

[³H]Chlorpromazine Photolabeling of the *Torpedo* Nicotinic Acetylcholine Receptor Identifies Two State-Dependent Binding Sites in the Ion Channel[†]

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ABSTRACT: Chlorpromazine (CPZ), a potent nicotinic acetylcholine receptor (nAChR) noncompetitive antagonist, binds with higher affinity in the ion channel in the desensitized state than in the closed channel state and with low affinity to additional sites in nAChR-rich membranes. For nAChR equilibrated with agonist, we confirm previous reports that [³H]CPZ occupies a site near the cytoplasmic end of the M2 ion channel domain, photolabeling positions M2–2, M2–6, and/or M2–9 in each subunit. We find that [³H]CPZ also binds at the extracellular end of the channel, photolabeling amino acids at positions M2–16 (α,γ), M2–17 (α,β,δ), and M2–20 (α,β,δ). The photolabeling at the cytoplasmic end of the channel is fully inhibitable by phencyclidine or proadifen, whereas neither drug inhibits [³H]CPZ photolabeling at the extracellular end, establishing that positively charged drugs can bind simultaneously at the cytoplasmic and extracellular ends of the ion channel. [³H]CPZ photolabeling is not detected in the transmembrane domain outside the ion channel, but it photolabels αMet-386 and αSer-393 in the cytoplasmic αMA helix. In the nAChR equilibrated with α-bungarotoxin to stabilize the nAChR in a closed state, [³H]CPZ photolabels amino acids at M2–5 (α), M2–6 (α,β,δ), and M2–9 (β,δ), with no labeling at M2–2. These results provide novel information about the modes of drug binding within the nAChR ion channel and indicate that within the nAChR transmembrane domain, the binding of cationic aromatic amine antagonists can be restricted to the ion channel domain, in contrast to the uncharged, allosteric potentiators and inhibitors that also bind within the δ subunit helix bundle and at subunit interfaces.

The Cys-loop superfamily of neurotransmitter-gated ion channels includes the excitatory nicotinic acetylcholine receptors (nAChR¹) and serotonin 5-HT₃ receptors, and the inhibitory GABA_A and glycine receptors (1–3). Our knowledge about the three-dimensional structure of these receptors is based upon models of a muscle-type nAChR derived from cryoelectron microscope images of the *Torpedo marmorata* nAChR (4, 5) together with X-ray diffraction models from crystals of molluscan homopentameric acetylcholine binding proteins that are homologous to a nAChR extracellular domain (6, 7). The nAChR structure, which was obtained in the absence of agonist and is assumed to represent the nAChR in the closed state, does not have the resolution necessary to accurately identify individual amino acids but defines the secondary and tertiary structures of the extracellular and transmembrane domains, which are conserved in higher resolution crystal structures of distantly

related prokaryotic channels (8–10). The N-terminal half of each subunit contributes to the extracellular domain, containing the neurotransmitter binding sites that are located at subunit interfaces (α–γ and α–δ in the α₂βγδ *Torpedo* nAChR) 30 Å above the level of the membrane. Each subunit's transmembrane domain is made up of a loose bundle of four α helices (M1–M4), with the amino acids from each M2 helix contributing to the lumen of the ion channel and M4 located most peripheral and in greatest contact with lipids.

A striking feature of the structure of the nAChR transmembrane domain is the presence of pockets within each subunit's helix bundle and at subunit interfaces that are potential binding sites for allosteric modulators, which contrasts with the compact structure of the transmembrane domain of the prokaryotic channels. This difference in structure may result because the nAChR is in its native lipid environment while the prokaryotic channels were purified in detergent and crystallized in detergent/lipid mixtures, or it may reflect a more fundamental difference between an nAChR which requires cholesterol for channel gating and the prokaryotic channels which function in the absence of cholesterol (11).

Photoaffinity labeling studies with [³H]chlorpromazine ([³H]-CPZ), a phenothiazine tertiary amine (Figure 1) that binds with high affinity to a site in the *Torpedo* nAChR in the desensitized state (12), provided initial evidence for a drug binding site in the nAChR transmembrane domain. [³H]CPZ photolabeled amino acids at position M2–6 in each subunit and at M2–2 and M2–9 in some subunits (numbering from the conserved positive charges at the N-terminal (cytoplasmic end) of each M2-helix) (13–16). Its binding site has not been localized in the closed

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Abbreviations: nAChR, nicotinic acetylcholine receptor; CPZ, chlorpromazine HCl; CPZsulfoxide, chlorpromazine sulfoxide; Carb, carbamylcholine; αBgTx, α-bungarotoxin; PCP, phencyclidine; Azietomidate, 2-(3-methyl-3*H*-diazirin-3-yl)ethyl 1-(phenylethyl)-1*H*-imidazole-5-carboxylate; TDBzl-Etomidate, 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl-1-(1-phenylethyl)-1*H*-imidazole-5-carboxylate; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; rpHPLC, reversed-phase high-performance liquid chromatography; V8 protease, *Staphylococcus aureus* endoproteinase Glu-C; EndoLys-C, *Lysobacter enzymogenes* endoproteinase Lys-C.

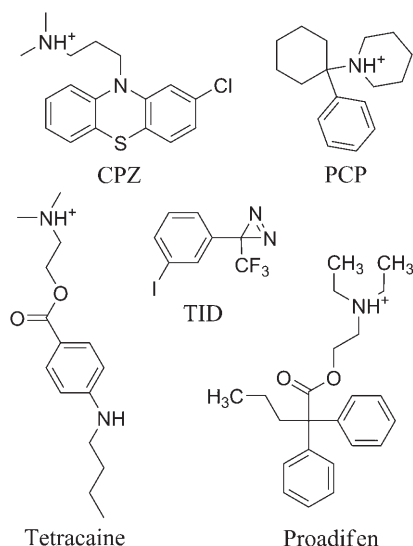


FIGURE 1: Structures of nAChR channel blockers.

channel state, when it binds with 10-fold lower affinity. Molecular dynamics simulations using the *Torpedo* nAChR structure predict that CPZ binds near the cytoplasmic end of the closed channel (17), while crystal violet, another aromatic amine, is predicted to bind at the extracellular end (18). Photolabeling with [³H]tetracaine, an aromatic tertiary amine inhibitor that binds preferentially to the channel in the closed state, establishes that it binds at the level of M2–5/6, M2–9, and M2–13 (19). State-dependent binding within the ion channel has been established for [¹²⁵I]TID, an uncharged, hydrophobic photoreactive drug, that binds at the level of M2–9 and M2–13 in the closed state and at the level of M2–2 and M2–6 in the desensitized state (20, 21). Photolabeling studies with uncharged, hydrophobic drugs have also identified drug binding sites in the nAChR transmembrane domain other than the ion channel, including agonist-dependent drug binding at the extracellular end of the δ subunit helix bundle (21–23), and a binding site for a positive allosteric modulator at the interface between γ and α subunits (24).

In this article, we photolabel the nAChR with [³H]CPZ to identify its binding site in the ion channel in the closed channel state and to determine whether it binds to additional sites in the transmembrane domain of the nAChR in the desensitized state. Photoactivated [³H]CPZ reacts with aliphatic as well as polar amino acid side chains, and this broad reactivity profile is advantageous for the identification of novel binding sites in the hydrophobic nAChR transmembrane domain for inhibitors that are positively charged at physiological pH.

EXPERIMENTAL PROCEDURES

Materials. nAChR-rich membranes were isolated from freshly dissected *Torpedo californica* electric organs as described (25) and stored in 36% sucrose and 0.02% NaN₃ at –80 °C until needed. Carbamylcholine chloride (Carb), phenothiazine, and the hydrochloride salts of chlorpromazine (CPZ), promazine, tetracaine, proadifen, and phencyclidine (PCP) were from Sigma-Aldrich. Chlorpromazine sulfoxide (CPZsulfoxide) was from the NIMH Chemical Synthesis and Drug Supply Program. α -Bungarotoxin (α BgTx) was from Biotoxins (St. Cloud, FL). *Staphylococcus aureus* endoproteinase Glu-C (V8 protease) was from MP Biomedicals. TPCK-treated trypsin was from Worthington Biochemical Corp. *Lysobacter enzymogenes*

endoproteinase Lys-C (EndoLys-C) was from Roche Diagnostics. [³H]Phencyclidine (27 Ci/mmol) was from Perkin-Elmer Life Science, and [³H]tetracaine (30 Ci/mmol) was from Sibtech (Newington, CT). [³H]Chlorpromazine (16–28 Ci/mmol in ethanol, depending on lot) was from ViTrax (Placentia, CA). The purity of [³H]CPZ was monitored by thin-layer chromatography on a silica gel plate (Kieselgel 60 F254) with the chromatogram developed in a solvent mixture made up of 1-butanol, methanol, water, and acetic acid (1:3:1:0.2). [³H]CPZ migrated as a single spot (R_f = 0.8) well resolved from CPZsulfoxide (R_f = 0.4) or promazine (R_f = 0.6). Stock solutions of [³H]CPZ in ethanol were unstable over time, with [³H]CPZsulfoxide the major degradation product. When needed, oxidized [³H]CPZ (375 μ Ci) in 0.11 mL methanol/0.01 N HCl was reduced by exposure to metallic zinc (15 mg) for 2 h at room temperature (26), and then [³H]CPZ was purified by thin layer chromatography. For preparative photolabeling experiments leading to the identification of photolabeled amino acids, freshly synthesized [³H]CPZ was used at a purity >95%.

Radioligand Binding Assays. A centrifugation assay (27) was used to measure the effects of CPZ or its analogues on the equilibrium binding of [³H]PCP or [³H]tetracaine to nAChR-rich membranes in phosphate-buffered *Torpedo* physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, and 5 mM sodium phosphate at pH 7.0). For [³H]PCP (6 nM) or [³H]tetracaine (7 nM), 0.2 mL aliquots at 0.7 mg protein/mL (~500 nM ACh binding sites) were incubated with the drugs for 2 h before centrifugation for 40 min at 15,000 rpm in a TOMY TX201 centrifuge. For nAChRs in the desensitized state (+Carb), nonspecific binding for [³H]PCP was determined in the presence of 300 μ M proadifen, while for nAChRs equilibrated with α BgTx, nonspecific binding of [³H]PCP or [³H]tetracaine was measured in the presence of 300 μ M tetracaine.

Data Analysis. The concentration dependence of the inhibition of [³H]PCP or [³H]tetracaine binding was fit to the following single site binding equation:

$$f_x = f_0 / (1 + x / IC_{50})$$

where f_x is the specific ³H-labeled radioligand bound in the presence of competing drug at concentration x , f_0 is the specific radioligand bound in the absence of inhibitor, and IC_{50} is the total inhibitor concentration associated with 50% inhibition of radioligand binding. Sigmaplot (SPSS) was used for the nonlinear least-squares fit of the data, and the standard errors of the parameter fits are indicated.

Photolabeling nAChR-Rich Membranes. Photolabeling experiments were carried out with 0.2 mg (analytical) or 10 mg (preparative) of membrane protein for each condition. Unless otherwise noted, nAChR-rich membranes were resuspended at a final concentration of 2 mg protein/mL (~2.5 μ M [³H]ACh binding sites) in TPS with oxidized glutathione (1 mM) added to serve as aqueous scavenger. Membrane suspensions were added to a tube containing the appropriate quantity of [³H]CPZ (20 μ Ci for analytical and 150–200 μ Ci for preparative photolabelings) which had been dried under an argon stream. After mixing for 30 min to allow solubilization of [³H]CPZ, the membrane suspensions were aliquoted and incubated on ice with appropriate drugs for an additional 45 min. For analytical photolabeling, aliquots of 100 μ L were placed in the wells of a 96-well microtiter plate, whereas 6 cm diameter plastic Petri dishes (Falcon 351007) were used for preparative photolabelings.

Membrane suspensions on ice were irradiated at 350 nm with a Spectronics EN-16 lamp (40 nm full width for half-maximal transmission) for 30 min for the preparative labeling (+Carb ± PCP) and in a Rayonet RPR-200 photochemical reactor (Southern New England Ultraviolet Co.) using RPR-3500 bulbs (350 nm, 40 nm full width for half maximal transmission) for 15 min (+Carb ± proadifen) or 30 min (+αBgTx ± tetracaine). Following irradiation, nAChR-rich membranes were pelleted and resuspended in sample buffer for SDS–PAGE. [³H]CPZ at 1.7 and 0.8 μM was used for preparative photolabeling of nAChRs in closed (+αBgTx) and desensitized (+Carb) states, respectively. Preliminary analytical photolabeling studies, quantified by liquid scintillation counting of excised nAChR subunit bands, revealed differences of < 10% in the subunit photolabeling in the presence of oxidized glutathione at 0, 1, and 10 mM, and no difference in the amount of proadifen inhibitable labeling.

Gel Electrophoresis and Proteolytic Digestions. nAChR subunits were separated on 11 cm long, 1.5 mm thick, 8% acrylamide gels (28). Protein bands were visualized using Coomassie blue stain (analytical gels) or GelCode Blue stain (Pierce). For analytical photolabeling, samples were run in duplicate on two gels, one of which was processed first for fluorography with Amplify (Amersham Biosciences) and exposed to Kodak Biomax XAR X-ray film at −80 °C. ³H incorporation into the nAChR subunits in the gels was quantified by liquid scintillation counting of the excised gel bands (25).

For preparative scale photolabeling, the gel bands containing the nAChR β and δ subunits were excised and passively eluted into 12 mL of elution buffer (100 mM NH₄HCO₃, 0.1% SDS, and 2 mM dithiothreitol at pH 8.4) over 3 days with gentle agitation. The eluates were filtered and concentrated to < 400 μL in Vivaspins 15 M_r 5,000 centrifugal concentrators (Vivascience), and the concentrates were acetone-precipitated (75% acetone, −20 °C, overnight) and resuspended in a minimal volume (50–200 μL) of resuspension buffer (15 mM Tris, 0.1% SDS, and 500 μM EDTA at pH 8.1). The α and γ subunit bands were excised and placed in wells of a 15 cm long, 1.5 mm thick 15% acrylamide gel with a 4% stacker (5 cm) for limited in gel digestion with V8 protease (28). After electrophoresis, this mapping gel was stained with GelCode Blue stain, and the α subunit proteolytic fragments of 20 kDa (αV8–20), 18 kDa (αV8–18), and 10 kDa (αV8–10), and the 24 kDa γ subunit fragment (γV8–24) (29) were excised, eluted, concentrated, and either filtered before direct reversed-phase high-performance liquid chromatography (rpHPLC) purification or acetone-precipitated and resuspended in digestion buffer. Proteolytic digestions of aliquots of β (trypsin) or δ (EndoLys-C) subunits were separated on 1.5 mm thick, 16.5% T, 6% C Tricine SDS–PAGE gels (29, 30). In preliminary experiments, we had found that labeled fragments from EndoLys-C and trypsin digests of [³H]CPZ-photolabeled γ subunit aggregated when fractionated by Tricine SDS–PAGE, while a fragment beginning at the N-terminus of γM2 could be isolated by rpHPLC from a trypsin digest of γV8–24.

Samples of αV8–20 and the δ subunit in resuspension buffer were digested with EndoLys-C (0.75 U per sample) at 25 °C for ~2 weeks. Aliquots of the β, γ, or δ subunit in resuspension buffer were digested for 3 days with V8 protease (100%, w/w). For trypsin digestion, samples of αV8–10, the β subunit, or γV8–24 in resuspension buffer were diluted 5-fold with 0.5% Genapol C-100 (Calbiochem) in 100 mM NH₄HCO₃ at pH 8.1. After a 10 min incubation, trypsin (~1:1 (w:w) in 20 mM CaCl₂

(10% sample volume)) was added to each sample for a 24 h digestion at 25 °C.

Reversed-Phase HPLC and N-Terminal Sequence Analysis. Samples were fractionated by rpHPLC as described (24). Material for sequence analysis was isolated from three independent preparative photolabelings: +Carb ± PCP; +Carb ± proadifen; and +αBgTx ± tetracaine. Most samples of interest were loaded onto glass fiber filters (Applied Biosystems #401111) for sequence analysis. HPLC fractions were slowly drop-loaded onto filters placed on a 45 °C heating block and treated with Biobrene followed loading to prevent unwanted cleavage at Trp residues (31). Filters loaded with samples containing detergent were treated with gas TFA (5 min), followed by washes with ethyl acetate (4 min) and N-butyl chloride (4 min) to remove excess detergent prior to sequencing. Because of their poor retention on glass fiber filters, HPLC samples containing αM4 or δM1 were loaded onto polyvinylidene fluoride filters using the Prosorb sample preparation cartridge (Applied Biosystems #401950). Samples were sequenced on an Applied Biosystems Procise 492 automated protein sequencer altered such that 1/6 of each cycle was injected into the amino acid analyzer, and 5/6 of each cycle was collected for scintillation counting. The amount of peptide detected was calculated using the following equation:

$$f(x) = I_0 \times R^x$$

where $f(x)$ is the background-subtracted pmol of the amino acid detected in cycle x (determined by peak height), I_0 is the initial amount of peptide, and R is the average repetitive yield. A dotted line representing this calculation is included in the sequencing graphs. Cys, Ser, His, and Trp were not used for this fit because of known problems with their detection and/or quantitation during Edman degradation. Incorporation into a specific residue (cpm/pmol) was calculated as follows:

$$(cpm_x - cpm_{x-1}) / (5 \times I_0 \times R^x)$$

where cpm_x is the cpm measured in cycle x .

Molecular Modeling. The *Torpedo californica* nAChR residues photolabeled by [³H]CPZ were examined in the closed-state *Torpedo marmorata* nAChR model (PDB #2BG9) derived from 4 Å cryo-electron microscopy electron density data (5). Forty-seven residue variations exist between the four subunits of the two *Torpedo* species, 16 of which are found in the transmembrane helices but only 2 in the M2 helices, βM2–11 (*T. cal.*, Val; *T. mar.*, Leu) and δM2–6 (*T. cal.*, Ser; *T. mar.*, Cys), and none in the MA helices that are the extensions of the M4 helices into the nAChR cytoplasmic domain. Energy-minimized, protonated models of CPZ, PCP, and tetracaine were constructed and docked to regions of the nAChR model using Discovery Studio Suite (Accelrys) CDOCKER. For channel docking, the ligands were placed at various levels of the M2 helices (M2–2, M2–6, M2–9, M2–13, M2–16/17, M2–20, M2–24, and M2–28), a docking sphere of 12 Å radius was placed around the ligands, and the program identified 150 solutions ranked by interaction energy from 25 randomly chosen initial orientations. CPZ, tetracaine, and PCP were each accommodated sterically within the ion channel from all initial positions, with the most favorable CDOCKER interaction energy for CPZ docked near M2–16/17 with its N⁺ forming a salt bridge with αGlu-262 (M2–20), an orientation shown in Figure 9D and E. Docking of CPZ at the level of M2–6 and M2–9, shown in Figure 9D and F, was less favorable energetically. For tetracaine, the strongest interaction

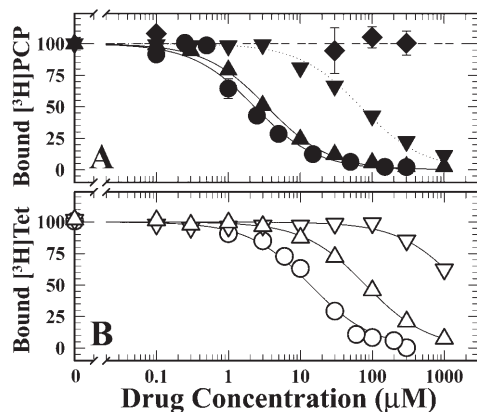


FIGURE 2: Inhibition of [3 H]PCP and [3 H]tetracaine binding to nAChR-rich membranes by CPZ and CPZ analogues. Equilibrium binding of [3 H]PCP (+Carb, closed symbols) (panel A) and of [3 H]tetracaine (+ α BgTx, open symbols) (panel B) was determined by centrifugation in the absence or presence of CPZ (●, $IC_{50} = 2.3 \pm 0.4 \mu M$; ○, $IC_{50} = 14 \pm 2 \mu M$), CPZsulfoxide (▼, $IC_{50} = 69 \pm 6 \mu M$; ▽, $IC_{50} = 1.7 \pm 0.2 mM$), promazine (▲, $IC_{50} = 3.5 \pm 0.2 \mu M$; △, $IC_{50} = 80 \pm 3 \mu M$), and phenothiazine (◆). For each competition experiment, the data were normalized to the specific binding in the absence of competitor, which for [3 H]PCP (+Carb) was 5530 ± 300 cpm (specific) and 700 ± 200 cpm (nonspecific), and for [3 H]tetracaine (+ α BgTx) was 3500 ± 300 cpm (specific) and 1700 ± 100 cpm (nonspecific).

energy was also for a molecule docked at the level of M2–16/17 in an orientation similar to that of CPZ, while for PCP, docking at the level of M2–13/16 was favored energetically. These simple calculations, which did not attempt to include phospholipid or energy minimization of the binding site structure (17), were used to identify the locations in the static nAChR that can accommodate CPZ and to visualize our experimental results.

RESULTS

CPZ and CPZsulfoxide Inhibition of [3 H]PCP and [3 H]Tetracaine Binding. Since [3 H]CPZsulfoxide was likely to be a contaminant of even recently synthesized/purified [3 H]CPZ, we first characterized its interactions with the nAChR in equilibrium binding assays using drugs that bind preferentially in the nAChR ion channel in the desensitized state ([3 H]PCP (32)) or a closed state ([3 H]tetracaine (33)). Assays were carried out in the presence of the agonist carbamylcholine (Carb), to stabilize nAChRs in the equilibrium desensitized state, or α -bungarotoxin (α BgTx), a peptide competitive antagonist that occupies the transmitter binding site stabilizing a closed channel state similar to the resting state. Consistent with previous reports (32), CPZ inhibited the binding of [3 H]PCP (+Carb) and [3 H]tetracaine (+ α BgTx) with IC_{50} values of $2 \mu M$ and $16 \mu M$, respectively. For each nAChR conformation, CPZsulfoxide bound with ~ 25 -fold lower affinity than CPZ (desensitized state, $IC_{50} = 55 \mu M$; closed state, $IC_{50} = \sim 1 mM$) (Figure 2). In parallel assays, we also determined that promazine (deschloro CPZ) inhibited the binding of [3 H]PCP (+Carb) and [3 H]tetracaine (+ α BgTx) with IC_{50} values of 3 and $80 \mu M$, while phenothiazine itself at concentrations up to $300 \mu M$ did not inhibit [3 H]PCP binding (+Carb). The low affinity of CPZsulfoxide for a site in the ion channel indicates that the presence of small amounts of this contaminant will be unlikely to contribute to observed photolabeling in the ion channel.

State-Dependent [3 H]CPZ Photoincorporation into the nAChR. The pharmacological specificity of nAChR photolabeling at the subunit level was determined by SDS–PAGE followed

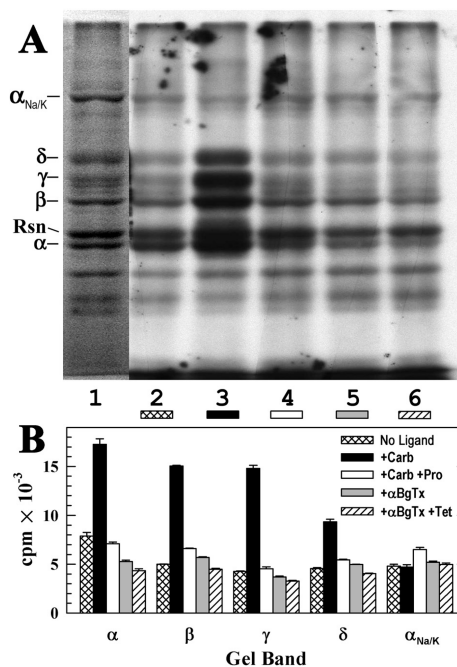


FIGURE 3: [3 H]CPZ photolabeling of the *Torpedo* nAChR in the closed and desensitized states. nAChR-rich membranes ($200 \mu g$ protein, 280 pmol of ACh binding sites in $100 \mu L$ of TPS supplemented with $1 mM$ oxidized glutathione) were equilibrated with $2.8 \mu M$ [3 H]CPZ alone (control, lane 2) or with $2 mM$ Carb (lane 3), $2 mM$ Carb and $0.1 mM$ proadifen (lane 4), $10 \mu M$ α BgTx (lane 5), or $10 \mu M$ α BgTx and $0.1 mM$ tetracaine (lane 6). After irradiation at $360 nm$ for $30 min$, aliquots of each condition were fractionated by SDS–PAGE on two gels, which were both stained with Coomassie blue with one processed first for fluorography, and the stained bands were excised from the second for 3H determination. (A) Coomassie blue stain (lane 1) and fluorograph (lanes 2–6; 14 day exposure at $-80^\circ C$). Indicated on the left are the mobilities of the nAChR subunits, rapsyn (Rsn), and the α subunit of the Na^+/K^+ -ATPase ($\alpha_{Na/K}$). (B) 3H incorporation in the gel bands containing the nAChR subunits and $\alpha_{Na/K}$, determined by liquid scintillation counting. Plotted are the mean cpm and range determined from bands in the two gels.

by fluorography and liquid scintillation of excised gel bands (Figure 3). nAChR-rich membranes were photolabeled with $3 \mu M$ [3 H]CPZ in the absence of other drugs, in the presence of the agonist Carb, or in the presence of α BgTx. Photolabeling of each nAChR subunit was 2–4-fold higher for nAChRs labeled in the desensitized state (+Carb) than those in a closed state (+ α BgTx), and for nAChRs in the desensitized state, proadifen, a desensitizing channel blocker (34), inhibited photolabeling of the δ subunit by 40%, the α and β subunits by 60%, and the γ subunit by 70%. For nAChRs photolabeled in the desensitized state, the 17,000 cpm incorporated in the α subunit indicated photolabeling of 0.8% of subunits, and the 15,000 cpm incorporated in the β and γ subunits indicated photolabeling of $\sim 1.4\%$ of subunits. Although subunit photolabeling in the presence of α BgTx was $< 50\%$ that seen for nAChRs in the desensitized state, at least a portion of that photolabeling was likely to be within the ion channel since tetracaine inhibited photolabeling of the α , β , and δ subunits by $\sim 20\%$.

The regions of PCP- or tetracaine-inhibitable photolabeling within the nAChR α subunit were further localized by subjecting the photolabeled subunits to in-gel digestion with V8 protease, which generates large, nonoverlapping subunit fragments of $20 kDa$ ($\alpha V8-20$, beginning at α Ser-173 and containing the M1, M2, and M3 membrane spanning helices as well as the ACh binding site segment C), $18 kDa$ ($\alpha V8-18$, beginning at α Thr-52

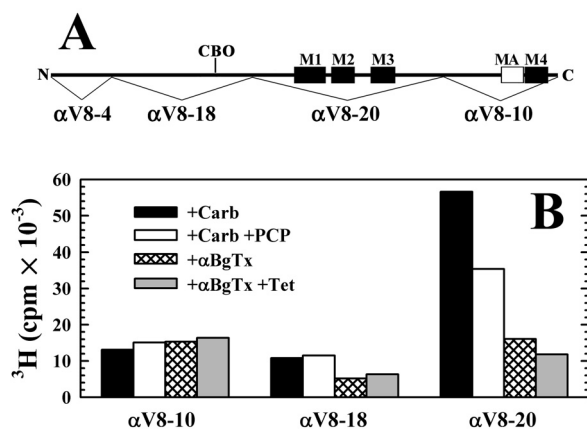


FIGURE 4: Distribution of [^3H]CPZ photoincorporation within large fragments of the nAChR α subunit. (A) A schematic indicating the location within the α subunit primary structure of the four α subunit fragments produced by in-gel digestion with V8 protease (28): $\alpha\text{V8-4}$, $\alpha\text{V8-18}$, containing most of the extracellular domain and the N-linked carbohydrate (CBO), $\alpha\text{V8-20}$, containing the M1-M3 membrane-spanning helices, and $\alpha\text{V8-10}$, containing M4 and the cytoplasmic amphipathic helix denoted MA. (B) [^3H]CPZ photoincorporation within the α subunit fragments isolated in separate preparative photolabelings of nAChRs either equilibrated with Carb $\pm 100 \mu\text{M}$ PCP (0.8 μM [^3H]CPZ) or with $\alpha\text{BgTx} \pm 100 \mu\text{M}$ tetracaine (1.7 μM [^3H]CPZ).

and containing ACh binding site segments A and B), and 10 kDa ($\alpha\text{V8-10}$, beginning at $\alpha\text{Asn-338}$ and containing the cytoplasmic MA helix and the M4 transmembrane helix) (28) (Figure 4). For membranes photolabeled in the presence of agonist, 70% of the ^3H was recovered in $\alpha\text{V8-20}$, and PCP reduced that labeling by 40%, with no inhibition of labeling in the gel bands containing $\alpha\text{V8-18}$ or $\alpha\text{V8-10}$.² For nAChRs photolabeled in the presence of $\alpha\text{-BgTx}$, 40% of ^3H was associated with $\alpha\text{V8-20}$, and tetracaine reduced that labeling by 25%.

To identify amino acids photolabeled by [^3H]CPZ in the nAChR in the desensitized state, nAChR-rich membranes were photolabeled on a preparative scale on two occasions in the presence of agonist, in the absence or presence of PCP or proadifen. To identify amino acids photolabeled in the nAChR in the closed channel state, membranes were equilibrated with αBgTx and photolabeled in the absence or presence of tetracaine. The experimental identification of the photolabeled amino acids are presented by representative sequencing data in the figures, and the average efficiencies of photolabeling at each position, obtained by sequencing multiple samples, are presented in Table 1 in order to compare the relative efficiencies of photolabeling in the different positions.

[^3H]CPZ Photoincorporation in the nAChR Ion Channel in the Presence of Agonist (Desensitized State). In a first experiment, nAChR-rich membranes equilibrated with agonist were photolabeled with [^3H]CPZ \pm PCP. Fragments beginning at the N-termini of βM2 ($\beta\text{Met-249}$) and δM2 ($\delta\text{Met-257}$) were isolated for sequence analysis by SDS-PAGE and rpHPLC

from trypsin digests of the β subunit and EndoLys-C digests of the δ subunit (Supporting Information Figure S1). Within βM2 (Figure 5A), there were peaks of ^3H release in cycles 6 and 9 that were fully inhibited by PCP, consistent with the labeling of $\beta\text{M2-6}$ ($\beta\text{Ser-254}$, 2 cpm/pmol) and $\beta\text{M2-9}$ ($\beta\text{Leu-257}$, 2 cpm/pmol), but the major peaks of ^3H release were in cycles 17 and 20, indicating photolabeling of $\beta\text{M2-17}$ ($\beta\text{Leu-265}$, 16 cpm/pmol) and $\beta\text{M2-20}$ ($\beta\text{Asp-268}$, 9 cpm/pmol). While PCP inhibited the photolabeling of $\beta\text{M2-6}$ and $\beta\text{M2-9}$, it did not inhibit the photolabeling of $\beta\text{M2-17}$ and $\beta\text{M2-20}$ near the extracellular end of the ion channel domain. Similarly, within δM2 (Figure 5B), there was PCP-inhibitable photolabeling of $\delta\text{M2-6}$ ($\delta\text{Ser-262}$, 0.8 cpm/pmol), but the major peaks of ^3H release were in cycles 17 ($\delta\text{Leu-273}$, 3 cpm/pmol) and 20 ($\delta\text{Gln-276}$, 12 cpm/pmol), neither inhibitable by PCP. While [^3H]CPZ photolabeling at the extracellular end of the ion channel has not been previously examined, the PCP-inhibitable [^3H]CPZ photolabeling of $\beta\text{M2-6}$, $\beta\text{M2-9}$, and $\delta\text{M2-6}$ is as reported previously by Changeux and co-workers (13, 14) in sequence analyses that extended only to M2-15.

To characterize the amino acids photolabeled in γM2 , we first attempted to fractionate EndoLys-C or trypsin digests of [^3H]CPZ-labeled γ subunits by Tricine SDS-PAGE. Since the labeled fragments in these digests migrated as intractable high molecular weight aggregates, as an alternative strategy we took advantage of the fact that V8 protease can be used to cleave the γ subunit at $\gamma\text{Glu-208}$ before γM1 , and we sequenced that fragment for 65 cycles to the next Glu after γM2 (Supporting Information Figure S2). While sequencing through γM2 , there was small peak of ^3H release in cycle 59 (20 cpm), consistent with the labeling of $\gamma\text{M2-16}$ ($\gamma\text{Phe-267}$), with lower peaks of ^3H release in cycles 45 and 52, consistent with the labeling of $\gamma\text{M2-2}$ ($\gamma\text{Thr-253}$) and $\gamma\text{M2-9}$ ($\gamma\text{Leu-260}$). In addition, there were more prominent peaks of ^3H release in earlier cycles of Edman degradation, but each of these could be accounted for by [^3H]CPZ photolabeling of $\gamma\text{Phe-267}$ and the presence of an acid-label Gln-Thr bond 5 cycles before that position.

In a second photolabeling experiment, [^3H]CPZ photolabeling within each of the M2 segments was characterized when nAChRs equilibrated with Carb were photolabeled \pm proadifen (Figure 6). Fragments beginning at the N-termini of βM2 and δM2 were isolated as described above, and sequence analysis established that [^3H]CPZ photolabeling of positions M2-6 and M2-9 was fully inhibited by proadifen, while photolabeling at M2-17 and M2-20 was partially inhibited (Figure 6A and B). Qualitatively, the photolabeling of positions M2-6 and M2-9 in this experiment was more prominent than that in the experiment in Figure 5. Quantitative comparison of the efficiencies of photolabeling (cpm/pmol) at each position indicates that $\beta\text{M2-20}$ and $\delta\text{M2-20}$ were each photolabeled with similar efficiency in the two experiments and that it was the photolabeling efficiency at the cytoplasmic end that differed (Table 1). Further studies would be required to identify the origin of this difference.³

²Further purification by rpHPLC established that only $\sim 20\%$ of the ^3H in the gel band containing $\alpha\text{V8-18}$ was associated with that fragment, which eluted as a sharp peak at $\sim 45\%$ organic, well resolved from the major peak of ^3H eluting at $\sim 80\%$ organic (not shown). Similarly, rpHPLC purification of the material in the gel bands containing $\alpha\text{V8-10}$ from nAChRs photolabeled either +Carb or + αBgTx established that only $\sim 50\%$ of ^3H was associated with $\alpha\text{V8-10}$, which elutes as a broad hydrophobic peak centered at 80% organic, with the remainder eluting as a hydrophilic peak at $\sim 50\%$ organic (see Figure 8).

³Photoexcitation of CPZ at 330 nm results almost exclusively in dechlorination and the formation of a reactive radical intermediate (35), while photoexcitation at 280 nm may result in the preferential loss of an electron and the formation of a radical cation intermediate (43), but see ref (35). Since the experiments of Figures 5 and 6 used two different Hg lamps, each with filters for optimal excitation at 350 nm, but with different half-widths, it is possible that subtle differences in the wavelength of photoexcitation may determine whether Site17/20 is photolabeled at a higher efficiency than Site2/6/9 (Figure 5) or at a similar overall efficiency (Figure 6).

Table 1: Pharmacological Specificity of [^3H]CPZ Photoincorporation into Residues in the nAChR M2 Ion Channel Domain in the Desensitized (+Carb) and Closed Channel (+ αBgTx) States^a

	+Carb -PCP/+PCP (Figure 5) (cpm/pmol)		+Carb -proadifen/+proadifen (Figure 6) (cpm/pmol)				+ αBgTx -tetracaine/+tetracaine (Figure 7) (cpm/pmol)		
	β	δ	α	β	γ	δ	α	β	δ
M2-2	<0.1/<0.1	<0.2/<0.3	3.4 / <0.1 (± 1.2)/	0.2 / <0.1 (± 0.1)/	52 / <2	0.3 / <0.05	<0.2/<0.1	<0.1/<0.05	<0.02/<0.01
M2-5	0.3 / <0.1 (± 0.1)/	<0.2/<0.05	2.0 / <0.2 (± 0.4)/	1.1 / <0.05 (± 0.3)/	<1/<2	<0.1/<0.02	0.6 / <0.2 (± 0.04)/	0.3 / <0.05	0.1 / <0.02 (± 0.002)/
M2-6	1.2 / 0.3 (± 0.3)/(± 0.03)	0.6 / <0.05 (± 0.02)/	12 / <0.2 (± 1.4)/	6.2 / 0.2 (± 1.2)/	<2/<2	4.9 / 0.2 (± 0.04)/	1.0 / <0.2 (± 0.04)/	0.2 / <0.05	4.4 / 0.1 (± 0.2)/(± 0.002)
M2-9	2.7 / 0.4 (± 0.3)/(± 0.03)	0.5 / <0.05 (± 0.05)/	3.2 / <0.1 (± 0.5)/	20 / 0.2 (± 4.7)/	<2/<2	2.0 / <0.02	1.3 / <0.2 (± 0.2)/	8.0 / 0.3	0.5 / <0.02 (± 0.01)/
M2-16	2.2 / 2.0 (± 1.0)/(± 0.7)	1.1 / 1.6 (± 0.1)/(± 0.2)	0.6 / 0.5 (± 0.5)/	2.7 / 0.5 (± 1.2)/	32 / 14	1.4 / 0.5	<0.05/<0.1	<0.1/<0.2	<0.05/<0.05
M2-17	17 / 23 (± 2)/(± 2)	3.4 / 5.3 (± 0.03)/(± 1.7)	1.5 / 0.7 (± 0.3)/	26 / 6.4 (± 5.5)/	<5/<3	13 / 3.0	<0.05/<0.1	0.3 / <0.2	0.2 / <0.05 (± 0.001)/
M2-20	11 / 11 (± 1.2)/(± 1.3)	10 / 14 (± 2)/(± 0.5)	4.1 / 2.1 (± 0.5)/	18 / 2.3 (± 7.2)/	10 / <5	13 / 6.4	0.6 / 1.2 (± 0.2)/(± 0.02)	0.5 / 1.5	0.6 / 0.6 (± 0.01)/(± 0.005)

^aThe ^3H incorporation in each residue (cpm/pmol of PTH-derivative) was calculated from the observed ^3H release and the initial and repetitive yields as described under Experimental Procedures. The results are from the three photolabeling experiments, including data from Figures 5–7. When three or two samples were sequenced, data are presented as a mean ($\pm\text{SEM}$) (data columns 1, 3, and 7) or as a mean ($\pm\text{range}$) (data columns 2, 4, and 9), respectively. For the other data, only a single aliquot of each sample was sequenced. The cpm/pmol values in bold text are the values for positions at which the peak of ^3H release was $>20\%$ over the background releases of ^3H in preceding cycles. The upper limits of photolabeling in the other cycles were determined from the random variation of the background release of ^3H in the adjacent sequencing cycles.

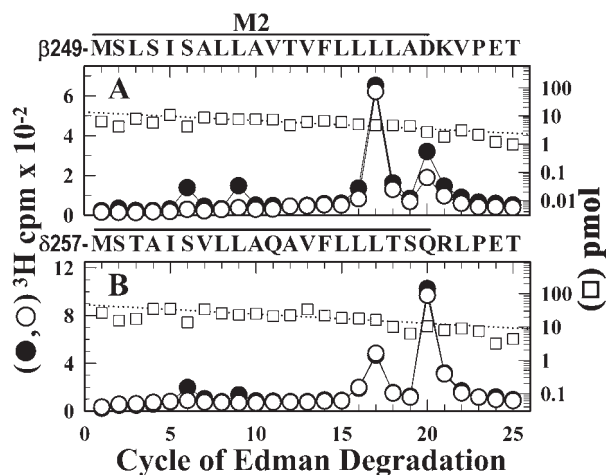


FIGURE 5: PCP modulation of [^3H]CPZ photoincorporation in the nAChR ion channel in the presence of agonist. ^3H (●, ○) and PTH-amino acids (□) released during sequence analysis through βM2 (A) and δM2 (B) in subunit fragments isolated from nAChR-rich membranes photolabeled with $0.8\ \mu\text{M}$ [^3H]CPZ after equilibration with Carb in the absence (●, ○) or presence (○, □) of $100\ \mu\text{M}$ PCP. Trypsin digests of β subunits and EndoLys-C digests of δ subunits were fractionated by Tricine SDS-PAGE and rHPLC to isolate fragments beginning at $\beta\text{Met-249}$ or $\delta\text{Met-257}$ (Supporting Information Figure S1). (A) The primary sequence began at $\beta\text{Met-249}$ (□, +PCP, 14 pmol; -PCP, 18 pmol), with any secondary sequence at <0.5 pmol. For nAChRs labeled +Carb (●), the peaks of ^3H release in cycles 6, 9, 17, and 20 indicated labeling of $\beta\text{Ser-254}$, $\beta\text{Leu-257}$, $\beta\text{Leu-265}$, and $\beta\text{Asp-268}$ at 2, 2, 16, and 9 cpm/pmol, respectively. Addition of PCP (○) reduced labeling of $\beta\text{Ser-254}$ and $\beta\text{Leu-257}$ to 0.3 cpm/pmol, while labeling of $\beta\text{Leu-265}$ was increased to 25 cpm/pmol, and labeling of $\beta\text{Asp-268}$ was unchanged (7 cpm/pmol). (B) The primary sequence began at $\delta\text{Met-257}$ (□, +PCP, 46 pmol; -PCP, 40 pmol), with a secondary sequence beginning at $\delta\text{Asn-437}$ (3 pmol both conditions). For nAChRs labeled +Carb (●), the peaks of ^3H release in cycles 6, 9, 17, and 20 indicated photolabeling of $\delta\text{Ser-262}$, $\delta\text{Leu-265}$, $\delta\text{Leu-273}$, and $\delta\text{Gln-276}$ at 1, 1, 3, and 12 cpm/pmol, respectively. Addition of PCP (○) reduced photolabeling of $\delta\text{Ser-262}$ and $\delta\text{Leu-265}$ to <0.1 cpm/pmol, while labeling of $\delta\text{Leu-273}$ (4 cpm/pmol) and $\delta\text{Gln-276}$ (13 cpm/pmol) was not affected.

Photolabeling within αM2 was characterized by sequencing an EndoLys-C digest of $\alpha\text{V8-20}$ (Figure 6C), which revealed a major peak of ^3H release in cycle 6, indicating labeling of $\alpha\text{M2-6}$ ($\alpha\text{Ser-248}$, 11 cpm/pmol), with smaller peaks of ^3H release in cycles 2 and 9, consistent with the labeling of $\alpha\text{Thr-244}$ (5 cpm/pmol) and $\alpha\text{Leu-251}$ (3 cpm/pmol). There were also small peaks of ^3H release in cycles 16, 17, and 20 (24, 26, and 60 cpm, respectively, expanded ordinate scale; Figure 6C') indicative of the photolabeling of $\alpha\text{Leu-258}$, $\alpha\text{Val-259}$, and $\alpha\text{Glu-262}$ at 1, 1, and 4 cpm/pmol. Photolabeling of $\alpha\text{M2-2}$, $\alpha\text{M2-6}$, and $\alpha\text{M2-9}$ was each reduced by $>95\%$ in the presence of proadifen. To characterize the photolabeling within γM2 (Figure 6D), the labeled γ subunit was digested in gel with V8 protease to produce a 24 kDa fragment ($\gamma\text{V8-24}$, beginning at $\gamma\text{-Ala167}$ (29)), and a fragment beginning at the γM2 N-terminus ($\gamma\text{Cys-252}$) was isolated by rHPLC at $\sim 55\%$ organic from a trypsin digest of $\gamma\text{V8-24}$ (Supporting Information Figure S3). Sequence analysis revealed the presence of the $\gamma\text{Cys-252}$ fragment at 0.5 pmol (along with a trypsin fragment at 5 pmol) and a major peak of ^3H release in cycle 2 indicating the labeling of $\gamma\text{Thr-253}$ at 50 cpm/pmol that was reduced $>95\%$ by proadifen. A minor peak of ^3H release in cycle 16 (Figure 6D') indicated the labeling of $\gamma\text{Phe-267}$ at ~ 30 cpm/pmol.

[^3H]CPZ Photoincorporation in the nAChR Ion Channel in the Presence of αBgTx (Closed State). nAChR-rich membranes were equilibrated with αBgTx and then photolabeled on a preparative scale with [^3H]CPZ in the absence or presence of tetracaine, a high affinity, closed-state selective channel blocker (19, 33). When the ^3H incorporation into the polypeptides of the nAChR-rich membrane preparation was determined by analytical SDS-PAGE and fluorography, rapsyn and the nAChR β subunit were the most highly photolabeled polypeptides (Supporting Information Figure S4). There was tetracaine-inhibitable labeling within the nAChR α , β , and δ subunits, with little, if any, in the γ subunit, in the α subunit of the $\text{Na}^+/\text{K}^+\text{-ATPase}$, or in rapsyn. Subunit fragments beginning at the

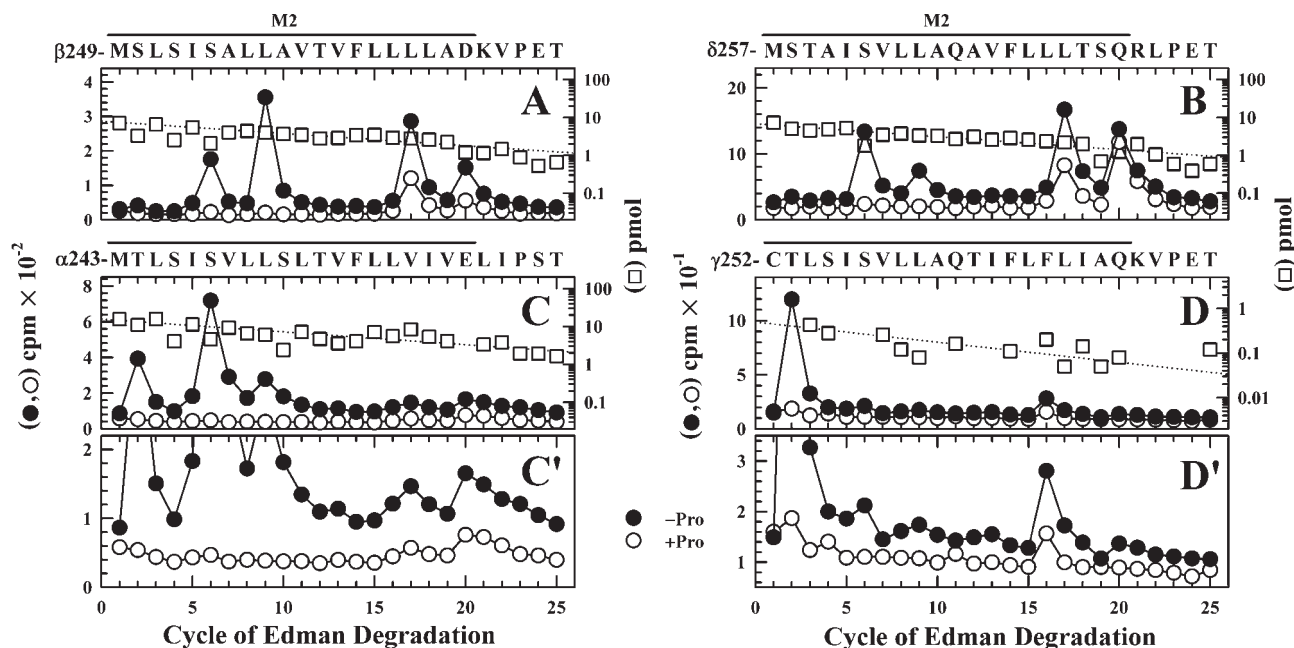


FIGURE 6: Proadifen modulation of [^3H]CPZ photoincorporation at the extracellular and cytoplasmic ends of the M2 ion channel domain in the presence of agonist. ^3H (●, ○) and PTH-amino acids (□) released during sequence analysis of fragments beginning at the N-termini βM2 (A), δM2 (B), αM2 (C, C'), and γM2 (D, D'), isolated from nAChR-rich membranes photolabeled with $0.8\ \mu\text{M}$ [^3H]CPZ after equilibration with Carb in the absence (●) or presence (○, □) of $100\ \mu\text{M}$ proadifen. Photolabeled subunits were isolated by SDS-PAGE, and the βM2 and δM2 fragments were isolated as described in Figure 5, while $\alpha\text{V8-20}$ and $\gamma\text{V8-24}$ were isolated by in gel digestion with V8-protease. Then an EndoLys-C digest of $\alpha\text{V8-20}$ was sequenced directly, and the γM2 fragment was isolated by rpHPLC from a trypsin digest of $\gamma\text{V8-24}$ (Supporting Information Figure S3). (A) The primary sequence began at $\beta\text{Met-249}$ ($I_0 = 8\ \text{pmol}$ – (□) and +proadifen), with a secondary sequence beginning at $\beta\text{Lys-216}$ ($\sim 0.5\ \text{pmol}$) ($10,800$ (●) and $2,600$ (○) cpm sequenced). (B) The only sequence detected began at $\delta\text{Met-257}$ ($I_0 = 7\ \text{pmol}$ – (□) and $11\ \text{pmol}$ +proadifen; $8,000$ (●) and $3,300$ (○) cpm sequenced). (C) The fragment beginning at $\alpha\text{Met-243}$ was the primary sequence ($I_0 = 16\ \text{pmol}$ – (□) and +proadifen), along with α subunit fragments beginning at $\alpha\text{Ser-173}$ ($10\ \text{pmol}$), $\alpha\text{His-186}$ ($8\ \text{pmol}$), and $\alpha\text{Tyr-277}$ ($5\ \text{pmol}$), as well as EndoLys-C (Gly-1, $8\ \text{pmol}$) and a V8 protease peptide (Val-180, $20\ \text{pmol}$). ($37,000$ (●) and $12,200$ (○) cpm sequenced). C' is an enlargement of C to show more clearly the level of labeling at $\alpha\text{M2-16}$, $\alpha\text{M-17}$, and $\alpha\text{M2-20}$. (D) While the primary sequence was trypsin ($6\ \text{pmol}$), the fragment beginning at $\gamma\text{Cys-252}$ was present ($I_0 = 0.5\ \text{pmol}$ – (□) and +proadifen; $1,280$ (●) and 510 (○) cpm sequenced). D' is an enlargement of D included to better show the level of labeling at $\gamma\text{M2-16}$. For all sequencing runs, the efficiencies of photolabeling (cpm/pmol) associated with the peaks of ^3H in cycles 2, 6, 9, 16, 17, or 20 are quantitated in Table 1.

N-termini of α -, β -, and δ -M2 were isolated for sequence analysis by the same procedures used for nAChRs photolabeled in the desensitized state.

For nAChRs photolabeled in the presence of αBgTx , the peaks of ^3H release when sequencing through αM2 indicated the photolabeling of $\alpha\text{M2-5}$ ($\alpha\text{Ile-247}$), $\alpha\text{M2-6}$ ($\alpha\text{Ser-248}$), and $\alpha\text{M2-9}$ ($\alpha\text{Leu-251}$), each at $\sim 1\ \text{cpm/pmol}$, while within βM2 and δM2 , the major peaks of ^3H release in cycle 9 and 6, respectively, indicated the photolabeling of $\beta\text{Leu-257}$ at $8\ \text{cpm/pmol}$ and $\delta\text{Ser-262}$ at $4\ \text{cpm/pmol}$ (Figure 7 and Table 1). Photolabeling at each of these positions was reduced by $> 90\%$ in the presence of tetracaine. In addition to these major peaks of ^3H release, in each subunit there was a low level release in cycle 20, consistent with the labeling of M2-20 at an efficiency $\sim 10\%$ that of position M2-6 or M2-9, and the photolabeling at M2-20 was not inhibited by tetracaine.

[^3H]CPZ Photoincorporation Outside of the Ion Channel. Upon the basis of the broad side chain reactivity observed for [^3H]CPZ photolabeling in the ion channel, including aliphatic (Val, Ile, and Leu), aromatic (Phe), and polar (Ser, Thr, Glu, Asp, and Gln) side chains, we extended our studies to determine whether [^3H]CPZ photoincorporated into other regions of the nAChR transmembrane domain. Since CPZ also binds to the agonist sites (12), we characterized photolabeling for nAChRs equilibrated with agonist (desensitized state) to search for binding sites distinct from those sites. Novel binding sites have been identified recently in the nAChR transmembrane domain for

uncharged hydrophobic drugs that bind within the ion channel and to sites in the δ subunit helix bundle (TID and benzophenone), or at the interfaces between subunits (benzophenone (γ - α and β - α); TDBzl-etomidate (γ - α) (21–24)).

[^3H]CPZ Does Not Bind/Photolabel within the δ Subunit Helix Bundle. In the desensitized state, there was no evidence that [^3H]CPZ photolabeled $\delta\text{M2-18}$ or $\delta\text{M2-22}$ (Figures 5B and 6B), residues that project toward the pocket formed by the δ subunit helix bundle and that are labeled by [^{125}I]TID in the open and desensitized states, along with $\delta\text{Ile-288}$ within the $\delta\text{M2-M3}$ loop and $\delta\text{Phe-232}$ and $\delta\text{Cys-236}$ within δM1 (21, 22). There was also no evidence of [^3H]CPZ photolabeling within δM1 , as we found only background ^3H in the rpHPLC fractions containing the fragment beginning at $\delta\text{Phe-206}$ and extending through δM1 , and no peaks of ^3H release when that fragment was sequenced through δM1 (labeling, if it occurred, was less than $0.4\ \text{cpm/pmol}$ or 3% the level of labeling of $\delta\text{M2-20}$). Also, sequence analysis of rpHPLC fractions from a solution digest of the δ subunit with V8 protease, which can be used to identify the photolabeling of $\delta\text{Ile-288}$ or amino acids in δM3 (23), established that $\delta\text{Ile-288}$ was not labeled by [^3H]CPZ ($< 0.1\ \text{cpm/pmol}$, data not shown).

[^3H]CPZ Photolabels within the α Subunit MA Helix but Not in αM4 . For nAChRs photolabeled in the desensitized state, fragmentation of the α subunit with V8 protease established that there was ^3H incorporation in $\sim 10\ \text{kDa}$ fragments that include $\alpha\text{V8-10}$, the fragment beginning at $\alpha\text{Asn-339}$ (28)

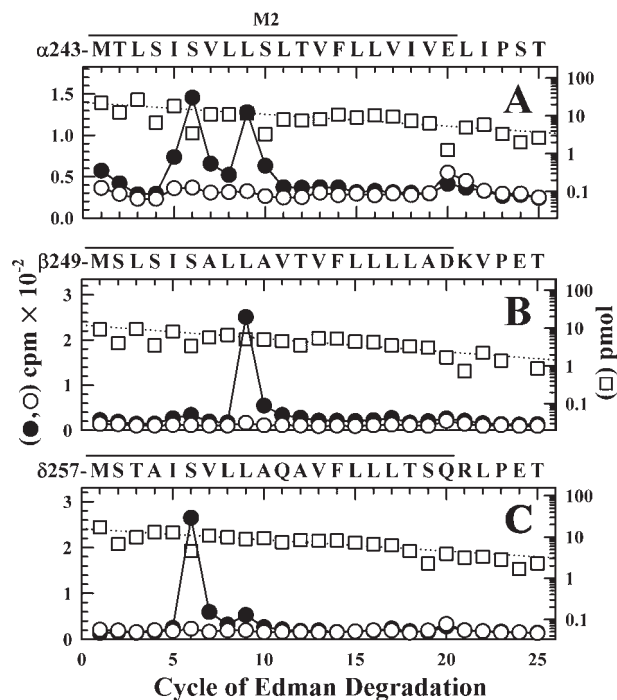


FIGURE 7: [^3H]CPZ photolabeling in the M2 ion channel in the presence of α -BgTx. ^3H (●, ○) and PTH-amino acids (□) released during sequence analysis of fragments beginning at the N-termini α M2 (A), β M2 (B), and δ M2 (C), isolated from nAChR-rich membranes photolabeled with 1.7 μM [^3H]CPZ after equilibration with 10 μM α BgTx in the absence (●, ○) or presence (□, □) of 100 μM tetracaine. Labeled subunits were separated by SDS-PAGE and processed as in Figure 5 to isolate peptides beginning at the N-termini of the M2 segments. (A) The fragment beginning at α Met-243 was the primary sequence (I_0 = 23 pmol –(□) and 17 pmol +tetracaine), along with α subunit fragments beginning at α Ser-173 (~12 pmol), α His-186 (~18 pmol), and α Tyr-277 (~2 pmol), as well as EndoLys-C (Gly-1, ~8 pmol) and a V8 protease peptide (Val-180, ~5 pmol) (10,200 (●) and 7,820 (○) cpm sequenced). (B) The primary sequence began at β Met-249 (I_0 = 12 pmol –(□) and 10 pmol +tetracaine), with any secondary sequence at <10% that level (1,900 (●) and 960 (○) cpm sequenced). (C) The only sequence detected began at δ Met-257 (I_0 = 15 pmol –(□) and +tetracaine; 5,300 (●) and 2,700 (○) cpm sequenced). For all sequencing runs, the efficiencies of photolabeling (cpm/pmol) associated with the peaks of ^3H in cycles 5, 6, 9, 16, 17, or 20 are quantitated in Table 1.

that contains the MA helix from the nAChR cytoplasmic domain (beginning at α Ile-376), and the hydrophobic M4 membrane spanning helix beginning at α Ile-406 (Figure 4). The amino acids in these fragments photolabeled by [^3H]CPZ were characterized by sequence analysis of the peaks of ^3H recovered when aliquots of that material were fractionated by rpHPLC before and after trypsin digestion (Figure 8A). Before digestion, ^3H was recovered in two broad peaks, one a hydrophobic peak centered at ~80% organic, where α V8–10 elutes (29), and a second hydrophilic peak centered at 50% organic, with <5% of ^3H eluting in the column flow-through. Sequence analysis of the hydrophobic peak confirmed the presence of the fragment beginning at α Asn-339 (α V8–10, ~20 pmol), with no peaks of ^3H release in 30 cycles of Edman degradation (not shown). When material from the hydrophilic peak was sequenced, there were no peaks of ^3H release in 10 cycles of Edman degradation, and the N-terminus of V8 protease was the only identifiable fragment (~2 pmol) (not shown). When the trypsin digest was fractionated by rpHPLC, ~50% of ^3H eluted in the column flow-through, whereas 15% was recovered in a broad hydrophobic peak

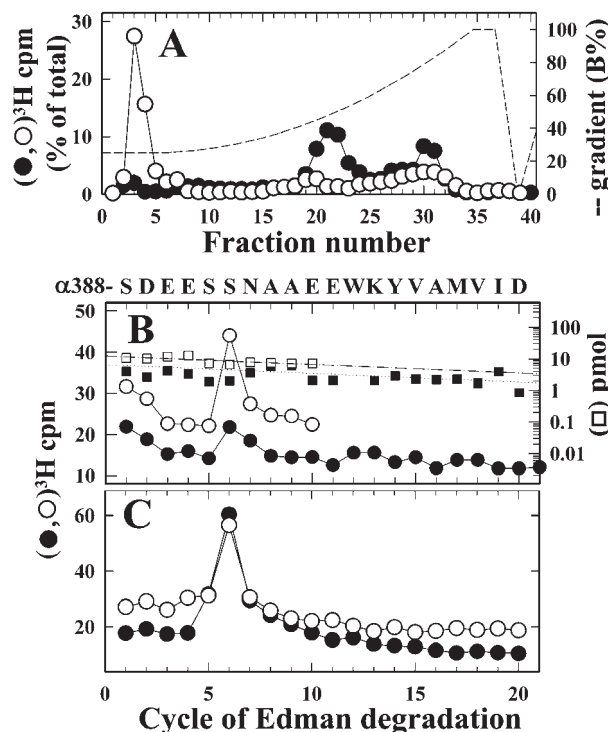


FIGURE 8: [^3H]CPZ photolabeling in the α subunit cytoplasmic (MA) helix. In gel digestion with V8 protease was used to isolate α subunit fragments of ~10 kDa from nAChRs from the photolabeling experiment used in Figure 4 (nAChRs photolabeled +Carb ± PCP). (A) rpHPLC fractionation of aliquots of this material, from nAChRs photolabeled –PCP, before (●) and after (○) digestion with trypsin. ^3H recovery (%) before and after trypsin digestion: fractions 3–5 (4%, 50%); fractions 20–23 (35%, 6%); fractions 28–32 (23%, 15%). (B and C) ^3H (●, ○) and PTH-amino acids (□, ■) released during sequence analysis of fractions 28–32 (B) and 3–5 (C) from the rpHPLC fractionations of the tryptic digests of material photolabeled –PCP (●, ■) and +PCP (○, □). (B) The hydrophobic peak of ^3H contained overlapping fragments beginning at α Ser-388 (■, I_0 = 7 pmol; □, I_0 = 12 pmol) and α Tyr-401 (not shown, –PCP, 5 pmol; +PCP, 8 pmol), with ^3H release at cycle 6 (4,400 (●) and 3,600 (○) cpm sequenced). For the sample not digested with trypsin, no peak of ^3H release was detected in 30 cycles of Edman degradation of fractions 28–32, which contained the fragment beginning at α Asn-339, the N-terminus of α V8–10 (–PCP, 19 pmol), or fractions 20–23, which contained V8 protease (~2 pmol). (C) ^3H release during sequence analysis of material in fractions 3–5 (11,100 (●) and 8,700 (○) cpm sequenced). Filters were washed before sequencing, as described in Experimental Procedures, to remove excess detergent.

centered at 80% organic (Figure 8A). Sequence analysis of the hydrophobic peak established the presence of fragments beginning at α Ser-388 and α Tyr-401 (~6 pmol of each), produced by trypsin cleavage at the two lysines N-terminal to M4, and a peak of ^3H release in cycle 6 (Figure 8B). In another photolabeling experiment, sequence analysis of the hydrophobic peak from the trypsin digest of α V8–10 established the presence of the α Tyr-401 fragment at 5 pmol and <0.3 pmol of the α Ser-388 fragment, with no peak of ^3H release in cycle 6 (not shown). The simplest interpretation of these results is that the peak of ^3H release in cycle 6 (Figure 8B) resulted from the low level labeling of α Ser-393 (~0.5 cpm/pmol). When the material in the rpHPLC column flow-through from the tryptic digest was sequenced for 20 cycles, the only peak of ^3H release was also in cycle 6 (Figure 8C), though individual fragment sequences were not identifiable. This release in cycle 6 is also likely to result, at least in part, from the photolabeling of α Ser-393, in the tryptic fragment extending

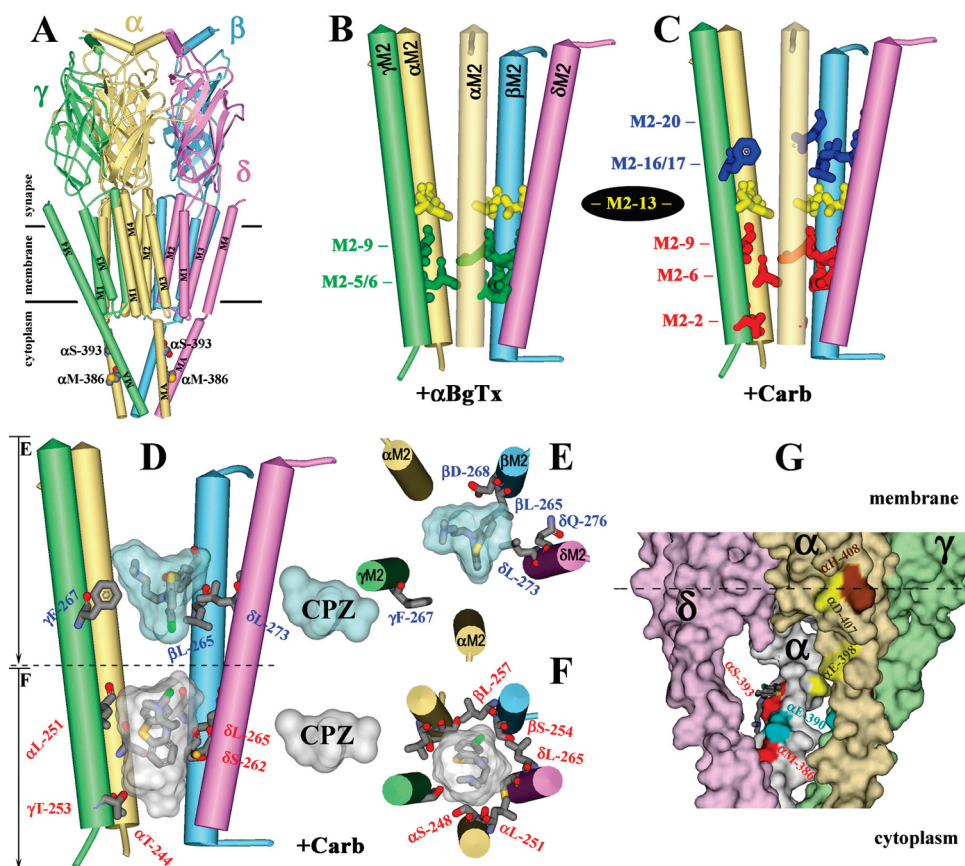


FIGURE 9: CPZ binding sites within the *Torpedo* nAChR ion channel. The model of the *Torpedo marmorata* nAChR (PDB # 2BG9) showing secondary structural elements (α -helices as cylinders and β -sheets as ribbons) color-coded by subunit (α , gold; β , blue; γ , green; and δ , magenta). (A) The entire structure is shown for orientation with respect to the membrane. The cytoplasmic residues labeled by [3 H]CPZ (α Met-286 and α Ser-393) are represented in CPK format. (B–F) Images of the M2 helices which form the ion channel domain, with the residues labeled by [3 H]CPZ represented in stick format. (B and C) The residues labeled by [3 H]CPZ in the closed state (B, green) or in the desensitized state (C) at the extracellular (blue, Site1/7/20) and cytoplasmic (red, Site2/6/9) ends of the channel, along with the ring of Val/Ile at M2–13 (yellow) that are not photolabeled by [3 H]CPZ. (D–F) The binding pockets for CPZ visualized as Connolly surface representations of the 10 lowest energy solutions when CPZ was docked within the ion channel at the level of M2–6 or M2–17 (see Experimental Procedures). In each site, the orientation of the lowest energy docked ligand and the residues labeled by [3 H]CPZ are shown in stick format, color coded by atom type (carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; and chlorine, green). The ion channel can accommodate two CPZ molecules simultaneously, as viewed from the side (D) or looking down the channel (E, upper binding site, blue pocket; F, lower binding site, white pocket). (G) The locations of [3 H]CPZ-photolabeled α Met-386 and α Ser-393 in the cytoplasmic basket (Connolly surface representation) formed by the MA helices of α , (beige), γ (pale green), α δ (white), δ (plum), and β (not shown) subunits, with color coding of selected amino acids: [3 H]CPZ-labeled α Met-386 and α Ser-393 (red); [3 H]azietomidate-photolabeled α Glu-390 (cyan) (40); [3 H]azidoctanol-photolabeled α His-408 (brown) (37); and [3 H]azidocholesterol-photolabeled α Glu-398 and α Asp-407 (yellow) (42). The lowest energy solution from docking CPZ near α δ Ser-393 (150 solutions total) is shown in stick format color-coded by atom type: carbon, gray; nitrogen, blue; sulfur, gold; and chlorine, green.

from α Ser-388 to α Lys-400 that would be expected to elute in the column flow-through. The 3 H in the rpHPLC column flow-through of the tryptic digest (Figure 8A) must also include digestion products of the labeled fragments that eluted in the hydrophilic peak of 3 H before trypsin digestion. Further studies are required to identify these photolabeled amino acids, but α Ser-393 is one likely candidate, as it could have been contained in a hydrophilic fragment of ~ 7 kDa produced by V8 protease cleavage of the nAChR α subunit (for example, α Asn-339 to α Glu-391/397).

To identify additional amino acids within α MA located N-terminal to α Ser-388, we carried out another [3 H]CPZ photolabeling of nAChRs (+Carb) and isolated, as above, α V8–10 for sequence analysis. When the fragment beginning at α Asn-339 (40 pmol) was sequenced for 60 cycles, there was a small peak of 3 H release in cycle 55, which confirmed the photolabeling of α Ser-393 (1 cpm/pmol), and in addition, there was a larger peak of 3 H release in cycle 48, which established that CPZ also photolabels α Met-386 (2 cpm/pmol) (Supporting Information Figure S5). Similar to α Ser-393, α Met-386 would be contained in

a small tryptic fragment extending from α Tyr-481 to α Lys-387, and labeling of that residue would also contribute to the peak of 3 H release seen in cycle 6 of the sequence analysis of the material in the column flow-through from the tryptic digest (Figure 8B).

DISCUSSION

In this work, we use [3 H]CPZ to provide new information about the diversity of binding sites in the nAChR transmembrane domain for aromatic amine noncompetitive antagonists. In early photolabeling studies, [3 H]CPZ was shown to bind to a site near the cytoplasmic end of the *Torpedo* nAChR ion channel in the desensitized state, where it photolabeled amino acids within each M2 helix at positions M2–2, M2–6, and/or M2–9, with the [3 H]CPZ binding (photolabeling) inhibitable by PCP (13–16). We extended those studies by determining whether [3 H]CPZ binds to additional sites in the transmembrane domain of the nAChR in the desensitized state and by identifying its binding site in the nAChR ion channel in the closed state.

For nAChRs in the desensitized state, we find only one additional site for [^3H]CPZ in the transmembrane domain: a site at the extracellular end of the channel, identified by the photolabeling of amino acids at positions M2-16 (γ), M2-17 (β , δ), and M2-20 (α , β , and δ), where [^3H]CPZ binding/photolabeling is enhanced, rather than inhibited, in the presence of a high concentration of PCP and only partially inhibited by proadifen. We will refer to the two CPZ binding sites as Site2/6/9 and Site17/20, upon the basis of the position of the photolabeled amino acids within the M2 helices. Our results establish that CPZ and PCP (or proadifen) bind in a mutually exclusive manner to Site2/6/9 and that occupancy of that site by PCP does not inhibit the binding of CPZ to Site17/20.⁴ Thus, the electrostatic constraints within the ion channel are of such a nature that two positively charged ligands can bind simultaneously at the cytoplasmic and extracellular ends of the ion channel. The potentiation of [^3H]CPZ photolabeling at Site17/20 seen in the presence of PCP and the partial inhibition of photolabeling seen in the presence of proadifen indicate that the binding of either drug at the cytoplasmic end of the ion channel results in subtle, but different, changes in structure at the extracellular end of the channel.

When nAChRs are stabilized in a closed channel state by the binding of αBgTx at the agonist sites, CPZ binds in the ion channel with 8-fold lower affinity than when the nAChR is in the desensitized state, and the [^3H]CPZ photolabeling of amino acids at M2-5 (α), M2-6 (α , β , δ), and M2-9 (β , δ) establishes for the first time that CPZ binds at a similar level in the ion channel in the closed and desensitized states. However, there are differences in the pattern of subunit labeling, such as the favored labeling of $\alpha\text{M2-2}$ and $\alpha\text{M2-6}$ in the desensitized state and of $\alpha\text{M2-6}$ and $\alpha\text{M2-9}$ in the closed state (Table 1), that must result from state-dependent differences in the local structure of the channel.

The nAChR amino acids photolabeled by [^3H]CPZ are identified (Figure 9) in the *Torpedo* nAChR structure (Protein Data Bank code 2BG9), obtained in the absence of agonist and assumed to be the closed state structure. The structural diversity of amino acids photolabeled by CPZ within the ion channel domain, including aliphatic (Leu, Val, and Ile), aromatic (Phe), and polar (Ser, Thr, Glu, Asp, and Gln) side chains, is noteworthy and indicates that [^3H]CPZ can photoincorporate into any binding site that it occupies. We highlight in Figure 9B and C the amino acids photolabeled by [^3H]CPZ in the closed channel (+ αBgTx , green) and desensitized (+Carb, red and blue) states, respectively. We also highlight the aliphatic side chains at position M2-13 (yellow) that are not photolabeled by [^3H]CPZ in either state but are photolabeled by [^3H]TDBzI-etomidate in the desensitized state (24) and contribute, along with the aliphatics at M2-9, to the binding site in the closed channel for the smaller uncharged, hydrophobic drugs [^{125}I]TID and [^3H]benzophenone (20, 23).

The lack of [^3H]CPZ photolabeling of the Val/Ile at M2-13 in conjunction with its photolabeling of the Leu at M2-9 and M2-16/17, as well as the fact that CPZ photolabels amino acids at the extracellular end of the channel even when PCP or proadifen occupies the site at the cytoplasmic end, provide strong

evidence that CPZ photolabeling at the extracellular end of the ion channel must result from occupancy of a site rather than a diffusional encounter of photoactivated CPZ, which is known to react efficiently with water (35).

Although CPZ only binds at the level of M2-6 and M2-9 in the closed channel, the lumen of the ion channel within the *Torpedo* nAChR structure can accommodate CPZ at binding sites between the levels of M2-6 and M2-20. We show in Figure 9D a side view of the M2 ion channel domain with one CPZ docked near M2-6 and M2-9, positions labeled in the closed and desensitized states, and a second CPZ docked at the level of M2-17 and M2-20, positions prominently photolabeled only in the desensitized state. While CPZ binding at the level of M2-6 and M2-9 will be in close proximity to side chains from each M2 helix (Figure 9F and Supporting Information Figure S6), a view looking down the extracellular half of the channel (Figure 9E) shows that one CPZ cannot be in simultaneous contact with all subunits at that level. In the most energetically favored orientation, the CPZ N^+ was within 3 Å of $\alpha\text{Glu-262}$, and its likely photoreactive C-Cl bond (35) was within 5 Å of the photolabeled $\beta\text{Leu-265}$ and $\delta\text{Leu-273}$, and 6.5 Å from $\gamma\text{Phe-267}$, consistent with the fact that the labeling of $\alpha\text{M2-17/20}$ was at < 25% the efficiency of those positions in each subunit (Table 1). Although positions M2-17 and M2-20 contribute to the lumen of the ion channel in the nAChR structure, it is significant that those residues are labeled only in the desensitized state by CPZ, by meproadifen mustard (36), or by uncharged, photoreactive anesthetics (37, 38). This state-dependent labeling establishes that there must be a substantial change in structure at the extracellular end of the ion channel between the closed channel and desensitized states, potentially a reduction in the diameter of the channel lumen to form a more compact binding site.

[^3H]CPZ Binding at the Extracellular End of the Ion Channel. Our photolabeling results establish that [^3H]CPZ binds at equilibrium to ion channel Site17/20 in the nAChR in the desensitized state, and the lack of inhibition by PCP establishes that this site has distinct pharmacological properties from ion channel Site2/6/9. However, the photolabeling data at a single [^3H]CPZ concentration are insufficient to determine the CPZ affinity for either site. Since PCP inhibits [^3H]CPZ photolabeling at Site2/6/9, CPZ must bind at that site with a K_D of $\sim 1\ \mu\text{M}$, upon the basis of its inhibition of [^3H]PCP binding. Since [^3H]CPZ photolabels amino acids in Site17/20 at the same or higher efficiency as that at Site2/6/9, it is possible that CPZ binds to the two sites with similar affinities. However, it is also possible that Site17/20 is one of the ~ 10 low affinity sites/nAChR detected by [^3H]CPZ equilibrium binding assays with *Torpedo* nAChR-rich membranes (12).

Additional [^3H]CPZ photolabeling studies will be required to identify drugs that compete with [^3H]CPZ binding at the site at the extracellular end of the ion channel. Meproadifen, a quaternary ammonium derivative of proadifen, is a likely candidate since it partially inhibits the photolabeling of $\alpha\text{M2-20}$ by two uncharged drugs, [^3H]azidoctanol and [^3H]azietomidate (37, 38), and [^3H]meproadifen mustard, its reactive aziridinium analogue, reacts selectively with $\alpha\text{M2-20}$ (36).

When [^3H]CPZ photolabels Site2/6/9 and Site17/20 in nAChRs equilibrated with agonist, it is most likely that all nAChRs are in the equilibrium desensitized state. However, [^3H]CPZ binds most rapidly to a state stabilized only transiently by an agonist (39), either the open channel state or a transient desensitized state, and the use of time-resolved photolabeling

⁴[^3H]CPZ photolabeling at the extracellular end of the ion channel was not characterized by Changeux and co-workers since most samples containing M2 segments were sequenced for fewer than 15 cycles of Edman degradation (13–15). However, the γ subunit samples were sequenced for 30 cycles (16), and in addition to peaks of PCP-inhibitable ^3H release in cycles 2, 6, and 9, the focus of the report, the data do include a peak in cycle 16, not inhibited by PCP.

techniques (22, 40) will allow the determination of the site that is occupied most rapidly when nAChRs are exposed transiently to agonist.

[³H]CPZ Binding Sites Outside the Ion Channel. We found no evidence that [³H]CPZ bound in the nAChR transmembrane domain in the δ subunit helix bundle, where [¹²⁵I]TID and [³H]benzophenone bind in an agonist-dependent manner (22, 23), and we found no evidence of labeling at the α M4 lipid interface. However, [³H]CPZ photolabeling of α Met-386 and α Ser-393 establishes that it binds in the nAChR cytoplasmic domain in the basket formed by the MA helices that are the extensions of the M4 helices (Figure 9G). Photolabeling of α Ser-393 was not inhibited by PCP, and though not examined, we assume that the photolabeling of α Met-386 will also be unaffected by PCP. However, the photolabeling of these residues is unlikely to be a result of a random collisional encounter of photolabeled [³H]CPZ since it did not photolabel other serines in α MA (α Ser-388 and α Ser-392) or of any of the acidic side chains, including α Glu-390, which is adjacent to α Ser-393 on the MA helix and photolabeled by the uncharged noncompetitive antagonist [³H]azetomidate (40). As the current nAChR structure provides only a very limited definition of the structure of the nAChR cytoplasmic domain and no definition of the nAChR regions interacting with the coiled-coil domain of cytoplasmic scaffolding protein rapsyn (41), insufficient structural information is available to interpret why CPZ and azetomidate each interact with the same region of nAChR, which may be a drug binding site at the interface between the nAChR and rapsyn (which was also photolabeled by [³H]CPZ).

SUPPORTING INFORMATION AVAILABLE

Six figures: (1) Isolation of [³H]CPZ-labeled fragments beginning at the N-termini of β M2 and δ M2; (2) sequence analysis of [³H]CPZ-labeled γ M1-M2; (3) isolation of [³H]CPZ labeled γ M2 by in gel digestion with V8 protease, trypsin digestion, and reversed-phase HPLC; (4) for nAChRs equilibrated with α BgTx, there is tetracaine inhibitable [³H]CPZ photoincorporation in the nAChR α , β , and δ subunits, with little if any photoincorporation in the γ subunit; (5) [³H]CPZ photolabeling in the α subunit cytoplasmic (MA) helix; and (6) two binding sites for CPZ within the nAChR ion channel. This material is available free of charge via the Internet at <http://pubs.acs.org>

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