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Interaction of a Benzomorphan Opiate with Acetylcholinesterase and the Nicotinic Acetylcholine Receptor

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SUMMARY

The benzomorphan opiate, (−)*N*-allylnormetazocine [(−)ANMC, (−)SKF10047], has been shown previously to bind two distinct sites on acetylcholine receptor (AChR)-rich membranes from *Torpedo* electroplaque. The low affinity site seems to correspond to the site for noncompetitive blockers on the AChR. The high affinity site, which can be photoaffinity labeled using UV irradiation, was distinct from this site. We show here, using a variety of techniques, that the high affinity binding site for (−)ANMC is on the acetylcholinesterase (AChE) associated with these membranes. The Triton X-100-solubilized peptide photolabeled with (−)[^{3}H]ANMC co-migrates with acetylcholinesterase activity on velocity sucrose gradient centrifugation and fast protein liquid chromatography. In addition, the labeled peptide cannot be precipitated with monoclonal or polyclonal antibodies raised against the nicotinic AChR but can be precipitated with anti-AChE antibodies. Localization of the binding site on AChE was confirmed by photolabeling of and reversible binding to the 11 S AChE purified from *Torpedo californica*. The binding and photolabeling had characteristics and affinity similar to those for the high affinity binding site in *Torpedo* electroplaque membranes. Competition studies with specific AChE inhibitors suggest that the binding site may be the catalytic site of the enzyme, which exists on the 66-kDa globular protein. The effect of (−) and (+)ANMC on AChE activity was also investigated. ANMC inhibited AChE activity at micromolar concentrations in a stereoselective fashion, with the (−) isomer exhibiting a 2-fold higher affinity than the (+) isomer. The inhibition was consistent with a competitive blockade of AChE activity.

Neuromuscular transmission involves the release of ACh from the presynaptic nerve terminal, ACh interaction with the postsynaptic nicotinic AChR which results in the opening of a relatively nonspecific cation channel, and the hydrolysis of ACh by AChE, (see Ref. 1 for a description of the kinetics of the process). The AChR at the neuromuscular junction or in *Torpedo* electroplaque is comprised of four distinct subunits of protein molecular weight (2–4): α (50,116), β (53,681), γ (56,279—or ϵ 52,568; see Ref. 5), and δ (57,565). These subunits form a glycosylated monomeric complex of 250,000 daltons in the ratio of $\alpha_2\beta\gamma$(or ϵ)δ(6–8). *In vivo*, the complex seems to exist as a disulfide-linked dimer formed by the oxidation of the half-cystines at position 500 of the δ subunit in each monomer. AChE of *Torpedo* electroplaque exists in two forms. The basal lamina contains an asymmetric complex of tetrameric subunits with a collagen-like tail which sediments in sucrose gradients with sedimentation coefficients of 13 S and 17 S (9). The disulfide-linked catalytic subunits sediment at 11 S when cleaved from their collagen-like tails by trypsin proteolysis (lytic form). A globular form of AChE exists as a membrane-bound protein dimer which sediments at 5.6 S (10). The individual catalytic units run slightly differently on sodium dodecyl sulfate-polyacrylamide gels with the asymmetric form appearing somewhat larger (68 kDa versus 66 kDa [10]).

Although a number of drugs and toxins have been shown to interact selectively with either the AChR (e.g., αBgt) or AChE (e.g., DFP), many compounds interact with both proteins, presumably due to similar binding sites. The most obvious example is the physiological ligand, ACh; however, numerous other examples exist including: 1) physostigmine, a therapeutically useful AChE inhibitor, which is both an agonist and channel blocker for the AChR (11); 2) gallamine, a competitive antagonist of the AChR, which also interacts with a site on the AChE (12–14); and 3)D-tubocurarine, an antagonist of the AChR which can also modulate AChE activity (12, 13).

Both proteins contain multiple binding sites for drugs and toxins. The AChR contains three classes of sites: 1) the acetylcholine binding sites, 2) the sites for snake α-neurotoxins, such as αBgt (which probably overlap the ACh binding sites but also include other sites of attachment), and 3) the site for noncompetitive blockers such as PCP and local anesthetics. The active

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ABBREVIATION: ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; ANMC, *N*-allylnormetazocine, SKF10047; αBgt, α-bungarotoxin; DFP, diisopropylfluorophosphate; EGTA, [ethylene bis(oxyethylenenitrilo]tetraacetic acid; FPLC, fast protein liquid chromatography; MOPS, 3[*N*-morpholino]propanesulfonic acid; PCP, phencyclidine.

site of AChE consists of both an esteratic and anionic subsite (15) which are involved in the binding and hydrolysis of ACh. A peripheral anionic site, capable of modulating enzyme activity, is also present on the AChE (12, 13). The binding of various compounds to sites other than those for ACh may affect responses of the ion channel or the enzyme.

We previously reported (16, 17) two distinct binding sites for the benzomorphan opiate (−)[^{3}H]ANMC on AChR-rich membranes from *Torpedo* electroplaque. Binding of (−)ANMC was decreased by carbamylcholine and other cholinergic effectors in an αBgt-insensitive fashion. This opiate was found to photoaffinity label efficiently a 66-kDa peptide in that 60–70% of the bound radioactivity was incorporated in a carbamylcholine-sensitive fashion. In the absence of β-mercaptoethanol, the label migrated as a 135-kDa dimer. Because of the sensitivity of binding and affinity labeling to cholinergic agents, the labeling of a peptide nominally co-migrating with the δ subunit, and the existence of the labeled peptide as a dimer in the absence of β-mercaptoethanol, the high affinity binding site was originally thought to be on the AChR (16, 17). We report here recent studies demonstrating that this site is actually located on the catalytic subunit of AChE. Like the AChR, the AChE catalytic subunit binds cholinergic ligands and, like the δ subunit of the AChR, migrates as a 66- or 68-kDa peptide which exists as a dimer in the absence of reducing agents (9, 10). The binding of (−) and (+)ANMC to the AChE inhibits the activity of AChE by an apparent competitive mechanism.

Experimental Procedures

Preparation of AChR-rich membrane fragments. Freshly dissected electric organ from *Torpedo californica* or frozen tissue from *Torpedo nobiliana* was minced, resuspended in an equal volume of cold 10 mM sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 10 mM iodoacetamide, and homogenized in 200-g batches in a Waring blender for 2 × 1 min at maximum speed at 4°. The homogenate was spun in a Beckman JA-10 rotor for 10 min at 6,500 rpm. Supernatants were filtered through two layers of gauze into a chilled flask. Pellets were rehomogenized with an equal volume of buffer and spun as above. Pooled supernatants were spun in a JA-10 rotor for 2 hr at 10,000 rpm at 4°. Pellets were rehomogenized with 20% w/v sucrose in 1 mM NaH_2PO_4 buffer (pH 7.0), 10 mM EDTA, and 0.02% NaN_3 at 4° using a Potter-Elvehjem homogenizer. Sucrose gradients in this resuspension buffer were prepared and run as previously described by Sobel *et al.* (18). The concentration of ^{125}I-αBgt sites was measured by the DE81 filter disc assay (19) using 0.1% w/v Triton X-100 in 10 mM Tris (pH 7.4) as the buffer.

Photoaffinity labeling. *Torpedo* membranes were incubated 45 min at room temperature with 50–150 nM [^{3}H]ANMC before being irradiated in polystyrene dishes by UV light (256 nm) from a distance of 7 cm. Free radioligand was removed by repeated washing of membranes pelleted in an Eppendorf microcentrifuge for 15 min. Incubation for 20 min with 1% v/v Triton X-100 in buffer solubilized most of the membrane fragments. Soluble proteins were recovered in the supernatant after insoluble material was pelleted by a 15-min centrifugation in an Eppendorf microcentrifuge.

Alternatively, 11 S AChE was incubated with 50 nM (−)[^{3}H]ANMC for 15 min and irradiated with UV light for 15 sec. The labeled protein was then prepared for analysis on 8% acrylamide/0.13% bisacrylamide sodium dodecyl sulfate gels as described by Laemmli (20). Gels were prepared for fluorography using Autofluor (National Diagnostics), dried, and exposed to Kodak XAR-5 film (preflashed to an absorbance of 0.2) at −80° for 1–2 days.

Binding assays. Equilibrium binding of (−) and (+)[^{3}H]ANMC to solubilized *Torpedo* membranes or purified 11 S protein was performed as previously described for membrane fragments (16) except that Schleicher & Schuell No. 32 filters, presoaked in 0.33% polyethylenimine (see Ref. 21), were used and that assays were performed at 4° to slow the dissociation of receptor-ligand complexes. In some cases, varying amounts of AChE inhibitors were included in the incubations. Nonspecific binding was determined by including a 200-fold excess of the unlabeled ANMC isomer. When used, carbamylcholine was present at 0.2 mM. Centrifugation assays were used to measure [^{3}H]ANMC binding to AChR-rich membranes as described previously (16).

Affinity gel. *T. californica* membranes, at 2 μM in αBgt binding sites were photoaffinity-labeled and solubilized as described. The preparation was then incubated with αBgt-linked Sepharose-4B beads (a 2-fold excess concentration) in the presence or absence of 0.2 or 10 mM carbamylcholine for 2 hr at 25° with mixing. Beads were removed by centrifugation for 15 min in an Eppendorf microcentrifuge. Aliquots of the supernatants were assayed for tritium radioactivity.

Alternatively, unlabeled membranes were solubilized and incubated

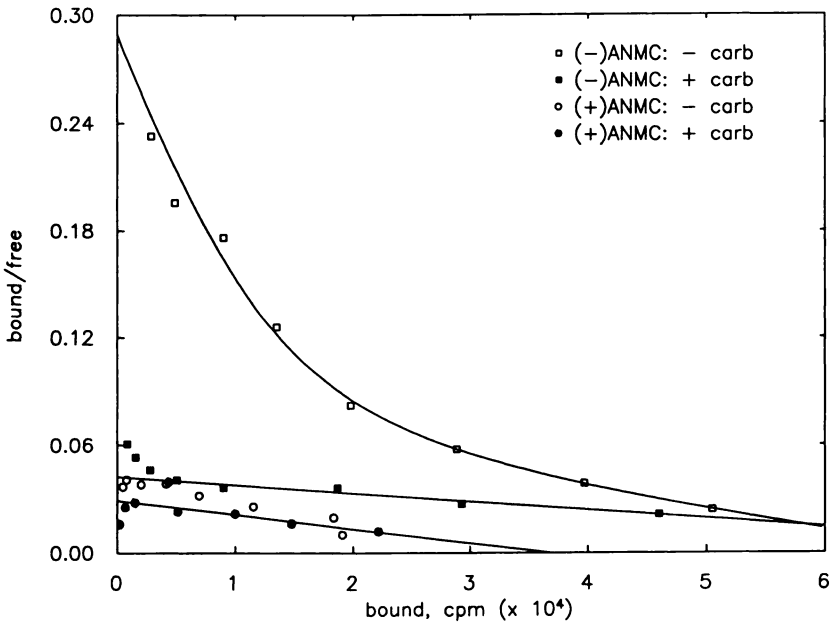

Fig. 1. Scatchard plot of the binding of (−) and (+)[^{3}H]ANMC to AChR-rich membranes from *T. nobiliana*. A centrifugation assay was used, and the concentration of carbamylcholine was 0.2 mM when present. The membrane concentration was 0.4 μM expressed in binding sites for α-^{125}I-Bgt.

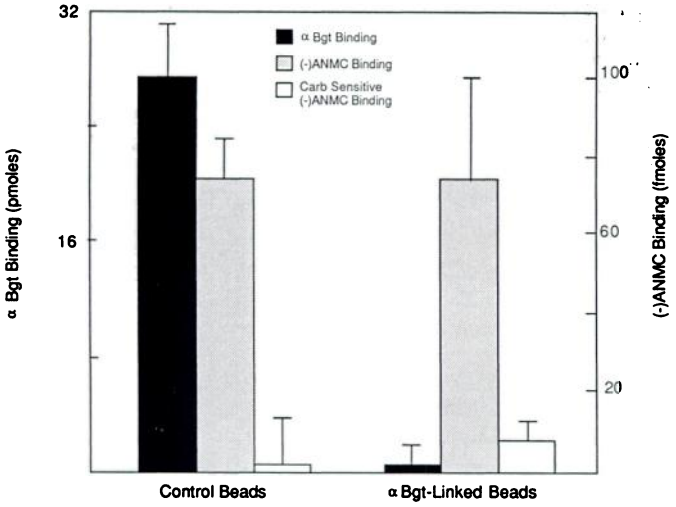

Fig. 2. Amounts of (−)[^{3}H]ANMC and α-^{125}I-Bgt which remained after incubation with affinity resin. Triton X-100-solubilized AChR-rich membranes from *T. nobiliana* at 0.14 μM were incubated either with control Sepharose (cyanogen bromide-activated Sepharose-4B that had been inactivated with ethanolamine) or αBgt-Sepharose. The (−)[^{3}H]ANMC concentration was 50 nM and the α-^{125}I-Bgt concentration was 15 nM for binding assays. When used, the carbamylcholine concentration was 0.2 mM.

with αBgt-linked beads (at least 2-fold excess concentration) ± excess αBgt or with an equivalent volume of control beads (i.e., cyanogen bromide-activated Sepharose-4B quenched with ethanolamine). After mixing for 3 hr at 25°, beads were centrifuged as described above, and specific binding of (−)[^{3}H]ANMC and α-^{125}I-Bgt to the supernatants was determined.

Immunoprecipitation. Solubilized membranes, unlabeled or labeled with α-^{125}I-Bgt or photoaffinity-labeled with (−)[^{3}H]ANMC, were mixed for 1 hr with excess monoclonal or polyclonal antibodies directed against the α or δ subunits of the AChR or against the catalytic subunit of AChE. Sheep anti-rat antibodies or fixed *Staphylococcus aureus* cells were then added and incubation proceeded for an additional 3 hr at 25°. Antibody complexes were pelleted by centrifugation for 15 min in an Eppendorf microcentrifuge. Supernatants were sampled for radioactivity or binding activity. *S. aureus* pellets were washed with buffer before being counted in a Beckman gamma counter (^{125}I) or solubilized with 10% Triton X-100 and assayed for radioactivity.

Sucrose gradients. *T. californica* membranes (500 nM in αBgt sites), labeled with α-^{125}I-Bgt or (−)[^{3}H]ANMC and solubilized as described above, were layered on 5–20% (w/v) sucrose gradients in 50 mM MOPS, 1 mM EGTA, 0.1% Triton X-100 (pH 7.5) and spun at 50,000 rpm for 2 hr in a Beckman VTi50 rotor at 4°. The gradients were then drained from the bottom and 0.6-ml fractions were collected.

FPLC separations. Triton X-100-solubilized membranes prepared and labeled as described above were loaded on a Superose 6 column in 50 mM MOPS, 1 mM EGTA, 0.1% Triton X-100 (pH 7.5), and the column was run at constant pressure in a Pharmacia FPLC. Fractions of 1 ml were collected and assayed for AChE activity and [^{3}H]ANMC radioactivity.

AChE assays. AChE activity was measured as described by Ellman *et al.* (22) using acetylthiocholine as a substrate. The development of the yellow reaction product was followed by absorption at 412 nm using an HP 8451A diode array spectrophotometer or a Turner Model 350 spectrophotometer. In the case of the Turner spectrophotometer, the signal was filtered at 0.5 Hz and the data were digitized using PDP 11/24 computer (AR11 analog to digital converter) at 1 Hz and displayed on a graphics terminal. The reaction rate was measured using a linear least squares fit to the data obtained within the first minute.

Materials. Live *T. californica* was purchased from Pacific Biomarine (Venice, CA) and frozen *T. nobiliana* electroplaque from Biofish Associates (Georgetown, MA). Polyclonal and monoclonal antibodies to the AChR were generously supplied by Dr. Jon Lindstrom (Salk Institute). Purified 11 S AChE as well as polyclonal and monoclonal antibodies to AChE were supplied by Prof. Palmer Taylor (University of California, San Diego). The National Institute on Drug Abuse provided both (−) and (+)ANMC as well as (−)[^{3}H]ANMC (45.9 Ci/mmol) and (+)[^{3}H]ANMC (43.6 Ci/mmol). [^{3}H]PCP (48 Ci/mmol) and α-^{125}I-Bgt (70–140 Ci/mmol) were purchased from New England Nuclear and nonradioactive PCP was supplied by the U. S. Pharmacopeial Convention, Inc. (Rockville, MD). Ultrapure sucrose for density gradients was purchased from Bethesda Research Laboratories. Fixed *S. aureus* cells were obtained from Boerhinger Mannheim and *Bungarus multicinctus* venom from Sigma. αBgt was purified from *B. multicinctus* venom as described previously (23) except that Mono Q and Superose 12 columns were used in a Pharmacia FPLC.

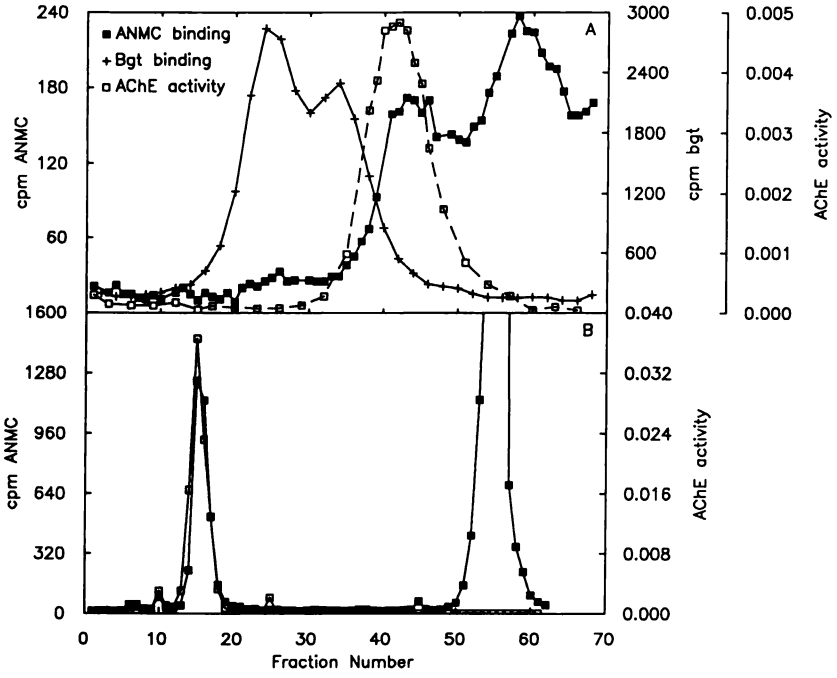

Fig. 3. Sucrose velocity gradient centrifugation (A) and Superose 6 chromatography (B) of Triton X-100-solubilized membranes from *T. nobiliana*. The membranes were prelabeled either with 15 nM α-^{125}I-Bgt or with 50 nM (−)[^{3}H]ANMC (covalently linked with UV light). AChE activity was measured by the hydrolysis of acetylthiocholine. Gradients were fractionated from the bottom. The peak of (−)[^{3}H]ANMC radioactivity at the *top* of the gradient represents the free drug.

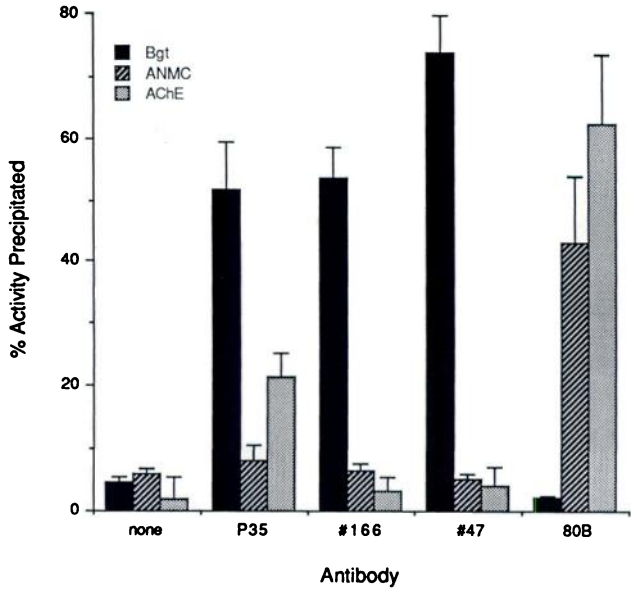

Fig. 4. Immunoprecipitation of α-^{125}I-Bgt-labeled protein, (−)[^{3}H]ANMC-labeled peptide, and AChE activity by anti-AChR and anti-AChE antibodies. *P35* is a polyclonal anti-AChR serum, *#166* is an anti-AChR monoclonal antibody specific for the δ subunit, *#47* is an anti-AChR monoclonal antibody specific for the α subunit, and *80B* is a polyclonal anti-AChE serum. The α-^{125}I-Bgt concentration was 15 nM, and the preparation was prelabeled with (−)[^{3}H]ANMC (50 nM) using UV cross-linking.

Results

Equilibrium Binding of [^{3}H]ANMC

As described previously using a centrifugation assay (16), (−)[^{3}H]ANMC binds to two sites on AChR-rich membranes from *Torpedo* electroplaque with the binding to the high affinity site being modified by carbamylcholine (see Fig. 1). As shown in Fig. 1, the affinity of the high affinity site is greater for (−)[^{3}H]ANMC than (+)[^{3}H]ANMC, and (+)[^{3}H]ANMC binds to fewer sites. The affinity for both isomers is decreased by carbamylcholine.

Following Triton X-100 solubilization of the membrane fragments, carbamylcholine-sensitive (−)[^{3}H]ANMC binding could be measured using a filtration assay. Incubation with αBgt-Sepharose removed all of the detectable αBgt binding activity from the preparation, but essentially none of the (−)[^{3}H]ANMC binding could be removed (Fig. 2). This suggests that the high affinity binding site for (−)[^{3}H]ANMC is *not* on the AChR.

Demonstration that the High Affinity Binding Site Is on AChE

A variety of purification techniques were employed to separate the high affinity (−)ANMC binding component from other proteins in the AChR-rich membranes. *Torpedo* membranes were labeled with (−)[^{3}H]ANMC as described under Experimental Procedures. Following washing of the membrane by centrifugation and solubilization of the pellet, greater than 70% of the remaining (−)[^{3}H]ANMC is incorporated into a peptide of 66 kDa. The remainder of the radioactivity co-migrates with free (−)[^{3}H]ANMC on a Sephadex G-10 column. (In some preparations, a second labeled peptide of 68 kDa was also observed, probably due to small contamination from the basal lamina form of AChE.) After solubilization, the Triton X-100 extract was loaded on a 5–20% w/v sucrose gradient and centrifuged for 2 hr at 50,000 rpm (206,360 × *g*) in a reorienting VTi50 rotor; the tube was drained from the bottom. As shown in Fig. 3A, the AChR, as measured by α-^{125}I-Bgt binding, sediments either as a 9 S monomer or a 13 S dimer. Because the fibrous material is filtered out in the preparation of these membranes, the 5.6 S globular form of the AChE is the predominant form (10) and migrates demonstrably slower than the 9 S AChR. The (−)[^{3}H]ANMC-labeled protein co-migrates with the 5.6 S AChE under these conditions.

As observed with sucrose gradient centrifugation, the (−)[^{3}H]ANMC label co-migrates with AChE activity on a Superose 6 column (Fig. 3B). This column separates proteins according to their diffusion constants, which suggests that the (−)[^{3}H]ANMC label is linked to a protein with the same Stokes radius as AChE.

Both photoaffinity-labeled peptide and unlabeled preparations were tested for interaction with polyclonal and monoclonal anti-AChR and anti-AChE antibodies. As shown in Fig. 4, anti-AChR antibodies are capable of precipitating >50% of the α-^{125}I-Bgt label but less than 15% of the (−)[^{3}H]ANMC radioactivity and AChE activity. The anti-AChR antibodies used included a polyclonal antibody (P35), a monoclonal antibody recognizing the δ subunit (mAb 166), and a monoclonal antibody recognizing the α subunit (mAb 47). A polyclonal anti-AChE antibody 80B did not precipitate α-^{125}I-Bgt-labeled protein but did precipitate about 75% of the AChE activity and (−)[^{3}H]ANMC radioactivity (Fig. 4). Unlabeled preparations, in which the loss of high affinity (−)[^{3}H]ANMC binding from

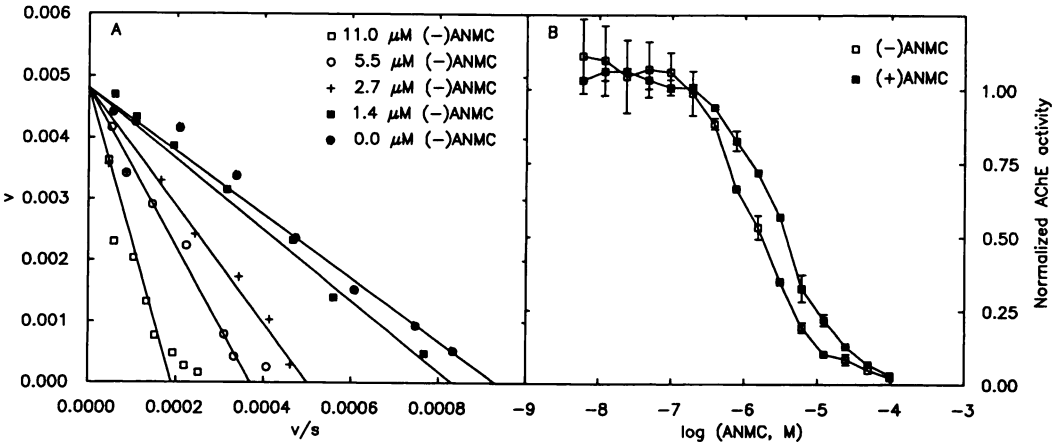

Fig. 5. The effect of ANMC of the activity of AChE from AChR-rich membranes from *T. nobiliana*. A, Eadie-Hofstee plot of AChE activity. Both the acetylthiocholine and the (−)ANMC concentrations were varied with constant dithiobisnitrobenzoic acid (0.32 mM) and membrane (6 nM expressed in α-^{125}I-Bgt binding sites) concentrations. B, inhibition of AChE activity by varying concentrations of (−) and (+)ANMC. Activity was normalized to the condition in which ANMC was omitted. The concentration of acetylthiocholine was 0.48 mM and that of the membranes was 6 nM expressed in binding sites for α-^{125}I-Bgt.

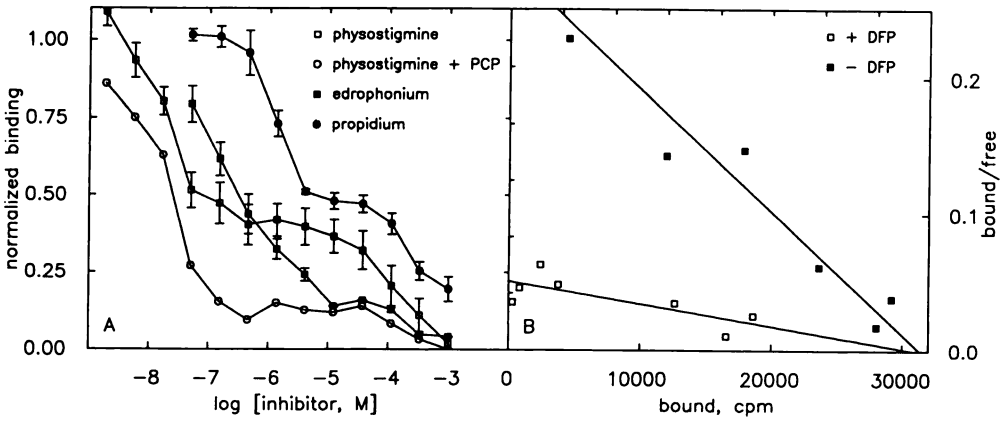

Fig. 6. A, inhibition of (−)[^{3}H]ANMC binding by various AChE inhibitors. In all cases, nonspecific binding, assessed in the presence of 200 μM (−)ANMC, was subtracted and the specific binding was normalized to binding in the presence of (−)[^{3}H]ANMC alone (i.e., no added effectors). The (−)[^{3}H]ANMC concentration was 50 nM and the membranes were 200 nM in α-^{125}I-Bgt sites. *B*, Scatchard transformations of (−)[^{3}H]ANMC binding to membranes treated with or without DFP. The concentration of membranes was 0.5 μM in sites for α-^{125}I-Bgt.

the supernatant was monitored as well as the loss of α-^{125}I-Bgt binding and AChE activity, gave similar results. Taken together, the results of these experiments and those of the previous three sections strongly suggest that the high affinity binding site for (−)[^{3}H]ANMC is on the AChE, in this case, the 5.6 S globular form of the enzyme.

Effect of ANMC on AChE Activity

The effect of (−) and (+)ANMC on the hydrolysis of acetylthiocholine by AChE from AChR-rich *Torpedo* electroplaque membranes was measured. The results, shown in Fig. 5B, demonstrate that the AChE activity is blocked by ANMC with the (−) isomer being approximately 2-fold more potent than the (+) isomer (1.6 versus 3.5 μM). The mechanism of the inhibition was studied further by measuring the apparent K_m of the enzyme in the presence of various concentrations of (−)ANMC. As shown in the Eadie-Hofstee plot of Fig. 5A, the V_{max} remains relatively constant whereas the K_m increases with (−)ANMC concentration, suggesting a competitive inhibition.

Inhibitors. The effect of specific AChE inhibitors on the binding of (−)[^{3}H]ANMC was investigated both to establish clearly that the binding site was on the AChE molecule and to determine the exact locus of the binding site on the AChE. In Fig. 6A, the inhibition of the interaction between (−)[^{3}H]ANMC (50 nM) and AChR-rich *T. californica* membranes (200 nM in α-^{125}I-Bgt binding sites) by specific AChE inhibitors is shown. Edrophonium and propidium were tested because other studies have demonstrated that they are highly specific ligands for the catalytic center and peripheral site, respectively (13). Edrophonium displaced (−)[^{3}H]ANMC binding with higher affinity than propidium, suggesting that the binding site was associated with the catalytic center. Physostigmine, an inhibitor of AChE capable of carbamylating the esteratic site in the catalytic center, is a potent blocker of (−)[^{3}H]ANMC binding. The blockade shows a biphasic concentration dependence with one portion of the inhibition curve showing extremely high affinity (10–20 nM) and another portion low affinity (100 μM). The low affinity binding seems due to interaction with the noncompetitive blocker site on the AChR, as this portion of the curve is not present when assays include 2.5 μM PCP. These results suggest that (−)[^{3}H]ANMC binds to at least the anionic portion of the catalytic center as judged by the inhibition by edrophonium. To determine whether (−)[^{3}H]ANMC could bind if only the esteratic site were blocked, the membranes were treated with DFP. As shown in Fig. 6B, essentially all of the (−)[^{3}H]ANMC binding activity is lost following phosphorylation of the esteratic site by DFP.

Interaction of (−)ANMC with Purified 11 S AChE

Purified catalytic subunits from the asymmetric form of AChE were generously provided for comparison by Prof. Palmer Taylor. When (−)[^{3}H]ANMC is incubated with 11 S AChE instead of membranes and UV irradiated as described under Experimental Procedures, radioactive label is covalently incorporated into a 68-kDa peptide that co-migrates with the catalytic subunit of AChE (Fig. 7). As with AChR-rich membrane fragments, approximately 70% of the bound radioactivity is incorporated. This labeling can be reduced by including either 200 μM carbamylcholine or 200 μM nonradioactive (−)ANMC. The labeled 68-kDa peptide is slightly larger than the 66-kDa peptide labeled in our *Torpedo* membranes. This is presumably due to the fact that the major AChE form in these membrane fragments is the 5.6 S form whose catalytic subunit migrates as a slightly smaller peptide than the catalytic subunit of the 11 S form (10).

Equilibrium binding. Equilibrium binding of both (−) and (+)[^{3}H]ANMC was performed using the purified 11 S form of the enzyme (see Fig. 8A). At 4°, both (−) and (+)[^{3}H]ANMC were capable of binding to the 11 S AChE with K_D values of 0.13 and 0.25 μM, respectively. In the presence of carbamylcholine, most of the binding is lost, presumably because the increased rate of dissociation in the presence of carbamylcholine leads to an artifactual loss of specific binding during the filtration and washing procedure (17).

Binding kinetics. In AChR-rich membranes, the association of (−)[^{3}H]ANMC is markedly modified by the presence or absence of carbamylcholine and by the order of addition of (−)[^{3}H]ANMC and carbamylcholine (17). *Prior* addition of carbamylcholine followed by the addition after 10–15 min of (−)[^{3}H]ANMC leads to a rapid followed by a slow rate of association of (−)[^{3}H]ANMC with the membrane fragments. *Simultaneous* addition of both carbamylcholine and (−)[^{3}H]ANMC leads to a marked stimulation of radioligand binding within 5 sec to a level greater than that observed at any time point under *prior* addition conditions. This is followed by a decrease in binding to the same equilibrium level observed under *prior* addition conditions (17). Similar results are observed with purified 11 S AChE, as shown in Fig. 8B. Rapid binding under conditions of simultaneous addition is not merely reflective of a slow association of carbamylcholine with the enzyme because binding of (−)[^{3}H]ANMC under these conditions reaches at least a 2-fold higher level than is observed in the absence of carbamylcholine. Since the binding of (−)[^{3}H]ANMC is probably to the catalytic center, the stimulation of

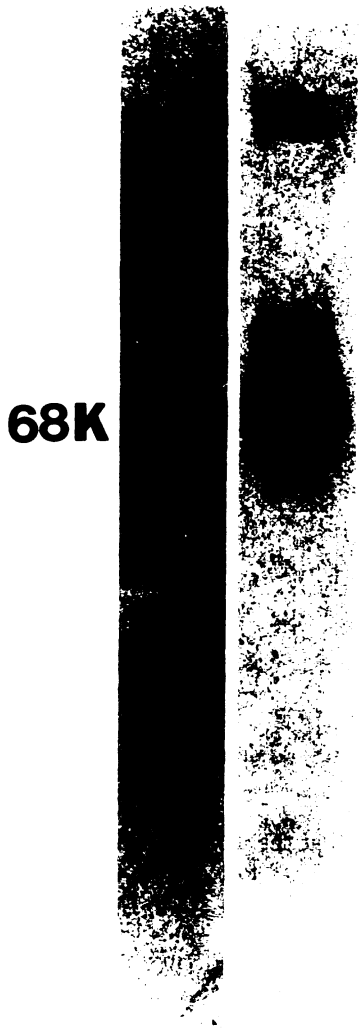

Fig. 7. Covalent incorporation of (−)[^{3}H]ANMC into the catalytic subunit of the 11 S AChE by UV irradiation. AChE (concentration of 120 μg/ml) was incubated with 50 nM (−)[^{3}H]ANMC for 15 min and the preparation was irradiated with 254 nm light for 15 sec. Shown are a Coomassie Blue stain of the protein (A) and a fluorogram (B) showing the position of the radioactive label.

binding may reflect the interaction of carbamylcholine with another site capable of allosterically regulating activity of the catalytic center.

Discussion

The interaction of (−) and (+)ANMC with AChE and the functional consequences of that interaction have been described. We have observed both (−) and (+)[^{3}H]ANMC binding to membrane bound and solubilized proteins of *Torpedo* electroplaque. The characteristics of the high affinity binding are not altered by selective removal of the AChR. Further evidence that the carbamylcholine-sensitive binding site was not on the AChR included the inability of a variety of antibodies directed against the AChR to precipitate ANMC binding activity. Antibodies against AChE did, however, precipitate the high affinity binding protein. Furthermore, (−)[^{3}H]ANMC binding comigrates with AChE activity in both sucrose gradients and FPLC column chromatography. Indications of ANMC binding to AChE were supported by similar kinetics of carbamylcholine-sensitive binding to purified AChE and efficient photolabeling of those catalytic subunits. ANMC also inhibits AChE activity in a stereoselective fashion by an apparent competitive mechanism. Results of competition studies employing ligands specific for different sites on the AChE suggest that (−)[^{3}H]ANMC binds to the catalytic center and is affected by agents specific for both the anionic and esteratic subsites.

Previous work has suggested that (−)ANMC binds to two distinct binding sites in *Torpedo* AChR-rich membranes (16, 17). The lower affinity site was identical to the PCP binding site and, as such, was assumed to be associated with the AChR. The higher affinity site was originally thought to be on the AChR due to several factors. Binding was sensitive to many types of cholinergic ligands, the photoaffinity-labeled product co-migrated with the δ subunit of the AChR, and, like the δ subunit of the AChR, the affinity-labeled product migrated as a dimer in the absence of β-mercaptoethanol. The catalytic subunit of AChE also binds cholinergic ligands and the globular form migrates as a 66-kDa peptide. Similiarly, it exists as a disulfide-linked dimer in the absence of reducing agents (9, 10). The studies described here conclusively demonstrate that the high affinity binding site for ANMC is on the catalytic subunit of the AChE.

The catalytic subunit of AChE contains two binding loci: 1) a catalytic center which is responsible for the enzymatic activity and which includes both an anionic and esteratic subsite and 2) a peripheral site which can allosterically regulate the activity of the catalytic center. The bulk of the data presented here suggest that (−)[^{3}H]ANMC interacts with the catalytic center. The apparent competitive inhibition of AChE activity by (−)ANMC and the relatively high affinity inhibition by physostigmine and edrophonium (but not propidium) support this conclusion. Assuming the binding is to the catalytic center, the rapid acceleration of binding immediately after carbamylcholine addition is presumably due to the interaction of carbamylcholine with another site on the enzyme which could in turn transiently increase the affinity of (−)[^{3}H]ANMC for the catalytic center. The decrease in binding following the initial stimulation could then either be attributed to a relaxation of the conformational state of the enzyme or the carbamylation of the esteratic subsite of the catalytic center by carbamylcholine. The time course of the carbamylation of AChE by carbamylcholine occurs with a half-time of approximately 1 min (24), which is approximately what is observed for a decrease in binding of (−)[^{3}H]ANMC to both the purified enzyme (Fig. 6B) and *Torpedo* membranes (17). The exact location of the (−)[^{3}H]ANMC binding site is particularly important because (−)[^{3}H]ANMC is a highly efficient photoaffinity label (16) and will be useful for defining the position of the binding site on the primary structure of the catalytic subunit of the AChE (25).

The binding of ANMC to the AChR seems to occur at the same site as PCP—the site for noncompetitive blockers (16, 17). The relatively rapid dissociation makes studying the kinetics of radioligand binding to this site extremely difficult. On

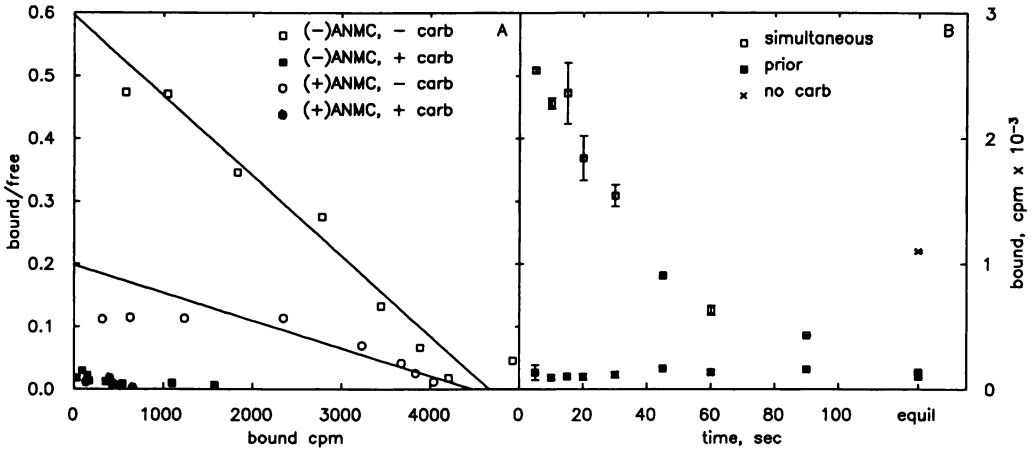

Fig. 8. A, equilibrium binding of (−) and (+)[^{3}H]ANMC to the purified 11 S form of the *T. californica* AChE. A filtration assay was used, and the concentration of AChE was 35 μg/ml. B, kinetics of (−)[^{3}H]ANMC binding to the purified 11 S *T. californica* AChE. The concentration of AChE was 35 μg/ml and, when used, the concentration of carbamylcholine was 0.2 mM. Carbamylcholine was either incubated with AChE *prior* to the addition of (−)[^{3}H]ANMC or added *simultaneously* with (−)[^{3}H]ANMC. A filtration assay was used.

the other hand, we (26),[1] and others (27) have studied the effects of ANMC on single channel events of the AChR and endplate currents. At concentrations greater than 10 μM, Kapai *et al.* (27) observed a decrease in the peak amplitude and a shortening of the mean decay time constant of the endplate current at the frog neuromuscular junction. At lower concentrations, a slight prolongation of the decay time constant was observed with the (+) isomer, suggestive of an effect on AChE. Kapai *et al.* (27) also observed a nonstereoselective shortening of the mean channel lifetime and burst duration. Our results (26[1]) suggest that ANMC is not a simple sequential blocker of the open channel (28) in that the channel seems capable of closing (as opposed to remaining blocked but open) while ANMC is bound.

In summary, both (−) and (+)ANMC are capable of interacting with both AChE and the nicotinic AChR. The binding affinity is greater for the AChE; however, effects on AChE and the AChR occur at similar concentrations. The efficient photolabeling of AChE by (−)[^{3}H]ANMC will provide a useful reagent for defining the structural characteristics of the important binding sites on the catalytic subunit of AChE.

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