Probing the Structure of the Nicotinic Acetylcholine Receptor with 4-Benzoylbenzoylcholine, a Novel Photoaffinity Competitive Antagonist*

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[3H]4-Benzoylbenzoylcholine (Bz2choline) was synthesized as a photoaffinity probe for the Torpedo nicotinic acetylcholine receptor (nAChR). [3H]Bz2choline acts as an nAChR competitive antagonist and binds at equilibrium with the same affinity (Kd = 1.4 μM) to both agonist sites. Irradiation at 320 nm of nAChR-rich membranes equilibrated with [3H]Bz2choline results in the covalent incorporation of [3H]Bz2choline into the nAChR γ- and δ-subunits that is inhibited by agonist, with little specific incorporation in the α-subunits. To identify the sites of photoincorporation, γ- and δ-subunits, isolated from nAChR-rich membranes photolabeled with [3H]Bz2choline, were digested enzymatically, and the labeled fragments were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and/or reversed-phase high performance liquid chromatography. For the γ-subunit, Staphylococcus aureus V8 protease produced a specifically labeled peptide beginning at Val-102, whereas for the δ-subunit, endoproteinase Asp-N produced a labeled peptide beginning at Asp-99. Amino-terminal sequence analysis identified the homologous residues γ-Leu-109 and δ-Leu-111 as the primary sites of [3H]Bz2choline photoincorporation. This is the first identification by affinity labeling of non-reactive amino acids within the acetylcholine-binding sites, and these results establish that when choline esters of benzoic acid are bound to the nAChR agonist sites, the para substituent is selectively oriented toward and in proximity to amino acids γ-Leu-109/δ-Leu-111.

The Torpedo nicotinic acetylcholine receptor (nAChR) is composed of four homologous subunits in a stoichiometry of αβγδ that associate pseudosymmetrically about a central axis that is the ion channel. Cryoelectron microscopic analyses of two-dimensional crystalline arrays of nAChRs provide a definition of three-dimensional structure approaching 5 Å resolution (1), including a definition of changes in structure in the transmembrane region between closed and open channel states (2). Since there is as yet no direct identification of the path of the polypeptide chains or of the structure of the acetylcholine (ACh)-binding sites, our knowledge of the structure of the ACh-binding sites has been derived primarily from the results of affinity labeling and mutational analyses.

The muscle type nAChR has two binding sites for agonists and competitive antagonists, located in the extracellular domain of the nAChR at the α-γ and α-δ-subunit interfaces (3, 4). At equilibrium, many competitive antagonists, such as d-tubocurarine (dTC), bind to the two sites with widely different affinities (5, 6). Affinity labeling and mutational analyses support a model of agonist site structure with contributing amino acids from three distinct regions of the α-subunit and from three (or more) discrete regions of the γ- (or δ-) subunit (reviewed in Refs. 7–9). For Torpedo nAChR, the competitive antagonist [3H]-p-(dimethylamino)benzeneazidium fluoroborate, which photoincorporated primarily into the nAChR α-subunit, labeled residues in three regions of α-subunit primary structure: α-Tyr-93 (Loop A), α-Trp-149 (Loop B), and α-Cys-192 (10, 11). Photoaffinity labeling with [3H]dTC and with [3H]nicotine, an agonist, identified residues in α-subunit Loop C as well as γ-Tyr-55 (Loop D), whereas [3H]dTC also reacted with δ-Trp-57, which is homologous to γ-Trp-55, and with γ-Tyr-111 and γ-Tyr-117 (Loop E) (12–15). Additionally, δAsp-180 (Loop F) has been identified by chemical cross-linking as a residue within 9 Å of the agonist site disulfide (α-Cys-192/193) (16). In embryonic skeletal muscle nAChR (α2β3γδ) mutational analyses have localized the binding site selectivity for dimethyltubocurarine to amino acid differences between γ- and δ-subunits in Loop E (γIle-116/δVal-118 and γTyr-117/δTyr-119) and in Loop F (γSer-161/δLys-163) (17, 18), whereas for adult mouse nAChR (α2β2δ) antagonist selectivity determinants were identified in Loop D (eGlu-57) and in other regions of non-α-subunit (19).

Although there is considerable information about the identity of amino acids contributing to the agonist-binding sites, little is known about the orientation of agonists or antagonists bound within the site. Exceptions include the labeling of Loop A by [3H]acetylcholine mustard which suggests an interaction between α-Tyr-93 in the desensitized nAChR and the quaternary ammonium group of ACh (20) and recent double mutant cycle analyses that have begun to identify likely pairwise interactions between amino acids within the peptide α-neurotoxins and amino acids of the nAChR, including residues in Loops C (21) and F (22). The structures of the reactive intermediates

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The abbreviations used are: nAChR, nicotinic acetylcholine receptor; dTC, d-tubocurarine; ACh, acetylcholine; Bz-choline, 4-benzoylbenzoylcholine; Carb, carboxymethyl; H2g-HTX, dl-dihydrohistrionicotoxin; Bz acid, benzoylbenzoic acid; CDI, 1,1-carbonyldimidazole; TPS, Torpedo physiological saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; V8 protease, Staphylococcus aureus glutamyl endopeptidase; EndoLys-C, endoproteinase Lys-C; EndoAsp-N, endoproteinase Asp-N; Endo H, endo-β-N-acetylglucosaminidase H; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; PTH, phenylthiohydantoin.
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formed upon UV irradiation of dTC and nicotine are unknown. Therefore, these labeling studies, while identifying residues contributing to the agonist sites, do not define the orientation of the drugs within the binding sites.

In this report, we demonstrate that 4-benzoylbenzoylcholine (Bz2choline), which possesses a well defined photochemistry, can be used as a photoaffinity probe of the agonist-binding sites. As with other benzophenones, Bz2choline is activated by ultraviolet light at >320 nm, producing a C–O diradical that can insert into C-H bonds but will not react with water (reviewed in Refs. 23–25). Thus, proximity alone should determine which residues are covalently labeled, independent of the inherent reactivity of the amino acid side chains, which should enable Bz2choline to identify amino acids in proximity to the para position of a benzoylcholine ester when it is bound in the ACh-binding sites. We report here that Bz2choline is a competitive antagonist of the Torpedo nAChR binding with equal affinity (Kd ~1.4 μM) at the two ACh-binding sites. [3H]Bz2choline specifically photoincorporates into the nAChR γ and δ-subunits with similar efficiencies, with specific incorporation within the α-subunit at no more than 10% the level in the γ or δ-subunit. The primary sites of specific photoincorporation for [3H]Bz2choline are the homologous residues within Loop E of the non-α-subunits, γLeu-109 and δLeu-111.

EXPERIMENTAL PROCEDURES

Materials—nAChR-rich membranes were isolated from the electric organs of Torpedo californica (Winkler Enterprises, San Pedro, CA) as described previously (26), and the final membrane suspensions were stored under argon in 36% sucrose, 0.02% sodium azide at ~80 °C. Choline p-toluene sulfonate, endoglycosidase H (Endo H), diisopropyl phosphofluoridate, and carbamylcholine chloride (Carb) were from Sigma. A-azidopyrene was purchased from Molecular Probes. 1,1-Carbonyl diimidazole (CDI) and 4-benzoylbenzoic acid (Bz2 acid) were from Aldrich. Staphylococcus aureus glutamyl endopeptidase (V5 protease) was from ICN Biomedical. Endoproteasin Lys-C (EndoLys-C) and Endoproteasin Asp-N (EndoAsp-N) were obtained from Roche Molecular Biochemicals. Pre-stained low molecular mass gel standards were from Life Technologies, Inc. (in kDa, ovalbumin (43), carbonic anhydrase (29), β-lactoglobulin (20), lysozyme (14), bovine trypsin inhibitor (5.5), and insulin (2.8)). Aluminumn-backed, high performance thin layer chromatography plates and glass-backed, preparative plates (silica gel, 60 F254) were from Merck. [3H]Choline chloride (85 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [3,5-3H]4-benzoyl diimidazole (CDI) and 4-benzoylbenzoic acid (Bz2 acid) were from Sigma. 1-Azidopyrene was purchased from Molecular Probes. 1,1-phosphofluoridate, and carbamylcholine chloride (Carb) were from Warner Instrument Corp.) at a holding potential of 80 mV.

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Electrophysiology—Torpedo nAChR subunit-specific cRNAs were transcribed in vitro as described (15). Isolated, follicle-free oocytes were microinjected with 10 ng of Torpedo nAChR subunit-specific RNA in a volume of 2 μl (a mixture of 2 μl water and 1 μl diluted cRNA). Oocytes were cultured in low-Ca2+ ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.6, and 50 μg/ml gentamicin for at least 48 h prior to use. Currents elicited by ACh were measured using a standard two-electrode voltage clamp (Oocyte Clamp OC-725B, Warner Instrument Corp.) at a holding potential of ~70 mV. The recording chamber (150 μl volume) was continually gravity perfused with low-Ca2+ ND96 solution (+1 μM atropine, pH 7.6). 3 μM ACh with various concentrations of Bz2choline was applied to the oocyte in the recording chamber for 5 s through solenoid valves.

Radioligand Binding Assays—The equilibrium binding of radioactive cholinergic ligands to nAChR-rich membranes in Torpedo saline (TPS, 250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, 5 mM sodium phosphate (pH 7.0) was measured by centrifugation in a Tomy MXT-150 microcentrifuge (30). Suspensions were pretreated with diisopropyl phosphofluoridate (~0.5 mM) to inhibit esterase activity. For [3H]ACh (30 nM) and [3H]dTC (11 nM), which bind with high affinity (Kd ~50 nM), binding was measured using dilute membrane suspensions (1.5 ml, 40 nM of protein/ml and 40 nM [3H]ACh sites), whereas binding of [3H]H12-HTX (8 nM) was measured at 0.3 μg protein/ml (0.2 ml volumes), and binding of [3H]Bz2choline was measured at 1.5 μg of protein/ml [3H]ACh sites with sample volumes varying between 30 and 200 μl. Membrane suspensions were equilibrated with drugs for 45 min before centrifugation, with the exception of [3H]H12-HTX which was incubated for 5 h. Non-specific binding of [3H]ACh, [3H]dTC, and [3H]Bz2choline was determined in the presence of 1 mM Carb and that of [3H]H12-HTX in the presence of 200 μM meropavidin. To measure the initial rates of binding, nAChR-rich membranes in TPS (5 mM ACh-binding sites) were incubated with various concentrations of Bz2choline or dTC for 20 min at room temperature. The binding reaction was initiated by adding 125I-BgTx to a final concentration of 10 nM, and after appropriate intervals, 125I-BgTx binding was terminated by removing aliquots and adding them to non-radioactive α-BgTx (5 μM final concentration). The quenched reaction solutions were centrifuged at 150,000 × g for 20 min in a Beckman 42.2Ti rotor. The supernatants were discarded, and the pellets were washed twice with TPS before counting. Under these assay conditions, 125I-BgTx bound to ~35% of the available sites in 10 min, and the non-specific binding was ~2% of the total 125I. Three time points were used to determine the initial rates of binding of 125I-BgTx for each condition.

Data Analysis—The concentration dependence of Bz2choline inhibition of ACh-induced currents or of radioligand equilibrium binding was fit to Equation 1,

\[ f(x) = f(0) + (f(0) - f_0) \times \frac{1}{1 + (x/C_{50})^n} + f_0 \]  

(Eq. 1)

where \( f(x) \) is the current or specific radioligand binding measured in the presence of Bz2choline concentration \( x \), \( f_0 \) is the current (or specific binding) in the absence of Bz2choline, \( C_{50} \) is the Bz2choline concentration associated with 50% inhibition; and \( n \) is the Hill coefficient. \( f_0 \) is the leak current in the absence of ACh or the nonspecific binding determined in the presence of 1 mM Carb (for [3H]ACh and [3H]dTC) or 200 μM meropavidin (for [3H]H12-HTX). The concentration-dependent inhibition of the initial rate of 125I-BgTx binding by Bz2choline or dTC
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was fit according to Equation 1 and also, for DTC, to a two-site model as shown in Equation 2,

$$f(x) = \frac{1}{2} f(1 + x/K_0) + \frac{1}{2} f(1 + x/K_1)$$  

where $f(x)$ is the initial rate of $^{125}$I-α-BgTx binding seen in the presence of inhibitor concentration $x$; $f_0$ is the rate of binding in the absence of inhibitor, and $K_0$ and $K_1$ are the dissociation constants for the high and low affinity binding sites, respectively. Equilibrium binding data for $[3H]$Bz2choline were fit to a single site model with a linear, nonspecific binding component as shown in Equation 3,

$$B_i (x) = B_{in} + (1 + (K_i/x)) + m \cdot x$$  

where $B_i(x)$ is the $[3H]$Bz2choline bound at a free concentration $x$; $B_{max}$ is concentration of $[3H]$Bz2choline-binding sites; $K_i$ is the dissociation constant, and $m$ is the slope of nonspecific binding derived from parallel experiments performed in the presence of excess Carb. SigmaPlot (Jandel Scientific) was used for nonlinear least squares fit of the data, and the standard errors of the parameter fits are indicated.

**Photolabeling of nAChR-rich Membranes with $[3H]$Bz2choline—**

Membrane suspensions (3 mg of protein/ml) in TPS were incubated with 0.5 mCi diisopropyl phosphofluoridate for 10 min to inactivate acetylcholinesterase prior to addition of $[3H]$Bz2choline. For labeling on an analytical scale, 75–1000 ng aliquots in 96-well plastic microtiter plates were equilibrated at room temperature with $[3H]$Bz2choline (15 μM) and additional cholinergic ligands for 20 min prior to photolysis, except for α-BgTx, which was incubated with the membranes for 60 min prior to addition of $[3H]$Bz2choline. The samples were then irradiated on ice for 60 min using a 100-watt, focused, short-arc, mercury lamp with a Pyrex glass filter and a 310-nm cut-off filter. Polypeptides were resolved by SDS-PAGE and visualized by Coomassie stain. The $3H$ incorporation into individual polypeptides was determined by scintillation counting of excised gel slices as described (12) or by fluorography as described previously (26), except that Amplify (Amersham Pharmacia Biotech) was used as the fluorographic reagent.

For labeling on a preparative scale, membrane suspensions (3 mg/ml; 3–7 ml in 2.5-cm diameter plastic Petri dishes) were equilibrated with $[3H]$Bz2choline (15 μM) and, where indicated, with 30 μM proadifen, a desensitizing aromatic amine noncompetitive antagonist (31). To visualize the nAChR subunits resolved by preparative SDS-PAGE without staining and destaining of the gel, after photolysis with $[3H]$Bz2choline, membrane suspensions were photolabeled with 1-azidopyrene, a fluorescent photoactive hydrophobic compound (32). 1-Azidopyrene solution (100 μg/ml in MeSO) was added after a concentration of 600 μM, and the sample was irradiated with a 365-nm hand-held lamp for 3 min. After SDS-PAGE, the unstained gel was visualized under UV light, and the nAChR subunits were excised based upon the fluorescence of incorporated 1-azidopyrene. The excised gel pieces containing γ or δ-subunit were diced, and the protein was passively eluted for 3 days into 30 ml of gel elution buffer (100 mM NH_4HCO_3, 0.1% SDS, pH 7.0). The eluate was clarified by centrifugation at 14,000 × g for 1 h at 4 °C and the protein pellets were resuspended to ~200 μl by Centricon-30 (Amicon). Excess SDS was removed by acetone precipitation (80% final concentration, overnight at ~20 °C). Starting from 15 mg of nAChR-rich membranes (typically ~15 nmol of ACh sites), ~500 μg of protein was recovered from the supernant bands. Based upon amino-terminal sequence analyses, the γ and δ-subunit preparations both contained a major contaminant the Na$^+_+$/K$^+_+$ ATPase β-subunit present at ~50–100% of the level of γ or δ-subunit, which was unexpected based upon previous reports (13). The δ-subunit preparations also contained γ-subunit at 10–30% the level of δ-subunit. This contamination by γ-subunit results because δ-subunit is the subunit most difficult to visualize based upon the fluorescence of the azidopyrene adduct.

**Enzymatic Digestions and Peptide Purification—**

For digestion with S. aureus V8 protease, the nAChR subunits or subunit fragments were resuspended in gel elution buffer and digested for 3 days with V8 protease (20% w/v). For digestion with EndoLyS-C, the nAChR subunits were resuspended in 100 mM Tris, 0.1% SDS, pH 8.6, and digestion conditions are described in the figure legends. Digests were fractionated on 1.5-mm thick 16% T, 6% C Tricine/SDS-PAGE gels (32, 33). To identify radioactive fragments, a strip was cut from the gel, cut into 2-mm slices, and counted, whereas the remainder of the gel was stored at 4 °C. This $^{3}H$ profile was used as a template to identify the regions of the preparative gel containing $^{3}H$. Material eluted from the gel was resuspended in gel elution buffer for further digestion with V8 protease or in the same buffer supplemented with 1% Genapol C-100 for digestion with EndoAsp-N (3 μg, 25 °C, 7 days). After purification by SDS-PAGE, eluted material (with or without additional protease treatment) was further purified by reversed-phase HPLC with a Brownlee Aquapore C-4 column (100 × 2.1 mm). Solvent A was 0.09% trifluoroacetic acid in water, and solvent B was 60% acetonitrile/40% isopropanol alcohol/0.09% trifluoroacetic acid. The gradients (in % solvent B) are indicated in the figures and are dashed lines. The columns (0.2 ml/min) were monitored by absorbance at 215 nm, by fluorescence emission at 432 nm (355 nm excitation), and by counting fraction aliquots (5–10%) for $^{3}H$. To determine whether the labeled γ-subunit fragment generated by V8 protease was glycosylated, protease digestion was terminated with 1 μl diisopropyl phosphofluoridate and then an aliquot of the digest (~15 ng) was loaded onto the HPLC fractions were reduced in volume to 50 μl and loaded directly onto the filters. One-third of each cycle was injected for mass determination by an on-line model 120A PTH-derivatizer analyzer, and two-thirds were collected for $^{3}H$ counting. Cycle mass yields for PTH-derivatives were estimated from chromatographic peak heights. Values plotted are actual pmol and, where appropriate, the mass (m) and repetitive yield ($R$) were calculated by nonlinear least squares regression (Sigma Plot) of the function $f(n) = f_0 \times R^n$ where $f(n)$ is the observed mass released in cycle $n$. PTH-derivatives of Ser, Cys, Arg, His, and Trp were excluded from the fits due to known problems with measuring mass yields of these residues. In some cases, sequencing of some peptides was blocked by treatment of the sample on the filter with o-phthalaldehyde (Pierce) or prior to cycles containing known proline residues. o-Phthalaldehyde reacts with primary, but not secondary, amines and can be used to block Edman degradation of any peptide not containing an amino-terminal proline (35). o-Phthalaldehyde treatment was carried out as described (12).

**RESULTS**

**Bz2choline Is an nAChR Antagonist—**

We first examined interactions of Bz2choline with Torpedo nAChRs expressed in Xenopus oocytes. Concentrations of Bz2choline up to 100 μM elicited no detectable currents, but it was a potent antagonist of ACh-induced currents (Fig. 1A). When co-applied with 3 μM ACh, Bz2choline produced a dose-dependent inhibition of ACh responses ($IC_{50} = 1 \mu M$) that was completely reversible after removal of the Bz2choline from the oocyte perfusion solution.

**Effects of Bz2choline on Equilibrium Binding of $[^{3}H]ACh$, $[^{3}H]dTC$, and $[^{3}H]H_{12}$-Histrionicotinotxin—**

Equilibrium binding experiments (Fig. 1B) demonstrated that Bz2choline was a potent inhibitor of the binding of both $[^{3}H]ACh$ ($IC_{50} = 5 \mu M$) and $[^{3}H]dTC$ ($IC_{50} = 3 \mu M$), with both inhibition curves well fit by a single site model (Equation 1, $n = 1$). Under the assay conditions used, with a low concentration of $[^{3}H]dTC$ (11 mM) and a higher concentration of nAChR (40 mM ACh sites), $[^{3}H]dTC$ would be bound only to the high affinity (α-γ) site (3). In contrast to its high affinity interaction with the ACh site, high concentrations of Bz2choline were necessary to inhibit the binding of the noncompetitive antagonist $[^{3}H]H_{12}$-histrionotoxin ($IC_{50} = 1 \mu M$) in the absence or presence of the agonist carbamylcholine (Carb).

**Inhibition of $^{125}$I-α-BgTx Binding—**

Initial rates of binding of $^{125}$I-α-BgTx were determined for nAChR-rich membranes equilibrated with Bz2choline or dTC (Fig. 2). For the assay conditions used, $^{125}$I-α-BgTx bound to the two ACh sites at equal rates (50 μl was treated with endoglycosidase H; 10 milliliters) for 3 days. This $^{3}H$ profile was used as a template to identify the regions of the preparative gel containing $^{3}H$. Material eluted from the gel was resuspended in gel elution buffer for further digestion with V8 protease or in the same buffer supplemented with 1% Genapol C-100 for digestion with EndoAsp-N (3 μg, 25 °C, 7 days). After purification by
FIG. 1. Benzoylbenzoylcholine, an nAChR photoaffinity probe that binds to the two ACh-binding sites with equal affinity. A. Xenopus oocytes expressing Torpedo nAChR were voltage-clamped at −70 mV. The currents (I) elicited by 3 μM ACh in the presence of Benzoylbenzoylcholine were normalized to the current in the absence of Benzoylbenzoylcholine (I0 = 5.20 ± 0.24 μA). The IC50 for Benzoylbenzoylcholine inhibition was 1.15 ± 0.05 μM, with a Hill coefficient of 0.91 ± 0.05. Three current measurements were made at each Benzoylbenzoylcholine concentration, with the means (±S.D.) shown. B. Benzoylbenzoylcholine inhibition of equilibrium binding of [3H]ACh (●), [3H]dTC (○), and [3H]HTX (▼, △) to nAChR-rich membranes. Membranes were incubated with Benzoylbenzoylcholine and 30 nM [3H]ACh (11 μM ACh sites), 10 nM [3H]dTC (40 μM ACh sites), or 8 nM [3H]HTX (420 μM ACh sites) in the presence (●) or absence (○) of 0.5 mM Carb. When the inhibition curves were fit to a single site model (see “Experimental Procedures,” Equation 1, n = 1), the IC50 values for Benzoylbenzoylcholine were as follows: [3H]ACh, 5.2 ± 0.6 μM; [3H]dTC, 3.2 ± 0.4 μM; [3H]HTX (−Carb), 0.75 ± 0.1 μM; [3H]dTC (+Carb), 1.0 ± 0.5 μM. C, inhibitory effect of Carb on racemate to nAChR-rich membranes by Benzoylbenzoylcholine (●) or dTC (○). Rates of binding (cpm/min) were determined in the absence (V0) and presence of inhibitor (Vf). For Benzoylbenzoylcholine IC50 = 4.9 ± 0.1 μM, n = 1. For dTC the data were fit by IC50 = 280 ± 50 nM, n = 0.68 ± 0.07, or by a two-site model (KD1 = 70 ± 23 nM, KD2 = 1.4 ± 0.4 μM). D, equilibrium binding of [3H]Bz2choline, nAChR-rich membranes (1.3 μM ACh sites, 160 μg of protein in 200 μl of TPS) were equilibrated with [3H]Bz2choline in the absence (●) or presence (○) of 1 mM Carb (to define nonspecific binding) and with (▼, △) 30 μM proadifen. The calculated KD values were 1.5 ± 0.1 and 0.8 ± 0.2 μM in the absence and presence of proadifen, respectively, with site concentrations of 1.7 ± 0.04 and 1.4 ± 0.05 μM.

single site model (IC50 = 4.9 μM, n = 1), consistent with its potency as an inhibitor of ACh-induced currents (Fig. 1A) or [3H]ACh equilibrium binding (Fig. 1B).

Equilibrium Binding of [3H]Bz2choline—The equilibrium binding of [3H]Bz2choline to nAChR-rich membranes was measured (Fig. 1D) in the absence and presence of proadifen (30 μM), a desensitizing noncompetitive antagonist (31). With nonspecific binding defined by the presence of excess Carb, the specific binding of [3H]Bz2choline was well fit by a simple hyperbolic binding function (−proadifen, KD = 1.4 μM; + proadifen, KD = 0.7 μM, Equation 3), and the site concentration was the same as the concentration of [3H]ACh sites (1.2 ± 0.2 Bz2choline sites/ACh site).

Photolabeling of [3H]Bz2choline into nAChR-rich Membranes—Photolabeling studies were carried out with Torpedo nAChR-rich membranes equilibrated with 15 μM [3H]Bz2choline, a concentration sufficient to occupy ~90% of the agonist sites. After 1 h of photolysis, the pattern of incorporation was assessed by SDS-PAGE followed by fluorography or by scintillation counting of bands excised from the gel. As seen in the fluorograph of an 8% acrylamide gel (Fig. 2), [3H]Bz2choline was incorporated primarily in the nAChR γ- and δ-subunits, with incorporation in α-subunit at lower efficiency. The incorporation in γ- and δ-subunits was inhibited by Carb, whereas incorporation in α-subunit appeared largely nonspecific. There was also Carb-inhibitable incorporation in a band with a mobility close to that of rapsyn (43K) in a region of the gel known to contain a proteolytic fragment of γ-subunit (36). Surprisingly, whereas incorporation in γ-subunit was inhibited by dTC or α-BgTx, dTC only partially inhibited the incorporation in δ-subunit, and in the presence of α-BgTx there was apparently increased incorporation in δ-subunit. The pharmacological specificity of the photolabeling at the level of the nAChR subunits was characterized by quantifying [3H]incorporation in excised gel bands in this and additional experiments. Incorporation in γ-subunit was reduced by 80–90% by Carb, dTC, or α-BgTx, whereas for δ-subunit, Carb reduced labeling by 70%, but dTC reduced labeling by only 30%, and α-BgTx actually increased labeling by 15–20%. The amount of Carb-inhibitable [3H]incorporation in γ- and δ-subunits indicated specific labeling of 6–8% of the nAChRs. Carb-inhibitable labeling of the α-subunit band varied between 3 and 20% that of the γ-band. Furthermore, when ACh sites were occupied by α-BgTx, [3H]Bz2choline incorporation in δ-subunit was inhibited 85% by tetracaine, an aromatic amine noncompetitive antagonist that binds with high affinity to the nAChR ion channel in the presence of α-BgTx (37).
Benzoylbenzoylcholine, an nAChR Photoaffinity Probe

Asn-linked carbohydrate in nAChR labeled fragment also contained the endoglycosidase H-sensitive carbohydrate sensitive to Endo H, which removes high mannose and some hybrid Asn-linked carbohydrates (39). As seen in the treated with endoglycosidase H, which removes high mannose and fractionated by SDS-PAGE, with a part of the digest also digested with Endo H (10 milliunits, 48 h, 25 °C). The 4 digests were fractionated by Tricine/SDS-PAGE, with the distribution of 3H within the gel determined by fluorography (6-week exposure). Indicated on the left are the mobilities of molecular mass markers.

To identify the site of labeling in the γ-subunit, [3H]Bz2choline labeled γ-subunit was isolated by preparative SDS-PAGE and digested with V8 protease in solution for 3 days. The digest was then fractionated by Tricine/SDS-PAGE, and a strip of the preparative gel was excised and cut into 2-mm slices for scintillation counting (Fig. 4A). There was a single major peak of 3H at 12 kDa. About 25% of the 3H loaded on the gel was recovered when this region was cut out from the preparative gel and eluted. When this material was further purified by reversed-phase HPLC (Fig. 4B), about 45% of 3H loaded on the column was recovered in a single peak of 3H centered at 60% solvent B, with the 3H in that peak reduced by 80% for the sample labeled in the presence of Carb. Material in that peak (fractions 26–29) was pooled and concentrated for amino-terminal sequence analysis (Fig. 4C). The only nAChR subunit sequence detected began at γVal-102 (–Carb, I₀ = 54 pmol; +Carb, I₀ = 28 pmol). In addition, in each sample there were three fragments from the β-subunit of the Na/K-ATPase beginning at Tyr-230 (I₀ = 50 pmol), Ser-142 (I₀ = 20 pmol), and Gly-172 (I₀ = 15 pmol). For the sample labeled in the absence of agonist, there was a prominent peak of 3H release in cycle 8 (260 pmol), which was reduced by 90% for the sample labeled in the presence of agonist. The 3H release in cycle 8, if originating from the nAChR γ-subunit fragment beginning at γVal-102, would correspond to [3H]Bz2choline photoincorporation in γVal-102. For the sample labeled in the presence of Carb, 15 pmol). For the sample labeled in the presence of Carb, 15 pmol). For the sample labeled in the presence of Carb, 58,000 cpm; (Carb, 219,000 cpm; +Carb, 112,000 cpm) were isolated by SDS-PAGE. The γ-subunits were digested with V8 protease and fractionated by Tricine/SDS-PAGE. A, 3H distribution determined by liquid scintillation counting of 2-mm gel slices cut from a 2-cm strip of the preparative slab gel. The peak of 3H, centered at 12 kDa, was excised and eluted (–Carb, 58,000 cpm; +Carb, 14,000 cpm) and then fractionated by reversed-phase HPLC. B, 3H HPLC elution profiles (15) determined by counting 5 (–Carb) or 10% (+Carb) of each fraction and the observed fluorescent bands (dotted line, +Carb). Fractions 27–30 were pooled, dried down, and resuspended in 100 μl of elution buffer (–Carb, 38,000 cpm; +Carb, 4,000 cpm) for direct sequence analysis (C) or for sequence analysis after further digestion with chymotrypsin (D). C, 3H release from sequencing 15-μl aliquots of the pooled fractions (–Carb, 5,700 cpm loaded, 950 cpm left on the filter; +Carb, 600 cpm loaded, 170 cpm left on the filter). A fragment of nAChR γ-subunit was present beginning at γVal-102 (–Carb, I₀ = 46 pmol, R = 92%; +Carb, I₀ = 19 pmol, R = 94%). Also present were peptides from the Na/K-ATPase β-subunit beginning at Tyr-230, Ser-142, and Gly-172 with I₀ (–Carb) = 55, 19, and 15 pmol, respectively, and the amino terminus of V8 protease (–Carb, I₀ = 18 pmol). Release of 3H in cycle 8 (260 pmol) was consistent with [3H]Bz2choline incorporation into nAChR γVal-102 in the peptide beginning at γVal-102. D, 3H release during sequence analyses of the chymotryptic digests. Aliquots of the pooled HPLC fractions in elution buffer supplemented with 1% Genapol C-100 were digested with chymotrypsin (50 μg, 48 h, 25 °C) and then sequenced directly without further purification (–Carb, 2550 cpm loaded, 310 cpm left on the filter; +Carb, 730 cpm loaded, 70 cpm left on the filter). Upon treatment with chymotrypsin, 3H release was seen in cycle 4, whereas release in cycle 8 was abolished, a result consistent with chymotrypsin cleavage after γTyr-105 and 3H incorporation at γVal-109.
Fractions 16

Specifically labeled material (43% of loaded cpm) eluted in a single peak, 23,000 cpm injected, 78% recovered; profiles (Carb) as well as the % solvent B (---).

Fluorescence (O) and the binding sites) were photolabeled with 15 g of 3H in the analytical lane was determined by gel slice analysis (Fig. 6). For the o-subunit isolated from membranes labeled with [3H]Bz2choline incorporation in the absence of 3H label in the preparative gels. Sequence analysis of this material identified a γ-subunit fragment beginning at γVal-47, with no detectable 3H release (<0.2 cpm/pmol) in or around cycle 9, corresponding to γTrp-55, the primary site of photolabeling by [3H]TTC (13). Aliquots of this material were subsequently digested with V8 protease (72 h at 25 °C), and the digest was fractionated by reversed-phase HPLC (Fig. 5B). About 45% of the loaded 3H was recovered in a single peak eluting at 38% solvent B (fractions 16–17). Sequence analysis of the material in fraction 16 (Fig. 5C) revealed the presence of a primary sequence beginning at γVal-102 (Carb, I₀ = 38 pmol; +Carb, I₀ = 40 pmol) with prominent, Carb-inhibitable release of 3H in cycle 8 consistent with incorporation at γLeu-109. Two secondary sequences at ~10% the level of the primary sequence were also detected, beginning at Val-1 of V8 protease and Arg-147 from the Na⁺/K⁺-ATPase (20%).

A primary site of photolabeling was at γVal-102 (Carb, I₀ = 38 pmol; +Carb, I₀ = 40 pmol; R = 92%), with secondary sequences from the Na⁺/K⁺-ATPase β-subunit (Arg-147 (Carb, I₀ = 3 pmol, R = 88%) and from V8 protease beginning at Val-1 (Carb, I₀ = 2 pmol, R = 92%). Release of 3H in cycle 8 (520 cpm) was consistent with [3H]Bz2choline incorporation into γ-Leu-109 (16 cpm/pmol) from the peptide beginning at γVal-102.

An alternative fragmentation strategy was developed to isolate the γ-Val-102 fragment in greater purity. Digestion of the γ-subunit in solution with endopeptidase Lys-C (EndoLys-C) produces a fragment of ~25 kDa extending from γValu-47 to γLys-192 (13) which could be isolated for subsequent digestion by V8 protease. [3H]Bz2choline-labeled γ-subunit was digested with EndoLys-C in solution, and the digest was then fractionated by preparative Tricine/SDS-PAGE with an aliquot of the digest separated in an adjacent analytical lane. The distribution of 3H in the analytical lane was determined by gel slice analysis (Fig. 5A), and the resulting profile was used as a template to isolate the ~26 kDa band containing the major fraction 16 (Fig. 5A), 4,400 cpm loaded, 1,500 cpm left on the filter; fraction 17 (Fig. 5B), 92%). Release of 3H in cycle 8 (520 cpm) was consistent with incorporation at γLeu-109. Two secondary sequences at ~10% the level of the primary sequence were also detected, beginning at Val-1 of V8 protease and Arg-147 from the Na⁺/K⁺-ATPase (20%).

[3H]Bz2choline incorporation in the absence of competing drugs (Control) digestion with EndoLys-C produced a major 3H band of ~20 kDa as well as a band of ~30 kDa, and 3H incorporation was not seen in either band for the digests of subunits isolated from nAChRs labeled in the presence of Carb or α-BgTx. For the o-subunit isolated from membranes labeled with [3H]Bz2choline in the absence of α-BgTx, there was a major 3H band of ~10 kDa that was not present in the other digests. This band had a mobility similar to a [3H]tetracaine-labeled fragment beginning at the NH₂ terminus of δM2 that was also isolated from EndoLys-C digests (38). Thus, in the presence of α-BgTx the inhibition of [3H]Bz2choline photoincorporation in one subdomain (the 20/30-kDa bands) accompanied by

FIG. 5 Identification of the site of [3H]Bz2choline photoincorporation in γ-subunit via sequential fragmentation with EndoLys C and V8 protease. nAChR-membranes (30 mg, 37 nmol ACh-binding sites) were photolabeled with 15 µM [3H]Bz2choline (700 Ci/mmol) in the presence of 30 µM propridin and in the absence (○) or presence (○) of 1 mM Carb. Labeled γ-subunits (~Carb, 456,000 cpm; +Carb, 255,000 cpm) were isolated by SDS-PAGE and resuspended in 0.1 M Tris, 0.1% SDS, pH 8.5, for digestion with EndoLys-C (1 unit, 3 weeks), with the digests fractionated by Tricine/SDS-PAGE. A, 3H distribution as determined by liquid scintillation counting of 4 mm gel slices cut from an analytical lane containing a 10% aliquot of the digest (~Carb). The peak of 3H, centered at 26 kDa, was excised and eluted from the preparative gels (~Carb, 117,000 cpm; +Carb, 13,200 cpm). Aliquots (20%) of the 26-kDa band (65 µl) were digested with 14 µg of V8 protease for 48 h at 25 °C and then fractionated by reversed-phase HPLC. B, HPLC 3H elution profiles with 10% of each fraction counted. Profiles (~Carb) as well as the % solvent B (---). Specifically labeled material (43% of loaded cpm) eluted in a single peak (fractions 16 and 17). C, 3H and mass release during sequence analysis of HPLC fraction 16 (○, 4,400 cpm loaded, 1,500 cpm left on the filter; ○, 2,300 cpm injected, 100% recovered). Also shown are the absorbance at 215 nm (---) and the fluorescence (---) profiles (---) as well as the % solvent B (---). About 45% of the loaded 3H was recovered in a single peak eluting at 38% solvent B (fractions 16–17). Sequence analysis of the material in fraction 16 (Fig. 5C) revealed the presence of a primary sequence beginning at γVal-102 (~Carb, I₀ = 38 pmol; +Carb, I₀ = 40 pmol) with prominent, Carb-inhibitable release of 3H in cycle 8 consistent with incorporation at γLeu-109. Two secondary sequences at ~10% the level of the primary sequence were also detected, beginning at Val-1 of V8 protease and Arg-147 from the Na⁺/K⁺-ATPase (20%).

[3H]Bz2choline incorporated in the site(s) of agonist-inhibitable photoincorporation and of the site of photoincorporation in the presence of α-BgTx, aliquots of the subunit isolated from nAChR-rich membranes labeled in three conditions (Control, +Carb, and +α-BgTx) were digested with EndoLys-C in solution (3 days, 25 °C). The digests were fractionated by Tricine/SDS-PAGE, with the distribution of 3H determined by gel slice analysis (Fig. 6). For the o-subunit isolated from membranes labeled with [3H]Bz2choline in the absence of competing drugs (Control) digestion with EndoLys-C produced a major 3H band of ~20 kDa as well as a band of ~30 kDa, and 3H incorporation was not seen in either band for the digests of subunits isolated from nAChRs labeled in the presence of Carb or α-BgTx. For the o-subunit isolated from membranes labeled in the presence of α-BgTx, there was a major 3H band of ~10 kDa that was not present in the other digests. This band had a mobility similar to a [3H]tetracaine-labeled fragment beginning at the NH₂ terminus of δM2 that was also isolated from EndoLys-C digests (38). Thus, in the presence of α-BgTx the inhibition of [3H]Bz2choline photoincorporation in one subdomain (the 20/30-kDa bands) accompanied by

FIG. 6 Mapping sites of [3H]Bz2choline photoincorporation in δ-subunit with EndoLys-C. δ-Subunits were isolated from nAChR-rich membranes photolabeled with 15 µM [3H]Bz2choline in the absence (●) or presence of 1 mM Carb (●) or 20 µM α-BgTx (●). Labeled subunits (50 µg of aliquots, 6,000–9,000 cpm), resuspended in gel elution buffer, were digested with 0.3 units of EndoLys-C for 3 days, and the digests were fractionated by Tricine/SDS-PAGE. The 3H distributions, as determined by gel slice analyses, are shown along with the mobilities of the molecular mass markers and the dye front (DF) (top).
increased $^3$H incorporation in the 10-kDa band, likely to contain the transmembrane domain of the nAChR, can account for the apparent lack of inhibition by $\alpha$-BTx of $[^3$H]$\text{Bz}_2\text{choline}$ labeling at the level of the intact $\delta$-subunit. When the 20-kDa $^3$H-labeled band was isolated from an EndoLys-C digest of labeled $\delta$-subunit and sequenced, $\delta$-subunit fragments beginning at $\delta$His-20, $\delta$His-26, and $\delta$Glu-47 were present at similar mass levels, as well as a fragment from the Na$^+$/K$^+$/ATPase $\beta$-subunit beginning at Arg-147. There was no $^3$H release above background during 15 cycles of Edman degradation, indicating that there was no detectable specific $^3$H incorporation in the region of $\delta$Tryp-57 (<0.1 cpm/pmol), the amino acid photolabeled by $[^3$H]HTC (13).

We wanted to determine whether $[^3$H]$\text{Bz}_2\text{choline}$ was photoincorporated in $\delta$Leu-111, the position homologous to $\gamma$Leu-109. Digestion with V8 protease would not be useful for this purpose, because it would be difficult to obtain sufficient yields at that position when sequencing a fragment beginning after $\delta$Glu-85, the first glutamate amino-terminal to $\delta$Leu-109. Digestion with endoproteinase Asp-N (EndoAsp-N), which cleaves before aspartates and sometimes before glutamates (40), could provide an alternative strategy if there was efficient cleavage at $\delta$Asp-99. EndoLys-C digests of labeled $\delta$-subunit were fractionated by preparative Tricine/SDS-PAGE (Fig. 7A), and the region of the gel between 20 and 29 kDa was excised and eluted. This material was then digested with EndoAsp-N (1 week, 25 °C), and the digest was separated by reversed-phase HPLC (Fig. 7B). When the principal peak of $^3$H (fraction 24) from the $-\text{Carb}$ sample was sequenced, the primary $^3$H release was in cycle 13 with lower release in cycle 9 (Fig. 7C). $^3$H release in those cycles was reduced by >90% for the sample isolated from nAChRs labeled in the presence of Carb. Two $\delta$-subunit peptides were detected beginning at $\delta$Asp-99 ($-\text{Carb}, I_0 = 39$ pmol; $+\text{Carb}, I_0 = 48$ pmol) and $\delta$Asp-76 ($-\text{Carb}, I_0 = 27$ pmol; $+\text{Carb}, I_0 = 42$ pmol). In addition there were fragments from nAChR $\gamma$-subunit beginning at $\gamma$Asp-76 and $\gamma$Glu-101 ($-\text{Carb}, I_0 = 40$ and 17 pmol, respectively) and a primary sequence from the $\beta$-subunit of the Na$^+$/K$^+$/ATPase beginning at Arg-147 ($-\text{Carb}, I_0 = 160$ pmol). Although the polypeptide composition of the sample was complex, the $^3$H release in cycle 13 was consistent with specific $[^3$H]$\text{Bz}_2\text{choline}$ photoincorporation in $\delta$Leu-111 in the fragment beginning at $\delta$Asp-99, and the release in cycle 9 would be consistent with labeling of $\gamma$Leu-109 in the fragment beginning at $\gamma$Glu-101.

Additional samples were sequenced to clarify the source of $^3$H release seen in cycles 9 and 13 (Fig. 8). Sequence analysis of the adjacent fractions (fractions 22, 23, and 25) revealed that the level of $^3$H release in cycle 13 was largest for fraction 24, which had the highest level of the $\delta$-Asp-99 fragment, whereas release in cycle 9 was greatest for fraction 23, which contained the $\gamma$Glu-101, $\gamma$Asp-76 and $\delta$Asp-76 fragments at the highest levels. Based upon the $^3$H release in cycle 13 and the masses of the $\delta$Asp-99 fragment in the fractions, the calculated $^3$H incorporation at $\delta$Leu-111 in fractions 23–25 was 10, 6, and 8 cpm/pmol, respectively. Although $^3$H release in cycle 9 correlated reasonably well with the mass levels of $\gamma$Glu-101, $\delta$Asp-76, or $\gamma$Asp-76 in the adjacent fractions, an additional experiment established that neither of the fragments beginning at $\delta$Asp-76 or $\gamma$Asp-76 could be the source of $^3$H release in cycle 9 (or 13). We took advantage of the fact that both fragments contain prolines prior to the observed $^3$H release in cycle 9 and that sequence analysis can be interrupted at any desired cycle of Edman degradation to treat the material on the sequencing filter with o-phenthaldehyde, which blocks Edman degradation of all peptides without an amino-terminal proline (12, 35). Thus, during sequence analysis of aliquots of fraction 23, samples were treated with o-phenthaldehyde prior to cycle 8 ($\delta$Pro-83) or cycle 6 ($\gamma$Pro-81). Following these treatments the only sequence present was $\delta$Asp-76 (45 pmol) or $\gamma$Asp-76 (65 pmol), depending upon the cycle of treatment, and neither had $^3$H release in cycle 9 or 13 (data not shown). Therefore, the $^3$H release in cycles 9 and 13 did not result from photolabeling of the fragments beginning at $\gamma$Asp-76 and $\delta$Asp-76. EndoAsp-N can cleave on the amino-terminal side of glutamates (40), and in separate experiments with labeled $\gamma$-subunit, we verified that EndoAsp-N does cleave at $\gamma$Glu-101 (not shown). Based upon the $^3$H release in cycle 9 and the masses of the $\gamma$Glu-101 fragment in the fractions, the calculated $^3$H incorporation at $\gamma$Leu-109 in fractions 23–25 was 15, 13, and 5 cpm/pmol, respectively, which was similar to the level of incorporation at $\gamma$Leu-109 seen in the $\gamma$-subunit band analyzed from the same labeling experiment (16 cpm/pmol, Fig. 5).

**DISCUSSION**

In this paper we introduce $[^3$H]$\text{Bz}_2\text{choline}$ as a novel antagonist photoaffinity probe of the nAChR. $\text{Bz}_2\text{choline}$ binds with

![Fig. 7. Identification of the site of $[^3$H]$\text{Bz}_2\text{choline}$ photoincorporation in $\delta$-subunit via sequential fragmentation with EndoLysC and EndoAsp-N. Labeled $\delta$-subunit samples, isolated from the $[^3$H]$\text{Bz}_2\text{choline}$ labeling described in Fig. 6, were digested (600 µl, $-\text{Carb}, 546,000$ cpm; $+\text{Carb}, 264,000$ cpm) with EndoLys-C (3 units, 1 week, 25 °C)). The digests were fractionated by Tricine/SDS-PAGE with aliquots (5%) electrophoresed in analytical lanes adjacent to the bulk digests. A, $^3$H distribution in the analytical lanes as determined by gel slice analysis is shown in relation to molecular mass markers (top). For the preparative lanes, the region from 20 to 29 kDa was excised and eluted (300 µl; $-\text{Carb}, 210,000; +\text{Carb}, 69,000$ cpm). This material, resuspended in gel elution buffer supplemented with 1% Genapol C-100, was digested with 2 µg of EndoAsp-N (1 week at 25 °C). B, reversed-phase HPLC purification of the digests with 8% of each fraction was counted. Also shown are the absorbance at 215 nm (---) and the fluorescence (○) profiles ($\text{Carb}$) as well as the % solvent B (- - -). C, $^3$H and mass release from sequence analysis of HPLC fraction 24 (85% cpm loaded, 3,900 cpm left on the filter; ○, 1,600 cpm loaded, 830 cpm left). Two $\delta$-subunit fragments were detected beginning at $\delta$Asp-99 (□, $-\text{Carb}, I_0 = 40$ pmol, $R = 92%$; +Carb, $I_0 = 51$ pmol, $R = 91%$) and at $\delta$Asp-76 ($-\text{Carb}, I_0 = 27$ pmol, $R = 84%$). Also present were fragments from nAChR $\gamma$-subunit beginning at $\gamma$Asp-76 ($-\text{Carb}, I_0 = 40$ pmol, $R = 87%$) and $\gamma$Glu-101 ($-\text{Carb}, I_0 = 17$ pmol, $R = 80%$) as well as from the Na$^+$/K$^+$/ATPase $\beta$-subunit beginning at Arg-147 ($I_0 = 170$ pmol, $R = 90%$). The sequence of the $\delta$Asp-99 peptide is shown at top.
Fraction 25, I

g values: Fraction 22,

linear, least squares fits of the residue masses yielded the following labels, photoincorporation in units (42), and for two competitive antagonist photoaffinity a unit fragments by sequence analysis of HPLC fractions from (43) and the initial masses (13, 9 (42) as in the fraction 25, 8 cpm/pmol, Fraction 22, 0.5 cpm/pmol, Fraction 23, 10 cpm/pmol, Fraction 24, 6 cpm/pmol, Fraction 25, 8 cpm/pmol. For the fraction 22, 0.5 cpm/pmol, Fraction 23, 10 cpm/pmol, Fraction 24, 6 cpm/pmol, Fraction 25, 8 cpm/pmol. For the γ-Val-109 sequence, non-linear, least squares fits of the residue masses yielded the following values: fraction 22, I = 15 ± 2 pmol, R = 90 ± 2%; fraction 23, I = 16 ± 3 pmol, R = 88 ± 4%; fraction 24, I = 38 ± 6 pmol, R = 92 ± 2%; fraction 25, I = 15 ± 1 pmol, R = 89 ± 1%. The calculated incorporation at δ-Leu-111 (cycle 13) for each fraction was as follows: fraction 22, 0.5 cpm/pmol; fraction 23, 10 cpm/pmol; fraction 24, 6 cpm/pmol; fraction 25, 8 cpm/pmol. For the γ-Val-101 sequence, non-linear, least squares fits of the residue masses yielded the following values: fraction 22, I = 25 ± 8 pmol, R = 82 ± 5%; fraction 23, I = 43 ± 11 pmol, R = 80 ± 5%; fraction 24, I = 17 ± 7 pmol, R = 80 ± 7%; fraction 25, I = 16 ± 4 pmol, R = 84 ± 4%. The calculated incorporation at γ-Leu-109 (cycle 9) for each fraction was as follows: fraction 22, 5 cpm/pmol; fraction 23, 15 cpm/pmol; fraction 24, 13 cpm/pmol; fraction 25, 5 cpm/pmol.

high affinity (K_D = 1 μM) to the two ACh sites, and its interaction with the ion channel domain is 1000-fold weaker, as judged by the inhibition of binding of [3H]12-HTX. Whereas Bz2choline binding to the ACh site at the α-γ interface is 40-fold weaker than that of dTC (K_D = 25 nM), it binds with similar affinity as dTC at the α-δ-binding site. Bz2choline is thus a potent competitive antagonist that upon UV irradiation will form a reactive intermediate of known structure (the excited state triplet ketone) that reacts via hydrogen abstraction and CH bond insertion (23). For Torpedo nAChR-rich membranes equilibrated with [3H]Bz2choline, UV irradiation results in selective photo incorporation into γ-Leu-109 and the homologous residue in δ-subunit, δ-Leu-111, amino acids within Loop E of the ACh-binding sites. These results, which define the orientation in the ACh-binding site of the para position of this substituted benzoylcholine ester, provide a first definition of the orientation of a choline ester competitive antagonist when bound in the ACh site.

When analyzed at the level of the intact subunits, [3H]Bz2choline was specifically photo incorporated into the nAChR γ- and δ-subunits at similar efficiencies, with specific incorporation into the α-subunit at less than 10% the efficiency of labeling of the γ- or δ-subunit. The inefficient labeling of the α-subunit was surprising. Whereas the sulfhydryl specificity of [3H]HTC (4-N-maleimido-α-benzylthiethylammonium) (41) restricts its reactivity to the α-subunits, containing the only known cysteines in the ACh-binding sites, the alkylating antagonist, [3H]lphotophane was found to react with the γ- and δ-subunits (42), and for two competitive antagonist photoaffinity labels, photocorporation in α-subunit either predominated ([3H]DDF (43)) or was at least at similar efficiency ([3H]dTC (3)) as in the γ- or δ-subunit.

Under equilibrium conditions with ACh sites fully occupied by [3H]Bz2choline, irradiation for 1 h resulted in specific photolabeling of ~3–5% of γ- or δ-subunit. This efficiency of photolabeling was ~10-fold higher than the labeling seen for [3H]dTC (3) or [3H]nicotine (12), but considerably lower than the stoichiometric labeling seen for benzophenone-based peptide photoaffinity probes of myosin light chain kinase (44) or of many peptide hormone receptors (reviewed in Refs. 23 and 25).

Within the nAChR γ-subunit, ~90% of [3H]photophane was inhibitable by carbamylcholine, and [3H]dTC and α-BgTx. Thus the primary site of photolabeling is within the agonist-binding site. For the δ-subunit, whereas ~70% of the [3H]labeling was inhibitable by carbamylcholine, it was not inhibited by α-BgTx. The origin of this difference was identified by analyzing the distribution of [3H] in the δ-subunit fragments produced by EndoLys-C (Fig. 6). The [3H] incorporation seen in the presence of α-BgTx was in an ~10-kDa fragment distinct from the ~20-kDa fragment that contains the Carb-inhibitable [3H] incorporation in δ-subunit. The δ-subunit labeling seen in the presence of α-BgTx is likely to be within the channel domain, as it is inhibitable by tetracaine, and EndoLys-C is known to produce a 10-kDa fragment beginning at the amino terminus of δM2 (38). These data suggest that for nAChRs in the resting state, [3H]Bz2choline can be photo incorporated into the channel domain, most prominently in the δ-subunit. The low levels of [3H] seen in the 10-kDa band for δ-subunit isolated from nAChRs labeled in the presence or absence of Carb indicate that there is little incorporation in this fragment when nAChRs are in the desensitized state (γ-Carb) and that, when [3H]Bz2choline occupies the agonist site, most nAChRs are probably in the desensitized state. Preferential photo incorporation in the ion channel domain in the closed channel state has also been seen for both [125I]2,3,4,5-tetrafluorinated-2-benzyltrimethylammonium and [3H]diazofluorene (46), whose efficiencies of photoincorporation are 10-fold higher in the resting state than in the desensitized state. Although [125I]TID was photo incorporated with similar efficiencies into the M2 segments of all subunits when nAChRs are in the resting state, [3H]diazofluorene was incorporated with high selectivity into δM2. Identification and characterization of these additional sites of [3H]Bz2choline photoprotein incorporation are still in progress.

To identify directly the amino acid(s) of the nAChR γ-subunit that were specifically photolabeled by [3H]Bz2choline, labeled γ-subunit was first digested with EndoLys-C which produced a 26-kDa fragment that was isolated and digested with V8 protease. This double digestion strategy was necessary to eliminate considerable contamination by the Na+/K+-ATPase β-subunit, which originates from contaminating membrane fragments present in the nAChR-rich membrane preparations and which has a mobility similar to the nAChR γ- and δ-subunits in our SDS-PAGE conditions (13). Reversed-phase HPLC of the V8 protease digested isolated a peak which contained a γ-subunit fragment beginning at γ-Val-102, and [3H] release in cycle 8 identified γ-Leu-109 as the primary site of [3H]Bz2choline photo incorporation in γ-subunit (Fig. 5). This conclusion was confirmed by radiosequence analysis of a chymotryptic digest of the labeled γ-Val-102 fragment which revealed [3H] release in cycle 4 (i.e. cleavage after γ-Tyr-105) and lack of [3H] release in cycle 8 (Fig. 4). In addition, it was consistent with the fact that the labeled ~12-kDa γ-subunit fragment produced by V8 protease also contained the site of endoglycosidase H-sensitive glycosylation in γ-subunit, γ-Asn-141 (15). A similar approach was utilized to identify δ-Leu-111 as the primary site of agonist-inhibitable [3H]Bz2choline photo incorporation within the δ-subunit. Digestion with EndoLys-C produced labeled fragments of 20–29 kDa, which were isolated. Since γ-subunit was known to contain the δ-subunit iso-
lation by preparative SDS-PAGE, the material isolated from the EndoLys-C digest could also contain the labeled γ-subunit fragment of similar mobility. The δ-subunit fragment was digested with EndoAsp-N to take advantage of the presence of δAsp-99. Sequence analysis of the peak of $^3$H from the reversed-phase HPLC separation of the EndoAsp-N digest revealed the presence of a fragment beginning at δAsp-99, and the major peak of $^3$H release was in cycle 13, consistent with labeling of δLeu-111, the homologous residue to γ-Leu-109 (Fig. 7). However, this HPLC fraction also contained fragments beginning at δAsp-76, γAsp-76, and γ-Glu-101 as well as a fragment from the Na$^+/K^+$-ATPase β-subunit, and there was $^3$H release in cycle 9 at a lower level than in cycle 13. Sequence analysis of the HPLC fractions on either side of the peak of $^3$H revealed that the mass levels of only the δAsp-99 fragment correlated with the level of $^3$H release in cycle 13 (Fig. 8). In addition, the levels of the fragment beginning at γ-Glu-101 did account for the $^3$H release seen in the ninth cycle of Edman degradation. Despite the fact that the δAsp-99 fragment was not isolated from the contaminating fragments, the data support the conclusion that δLeu-111 is the primary site of specific $[^3]H$Bz-choline photoincorporation in the δ-subunit.

The levels of $^3$H incorporation in γ-Leu-109 (16 cm/μmol) and δLeu-111 (~8 cm/μmol) in subunit fragments isolated from the same labeling experiment indicate that they are labeled at similar efficiencies and that $[^3]H$Bz-choline is incorporated into ~2−4% of the subunits at that position. The labeled 20−29-kDa fragments of the γ- and δ-subunits produced by EndoLys-C began at γ-Glu-47 and δ-Glu-47, and sequence analysis of those fragments allow direct estimation of the levels of $[^3]H$Bz-choline incorporation in ACh-binding site Loop D, defined by γTrp-55 and Trp-57, the primary sites of $[^3]H$TC photoincorporation in the γ- and δ-subunits. No $^3$H release above background was detected while sequencing through γAsn-61 or δAla-61, which indicated that any incorporation in binding site Loop D is at <2% the efficiency of labeling of γ-Leu-109/δLeu-111.

The selective photoincorporation of $[^3]H$Bz-choline in γ-Leu-109 and δLeu-111 is striking. These are the first amino acids identified by affinity labeling within the nAChR agonist-bind-}


ting sites other than Tyr, Trp, or Cys, amino acids with intrinsic side chain reactivity. Benzophenone affinity probes have been shown to react especially well with Met, but also with a wide variety of amino acids including Tyr, Ser, Gly, and Pro (24). To our knowledge this is the first example of a benzophenone derivative reacting with a leucine residue within a native protein, presumably via CH bond insertion in the leucine side chain. Thus, the covalent incorporation of $[^3]H$Bz-choline into γ-Leu-109/δLeu-111 certainly results from the proximity of the bound, excited state ketone with those amino acids. It is notable that there is no photolabeling of the adjacent γTyr-111/ δArg-113 which in Torpedo nAChR have been identified by $[^3]H$TC affinity labeling and mutational analyses (15) as contributors to ACh-binding site Loop E. Also there is no photoincorporation in γMet-116/γTyr-117, positions that have been identified as δTC affinity determinants in the mouse nAChR γ-subunit (17).

In addition to the photo incorporation in γ-Leu-109/δLeu-111, there was also agonist-inhibitable photolabeling within α-subunit, albeit at <10% the efficiency in γ- or δ-subunit. Identification of the amino acids in the α-subunit that are in proximity to photoactivated Bz-choline will further define proximity relations in the ACh-binding site.

The results of photoaffinity labeling and mutational analyses have both provided consistent evidence that the ACh-binding sites are at subunit interfaces, with amino acids from γ- (or δ-) subunit making important contributions to binding sites for agonists and competitive antagonists. In contrast, available data concerning the structure of the nAChR based upon cryo-electron microscopy (1) have been interpreted to suggest that ACh sites are within the α-subunits, with the non-α-subunits possibly contributing to the access way to the binding sites. The highly selective labeling of γ-Leu-109/δLeu-111, in conjunction with the lack of efficient specific labeling of any amino acids in the α-subunit, provides further evidence that amino acids from the non-α-subunits are directly involved in the structure of the ACh-binding sites.
Probing the Structure of the Nicotinic Acetylcholine Receptor with 4-Benzoylbenzoylcholine, a Novel Photoaffinity Competitive Antagonist
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