Identification of Sites of Incorporation in the Nicotinic Acetylcholine Receptor of a Photoactivatable General Anesthetic*

Megan B. Pratt, S. Shaukat Husain, Keith W. Miller, and Jonathan B. Cohen

From the ‡Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115 and the §Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, Massachusetts 02114 and $Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Most general anesthetics including long chain aliphatic alcohols act as noncompetitive antagonists of the nicotinic acetylcholine receptor (nAChR). To locate the sites of interaction of a long chain alcohol with the Torpedo nAChR, we have used the photoactivatable alcohol 3-[3H]azioctanol, which inhibits the nAChR and photo-incorporates into nAChR subunits. At 1 and 275 μM, 3-[3H]azioctanol photoincorporated into nAChR subunits with increased incorporation in the α-subunit in the desensitized state. The incorporation into the α-subunit was mapped to two large proteolytic fragments. One fragment of ~20 kDa (αV8-20), containing the M1, M2, and M3 transmembrane segments, showed enhanced incorporation in the presence of agonist whereas the other of ~10 kDa (αV8-10), containing the M4 transmembrane segment, did not show agonist-induced incorporation of label. Within αV8-20, the primary site of incorporation was αGlu-262 at the C-terminal end of αM2, labeled preferentially in the desensitized state. The incorporation at αGlu-262 approached saturation between 1 μM, with ~6% labeled, and 275 μM, with ~30% labeled. Low level incorporation was seen in residues at the agonist binding site and the protein-lipid interface at ~1% of the levels in αGlu-262. Therefore, the primary binding site of 3-azioctanol is within the ion channel with additional lower affinity interactions within the agonist binding site and at the protein-lipid interface.

The molecular sites of actions of general anesthetics are currently unknown. However, in recent years, evidence for the direct interaction between general anesthetics and specific proteins has accumulated (1). In particular, at clinically effective concentrations most volatile general anesthetics perturb ligand-gated ion channels. For example, they enhance agonist action on inhibitory receptors including most GABA A and glycine receptors. They also noncompetitively inhibit excitatory receptors such as nicotinic acetylcholine receptors (nAChR) and serotonin 5HT3 receptor. These ion channels belong to a superfamily that is composed of five homologous subunits arranged as a pseudopentamer with each subunit composed of a large N-terminal extracellular segment and four transmembrane segments, M1-M4. The two agonist binding sites are located in the N-terminal extracellular segment at subunit interfaces. Based on photoaffinity labeling and mutational analyses of the nAChR, the M2 segments of each subunit are α-helices arranged around the central axis and contribute to the lumen of the nAChR ion channel. Additionally, several nAChR noncompetitive antagonists have been shown to bind within the lumen of the ion channel (2–4).

Although evidence exists for direct binding of anesthetics on ligand-gated ion channels, the binding sites have not been clearly located. In the GABA A and glycine receptors, mutational analyses have identified two residues, one each in the transmembrane segments M2 and M3, which modulate the action of a variety of general anesthetics, including long chain alcohols (5–7). These residues have been hypothesized to contribute to an anesthetic site (8), but other groups (1, 9, 10) have suggested that these and neighboring residues act allosterically on anesthetic sites elsewhere in the receptor.

In muscle nAChR, single channel studies with long chain alcohols and other anesthetics, such as isoflurane, suggest that these anesthetics bind within the ion channel. The open channel state in the presence of these drugs is characterized by flickering, similar to that seen with QX-222, an aromatic amine channel blocker (11). However, butanol and hexanol do not compete with QX-222 for a common binding site (12). In flux studies with nAChR-rich membranes from Torpedo electric organ, octanol and heptanol do compete with each other but not with procaine (13). Site-directed mutagenesis of muscle nAChR has shown that the nature of the residue at the M2 position 10' (based on numbering from the conserved positive charge at the N terminus of M2), facing the lumen of the ion channel, can increase the potency of long chain alcohols and isoflurane as channel blockers (14).

Because of the difficulty of interpreting mutational studies in this highly allosteric family of receptors, a complementary approach, photoaffinity labeling, is attractive. The photoaffinity general anesthetic 3-azioctanol was developed (15) as a probe of the binding sites of long chain alcohols. This compound acts as an anesthetic in tadpoles, producing a loss of righting reflex with an EC50 of ~160 μM, an EC50 that is about one-third of the potency of octanol. For the GABA A receptor, 3-azioctanol potentiates the response to submaximal concentrations of GABA, and it inhibits agonist activation of muscle-type nAChR. Additionally, 1 μM 3-[3H]azioctanol was shown to photoincorporate into subunits of the Torpedo nAChR with preferential incorporation into the α-subunit in the presence of agonist. This agonist-
dependent incorporation was localized to a 20-kDa fragment containing the first three transmembrane fragments.

In the present work, 3-[3H]Azioctanol has been used as a photoaffinity probe to localize further the sites of interaction of a long chain alcohol with Torpedo nAChR-rich membranes. The primary site of incorporation in the presence of agonist was mapped to αGlu-262, at the C terminus of αM2. Additional sites of incorporation were found although at lower efficiency than the incorporation at αGlu-262. The levels of incorporation at 1 and 275 μM indicated that the incorporation at αGlu-262 approached saturation across this concentration range, whereas the incorporation at the other sites increased linearly. Therefore, αGlu-262 is within the high affinity binding site of long chain alcohol anesthetics.

EXPERIMENTAL PROCEDURES

Materials—nAChR-enriched membranes were isolated from Torpedo californica electric organ (16). The final membrane suspensions were stored in 38% sucrose at −80 °C under argon. The membranes used here contained 0.5–2.0 nmol of acetylcholine binding sites per milligram of protein. 3-[3H]Azioctanol and nonradioactive 3-azioctanol were synthesized as described previously (15). The specific activity of the 3-[3H]Azioctanol (11 Ci/mmol) was stored at 90 °C in CH2Cl2, which was removed via evaporation immediately prior to the addition of membranes or isotopic dilution. For studies of incorporation at concentrations higher than 1 μM 3-[3H]Azioctanol, this stock was isotonically diluted with a stock of nonradioactive 3-azioctanol, 11 mM (concentration determined by the absorbance of 3-azioctanol at 350 nm (15)) in Torpedo physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, 5 mM sodium phosphate, pH 7.0), to a final specific activity of ~0.04 μCi/mm. This dilution was prepared immediately before addition to membranes. Staphylococcus aureus glutamylendopeptidase (V8 protease) was from ICN Biomed Inc, endoproteinase Lys C (EndoLysC) from Roche Molecular Biochemicals, and Staphylococcus aureus BgTx was purchased from Calbiochem. Nicotine, d-tubocurarine, and carbamylcholine were from Sigma. Pancuronium was from Organon; α-bungarotoxin (αBgtX) was purchased from Biotoxins, Inc.

Photoaffinity Labeling of nAChR-enriched Membranes with 3-[3H]Azioctanol—For analytical labeling experiments, freshly thawed Torpedo nAChR-rich membranes were diluted with TPS and pelleted (15000 ×g) for 30 min, resuspended in sample buffer and submitted to SDS-PAGE.

Gel Electrophoresis—SDS-PAGE was performed as described by Laemmli (20), with modifications (18). For analytical gels, the polyacrylamide gels were resolved on a 1-mm thick 8% acrylamide gel visualized with Coomasie Blue, and destained to allow visualization of the subunits. The α-subunits were then excised and placed directly into individual vials of a 1.5-mm mapping gel, composed of a 5-cm 4.5% acrylamide stacking gel, and a 15-cm 15% acrylamide separating gel. Into each well was added 1:1 gram subunit:gram S. aureus V8 protease in overlay buffer (5% sucrose, 125 mM Tris-HCl, 0.1% SDS, pH 6.8). The gel was run at 150 V for 2 h, and then the current was turned off for 1 h. The gel was then run at constant current overnight until the dye front reached the end of the gel. The gel was stained, and 3H was quantified by liquid scintillation counting. For preparative labeling, the polyacrylamide gels were resolved on a 1.5-mm thick 8% acrylamide gel. The α-subunit was identified in 8% gels by 1-AP fluorescence and then excised and loaded directly onto the 1.5-mm mapping gel. The α-subunit proteolytic fragments of ~20 kDa (αV8-20) and ~10 kDa (αV8-10) were identified by fluorescence and excised. The region between αV8-20 and αV8-10 was excised to isolate αV8-18. The excised proteolytic fragments were isolated by passive elution into 0.1 n NH4HCO3, 0.1% SDS (19). The eluate was filtered (Whatman No. 1) and concentrated using Milipore M 5,000 concentrators. To remove excess SDS, acetone was added to the concentrate. Following incubation at −20 °C overnight, the peptides were pelleted.

Proteolytic Digestion—For EndoLysC digestion, aceton-precipitated subunits or subunit fragments were resuspended in 15 mM Tris, pH 8.1, 0.1% SDS. EndoLysC (1.5 million in resuspension buffer) was added to a final volume of 100 μl. The digestion was allowed to proceed for 7–9 days before separation of fragments by HPLC. For S. aureus V8 protease digestion in solution, aceton-precipitated peptides were resuspended in 15 mM Tris, pH 8.1, 0.1% SDS. V8 protease in resuspension buffer was added to a final concentration of 1:1 (w/w) and incubated at room temperature for 3–4 days before separation of fragments by HPLC. For trypsin digestion, aceton-precipitated peptides were resuspended in a small volume (40 μl) of 100 mM NH4HCO3, 0.1% SDS, pH 7.8. Gel-cast final concentration was added to a final volume of 0.02% SDS, 0.5% Genapol C-100, and 1:1 (w/w) trypsin. The digestion was allowed to proceed 3–4 days at room temperature prior to separation of the fragments by HPLC.

HPLC Purification—Proteolytic fragments from enzymatic digestion of αV8-20 and αV8-10 fragments labeled with 3-[3H]Azioctanol were further purified by reverse-phase HPLC (22), using a Brownlee C4-Acquapore column (100 × 2.1 mm, 7-μm particle size). Solvent A was 0.08% trifluoroacetic acid in water, and solvent B was 0.05% trifluoroacetic acid in 60% acetonitrile, 40% 2-propanol. A nonlinear gradient (Waters Model 680 gradient controller, curve No. 7) from 25 to 100% solvent B in 80 min was used. The rate of flow was 0.2 ml/min, and 0.5-ml fractions were collected. The elution of peptides was monitored by absorbance at 215 nm, and the fluorescence from 1-AP was detected by fluorescence emission (357-nm excitation, 432-nm emission). Additionally, aliquots from the fractions were taken to determine the distribution of 3H by liquid scintillation counting.

Intact αV8-18 and αV8-18 proteolytic fragments were also purified by HPLC (23), using a Brownlee C4-Acquapore column. Solvent A was 0.09% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. A linear gradient with several steps was used: 0 min, 10% solvent B; 10 min, 10% solvent B; 25 min, 25% solvent B; 45 min, 40% solvent B; 65 min, 60% solvent B; 75 min, 100% solvent B. The rate of flow was 0.25 ml/min, and 0.5-ml fractions were collected. Measurements were determined as for the purification of the fragments of enzymatic digestion.

Screening: Automated N-terminal sequence analysis was performed on an Applied Biosystems Model 477A protein sequencer with an in-line 120A PTH analyzer. HPLC samples (450-μl fractions) were directly loaded onto chemically modified glass fiber disks (Beckman) in 20-μl aliquots, allowing the solvent to evaporate at 40 °C between loads. Sequencing was performed using gas-phase trifluoroacetic acid to minimize possible hydrolysis. After conversion of the released amino acids to PTH-amino acids, the sequencing was divided (15,000 × g) for 30 min, resuspended in sample buffer, and submitted to SDS-PAGE.
RESULTS

Photoincorporation of $[^3\text{H}]$Azioctanol into nAChR-rich Membranes—Initial experiments were designed to characterize the general pattern of photoincorporation of $[^3\text{H}]$Azioctanol and to test the sensitivity of photoincorporation to various ligands. For these initial experiments, two concentrations of $[^3\text{H}]$Azioctanol were used, $1 \text{ mM}$ ($11 \text{ Ci/mmol}$) and $275 \text{ mM}$ ($0.04 \text{ Ci/mmol}$). For inhibition of Torpedo nAChR using flux assays, the IC$_{50}$ of $[^3\text{H}]$Azioctanol is approximately $100 \text{ mM}$. Therefore, $1 \text{ mM}$ $[^3\text{H}]$Azioctanol was well below the concentration necessary for inhibition of $50\%$ of the nAChR, whereas $275 \text{ mM}$ $[^3\text{H}]$Azioctanol was a concentration sufficient to produce greater than $50\%$ inhibition. Isotopic dilution of $[^3\text{H}]$Azioctanol resulted in the presence of similar levels of $[^3\text{H}]$ in the samples containing $1 \text{ mM}$ and $275 \text{ mM}$ $[^3\text{H}]$Azioctanol. Membranes (2 mg/ml protein) were equilibrated with $[^3\text{H}]$Azioctanol in the presence and absence of $2 \text{ mM}$ carbamylcholine. After irradiation for 10 min at 365 nm, the pattern of incorporation was assessed by SDS-PAGE followed by fluorography or scintillation counting of gel slices.

As seen in the fluorograph of the $8\%$ polyacrylamide gel (Fig. 1A) at both $3\text{ mM}$ $[^3\text{H}]$Azioctanol concentrations in the absence of carbamylcholine, the principal polypeptide labeled was a 34-kDa polypeptide identified as a mitochondrial chloride channel (VDAC) (24). The $[^3\text{H}]$ incorporation in VDAC, although not affected by the presence of carbamylcholine, was reduced by $50\%$ at the higher concentration of $[^3\text{H}]$Azioctanol. This decrease suggests specific incorporation of $[^3\text{H}]$Azioctanol in VDAC, which is inhibited by excess non-radioactive 3-azioctanol. The $[^3\text{H}]$ incorporation in other non-receptor polypeptides (rapsyn, $43 \text{ kDa}$) and the $\alpha$-subunit of (Na$^{+}$/K$^{+}$)-ATPase ($\alpha_{\text{NK}}$), was not altered by the presence of carbamylcholine and appeared similar at 1 and $275 \text{ mM}$ $[^3\text{H}]$Azioctanol.

Of the nAChR subunits, $\alpha$ was labeled most strongly. Incorporation of $[^3\text{H}]$Azioctanol into the $\alpha$-subunit was dependent on the conformational state of the nAChR, as the presence of agonist resulted in enhanced incorporation into the $\alpha$-subunit but not in non-nAChR polypeptides. Based on scintillation counting of excised gel slices, the increase in incorporation was on average $5\text{-fold}$ at $1 \text{ mM}$ $[^3\text{H}]$Azioctanol (Fig. 1B) and $3\text{-fold}$ at $275 \text{ mM}$. The presence of agonist also increased the incorporation in the $\beta$-subunit, although only by $1.4\text{-fold}$ at $1 \text{ mM}$. Because the $[^3\text{H}]$Azioctanol at $275 \text{ mM}$ had an $275\text{-fold}$ lower specific activity, the observed similarity in the $[^3\text{H}]$ incorporation in the $\alpha$-subunit at the two conditions indicated that $[^3\text{H}]$Azioctanol is a selective inhibitor of certain nAChR subunits.

$[^3\text{H}]$Azioctanol Incorporation in the nAChR

$[^3\text{H}]$Azioctanol Incorporation in nAChR-rich membranes. nAChR-rich membranes (100 $\mu\text{g}$ at 2 mg/ml) were equilibrated with 1 $\mu\text{M}$ $[^3\text{H}]$Azioctanol in TPS in the absence of other drugs or in the presence of 2 $\text{ mM}$ carbamylcholine (carb), 200 $\mu\text{M}$ phenyltrimethylammonium (PTA), 100 $\mu\text{M}$ nicotine, 100 $\mu\text{M}$ pancuronium, 1 $\text{ mM}$ gallamine, or 30 $\mu\text{M}$ d-tubocurarine and irradiated for 10 min at 365 nm. After photolysis, samples were subjected to SDS-PAGE and visualized by Coomassie Blue. Bands corresponding to the $\alpha$-subunit as well as the 37-kDa (calelectrin) and 43-kDa (rapsyn) bands were excised. $[^3\text{H}]$ incorporation was quantified by scintillation counting. Bars indicate the mean $\pm$ S.D. of duplicate samples.

$[^3\text{H}]$Azioctanol Incorporation in nAChR-rich membranes. nAChR-rich membranes (100 $\mu\text{g}$ at 2 mg/ml) were equilibrated with 1 $\mu\text{M}$ $[^3\text{H}]$Azioctanol in TPS in the absence of other drugs or in the presence of 2 $\text{ mM}$ carbamylcholine (carb), 200 $\mu\text{M}$ phenyltrimethylammonium (PTA), 100 $\mu\text{M}$ nicotine, 100 $\mu\text{M}$ pancuronium, 1 $\text{ mM}$ gallamine, or 30 $\mu\text{M}$ d-tubocurarine and irradiated for 10 min at 365 nm. After photolysis, samples were subjected to SDS-PAGE and visualized by Coomassie Blue. Bands corresponding to the $\alpha$-subunit as well as the 37-kDa (calelectrin) and 43-kDa (rapsyn) bands were excised. $[^3\text{H}]$ incorporation was quantified by scintillation counting. Bars indicate the mean $\pm$ S.D. of duplicate samples.
incorporation in the nAChR

The effects of several noncompetitive antagonists on the incorporation of 3-[3H]aziotanol at 1 µM were also tested (Fig. 3A). For membranes equilibrated with carbamylcholine, the 3H incorporation in the nAChR α-subunit was insensitive to the presence of 1 mM octanol. At 100 µM, meproadiifen, an aromatic amine noncompetitive antagonist, reduced the incorporation by ~50%. Two other aromatic amine noncompetitive antagonists, phenicyclidine and QX-222, failed to inhibit the incorporation of 3-[3H]aziotanol in the α-subunit (data not shown). The presence of these noncompetitive antagonists did not affect the incorporation in the other nAChR subunits (data not shown) nor the incorporation in non-nAChR polypeptides including rapsyn (43 kDa), VDAC (34 kDa), and calelectrin (37 kDa) (data not shown).

The incorporation of 3-[3H]aziotanol in nAChR α-subunit was measured over a range of 3-[3H]aziotanol concentrations, using a constant specific activity of 3-[3H]aziotanol (Fig. 4). The incorporation in the (Na+/K+)-ATPase α-subunit (open symbols) increased linearly across the range of concentrations tested and was not affected by the presence of cholinergic drugs. For membranes equilibrated with carbamylcholine, the incorporation in the α-subunit increased up to ~1 mM and then appeared to saturate. At all concentrations, the incorporation in the presence of aBgTx was less than that seen in the absence of added drugs although at ~2 mM the incorporation in the presence of aBgTx was similar to that seen in the presence of carbamylcholine. In the absence of drug, the incorporation appeared to increase nearly linearly up to 1 mM, and then the incorporation increased sharply, surpassing the incorporation in the presence of carbamylcholine at 2 mM. However, the incorporation of 3-[3H]aziotanol in nAChR α-subunit membranes (100 µg at 2 nM) were equilibrated with varying concentrations of 3-[3H]aziotanol (~0.04 Ci/mmol) in the absence of other drugs (●, ○), or in the presence of 2 mM carbamylcholine (●, ○), or in the presence of 10 μM aBgTx (□, □). After irradiation at 365 nm for 10 min, samples were subjected to SDS-PAGE and visualized with Coomassie Blue. Bands corresponding to nAChR α-subunit (solid symbols), as well as the 90-kDa band containing the α-subunit of (Na+/K+)-ATPase (open symbols) were excised, and 3H incorporation was quantified by scintillation counting.

Error bars are from the average of four separate experiments normalized to a common specific activity by assuming common level of incorporation in α-subunit in the presence of carbamylcholine at 2.2 mM 3-[3H]aziotanol.
higher incorporation in the absence of carbamylcholine showed high variability. The total 3H incorporation at 2 mM 3-[3H]azioctanol was $\sim 0.25$ mol of 3-[3H]azioctanol/mol of a, based on the reported counting efficiency (25%) of the toluene-based gel mixture used (21).

**Mapping of 3-[3H]Azioctanol Photoincorporation into a Subunit Proteolytic Fragments**—The distribution of 3-[3H]azioctanol incorporation within the a-subunit was examined by digestion of the labeled subunit with *S. aureus* V8 protease under conditions that are known to generate four large non-overlapping fragments resolvable by SDS-PAGE (Fig. 5). The largest fragment, a 20-kDa peptide (aV8-20), begins at aSer-173 and contains the first three membrane spanning regions, aM1, aM2, and aM3 (18). The 10-kDa peptide (aV8-10) contains the fourth membrane spanning region, aM4, and begins at aAsn-339. The 18-kDa (aV8-18) and 4-kDa (aV8-4) peptides begin at aVal-46 and aSer-1, respectively. Membranes labeled with 3-[3H]azioctanol were subjected to SDS-PAGE, and the a-subunit was excised. This gel piece was loaded onto a mapping gel along with V8 protease. The a-subunit was cleaved in the gel with the protease, and the fragments were separated on the gel. Again, like the studies of the incorporation in the intact subunits, the incorporation was measured at both 1 mM and 275 $\mu$M aM 3-[3H]azioctanol. Similar levels of 3H were used at these two concentrations, with a specific activity of 11 Ci/mmol at 1 mM and 0.04 Ci/mmol at 275 $\mu$m, an ~275-fold reduction in specific activity in the samples labeled in the presence of 275 $\mu$m 3-[3H]azioctanol compared with those labeled under the 1 mM condition. Based on liquid scintillation counting of these a-subunit proteolytic fragments, the main sites of photoincorporation in the absence of agonist were within the aV8-20 and aV8-10 fragments (Fig. 5). The 3H incorporation in each fragment was similar at both concentrations of 3-[3H]azioctanol. In the absence of agonist, the incorporation in aV8-10 was ~60% that of aV8-20. The addition of agonist increased the labeling of the aV8-20 fragment, 9-fold at 1 $\mu$m and 5-fold at 275 $\mu$m, whereas the $^{3}$H incorporated in aV8-10 was unchanged by the presence of carbamylcholine. In the presence of carbamylcholine, the incorporation in aV8-20 accounted for ~90% of the incorporation at both 3-[3H]azioctanol concentrations, whereas aV8-10 contained ~6% of the total 3-[3H]azioctanol incorporation within the a-subunit fragments. The similar levels of 3H incorporation in the fragments between the two concentrations, with 3-[3H]azioctanol at an ~275-fold lower specific activity at 275 $\mu$m, indicated that ~275-fold more molecules of 3-azioctanol were incorporated at 275 $\mu$m 3-[3H]azioctanol. In all conditions, the incorporation in aV8-18 and aV8-4 appeared similar and was lower than the incorporation in aV8-10.

The carbamylcholine-dependent labeling of nAChR with 3-[3H]azioctanol was in the aV8-20 fragment containing aM1, aM2, and aM3. To further localize the site of labeling, 10 mg of membranes were labeled with 1 $\mu$m or 275 $\mu$m 3-[3H]azioctanol in the presence or absence of carbamylcholine, methpordifen, or aBgTx. Additionally, these membranes were labeled with 1-azidopyrene, a fluorescent compound that photoincorporates in transmembrane segments, to aid in the localization of transmembrane segments. Following the digestion of a-subunit with V8 protease, the aV8-20, aV8-18, and aV8-10 fragments were excised and eluted. To quantify the 3H incorporation, the eluted aV8-20 and aV8-10 fragments were subjected to sequence analysis. Based on sequence analysis of the fragments, at 1 $\mu$m 3-[3H]azioctanol, in the absence of carbamylcholine ~0.008 moles of 3-[3H]azioctanol incorporated into a mole of aV8-20 and ~0.004 moles into aV8-10. In the presence of carbamylcholine, 0.06 moles incorporated into aV8-20 and 0.004 moles into aV8-10. At 275 $\mu$m 3-[3H]azioctanol, the incorporation increased with ~0.55 moles incorporated per mole of aV8-20 and 0.24 moles per mole aV8-10 in the absence of carbamylcholine. In the presence of carbamylcholine at 275 $\mu$m 3-[3H]azioctanol, ~1.3 moles 3-[3H]azioctanol incorporated into aV8-20 and ~0.40 moles into aV8-10.

3-[3H]Azioctanol Photoincorporation within the aM2 Segment—To determine whether there was incorporation in the aM2 segment, the eluted aV8-20 fragment, labeled with 3-[3H]azioctanol, was digested with EndoLysC. Digestion with EndoLysC is known to create an ~10-kDa fragment starting at aMet-243, the N terminus of the aM2 segment, that can be purified by reverse-phase HPLC (16). When the EndoLysC-digested aV8-20, which had been labeled with 275 $\mu$m 3-[3H]azioctanol in the presence of carbamylcholine, was fractionated by reverse-phase HPLC, ~80% of the 3H eluted at a peak centered at fraction 33 (~88% organic) (Fig. 6A). For the samples labeled in the presence of aBgTx or the absence of other drugs, the 3H in fraction 33 was only ~20% that seen for the sample labeled in the presence of carbamylcholine.

For each labeling condition, fraction 33, which contained the peak of 3H from the sample labeled in the presence of carbamylcholine, was subjected to Edman degradation (Fig. 6B) showing that the only sequence present was that beginning at aMet-243 (~carb: I0 = 23 pmol; +carb: I0 = 30 pmol). No other sequences were present at more than 10% of the mass of the 3-[3H]azioctanol in the presence of carbamylcholine (data not shown). The band was excised, and moreover, the 22-kDa region was also excised in other conditions. This band was assumed to be aV8-20 that was highly labeled with 3-[3H]azioctanol, resulting in reduced mobility. Therefore, the 3H present in this region was attributed to labeling in aV8-20 and was added to that of the aV8-20 band.
samples labeled with 1 μM 3-[3H]azioctanol revealed the presence of a single sequence beginning at αMet-243 with release of 3H in cycle 20 (data not shown). In the presence of carbamylcholine, the release in cycle 20 was equivalent to 0.025 mol per mol of αGlu-262 and that labeling was reduced by ~40% for the sample labeled in the presence of meprafidin and carbamylcholine (0.014 mol of 3-[3H]azioctanol per mol of αGlu-262). In the absence of carbamylcholine, the incorporation of 3-[3H]azioctanol at αGlu-262 (0.0012 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262) was ~5% that seen in the presence of carbamylcholine.

3-[3H]Azioctanol Photoincorporation within the αM1 and αM3 Segments—When EndoLysC cleaves αV8-20 at αLys-242, before αM2, it can also cleave the fragment between the N terminus of αV8-20 and αLys-242. There are two lysines in this fragment, αLys-179 and αLys-185. Cleavage at either of these two sites will generate a fragment that contains a portion of the Ach binding site (α(190–200)) as well as the αM1 segment. This fragment can be resolved from the fragment containing αM2 by HPLC purification. The fragment containing αM1 elutes in a peak of absorbance and fluorescence near fraction 29 (69% organic) (Fig. 6A). Sequence analysis of this fraction from the sample labeled at 275 μM 3-[3H]azioctanol revealed that the sequence of the peak of 3H from the peptide beginning at αMet-243. For the sample labeled in the presence of carbamylcholine, there was a peak of 3H release in cycle 20, corresponding to incorporation at αGlu-262, and that release was reduced by ~60% in the sample labeled in the absence of carbamylcholine or in the presence of αBgTx (data not shown). Based upon the 3H release in cycle 20, in the presence of carbamylcholine, there was ~0.33 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262. In the absence of other drugs or in the presence of αBgTx, there was ~0.14 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262.

The HPLC profile of the EndoLysC-digest of αV8-20 labeled in the presence of 1 μM 3-[3H]azioctanol was similar to that at 275 μM (data not shown). For the sample labeled in the presence of carbamylcholine, ~70% of the 3H eluted as a single peak at ~90% organic. As with fraction 33 from the sample labeled in the presence of 275 μM 3-[3H]azioctanol, sequence analysis of the fraction containing the peak of 3H from the samples labeled with 1 μM 3-[3H]azioctanol revealed the presence of a single sequence beginning at αMet-243 with release of 3H in cycle 20 (data not shown). In the presence of carbamylcholine, the release in cycle 20 was equivalent to 0.025 mol per mol of αGlu-262 and that labeling was reduced by ~40% for the sample labeled in the presence of meprafidin and carbamylcholine (0.014 mol of 3-[3H]azioctanol per mol of αGlu-262). In the absence of carbamylcholine, the incorporation of 3-[3H]azioctanol at αGlu-262 (0.0012 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262) was ~5% that seen in the presence of carbamylcholine.

3-[3H]Azioctanol Photoincorporation within the αM1 and αM3 Segments—When EndoLysC cleaves αV8-20 at αLys-242, before αM2, it can also cleave the fragment between the N terminus of αV8-20 and αLys-242. There are two lysines in this fragment, αLys-179 and αLys-185. Cleavage at either of these two sites will generate a fragment that contains a portion of the Ach binding site (α(190–200)) as well as the αM1 segment. This fragment can be resolved from the fragment containing αM2 by HPLC purification. The fragment containing αM1 elutes in a peak of absorbance and fluorescence near fraction 29 (69% organic) (Fig. 6A). Sequence analysis of this fraction from the sample labeled at 275 μM 3-[3H]azioctanol revealed that the sequence of the peak of 3H from the peptide beginning at αMet-243. For the sample labeled in the presence of carbamylcholine, there was a peak of 3H release in cycle 20, corresponding to incorporation at αGlu-262, and that release was reduced by ~60% in the sample labeled in the absence of carbamylcholine or in the presence of αBgTx (data not shown). Based upon the 3H release in cycle 20, in the presence of carbamylcholine, there was ~0.33 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262. In the absence of other drugs or in the presence of αBgTx, there was ~0.14 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262.

The HPLC profile of the EndoLysC-digest of αV8-20 labeled in the presence of 1 μM 3-[3H]azioctanol was similar to that at 275 μM (data not shown). For the sample labeled in the presence of carbamylcholine, ~70% of the 3H eluted as a single peak at ~90% organic. As with fraction 33 from the sample labeled in the presence of 275 μM 3-[3H]azioctanol, sequence analysis of the fraction containing the peak of 3H from the peptides labeled with 1 μM 3-[3H]azioctanol revealed the presence of a single sequence beginning at αMet-243 with release of 3H in cycle 20 (data not shown). In the presence of carbamylcholine, the release in cycle 20 was equivalent to 0.025 mol per mol of αGlu-262 and that labeling was reduced by ~40% for the sample labeled in the presence of meprafidin and carbamylcholine (0.014 mol of 3-[3H]azioctanol per mol of αGlu-262). In the absence of carbamylcholine, the incorporation of 3-[3H]azioctanol at αGlu-262 (0.0012 mol of 3-[3H]azioctanol incorporated per mol of αGlu-262) was ~5% that seen in the presence of carbamylcholine.
TABLE I
Incorporation of 3-[^3H]Azioctanol into fragments and residues of the α-subunit

| Concentration (μM) | V8 fragment | V8-20 – Carba + Carba | V8-10 – Carba + Carba | V8-18 – Carba + Carba |
|-------------------|-------------|-----------------------|-----------------------|-----------------------|
|                   |             | 0.55 ± 0.03           | 0.24 ± 0.01           | 0.06 ± 0.00           |
| 275               |             | 0.008/0.06            | 0.004/0.004           |                       |
| 1                 |             |                       |                       |                       |

| Subfragment       | V8-18       | V8-20 – Carba + Carba | V8-18 – Carba + Carba |
|-------------------|-------------|-----------------------|-----------------------|
| αM243–αK340       |             | 0.7 ± 0.1             | 0.19                  |
| αH186–αK242       |             | 0.14 ± 0.01           | 0.004/0.003           |
| αY401–αG437       |             | 0.27 ± 0.19           | 0.0017                |
| αK77–αK7          |             | 1.0 ± 0.1             | 0.10                  |

| Residue           | V8-18       | V8-18 – Carba + Carba | V8-18 – Carba + Carba |
|-------------------|-------------|-----------------------|-----------------------|
| αGlu-262          |             | 0.16 ± 0.02           | 0.003                 |
| αTyr-190          |             | 0.35 ± 0.07           | 0.003                 |
| αHis-408          |             | 0.06 ± 0.04           | 0.0003                |
| αCys-412          |             | 0.00012               | 0.00003               |
| αGlu-51/αArg-57   |             | 0.00004               | 0.00004               |

3-[^3H]Azioctanol Incorporation in the nAChR

The ratio of moles 3-[^3H]Azioctanol incorporated per mol of the fragments or residues labeled was calculated from the[^3H] incorporation and the known specific activity of 3-[^3H]Azioctanol. The[^3H] incorporation in each fragment and residue was calculated as described under “Experimental Procedures.” For the incorporation into fragments, the mass levels were based on the observed mass sequenced and the total radioactivity loaded.

For the incorporation into specific residues, the mass was based on the initial and repetitive yields, and the radioactivity was based on the observed release. Averages shown are from duplicate preparative-labeling experiments.

These results indicate that the fragment beginning at αLeu-263, cleavage must occur at αGlu-262, which is labeled by 3-[^3H]Azioctanol. Therefore, it was expected that only fragments not labeled at αGlu-262 would be digested to generate the fragment beginning at αLeu-263. In the sample labeled in the presence of carbamylcholine, ~85% of the[^3H] eluted at fraction 33 (Fig. 7, inset). This fraction, based on sequence analysis, contained a fragment beginning at the N terminus of αV8-20, and based on the high levels of[^3H] in the fraction, this fragment should have contained the αM2 segment. This fragment beginning at αLeu-263 was expected to elute at ~55% organic (19). A small peak of[^3H] was present in fraction 23 (~50% organic), and one-half of this fraction from each condition was subjected to Edman degradation (data not shown).

Two sequences were present, the first fragment beginning at αLeu-263 (~carb: I₀ = 4.8 pmol; +αGABA: I₀ = 3.4 pmol; +carb: I₀ = 1.5 pmol) and a fragment beginning at αThr-52 (~carb: I₀ = 72 pmol; +αGABA: I₀ = 24 pmol; +carb: I₀ = 37 pmol), an N terminus of the αV8-18 fragment arising from contamination of the αV8-20 sample with αV8-18. Based upon the mass levels present, if the[^3H] in this fraction were attributable only to the sequence beginning at αLeu-263, then, in the presence of carbamylcholine, ~0.08 mol of 3-[^3H]Azioctanol incorporated per mol of fragment, ~6% of the incorporation in the fragment beginning at αMet-243. Therefore, the αM3 segment was labeled at less than 6% the levels of incorporation in the αM2 segment.

3-[^3H]Azioctanol Photoincorporation within the Agonist Binding Site—For the αV8-20 fragment isolated from nAChRs labeled with either 1 or 275 μM 3-[^3H]Azioctanol in the absence of carbamylcholine, the HPLC chromatogram of the EndoLysC digest of αV8-20 (Fig. 6A) contained a peak of[^3H] at fraction 29 (68% organic) in addition to the peak at fraction 33. When the material in fraction 29 was sequenced, the primary sequence began at αHis-186 (~carb: I₀ = 35 pmol; +αBGTx: I₀ = 55 pmol; +carb: I₀ = 36 pmol) (Fig. 6C). This fragment contains residues contributing to the ACh site (α(190–200)) as well as the αM1 segment, because there is no lysine between αHis-186 and αLys-242 prior to αM2. At 275 μM 3-[^3H]Azioctanol,[^3H] release was evident in cycles 5 and 13 for the fragment labeled in the absence of carbamylcholine but not for the samples labeled in the presence of carbamylcholine or αBGTx. Release of[^3H] in these cycles correspond to αTyr-190 and αTyr-198, residues known to contribute to the agonist binding site (2). The amount of incorporation in these residues was ~10% that in αGlu-262 in the absence of carbamamylcholine, with 3-[^3H]Azioctanol only incorporating at ~0.013 mol per mol of αTyr-190 and ~0.017 mol per mol of αTyr-198. A similar pattern of release, although with lower levels of[^3H] incorporation, was seen in the sample labeled with 1 μM 3-[^3H]Azioctanol in the absence of carbamylcholine (see Table I under “Discussion”). In the presence of carbamylcholine, whereas there was no release in cycle 5 or 13, there was release evident in cycle 3, which, if originating from the fragment beginning at αHis-186, indicated ~0.003 mol incorporated per mol of αVal-189.
accounts for most of the incorporation in αV8-18 because there was ~6% incorporation in αV8-18. Because the radioactive release in the cycle containing aTyr-93, a residue contributing to the agonist binding site, was at background levels, this position was labeled by no more than 0.00003 mol of 3-[3H]azioctanol per mol of residue (data not shown).

3-[3H]Azioctanol Photoincorporation within αV8-10—At 275 μM 3-[3H]azioctanol, αV8-10 fragments labeled in the presence or absence of other cholinergic drugs showed similar levels of 3H incorporation. Additionally, the levels of incorporation in αV8-10 labeled with 1 μM 3-[3H]azioctanol were similar in the presence and absence of other drugs. HPLC purification of intact αV8-10 labeled with 275 μM 3-[3H]azioctanol revealed that ~60% of the incorporated 3H eluted in the flow-through (Fig. 9A, inset), whereas only ~20% eluted in a broad peak between fractions 32–35 where intact αV8-10 was known to elute (22). Sequence analysis confirmed the presence of αV8-10 in these fractions. The presence of 3H in the flow-through indicated that most of the 3-[3H]azioctanol incorporated into αV8-10 was not stably incorporated under the conditions of HPLC.

To localize the 3H incorporation within αV8-10 that was stably incorporated, 3-[3H]azioctanol labeled αV8-10 that had been eluted from gel was digested with trypsin, under conditions known to cleave the fragment at αLys-400 (22). HPLC purification of the digest showed the major peak of 3H in the flow-through, as well as a peak of 3H at fractions 30–33 (Fig. 9A). Based upon the 3H elution profile seen when intact αV8-10 was purified by HPLC, the 3H in the flow-through, ~60% of the eluted 3H, was assumed to result from 3-[3H]azioctanol incorporation, which was unstable to HPLC conditions. The 3H present between fractions 30–33 accounted for ~15% of the total eluted 3H. Sequence analysis of the pooled fractions 30–33 showed the presence of a primary sequence beginning at aTyr-401 and a secondary sequence beginning at aSer-388 (Fig. 9B). In all concentrations tested, 3H release was observed in cycles 8 and 12, indicating incorporation in αHis-408 and αCys-412. Additionally, low level release was seen reproducibly in cycle 3, corresponding to αAla-403. 3-[3H]Azioctanol incorporated into αHis-408 and αCys-412 at ~0.0025 mol per mol of residue at 275 μM and at ~1% that level at 1 μM. However, at both concentrations most of the 3H eluted with the flow-through of the HPLC, and this 3H could have been incorporated into these residues but labile under HPLC conditions. Alternatively, there could have
been another residue or residues in αV8-10 that were labeled more prominently, but the incorporation at this site(s) was highly labile under the conditions of HPLC.

DISCUSSION

3-[3H]Azioctanol photoincorporates with high efficiency into the α-subunit of the nAChR, with the primary site of incorporation being αGlu-262, within the ion channel at the extracellular end of αM2. Additional incorporation was present in αHis-408 and αCys-412, residues previously identified as being situated at the lipid protein interface (19, 24), and in αTyr-190 and αTyr-198, residues at the agonist binding site (2), as well as minor incorporation elsewhere. Whereas the incorporation in αM4 was independent of the presence of other drugs, the incorporation at αGlu-262 increased for nAChR in the desensitized state, and incorporation at αTyr-190/αTyr-198 was seen only in the absence of carbasymicholine or αBgTx.

When labeling was analyzed at the level of the subunit, the most prominent pharmacology of labeling was the dependence of the α-subunit incorporation on the presence of carbasymicholine. This increased incorporation was because of the desensitization of the nAChR because other agonists also increased the incorporation, whereas the incorporation was lowest in the presence of pancuronium or αBgTx. The competitive antagonists d-tubocurarine and gallamine caused only a partial increase in the incorporation in the α-subunit. In the presence of carbasymicholine, the aromatic amine noncompetitive antagonist meproaifden partially (−60%) inhibited the incorporation in the α-subunit, although two other aromatic amine noncompetitive antagonists, phencyclidine and QX-222, did not.

3-[3H]Azioctanol incorporated into the α-subunit at several sites. The levels of incorporation at these sites are summarized in Table I. The incorporation was calculated from mass levels and radioactivity incorporation from duplicate labeling experiments, although the values were determined differently for the large subunit fragments produced by V8 protease, their subfragments, and individual labeled amino acids. To estimate incorporation by sequence analysis in large subunit fragments (αV8-20, αV8-18, and αV8-10) or in fragments isolated by HPLC, incorporation was calculated as the [3H] loaded on the sequencer filter divided by three times the observed initial yield of the sequence. This calculation is likely an overestimate because it is unknown what percent of the loaded material was sequencable, perhaps as little as 10% or maybe up to about 50%. An additional source of error for the calculation of incorporation in the αV8-20 and αV8-10 fragments was the necessity of treating the samples on the filter to remove excess SDS before sequencing. For incorporation at individual amino acids, the mass of that residue was calculated from the initial and repetitive yields. In this calculation, the radioactivity released and the mass levels reflect only the sequenced material. Because of the differences in the calculations, comparisons should only be made between values determined by the same method. For example, for the incorporation in the subfragment containing αM2 from αMet-243 to αLys-340, was calculated to be −1.4 mol/mol whereas the incorporation at αGlu-262 was only −0.35 mol/mol. However, the lack of significant incorporation in any other amino acids in this fragment indicate that it is unlikely that the incorporation at αGlu-262 accounts for only one-third of the incorporation in the fragment. Additionally, the incorporation in αV8-10 is much greater than the incorporation in the isolated fragments. As is discussed later, this discrepancy is because of lack of stability of incorporation to HPLC conditions.

The primary site of incorporation of 3-[3H]Azioctanol was αGlu-262. This residue was the only residue labeled whose incorporation at 1 and 275 μM 3-[3H]Azioctanol (Table I) did not increase approximately linearly with concentration. In the de-
other atoms.

chains near labeled side chains in other fragments showed no octanol photoincorporated into a variety of side chains, includ-

ing arginine, 3-(trifluoro-methyl)-3-((

channel domain, and the preferential incorporation into the M2 segment, the other in M3, are known to confer sensitivity to several classes of anesthetics, including long chain alcohols, volatile anesthetics (isoflurane and enflurane), and intravenous anesthetics (etomidate) (reviewed in Ref. 34). The position in M2, 15’ (see Fig. 10), is located in the extracellular half of the M2 segment on the face of the M2 α-helix opposite the lumen of the ion channel. The position in M3 is about seven amino acids from the N terminus of M3, but the orientation of this segment is not clearly established. However, this residue has been predicted to face the M2 helix, positioning it near the M2 15’ residue (5). Neither of these residues was labeled in the nAChR by 3-[3H]aziotanol. The lack of labeling would be consistent with different binding sites on the two receptors, reflecting the different actions of long chain alcohols on them.

Fig. 10 shows a model of the nAChR αM2 segment as an α-helix including the positions implicated by mutational work on the nAChR and GABA<sub>A</sub> receptor and the photolabeled residue reported here. The azi group of 3-aziotanol was positioned near αGlu-262 (20’). The carbon chain of 3-aziotanol reaches to the 13’ residues but does not reach the 10’ position which is a determinant of alcohol potency in the nAChR. For the nAChR then, the photolabeling and mutagenesis results are in apparent disagreement. However, the nAChR mutagenesis studies measure octanol inhibition of the open state of the receptor, whereas the photoaffinity labeling studies are done with a desensitized receptor. It is possible that octanol binds at different positions in the two states. Indeed, differential labeling of the M2 ion channel in the resting and desensitized states has been seen with two other photoaffinity probes, 3-(trifluoro-methyl)-3-(m-[[125I]iodophenyl)diazirine (35) and [3H]diazofluorene (24). 3-Aziotanol might bind closer to the 10’-position in the open state, and closer to αGlu-262 in the desensitized state. Alternatively, the mutations studied may have changed the structure of the region near αGlu-262.

The studies presented here provide strong evidence that, in the desensitized state of the nAChR, the highest affinity binding site of 3-[3H]aziotanol is within the ion channel domain near αGlu-262. Further studies, such as photoincorporation of 3-[3H]aziotolan in the open channel and the effects of site-directed mutagenesis of the N-terminal end of the M2 segment on the inhibition of the nAChR by alcohols, will be necessary to further define the site of action of long chain alcohols on the nAChR.

Acknowledgments—The authors thank Aimeé Powelka for contributions to the studies of the effects of agonists and competitive antagonists on 3-[3H]aziotolan incorporation in nAChR subunits.

REFERENCES
1. Franks, N. P., and Lieb, W. R. (1994) Nature 367, 607–614
2. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
3. Hucu, F., Tsetlin, V. I., and Machold, J. (1996) Eur. J. Biochem. 239, 539–557
4. Corringer, P.-J., Le Novere, N., and Changeux, J.-P. (2000) Annu. Rev. Pharma
col. 40, 431–458
5. Wick, M. J., Mihic, S. J., Ueno, S., Mascia, M. P., Trudell, J. R., Brozowski, S. J., Ye, Q., Harrison, N. L., and Harris, R. A. (1998) Proc. Natl. Acad. Sci.
U. S. A. 95, 6504–6509
6. McGurk, K. A., Pistas, M., Belelli, D., Hope, A. G., and Lambert, J. J. (1998) Br. J. Pharmacol. 124, 13–20
7. Mosdy, E. J., Krauer, C., Granja, R., Strakhova, M., and Skolnick, P. (1997) J. Neurochem. 69, 1310–1313
8. Mihic, S. J., Ye, Q., Wick, M. J., Kejchines, V. K., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., Harris, R. A., and Harrison, N. L. (1997) Nature 389, 385–389
9. Pistas, M., Belelli, D., McGurk, K., Peters, J. A., and Lambert, J. J. (1999) J. Physiol. (Lond.) 515, 3–18
10. Birnir, B., Tierney, M. L., Dalez, J. E., Cox, G. B., and Gage, P. W. (1997).
11. Dilger, J. P., Liu, Y., and Vidal, A. M. (1995) Eur. J. Anaesthesiol. 12, 31–39
12. Dilger, J. P., and Vidal, A. M. (1994) Mol. Pharmacol. 46, 169–175
13. Wood, S. C., Tonner, P. H., Dearmendi, A. J., Bugge, B., and Miller, K. W. (1995) Mol. Pharmacol. 47, 121–130
14. Forman, S. A., Miller, K. W., and Yellen, G. (1995) Mol. Pharmacol. 48, 574–581
15. Husain, S. S., Forman, S. A., Kloczewiak, M. A., Addona, G. H., Olsen, R. W., Pratt, M. B., Cohen, J. B., and Miller, K. W. (1999) J. Med. Chem. 42, 3300–3307
16. Pedersen, S. E., Sharp, S. D., Liu, W.-S., and Cohen, J. B. (1992) J. Biol. Chem. 267, 10489–10499
17. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106
18. White, B. H., and Cohen, J. B. (1988) Biochemistry 27, 8741–8751
19. Blanton, M. P., and Cohen, J. B. (1994) Biochemistry 33, 2859–2872
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Wood, S. C., Tonner, P. H., Dearmendi, A. J., Bugge, B., and Miller, K. W. (1995) Mol. Pharmacol. 47, 121–130
22. Blanton, M. P., and Cohen, J. B. (1992) Biochemistry 31, 6987–6997
23. Blanton, M. P., and Cohen, J. B. (1992) Biochemistry 31, 3738–3750
24. Gallagher, M. J., and Cohen, J. B. (1999) Mol. Pharmacol. 56, 300–307
25. Akabas, M. H., Kaufmann, C., Archdeacon, P., and Karlin, A. (1994) Neuron 13, 919–927
26. Zhang, H., and Karlin, A. (1998) Biochemistry 37, 7952–7964
27. Blanton, M. P., and Cohen, J. B. (1992) J. Biol. Chem. 267, 15770–15783