate on the AcChR concentration, and the lower B_{max} observed in the presence of carbamoylcholine are consistent with the following mechanism:

$$R + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R'A$$

where R is the AcChR and RA and $R'A$ are two species of (−)-[^{3}H]ANMC complexes. The apparent K_D measured for this mechanism is:

$$K_{app} = \frac{K_1 K_2}{K_2 + 1}$$

where $K_1 = k_{-1}$ and $K_2 = k_{-2}/k_2$. The fourth order Runge-Kutta technique with a step size of 100 msec was used to solve numerically the differential equations for this mechanism. Because a filtration assay was used, some dissociation of the ligand would be expected during the 2-sec filtration and wash. This was simulated at each time point by setting k_1 equal to zero and allowing "dissociation" to proceed for 2 sec. Attempts were made to model the data obtained in the absence of carbamoylcholine (Figs. 1, 5, and 6) or after prior addition of carbamoylcholine or *d*-tubocurarine (Fig. 1). No attempt was made to model the conditions of simultaneous addition of carbamoylcholine (Figs. 1 and 6) because the temporal resolution was not adequate to determine the 10 kinetic constants that would be dictated by a minimal model. In the absence of carbamoylcholine, the kinetic constants that fit the data were: $k_1 = 4 \times 10^5$ M^{-1} sec^{-1}, $k_{-1} = 0.26$ sec^{-1}, $k_2 = 1.5 \times 10^{-3}$ sec^{-1}, and $k_2 = 4.8 \times 10^{-3}$ sec^{-1}. This gives rise to equilibrium and isomerization constants of $K_1 = 0.65$ μM, $K_2 = 3.2$, and $K_{app} = 0.5$ μM. Given these constants and assuming a 2-sec dissociation during filtration and wash, a Scatchard plot of the data should measure 69% of the total number of binding sites (B_{max}). In the presence of carbamoylcholine ("prior addition"), the kinetic constants determined were: $k_1 = 4 \times 10^6$ M^{-1} sec^{-1}, $k_{-1} = 4$ sec^{-1}, $k_2 = 4 \times 10^{-3}$ sec^{-1}, and $k_{-2} = 1.6 \times 10^{-2}$ sec^{-1}. The corresponding equilibrium and isomerization constants are: $K_1 = 1$ μM, $K_2 = 4$, and $K_{app} = 0.8$ μM. In this case, 20% of the total number of sites would be measured with saturation analysis. These constants were used to generate the *solid lines* in Figs. 1, 5, and 6 and seem to be adequate to describe the data quantitatively.

One reasonable interpretation for the differences in affinity in the presence versus the absence of carbamoylcholine is that the AcChR exists in different conformational states under these conditions. A number of models for the conformational equilibria of the AcChR have been proposed (reviewed in Refs. 40 and 41). In general, four conformational states have been assumed: 1) the resting state, which comprises 80–90% of the receptor molecules in the absence of agonist; 2) the active state, which exists transiently after agonist addition and which is characterized by an open ion channel; 3) the intermediate desensitized state, which exists transiently after channel activation and which is characterized by a closed ion channel; and 4) the desensitized state, which comprises more than 95% of the total number of receptors after equilibration with agonist. The binding of (−)-[^{3}H]ANMC in the absence of carbamoylcholine may reflect binding largely to the resting state, whereas binding in the presence of carbamoylcholine may reflect binding to the desensitized state. The isomerization to the $R'A$ state could reflect a state induced by the ligand other than the four states described above. The enhanced binding under the simultaneous addition protocol could reflect the interaction of (−)-[^{3}H]ANMC with the active or intermediate states. The relaxation of the level of binding following simultaneous addition of carbamoylcholine and (−)-[^{3}H]ANMC is consistent with the time course of the transition from the intermediate or active states to the desensitized states. A more detailed description of the interaction of benzomorphans with states of the AcChR will require investigations of the effect of benzomorphans on ion flux and agonist binding.

The relationship between the high affinity benzomorphan site and the site for noncompetitive blockers (i.e., PCP, histrionicotoxin) has been studied by radioligand binding (Fig. 4, Ref. 9) and by photoaffinity labeling (9). The conclusion was that the sites seem to be at least partially distinct. Using a centrifugation assay, two components have been observed for (−)-[^{3}H]ANMC binding: a high affinity site (K_D of 0.4 μM) and a low affinity site (K_D of 2 μM). PCP decreases the B_{max} of the high affinity site without affecting the K_D (9), and, to the extent that accurate measurements can be made on the low affinity site, both PCP and tetracaine seem to decrease the K_D of this site. In addition, the K_I for the inhibition of [^{3}H]PCP binding by (−)-ANMC (characterized by an increase in the K_D with no change in the B_{max}) is approximately 7 μM, which is the same order of magnitude as the K_D of (−)-[^{3}H]ANMC binding to its low affinity site (9). Both nonradioactive (−)-ANMC and (−)-pentazocine decrease the affinity of (−)-[^{3}H]ANMC for the AcChR with a K_I on the order of 0.5 μM. Taken together, these results suggest that the high affinity site for benzomorphans is distinct from the site for noncompetitive blockers but that the low affinity site for benzomorphans may be identical to the site for noncompetitive blockers. The distinction between the noncompetitive blocker site and the benzomorphan site was supported further by photoaffinity labeling experiments (9). Using *Torpedo marmorata* AcChR, the noncompetitive blocker, 5-azido-[^{3}H]trimethisoquin, and (−)-[^{3}H]ANMC, both labeled the δ-subunit of the AcChR. However, cleavage of the subunit with trypsin demonstrated that the two labels were on different portions of the subunit, with 5-azido-[^{3}H]trimethisoquin being closer to the N-terminus and (−)-[^{3}H]ANMC closer to the C-terminus.

The effect of carbamoylcholine on (−)-[^{3}H]ANMC binding has a number of unique characteristics. The inhibition of binding has an IC_{50} of approximately 40 μM and the effect seems to be modified by α-Bgt but not completely inhibited. Under identical conditions, the binding of α-[^{125}I]Bgt is decreased by approximately 90% and the effect of carbamoylcholine on [^{3}H]PCP is completely abolished (9). The simplest interpretation of these results is that the effect of carbamoylcholine on (−)-[^{3}H]ANMC reflects an interaction of carbamoylcholine with a site or sites distinct from its high affinity binding sites that are blocked by α-Bgt. Additional binding sites for carbamoylcholine on the AcChR have been proposed by others (42, 43), but the relationship between the results of those studies and those presented here is not clear.

In conclusion, we have presented evidence that the benzomorphan opiate, (−)-[^{3}H]ANMC, binds to the AcChR with a complex mechanism that most likely involves an isomerization of the receptor-ligand complex. The high affinity binding site seems to be distinct from the binding sites for PCP and tetracaine; however, the sites seem to be allosterically coupled. The definition of the relationship between the model for the binding mechanism and the known conformational transitions of the AcChR will require further studies of the effects of benzomorphans on AcCh-induced ion flux and on the binding of cholinergic agonists.

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Send reprint requests to: Dr. Robert E. Oswald, Department of Pharmacology, New York State College of Medicine, Cornell University, Ithaca, NY 14853.