

Photoaffinity Labeling the Torpedo Nicotinic Acetylcholine Receptor with [³H]Tetracaine, a Nondesensitizing Noncompetitive Antagonist

RICHARD E. MIDDLETON,¹ NINA P. STRNAD, and JONATHAN B. COHEN

Department of Neurobiology, Harvard Medical School, Boston, Massachusetts

Received March 23, 1999; accepted May 18, 1999

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Tetracaine (*N,N*-dimethylaminoethyl-4-butylaminobenzoate) and related *N,N*-dialkylaminoethyl substituted benzoic acid esters have been used to characterize the high-affinity binding site for aromatic amine noncompetitive antagonists in the *Torpedo* nicotinic acetylcholine receptor (nAChR). [³H]Tetracaine binds at equilibrium to a single site with a K_{eq} value of 0.5 μ M in the absence of agonist or presence of α -bungarotoxin and with a K_{eq} value of 30 μ M in the presence of agonist (i.e., for nAChR in the desensitized state). Preferential binding to nAChR in the absence of agonist is also seen for *N,N*-DEAE and *N,N*-diethylaminopropyl esters, both binding with 10-fold higher affinity in the absence of agonist than in the presence, and for the 4-ethoxybenzoic acid ester of *N,N*-diethylamin-

oethanol, but not for the 4-amino benzoate ester (procaine). Irradiation at 302 nm of nAChR-rich membranes equilibrated with [³H]tetracaine resulted in covalent incorporation with similar efficiency into nAChR α , β , γ , and δ subunits. The pharmacological specificity of nAChR subunit photolabeling as well as its dependence on [³H]tetracaine concentration establish that the observed photolabeling is at the high-affinity [³H]tetracaine-binding site. Within α subunit, $\geq 95\%$ of specific photolabeling was contained within a 20-kilodalton proteolytic fragment beginning at Ser¹⁷³ that contains the M1 to M3 hydrophobic segments. With all four subunits contributing to [³H]tetracaine site, the site in the closed channel state of the nAChR is most likely within the central ion channel domain.

The muscle nicotinic acetylcholine receptor (nAChR) consists of four homologous subunits ($\alpha_2\beta\gamma\delta$) arranged pseudosymmetrically around a central axis that is a cation-selective ion channel. Each subunit has a common primary structure motif: a hydrophilic, extracellular N-terminal half containing amino acids of the agonist-binding sites, followed by three hydrophobic membrane spanning segments (M1–M3), a cytoplasmic domain, a fourth transmembrane segment (M4), and short extracellular C-terminal tail. Affinity labeling studies, site-directed mutagenesis, and low-resolution (9 Å) cryoelectron microscopy provide considerable information about nAChR structure (reviewed in Karlin and Akabas, 1995; Hucho et al., 1996; Unwin, 1998). The two agonist-binding sites, which are located extracellularly at the α - γ and α - δ subunit interfaces, are composed of multiple loops of primary structure from α and γ (or δ) subunits (reviewed in Prince and Sine, 1998). M2 domains from each subunit line

the pore of the ion channel (Imoto et al., 1988; Unwin, 1995), with additional contributions from the extracellular ends of the M1 segments (Zhang and Karlin, 1997), whereas M3 and M4 segments are more peripheral and in contact with lipid (Blanton and Cohen, 1994).

Noncompetitive antagonists (NCAs) block the nAChR permeability response without preventing the binding of ACh (acetylcholine). A structurally diverse group of drugs act as NCAs, including many aromatic amines, general anesthetics, fatty acids, steroids, and neuropeptides such as Substance P (reviewed in Arias, 1998). Studies of the binding of the aromatic amines [³H]meproadifen and [³H]phencyclidine (PCP) or of the spiroperidine [³H]histrihnicotoin ([³H]HTX) to nAChR-rich membranes from *Torpedo* electric organ establish that each binds with high affinity ($K \sim \mu$ M) to one site per nAChR and to additional lower-affinity sites (Heidmann et al., 1983). The high-affinity site is linked allosterically to the ACh site, with most aromatic amines binding at equilibrium with highest affinity in the presence of agonist [i.e., to the desensitized state of the nAChR (Cohen et al., 1985; Arias, 1996; Lurtz and Pedersen, 1999)].

This work was supported in part by U.S. Public Health Service Grant NS19522.

¹ Present address: Merck Research Laboratories, Rahway, New Jersey 07065.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; NCA, noncompetitive antagonist; V8 protease, *Staphylococcus aureus* glutamyl endopeptidase; GSSG, oxidized glutathione; [³H]HTX, [³H]histrihnicotoin; [¹²⁵I]TID, 3-(trifluoromethyl)-3-(*M*-[¹²⁵I]iodophenyl) diazine; HTX, *dl*-perhydrohistrihnicotoin; H₁₀-HTX, *dl*-decahydro(pentenyl)histrihnicotoin; PAGE, polyacrylamide gel electrophoresis; PCP, phencyclidine; TPS, *Torpedo* physiological saline.

Affinity-labeling studies have identified homologous residues near the cytoplasmic end of each M2 segment that contribute to the high-affinity binding site in desensitized *Torpedo* nAChRs for the aromatic amine NCAs [³H]chlorpromazine (Revah et al., 1990) and [³H]trimethylphenylphosphonium (Hucho et al., 1986). Mutational analyses also implicate these amino acids as affinity determinants for the aromatic amine QX-222 (Charnet et al., 1990) and for aliphatic alcohols (Forman, 1997) acting as open channel blockers. However, in each conformational state, there may be distinct, nonoverlapping sites for positively charged NCAs, and different regions within the ion channel may contribute to the binding site for a single ligand in different conformational states. In the desensitized nAChR, [³H]meproadifen mustard reacts with αGlu²⁶² at the extracellular end of M2 (Pedersen et al., 1992), and based on fluorescence energy transfer, the binding site for ethidium is located above the level of the bilayer in the vestibule of the channel (Johnson and Nuss, 1994, but see Lurtz et al., 1997). In the open channel state, [³H]quinacrine azide is photoincorporated into amino acids within αM1 (DiPaola et al., 1990), and mutations within αM1 affect quinacrine potency as an NCA but not chlorpromazine (Tamamizu et al., 1995). Photoaffinity labeling studies with 3-(trifluoromethyl)-3-(*M*-[¹²⁵I]iodophenyl) diazirine ([¹²⁵I]TID; White and Cohen, 1992) and [³H]diazofluorene (Blanton et al., 1998), uncharged, hydrophobic NCAs, have identified a binding site in the M2 domain in the absence of agonist (closed channel), as well as changes in structure of the M2 domain between resting and desensitized states. However, the TID site in the closed channel appears distinct from the binding site for PCP because PCP does not inhibit [¹²⁵I]TID photoincorporation and TID does not inhibit [³H]PCP binding (White et al., 1991).

Tetracaine (dimethylaminoethyl-*p*-butylaminobenzoate) is an unusual aromatic amine NCA because it is 100-fold more potent as an inhibitor of [³H]HTX binding ($K \approx 1 \mu\text{M}$) in the absence of agonist than in the presence (Blanchard et al., 1979), and it stabilizes the resting state rather than the desensitized state of the nAChR (Boyd and Cohen, 1984). Here, we characterize the binding properties of several structural analogs of tetracaine to further define the requirements for preferential binding to the closed channel state, and we use [³H]tetracaine itself as an intrinsic photoaffinity reagent to define the structure of its high-affinity binding site in the nAChR in the absence of agonist. [³H]Tetracaine is specifically photoincorporated with similar efficiency into each nAChR subunit. In the following report (Gallagher and Cohen, 1999), we identify the homologous amino acids in the M2 segment of each subunit that contribute to this high-affinity [³H]tetracaine site.

Experimental Procedures

Materials. [*ring*-3,5-³H]Tetracaine ([³H]tetracaine, 36 Ci/mmol) and [³H]HTX (60 Ci/mmol) were prepared at New England Nuclear Research Products (Boston, MA) by tritium gas catalytic reduction of 3,5-dibromotetracaine and *dl*-decahydro(pentenyl)histronicotoxin (H₁₀-HTX), respectively. For binding experiments, [³H]tetracaine was purified to >95% by silica thin-layer chromatography (5:4:1 cyclohexane/chloroform/diethylamine; $R_f = 0.17$). When stored in ethanol, [³H]tetracaine decomposed at ~10% per month, forming tritiated degradation products that did not partition into nAChR-rich membranes or bind to glass filters. Also, these degradation

products did not appear to photoincorporate into nAChR-rich membranes because the same [³H]tetracaine photolabeling patterns were seen with [³H]tetracaine of 95 or 50% radiochemical purity (not shown).

H₁₀-HTX and *dl*-perhydrohistronicotoxin (H₁₂-HTX) were kindly provided by Dr. Y. Kishi (Harvard University, Cambridge, MA). Proadifen was obtained from SmithKline Beecham (Philadelphia, PA). Piperocaine was obtained from Eli Lilly (Indianapolis, IN). Tetracaine, procaine, carbamylcholine chloride, *d*-tubocurarine chloride, oxidized glutathione (GSSG), and endoglycosidase H were obtained from Sigma Chemical Co. (St. Louis, MO). α-Bungarotoxin was purchased from Biotoxins Inc. (St. Cloud, FL). PCP was obtained from Alltech Associates. *Staphylococcus aureus* V8 protease (V8 protease) was obtained from ICN Biochemicals (Costa Mesa, CA).

The 2-(diethylamino)ethyl- and 3-(diethylamino)propyl esters of *p*-butylaminobenzoic acid were synthesized by coupling with diisopropylcarbodiimide using chemicals from Aldrich Chemical (Milwaukee, WI). For synthesis of the DEAE ester, 2 g (0.01 mol) of 4-(butylamino)benzoic acid, 1.8 ml (0.011 mol) of diisopropylcarbodiimide, and 0.2 g (0.0013 mol) of 4-pyrrolidinopyridine were stirred for 5 min at room temperature in 10 ml of methylene chloride, and then 3 equivalents (6 ml) of *N,N*-diethylethanamine were added and allowed to react overnight. After removal of methylene chloride under vacuum, the residue was resuspended in anhydrous ether, with the insoluble diisopropyl urea removed by filtration. The filtrate was washed with 5% NaHCO₃ and water, and the organic layer was dried over anhydrous Na₂SO₄. HCl gas was bubbled into the ethereal solution to precipitate the dihydrochloride salt that was collected by filtration and then recrystallized three times from ethanol ether. Two grams of product (55% yield) was obtained. A similar synthesis starting with 3-diethylamino-1-propanol yielded 3.4 g of product (60% yield). Appropriate elemental analyses and NMR spectra were obtained for each compound. Meproadifen iodide was prepared by reaction of proadifen base with methyl iodide.

nAChR-rich membranes were isolated from the electric organs of *Torpedo californica* (Marinus, Inc., Westchester, CA) as described (Pedersen and Cohen, 1990). The final membrane pool was stored in 38% sucrose, 0.02% NaN₃ at -80°C under argon and contained 1 to 2 nmol of acetylcholine-binding sites/mg of protein, as measured by a [³H]ACh centrifugation assay (Pedersen et al., 1986).

Radioligand Binding Assays. The equilibrium binding of [³H]tetracaine to *Torpedo* nAChR-rich membranes in *Torpedo* physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM sodium phosphate, pH 7.0) was assayed by centrifugation. Membrane suspensions (~400 nM ACh sites) were equilibrated at 4°C for 4 h with varying concentrations of [³H]tetracaine (0.5 Ci/mmol). Membranes were also preequilibrated with agonist or competitive antagonist, or with excess nonradioactive tetracaine (50–100 μM) to define nonspecific binding. Aliquots (0.1 ml) were then centrifuged in a Beckman Airfuge at 100,000g for 10 min at ~8°C. After removal of the supernatants, membrane pellets were resuspended in 0.1 ml of 10% SDS, and pellet and supernatant ³H were determined by liquid scintillation counting. This centrifugation assay was also used to quantify the concentration of high-affinity [³H]tetracaine sites compared with the concentration of [³H]ACh and/or [³H]HTX sites measured simultaneously with the same membrane suspension. For studies with [³H]ACh, membrane suspensions were pretreated with 0.3 mM diisopropylphosphofluoridate to inhibit cholinesterase activity. [³H]HTX was diluted isotopically with H₁₂-HTX to produce a final radiochemical specific activity of 2 Ci/mmol for binding assays.

[³H]HTX and [³H]tetracaine binding were also determined by a filtration assay using glass-fiber filters (2.5 cm, No. 32; Schleicher & Schuell, Keene, NH) that had been pretreated with an organosilane (1.0% Prosil; Lancaster Synthesis, Windham, NH). Membrane aliquots (typically 100–200 μl) were applied to the filters in the absence of vacuum to achieve a uniform spread of the suspension over the

filter surface; then, vacuum was applied, and the filter was washed with 5 ml of TPS at 4°C.

Photoaffinity Labeling of nAChR-Rich Membranes with [³H]Tetracaine. Membrane suspensions (30 μl, 1.5–1.8 mg protein/ml) in TPS were equilibrated at room temperature with [³H]tetracaine (4 Ci/mmol) and cholinergic ligands and then irradiated for 30 min with a 302-nm lamp (Spectroline EB-280C, 1150 μW/cm²) at a distance of 12 cm as described (Pedersen and Cohen, 1990). Unless indicated otherwise, suspensions also included 50 mM GSSG as an aqueous photochemical scavenger. The 96-well microtiter plate containing the samples was placed in a water bath during photolysis, so the temperature increased <2°C during 30 min of irradiation. After photolysis, samples were usually prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by the direct addition of 10 μl of 4× sample loading buffer to reaction mixtures. To quantify the dependence of ³H incorporation as a function of free [³H]tetracaine, after photolysis the reaction mixtures were centrifuged, with the supernatants assayed to determine free [³H]tetracaine, and the pellets dissolved in sample loading buffer. In preliminary experiments, the efficiency of [³H]tetracaine photoincorporation into nAChR-rich membranes was found to be 10 times higher for the 302-nm lamp than for lamps of 254 or 365 nm of similar radiant flux density (not shown).

Gel Electrophoresis. Polypeptides were resolved by SDS-PAGE on 8% acrylamide gels with a modified Laemmli buffer system (White and Cohen, 1988), and the incorporation of [³H]tetracaine was determined by fluorography or quantified by scintillation counting of gel slices as described previously (Middleton and Cohen, 1991). Proteolytic mapping of the [³H]tetracaine-labeled α subunit with *S. aureus* V8 protease was performed according to the procedure of Cleveland et al. (1977) as described by White and Cohen (1988). Membrane suspensions (540-μg aliquots) were photolabeled with [³H]tetracaine and then electrophoresed on an 8% acrylamide gel. After a brief staining with Coomassie brilliant blue and destaining, the band containing α subunit was excised and transferred to the well of a mapping gel (15% acrylamide) to which V8 protease (3 μg) was added. ³H incorporation into the proteolytic fragments of [³H]tetracaine-labeled α subunit was analyzed by fluorography and by scintillation counting of gel slices.

Data Analysis The equilibrium binding of [³H]tetracaine and the concentration dependence of ³H incorporation into nAChR subunits were fit by nonlinear least-squares (SigmaPlot; Jandel Scientific, San Rafael, CA) to the function $[B = A/[1 + (K_{eq}/L)] + m * L]$, where

B is the bound [³H]tetracaine (or cpm incorporated), *A* is the maximum specific binding (or cpm incorporated), *K_{eq}* is the dissociation constant (or apparent dissociation constant, *K_{AP}*), *L* is the measured free [³H]tetracaine concentration, and *m* is the slope of nonspecific binding (or labeling), which was usually determined in parallel experiments performed in the presence of excess nonradioactive tetracaine or H₁₀-HTX. For labeling experiments, the measured free ³H cpm was adjusted to account for the ≈50% radioimpurities known to be present in the [³H]tetracaine used for photolabeling.

The concentration dependence of the inhibition of reversible binding of [³H]HTX or [³H]tetracaine and of [³H]tetracaine photolabeling of nAChR subunits was fit to a function $[B = A/[1 + (I/K_i)^n] + NSP]$, where *B* is the ³H cpm bound (or incorporated) in the presence of inhibitor at a total concentration, *I*; *A* is the specific ³H cpm bound (or incorporated) in the absence of inhibitor; *K_i* is the apparent inhibitor dissociation constant; *n* is the Hill coefficient, and NSP is the observed nonspecific binding or labeling, which was not treated as an adjustable parameter. The parameter *n_H* was treated as adjustable only if inhibition curves deviated from a single-site model (*n_H* = 1). Under the assay conditions used, with the concentration of free [³H]HTX or [³H]tetracaine much less than their *K_{eq}* value, *K_i* will be close to the inhibitor equilibrium dissociation constant when *n_H* = 1 and the total inhibitor concentration is a good approximation of the free concentration.

Results

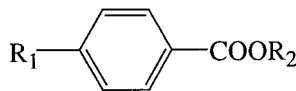
Inhibition of [³H]HTX Binding by Tetracaine Analogs. Because tetracaine's preferential binding to the closed channel state of the nAChR appeared unusual for aromatic amine NCAs, we first examined other benzoic acid esters as inhibitors of [³H]HTX binding to identify structural features responsible for tetracaine's binding properties (Table 1). All drugs at high concentrations completely inhibited specific binding of [³H]HTX, with the concentration dependence of inhibition for each drug consistent with competition at a single site. With the exception of procaine (V), all bound with highest affinity in the absence of agonist. For the esters of *p*-butylaminobenzoic acid, replacement of the dimethylaminoethyl of tetracaine (I) by DEAE (II) resulted in a ~6-fold increase in binding affinity in both the absence and presence

TABLE 1

Equilibrium binding of tetracaine analogs to *Torpedo* nAChR NCA site

Values of *K_i* were determined as described in the text by nonlinear regression fit (*n_H* = 1) of the observed concentration dependence of the inhibition of equilibrium binding at 20°C of [³H]HTX (20 nM) by *Torpedo* nAChR-rich membranes (200 nM ACh sites) in the absence (–) or presence of 0.1 mM carbamylcholine (+ Carb) or *d*-tubocurarine (+dTC). Parameter uncertainties were 5 to 15% of *K_i*.

Compound	R ₁	R ₂	Inhibition of [³ H]HTX Binding <i>K_i</i>		
			–	+ Carb	+dTC
				μM	
I	C ₄ H ₉ NH–	–CH ₂ CH ₂ N(CH ₃) ₂	2.2	43	3.8
II	C ₄ H ₉ NH–	–CH ₂ CH ₂ N(C ₂ H ₅) ₂	0.4	5.7	0.5
III	C ₄ H ₉ NH–	–CH ₂ CH ₂ CH ₂ N(C ₂ H ₅) ₂	0.3	2.6	0.2
IV	C ₂ H ₅ O–	–CH ₂ CH ₂ N(C ₂ H ₅) ₂	1.6	30	3.3
V	NH ₂ –	–CH ₂ CH ₂ N(C ₂ H ₅) ₂	2000	1500	2000
VI	H–	–(CH ₂) ₃ N	0.8	55	2



of agonist. Comparison of procaine (**V**) with compound **II** established that aliphatic substitution at the aryl nitrogen increased affinity for the closed channel conformation by 4000-fold but by only 300-fold for the desensitized conformation. However, high-affinity binding in the absence of agonist did not depend uniquely on the butylamino group because the *p*-ethoxybenzoic acid ester (**IV**) also bound preferentially to the resting state ($K_1 = 1.6 \mu\text{M}$), and piperocaine (**VI**), an unsubstituted benzoic acid ester, was bound with 50-fold higher affinity in the absence of agonist. Thus, selective, high-affinity binding in the absence of agonist appeared to be the rule for many simple benzoic acid esters, with procaine a notable exception.

Equilibrium Binding of $[^3\text{H}]$ Tetracaine. The equilibrium binding of $[^3\text{H}]$ tetracaine to nAChR-rich membranes in TPS at 4°C was determined by centrifugation and filtration assays (Fig. 1A). For $[^3\text{H}]$ tetracaine concentrations to $4 \mu\text{M}$, in each assay the total binding was well fit by a hyperbolic binding function with a linear, nonspecific component. The nonspecific binding calculated from the fit of the total binding function was approximately the same as that measured in the presence of $50 \mu\text{M}$ nonradioactive tetracaine. $[^3\text{H}]$ Tetracaine bound to the same number of high-affinity sites in both assays, with the ratio of sites determined by filtration and centrifugation equal to 1.2 ± 0.2 in three experiments. The same value of the dissociation constant was determined by centrifugation ($K_{\text{eq}} = 0.5 \pm 0.1 \mu\text{M}$) and filtration ($K_{\text{eq}} = 0.6 \pm 0.1 \mu\text{M}$). The nonspecific binding determined by filtration, with a brief (5 s) wash, was only 20% that determined by centrifugation. The partition coefficient, determined as the ratio of the nonspecifically bound to the free tetracaine concentration normalized to the membrane protein concentration was $0.08 \pm 0.02 (\text{mg/ml})^{-1}$ by centrifugation and 0.014 ± 0.005 by filtration. Thus, in the absence of agonist, specific $[^3\text{H}]$ tetracaine binding to the nAChR-rich membranes was characterized by a single K_{eq} , and a filtration assay can be used to quantify equilibrium binding of $[^3\text{H}]$ tetracaine to its high-affinity site.

The filtration assay was used to examine the effects of an agonist (carbamylcholine) and competitive antagonists (α -bungarotoxin and *d*-tubocurarine) on the binding to nAChR-rich membranes of $[^3\text{H}]$ tetracaine at concentrations to $20 \mu\text{M}$ (Fig. 1B). None of the ligands altered the nonspecific binding determined in the presence of $100 \mu\text{M}$ tetracaine (not shown), and $[^3\text{H}]$ tetracaine was bound with similar affinity ($K_{\text{eq}} = 0.5 \pm 0.1 \mu\text{M}$) and to the same number of sites (ratio = 1.1 ± 0.2 , $n = 19$) in the absence and presence of α -bungarotoxin. Partial desensitization of the nAChR with *d*-tubocurarine resulted in $[^3\text{H}]$ tetracaine binding to the same number of sites but with a K_{eq} value of $1.1 \pm 0.6 \mu\text{M}$ ($n = 23$). In the presence of carbamylcholine, which converts nAChRs fully to the desensitized state, $[^3\text{H}]$ tetracaine binding affinity was decreased dramatically, with only $\sim 80\%$ of the expected tetracaine-binding sites occupied at $20 \mu\text{M}$ $[^3\text{H}]$ tetracaine. When the data were fit using the site concentration determined in the presence of α -bungarotoxin or *d*-tubocurarine, K_{eq} was $29 \pm 7 \mu\text{M}$ ($n = 4$).

To determine the number of $[^3\text{H}]$ tetracaine-binding sites per nAChR, $[^3\text{H}]$ tetracaine binding was measured in parallel with binding assays of $[^3\text{H}]$ HTX (in the presence of $100 \mu\text{M}$ carbamylcholine) and $[^3\text{H}]$ ACh. The ratio of $[^3\text{H}]$ tetracaine sites to $[^3\text{H}]$ ACh sites was 0.43 ± 0.07 ($n = 19$), and the ratio

of $[^3\text{H}]$ HTX to $[^3\text{H}]$ ACh sites was 0.5 ± 0.1 . Thus, $[^3\text{H}]$ tetracaine and $[^3\text{H}]$ HTX each bound to the same number of sites, and this number was half the number of $[^3\text{H}]$ ACh sites.

Inhibition of $[^3\text{H}]$ Tetracaine Binding by NCAs. The desensitizing NCAs PCP, meproadifen, proadifen, and H_{10} -HTX were examined as inhibitors of the equilibrium binding of $[^3\text{H}]$ tetracaine at 4°C in the absence of other cholinergic ligands or in the presence of either α -bungarotoxin or *d*-tubocurarine (Fig. 2). Although HTX binds with similar affinity ($K_{\text{eq}} = 0.3 \mu\text{M}$) in the absence or presence of agonist at 20°C (Blanchard et al., 1979), at 4°C it binds with high affinity ($K_{\text{eq}} = 0.3 \mu\text{M}$) in the presence of agonist but only weakly ($K_{\text{eq}} = 8 \mu\text{M}$) in the absence (Cohen et al., 1985). The four NCAs inhibited $[^3\text{H}]$ tetracaine binding in a concentra-

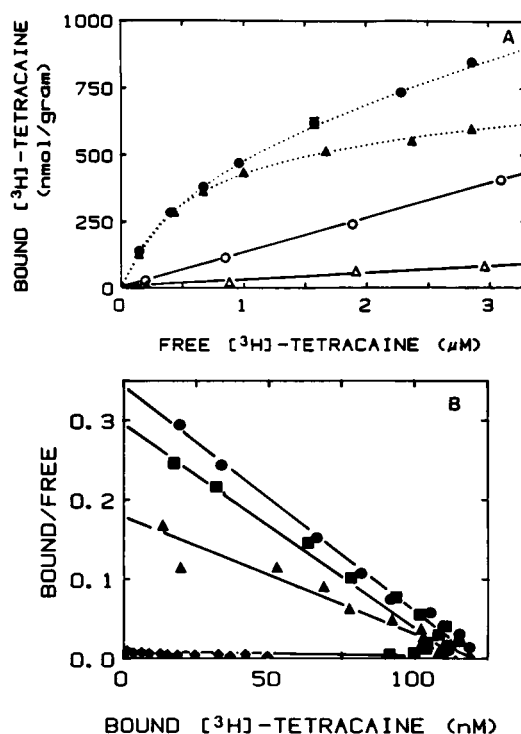


Fig. 1. Equilibrium binding of $[^3\text{H}]$ tetracaine to nAChR-rich membranes. A, nAChR-rich membranes ($0.5 \mu\text{M}$ $[^3\text{H}]$ ACh sites, 0.5 mg/ml) were incubated with $[^3\text{H}]$ tetracaine up to $4 \mu\text{M}$ for 4 h at 4°C in the absence (\bullet , \blacktriangle) or presence (\circ , \triangle) of $50 \mu\text{M}$ nonradioactive tetracaine, and binding was determined by centrifugation (\bullet , \circ) or filtration (\blacktriangle , \triangle) as described in *Experimental Procedures*. Dashed lines are the nonlinear least-squares fit to a hyperbolic function as a single class of sites (B_{max} , K_{eq}) and a linear nonspecific component (slope, m). For total binding by centrifugation, $B_{\text{max}} = 0.23 \pm 0.03 \mu\text{M}$, $K_{\text{eq}} = 0.59 \pm 0.07 \mu\text{M}$, and $m = 0.064 \pm 0.008$. For total binding by filtration, $B_{\text{max}} = 0.31 \pm 0.02 \mu\text{M}$, $K_{\text{eq}} = 0.59 \pm 0.07$, and $m = 0.005 \pm 0.0006$. Calculated specific binding (total binding – directly measured nonspecific binding) by centrifugation was fit by $B_{\text{max}} = 0.25 \pm 0.01 \mu\text{M}$ and $K_{\text{eq}} = 0.61 \pm 0.05 \mu\text{M}$, whereas for filtration, $B_{\text{max}} = 0.27 \pm 0.004 \mu\text{M}$ and $K_{\text{eq}} = 0.51 \pm 0.03 \mu\text{M}$. B, nAChR-rich membranes were preincubated for 30 min in the absence (\bullet) or presence of $100 \mu\text{M}$ carbamylcholine (\blacklozenge) or $50 \mu\text{M}$ *d*-tubocurarine (\blacktriangle) or for 60 min with $4 \mu\text{M}$ α -bungarotoxin (\blacksquare) before the addition of $[^3\text{H}]$ tetracaine at concentrations to $20 \mu\text{M}$. Parallel samples also contained $100 \mu\text{M}$ nonradioactive tetracaine to determine nonspecific binding. After an additional 4 h of incubation at 4°C , bound and free $[^3\text{H}]$ tetracaine were determined by filtration, and at each concentration, specifically bound $[^3\text{H}]$ tetracaine was calculated as the difference between total and nonspecific binding. Specific binding in the absence of cholinergic ligands was characterized by $B_{\text{max}} = 120 \pm 2 \text{ nM}$, $K_{\text{eq}} = 340 \pm 30 \text{ nM}$; with α -bungarotoxin, $B_{\text{max}} = 110 \pm 2 \text{ nM}$, $K_{\text{eq}} = 280 \pm 40 \text{ nM}$; with *d*-tubocurarine, $B_{\text{max}} = 120 \pm 3 \text{ nM}$, $K_{\text{eq}} = 560 \pm 60 \text{ nM}$; and with carbamylcholine, $B_{\text{max}} = 86 \pm 13 \text{ nM}$, $K_{\text{eq}} = 12 \pm 3 \mu\text{M}$. When the data in the presence of carbamylcholine were fit with B_{max} constrained to 120 nM , $K_{\text{eq}} = 21 \pm 1 \mu\text{M}$.

tion-dependent manner, with high concentrations inhibiting [^3H]tetracaine binding by the same extent as 100 μM tetracaine. K_i values for inhibition of [^3H]tetracaine binding were 6- to 10-fold lower in the presence of *d*-tubocurarine than in its absence, consistent with the preferential binding of these NCAs to desensitized nAChR (Heidmann et al., 1983; Cohen et al., 1985). The concentration dependence of inhibition by H_{10} -HTX (Fig. 2A) and PCP (Fig. 2B) was well fit by Hill coefficients of 1 in both the absence and presence of *d*-tubocurarine or α -bungarotoxin, consistent with simple competitive inhibition of [^3H]tetracaine binding. For proadifen (Fig. 2C), the Hill coefficients for inhibition were unitary except in the presence of α -bungarotoxin ($n_H = 1.6$). For meproadifen (Fig. 2D), the dose dependence of inhibition was characterized by $n_H = 1$ in the presence of *d*-tubocurarine but by $n_H = 1.5$ in the absence of ACh site ligand and by $n_H = 1.8$ in the presence of α -bungarotoxin.

Photoincorporation of [^3H]Tetracaine into nAChR-Rich Membranes. To determine whether [^3H]tetracaine could be specifically photoincorporated into its high-affinity binding site, nAChR-rich membranes were equilibrated with 5 μM [^3H]tetracaine and then irradiated at 302 nm for 30 min in the presence or absence of the NCA H_{10} -HTX (30 μM). When the proteins were resolved by SDS-PAGE and the gel was processed for fluorography, it was seen (Fig. 3, lanes 2 and 3) that H_{10} -HTX decreased [^3H]tetracaine incorporation not only in the nAChR subunits but also into another protein of the nicotinic postsynaptic membrane [rapsyn/43 kilodaltons (kDa) protein] and into polypeptides of contaminating membrane fragments such as the α subunit of the Na^+, K^+ -ATPase (90 kDa; White and Cohen, 1988). Photoincorporation into nonreceptor polypeptides was also decreased by the agonist carbamylcholine, which allosterically inhibits [^3H]tetracaine binding (see Figs. 6 and 8). It was improbable that HTX and carbamylcholine actually inhibited [^3H]tetra-

caine binding to specific sites on the Na^+, K^+ -ATPase. A more likely explanation was that a reactive [^3H]tetracaine intermediate generated primarily when [^3H]tetracaine was bound to its high-affinity site in the nAChR could dissociate from its NCA site and then react with other membrane proteins. This pseudospecific photoincorporation should be sensitive to aqueous compounds that could quench the free reactive species.

GSSG was examined as a potential aqueous scavenger, and indeed high concentrations (50 mM) were effective in dramatically reducing the pseudospecific [^3H]tetracaine photoincorporation into nonreceptor polypeptides (Fig. 3, lanes 4–7). With 50 mM GSSG, the H_{10} -HTX-sensitive labeling was primarily associated with the four nAChR subunits. There also remained specific photolabeling of an nAChR β subunit proteolytic fragment, which appears as a band migrating with slightly greater mobility than the α subunit (Pedersen and Cohen, 1990), as well as of bands migrating between the α and β subunits in a gel region known to contain proteolytic fragments of nAChR γ and δ subunits (Pedersen and Cohen, 1990).

The effects of GSSG at concentrations up to 100 mM on specific and nonspecific [^3H]tetracaine photoincorporation into nAChR subunits and nonreceptor polypeptides were quantified by analyzing ^3H incorporation in gel slices excised from stained gels (Fig. 4). For nAChR subunits and nonreceptor polypeptides, the effects of GSSG were most pronounced at concentrations up to 10 mM. For nAChR subunits, the [^3H]tetracaine photolabeling inhibitable by H_{10} -HTX (the difference between ^3H incorporation for samples labeled in the absence and presence of HTX) was essentially constant at GSSG concentrations of >10 mM. In the presence of 50 mM GSSG, the H_{10} -HTX-sensitive ^3H incorporation into the nAChR α , β , γ , and δ subunits was 792, 730, 301, and 715 cpm, respectively, with only 21 and 54 cpm in the non-

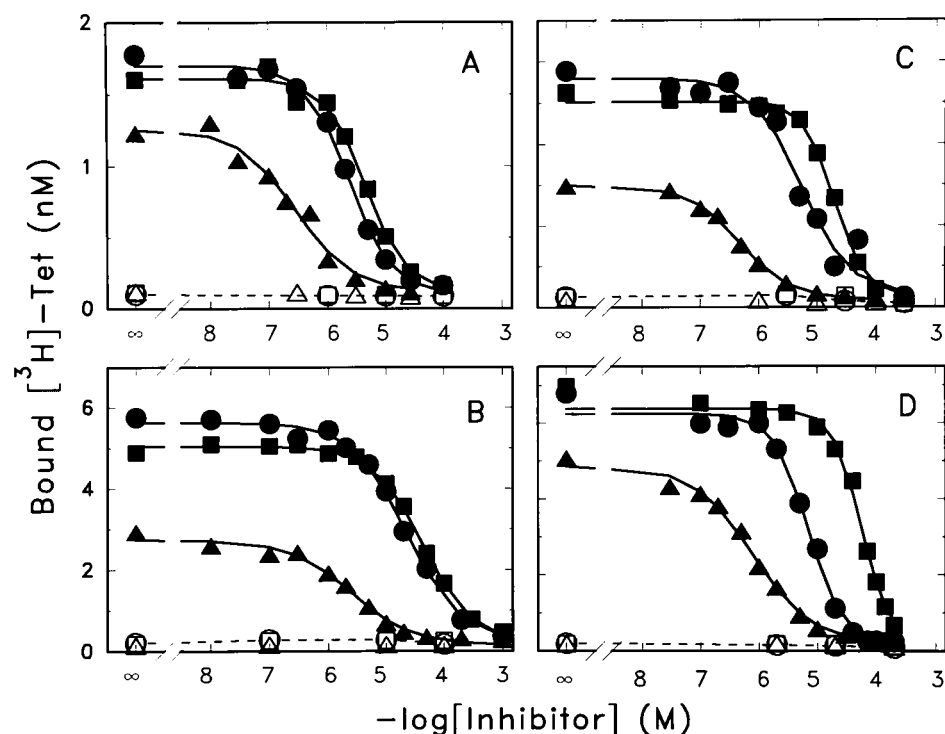


Fig. 2. Inhibition of [^3H]tetracaine binding to nAChR-rich membranes by NCAs. nAChR-rich membranes (0.2 μM tetracaine sites) were preincubated at 4°C for 30 min without competitive antagonist (\bullet), with 50 μM *d*-tubocurarine (\blacktriangle), or with α -bungarotoxin (\blacksquare , 4 μM , 90 min) before the addition of 10 nM [^3H]tetracaine. Aliquots were immediately mixed with (A) H_{10} -HTX, (B) PCP, (C) proadifen, or (D) meproadifen, at concentrations ranging from 10 nM to 1 mM. After 4-h incubation at 4°C , binding was determined by filtration. Parallel samples contained 100 μM nonradioactive tetracaine for determination of nonspecific binding (open symbols). Inhibition curves were fit as described in *Experimental Procedures*, with $n_H = 1$ unless noted. For H_{10} -HTX (A): in the absence of cholinergic ligands, $K_i(-) = 2.2 \pm 0.2$ μM ; with dTC present, $K_i(+\text{dTC}) = 0.3 \pm 0.1$ μM ; and with α -bungarotoxin, $K_i(+\alpha\text{Btx}) = 4.4 \pm 0.5$ μM . For PCP (B): $K_i(-) = 22 \pm 2$ μM , $K_i(+\text{dTC}) = 2.4 \pm 0.4$ μM , and $K_i(+\alpha\text{Btx}) = 44 \pm 2$ μM . For proadifen (C): $K_i(-) = 6 \pm 1$ μM , $K_i(+\text{dTC}) = 0.5 \pm 0.04$ μM , $K_i(+\alpha\text{Btx}) = 18 \pm 3$ μM , and $n_H = 1.6 \pm 0.1$. For meproadifen (D): $K_i(-) = 7 \pm 1$ μM , $n_H = 1.5 \pm 0.2$; $K_i(+\text{dTC}) = 0.9 \pm 0.1$ μM , $n_H = 1$; and $K_i(+\alpha\text{Btx}) = 56 \pm 4$ μM , $n_H = 1.8 \pm 0.2$.

receptor polypeptides of 37 kDa (calelectrin) and 90 kDa (Na^+, K^+ -ATPase α subunit), respectively. GSSG reduced the pseudospecific incorporation into nonreceptor polypeptides by $\approx 85\%$, and all subsequent experiments included 50 mM GSSG in the reaction mixture.

$[^3\text{H}]$ Tetracaine photoincorporation into nAChR subunits was quantified by measuring ^3H incorporation as a function of free $[^3\text{H}]$ tetracaine concentration (Fig. 5). The nonspecific labeling of each nAChR subunit in the presence of 50 μM H_{10} -HTX increased linearly with free tetracaine (Fig. 5, open symbols), and for each subunit, the total ^3H incorporation (Fig. 5, filled symbols) was well fit by a simple hyperbolic binding function plus a linear, nonspecific component. For each subunit, K_{AP} was $\sim 1.4 \mu\text{M}$, a value close to the K_{eq} value of $0.5 \mu\text{M}$ determined directly for the reversible, equilibrium binding of $[^3\text{H}]$ tetracaine (Fig. 1A) and well below the affinity of $[^3\text{H}]$ tetracaine for the agonist site ($K \sim 800 \mu\text{M}$;

Blanchard et al., 1979). For the conditions of photolabeling used, the maximum specific cpm incorporated was equivalent to labeling of 0.6, 1.1, 0.9, and 1.0% of the α , β , γ , and δ subunits, respectively, which was increased by 50 to 75% when the time of irradiation was increased from 30 to 60 min (not shown).

Effects of Cholinergic Ligands on $[^3\text{H}]$ Tetracaine Photoincorporation into nAChR-Rich Membranes. $[^3\text{H}]$ Tetracaine photoincorporation was examined in the presence of agonists, competitive antagonists, and NCAs (Fig. 6) for comparison with the known pharmacology of $[^3\text{H}]$ tetracaine binding to the NCA site. The NCAs proadifen, PCP, and H_{10} -HTX each inhibited the photoincorporation of $[^3\text{H}]$ tetracaine into all four nAChR subunits (Fig. 6, lanes 2 versus 3–5). PCP was the least potent, consistent with its relative potency as an inhibitor of $[^3\text{H}]$ tetracaine equilibrium binding in the absence of agonist (Fig. 2). $[^3\text{H}]$ Tetracaine photoincorporation into the nAChR subunits was not inhibited by excess α -bungarotoxin (Fig. 6, lanes 2 versus 9 and 16), and the three NCAs still inhibited subunit photolabeling in the presence of α -bungarotoxin (lanes 6–8). Carbamylcholine (10 and 300 μM) reduced $[^3\text{H}]$ tetracaine incorporation into all four nAChR subunits by 70 to 80% (Fig. 6, lanes 2 versus 17 and 18, and counting of excised gel bands), but this inhibition was not seen when α -bungarotoxin was present, preventing carbamylcholine from binding to the agonist site (lanes 14 and 15). Although d -tubocurarine at 2 μM had little effect on $[^3\text{H}]$ tetracaine photoincorporation (lane 13), d -tubocurarine at 50 μM reduced labeling by 25 to 40% (lane 12), an inhibition not seen in the presence of α -bungarotoxin (lane 10). Thus, the inhibition of $[^3\text{H}]$ tetracaine photolabeling by the NCAs was consistent with a competitive inhibition of

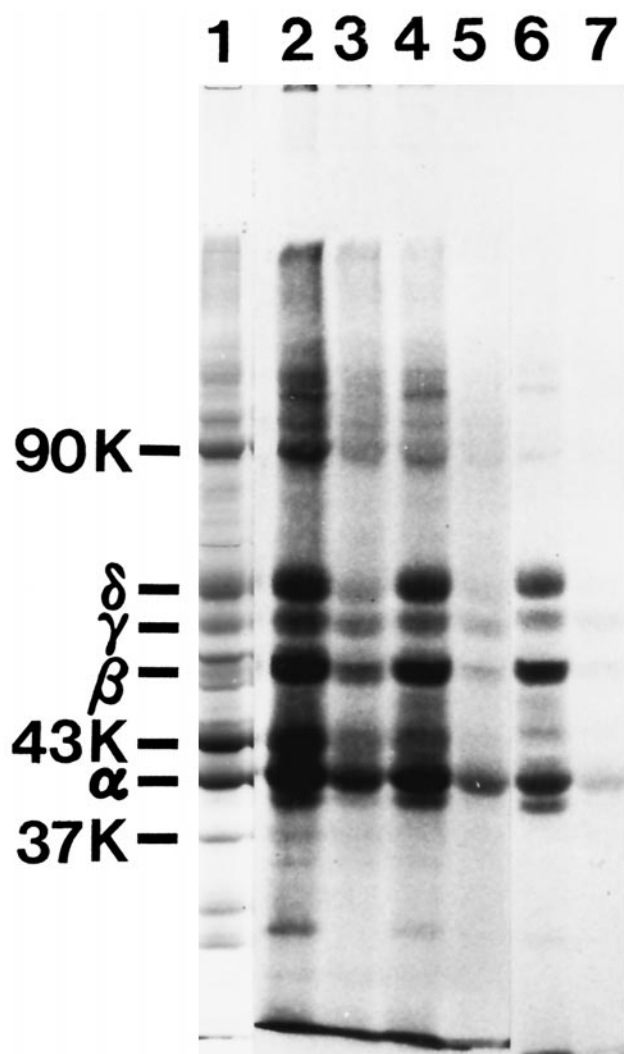


Fig. 3. $[^3\text{H}]$ Tetracaine photoincorporation into nAChR-rich membranes. nAChR-rich membranes (53 μg) were suspended in 30 μl of TPS (2.7 μM ACh sites)/5 μM $[^3\text{H}]$ tetracaine with (lanes 3, 5, and 7) or without (lanes 2, 4, and 6) 30 μM H_{10} -HTX. Membrane suspensions also contained GSSG at 0 mM (lanes 2 and 3), 1 mM (lanes 4 and 5), or 50 mM (lanes 6 and 7). Samples were irradiated for 30 min at 302 nm and polypeptides were resolved by SDS-PAGE as described in *Experimental Procedures*. Coomassie blue stain (lane 1) and fluorograph (lanes 2–7) exposed for 4 days are shown.

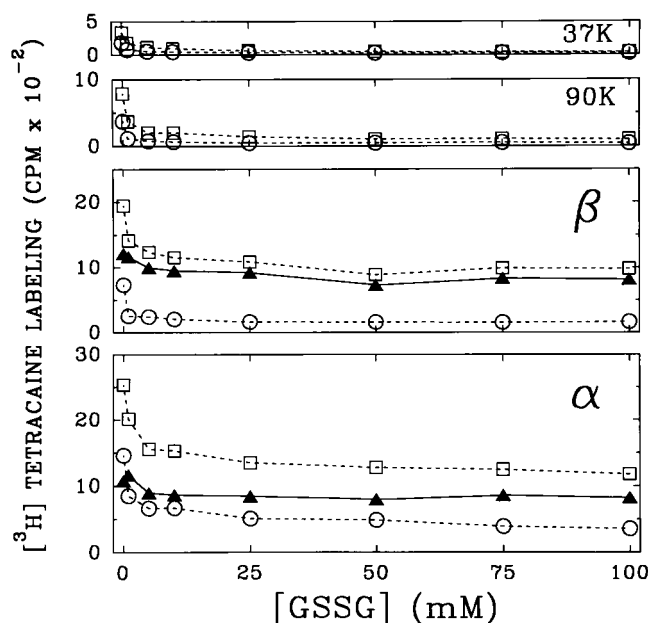


Fig. 4. Effects of GSSG on $[^3\text{H}]$ tetracaine photoincorporation into nAChR-rich membranes. Membranes were photolabeled as described in Fig. 3 with 5 μM $[^3\text{H}]$ tetracaine in the absence (\square) or presence (\circ) of 30 μM H_{10} -HTX and with GSSG from 0 to 50 mM. nAChR α and β subunits and nonreceptor polypeptides of 37 kDa (calelectrin) and 90 kDa (Na^+, K^+ -ATPase α subunit) were excised from the stained gel, and the incorporated ^3H cpm was determined by liquid scintillation counting. The H_{10} -HTX inhibitable labeling (\blacktriangle) was calculated as the difference between ^3H incorporation in the absence (\square) and presence (\circ) of H_{10} -HTX.

high-affinity [^3H]tetracaine binding, whereas the inhibition of photolabeling by carbamylcholine and *d*-tubocurarine resulted from the allosteric inhibition of [^3H]tetracaine binding when the nAChR was desensitized by drugs binding to the agonist site.

The concentration dependence for the inhibition of [^3H]tetracaine photolabeling of nAChR subunits by H_{10} -HTX (Fig. 7) and carbamylcholine (Fig. 8) was determined by quantification of ^3H incorporation in gel slices. For H_{10} -HTX, the inhibition data for each subunit were well fit by a single-site inhibition function with IC_{50} values ranging from 3 to 4 μM and maximal inhibition of 81, 94, 65, and 93% for the α , β , γ , and δ subunits, respectively. The observed IC_{50} values exceeded the directly measured K_{eq} value of 0.3 μM at 20°C (Heidmann et al., 1983), but they were consistent with that value for the assay conditions used: 1) the site concentration (1.3 μM) exceeds the K_{eq} value for H_{10} -HTX, 2) a significant percentage of the H_{10} -HTX is bound nonspecifically [partition coefficient = 0.3 (mg/ml) $^{-1}$], and 3) the free concentration of [^3H]tetracaine was greater than its K_{eq} value. For the known nAChR concentration, the approximate free [^3H]tetracaine concentration, and the known partition coefficient for H_{10} -HTX, the observed IC_{50} value leads to a calculated dis-

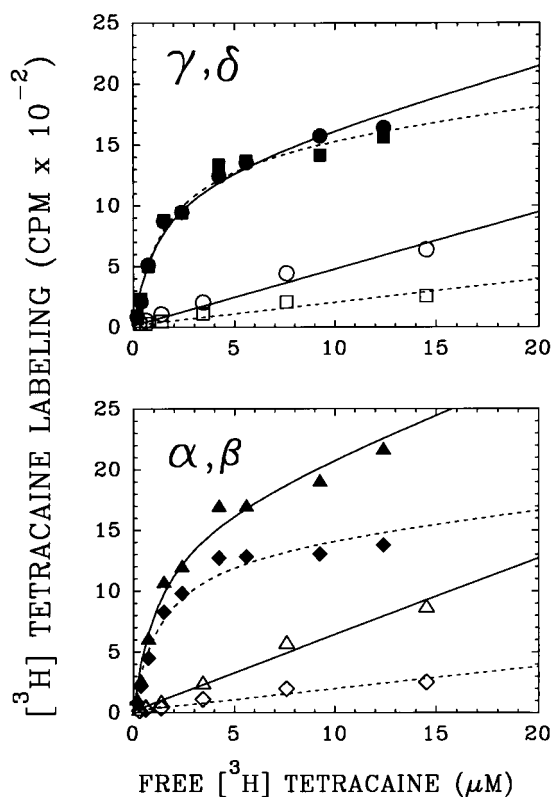


Fig. 5. Concentration dependence of [^3H]tetracaine incorporation into the nAChR subunits. nAChR-rich membranes (53 μg) were suspended in 30 μl of TPS (2.7 μM ACh sites)/50 mM GSSG with various concentrations of [^3H]tetracaine, in the absence (solid) or presence (open) of 50 μM H_{10} -HTX. Membrane suspensions were irradiated at 302 nm, and the [^3H]tetracaine incorporation into the α (\blacktriangle , \triangle), β (\blacklozenge , \lozenge), γ (\bullet , \circ), and δ (\blacksquare , \square) subunits determined as described in *Experimental Procedures*. Free [^3H]tetracaine was measured as supernatant ^3H cpm after centrifugation of irradiated samples. Lines represent nonlinear least-squares fits to the data as described in *Experimental Procedures*, with K_{AP} values of 1.3 ± 0.3 , 1.3 ± 0.3 , 1.3 ± 0.3 , and 1.5 ± 0.3 μM for the α , β , γ , and δ subunits, respectively. The maximum specific incorporation was 1610, 1370, 1270, and 1520 cpm for the α , β , γ , and δ subunits, respectively.

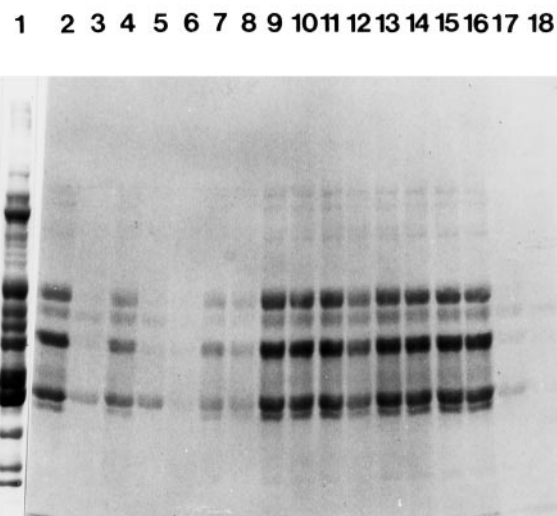


Fig. 6. Effects of cholinergic ligands on [^3H]tetracaine incorporation into nAChR-rich membranes. nAChR-rich membranes (53 μg) were suspended in TPS (2.7 μM)/50 mM GSSG for preincubation (60 min) in the absence (lanes 2–5, 12, 13, 17, and 18) or presence (lanes 6–11 and 14–16) of α -bungarotoxin (10 μM) and the following cholinergic ligands: lane 2, no further additions; lane 3, 30 μM H_{10} -HTX; lane 4, 50 μM PCP; and lane 5, 100 μM proadifen. Lanes 6 to 11, 10 μM α -bungarotoxin with 30 μM H_{10} -HTX (lane 6), 50 μM PCP (lane 7), 100 μM proadifen (lane 8), no other drug (lane 9), 50 μM *d*-tubocurarine (lane 10), and 2 μM *d*-tubocurarine (lane 11). Lane 12, 50 μM *d*-tubocurarine; lane 13, 2 μM *d*-tubocurarine. Lanes 14 to 16, 10 μM α -bungarotoxin with 300 μM carbamylcholine (lane 14), 10 μM carbamylcholine (lane 15), and no other drug (lane 16). Lane 17, 300 μM carbamylcholine; lane 18, 10 μM carbamylcholine. [^3H]Tetracaine was added, the samples were irradiated at 302 nm, and the polypeptides were resolved by SDS-PAGE as described in *Experimental Procedures*. Coomassie blue stain of a gel lane (lane 1) and the fluorograph (lanes 2–17) exposed for 2 weeks are shown.

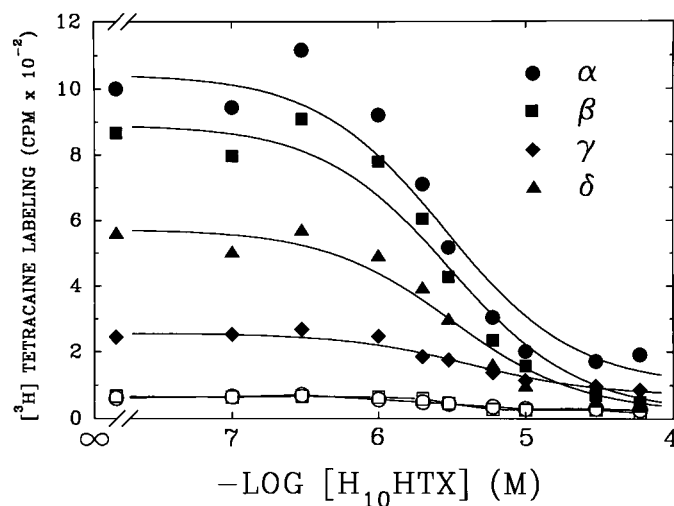


Fig. 7. Inhibition of [^3H]tetracaine photoincorporation into nAChR-rich membranes by H_{10} -HTX. nAChR-rich membranes (53 μg) were suspended in 30 μl of TPS (2.7 μM ACh sites) with 2 μM [^3H]tetracaine/50 mM GSSG and various concentrations of H_{10} -HTX. After irradiation, membrane polypeptides were fractionated by SDS-PAGE. The nAChR α (\bullet), β (\blacksquare), γ (\blacklozenge), and δ (\blacktriangle) subunits, as well as nonreceptor polypeptides of 37 kDa (\circ , calelectrin) and 90 kDa (\square , Na^+ , K^+ -ATPase α subunit), were excised from the stained gel, and the incorporated ^3H cpm was determined by liquid scintillation counting. Data were fit with the inhibition function described in *Experimental Procedures* with K_i values of 2.8 ± 0.6 , 3.0 ± 0.5 , 4.1 ± 0.9 , and 3.1 ± 0.5 μM for α , β , γ , and δ subunits, respectively.

sociation constant of $\approx 0.8 \mu\text{M}$. For carbamylcholine, the subunit inhibition data were fit by IC_{50} values between 1.7 and $2.6 \mu\text{M}$, with maximal inhibition of labeling of 90, 91, 61, and 91% for the α , β , γ , and δ subunits. Again, the observed total concentration of carbamylcholine for 50% inhibition exceeded the directly measured K_{eq} value of $0.1 \mu\text{M}$ (Boyd and Cohen, 1980), which was not surprising for an assay using $2.7 \mu\text{M}$ ACh sites. When the inhibition curves were fit to determine the free concentration of carbamylcholine associated with 50% inhibition (K_{AP} ; White and Cohen, 1988), the K_{AP} values for each subunit were between 0.3 and $0.9 \mu\text{M}$.

Mapping the $[^3\text{H}]$ Tetracaine-Labeled Site in nAChR α Subunit with *S. aureus* V8 Protease. Digestion of the nAChR α subunit by *S. aureus* V8 protease using the procedure of Cleveland et al. (1977) produces four nonoverlapping fragments of 20 ($\alpha\text{V8-20}$), 18 ($\alpha\text{V8-18}$), 10 ($\alpha\text{V8-10}$), and 4 kDa ($\alpha\text{V8-4}$) that are readily resolved by SDS-PAGE (Pedersen et al., 1986). The N termini of $\alpha\text{V8-20}$, $\alpha\text{V8-18}$, $\alpha\text{V8-10}$, and $\alpha\text{V8-4}$ begin at αSer^{173} , αVal^{46} , αAsn^{339} , and αSer^1 , respectively (Pedersen et al., 1986; White and Cohen, 1988). $\alpha\text{V8-18}$ contains the only Asn-linked carbohydrate α subunit, and this fragment is shifted to a band of 12 kDa ($\alpha\text{V8-12}$) when the carbohydrate is removed by digestion with endoglycosidase H (Pedersen et al., 1986). nAChR-rich membranes were labeled with $[^3\text{H}]$ tetracaine in the absence or presence of 50 mM GSSG and with or without $30 \mu\text{M}$ H_{10} -HTX. After treatment of the labeled membranes with endoglycosidase H, α subunits were isolated by SDS-PAGE and then digested with V8 protease as described in *Experimental Procedures* to determine the ^3H incorporation within each α subunit proteolytic fragment (Fig. 9). In the presence of 50 mM GSSG (lanes 5–8), $\alpha\text{V8-20}$ was the only fragment labeled specifically. When gel pieces corresponding to the proteolytic fragments were excised and the ^3H incorporation was quantified by scintillation counting, it was found that 96% of the specific

incorporation was in $\alpha\text{V8-20}$, with specific labeling in $\alpha\text{V8-18}$, $\alpha\text{V8-10}$, and $\alpha\text{V8-4}$ each $<2\%$ that of $\alpha\text{V8-20}$. The principal effect of 50 mM GSSG was to reduce the nonspecific incorporation into $\alpha\text{V8-20}$ by 80%, whereas the specific labeling was not decreased at all. In contrast, H_{10} -HTX-sensitive labeling of $\alpha\text{V8-18}$, $\alpha\text{V8-10}$, and $\alpha\text{V8-4}$ was reduced by 78, 69, and 97%, respectively.

Discussion

The study presented here concerns the nature of the binding site for amine NCAs that bind selectively to the nAChR in the absence of agonist (i.e., to the closed channel state of the nAChR). In contrast to the selectivity for the desensitized state seen for most bulky amine NCAs containing fused aromatic rings or multiple aromatic or aliphatic rings, we found that most *N,N*-substituted ethanolamine esters of benzoic acid actually bind selectively in the absence of agonist (Table 1). With the exception of procaine, which binds weakly and with similar affinity in the absence and presence of agonist, 4-butylamino and 4-ethoxybenzoate esters bind preferentially to the resting state, as does piperocaine, an unsubstituted benzoate. The selectivity of tetracaine for the resting

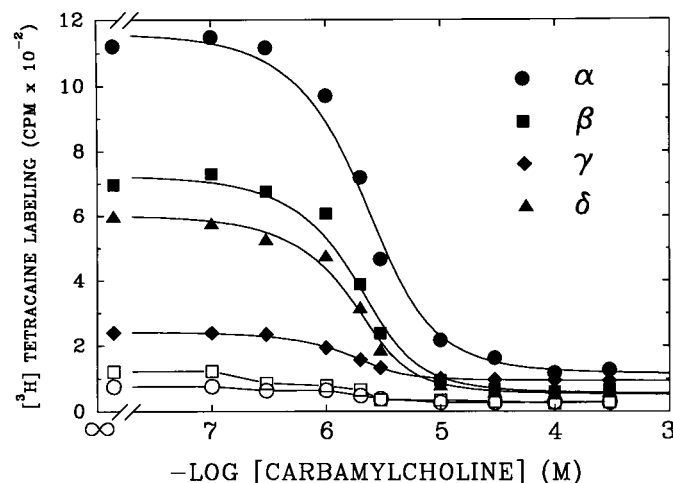


Fig. 8. Inhibition of $[^3\text{H}]$ tetracaine photoincorporation into nAChR-rich membranes by carbamylcholine. nAChR-rich membranes ($53 \mu\text{g}$) were suspended in $30 \mu\text{l}$ of TPS ($2.7 \mu\text{M}$ ACh sites) with $2 \mu\text{M}$ $[^3\text{H}]$ tetracaine/50 mM GSSG and various concentrations of carbamylcholine. After irradiation, membrane polypeptides were fractionated by SDS-PAGE. The nAChR α (\bullet), β (\blacksquare), γ (\blacklozenge), and δ (\blacktriangle) subunits, as well as nonreceptor polypeptides of 37 kDa (\square , calelectrin) and 90 kDa (\square , Na^+ , K^+ -ATPase α subunit), were excised from the stained gel, and the incorporated ^3H cpm was determined by liquid scintillation counting. Data were fit to calculate K_{AP} value for free carbamylcholine as described (White and Cohen, 1988), with $K_{\text{AP}} = 0.9 \pm 0.2$, 0.6 ± 0.2 , 0.4 ± 0.1 , and $0.5 \pm 0.1 \mu\text{M}$ for the nAChR α , β , γ , and δ subunits, respectively.

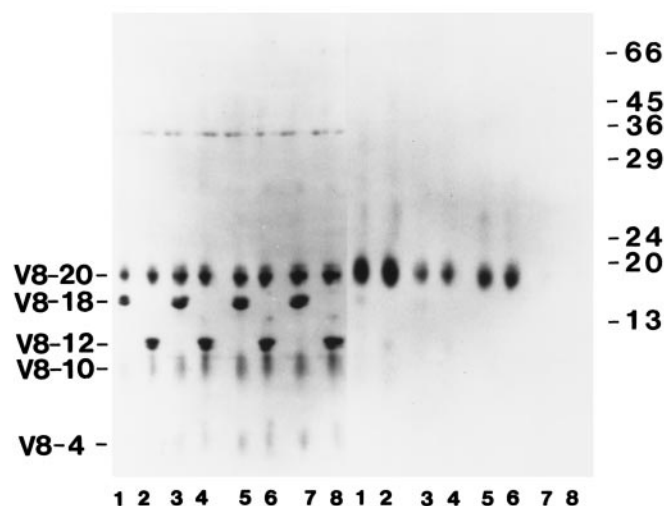


Fig. 9. Proteolytic mapping of $[^3\text{H}]$ tetracaine photoincorporation into the nAChR α subunit using *S. aureus* V8 protease. nAChR-rich membranes ($540 \mu\text{g}$) were suspended in $310 \mu\text{l}$ of TPS ($2.7 \mu\text{M}$) with $5 \mu\text{M}$ $[^3\text{H}]$ tetracaine in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of $30 \mu\text{M}$ H_{10} -HTX and with (lanes 1–4) or without (lanes 5–8) 50 mM GSSG. Membrane suspensions were irradiated for 30 min at 302 nm and then pelleted, and the pellets were resuspended in 50 mM sodium phosphate, pH 7.0/1.0% SDS ($47 \mu\text{l}$) and incubated overnight in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 4.3 mU endoglycosidase H. $[^3\text{H}]$ Tetracaine-labeled α subunit was resolved by SDS-PAGE and then excised for digestion with $3 \mu\text{g}$ of V8 protease in a proteolytic mapping gel as described in *Experimental Procedures*. The mapping gel was stained with Coomassie blue (A) and subjected to fluorography for 4 weeks (B). The α subunit proteolytic fragments are labeled by the nomenclature of White and Cohen (1988), and the molecular weight standards are bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and cytochrome *c* (13 kDa). Based on counting of excised gel slices, in the presence of 50 mM GSSG the incorporation into $\alpha\text{V8-20}$ in the absence and presence of HTX was 3433 and 622 cpm, respectively, whereas incorporation in $\alpha\text{V8-18}$ was 63 and 30 cpm; in $\alpha\text{V8-10}$, 154 and 75 cpm; and in $\alpha\text{V8-4}$, 33 and 27 cpm. After α subunit deglycosylation with endoglycosidase H, incorporation in $\alpha\text{V8-20}$ was 3574 and 664 cpm. In the absence of GSSG, incorporation in $\alpha\text{V8-20}$ was 4824 and 2736 cpm in the absence and presence of HTX.

state results from contributions both from the 4-butylamino substitution and from the presence of the *N,N*-dimethyl rather than *N,N*-diethyl. With reference to procaine (compound **V**, Table 1), the 4-butylamino substitution (compound **II**) enhanced binding to the resting state by 10-fold more than to the desensitized state, with the further methyl replacements in tetracaine (compound **I**) weakening binding to the desensitized state by slightly more than to the resting state. In addition to the benzoic acid esters, at least one phenylacetic acid ester of *N,N*-diethylaminoethanol binds preferentially in the absence of agonist (Cohen et al., 1986). Although proadifen (the ester of 2,2-diphenylpropionic acid) bound with 10-fold higher affinity to the desensitized state and adiphenine (the ester of diphenylacetic acid) bound with similar affinity ($K_1 = 4 \mu\text{M}$) in the presence or absence of agonist, butethemate (the 2-phenylbutyryl ester) bound with 8-fold higher affinity to the resting state. The only other drug known to have high resting state selectivity is amobarbital, which binds with 500-fold higher affinity to the resting state, whereas other barbiturates bind preferentially to the desensitized or open channel states (Cohen et al., 1986; de Armendi et al., 1993).

In the absence of agonist, [^3H]tetracaine was bound at equilibrium with high affinity ($K_{\text{eq}} = 0.5 \mu\text{M}$) to one site per nAChR monomer, and [^3H]tetracaine bound with the same high affinity when ACh sites were occupied with α -bungarotoxin. [^3H]Tetracaine also bound to a single site in the desensitized state of the nAChR but with 60-fold lower affinity. In contrast to other, more hydrophobic aromatic amine NCAs that bind to as many as 30 low-affinity sites as well as to the high-affinity site (Heidmann et al., 1983), there was no evidence that [^3H]tetracaine (up to $20 \mu\text{M}$) binds to additional low-affinity sites in the nAChR-rich membranes. [^3H]Tetracaine bound to the same number of high-affinity sites as [^3H]HTX, and as seen previously for [^3H]PCP and [^3H]HTX (Heidmann et al., 1983), this number was half the number of [^3H]ACh sites in the nAChR-rich membranes. Because [^3H]ACh binds to two sites per nAChR (Neubig and Cohen, 1979), these NCAs each bind with high affinity to one site per nAChR.

HTX, PCP, proadifen, and meproadifen each completely inhibited the specific [^3H]tetracaine binding to nAChR-rich membranes in the absence as well as in the presence of the competitive antagonists *d*-tubocurarine and α -bungarotoxin. For HTX and PCP, in each condition the concentration dependence of inhibition was well fit by a simple, single-site model ($n_{\text{H}} = 1$). In the presence of *d*-tubocurarine, the concentration dependence of inhibition by meproadifen or proadifen was also consistent with competition at a single site ($n_{\text{H}} = 1$). However, at the higher concentrations ($>10 \mu\text{M}$) of these drugs necessary to inhibit [^3H]tetracaine binding in either the presence of α -bungarotoxin or the absence of other agonist or competitive antagonists, the inhibition curves were characterized by Hill coefficients between 1.5 and 2.0. The steep concentration dependence seen in the presence of α -bungarotoxin suggests that high concentrations of meproadifen and proadifen desensitize the nAChR by a mechanism unrelated to their binding to the high-affinity NCA site or to the ACh sites (Heidmann et al., 1983; Boyd and Cohen, 1984). Of the four NCAs studied, meproadifen binds with highest affinity to the ACh site ($K_{\text{eq}} = 50 \mu\text{M}$; Heidmann et al., 1983), so in the absence of α -bungarotoxin or *d*-tubocu-

rarine, it may also inhibit [^3H]tetracaine binding by binding to the agonist site and desensitizing the nAChR.

To characterize the structure of the aromatic amine NCA site in the closed channel state of the nAChR, we tested whether [^3H]tetracaine itself could be used as a photoaffinity probe. Irradiation of nAChR-rich membranes equilibrated with [^3H]tetracaine resulted in covalent incorporation into membrane polypeptides. However, the observed photolabeling in the absence of aqueous scavenger was surprising, with photoincorporation into all polypeptides in the membrane suspensions inhibited by the presence of the NCA H_{10} -HTX (Figs. 3 and 4) or the agonist carbamylcholine, drugs that inhibit [^3H]tetracaine binding to its high-affinity site in the nAChR. Because the H_{10} -HTX-sensitive [^3H]tetracaine incorporation into polypeptides other than the nAChR subunits was reduced by at least 85% in the presence of the aqueous scavenger GSSG (50 mM), this labeling probably occurred after photoactivated [^3H]tetracaine dissociated from its nAChR binding site. GSSG was also an effective aqueous scavenger of the nonspecific photolabeling by [^3H]d-tubocurarine (Pedersen and Cohen, 1990) and [^3H]nicotine (Middleton and Cohen, 1991). However, the specific component of [^3H]tetracaine photolabeling was insensitive to GSSG concentrations to 50 mM, whereas for the photolabeling at the agonist site, concentrations of GSSG of more than 1 mM inhibited the specific as well as the nonspecific components. Thus, it appears that the [^3H]tetracaine binding site in the closed channel state of the nAChR is substantially more protected from the aqueous environment than the ACh site. Another difference between the photolabeling by [^3H]tetracaine and that by [^3H]d-tubocurarine or [^3H]nicotine is the wavelength dependence, with tetracaine photoincorporation more efficient for irradiation above 300 nm, whereas the other drugs required irradiation below 300 nm. Because tetracaine's long wavelength absorption maximum is 300 nm in benzene and 310 nm in water, it is tetracaine and not the nAChR that is photoactivated.

In the presence of 50 mM GSSG, nearly all [^3H]tetracaine photoincorporation sensitive to H_{10} -HTX was restricted to the four nAChR subunits. The specific photolabeling of the four subunits resulted from activation of [^3H]tetracaine bound to its high-affinity NCA site, as evidenced by the dependence of this specific photolabeling on free [^3H]tetracaine concentration (Fig. 5) and the concentration dependence of inhibition by H_{10} -HTX (Fig. 7) or carbamylcholine (Fig. 8). Also, as expected from the equilibrium binding data, the subunit photolabeling was inhibitable by other aromatic amine NCAs but not by α -bungarotoxin (Fig. 6). Thus, [^3H]tetracaine bound to its single high-affinity binding site in the nAChR in the absence of agonist is photoincorporated into all four subunits with similar efficiency, which indicates that the binding site is located within the central ion channel domain at the interface of all five subunits. Within α subunit, the amino acids specifically labeled by [^3H]tetracaine are restricted to a 20-kDa fragment containing the M1–M3 hydrophobic segments. As described in Gallagher and Cohen (1999), amino acids from the M2 segments of each subunit are specifically photolabeled by [^3H]tetracaine, and the binding site for [^3H]tetracaine in the closed state of the ion channel overlaps and extends beyond the binding site for [^{125}I]TID in the M2 ion channel domain (White and Cohen, 1992).

Acknowledgments

We thank Dr. Wu Schyong Liu for synthesis of the *p*-butylamino-benzoic acid esters and Drs. Benjamin White and Steen Pedersen for helpful suggestions during the course of these experiments.

References

- Arias HR (1996) Luminal and non-luminal non-competitive inhibitor binding sites on the nicotinic acetylcholine receptor (review). *Mol Membr Biol* **13**:1–17.
- Arias HR (1998) Binding sites for exogenous and endogenous non-competitive inhibitors of the nicotinic acetylcholine receptor. *BBA Rev Biomembr* **1376**:173–220.
- Blanchard SG, Elliott J and Raftery MA (1979) Interaction of local anesthetics with *Torpedo californica* membrane-bound acetylcholine receptor. *Biochemistry* **18**:5880–5884.
- Blanton MP and Cohen JB (1994) Identifying the lipid-protein interface of the *Torpedo* nicotinic acetylcholine receptor: Secondary structure implications. *Biochemistry* **33**:2859–2872.
- Blanton MP, Dangott LJ, Raja SK, Lala AK and Cohen JB (1998) Probing the structure of the nicotinic acetylcholine receptor ion channel with the uncharged photoactivatable compound [³H]diazofluorene. *J Biol Chem* **273**:8659–8668.
- Boyd ND and Cohen JB (1980) Kinetics of binding of [³H]-acetylcholine and [³H]-carbamylcholine to *Torpedo* post-synaptic membranes: Slow conformational transitions of the cholinergic receptor. *Biochemistry* **19**:5344–5353.
- Boyd ND and Cohen JB (1984) Desensitization of membrane-bound *Torpedo* acetylcholine receptor by amine noncompetitive antagonists and aliphatic alcohols: Studies of [³H]-acetylcholine binding and ²²Na⁺ ion fluxes. *Biochemistry* **23**:4023–4033.
- Charnet P, Labarca C, Leonard RJ, Vogelaar NJ, Czyzyk L, Gavin A, Davidsen N and Lester HA (1990) An open-channel blocker interacts with adjacent turns of α -helices in the nicotinic acetylcholine receptor. *Neuron* **2**:87–95.
- Cleveland DW, Fischer SG, Kirschner MW and Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* **252**:1102–1106.
- Cohen JB, Correll LA, Dreyer EB, Kuisk IR, Medynski DC and Strnad NP (1986) Interactions of local anesthetics with *Torpedo* nicotinic acetylcholine receptors, in *Molecular and Cellular Mechanisms of Anesthetics* (Roth SH and Miller KW eds) pp 111–124, Plenum Press, New York.
- Cohen JB, Medynski DC and Strnad NP (1985) Interactions of local anesthetics with nicotinic acetylcholine receptors, in *Effects of Anesthesia* (Covino B, Fozzard H, Redher K and Strichartz G eds) pp 53–64, American Physiological Society, Bethesda, MD.
- de Armendi AJ, Tonner PH, Bugge B and Miller KW (1993) Barbiturate action is dependent on the conformational state of the acetylcholine receptor. *Anesthesiology* **79**:1033–1041.
- DiPaola M, Kao PN and Karlin A (1990) Mapping the α subunit site photolabeled by the noncompetitive inhibitor [³H]quinacrine azide in the active state of the nicotinic acetylcholine receptor. *J Biol Chem* **265**:11017–11029.
- Forman SA (1997) Homologous mutations on different subunits cause unequal but additive effects on *n*-alcohol block in the nicotinic receptor pore. *Biophys J* **72**:2170–2179.
- Gallagher MJ and Cohen JB (1999) Identification of amino acids involved in the binding of [³H]tetracaine to the *Torpedo* nicotinic acetylcholine receptor. *Mol Pharmacol* **56**:300–307.
- Heidmann T, Oswald RE and Changeux J-P (1983) Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from *Torpedo marmorata*. *Biochemistry* **22**:3112–3127.
- Hucho F, Oberthur W and Lottspeich F (1986) The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M II of the receptor subunits. *FEBS Lett* **205**:137–142.
- Hucho F, Tsetlin VI and Machold J (1996) The emerging three-dimensional structure of a receptor: The nicotinic acetylcholine receptor. *Eur J Biochem* **239**:539–557.
- Imoto K, Busch C, Sakmann B, Mishina M, Konno T, Nakai J, Bujo H, Mori Y, Fukuda K and Numa S (1988) Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature (Lond)* **335**:645–648.
- Johnson DA and Nuss JM (1994) The histrionicotoxin-sensitive ethidium binding site is located outside of the transmembrane domain of the nicotinic acetylcholine receptor: A fluorescence study. *Biochemistry* **33**:9070–9077.
- Karlin A and Akabas MH (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* **15**:1231–1244.
- Lurtz MM, Hareland ML and Pedersen SE (1997) Quinacrine and ethidium bromide bind the same locus on the nicotinic acetylcholine receptor from *Torpedo californica*. *Biochemistry* **36**:2068–2075.
- Lurtz MM and Pedersen SE (1999) Aminotriarylmethane dyes are high-affinity noncompetitive antagonists of the nicotinic acetylcholine receptor. *Mol Pharmacol* **55**:159–167.
- Middleton RE and Cohen JB (1991) Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [³H]-Nicotine as an agonist photoaffinity label. *Biochemistry* **30**:6987–6997.
- Neubig RR and Cohen JB (1979) Equilibrium binding of [³H]tubocurarine and [³H]acetylcholine by *Torpedo* post-synaptic membranes: Stoichiometry and ligand interactions. *Biochemistry* **18**:5464–5475.
- Pedersen SE and Cohen JB (1990) d-Tubocurarine binding sites are located at α - γ and α - δ subunit interfaces of the nicotinic acetylcholine receptor. *Proc Natl Acad Sci USA* **87**:2785–2789.
- Pedersen SE, Dreyer EB and Cohen JB (1986) Location of ligand binding sites on the nicotinic acetylcholine receptor α subunit. *J Biol Chem* **261**:13735–13743.
- Pedersen SE, Sharp SD, Liu W-S and Cohen JB (1992) Structure of the noncompetitive antagonist binding site in the *Torpedo* nicotinic acetylcholine receptor: [³H]Meproadifen mustard reacts selectively with α subunit Glu-262. *J Biol Chem* **267**:10489–10499.
- Prince RJ and Sine SM (1998) The ligand binding domains of the nicotinic acetylcholine receptor, in *The Nicotinic Acetylcholine Receptor: Current Views and Future Trends* (Barrantes FJ ed) pp 31–59, RG Landes, Austin.
- Revah F, Galzi JL, Giraudat J, Haumont P-Y, Lederer F and Changeux J-P (1990) The noncompetitive blocker [³H]chlorpromazine labels three amino acids of the acetylcholine receptor γ subunit: Implications for the α -helical organization of regions MII and for the structure of the ion channel. *Proc Natl Acad Sci USA* **87**:4675–4679.
- Tamamizu S, Todd AP and McNamee MG (1995) Mutations in the M1 region of the nicotinic acetylcholine receptor alter the sensitivity to inhibition by quinacrine. *Cell Mol Neurobiol* **15**:427–438.
- Unwin N (1995) Acetylcholine receptor channel imaged in the open state. *Nature (Lond)* **373**:37–43.
- Unwin N (1998) The nicotinic acetylcholine receptor of the *Torpedo* electric ray. *J Struct Biol* **121**:181–190.
- White BH and Cohen JB (1988) Photolabeling of membrane-bound *Torpedo* nicotinic acetylcholine receptor with the hydrophobic probe 3-trifluoromethyl-3-(m-[¹²⁵I]iodophenyl)diazirine. *Biochemistry* **27**:8741–8751.
- White BH and Cohen JB (1992) Agonist-induced changes in the structure of the acetylcholine receptor M2 regions revealed by photoincorporation of an uncharged nicotinic non-competitive antagonist. *J Biol Chem* **267**:15770–15783.
- White BH, Howard S, Cohen SG and Cohen JB (1991) The hydrophobic photoreagent 3-(trifluoromethyl)-3-(m-[¹²⁵I]iodophenyl)diazirine is a novel noncompetitive antagonist of the nicotinic acetylcholine receptor. *J Biol Chem* **266**:21595–21607.
- Zhang H and Karlin A (1997) Identification of acetylcholine receptor channel-lining residues in the M1 segment of the beta subunit. *Biochemistry* **36**:15856–15864.

Send reprint requests to: Dr. Jonathan B. Cohen, Department of Neurobiology, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115. E-mail: jonathan_cohen@hms.harvard.edu