Demonstration and affinity labeling of a stereoselective binding site for a benzomorphan opiate on acetylcholine receptor-rich membranes from *Torpedo* electroplaque

(cholinergic receptor/ion channel/photoaffinity labeling/N-allyl-N-normetazocine/phencyclidine)

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**ABSTRACT** The interaction of an optically pure benzomorphan opiate, (−)-N-allyl-N-normetazocine [(−)-ANMC], with the nicotinic acetylcholine receptor from *Torpedo* electroplaque was studied by using radioligand binding and affinity labeling. The binding was complex with at least two specific components having equilibrium dissociation constants of 0.3 μM and 2 μM. The affinity of the higher affinity component was decreased by carbamoylation but not by α-bungarotoxin. The effect of carboxamoylation was not blocked by α-bungarotoxin. In comparison, the affinity of [3H]phencyclidine, a well-characterized ligand for a high-affinity site for noncompetitive blockers on the acetylcholine receptor, is increased by carboxamoylation and the increase is blocked by α-bungarotoxin. The binding of (−)-[3H]ANMC was inhibited by a number of other benzomorphans, with (−) isomers being 4- to 5-fold more potent than (+) isomers. Phencyclidine inhibits the binding of (−)-[3H]ANMC to its high-affinity site by a mechanism that is not competitive. UV-catalyzed affinity labeling indicated that the high-affinity-binding site for (−)-[3H]ANMC is at least partially associated with the δ subunit. Tryptic degradation of the *Torpedo marmorata* δ chain suggested that (−)-ANMC labeled a 16,000-dalton COOH-terminal portion of the subunit. In contrast, 5-azido-[3H]trimethisinoquin, a photoaffinity label of the high-affinity site for noncompetitive blockers, labels a 47,000-dalton NH₂-terminal fragment of the δ subunit. These results suggest that (−)-[3H]ANMC binds to sites completely distinct from the binding sites for acetylcholine. The high-affinity-binding site for (−)-ANMC and that for phencyclidine and 5-azidotrimethisoin are allosterically coupled but are regulated differently and are probably physically distinct.

The nicotinic acetylcholine receptor (AcChoR) from *Torpedo* electroplaque is a multisubunit protein that mediates ion flux across the cell membrane in response to the binding of acetylcholine (AcCho). The receptor complex consists of four different subunits of 50,116 (α), 53,681 (β), 56,279 (γ), and 57,565 (δ) daltons (measured by sequence analysis; ref. 1), existing in a stoichiometry of 2:1:1:1 (2–4). These subunits migrate in NaDodSO₄/polyacrylamide gels with apparent molecular masses of 40,000, 50,000, 60,000, and 66,000 daltons, respectively. A high-affinity-binding site for AcCho is associated, at least in part, with each of the α subunits (reviewed in ref. 5). In addition, a series of compounds, known collectively as noncompetitive blockers, has been shown to bind to the AcChoR (6–13). These compounds, which include phencyclidine (PCP), histrionotoxin, chlorpromazine, and some aminated local anesthetics, bind to one high-affinity site per AcChoR monomer (reviewed in ref. 14) as well as to a variable number of low-affinity sites associated with the membrane lipids (10, 11).

Benzomorphan opiates have been shown to inhibit the binding of [3H]PCP to central nervous system synaptic membranes (15, 16) and to modify the activity of serum choline terase (17). Some opiate derivatives, in particular the benzomorphans, can inhibit the binding of [3H]perhydrohistrionotoxin and [3H]PCP to the *Torpedo* AcChoR (18, 19). In the present studies, a radioactive optically pure benzomorphan, (−)-[3H]-N-allyl-N-normetazocine [(−)-[3H]ANMC], was used to measure reversible binding to and affinity labeling of the *Torpedo* electroplaque AcChoR. Specific binding was shown to be complex with at least one component having lower affinity in the presence rather than the absence of cholinergic effectors. Binding to the high-affinity site was inhibited by PCP with a mechanism that is not competitive. α-Bungarotoxin (α-BuTx) had no effect on the decrease in affinity induced by cholinergic effectors. UV-catalyzed affinity labeling (20) resulted in the labeling of the δ subunit and, in some cases, the α subunit. These results may indicate a unique binding site for benzomorphans on the AcChoR.

**MATERIALS AND METHODS**

AcChoR-rich membranes were prepared from the electroplaque of *Torpedo californica*, *Torpedo marmorata*, and *Torpedo nobiliana* as described (21). The binding of (−)-[3H]ANMC was measured by either a centrifugation (10) or a filtration assay (22, 23) in a total volume of 125 μl. The buffer used in all cases was 50 mM 3-(N-morpholino)propanesulfonic acid (Mops)/NaOH and 1 mM EGTA, pH 7.5 (Mops/EGTA).

Cross-linking of (−)-[3H]ANMC and 5-azido-[3H]trimethisinoquin (5A[3H]HT) induced by irradiation with UV light was performed as described (20, 24) except that Mops/EGTA was the buffer. Trypsin degradation was performed as described by Wennogle et al. (25).

Benzomorphan opiates (ANMC, metazocine, pentazocine, cyclazocine, etorphine, naloxone, and (−)-[3H]ANMC (49.5 Ci/mmol; 1 Ci = 37 GBq) were supplied by the National Institute on Drug Abuse. Phencocine, thebaine, and oxymorphone were gifts of A. E. Jacobson (National Institutes of Health). A. Gero (Hanenmann University) generously supplied metazocine, pentazocine, cyclazocine, 5-ethyl-2'-hydroxy-2-methyl-6,7-benzomorphan, and 5-(m-hydroxyphenyl)-2-methylmorphin. PCP was obtained from U.S. Pharmacopeial Convention (Rockville, MD), and [3H]PCP (48 Ci/mmol), [3H]AcCho (90 mCi/mmol), and [125I]-labeled α-BuTx ([125I]-α-BuTx) were purchased from New England Nuclear (Boston, MA). 5A[3H]HT was a gift of J. P. Changeux (Institut Pasteur, Paris). Live *T. californica* were purchased from Pacific Biomarine (Venice, CA), and frozen *T. nobiliana* were purchased from Biofish Associates.

Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; (−)-ANMC, (−)-N-allyl-N-normetazocine; 5A[3H]HT, 5-azido-[3H]trimethisinoquin; α-BuTx, α-bungarotoxin; MPTA, [4-(N-maleimidophenyl)trimethyllammonium; PCP, phencyclidine.

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RESULTS

Equilibrium Binding of (−)[3H]ANMC to AcChoR-Rich Membrane Fragments. As illustrated in Fig. 1, the specific binding of (−)[3H]ANMC to AcChoR-rich membrane fragments from T. californica is saturable. (Identical results were obtained with T. marmorata and T. nobiliana.) The curve drawn through the experimental points represents a nonlinear least squares fit of the data to a two-site model. The equilibrium dissociation constant (Kd) of the high-affinity component is 0.36 ± 0.07 μM and that of the low-affinity component is 2.2 ± 0.4 μM. The high-affinity component comprises between 40% and 50% of the specific binding. The total concentration of sites in the preparation shown in Fig. 1 was 0.1 μM, which is within 10% of the number of AcCho binding sites measured by [3H]AcCho binding and α-BuTx binding. This suggests that two binding sites for (−)-ANMC may exist on each 250,000-dalton AcChoR monomer. Both components were lost following heating to 100°C for 3 min.

Fig. 2 shows a comparison of the binding of (−)-[3H]ANMC and of [3H]PCP in the presence and absence of carbamoylcholine and α-BuTx. The affinity and number of binding sites for (−)-[3H]ANMC are unaffected by the presence of α-BuTx; however, carbamoylcholine decreases the affinity of the high-affinity component without changing the number of binding sites (Fig. 2A), with an IC50 of 40 ± 8 μM. This effect is not blocked by α-BuTx. Under identical conditions, α-BuTx blocks completely the agonist-induced increase in affinity for [3H]PCP and inhibits completely the binding of 125I-α-BuTx. d-Tubocurarine and decamethonium also decrease the affinity of (−)[3H]ANMC (IC50 values of 47 ± 10 μM and 7.6 ± 1.1 μM, respectively). The effects of d-tubocurarine and decamethonium are not inhibited by α-BuTx. This is in contrast to the binding of [3H]PCP, which shows an increased affinity in the presence of carbamoylcholine (EC50 = 0.15 μM), d-tubocurarine (EC50 = 0.08 μM), and decamethonium (EC50 = 0.1 μM) and a decreased affinity in the presence of α-BuTx. In the case of decamethonium, a decrease in [3H]PCP binding was observed at higher concentrations (IC50 = 7 μM). In the case of PCP, α-BuTx blocks the increase in affinity induced by carbamoylcholine, d-tubocurarine, and decamethonium.

Interaction Between Sites for PCP and (−)-ANMC. (−)-ANMC is a competitive inhibitor of [3H]PCP binding both in the presence (Fig. 3D) and absence (Fig. 3C) of 0.2 μM carbamoylcholine. The Kd for (−)-ANMC, calculated from Dixon plots, was 7.7 μM in the presence of carbamoylcholine and 4.8 μM in its absence. Nonlinear least squares analysis of the binding isotherms for (−)[3H]ANMC in the presence of carbamoylcholine indicates that the Kd of the high-affinity component is unchanged in the presence of PCP, but the apparent Bmax is decreased. On the other hand, the Kd of the low-affinity component is increased and the apparent Bmax remains unchanged. These data indicate that the high-affinity component is decreased by an apparent noncompetitive mechanism. The low-affinity component in the absence of carbamoylcholine is inhibited competitively by PCP. The IC50 for PCP was calculated for each concentration of (−)-[3H]ANMC and was found to be constant (6.9 ± 0.5 μM). In the presence of carbamoylcholine, PCP inhibited the binding

(georgetown, MA). Purified T. marmorata membranes were a gift of B. Holton and A. Ribera (Institut Pasteur).
of (−)-[3H]ANMC with a $K_i$ of 0.8 μM. The most likely interpretation of these results is that the high-affinity-binding site for (−)-ANMC is distinct from that for PCP but is allosterically coupled. The concentration of (−)-ANMC required to inhibit [3H]PCP binding and the competitive nature of the interaction between the low-affinity (−)-ANMC site and the PCP site suggest that these may be the same site.

Interaction Between Sites for α-BuTx and (−)-ANMC. The effect of (−)-ANMC on the initial rate of binding of 15 nM $^{125}$I-α-BuTx to AcChoR-rich membrane fragments (5 nM in $^{125}$I-α-BuTx sites) was measured. The IC$_{50}$ for (−)-ANMC was 440 μM, indicating that the sites described in Figs. 1–3 are not the high-affinity sites for AcCho and α-BuTx.

Stereoselectivity of Benzomorphan Binding. (−) Isomers of benzomorphans are 4- to 5-fold more potent inhibitors of (−)-[3H]ANMC binding than (+) isomers (Table 1). This indicates that, in each case, the (−) isomer exhibits a decrease in the free energy of binding 0.8–0.9 kcal/mol (1 cal = 4.184 J) greater than the (+) isomer.

Changes in the substituents on the three benzomorphan rings had only minor effects. However, the addition of another ring and ether linkage in morphine derivatives such as naloxone, etorphine, thebaine, and oxymorphone (data not shown) or the removal of the 6,7-aromatic ring in the methylmorphinan resulted in drastically decreased affinity but retention of stereospecificity, with approximately the same ratio of affinities between the (−) and (+) isomers.

Photoaffinity Labeling. (−)[3H]ANMC can be covalently cross-linked to the AcChoR by irradiating AcChoR-rich membrane fragments with 254-nm light in the presence of (−)[3H]ANMC. Slight differences in the labeling patterns were observed for AcChoR-rich membrane fragments prepared from T. marmorata and from T. nobiliana and T. californica. In general, the δ subunit was the major cross-linked product, with the α subunit labeled relatively greater in the case of T. marmorata. Fig. 4 shows the labeling of peptides from T. nobiliana AcChoR-rich membrane fragments. Approximately 60% of the specifically bound radioactivity measured by a filtration assay was incorporated into the δ subunit. Consistent with the reversible binding of (−)-[3H]ANMC (Fig. 2), carbamoylcholine decreases the labeling of the δ subunit (Fig. 4, lane B vs. lane E). Again α-BuTx has no effect either in the presence (lane I) or absence (lane E) of carbamoylcholine. PCP (150 μM) decreased the labeling of the δ chain (lanes D and H) in proportion with the decrease in the reversible binding measured with a filtration assay. These results suggest that the binding site(s) for (−)-[3H]ANMC is associated, at least partially, with the δ subunit.

The labeling of the T. marmorata AcChoR was studied further by trypsin degradation of the (−)[3H]ANMC-labeled membrane-bound AcChoR. T. marmorata was used in this experiment to allow direct comparisons with previous data (25, 26). As described by Wennogle and Changeux (26), the α subunit is degraded sequentially into large NH$_2$-terminal fragments of 38,000, 35,000, and 32,000 daltons, which retain the site of [3H]4-(N-maleimido)phenyltrimethylammonium ([3H]MPTA) labeling (an affinity label for the AcCho binding site). The site of (−)[3H]ANMC labeling on the α subunit, like the site of [3H]MPTA labeling, remains with these large fragments (Fig. 5). The δ chain has been shown to be degraded sequentially by trypsin into NH$_2$-terminal fragments of 50,000, 49,000, and 47,000 daltons, which retain the site of labeling for the local anesthetic 5A3[H]T (25). Although an increase in radioactivity at level of the tracking dye with trypsin treatment was observed, this is apparently not related to fragments of the δ chain because >97% of the radioactivity incorporated into the δ chain can be recovered in the
NH₂-terminal fragments. The labeling of the β chain by (−)-[13H]ANMC does not appear in the NH₂-terminal tryptic fragments of the β chain (Fig. 5). Instead, the site of (−)-[13H]ANMC labeling is lost upon the cleavage of the δ chain to its 50,000-dalton fragment with the loss of the 16,000-dalton COOH-terminal portion of the subunit. This suggests that (−)-[13H]ANMC may label a domain of the δ subunit that is distinct from the site labeled by 5A[3H]T.

DISCUSSION

The stereoselective binding of a benzomorphan opiate, (−)-[13H]ANMC, to AcChoR-rich membrane fragments from Torpedo has been demonstrated. Several characteristics of the binding distinguish this ligand from others that bind to the AcChoR (reviewed in refs. 5 and 14). (i) The reversible equilibrium binding is complex, with a high-affinity component

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**Table 1. Inhibition of (−)-[13H]ANMC binding by opiate derivatives**

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The binding of 50 nM (−)-[13H]ANMC to AcChoR-rich membrane fragments (200 nM in 125I-α-BuTx sites) was measured by using the rapid filtration assay following a 45-min incubation at 25°C. No carbamoylcholine or α-BuTx was present. *The 6,7-benzyl group is absent.

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**Fig. 4.** NaDdSO₄ electrophoretic analysis (10% acrylamide) of UV-catalyzed labeling of T. nobiliana AcChoR-rich membranes by 50 nM (−)-[13H]ANMC (700 nM in 125I-α-BuTx sites, 0.6 nmol of 125I-α-BuTx sites per mg of protein). A Coomassie blue stain of the protein (lane A) is shown next to fluorograms of labeled membranes cross-linked under the following conditions: absence of effectors ((−)-[13H]ANMC only) (lane B); 0.15 mM nonradioactive (−)-ANMC (lane C); 0.15 mM nonradioactive PCP (lane D); 2 μM α-BuTx (lane E); 0.2 mM carbamoylcholine (lane F); 0.2 mM carbamoylcholine, 0.15 mM nonradioactive (−)-ANMC (lane G); 0.2 mM carbamoylcholine, 0.15 mM nonradioactive PCP (lane H); and 0.2 mM carbamoylcholine, 2 μM α-BuTx (lane I). The apparent molecular mass of the receptor subunits is given in kilodaltons (40,000 daltons, α; 50,000 daltons, β; 60,000 daltons, γ; 66,000 daltons, δ). The 132,000-dalton band represents a small population of unreduced δ-δ dimer; t indicates the position of the tracking dye.

**Fig. 5.** Degradation by trypsin of alkaline-treated T. marmorata AcChoR-rich membrane proteins labeled with either 5A[3H]T (Left) or (−)-[13H]ANMC (Right) (700 nM in 125I-α-BuTx sites). Incubations with varying concentrations of trypsin were for 15 min at 25°C; the reactions were terminated by the addition of diisopropyl fluorophosphate to 1 μM. Shown are the fluorograms of NaDdSO₄/10% acrylamide gels of 5A[3H]T-labeled peptides (Left) and (−)-[13H]ANMC-labeled peptides (Right). The concentrations of trypsin were no trypsin (lanes 1), 0.16 μg/ml (lanes 2), 1.3 μg/ml (lanes 3), and 10 μg/ml (lanes 4). 5A[3H]T labeling was performed in the presence of 0.2 mM carbamoylcholine. This results in a more intense labeling than in the absence of carbamoylcholine but produces the same labeled tryptic fragments. Molecular masses are given in kilodaltons. The 50,000- and 47,000-dalton bands are fragments of the δ subunit and the 32,000-dalton band is a fragment of the α subunit; t indicates the position of the tracking dye.
having a $K_d$ of $\approx 0.3 \mu M$ and a low-affinity component of 2 $\mu M$. In the previous report by Heidmann et al. (10), multiple components were reported but the low-affinity sites were heat insensitive and, except in the case of PCP, were not inhibited by PCP, suggesting a lipid-associated binding site. Both low- and high-affinity-binding sites for (--)ANMC reported here are heat sensitive and inhibited by PCP, suggesting that the sites are associated with protein rather than lipid. (ii) Equilibrium binding simplifies to a single low-affinity component in the presence of carbamoylcholine and this effect is not blocked by $\alpha$-BuTx. (iii) The high-affinity component is noncompetitively inhibited by PCP and the low-affinity component is competitively inhibited by PCP. (iv) Trypsin cleavage suggests that the portion of the T. marmorata $\delta$ subunit labeled by (--)HIANMC may be associated with a 16,000-dalton COOH-terminal portion of the molecule. The results demonstrate a unique, high-affinity-binding site for benzomorphans that is allosterically coupled to the site for noncompetitive blockers (i.e., the PCP binding site).

The equilibrium binding data are consistent with two binding sites per AcChoR monomer. This is in contrast to the binding of [11H]PCP, which exhibits one high-affinity site per AcChoR monomer (10). The regulation of the binding of (--)HIANMC by cholinergic effectors differs dramatically from that of [11H]PCP. Carbamoylcholine, d-tubocurarine, and decamethonium increase the affinity of the AcChoR for [11H]PCP but decrease the affinity for (--)HIANMC. The concentrations of cholinergic effectors required to increase the affinity for PCP (ED$_{50}$) are lower by a factor of 75–600 than the IC$_{50}$ values for inhibition of (--)ANMC binding. A decrease in affinity in the presence of carbamoylcholine has also been reported for the local anesthetic tetracaine (27, 28).

The number of these effectors is different in two high-affinity, $\alpha$-BuTx-sensitive sites for carbamoylcholine that reside on the $\alpha$ subunits. Additional sites for cholinergic agonists and antagonists have been proposed (29, 30), although the presence of these receptor subunits may be controversial. The noncompetitive interaction between the high-affinity (--)ANMC site and the PCP site, the differential regulation by cholinergic effectors, and the differential affinity labeling results (see below) suggest that the binding site for PCP and the high-affinity-binding site for (--)ANMC are distinct. The competitive interaction between the low-affinity site for (--)ANMC and for the site for PCP suggests that these sites may be identical. The weak inhibition of the initial rate of $\alpha$-BuTx binding by (--)ANMC and the heat sensitivity of both (--)ANMC sites indicate that these sites are neither the high-affinity sites for AcCho and $\alpha$-BuTx nor the lipid-associated sites reported previously for some noncompetitive blockers (10).

The UV-catalyzed cross-linking of (--)HIANMC to the AcChoR results in the specific labeling of the $\alpha$ and $\delta$ subunits for T. marmorata and of the $\delta$ subunit for T. nobiliana and T. californica. The labeling is controlled in a manner identical to that of the reversible binding of (--)HIANMC. The site of labeling on the T. marmorata $\delta$ chain may be on a 16,000-dalton COOH-terminal portion of the subunit that contains a cysteine, which is thought to be involved in disulfide-linked $\delta$-$\delta$ dimers of the receptor (25). This portion of the subunit most likely includes the 12,000-dalton tryptic peptide described by Anderson et al. (31), which contains the major antigenic sites on the $\delta$ subunit.

In conclusion, the benzomorphan (--)ANMC has been shown to bind stereoselectively to the nicotinic AcChoR. The high-affinity-binding site is regulated by cholinergic effectors in a manner opposite to that of PCP and most other noncompetitive blockers and is allosterically coupled to the PCP site. Affinity labeling suggests that PCP and other noncompetitive blockers interact with a site distinct from that for (--)ANMC. These results indicate that benzomorphans opiate bind to a unique site on the AcChoR.

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