Nicotinic acetylcholine receptor (nAChR) agonists, such as epibatidine and its molecular derivatives, are potential therapeutic agents for a variety of neurological disorders. In order to identify determinants for subtype-selective agonist binding, it is important to determine whether an agonist binds in a common orientation in different nAChR subtypes. To compare the mode of binding of epibatidine in a muscle and a neuronal nAChR, we photolabeled Torpedo α3β2δ and expressed human α4β2 nAChRs with [3H]epibatidine and identified by Edman degradation the photolabeled amino acids. Irradiation at 254 nm resulted in photolabeling of αTyr198 in agonist binding site segment C of the principal (+) face in both α subunits and of γLeu109 and γTyr117 in segment E of the complementary (−) face, with no labeling detected in the δ subunit. For affinity-purified α4β2 nAChRs, [3H]epibatidine photolabeled α4Tyr195 (equivalent to Torpedo αTyr190) in segment C as well as β2Val111 and β2Ser113 in segment E (equivalent to Torpedo γLeu109 and γTyr111, respectively). Consideration of the location of the photolabeled amino acids in homology models of the nAChRs based upon the acetylcholine-binding protein structure and the results of ligand docking simulations suggests that epibatidine binds in a single preferred orientation within the α-γ transmitter binding site, whereas it binds in two distinct orientations in the α4β2 nAChR.

Nicotinic acetylcholine receptors (nAChRs) are prototypical members of the Cys loop superfamily of neurotransmitter-gated ion channels that mediate the actions of the neurotransmitter acetylcholine (1). nAChRs from vertebrate skeletal muscle and the electric organs of Torpedo rays are heteropentamers of homologous subunits with a stoichiometry of 2α:γ(ε):δ that are arranged pseudosymmetrically around central cation-selective ion channels (1, 2). There are 12 mammalian neuronal nAChR subunit genes: nine neuronal α subunits (α2–α10) and three neuronal β subunits (β2–β4). The α4β2 nAChR is the most abundant and widely distributed nAChR subtype expressed in the brain and is a major target for potential therapeutic agents for neurological diseases and conditions, including nicotine dependence and Alzheimer and Parkinson diseases (3, 4). Although the ratio of α4 to β2 subunit in vivo is uncertain, expressed receptors containing either three α4 or three β2 subunits have distinct pharmacological properties (5, 6).

The agonist binding sites (ABS) of nAChRs are located within the amino-terminal extracellular domain at the interface of adjacent subunits (α-γ and α-δ in the Torpedo nAChR), and different nAChR subunit combinations form ABS with distinct physical and pharmacological properties (3, 7). Affinity labeling studies with Torpedo nAChR and site-directed mutational analyses of muscle and neuronal nAChRs identified key amino acids delineating the ABS from three noncontiguous stretches of the α subunit (Segments A–C, the principal component (+ face)) and three noncontiguous regions of the non-α subunit (Segments D–F, the complementary component (− face)) (8, 9). The three-dimensional structure of the ABS in the absence and presence of nAChR agonists or competitive antagonists has been determined for snail acetylcholine-binding proteins (AChBPs) that are soluble homopentamers homologous to the extracellular (amino-terminal) domain of a nAChR (10–12). In the AChBP, four aromatic amino acids from Segments A–C that are conserved within α subunits, along with a conserved Trp in Segment D, form a core aromatic “pocket” with a dimension optimal for accommodation of a trimethylammonium group. The other amino acids in the non-α subunits closest to the aromatic pocket, which are generally not conserved among γ, δ, or neuronal β subunits, are on three antiparallel β strands. The AChBP structure was used to refine the structure of the Torpedo nAChR in the absence of agonist to 4 Å resolution (13). In this structure, there is a reorientation of Segments A–C, resulting in the absence of a well defined core aromatic binding pocket.
Analysis of agonist interactions with mutant nAChRs containing fluorine-substituted core aromatic residues provides evidence that cation–π interactions, particularly with α-Trp in Segment B, are important determinants of agonist binding affinity (14) and for the higher affinity binding of nicotine to α4β2 nAChRs compared with α2βγδ nAChRs (15). Mutational analyses and molecular docking calculations have also provided evidence that two molecules of very similar structure may actually bind to a single receptor in very different orientations, as seen for two high affinity antagonists, t-tubocurarine and its quaternary ammonium analog metocurine, binding to the AChBP and to the muscle nAChR (16, 17).

Photoaffinity labeling provides an alternative means to identify amino acids contributing to a drug binding site (18, 19) and has been used to determine the orientation of drugs bound in the ABS of Torpedo nAChR (20). Epibatidine binds with very high affinity (~10 pm) to heteromeric neuronal nAChRs (e.g. α4β2) and with nanomolar affinity to α7 and muscle-type/Torpedo nAChRs (3). Utilizing a photoactive analogue of epibatidine (azideopibatidine; Fig. 1) and mass spectrometry, Tomizawa et al. (21) identified photolabeled amino acids in the Aplysia AChBP (Tyr495 in Segment C and Met116 in Segment E), establishing an orientation for bound azideopibatidine consistent with the orientation of epibatidine in an AChBP crystal structure (12).

In this report, we use [3H]epibatidine as a photoaffinity reagent to identify the amino acids photolabeled in an expressed α4β2 nAChR and in the Torpedo α2βγδ nAChR. Comparisons of the labeled amino acids seen in the Torpedo nAChR α-γ binding site and in the α4β2 nAChR, in conjunction with the results of docking calculations for epibatidine binding to homology models of the α2βγδ and α4β2 nAChRs, suggests that epibatidine binds in a single orientation in the α-γ site but in two orientations in the α4β2 ABS.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Epibatidine (45 Ci/mmol) was obtained from PerkinElmer Life Sciences and stored in 95% ethanol at 4 °C. Carbamylcholine chloride, bromaacetylcholine bromide, and diisopropylfluorophosphate were from Sigma. Epibatidine and t-tubocurarine (dTC) were from Tocris (Ellisville, MO), and Staphylococcus aureus endoproteinase Glu-C (V8 protease) was from Worthington. Endoproteinase Lys-C (EndoLys-C) and protease inhibitor mixture set III were from Calbiochem. Sodium cholate and CHAPS were from U.S. Biochemical Corp. Afni-Gel 10 was from Bio-Rad. Synthetic lipids (dioleoyl phosphatidic acid and dioleoyl phosphatidy choline) as well as cholesterol, asolectin, and total lipid extract from porcine brain were obtained from Avanti Polar Lipids (Alabaster, AL).

**Preparation of Torpedo nAChR—** Torpedo californica nAChR-rich membranes for radioligand binding studies and for affinity purification were isolated from frozen electric organs (Aquatic Research Consultants, San Pedro, CA), as described previously (22). Torpedo nAChR-rich membranes at 1 mg/ml protein were solubilized in 1% sodium cholate in vesicle dialysis buffer (VDB; 100 mM NaCl, 0.1 mM EDTA, 0.02% NaN3, 10 mM MOPS, pH 7.5) and treated with 0.1 mM diisopropylfluorophosphate after insoluble material was pelleted by centrifugation (91,000 × g for 1 h). The nAChR was affinity-purified on a bromaacetylcholine bromide-derivatized Afni-Gel 10 column and then reconstituted into lipid vesicles composed of dioleoyl phosphatidic acid/dioleoyl phosphatidy choline/cholesterol (at a molar ratio of 3:1:1), as described (23, 24). The lipid/nAChR ratio was adjusted to molar ratio of 400:1. Based upon SDS-PAGE, after purification, the nAChR comprised more than 90% of the protein in the preparation. Both the nAChR-rich membranes and purified nAChRs were stored at −80 °C.

**Preparation of α4β2 nAChR—** HEK-293 cells stably transfected with human α4β2 nAChRs (HEK-ho4β2) were kindly provided by Dr. Joseph H. Steinbach (Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO) (25). HEK-ho4β2 cells were grown at 37 °C in a humidified incubator at 5% CO2 in 140-mm dishes and maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 450 μg/ml Geneticin (G418; A.G. Scientific, San Diego, CA) as a selection agent (26). For membrane preparation, HEK-ho4β2 cells were homogenized in VDB in the presence of 0.2 μl/ml protease inhibitor mixture set III (Calbiochem). Membrane fractions were isolated by centrifugation (39,000 × g for 1 h) and then resuspended in VDB containing 0.2 μl/ml protease inhibitor mixture III and stored at −80 °C. For affinity purification, HEK-ho4β2 cell membranes (typically 2 g of protein from ~600 dishes) at 1 mg/ml protein in VDB containing 0.2 μl/ml protease inhibitor mixture III and stored at −80 °C. For affinity purification, HEK-ho4β2 cell membranes were solubilized with 1% CHAPS, centrifuged (91,500 × g for 1 h) to pellet insoluble material, and then dialyzed for 5 h against 1% cholate. The ho4β2 nAChRs were affinity-purified on a bromaacetylcholine bromide-derivatized Afni-Gel 10 column and reconstituted into lipid vesicles composed of asolectin/total lipid extract from porcine brain, as described previously (26). The purity of ho4β2 nAChRs was >50%, as estimated by densitometric quantification of Coomassie Blue-stained gels.

**Radioligand Binding to Torpedo nAChR-rich Membranes—** The effect of epibatidine and dTC on the binding of [3H]epibatidine to Torpedo nAChR-rich membranes was determined using a centrifugation assay with duplicate samples. Membranes at 0.1 mg/ml protein (16 nM ACh binding sites) in Torpedo physiological saline (250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, and 5 mM sodium phosphate, pH 7.0) were incu-
bated for 1 h at room temperature with either 0.5 nM [3H]epibatidine and increasing concentrations of epibatidine (final concentrations 0.3 nM to 3 μM) or 2 nM [3H]epibatidine in the absence and presence of 6 μM proadifen and increasing concentrations of dTC (final concentrations 1 nM to 1 mM). Bound and free [3H]epibatidine were separated by centrifugation (39,000 × g for 1 h) and then quantified by liquid scintillation counting. Nonspecific binding was determined in the presence of 1 mM carbachol.

Data Analysis—The concentration-dependent inhibition of [3H]epibatidine binding by epibatidine and dTC were fit according to a single-site model as follows,

\[ f(x) = \frac{NS}{1 + (x/IC_{50})} \quad (\text{Eq. 1}) \]

and to a two-site model,

\[ f(x) = \frac{NS}{1 + (x/IC_{50,1})} + \frac{NS}{1 + (x/IC_{50,2})} \quad (\text{Eq. 2}) \]

where \( f(x) \) is the [3H]epibatidine binding in the presence of competitor concentration, \( T \) is the total specific binding, \( NS \) is the nonspecific binding determined in the presence of 1 mM carbachol, and \( IC_{50} \) is the concentration of competitor that inhibits 50% of the total specific binding. Sigmaplot version 11 (Systat Software) was used for non-linear least squares fit of the data, and the S.E. values of the parameter fits are indicated.

[3H]Epibatidine Photolabeling of Torpedo nAChRs—For analytical labelings, ~50-μg samples of affinity-purified Torpedo nAChRs in 1 ml of VDB were incubated with 0.13 μM [3H]epibatidine in the absence or presence of epibatidine (40 μM) or increasing concentrations of dTC (final concentrations 0.2–20 μM). After a 2-h incubation at room temperature, the samples in glass test tubes were irradiated for 30 min with a 254-nm UV lamp (Spectrolite EN-280L). The labeled membranes were then pelleted by centrifugation (39,000 × g for 1 h), resuspended in electrophoresis sample buffer (12.5 mM Tris-HCl, 2% SDS, 8% sucrose, 1% glycerol, 0.01% bromphenol blue, pH 6.8), and resolved on 1-mm-thick, 8% polyacrylamide, 0.33% bisacrylamide gels (27). After staining with Coomassie Blue R-250 and destaining to visualize bands, gels were impregnated with fluor (Amplify; GE Biosciences) for 30 min, dried, and exposed to Eastman Kodak Co. X-Omat LS film at −80 °C (1–4-week exposure). For some 8% gels, following staining and destaining, gels were soaked in distilled water overnight, and bands corresponding to the α and γ subunits were excised, soaked in overlay buffer (5% sucrose, 125 mM Tris-HCl, 0.1% SDS, pH 6.8) for 30 min, transferred to the wells of a 15% acrylamide mapping gel, and digested in gel with 10 μl of V8 protease (28). After electrophoresis, mapping gels were processed for fluorography (3–6-week exposure) as described above. For both 8 and 15% mapping gels, to quantify the amount of [3H]epibatidine incorporated into nAChR subunits or subunit proteolytic fragments, bands were excised from the gel, transferred to 5-ml scintillation vials, and soaked in 0.5 ml of 0.1% SDS for 4 days with occasional mixing. Then 3 ml of liquid scintillation mixture was added, and samples were counted for 5 min.

For labeling on a preparative scale, affinity-purified Torpedo nAChRs (~3 mg of protein in 8 ml) were labeled with 550 nM [3H]epibatidine, and the α and γ subunits were isolated by SDS-PAGE and then subjected to in-gel digestion with V8 protease. Based on fluorographs of mapping gels from analytical scale labeling experiments, labeled proteolytic fragments from each subunit (αV8-20 and γV8-14) were excised, and the polypeptides were retrieved by passive diffusion into 25 ml of elution buffer (0.1 mM NH₄HCO₃, 0.1% (w/v) SDS, 1% β-mercaptoethanol, pH 7.8) and concentrated in Centriprep-10 and Centri-3 concentrators (10/3 kDa cut-off; Amicon) to a final volume of ~150 μl. For digestion with EndoLys-C, αV8-20-labeled peptides were aceton-precipitated (75% acetone at −20 °C overnight) to remove SDS, resuspended in 150 μl of 25 mM Tris-HCl, 0.5 mM EDTA, 0.1% SDS, pH 8.6, and then digested with three units of EndoLys-C for 7 days.

[3H]Epibatidine Photolabeling of αβ2 nAChR—For analytical labelings, ~50-μg samples of affinity-purified αβ2 nAChRs in 1 ml of VDB were photolabeled with 880 nM [3H]epibatidine in the absence or presence of epibatidine (40 μM). For labeling on a preparative scale, αβ2 nAChRs (1.2 mg of protein in 6 ml) were photolabeled with 880 nM [3H]epibatidine. Using the same protocols described above for Torpedo nAChR, samples from analytical labelings were processed to determine [3H]epibatidine incorporation into the α4 and β2 nAChR subunits (as well as a 36-kDa proteolytic fragment), and the α4 and β2 nAChR subunits and the 36-kDa proteolytic fragments from preparative scale labeling were retrieved, aceton-precipitated, and resuspended in 150 μl of 0.1 mM NH₄HCO₃, 0.1% SDS, pH 7.8, for digestion with V8 protease (100 μg of protease for 4 days).

Reversed-phase HPLC Purification and Sequence Analysis—Prior to sequence analysis, all of the [3H]epibatidine-labeled peptides were purified using reversed-phase HPLC (rHPLC) on a Shimadzu LC-10A binary HPLC system, using a Brownlee Aquapore C₄ column (100 × 2.1 mm). Solvent A was composed of 0.08% trifluoroacetic acid in water, and Solvent B contained 0.05% trifluoroacetic acid in 60% acetonitrile, 40% 2-propanol. A non-linear elution gradient at 0.2 ml/min was employed (25–100% Solvent B in 100 min, shown as dotted line in the figures), and fractions were collected every 2.5 min (40 fractions/run). The elution of peptides was monitored by the absorbance at 210 nm, and the amount of [3H]epibatidine associated with each fraction was determined by liquid scintillation counting of % aliquots.

For sequence analysis, rHPLC fractions containing peaks of [3H]epibatidine were pooled, diluted 3-fold with 0.1% trifluoroacetic acid, and loaded onto polyvinylidene difluoride filters using Porsorb® sample preparation cartridges (catalog number 401959; Applied Biosystems). The filters were then treated with Biobrene, as recommended by the manufacturer. Sequencing was performed on an Applied Biosystems PROCISE™ 492 protein sequencer configured to utilize one-sixth of each cycle of Edman degradation for amino acid quantification and to collect the other five-sixths for [3H] counting. To determine the amount of sequenced peptide, the pmol of each amino acid in a detected sequence was quantified by peak height and fit to the equation \( f(x) = I_o R^x \), where \( I_o \) represents the initial amount of the peptide sequenced (in pmol), \( R \) is the repetitive yield, and \( f(x) \) is the pmol detected in cycle x. Ser, His, Trp, and Cys were not included in the fits due to known problems with their accurate detection/quantification. The fit was calculated in Sigma-

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Plot 11 using a non-linear least squares method, and figures containing 3H release profiles include this fit as a dotted line. Some sequencing samples were treated with o-phthalaldehyde (OPA) prior to a cycle known to contain a proline (29). OPA reacts with all amino-terminal amino acids (but not with the imino acid proline) and blocks further Edman degradation (30). Thus, release of 3H in a cycle after an OPA treatment establishes that the 3H release originates from a peptide with a proline in the OPA-treated cycle. Quantification of 3H incorporated into a specific residue (cpm/pmol) was calculated by (cpm_i - cpm_{i-1})/51, R^2.

**Molecular Modeling**—Models of the extracellular domain of the human (α4)3(β2)_3 and Torpedo nAChR were constructed from the x-ray structure of the epibatidine-bound form of the *Aplysia* AChBP (12) (Protein Data Bank code 2BYQ) using the Discovery Studio (Accelrys, Inc.) software package. Epibatidine (volume 156 Å^3) was docked into the AChBP crystal structure and in the models of the *Torpedo* α-γ and human α4-β2 ABS using CDOCKER (31, 32), a CHARMM-based (33) molecular dynamics simulated annealing program that treats the ligand as fully flexible while maintaining a rigid receptor. In each docking experiment, 50 replicas of epibatidine (protonated form) were seeded in random orientations within the ABS defined by a binding site sphere of 12 Å radius. For each starting seed, CDOCKER was used to generate 10 ligand conformations using high temperature molecular dynamics, and then the 10 lowest energy orientations were identified using random rigid body rotations, followed by simulated annealing and a full potential final minimization step. For visualization, we show in Fig. 7 the Connolly surface representations, defined by a 1.8-Å diameter probe, of the ensemble of the 20 docking solutions with the lowest CDOCKER interaction energies.

**RESULTS**

**[^3]H**Epibatidine Binding to Torpedo nAChR—In equilibrium binding studies with Torpedo nAChR-rich membranes, epibatidine fully inhibited the binding of 0.5 nM[^3]H]epibatidine, with the data well fit by a single-site model with an IC_{50} of 11 ± 2 nM (Fig. 2A). We used the displacement of [^3]H]epibatidine binding by dTC to explore the agonist binding site selectivity of epibatidine. dTC binds with higher affinity (K_A = 35 nM) at the Torpedo α-γ agonist binding site than the α-δ site (K_A = 8 μM) (34). The dTC displacement data (Fig. 2B, closed circles) were well fit by a model of two sites, equally occupied by [^3]H]epibatidine, with IC_{50} values of 42 ± 7 nM and 1.8 ± 0.2 μM, consistent with dTC displacement of [^3]H]epibatidine bound to the α-γ and α-δ sites, respectively. The addition of proadifen, a desensitizing noncompetitive antagonist, enhanced [^3]H]epibatidine binding (Fig. 2B, open circles) with little effect on the relative occupancy by epibatidine of the α-γ and α-δ sites.

**Photoincorporation of[^3]H**Epibatidine into Torpedo nAChR—We used SDS-PAGE followed by fluorography (Fig. 3A) and scintillation...
counting of excised gel bands (Fig. 3B) to provide an initial characterization of the subunit and pharmacological specificity of [3H] incorporation when affinity-purified *Torpedo* nAChRs were photolabeled on an analytical scale (∼50-µg samples). As seen in the fluorograph, [3H]epibatidine was incorporated primarily in the nAChR α and γ subunits (−Epi lane). Photo- labeling was eliminated by the addition of an excess (40 µM) of nonradioactive epibatidine (+ Epi lane) and inhibited in a concentration-dependent manner by dTC. Based upon scintillation counting, the concentration dependence of dTC inhibition of [3H] photoincorporation in the γ subunit (Fig. 3B, open circles) was well fit by a single-site model with an IC50 value of 0.45 ± 0.07 µM, whereas inhibition of labeling in the α subunit (Fig. 3B, solid circles) was better fit by a two-site model (IC50 values of 35 ± 12 nM and 2 ± 0.4 µM) than a one-site model (IC50 = 0.6 ± 0.2 µM), where there were systematic deviations of the data from the fit at concentrations below 0.3 µM. Pharmacologically specific (epibatidine-inhibitable) [3H] photoincorporation within the δ subunit, if it occurred, was at <10% the level in the γ subunit. Although these labeling studies were conducted with affinity-purified nAChRs, an [3H]epibatidine photolabeling of native *Torpedo* nAChR-rich membranes under similar conditions also resulted in labeling of α and γ subunits, with little if any labeling in the δ subunit (data not shown).

Identification of Amino Acids Photolabeled by [3H]Epibatidine in the Torpedo α and γ Subunits—To identify amino acids photolabeled by [3H]epibatidine within the α and γ subunits, the incorporation in each subunit was first mapped by in-gel digestion with V8 protease, which for the α subunit produces fragments of ∼20 kDa (αV8-20, beginning at αSer173 and containing ACh binding site Segment C), ∼18 kDa (αV8-18, beginning at αThr22 and containing binding site Segments A and B), and ∼10 kDa (αV8-10, beginning at αAsn338 and containing the M4 membrane-spanning helix (35)). The corresponding fluorograph of a mapping gel (Fig. 4) established that all detectable (and specific) labeling in the α subunit was contained within a Coomassie-stained band of ∼20 kDa (αV8-20), whereas, based upon liquid scintillation counting, the labeling in αV8-18 was less than 5% that of αV8-20 (supplemental Fig. S1). For the γ subunit, all [3H] incorporation was contained within an ∼14 kDa band (γV8-14).

To identify the residues photolabeled by [3H]epibatidine, αV8-20 and γV8-14 were isolated from affinity-purified *Torpedo* nAChRs photolabeled on a preparative scale (3.5 mg). Labeled αV8-20 was digested with EndoLys-C, which cleaves αV8-20 after αLys285 (36). When the digest was fractionated by rpHPLC, ∼90% of the recovered [3H] eluted in a single peak at ∼87% solvent B (supplemental Fig. S1). Sequence analysis of the peak fractions (Fig. 5A) revealed a primary sequence beginning at αHis199 (23 pmol) and a single peak of [3H] release in cycle 13, corresponding to labeling of αTyr198 (28 pmol) (32 pmol), one of two tyrosines in Segment C contributing to the core aromatic binding pocket for ACh. During sequencing, the sample was treated after cycle 8 (see the arrow in Fig. 5A) with OPA, which reacts with primary, but not secondary, amines and blocks Edman degradation of any peptide without an amino-terminal proline at the time of addition (29, 30). The only sequence detected after cycle 8 was the primary sequence (with αPro194 in cycle 9), providing additional evidence that the [3H] release in cycle 13 resulted from [3H]epibatidine incorporation into αTyr198.

When [3H]epibatidine-labeled γV8-14 was further purified by rpHPLC, ∼80% of the recovered [3H] eluted in a single peak at ∼57% solvent B (supplemental Fig. S1). Sequence analysis of the pooled peak fractions (Fig. 5B) revealed a single sequence beginning at γVal102 (55 pmol) with [3H] release in cycles 8 and 16, corresponding to labeling of γLeu109 (23 pmol) and γTyr117 (13 pmol), residues located within Segment E of the agonist binding site previously identified by photoaffinity labeling (20). Photoincorporation of [3H]Epibatidine into αβ2 nAChR—Affinity-purified neuronal αβ2 nAChRs were photolabeled on an analytical scale (∼50-µg samples) with 880 nM [3H]epibatidine in the absence and presence of excess nonradioactive epibatidine (40 µM), and the [3H] incorporation was assessed by SDS-PAGE and fluorography (Fig. 6). [3H]epibatidine photoincorporated primarily into the α4 subunit and in a broad band migrating with an apparent molecular mass of ∼36 kDa, with low level labeling of the β2 subunit, and labeling in each band was fully inhibited by epibatidine. Based upon liquid scintillation counting of excised gel bands, 1,600, 550, and 2,400 cpm were incorporated into the α4 and β2 subunits and 36 kDa band, respectively, and photolabeling in each band was inhibited by >95% by excess nonradioactive epibatidine. Based on previous studies with purified αβ2 nAChRs (26), the [3H]epibatidine-labeled material migrating at ∼36 kDa probably included proteolytic fragments of both the α4 and β2 subunits, which was confirmed by protein sequencing (supplemental Fig. S2).
Identification of Amino Acids Photolabeled by [3H]Epibatidine in the α4β2 nACHR—To identify specific residues labeled by [3H]epibatidine in the α4 and β2 subunits, each subunit was isolated from α4β2 nACHRs labeled on a preparative scale (1.2 mg of nAChR; 800 nM [3H]epibatidine). Labeled subunits were digested with V8 protease (Fig. 7A), the [3H] eluted in peaks centered at 29, 30. The only sequence detected after cycle 8 was the primary sequence, establishing that the [3H] release in cycle 15 resulted from [3H]epibatidine incorporation into 4Trp181 (5 pmol), residues that are contained within Segment C of the agonist binding domain.

DISCUSSION

In this report, we use the intrinsic photoreactivity of the nACHR agonist [3H]epibatidine to compare its mode of binding in the Torpedo and neuronal α4β2 nACHRs. Although the reactive intermediates formed upon photolysis of epibatidine have not been directly identified, halo pyridines, such as the chlorinated pyridine ring of epibatidine, are known to undergo photoaddition reactions initiated by the cleavage of the C–Cl bond (37–39), and it is that carbon that is probably reactive in epibatidine. Irradiation at 254 nm results in [3H]epibatidine photoincorporation into the α4-β2 and Torpedo α-γ ABS, with little or no labeling detected in regions outside of that domain. Likely contributors to the high specificity of [3H]epibatidine labeling

Identification of [3H] Epibatidine in the α4β2 nACHR—Affinity-purified Torpedo α4β2 nACHRs (3.5 mg in 8 ml) was photolabeled with 550 nM [3H]epibatidine, nACHR subunits were separated by SDS-PAGE (8% acrylamide gel), and the stained α and γ subunits bands were excised and digested in gel with V8 protease on a second gel, as described under Experimental Procedures. Material eluted from V8-20 was digested with Endolys-C and then fractionated by HPLC, whereas material eluted from V8-14 was purified directly by HPLC (supplemental Fig. S1). A, [3H] (●) and PTH-derivatives (□) released during amino acid sequence analysis of the [3H] peak (fractions 34–36; 31,000 cpm) from the HPLC purification of the Endolys-C digest of αV8-20. During sequencing, the filter was treated with OPA before cycle 9 (indicated by an arrow) to chemically isolate the primary peptide detected (IαHis460b, Iγ433 = 23 ± 9 pmol, r = 96%, with αPro176 in cycle 9) by preventing further sequencing of fragments not containing a proline in this cycle (29, 30). The only sequence detected after cycle 8 was the primary sequence, establishing that the [3H] release in cycle 15 resulted from [3H]epibatidine incorporation into 4Trp181 (28 cpm/pmol), 5, [3H] (●) and PTH-derivatives (□) released during amino acid sequence analysis of the [3H] peak (fractions 28–30; 22,500 cpm) from the HPLC purification of γV8-14. The only fragment detected began at γVal180 (Iγ433 = 55 ± 5 pmol, r = 94%) and was present at >20-fold higher amount than any other sequence. The [3H] releases in cycles 8 and 16 correspond to labeling of γ-Leu189 (23 cpm/pmol) and γTyr117 (13 cpm/pmol).

DISCUSSION

In this report, we use the intrinsic photoreactivity of the nACHR agonist [3H]epibatidine to compare its mode of binding in the Torpedo and neuronal α4β2 nACHRs. Although the reactive intermediates formed upon photolysis of epibatidine have not been directly identified, halo pyridines, such as the chlorinated pyridine ring of epibatidine, are known to undergo photoaddition reactions initiated by the cleavage of the C–Cl bond (37–39), and it is that carbon that is probably reactive in epibatidine. Irradiation at 254 nm results in [3H]epibatidine photoincorporation into the α4-β2 and Torpedo α-γ ABS, with little or no labeling detected in regions outside of that domain. Likely contributors to the high specificity of [3H]epibatidine labeling

Identification of Amino Acids Photolabeled by [3H]Epibatidine in the α4β2 nACHR—To identify specific residues labeled by [3H]epibatidine in the α4 and β2 subunits, each subunit was isolated from α4β2 nACHRs labeled on a preparative scale (1.2 mg of nAChR; 800 nM [3H]epibatidine). Labeled subunits were digested with V8 protease for 4 days, and the digests were fractionated by rpHPLC. When the α4 subunit digest was fractionated by rpHPLC (Fig. 7A), the [3H] eluted in peaks centered at fraction 25 (~50% solvent B) and fraction 28 (~57% solvent B) and in the column flow-through. 4 Sequence analysis of the pool of fractions 24–26 (Fig. 7B) revealed a fragment beginning at α4Trp181 (5 pmol) as well as the amino terminus of V8 protease, which was the primary sequence. The major peak of [3H] release in cycle 15 corresponded to labeling of α4Tyr195 (103 cpm/pmol), a residue that is contained within Segment C of the agonist binding domain. Sequence analysis of the V8 protease digest of the [3H] epibatidine-labeled 36 kDa band (supplemental Fig. S2) revealed the presence of nACHR subunit fragments beginning at α4Tyr181 and at β2Val104, with [3H] cpm release in cycles 8, 10, and 15, consistent with labeling of β2Val111, β2Ser113, and α4Tyr195, respectively.

DISCUSSION

In this report, we use the intrinsic photoreactivity of the nACHR agonist [3H]epibatidine to compare its mode of binding in the Torpedo and neuronal α4β2 nACHRs. Although the reactive intermediates formed upon photolysis of epibatidine have not been directly identified, halo pyridines, such as the chlorinated pyridine ring of epibatidine, are known to undergo photoaddition reactions initiated by the cleavage of the C–Cl bond (37–39), and it is that carbon that is probably reactive in epibatidine. Irradiation at 254 nm results in [3H]epibatidine photoincorporation into the α4-β2 and Torpedo α-γ ABS, with little or no labeling detected in regions outside of that domain. Likely contributors to the high specificity of [3H]epibatidine labeling
include the very high binding affinity to αβ2 nACHRs (∼100 pm) (40) and the molecular rigidity of epibatidine. In contrast to photolabeling studies conducted with the iodo-analog of epibatidine ([125I]epibatidine) (41), the amino acids in the ABS of the photolabeling studies conducted with the iodo-analog of epibatidine binds to both the Torpedo nAChR (11 nM). Although epibatidine may bind with 3–4-fold higher affinity to one of the two sites in the Torpedo nAChR, as does ACh (42), it did not have the high selectivity between the sites seen in the mouse muscle nAChR (>170-fold higher affinity for the α-γ over the α-δ site in the desensitized state (43)). Despite its high affinity binding to the α-γ and α-δ ABS in the Torpedo nAChR, [3H]epibatidine photoincorporated into the α and γ subunits with little, if any, in the δ subunit. The concentration dependence of dTc inhibition of [3H]epibatidine photolabeling of the α subunit indicated that both α subunits may be photolabeled, but further studies at higher [3H]epibatidine concentrations would be necessary to identify possible photolabeling in the δ subunit. Within the α and γ subunits, photolabeling was restricted to amino acids within binding site Segments C (α) and E (γ), with αTyr199 (28 cpm/pmol), γLeu109 (23 cpm/pmol), and γTyr117 (13 cpm/pmol) labeled most efficiently. [3H]Epibatidine photolabeling at the subunit level and within the α subunit mirrors that observed with [3H]nicotine photoincorporation into the Torpedo nAChR (29) but not within the γ subunit. [3H]Epibatidine did not photolabel γTrp55, the primary amino acid labeled by [3H]nicotine (44).

In the α4β2 nAChr, [3H]epibatidine photolabeled the α4 subunit at ∼3-fold higher efficiency than the β2 subunit, and (γLeu109/β2Val111 most efficiently. The position equivalent to the Aplysia AChBP Met116, which was labeled by azidoepibatidine, was labeled in the Torpedo nAChr (γTyr117) at 50% of the efficiency of γLeu109, but any labeling of the corresponding position in the αβ2 nAChr (β2Phe119), if it occurred, was at <10% the efficiency of β2Val111.

Proposed Orientations of Epibatidine in the Torpedo and α4β2 nAChr Agonist Binding Sites from Photolabeling and Molecular Modeling—As one approach to identify factors that could explain the selective labeling of non-equivalent core aromatic amino acids within Segment C of the Torpedo (αTyr198) and α4β2 (α4Tyr195) ABS by what must be the same epibatidine photoreactive intermediate, we used computational methods to predict favored epibatidine docking orientations in the ABS in homology models of the Torpedo and α4β2 nAChRs based on the structure of the epibatidine-bound form of Aplysia AChBP (12). When epibatidine was docked in the Torpedo α-γ ABS, a single binding orientation was highly favored (Fig. 8A) that was essentially the same as epibatidine in the crystal structure of the ligand-bound form of AChBP (12) and as we found for epibatidine docked in the AChBP crystal structure (not shown) or had been found for epibatidine docked in a structural model of chick α7 nAChr (45). In this orientation, the azacycloheptane ring occupies the “aromatic box” formed by αTyr99, αTyr198, αTyr199, αTrp55, and γTrp55 and the chloropyridyl ring is oriented toward Segment E, with C6 of the pyridine ring (the most likely reactive site) positioned within 6 Å of the labeled αTyr198, γTyr117, and γLeu109 but 11 Å from the unlabeled αTyr190 (Fig. 8B). When epibatidine was docked into the ABS of the α4β2 nAChr homology model, two distinct binding orientations consistent with this, [3H]epibatidine photolabeled α4Tyr195 (10 cpm/pmol; equivalent to Torpedo αTyr198) in Segment C more efficiently than the two labeled amino acids in the β2 subunit: β2Val111 (2 cpm/pmol) and β2Ser113 (∼0.6 cpm/pmol) in Segment E (equivalent to Torpedo γLeu109 and γTyr111, respectively). Tomizawa et al. (21) reported that [3H]azideoepibatidine also photolabeled the α4 subunit more efficiently (no labeling of the β2 subunit was evident by fluorography), although the photolabeled amino acids were not identified. Although [3H]epibatidine photolabeled αTyr198 in the Torpedo nAChr and azideoepibatidine photolabeled the corresponding position (Tyr195) in the Aplysia AChBP (21), that position was not labeled in the α4 subunit, whereas the position corresponding to αTyr190 was labeled. Within the γ and β2 subunits, [3H]epibatidine labeled equivalent positions
were predicted with similar energy and frequency: one orientation similar to that seen in the *Torpedo* α-γ (A and B) and human α4-β2 (C–F) homology models using CDOCKER, as described under "Experimental Procedures." A, C, and E, views of the α-γ (A) and α4-β2 (C and E) ABS in a flat ribbon representation (gold, α and α4; cyan, γ; pink, β2) showing Connolly surface of the ensemble of the 20 lowest energy solutions of epibatidine, which docked in a single orientation in the α-γ ABS and in two orientations in the α4-β2 ABS, one orientation (C) similar to that in the α-γ ABS, and a second orientation rotated by ~180° and translated by 3 Å. In each image, the lowest energy epibatidine orientation and amino acids within the ABS are shown in stick format with the [3H]epibatidine-labeled amino acids colored red and unlabeled amino acids in blue. B, D, and F, the distances in Å between the C₆ of the chloropyridyl ring of epibatidine and amino acids within the ABS for A, C, and E, respectively. The colors reflect atom type: carbon (black), oxygen (red), nitrogen (blue), and chlorine (green). See supplemental Fig. S3 for an alternative view of α4β2 ABS that highlights the orientations of epibatidine relative to α4Trp₁⁴³.

FIGURE 8. Molecular models of epibatidine docked in the *Torpedo* α-γ and α4β2 nAChR agonist binding sites. Epibatidine was docked into the ABS of the *Torpedo* α-γ (A and B) and human α4-β2 (C–F) homology models using CDOCKER, as described under “Experimental Procedures.” A, C, and E, views of the α-γ (A) and α4-β2 (C and E) ABS in a flat ribbon representation (gold, α and α4; cyan, γ; pink, β2) showing Connolly surface of the ensemble of the 20 lowest energy solutions of epibatidine, which docked in a single orientation in the α-γ ABS and in two orientations in the α4-β2 ABS, one orientation (C) similar to that in the α-γ ABS, and a second orientation rotated by ~180° and translated by 3 Å. In each image, the lowest energy epibatidine orientation and amino acids within the ABS are shown in stick format with the [3H]epibatidine-labeled amino acids colored red and unlabeled amino acids in blue. B, D, and F, the distances in Å between the C₆ of the chloropyridyl ring of epibatidine and amino acids within the ABS for A, C, and E, respectively. The colors reflect atom type: carbon (black), oxygen (red), nitrogen (blue), and chlorine (green). See supplemental Fig. S3 for an alternative view of α4β2 ABS that highlights the orientations of epibatidine relative to α4Trp₁⁴³.

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were predicted with similar energy and frequency: one orientation similar to that seen in the *Torpedo* α-γ ABS (Fig. 8C; *up orientation* with reference to the position of chlorine) and a second orientation with epibatidine rotated by ~180° and translated by 3 Å, orienting the chloropyridyl ring toward the core aromatic side chains (Fig. 8E; *down orientation*). In both orientations, the positively charged nitrogen in the azabicycloheptane ring is located 2.6 Å from the backbone carbonyl group of α4Trp₁⁴³ and ~4.6 Å from the aromatic side chain (supplemental Fig. S3), preserving equivalent potential for the hydrogen bonding and/or cation-π interactions predicted to be important determinants of binding affinity (9, 12, 14, 15). In both orientations, the positively charged nitrogen is also ~4 Å from α4Tyr₂⁰², which was not photolabeled by [3H]epipabatidine. In the *up orientation* (Fig. 8C), the pyridyl C₆ is positioned ~5 Å from the labeled β2Val¹¹¹ but 11 Å from the labeled α4Tyr¹⁹⁵ (Fig. 8D). In the *down orientation* (Fig. 8E), the pyridyl C₆ is positioned ~5 Å from the labeled α4Tyr¹⁹⁵ but 12 Å from the labeled β2Val¹¹¹ (Fig. 8F). These simple proximity relations suggest that epibatidine is likely to bind in an *up orientation* when β₂Val¹¹¹ is labeled and in a *down orientation* when α₄Tyr¹⁹⁵ is photolabeled.

Although only a single binding orientation has been seen for epibatidine and other agonists and antagonists in the *Aplysia* AChBP crystal structures (11, 12), mutational analyses have provided evidence that the competitive antagonist dTC and its
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quaternary ammonium analog, metocurine, bind in distinctly different orientations in both the AChBP and the muscle nAChR (16, 17). Moreover, the prediction of two distinct epibatidine binding orientations in the $\alpha 4\beta 2$ AChBP parallels the prediction that dTC and metocurine can bind in distinct orientations in the AChBP and human $\alpha 2-\varepsilon$ AChR, respectively (16, 17). Our interpretation is based upon a plausible, but unproven, assumption that cleavage of the C–Cl bond of epibatidine produces the only reactive intermediate, and it is also possible that the differences in the patterns of $[^3]$H]epibatidine photolabeling between the Torpedo and $\alpha 4\beta 2$ nAChR result from differences in the structures of the transmitter binding sites between the two nAChRs, including the position and orientations of the core aromatic amino acids. However, the proposal that nAChR ligands bind in a “normal” and an “inverted” orientation provides a plausible explanation for our photolabeling results, as for photolabeling studies of neonicotinoids binding to the Lyn- naca AChBP (46).

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